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Integrating laccase–mediator treatment into an industrial-type sequence for totally chlorine-free bleaching of eucalypt kraft pulp

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Abstract: Enzymatic delignification using the high-redox potential thermostable laccase from the fungus *Pycnoporus cinnabarinus* and a chemical mediator (1-hydroxybenzotriazole) was investigated to improve totally chlorine-free (TCF) bleaching of *Eucalyptus globulus* kraft pulps. Different points of incorporation of the enzyme treatment into an industrial-type bleaching sequence (consisting of double oxygen, chelation and peroxide stages) were investigated in pressurized laboratory reactors. The best final pulp properties were obtained using an O–O–L–Q–PoP sequence, where a laccase–mediator stage (L) was incorporated between double oxygen and chelation. The worse results, when the enzymatic and chelation treatments were combined in a unique stage, seemed related to partial inhibition of laccase-mediator activity by the chelator. The new TCF sequence including the laccase stage permitted to improve eucalypt pulp delignification to values around kappa 5 (hexenuronic acid contribution over 50%) compared to kappa 7 using only TCF chemical reagents. In a similar way, the final brightness obtained, over 91% ISO, was 3–4 points higher than that obtained in the chemical sequences. Although technical and economic issues are to be solved, the results obtained show the feasibility of integrating a laccase–mediator treatment into a TCF sequence for bleaching eucalypt kraft pulp.

Keywords: biobleaching; laboratory reactor; laccase–mediator system; totally-chlorine free sequence; eucalypt kraft pulp; delignification

INTRODUCTION

Removal of lignin from wood is the first step in the manufacturing of chemical paper pulps, kraft alkaline pulping being the most common process.[1,2] Although most lignin is removed during cooking, some residual lignin remains in pulp that must be removed in oxidative bleaching reactions. Historically, pulp bleaching has been accomplished using chlorinated reagents (Cl_2, ClO_2, and NaOCl), but nowadays peroxide, oxygen, ozone, and lignocellulose-degrading enzymes constitute environmentally friendly alternatives to develop totally chlorine-free (TCF) sequences.[3,4]

The use of fungal laccases in the presence of redox mediators, the so-called laccase–mediator system described 15 years ago,[5] offers the possibility to bleach different types of pulp in TCF sequences due to their ability to degrade lignin,[6,7] compared with already commercialized xylanases that only remove lignin in an indirect way.[8] A recent comparison of different wild and recombinant enzymes has shown that the laccase–mediator system has the highest potential for delignifying paper pulps.[9] From its description, laccase–mediator systems have been included in a variety of patents,[10–13] and investigated for delignification and bleaching of different paper pulps.[14–23]

Chlorine has been eliminated from industrial bleaching of most pulp types because of its negative environmental effects due to the release of chlorinated compounds.[24] Modern eucalypt pulp bleaching includes the use of chlorine dioxide in elemental chlorine-free (ECF) sequences, and chlorine-free reagents in TCF sequences. However, most TCF chemical reagents, such as hydrogen peroxide and oxygen, are less efficient than chlorine reagents in attaining high and stable pulp brightness degrees. This is due to their lower delignification power that not only results in lower brightness but also in higher colour reversion, owing to their higher residual lignin content.

Since laccases are oxidized by molecular oxygen, which acts as the final electron acceptor,[25,26] laccase-based bleaching can be considered as an enzyme-catalyzed oxygen delignification stage. However, the high redox potential of the activated copper species at the enzyme catalytic site enables a strong

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delignification of pulp, which is mediated by the stable radicals formed during enzyme oxidation of suitable chemical mediators.[27,28]

Once the potential of the laccase–mediator systems for delignifying and bleaching paper pulps has been demonstrated, a crucial aspect for their industrial implementation is to optimize the integration of the enzymatic delignification systems into ECF and TCF sequences. The objective is to exploit the synergies between enzymatic and chemical reagents, and to define an optimal sequence from the point of view of product quality. Additionally, economic and environmental aspects are to be considered to establish the feasibility of the new bleaching processes.

