Enzyme and Microbial Technology 46 (2010) 534–539

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Copper inducing effect on laccase production of white rot fungi native from Misiones (Argentina)

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ARTICLE INFO

Article history:
Received 9 December 2009
Accepted 23 December 2009

Keywords:
White rot fungi
Laccase
Copper induction

ABSTRACT

Fungi may be selected as models for gene expression studies and further adaptation for biotechnological enzyme production. The aim of this work was to evaluate laccase production and to analyze the effect of Cu^{2+} on selected fungi natives of Misiones, *Ganoderma applanatum* (strain F), *Peniophora* sp. (BAFC 633), *Pycnoporus sanguineus* (BAFC 2126) and *Coriolus versicolor* f. antarcticus (BAFC 266).

Fungi secretion system of *G. applanatum*, *Peniophora* sp., *P. sanguineus* and *C. versicolor* f. antarcticus is sensitive to stimulation by copper. Biomass values of *G. applanatum*, *Peniophora* sp. and *C. versicolor* f. antarcticus did not show differences between treatments. *P. sanguineus* biomass underwent a dramatic growth inhibition with 1 mM Cu^{2+} and marked delay in growth with 0.5 mM Cu^{2+}. Proteins were increased with copper in *Peniophora* sp., *C. versicolor* and *G. applanatum*. *G. applanatum* and *Peniophora* sp. reached the highest enzyme activity at 10th day equivalent to 49.2-fold and 19.7-fold higher than the control samples, respectively. Copper produced an increase of constitutive laccases in all fungi and an additional inducible isoenzyme in *Peniophora* sp., *C. versicolor* f. antarcticus and *G. applanatum*.

1. Introduction

Conventional systems of pulp production for paper manufacture comprise mainly of mechanical and chemical methods. The drawbacks of these traditional procedures are the high energy consumption and effluent contamination, making thus necessary the development of friendly environmental technologies, such as biopulping [1,2]. White rot fungi (WRF) are the most efficient lignin degrader basidiomycetes capable of wood degradation through ligninolytic enzymes secretion such as laccase [3–5]. In Argentina, several WRF have been described leading to interesting possibilities for biotechnology use [6–8] and accordingly, more studies are needed to deepen in the molecular aspects of enzymes involved.

Laccase (EC 1.10.3.2) secreted by most of the WRF, is a ligninolytic enzyme belonging to the multicopper oxidases family (MOC) [9]. This enzyme is generally extracellular and catalyzes the oxidation of several phenolic compounds, aromatic amines, thiols and some inorganic compounds using molecular oxygen as electron acceptor [10]. The low substrate specificity makes this enzyme interesting for biotechnology purposes in various industries such as pulp and paper and textiles, and bioremediation of industrial pollutants [11]. However, the slow growth and poor enzyme secretion under natural conditions are limitations to the technological use of WRF [12]. In this regard, different strategies such as heterologous enzyme production [13,14] or purification and direct application are currently developed [15,16].

Previous degradation studies in solid culture revealed the occurrence of laccase secreted by WRF native of Argentine [7]. Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{3+} by gene expression induction or through translational or post-translational regulation [17–20]. However there are still relatively little data about the enzyme stimulation and activation mechanisms of WRF native of the province of Misiones.

The aim of this work was to evaluate laccase production and to analyze the effect of Cu^{2+} on selected fungi natives of Misiones, *Ganoderma applanatum* (strain F), *Peniophora* sp. (BAFC 633), *Pycnoporus sanguineus* (BAFC 2126) and *Coriolus versicolor* f. antarcticus (BAFC 266).

2. Materials and methods

2.1. Microorganisms

G. applanatum (strain F) was provided by the Culture Collection of the Faculty of Forestry, National University of Misiones, Argentina. *C. versicolor* f. antarcticus (BAFC 266), *Peniophora* sp. (BAFC 633) and *P. sanguineus* (BAFC 2126) were provided by the

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doi:10.1016/j.enzmictec.2009.12.017

Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina.

