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M. Speranza [ab]; D. Ibarra [b]; J. Romero [a]; A. T. Martínez [b]; M. J. Martínez [b]; S. Camarero [b]
[a] Centro de Investigación y Tecnología, Pontevedra, Spain
[b] Centro de Investigaciones Biológicas, Madrid, Spain

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ORIGINAL ARTICLE

Microscopy studies reveal delignification and sterol removal from eucalypt kraft pulps by laccase–HBT

M. SPERANZA[1,2], D. IBARRA[2], J. ROMERO[1], A. T. MARTÍNEZ[2], M. J. MARTÍNEZ[2], & S. CAMARERO[2]

[1]*Centro de Investigación y Tecnología, ENCE, Carretera de Campañó s/n, E-36157 Pontevedra, Spain and* [2]*Centro de Investigaciones Biológicas, CSIC, Ramiro de Maetzu 9, E-28040 Madrid, Spain*

Abstract
Fungal laccases in the presence of mediators are powerful biocatalysts to degrade lignin. *Pycnoporus cinnabarinus* laccase and 1-hydroxybenzotriazole (HBT) have been successfully used to delignify eucalypt kraft pulp once integrated in a totally chlorine-free bleaching sequence. Real time delignification of kraft pulp by laccase–HBT was verified *in situ* by monitoring the loss of lignin autofluorescence during the enzymatic treatment using confocal laser scanning microscopy. The highest delignification of pulp fibers occurred over a very short time-span (5 min). Moreover, we demonstrate the removal of sterols, responsible for pitch deposits in hardwood kraft pulps, as an additional effect of laccase–HBT. Spherical structures between pulp fibers localized by low temperature scanning electron microscopy were removed by laccase–HBT. The use of filipin, a specific stain, revealed the sterol nature of many of these structures. At the end of the enzyme-aided bleaching sequence, the fluorescent sterols–filipin signals were almost completely absent.

Keywords: *Laccase–HBT, lignin, sterols, pitch, eucalypt pulp, filipin staining, microscopy*

Introduction

The pulp and paper industry's concern to reduce consumption of chemicals and improve pulp quality has led to the introduction of enzyme-aided technologies in different parts of the manufacturing process (Bajpai 2004). During kraft pulping, most lignin from wood is removed, but residual lignin remains in the cellulose fibers. This residual lignin, responsible for the kraft pulp color, must be removed during the bleaching process. Traditionally, chlorine and chlorine dioxide have been used as bleaching agents, but their use in industrial bleaching sequences of most pulp types has been eliminated or considerably reduced due to the toxicity of the chlorinated compounds released. Modern, totally chlorine-free (TCF) bleaching is environmentally friendly, but less efficient in attaining high and permanent pulp brightness due to the lower delignification power of oxygen and hydrogen peroxide compared to chlorine reagents.

However, the problem of so-called pitch deposits in pulp increased with the use of TCF bleaching because wood extractives survive this type of process. The accumulation of wood extractives in pitch deposits reduces pulp quality and produces problems at the mill (Back & Allen 2000). Lipases are being used successfully for pitch biocontrol in softwood pulps, but are not effective in hardwood pulps, such as eucalypt pulps, since in this case, sterols (mainly sitosterol and stigmastanol) are the principal responsible for pitch deposits (del Río et al. 1999). A challenge of recent years has been to find biological agents for delignification and control of pitch deposits in paper pulps.

Laccases are multicopper oxidases secreted by white-rot fungi (among other organisms) that oxidize a wide range of aromatic compounds using molecular oxygen as final electron acceptor (Thurston 1994; Claus 2004). However, the oxidation capability of laccases is limited by their relatively low redox potential (0.4–0.8 V). This limitation is overcome in the presence of certain compounds acting as redox mediators that expand laccase activity towards high redox potential substrates. Once oxidized by the

Correspondence: Susana Camarero, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain. E-mail: susanacam@cib.csic.es

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enzyme, the mediator radicals undertake the oxidation of the target aromatic substrate by different mechanisms (Baiocco et al. 2003). Laccase–mediator systems constitute powerful biocatalysts for lignin degradation, and have been thoroughly assayed for delignification of paper pulps (Bourbonnais et al. 1997; Call & Mücke 1997). In this sense, *Pycnoporus cinnabarinus* laccase, in the presence of 1-hydroxybenzotriazole (HBT) as mediator, has been used successfully as a delignifying and bleaching agent to produce high quality paper pulp from non-wood (Camarero et al. 2004) and eucalypt pulps (Ibarra et al. 2006a). The integration of this system in a TCF industrial-like bleaching sequence in combination with chemicals produced significant improvement of pulp properties, including higher brightness and lower kappa number values than those attained by the standard chemical bleaching sequence (Ibarra et al. 2006b).

