Screening for ligninolytic enzymes in autochthonous fungal strains from Argentina isolated from different substrata

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Summary

The production of different extracellular ligninolytic enzymes was studied in autochthonous fungal strains from Argentina isolated from litter derived from hydrocarbon-polluted sites and from basidiocarps frowing on wood in forests. The strains tested were cultivated in a carbon-limited medium with shaking. Laccase activity reached higher levels than aryl-alcohol oxidase and manganese-dependent peroxidase activities in liquid cultures from different fungi. No lignin peroxidase activity was found in any strain assayed. Some species are reported for the first time as producers of different ligninolytic enzymes.

Key words

Aryl-alcohol oxidase, Basidiomycetes, Deuteromycetes, Laccase, Lignin peroxidase, Manganese-peroxidase

Enzimas ligninolíticas de cepas fúngicas autóctonas de Argentina aisladas de diferentes sustratos

Resumen

Se estudió la producción de diferentes enzimas ligninolíticas extracelulares en cepas fúngicas autóctonas de Argentina aisladas a partir de materia orgánica de sitios contaminados con hidrocarburos y basidiocarpos desarrollados sobre restos leñosos de bosques. Las diferentes cepas estudiadas se cultivaron en un medio limitado en carbono bajo agitación. Se detectaron niveles superiores de actividad lacasa en relación a los correspondientes para las actividades extracelulares de aril-alcohol oxidasa y peroxidasa dependiente de manganeso en los cultivos líquidos de diferentes hongos. No se detectó actividad lignina peroxidasa en ninguno de los aislamientos analizados. Diferentes especies son citadas por primera vez como productoras de diferentes enzimas ligninolíticas. *Palabras clave:* Aril-alcohol oxidasa, basidiomycetes, deuteromycetes, lacasa, lignina peroxidasa, peroxidasa dependiente de manganeso.

Palabras clave

Aril-alcohol oxidasa, Basidiomycetes, Deuteromycetes, Lacasa, Lignina peroxidasa, Peroxidasa dependiente de manganeso

Lignin is a recalcitrant heteropolymer of phenylpropanoid units present in woody plant tissues, that confers them rigidity and resistance to biological attack [1]. In order to depolymerize and mineralize lignin, white-rot fungi have developed an oxidative and unspecific system including extracellular enzymes, low molecular weight metabolites and activated oxygen species [2-5]. Due to the lack of specificity of the system involved in the lignin depolymerization, white-rot basidiomycetes and their enzymes are being studied for their application on the degradation of aromatic pollutants causing environmental problems [6-9].

The extracellular enzymatic systems include ligninolytic peroxidases, laccases and oxidases responsible for the production of extracellular hydrogen peroxide (H_2O_2) [10]. Those enzyme systems exhibit differential characteristics depending on the species, strains and culture conditions [11-13]. Among peroxidases, lignin peroxidase (LiP) is able to oxidize directly non-phenolic units whereas manganese peroxidase (MnP) and laccase oxidize prefe-

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Aceptado para publicación el 23 de Mayo de 2002

1130-1406/01/10.00 Euros

rentially phenolic units, but also act on non-phenolic units in the presence of mediators [14,15]. Recently there have been reports of a new peroxidase type in *Pleurotus* and *Bjerkandera* species which shares catalytic properties with LiP and MnP [16-17] and is also able to oxidize azo-dyes not oxidized by MnP or LiP [18]. On the other hand, two extracellular oxidases, glyoxal and aryl-alcohol (AAO) oxidases, have been reported for extracellular H_2O_2 production [19,20].

Several screening works about ligninolytic enzymes have been carried out mainly in white-rot basidiomycetes [21-24]. However, other fungi, representatives of different taxonomic and ecophysiological groups, are able to degrade lignocellulosic substrata, mineralize ^{14}C-milled wood lignin [25] and produce ligninolytic enzymes [13,26].

