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Process Biochemistry 43 (2008) 368–375

Process Biochemistry

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Ligninolytic enzyme ability and potential biotechnology applications of the white-rot fungus *Grammothele subargentea* LPSC no. 436 strain

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Received 14 November 2007; accepted 21 December 2007

Abstract

To get a better insight into the ligninolytic system of *Grammothele subargentea*, extracellular ligninolytic enzyme activities and ability to degrade synthetic dyes as well as *Eucalyptus globulus* wood were assayed in cultures grown on an agar medium with Cu^{2+} or dyes and on *E. globulus* wood chips. Laccase was the only ligninolytic enzyme detected. The fungus was able to decolorize different dyes, being the highest levels of laccase activity in cultures with Brilliant Green. Cultures on wood showed both ligninolytic activity and degradative ability on lipophilic extractives. An extracellular laccase with pI 3.5 and maximal activity at pH 4.0 and 50–55 °C was detected on liquid cultures containing 0.6 mM Cu^{2+}. The enzyme extract was stable at pH 6.0–7.0 and up to 60 °C. A laccase-mediator system using a *G. subargentea* laccase crude extract and 1-hydroxybenzotriazole as mediator improved the tensile strength of a paper from recycled high-kappa-number pulp.

Keywords: Dye decolorization; Extracellular ligninolytic enzymes; Laccase; Paper industry; White-rot fungus; Wood degradation

1. Introduction

Lignin is probably one of the most recalcitrant compounds synthesized by plants, which is mostly abundant in trees and the main contributor to wood strength [1], being only degraded by a few microorganisms. White-rot fungi mineralize lignin by means of complex systems made up by extracellular oxidoreductases, such as laccases and peroxidases, low-molecular-mass metabolites and active species of oxygen [2–4]. The ability to degrade lignin and other recalcitrant compounds such as single aromatic molecules and other xenobiotics confirms the unspecific nature of these oxidative enzymes [5–8]. Because of this, their potential application on areas such as pulp industry and/or bioremediation are currently under study [4,5,8–12]. The use of fungi along with their enzymes to degrade lignin at the industrial level should lead to a reduction in manufacturing costs as well as pollution, contributing to the use of new environmentally sound bleaching sequences and the improvement of paper and related products quality as well, such as those derived from recycled pulp [4,5,10,11,13–15].

Among fungal extracellular oxidative enzymes involved in lignin degradation, laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are phenol-oxidases currently under study for their use in transforming aromatic compounds [2,5–7,16]. These enzymes oxidize aromatic amines, a wide number of phenolic compounds including chlorophenols, secondary aliphatic polyalcohols, anthraquinone dyes and to a certain extent, some polycyclic aromatic hydrocarbons (PAHs), such as anthracene, as well as some inorganic ions (like Mn^{2+}),

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doi:10.1016/j.procbio.2007.12.016

inorganic and organic metal ion complexes such as ferrocyanide, ferrocenes and cytochrome c or an electron itself transferred directly from an electrode [4,5,16]. These substrates also are oxidized by peroxidases. However, laccases, unlike peroxidases, do not require hydrogen peroxide, raising this the interest of biotech-companies upon them. Furthermore, laccases can participate in the production of active oxygen species and the oxidation of non-phenolic units of lignin, azo and indigo dyes, and other PAHs, compounds that cannot be oxidized by laccases on their own [3,5–7]. The laccase–substrate couple constitutes the laccase-mediator system (LMS), which oxidize compounds by the laccase generated free radicals, suggesting that LMS might have a more powerful catalytic activity than peroxidases.

In a survey made in a subtropical area in Northern Argentina, aimed at isolating lignin degrading organisms, a highly active fungus, *Grammothele subargentea* (Speg.) Rajch. (Stereales, Basidiomycota) strain LPSC no. 436, was isolated and identified. It is a white-rot fungus distributed in temperate and tropical regions of America and East Africa [17], that showed the highest laccase activity among several fungi [18]. Recently, Saparrat [19] described the culture conditions that enhanced fungal enzyme synthesis and activity. Therefore, the aim of this study was to get a better insight into the extracellular ligninolytic system of *G. subargentea* by evaluating its ability to degrade *Eucalyptus globulus* Labill. wood chips as well as synthetic dyes. Furthermore, the effect of a laccase extract from G. *subargentea* in a LMS using 1-hydroxybenzotriazole (HBT) as mediator upon the chemical and physical properties of a recycled unbleached kraft pulp was also analyzed.

