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Holly Ravalason, Frédérique Bertaud, Isabelle Gimbert, Valérie Meyer, Katia Ruel, et al.. Laccase/HBT and laccase-CBM/HBT treatment of softwood kraft pulp: impact on pulp bleachability and physical properties. *Bioresource Technology*, 2012, 121, pp.68-75. 10.1016/j.biortech.2012.06.077 . hal-01268169

HAL Id: hal-01268169

<https://hal.science/hal-01268169>

Submitted on 29 May 2020

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Laccase/HBT and laccase-CBM/HBT treatment of softwood kraft pulp: Impact on pulp bleachability and physical properties

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A B S T R A C T

Pycnoporus cinnabarinus laccase and a chimeric laccase-CBM were applied in softwood kraft pulp biobleaching in the presence of 1-hydroxybenzotriazole (HBT). The presence of CBM could enhance the laccase biobleaching potential as a decrease in the enzymatic charge and chlorine dioxide consumption, as well as an increase in pulp brightness were observed. Laccase/HBT treatment could be improved by increasing oxygen pressure from 1 to 3 bar and pulp consistency from 5% to 10%. Conversely, under the same conditions, no improvement of laccase-CBM/HBT treatment was observed, indicating a different behavior of both systems. However, laccase-CBM/HBT treatment led to a better preservation of pulp properties. This effect was probably due to fiber surface modifications involving the action of the CBM. Transmission electron microscopy examination of pulp fibers indicated a retention of laccase-CBM inside the pulp fibers due to CBM binding and an increased external microfibrillation of the fibers due to enzymatic treatments.

Keywords:

Laccase
CBM
Biobleaching
Softwood kraft pulp
Chlorine dioxide

1. Introduction

Bleached kraft pulp production is a dominant process contributing to more than 50% of the global pulp production. Traditionally, bleaching was performed using chlorine until it was shown that chlorine bleaching could lead to the production of halogenated organic compounds, measured as adsorbable organic halides (AOX), known to be particularly toxic substances (Muna and Sreerishnan, 2001). Therefore, due to environmental concerns, pulp mills have

gradually eliminated chlorine from bleaching sequences. Nowadays, in most bleaching processes, chlorine dioxide is used as a substitute for chlorine, in elemental chlorine free (ECF) bleaching sequences, and demand for the use of non-chlorine based bleaching agents, such as oxygen, ozone or hydrogen peroxide, has increased. New technologies for pulp processing, such as enzyme-based pulp treatments, have also been developed to further decrease the environmental impact of pulp bleaching and the production costs. Indeed, some pulp mills have already included a hemicellulase stage, such as a xylanase stage, in their bleaching sequence (Beg et al., 2001). It was shown that xylanase pre-treatment of pulp could lead to a significant decrease in bleaching chemical consumption, by removing hemicelluloses from pulp, thus enhancing lignin accessibility to the bleaching agents. However, as the xylanase

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action does not directly target lignin, its potential in pulp biobleaching is limited. The use of lignin degrading enzymes, as efficient delignification agents, has therefore been investigated. In particular, laccases were shown to have a great potential for pulp delignification. Laccases (EC 1.10.3.2) are multicopper oxidases able to catalyze the oxidation of various phenolic compounds with the concomitant reduction of oxygen to water. Laccases are only able to directly oxidize phenolic lignin moieties. However, lignin degradation can be extended to the non-phenolic lignin units in the presence of a low molecular weight compound, acting as a redox mediator (Bourbonnais and Paice, 1990). Laccase/mediator systems (LMS) have been investigated in numerous studies for pulp delignification. *Pycnoporus cinnabarinus* laccase was shown to be particularly efficient for this kind of application (Herpoël et al., 2002; Sigoillot et al., 2004; Ibarra et al., 2006b). In a previous study, the fusion of a family 1 carbohydrate-binding-module (CBM), referring to the CAZy database (Cantarel et al., 2008; <http://www.cazy.org>), to the *P. cinnabarinus* laccase was shown to greatly improve the biobleaching potential of the enzyme (Ravalason et al., 2009). It was suggested that conferring a binding capability to the laccase could promote enzyme and substrate proximity, leading to an enhancement of laccase efficiency. Here, the impact of *P. cinnabarinus* laccase and laccase-CBM on softwood kraft pulp treatment, in the presence of 1-hydroxybenzotriazole (HBT) was further investigated. Pulp bleachability following the enzymatic treatments, as well as physical properties of the bleached pulp, were also analyzed. The interaction between the enzymes and the fibers and the resulting ultrastructural modifications of the pulp fibers were observed by transmission electron microscopy (TEM).

2. Methods

2.1. Chemicals and enzymes

2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonate (ABTS) and 1-hydroxybenzotriazole (HBT) were purchased from Sigma-Aldrich (Lyon, France). *P. cinnabarinus* laccase was obtained from cultures of the monokaryotic strain ss3, now deposited as BRFM137 (Banque de Ressources Fongiques, Marseille, France), in 250-ml flasks, according to Herpoël et al. (2000). Laccase-CBM was obtained from cultures of an *Aspergillus niger* recombinant strain (Ravalason et al., 2009). Cultures were performed in 250-ml flasks according to Record et al. (2002). Laccase activity was measured by monitoring A_{420} ($\epsilon_{420} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) with respect to the rate of oxidation of 500 μM ABTS in sodium tartrate buffer (50 mM, pH 4.0) at 30 °C. Activities were expressed in international units (IU), 1 unit being defined as the amount of laccase that oxidizes 1 μmol of substrate per min. The experiments were performed in triplicate and the standard deviation was lower than 10% of the average value.

