CHARACTERIZATION OF THE OXIDATIVE ENZYME POTENTIAL IN WILD WHITE ROT FUNGI FROM THE SUBTROPICAL FOREST OF MISIONES (ARGENTINA)

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ABSTRACT

This research aimed to evaluate the potential of several native white rot fungi (WRF) isolated from subtropical environments of Misiones (Argentina) to produce different ligninolytic enzymes. *Coriolus versicolor* f. *antarcticus* BAFC 266, *Pycnoporus sanguineus* BAFC 2126 and *Phlebia brevispora* BAFC 633 showed the highest phenoloxidase activity. *Ganoderma applanatum* strain E, *P. sanguineus* BAFC 2126 and *P. brevispora* BAFC 633 revealed marked laccase and peroxidase activity. *C*. *versicolor* f. *antarcticus, G. applanatum* (strain A) and *Trametes villosa*, gave high positive reactions with 2,6-dimethoxyphenol oxidation at the lowest tested pH. *C. versicolor* f. *antarcticus*, *G. applanatum* strains D and F, *T. elegans* BAFC 2127and *T. villosa*, showed the highest manganese peroxidase activity. *C*. *versicolor* f. *antarcticus* also produced the highest lignin peroxidase activity. Tyrosinase activity was mostly evident in *G. applanatum* strains (D and F) and *Phanerochaete chrysosporium* HHB 11741. Kraft liquor decolorization results were variable and depended on the fungus and the liquor concentration. Some fungi with moderate ligninolytic activity showed high decolorization rates (e.g. *Pleurotus sajor-caju* and *Steccherinium* sp. BAFC 1171) indicating the significance of additional approach to evaluate a potential biotechnological application.

Keywords: oxidative enzymes, screening, white-rot fungi.

INTRODUCTION

The major component of biomass is lignocellulose and it represents the most abundant renewable organic resource in soil. A large amount of cellulose, hemicellulose and lignin by-products from agriculture and forestry are usually wasted (Sánchez, 2009). Lignocellulosic plant material can be efficiently decomposed by wood-decaying filamentous fungi (Basidiomycota and Ascomycota), which are indispensable for the Earth's carbon cycle, generation of soil humic matter and formation of soil fine structure. These fungi can be frequently found in Nature forming brackets, caps or resupinaceous (corticioid) fruiting bodies on wood, thus facilitating their sexual basidiospores dissemination (Lundell *et al*., 2010).

The ability to break down lignin, the most recalcitrant component of cell walls, to CO_2 has become an essential role in the carbon cycle played by a small group of basidiomycetes commonly called white-rot fungi (WRF). This potential, mainly based on their strong oxidative activity and low substrate specificity of their ligninolytic enzymes, has been also proposed for the degradation of a variety of persistent environmental pollutants (Tekere *et al*., 2001a; Levin *et al*., 2004). As a secondary benefit, promising WRF may be used not only to alleviate environmental deterioration derived from lignocellulosic

residue generation, but also to promote its bioconversion to different added-value products (Millati *et al.*, 2011).

The enzyme production of phenol-type oxidases differentiates WRF behavior from other wood-rotting basidiomycetes and different wood decomposers (Cañas and Camarero, 2010).

Extracellular enzymatic systems of WRF display differential features depending on the species, strains and culture conditions. Nevertheless, they usually involve enzymes such as lignin peroxidase (LiP, EC 1.11.1.14), able to directly oxidize non-phenolic units, whilst manganese peroxidase (MnP, EC 1.11.1.13) and laccase (Lac, EC 1.10.3.2) preferentially oxidize phenolic compounds, although non-phenolic units may eventually be degraded in the presence of mediators (Saparrat *et al*., 2002; Lundell *et al*., 2010).

Biotechnological applications developed in the postgenomic era are associated with the use of isoenzymes (or the genes involved) with different physicochemical properties such as *pI*, optimal pH, response to inducers and repressors, etc. These particular features make them interesting to be used in "tailor made processes". On the other hand, there is some evidence that same species from different habitats may show different genomic and proteomic features that may be exploited for relevant biotechnological applications (Tekere *et al*., 2001b; Fonseca *et al*., 2010).

