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Preliminary studies on the biobleaching of loblolly pine Kraft pulp with *Trametes trogii* crude extracts

Verónica Da Re [a], Leandro Papinutti [a,*], Laura Villalba [b], Flavia Forchiassin [a], Laura Levin [a]

[a] *Lab. de Micología Experimental, Dpto. de Biodiversidad y Biología Experimental, Fac. Cs. Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, 4to piso, C1428EHA Ciudad Universitaria, CABA, Argentina*

[b] *Programa de Investigación en Celulosa y Papel, Fac. Cs, Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552, N3300LQH Posadas, Misiones, Argentina*

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Abstract

Trametes trogii (BAFC 463), *Coriolus versicolor* var. *antarcticus* (BAFC 266) and *Trametes villosa* (BAFC 2755) are Argentinean white-rot fungi that proved to be efficient ligninolytic enzyme producers in previous studies. The aim of the present work was investigating the ability of their crude extracellular extracts for the biobleaching of loblolly pine Kraft pulp. The production of laccase, manganese peroxidase, endoxylanase and endoglucanase was also evaluated. Out of the three fungi studied, the best biobleaching results were obtained with culture supernatants from *T. trogii* grown for 21 days in a synthetic medium with glucose and asparagine as carbon and nitrogen sources, respectively, with the addition of 1 mM copper sulfate. Such medium rendered high level of ligninolytic enzymes: 0.25 U ml^{-1} of manganese peroxidase and 44 U ml^{-1} of laccase. A strong brightness increase was attained after the enzymatic treatment followed by H_2O_2. Bleaching up to 82% ISO brightness (compared with 37% in the peroxide-bleached control) was obtained. Moreover, after the enzymatic treatment, hydrogen peroxide requirements decreased (as reflected by the higher residual peroxide), and the pulp luminance reached a value of 92.5. Addition of known laccase mediators such as HBT, ABTS, and HBA did not improve the process. The supernatant did not significantly lose its bleaching capacity even after successive recovery and reapplication to unbleached pulp. Correspondingly, laccase and manganese peroxidase activities in the recovered supernatant, exhibited near 100% of their initial values.

Keywords: Biobleaching; Enzyme stability; Kraft pulp; Laccase; Mediator; Xylanase

1. Introduction

The importance of microbial enzymes in pulp and paper manufacturing has grown significantly in the last two decades. Currently the most important application of enzymes in the pulp and paper industry is in the prebleaching of Kraft pulp. Enzymes provide a very simple and cost-effective way to reduce the use of chlorine compounds, and other bleaching chemicals. Manganese peroxidase (MnP), laccase plus ABTS or other mediators, can partially reproduce the delignifying effect of the fungus and substantially increased the bleachability of Kraft pulps. Pulp bleaching with a laccase-mediator system has reached pilot plant stage and is expected to be commercialized soon. While lignases attack the lignin, xylanases degrade hemicelluloses and make the pulp more permeable for the removal of residual lignin [1]. Xylanase prebleaching technology is now in use at several mills worldwide [2].

Laccase is an enzyme secreted by most of the lignin degrading basidiomycetes [3,4]. It belongs to a family of multicopper oxidases that catalyze the oxidation of phenolic compounds with a concomitant reduction of oxygen to water. It was demonstrated that laccase requires the presence of a mediator to efficiently degrade residual lignin in Kraft pulp [5–7]. Several articles described purified laccase-mediator systems for bleaching and improving paper strength properties [8–11]. The main constraints in the use of laccases in industrial processes are the large-scale availability required, and production costs of the enzymes. Synthetic laccase mediators have also a high cost and a growing concern exist about their possible toxicity.

Trametes trogii (BAFC 463), *Coriolus versicolor* var. *antarcticus* (BAFC 266) and *Trametes villosa* (BAFC 2755) are Argentinean strains of white rot fungi that proved to be efficient

* Corresponding author.
E-mail address: leandru@bg.fcen.uba.ar (L. Papinutti).

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ligninolytic enzyme producers in previous works [3,12,13]. *T. trogii* strain BAFC 463, showed efficient wood lignin degradation [14] and also provided good xylanases [15] and ligninases [12] production. It produces high amounts of MnP, and higher laccase levels than those reported for most other white rot fungi under favorable conditions, accompanied by high levels of the hydrogen peroxide-producing enzyme glyoxal oxidase (GLOX). The simultaneous occurrence of high ligninolytic and hydrogen peroxide-producing activities, essential for peroxidase activity and rate limiting for pollutant degradation, makes it an attractive microorganism towards future biotechnological applications. On the other hand, in contrast to the well-studied model-organism *Phanerochaete chrysosporium*, *T. trogii* is N-unregulated. Sufficient or excess N-nutrients stimulate high MnP and laccase titres in parallel with the high biomass production. This characteristic of the fungus makes it an outstanding candidate for large-scale fermentation to produce ligninolytic enzymes in bulk for bioremediation [12].