2.2. Enzymatic treatment of softwood kraft pulp

A mixed softwood kraft pulp (kappa number: 22.7 and brightness: 29.9% ISO) was provided by Fibre Excellence's Tarascon mill (France).

2.2.1. Laccase-CBM binding to the pulp

Laccase-CBM was bound to the pulp by mixing 1400 U of enzyme with 50 g (dry weight) of pulp for 2 h at 45 °C in a water bath. The pulp consistency was adjusted to 5% using sodium acetate buffer (50 mM, pH 5.0). After incubation, the supernatant was separated from the pulp by filtration for residual laccase activity measurement. The fraction of enzyme bound to the substrate was calculated by subtracting the residual activity from the activity of

laccase-CBM incubated under the same conditions but without substrate. Under those conditions 5 U of laccase-CBM were bound per gram of pulp (dry weight). After the binding step the pulp was thoroughly washed with water to remove the unbound fraction. As a control, pulp was treated under the same conditions with *P. cinnabarinus* laccase.

2.2.2. Laccase/HBT and Laccase-CBM/HBT treatments

Laccase/mediator system (LMS) treatments were further carried out in a 4-L rotating laboratory pressure reactor. 1.5% odp HBT was added to 50 g (dry weight) of pulp pre-treated with laccase or laccase-CBM. For the laccase/HBT treatment, 20 U of laccase were further added per gram of pulp. The pulp consistency was adjusted to 5% or 10% using sodium acetate buffer (50 mM, pH 4.0). The reactor was filled with oxygen to a pressure of 1 or 3 bar and the temperature was adjusted to 45 °C. After 3 h treatment, the pulp was thoroughly washed with water to stop the reaction. An abiotic control experiment was performed under the same conditions in the absence of enzyme and mediator (LMS abiotic control).

2.3. ECF bleaching of pulp

Subsequently to the enzymatic treatments, the pulps were submitted to an alkaline extraction stage (E) at 10% pulp consistency, using 1.5% odp NaOH at 70 °C for 1 h.

The pulps were further subjected to a conventional ECF bleaching sequence as $D_0E_1D_1E_2D_2$ (D: chlorine dioxide stage and E: alkaline extraction stage). The bleaching stages were carried out in sealed polyethylene bags placed in water baths maintained at the required temperature. Chemical charges (expressed as% of dry weight of pulp) were as follows: for the D_0 stage, the ClO_2 charge was calculated on the basis of the kappa number of the pulp using the following relationship: $\% \text{ClO}_2 = (\text{kappa factor}) \times (\text{kappa number})/2.63$. Two kappa factor values (0.18 and 0.21) were applied for each pulp. The ClO_2 charge applied in the D_1 stage was half of the D_0 stage charge. 0.3% ClO_2 was applied in the D_2 stage charge. The NaOH charges were 1.5% in E_1 and 0.8% in E_2 . The bleaching stages were performed at 10% consistency and 70 °C, except for the D_0 stage which was carried out at 55 °C. The duration was 1 h for the D_0 and E stages and 2 h for the D_1 and D_2 stages. Pulp bleachability was compared to the initial pulp submitted to the same bleaching sequence without the enzymatic treatment step (ECF bleaching control).

2.4. Analysis of pulp properties

Pulp kappa number was determined according to standard ISO 302:1981.

Pulp brightness was evaluated according to standard ISO 2470:1999.

Residual amounts of ClO_2 were measured by titration with 0.1 N sodium thiosulfate.

Pulp color was measured according to international standard ISO 5631:2000. The measurements were performed in quadriplate and the standard deviation was lower than 5% of the average value. Results were expressed in the three-dimensional CIE Lab color system (L^* , a^* , b^*). L^* corresponds to lightness (black/white coordinate), a^* to the green/red coordinate and b^* to the blue/yellow coordinate. Color difference, taking the unbleached kraft pulp as a reference, was calculated according to the following formula (Ohno, 2000):

$$\Delta E_{ab}^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (1)$$

Intrinsic viscosity was determined according to standard ISO 5351-1:1981.

Bleached pulp fiber morphology was measured using a MorFi LB-01 Fiber Analyzer (Techpap, France), on 0.3 g (dry weight) of pulp. Analyses were performed in duplicate.

Bleached pulp physical properties were measured according to the following Tappi standards: T404 cm92 (tensile index) and T496 cm85 (tear index), on Rapid-Köthen handsheets manufactured according to ISO 5269-2:2004 standard.

2.5. Transmission electron microscopy

Pulp samples were fixed by two methods, depending on the kind of analysis subsequently performed.

