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Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system

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Abstract

High-quality flax pulp was bleached in a totally-chlorine-free (TCF) sequence using a laccase-mediator system. Three fungal laccases (from *Pycnoporus cinnabarinus*, *Trametes versicolor* and *Pleurotus eryngii*) and two mediators, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and 1-hydroxybenzotriazole (HBT), were compared. *P. cinnabarinus* and *T. versicolor* laccases in the presence of HBT gave the best results in terms of high brightness and low lignin content (kappa number). The former laccase also resulted in the best preservation of cellulose and the largest removal of residual lignin as revealed by analytical pyrolysis, and was selected for subsequent TCF bleaching. Up to 90% delignification and strong brightness increase were attained after a laccase-mediator treatment followed by H_2O_2 bleaching. This TCF sequence was further improved by applying H_2O_2 under pressurized O_2. In this way, we obtained up to 82% ISO brightness (compared with 37% in the initial pulp, and 60% in the peroxide-bleached control) and very low kappa number (near 1). Good results were also found when the laccase-mediator treatment was performed in a bioreactor under pressurized oxygen. The pulp properties obtained, which could not be attained by conventional TCF bleaching of flax pulp, demonstrate the feasibility of enzymatic bleaching to substitute chlorine-containing reagents in manufacturing of these high-price paper pulps.
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Keywords: Laccase; *Pycnoporus cinnabarinus*; 1-Hydroxybenzotriazole; Paper pulp; Flax; Enzymatic bleaching; Delignification; Totally-chlorine-free

1. Introduction

Use of non-wood fibers in paper manufacturing is being recommended by different world organizations due to changes in agricultural policies, wood supply issues, and environmental concerns [1,2]. Despite wood is still by far the main raw material for paper pulp manufacturing, non-wood lignocellulosic fibers from cereal straw and other sources are widely used in many developing countries [3,4]. Moreover, a world market exits for high-value added papers from textile-type fibers obtained from non-wood plants (such as flax, hemp, kenaf, jute or sisal) [3]. These high-quality chemical pulps are often more difficult to bleach than wood chemical pulps due to anatomical and chemical characteristics, including the presence in most of them of lignified core fibers (xylem) together with the long bast (phloem) fibers that have the best properties for textile and paper applications [5]. Modern totally-chlorine-free (TCF) chemical bleaching sequences (using H_2O_2, O_2 and/or O_3) often do not confer adequate brightness to these pulps, and Cl_2 and ClO_2 are being used for industrial bleaching. Therefore, the development of environmentally-friendly technologies for paper manufacturing from non-woody fibers is an urgent need in both industrialized and developing countries. This should include new bleaching sequences limiting the use of chlorine-containing reagents that exert a very negative impact in the aquatic environment [4,6].

Biotechnology has high potential in different aspects of pulp and paper manufacturing with the aim of reducing costs, improving products or limiting the environmental impact of an industry that has been traditionally considered as highly contaminant [7,8]. Enzymatic pre-bleaching with xylanases is established at the mill scale, enabling an increase of brightness or reduction in the consumption of chemicals. Lignin-degrading oxidoreductases, laccases

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and peroxidases, have much higher potential in paper pulp bleaching [9]. However, their performances to delignify and bleach paper pulp are still to be demonstrated at the mill scale. The introduction of the laccase-mediator concept, based on the use of low-molecular weight redox mediators that generate stable radicals, has enlarged the potential of laccases in different applications including the paper pulp industry [10]. These mediators enhance the oxidative capabilities of the enzyme enabling oxidation of non-phenolic lignin [11]. During last decade, much research has focused on the laccase-mediator system for pulp bleaching including understanding the action mechanisms [12–14] and description of more suitable mediators [15–18]. Several patents on laccase-mediator bleaching have been deposited since the first Call's patent [19]. However, all these biobleaching studies focused on wood pulps, and little is known about the efficiency of the laccase-mediator systems on non-wood pulps including those used for manufacturing specialty papers.

In the present paper, we explore the potential of laccase-mediator systems to remove lignin-derived products responsible for color from a high-quality flax pulp. Three fungal laccases, from *Pycnoporus cinnabarinus*, *Trametes versicolor* and *Pleurotus eryngii*, and two mediators, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) and HBT (1-hydroxybenzotriazole), are compared in terms of final pulp properties. Moreover, analytical pyrolysis was applied for the analysis of the residual lignin in these highly delignified pulps. This information was used to select the most efficient laccase-mediator system, and then develop a TCF sequence for the environmentally-sound bleaching of flax pulp.