The aim of the present work was to produce different extracellular enzymes involved in lignin degradation and detoxification of aromatic pollutants and to determine the ability of several argentinian autochthonous basidiomycetes and deuteromycetes strains, isolated from organic matter of hydrocarbon-polluted sites and basidiomes developed on wood, to produce these enzymes.

Materials and methods

Fungal strains. The strains used for this work were isolated from different sources/substrata and habitats from Argentina (Table 1). They belong to the culture collection of Spegazzini Institute (CLPS). Stock cultures of the basidiomycetes strains were kept at 4 °C on 2% (w/v) malt-agar slants supplemented with yeast extract (0.4%) and *Populus* spp. wood chips. Cultures of the deuteromycetes were maintained on agar slants (Table 1) at 4 °C.

Medium and culture conditions. The production of extracellular ligninolytic enzymes was carried out in the modified Czapek Dox medium [23]. Homogenized mycelium from 5-10 day-old cultures was used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of medium (3-5 mg of dry weight/ml of inoculum). Four replicate flasks were incubated at 25 °C in a rotary shaker at 150 rev/min.

Analytical methods. The cultures were harvested at the 7th and 21st day of incubation. Each sample was centrifuged (10,000 x *g* for 30 min) at 4 °C. The mycelial pellet was dried at 60 °C and weighed to estimate the fungal biomass. The supernatant of the liquid culture was kept for enzyme assays.

Enzyme assays. AAO (EC 1.1.3.7) activity was estimated by the veratraldehyde formation from 5 mM veratryl alcohol (VA) (Fluka) in 100 mM phosphate buffer at pH 6.0 (ε_{310}: 9,300/Mcm) [20]. Laccase (EC 1.10.3.2) activity was measured with 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma) in 100 mM acetate buffer at pH 5.5 (ε_{436} of the ABTS cation radical: 29,300/Mcm) [23]. LiP (EC 1.11.1.14) activity was determined by H_2O_2-dependent veratraldehyde formation from 2 mM VA in 100 mM sodium tartrate buffer at pH 3.0, with H_2O_2 0.4 mM [27]. MnP (EC 1.11.1.13) activity was estimated using as substrate 0.01 % phenol-red (Sigma) in 100 mM sodium tartrate, pH 5.0 [28], in presence of 0.1 mM H_2O_2 and 1 mM $MnSO_4$.

The enzyme reactions were carried out in duplicate and controls were performed without the addition of the enzyme or H_2O_2 for oxidase and peroxidase assays, respectively. Based on preliminary studies, optimal assay times were known to fall in the linear range of enzyme kinetics. All oxidation rates were determined at 25 °C using a Beckman DU 640 u.v.-visible spectrophotometer. One enzymatic activity unit (U) was defined as the amount of enzyme that transforms 1 µmol of substrate/min.

Table 1. Fungal strains used in this study.