2. Materials and methods

2.1. Fungal strain

G. subargentea LPSC (culture collection of the Instituto Spegazzini, UNLP, Argentina) strain 436 was isolated from a fruiting body collected from the trunk of a tree growing in the rain forest of a subtropical area in Garupá, Province of Misiones, Argentina (27°29′S, 55°50′W). Stock cultures were maintained on malt extract agar supplemented with yeast extract (0.4%) and *Populus nigra* L. wood chips at 4 °C.

2.2. Growth and extracellular oxidative enzyme production on agar medium supplemented with Cu^{2+}

The effect of Cu^{2+} on fungal growth and synthesis of oxidative enzymes was studied on solid media by inoculating mycelial plugs onto plates containing the modified Czapek Dox agar (2%, w/v) basal medium [18] supplemented with $CuSO_4{\cdot}5H_2O$ at concentrations ranging from 0.05 μM, which was the level of Cu^{2+} in the basal medium, to 1.8 mM. A 6-mm diameter agar plug of a culture grown on basal medium, was used to inoculate agar plates. Three replica plates per treatment were incubated in the dark at 25 ± 1.5 °C. The effect of Cu^{2+} on growth was estimated after 7 days of incubation, calculating the percentage of reduction in colony diameter in response to the media Cu content, as described by Saparrat and Hammer [20]. Ligninolytic enzyme activity was determined after a 7-day incubation period in extracts obtained from the culture medium areas beneath the mycelium [21]. The data were analyzed by a one-way ANOVA and means were contrasted by Tukey's test. Linear regressions were performed using STATGRAPHICS Plus Version 4 software for Windows (Microsoft, USA).

2.3. Extracellular enzyme production and decolorization on agar-dye medium

The ability of *G. subargentea* to decolorize synthetic dyes supplemented to the modified Czapek Dox agar (2%, w/v) basal medium was evaluated. A total of 12 synthetic dyes (0.01%, w/v) representing azo, heterocyclic and triphenylmethane groups were tested (Table 1). One percent stock solutions of the dyes were sterilized by filtration and added to autoclaved basal medium. Bengal Rose, Brilliant Green, Congo Red were from Fluka. Crystal Violet, Eosin Y, Fuchsin, Methylene Blue B, Methyl Red and Phloxine were from E. Merck. Neutral red was obtained from Riedel-de Haen. Toluidine Blue was purchased from Anedra. Trypan Blue was from Sigma Chemical. Three replicates per treatment were inoculated and incubated as described before. The scale used was: (1) plates decolorized after 7 days of incubation; (2) plates decolorized after 14 days; (3) plates decolorized after 21 days; (4) no decolorization. Colony size and oxidative enzyme activity were determined as described before after 7 and 21 days of incubation.

2.4. Solid-state fermentation (SSF)

G. subargentea LPSC strain 436 was cultivated in 100 ml Erlenmeyer flasks containing 2 g (dry mass) of *E. globulus* wood chips (1–2 mm × 10–20 mm) in 5 ml water, which were sterilized twice at 121 °C for 30 min. Two mycelium plugs (6 mm diameter) from 2% malt extract agar cultures were added as inoculum. Six replicates of inoculated and uninoculated control flasks were incubated at 25 ± 1.5 °C. Ligninolytic enzyme activity (expressed as mU/g wood) was evaluated on each of three flasks after 15 and 30 days of incubation. Enzyme extracts were prepared as described by Saparrat and Guillén [8]. Wood mass loss and degradation of Klason lignin, lipophilic compounds and free and esterified sterols of eucalypt wood were determined on the three remaining replicates. Wood dry mass was measured by weighing the flasks content after drying them in an aerated oven at 60 ± 5 °C for 12 h. Lipophilic compounds were extracted from sawdust (<0.4 mm) in a Soxhlet using acetone as solvent and the extracts were dried for mass estimation [9]. Chromatographic analysis of acetone extracts was carried out according to Martínez et al. [9]. The Klason lignin content was determined according to TAPPI Standard T222 om-88 [22]. Degradation of free and esterified sterols was quantified by estimating the difference between lipophilic compounds after a 15- and 30-day incubation period in uninoculated controls and in wood inoculated with the fungus.