2. Experimental procedures

2.1. Materials

Flax (*Linum usitatissimum*) alkaline pulps with 36–37% ISO brightness, 11 kappa number and 900–1000 mL/g viscosity, from a soda anthraquinone cooking process [20], were provided by CELESA (Spain). The flax used as raw material for paper pulp manufacturing contained approximately 15% core fibers. ABTS and HBT purchased from Roche and Sigma–Aldrich, respectively, were used as enzyme mediators.

2.2. Enzyme production and activity estimation

Laccases from *T. versicolor* IJFM A136, *P. cinnabarinus* IJFM A720 and *P. eryngii* IJFM A169 (ATCC 90787) were produced in glucose-peptone [21] or glucose–ammonia media [22]. Production was induced by addition of 150 μM $CuSO_4$. In the case of *P. eryngii*, the medium was supplemented with 100 μM $MnSO_4$ that inhibits versatile peroxidase production by this fungus [23]. Cultures were harvested at the time of maximal activity. Laccase activity was determined by 5 mM ABTS oxidation to its cation radical ($\varepsilon_{436\,nm}$: 29,300 $mM^{-1}\,cm^{-1}$) in 0.1 M acetate buffer, pH 5. One activity unit was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute.

2.3. Laccase-mediator treatments

Flasks treatments were carried out in duplicate using 10 g of flax pulp, at 2–3% consistency in 50 mM tartrate buffer, pH 4. Pulp was kept under O_2 bubbling for 24 h, at 160 rev/min, and 30 °C. In the first experiment with different laccases, the whole cultures were used and the enzyme and mediators dosages were 10–20 U of laccase and 20 mg of ABTS or HBT per gram of pulp (with the following initial properties: 37% ISO brightness, 11 kappa number and 900 mL/g viscosity). In the subsequent experiments, the enzymatic treatment was performed using 20 U/g of partially-purified laccase from *P. cinnabarinus* and 4% (w/w) HBT relative to pulp. Tween 80 (0.05%, w/v) was added as surfactant. The initial pulp used in these experiments had 36% ISO brightness, 11 kappa number and 1000 mL/g of viscosity. Laccase was prepared after ultrafiltration by 3 kDa cut-off membrane, or precipitated with ammonium sulfate followed by diafiltration and lyophilization with 30% lactose. Laccase-mediator treatments in bioreactor under pressurized O_2 (6 bar) were carried out on 40 g of pulp under the latter conditions (but 3%, w/w, HBT) for two different times: (1) 24 h, 30 °C, and 60 rev/min, using 3% consistency pulp; and (2) 4 h, 40 °C, 60 rev/min, using 8% consistency pulp. Pulps treated under identical conditions (pH, temperature, oxygen, shaking and time of reaction) but without enzyme or mediator (control pulps) were included in all experiments (similar results were obtained in controls without enzyme in the presence of mediator).

2.4. Optimization of a TCF sequence and evaluation of the resulting pulp

For development of a flax pulp bleaching TCF sequence, the laccase-mediator treatment (L stage) was followed by an alkaline extraction of the pulps (E stage) using 1.5% NaOH for 1 h at 60 °C. Subsequent bleaching with H_2O_2 (P stage) consisted of 3% H_2O_2 in 1.5% NaOH, for 2 h at 90 °C. The E′ stage used for comparison included the latter alkaline conditions without H_2O_2. Pressurized H_2O_2 bleaching (P_O stage) was carried out in a reactor with 3% H_2O_2 in 1.5% NaOH, for 2 h at 90 °C, under 5 bar O_2 atmosphere. Reductive treatment of pulp (R stage) was performed with 2% $NaBH_4$, for 30 min at 20 °C. All pulp treatments after the laccase-mediator bleaching were carried out at 5% consistency. Brightness, kappa number (lignin content can be roughly estimated by multiplying kappa number by a factor of 0.15) and viscosity were measured at the different

stages according to ISO 3688, ISO 302 and ISO 5351/1 standards [24], respectively. Pulps were refined at 20 000 rev in a PFI laboratory equipment (ISO 5264-2) to the same refining degree (°SR) (ISO 5267-1), and papermaking properties, breaking length, tensile index (ISO 5270/1924-1), burst index (ISO 5270/2758), tear index (ISO 5270/1974) and folding endurance (ISO 5270/5626) [24], were evaluated.

