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Boosting the effect of a laccase–mediator system by using a xylanase stage in pulp bleaching

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ABSTRACT

Using an enzyme-based stage involving a xylanase (X) or laccase (as part of a laccase–mediator system, L) in a bleaching process can help reduce reagent consumption and hence its environmental impact. In this work, both types of enzymes were applied to eucalypt pulp. The influence of process variables in the laccase–mediator treatment (*viz.* laccase dose, HBT dose and reaction time) was assessed by using a three-variable sequential statistical plan. The effect of a pretreatment with X on the previous variables was also assessed. Kappa number and brightness models for the L stage and XL sequence were found to perform disparately, which suggests the formation of lignin derivatives interfering with brightness measurements. The L system oxidized readily accessible lignin within the first hours of treatment and affected the contents in cellulose and hexenuronic acids (HexA) of the resulting pulp. Xylanase facilitated access of the laccase–HBT system to lignin and HexA in cellulose fibres. The L treatment increased effluent properties such as Microtox toxicity, COD and colour, and led to strong inactivation of the enzyme. The increased toxicity of the effluents was due to HBT; based on statistical data, however, the effect can be reduced by lowering the mediator dose.

1. Introduction

The bleaching plant is among the sections in the pulp and paper industry which have undergone the greatest changes in response to environmental concerns arising from the formation of chlorinated dioxins and other chlorinated compounds during pulp production. Such concerns have brought about a major technological revolution in the pulp and paper industry which has led to the production of elemental chlorine free (ECF) and totally chlorine free (TCF) pulp [1,2]. The use of biotechnological methods in industrial processes can provide enormous benefits. Xylanases (X) are hydrolytic enzymes that catalyse xylan degradation. Their favourable effect has been ascribed to partial removal of xylans from fibre surfaces and to fibres thus being made more accessible to the reagents in subsequent bleaching stages [3,4]. The industrial implementation of xylanases is simple and economically feasible [5,6]. The use of laccases in the form of laccase–mediator systems (LMS) provides an effective alternative to xylanases acting directly on lignin. However, LMS must be fine-tuned before their final industrial implementation. Thus, their application conditions must be optimized in order to reduce enzyme and mediator consumption, and also reaction times. 1-Hydroxybenzotriazole (HBT) is currently deemed the most effective mediator [7–11]. Carefully designed statistical plans have been used for dye decolourization [12,13] and also to optimize LMS usage in the bleaching of softwood kraft [14] and flax [15–17] pulp. Recently, the laccase–mediator system was optimized for application to eucalypt kraft pulp after alkaline extraction [18].

In this work, the influence of process variables in an LMS treatment (*viz.* laccase and HBT doses, and reaction time) was evaluated in eucalypt kraft pulp subjected to the same enzyme stage. Models for predicting kappa number and brightness in the pulp were constructed and the effects of a xylanase pretreatment assessed. Also, pulp viscosity and hexenuronic acid content, and various effluent properties, were determined at different points of the process in order to evaluate the environmental impact of LMS.

2. Materials and methods

2.1. Raw material

The raw material used was oxygen-delignified kraft pulp from *Eucalyptus globulus* produced by the Torraspapel S.A mill in Zaragoza, Spain. Before treatment, the pulp was washed with a solution consisting of 50 mM Tris–HCl buffer at pH 7 at room temperature for 30 min. The initial kappa number, brightness and viscosity of the pulp after washing were 8.4, 51.2% ISO and $972 \pm 20\,mL\,g^{-1}$, respectively. The initial hexenuronic acid (HexA) content was $38.0 \pm 1.1\,\mu mol\,g^{-1}$ oven-dried pulp (odp).

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Table 1
Variables distribution with the factor of each level.

	Variables	−1	0	+1
x_1	Laccase dose (U g^{-1} odp)	1	10.5	20
x_2	HBT dose (% odp)	0.5	1.5	2.5
x_3	Reaction time (h)	1	4	7

2.2. Xylanase pretreatment (X stage)

The enzyme used in the X stage was a xylanase from the bacterial strain *Bacillus* sp. BP-7 that was isolated and characterized in previous work [19]. The X treatment was conducted by using 3 U xylanase g^{-1} odp in Tris–HCl buffer pH 7 and pulp of 10% consistency at 50 °C for 2 h. The resulting pulp was washed with decalcified water three times and distilled water once. The final kappa number, brightness, viscosity and hexenuronic acid (HexA) content of X pulp were 8.4, 53.7% ISO, 986 ± 31 mL g^{-1} and 32.5 ± 0.8 μmol g^{-1} odp, respectively.