2.2. Culture conditions

To prepare the liquid inoculums, 36 mm^2-agar plugs from each fungus grown 5–7 days in malt agar plates (agar 20 g/L, malt extract 12.7 g/L) were cut and then transferred to 250 mL-Erlenmeyer flasks containing 50 mL of medium (12.7 g/L malt extract and 5 g/L corn steep liquor) and incubated at 29 ± 1 °C in static conditions. Stock cultures were maintained on malt agar at 4 °C.

2.3. Oxidation assay on solid culture

Dye oxidation ability was assayed in malt agar supplemented with 5 mM 2,6-dimethoxyphenol (DMP) in sodium acetate buffer 0.1 M (pH 4). A mycelial disc (6 mm) was placed in the middle of the dye-agar plate and incubated at 30 °C in the darkness. Plates were examined every 24 h to monitor growing and color changes. All fungi were tested with DMP in solid cultures for 5 days. Growth and color changes were expressed in cm.

Effluent experiments were performed on plates containing 20 g/L agar and 10 g/L glucose malt agar supplemented with 6.6% or 3.3% black liquor. The effluent was obtained from laboratory scale as black liquor, a by-product of the kraft cooking process, consisting of the remaining substances after wood components have been dissolved in the cooking chemicals. Dephenolization of black liquor was followed by measuring changes every 3 days and expressed in cm.

2.4. Copper addition assays

The copper addition assays were carried out at concentrations of 0.5 mM and 1 mM of $CuSO_4$ in liquid medium and incubated for 5, 7, 10 or 14 days under the conditions previously described. Each experiment included a control without inducer. All experiments were conducted in duplicate.

2.5. Biomass and protein determination

Biomass growth was determined by measuring the mycelium dry weight, while proteins were measured by the Bradford method on the conditioned medium.

Liquid media was separated from the supernatant mycelia by filtering in a Büchner funnel using fiberglass filters (GF/C) and frozen at −20 °C until use. Biomass dry weight was determined by the difference between the fiberglass filters (GF/C) weight before and after filtration through a Büchner funnel and subsequent drying at 80 °C till constant weight.

Protein was determined by micro-test using the Bradford technique (BioRad) following manufacturer's instructions with bovine serum albumin as the standard.

2.6. Enzyme assays

Laccase (EC 1.10.3.2) activity was measured at 30 °C using 5 mM of DMP in sodium acetate buffer 0.1 M (pH 3.6) [21]. The absorbance increase of assay mixture was monitored at 469 nm (E_{469} = 27.5 mM^{-1} cm^{-1}) in a spectrophotometer Shimadzu UV-3600. The enzyme activities were expressed as international units (U), defined as the amount of enzyme needed to produce 1 μmol product/min at 30 °C and reported as L of conditioned medium.

To compare the effect of the inducer addition on the specific laccase activity of the fungi, the specific laccase activation factor, K_{EA}, was defined as the ratio of stimulated specific laccase activity (E_{SA}) related to basal specific laccase activity (E_{BA}). Specific laccase activity was expressed as U g^{-1} biomass.

2.7. Polyacrylamide gel electrophoresis

In order to identify the number of isoenzymes involved in copper induction, the crude enzyme was subjected to polyacrylamide gel electrophoresis on native PAGE (7.5%) and SDS-PAGE (7.5%). After separation of proteins on native PAGE (ND-PAGE) the gel was incubated in 0.1 M sodium acetate buffer containing 5 mM DMP for the laccase activity detection [22]. After 5 min incubation, dye solution was discarded and gel was immediately scanned by Scanner HP Deskjet F300 All-in-One series.

The molecular weight of the isoenzymes related to copper response was evaluated by 7.5% SDS-PAGE and compared to a molecular weight marker (Kaleidoscope, BioRad). The SDS was removed by gel incubation in 50 mM sodium acetate with 0.2% Triton X-100 followed by staining with 5 mM DMP for laccase activity detection [23].