2.5. Analytical pyrolysis

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) was performed in duplicate with a Curie-point pyrolyzer coupled to a Varian Saturn 2000 GC-MS equipment, using a 30 m × 0.25 mm DB-5 column (film thickness 0.25 μm). Approximately 1–2 mg of sample were deposited on a ferromagnetic wire, inserted into the glass liner, and pyrolyzed at 610 °C for 3.5 s. The oven was programmed from 40 °C (1 min) to 300 °C at a rate of 6 °C/min, and the final temperature was held for 20 min. The injector, equipped with a liquid CO_2 cryogenic unit, was programmed from −30 °C (1 min) to 300 °C at 200 °C/min, and the GC-MS interface was kept at 300 °C. Pyrolysis products were identified by mass spectra comparison with those in the Wiley and Nist computer libraries and reported in the literature. Since some lignin-derived compounds were minor peaks in pyrograms, their areas were integrated in single-ion chromatographic profiles corresponding to their molecular ions and converted into total-ion areas. Selected compounds were quantified as markers for cellulose and different lignin units. In this way, decrease of lignin/cellulose ratio and changes of ratio between lignin syringylpropanoid (S) to guaiacylpropanoid (G) units (S/G ratio) were calculated from peak areas of 4-hydroxy-5,6-dihydro(2*H*)-pyran-2-one (*m*/*z* 114) as cellulose marker; 4-methylguaiacol (*m*/*z* 138), 4-ethylguaiacol (*m*/*z* 152), 4-vinylguaiacol (*m*/*z* 150) and *trans*-4-propenylguaicol (*m*/*z* 164) as G-lignin markers; and 4-methylsyringol (*m*/*z* 168), 4-ethylsyringol (*m*/*z* 182), 4-vinylsyringol (*m*/*z* 180) and *trans*-4-propenylsyringol (*m*/*z* 194) as S-lignin markers [25].

3. Results

The results obtained show the feasibility of bleaching high-quality flax pulp in a chlorine-free sequence based on the use of laccase in the presence of a redox mediator followed by H_2O_2 treatment as described in the following sections.

3.1. Flax pulp bleaching with different laccases and mediators

Table 1 shows the modification of flax pulp properties after treatment with three fungal laccases in the presence of two synthetic mediators. Brightness and kappa number analyses showed the high effectiveness of laccases from *P. cinnabarinus* and *T. versicolor* for delignifying and bleaching flax pulp when HBT was used as mediator. Up to 20% ISO brightness increase with respect to the initial pulp was attained after the laccase-HBT treatment (both followed by alkaline extraction). A decrease of kappa number from 9 to 3 was simultaneously produced. *P. cinnabarinus* laccase plus HBT gave the best selectivity in lignin removal, determined as the ratio between the decreases of kappa number (an estimation of lignin content) and pulp viscosity (an estimation of cellulose integrity).

Pulp samples were also analyzed by Py-GC-MS. The main chromatographic peaks corresponded to carbohydrate-derived compounds whereas the peaks of lignin-derived compounds were much lower. However, by monitoring individual ions corresponding to the M_w of selected lignin markers (see Section 2.5), information on relative lignin content and composition [26] could be obtained, as illustrated in Fig. 1. This revealed the presence in flax pulp of a GS-type lignin, with predominance of G-units, which was preferentially removed against cellulose during laccase-mediator treatment. In this process, a preferential removal of lignin S-units was also observed. The Py-GC-MS data shown in Table 1 confirmed that laccases from *P. cinnabarinus* and *T. versicolor*, in the presence of HBT, produced the largest lignin removal from flax pulp.

Table 1
Pulp properties and Py-GC-MS analysis of flax pulp treated with different laccase-mediator systems, compared with the initial pulp and the corresponding control (all samples were analyzed after alkaline extraction)

	Pulp properties			Pyrolysis analyses	
	Brightness (%)	Kappa number	Viscosity (mL/g)	Lignin/cellulose decrease (%)	S/G ratio
Initial pulp	39 ± 0	9.0 ± 0.2	750 ± 1	0	0.23
Control pulp[a]	45 ± 4	8.3 ± 2.0	710 ± 28	0	0.17
T. versicolor laccase + ABTS	48 ± 2	6.3 ± 0.7	430 ± 51	15	0.07
T. versicolor laccase + HBT	60 ± 1	3.3 ± 0.2	600 ± 14	56	0.03
P. cinnabarinus laccase + ABTS	34 ± 1	8.5 ± 0.5	570 ± 44	0	0.12
P. cinnabarinus laccase + HBT	55 ± 4	2.9 ± 0.4	680 ± 4	69	0.02
P. eryngii laccase + ABTS	33 ± 2	9.8 ± 1.3	590 ± 3	18	0.11
P. eryngii laccase + HBT	36 ± 0	8.0 ± 0.8	610 ± 1	30	0.11

Mean values and 95% confidence limits for the different pulp properties determined after treatment with the different laccase-containing cultures in the presence of ABTS or HBT.