2.3. Laccase–mediator treatment (L stage)

The enzyme used was commercial laccase from *Trametes villosa* (NS-51002) supplied by NOVOZYMES® (Bagsvaerd, Denmark). The mediator, HBT, was obtained from Sigma–Aldrich. Enzymatic runs were performed in a pressurized reactor at 590 kPa, 30 °C and a stirring rate of 60 rpm, using 50 mM sodium tartrate buffer at pH 4 and 5% consistency. A few drops of 0.05% w/v of the surfactant Tween 80 were also added. For each experiment 25 g odp of eucalypt pulp were treated. The experimental design used involved the laccase dose, mediator dose and reaction time as variables.

2.4. Experimental design

Enzyme treatments were applied in accordance with a 2^3 experimental design involving three variables at two levels each and three replicates at the central point, which required a total of 11 tests. Based on previous results for flax [15] and eucalyptus pulp [18], the tentative optimum ranges for the independent variables were taken to be 1–20 U g^{-1} odp (x_1), 0.5–2.5% odp (x_2) and 1–7 h (x_3). The minimum effective HBT dose was 0.5% odp—no reaction occurred at lower proportions [16]. The results for the variables were coded as −1 or +1 (Table 1), both for direct comparison of coefficients and for easier understanding of the effect of the variables on the responses. The independent variables were zeroed at the central point. The results of the three replicates at the central point, and their variance, were used in combination with the variance of the saturated model to calculate Snedecor's *F*-value in order to determine whether the variance was homogeneous or heterogeneous. The variance was homogeneous in all cases, so a linear model was constructed, its significant terms identified and potential curvature detected. Linear multiple regression was applied by using an Excel spreadsheet to implement the stepwise backward regression method and discard all terms with a probability $p < 0.05$.

2.5. Pulp properties

Treated pulp samples were characterized in terms of kappa number and brightness according to ISO 302 and 3688, respectively, using 2 replicates for the former and 3 for the latter. The standard deviation was 0.1 for both.

Pulp viscosity and the hexenuronic acid content were determined at low (1 U laccase g^{-1} odp, 0.5% HBT odp, 1 h), medium (10.5 U laccase g^{-1} odp; 0.5% HBT odp, 4 h) and high levels (20 U laccase g^{-1}, 2.5% HBT odp, 7 h) of the operating conditions. Viscosity was measured in accordance with ISO 5351-1 and the hexenuronic acid (HexA) content with UV detection [20].

2.6. Effluent properties

The toxicity, chemical oxygen demand (COD), colour and residual enzyme activity of the effluents were also determined at low, medium and high levels of the process variables for L and XL sequences. Toxicity, however, was also assessed at some additional points of the process in order to evaluate the effect of an increased HBT dose on it.

Effluent toxicity was determined with the Microtox method, which measures light reduction caused by the microbe *Vibrio fischeri* in contact with toxins (UNE-EN ISO 11348-3). One equitox·m^3 is defined as the reciprocal of the wastewater dilution (expressed in parts per one) resulting in 50% inhibition within 15 min under typical biotesting conditions. Toxicity measurements were colour-corrected as per the recommendations of Azur Environmental, the manufacturer of the MicrotoxOmni equipment used. COD and colour values were calculated in accordance with ASTM D1252-00 and ASTM D1029-00, respectively. Absorbance data were obtained at 600 nm for COD and 465 nm for colour. The enzymatic activity of laccase was defined as the amount of enzyme needed to convert 1 μmol of the substrate ABTS per minute. Oxidation of ABTS was followed by an increase in the absorbance at 436 nm (ε_{436} = 29300 M^{-1} cm^{-1}) as measured on a Shimadzu 1603 UV–vis spectrophotometer. The reaction mixture contained 5 mM ABTS, 100 mM sodium acetate buffer at pH 5 and 10–50 μL of enzyme solution. Two replicates for toxicity, COD and residual enzyme activity were performed and four replicates for colour.

