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Exploring the enzymatic parameters for optimal delignification of eucalypt pulp by laccase-mediator

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Abstract

Pycnoporus cinnabarinus laccase was compared with commercial laccases from *Trametes villosa* and *Myceliophthora thermophila* in terms of stability and mediator oxidation rates. Because of its high thermal stability and efficiency oxidizing 1-hydroxybenzotriazole (HBT), the *P. cinnabarinus* laccase was selected for totally chlorine free (TCF) bleaching of paper pulp, using HBT as mediator. Inactivation of laccase by HBT (50% in 4 h) decreased 20% in the presence of eucalypt kraft pulp. Laccase–HBT delignified (four points-decrease of kappa number with respect to the control) and bleached eucalypt pulp (6% ISO brightness increase) and subsequent alkaline treatment with hydrogen peroxide enhanced pulp brightness in 16% ISO. Short enzymatic treatment applied between oxygen delignification and peroxide bleaching stages enabled to obtain a final pulp with 90.3% ISO brightness and kappa number 5. The most significant improvement of pulp properties was produced during the first 2 h of laccase–HBT treatment, and no differences between enzyme performances at 65 and 50 °C were seen. Determination of hexenuronic acids, which contributed up to 50% to kappa number value after laccase-mediator treatment of eucalypt kraft pulp, provided a more realistic estimation of the final delignification rate that attained 81%. FTIR analysis of residual lignins from the treated pulps revealed strong modifications during laccase–HBT treatment.

Keywords: Laccases; *Pycnoporus cinnabarinus*; Thermal stability; 1-Hydroxibenzotriazole; Eucalypt kraft pulp; TCF-bleaching

1. Introduction

Enzymes constitute environmentally friendly alternatives to the use of chlorinated chemicals in pulp and paper manufacturing. Several pulp mills in Europe and North America already incorporate enzyme-aided processes to reduce consumption of chemicals and contamination in their bleaching sequences by using xylanases [1]. These enzymes hydrolyze the xylan reprecipitated on the surface of cellulosic fibres after kraft cooking, contributing to release lignin, but the effect on pulp bleachability is limited. On the other hand, the efficiency of fungal laccases in the presence of mediators, the so-called laccase-mediator systems, to delignify different types of pulps has been demonstrated [2–5], being the most promising enzymatic systems for environmentally sound bleaching. The most effective laccase mediators described elsewhere for pulp delignification are those containing an N–OH functional group, like 1-hydroxybenzotriazole (HBT) [2,6]. The application of laccases in industrial bleaching sequences would not significantly increase costs since laccases use oxygen as electron acceptor and could be produced in large amounts at reasonable price. However, there are several constraints that should be overcome before the industrial implementation of the laccase-mediator systems, such as the choice of the best enzyme and mediator in terms of efficiency, cost and environmental safety.

Kraft pulping represents more than 50% of the worldwide pulp production. Eucalypt wood provides a fibre of strategic interest for Spain and other Southern countries in Europe and America, and is having increasing interest for pulp and paper industry due to its excellent characteristics for writing and printing paper, tissue paper, etc. The laccase from *Pycnoporus cinnabarinus* is compared here with two commercial laccases, in terms of their thermal and pH activity and stability for enzymatic pulp bleaching. Then, it is used to delignify (and bleach)

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eucalypt kraft pulp in combination with oxygen and peroxide treatments, resulting in significantly higher brightness and lower kappa number values than obtained when applying only chemical bleaching agents.

2. Materials and methods

2.1. Pulp samples

Eucalyptus globulus kraft pulps were produced at the ENCE mill in Pontevedra (Spain). Brown (unbleached) pulp with 15.8 kappa number, 36.7% ISO brightness and 1239 mL/g viscosity, and oxygen delignified pulp with 9.7 kappa number, 61.0% ISO brightness and 963 mL/g viscosity were used for the optimization of laccase–HBT treatment.

2.2. Enzymes

P. cinnabarinus laccase, produced by Beldem (Andenne, Belgium) from the monokaryotic hyperproducing strain ss3 [7], was compared with two commercial laccases from *Trametes villosa* and *Myceliophthora thermophila* supplied by Novozymes (Denmark). Laccase activity was determined by measuring the oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) buffered with 100 mM sodium acetate (pH 5) at 24 °C. Formation of the ABTS cation radical was monitored at 436 nm ($\varepsilon_{436} = 29{,}300\,M^{-1}\,cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme that transforms 1 μmol of substrate per minute. All spectrophotometric measurements were carried out on a Shimatzu UV–vis 160.

2.3. Enzyme stability and optimal pH and temperature

Laccases (800 mU/mL initial activity) were incubated in 100 mM citrate–phosphate–borate buffer (pH range from 2 to 10) during 24 h at 24 °C to determine their stability at those pH. The activities of the respective laccases at different times were determined with 5 mM ABTS as substrate, in sodium acetate buffer, pH 5, as described in Section 2.2.

The optimum pH was investigated in the same range (pH 2–10). The initial activity of each enzyme at the different pH was analyzed using 5 mM ABTS, in 100 mM citrate–phosphate–borate buffer, as substrate.

Thermal stability was determined using the same dose of enzyme in 100 mM citrate–phosphate–borate buffer (at pH 4 for *P. cinnabarinus* and *T. villosa* laccases, and pH 7 for *M. thermophila* laccase) at different temperatures (30, 40, 50, 60, 70 and 80 °C) during 24 h.

The optimum temperature for the three laccases was measured in the same range of temperatures (30–80 °C) in 100 mM acetate buffer, pH 5, as described in Section 2.2.