To visualize laccase and laccase-CBM by immunolabeling, pulp samples were taken at the end of the enzymatic treatments, washed with distilled water and fixed in a freshly prepared mixture of 0.2% glutaraldehyde (v/v), 2% paraformaldehyde (w/v) in 0.05 M phosphate buffer (pH 7–7.2), for 2 h at room temperature and overnight at 4 °C. For lignin and micromorphological analysis, samples were taken at the end of the first alkaline extraction stage and fixed with KMnO_4 2.5% (w/v) in water during 1.5 h (Kerr and Goring, 1975).

All the samples were further submitted to dehydration in a series of aqueous solutions of increasing ethanol concentration. Progressive infiltration with LR White resin (Electron Microscopy Sciences, Hatfield, USA) was carried out by serial incubation in ethanolic solutions of increasing LR White resin concentration followed by several incubations in 100% LR White. The pulp samples were then embedded in gelatine capsules and the resin allowed to polymerize for 24 h at 50 °C. Ultrathin sections (50 nm thickness) were prepared and either transferred on copper-grids for direct examination after KMnO_4 staining, or collected on plastic rings for immunolabeling. For the detection of laccase and laccase-CBM, the sections were floated on several dilutions of specific antibodies raised against *P. cinnabarinus* laccase (Record et al., 2002) in 10 mM Tris buffered saline (500 mM NaCl) to determine the optimal ratio of labeling and background (Ruel et al., 1994). The secondary marker was Auroprobe EM protein A conjugated with 5 nm colloidal gold (BB International, Cardiff, UK). The gold particles were further enhanced using a silver enhancing kit (Amersham Bioscience, Buckinghamshire, UK). Finally, the thin-sections were transferred on copper-grids and post-stained with 2.5% aqueous uranyl acetate. To verify the specificity of the antibodies and the method used, control pulp (LMS abiotic control) was treated under the same conditions. All the samples were further examined with a transmission electron microscope (Philips CM 200-Cryo) at an accelerating voltage of 80 kV and pictures taken on 4489 Kodak film plates.

3. Results and discussion

3.1. Laccase/HBT and laccase-CBM/HBT biobleaching of softwood kraft pulp

In a previous study, it was shown that the fusion of a CBM to the *P. cinnabarinus* laccase could greatly enhance the delignification potential of the enzyme and led to a decrease in the enzymatic charge required for efficient softwood kraft pulp biobleaching (Ravalason et al., 2009). Indeed, compared to a laccase/HBT treatment with 20 U of enzyme per gram of pulp and 3% HBT, laccase-CBM/HBT treatment with 5 U/g of laccase-CBM led to an increase in pulp brightness after a DEDED bleaching sequence and a decrease in ClO_2 consumption.

Here, laccase/HBT and laccase-CBM/HBT treatments of softwood kraft pulp were performed under the same conditions but HBT charge was decreased to 1.5%. Compared to the unbleached

pulp, a similar decrease in kappa number, of around 15%, was measured after both enzymatic treatments and an alkaline extraction stage (Fig. 1A). The decrease in pulp kappa number was similar to that observed using a 3% HBT charge (Ravalason et al., 2009). An ECF bleaching sequence was further applied to the pulps, and bleaching responses were evaluated by measuring the final brightness reached and the ClO_2 consumed for each pulp. Pulp bleachability was defined as the amount of ClO_2 consumed to obtain a target brightness (Fatehi et al., 2009). To allow comparison, two ClO_2 charges, adjusted to pulp kappa numbers and expressed as kappa factors, were applied to the pulps. Final brightness levels, by applying an initial kappa factor of 0.21, were around 85% ISO for the ECF bleaching control, the LMS abiotic control and the laccase/HBT treated pulp (Fig. 1B). The final brightness was enhanced after the laccase-CBM/HBT treatment and reached a value of 85.6 and 86.5% ISO for initial kappa factors of 0.18 and 0.21, respectively. The laccase-CBM/HBT treatment also led to a decrease in chlorine dioxide consumption. Indeed, for a targeted 85% ISO brightness, ClO_2 savings of 4.2 kg/T of pulp and 8.5 kg/T of pulp were calculated for the laccase/HBT and laccase-CBM/HBT treated pulps, respectively. These values were calculated according to the