3. Results

The first pulp properties to be optimized were kappa number and brightness. The results of these properties from L and XL sequences are shown in Table 2.

3.1. Modelling

Experimental data were fitted to a second-order polynomial equation with the kappa number (Y_{KN}) and brightness (Y_{Br}) as responses. A preliminary test with the four models (*viz.* kappa number and brightness in the L and XL sequences) revealed that the quadratic term was significant ($p < 0.05$). Two additional tests were therefore required in order to identify the variables possessing a significant term and ensuring their accurate discrimination. A second analysis of the modelling equations provided the following responses, where all significant terms had $p < 0.05$:

Kappa number model for L

$$Y_{KNL} = 6.95 - 0.35x_1 - 0.27x_2 - 0.54x_3 - 0.18x_1x_3 + 0.18x_1x_2x_3 - 0.77x_2^2 + 1.3x_3^2 \quad (1)$$

with $R^2 = 0.99$.

Brightness model for L

$$Y_{Br(\%ISO)L} = 58.85 + 2.25x_1 + 0.39x_2 + 2.4x_3 - 4.77x_1^2 - 4.45x_2^2 + 5.56x_3^2 \quad (2)$$

with $R^2 = 0.99$.

Kappa number model for XL

$$Y_{KN\,XL} = 5.25 - 0.26x_1 - 0.23x_2 - 0.56x_3 + 0.65x_3^2 \quad (3)$$

with $R^2 = 0.94$.

Table 2
Applied conditions of L experiences and kappa number and brightness results after L and XL sequences.

x_1	x_2	x_3	Laccase dose (U g^{-1})	HBT dose (%)	Time (h)	L		XL	
						Kappa number	Brightness (%ISO)	Kappa number	Brightness (%ISO)
−1	−1	−1	1	0.5	1	8.3	50.3	7.1	50.4
1	−1	−1	20	0.5	1	8.4	54.8	6.2	55.4
−1	1	−1	1	2.5	1	8.1	50.6	6.5	54.0
1	1	−1	20	2.5	1	7.3	55.5	6.0	54.9
−1	−1	1	1	0.5	7	7.9	54.6	5.9	57.2
1	−1	1	20	0.5	7	6.5	59.5	5.2	58.2
−1	1	1	1	2.5	7	7.1	56.2	5.1	59.3
1	1	1	20	2.5	7	6.3	60.0	5.1	60.2
0	0	0	10.5	1.5	4	7.0	59.3	5.4	59.7
0	0	0	10.5	1.5	4	7.1	58.2	5.0	58.3
0	0	0	10.5	1.5	4	6.8	59.1	5.3	58.4
1	0	0	20	1.5	4	6.6	56.3	5.7	57.7
0	−1	0	10.5	0.5	4	6.5	54.0	5.5	56.3

Brightness model for XL

$$Y_{Br(\%ISO)XL} = 58.46 + 1.04x_1 + 1.04x_2 + 2.52x_3 - 2.23x_1^2 \quad (4)$$

with $R^2 = 0.89$.

In the previous equations, $x_1 = (L - 10.5)/9.5$ (L denoting the laccase dose, in U g^{-1}), $x_2 = (M - 1.5)/1$, (M denoting the HBT dose, as a percentage) and $x_3 = (t - 4)/3$ (t being the reaction time, in hours). As can be seen from the R^2 values obtained, the fit was quite good in all cases.

3.2. Model fitting

Experimental kappa number and brightness values were compared with the calculated values provided by the models (Fig. 1). The differences were all minimal and fell within the acceptable ranges in the respective ISO standards. Therefore, the proposed models provide an accurate depiction of the actual process. The models for the L stage exhibited better fitting than those for the XL sequence.

3.3. Models for L pulp

Fig. 2 shows the predicted response surfaces for kappa number and brightness obtained from Eqs. (1) and (2) at a constant laccase dose. Both properties were influenced by the three variables, the reaction time having the strongest effect and the mediator dose the weakest. The laccase dose exhibited a linear influence on kappa number (Fig. 2a) and a quadratic influence on brightness (Fig. 2b). On the other hand, the mediator dose and reaction time exhibited a quadratic influence on both pulp properties.