Structural studies of cellulose pulps using different microscopic techniques provide useful information about the physical and chemical properties of this material submitted to different treatments (Daniel et al. 2002). Low temperature scanning electron microscopy (LTSEM) allows structural studies avoiding the artifacts caused by the pretreatment of samples as in conventional SEM. In addition, it is possible to detect pitch deposition in eucalypt pulps by fluorescence microscopy (FM) using filipin staining (Speranza et al. 2002). Filipin is a polyene antibiotic used as a probe to detect cholesterol in animals and ergosterol in fungal membranes (Milhaud et al. 1988; Severs 1997). This antibiotic reacts specifically with those 3β-hydroxysterols, including an 8–10 carbon side-chain and a flat tetracyclic nucleus (Elias et al. 1979; Clejan & Bittman 1985), and, more efficiently, when a double bond is present at C_5 (Milhaud et al. 1988). The complexes formed with filipin have absorption and emissions bands at 357 and 480 nm, respectively. Therefore, they can be localized after ultraviolet excitation using FM. Since these complexes are formed in a 1:1 stoichiometric reaction (Milhaud et al. 1988), the presence and distribution of free sterols can be quantitatively analyzed.

In this study, we proposed to disclose a dual effect of the laccase–HBT system, the removal of sterols together with the removal of lignin from eucalypt kraft pulps, by means of different microscopic techniques.

Materials and methods

Pulp samples

Eucalyptus globulus kraft pulp was produced at the ENCE mill in Pontevedra (Spain). Brown (unbleached) pulp with 15.8 kappa number and oxygen delignified pulp with 9.7 kappa number were used for laccase–HBT treatments.

Laccase

Laccase was produced by Beldem (Andenne, Belgium) from *P. cinnabarinus* strain ss3 (Herpoël et al. 2000). 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. One unit of activity was defined as the amount of enzyme that transforms 1 μmol of ABTS to its cation radical ($\varepsilon_{436} = 29{,}300\ M^{-1}\ cm^{-1}$) per minute.

Pulp bleaching

Pulp treatment with *P. cinnabarinus* laccase in the presence of HBT (Sigma-Aldrich), once optimized for eucalypt pulp (Ibarra et al. 2006a), was integrated in an industrial-type TCF bleaching sequence (Figure 1) (Ibarra et al. 2006b). Pulp bleaching was carried out in 4-L reactors with 200 g of eucalypt pulp (dry weight) at 10% consistency. The TCF industrial-type bleaching sequence O-O-Q-PoP included: two alkaline oxygen stages (O-O), a chelation stage (Q) with diethylenetriaminepenta-acetic acid (DTPA) and an alkaline peroxide stage (PoP) including a first step under pressurized O_2 and an atmospheric step. Laccase (20 U g^{-1} of pulp) and HBT (1.5% of pulp dry weight) were applied after the double oxygen stage (O-O-L-Q-PoP) at pH 4 for 2 h at 50°C, with stirring for 1 min (60 rev min^{-1}) each 30 min. The same bleaching sequence with stage under the same conditions as the L stage but without enzyme was used as control. Oxygen delignified pulp treated with laccase–HBT (O-O-L) and full bleached pulp at the end of the enzyme-aided bleaching sequence (O-O-L-Q-PoP) were used for microscopy studies, and compared with their corresponding controls (O-O and O-O-Q-PoP pulps). Laccase–HBT treatment was also applied on the unbleached (brown) kraft pulp (kraft-L). Pulp samples were filtered and washed with distilled water before storing at −20°C.

