BIOBLEACHING OF ORANGE TREE PRUNING CELLULOSE PULP WITH XYLANASE AND LACCASE MEDIATOR SYSTEMS

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The biobleachability of soda-anthraquinone pulp from orange tree pruning has been evaluated in this study for the first time. Three different laccase-mediator systems (LMS) have been tested: laccase from *Trametes villosa* (Tv), either in combination with 1-hydroxybenzotriazole (HBT) or with acetosyringone (AS), and laccase from *Myceliophthora thermophila* (Mt) in combination with AS. After the LMS treatment (L), a standard bleaching sequence, comprising an alkaline extraction (E) and a hydrogen peroxide stage (P), was carried out, altogether resulting in a LEP sequence. The three LMS improved the bleaching sequence, L-Tv+AS being the LMS that provided the highest delignification and improvement of optical properties. In contrast, no significant variations were observed in the viscosity and mechanical properties of the handsheets. In order to boost the optimal L-Tv+AS sequence, a xylanase pretreatment was performed; however, no improvements in delignification, optical properties or brightness stability were observed compared with the same sequence without xylanase pretreatment.

Keywords: orange tree-pruning, *Myceliophthora thermophila*, *Trametes villosa*, acetosyringone, laccase

INTRODUCTION

As a result of intensive agriculture associated to population growth and improved living standards, the rapid increase in the volume and types of agricultural biomass residues is becoming a growing problem in terms of pollution, pests, interference with soil cultivation and occupation of large areas. In order to avert these problems, residues can be simply disposed of or they can be used for the production of fibers, energy or added-value chemicals. The latter option, the so-called lignocellulose biorefinery, is a promising route for the development of a competitive, innovative and sustainable biobased economy.

An interesting and abundant agricultural residue is orange tree pruning, the result of trimming away unneeded branches of orange trees. Oranges are one of the major crops in Brazil, United States and most Mediterranean countries, including Spain and Greece.[1] Spain alone produces about 2.82 x 10^6 tons each year.[2] Considering that the "pruning to fruit weight" ratio of orange trees is 0.8, orange tree pruning residue in Spain is estimated to be more than 2.25 x 10^6 tons each year. This lignocellulosic residue is composed mainly of wood and the remaining fraction consists of leaves, bark, pith and young stems with relatively low cellulose content.

Recently, a conceptual integrated biorefinery based on orange tree pruning has been evaluated.[3] The main woody fraction was cooked with soda-anthraquinone to produce pulp of acceptable quality. Additionally, the remaining fraction was evaluated as fuel, resulting in a suitable material for cheap energy production. In the development of this biorefinery, biotechnology could play an important role by providing efficient and ecofriendly biocatalysts.[4] In connection to this, lignocellulose-degrading enzymes – which include hydrolases (such as xylanases) acting on

hemicelluloses, and oxidoreductases (such as laccases) involved in lignin degradation – are of enormous interest, especially in the field of pulp production, where they are introduced as substitutes for chlorine-containing reagents used in pulp bleaching.

Xylanases have been the type of enzymes most commonly incorporated into pulp bleaching sequences performed in pulp mills.[5] These enzymes mainly hydrolyze the xylan reprecipitated on the surface of cellulosic fibers after pulp cooking, contributing to an indirect release of lignin. As a result, the bleaching effect of chemical reagents is enhanced and the chemical consumption decreases. An alternative to xylanases have been laccases, which in the presence of mediators, the so-called laccase-mediator systems (LMS), act directly on lignin.[6] The potential of various laccases and synthetic mediators has been successfully shown in several bleaching sequences, 1-hydroxybenzotriazole (HBT) being one of the most efficient laccase mediators.[7,8] However, potential drawbacks of this and other synthetic mediators, such as high cost and dose, limited biodegradability and potential toxicity, have prompted an active search for alternative mediators with environmental and economic advantages. With this end in view, it has been suggested that free radicals of some fungal metabolites and lignin derived products from plants and cooking effluents could act as natural mediators of laccases. Some of these compounds, such as acetosyringone and syringaldehyde, have been already identified as efficient laccase mediators in pulp bleaching.[7,9]

Numerous studies on pulp enzymatic bleaching have evaluated either xylanases or laccases.[7,8,10] However, fewer reports address the use of both enzymes in sequence or jointly.[11-16]

Once the feasibility of producing pulp from orange tree pruning has been established,[3] the present study aimed at evaluating, for the first time, its bleachability by means of different enzyme-containing bleaching sequences. To this end, soda-anthraquinone pulp from orange tree pruning was treated sequentially with xylanase and LMS prior to a standard bleaching sequence (consisting of alkaline extraction and peroxide stages).

