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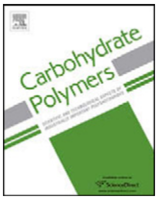

Xylanase- and laccase-aided hexenuronic acids and lignin removal from specialty sisal fibres

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This work was conceived to investigate for the first time the effectiveness of the combined use of xylanase and laccase for the removal of hexenuronic acids (HexA) and lignin from sisal pulp fibres. To this end, xylanase (X) and laccase (L) treatments were used in an XLQPo sequence (where Q denotes a chelating stage and Po an oxygen-reinforced peroxide multi-step treatment) that was applied to pulp in order to obtain sisal fibres with a high cellulose content. The results of the XLQPo sequence were compared with those of an LQPo sequence. The L stage of both sequences was performed in the presence of either the natural compound sinapyl aldehyde (SLD) or the synthetic compound violuric acid (VA), employed as mediators, in order to compare their efficiency in aiding pulp bleaching and HexA removal. Changes in HexA content and the contributions of lignin and HexA to kappa number during each sequence were examined. The xylanase treatment was found to remove 47% of lignin, 15% of xylan and 27% of HexA from the initial pulp, whereas the laccase–VA system removed 28% of HexA and exhibited higher efficiency than the laccase–SLD system in reducing kappa number and increasing brightness. In any case, when the X treatment was applied, the sequence including laccase–SLD treatment resulted in the strongest delignification effect. The effluents from each stage of the bleaching sequences were analysed for COD, colour and toxicity, which peaked after the L stage and were significantly higher with SLD than with VA.

1. Introduction

Increasing pressure from environmental legislation has led the pulp and paper industry to seek cleaner production methods aimed at minimizing the use of polluting chemicals in the bleaching process (Roncero & Vidal, 2007). The use of enzymes has emerged as a very promising choice not only for implementing clean bleaching processes, but also for developing novel, high-added value products. In fact, xylanases have already proved effective for boosting pulp bleachability and saving chemical bleaching reagents at the mill scale (Roncero, Torres, Colom & Vidal, 2002; Viikari, Kantelinen, Sundquist & Linko, 1994). Xylanases are hydrolytic enzymes that catalyse xylan degradation. Their beneficial effect has been ascribed to their selectively hydrolysing xylans re-precipitated on fibre surfaces, thereby contributing to releasing lignin and facilitating reagent penetration in subsequent bleaching stages (Roncero, Torres, Colom & Vidal, 2003; Torres et al., 2000). Recently, an innovative aspect of xylanases has been identified: their ability to reduce the content of hexenuronic acids (HexA) in pulp (Valls & Roncero, 2010). HexA are known to form during alkaline cooking of wood by elimination of methanol from the 4-*O*-methylglucuronic acid group bonded as a side group to xylans (Daniel, Neto, Evtuguin & Silvestre, 2003). The significance of HexA relies on their adverse effects on pulp bleaching; thus, they contribute to kappa number, increase the consumption of bleaching agents, retain metal ions by chelation, cause brightness reversion and facilitate the formation of oxalic acid and the scaling of process circuits by calcium oxalate (Cadena, Vidal & Torres, 2010; Valls & Roncero, 2009). Because xylanases hydrolyse xylans on fibre surfaces and such xylans contain HexA, these compounds may be removed by enzymatic treatment with xylanases (Valls & Roncero, 2009; Valls, Vidal & Roncero, 2010).

The combination of a fungal laccase with a chemical a mediator, which is called a "laccase–mediator system" (LMS), has proved more effective than the use of xylanases alone for the biobleaching of various types of pulp by virtue of their direct action on lignin; however, their effectiveness at the mill scale remains to be assessed (Barreca, Fabbrini, Galli, Gentili & Ljunggren, 2003; Bourbonnais & Paice, 1990; Camarero et al., 2004; Fillat & Roncero, 2010; Ibarra, Camarero, Romero, Martínez & Martínez, 2006). Some synthetic mediators such as 1-hydroxybenzotriazole (HBT) and violuric acid (VA) have proved highly effective in promoting pulp delignification; however, concerns about their possible toxicity and high cost have so far hindered broad industrial use (Bourbonnais,

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Paice, Freiermuth, Bodie & Borneman, 1997; Moldes, Díaz, Tzanov & Vidal, 2008; Shleev et al., 2006; Valls, Colom, et al., 2010). This has promoted a search for natural, potentially cost-effective mediators such as lignin-derived phenols, which can be readily obtained from plants and spent pulping liquors or, directly, from fungal metabolism (Aracri, Colom & Vidal, 2009; Camarero, Ibarra, Martínez & Martínez, 2005; Eggert, Temp, Dean & Eriksson, 1996; Johannes & Majcherczyk, 2000; Moldes et al., 2008). Recent studies have shown the effectiveness of some natural phenols to mediate laccase catalysis towards recalcitrant substrates such as pulp lignin (Camarero et al., 2007; Fillat, Colom & Vidal, 2010) and lipids (Gutiérrez et al., 2007). Valls and Roncero (2010) examined the effect of HexA content reduction in eucalyptus kraft pulp by a laccase–HBT system and found it to be strongly boosted by a xylanase pretreatment. However, no study has so far examined the removal of HexA by LMS from non-wood pulp, the potential for which of natural mediators remains unknown. The aim of this work was to investigate for the first time changes in HexA and lignin in sisal pulp during two different TCF bleaching sequences performed with and without a xylanase pretreatment (an X stage) and including an LMS treatment (an L stage) where either the natural compound sinapyl aldehyde (SLD) or the synthetic compound violuric acid (VA) was used as laccase mediator. The resulting effluents were characterized with a view to assessing the environmental impact of the bleaching treatments and the feasibility of closing circuits in a future industrial implementation, a scarcely explored aspect of bleaching sequences (Aracri et al., 2009; Fillat & Roncero, 2010; Fillat et al., 2010).