Low temperature scanning electron microscopy

Ultrastructural characterization of kraft-L, O-O-L and O-O-L-Q-PoP pulps, with their respective controls, was carried out using LTSEM. Hydrated fibers were mounted with O.C.T. (Optimal Cutting Temperature BDH) on a specimen holder at room temperature and plunged into subcooled liquid nitrogen (−204°C) under vacuum using an Oxford CT 1500 Cryotransfer system. The specimens

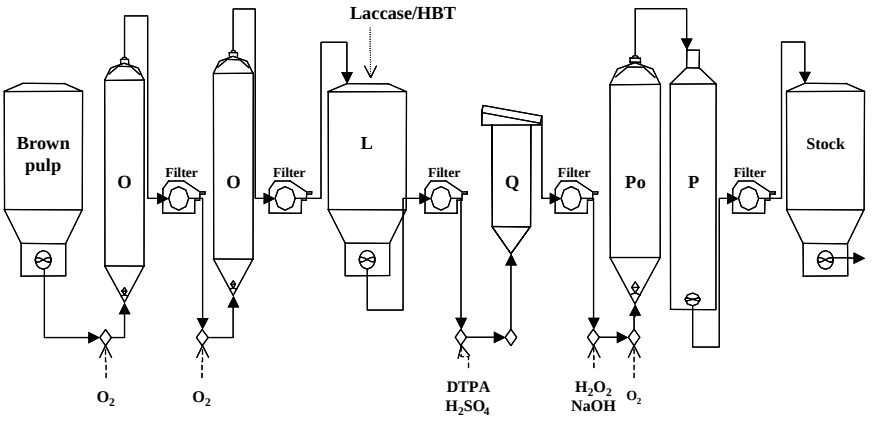

Figure 1. Scheme of the enzyme-aided TCF sequence used for bleaching eucalypt kraft pulp, resulting from the integration of a laccase–mediator stage (L) in an industrial-type sequence (O-O-Q-PoP), adapted from Ibarra et al. (2006a). The L stage was integrated after the double oxygen delignification (O-O) and followed by chelation (Q) and hydrogen peroxide (PoP) stages.

were then immediately transferred to the cryo-preparation chamber, etched (-90°C for 2 min), and gold sputter-coated under pressure (4×10^{-1} thorr). The observations were carried out on a LT-SEM Zeiss DSM960, at $-135/-150^{\circ}$C, operated at 15 kV and varying working distances (8–20 mm).

Sterol staining

Filipin, a specific stain for sterols, was used to probe and localize the action of laccase–HBT using FM, and to determine the spatial distribution of the main lipophilic extractives during the bleaching process (Speranza et al. 2002). Filipin solution was prepared by dissolving 2.5 mg of filipin (Sigma) in 1 mL of dimethylformamide (Merck), and mixing with 50 mL of PBS (Sarig et al. 1994). Filipin solution was applied to the hydrated pulp samples with their respective controls, mounted in glass slides, and incubated in the dark for 20 min at room temperature. The preparations were rinsed with PBS to remove the stain, and mounted in glycerol containing 1% Mowiol 40–88 (Aldrich) as antifade agent. They were immediately examined with transmitted light, using phase contrast, and epifluorescence with an Axioplan Zeiss microscope. Filipin was excited with UV light using a 360/40D filter, the fluorescence emitted was analyzed through a 460-nm barrier filter, and the image captured digitally with a CCD device (Leica DFC 350 FX). Similar exposure times were used in all cases to facilitate sterol distribution analysis. Extractive-free controls were prepared by extracting pulp samples with acetone in a Soxhlet apparatus for 8 h, followed by water and PBS washing. Positive controls for filipin staining were prepared using sitosterol and stigmastanol solutions (1 mg mL^{-1}) in acetone.

Confocal microscopy

Real time delignification of brown kraft pulp by laccase–HBT was monitored *in situ* for 45 min using a CLSM Leica TCS microscope with a spectrophotometer detection system (SP2) (Leica Microsystems, Mannheim, Germany). Eucalypt fibers were deposited in a glass bottom culture dish (P35G-1.5-14C, MatTek Corporation, USA), and treated with 20 U laccase and 1.5% HBT per gram of pulp, in tartrate buffer pH 4, at room temperature. Pulp buffered with sodium tartrate (pH 4) in the presence of 1.5% HBT was used as control. The culture dish was placed immediately in the CLSM inverted microscope, and lignin autofluorescence emission between 530 and 560 nm was collected using an FITC filter, and using blue Ar (488, 20 mw) ion laser as excitation line. Laser excitation power and emission detection conditions were selected to prevent photo-induced quenching, and were not altered throughout the experiments (Barsberg & Nielsen 2003). Digitally transmitted light signal was collected simultaneously with the confocal images. Time course observations of the same fibers from 0 time (3–5 min after enzyme–mediator was applied to pulp) and each 5 min during 45 min of incubation were carried out. Before real time experiments, pulp samples were analyzed using Lambda scan function to determine the emission spectrum of autofluorescence for emission wavelengths between 385 and 700 using UV Ar (351 and 364 nm) and blue Ar (488) as light sources. Lambda image stack was obtained by scanning the same x–y optical section with a bandwidth of 20 nm.