2.5. Enzyme assays

Unless otherwise stated, laccase (EC 1.10.3.2) activity was measured using as substrate 5 mM 2,6-dimethoxyphenol (DMP, Fluka) in 0.1 M sodium tartrate buffer, pH 4 at 25 °C [7]. Aryl-alcohol oxidase (EC 1.1.3.7), lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13) and manganese-independent peroxidase (EC 1.11.1.7) activities were determined as described by Saparrat and Guillén [8]. One activity unit (U) was defined as the amount of enzyme releasing 1 μmol reaction product/min.

2.6. Preparation and characterization of a crude extract with laccase activity of G. subargentea

The fungus was grown for 20 incubation days in the modified Czapek Dox liquid medium supplemented with 0.6 mM Cu^{2+} at third incubation day [19]. Then the mycelium was removed by centrifugation at 20 000 × *g* and 4 °C for 10 min. The supernatant was collected and concentrated by using an Amicon ultrafiltration cell (model 8050) equipped with a PM10 Diaflo ultrafilter (cut-off 10 kDa) to achieve a 7.6-fold concentration factor for laccase. Then, the crude extract was fractionated and conserved at −20 °C. pH and temperature effects on enzyme stability were estimated by preincubating crude extracts at pHs between 2.0 and 7.0 and temperatures ranging between 40 and 70 °C for 1, 24 and 48 h and 0–4 h, respectively, measuring enzyme activity as described before. The effect of the pH and temperature in the catalytic activity of laccase was assayed by performing the reaction at pHs and temperatures ranging between pH 2.0–7.0 and 22–55 °C, respectively. The effect of ionic strength on laccase activity was evaluated at NaCl concentrations between 0 and 1 M.

Table 1
Decolorization of synthetic dyes at 0.01% (w/v) by *Grammothele subargentea*, their effect on growth and extracellular laccase production

Synthetic dye		Growth reduction[a,b]	Decolorization of medium[c]	Laccase activity[d,b] (mU/cm^3)
Common name	C.I. number			
Azo type				
Congo Red	22 120	34.8 ± 1.7	1	24.1 ± 4.4
Methyl Red	13 020	42.4 ± 3.2	1	52.2 ± 2.5
Trypan Blue	23 850	1.3 ± 1.3	1	8.6 ± 3.0
N-Heterocyclic type				
Methylene Blue B	52 015	26.9 ± 4.1	4	13.8[e] ± 1.3
Neutral Red	50 040	17.4 ± 1.8	2	13.5 ± 10.0
Toluidine Blue	52 040	19.5 ± 2.4	2[f]	8.9 ± 1.0
O-Heterocyclic type				
Bengal Rose	45 440	50.6 ± 3.2	1	75.0 ± 10.6
Eosin Y	45 380	38.7 ± 2.2	2	9.2 ± 1.6
Phloxine	45 410	36.8 ± 4.2	1	14.1 ± 7.3
Triphenylmethane type				
Brilliant Green	42 040	90.3 ± 2.5	3	292.0 ± 43.2
Crystal Violet	42 555	84.8 ± 3.9	3	143.5 ± 42.8
Fuchsin	42 510	23.9 ± 2.4	2	55.9 ± 10.0

[a] Percentage of growth reduction caused by each dye compared to control cultures after 7 days of incubation.
[b] Mean ± S.D. of three replicates.
[c] 1, plates decolorized after 7 days of incubation; 2, plates decolorized after 14 days; 3, plates decolorized after 21 days; 4, no decolorization.
[d] Laccase activity of decolorized areas of agar cultures after 21 days of incubation.
[e] Extracellular laccase activity of the medium close to the mycelium after 21 days of incubation.
[f] Diffuse decolorization of agar medium.

Proteins were precipitated by adding to crude extracts 5 volumes of acetone, then were pelleted by centrifugation (10 000 × *g* for 15 min), and the pellets were redissolved in deionized water. The whole procedure was repeated one time and proteins were pooled. Isoelectric focusing (IEF) was performed on an immobilized pH gradient on 5% polyacrylamide gels (1 mm thickness) at pIs ranging from 3.0 to 10.0 (Bio-Rad Ampholine) in a mini IEF cell (Model 111, Bio-Rad) at: 100 V for 15 min, 200 V for the following 15 min, and 450 V for the last 60 min. Protein bands were stained with Coomassie Brilliant Blue R-250. Bands with laccase activity were revealed by incubating the gel in 0.1 M sodium tartrate buffer, pH 4.0, containing 5 mM DMP.