Exploration and description of new microorganisms from rich and vast biodiversity of poorly explored natural environments can reveal innovative capacities for potential biotechnological applications such as bioremediation and biomass conversion, areas where WRF play an essential role (Saparrat *et al*., 2002; Levin *et al*., 2004; Sánchez, 2009; Giorgio *et al*., 2012). Moreover, the relevance of screening new isolates in addition to culture collection strains has been already highlighted (Tekere *et al*., 2001a).

The aim of this study was to disclose qualitative differences in the production of oxidative enzymes in subtropical WRF native from Misiones (Northeast, Argentina) and to categorize most promising strains for future biotechnological applications on biomass conversion.

MATERIAL AND METHODS

Microorganisms

This work includes strains from the United States, such as *Phanerochaete* and *Ceriporiopsis* of recognized efficiency on biomass conversion such as biopulping (Blanchette and Burnes 1988; Akhtar *et al*., 1993; Ferraz *et al*., 2003; Villalba *et al*., 2006), in order to compare them with the native strains in the Misiones province.

The fungal strains used in the present work were previously isolated from the subtropical rainforest of Misiones (Argentina) and properly deposited in public culture collections. *Ganoderma applanatum* (strains A, B, C, D, E, F) and *Pleurotus sajor-caju* were deposited at the Culture Collection of the Faculty of Forestry, Universidad Nacional de Misiones, Argentina. *Coriolus versicolor* f. *antarcticus* BAFC 266, *Phlebia brevispora* BAFC 633, *Pycnoporus sanguineus* BAFC 2126, *Trametes elegans* BAFC 2127, *Trametes villosa* BAFC 2755 and *Steccherinium* sp. BAFC 1171 were deposited at the Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, Universidad de Buenos Aires, Argentina.

On the other hand, fungi used for comparison purposes such as *Ceriporiosis subvermispora* FP 90031, *Phanerochaete chrysosporium* ME 446, *P. chrysosporium* BKMF

1767, *Ceriporiosis subvermispora* L 6332, *P. chrysosporium* HHB 11741 and *C. subvermispora* FP 105752 were provided by the Center for Forest Mycology Research, Forest Products Laboratory, USDA, USA.

Culture conditions

Stock cultures of fungal strains were maintained by periodic sub-culturing on malt extract agar plates containing 12.7 g/l malt extract and 20 g/l agar (MEA) incubated for seven days at 28°C. Sub-cultures were carried out every 30 days followed by storage at 4°C. Inocula consisted in 0.5-mm^2agar plugs covered with mycelium from five-seven-day-old colonies previously grown at 28°C on MEA plates. Mycelium plugs were aseptically cut from actively growing zones and then, transferred to the corresponding plates for screening.

Tests were performed in triplicate and repeated when the target reaction was not induced.

Enzyme detection on solid media

Enzymes are assumed as extracellular form if they diffuse widely into the agar which was not yet overgrown (Gramss *et al*., 1998).

Phenoloxidase activity (PhOX) is noticed by the agar color change of samples grown on solid MEA supplemented with 0.5% w/v tannic acid (Bavendamm 1928, Serrano Silva *et al*. 2010). After seven days of incubation at 28°C, a dark brown color around the mycelium is considered a positive result (Rayner and Boddy, 1988).

Peroxidase (POX) activity was also revealed by pouring equal parts of 0.4% v/v H_2O_2 and 1 % w/v pyrogallol on MEA growth plates. A yellowish-brown color in the solid media indicates a positive result. POX is rated positive when staining with H_2O_2 is clearly browner than those without H_2O_2. The high rate of applied H_2O_2 ensured the partial inhibition of most laccase activities, as previously recommended (Gramss *et al*., 1998).

To reveal laccase activity (Lac), fungi were grown at 28°C on plates with MEA medium supplemented with the textile dye Vilmafix® Blue RR-BB dye (0.2 g/l) (Pajot *et al*., 2007). After complete decolorization (five days), the plate was covered with 1.2 mM ABTS [2,2'-azino-bis(3ethylbenzothiazoline-6-sulphonic acid)] in acetate buffer pH 4.5 and incubated in the dark for 20 min. The appearance of green color in the solid media indicates a positive result. A solution containing 0.7 mM H_2O_2 was subsequently added to the previous plate in order to reveal peroxidase activity (POX). The intensification of green color is considered a positive reaction (Murugesan *et al*., 2007).

