Rodriguésia 73: e01332020. 2022
http://rodriguesia.jbrj.gov.br
DOI: http://dx.doi.org/10.1590/2175-7860202273033

Original Paper

Biotransformation of *Araucaria araucana* lignans: solid-state fermentation with a naturally occurring *Pleurotus ostreatus* strain

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Abstract

The effects of a naturally occurring Patagonian strain of the white-rot fungus *Pleurotus ostreatus* on *Araucaria araucana* wood lignans was evaluated. Lignans of colonized and non-colonized wood shavings and the activity of fungal ligninolytic enzymes were studied. Lignans were identified using gas chromatography with a mass spectrometry detector. Only eudesmin lignan resisted biological degradation. The highest laccase activity was 0.111 ± 0.067 IU.g^{-1} dry matter substrate, which was reached after 60 days, whereas the highest manganese peroxidase (MnP) activity was 0.220 ± 0.109 IU.g^{-1} dry matter substrate, which was reached after 25 days, when the fungus was grown in a solid-state culture on wood shavings. The degradation properties of this fungal strain may be useful for not only treating resinous wastes from the regional forest industry to produce biofuels but also improving paper production. Moreover, the capacity of this white-rot fungus to grow on resinous *A. araucana* materials as substrate suggests the possibility of using the wood shavings or sawdust of this and other conifers as a food source to culture *P. ostreatus*, an edible mushroom.

Key words: *Araucaria*, GC-MS, laccase, lignans, *Pleurotus*.

Resumen

Se evaluaron los efectos de una cepa patagónica de *Pleurotus ostreatus* sobre la composición de los lignanos de madera de *Araucaria araucana*. Se estudiaron los lignanos presentes en virutas de madera colonizada y no colonizada por el hongo y se evaluaron las actividades de las enzimas ligninolíticas. Los lignanos se identificaron mediante cromatografía de gases acoplada a espectrómetro de masas (GCMS). Solamente el lignano eudesmin resistió la degradación biológica. La mayor actividad de la enzima lacasa detectada fue de $0{,}111 \pm 0{,}067$ UI.g^{-1}, que se alcanzó luego de 60 días, mientras que la mayor actividad de la enzima manganeso peroxidasa (MnP) fue de $0{,}220 \pm 0{,}109$ UI.g^{-1} a los 25 días de cultivo. Las propiedades degradativas de esta cepa fúngica pueden ser útiles, no solamente para tratar los desechos resinosos de la industria forestal regional sino también para producir biocombustibles a partir de estos, o aplicarlos en la producción de papel. Además, la capacidad de este hongo de podredumbre blanca para crecer en un sustrato resinoso como *A. araucana* sugiere la posibilidad de usar las virutas o aserríes de coníferas como sustrato para cultivar un alimento de valor como *P. ostreatus*.

Palabras clave: *Araucaria*, GC-MS, lacasa, lignanos, *Pleurotus*.

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Introduction

Only two species of the genus *Araucaria* Juss. occur in South America: *A. araucana* (Mol.) K. Koch, present in the Patagonian Andes of Argentina and Chile, and *A. angustifolia* (Bert.) O. Kuntze, present in northeastern Argentina, Brazil, and eastern Paraguay (Veblen 1982). *Araucaria araucana* is a coniferous tree that can reach a height of 50 m and a diameter of 2 m. The Andean populations of this species are distributed between 37°24'S and 40°03'S at 900–1,700 m above sea level; another population is located in the Chilean coastal range between 37°30'S and 38°30'S at altitudes ranging from 600 to 1,400 m and receiving an annual precipitation of 1,500–2,500 mm. They are long-lived conifers that have survived the many glaciation events that have led to the various present-day distributions of araucaria forests (Villagrán & Armesto 2005).

Araucaria araucana wood is of excellent quality, light, easy to work, and has a yellowish-white color. For these reasons, it is highly valued for construction and carpentry work, and straight and cylindrical logs are used in the manufacture of ship masts. Its main defense against pathogens is its thick bark (which reaches 10 cm thick in adults) and the abundant resin they secrete when damaged. However, when exposed to the elements, its wood can be easily damaged by biotic and abiotic factors.