In the present study, the effect of including a laccase–mediator treatment at different points of an industrial-type eucalypt pulp TCF sequence is investigated using pressurized reactors, and a new enzyme-containing sequence is defined resulting in pulp brightness and kappa number values that could not be obtained using only chemical bleaching reagents.

MATERIALS AND METHODS

Pulp and enzyme samples

Brown (unbleached) kraft pulp from eucalypt (*Eucalyptus globulus*) with 14.2 kappa number, 41.2% ISO brightness and 1188 $mL\,g^{-1}$ viscosity was obtained from the ENCE mill in Pontevedra, Spain. The crude laccase preparation used in TCF bleaching was provided by Beldem (Belgium). It was obtained from 1 m^3 fermentor cultures of a laccase-hyperproducing monokaryotic strain (ss3) of the fungus *Pycnoporus cinnabarinus* provided by INRA (Marseille, France).[29] One activity unit was defined as the amount of enzyme transforming 1 μmol of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) per minute to the corresponding cation radical (ε_{436} 29 300 $M^{-1}\,cm^{-1}$) in 0.1 M sodium acetate buffer (pH 5). 1-Hydroxybenzotriazole (HBT; Sigma-Aldrich) was used as laccase mediator. Diethylenetriaminepentaacetic acid (DTPA; Sigma-Aldrich) inhibition (at concentrations between 75 μM and 15 mM) of decolorization of 25 μM Aniline Blue (Sigma-Aldrich) by laccase (100 $U\,mL^{-1}$) alone and in the presence of 0.1 mM HBT in 50 mM sodium citrate, pH 5, was followed at 592 nm for 6 h at 24 °C.

Pulp bleaching sequences

The different pulp bleaching sequences were carried out in 4 L stainless steel reactors with shaking, temperature and pressure controls, using 200 g of eucalypt pulp (dry weight) at 10% consistency (in all stages).

A standard (industrial type) TCF bleaching sequence O–O–Q–PoP was performed including: (1) double oxygen stage (O) using 6 $kg\,cm^{-2}$ O_2 pressure, 1.5% NaOH and 0.5% $MgSO_4$ for 60 min at 98 °C; (2) chelation stage (Q) using 0.3% DTPA for 60 min at 85 °C (pH 5–6); and (3) alkaline peroxide stage (PoP) using 3% H_2O_2, 2% NaOH, 0.1% $MgSO_4$ and 0.5% $Na_2Si_2O_3$ for 140 min at 105 °C under 6 $kg\,cm^{-2}$ O_2, followed by 180 min at 98 °C without O_2 pressure (above percentages referred to pulp dry weight). The pulp samples were exhaustively washed with distilled water after each stage.

Laccase–mediator treatment was assayed at two points of the TCF sequence by: (1) incorporating a laccase (20 $U\,g^{-1}$ pulp) and HBT (1.5%, referred to pulp) stage (L) at pH 4, for 120 min at 50 °C, with 1 min stirring (60 rpm) every 30 min (O–O–L–Q–PoP sequence); and (2) substituting the Q stage by a stage combining laccase-HBT (20 $U\,g^{-1}$ of enzyme and 1.5% mediator) and DTPA (0.3%, referred to pulp) treatments at pH 4 for 120 min at two temperatures (50 °C and 65 °C) with stirring as described above (O–O–L/Q–PoP sequence).

A second chemical sequence (O–O–a–Q–PoP) was applied including a control stage under the conditions of stage L, but without laccase and mediator. Because of its slightly acidic conditions, this stage was called stage a. A third chemical sequence included DTPA chelation at lower temperature (50 °C or 65 °C) and pH(pH4) without laccase, and was used as a control to identify the effect of laccase–mediator in the sequence combining enzymatic and chelator treatments.