2.8. Statistics analysis

Two-way ANOVA with Bonferroni post-test was performed using GraphPad Prism Program version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

Table 1
Fungus growth and oxidation of DMP in solid media.

Fungi	DMP growth	DMP oxidation
Ganoderma applanatum strain F	0	0
Peniophora sp. BAFC 633	2	3
Pycnoporus sanguineus BAFC 2126	1	2
Coriolus versicolor f. antarticus BAFC 266	3	4

Note: Results are expressed in cm of growth or oxidation after 5 culture days.

3. Results

3.1. Screening white rot fungi for laccase production

Oxidation zones with different intensities were clearly observed in all fungi (Table 1 and Fig. 1).

3.2. Effect of Cu^{2+} addition on mycelia biomass weight and protein secretion

Previous works showed that all fungi tested produce laccase; however this constitutive enzyme production might be changed by the action of metals such as copper. To test this theory, the growth and the amount of secreted protein in liquid medium with and without copper was assessed (Fig. 2).

Biomass values of *G. applanatum*, *Peniophora* sp. and *C. versicolor* f. antarcticus did not show differences between treatments ($p > 0.05$). Nevertheless, *P. sanguineus* at different incubation days showed a marked delay in growth with 0.5 mM $CuSO_4$ ($p < 0.01$) and a dramatic growth inhibition with the addition of 1 mM $CuSO_4$.

G. applanatum showed a significative proteins increase only with 0.5 mM $CuSO_4$ at 10 days culture ($p < 0.05$). Both copper treatments produced a significative proteins increase in *Peniophora* sp. ($p < 0.001$) starting at the 7th culture day and in *C. versicolor* ($p < 0.05$) at the 14th day. *P. sanguineus* did not showed differences with or without copper ($p < 0.05$).

3.3. Effect of Cu^{2+} addition on laccase enzymatic activity and specific laccase activity

Laccase enzymatic activity in conditioned medium with and without copper was quantified and the specific laccase activity was calculated at the day of maximum production (Table 2).

Laccase secretion increased significantly ($p < 0.001$) in cultures supplemented with Cu^{2+} for all fungi analyzed in this work. These increments were dose dependent and significantly higher ($p < 0.001$) with 0.5 mM of $CuSO_4$ for all fungi.

G. applanatum response to stimulation was significantly different from the 7th day ($p < 0.001$) for all treatments, reaching the highest enzyme activity at 10th day (18,830 U g^{-1}), equivalent to 49.2-fold higher than the production without copper (383 U g^{-1}). *Peniophora* sp. achieved a significant enzyme activity increase from 7th culture day ($p < 0.001$) with the highest value at 10th culture day (11,462 U g^{-1}), equivalent to 19.7-fold higher than the production without copper (580 U g^{-1}). *P. sanguineus* showed maximum significant increase ($p < 0.001$) at 14th day (27,132 U g^{-1}) 27.7-fold higher than without copper (978 U g^{-1}). *C. versicolor* f. antarcticus laccase activity at 14th day (13,304 U g^{-1}) was 7.6-fold higher than without copper (1750 U g^{-1}).

3.4. Induction of laccase isoenzymes with Cu^{2+} addition

To verify the possible presence of laccase isoforms and their differential response to copper induction, ND-PAGE and SDS-PAGE analyses with DMP were carried out.