[a] Pulp treated under the same conditions (pH, temperature and O_2) but without enzyme or mediator.

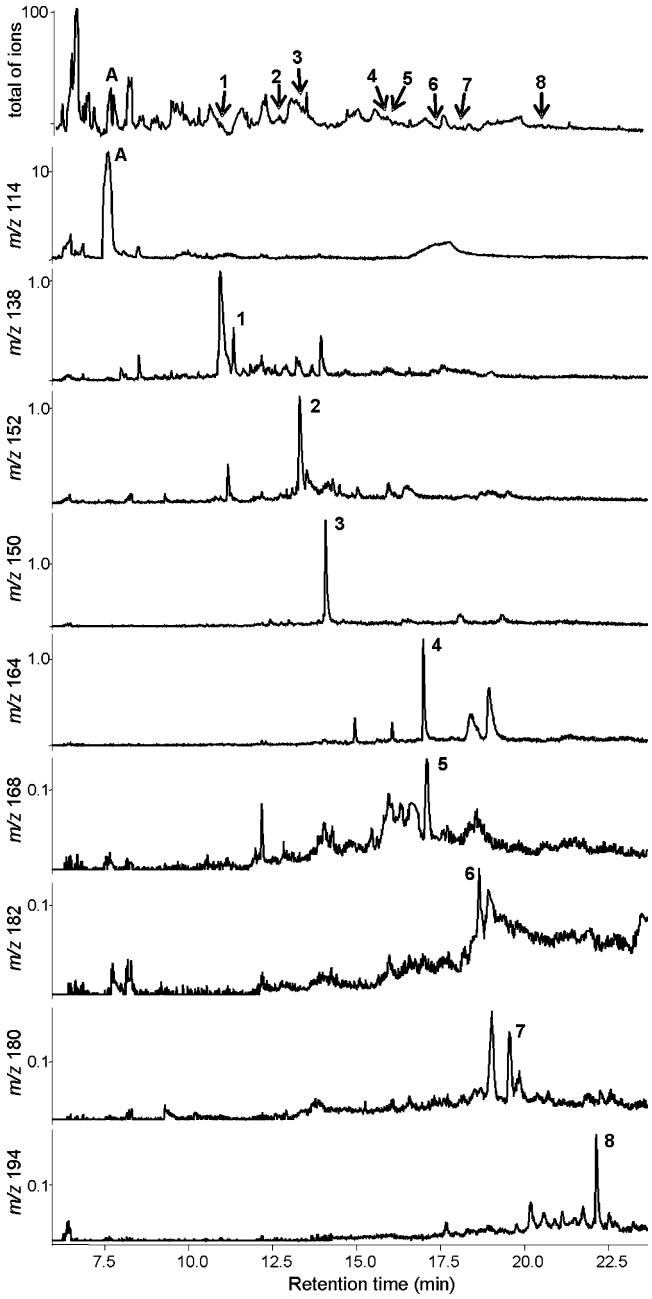

Fig. 1. Analysis of residual lignin in the initial flax pulp by Py-GC-MS using marker compounds. Total-ion chromatographic trace (top) showing carbohydrate and lignin derived compounds, and selected ion chromatographic profiles (bottom) enabling identification of four G-lignin markers (1–4), four S-lignin markers (5–8), and one cellulose marker (A). Peak identification: (1) 4-methylguaiacol (*m*/*z* 138); (2) 4-ethylguaiacol (*m*/*z* 152); (3) 4-vinylguaiacol (*m*/*z* 150); (4) *trans*-4-propenylguaicol (*m*/*z* 164); (5) 4-methylsyringol (*m*/*z* 168); (6) 4-ethylsyringol (*m*/*z* 182); (7) 4-vinylsyringol (*m*/*z* 180); (8) *trans*-4-propenylsyringol (*m*/*z* 194); and (A) 4-hydroxy-5,6-dihydro(2*H*)-pyran-2-one (*m*/*z* 114).