2. Experimental

2.1. Raw material

The raw material was sisal (*Agave sisalana*) alkaline pulp obtained by soda–anthraquinone cooking at the CELESA mill (Tortosa, Spain). Prior to characterization, the pulp was washed with acidified water at pH 4 at 2% pulp consistency for 30 min, followed by filtration and thorough washing with de-ionized water. This procedure was needed to remove contaminants and metals, and also to bring the pulp pH closer to that for the enzyme treatments. The main properties of the initial pulp were as follows: 37.9 ± 0.1% ISO brightness, 7.87 ± 0.05 kappa number, 733 ± 1 mL/g viscosity and a hexenuronic acid (HexA) content of 41.36 ± 1.08 μmol/g oven-dried pulp (odp).

2.2. Bleaching sequences

Two different TCF bleaching sequences were applied to the pulp, namely: LQPo and XLQPo, where L denotes the laccase–mediator treatment, Q a chelating treatment, Po an oxygen-reinforced hydrogen peroxide multi-step treatment and X an enzyme pretreatment with xylanase. Control sequences were performed without addition of the mediator in the L stage for comparison. At the end of each stage, the pulp was filtered and thoroughly washed with de-ionized water.

The enzyme used in the X stage was a commercial xylanase (Pulpzyme® HC) supplied by NOVOZYMES® (Bagsvaerd, Denmark). The X treatment was performed in polyethylene bags, using 3 U/g odp xylanase in Tris–HCl buffer (pH 7), at 10% pulp consistency, at 50 °C for 2 h.

Laccase from *Trametes villosa* (NS-51002) was supplied by NOVOZYMES®. One activity unit was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical ($\varepsilon_{436\,\mathrm{nm}} = 29{,}300\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. Sinapyl aldehyde (SLD) and violuric acid (VA) purchased from Sigma–Aldrich were used as laccase mediators. Laccase treatments were carried out at 5% pulp consistency in an oxygen pressurized (0.6 MPa) reactor at 50 °C, using a stirring rate of 30 rpm for 4 h; the reactor was supplied with 50 mM sodium tartrate buffer at pH 4 and 20 or 40 U/g odp laccase in addition to Tween 80 (0.05%, w/v) as surfactant.

The L treatment was followed by a Q stage involving the use of chelating agents to reduce the contents in metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}) liable to degrade the bleaching agents and cellulose during the peroxide bleaching treatment (Heijnesson, Simonson & Westermark, 1995). The Q treatment was conducted in the presence of 1% DTPA (diethylenetriaminepentaacetic acid) at 5% consistency, pH 5–6 and 85 °C for 1 h.

The last step of the TCF sequence was a Po stage carried out at 5% consistency in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm, using 3% odp H_2O_2, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$, at 90 °C for 4 h. This stage was performed in 3 steps ($t_1 = 1$ h, $t_2 = 1$ h, $t_3 = 2$ h) each involving the addition of 1% odp H_2O_2 and withdrawal of a small amount of pulp from the reactor to determine brightness and kappa number at the end.

2.3. Pulp properties

Pulp properties were determined after each stage in all sequences. Treated pulp samples were characterized in terms of kappa number, brightness and viscosity according to ISO 302, 3688 and 5351-1, respectively. The hexenuronic acid (HexA) content was determined by UV detection (Chai, Zhu & Li, 2001). An estimate of the actual lignin content of the pulp was obtained by determining the kappa number due to lignin (KN_{lig}) (Li, Sevastyanova & Gellerstedt, 2002; Valls & Roncero, 2010). This involved measuring kappa number following removal of HexA by acid hydrolysis with mercury acetate and efficient washing with distilled water. The KN values thus obtained were used to calculate the actual degree of pulp delignification. Analyses were performed in duplicate for kappa number, viscosity and HexA content, and in quadruplicate for brightness.