Another useful technique for Lac detection was used as follows. Fungi were grown for five days at 28#ºC on MEA plates. Lac activity was evidenced by adding 12.4 g/l guaiacol in 96% v/v ethanol. The appearance of an orange coloration after 4 h is considered a positive result (López *et al*., 2006).

A third test carried out to detect Lac activity was performed by inoculating fungal strains on MEA plates at pH 4, 5 or 6, containing 0.8 g/l 2,6-dimethoxyphenol (DMP), as an adaptation of previous protocols for Lac evaluation in liquid media (Park *et al*., 2008; Flores *et al*., 2009). Dark orange oxidation zones were daily measured during 5 days.

To detect manganese peroxidase activity (MnP) fungi were grown on MEA plates for five days at 28°C. Plates were developed by the addition of 0.03 mM N,N,N',N'tetramethyl-p-phenylenediamine (TMPD), 1 mM H_2O_2, 0.7 mM $MnSO_4 \cdot 7H_2O$, in citrate-phosphate buffer

pH 5.0. Enzyme activity is evidenced by the occurrence of a purple color on the agar plates (Toh *et al*., 2003).

For lignin peroxidase activity (LiP), WRF were grown on MEA for five days at 28°C and plates were then revealed with 32 mM Azure II in tartrate buffer pH 4.5 plus 0.1 mM H_2O_2 (Archibald, 1992; Zhao *et al*., 1996). The discoloration after ten min is considered a positive result.

Tyrosinase (Tyr) activity was assessed according to a modified method by Rayner and Boddy (1988). After fungal growth on MEA for five days at 28°C, Tyr was revealed with 1 % w/v pyrocatechol in phosphate buffer pH 7.4. A brownish pink color on the agar plates after ten min is considered positive.

Black liquor decolorization assays on solid medium

In order to test the fungal ability for decolorization usually associated with ligninolytic activity, fungi were inoculated onto plates containing a culture medium supplemented with two different concentrations of Kraft liquor effluent.

Lignin degradation was assayed on plates containing 10 g/l glucose malt agar supplemented with either 3.3 or 6.6 % v/v black liquor. Black liquor, a lignin-rich effluent of the Kraft cooking process, was obtained at laboratory scale and consisted mainly of remaining substances after dissolving wood components in the cooking chemicals. Degradation was followed by measuring changes from 3rd to 14th culture day (Fonseca *et al*., 2010).

RESULTS
Enzyme detection on solid media

Many microorganisms producing ligninolytic enzymes have been selected using solid media containing substrates or dye indicators that allow direct visualization of enzyme production (Nishida *et al*., 1988, De Jong *et al*., 1992; Barbosa *et al*., 1996).

Data corresponding to ligninolytic activities detection on solid media for all tested fungal strains are presented in Table 1 and are representative of three independent experiments. Screening criteria for ligninolytic enzyme activities on solid media (MEA) with different indicators are shown in Fig. 1.

Table 1. Screening for oxidative enzyme activities of white-rot fungi on solid medium (MEA)