EXPERIMENTAL

Chemicals and raw material

Hydroxybenzotriazole (HBT) was purchased from Fluka (Ref. 54802). Acetosyringone (AS), NaOH and H_2O_2 were reagent-grade and obtained from Merck or Sigma–Aldrich. Novozyme® 51003 (laccase from the ascomycete *M. thermophila*), Novozyme® 51002 (laccase from the basidiomycete *T. villosa*) and Pulpzyme® HC (xylanase produced from a genetically modified *Bacillus* species) were donated by Novozymes® (Bagsvaerd, Denmark).

Orange tree pruning (OTP) was obtained directly from an orange tree plantation in Palma del Río, Córdoba, Spain. The chemical composition of this raw material was 3.6% extractives, 3.4% ash, 19.9% lignin and 73.2% holocellulose. The analyses were carried out according to: TAPPI T 204 om-88, TAPPI T 211 om-93, TAPPI T 222 om-88 and Wise *et al.*[17] respectively.

Pulp production

OTP pulp was obtained using a cylindrical 15-L batch reactor with a jacket-type electrical heater controlled by a computer. The reactor was fitted with a rotating axle to ensure proper agitation, and was connected to a control unit equipped with an appropriate mechanism for measuring and controlling pressure and temperature. The raw material was cooked with soda-anthraquinone in the reactor under the following conditions: 185 ºC, 60 min, 20% NaOH, 1% anthraquinone and 8:1 liquid/solid ratio. The resulting cooked material was fiberized in a wet disintegrator at 1200 rpm for 30 min, and particles were separated by sieving through a screen of 0.16 mm mesh ^{3}. Before applying any enzymatic treatments, the obtained pulp was acidified to the different values of pH required for the subsequent bleaching stages. This acidification consisted in the addition of drops of H_2SO_4 4N to a 3% pulp suspension until reaching the required pH.

Enzymatic treatments

Experiments were performed in duplicate using 70 g of acidified OTP pulp, which was introduced into 500-mL pressurized reactors together with the commercial laccases and chemicals. The laccase-mediator systems used were: i) *T. villosa* laccase with HBT (L-Tv+HBT), ii) *T. villosa* laccase with AS (L-Tv+AS), and iii) *M. thermophila* laccase with AS (L-Mt+AS). The mixture was blended intensively before adding oxygen at a pressure of 6 kg per cm^2 and submerging the reactors in a thermostatic bath. Treatment duration was fixed at 4 h. In accordance with what has been previously specified by Martín-Sampedro *et al.*,[11,12] laccase dose, temperature, mediator concentration and consistency were fixed during all laccase treatments at 17.5 U/g oven dried pulp (odp), 45 ºC, 0.01 g/g odp and 10%, respectively (1 U of laccase is defined as the amount of laccase enzyme required to convert 1 µmol/min of ABTS to its cationic radical in 0.1 M sodium acetate buffer, pH 5.2 at 24 ºC). The enzymatic activity of commercial laccases was 400 U/mL and 250 U/mL, for *T. villosa*

laccase and *M. thermophila* laccase, respectively. Treatments with *T. villosa* laccase were carried out in 100 mM sodium tartrate buffer at pH 4, and treatments with *M. thermophila* laccase were performed in 100 mM sodium phosphate at pH 7. The pH was fixed at these particular values because enzymes remained sufficiently stable at least for 8 h at room temperature, and without significant loss of their maximum activity levels, taking into account their optimal pH.[18]

A few drops of Tween 80 were added to all assays in order to improve the interaction between enzyme and substrate.[19] Controls without enzyme and mediator (Lc-Tv and Lc-Mt) were also assayed for each case. After each laccase-based bleaching stage (L), pulps were filtered and washed with distilled water until reaching neutral pH, and air dried at room temperature.

Laccase treated pulps and controls were subjected to an alkaline extraction (E stage), in order to remove the lignin modified in the previous step. The conditions of alkaline extraction were selected as follows: NaOH, 1.5% odp; consistency, 5%; temperature, 90 ºC; and treatment time, 120 min. Thereafter, the pulps were filtered and thoroughly washed with distilled water and air dried at room temperature. Finally, a hydrogen peroxide bleaching stage (P stage) was applied to evaluate the influence of the enzymatic treatments under the following conditions: H_2O_2, 3% odp; NaOH, 1.5% odp; DTPA, 1% odp; $MgSO_4 \cdot 7H_2O$, 0.2% odp; consistency, 5%; temperature, 90 ºC; and treatment duration, 120 min; resulting in a LEP biobleaching sequence.

With a view to assess whether it resulted in a better performance of the laccase biobleaching, a xylanase pretreatment (X) was included before the optimal laccase mediator system. The conditions used during the xylanase pretreatment were as follows: consistency, 10%; incubation time, 2 h; 20 AXU/g odp; temperature, 45 ºC; and pH 7 (as per the product specification sheet). One xylanase unit is defined as the amount of enzyme which, under standard conditions (pH 9.0, 50 °C, 30 min incubation), releases a defined amount of dye from dyed RBB xylan. After the xylanase and laccase treatments, an alkaline extraction and a hydrogen peroxide bleaching stage were also performed as described above, resulting in a XLEP biobleaching sequence.