3.2. Development of a TCF sequence for flax pulp bleaching including laccase

In the light of the results described above, *P. cinnabarinus* laccase and HBT were selected for further bleaching studies. First, a peroxide bleaching step (P stage) was added after the alkaline extraction (E stage) that followed the laccase-HBT treatment (L stage). Eighty-nine percent delignification rate, resulting in a final kappa number around 1, and a strong increase of brightness, from 37 to 81% ISO, were attained after the LEP sequence as shown in Table 2 (the 95% confidence limits in this and following tables are all below 10% of the mean values presented).

Analytical pyrolysis of pulp samples from the LEP sequence showed the removal of lignin markers during the laccase-HBT treatment (followed by alkaline extraction) and the subsequent peroxide bleaching. This is illustrated in Fig. 2 for two marker compounds of G and S lignin units (4-vinylguaiacol and 4-vinylsyringol, respectively). As shown in the single-ion chromatographic profiles, G and S lignin units were easily detectable in the crude pulp but their relative abundance dramatically decreased during successive bleaching with laccase-mediator and H_2O_2. These changes were quantified using the nine lignin marker compounds shown in Fig. 1, and the results are shown in Table 3. At the end of the LEP sequence, a 92% decrease of lignin/cellulose ratio and the complete removal of lignin S units were attained. A significant correlation between the lignin removal values estimated from kappa number and the decreases of lignin/cellulose ratio from pyrolysis was found in the course of the LEP sequence (R^2: 0.90).

Further studies focused on the optimization of the above TCF sequence for flax pulp bleaching. It was found that the LEP sequence could be substituted by a simple LP sequence, since similar brightness and kappa number and better viscosity were obtained after removing the E stage (Table 4). The viscosity of the pulps treated with laccase-HBT was lower than that of the corresponding controls. However, when a reductive treatment with $NaBH_4$ (R stage) was applied after laccase-HBT, the viscosity was much better conserved (data not shown) as described below for the bioreactor treatment.

The effect of pressurized O_2 during peroxide bleaching (Po stage) was also examined. No significant improvement of kappa number was attained with the LPo sequence (Table 5) compared with the LP one (Table 4), but higher brightness was attained (up to 82% ISO). To obtain a similar brightness using TCF chemical reagents, a long bleaching sequence including the use of O_2, O_3 and H_2O_2 was required (Table 5). When other pulp properties were compared after enzymatic and chemical bleaching, the highest viscosity and the lowest kappa number were obtained after the LPo sequence. Furthermore, papermaking tests revealed that mechanical properties estimated by tear, tensile and burst indices, and folding endurance were similar or better for the paper handsheets prepared after the LPo sequence than after the chemical sequence.

3.3. Bioreactor bleaching of flax pulp using laccase-HBT

Successful pulp delignification and brightness increase were obtained when the L stage was performed in a closed

Table 2
Properties of flax pulp after treatment with *P. cinnabarinus* laccase and HBT (L) followed by alkaline extraction alone (E) or combined with peroxide bleaching (EP)

	E			EP		
	Brightness (% ISO)	Kappa number	Viscosity (mL/g)	Brightness (% ISO)	Kappa number	Viscosity (mL/g)
Initial pulp	37	10	855	57	6.9	630
Control pulp[a]	42	8.9	920	61	5.5	680
Laccase + HBT (L)	59	2.8	610	81	1.3	470

[a] Pulp treated under the same conditions (pH, temperature and O_2) but without laccase and mediator.

Table 3
Relative removal of lignin and changes of its S/G ratio estimated by Py-GC-MS after flax pulp treatment with *P. cinnabarinus* laccase and HBT (L) followed by alkaline extraction alone (E) or combined with peroxide bleaching (EP)

	E		EP	
	Lignin/cellulose decrease (%)[a]	Lignin S/G ratio	Lignin/cellulose decrease (%)	Lignin S/G ratio
Control pulp[b]	0	0.35	16	0.29
Laccase + HBT (L)	85	0.03	92	0

[a] From lignin/cellulose ratio of control before peroxide.
[b] Pulp treated under the same conditions (pH, temperature and O_2) but without laccase or mediator.

reactor under pressurized O_2 (Table 6) instead of using open flasks with O_2 bubbling as in previous experiments (see Table 4). Pulp properties after 24 h treatment with laccase-HBT under pressurized O_2, followed by peroxide bleaching, were similar to those obtained in experiments under atmospheric pressure. When the L stage was limited to 4 h, to approach mill operation conditions, no significant improvement of pulp properties was observed after the enzymatic treatment compared with the control. However, when peroxide bleaching was applied to this pulp, brightness approaching that obtained after 24 h and higher viscosity was found (Table 6).