Fungal strain	PhOX (tannic acid)	Lac (ABTS)	POX (ABTS/ H_2O_2)	Lac (guaiacol)	POX (pyrogallol/ H_2O_2)	MnP (TMPD/ Mn^{2+}/H_2O_2)	LiP (Azure II/ H_2O_2)	Tyr (pyrocathecol)
C. subvermispora FP 105752	++ NG	++	++	+++	++	-	+	V
C. subvermispora FP 90031	++ NG	++	++	+++++	+++	++	++	V
C. subvermispora L 6332	– NG	-	-	+	-	-	+	+
C. versicolor f. antarcticus BAFC 266	+++ G	++	++	++++	++	+++	+++	+
G. applanatum (strain A)	++ NG	++	++	++++	++	+	++	+
G. applanatum (strain B)	++ NG	+	+	-	++	+	+	-
G. applanatum (strain C)	++ NG	+	+	++	++	+	+	-
G. applanatum (strain D)	++ G	+	++	-	++	+++	++	++
G. applanatum (strain E)	++ NG	+++	+++	-	++	+	+	-
G. applanatum (strain F)	++ G	+	++	-	++	+++	++	++
P. brevispora BAFC 633	+++ NG	+++	+++	+++	++	+	+	V
P. chrysosporium BKM-F 1767	– NG	-	+	+	-	-	++	V
P. chrysosporium HHB 11741	– NG	-	-	-	+	-	+	+++
P. chrysosporium ME 446	– NG	-	-	-	-	-	+	+
P. sajor-caju	– NG	++	++	++++	++	+	+	-
P. sanguineus BAFC 2126	+++ G	+++	+++	+++	++	++	+	V
Steccherinium sp. BAFC 1171	++ G	++	++	-	++	-	+	-
T. elegans BAFC 2127	++ G	+	++	++	+++	+++	++	+
T. villosa BAFC 2755	++ NG	++	+++	++++	++	+++	++	V

Note: number of crosses (+ to +++++) indicates positive reaction intensity (weak to very strong); (-) implies no detectable reaction; (G) denotes unrestricted growth; (V) indicates variable result; (NG) indicates growth inhibition. Shading indicates the fungi native of the Province of Misiones.

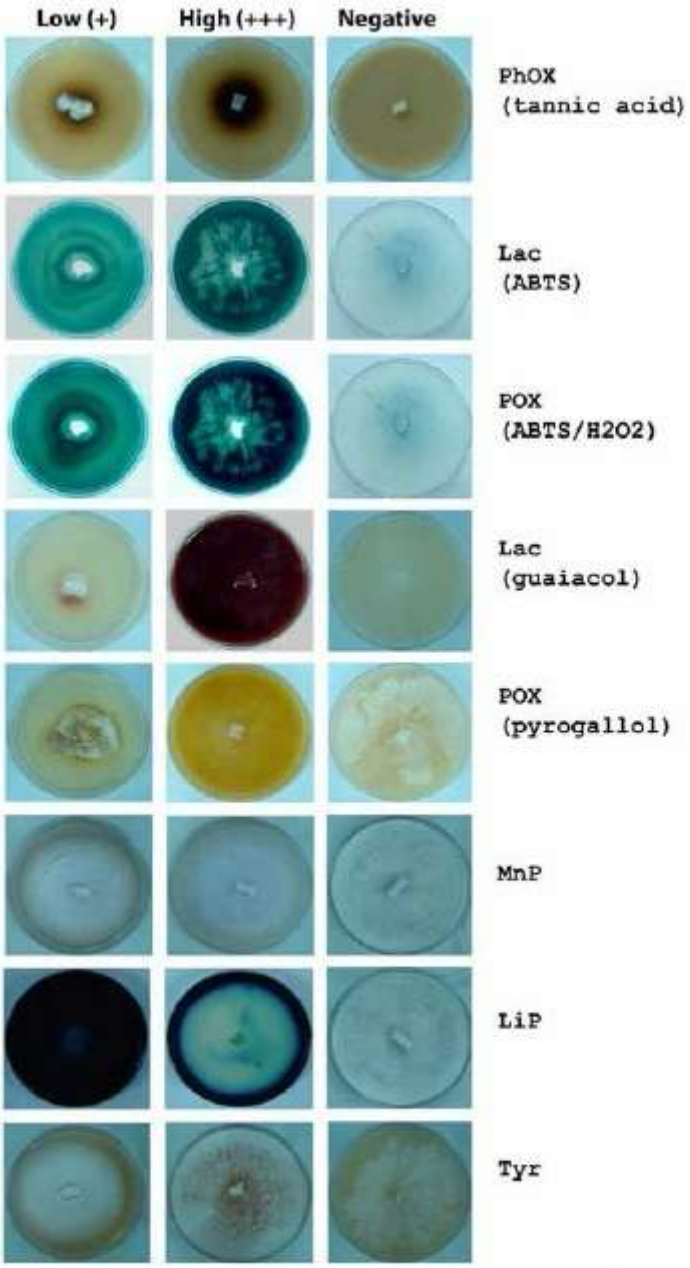

Figure 1. Screening criteria for the detection of ligninolytic enzyme activities on solid media (MEA) with different indicators. Columns depict: left, low levels (+); centre, high levels (+++); right, negative reaction.