In addressing the durability of the species, Eaton & Hale (1993) defined natural durability as the wood's ability to resist biological degradation. Plants defend against pathogenic attacks via structural, physical and biochemical barriers that inhibit pathogen invasion and propagation. The lignins fulfill essential functions by providing structural reinforcement to plant tissues, thereby allowing all vascular plants to stand upright (Lewis & Sarkanen 1998). Lignans, are an omnipresent group of closely related non structural phenolic secondary metabolites, biosynthesized via the phenylpropanoid pathway and defined by IUPAC as dimeric C_6C_3 motifs linked at carbons 8 and 8' (Moss 2000). These and other compounds are responsible for the natural resistance of wood against the actions of insects, fungi, and bacteria. It is important to analyze the antimicrobial activity of lignans as potential inhibitors of fungal/bacterial cytotoxins and enzymes and as inhibitors of microbial growth (Rowe 1989; Castro *et al*. 1996). Cespedes *et al*. (2006) revealed that lignans found in *A. araucana* extracts show antifungal activities against white-rotting and wood-staining fungi. Lignans derivatives have high antioxidative potency and radical scavenging capacity (Heitner *et al*. 2010; Valette *et al*. 2017). However, lignans are not involved in the formation of physical barriers, such as the plant cell wall, for protection against fungal attacks (Lewis & Sarkanen 1998).

Pleurotus ostreatus (Jacq.) P. Kumm. (Agaricales, Basidiomycota) is an edible white-rot fungus that is capable of growing on the trunk of *A. araucana.* In addition, a few species of xylophagous fungi, such as ones belong to Ascomycota and Basidiomycota, have been reported growing on *Araucaria* trees (Lechner *et al*. 2002), which possess strong antifungal activity (Cespedes *et al*. 2006). The most common pattern of white-rot fungi attack consists of the simultaneous decay of all wood components. This fungus is able to secrete specific ligninolytic enzymes that cause significant phenolic degradation (Shah & Nerud 2002; Ruiz-Rodríguez *et al*. 2011; Rugolo *et al*. 2016). The enzyme manganese peroxidase (MnP), produced by white-rot fungi, is capable of oxidizing phenolic lignin units and other phenolic compounds. It has been shown that the native ferric MnP enzyme donates two electrons to H_2O_2, allowing the formation of a porphyrin cation radical (Wariishi *et al*. 1988). Other enzymes produced by the white-rot fungus include laccases, a group of oxidoreductases that catalyze the oxidation of unspecific substrates (*p*-diphenols, *o*-diphenols, aryldiamines, aminophenols, and hydroxyindols) with the concurrent reduction of dioxygen to water (Cai *et al*. 1993). Despite lignin peroxidase has not reported in *Pleurotus* species, a versatile peroxidase (VP) has been described in genus *Pleurotus* as a third type of ligninolytic peroxidase that has both catalytic properties of LiP and MnP (Martinez *et al.* 2005).

Although *Pleurotus ostreatus* is typically isolated from nonresinous species, several specimens have been found growing and producing basidiomes on *Araucaria.* The above-described properties of *Pleurotus ostreatus* have stimulated interest in the degradation patterns of strains of this fungus isolated from conifers in Patagonia, with the goal of developing potential applications to treat waste produced by the regional forestry industry, which is dominated by *Pinus* exploitation. Presently, the focus is on the development of biological technologies due to their low cost and environmental advantages over existing chemical methods. The pretreatment

of agricultural and forestry waste using fungi has attracted considerable interest with respect to biofuel production (Madadi & Abbas 2017). Moreover, in the process of wood pulping, a portion of the unwanted lignocellulosic material is carried over to the following stages of processing and causes problems, such as the settling of deposits, interference with chemical processes, and reduced efficiency. The use of enzymes, such as laccases, improves efficiency and decreases production costs in the paper industry (Buchert *et al*. 2002). Laccase enzymes have also been shown to maintain their stability and reactivity even in systems with aqueous organic solvents, increasing the potential use of these enzymes in the modification and valorization of lignin (Mattinen *et al*. 2011).