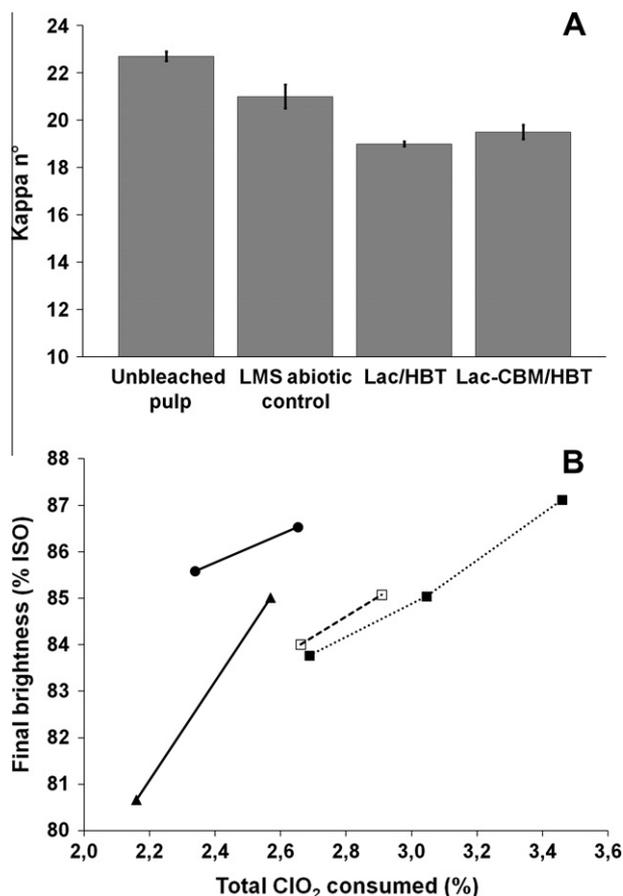


Fig. 1. Effect of laccase/HBT and laccase-CBM/HBT treatments on softwood kraft pulp biobleaching. (A) Effect of the enzymatic treatments on pulp kappa number. The pulp was treated with 20 U/g of laccase (Lac) or 5 U/g of laccase-CBM (Lac-CBM) in the presence of 1.5% HBT, under 1 bar O_2 pressure and at 5% pulp consistency. Kappa numbers were measured after an alkaline extraction stage. (B) Final brightness obtained at the end of the DEDED bleaching sequence as function of the total chlorine dioxide consumed. Laccase/HBT (—▲—), laccase-CBM/HBT (—●—) and LMS abiotic treatments (—□—) were followed by an alkaline extraction stage and a DEDED bleaching sequence. Initial pulp (—■—) was submitted to the same bleaching sequence (ECF bleaching control). Two initial chlorine dioxide charges (kappa factors of 0.18 and 0.21) were applied for each pulp. An additional chlorine dioxide charge (kappa factor of 0.24) was applied to the ECF bleaching control.

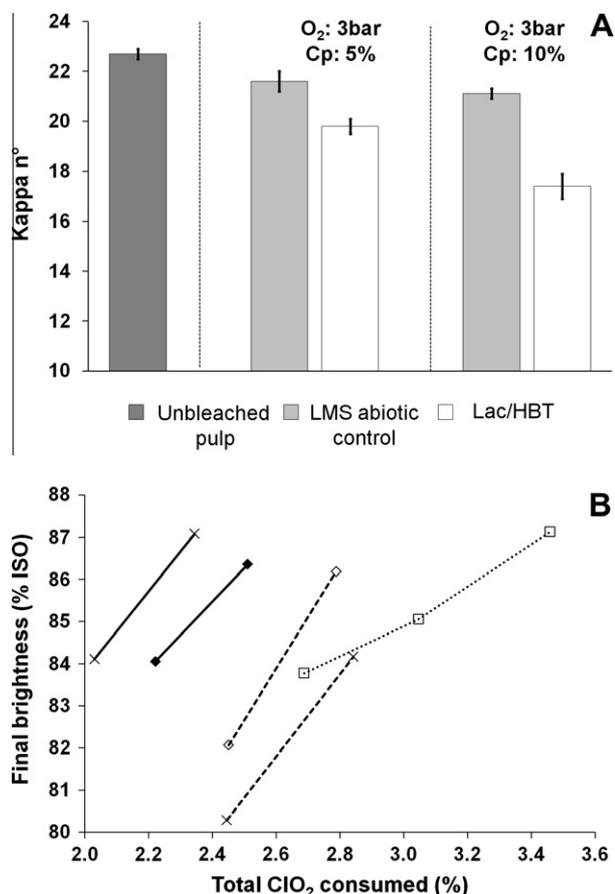


Fig. 2. Impact of an increase in oxygen pressure and pulp consistency on laccase/HBT softwood kraft pulp biobleaching. (A) Effect of the enzymatic treatments on pulp kappa number. The pulp was treated with 20 U/g of laccase in the presence of 1.5% HBT, under 3 bar O₂ pressure and at 5 or 10% pulp consistency. Kappa numbers were measured after an alkaline extraction stage. (B) Final brightness obtained at the end of the DEDED bleaching sequence as function of the total chlorine dioxide consumed. Softwood kraft pulp was submitted to a laccase/HBT treatment, under 3 bar oxygen pressure and 5 (—◆—) or 10% (—×—) pulp consistency. LMS abiotic treatments were performed under 3 bar oxygen pressure and 5 (—◇—) or 10% (—◇—) pulp consistency. Enzymatic and abiotic treatments were followed by an alkaline extraction stage and a DEDED bleaching sequence. Two initial chlorine dioxide charges (kappa factors of 0.18 and 0.21) were applied for each pulp. Results obtained for the ECF bleaching control (kappa factors of 0.18, 0.21 and 0.24) are also indicated (—■—).

amount of ClO₂ consumed by the ECF bleaching control pulp. To reach a final brightness value of 87.1, i.e. in the same range than the laccase-CBM/HBT treated pulp, an initial kappa factor of 0.24 had to be applied to this control pulp. In this case ClO₂ savings of 3.5 kg/T of pulp could be calculated for a targeted 86.5% ISO brightness, for the laccase-CBM/HBT treated pulp.