Fluorescence analyses

Due to the displacement of the focal plane in the *z* axis during the experiments, a single fluorescence measurement included 15–20 2D-optical sections successively covering the *z* direction. The fluorescence intensity was measured with Leica Confocal Software (version 2871-7A), using the region of interest (ROI) function, the fluorescence intensity of 5–10 ROIs were measured, and the mean fluorescence intensity (MFI) was calculated for each cell.

Results and discussion

Integration of an enzymatic treatment with *P. cinnabarinus* laccase in the presence of HBT as mediator in a industrial-type TCF bleaching sequence produces a significant decrease in the final lignin content of eucalypt kraft pulp together with higher and more permanent pulp brightness (Ibarra et al. 2006b). The industrial TCF sequence (O-O-Q-PoP) was based on the use of oxygen and hydrogen peroxide as delignifying and bleaching agents. The point where the enzymatic stage was integrated in the bleaching sequence is indicated in Figure 1. Pulp samples after different bleaching stages were analyzed by different microscopic techniques (see Materials and methods for pulp nomenclature). Brown (unbleached) kraft pulp treated with laccase–mediator system was also analyzed for comparison.

Structural properties of the different eucalypt kraft pulps were analyzed by LTSEM. Brown kraft and oxygen delignified (O-O) pulps showed spherical structures between fibers (Figure 2a, b). These structures were similar to sterol deposits previously detected in *E. globulus* wood that survive the kraft cooking process (Speranza et al. 2002). These structures were more abundant in oxygen delignified pulp than in brown kraft pulp, probably due to the release of part of the intracellular deposits from parenchyma cells during the oxygen treatment. Most of these free spherical structures in pulp were unexpectedly removed after the laccase-HBT

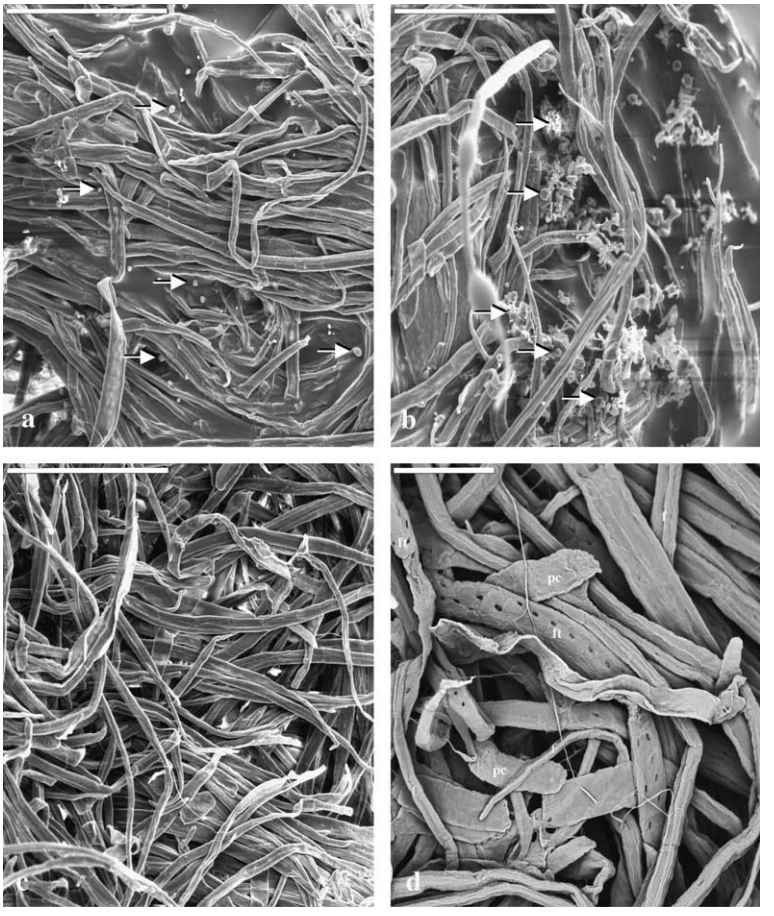

Figure 2. Eucalypt kraft pulp images obtained by low temperature scanning electron microscopy. (a) Brown kraft pulp showing abundant spherical structures between pulp fibers (arrows). (b) Control O-O pulp showing an increase of the spherical structures (arrows) precipitated between fibers. (c) O-O-L pulp, after laccase–HBT treatment most of these structures were completely removed from pulp. (d) O-O-L-Q-PoP pulp, at the end of the enzyme-aided bleaching sequence no spherical structures were observed. Parenchyma cells (pc) and fiber tracheids (ft) were seen between fibers (f). Bars: 200 μm in (a–c), 50 μm in (d).

treatment (Figure 2c), and at the end of the enzyme-aided bleaching sequence (O-O-L-Q-PoP) were completely absent (Figure 2d).