2.4. Oxidation of HBT/ABTS

Oxidation of HBT (from Sigma–Aldrich) by *P. cinnabarinus*, *T. villosa* and *M. thermophila* laccases was assayed with 600 mU/mL of enzyme in 100 mM sodium tartrate buffer (pH 4). Two different HBT concentrations were assayed: 3.33 mM HBT, the molar concentration used in the bleaching assays, or 100 mM, near saturating concentration (more concentrated solutions are difficult to handle). Oxidation of 3.3 mM ABTS with 5 mU/mL of laccase in the same buffer was assayed for comparison.

2.5. Laccase inactivation by the mediator

Activity of *P. cinnabarinus* laccase (600 mU/mL initial activity) was followed during 12 h at 50 °C, in 50 mM sodium tartrate buffer (pH 4) in the absence or presence of 3.33 mM HBT. The effect of pulp was evaluated by following the activity (20 U initial activity/g of pulp) during 4 h in the presence (1.5% w/w, equivalent to 3.33 mM) or absence of HBT using 1 g (dry weight) of brown kraft pulp (3% consistency) at 50 °C, in 50 mM sodium tartrate buffer (pH 4).

2.6. Laccase-mediator treatments

Optimization of the laccase-mediator treatment was carried out in duplicate with 10 g (dry weight) of brown pulp at 3% consistency in 50 mM sodium tartrate buffer (pH 4), using 20 U/g of *P. cinnabarinus* laccase and 1.5% or 3% (w/w) HBT (all relative to pulp dry weight). Tween 80 (0.05% w/v) was added as surfactant. The treatments were carried out in flasks under O_2 atmosphere (continuous bubbling) in a thermostatic shaker, at 160 rev/min and 50 °C, for 12 h. As controls, pulps were treated under identical conditions but without enzyme.

2.7. Combination of enzymatic and chemical treatments

The laccase-mediator treated eucalypt pulp was extracted with alkali (1.5% NaOH, for 1 h at 60 °C and 5% consistency) or treated with hydrogen peroxide (3% H_2O_2 in 1.5% NaOH, for 2 h at 90 °C and 5% consistency).

A complete bleaching TCF sequence was also performed onto both brown and oxygen-delignified eucalypt kraft pulps treated with laccase-mediator (10% consistency), where a chelation treatment, using 0.3% diethylentriaminepentaacetic acid (DTPA from Sigma–Aldrich; 1 h at 85 °C, pH 5–6), was included to improve the performances of the hydrogen peroxide (3.0% H_2O_2, 1.5% NaOH, 0.1% SO_4Mg and 0.5% $Si_2O_3Na_2$). This peroxide treatment was applied in two steps, the first one under pressurized oxygen (2 h at 105 °C, under 6 kg/cm^2 O_2 pressure) and the second one under atmospheric pressure (3.5 h at 98 °C).

2.8. Evaluation of pulp properties

Pulp brightness, kappa number (as estimation of lignin content) and viscosity (determined as the intrinsic viscosity of a sample of cellulose dissolved in a diluted solution of cupri-ethylenediamine) were estimated by ISO standard methods [8]. The content of hexenuronic acids (HexA) in pulps was measured by spectrophotometric quantitation of the furan derivatives produced by the acidic hydrolysis of hexenuronic acids [9]. The contribution of lignin to kappa number was calculated from the following equation:

$$K_a = 0.086\,\mathrm{HexA} + K_r$$

where the real kappa number, which corresponds to the lignin content (K_r), is calculated from the difference between the apparent kappa number measured by standard methods (K_a) and the kappa number due to hexenuronic acid content. The latter is estimated after calculating the corresponding conversion factor according to the contribution of 0.86 units to the kappa number by every 10 μmol of HexA.

Two selectivity indexes were calculated on the basis of decrease of kappa number (Slc_K) or increase of brightness (Slc_B) with respect to integrity of initial pulp estimated from cellulose viscosity, according to the following equations:

$$Slc_K = \left(\frac{K_i - K_f}{V_i - V_f}\right)$$

$$Slc_B = \left(\frac{B_f - B_i}{V_i - V_f}\right)$$

where K_i and K_f correspond to initial and final kappa number, B_i and B_f to initial and final brightness and V_i and V_f to initial and final pulp viscosity, respectively.

2.9. Spectroscopic analysis of residual lignin in the brown and bleached pulp

Residual lignins were isolated from brown and bleached pulps by enzymatic hydrolysis of cellulose with *Trichoderma reesei* cellulase (Econase CEP, from AB-Enzymes) and *Aspergillus niger* β-glucosidase (Novozym 188, from Novozymes), for 48 h at pH 5 (50 mM sodium acetate buffer), 50 °C, and 180 rpm, using 5% pulp consistency [10]. These residual lignins contained contaminating protein from the enzymatic hydrolysis, and were purified using an alkaline protease from *Bacillus licheniformis* (Subtilisin type VIII, Sigma) in the first purification step, and dimethylacematide (DMAC) and NaOH for the subsequent solvent extraction purification.

FTIR spectra were obtained with a Bruker IF-28 spectrometer using 1 mg of lignin in 300 mg of KBr. A total of 50 interpherograms were accumulated, and the spectra were corrected by baseline subtraction between valleys ca. 1850 and 900 cm^{-1}.

3. Results

3.1. Comparison of three fungal laccases

The stability and optimal temperature and pH of the *P. cinnabarinus* laccase were compared with those of two commercial fungal laccases from *T. villosa* and *M. thermophila*. Laccases from *P. cinnabarinus* and *T. villosa* were highly stable in a wide pH range from 4 to 9 (Fig. 1A and B). In contrast, *M. thermophila* laccase was unstable below pH 6 (Fig. 1C) but highly stable at alkaline pH, showing total activity after 24 h at pH 10 (compared to 40% and 20% of initial activity of the other two laccases). The three laccases were completely inactivated after 24 h at pH 2, and only *P. cinnabarinus* laccase was 100% stable at pH 3 for 24 h.