As bleaching responses of treated pulps, using 1.5% HBT, were similar to those observed using 3% HBT (Ravalason et al., 2009), a charge of 1.5% HBT was used for all the following LMS treatments.

3.2. Effect of operating conditions on laccase/HBT and laccase-CBM/HBT treatments

3.2.1. Effect of an increase in oxygen pressure and pulp consistency on laccase/HBT biobleaching

To evaluate the impact of LMS treatment conditions on laccase/HBT biobleaching of softwood kraft pulp, oxygen pressure was increased from 1 to 3 bar and pulp consistency was increased from 5% to 10%. Compared to the kappa number value obtained under 1 bar oxygen pressure and 5% pulp consistency (Fig. 1A), an increase

in oxygen pressure from 1 to 3 bar during LMS treatment did not significantly modify pulp kappa number decrease after an alkaline extraction stage (Fig. 2A). However, when both oxygen pressure and pulp consistency were increased (3 bar and 10% consistency), a further decrease in pulp kappa number was observed. Kappa number decrease was about 50% higher than that measured under the initial LMS treatment conditions. The modification of LMS treatment conditions also led to an improvement in pulp bleachability (Fig. 2B). Indeed, by increasing oxygen pressure at 5% pulp consistency, higher final brightness values were obtained, for similar values of chlorine dioxide consumption. This result could partially be due to the delignifying effect of oxygen itself, as shown by the bleaching response of abiotic control pulp when O₂ pressure was increased. Nevertheless a further bleachability improvement was observed in the presence of laccase and HBT. This could not be expected considering the kappa number values after an alkaline extraction stage. It is likely that pulp physico-chemical modifications occurred and might not be reflected by a simple measurement of kappa number. By increasing pulp consistency at 3 bar oxygen pressure, final brightness was not drastically affected, but a decrease in chlorine dioxide consumption was observed. Hence, the final brightness reached a value of 84.1% and 87.1% ISO for an initial kappa factor of 0.18 and 0.21, respectively. For a targeted 85% ISO brightness, ClO₂ savings of 8.7 kg/T of pulp were calculated, as compared to the ECF bleaching control consumption. This value was increased by 4.4 kg/T of pulp, considering the savings obtained under 1 bar oxygen pressure and 5% pulp consistency.

3.2.2. Comparison of laccase/HBT and laccase-CBM/HBT biobleaching under modified LMS treatment conditions

The impact of oxygen pressure and pulp consistency was investigated for laccase-CBM/HBT treatment. Both parameters were increased, to reproduce the best conditions observed for the laccase/HBT treatment. Trials were performed with laccase and laccase-CBM, using 20 and 5 U of enzyme per gram of pulp, respectively, under 3 bar oxygen pressure and at 10% pulp consistency. A LMS abiotic control was considered under the same conditions. The treated pulps were then submitted to an alkaline extraction stage and to an ECF bleaching using an initial kappa factor of 0.21. Table 1 shows the kappa number of the treated pulps after an alkaline extraction stage, and the pulp brightness and ClO₂ consumed at the end of the D₀ stage. The results obtained under 1 bar oxygen pressure and at 5% pulp consistency are also indicated. By applying modified LMS treatment conditions, both laccase/HBT and laccase-CBM/HBT treatments led to a decrease in pulp kappa number, as well as an increase in pulp brightness at the end of the D₀ stage, compared to the ECF bleaching control and LMS abiotic control. However, only the laccase/HBT treatment was improved by the modification of operating conditions, as a 12% decrease in pulp kappa number, a 19% decrease in ClO₂ consumption and a 5 points increase in brightness were observed. As a comparison, slight modifications of the effect of laccase-CBM/HBT treatment were noted.

It was expected that an increase in oxygen pressure would lead to an increase in pulp delignification in the presence of laccase-CBM/HBT. Indeed, an increase in oxygen pressure can positively affect LMS biobleaching of pulps as it leads to an increase in oxygen solubility, therefore facilitating the oxidation reactions catalyzed by laccase (Moldes and Vidal, 2008; Oudia et al., 2008; Fillat and Roncero, 2009). Concerning the other operating parameter tested here, it could be expected that an increase in pulp consistency would contribute to a better contact between laccase/mediator systems and pulp fibers, leading to the observed increase in biobleaching efficiency. However, if the increase in pulp consistency might have enhanced the efficacy of unbound laccase by increasing the amount of substrate near the enzyme, this result was already obtained by the binding of laccase-CBM to the pulp. As a conse-

Table 1
 Comparison of laccase/HBT and laccase-CBM/HBT treatment of softwood kraft pulp.

Treatment conditions		Pulp kappa number ^a	Pulp brightness after D ₀ (%ISO) ^b	ClO ₂ consumed in the D ₀ stage (%)
Initial pulp		22.7 ± 0.2	39.2 ± 0.3	1.8
LMS stage: PO ₂ : 1 bar Cp: 5%	Abiotic control	21.0 ± 0.2	38.8 ± 0.1	1.7
	Laccase/HBT	19.0 ± 0.1	40.4 ± 0.3	1.6
	Laccase-CBM/HBT	19.5 ± 0.5	42.7 ± 0.2	1.6
LMS stage: PO ₂ : 3 bar Cp: 10%	Abiotic control	21.1 ± 0.2	38.0 ± 0.3	1.7
	Laccase/HBT	16.8 ± 0.4	45.4 ± 0.1	1.3
	Laccase-CBM/HBT	19.5 ± 0.1	41.7 ± 0.1	1.6

^a Pulp kappa number was measured after an alkaline extraction stage, except for the initial pulp.