Finally, it was found that a reductive treatment with $NaBH_4$ (R stage) efficiently prevented the viscosity loss produced after the 24 h treatment with laccase-HBT (Table 6). However, a reductive treatment was not required after 4 h treatment since viscosity was much more conserved. On the other hand, the effect of H_2O_2 alone can be deduced by comparing the P stage and an alkaline treatment under the same conditions used for peroxide bleaching (E′ stage) (Table 6). In this way, it was found that H_2O_2 not only increases pulp brightness after the enzymatic treatment, but also reduces kappa number and viscosity.

Table 4
Improvement of properties of laccase-HBT treated flax pulp (L) by subsequent alkaline extraction (LE), peroxide bleaching (LP) or a combination of both (LEP)

	Brightness (% ISO)	Kappa number	Viscosity (mL/g)
Initial pulp	36	11	1025
Control	38	14	1065
Laccase + HBT (L)	42	9.5	840
E control	39	9.4	1000
LE sequence	49	5.0	780
EP control	62	5.5	530
LEP sequence	76	1.4	470
P control	63	5.4	690
LP sequence	80	1.6	730

The controls correspond to pulp treated under the same conditions (pH, temperature and O_2) but without enzyme or mediator, followed by the different chemical stages.

Table 5
Pulp and papermaking properties of flax pulp bleached with laccase-HBT followed by pressurized H_2O_2 (LPo sequence) compared with a chemical sequence including O_2 (O), O_3 (Z) and H_2O_2 bleaching (P), acidic washing (W_A), and a reductive step (R)

	Po control[a]	Bleaching sequences	
		LPo	W_AOZRPW_A
Pulp properties			
Consumed energy (Wh)[b]	260	250	250
Kappa number	4.6	1.3	3.0
Brightness (% ISO)	65	82	83
Viscosity (mL/g)	610	640	570
Paper properties			
Breaking length (km)	6.5	6.0	5.7
Tensile index (N m/g)	64	59	56
Burst index (kN/g)	3.0	3.5	3.2
Tear index (mN m^2/g)	6.7	7.6	5.1
Folding endurance	2.7	3.1	2.9

[a] Under the same conditions (pH, temperature and O_2) of the L stage but without laccase or mediator, followed by Po.
[b] For the same refining degree of 91–92 °SR.