2.4. Carbohydrate analysis by HPLC

The sugar composition of the initial pulp and the enzymatically treated pulp (X and L) from all sequences was determined by high performance liquid chromatography (HPLC) of samples previously Soxhlet-extracted with acetone and ground to a particle size <0.5 mm. Two replicates of the resulting samples were hydrolysed by using a modified version of the TAPPI 249 cm-09 test method. The hydrolysis process involved the following two steps: (*a*) *prehydrolysis with concentrated sulphuric acid.* Approximately 50 mg of sample was placed in a test tube and impregnated with 5 mL of 72% sulphuric acid, after which the tube was placed in a water bath at 30 ± 0.5 °C for 1 h with occasional stirring. (*b*) *Final hydrolysis with dilute sulphuric acid.* The tube contents were washed in a 250-mL flask in order to obtain a final solution 4% in sulphuric acid and the flask was placed in an autoclave at 103 ± 7 kPa for 1 h. Once the reaction was complete the specimen solution was cooled at room temperature and passed through a glass filter to remove lignin insoluble in sulphuric acid. Prior to HPLC analysis, the solution was filtered through a Whatman membrane of 0.45 μm pore size. The high performance liquid chromatograph was fitted with a refractive index detector. The chromatographic determination was performed with an Agilent 1100 HPLC instrument furnished with column packed with Aminex HPX-87H ion-exchange resin under the following operating conditions: mobile phase, 0.006 mol/L sulphuric acid; flow rate, 0.6 mL/min; column temperature, 60 °C. Measurements were interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all from

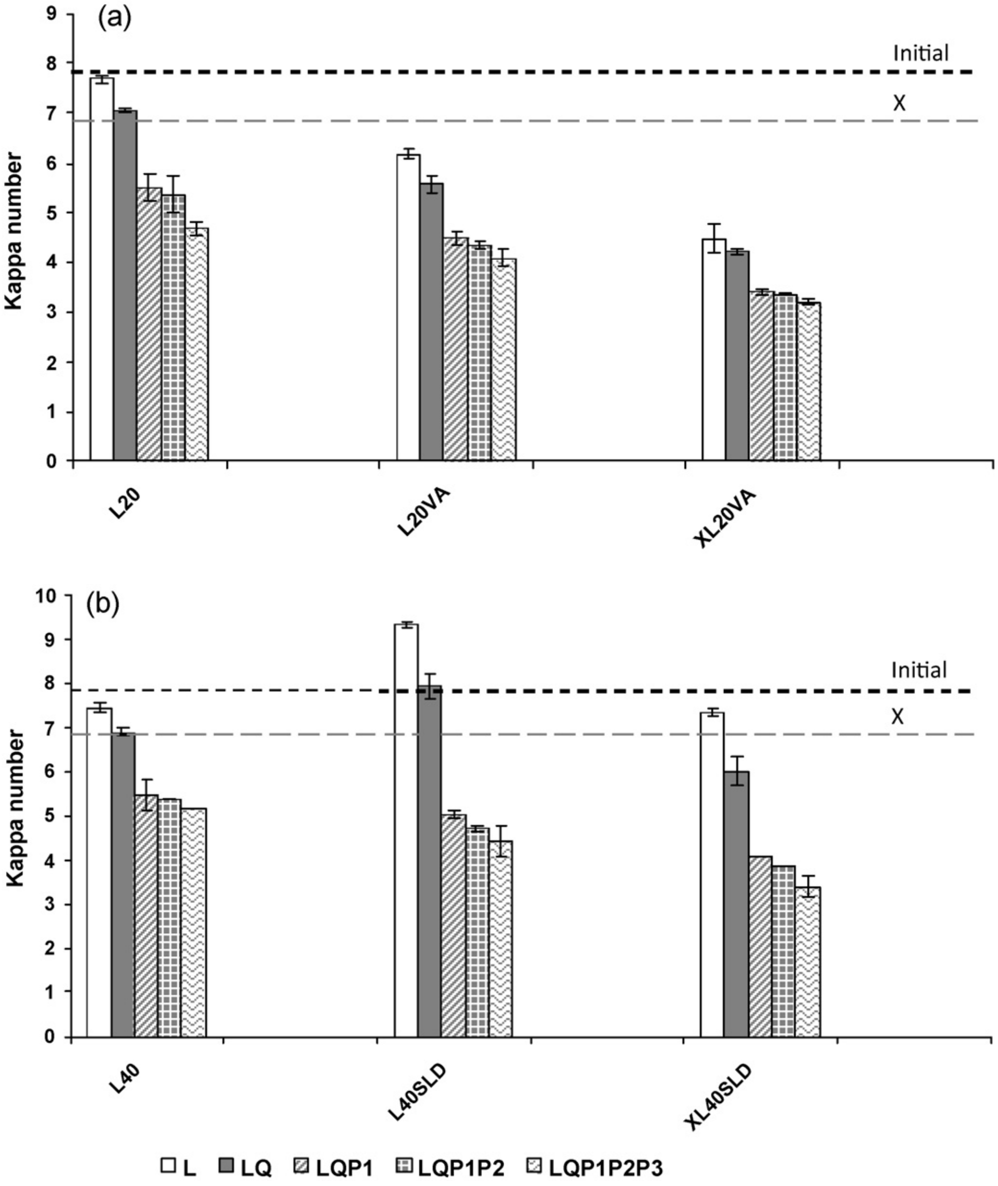

Fig. 1. Kappa number of pulps obtained from each stage of the sequences involving VA (a) and SLD (b), including the three steps of H_2O_2 addition in the Po stage. Dashed lines indicate the values of the initial pulp and the xylanase treated pulp (X).