^b Kappa factor applied in the D₀ stage: 0.21.

quence, increasing pulp consistency may have no significant impact on laccase-CBM. This should be verified by an independent evaluation of the impact of pulp consistency on laccase-CBM/HBT treatment, and also by increasing the amount of laccase-CBM bound to the pulp fibers.

3.3. Pulp color after enzymatic treatments and E stage

Pulp color and brightness were measured after the 3 h enzymatic stages and after the first alkaline extraction stage. Laccase/HBT and laccase-CBM/HBT treatments led to a decrease in pulp brightness (Table 2). This effect should be due to the action of enzyme and mediator, as an increase in brightness was observed after the LMS abiotic control treatments. As indicated by the color difference values (ΔE^*), enzymatic and LMS abiotic treatments led to a modification of pulp color, compared to the unbleached kraft pulp. The highest color difference values were observed for the laccase/HBT treated pulps. Surprisingly, ΔE^* values were higher after the LMS abiotic treatments than after the laccase-CBM/HBT treatment. However, differences in the contribution of each color coordinate could be observed. LMS abiotic treatments led to an increase in lightness, which contributed to a large extent for the color difference observed. Conversely, laccase/HBT and laccase-CBM/HBT treatments led to a decrease in pulp lightness, compared to the LMS abiotic control, as well as a red and yellow shift of pulp color, as shown by the increase in a^* and b^* coordinates. Red shift was particularly pronounced for both treatments. Increasing oxygen pressure and pulp consistency led to a further increase in color difference for the laccase/HBT treated pulp, mainly due to an increase in redness. As a comparison, no significant differences in pulp color coordinates were observed when these modified treatment conditions were applied to the laccase-CBM/HBT system. Pulp brightness and color were also measured after the first alkaline extraction

stage (Table 2). The extraction stage led to an increase in pulp brightness and lightness for the laccase/HBT and laccase-CBM/HBT treated pulps, whereas the values decreased for the abiotic controls. The alkaline extraction also led to a significant decrease in pulp redness for all LMS-treated pulps, and only to a slight modification of the b^* coordinate. The redness values were comparable between laccase/HBT and laccase-CBM/HBT-treated pulps under 1 bar oxygen pressure and 5% pulp consistency. This value was still high for the laccase/HBT-treated pulp when both oxygen pressure and pulp consistency were increased during LMS treatment. Conversely, no significant modification of the a^* coordinate was observed for the laccase-CBM/HBT-treated pulp under modified LMS-treatment conditions.

Optical analyses has already been suggested to be a way to study LMS treatments of pulps, as modifications of pulp color were found to be correlated to biobleaching results (Moldes et al., 2010; Moldes and Vidal, 2011). Pulp darkening has previously been observed after LMS treatment of pulps, and was thought to be due to covalent bonds between the mediator and the pulp fibers, or the result of quinone formation during laccase/HBT oxidation of pulp lignin (Chakar and Ragauskas, 2000, 2001; Moldes et al., 2010). Pulp color, and in particular a^* coordinate, was also suggested to be correlated with quinone content of chemical pulp (Lachenal et al., 2005). The red shift observed after LMS treatment of pulps could therefore be partially due to quinone formation. It has been shown that quinonoid structures were particularly reactive towards sodium hydroxide and could be removed by an alkaline extraction stage (Zawadzki et al., 1999; Chakar and Ragauskas, 2000), which could lead to the decrease in redness observed for the LMS-treated pulp. Interestingly, the results obtained also suggest that there may be a correlation between the red shift observed after laccase/HBT and laccase-CBM/HBT treatments, and the extent of kappa number decrease after an alkaline extraction stage. In the

Table 2
 Impact of laccase/HBT and laccase-CBM/HBT treatment on pulp brightness and color after LMS treatment and an alkaline extraction stage.

Treatment conditions		Pulp brightness (% ISO)		Pulp color			
				ΔE^*	L^*	a^*	b^*
Unbleached pulp			29.90	–	72.11	3.25	18.85
LMS stage: PO ₂ : 1 bar Cp: 5%	Abiotic control	After LMS	32.84	3.3	75.27	3.72	19.81
		After E	30.31	1.9	73.35	2.75	20.18
	Laccase/HBT	After LMS	25.05	4.9	69.36	5.76	21.97
		After E	26.09	3.0	70.09	3.37	21.10
	Laccase-CBM/HBT	After LMS	27.98	2.4	71.48	5.15	20.21
		After E	28.98	3.1	73.41	2.91	21.60
LMS stage: PO ₂ : 3 bar Cp: 10%	Abiotic control	After LMS	32.22	3.4	75.05	3.74	20.38
		After E	29.26	0.9	71.74	3.65	19.59
	Laccase/HBT	After LMS	24.24	7.0	68.91	8.15	22.68
		After E	26.17	3.8	69.44	5.36	20.43
	Laccase-CBM/HBT	After LMS	28.52	1.9	72.29	4.83	19.85
		After E	29.54	2.1	72.92	2.66	20.68