To verify the removal of sterols during the enzymatic treatment, FM studies were carried out using a methodology previously developed for sterol localization in pulp (Speranza et al. 2002). The use of filipin staining allowed the localization of sitosterol and stigmastanol, two of the main wood extractives responsible for pitch deposits in eucalypt pulp. During chemical bleaching, the strong-fluorescent signals from the sterols–filipin complexes remained (Figure 3a, c, e), confirming that these compounds can survive TCF bleaching (del Río et al. 1999). However, in the eucalypt pulps treated with laccase-HBT, reduction of fluorescent sterols–filipin signals was evident, revealing sterol removal by the enzymatic system (Figure 3b, d, f).

In brown kraft pulp, the strongest fluorescent signals of the sterol–filipin complexes were found in the parenchyma cells and free deposits (Figure 4a), but fluorescence was also localized in the fiber lumen (Figure 4c) and fiber cell wall surface (Figure 4b). During laccase-HBT treatment of kraft pulp, significant reduction of sterol-filipin fluorescence in fibers was observed, with the signals inside parenchyma cells remaining (data not shown).

In the control oxygen delignified pulp, the strongest sterol–filipin signals were also found inside parenchyma cells. However, a high level of fluorescence was also detected in free deposits, correlating with the spherical structures released during O-O treatment previously observed by LTSEM (Figure 5a). This fluorescence pattern was maintained until the end of the chemical bleaching sequence (Figure 5c). During laccase-HBT treatment of O-O pulp, significant reduction in the general fluorescence corresponding to sterol–filipin signals in free deposits was observed (Figure 5b). Additionally, the less accessible internal sterol deposits from unbroken parenchyma cells, that retained their fluorescence intensity after laccase-HBT treatment of brown kraft pulp, were removed to a greater extent from oxygen delignified pulp by the enzymatic treatment (Figure 5b). It seems as if structural changes of the parenchyma cells (such as