Pulp and paper-making evaluation

Pulp brightness, kappa number and viscosity were evaluated by standard methods (ISO 3688:1999, ISO 302:1981 and ISO 5351/1:1981, respectively).[30] Mean and 95% confidence limits (from up to four replicates) were calculated. Brightness reversion was estimated after 48 h at 80 °C under 65% relative humidity (ISO 5630/3:1996). Hexenuronic acids were estimated spectrophotometrically,[31] and a factor of 0.086 was used to estimate their contribution to kappa number in eucalypt pulps. Pulps were refined using a PFI refiner (ISO 5264/2:2002) operating at 1400 rpm for different times (0, 1000, 2000, 3000 and 4000 total revolutions). The refining degree (freeness) was measured by the Schopper–Riegler method (ISO 5267/1:1999). Then, laboratory handsheets were prepared with a grammage of 65 $g\,cm^{-2}$, and different mechanical properties, namely thickness, apparent bulk density, burst index, tensile index, tear index and air porosity (Gurley index), and optical properties, namely light scattering coefficient and opacity, were evaluated (ISO 536:1995, ISO 534:1998, ISO 2758:2001, ISO 1924/2:1994, ISO 1974:1990, ISO 5636/5:2003, ISO 9416:1998 and ISO 2471:1998, respectively).[30]

RESULTS AND DISCUSSION

After showing efficient bleaching of flax pulp by *P. cinnabarinus* laccase in the presence of HBT,[21,22] this enzyme, which combines high redox potential (0.75–0.79 V) and thermal stability, is being assayed

to bleach eucalypt kraft pulp.[32] In the present study the enzymatic treatment of eucalypt pulp was scaled up, and the best way to integrate the laccase–mediator treatment into an industrial-type TCF sequence was investigated using laboratory reactors.

Integrating enzyme treatment into a TCF sequence for bleaching eucalypt pulp

Figure 1 shows a scheme of the industrial process of manufacturing paper pulp from eucalypt wood by kraft pulping and TCF bleaching. The latter included the following stages: (1) double oxygen delignification (O–O); (2) chelation stage (Q) to remove metals that destroy peroxide; and (3) alkaline hydrogen peroxide stage (PoP), which includes a step under pressurized oxygen, and a second atmospheric step. The pulping and bleaching sections also include inter-stage washing, and different tanks where the pulp is stocked. An analysis of this sequence revealed three possible points for including a laccase–mediator treatment: (1 and 2) adding the enzyme and mediator to the stock tank where the brown pulp is stored, or to a second tank between the stages O–O and Q; and (3) adding the enzyme and mediator to a modified chelation stage.

Previous small-scale assays had shown that application of a laccase–mediator treatment to brown eucalypt pulp followed by double oxygen and peroxide stages resulted in lower brightness (87% ISO brightness) than obtained when the laccase–mediator treatment was applied to previously oxygen-delignified pulp followed by peroxide bleaching (90% ISO brightness),[32] and the same tendency was observed for the decrease of kappa number. Therefore, point 2 in Fig. 1 was considered more adequate than point 1 for enzyme application. In the present study we scaled up the enzymatic bleaching by using 4 L pressurized reactors for both the enzymatic and chemical stages. Moreover, we investigated the possibility to apply the laccase–mediator treatment in combination with the chelation stage. The corresponding sequence, which we called O–O–L/Q–PoP, was compared with the O–O–L–Q–PoP sequence, where the laccase–mediator (L) and chelation treatments were applied as two separate stages (points 2 and 3 of Fig. 1).