On the other hand, *P. cinnabarinus* and *M. thermophila* laccases showed higher thermal stability than *T. villosa* laccase. Full or almost full activity was observed after 1 h at 60 °C for the two former laccases (Fig. 1D and F), whereas the activity of *T. villosa* laccase began to decrease significantly at 50 °C (only 30% of activity remained after 8 h) (Fig. 1E). This laccase

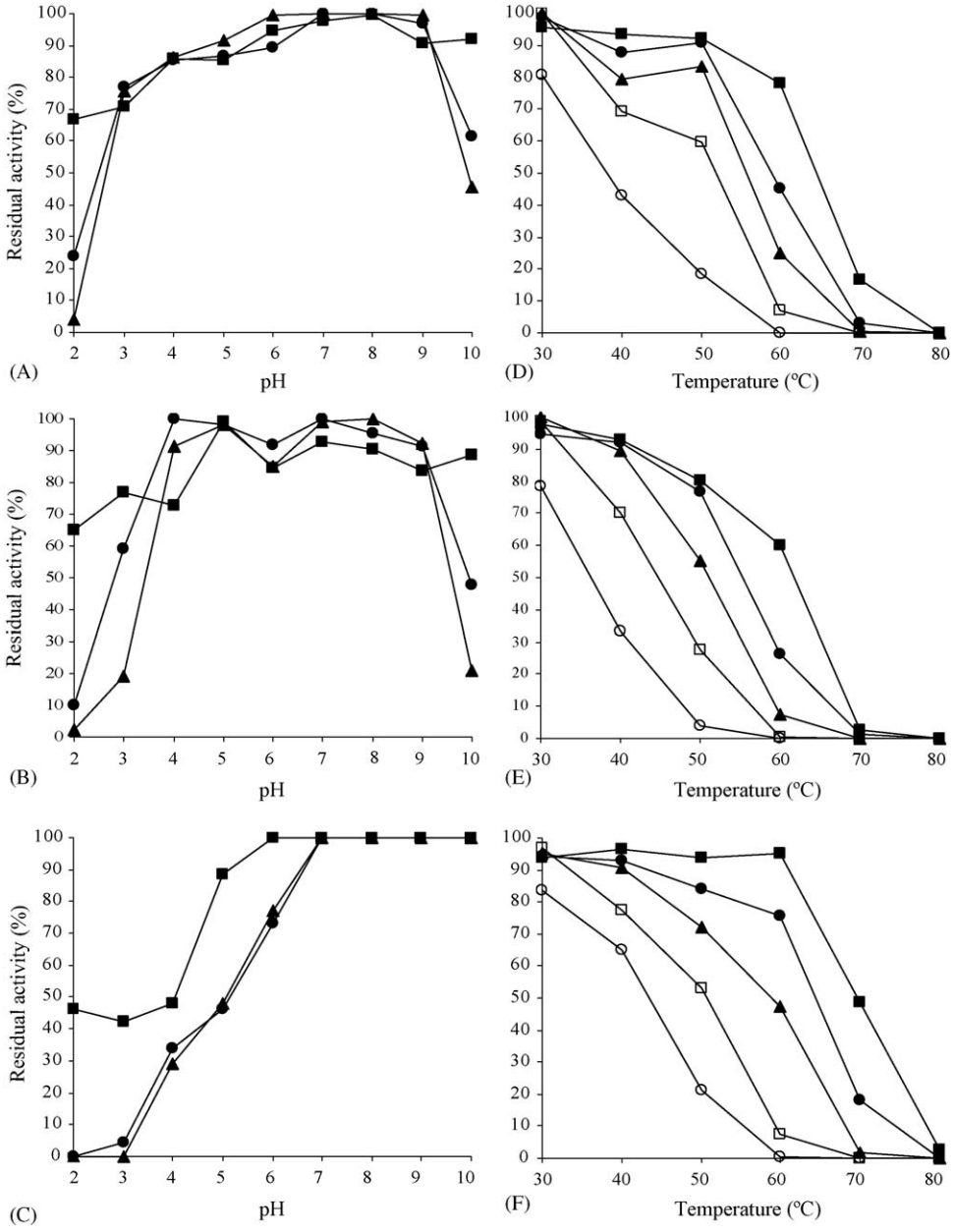

Fig. 1. Comparison of pH stability (left) and temperature stability (right) of laccases from *P. cinnabarinus* (A and D), *T. villosa* (B and E) and *M. thermophila* (C and F) laccases. Enzyme stability towards pH was estimated at 24 °C in 100 mM citrate–phosphate–borate buffer (pH 2–10) after 0 (■), 8 (●) and 24 h (▲) of incubation. Thermostability was calculated after incubation of laccases in 100 mM citrate–phosphate–borate buffer (pH 4 for *P. cinnabarinus* and *T. villosa* laccases, and pH 7 for *M. thermophila* laccase) at different temperatures (30–80 °C), during 1 (■), 2 (●), 4 (▲), 8 (□) and 24 h (◯).