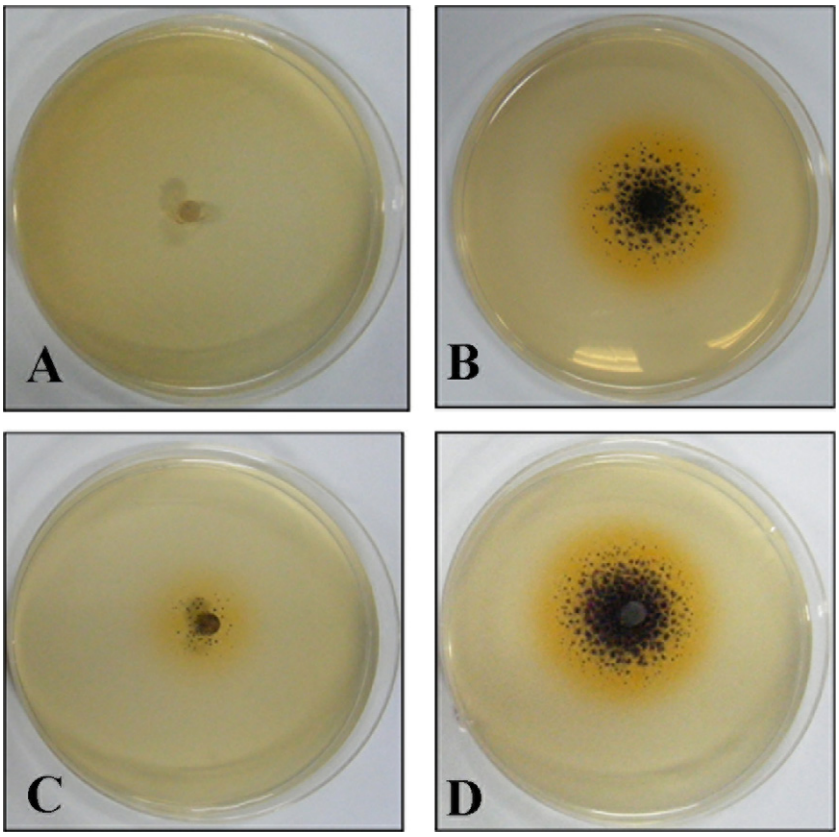

Fig. 1. Fungus growth and oxidation of DMP in solid media. Growth and oxidation zones were evaluated in solid cultures with DMP for 5 days for (A) *Ganoderma applanatum* (strain F), (B) *Peniophora* sp. (BAFC 633), (C) *Pycnoporus sanguineus* (BAFC 2126) and (D) *Coriolus versicolor* f. antarcticus (BAFC 266). Data are representative of two similar experiments.

ND-PAGE showed a clear increment of a laccase band (lower band, Fig. 3), for all fungi at both Cu^{2+} concentrations at 10th and 14th culture day, although bands were also evident in the samples without copper (constitutive isoenzyme).

Our data also revealed the presence of other laccase bands (upper band, Fig. 3) in *Peniophora* sp., *C. versicolor* f. antarcticus and *G. applanatum*. These bands were present only with copper treatment (inducible isoenzyme).