The proposed models were used to identify the points leading to the smallest kappa number and highest brightness. In those cases

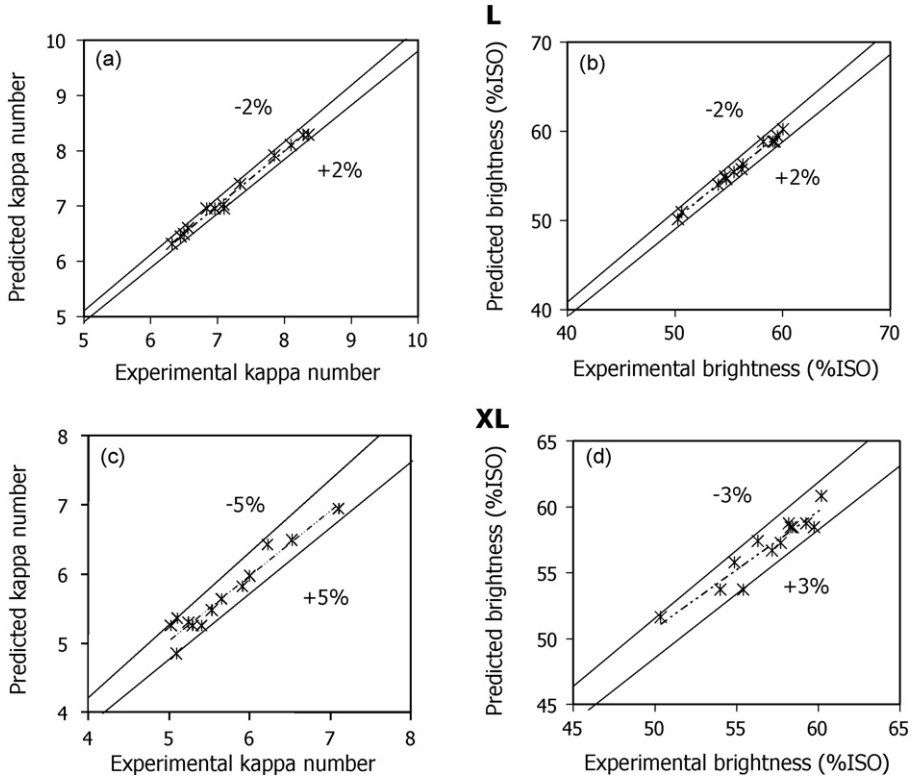

Fig. 1. Experimental *vs.* predicted results of kappa number (a) and brightness (b) for L sequence and of kappa number (c) and brightness (d) for XL sequence, with its corresponding errors.

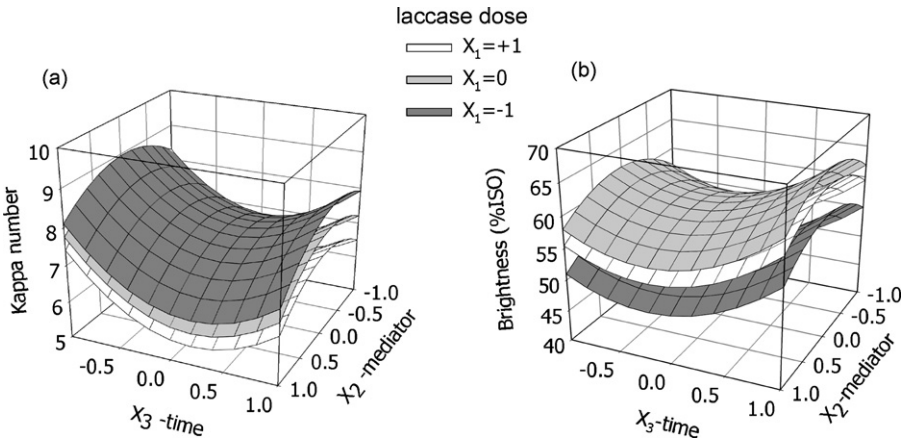

Fig. 2. Predicted surfaces for L sequences. Kappa number (a) and brightness (b) with constant laccase doses-x_1. $x_1 = +1$ (□); $x_1 = 0$ ( ) and $x_1 = -1$ (■).