The present study was undertaken to evaluate the feasibility of loblolly pine Kraft pulp biobleaching applying *T. trogii*, *C. versicolor* var. *antarcticus* and *T. villosa* crude extracts.

Crude culture filtrates offer several advantages, their production process is not expensive, they may include natural laccase-mediators secreted by the fungus [16], and on the other hand, proteins or other factors present in the medium may stabilize crude enzymes.

2. Materials and methods

2.1. Organisms

T. trogii BAFC 463, *C. versicolor* var. *antarcticus* BAFC 266, *Trametes villosa* BAFC 2755 (Polyporaceae, Aphyllophorales, Basidiomycetes) were obtained from the culture collection of the Faculty of Exact and Natural Sciences, Universidad de Buenos Aires.

2.2. Culture conditions

Medium for fungal cultures (GA medium) contained glucose, 10 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $MnCl_2{\cdot}4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4{\cdot}H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; asparagine monohydrate, 4 g; distilled water up to 1 l, supplemented with 1 mM copper sulfate. One hundred ml Erlenmeyer flasks with 20 ml of medium were inoculated with a 25-mm^2 surface agar plug from a 7-day-old culture grown on malt agar (1.3% malt extract, 1% glucose, Bacto-agar 2%). Incubation was carried out statically at $28 \pm 1\,^{\circ}C$. Cultures were harvested at different incubation periods and filtered through a filter paper using a Büchner funnel, the culture supernatants were used as enzyme sources. All chemicals were of analytical grade and were used without further purification.

2.3. Enzymatic treatment of unbleached softwood Kraft pulp (USKP)

The mixture of 100 mg of an industrial unbleached softwood loblolly pine (*Pinus taeda*) Kraft pulp (USKP) (Kappa number: 24.8), previously rinsed with distilled water, plus 2.5 ml supernatant (final pH 6.5) was incubated 12 h at 50 °C and 50 rpm. Then, the enzyme-treated SKP (ESKP) was rinsed 3 times with 50 ml distilled water, and dried in open air at room temperature (25 °C).

2.4. Bleaching

All bleaching experiments were carried out in Erlenmeyer flasks with different bleaching sequences. One hundred milligrams dried ESKP plus 10 ml H_2O_2 2% was incubated 2 h at 80 °C and 50 rpm. Then, the ESKP was rinsed 3 times with 20 ml distilled water. The recovered supernatants from the first cycle were collected and applied without further manipulations under similar conditions to a new pulp (second cycle). In the control samples, the supernatant was replaced by distilled water. A medium-only control was also conducted. For all experiments, measurements were carried out in triplicate parallel incubations. The enzyme activity values are reported as the mean with a standard deviation less than 5%.

2.5. Effect of mediators

The effect of laccase mediators (1.5%, w/v) was assayed at the enzymatic treatment step. Synthetic (ABTS [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] and HBT (1-hydroxybenzotriazole)) and natural mediators (anisaldehyde and 4-hydroxybenzoic acid (HBA)) were tested.

2.6. Evaluation of pulp properties

Luminance (L^*) was measured against a white reference standard (titanium oxide $L^* = 94.5$) with a SP62 Portable Sphere Spectrophotometer (D65/10°). Pulp sheets were prepared on a Büchner funnel to determine their brightness. Pulp brightness was determined with a colorimeter Color Touch Model ISO Technidyne.

2.7. Enzyme activities and H_2O_2 concentration estimations

All the enzyme activities were determined at 50 °C. Laccase activity (E.C:1.10.3.2) was measured using 2,2′-azino bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 0.1 M sodium acetate buffer (pH 3.4). Oxidation of ABTS was determined by the increase in A_{420} ($\varepsilon_{420} = 36/\text{mM cm}$) [7]. Manganese peroxidase activity (MnP) (E.C:1.11.1.13) was measured using phenol red as the substrate in 0.1 M sodium dimethylsuccinate buffer (pH 4.5) ($\varepsilon_{610} = 22/\text{mM cm}$) [17]. Endo-ß-D-1,4-glucanase (E.C:3.2.1.4) and Endo-ß-D-1,4-xylanase (E.C:3.2.1.8) activities were determined by measuring the reducing sugars released from carboxymethylcellulose or oat xylan, respectively, as substrates, in 0.1 M sodium acetate buffer, pH 4.8. Liberated reducing sugars were quantified by the Somogyi-Nelson method [18], using either glucose or xylose as standards. Glyoxal oxidase activity was determined by using a peroxidase-coupled assay with methylglyoxal as GLOX substrate and phenol red as the peroxidase substrate [19]. Enzyme activity is expressed in International Units (U), as the amount of enzyme needed to release 1 μmol of product per min. H_2O_2 concentration was measured by using a peroxidase-coupled assay, with phenol red as the peroxidase substrate. Reaction mixture consisted of 2.5 U of horseradish peroxidase (HRP, Sigma) per ml of culture supernatant and phenol red 0.01% as an electron donor. After 10 min NaOH was added (0.2 M final concentration), and the absorbance was read at 610 nm. Samples lacking HRP were used as blanks [20].