The aim of the present work was to evaluate the degradation, as measured by gas chromatographic/mass spectrometric (GC-MS) analysis, of lignans in wood extractives of *A. araucana* by a naturally occurring *P. ostreatus* strain.

Material and Methods

Fungal strain and culture conditions

Pleurotus ostreatus LPSC 1254 strain (La Plata Spegazzini Collection) was used in this study. The strain was maintained in Petri dishes at 4 °C on ME agar (malt extract 1.2%, glucose 1% and agar 2%).

Spawn and solid-state cultures

Spawn production was prepared in 350 mL glass flasks filled with 200 g of wet boiled (for 20 min at 90–100 °C) oat grains (*Avena sativa*) and 1% (w/w) $CaCO_3$. Flasks were sterilized for 1.5 h at 121 °C, cooled to 23 °C, inoculated with a 1 cm diameter plug of mycelium and then incubated at 23 °C in the dark for 20 days with periodic shaking.

Another set of glass flasks were filled with 10 g (dry weight) of *Araucaria araucana* wood shavings as a substrate. The samples of wood shavings were produced in our laboratory and were collected from different trees. The humidity was adjusted to 75% (w/w) with distilled water. The flasks were covered with cotton plugs to allow air circulation and autoclaved at 121 °C for 2 h. After cooling, they were inoculated with 5% w/w of the spawn (colonized oat grains) and then incubated at 23 °C in the dark until total substrate colonization (30–40 days) with *P. ostreatus* mycelia. Wood shavings without fungus were used as controls.

Chromatographic techniques

Pleurotus ostreatus-colonized and control *A. araucana* wood shavings (in samples of 10 g each) were harvested and extracted with maceration three times using ethyl acetate at 30 °C. The crude extract was then filtered and evaporated to dryness under vacuum. The total crude extract was separated using the TLC technique (Willför *et al*. 2006) in normal phase Merck silica gel with an ethanol-dichloromethane (7:93, v/v) solution as eluent. The separated analytes were detected by spraying TLC plates with a sulfuric acid-ethanol mixture (95:5, v/v) followed by a hold at 150 °C for 2 min in a hot plate.

The isolation and identification of lignans were performed according to Willför *et al*. (2006) by preparative layer chromatography (PLC) silica gel 60 F_{254} plates of 2 mm thickness (Merck) using ethanol-dichloromethane (7:93, v/v) solution as eluent. Bands were injected on the plate with a micropipette containing 500 mg of extract. Visible bands under UV light were removed manually, washed with ethyl acetate, filtered and injected into GC-MS equipment for identification.

GC-MS analysis was performed by gas chromatography/mass spectrometry (GC-MS) instrument (Agilent 7890, California, USA) equipped with an Agilent 5975 mass detector and a silica HP5-MS capillary column of 30 m x 0.25 mm internal diameter and 0.25 μm film thickness under the following conditions: temperature: 250 °C; detector (mass): 280 °C; oven: 100 °C for 5 min, increased at 8 °C/min to 250 °C, and maintained at 250 °C for 15 min. The detector was set in scan mode from 50 to 500 amu. The carrier gas (electronic grade helium) flow rate was 1 $mL.min^{-1}$. Mass spectra were obtained from the total ion current (TIC), and identification of the constituents was based on comparison of the retention times with those of reference standards, with their linear retention indices determined relative to a series of n-hydrocarbons, and on computer matching against commercial libraries (NIST05 National Institute of Standards and Technology 2008).

Identification of compounds was performed based on comparisons with mass spectra in the literature (Yamamoto *et al*. 2004, 2010) and database interpretation of mass spectrometric fragmentation patterns (NIST05a).