Fungal species and taxonomic groups	CLPS[a]	Substrate/habitat and collection site
Basidiomycetes		
Amauroderma boleticeum (Pat. And Gaill.) Torr.	157	Decaying wood of subtropical rain forests[Ga]
Auricularia sp.	550	Decaying wood of subtropical rain forests[A]
Coriolopsis rigida (Berk. Et Mont.) Murrill	232	Rotten wood of subtropical rain forests[Ga]
Cyathus striatus (Hudson) Hoffm.	381	Rotten wood of subtropical rain forests[Sa]
Grammothele subargentea (Speg.) Rajch.	436	Trunk of living tree *Angiosperm* of subtropical rain forests[Ga]
Loweporus lividus (Kalchbrenner) Wright	289	Rotten wood of subtropical rain forests[Ga]
Peniophora albobadia (Schw.: Fr.) Boidin	285	Decaying wood of subtropical rain forests[Sa]
Phanerochaete septocystidia (Burt.) Erikss	288	Rotten wood of subtropical rain forests[Sa]
Phanerochaete tuberculata (Karst.) Parm.	435	Rotten wood of subtropical rain forests[Sa]
Phellinus gilvus (Schw.) Pat. var. *licnoides* (Mont.)	156	Rotten wood of subtropical rain forests[Sa]
Phellinus linteus (Berk. Et Curt.) Teng.	338	Trunk of living tree of subtropical rain forests[Sa]
Pleurotus laciniatocrenatus (Speg.) Speg.	39	Trunk of living tree *Taxodium* sp. of urban forest area[Pe]
Pycnoporus sanguineus (L.: Fr.) Murr.	163	Trunk of living tree *Leguminosae* of subtropical rain forests[Ga]
Trametes pavonia (Hook.) Ryv.	437	Rotten trunk of subtropical rain forests[Sa]
Trametes subectypus (Murr.) Gibn.	342	Rotten wood of subtropical rain forests[Ga]
*Trametes villosa (*Fr.) Kreisel	233	Rotten trunk of subtropical rain forests[Sa]
Deuteromycetes		
Alternaria alternata (Fries: Fries) Keissler	267[b]	Floating litter collected in hydrocarbon-polluted water[S]
Beltrania rhombica Penz	272[e]	Floating litter collected in hydrocarbon-polluted water[S]
Cladosporium cladosporioides (Fresen.) de Vries	556[d]	Hydrocarbon-polluted soil[E]
Epicoccum purpurascens Ehrenb. Ex Schlecht.	554[d]	Hydrocarbon-polluted soil[E]
Fusarium solani (Martius) Saccardo	493[d]	Hydrocarbon-polluted soil[E]
Graphium putredinis (Corda) Hughes	423[c]	Hydrocarbon-polluted soil[E]
Minimidochium parvum Cabello, Arambarri and Cazau	548[d]	Floating litter collected in hydrocarbon-polluted water[S]
Penicillium chrysogenum Thom	495[c]	Crude oil[E]
Phaeoisaria clematidis (Fuckel) Hughes	154[b]	Floating litter collected in hydrocarbon-polluted water[S]
Tetraploa aristata Berkeley et Broome	419[b]	Hydrocarbon-polluted organic matter floating in freshwater[S]
Trichoderma saturnisporum Hammill	264[d]	Floating litter collected in hydrocarbon-polluted water[S]

[a]CLPS strain no. Culture media deuteromycetes type: [b]corn-meal agar medium; [c]czapek with 1% (v/v) crude oil; [d]2% (w/v) malt-agar; [e]potato extract-agar.
Collection source: [A]Aristóbulo del Valle (Province of Misiones), Argentina; [E]Ensenada (Province of Buenos Aires), Argentina; [Ga]Garupá (Province of Misiones), Argentina; [Pe]Pereyra Park (Province of Buenos Aires), Argentina; [S]Santiago river (Province of Buenos Aires), Argentina; [Sa]Santa Ana (Province of Misiones), Argentina.

Results and discussion

The ability of different argentinian fungal strains belonging to basidiomycetes and deuteromycetes to produce extracellular ligninolytic enzymes in the modified Czapek Dox medium under shaking was screened. All the fungal strains studied produced abundant growth under the culture conditions assayed with a yield between 200 and 600 mg/100 ml of medium. The highest biomass values were obtained with the cultures of *Cyathus striatus* - at 21 days of incubation -. As a consequence of the utilization of a C-limited medium, the biomass obtained at 7 days (trophophase) was higher than at 21 days (idiophase).

No ligninolytic enzymatic activities were detected in the basidiomycetes *Cyathus striatus*, *Loweporus lividus*, and *Phellinus linteus* and the deuteromycetes *Alternaria alternata*, *Beltrania rhombica*, *Cladosporium cladosporioides*, *Epicoccum purpurascens*, *Penicillium chrysogenum* and *Trichoderma saturnisporum* in the culture conditions assayed.