Sigma–Aldrich). Because the column failed to resolve xylose, mannose and galactose, their combined content was expressed as xylose (Garrote, Domínguez & Parajó, 2001).

2.5. *Effluent properties*

The effluents from each stage in the bleaching sequences were analysed for chemical oxygen demand (COD) and colour following ASTM D1252-00 and ASTM D1209-00, respectively. Absorbance data were taken at 600 nm for COD and 465 nm for colour. A Thermo Scientific Evolution 600 spectrophotometer was used in both cases.

Effluent toxicity was determined with the Microtox method, using the marine luminescent bacterium *Vibrio fischeri* in a Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental) in accordance with the UNE-EN ISO 11348-3: 1999 standard. The difference between the amount of light emitted before and after addition of the sample was used to measure toxicity. In order to prevent pH effects, each sample was adjusted to pH 6–8 with an NaOH solution. Ecotoxicity was quantified as EC_{50}, which is defined as the effective concentration of sample reducing the light emission intensity by 50% after 15 min of contact. EC_{50} is inversely proportional to biological toxicity, expressed in toxicity units (TU). The reference toxicant $ZnSO_4 \cdot 7H_2O$ was used to control *V. fischeri* batch quality in accordance with the Basic Test procedure. Toxicity measurements were colour-corrected as per the recommendations of the equipment manufacturer. COD, colour and toxicity tests were conducted on duplicate samples.

3. Results and discussion

3.1. *Pulp properties*

This work was designed to assess the potential of a xylanase pretreatment on sisal pulp for boosting bleaching and HexA removal during a bleaching sequence, as well as to compare the effectiveness of VA and SLD as laccase mediators in aiding lignin and HexA removal. Six different bleaching sequences were used for this purpose, namely: L20 and L40, two control sequences where the L stage was performed with 20 and 40 U/g odp laccase, respectively, in the absence of mediator (VA and SLD, respectively); L20VA and L40SLD, where L was performed in the presence of VA and SLD, respectively; and XL20VA and XL40SLD, both of which included a xylanase pretreatment as additional stage. A previous study (Aracri et al., 2009) revealed SLD to be the best choice for aiding pulp delignification among various *p*-hydroxycinnamic compounds assayed as natural mediators of laccase. This led us to select it for further testing here, where it was used in the L treatment under the optimum conditions established in previous tests – this is why we used a different laccase dose with each mediator. Fig. 1 shows kappa number of the pulp obtained after each stage of the bleaching sequences including the L treatments with VA and SLD and their corresponding controls. P1, P2 and P3 represent the three steps of H_2O_2 addition during the Po stage. As can be seen, the xylanase reduced the kappa number of the initial pulp from 7.87 to 6.78, which can be ascribed to a delignifying effect resulting from the removal of lignin trapped between xylan chains (Roncero, Torres, Colom & Vidal, 2005), as well as to the removal of HexA bonded as side groups to xylans (Valls, Vidal, Gallardo, et al., 2010). The L20VA sequence proved efficient in decreasing kappa number with respect to the control sequence, particularly in the L stage (1.51 points decrease); thus, it provided a kappa number 15% smaller than that yielded by the control sequence. The reduction in kappa number caused by the L stage was higher in the xylanase-pretreated pulp than in the initial pulp. This suggests that the X stage increased the accessibility of the laccase–VA system into pulp fibres, thereby facilitating removal of lignin and hexenuronic acids contributing to kappa number, which is consistent with the bleach boosting effect of xylanase treatments previously observed by other authors. The beneficial effect of xylanase resulted in a 22% decrease in final kappa number with respect to the sequence excluding the X stage. In contrast to the effect observed with the laccase–VA system, the L stage carried out in the presence of the laccase–SLD system resulted in a significant increase in kappa number – which exceeded the values for the control sequence after the L and Q stages. This suggests a disparate behaviour of the natural compound in relation to VA after it is oxidized to a phenoxy radical by the enzyme. When phenolic compounds are used as laccase mediators for pulp bleaching, the delignification effect may be hindered by adverse reactions involving the phenoxy radicals generated by enzymatic oxidation of the mediator; such is the case with depleting reactions (*e.g.* homopolymerization and cross-coupling in the lignin structure) or fragmentations (Aracri et al., 2009; Moldes et al., 2008). The increase in kappa number observed upon treatment with the laccase–SLD system may have resulted from adsorption or partial condensation of the phenol on the pulp via radical-coupling reactions. The increase caused by the L stage was more marked in the initial pulp (from 7.87 to 9.33) than in the xylanase-treated pulp (from 6.78 to 7.34), probably as a result of the higher content of reactive sites (*e.g.* lignin, hexenuronic acids) promoting binding of SLD in the former. Despite the adverse effect on the enzyme treatment, the final bleaching stage yielded a kappa number smaller than the control value (14%), which suggests that the natural mediator may be simultaneously involved in coupling and oxidative degradation reactions during the L stage, the effect of the latter only being observed at the end of the bleaching sequence. Similarly to VA, application of the X stage prior to the laccase–SLD treatment led to a final kappa number 24% smaller than in the absence of an X stage and similar to that for the XL20VA sequence.