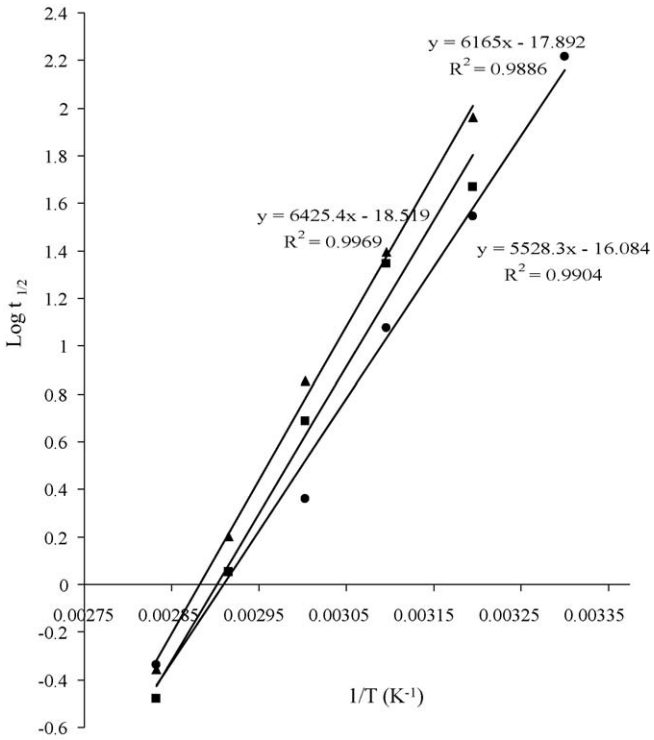

Fig. 2. Arrhenius plots showing the straight-line relationships between the 50% deactivation times ($t_{1/2}$) of *M. thermophila* (▲), *P. cinnabarinus* (■) and *T. villosa* (●) laccases compared with the exponential of the inverse of temperature, 1/*T* (K^{-1}).

was completely inactivated after 1 h at 70 °C, whereas *M. thermophila* and *P. cinnabarinus* laccases showed, respectively, 50% and 20% of the initial activity. Thermal deactivation of the three laccases followed the exponential decay model as a function of time. The deactivation constants (K_d) for each temperature, obtained from the slopes of the adjusted straight-lines, were notably higher in the case of *T. villosa* laccase for temperatures above 50 °C. The enzymes half-life ($t_{1/2}$), which corresponds to the time period necessary for the residual enzyme activity to decrease to 50% of its initial value at a certain temperature, were calculated and Arrhenius plots were drawn for comparison of the effect of temperature on the $t_{1/2}$ of each laccase (Fig. 2), permitting the calculation of the corresponding deactivation energies (E_d). The E_d of the three laccases are shown in Table 1 together with their respective T_{50}, temperature needed for 50% deactivation of the enzyme (in 1 h). The values for both E_d and T_{50} were higher for *M. thermophila* laccase > *P. cinnabarinus* laccase > *T. villosa* laccase, showing the order of their thermal stabilities.

The three laccases showed their optimal activity in the range of pH 3–4.5 (Fig. 3A), although *M. thermophila* laccase differed from the other laccases in its capacity to work at pH 6.5–8. The activities of the *M. thermophila* and *P. cinnabarinus* laccases increased with temperature in the range of 40–90 °C, however this was not the case with the *T. villosa* laccase (Fig. 3B).

Table 1
Deactivation energies (E_d) and temperatures needed for 50% deactivation (T_{50}) of laccases from *M. thermophila*, *P. cinnabarinus* and *T. villosa*

Laccases	E_d (kJ mol^{-1})	T_{50} (1 h) (°C)
M. thermophila	123	70
P. cinnabarinus	118	66
T. villosa	106	61

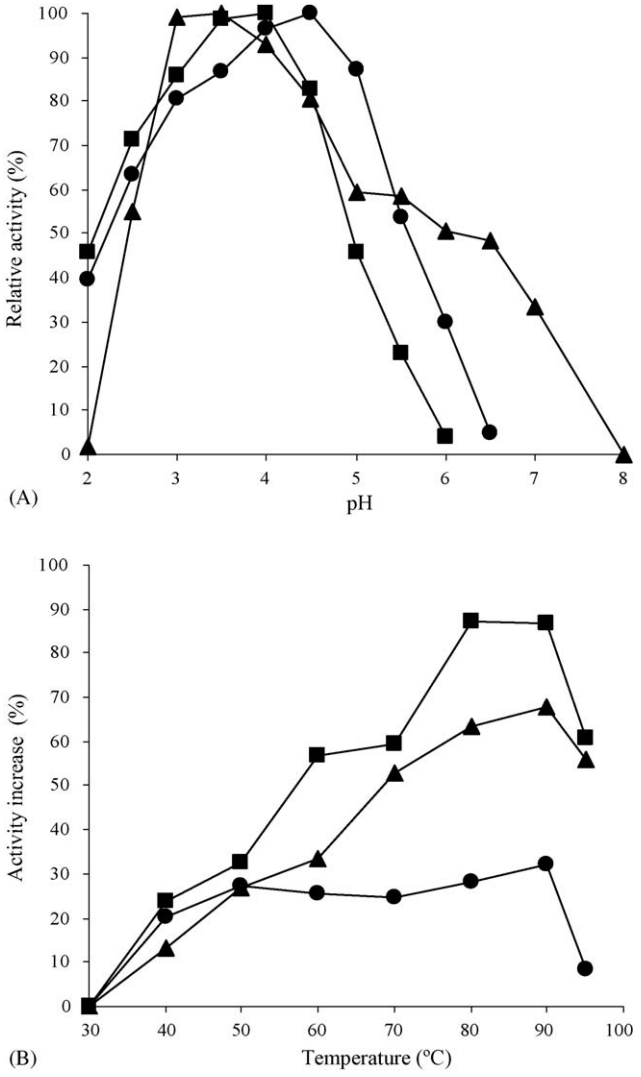

Fig. 3. Optimal pH (A) and temperature (B) of *P. cinnabarinus* (■), *T. villosa* (●) and *M. thermophila* (▲) laccases. Laccase activity as a function of pH was normalized to the optimum pH. Increase of laccase activity as a function of temperature was referred to the activity found at 30 °C.