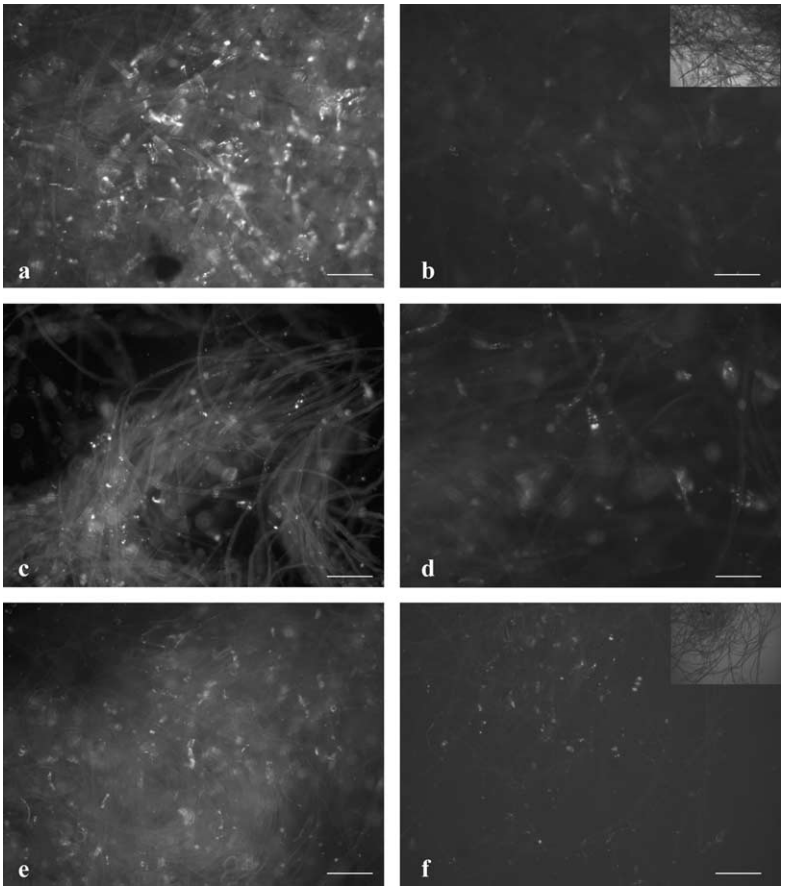

Figure 3. Images from fluorescence microscopy showing a general aspect of sterol–filipin complexes in different eucalypt kraft pulps and the loss of fluorescent signals after laccase–HBT treatment (L). Brown-L (b), O-O-L (d) and O-O-L-Q-PoP (f) pulps were compared with their corresponding controls: brown kraft pulp (a), O-O pulp (c) and O-O-Q-PoP pulp (e). Inserts correspond to transmission images of the same fields. Bars: 200 μm.

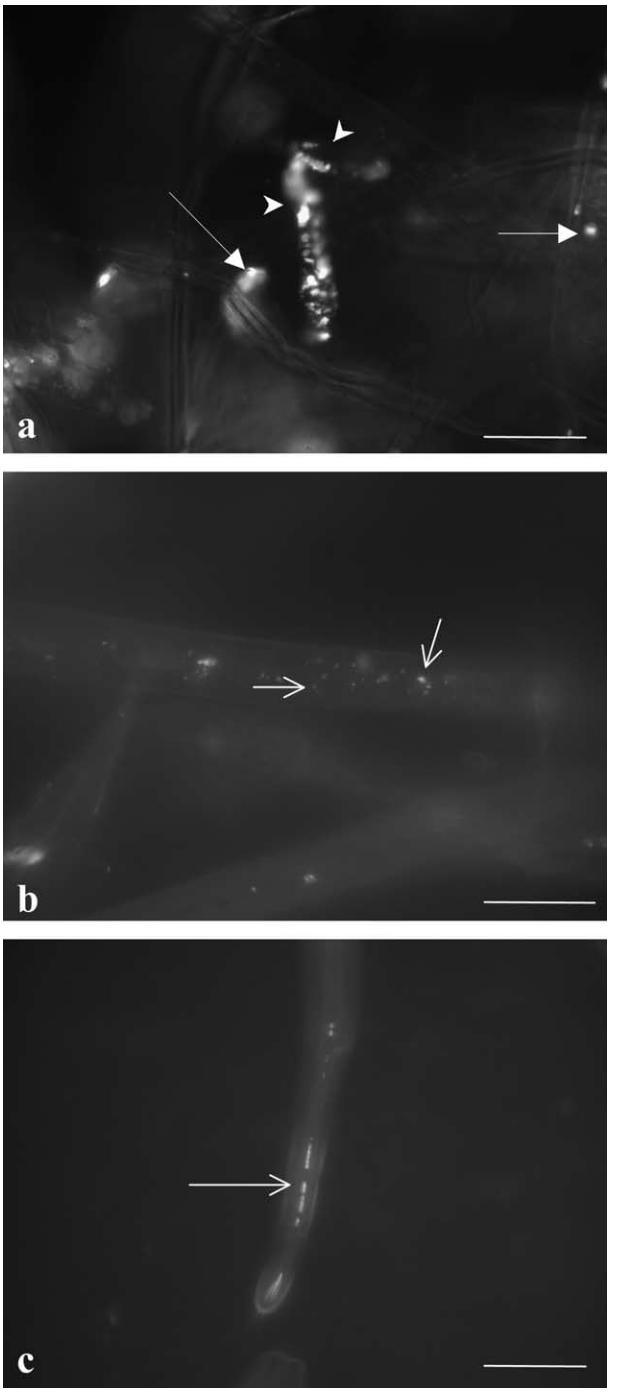

Figure 4. Fluorescence microscopy images showing the distribution of sterols in the different components of eucalypt brown kraft pulp using filipin staining. (a) Detail showing the fluorescent sterol–filipin signals in parenchyma cells (head arrows) and free deposits (arrow); (b) small sterol–filipin spots localized in the fibers surface; (c) sterol–filipin signals in fiber lumen. Bars: 50 μm in (a), 20 μm in (b), and 30 μm in (c).