Enzyme activities

Laccase activity (E.C.: 1.10.3.2) was measured using 0.5 mM 2,2'-azino-bis-(3-ethylbenzthiazo-

linesulphonate) (ABTS) in 0.1 M sodium acetate buffer (pH 3.6) at 30 °C. Oxidation of ABTS was determined by monitoring the increase in absorbance at 420 nm (ε 420 = 36 $mM^{-1}.cm^{-1}$). Manganese peroxidase activity (MnP) (E.C.:1.11.1.13) was measured using phenol red and $MnSO_4$ as substrate in 0.1 M sodium dimethyl succinate buffer (pH 4.5) (ε 610 = 22 $mM^{-1}.cm^{-1}$) at 50 °C. Enzyme activity was expressed in International Units (U) standardized as the amount of enzyme required to release 1 µmol of product in 1min. In terms of production, the activity was defined as $IU.g^{-1}$ dry residue. Samples from solid-state cultures were harvested after 25, 35 and 60 days. Crude extracts were obtained by adding distilled water to the samples from each freshly harvested culture (5:1, w/w), stirring for 20 min, filtering and centrifuging [modified from Vares *et al*. (1995)]. The supernatants were stored at -10°C until needed.

Results and Discussion

TLC analysis of non-colonized *Araucaria araucana* wood shavings (control), referred to as Aa, revealed the presence of four highly visible spots that were identified based on their retention factor (Rf) values as eudesmin (Rf = 0.91), pinoresinol monomethyl ether (Rf = 0.82), lariciresinol (Rf = 0.47), and secoisolariciresinol (Rf = 0.32). Two bands with Rf values of 0.54 and 0.24 were not identified but corresponded to derivatives of lariciresinol (having molecular weights of 374 and 360). A similar pattern was observed in colonized wood, referred to as Aa Col, but the spots were less intense than those of the controls (Fig. 1).

The GC-MS chromatogram of the total extract of *Araucaria araucana* wood shavings (Aa) is shown in Figure 2, and that of the total extract of *A. araucana* wood shavings colonized with *P. ostreatus* (Aa Col) is shown in Figure 3. Extracted ion chromatograms at m/z 151 were obtained for better detection of lignans. The identified lignans and derivatives and their molecular weights, molecular formulas, abundance percentages (relative peak area %) and retention times (min) in Aa and Aa Col are listed in Table 1. The chemical structures of the detected lignans are shown in Figure 4.

In Aa, the major components were eudesmin (28%), secoisolariciresinol-4-methyl ether-9'-acetate (25.8%), and lariciresinol (21.5%). The minority component corresponded to pinoresinol monomethyl ether (5.72%). Other components accounting for less than 2% included shonanin (1.95%) and isovanillin (1.34%). In contrast, in Aa Col, the number of lignans and derivatives was lower; they included isovanillin (26.2%), eudesmin (19.41%), and (E)-conipheryl alcohol (3.66%). The minority components (< 2%) were shonanin (1.67%) and secoisolariciresinol-4-methyl ether-9'-acetate (1.61%). However, three lignans detected in Aa, secoisolariciresinol, pinoresinol monomethyl ether, and lariciresinol, were not detected in the Aa Col sample. The GC-MS analysis revealed the presence of 6 lignans, including the dibenzylbutanediol-type lignans secoisolariciresinol and secoisolariciresinol-4-methyl ether-9'-acetate, the tetrahydrofuran-type lignans lariciresinol and shonanin, and the furofuran-type lignans pinoresinol monomethyl ether and eudesmin. All of these lignans have also been reported in *Araucaria angustifolia* resin extracts (Yamamoto *et al.* 2004).