Eucalypt pulp delignification/bleaching in an enzyme-containing sequence

Figure 2 shows the changes of kappa number (mean and 95% confidence limits), an estimation of lignin content in eucalypt pulps along two enzyme-containing sequences (A and B) and two chemical sequences (C and D). The former correspond to the sequences including the combined L/Q stage, or the successive L and Q stages. The latter correspond to the control sequence O–O–a–PoP, where a slightly acidic treatment (stage a) was applied under the conditions of stage L but without enzyme and mediator, and the standard sequence O–O–Q–PoP. The acidic stage caused a slight decrease of the kappa number compared with the standard sequence, but the difference was not significant (as shown by the mean confidence limits). Since these sequences (Fig. 2C and D) were equivalent, the differences in B could be attributed to the effect of the laccase–mediator system. In the chemical sequences, similar decreases of kappa number were observed after the double-oxygen and peroxide stages. In the two other sequences (A and B) a third decrease of kappa number was observed

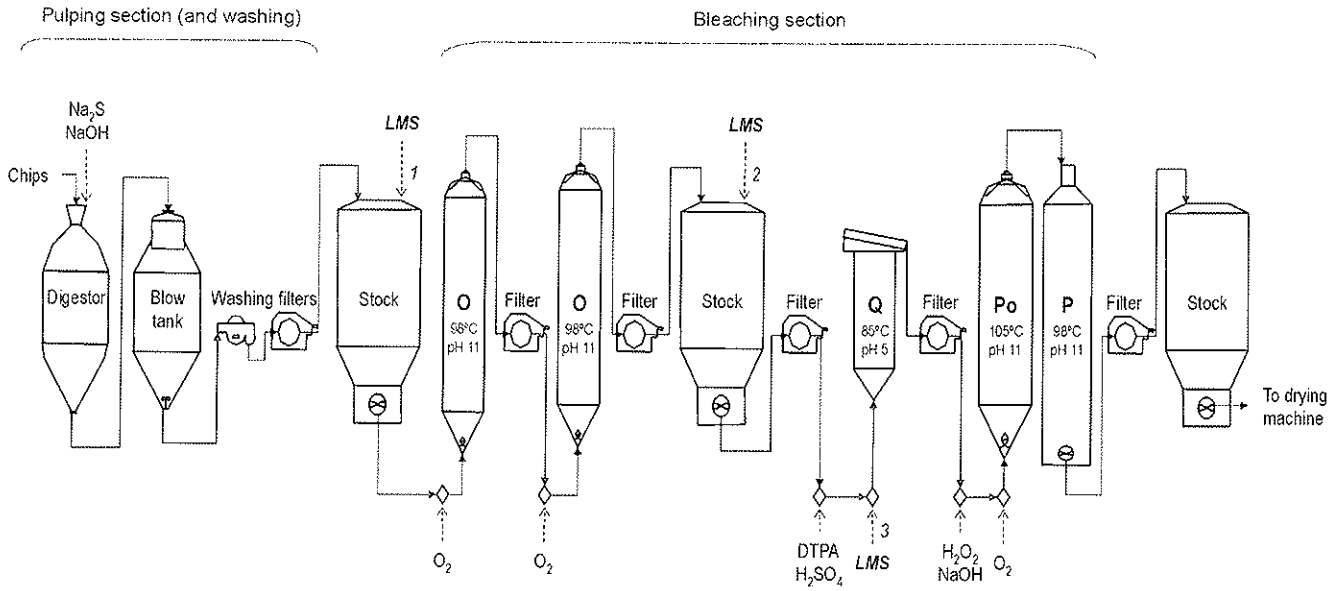

Figure 1. Scheme of industrial manufacturing of eucalypt (*Eucalyptus globulus*) kraft pulp showing the standard TCF bleaching sequence (O–O–Q–PoP). In addition to the pulping and bleaching reactors, several washing filters and tanks are indicated, as well as the positions where reagents are added. Three possible points to include the laccase–mediator system (LMS) are also shown: (1 and 2) stock tanks before and after the oxygen bleaching; and (3) modified chelation stage. TCF sequence stages: O, oxygen stage (double); Q, chelation stage using DTPA; PoP, alkaline peroxide stage including a first step under pressurized oxygen and a second treatment under atmospheric pressure. The pH and temperature conditions of the different bleaching stages are indicated (the residence time in each stage is of 1–3 h).