2.7. Effect of the G. subargentea laccase extract–HBT system on the properties of a recycled pulp

Virgin softwood kraft pulp, which is a mixture of *Pinus elliotti* and *Pinus taeda* (kappa number 85.0, Canadian standard freeness (CSF) 810 ml, supplied by Papel Misionero S.A., Misiones, Argentina), was refined in a PFI mill at 20,000 revolutions in two steps using a 1.8 N/mm load (final freeness: 640 ml CSF). Handsheets of 214 g/m^2 were made, dried under tension according to the SCAN-P 2:75 Test Method [23], repulped according to Mocchiutti et al. [10] and classified using a Bauer–McNett classifier according to the SCAN-M6:69 Test Method [23]. The only fraction collected, R30, was treated with HCl 0.1 M at 4% pulp consistency (4 g dry pulp/100 g of suspension) during 45 min, was washed with distilled water until a pH of 4.5 and was stored at 4 °C.

An aliquot of supernatants from 20 days old liquid cultures of *G. subargentea* LPSC no. 436 strain with laccase activity (at a dosage of 350 mU laccase activity/g dry pulp using guaiacol as substrate according to Guillén et al. [24]) and a solution of HBT mediator (1-hydroxybenzotriazole, monohydrate; ICN Biomedicals; 3% on dry pulp) were used to treat a pulp suspension at 2% consistency. The mixture was incubated at pH 4.5 (adjusted with 55 mM sodium acetate buffer) and 40 ± 1 °C. Oxygen was incorporated by agitation (200 rpm) and by bubling it under atmospheric pressure for 4.5 h. Laccase activity was assayed at 20 min intervals and when reduction in activity level was observed an additional aliquot of the enzyme was added. Three controls were run, one with no enzyme and HBT, another that had only enzyme and a third one that had only HBT. The enzyme adsorbed onto the pulps was released by treating them with 0.1% Tween 20 (T) for 30 min. Then the pulps were filtered and washed until no enzyme activity was detected on the supernatant. Then, an alkaline extraction (AE) of the solid residue was performed at 7% pulp consistency, using 2% NaOH on dry pulp at 70 ± 1 °C for 30 min. The kappa number [25], total acidic groups [26] and water retention value in the calcium form (3000 × *g*; 15 min) of the treated pulps were determined. Handsheets with a grammage of 60 g/m^2 were prepared according to the SCAN-C 26:76 Test Method [23] to determine the tensile index [25], apparent density [25], light-scattering coefficient "*s*" (using a R68 filter; effective wavelength of 681 nm) and specific light absorption coefficient "*k*" at 681 nm, which was determined according to Kubelka–Munk theory [27]. Three up to 10 measurements were taken for each pulp properties after different treatment sequences. Results were analyzed by a one-way ANOVA and means of all variables were contrasted by Tukey's test ($P = 0.05$) [28].

3. Results

3.1. Growth and extracellular oxidative enzyme production on agar medium supplemented with Cu^{2+}

Copper inhibited growth of *G. subargentea*. There is a linear relationship between the Cu content in the media and fungal growth inhibition (R^2: 0.98; % growth reduction = 0.0193 × μM Cu^{2+} + 0.815). The largest reduction (35.4%) occurred at a concentration of 1.8 mM. Regarding the extracellular activity of aryl-alcohol oxidase, laccase and peroxidase enzymes, only laccase activity was detected and its levels increased together with the amount of Cu^{2+} in the media, though the highest enzyme activity (302 mU/cm^3 of agar medium) was found at 1.5 mM Cu^{2+}.

Table 2
Eucalyptus globulus wood treatment with *G. subargentea*

Incubation time (day)	Laccase activity[a] (mU/g wood)	Wood mass loss[a,b] (%)	Degradation[a,b] (%)			
			Lignin[c]	Total lipophilic compounds	Free sterols	Sterol esters
15	291.9 ± 31.6	5.5 ± 3.3	1.1 ± 0.4	17.3 ± 7.0	70.1 ± 0.3	60.6 ± 0.3
30	336.6 ± 25.4	8.2 ± 5.6	13.5 ± 8.7	6.2 ± 3.4	73.6 ± 4.5	74.6 ± 2.7

[a] Mean ± S.D. of three replicates.
[b] % Degradation in relation to the content of uninoculated sterilized wood chips (control).
[c] Analyzed by the Klason method.