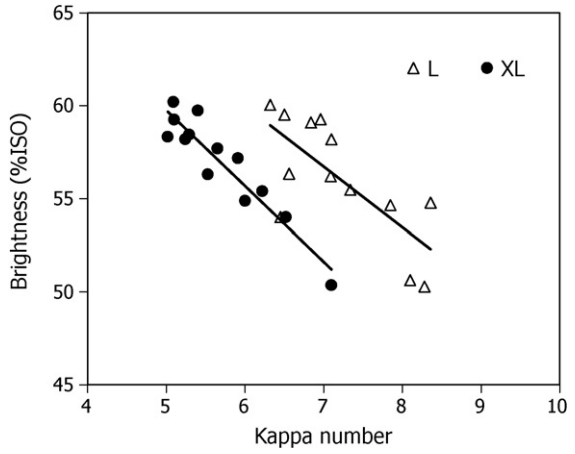

Fig. 3. Brightness vs. kappa number of L and XL pulps.

where a quadratic term for some variable had any effect, the inflection point was estimated from the derivative of the model with respect to the particular variable, and the equation equalled to zero. The combination of a laccase dose of 20 U g^{-1} odp, an HBT dose of 2.5% odp and a time of 4.6 h (20, 2.5, 4.6) was expected to provide the smallest kappa number (5.5), and that of a laccase dose of 13 U g^{-1} odp, an HBT dose of 1.5% odp and 7 h (13, 1.5, 7) the highest brightness (67.1%ISO). Although the HBT dose used in both was in its high or middle segment of variation, it was scarcely influential, which suggests that it can be reduced without detracting from the results.

3.4. Effect of an enzyme pretreatment with xylanase

XL pulp had a kappa number 1–2 units smaller than L pulp, but similar brightness (Fig. 3). In order to evaluate the effects of xylanase on the different variables of the LMS treatment, the models obtained for pulp samples subjected to an L stage and an XL sequence were compared (Fig. 4). Based on the XL models, the point at (20, 2.5, 5.3) provided the smallest kappa number (4.6) and that at (8.3, 2.5, 7) the highest brightness (62.1%ISO).

3.5. Effects on viscosity and hexenuronic acids

The viscosity of some pulp samples after the enzyme treatment (L) was assessed and compared with that of the initial eucalyptus pulp (972 ± 20 mL g^{-1} odp). Low levels of the process variables had no effect on viscosity, whereas medium and high levels reduced it slightly (by 100 mL g^{-1} odp, results not shown).

The effect on HexA was assessed in various pulp samples (Fig. 5). The laccase–mediator treatment reduced the HexA content of the pulp after the L stage and XL sequence, whichever the application conditions. As can be seen from Fig. 5, XL pulp had a lower HexA content than L pulp.

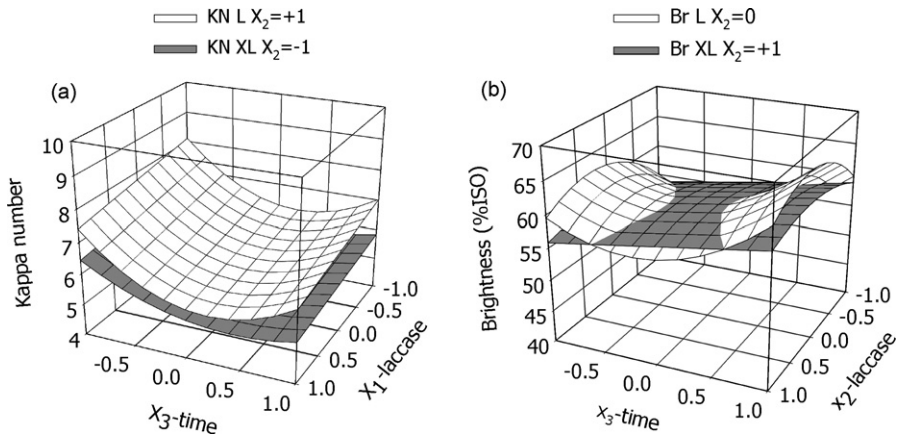

Fig. 4. Surfaces estimated for kappa number at high HBT dose in L (□) and at low HBT dose in XL (■) (a). Surfaces estimated for brightness at medium HBT dose in L (□) and at high dose in XL (■) (b).