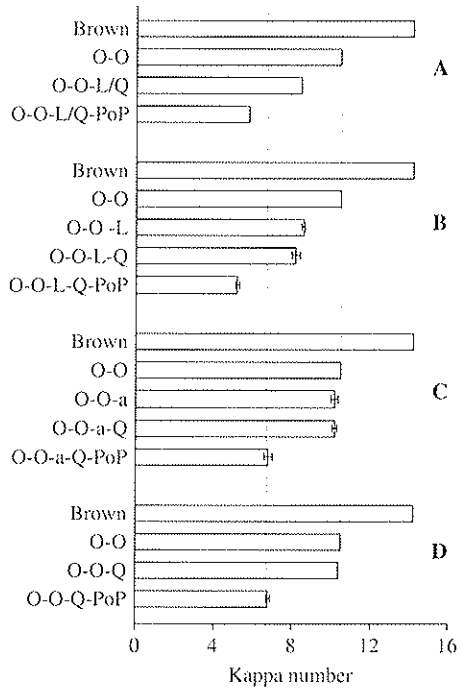

Figure 2. Comparison of kappa number along the enzyme-containing (A and B) and chemical (C and D) TCF bleaching sequences of eucalypt kraft pulp. Kappa values were estimated after the different stages of the two enzyme-containing sequences O–O–L/Q–PoP and O–O–L–Q–PoP, compared with the standard chemical sequence O–O–Q–PoP, and the control chemical sequence O–O–a–Q–PoP, which includes a stage without laccase and mediator (stage a) under the same conditions of stage L. Sequence stages: O, oxygen stage (double); L, laccase–mediator stage; a, control stage under mild acidic conditions; Q, chelation stage; L/Q, modified chelation stage including laccase–mediator; PoP, alkaline peroxide stage including a first step under pressurized oxygen. Vertical doted lines correspond to kappa values after double-oxygen and peroxide stages in chemical TCF bleaching. Mean values and 95% confidence limits are shown.

after the enzymatic stage. Kappa decrease after the stage L/Q seemed slightly higher than after stage L but the difference was not significant. As found in the chemical sequences, the subsequent Q stage did not affected kappa number in B significantly. However, application of the PoP stage resulted in lower kappa in O–O–L–Q–PoP (final kappa of 5.2 ± 0.1) than in O–O–L/Q–PoP (final kappa of 5.7 ± 0.0). Both values are lower than obtained after chemical bleaching (final kappa of 6.8 ± 0.2). These differences are important for low kappa number pulps, especially when the contribution of hexenuronic acids is considered.

Estimation of lignin content in highly delignified pulps by the kappa method suffers from interferences due to the presence of hexenuronic acids,[31,33] the amount of these polysaccharide oxidation products in pulps increasing during TCF bleaching. Other compounds that could interfere in kappa number estimation were not considered here.[34,35] The contribution of hexenuronic acids to eucalypt pulp kappa number was estimated after different stages of the O–O–L–Q–PoP sequence and the control chemical sequence: brown pulp (24%), O–O pulp (37%), O–O–a pulp (39%), O–O–L pulp (44%), O–O–a–Q–PoP pulp (49%) and O–O–L–Q–PoP (54%). The final kappa and hexenuronic acid values are shown in Table 1. Differences in hexenuronic acid contribution to kappa number after the L stage and its control represented 5%, and remained unchanged after the peroxide stage. It is remarkable that the hexenuronic acid contribution attained around 50% at the end of both sequences.

Table 1. Pulp and paper-making properties of eucalypt pulps bleached in a sequence containing a laccase stage (L) compared with the control sequence containing an acidic stage (a) and the standard TCF sequence (O–O–Q–PoP) (mean values)