Additionally, some differences were observed in the relative abundances of compounds between Aa and Aa Col that can be attributed to the fungus. In particular, the presence of eudesmin decreased from 28% in Aa to 19.41% in Aa Col, whereas the amount of isovanillin increased from 1.34% in Aa to 26.62% to Aa Col. The presence of (E)-coniferyl alcohol and isovanillin in the Aa Col sample indicate wood degradation. Both compounds are not only precursors and abundant components in

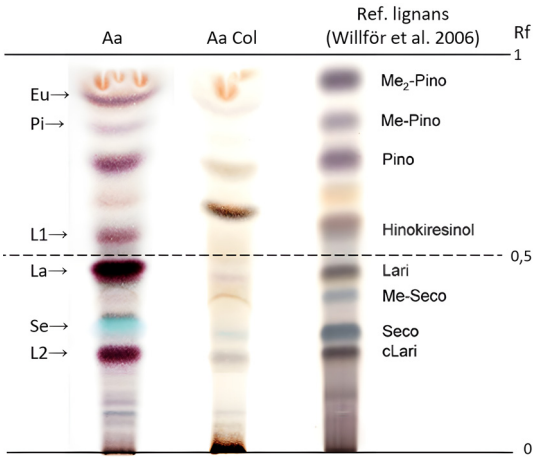

Figure 1 – TLC analysis of white-rot-colonized *Araucaria araucana* wood shavings showing bands of ethyl acetate soluble compounds. Bands of colonized samples are less intense than those of non-colonized samples. Aa Col = colonized wood shavings; Aa = non-colonized wood shaving. Reference bands correspond to eudesmin (Eu), pinoresinol monomethyl ether (Pi), secoisolariciresinol (Se), lariciresinol (La), and derivatives of lariciresinol (L1 and L2) are shown.

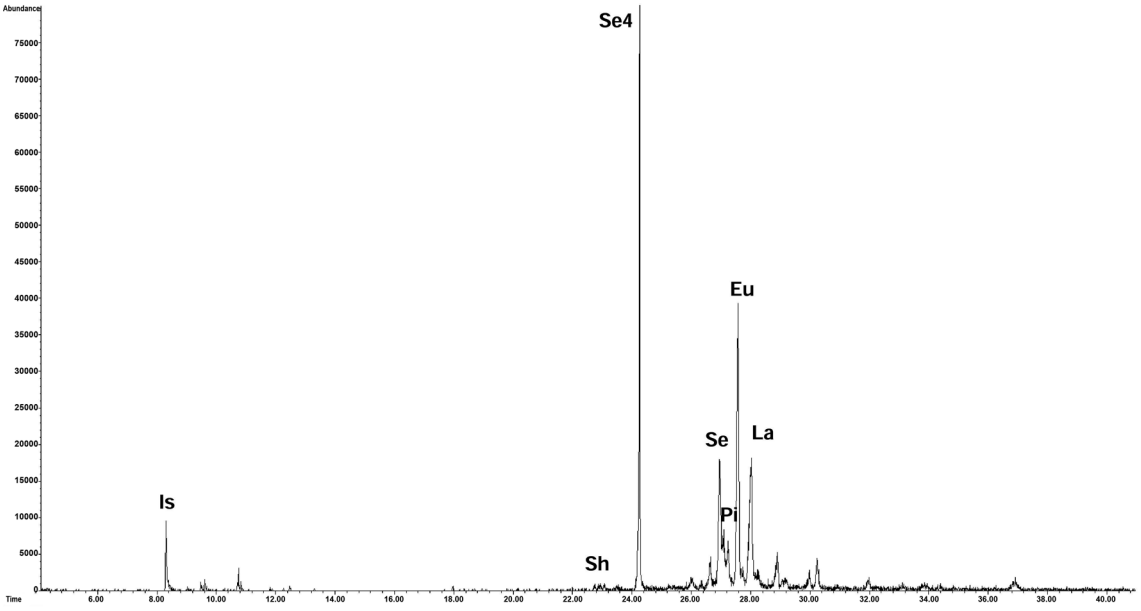

Figure 2 – GC-MS chromatogram of the total extracts from non-colonized *Araucaria araucana* wood shavings.

lignin polymer but also products of ligninolytic degradation. In fact, these products result from the activities of enzymes such as laccases (Su *et al*. 2018), MnP and aryl-alcohol oxidases, a small group of enzymes detected in *P. ostreatus* that are likely involved in lignan degradation (Lupo *et al*. 2009).