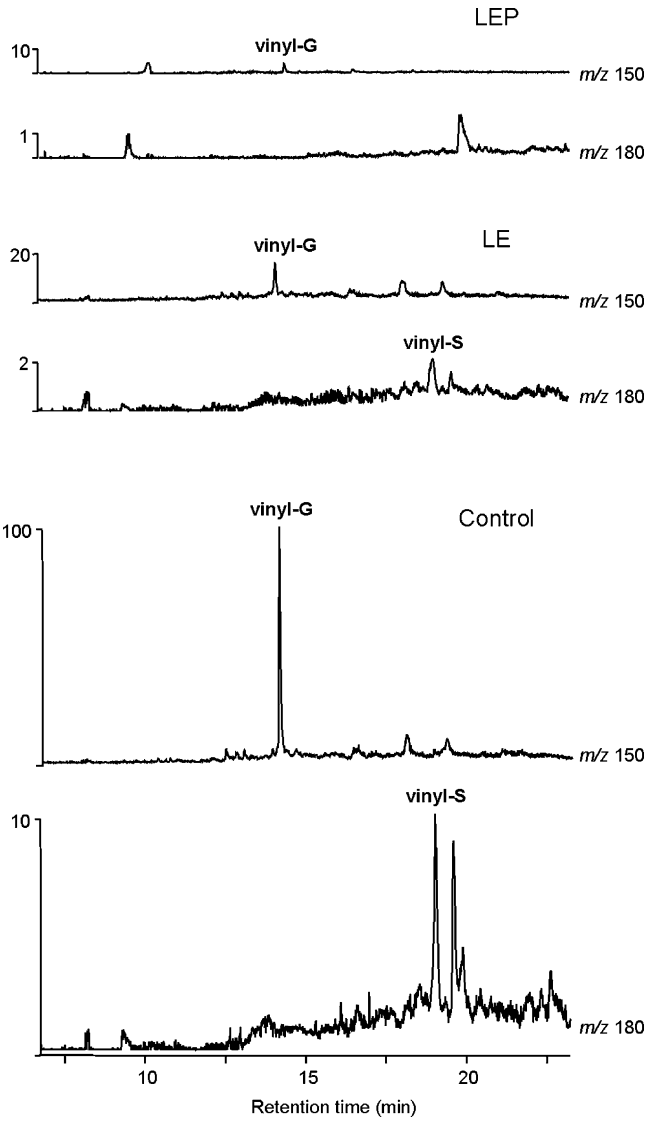

Fig. 2. Py-GC-MS analysis of residual lignin during TCF bleaching of flax pulp using a laccase-based LEP sequence. Single-ion chromatographic profiles are presented showing decrease of two representative G and S lignin markers (4-vinylguaiacol, *m*/*z* 150; and 4-vinylsyringol, *m*/*z* 180) after enzymatic treatment with *P. cinnabarinus* laccase in the presence of HBT followed by alkaline extraction (LE), and subsequent H_2O_2 bleaching (LEP), compared with the control pulp. Traces were previously normalized for the same area of the cellulose marker, 4-hydroxy-5,6-dihydro(2*H*)-pyran-2-one.

Table 6
Pulp properties after 4 h (40 °C) and 24 h (30 °C) treatment with laccase-HBT in pressurized O_2 reactor (L) followed by peroxide bleaching (LP), and a combination of $NaBH_4$ reduction and peroxide bleaching (LRP)

	Bleaching sequences				
	L	LP	LRP	LR	LE′
Brightness (% ISO)					
Control (4 h)[a]	39	61	62	43	44
Laccase + HBT (4 h)	41	72	71	48	53
Control (24 h)[a]	40	61	62	43	45
Laccase + HBT (24 h)	53	78	78	61	65
Kappa number					
Control (4 h)	10	5.1	5.7	9.1	7.3
Laccase + HBT (4 h)	7.3	3.2	3.1	6.6	4.2
Control (24 h)	9.4	5.7	6.0	8.4	7.1
Laccase + HBT (24 h)	3.9	1.2	1.0	2.8	1.9
Viscosity (mL/g)					
Control (4 h)	1000	910	910	1000	1000
Laccase + HBT (4 h)	930	820	820	950	870
Control (24 h)	1100	810	800	1000	980
Laccase + HBT (24 h)	760	640	860	920	670

The effects of $NaBH_4$ reduction (LR) and the alkaline conditions used in peroxide bleaching (LE′) after laccase-HBT treatment are included.

[a] Under the same conditions (temperature, pH and pressurized O_2) of the corresponding L stages but without laccase or mediator.

4. Discussion

The present study shows efficient bleaching of high-quality paper pulp using enzymes as an alternative to Cl_2 or ClO_2, which exert a negative environmental impact due to the release of a variety of chlorinated compounds that can be incorporated to trophic chains [27]. Laccases from *P. cinnabarinus* and *T. versicolor* in the presence of HBT strongly delignified alkaline flax pulps, whereas worst results were obtained with the *P. eryngii* laccase probably due to its lower redox potential [16]. Evaluation of lignin removal by pyrolysis could be more reliable than obtained from wet chemistry methods, since hexenuronic acids formed during alkaline pulping interfere kappa number estimation [28,29]. The 63–68% decrease of kappa number obtained with the above laccases is much higher than previously reported after similar LE treatments of chemicals pulps from different woods [28,30–32]. The same could be said for the significant increase of pulp brightness attained. It can be emphasized that brightness already increased after treatment with laccase (from *P. cinnabarinus*) and HBT, whereas an alkaline extraction is generally required to see an increase of brightness in most studies reported in the literature [31].

The efficiency of HBT, which was selected as mediator for flax pulp bleaching, was in all cases higher than that of ABTS in agreement with previous reports [16]. It has been suggested that laccase mediators could be toxic or scarcely biodegradable. In seems that HBT at the doses used has a toxicity below the limits accepted by Spanish regulations for metropolitan waters [33]. However, each bleaching effluent must be evaluated since new toxic compounds could be generated during the reactions of mediators with laccase and residual lignin in pulp. On the other hand, the laccase from *P. cinnabarinus* was selected for flax bleaching because of its higher selectivity removing residual lignin. This is a high redox potential laccase [17,18] that can be produced in high levels for different applications using selected monokaryotic strains of *P. cinnabarinus* [34]. This

laccase has been extensively investigated during last years including gene cloning [35], protein crystallization [36], and expression in different host systems [37].