Malt agar medium supplemented with tannic acid is frequently used to detect the presence of extracellular oxidases which are generally characteristic of ligninolytic activity (Bavendamm, 1928; Morozova *et al*. 2007; Herter *et al*. 2012). Many of the tested fungi showed activity when grown in tannic acid-amended medium (Table 1). *C. versicolor* f. *antarcticus* BAFC 266, *P. sanguineus* BAFC 2126 and *P. brevispora* BAFC 633 (with no evident growth) showed the maximal oxidation of tannic acid. On the other hand, *G. applanatum* (strains D and F), *Steccherinium* sp. BAFC 1171 and *T. elegans* BAFC 2127 were able to grow in this medium with moderate PhOX activity. Conversely, *C. subvermispora* FP 90031 and *C. subvermispora* FP 105752, *G. applanatum* strains A, B, C, E, and *T. villosa* BAFC 2755, showed PhOX release under these conditions but were unable to grow, a fact probably related to the high tannic acid concentration applied (Gramss *et al*., 1998).

In the present work, textile dye Vilmafix® Blue RR-BB was totally decolorized after five days of cultivation with *C. versicolor* f. *antarcticus* BAFC 266, *P. brevispora* BAFC 633 and *C. subvermispora* FP 105752. Fungi *G. applanatum* strain E, *P. sanguineus* BAFC 2126 and *P. brevispora* BAFC 633 revealed high Lac activity when confronted to ABTS and the blue-green intensity of haloes was increased after addition of H_2O_2 due the presence of POX (Table 1). Other fungi like *C. subvermispora* FP 105752, *C. subvermispora* FP 90031, *C. versicolor* f. *antarcticus* BAFC 266*, G. applanatum* cepa A, *P. sajor-caju* and *Steccherinium sp.* BAFC 1171 showed moderate Lac and POX activity. *T. villosa* BAFC 2755 showed moderate Lac activity while showed high POX activity (Table 1). Additionally *T. elegans* 2127 and *G. applanatum* (strain D and F) showed low activity Lac and revealed POX activity more intense (Table 1). *C. subvermispora* L6332, *P. chrysosporium* ME 446, *P. chrysosporium* HHB 11741 did not show ABTS oxidation (Table 1.). In the results analysis, it was important to consider the degree of discoloration achieved during growth, prior to the addition of ABTS ± H_2O_2, since it could be interpreted as false positives; therefore discoloration plates were taken as positive controls to determine the Lac and POX real activities during development.

The detection of POX with pyrogallol plus H_2O_2 appeared a quite sensitive technique and showed activity in most of the tested fungi with the exception of *P. chrysosporium* ME 466, *C. subvermispora* L 6332 and *P. chrysosporium* BKMF 1767, latter two confirmed previous results with ABTS/ H_2O_2 (Table 1). The wide distribution of POX reaction found virtually in all tested fungi, particularly when assayed by pyrogallol/ H_2O_2 (Table 1), has been also documented for different ecological groups of fungi (Gramss *et al*., 1998). The extracellular nature of detected enzymes was also herein observed. The role of POXs could be essential for wood degradation, as already emphasized for the degradation of litter (Gramss *et al*., 1998).

When the guaiacol modified technique was applied, most of the above results for Lac activity could be confirmed, being *C. subvermispora* FP 90031 the highest positive (Table 1). Meanwhile, *P. brevispora* BAFC633, *G. applanatum* (strains A and C), *P. sanguineus* BAFC 2126, *T. villosa* BAFC 2755, *T. elegans* BAFC 2127, *C. versicolor* f. *antarcticus* BAFC 266, *P. sajor-caju* and *C. subvermispora* FP 105752 showed qualitatively lower guaiacol oxidation. Negatives as those of *G. applanatum* (strain B, D, E and F), *P. chrysosporium* HHB 11741, *P. chrysosporium* ME 466 and *Steccherinium* sp. BAFC 1171 might be related to the inability of some laccases to oxidize certain substrates depending on their redox potential (Pajot *et al*., 2007). The presence of Lac as one of the main ligninolytic activities was not unexpected, in agreement with previous screening reports on native argentinian fungi (Saparrat *et al*., 2002).