LiP activity was not detected in any of the strains studied. Previous screening, including the basidiomycete *Phanerochaete chrysosporium*, reported similar results in shake or static conditions [21,23]. It is known that *P. chrysosporium* needs specific growth conditions to produce LiP, including nitrogen limited medium and O_2 saturated atmosphere [27]. It is possible that the culture conditions used in this study could affect LiP expression although other fungi produce LiP in air-atmosphere and non-limited nitrogen medium [29]. In this sense, it is possible that the absence of VA as LiP inductor in the culture medium, or the inhibition of this activity by aromatic compounds present in the culture, are responsible for the negative results [30-31].

The ligninolytic enzymatic activities, AAO, laccase and MnP, detected after 7 and 21 days of incubation in the different strains studied are shown in figures 1, 2 and 3, respectively. Note that the activity levels obtained did not necessarily reflect the optimum production for each strain.

The AAO activity was observed only in the 25 % of the strains tested (Figure 1), reaching the highest levels - more than 5 mU/ml – in *Amauroderma boleticeum* at 7 days and in *Pleurotus laciniatocrenatus* at 21 days of incubation. Extracellular AAO has been characterized from the culture liquid of *Pleurotus* and *Bjerkandera* species [20,32-35] and from the mycelial extract of *Phanerochaete chrysosporium* [36]. The AAO catalyzes the oxidation of a broad number of aromatic alcohols, chemically related to lignin, to aldehydes [20] and it participates in the continuous H_2O_2 production through a system based on aromatic aldehydes redox cycling [37-38].

Extracellular laccase activity was detected in most of the basidiomycetes strains studied (Figure 2); *Grammothele subargentea* reached the highest activity (170 mU/ml). According to the results obtained in previous screening works [21,23], laccase was the main ligninolytic activity detected. This activity was also produced by some of the deuteromycetes strains tested (*Minimidochium parvum* and *Tetraploa aristata*). Laccase is a copper-containing oxidase involved in lignin biodegradation and secreted by most basidiomycetes [10,21,23]. However, this enzyme also participates in other physiological processes, as conidial pigmentation, morphogenesis, pathogenesis and detoxification of phenolic compounds, and it has also been characterized from different ascomycetes and deuteromycetes [39]. Recently, the participation of this enzyme in oxygen activation during the oxidation of hydroquinones [40] and its synergistic action with AAO to produce hydroxyl radical [41], a low molecular weight compound involved in the initial attack to lignocellulose when ligninolytic enzymes can not penetrate plant cell walls [42], has been reported. On the other hand, the addition of aromatic compounds to the fungal cultures may induce the expression of different laccase isoforms [43,44]. In this work, the ligninolytic enzymatic activities have been studied in a carbon-limited medium, in absence of inducers, suggesting that the ligninolytic enzymes reported here could be constitutive.

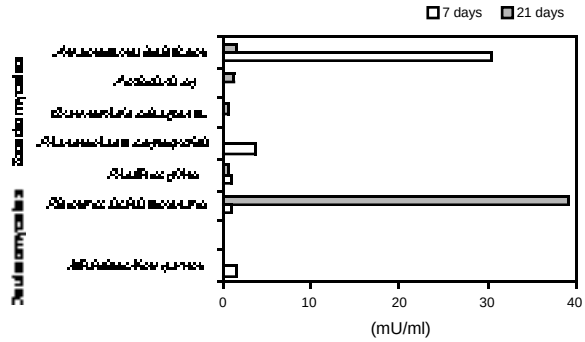

Figure 1. AAO activities in the culture fluid of different fungi grown in the modified Czapek Dox medium (mean values).

