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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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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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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.