Fig. 2 shows the brightness values obtained with the sequences involving VA and SLD – and their corresponding controls. As can be seen, the laccase–VA system exhibited a high efficiency in raising pulp brightness in all stages, especially when a xylanase pretreatment was applied; thus, the L20VA and XL20VA sequences raised brightness by 11% and 17%, respectively, with respect to the control sequence. Similarly to kappa number, pulp brightness was adversely affected by the laccase–SLD system in the L and Q stages, especially in the absence of an X stage. The loss of brightness in the laccase–SLD treated samples was accompanied by a markedly increased *k*/*s* ratio (Jordan, 1996) and by changes in the CIE $L^*a^*b^*$ colour coordinates (Hunt, 1998) (results not shown), which suggests the formation of a substantial amount of chromophoric groups as a result of the enzyme treatment (Fillat & Roncero, 2009a; Moldes et al., 2008). After the Po stage, however, the laccase–SLD treated pulp samples from both sequences (with and without an X stage) exhibited increased brightness with respect to the control pulp (3% and 5% higher, respectively) as a result of the oxidation and dissolution of chromophoric species, and lignin degradation products, in the alkaline bleaching medium used.

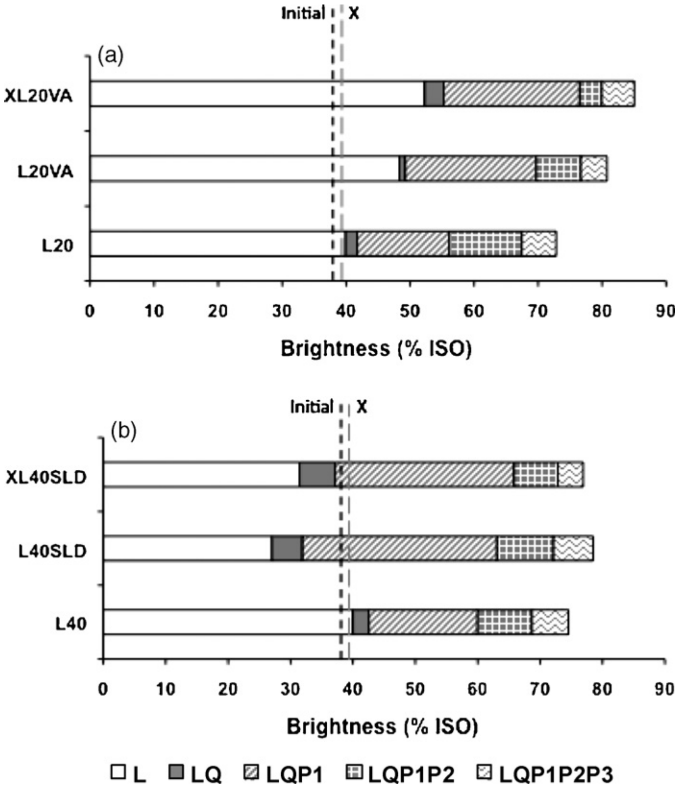

Fig. 2. Brightness of pulps obtained from each stage of the sequences involving VA (a) and SLD (b), including the 3 steps of H_2O_2 addition in the Po stage. Dashed lines indicate the values of the initial pulp and the xylanase treated pulp (X). Standard deviation = 0.1.

Pulp samples from each stage of the six bleaching sequences were subjected to viscosity measurements in order to assess the effect of each treatment on cellulose integrity (Table 1). The L and Q stages in all bleaching sequences led to similar pulp viscosity values, with no appreciable losses from the initial pulp. The viscosity reduction caused by the Po stage was more marked with VA than with SLD; probably, the mediators oxidized carbohydrate chains in cellulose to carbonyl groups during the L stage, thus making the pulp vulnerable to degradation by the strong alkaline medium used in the bleaching stage. The increased viscosity values obtained at the end of the sequences including an X stage may have resulted from partial removal of xylans from the pulp increasing the average degree of polymerization of carbohydrates in the fibres (Roncero et al., 2002).

The xylan contents of the initial and enzymatically (X and L) treated pulp samples as calculated after acid hydrolysis and HPLC analysis are reported in Table 2. As can be seen, the L–VA and

Table 1
Viscosity of initial and xylanase-treated pulps, and pulps obtained from each stage of each bleaching sequence.

	Viscosity (mL/g)
Initial	733 ± 1
X	756 ± 16

	Viscosity (mL/g)		
	L	Q	Po
L20	774 ± 13	745 ± 13	620 ± 8
L40	729 ± 3	758 ± 35	663 ± 37
L20VA	721 ± 26	750 ± 15	600 ± 33
L40SLD	739 ± 39	748 ± 10	592 ± 25
XL20VA	765 ± 21	801 ± 9	691 ± 41
XL40SLD	703 ± 30	788 ± 32	691 ± 10

L–SLD treatments reduced xylan contents only slightly in comparison with the initial pulp; by contrast, their application after the X stage reduced the xylan content by 20% and 25%, respectively, the decrease occurring largely (66% and 54%, respectively) during the xylanase treatment. This result confirms that xylanase acts by hydrolysing xylans present in the pulp and shows that xylan removal is boosted by the subsequent L stage with both mediators.