Comparison of HBT oxidation by the three laccases showed that *P. cinnabarinus* and *T. villosa* laccases efficiently oxidized HBT whereas laccase from *M. thermophila* did not. The two former laccases showed similar HBT oxidation rates, slightly higher for *P. cinnabarinus* laccase. When assaying 3 mM HBT, the molar concentration to be utilized in the bleaching assays, the activity of *P. cinnabarinus* and *T. villosa* laccases decreased to values around 16–18% with respect to the activities obtained using 100 mM HBT (a concentration near saturating conditions). The activities with 3.3 mM HBT were maintained during the first 3 h of reaction. On the contrary, no HBT oxidation could be observed by *M. thermophila* laccase throughout 6 h period. It was possible to conclude that *P. cinnabarinus* laccase exhibited

better properties than the two commercial laccases for enzymatic pulp bleaching, and therefore, it was selected for TCF bleaching of eucalypt kraft pulp in the presence of HBT.

Oxidation of HBT by *P. cinnabarinus* laccase is notably poorer than ABTS oxidation and requires significantly higher substrate concentrations to saturate the enzyme, according to the respective K_m, in the mM range for HBT and μM for ABTS [11]. For this reason, the presence of HBT might not interfere in the measurement of laccase activity using ABTS as substrate (see Section 3.2).

3.2. Laccase inactivation

Enzyme inactivation by the oxidized species of some mediators is a general drawback of the laccase-mediator systems. Before initiating the bleaching experiments with eucalypt pulp, evolution of the activity of *P. cinnabarinus* laccase was followed along 12 h incubation period, in the presence or absence of HBT. Fifty percent of residual laccase activity remained after 12 h at 50 °C in the absence of HBT. However, in the presence of 3.3 mM HBT (the molar concentration to be used in pulp treatments), laccase was strongly inactivated, and only 9% activity could be recovered after 12 h of incubation (Fig. 4A). The most important inactivation of laccase by HBT (more than 50%) was produced during the first 4 h. In order to determine the extension of inactivation of the enzyme that would be produced during pulp treatment with laccase–HBT, residual activity was measured in the presence of 1.5% (w/w) HBT (equal to 3.3 mM) and kraft pulp (Fig. 4B). In the presence of pulp, the inactivation of laccase by HBT was lowered around 20% compared to results obtained when pulp was not present.

3.3. Effect of the enzymatic treatment

The capability of the laccase–HBT system to delignify and bleach eucalypt kraft pulp was determined by treating brown pulp with *P. cinnabarinus* laccase in the presence of HBT. Laccase treatment (12 h) was assayed with two different HBT concentrations (1.5% and 3% w/w) and followed by alkaline extraction. The kappa number (an estimation of the lignin content in pulp), brightness and viscosity (an estimation of the integrity of cellulose) of the resulting pulps are shown in Table 2. The enzymatic treatment produced four points decrease of kappa number and 6% ISO brightness increase (with respect to the control pulp without enzyme).

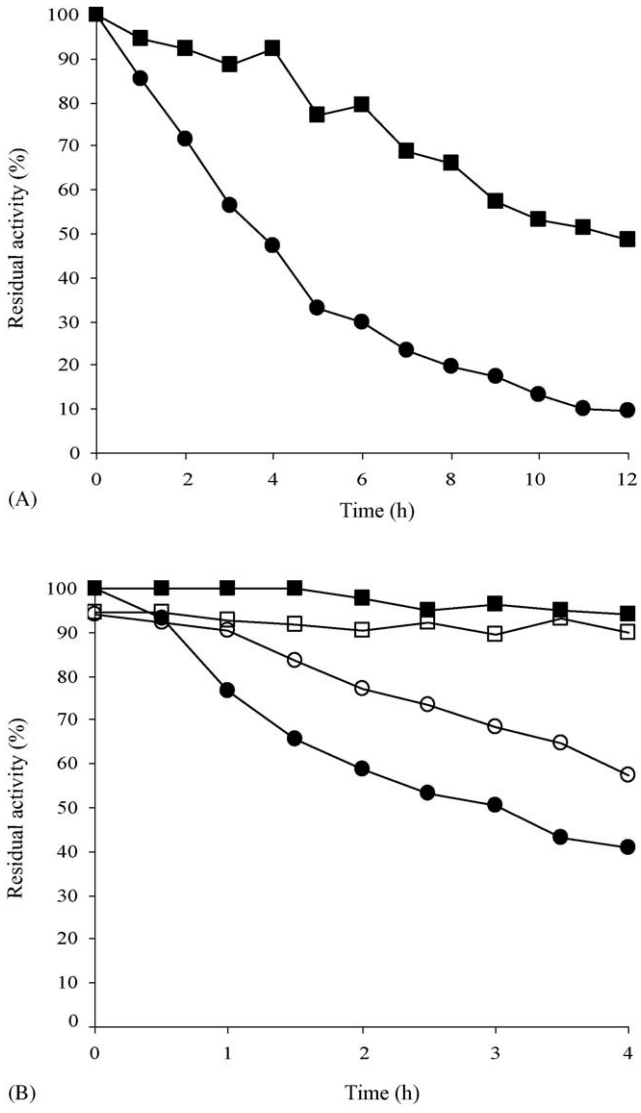

Fig. 4. Evolution of activity of *P. cinnabarinus* laccase in the absence (■) or presence (●) of HBT during 12 h (A) and effect of pulp addition to the inactivation of laccase by HBT during 4 h (B); Residual activities after 4 h incubation of laccase alone (■), laccase with HBT (●), laccase in the presence of brown eucalypt kraft (□) and laccase with HBT in the presence of brown eucalypt kraft pulp (○), are indicated.