Pulp and paper characterization

Treated pulps and controls were characterized according to the corresponding standards in terms of their kappa numbers (UNE 57034), and viscosities (UNE 57–039-92). Hexenuronic acid (HexA) content was analyzed in pulps obtained from the LEP, XLEP and conventional EP sequences tested according to Gellerstedt and Li.[20]

Handsheets were obtained from the treated pulps in accordance with UNE-EN ISO 5269–2 and characterized in terms of tensile index, stretch and tear index (UNE-EN ISO 5270). Brightness (UNE 57062) and CIE L*a*b* and CIE L*C* coordinates (T 527) were also determined in the handsheets using a spectrophotometer ELREPHO 070 (Lorentze and Wettre).

Accelerated ageing

The bleached and the unbleached handsheets were subjected to accelerated ageing to analyze changes in their optical properties.

This accelerated ageing was carried out in a climatic test cabinet CTS (model C-20/250/S), and consisted in a moist heat treatment at 80 ºC and 65% relative humidity for 6 days, as specified by the standard UNE 57092–4. After accelerated ageing, pulps were characterized in terms of brightness and CIE L*a*b* and CIE L*C* coordinates, also in accordance with the aforementioned standards.

RESULTS AND DISCUSSION

Soda-anthraquinone pulping was carried out using orange tree pruning as raw material. The resulting unbleached pulp showed a kappa number, viscosity, and brightness of 22.0, 430 mL/g and 33.5% ISO, respectively. This unbleached pulp was subjected to several biobleaching sequences using three different laccase mediator systems. Once the most efficient LMS was selected, a pretreatment with xylanase was included in the biobleaching sequence as first stage, in order to evaluate if this type of hydrolytic enzyme enhances the subsequent biobleaching sequence.

Biobleaching with laccase mediator systems

Figure 1 shows the evolution of kappa number (Fig. 1a) and brightness (Fig. 1b) through the different LEP biobleaching sequences assayed and the color coordinates (CIE L*a*b* and CIE L*C*) at the end of the LEP sequences (Fig. 1c and 1d). It should be mentioned that enzymatic treatments using laccase from *T. villosa* were carried out at pH 4, and this lower pH could enhance the hydrogen peroxide bleaching stage, as suggested by the lower kappa number and the higher brightness obtained in the Lc-Tv control sequence (pH 4 during the L stage), compared to that observed in the Lc-Mt control sequence (pH 7) (Fig. 1a). Similar effects of the pH on control sequences have been reported by other authors.[13,21] A plausible explanation for this finding could be the elimination of metallic ions from pulps (such as Fe^{2+}, Mn^{2+} and Cu^{2+}) during enzymatic or control-buffer stages under conditions of acidic pH (pH 4 in Lc-Tv).[22] When present, these metallic ions could result in

hydrogen peroxide degradation to form hydroxyl radicals, which would reduce the efficiency of the hydrogen peroxide stage,[23] a phenomenon that possibly occurs in the Lc-Mt control sequence (pH 7). Furthermore, these metallic ions could also decrease the brightness and contribute to the reversion of brightness resulting from the formation of colored complexes.[24] Finally, the acidic pH also contributes to the removal of HexA, thus enhancing the bleaching process.[7,13]

When the L treatment was applied, with laccase either from *T. villosa* or from *M. thermophila*, higher brightness and lightness (L*) and lower color (C*, a* and b* coordinates) and kappa number were obtained after the P stage, compared with the respective control sequences. Nevertheless, the highest enhancements in bleaching were observed in the sequences with *T. villosa* laccase, likely as a result of its higher redox potential. Consistently, Valls *et al.*[13] observed lower kappa numbers and higher brightness in eucalyptus kraft pulp treated with laccase from *T. villosa* (combined with violuric acid at pH 4 and 50 ºC, VATvL), compared to laccase from *M. thermophila* (combined with methyl syringate at pH 7 and 50 ºC, MeSMtL). These authors reported that the VATvL system removes both lignin and hexenuronic acids (two major contributors to the kappa number) from fibers, while the MeSMtL system only removed more lignin than the VATvL system, but not hexenuronic acids.