Pulp samples from the L and Po stages in all sequences were analysed for HexA. As can be seen from Table 2, the xylanase treatment successfully removed a substantial fraction of HexA in the initial pulp (27%) by releasing xylans chains from fibre surfaces. Interestingly, laccase reduced the HexA content in the control treatment, especially at the higher enzyme dose. HexA reduction was more marked in the laccase–VA treated pulp, to the same extent as that produced by xylanase, which suggests the laccase–VA system can destroy HexA by oxidizing their double bonds similarly to electrophilic bleaching agents (Ventorim, Colodette, Gomes & da Silva, 2008). The pulp samples treated with the laccase–SLD system exhibited no reduction in HexA content, which confirms the disparate behaviour of the enzymatically oxidized natural mediator towards fibres. Finally, the pulp samples from the sequences including an X stage exhibited a reduction in HexA with respect to those subject to no X pretreatment, which can be ascribed to the xylanase treatment since no synergistic effect between the xylanase and the laccase–mediator systems was observed.

Residual lignin and HexA are known to be the main substances contributing to kappa number in pulp fibres (Costa & Colodette, 2007; Li et al., 2002; Valls & Roncero, 2010). In this work, their contribution was estimated in pulp samples from the L and Po stages in all bleaching sequences, as well as in the initial and xylanase-pretreated pulp (Fig. 3), in order to assess the actual delignifying effect of the different treatments. The xylanase treatment decreased KN_{lig} for the initial pulp from 3.93 to 2.07, which indicates substantial delignification by effect of the enzyme. This reflected in the decreased KN_{lig} value obtained in both the L and the Po stage after the X treatment. Interestingly, the final KN_{lig} value for the XL40SLD sequence was slightly smaller than that for the XL20VA sequence in spite of the significant increase due to the laccase–SLD system in the L stage. This indicates that oxidative degradation of lignin by the laccase–natural mediator system is enhanced after the xylanase treatment and results in the best delignifying performance at the end of the bleaching sequence.

Table 2
Xylan content (% odp) of initial and enzymatically (X and L) treated pulps from the bleaching sequences. HexA content of initial and xylanase-treated pulps, and pulps after L and Po stages.

	% XYL	HexA (μmol/g odp)
Initial	16.3 ± 0.4	41.36 ± 1.08
X	14.1 ± 0.3	30.08 ± 0.66

	% XYL	HexA (μmol/g odp)	
	L	L	Po
L20	16.2 ± 0.4	36.44 ± 4.83	36.19 ± 0.27
L40	15.7 ± 0.5	31.50 ± 0.06	30.63 ± 1.46
L20VA	15.8 ± 0.3	26.59 ± 0.38	25.22 ± 0.69
L40SLD	15.2 ± 0.3	32.66 ± 0.40	31.83 ± 3.34
XL20VA	13.0 ± 0.4	23.69 ± 1.85	23.83 ± 2.71
XL40SLD	12.1 ± 0.5	26.72 ± 0.73	25.84 ± 0.82

Table 3
COD and colour values of effluents obtained from xylanase treatment, and L, Q and Po stages of each bleaching sequence.

	COD (kg O_2/t_{pulp})	Colour (kg Pt/t_{pulp})
X	100 ± 3	4.4 ± 0.1

	COD (kg O_2/t_{pulp})			Colour (kg Pt/t_{pulp})		
	L	Q	Po	L	Q	Po
L20	94 ± 2	10 ± 1	11 ± 2	3.3 ± 0.1	2.6 ± 0.1	2.1 ± 0.1
L40	117 ± 13	3 ± 1	8 ± 1	4.2 ± 0.1	1.8 ± 0.1	1.4 ± 0.0
L20VA	99 ± 4	6 ± 3	13 ± 5	15.3 ± 0.5	3.0 ± 0.1	1.4 ± 0.0
L40SLD	131 ± 4	15 ± 3	11 ± 2	78.2 ± 2.3	13.7 ± 0.4	2.0 ± 0.1
XL20VA	103 ± 4	4 ± 1	8 ± 1	16.3 ± 0.5	3.3 ± 0.1	0.8 ± 0.0
XL40SLD	136 ± 1	7 ± 1	8 ± 1	90.9 ± 2.7	10.7 ± 0.3	2.0 ± 0.1