The *Araucaria araucana* wood showing white-rot contained mainly eudesmin, which was less reactive/more recalcitrant than the other lignans. This result could have been due to the absence of hydroxyl groups and the presence of β-β (α-O-γ) linkages in eudesmin. Moreover, the aryl-

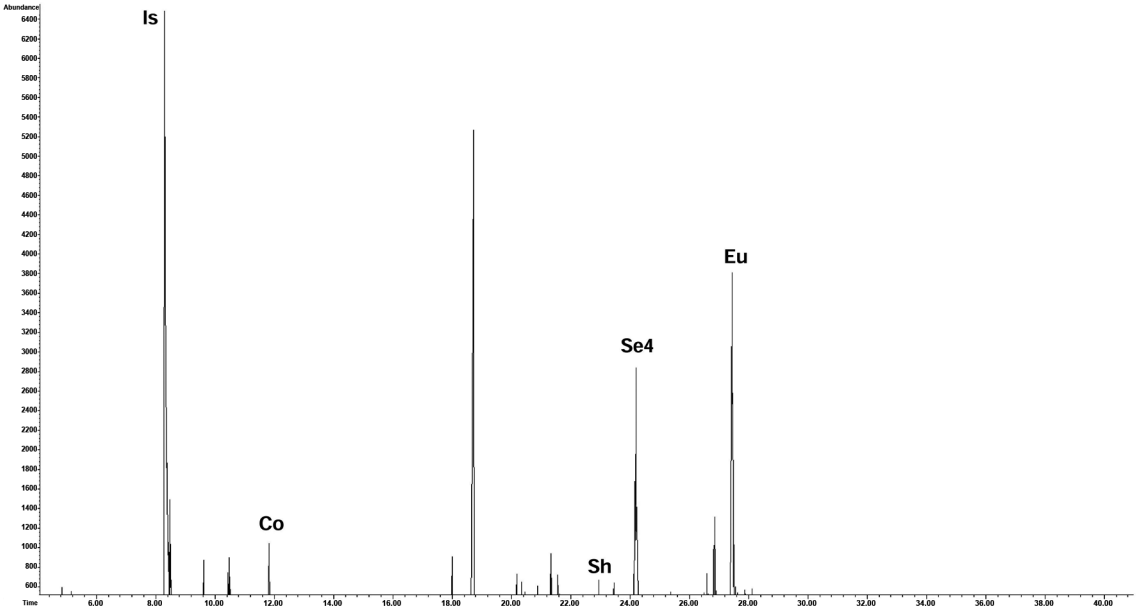

Figure 3 – GC-MS chromatogram of the total extracts from *Araucaria araucana* wood colonized with the white-rot fungus *Pleurotus ostreatus*.

Table 1 – Lignans and derivatives detected in *Araucaria araucana* wood shavings colonized or non-colonized with the white-rot fungus *Pleurotus ostreatus*.

	Compound	Molecular formula	Molecular weight	Retention time (min)	Percentage of total in non-colonized wood	Percentage of total in colonized wood
Is	isovanillin	$C_8H_8O_3$	152	8.32	1.34%	26.62%
Co	(E)-conipheryl alcohol	$C_{10}H_{12}O_3$	180	11.81	-	3.66%
Sh	shonanin	$C_{20}H_{24}O_5$	344	22.92	1.95%	1.67%
Se4	secoisolariciresinol-4-methyl ether-9'-acetate	$C_{23}H_{30}O_7$	418	24.21	25.83%	1.61%
Se	secoisolariciresinol	$C_{20}H_{26}O_6$	362	26.92	13.01%	-
Pi	pinoresinol monomethyl ether	$C_{21}H_{24}O_6$	372	27.17	5.72%	-
Eu	eudesmin	$C_{22}H_{26}O_6$	386	27.432	28.00%	19.41%
La	lariciresinol	$C_{20}H_{24}O_6$	360	27.910	21.51%	-
	not identified				2.60%	47.03%
	Total				**100%**	**100%**