Very high delignification rate, estimated as reduction of kappa number (89%) or Py-GC-MS cellulose/lignin ratio (92%), and strong increase of brightness (up to 81–82% ISO) were obtained when peroxide bleaching was added to the L + E treatment. This improvement of pulp properties is much better than reported after LEP bleaching of other pulps [31], even when ClO_2 was applied after the laccase treatment [38]. H_2O_2 treatment of flax pulp treated with laccase and HBT resulted in extremely low kappa number. However, lignin removal from pulp was mainly produced by the laccase-mediator treatment. The same could be said for the modification of residual lignin, since the most significant reduction of its S/G ratio occurred during the L and E stages. Meanwhile, the P stage significantly enhanced the brightness improvement already obtained after the enzymatic treatment. Moreover, suppression of the E stage often included in biobleaching sequences [28,30,31], resulted in pulp with similar brightness and kappa number, and improved viscosity. It seems that quinones and other products responsible for pulp color and kappa number can be efficiently removed during the alkaline peroxide stage [40].

Pulps from flax and other non-wood fibers are recalcitrant towards bleaching. However, the 81–82% ISO brightness attained after the LEP or LPo sequences is a very good result, comparable with those obtained using chlorine-containing reagents. Moreover, the extremely low lignin content after TCF bleaching using laccase-HBT had never been attained in conventional TCF sequences using chemical reagents. As shown here, a long non-enzymatic TCF sequence (e.g. W_AOZRPW_A) was necessary to bleach flax pulp at the same brightness degree. Similar brightness but higher kappa number have been recently reported after bleaching flax pulp in a multistage TCF sequence using TAED as H_2O_2 activator [39].

To approach industrial application conditions, flax pulp bleaching with laccase-HBT was performed for 4 or 24 h in a closed bioreactor under pressurized O_2, the final electron acceptor in laccase-mediator oxidation of lignin [9]. A positive effect of pressurized O_2 on the enzymatic treatment was observed by comparing the 24 h flask (42% ISO brightness) and bioreactor treatments (53% ISO brightness) just after the enzymatic stage. The effect of L stage on flax lignin and cellulose depended on the duration of the treatment [30]. Despite only a moderate effect on pulp brightness and kappa number was observed after 4 treatment, the subsequent peroxide bleaching narrowed the brightness difference between the two treatments. Therefore, satisfactory bleaching of flax pulp (up to 72% ISO brightness) can be attained by a TCF sequence consisting of a short laccase-HBT treatment under pressurized O_2 followed by peroxide bleaching. Alternatively, the enzymatic treatment can be extended and a reductive stage introduced to prevent the oxidative degradation of cellulose produced after long enzymatic treatment.

It is known that cellulose degradation occurs when paper pulp bleached with O_3 undergoes alkaline conditions [41]. The results obtained suggest that extended laccase-mediator treatment produces a similar effect on flax pulp, due to formation of carbonyl groups that could cause the scission of cellulose chains by β-elimination in alkaline medium. However, viscosity drop could be avoided by reducing the carbonyls to alcohols before the alkaline stage. The complete recovery of initial viscosity after the reductive stage shows that viscosity drops are due to the action of the laccase-mediator system by the mechanism described (and not to the action of eventual contaminating hydrolases). Under these conditions (use of an LRP sequence including extended enzymatic treatment), higher pulp delignification and brightness can be attained, but the cost of the reducing agent consumed and the larger equipment required should be taken into account.

It is possible to conclude that the laccase-mediator system is especially promising for bleaching high-quality pulps from flax and other non-wood fibers. Once integrated in a TCF sequence, satisfactory brightness and delignification rates can be obtained. It is necessary to emphasize that such high delignification degree has not been reported after enzymatic treatment of other pulps, and are difficult to obtain by TCF bleaching of flax pulp with chlorine-free chemical reagents. A patent on this laccase application has been deposited and some treatment parameters have been recently optimized [42,43]. TCF sequences based on laccase-mediator treatment could permit substitution of chlorine-containing reagents for bleaching high-quality pulps. The cost of the enzymatic bleaching would be affordable because: (i) low investment for new installations would be required; (ii) laccase hyperproducing strains are available or could be obtained using industrial hosts; (iii) enzyme and mediator costs would be compensated by the high price of these pulps (e.g. the market price of flax pulp is around four-times that of kraft pulp); (iv) reagents would be saved due to the shortening of the bleaching sequence; and (v) environmental advantages would be produced by avoiding Cl_2 and ClO_2.

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