3.2. Extracellular enzyme production and decolorization on agar-dye medium

The ability of the strain to grow, produce extracellular oxidative enzymes and metabolize chromophores was also evaluated (Table 1). Along a 21-day period, the fungus grew in the presence of dyes which were all metabolized except for Methylene Blue B. The largest growth reduction (85–90%) after 7 days of incubation was provoked by media supplemented with triphenylmethane dyes, such as Brilliant Green or Crystal Violet. Only one *O*-heterocyclic dye, Bengal Rose, provoked as much as a 50% reduction in growth in 7 days. Laccase activity was detected in both decolorized and non-decolorized areas close to the mycelium and while the highest activity was detected in cultures supplemented with Brilliant Green, no aryl-alcohol oxidase and peroxidase enzymes activities were found (Table 1).

3.3. Solid-state fermentation (SSF)

Laccase activity in solid-state fermentation (SSF) cultures of the fungus and the degradation of *E. globulus* wood components are shown in Table 2. While no aryl-alcohol oxidase and peroxidase activity was detected in SSF extracts (data not shown), the fungus degraded *E. globulus* wood mass by 5.5 and 8.2% after 15 and 30 days of incubation, respectively, also reducing the level of lignin and total lipophilic compounds, including free and esterified sterols (Table 2).

3.4. Characterization of a crude extract with laccase activity of G. subargentea

Crude extracellular extracts of *G. subargentea* cultures grown on medium supplemented with copper were characterized. While the enzyme-complex was completely stable for 48 h within a pH range 6.0–7.0 at 25 °C, at pH 4.0–5.0 there was a 35–40% reduction in activity (Fig. 1A). Shorter incubations at low pH also affected enzyme stability, since incubation for 1 h at pH 2.0 provoked a 50% reduction in activity.

Zymograms obtained after IEF of the crude extract, which was obtained from liquid cultures, showed that laccase activity was concentrated on a band that had a pI 3.5 (Fig. 2).

Although laccase activity spanned along a broad pH range (pH 2–6), when 100 mM sodium tartrate (2.0–7.0) was used as reaction buffer, the highest levels of activity were detected at pH 4.0 (Fig. 1B).

Pre-incubation of the enzyme extract at 40–50 °C for 4 h resulted in a considerable increment in activity (150%). However, at 70 °C it's half-life was only 1 h, and after 4 h only 8% of activity remained. The enzyme was stable at 60 °C (Fig. 3A), and in accordance with this the highest laccase activity occurred at incubation temperatures between 50 and

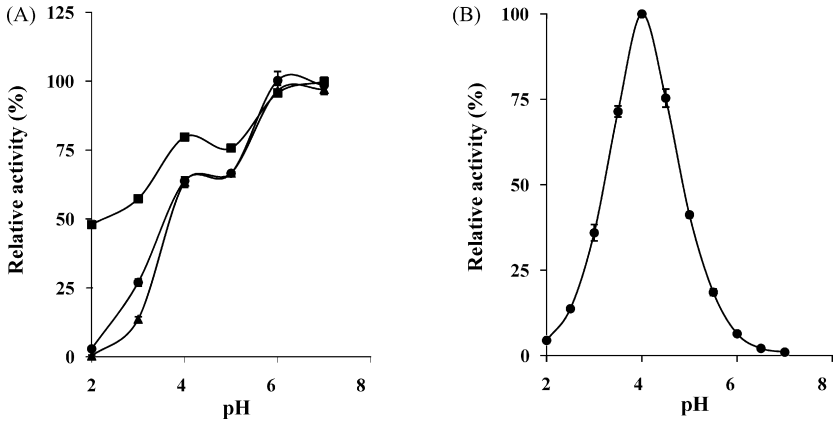

Fig. 1. Effect of pH on laccase stability (A) and activity (B). Values are means of three replicates. Error bars represent standard deviation. Stability of the enzyme was tested by preincubating enzyme extracts at the desired pH for 1 h (squares), 24 h (circles) and 48 h (triangles). Laccase enzyme activity was determined using 100 mM sodium tartrate buffer at pH between 2.0 and 7.0.