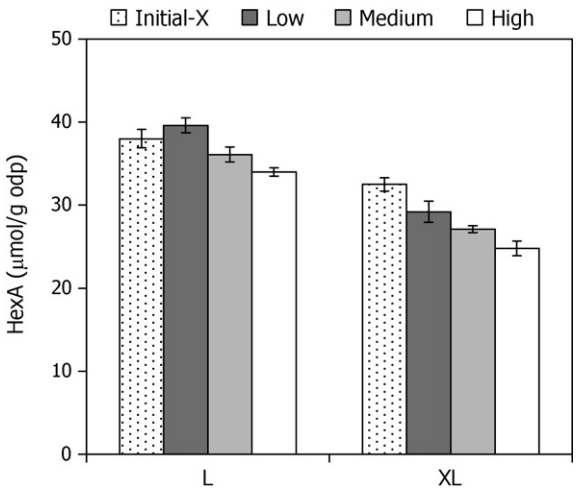

Fig. 5. Hexenuronic acids of initial pulps and pulps treated at low, medium and high application conditions in L and XL sequences.

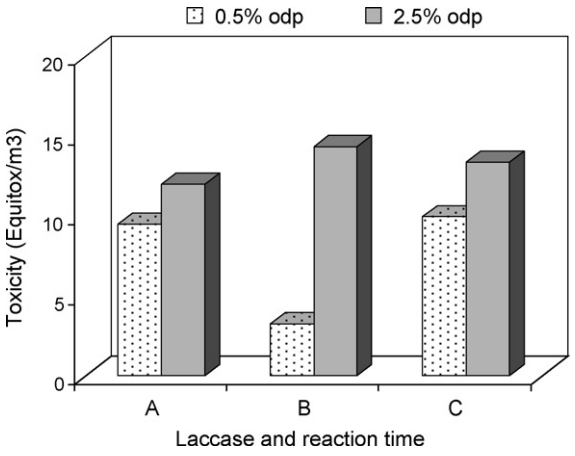

Fig. 6. Effect of increasing the HBT dose (from 0.5 to 2.5% odp) in the Microtox toxicity. A: 1 U g^{-1} odp laccase dose, 1 h; B: 20 U g^{-1} odp laccase dose, 1 h; C: 20 U g^{-1} odp laccase dose, 7 h.

3.6. Effects on effluent properties

Table 3 summarizes the effects of the L treatment on various effluent properties. As can be seen, toxicity, COD, colour and laccase inactivation increased with increasing value of the process variables. Also, Microtox toxicity was affected by a rise in HBT dose from 0.5 to 2.5% odp (see Fig. 6).

4. Discussion

4.1. Models for L pulp

The models used to predict kappa number and brightness revealed that both properties were affected by the three studied variables (see Fig. 2a and b). Increasing the laccase dose from 1 to 20 U g^{-1} odp reduced the kappa number; by contrast, brightness only improved at laccase doses up to 13 U g^{-1} odp and then decreased. As previously suggested by some authors [21], this may have resulted from the formation of quinones at high laccase levels. From 0.5 to 1.4% odp of HBT dose, kappa number remained invariable; however, it caused brightness to rise. From that threshold to the maximum mediator dose studied (2.5% odp), the kappa number decreased but brightness failed to increase further. The effect of the mediator is difficult to explain. Also, although lignin was partially extracted in the aqueous medium after L, the overall delignifying effect was easier to envisage after an alkaline stage. In previous work involving application of the same experimental after alkaline extraction [18], the HBT dose exhibited a linear influence on both properties. This suggests that something (e.g. the presence of lignin or HBT attaching to pulp fibres) interferes with measurements of kappa number after L. In any case, it was efficiently removed in the subsequent alkaline extraction stage.

The reaction time exhibited two intervals of influence. During the first (from 1 h to 3.4–4.6 h), the kappa number decreased rapidly while brightness remained constant or decreased only slightly. During the second (until 7 h), brightness increased whereas the kappa number remained constant, which indicates that the system reached a point beyond which the latter could not be further reduced.

Some authors believe that all lignin in pulp is available for reaction, while others think that a certain amount always remains unreacted [22–24]. Our results suggest that all accessible lignin was oxidized in the first reaction interval but a further increase in brightness was precluded by the formation of quinones [16,21]. Although no additional lignin was removed during the second interval, brightness increased by effect of a modification in previously oxidized lignin or the system removing other coloured compounds containing carbonyl or carboxyl groups [25,26]. A similar effect of the reaction time was recently observed elsewhere [27].