Many authors have used DMP for laccase quantification (Park y Park, 2008; Flores *et al*., 2009). In this paper, the method was applied to solid medium. In agreement with ABTS and guaiacol data (Table 1), *C. versicolor* f. *antarcticus* BAFC 266, *P. brevispora* BAFC 633, *C. subvermispora* FP 105752, *T. villosa* BAFC 2755, and *C. subvermispora* FP 90031 gave highly positive results for DMP oxidation at 4 and 5 pH values (Table 2). On the other hand, marked Lac activities for *G. applanatum* strain A, *P. sajor-caju* and *P. sanguineus* BAFC 2126 were also coincident with results from ABTS and guaiacol tests (Table 1), but in the case of *Steccherinium* sp. BAFC 1171, negative results from DMP (Table 2) showed more correlation with guaiacol oxidation (Table 1).

Table 2. Comparative growth and oxidation ability of tested fungi on MEA/DMP plates at different pH.

Fungi	DMP Growth			DMP Oxidation		
	pH 4	pH 5	pH 6	pH 4	pH 5	pH 6
C. subvermispora FP 105752	+++	++	-	+++	++	-
C. subvermispora FP 90031	++	++	-	+++	+++	-
C. subvermispora L 6332	-	-	-	-	-	-
C. versicolor f. antarcticus BAFC 266	+++ [a]	++	-	++++ [a]	+++	-
G. applanatum (strain A)	++++	++	-	++++	+++	-
G. applanatum (strain B)	-	++	-	-	++	-
G. applanatum (strain C)	+	+++	+	++	+++	-
G. applanatum (strain D)	-	-	+	-	-	-
G. applanatum (strain E)	-	+	-	-	++	-
G. applanatum (strain F)	- [a]	-	-	- [a]	-	-
P. brevispora. BAFC 633	++ [a]	++	-	+++ [a]	++	-
P. chrysosporium BKMF 1767	-	-	-	-	-	-
P. chrysosporium HHB 11741	-	-	-	-	-	-
P. chrysosporium ME 446	-	-	-	-	-	-
P. sajor-caju	+++	-	+++	+++	-	++++
P. sanguineus BAFC 2126	+ [a]	++	-	++ [a]	+++	-
Steccherinium sp. BAFC 1171	-	-	+	-	-	-
T. elegans BAFC 2127	-	-	+	-	-	-
T. villosa BAFC 2755	+++	+	-	++++	++	-

Note: number of crosses (+ to ++++) indicates positive reaction intensity (weak to very strong); (-) implies no detectable reaction.
[a] Data from Fonseca *et al*., (2010).

Concerning MnP, *G. applanatum* (strains D and F), *T. elegans* BAFC 2127, *T. villosa* BAFC 2755 and *C. versicolor* f. *antarcticus* BAFC 266 showed the highest MnP activity. Likewise, moderate activities were found in *C. subvermispora* FP 90031 and *P. sanguineus* BAFC 2126 (Table 1).

The highest LiP activity was found in *C. versicolor* f. *antarcticus* BAFC 266, followed by *G. applanatum* (strains A, D and F), *T. elegans* BAFC 2127, *T. villosa* BAFC 2755 *C. subvermispora* FP 90031 and *P. chrysosporium* BKM-F 1767, as indicated by the marked degradation of Azure II (Table 1). These findings resulted particularly relevant considering a previous screening on the ligninolytic potential of other argentinian fungi where LiP activity could not be detected (Saparrat *et al*., 2002). However, they showed correlation with positive results from Zhao *et al*. (1996) and Levin *et al*. (2004). As these authors already found, the herein applied LiP technique was highly successful to this purpose.