	O–O–L–Q–PoP	O–O–a–Q–PoP	O–O–Q–PoP
Pulp properties			
Kappa number	5.2	6.7	6.8
Hexenuronic acids (mEq kg^{-1})	32.7	38.4	32.1[a]
Brightness (% ISO)	91.2	87.9	87.9
Colour reversion (%)	29.2	33.6	36.0[a]
Viscosity (mL g^{-1})	693	788	758
Papermaking properties[b]			
Freeness (°SR)	78	79	78
Thickness (μm)	85.0	84.8	84.0
Apparent bulk density (g cm^{-3})	0.76	0.78	0.77
Burst index (kPa m^2 g^{-1})	6.9	6.8	7.3
Tensile index (N m g^{-1})	92.0	92.5	98.5
Tear index (mN m^2 g^{-1})	8.6	8.6	8.6
Gurley index (s)	290	490	610
Scattering coefficient (m^2 kg^{-1})	23.0	21.5	22.0
Opacity (%)	64.6	65.0	64.0

[a] From mill TCF pulps.
[b] Paper-making properties after 4000 revolutions PFI refining.

Figure 3 shows the brightness obtained in the above sequences. As in the case of kappa number, no significant differences were found between the two chemical sequences (C and D) although the control sequence included an additional step (stage a). Therefore, the brightness differences with respect to B are assignable to the enzymatic treatment. In all cases, the strongest increase of brightness, more than a 30-point improvement in B–D, corresponded to the final peroxide stage. Brightness after the combined L/Q stage was higher than after the simple L stage, but the difference was inverted when stage Q was applied in B. Laccase application in the chelation stage (O–O–L/Q–PoP sequence) resulted in a lower final brightness (only $84.4 \pm 0.0\%$ ISO) than obtained in the chemical bleaching sequences (around 87.8% ISO). By contrast, the brightness improvement due to stage L was increased at the end of the O–O–L–Q–PoP sequence compared with the chemical sequences (Table 1). In this way, up to $91.2 \pm 0.2\%$ ISO brightness was attained. The introduction of the laccase stage also decreased the reversion of pulp brightness at the end of the O–O–L–Q–PoP sequence compared with the control sequence (Table 1).

Some decrease of pulp viscosity is inherent to most oxidative pulp delignification and bleaching

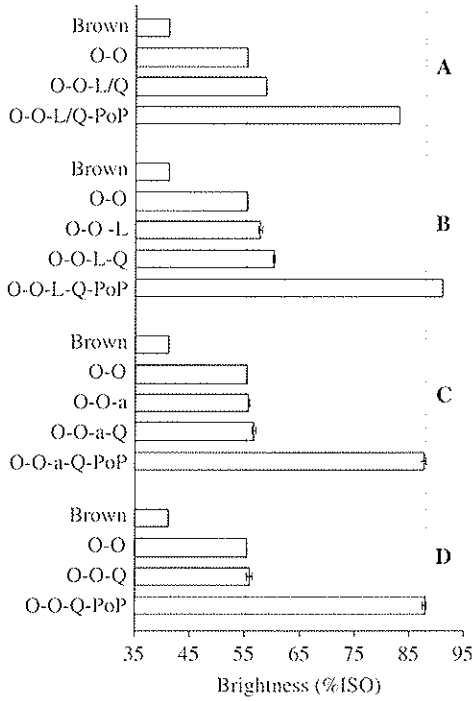

Figure 3. Comparison of pulp bleaching along the enzyme-containing (A and B) and chemical (C and D) TCF bleaching sequences of eucalypt kraft pulp. ISO brightness values were estimated after the different stages of the two enzyme-containing sequences O–O–L/Q–PoP and O–O–L–Q–PoP, compared with the standard chemical sequence O–O–Q–PoP, and the control chemical sequence O–O–a–Q–PoP, which includes a stage without laccase and mediator (stage a) under the same conditions of stage L. Sequence stages: O, oxygen stage (double); L, laccase–mediator stage; a, control stage under mild acidic conditions; Q, chelation stage; L/Q, modified chelation stage including laccase–mediator; PoP, alkaline peroxide stage including a first step under pressurized oxygen. Vertical dotted lines correspond to brightness values after double-oxygen and peroxide stages in chemical TCF bleaching. Mean values and 95% confidence limits are shown.