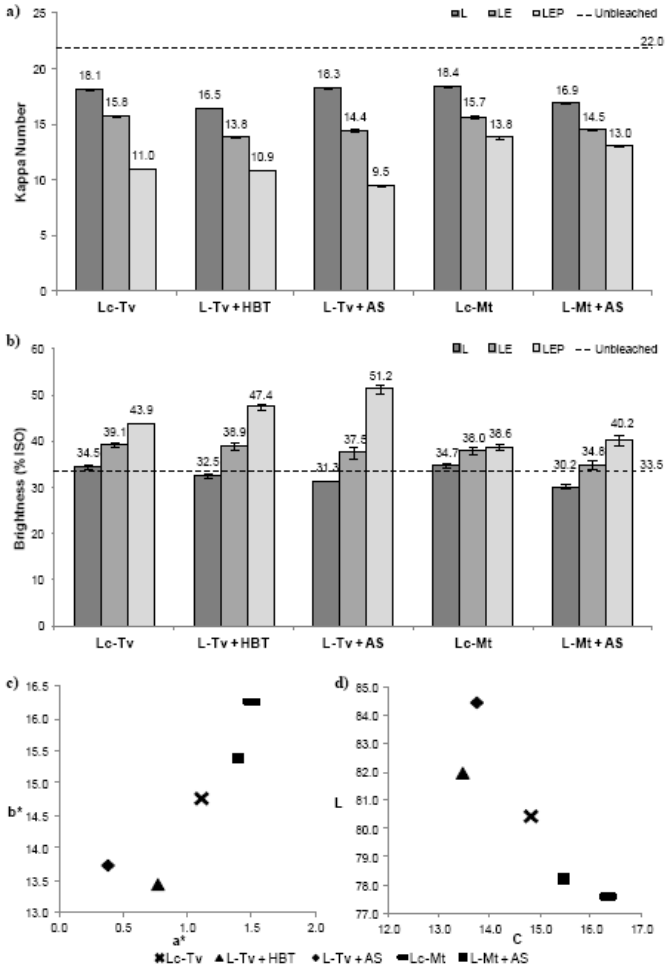

Figure 1: Evolution of pulp properties after each stage in biobleaching sequences: kappa number (a); brightness (b) and color coordinates of biobleached pulps: CIE L*a*b* (c) and CIE L*C* (d)

Fillat *et al*.[21] also compared a high-redox potential laccase from *Pycnoporus cinnabarinus* (combined with violuric acid or syringaldehyde at pH 4 and 50 ºC) with laccase from *M. thermophila* (combined with methyl syringate at pH 7 and 50 ºC) in a TCF sequence applied to flax soda-anthraquinone pulp. They found that laccase from *M. thermophila* was associated to smaller reductions in kappa number and increases in brightness, which is consistent with our results. However, when Babot *et al*.[25] compared the same enzymes, combined with methyl syringate or syringaldehyde, on eucalyptus kraft pulp, laccase from *P. cinnabarinus* resulted in poorer delignification and similar or lower increase in brightness (depending on the mediator added) at pH 4 and 50 ºC. It must be pointed out, however, that these treatments were longer (12 h) and *M. thermophila* laccase was used at pH 6.5, while in our study and in those of Valls *et al*.[13] and Fillat *et al*.,[21] this laccase was used at pH 7 and for shorter times (4 h). Therefore, the efficiency of the laccases could have been influenced by the type of pulp, the mediator and the treatment conditions.

The evaluation of the mediators used with the *T. villosa* laccase shows that the natural mediator AS led to a lower delignification and brightness after the L stage, but to a higher delignification and brightness at the end of the LEP biobleaching sequence compared to HBT. Delignification after the L stage with AS may have been impaired by simultaneous coupling reactions (homopolymerization and/or coupling with lignin) and also by oxidation of lignin,[26] as suggested by the slightly higher kappa number found after L-Tv+AS, compared to that of the Lc-Tv control pulp. Barneto *et al*.[27] reported that, when the mediator structure is based on a syringyl unit (with two methoxy groups in *ortho*-phenol positions), laccase-induced reactions slightly increase the kappa number, which is accompanied by a marked increase in color and reduction in brightness, suggesting enzymatic polymerization of the syringyl derivatives, which cause the formation of chromophore groups. However, when HBT was used, neither polymerization nor grafting reactions were found to take place, with a 1.6 point reduction in kappa number and no significant change in brightness, compared with the control after the L stage. When a subsequent alkaline extraction and a hydrogen peroxide bleaching stage were included in the process, the adverse effect on L-Tv+AS was reversed, observing the lowest kappa number and the highest brightness with this LEP sequence, as a result of the oxidation and dissolution of chromophoric species and lignin degradation products.[26] Thus, higher delignification (56.9%) and brightness (51.2% ISO) were achieved at the end of the L-Tv+AS sequence compared to the L-Tv+HBT sequence (delignification of 50.5% and brightness of 47.4% ISO) and the Lc-Tv control sequence (delignification of 49.8% and brightness of 43.9% ISO). Barneto *et al*.[27] also observed a higher reduction in kappa number and increase in brightness during the P stage when natural mediators had been used in a previous laccase stage, while, with HBT, the subsequent kappa number reduction was similar to that of the control pulp. Despite these reductions, when the whole biobleaching sequence was taken into account, these authors observed a higher increase in delignification and brightness associated to HBT, which is contrary to our results. Nevertheless, a different enhancement in bleaching was reported by the same authors using different raw materials (kenaf and sisal pulps), therefore, it would be reasonable to think that the different behavior observed in this study might have been the consequence of using OTP pulp. A plausible explanation could be the low HexA content in OTP pulp (9.4 μmol/g odp), as the greater reduction of the kappa number typically caused by HBT is mostly mediated by a much deeper elimination of HexA, compared with that caused by natural mediators based on a syringyl unit (*e.g.* AS).[13,21]