OCH_3 groups in these lignans might increase the stability of this molecule under fungal degradation. These phenomena likely explain why the LPSC 1254 strain of *P. ostreatus* was not able to easily degrade eudesmin. However, it is unclear how this compound is integrated into the lignin structure (potentially masked) and whether the mycelium lacks the enzymatic reactions that favor the degradation of this compound. Eudesmin has been reported to have high antifungal activity against

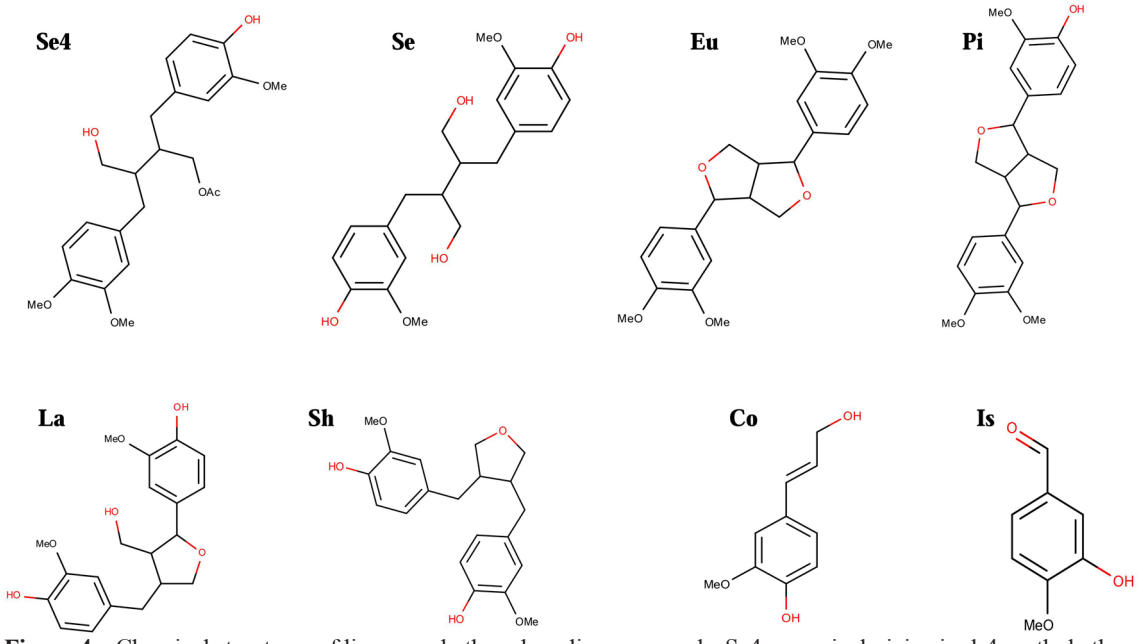

Figure 4 – Chemical structures of lignan and other phenolic compounds. Se4 = secoisolariciresinol-4-methyl ether-9'-acetate; Se = secoisolariciresinol; Eu = eudesmin; Pi = pinoresinol monomethyl ether; La = lariciresinol; Sh = shonanin; Co = (E)-conipheryl alcohol; Is = isovanillin.

some phytopathogens, such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Phomopsis obscurans* (Ell. & Ev.) B. Sutton *and Fusarium oxysporum* Schltdl., at concentrations between 50 and 150 μM (Cantrell *et al.* 2005). In another study (Martínez *et al.* 2005), GC-MS analysis of *Eucalyptus globulus* Labill. hardwood degraded by the white-rot fungus *Ceriporiopsis subvermispora* (Pilát) Gilb. & Ryvarden showed a decrease in lignin peaks and an increase in carbohydrate peaks relative to fungus-treated wood but no difference in the total degradation of lignin-derived compounds.