In this work, effects were assessed after the L treatment in order to better understand the implications of the enzyme stage, even though a P or E stage is usually employed to remove insoluble lignin from pulp [28]. In previous work [18], an alkaline extraction stage was applied to all pulp samples exhibiting high delignification (55%) and brightness (70%ISO) after an XLE sequence. The results confirmed that some lignin fractions insoluble at the pH used in the enzyme stage remained in the pulp after the L treatment.

The application conditions leading to the smallest kappa number differed from those providing the highest brightness as a result of a decrease in kappa number giving rise to no increase in brightness after L. Although such application conditions involved the highest HBT dose used, this variable had little effect on the models. This suggests that the mediator dose can be reduced in order to lessen contamination problems. Reducing the HBT dose to 0.5% odp was proposed in previous work [18,29].

Table 3
Effluent properties at different application conditions.

	Low	Medium	High
Microtox toxicity (Equitox·m^3)	10 ± 0	12 ± 1	13 ± 2
COD (kg O_2·t $pulp^{-1}$)	24 ± 7	106 ± 14	210 ± 5
Colour (kg Pt·t $pulp^{-1}$)	1 ± 0	16 ± 1	28 ± 2
Laccase activity loss (%)	26 ± 1	90 ± 1	97 ± 1

4.2. Effect of an enzyme pretreatment with xylanase

The favourable effect of xylanase has been widely ascribed to its removing xylans, thereby breaking the link between cellulose and lignin and facilitating removal of the latter in subsequent bleaching stages [3,30–33]. As can clearly be seen from Fig. 3, xylanase decreased the kappa number.

The proposed models were used to assess the effects of xylanase on the different process variables (Fig. 4a and b). The kappa number and brightness models for the L sequence were both more complex than those for the XL sequence. This suggests that xylanase makes fibres more accessible for penetration of the laccase–mediator system.

The effect of the laccase dose on kappa number and brightness was not affected by xylanase. Thus, the kappa number exhibited

a similar, linear influence in both models, while brightness only increased up to a laccase dose of 13 U g^{-1} odp and decreased above that level. This result can be ascribed to the formation of lignin derivatives such as quinones at high laccase doses.

The effect of the mediator dose in decreasing the kappa number in the L sequence was only observed over the range 1.4–2.5% odp, and that in increasing brightness from 0.5–1.4% odp. On the other hand, the effect of HBT in decreasing the kappa number and increasing brightness in the XL sequence was observed over the mediator range 0.5–2.5% odp. These results can be ascribed to the xylanase pretreatment facilitating penetration of HBT into pulp fibres.

In both models, the reaction time exhibited two intervals of influence. During the first few hours (1–5 h), the system oxidized reactive or readily accessed lignin; during the last few (5–7 h), a kappa number limit was reached where this property levelled off. During the first interval, the decrease in kappa number was more marked in the L sequence. However, the smallest kappa number obtained was smaller in XL (4.6) than it was in L (5.5). This suggests that the xylanase pretreatment makes previously unreached lignin more accessible. A similar effect of xylanase was previously observed with ozone as reagent [4].

Fig. 4a shows the calculated response surfaces for the kappa number in the L and XL sequences at a constant mediator dose that was the highest (x_2 = +1; 2.5% odp) for the L sequence and lowest (x_2 = −1; 0.5% odp) for the XL sequence. Although the HBT dose was lower in XL than in L, the kappa number predicted by the model was also smaller for XL. A similar effect was observed in the laccase dose and reaction time. Fig. 4b shows the calculated surfaces for brightness in the L and XL sequences at a constant HBT dose (x_2) with brightness at its highest level in both L (x_2 = 0; 1.5% odp) and XL (x_2 = +1; 2.5% odp). Unlike kappa number, brightness was not increased by the XL sequence. At any rate, the ultimate effect of L was delignification rather bleaching.

The xylanase pretreatment made fibre more accessible for penetration of laccase and HBT. This facilitated oxidation of some previously inaccessible lignin, which resulted in a smaller kappa number in all XL pulp samples. Moreover, the use of an X pretreatment allows similar brightness and a smaller kappa number to be obtained by using 30% less laccase (14.3 U g^{-1} odp), 80% less HBT (0.5% odp) and a 45% (4 h) shorter reaction time [18].