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Ravalason, H. (Auteur de correspondance), Bertaud, F., Herpoël-Gimbert, I., Meyer, V., Ruel, K., Joseleau, J.-P., Grisel, S., Olivé, C., Sigoillot, J.-C., Petit-Conil, M. (2012). Laccase/HBT and laccase-CBM/HBT treatment of softwood kraft pulp: impact on pulp bleachability and physical properties. *Bioresource Technology*, 121, 68-75. DOI : 10.1016/j.biortech.2012.06.077

same way, pulp optical properties were consistent with the differences in bleachability observed for both systems, under initial and modified LMS treatment conditions. However it must be pointed out that optical properties and decrease in kappa number could not be used to directly compare laccase/HBT and laccase-CBM/HBT treatments, as indicated by the results obtained under 1 bar oxygen pressure and at 5% pulp consistency. Indeed, despite a comparable red shift and decrease in kappa number, laccase-CBM/HBT treatment led to a better pulp bleachability than laccase/HBT treatment. In this case, besides LMS-induced lignin oxidation, the action of CBM might also be involved in the differences observed.

3.4. Physical properties of bleached pulp

Determination of physical properties is important to evaluate the impact of an enzymatic treatment on pulp quality. Pulp viscosity and average fiber length were measured for the different treatments performed, at the end of the bleaching sequence. Table 3 shows the values obtained for the treatments leading to the same range of brightness values, i.e. 86.5–87% ISO brightness, as well as the values obtained for the corresponding LMS abiotic controls. To compare laccase/HBT and laccase-CBM/HBT treatments performed under the same conditions, results obtained for the laccase/HBT treatment under 1 bar oxygen pressure and at 5% pulp consistency were also indicated. All the enzymatic treatments led to a slight decrease in pulp viscosity, as previously shown for laccase/HBT treatment of chemical pulps (Camarero et al., 2004; Ibarra et al., 2006a; Moldes et al., 2010). Laccase/HBT treatment under 3 bar oxygen pressure and at 10% pulp consistency led to the highest decrease in pulp viscosity. Average fiber length was not significantly modified after the different treatments. Mechanical properties were also evaluated for the different bleached pulps. As shown in Fig. 3A, no significant modification of tear index was observed for the enzyme-treated pulps. As tear index was shown to be mainly dependent on fiber length (Seth and Page, 1988), these results are consistent with the measured average fiber length values. Fig. 3B shows the tensile index measured at the end of the bleaching sequence. Compared to the ECF bleaching control pulp, laccase/HBT treatments led to a decrease of about 15% in tensile index. This decrease could partially be explained by the impact of treatment conditions, as shown by the values obtained for the corresponding LMS abiotic controls. These results suggest a weakening of cellulose fibrils involved in interfiber bonding. Conversely, no significant modification was observed for the laccase-CBM/HBT treated pulp. This effect may be due to the action of the CBM. Indeed, it was shown that some fungal CBMs could promote the non-hydrolytic disruption of cellulose fibers (Xiao et al., 2001; Gao et al., 2001). Fungal CBMs have also already been shown to improve pulp properties, and in particular to increase tensile strength of paper sheets (Suurnakki et al., 2000; Pala et al., 2001). It was suggested

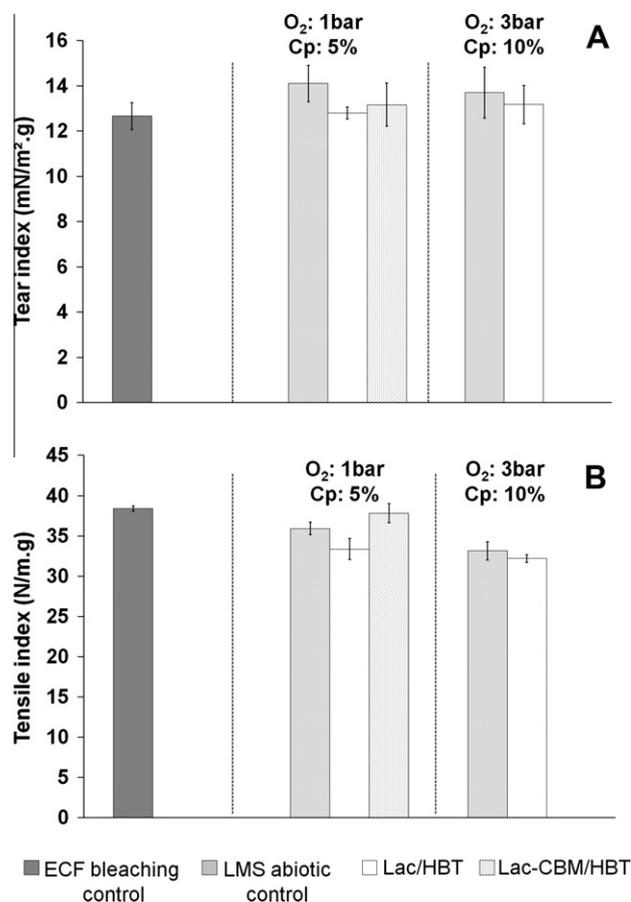


Fig. 3. Tear index (A) and tensile index (B) of LMS-treated pulps and corresponding controls at the end of the bleaching sequence, using an initial kappa factor of 0.24 for the ECF bleaching control and 0.21 for the other pulps.

that this effect could be due to fiber surface and interfacial properties modifications induced by the CBM action (Pala et al., 2001). Fiber surface modifications could therefore have compensated the effect of laccase and HBT as well as treatment conditions.