A hydrogen peroxide treatment after the enzymatic treatment significantly extended pulp bleaching attaining 16% ISO brightness increase (whereas the four-point reduction of kappa number was maintained). Final viscosity was 120 points lower than in the

Table 2
Properties of eucalypt brown pulp after laccase–HBT treatment (using two different concentrations) followed by alkaline extraction or alkaline hydrogen peroxide treatment

	After alkaline extraction			After H_2O_2 treatment		
	Brightness (%ISO)	Kappa number	Viscosity (mL/g)	Brightness (%ISO)	Kappa number	Viscosity (mL/g)
Initial brown pulp	38.6 ± 0.1	14.4 ± 0.1	1227 ± 1.0	54.5 ± 0.2	11.5 ± 0.1	1056 ± 11.0
Control pulp[a]	45.4 ± 0.0	13.1 ± 0.0	1249 ± 0.0	57.6 ± 0.0	11.1 ± 0.0	1051 ± 0.0
Laccase + 1.5% HBT	51.4 ± 0.0	8.9 ± 0.0	1021 ± 6.0	73.6 ± 0.4	7 ± 0.2	932 ± 23.0
Laccase + 3% HBT	46.7 ± 0.8	9.6 ± 0.4	1056 ± 4.0	70.2 ± 1.1	7.1 ± 0.4	941 ± 11.0

[a] Control pulp: pulp treated under the same conditions (1.5% HBT, pH 4, 50 °C, and 12 h) without laccase.

Table 3
Effect of laccase–HBT treatment on oxygen-delignified eucalypt pulp, and after subsequent chelation and peroxide treatment

	After enzymatic treatment			After chelation and H_2O_2 treatments		
	Brightness (%ISO)	Kappa number	Viscosity (mL/g)	Brightness (%ISO)	Kappa number	Viscosity (mL/g)
Initial oxygen pulp	61.0 ± 0.0	9.7 ± 0.0	963 ± 0.0	85.8 ± 0.0	6.3 ± 0.0	771 ± 0.0
Control pulp[a]	63.2 ± 0.0	8.5 ± 0.0	963 ± 0.0	86.5 ± 0.9	6.2 ± 0.0	649 ± 28.0
Laccase–HBT	67.0 ± 0.0	7.0 ± 0.0	886 ± 0.0	90.1 ± 0.4	5.0 ± 0.1	589 ± 13.0

[a] Control pulp: pulp treated under the same conditions (1.5% HBT, pH 4, 50 °C, and 12 h) without laccase.

control pulp. The decrease of viscosity was, however, lesser than obtained after alkaline extraction of the laccase-treated pulp. No improvement of the above pulp properties was found when HBT concentration was increased to 3% (w/w). Therefore, 1.5% (w/w) HBT was used in the subsequent treatments. Finally, it was observed that laccase activity decreased during pulp treatment, being 44% of the initial activity after 4 h treatment and 19% after 12 h.

3.4. Optimization of pulp treatment conditions

Laccase-mediator treatment was combined with additional oxygen, chelation and peroxide treatments to improve the final properties of the eucalypt pulps. Laccase–HBT was firstly applied to an oxygen-delignified pulp and followed by chelation (to remove metals destroying H_2O_2) and peroxide treatment. Pulp brightness, kappa number and viscosity after the enzymatic treatment and after full bleaching are presented in Table 3. Final pulp brightness and kappa number were markedly better than obtained in the previous bleaching where only laccase-mediator and peroxide treatments were applied (Table 2), but the delignification produced by the enzymatic treatment was lower. In a similar way, loss of pulp viscosity (60-points decrease) was lower when treating oxygen-delignified pulp than when using brown pulp (referred both to the controls without enzyme). On the other hand, residual laccase activity was lower than in brown pulp treatment (35% of the initial activity after 4 h, and 12% after 12 h).

In order to determine the best point to incorporate the enzymatic treatment into an industrial-type TCF bleaching sequence (consisting of oxygen, chelation and peroxide stages), the performances of laccase–HBT treatment before or after the oxygen delignification step were compared. Industrial brown and oxygen-delignified eucalypt kraft pulps were used, respectively. Comparison of the resulting final pulp properties showed similar decrease of viscosity and similar final kappa number in both cases, but the highest brightness was obtained when the enzymatic treatment was applied after the double oxygen stage (Table 4).

Increase of temperature and reduction of reaction time are important to incorporate the enzymatic treatment into industrial bleaching processes where reaction times are short and temperatures high. Thus, laccase–HBT treatment was assayed at shorter times (from 1 to 8 h) using two different temperatures (50 and 65 °C), and the evolution of final pulp brightness and kappa number was monitored. Fig. 5 shows the final pulp properties after different times of laccase-mediator treatment (of oxygen delignified pulp) followed by peroxide bleaching. The most significant enhancement of pulp properties by the laccase–HBT system was produced during the first 2 h of treatment, and no significant differences in final pulp properties were observed when the enzymatic treatment was carried out at 65 °C instead of 50 °C.

Table 4
Comparison of final pulp properties when applying the laccase–HBT treatment before or after oxygen delignification, followed (in both cases) by chelation and peroxide treatments

	Brightness (%ISO)	Kappa number	Viscosity (mL/g)
Control[a]-oxygen	85.1	6.4	914
Laccase-oxygen	87.5	5.3	866
Oxygen-control[a]	85.1	5.8	728
Oxygen-laccase	90.3	5	682

[a] Control: pulp treated under the same conditions of the enzymatic treatment (1.5% HBT, pH 4, 8 h and 50 °C) without laccase.

Selectivity of the laccase–HBT treatment was calculated on the basis of viscosity preservation during the delignification (decrease of kappa number) and bleaching (increase of brightness) process, as regards to initial pulp. Selectivity referred to kappa decrease (Slc_K) or brightness increase (Slc_B), was higher when laccase–HBT system was applied onto brown pulp (Fig. 6). Moreover, laccase–HBT system was, in general, more selective during short-term treatments (first 2 h).