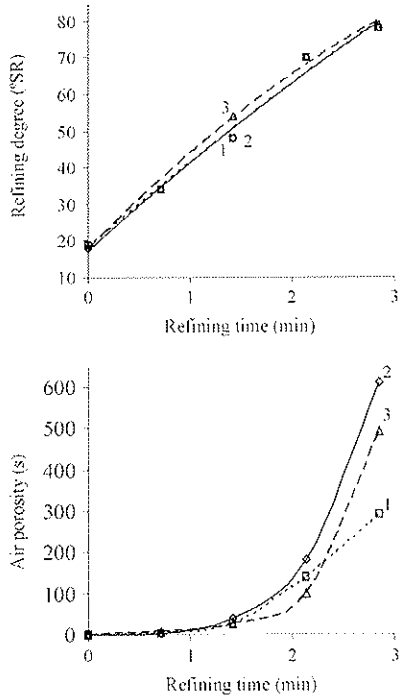

Figure 4. Comparison of some refining and paper-making properties of eucalypt kraft pulps after enzymatic and chemical TCF bleaching. Pulp-refining degree (estimated as Schopper–Riegler index, A), and air porosity of handsheets (estimated by Gurley index, B) plotted against refining energy (as refining time). Lines 1–3 correspond to pulps bleached using the enzyme-based sequence O–O–L–Q–PoP, and the chemical sequences O–O–Q–PoP and O–O–a–PoP, respectively.

treatments. The strongest decreases of eucalypt pulp viscosity (around 200 $mL\,g^{-1}$) were produced after the double-oxygen and peroxide stages. A lower viscosity decrease (around 100 $mL\,g^{-1}$) was found after the enzymatic stages. The viscosity decrease was maintained at the end of the O–O–L/Q–PoP sequence compared with the standard sequence. However, the difference with the chemical sequences was lower in the case of the O–O–L–Q–PoP sequence (Table 1). Any decrease in cellulose viscosity can affect negatively some pulp applications. However, the decreases by the laccase–mediator treatment were moderate, compared with those caused by the hydrogen peroxide and oxygen stages. Moreover, it has been shown that the oxidative alteration of cellulose by the laccase–mediator systems can be reversed by a reductive stage.[36]

Pulp and paper-making properties after enzyme-containing TCF bleaching

In addition to final pulp properties, Table 1 shows the comparison of some refining, paper-making and optical properties of the bleached pulps at the end of the best enzyme-containing sequence (O–O–L–Q–PoP) compared with the two chemical sequences (O–O–a–Q–PoP and O–O–Q–PoP). The latter properties were evaluated at different refining degrees (as shown in Fig. 4) but only those corresponding to 4000 revolutions are shown in Table 1. Scarce differences were observed in most of the parameters determined, as illustrated in Fig. 4(A) for the refining degree. However, a significant increase in the air porosity of the handsheets prepared from the enzyme-treated pulps was obtained, as shown in Fig. 4(B). This characteristic can be desirable for some paper types, and can also result in improved drying in the paper machine. Other mechanical and optical parameters were scarcely modified, revealing that the enzymatic delignification did not cause detrimental effects in the eucalypt pulp properties. Moreover, the small decrease of burst and tensile indices with respect to the O–O–Q–PoP sequence seems related to introduction of an additional (acidic) stage more than to the action of the laccase–mediator system, since the values were similar in both the O–O–L–Q–PoP and the O–O–a–PoP sequences.

Comparison of O–O–L/Q–PoP and O–O–L–Q–PoP TCF sequences

The objective of the study was to identify the best position to incorporate the laccase–mediator treatment to the standard O–O–Q–PoP sequence. The results