Considering LiP positive results on *P. chrysosporium* strains (Table 1), it would interesting to note that differences on the detection methodology, as well as the influence of culture medium and/or conditions should not be neglected at the time of evaluating LiP production (Zhao *et al*., 1996; Tekere *et al*., 2001a; Saparrat *et al*., 2002; Levin *et al*., 2004).

With regard to Tyr activity, it was distinctly detected in *P. chrysosporium* HHB 11741, and to a lesser extent, in *G. applanatum* (strains D and F) (Table 1).

Black liquor decolorization assays on solid medium

Approximately half of the fungi tested showed discoloration at both concentrations tested. In many cases, reasonably, the discoloration was lower at the highest concentration of black liquor tested. *P. sajor-caju* showed the highest decolorization rates at both concentrations used, followed by *Steccherinium* sp. BAFC 1171, *T. elegans* BAFC 2127*, G. applanatum* strain A and *P. sanguineus* BAFC 2126. Other fungi showed very good discoloration, but mostly at the lowest concentration of black liquor, such as *G. applanatum* strain D and *C. versicolor* f. *antarcticus* BAFC 266. Meanwhile, *P. brevispora* BAFC 633 was able to completely decolorize the black liquor only at a concentration of 3.3 % (v/v), and 6 of the 17 fungi tested showed no discoloration at any of the concentrations tested, especially for strains of *P. chrysosporium* and *C. subvermispora*.

The demonstrated Kraft liquor degradation capability achieved by some tested fungi along 14 days of cultivation thus suggested their potential use for bioremediation of pulping industry effluents (Fonseca et al., 2010). The reduced peak at 280 nm of the spectral scanning performed in broth of *P. brevispora* BAFC 633 supplemented with black liquor indicated fungal ligninolytic activity (Shimizu et al., 2009). The intensity of the absorbance is related to the lignin concentration, being significantly lower for the fungus treated samples.

The apparent lack of correlation between ligninolytic enzyme profile and Kraft liquor discoloration was not completely unusual. Previous reports on dye decolorization by WRF also described similar results frequently attributed to other degradative mechanisms rather than ligninolytic system (Tekere *et al*., 2001a; Levin *et al*., 2004).

The herein described screening tests allowed to reveal a hidden and up-to-date unexplored potential of wild fungi isolated from subtropical environments, which may behave as competent ligninolytic enzyme-producers, such as *C. versicolor* f. *antarcticus* BAFC 266, *G. applanatum* strains A, D and F, *P. sanguineus* BAFC 2126, *P. brevispora* BAFC 633, *T. elegans* BAFC 2127 and *T. villosa* BAFC 2755. The ligninolytic enzyme machinery of these fungi might be considered for the residual lignin oxidation during biomass conversion, representing a promising and low cost bioremediation and bioconversion alternative (Fonseca *et al*., 2010; Giorgio *et al*., 2012).

DISCUSSION

As already described for different ecological groups such as litter decomposing, wood degrading and ectomycorrhizal fungi (Sinsabaugh *et al.,* 2002), PhOX activity seemed to be common in the herein tested fungi.

Unsurprisingly, only some fungi are able to grow in tannic acid-containing medium because tannins are usually toxic to microorganisms, mainly due to enzymatic inhibition, substrate deficiency, action on membranes and lack of metal ions (Ngono Ngane *et al*., 2006). Nevertheless, some fungi, bacteria and yeasts are quite resistant and capable of growing in the presence of tannins making its use justifiable to the screening purpose under stringent conditions (Bhat *et al*., 2007). Eventually, as previously proposed, the use of lower (1/10) tannic acid concentrations was proved to still induce browning in the presence of oxidative activities without inhibiting fungal growth (Gramss *et al*., 1998).

Dye decolorization potential of different fungi has been extensively studied and associated to lignin degrading ability (Kersten y Cullen, 2007). It has been already documented that laccase enzyme of white rot fungi such as *T. versicolor* 951022 showed affinity and specific activity for ABTS (Moon-Jeong *et al*., 2005).

The differences observed with the three substrates used for Lac screening could be attributed at different positions of chemicals groups and different affinities of Lacs for each compound (Min *et al*., 2001).