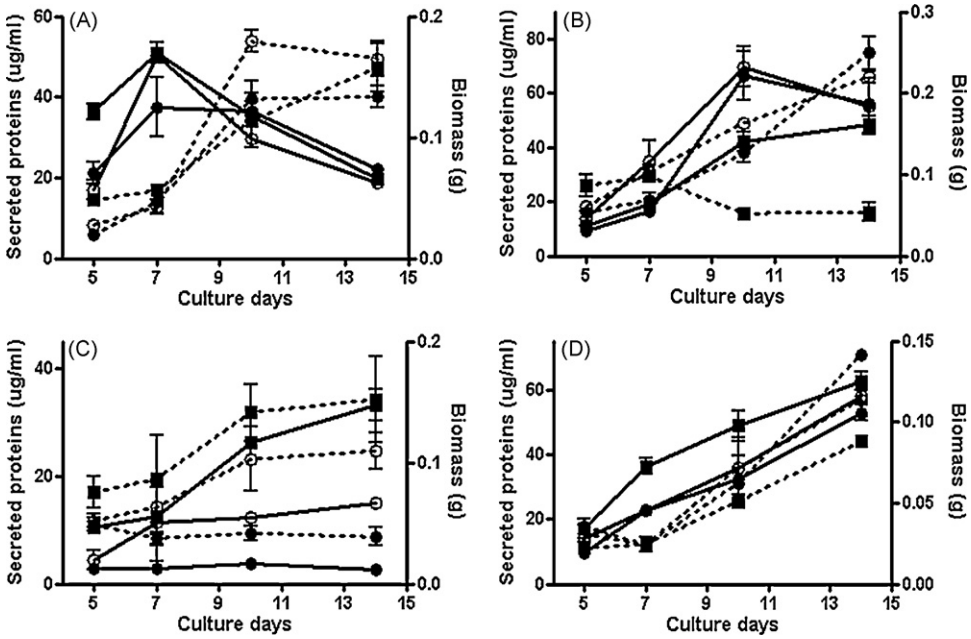

Fig. 2. Effect of Cu^{2+} addition on mycelial biomass and secreted proteins. Mycelial biomass expressed in g (—) and secreted proteins expressed in μg/ml (- - -) were determined from 5, 7, 10 and 14 culture days without (■) or with 0.5 mM (○) or 1 mM (●) of $CuSO_4$ for (A) *Ganoderma applanatum* (strain F), (B) *Peniophora* sp. (BAFC 633), (C) *Pycnoporus sanguineus* (BAFC 2126) and (D) *Coriolus versicolor* f. antarcticus (BAFC 266). Data are presented as the average from duplicate experiments.

Table 2
Specific laccase activity and specific laccase activation factor, K_{EA}.

Fungi	Without copper			With 0.5 mM copper			K_{EA}
	Enzymatic activity (U/L)	Biomass (g)	Specific enzymatic activity (U/g)	Enzymatic activity (U/L)	Biomass (g)	Specific enzymatic activity (U/g)	
Ganoderma applanatum strain F	44.89	0.1173	382.69	1856.61	0.0986	18,829.72	49.20
Peniophora sp. BAFC 633	81.32	0.1402	580.24	4139.55	0.3612	11,462.13	19.75
Pycnoporus sanguineus BAFC 2126	122.53	0.1252	978.67	3143.21	0.1159	27,131.72	27.72
Coriolus versicolor f. antarticus BAFC 266	163.30	0.0934	1749.33	679.82	0.0511	13,303.72	7.61

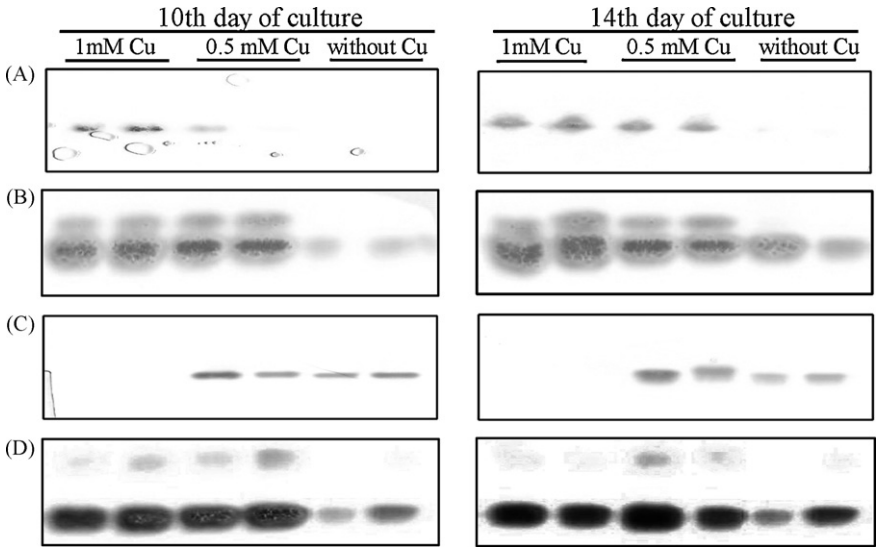

Fig. 3. Laccase activity in culture media on native PAGE. Twenty micrograms of secreted protein from fungi cultured with 0.5 mM, 1 mM or without $CuSO_4$, was analyzed with 7.5% ND-PAGE gels and incubated with DMP. (A) *Ganoderma applanatum* (strain F), (B) *Peniophora* sp. (BAFC 633), (C) *Pycnoporus sanguineus* (BAFC 2126) and (D) *Coriolus versicolor* f. antarcticus (BAFC 266).