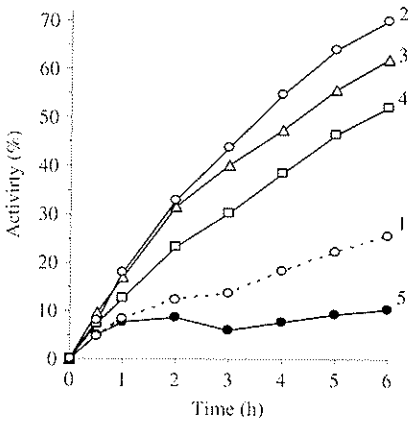

Figure 5. Effect of different concentrations of DTPA on the activity of the laccase–mediator system. Activity was estimated by a dye (Aniline Blue) decolorization assay. Line 1 shows decolorization by laccase in the absence of HBT and DTPA, line 2 in the presence of mediator, and lines 3–5 in the presence of mediator and 1.5, 3.0, and 15.0 mM DTPA, respectively.

obtained show that the improvement in pulp delignification (with respect to the chemical sequences) was lower when the laccase and chelation treatments were combined (stage L/Q) than when they were separately applied in a O–O–L–Q–PoP sequence. This sequence also improved pulp brightness, and caused only a moderate decrease of cellulose viscosity.

The worse results of the O–O–L/Q–PoP sequence do not seem to be due to a decreased efficiency of the chelating agent (DTPA) when applied under lower temperature and pH conditions (50 °C and 65 °C and pH 4, to facilitate the action of laccase). This was confirmed by applying an O–O–Q–PoP sequence, where stage Q was under the above temperature and pH conditions (data not shown). We also know that the difference is not due to a decreased activity of the laccase–mediator system at a higher temperature (65 °C) than used in stage L (50 °C), since a whole O–O–L/Q_{50}–PoP sequence (including a 50 °C L/Q stage) was applied with identical results.

Successful combination of the enzymatic and chelation treatments seems prevented by a partial inhibition of the activity of the laccase–mediator system by DTPA (Fig. 5). Using Aniline Blue it was shown that low DTPA concentrations (up to 3 mM) partially inhibited the laccase–mediator activity, and that higher DTPA concentrations (e.g. 15 mM) inhibited both the direct and mediated laccase activities. Inhibition of laccase–mediator activity was also found with higher redox potential substrates (which are oxidized only in the presence of mediators) and was similar to other chelators like ethylenediaminetetraacetic acid (EDTA) (data not shown). Although DTPA concentration at the L/Q stage (0.76 mM) should only result in a partial inhibition, this seems enough to result in worse pulp delignification and bleaching results than found when a separate L stage was applied in the O–O–L–Q–PoP sequence.

CONCLUDING REMARKS

We show that the laccase–mediator system is an effective biological reagent catalyzing the oxidative delignification and bleaching of eucalypt kraft pulp in TCF sequences. The enzymatic treatment can be scaled to reactors with better results than previously obtained in laboratory atmospheric bleaching,[32] due to the use of pressurized oxygen, which improved the efficiency of both the enzymatic and peroxide treatments. The improvements of eucalypt pulp kappa number and brightness were better than reported in most studies on enzymatic bleaching of other wood and non-wood pulps.[14–23] Moreover, it is shown that integrating the laccase–mediator stage in a TCF bleaching sequence results in improved delignification, with a final 'lignin kappa' (hexenuronic acids excluded) below 2.5. The same enzyme-containing sequence provided a significant increase of the final brightness to values over 91% ISO, which are several points above the highest brightness obtained by bleaching eucalypt pulp using only TCF chemical reagents.

Several aspects need to be solved before mill implementation of an enzymatic stage in TCF bleaching eucalypt and other pulps. One aspect to be addressed is the large-scale production and low price commercialization of suitable laccases. Most probably, the improvement of gene vectors and host systems will overcome this problem, as in the case of other enzymes currently in the mill. A second aspect to be solved is the availability of efficient and economic mediators for the above applications. The eventual toxicity of most laccase mediators and their reaction products constitutes an important issue that is still to be investigated, despite its environmental importance. A future alternative will be the use of 'natural' (potentially cheap and safe) mediators. In this sense, lignin-derived phenolic compounds have been recently described as laccase mediators with similar or even better performance than the best synthetic mediators including higher activities and lower dose requirements.[37]

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