Preceding results showed the relevance of investigating these ligninolytic activities according to different protocols and involving varied substrates in order to achieve more conclusive results. Additionally, data from DMP oxidation also gave evidence of the importance of pH at the time of screening these activities, being the highest values generally obtained at pH 4-5 (Table 2). These findings were in agreement with those from Tekere *et al*. (2001a), who also noted a preference for acidic pHs (< 4.5) for dye degradation by ligninolytic fungi.

It should be however noted that, as previously emphasized, if detected ligninolytic activities were constitutive, titers may be subjected to the influence of inducers such as aromatic compounds or Cu^{2+} (Saparrat *et al*., 2002; Dhouib *et al*., 2005). These results were in agreement with others previously reported by Tekere *et al*. (2001a), Saparrat *et al*. (2002), Matos *et al*. (2007), Lin *et al*. (2003) and Basto *et al*. (2006) who also described high ligninolytic activities in closely related fungal specimens such as *T. villosa*, *C. versicolor*, *G. applanatum* and *P. sanguineus*, consequently proposed for different biotechnological applications.

Different authors have earlier reported high MnP activity in other *Trametes* and *Ganoderma* species, e.g. in *T. versicolor* when grown with high carbon and nitrogen sources (Tekere *et al*., 2001b), and in *G. lucidum* IBL-05 as the only secreted enzyme (Bibi *et al*., 2009). The simultaneous presence of MnP and Lac has been already described as a typical feature in basidiomycetes (Tekere *et al*., 2001a; Saparrat *et al*., 2002).

The simultaneous secretion of LiP and MnP in the studied fungi may represent a valuable potential as this enzyme association has been previously related to efficient degraders (Levin *et al*., 2004).

Compared to other work on Tyr secretion, different chromogenic phenolic solutions were used for its detection, and these results were somehow divergent to the wide distribution of Tyr previously found in different ecological groups by Gramss *et al*. (1998). The extracellular nature of the Tyr activities described in the present work was also different with respect to previous reports on intracellular Tyr (Gramss *et al*., 1998), and this property was herein additionally confirmed since no ethanol was applied to dissolve the indicator substance.

Considering the oxidative strains commonly used in USA in biopulping experiments, we observed that in general strains of local origin showed higher oxidative potential that *Phanerochaete* strains and similar to *Ceriporiopsis* strains. These finding described ligninolytic activities in isolates from the subtropical rainforest of Misiones are not unexpected. Previous reports on similar screening campaigns already drawn the attention to the highly adaptable physiology and heterogeneous enzyme systems of WRF isolated from harsh subtropical environments where a wide host range and tolerance are usually observed (Tekere *et al*., 2001*a*).

The secreted oxidative enzymes may alleviate the environmental impact of industrial processes and chemical manufacturing during biomass conversion, among other applications (Sánchez, 2009). Special interest is based on their undeniable advantages and promising application in specific bioprocesses such as pre-treatment of lignocellulosic materials for bioethanol production, biosensors, bioremediation (Levin *et al*., 2004) and several processing steps related to the paper industry such as pulping (Villalba *et al*. 2006), bleaching, deinking, depitching and effluent treatment.

Accordingly, next steps will pursue the knowledge of the molecular basis for the production of these biotechnology relevant enzymes, considering their prospective commercial insertion.

At the same time, this work allowed to disclose certain differences in the enzymatic profiles of fungi belonging to the same genus and/or species. This fact highlighted once again, the relevance of evaluating the enzymatic potential of fungal isolates for each particular case, as a desired activity may not be always associated to a given microbial identity. This study also denoted that different methodologies for enzyme assessment would be not excluding but complementary, and may successfully help at the time of selecting fungal strains from nature in order to be applied with specific biotechnological purposes.

CONCLUSIONS

The screening tests showed that our subtropical environments yield effective ligninolytic enzymes producers such as *C. versicolor* f. *antarcticus* BAFC 266, *G. applanatum* strain F, *Peniophora sp*. BAFC 633, *P. sanguineus* BAFC 2126 and *T. villosa* BAFC 2755, that can be used on residual lignin oxidation during biomass conversion.

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