pit enlarging) or chemical modification of their extractives (increased dispersion/dissolution of sterols) occurred during oxygen delignification of eucalypt kraft pulp (Dinesh & Daniel 2005; Freire et al. 2006). These changes would facilitate the action of the enzyme–mediator system on sterols, as also observed for lignin degradation (Ibarra et al. 2006a). Finally, at the end of the complete enzyme-aided bleaching sequence (O-O-L-Q-PoP), all fluorescent signals had virtually disappeared, with only a few remaining signals inside parenchyma cells (Figure 5d), in contrast to the control chemical bleaching sequence (Figure 5c). The recalcitrance of sterols from parenchyma cells has been demonstrated by the accumulation of these cells in the pitch deposits found in eucalypt TCF pulps (Speranza et al. 2001). The presence of saturated sterols, such as stigmastanol, which have been described as more recalcitrant towards chemical (Jansson et al. 1995) and enzymatic bleaching could also be involved. The results presented here, on the action of the laccase–HBT system on eucalypt pulp lipids, agree with those obtained from chemical analyses (Gutiérrez et al. 2006), and provide additional information on the distribution of sterols in the different pulp elements and their differential removal.

To verify the delignification of eucalypt kraft pulp *in situ* during the laccase–HBT treatment, a real time CLSM assay was carried out. Before the real time experiments, pulp samples were analyzed to verify the emission spectrum of lignin autofluorescence in eucalypt pulp fibers using a wide range of wavelengths. Lignin autofluorescence in fibers had an emission maximum at 530–560 nm. Transmission and fluorescence CLSM images of eucalypt before the reaction showed the general aspect of kraft pulp fibers (Figure 6a), and the autofluorescence of lignin (Figure 6b). During enzymatic treatment, a progressive loss of fluorescence intensity in fiber cell walls was observed, as shown by comparison of CLSM images after 0 and 45 min of reaction (Figure 6b, d). Lignin is an aromatic polymer in which different excitation states among the phenylpropanoid units take place, allowing the excitation energy transfer (EET) from one lignin moiety emission to another in the vicinity. The emission spectra of these chromophores (corresponding to the different chemical structures from lignin units) seem to be responsible for lignin autofluorescence (Barsberg & Nielsen 2003; Barsberg et al. 2003). The oxidative degradation of side-chains in lignin units, among other reactions produced by laccase–HBT, generates quinones and results in loss (quenching) of the natural autofluorescence of lignin (Zhou et al. 1995; Barsberg & Nielsen 2003).

The fluorescence intensity of pulp fibers is proportional to their lignin content, and a correlation between both can be established (Li & Reeve 2004). Hence, the loss of fluorescence intensity of fiber cell

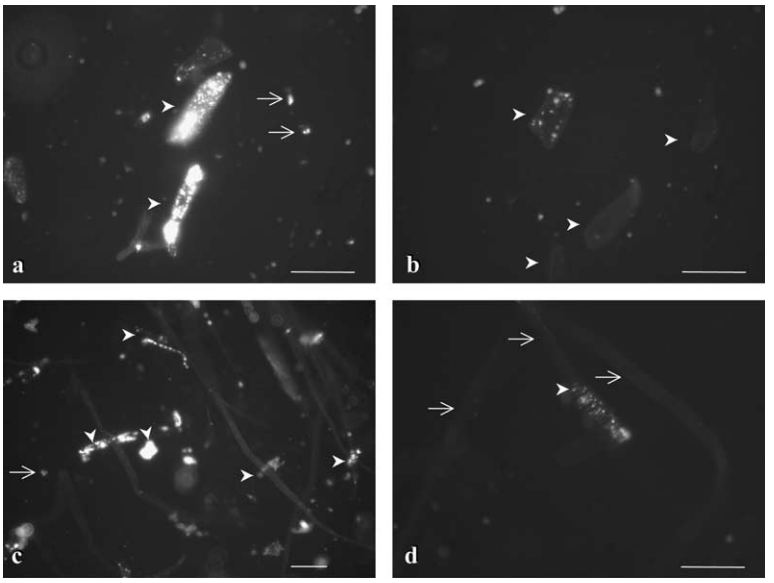

Figure 5. Fluorescence microscopy images of oxygen delignified kraft pulps showing the evolution of the filipin–sterol complexes after chemical or enzymatic bleaching. Pulps: (a) control O-O pulp, (b) O-O-L pulp, (c) control O-O-Q-PoP pulp, and (d) O-O-L-Q-PoP pulp. After oxygen delignification an increase in free sterol deposits was observed (arrows in a and c). These deposits were removed by the enzymatic treatment (L) (b), and were completely absent after the full enzyme-aided bleaching sequence (d). Laccase-HBT action was also evident in fibers (arrows in d) and inside parenchyma cells (head arrows in b). Only some residual signals (of the strong initial signals, head arrows in a) inside parenchyma cells were observed at the end of the O-O-L-Q-PoP bleaching sequence (head arrow in d). (A) Bars: 50 μm in (a), (b), (d), and 100 μm in (c).