To confirm the molecular weight of isoenzymes, SDS-PAGE was carried out for the proteins secreted at the maximum stimulation day (Fig. 4). Molecular weights of laccase isoenzymes from samples with and without copper were estimated to be 62.5 kDa for *G. applanatum*, 60 kDa for both *Peniophora* sp. and *C. versicolor* f. antarcticus, and 54 kDa for *P. sanguineus*. In addition, *Peniophora* sp., *C. versicolor* f. antarcticus and *G. applanatum* showed isoenzyme bands only with copper treatment corresponding to the molecular weight of 75 kDa, 120.5 kDa and 103 kDa, respectively (black arrow in Fig. 4).

3.5. Biotechnology application

To test biotechnology applications, all fungi were inoculated onto plates containing solid media supplemented with black liquor effluent (Table 3). The fact that all fungi caused black liquor dephenolization at 14 days of cultivation indicates potential uses for bioremediation of pulping industries effluents. *Peniophora* sp., *C. versicolor* f. antarcticus and *G. applanatum* turned out to be the most efficient in this screening.

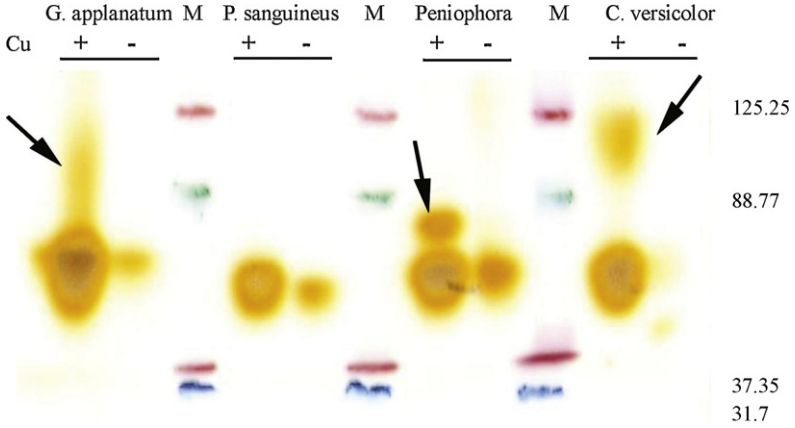

Fig. 4. Estimation of the molecular mass (by SDS-PAGE) of laccase isoenzymes. Twenty micrograms of protein from fungi cultured 10 days with 0.5 mM (+) or without (−) $CuSO_4$ was analyzed with 7.5% SDS-PAGE gels and incubated with DMP. Laccase inducible isoenzymes bands shown with black arrow. (A) *Ganoderma applanatum* (strain F), (B) *Pycnoporus sanguineus* (BAFC 2126), (C) *Peniophora* sp. (BAFC 633) and (D) *Coriolus versicolor* f. antarcticus (BAFC 266).

Table 3
Dephenolization on solid plates with effluent black liquor.

Fungi	3.3% LN	6.6% LN
Ganoderma applanatum strain F	5	4
Peniophora sp. BAFC 633	–	7
Pycnoporus sanguineus BAFC 2126	5	6
Coriolus versicolor f. antarticus BAFC 266	4	7

Note: Results are expressed in cm of dephenolization after 14 culture days.