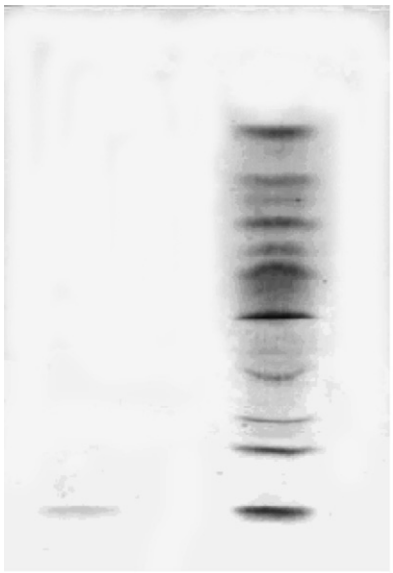

Fig. 2. Isoelectric focusing of the laccase from *Grammothele subargentea* at pH 3.0–10.0. The gel was stained with Coomassie Blue R-250. Lane 1, crude extract; lane 2, pI standards (BioRad).

55 °C, though it remained at high levels at temperatures between 22 and 55 °C (Fig. 3B).

Ionic strength also affected laccase activity. Though pre-incubation of the enzyme extract in 0.1 M of NaCl did not alter activity, it was reduced by approximately 71% with 1 M NaCl (data not shown).

3.5. Effect of the G. subargentea laccase extract–HBT system on the properties of a recycled pulp

We analyzed the chemical and physical properties of a pulp after treating it with a laccase crude extract in a LMS. Treatment with laccase extract alone had the same effect than the C-T-AE sequence (data not shown). The EH-T-AE sequence increased the paper tensile index by 8.0 and 11.7% compared to the H-T-AE and C-T-AE sequences, respectively (Fig. 4). The light-scattering coefficient of the pulp and its apparent density, after EH-T-AE sequence and those of the pulp treated only with HBT (H-T-AE sequence) were similar though significantly different from untreated control pulp ($P < 0.05$). Other characteristics such as kappa number, water retention and total content of acid groups remained unchanged. However, as evidenced by the *k*-coefficient, the pulp treated with laccase extract–HBT was darker than the others ($P < 0.05$), which might be due to the oxidation of the pulps components treated with LMS.

4. Discussion

Previous studies have shown that *G. subargentea* produce low levels of extracellular manganese-dependent peroxidase activity on phenol red [18]. Saparrat [19] suggested that this was due to a laccase activity. Here we confirmed that extracellular laccase was the only enzyme detected by *G. subargentea* under the conditions studied, since it's activity was associated to a single acidic protein, like in *Coriolopsis rigida* [7], *Lentinula edodes* [6] and *Pycnoporus cinnabarinus* [29]. As described before by Saparrat [19], the enzyme activity was induced by Cu, increasing in direct relation with concentration until 1.5 mM. While Saparrat [19] evaluated the enzyme response to Cu^{2+} in liquid media, we did so on solid media. Therefore, the differences found might be due to the fact that agar sequestered copper. Two evidences support this, one is that agar is known to complex a variety of metal ions [30–32] and the other one is that a higher Cu^{2+} concentration was required in solid media to produce as much enzyme as in liquid medium. Bollag and Leonowicz [33], Levin et al. [34] and Saparrat et al. [7] found that laccase was the major extracellular protein fraction in fungal cultures supplemented with several inducers, including copper. Furthermore, Collins and Dobson [35] and Palmieri et al. [36] found that laccase expression of Basidiomycetes is induced by Cu at a transcriptional level.

G. subargentea growth was not fully inhibited by the dyes, which were decolorized as a result of biotransformation and/or