walls produced during treatment of eucalypt kraft pulp with laccase–HBT correlates with lignin degradation. The progressive loss of fluorescence intensity of fiber cell walls during the 45-min real time assay is represented in Figure 6(c). The most significant fiber delignification by laccase-HBT (indicated as pixel intensity, a measure of the fluorescence intensity) was produced during the first 5 min (20–40% of fluorescence intensity loss), reaching a maximum after 20 min of reaction (70% loss). The real time experiment designed here could be a useful tool for optimizing conditions (concentration and time of reaction) of the laccase–mediator delignification stage before integration into an industrial bleaching process.

Integration of a laccase–mediator stage in the industrial TCF bleaching of eucalypt kraft pulps

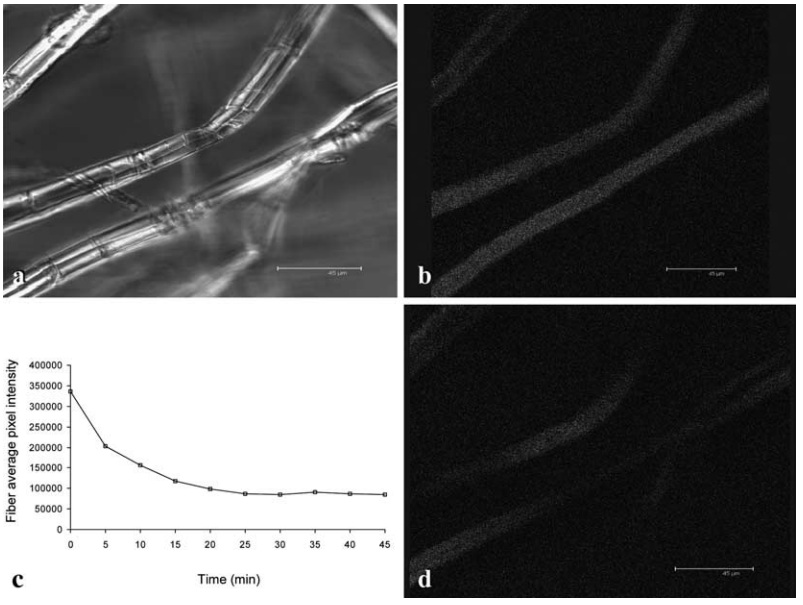

Figure 6. Real time delignification of kraft pulp with laccase–HBT followed by CLSM. Transmission (a) and fluorescence (b, d) images of kraft pulp at initial time (a, b) and after 45 min (d) of enzymatic treatment are shown. The delignification kinetics followed by the progressive loss of lignin autofluorescence in pulp fibers along the enzymatic treatment is also illustrated (c).

could be advantageous from two points of view: (i) to decrease lignin content of pulp, providing higher degrees of brightness and lower brightness reversion than obtained by standard sequences, and (ii) for pitch control, by removing the sterols accumulated in pulp during TCF bleaching sequences. Both enzymatic effects should enhance pulp quality with respect to standard chemical TCF bleaching, as revealed by chemical analyses (Gutiérrez et al. 2006). Moreover, the use of natural mediators obtained from cheap sources, such as lignin (Camarero et al. 2005), instead of synthetic ones, might significantly increase the feasibility of laccase–mediator systems in biotechnological applications of environmental interest (Camarero et al. 2007; Gutiérrez et al. 2007).

Conclusions

- Free HBT radicals generated by oxidation with laccase are able to concomitantly remove both lignin and lipids in eucalypt kraft pulps, as revealed by microscopic techniques.
- Filipin staining localization of sterols in the different pulp components after laccase–HBT treatment, demonstrated the ability of the enzymatic system to degrade the recalcitrant spherical structures from parenchyma cells, that are mainly responsible for the pitch problem in eucalypt pulps.
- CLSM analyses allowed for the first time *in situ* demonstration of lignin degradation in eucalypt fibers by the laccase–HBT system, and determination of the process kinetics.

Acknowledgements

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