4. Discussion

The Paranaense forest region (Misiones) is characterized by its biodiversity, and within this environment a large number of microorganisms can be isolated. Enzymes secretion ability is of upmost importance and especially laccase among the oxidative enzymes. In this work, we report four native fungi as outstanding laccase producers. Regarding the effect of the inducer addition on enzyme secretion, we found that constitutively produced laccase was susceptible to treatment in all fungi and inducible laccase isozyme only in selected fungi (*Peniophora* sp., *G. applanatum* and *C. versicolor* f. antarcticus).

In relation to the inducing effect of Cu^{2+} on laccase production, our results were comparable to those described by other authors, who have reported increases of enzyme activity with 1–2 mM Cu^{2+} in *Trametes trogii* [24], *Pleurotus ostreatus* [18,25] and *Trametes pubescens* [26]. Our results indicate that smaller amounts of Cu^{2+} gave better results achieved in shorter times. *G. applanatum* and *Peniophora* sp. reached the highest response on laccase activity. This remarkable effect suggests that these fungi could be a suitable model to study the effect of the inducer in the flow of genetic information.

Laccase expression in white rot fungus *C. versicolor* is regulated by copper and nitrogen at the level of gene transcription [27]. Recently, Alvarez et al. investigated the effect of copper addition on gene expression encoding the ligninolytic enzymes laccase and manganese peroxidase in *Ceriporiopsis subvermispora*. They isolated and characterized an ACE1-like transcription factor from *C. subvermispora* (Cs-ACE1) essential to laccase induction with copper addition. Therefore, further studies are required to determine whether these changes are due to translational and/or transcriptional increase [28].

ND-PAGE/SDS-PAGE and enzyme activity matching results reveal the occurrence of different molecular weight isoenzymes. The increase on enzymatic activity was attributed to an increase of constitutive and inducible enzyme levels. Some authors have reported the existence of laccase isoenzymes in various WRF species with molecular weights ranging from 60 kDa to 80 kDa [29–36]. We report here new laccase data for *Peniophora* sp., *G. applanatum* and *C. versicolor* f. antarcticus. Moreover, we provide evidence on the existence of a copper inducible isoenzyme in these fungi.

WRF were reported to have a great potential for biotechnological applications [37,38] and laccase enzyme is much involved with this effect [39]. Matos et al. demonstrated that *G. applanatum* proved to be effective for the decolorization and dephenolization for removal of both color and phenolic compounds from olive mill wastewaters [40]. Diorio et al. investigated decolorization of malachite green by *C. versicolor* f. antarcticus in a two-phase bioreactor. Our studies indicate interesting dephenolization properties for Kraft liquor in all fungi tested, so laccase contribution to this process should be tested in further biotechnological applications [41].

In conclusion, this work proves that fungi secretion system of *G. applanatum* (strain F), *Peniophora* sp. (BAFC 633), *P. sanguineus* (BAFC 2126) and *C. versicolor* f. antarcticus (BAFC 266) is sensitive to stimulation by Cu^{2+}. *G. applanatum* and *Peniophora* sp. gave the highest response to copper stimulation even at low levels of the inducer and short times. Copper produced an increase of constitutive laccases in all fungi and an additional inducible isoenzyme in *Peniophora* sp., *C. versicolor* f. antarcticus and *G. applanatum*. These are the underlying principles to select fungi as models for gene expression studies and further adaptation for biotechnological enzyme production.

Acknowledgements

The authors thank the Experimental Mycology Department at the University of Buenos Aires and the Culture Collection of the Faculty of Forestry Eldorado of the Universidad Nacional de Misiones for the kind fungi supply.

Part of the experimental work was funded by the Fundación Banco Río and Secretaría de Ciencia y Tecnología de la Universidad Nacional de Misiones, through the grants for innovation projects.

M.I. Fonseca has a fellowship for doctoral studies of CONICET, Argentina. E. Shimizu has completed part this work through a scholarship from the Comité Ejecutivo de Desarrollo e Innovación Productiva (CEDIT) from Misiones, Argentina.

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