3.5. Transmission electron microscopy analysis of softwood kraft pulp fibers

Localization of laccase and laccase-CBM in softwood kraft pulp fibers taken at the end of the enzymatic treatments was observed after immunogold labeling followed by transmission electron microscopy, using antibodies directed against *P. cinnabarinus* laccase (Fig. S1). Almost no gold particle was detected for laccase-treated pulps (Fig. S1,

Table 3
Properties of the treated pulps at the end of the bleaching sequence.

Treatment conditions ^a	Final brightness (%ISO)	Total ClO ₂ consumed (%)	Bleached pulp viscosity (ml/g)	Average area-weighted fiber length (mm)
ECF bleaching control (KF in D ₀ : 0.24)	87.1 ± 0.1	3.5	1004 ± 12	2.09 ± 0.02
LMS stage:				
Abiotic control (KF in D ₀ : 0.21)	85.1 ± 0.1	2.9	995 ± 1	2.00 ± 0.02
PO ₂ :				
Laccase/HBT (KF in D ₀ : 0.21)	85.1 ± 0.1	2.6	974 ± 3	2.03 ± 0.01
1 bar				
Laccase-CBM/HBT (KF in D ₀ : 0.21)	86.5 ± 0.1	2.5	946 ± 4	2.03 ± 0.04
Cp: 5%				
LMS stage:				
Abiotic control (KF in D ₀ : 0.21)	84.2 ± 0.1	2.8	982 ± 4	2.01 ± 0.05
PO ₂ :				
Laccase/HBT (KF in D ₀ : 0.21)	87.1 ± 0.1	2.3	929 ± 6	2.03 ± 0.04
3 bar				
Cp: 10%				

^a KF: kappa factor. Cp: pulp consistency.

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B) but labeling was significantly increased after laccase-CBM treatment (Fig. S1, C and D), indicating a retention of the enzyme inside the fiber wall as a result of CBM binding. Gold particles were distributed throughout the cell wall demonstrating the penetration of laccase-CBM inside the fibers, with apparently no preferential localization of the enzyme. The ultrastructure of pulp fibers and distribution of lignin were also analyzed after the first alkaline extraction stage, by KMnO₄ staining (data not shown). Compared to the unbleached pulp, no significant differences in lignin distribution were observed between the samples. However, as a result of kraft pulping process, general predominant characteristics could be noted despite fiber heterogeneity. Fibers were individualized consequently to lignin dissolution especially from middle lamella, and S1 and S3 layers were detached and lightened due to a superficial penetration of cooking liquor. The opening of the structure induced by the chemical process therefore allowed the enzymes to penetrate and diffuse inside the fiber wall. Even if no clear differences in lignin distribution could be observed between the samples, laccase/HBT and laccase-CBM/HBT treatments clearly led to an increased external microfibrillation of the fibers, as previously observed for pulps treated with lignin-oxidizing enzymes (Sigoillot et al., 2001; Vivekanand et al., 2008).

3.6. Overall comparison of laccase/HBT and laccase-CBM/HBT systems

In this study laccase/HBT and laccase-CBM/HBT treatments were integrated into an ECF bleaching sequence. Under 1 bar oxygen pressure and at 5% pulp consistency, 28% ClO₂ savings were obtained with a laccase-CBM/HBT pre-treatment, for a targeted final brightness of 85% ISO. With the laccase/HBT system, the same results could be achieved with a higher laccase charge, when both oxygen pressure and pulp consistency were increased. However these modifications also led to a decrease in bleached pulp tensile index. The fusion of a CBM to the *P. cinnabarinus* laccase therefore allowed keeping the same chlorine dioxide savings while still achieving a high brightness and good pulp properties with decreased enzymatic charge. To confirm these promising results, a scaled-up production of laccase-CBM would be needed to validate the process at a pilot-scale, for a potential industrial implementation. Studies are currently being conducted to optimize enzyme production.

4. Conclusion

The fusion of a CBM to the *P. cinnabarinus* laccase could improve softwood kraft pulp biobleaching under specific LMS treatment conditions. Laccase/HBT and laccase-CBM/HBT treatments led to a red shift of pulp color, which seemed to be correlated to the extent of kappa number decrease after an alkaline extraction stage. Laccase-CBM/HBT treatment also led to a better preservation of pulp properties, which could be due to surface modifications induced by the CBM action. Microscopic examination of pulp fibers indicated a retention of laccase-CBM inside the pulp fiber, and an increased external microfibrillation after enzymatic treatments.

Acknowledgements

This study was partly funded by the Biorenew European project (NMP2-CT-2006-026456). H. Ravalason is grateful to the Centre Technique du Papier for her PhD Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2012.06.077>.

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