3. Results and discussion

Crude culture filtrates from *T. trogii*, *C. versicolor* var. *antarcticus* and *T. villosa* were assayed for the enzymatic treatment of Kraft pulp (without subsequent addition of NaOH, and minimizing H_2O_2 requirements). The main object of this preliminary screening test was to search for the more efficient culture filtrate for biobleaching. The experiment was focused on screening rapidly while preserving assay reagents. With this in mind, the efficiencies of the supernatants were tested by using 100 mg samples and luminance as parameter (which is fast and easy to measure). For further assays, brightness of the pulps will be

Table 1
Time course of enzyme activities (U ml^{-1}) from *T. trogii* cultured statically on GA supplemented with 1 mM $CuSO_4$, and bleaching capacity of the culture filtrates (in terms of luminance of the pulp after treatment)

Time (day)	MnP	Laccase	Endoglucanase	Endoxylanase	Luminance
7	0	0.1	0	0	77.98
11	0	0.86	0.57	1.12	78.16
14	0.22	75.00	3.99	11.82	90.84
21	0.25	43.53	2.56	12.19	92.45
23	0.18	27.77	2.85	11.33	90.34
30	0.19	33.33	2.85	11.82	89.74

Luminance of unbleached pulp was 77.93, and that of water treated was 78.94.

determined and the pulp sample sizes will be increased to give more reliable data.

The values of luminance attained were 92.5, 74.4 and 74.1 for *T. trogii*, *C. versicolor* var. *antarcticus* and *T. villosa*, respectively. Laccase and MnP activities were detected in all the strains, but *T. trogii* rendered the highest titers of both enzyme activities along with the uppermost luminance values. This improved biobleaching efficiency could be attributed to the redox potential (E° 0.79) of *T. trogii* laccase, which belongs to the group of high redox potential laccases [21]. On the other hand, high levels of GLOX activity were produced by *T. trogii* with 1 mM Cu addition (0.19 U ml^{-1}, at day 21 of growth). Only a few of the 67 strains analyzed by de Jong et al. [22] tested up to 0.003 U ml^{-1} of GLOX activity. Kersten [19], using an optimized liquid medium, obtained 0.032 U ml^{-1} of GLOX activity in *P. chrysosporium*. Even though H_2O_2 is the electron acceptor required for catalysis, peroxidases including MnP are sensitive to inactivation by H_2O_2. In order to prevent this type of inactivation in biobleaching experiments, H_2O_2 can be supplied continuously on demand. When assaying MnP of *Bjerkandera* sp. strain BOS55 in biobleaching, an alternative approach towards H_2O_2 addition was its continuous enzymatic generation with glucose oxidase and glucose [23]. Likewise, the continuous supply of H_2O_2 by GLOX, may improve the biobleaching effect of *T. trogii* crude culture filtrates.

Table 1 illustrates the time course of enzyme activities and bleaching capacities of *T. trogii* crude filtrates, when the fungus was cultured statically on GA supplemented with 1 mM $CuSO_4$. The highest luminance was achieved with crude culture filtrates from day 21 (containing laccase (44 U ml^{-1}), MnP (0.25 U ml^{-1}) and endoxylanase (12 U ml^{-1})). Moreover, after the enzymatic treatment, H_2O_2 requirement decreased, as reflected by the higher residual peroxide (data not shown), and the luminance of the pulp increased significantly to a value of 92.5 (measured against a white reference standard (titanium oxide $L^* = 94.5$)) (Table 1). In comparison, luminance of the original unbleached pulp was 77.9, and the value of the control (treated with water) was 78.9. A medium-only control was also conducted, but the pulp luminance did not differ significantly from that of the water treated. Because of the presence of hydrolases (cellulases and hemicellulases) and oxidases (lignases) in different proportions as function of culture age, the resulted bleaching of the pulp differed each day. Another influencing factor for the bleaching could be the concentration of the mediator secreted by the fungus. The required presence of mediators may have extended the range of substrates susceptible to oxidation which in turn, may have led to the improved bleaching efficiency compared to the previously reported [5,6].