As aforementioned, at the end of the L-Mt+AS sequence, higher kappa and lower brightness were observed compared to all L-Tv sequences. The reasons are probably not only a higher amount of chromophores formed with this enzyme during the L stage (corresponding with the highest decrease in brightness observed during the L stage), but also the fact that the laccase from *M. thermophila* may induce the formation of chromophores, which are more difficult to remove from the pulp in subsequent stages, as Fillat *et al*.[21] have reported. When the same LMS (L-Mt+AS) was used at 45 ºC and pH 6.5 with soda-anthraquinone pulps from olive tree pruning residue[11] or oil palm empty fruit bunches (EFB),[12] brightness increases found at the end of the LEP sequence were of 3.7 and 3.0% ISO, respectively; whereas, when the same LMS was applied at pH 7 to OTP pulp in the same LEP sequence, there was an increase of 1.6% ISO. These results

confirm that the type of pulp plays a role in the effectiveness of the biobleaching sequence.

No big differences in viscosity after each stage of the LEP biobleaching were found, values between 395 and 430 mL/g suggest that the biobleaching process causes no significant degradation of the polysaccharide chains, which is in agreement with previous reports.[7,28,29] Thus, all the sequences provided bleached pulps with similar mechanical properties (Table 1) to those of the unbleached pulp reported by Gonzalez *et al*.[3] when OTP were subjected to soda-anthraquinone pulping under different conditions. Therefore, the LEP biobleaching used in this study did not significantly reduce the mechanical strength of the obtained pulps. This is consistent with what has been observed in experiments with different raw materials and has been reported elsewhere.[11,12]

Although all the different LEP sequences assayed resulted in fairly similar mechanical properties, a slight increase (mainly in tear index) was found when LMS containing AS was applied. Moldes *et al*.[9] also found a slightly increased tear index when laccase from *T. villosa* was applied jointly with HBT or violuric acid. However, they did not find any improvement when a natural mediator was applied, although perhaps it should be pointed out that they used syringaldehyde instead of AS. Martín-Sampedro *et al*.[11] also found an increase of the tear index when laccase from *M. thermophila* and AS were applied to olive tree pruning pulp. This improvement of pulps treated with LMS is likely caused by the increased flexibility of the enzyme-treated pulps as a result of delignification and delamination of cell walls.[14]

Regarding the burst index, no significant differences between the LMS experiments and their respective controls were observed. Contrarily, other authors have reported a slight decrease in the burst index when the LMS was applied,[9] although using different mediators (violuric acid or syringaldehyde) and also a different raw material (eucalypt kraft pulp). On the other hand, the effect of the pH at which the enzymatic stage is carried out, seems to have a significant effect on the burst index. Experiments carried out at pH 7 (Lc-Mt and L-Mt+AS) provided pulps with lower burst index than those performed at pH 4 (Lc-Tv, L-Tv+HBT and L-Tv+AS). As it has been explained above, during the control or the enzymatic stage at pH 7, the metallic ions present in the pulp could degrade the cellulose.[22]

Biobleaching including a xylanase pretreatment

After selecting the optimal LMS for OTP pulp biobleaching, a pretreatment with xylanase was assayed to evaluate the potential for enhancement of this enzyme over the subsequent LEP biobleaching sequence. Several authors have reported how xylanases can boost a subsequent biobleaching using LMS by means of hydrolyzing the xylans that have reprecipitated on the surface of cellulosic fibers after pulp cooking and releasing lignin in an indirect way.[11-15,30]

Table 1
Tear and burst indexes of handsheets from unrefined pulps obtained at the end of LEP biobleaching sequences with or without xylanase pretreatment, and an EP sequence

	Tear index (mNm^2/g)	Burst index ($KPam^2/g$)
Lc-Tv	1.36 ± 0.08	0.47 ± 0.02
L-Tv+HBT	1.37 ± 0.06	0.47 ± 0.01
L-Tv+AS	1.45 ± 0.08	0.50 ± 0.01
Lc-Mt	1.18 ± 0.10	0.40 ± 0.02
L-Mt+AS	1.28 ± 0.07	0.38 ± 0.03
XcLc-Tv+AS	1.55 ± 0.09	0.54 ± 0.04
XL-Tv+As	1.36 ± 0.10	0.40 ± 0.03
EP	1.43 ± 0.07	0.48 ± 0.02