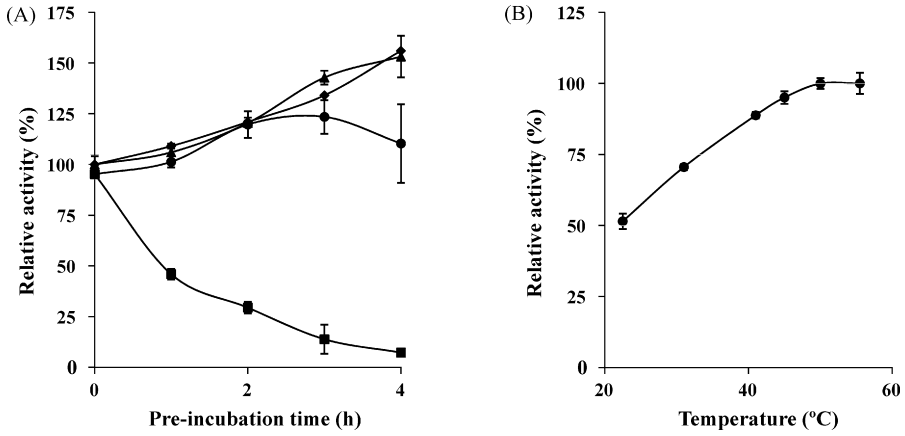

Fig. 3. Stability (A) and activity of laccase (B) at different temperatures. Values are means of three replicates. Error bars represent the standard deviation. Stability of the enzyme was tested by preincubating enzyme extracts for 4 h at 40 °C (diamonds), 50 °C (triangles), 60 °C (circles) and 70 °C (squares), then enzyme activity was determined at 50 °C.

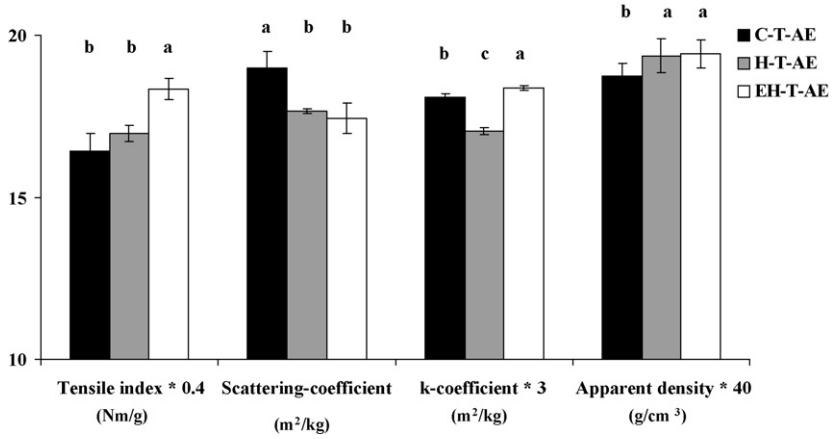

Fig. 4. Effect of the *G. subargentea* laccase crude extract–HBT system on the properties of a recycled unbleached softwood kraft pulp after a treatment sequence. Values are means of two replicates. Error bars represent the standard deviation; data for each property followed by the same letter are not significantly different (Tukey test, $P < 0.05$). The *s*-coefficient is reported to the nearest 0.5 m^2/kg according to the SCAN-C 27:69 Test Method [23]. EH-T-AE, treatment with *G. subargentea* enzyme extract in the presence of HBT followed by washing with Tween 20 and alkaline extraction; H-T-AE, treatment with HBT alone followed by washing with Tween 20 and alkaline extraction; C-T-AE, control pulp which was treated in the same conditions as the treatments with enzyme and/or HBT, except that laccase crude and the mediator were not added.

mycelial adsorption [20,37]. The mycelium of *G. subargentea* was white when grown on dye supplemented media and extracellular laccase activity was found mainly in decolorized areas. Calvo et al. [38], Eichlerová et al. [39], Pointing et al. [40] and Revankar and Lele [12] found that the oxidative activity of white-rot Basidiomycetes transformed a broad spectrum of chromophores. A similar pattern was observed in *G. subargentea*, which could not transform Methylene Blue B.

G. subargentea degraded lignin and lipophilic compounds from *E. globulus* wood, including free and esterified sterols, which are the main compounds responsible for ''pitch'' deposition in pulp and paper manufacture. Lipophilic compounds are composed mainly of byproducts, wood constituents and also of fungal metabolites synthesized along the incubation period [9]. The lower level of degradation of lipophilic compounds by *G. subargentea* after a longer incubation period, such as 30 days, could be due to an increase in low-molecular-mass compounds as a result of lignin degradation. Similar results were found when *Bjerkandera adusta* was cultivated on *E. globulus* wood under SSF conditions [9]. The concomitant laccase activity and *E. globulus* wood degradation in culture, suggest a role for the enzyme complex in degradation. Furthermore, in fungi lacking peroxidase activity, laccase might play one of the most relevant roles in lignin biodegradation [4,29]. Several fungal species, including *Marasmius quercophilus*, *Pleurotus eryngii* and *P. laciniatocrenatus*, degrade lignin by means of the participation of laccases [8,16,41]. These oxidative enzymes along with others such as esterases, also are involved in the transformation of lipophilic extractives of *E. globulus* wood [11].