4.3. Effects on viscosity and hexenuronic acids

The laccase–mediator treatment produces free radicals ($OH^{\bullet}$) which not only facilitate delignification, but also can act unselectively on cellulose; in fact, hydroxyl radicals cause the formation of carbonyl groups which can break the cellulose chain and reduce its degree of polymerization in subsequent alkaline stages [34]. A slight decrease in viscosity (100 mL g^{-1} odp) was observed at medium to high levels of the process variables. In any case, oxidative alteration of cellulose by the laccase–mediator system can be reversed by using of a reducing stage [8,15,35].

Hexenuronic acids were also analysed under the different application conditions studied on account of their increasing significance to bleaching processes and paper properties [36,37]. HexA decreased by effect of both X and L. During X, the HexA content decreased by 15% owing to the removal of xylans. Such a content was also decreased by the L stage, to an extent dependent on the particular application conditions. Thus, increasing the values of the process variables in L from low to medium or medium to high levels reduced the HexA content of pulp in the L stage and XL sequence. Furthermore, the X pretreatment stage boosted HexA removal by the laccase–HBT system. Using high levels of the process variables in L reduced the HexA content by 10%; however, the X pretreatment stage (XL sequence) boosted HexA removal to 35%.

4.4. Effects on effluent properties

As can be seen from Table 3, Microtox toxicity was not significantly affected by the increase in the values of the process variables in L. HBT is potentially toxic to the environment by itself [38] or through its reactions products [17,39,40]. This led us to examine its effect under variable application conditions (Fig. 6). Raising the HBT dose from 0.5 to 2.5% odp increased toxicity in all instances. This was probably the result not only of the presence of the mediator (HBT), but also to the formation of degradation by-products that are even more toxic than the mediator itself.

COD also increased with increasing value of the process variables (Table 3), possibly through the formation of certain products during the reaction and, as pointed out by some authors [17], as a result of using commercial laccase. Effluent colour was insubstantial at low levels of the process variables (Table 3), but substantial at medium and high levels. This can be ascribed to the formation of coloured oxidation products of the mediator and also to an increased content of degraded lignin in the effluents. HBT in a laccase–mediator system is partially converted into benzotriazole (BT) [7,41,42], which might be responsible for the increased red colour of the effluents at long treatment times.

Based on the results (Table 3), laccase was strongly inactivated specially at high levels of the process variables. This can be produced by the oxidized HBT—in fact, laccase is known to be inactivated by oxidized species of some mediators [17,43–45].

The effect of the X pretreatment stage on the effluent properties was also analysed. Results showed that the effluent properties were not significantly affected by the xylanase pretreatment stage. Toxicity, COD, Colour and the laccase activity loss ranged from 6 to 13 Equitox·m^3, 38–143 kg O_2·t $pulp^{-1}$, 1–28 kg Pt·t $pulp^{-1}$ and 36–98%, respectively. These properties showed similar trends than in the L sequence.

Kappa number and brightness were slightly affected by the increase in HBT dose. Also, low HBT doses were recently found to provide pulp with good properties [18]. The use of an HBT dose of 0.5% odp reduces laccase inactivation and effluent toxicity, thereby facilitating recycling.

5. Conclusions

The influence of LMS process variables (*viz.* laccase and HBT doses, and reaction time) was examined in eucalyptus kraft pulp, and so were the effects of a xylanase pretreatment. The application of models for kappa number and brightness showed the two properties to behave differently, and suggest the formation of lignin derivatives that interfere with brightness measures. All reactive lignin was removed during the first few hours of treatment, the residual lignin being inaccessible to the reagents. LMS was found to affect the properties of the resulting pulp and its content in hexenuronic acids (HexA), and a xylanase pretreatment to facilitate access of laccase and HBT to previously inaccessible lignin and HexA.

As regards effluent properties, Microtox toxicity rose with increasing HBT dose. COD and colour increased with increasing values of the process variables in L, and laccase was strongly inactivated during the treatment. The ability to use a lower HBT dose can help reduce laccase inactivation and effluent toxicity, thereby facilitating recycling and making the process more environmentally friendly.

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