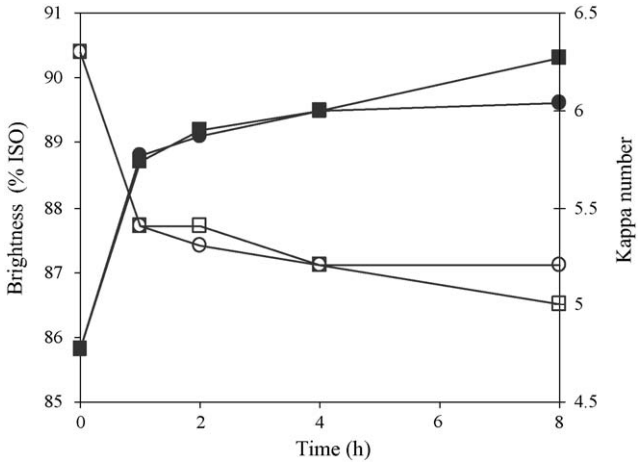

Fig. 5. Optimization of O–O–L–Q–PoP sequence: Evolution of brightness (%ISO) at 50 °C (■) or 65 °C (●) and kappa number at 50 °C (□) or 65 °C (○) during 8 h of treatment, using oxygen-delignified eucalypt kraft pulp in presence of *P. cinnabarinus* laccase (20 U/g) plus 1.5% of HBT.

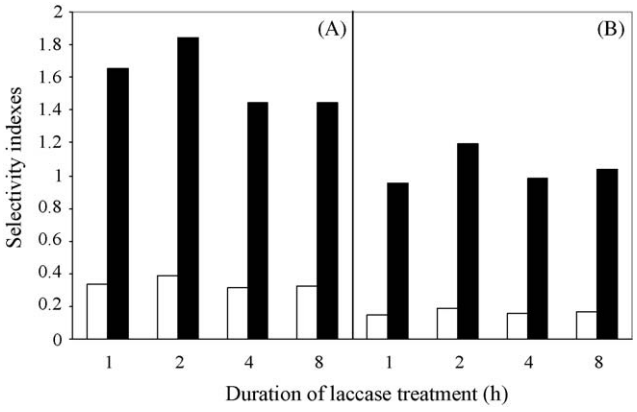

Fig. 6. Selectivity indexes estimated as kappa number decrease (Slc_K, in white bars) or brightness increase (Slc_B, in black bars) with regards to viscosity preservation, during the laccase–HBT treatment (1–8 h) of brown pulp (A) or oxygen-delignified pulp (B), followed in both cases by chelation and peroxide treatments.

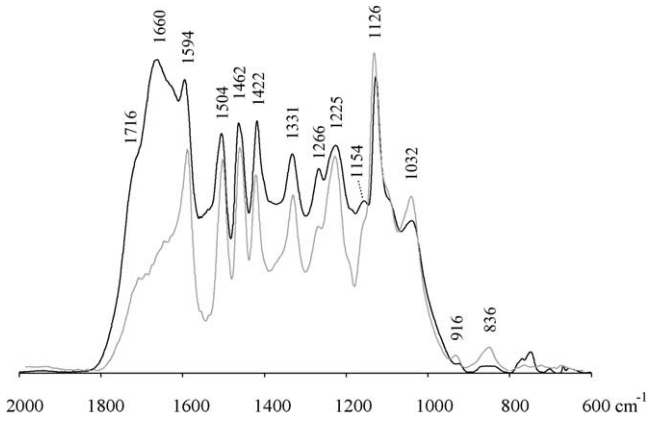

Fig. 7. Modification of residual lignin from eucalypt kraft pulps by laccase–HBT treatment. The 2000–600 cm^{-1} region of the FTIR spectra of the residual lignins from the oxygen delignified pulp after the laccase-mediator treatment (in black) and its corresponding control (in grey) are shown.

3.5. Lignin in the bleached pulps

It is known that the presence of hexenuronic acid groups in pulps interfere the estimation of lignin content by kappa number measurement, increasing the apparent kappa number (K_a) values. The content of hexenuronic acids decreased slower than the content of lignin (estimated by K_r) during standard pulp bleaching. In this way, the contribution of hexenuronic acids to kappa number increased from eucalypt brown pulp (24%) to TCF bleached pulp (49%), especially when an enzymatic treatment being selective for lignin was included (54%).

Residual lignins from the laccase–HBT treated pulps were isolated and analyzed by FTIR spectroscopy for comparison. The FTIR spectra of eucalypt kraft pulps showed the typical lignin patterns, with the aromatic bands around 1600 cm^{-1} and the characteristic triplet between 1422 and 1504 cm^{-1} [12], and presented a higher intensity of signals assigned to syringyl (S) type lignin units (1331 cm^{-1} band) than to guaiacyl (G) units (1266 cm^{-1} shoulder) [13]. Strong modification of lignin was observed after the laccase-mediator treatment compared with the corresponding control (oxygen-delignified pulp treated under the same conditions without enzyme) (Fig. 7). Important increases of the 1660 cm^{-1} band corresponding to carbonyl groups conjugated with the aromatic ring was observed. Decrease of lignin S/G ratio was also observed by the increase of the band at 1266 cm^{-1} (G units) and the slight decrease of that at 1331 cm^{-1} (S units), together with changes in other bands.

4. Discussion

The three laccases under study showed optimal activity in the acidic pH range as described for other fungal laccases [14]. However, the capability of *M. thermophila* laccase to work at alkaline pH together with its thermal stability would be excellent properties for the enzyme to be applied on industrial pulp bleaching. Nevertheless, its extremely low activity on HBT, in agreement to its reported low redox potential (0.45 V) [6], limits the applicability of *M. thermophila* laccase using this mediator. On the contrary, *P. cinnabarinus* laccase has one of the highest redox potentials (0.8 V) described for a fungal laccase [11] enabling rapid oxidation of HBT. Moreover, *P. cinnabarinus* laccase posses high thermal stability, with T_{50} and E_d values close to those observed for the highly thermostable *M. thermophila* laccase. The high-redox potential *T. villosa* laccase showed, by contrast, low thermostability (and activity at high temperature). For the above reasons, the *P. cinnabarinus* laccase would be the enzyme of choice for biotechnological industrial applications as enzyme-aided TCF pulp bleaching.