Table 2
Effect of mediator (1.5%, w/v to the supernatant) on residual enzyme activities after the treatment of pulp 12 h at 50 °C

	Laccase (%)	MnP (%)	Luminance
Control	96	100	92.76
HBT	48	48	78.89
HBA	37	84	87.78
Anisaldehyde	49	84	83.07
ABTS	30	100	85.23

Luminance of Kraft pulp after treatment with *T. trogii* supernatants and peroxide bleaching (H_2O_2 2% stage: 2 h at 80 °C). Supernatants from *T. trogii* cultured statically 21 days on GA supplemented with 1 mM $CuSO_4$ were applied (laccase activity: 45.53 U ml^{-1}; MnP activity: 0.25 U ml^{-1}).

Thus, in an attempt to gain further efficiency in the bleaching process, laccase mediators were artificially added to the supernatants (HBT, HBA, ABTS or anisaldehyde); surprisingly, none of them improved biobleaching. Moreover, their addition decreased the efficiency of the process. Furthermore, measurements of residual laccase activities showed an important destabilization effect caused by these mediators (up to 30% residual activity with respect to the initial activity) (Table 2). Enzyme inactivation by the oxidized species of some mediators is a general drawback of the laccase-mediator systems [24,25]. Likely, such deactivation effect was responsible for the lower luminance values compared to the control without mediator addition. Hydrolytic enzymes showed complete inactivation in all the experiments regardless of the addition of mediators (data not shown). The production of anisaldehyde by *T. trogii* has been previously demonstrated [26], this or other natural mediators may be present in the extracellular fluids of the fungus, and play a role in the bleaching process without the concomitant undesired effect of enzyme deactivation.

Considering that laccase of *T. trogii* demonstrated to be completely stable for 24 h at 50 °C [12], the efficiency of the crude culture filtrate was assayed through a second cycle of incubation (Table 3). A strong brightness increase was achieved after the enzymatic treatment followed by H_2O_2 bleaching. We obtained up to 82% ISO brightness (compared with 37% in the peroxide-bleached control). The supernatant did not significantly lose its

Table 3
Brightness of Kraft pulp after treatment with *T. trogii* supernatants and peroxide bleaching, and residual laccase and MnP activities after both cycles (supernatant treatment conditions: 12 h at 50 °C; subsequent H_2O_2 2% stage: 2 h at 80 °C)

	Brightness (% ISO)	Laccase (%)	MnP (%)
Control pulp (water treated)	37.3		
Crude extracts (first cycle)	82.26	96	100
Crude extracts (second cycle)	73.61	91	100

Supernatants from *T. trogii* cultured statically 21 days on GA supplemented with 1 mM $CuSO_4$ were applied (laccase activity: 45.53 U ml^{-1}; MnP activity: 0.25 U ml^{-1}; GLOX activity: 0.19 U ml^{-1}; initial hydrogen peroxide concentration in the supernatant: 0.035 mM).

bleaching capacity after two cycles of incubation, (after the second cycle of incubation the treated pulp attained 73.6% ISO brightness compared with 82.3% obtained after the first cycle). Furthermore, laccase and MnP retained approx. 100% of their activities (Table 3). MnP activity also showed to be involved in biobleaching; this enzyme can use unsaturated fatty acids as mediators through lipid peroxidation to degrade synthetic lignins efficiently [27,28]. Thus, the synergistic effect of laccase and MnP in biobleaching by *T. trogii* crude culture filtrates cannot be ruled out. MnP and laccase proved to act synergistically in lignin and dye degradation [29,30]. Xylanases are commercially used for biobleaching, but their effect on this process is limited [2]. Xylanases do not seem to contribute appreciably to the biobleaching efficiency of *T. trogii* crude filtrates, taking into account that only 12% of the initial endoxylanase activity remained after the first cycle of incubation in the supernatants, but they did not lose their bleaching capacity when used in a second cycle. The endoglucanase activity represents a drawback when applying crude supernatants. In order to reduce the effects on the cellulose fraction of lignocellulose, we are searching for experimental biobleaching conditions that do not affect ligninolytic activities but deactivate endoglucanase. In fact, laccase and manganese peroxidase activities in the recovered supernatant, exhibited near 100% of their initial values even after successive recovery and reapplication to unbleached pulp, but endoglucanase was completely inactivated after the first cycle of incubation (data not shown).

Furthermore, in order to obtain supernatants suitable for biobleaching purposes, response surface optimization techniques were applied, aimed at simultaneously minimizing cellulase activity and maximizing ligninolytic enzyme production by *T. trogii* [31].

Although, suitable laccases could be produced at large-scale and low prices, the cost of the mediator is the determining factor for the commercialization of the laccase-mediator bleaching system. Therefore, the fact that crude extracellular extracts of *T. trogii* do not require the addition of synthetic mediators for biobleaching, seems to be a promising alternative.

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