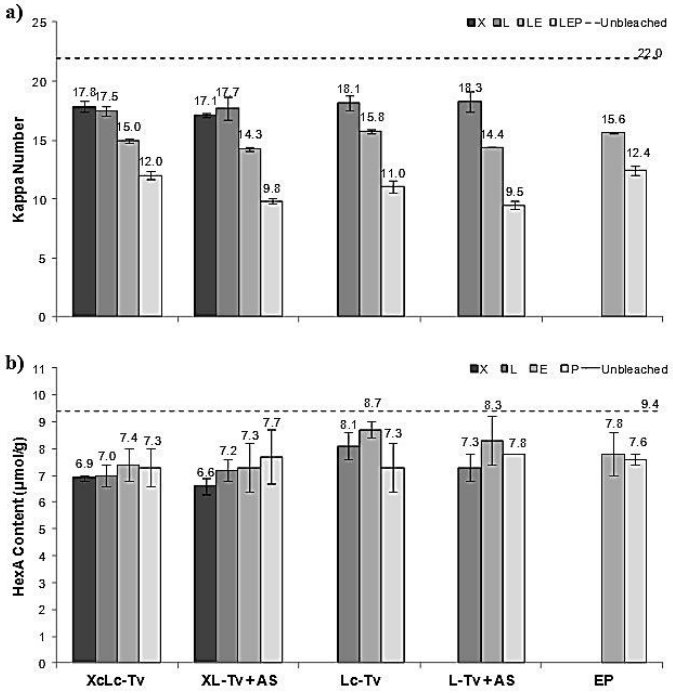

Figure 2: Evolution of kappa number (a) and hexenuronic acid content (b) after each stage in biobleaching sequences with xylanase pretreatments and their respective controls

Figure 2 shows the evolution of the kappa number and HexA content along with the optimal LEP biobleaching sequence with and without a xylanase pretreatment (XL-Tv+AS and L-Tv+AS, respectively), their respective control sequences (XcLc-Tv+AS and Lc-Tv+AS) and a simple TCF sequence including alkaline extraction and a hydrogen peroxide stage without any enzymatic or control treatment (EP). Comparing kappa number values, the addition of a xylanase pretreatment did not cause a significant increase in delignification (kappa number of 9.5 and 9.8 for L-Tv+AS and XL-Tv+AS, respectively). When the HexA content was analyzed (Fig. 2b), similar values were found in the xylanase pretreated pulp and in the control pulp. In fact, no significant differences in HexA content were found comparing all the bleached pulps. Contrarily, several authors have reported an enhancement in delignification when a xylanase pretreatment was performed prior to LMS biobleaching. This reduction of the kappa number can be put down to a delignifying effect resulting from the removal of lignin trapped between xylan chains.[31] Some authors have also attributed this reduction of the kappa number to the removal of HexA linked to xylans as side groups.[30] To illustrate this last point: Valls *et al.*[16] found an enhancement in delignification from 44% to 55% using eucalyptus kraft pulp; Martín-Sampedro *et al.*[12] observed an enhancement from 48.4% to 53.9% when EFB soda-anthraquinone pulp was used; and Oksanen *et al.*[15] reported an enhancement from 39% to 44% using pine kraft pulp. All these authors did observe an elimination of HexA during xylanase pretreatment, which is known to contribute to kappa number determination.[32] This is probably the cause of the observed reduction in kappa number (interpreted as enhancement in delignification). For this reason, Valls *et al.*[13,16] reported that the xylanase pretreatment boosted the removal of HexA without affecting the content of lignin in the fibres. Therefore, when OTP pulp was used, the xylanase pretreatment did not boost the LMS biobleaching, as the xylanase did not improve HexA removal. This finding is likely the result of a low initial HexA content in the OTP soda-anthraquinone pulp (9.4 µmol/g odp) compared with unbleached pulps obtained from other raw materials, such as eucalyptus kraft pulp (~40 µmol/g odp), EFB soda-anthraquinone pulp (~20 µmol/g odp), pine kraft pulp (30 µmol/g odp) or

sisal soda-anthraquinone pulp (~40 µmol/g odp), as well as different raw materials studied.

The evaluation of brightness evolution (Fig. 3a) and of the color coordinates observed at the end of the different biobleaching sequences (Fig. 3b and 3c) provided similar results to the analysis of the kappa number. Xylanase pretreatment did not add any significant improvement. In fact, pulp from the XL-Tv+AS sequence had lower brightness (48.2% ISO) than pulp from the L-Tv+AS sequence (51.2% ISO). Similarly, the best color coordinates (lowest color [a*, b* and C*] and the highest lightness [L*]) were obtained with the L-Tv+AS sequence (where there is no xylanase pretreatment). In contrast with these findings, several authors have reported an improvement of pulp optical properties (1.5-6% ISO increase in brightness) when xylanase pretreatment was included prior to the LMS treatment.[11-16] However, these authors used raw materials with a higher HexA content than that of the OTP pulp. Fillat *et al.*[21] also used a raw material with low HexA content (flax soda-anthraquinone pulp, with initial HexA content of 10.7 µmol/g odp), observing no improvement in brightness when the xylanase pretreatment was added prior to LMS biobleaching, which is consistent with what we report here. Nevertheless, these authors did observe the removal of HexA and a reduction of the kappa number mediated by the xylanase pretreatment.