3.2. *Effluent properties*

Table 3 shows the COD and colour values, respectively, as determined in the effluents from each stage of the studied bleaching sequences. As can be seen, the xylanase treatment produced a high COD accounting for more than 40% of the total value for the sequences including an X stage. This can be ascribed to substantial carbohydrate degradation and lignin removal at this stage, which testifies to the efficiency of the enzyme (Fillat & Roncero, 2009b; Ventorim et al., 2008). The substantial contribution of X to the total COD for the bleaching sequence is consistent with previously reported results (Siles, Torres, Colom & Vidal, 1996; Ventorim et al., 2008) and is the greatest disadvantage of using xylanases in bleaching processes. In all instances, COD after the L stage was markedly higher than it was after Q and Po; this was mainly a result of the presence of sodium tartrate buffer and the use of commercial laccase (Fillat & Roncero, 2009b; Fillat et al., 2010), as suggested by the high values obtained in the control treatments. The effluents from the L stage carried out in the presence of the mediator exhibited higher COD values than the control process by effect of the presence of the mediator and of degradation products of the mediator and lignin (Aracri et al., 2009; Fillat & Roncero, 2009a). When the laccase–mediator treatments were inserted after the X stage, the resulting effluents had higher COD levels than those obtained in the absence of an X pretreatment. This increase can be ascribed to further dissolution of xylans during the L stage when performed after the xylanase treatment, as confirmed by the carbohydrate analysis of the pulp samples. COD for the effluents from the Q and Po stages performed after the laccase–mediator treatments were lower in the sequence including an X stage; however, the combined COD for all stages was significantly higher when a xylanase treatment was applied.

Similarly to COD, the colour of the effluents produced in the L stage was higher than the values measured after the Q and Po stages in all cases, and decreased in the sequence L > Q > Po. The effluents from the L stage performed in the presence of a laccase–mediator system exhibited a marked increase in colour relative to the control effluents by effect of the increased amount of chromophoric species resulting from oxidation and/or degradation of lignin and

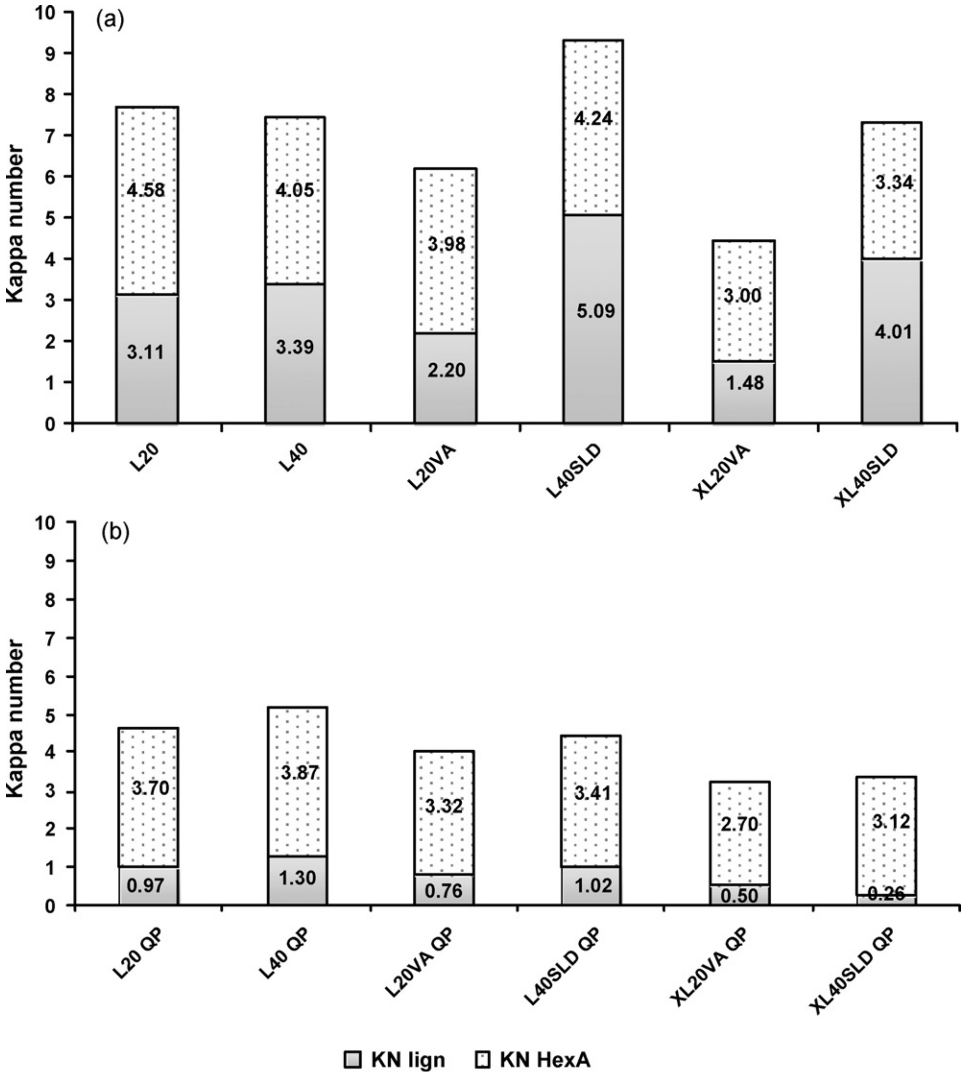

Fig. 3. Contributions to kappa number of pulps obtained from the L (a) and Po (b) stages of each bleaching sequence due to lignin and to HexA.

the mediators. This effect was particularly strong in the presence of the laccase–SLD system, which was also responsible for the marked drop in brightness and increase in *k*/*s* ratio in the treated pulp (Aracri et al., 2009). The effluents from the Q and Po stages involving SLD-treated pulp samples continued to exhibit some effect of the presence of chromophoric species produced by the natural mediator in the L stage, their colour being significantly higher than in the effluents from the VA treatments. Similarly to COD, the colour of the effluents from the L stage performed after xylanase treatment was higher than in the absence of an X stage.