Lignans have high chelating capacity, a feature that is especially effective in combating the degradation by xylophagous fungi that use peroxidases, Cu-dependent laccases, and iron-promoted Fenton reactions. In addition to having metal chelating properties, lignans are capable of reducing the levels of reactive oxygen species, which are necessary for the enzymatic or oxidative processes that lead to wood decomposition (Donoso-Fierro *et al*. 2009).

The laccase and MnP activities in solid culture are shown in Table 2. Enzymatic activity appeared first as high laccase activity, and the maximum level of enzymatic activity was observed on day 25 (0.148 ± 0.023 IU.g^{-1} dry matter substrate). Thereafter, the level of activity decreased rapidly, although low levels of laccase activity continued to be detected throughout the incubation period. MnP was first detected in the extracts after 25 days of fermentation. The level of activity increased throughout the experiment, reaching its maximum level on day 60 (0.220 ± 0.010 IU.g^{-1} dry matter substrate).

Elisashvili *et al.* (2006) reported that the presence of a lignocellulosic substrate is necessary for MnP production by *Pleurotus dryinus* (Pers.) P. Kumm. When *P. ostreatus* was cultured in wheat straw and tree leaves of *Fagus sylvatica* L., it showed laccase activity values of approximately 1.75–4.00 and 1.75–3.50 IU.g^{-1} dry matter substrate, respectively, using ABTS, and MnP activities of 1.75–3.75 and 0.03–1.48 IU.g^{-1} dry matter substrate, respectively (Elisashvili *et al*. 2008). In a study of grapevine sawdust, Stajic *et al*. (2004) reported a laccase activity of 3.49 IU.g^{-1} dry matter substrate using syringaldazine for laccase detection.

The white-rot fungi *Pleurotus* spp. possess the ability to degrade the lignin polymers of wood tissues. Solid-state fermentation is an efficient method to study the biotransformation of *P. ostreatus* substrates and simultaneously produce hydrolytic and ligninolytic enzymes when fermenting lignocellulosic materials (Ozcirak Ergun & Ozturk Urek 2017). Our main aim in this study was to investigate the biotransformation of *A. araucana* extractives by the white-rot fungus *P. ostreatus* under solid-state fermentation conditions.

Lignans can be efficiently oxidized by *Pleurotus ostreatus* enzymes. Among the lignans detected in *Araucaria araucana* wood in this study, only the lignan eudesmin showed little degradation. Of the lignans analyzed in this study, eudesmin seems to be the most recalcitrant to lignocellulolytic action by *P. ostreatus*. Nevertheless, all of the lignans seemed to be influenced; no unreacted lignan molecules in Aa Col were detected in the GC-MS analysis. In addition, our study suggests that this white-rot strain can degrade other phenolic groups present in *A. araucana* lignans. This is the first study to culture *P. ostreatus* on *A. araucana* wood as a substrate. Future research should explore the ability of native strains of *P. ostreatus* to grow on, degrade and develop basidiomes on substrates generated from conifer debris from the regional forest industry, which is dominated by the use of *Pinus* species.

To determine the *in vitro* and *in vivo* roles of various lignans, further assays should be undertaken with purified molecules and mixtures to determine their independent and interactive effects.

Table 2 – Activities of laccase and manganese peroxidase (IU.g^{-1} dry matter) of *Pleurotus ostreatus* strain 1254 growing on *Araucaria araucana* wood shavings.

Number of culture days	Enzyme activity (IU.g^{-1} dry matter)	
	Laccase	MnP
25	0.148 ± 0.023	0.030 ± 0.006
35	0.087 ± 0.006	0.054 ± 0.006
60	0.069 ± 0.015	0.220 ± 0.010

Acknowledgements

The authors thank "Proyecto PCCI130036 de Cooperación Bilateral MinCyT (Argentina) - CONICYT (Chile)" (MR and JB) and PIP-CONICET 11220110100388 (MR), for financial assistance.

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Area Editor: Dr. Anibal de Carvalho Junior
Received in July 11, 2020. Accepted in April 21, 2021.