HBT radicals inactivate laccase by oxidation of aromatic amino acid residues on the protein surface [15]. In the present study, the presence of pulp reduced the inactivation of *P. cinnabarinus* laccase by HBT because pulp would act as a reducing substrate for the nitroxyl radicals [11]. This protective effect of pulp could be seen despite a decrease around 10–15% of initial laccase activity was produced, probably due to the adsorption of the enzyme to the pulp surface. Furthermore, in the presence of brown pulp laccase was inactivated by HBT radicals in a lesser extend than in the presence of oxygen-delignified pulp, probably because the higher lignin content would contribute to faster consumption of HBT free radicals [16], enabling also higher delignification rates [2]. Laccase–HBT system produced, in fact, higher delignification rates when applied to brown pulp (32%) than when applied to double oxygen-delignified pulp (18%) (values referred to their corresponding controls without enzyme). The decrease of lignin content (kappa number from 15.8 to 9.7) occurred during the oxygen treatment, would limit the degradation to be produced during the following laccase treatment. Moreover, values from Table 2 include alkaline extraction of pulp after laccase–HBT treatment, removing the lignin degradation products that could interfere in the kappa number measurement.

On the other hand, a hydrogen peroxide stage after the enzymatic treatment significantly enhanced pulp brightness and lowered the final kappa number. However, and by contrast to results obtained with non-wood fibres wherein consecutive laccase–HBT and alkaline peroxide treatments gave excellent final pulp properties [17], the resulting values should be

considerably improved. Combination of laccase–HBT with oxygen, peroxide and chelation treatments in the O–O–L–Q–PoP TCF industrial-like bleaching sequence gave the best final pulp properties. However, the selectivity of the whole bleaching process was higher when the enzyme was applied before the double oxygen. Thus, laccase–HBT is more selective delignifying pulp than oxygen at high temperature, and what is more important, this selectivity is transferred and maintained all along the bleaching process. Nevertheless, the higher final brightness attained after O–O–L–Q–PoP proves that the laccase–HBT treatment particularly facilitates the subsequent bleaching with hydrogen peroxide [5]. Similar brightness and kappa values have been described in TCF bleaching of eucalypt kraft pulps but two laccase stages (L(EO)LQPO) were needed and the viscosity loss was notably higher [18].

A short and high-temperature laccase-mediator stage would be advantageous for its industrial application from two points of view: (i) decreasing the reactor volume required for a more extended treatment period; (ii) reducing the need to decrease the pulp temperature between two high-temperatures stages (e.g. oxygen and chelation stages). It was demonstrated here that the laccase-mediator treatment could be performed with the same efficiency at shorter times (2 h) and higher temperatures (65 °C). Moreover, laccase–HBT system seems to be more selective for bleaching and delignifying eucalypt pulp during the first 2 h.

The kappa number of chemical pulps is usually used to show the extent of delignification in pulping and bleaching processes. However, the kappa number measured by standard (ISO) methods not only reflects the lignin content but also the carbohydrate structures sensitive to oxidation by permanganate, mainly hexenuronic acid residues [19]. These hexenuronic acids, derived from the 4-*O*-methylglucuronic acid residues forming xylan branches, are produced in notable amounts during kraft cooking [20] and increase the kappa number of these pulps. Therefore, the kappa number recalculated (K_r) by subtracting the hexenuronic acids contribution reflects more realistically the lignin content of pulps. In the present study a contribution of hexenuronic acids to kappa number from 24% to 54% could be observed. The percentage increased when lignin content decreased, being a major contributor in the bleached pulps, in agreement with related studies [21]. Consequently, the real delignification rate produced after laccase–HBT and peroxide stages should be significantly higher than the estimated by the apparent kappa number (K_a), since around 50% of the kappa number value is not due to lignin. In fact the final delignification of pulp after successive oxygen, laccase-mediator, chelation and peroxide stages was 68% when estimated from total kappa, but up to 81% when estimated by the real kappa number (K_r) (both referred to initial brown pulp). Pulps with high hexenuronic acids content are harder to bleach by chemical reagents [22] and exhibit a higher tendency to brightness reversion [23,24].

Study of the chemical modification of lignin by laccase–HBT treatment requires the proper isolation of the residual lignins from the so treated-pulps [10]. The higher intensity of S-units versus G-units signals found in the FTIR spectrum of the control pulp lignin is in concordance with the higher abundance of S units in eucalypt [25–27]. The decrease of S/G ratio in lignin isolated from treated-pulp revealed an easier degradation of S units, less condensed than G units, by the laccase-mediator treatment. The notable increase of the band around 1660 cm^{-1} is probably due to the formation of conjugated carbonyl groups (or quinones) by oxidation of lignin side-chains during the enzymatic treatment of pulp. Oxidative degradation of lignin side-chains has been reported during lignin biodegradation [28]. The formation of quinones during reaction of laccase–HBT with lignin model compounds [29] and increase of carbonyl FTIR signals in lignins from pulps treated with laccase–HBT have been also described [30].

On the light of the results presented here, and with the aim to proceed towards the application of laccase-mediator in eucalypt kraft pulp bleaching, short laccase–HBT treatments in reactor under industrial-like conditions and optimization of enzyme-aided TCF bleaching sequence, have been investigated. Evaluation of pulp and paper properties and study of the chemical modifications of lignin produced by the enzymatic treatment integrated in the industrial TCF sequence, are currently being carried out [31].

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