One of the major hurdles for industrial implementation of LMS-based processes is concern about the potential toxicity of the synthetic mediator, which recently promoted a search for alternative, environmentally safe natural compounds (Camarero et al., 2005, 2007; Gutiérrez et al., 2007; Moldes et al., 2008). However, little research has previously been conducted to evaluate the toxicity of effluents from LMS-based bleaching processes and compare natural and synthetic mediators in this respect (Aracri et al., 2009; Fillat & Roncero, 2009b; Fillat et al., 2010). Toxicity tests provide a valuable tool for assessing the environmental hazards of industrial wastewater. Spanish legislation has imposed a limit on the composition of discharged inhibitory matter (IM); thus, the Catalonian regional government has passed a specific regulation (Decree 130/2003) on public sewage services to establish an emission limit value (ELV) for IM discharges at 25 equitox/m^3 [1 equitox/m^3 = 1 T.U. = $(1/EC_{50}) \times 100$]. The bioassay with *V. fisheri* is of technical interest according to Spanish regulations. In this work, we used it to assess toxicity in the effluents produced during the different bleaching sequences with emphasis on the contribution of the mediators used in the L stage. Toxicity data are reported in Table 4. The effluents analysed were those from the X and L stages, which had the strongest impact on COD and colour of the bleaching sequences. As can be seen, the treatments with xylanase and laccase in the absence of a mediator led to low toxicity values, which were raised above the Catalonian ELV by the LMS treatment – to a similar extent in the presence and absence of a xylanase pre-treatment. The increase was especially marked with the laccase–SLD treatment, the resulting effluent being roughly 6 times more toxic than that from the laccase–VA treatment – the latter being only slightly higher than the ELV. In order to determine whether the impact on effluent toxicity was due to the laccase-oxidized or reduced form of the mediator, and to evaluate the influence of pulp, bioassays were performed on effluents from L stages without pulp and on solutions of the mediators at the same concentrations as in the reaction medium. The results showed that VA, whether in its reduced or oxidized form, introduced no toxicity in the effluents; by contrast, reduced SLD led to a toxicity level slightly below the ELV that was dramatically increased after laccase

Table 4
Toxicity values of effluents obtained from X and L stages of each bleaching sequence, effluents obtained from L treatments performed without pulp of the sequences L20VAQPo and L40SLDQPo, and of solutions of mediators at the same concentration as in the L treatment.

Toxicity (T.U.)	
Enzymatic stages	
X	4 ± 0
L20	11 ± 3
L40	7 ± 0
L20VA	33 ± 4
L40SLD	208 ± 28
XL20VA	38 ± 2
XL40SLD	165 ± 5
L stages without pulp	
L20VA (no pulp)	1 ± 0
L40SLD (no pulp)	195 ± 57
Mediator solution	
VA	2 ± 0
SLD	20 ± 10

oxidation, probably through the formation of stable radicals and by-products from the mediator (Aracri et al., 2009). The addition of pulp to the reaction medium at the L stage increased toxicity in the effluents, particularly with the laccase–VA treatment; this can be ascribed to the dissolution of degradation products from lignin. The effluents from the stages following the L treatment were expected to exhibit very low toxicity as previously found by Fillat et al. (2010). In fact, only the effluents from the Q and Po stages involving LMS-treated pulp were analysed to assess the effect of the mediators throughout the bleaching sequences, and they showed very low toxicity levels with both mediators (*ca.* 2 T.U.). The previous results are all consistent with those reported by other authors for effluents from LMS-based processes (Fillat & Roncero, 2009b; Fillat et al., 2010).

4. Conclusions

This work was designed to assess the potential of a xylanase pretreatment of sisal pulp for boosting bleaching and HexA removal during a bleaching sequence, as well as to compare the effectiveness of VA and SLD as laccase mediators for aiding lignin and HexA removal. The pretreatment with xylanase exhibited substantial delignification and HexA removal effects on the initial sisal pulp. The laccase–VA system was efficient in decreasing kappa number and raising brightness in all stages, as well as in significantly reducing the HexA content. On the other hand, the laccase–SLD system increased kappa number and decreased brightness in the L stage through adsorption and/or covalent binding to fibres, thereby failing to reduce the HexA content; if an X pretreatment was applied, however, the laccase–SLD treatment exhibited the strongest delignification effect at the end of the bleaching sequence and provided sisal fibres with a high cellulose content.

Analysis of the effluents showed the enzymatic stages to provide the most significant contribution to total COD and colour in the bleaching sequences. The effluents from the LMS stages exhibited the highest toxicity, which clearly exceeded ELV in the presence of SLD. Toxicity was due mainly to the presence of pulp in the laccase–VA treatment and to oxidation of the mediator by laccase in the laccase–SLD treatment.

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