Crude extracellular extracts from liquid cultures of the fungus were analyzed and characterized. The enzyme pI, optimum pH and temperature range were determined together with the ionic strength effect on enzyme stability and activity, which by the way were similar to those of other isolated laccases [2,4,33]. The laccase crude extract from *G. subargentea* was highly stable to high temperature compared to laccases from *Pleurotus eryngii* [16], *P. cinnabarinus* [29], *Thelephora terrestris* [42] and *Trametes trogii* [34]. Furthermore, pre-incubation of the enzyme extract at 40–60 °C increased the level of activity, in a way similar to the laccase isolated from *Chaetomium thermophilium* [43]. This type of response to preincubation can be due to conformational changes, that might increase flexibility, hence, the catalytic activity of the enzyme. This has been described for the carboxylesterase of the extremely thermoacidophilic archaeon *Sulfolobus solfataricus* as a response to temperature and cosolvents [44]. Dawson et al. [45] and Taniguchi et al. [46] suggested that conformational changes in metalloproteins, such as cytochrome c and laccases, can lead to more efficient electron transfer rates in reactions involving those proteins. Other laccases such as those of the basidiomycete PM1 and *Polyporus versicolor* were activated by temperature [46,47]. Since *G. subargentea* was isolated from a subtropical area, it is not surprising the stability of its laccase to high temperatures, though the ecological role of this remains to be determined.

Recently da Silva et al. [48] reported the effect of the *Trametes hirsuta* laccase extract/HBT system on the chemical composition and strength properties of the fibrous fraction of an unbleached recycled softwood kraft pulp. However, not much is known about recycling of unbleached pulps and its modification by treatment sequences involving enzyme preparations such as one with laccases from *G. subargentea* [49]. The *G. subargentea* laccase extract/HBT system did not delignified the pulp efficiently, however its activity increased the tensile index and the *k*-coefficient of the pulp. The chemical modifications of pulp-lignin provoked by the *G. subargentea* laccase extract/HBT system, due to the production of radicals, quinones and other polar compounds, might make lignin more hydrophilic and likely to polymerize with the surfaces of adjacent fibers, leading this to an increase in fiber bonding capacity. Lund and Felby [50] suggested that oxidized lignin can undergo polymerization, which may act as a wet-strength agent of paper by encapsulation of the fibers in the sheet. Therefore, our

results suggest that the LMS using a *G. subargentea* laccase extract, oxidized lignin to higher levels than controls, which leads to higher levels of chromophores. Chakar and Ragauskas [51] and Wong et al. [15] found that lignin containing pulps developed, as a result of LMS activity, chromophores, which are mainly aromatic ketones, including quinones of oxidized lignin [50]. Specific modifications of lignin from recycled high-kappa-number pulps may be a way of improving their papermaking properties [49,52]. It has already been shown that LMS is an efficient system for demethylation and delignification of kraft pulp, transformation of PAHs, decolorization of synthetic dyes and wet-strength improvement of unbleached kraft pulp [5,6,50,51].

5. Conclusions

The ability of the *G. subargentea* LPSC 436 strain to degrade lignin of *E. globulus* wood, its resistance to the inhibitory action of synthetic dyes and its ability to decolorize different chromophore types, as well as the improvement in the strength properties of a recycled high-kappa-number pulp after treating it with a *G. subargentea* laccase extract/HBT system, suggest that this fungus might be a useful tool in the biotechnology field such as the pulp industry and bioremediation.

Acknowledgments

M.C.N. Saparrat is a researcher from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. P. Mocchiutti is recipient of a scholarship from CONICET, Argentina. N.O. Caffini and P.A. Balatti are researchers from Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICBA), Argentina. This work was supported by Fundación Antorchas (Proy. 14188-64) Argentina, and Agencia Nacional de Promoción Científica y Tecnológica (PICT 14-06962, BID 1201/OC-AR, ANPCYT 13404 BID 1201/OCAR), Argentina.

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