No significant differences in viscosity after each stage of the XLEP biobleaching were found, with values between 395 and 415 mL/g. This result indicates that neither the LMS nor the xylanase treatments are so aggressive to degrade the cellulose chains. Similar results have been described elsewhere.[11,12,14] Conversely, other authors have reported increases in viscosity after the xylanase pretreatment, suggesting the elimination of hemicelluloses of low molar mass as a potential explanation.[13,30]

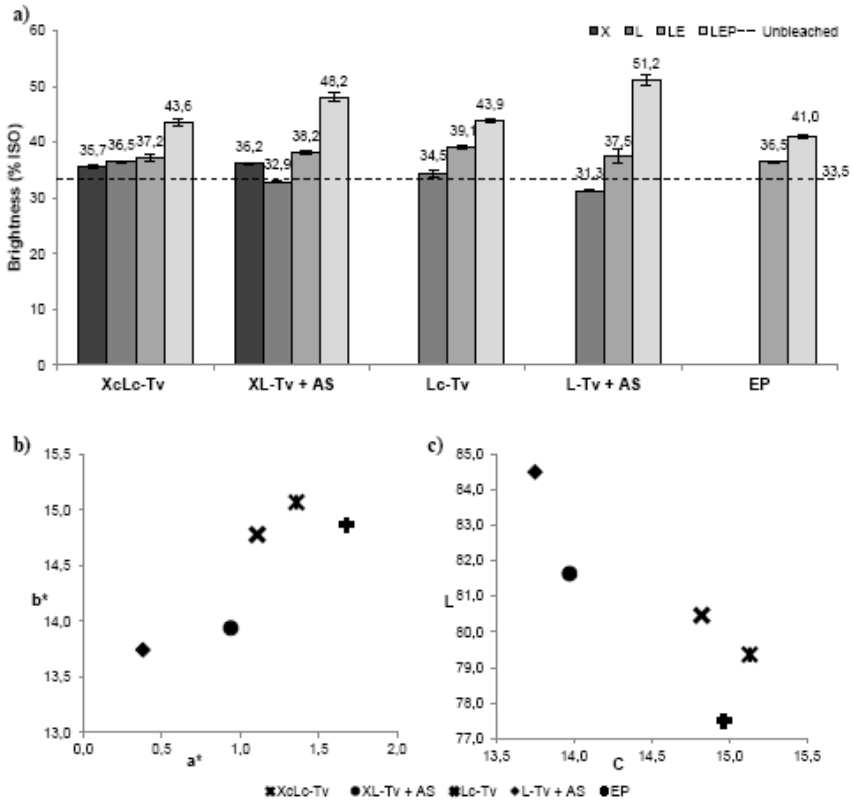

Figure 3: Evolution of brightness after each stage in biobleaching sequences with xylanase pretreatments and their respective controls (a)

Table 2
Changes in brightness and yellowness after accelerated ageing of OTP pulps coming out of several laccase biobleaching sequences, with or without xylanase pretreatment, and an EP sequence

	Brightness (% ISO)			Yellowness (%)		
	Original[a]	Aged[b]	Reduction	Original[a]	Aged[b]	Increase
Lc-Tv	43.9	43.7	0.2	31.5	32.7	1.2
L-Tv+HBT	47.4	47.1	0.3	28.3	29.2	0.9
L-Tv+AS	51.2	50.5	0.7	27.8	28.8	1.0
Lc-Mt	38.6	38.5	0.1	32.2	33.5	1.3
L-Mt+AS	40.2	39.3	0.9	35.4	36.0	0.6
XcLc-Tv+AS	43.6	42.8	0.8	31.4	32.5	1.1
XL-Tv+As	48.2	46.3	1.9	28.3	29.9	1.6
EP	41.0	40.2	0.8	31.9	33.5	1.7

[a] Before accelerated ageing treatment; [b] After accelerated ageing treatment

Table 1 shows the mechanical properties of handsheets formed from the bleached pulps produced with the different biobleaching sequences. Although all bleached pulps showed similar mechanical properties, slightly worse mechanical strength was found for pulps pretreated with xylanases, mainly in regard to the burst index. As the burst index is related to interfiber bonding, this reduction could be the result of hemicelluloses being lost during the xylanase pretreatment. A similar but more pronounced effect (probably because the hemicellulose content in the initial pulp was greater) has been observed in the tensile index (also related to interfiber bonding) by Martin-Sampedro *et al*. using EFB pulp[12] and olive tree pruning pulp;[11] and by Herpoel *et al*.[14] using wheat straw soda-anthraquinone pulp.