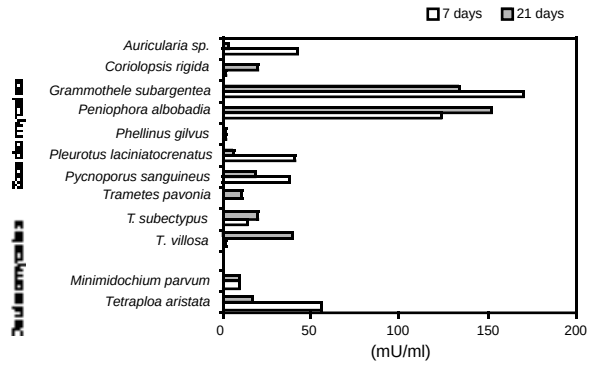

Figure 2. Laccase activities in the culture fluid of different fungi grown in the modified Czapek Dox medium (mean values).

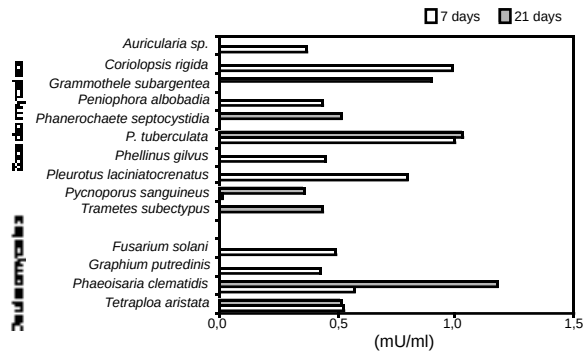

Figure 3. MnP activities in the culture fluid of different fungi grown in the modified Czapek Dox medium (mean values).

Laccase production, induced by xylidine, has been already reported in *Pycnoporus sanguineus* [45]. However, it is necessary to mention that some fungi, as *Cladosporium cladosporioides* and *Penicillium chrysogenum*, only secrete laccase in presence of inducers such as lignin derived compounds, humic acids or xylidine [46-47].

As laccase, MnP production was detected in numerous strains studied (Figure 3). However, the MnP levels were comparatively lower. These results agree with those reported from other fungi using phenol red as substrate [23,26]. This study is the first report on the production of peroxidases related to ligninolytic systems by fungal species belonging to different taxonomic and ecophysiological groups. Except *Auricularia* sp. [48], *Fusarium solani* [26] and *Pycnoporus sanguineus* [49], the fungi listed in figure 3 are reported for the first time as producers of extracellular MnP. This enzyme could play an important role in lignin biodegradation in fungi lacking LiP activity. *Pleurotus* species can mineralize lignin from ^{14}C-wheat straw and a manganese oxidizing peroxidase is involved in the process [50].

Most basidiomycetes with ligninolytic enzymatic activity secrete laccase and MnP simultaneously. However, the production of AAO, laccase and MnP has been detected only in *Auricularia* sp., *Grammothele subargentea*, *Phellinus gilvus* and *Pleurotus laciniatocrenatus*. In deuteromycetes with enzymatic activity, MnP has been the enzyme found in most species, although laccase and AAO are also secreted by some fungi.

Basidiomycetes, which produce ligninolytic enzymes, are associated with wood rots and lignin degradation [10]. However, the presence of laccase and peroxidase activities in *Minimidochium parvum* and *Tetraploa aristata*, deuteromycetes isolated from soil and floating litter in water polluted with crude-oil from Argentina, could be involved in the degradation of xenobiotics and detoxification of polluted systems since both enzymes are involved in the biodegradation of recalcitrant aromatic compounds [6-7,51].

The authors wish to thank Lic. Bucsinszky, A.M.M. (CONICET technician) who is in charge of the culture collection at the Spegazzini Institute, and Lic. Tournier, H.A. (Fac. Ciencias Médicas UNLP) for their excellent technical assistance. This chapter has benefited from the constructive criticism of anonymous reviewers. Saparrat, M.C.N. is a recipient of a scholarship from CONICET. Cabello, M.N. is a researcher from CIC. Arambarri, A.M. is a researcher from CONICET. This research was supported by Grants from CONICET and CIC, Argentina.

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