Effect of accelerated ageing on optical properties

Accelerated ageing of bleached pulps was conducted in order to evaluate how enzymatic treatments affect the stability of brightness and yellowness (Table 2). As expected, accelerated ageing decreased brightness and increased yellowness of all bleached pulps tested in our study. However, a more pronounced brightness reversion has been reported in eucalyptus kraft pulps (around 4-5% ISO),[33,34] probably because of their higher HexA content (~ 40 μmol/g odp in eucalyptus pulp; 9.4 μmol/g odp in OTP pulp). The HexA content is known to be a contributing factor to yellowness and brightness reversion.[32]

Overall, brightness reversion in all LEP sequences tested here resulted to be bigger than it was in the controls (Table 2). Similarly, Martín-Sampedro *et al*.[11,12] reported a loss of stability of the optical properties when pulps from EFB or olive tree pruning were treated with L-Mt+AS and compared with their respective controls. Biobleaching of an industrial eucalyptus pulp with the same LMS [29] or with a laccase from *Trametes* sp. I-62 and AS (L-Tsp+AS),[35] also resulted in brightness reversion. Both these LMS (L-Mt+AS and L-Tsp+AS) were unable to significantly remove HexA, according to the results showed by Martín-Sampedro *et al*.[12,35] In contrast with this body of evidence, other authors have reported an improvement of brightness stability after a LMS treatment of an eucalyptus pulp; however, all these authors reported the removal of HexA during the enzymatic treatments.[7,34] Basal HexA content in all OTP pulps assayed in our study was very low, and no significant removal of HexA was observed during any biobleaching sequence, which explains why optical stability did not improve in our experiments.

Finally, if L-Tv+HBT and L-Tv+AS sequences are compared, higher reversion of optical properties was observed when the natural mediator was used, probably on account of the higher amount of chromophores formed. Therefore, the type of enzyme, mediator and pulp used would determine the stability of the optical properties, since not only the HexA content, but also the lignin and hemicelluloses content can modify the response to accelerated ageing.[32]

When a xylanase was used as pretreatment (XLEP), higher reversion of brightness and yellowness was observed, compared with LEP, control, and EP sequences. Similar results have been described by Martín-Sampedro *et al*.[11,12] although Roncero *et al*.[31] reported less brightness reversion after a xylanase pretreatment. However,

these last authors used eucalyptus kraft pulp and observed a reduction in HexA content due to the action of xylanases.

CONCLUSION

Orange tree pruning soda-anthraquinone pulp seems to be a suitable material to be biobleached with a LEP sequence, consisting of laccase-mediator, alkaline extraction and hydrogen peroxide stages. Laccase from *T. villosa*, in combination with acetosyringone, was the LMS that reached the greatest delignification and increase of optical properties in the resulting bleached pulps. The viscosity and mechanical properties of the obtained handsheets were not significantly changed. The addition of a xylanase pretreatment before the optimal LEP sequence did not improve any of the pulp and handsheet properties tested, probably because of the low HexA content in orange tree pruning.

ABBREVIATIONS

AS	Acetosyringone
AXU	Xylanase activity unit
DTPA	Diethylene triamine pentaacetic acid
E	Alkaline extraction
EFB	Empty fruit bunches
EP	Bleaching sequence consisted of alkaline extraction and hydrogen peroxide stages
HBT	1-hydroxybenzotriazole
HexA	Hexenuronic acid
L	Laccase-mediator pretreatment
Lc-Mt	Biobleaching sequence including a control pretreatment without laccase from *Myceliophthora thermophila* nor mediator
Lc-Tv	Biobleaching sequence including a control pretreatment without laccase from *Trametes villosa* nor mediator
LMS	Laccase-mediator system
L-Mt+AS	Biobleaching sequence including a pretreatment with laccase from *Myceliophthora thermophila* and acetosyringone
L-Tv+AS	Biobleaching sequence including a pretreatment with laccase from *Trametes villosa* and acetosyringone
L-Tv+HBT	Biobleaching sequence including a pretreatment with laccase from *Trametes villosa* and 1-hydroxybenzotriazole
MeSMtL	LMS consisted of laccase form *Myceliophthora thermophila* and methyl syringate
Mt	*Myceliophthora thermophila*
odp	Oven dried pulp
OTP	Orange tree pruning
P	Hydrogen peroxide stage
RBB	Remazol Brilliand Blue
TCF	Total chlorine free
Tv	*Trametes villosa*
VATvL	LMS consisted of laccase from *Trametes villosa* and violuric acid
X	Xylanase treatment
XcLc-Tv+AS	Biobleaching sequence including a control pretreatment without xylanase and a control pretreatment without laccase from *Trametes villosa* nor acetosyringone
XL-Tv+AS	Biobleaching sequence including a pretreatment with xylanase and a pretreatment with laccase from *Trametes villosa* and acetosyringone

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