Flax pulp bleaching and residual lignin modification by laccase-mediator systems*

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Enzymatic bleaching of flax alkaline pulp is intended here by using laccase-mediator systems (LMS) based on *Pleurotus eryngii, Trametes versicolor* and *Pycnoporus cinnabarinus* laccases and two mediators. The efficiency of the different LMS treatments was compared in terms of brightness, kappa number and viscosity modifications. Furthermore, changes in the molecular structure of residual lignin and lignin/carbohydrate ratio were analyzed by pyrolysis-gas chromatography-mass spectrometry of the treated pulps. *Pycnoporus cinnabarinus* and *T. versicolor* laccases in the presence of 1-hydroxybenzotriazole (HBT), were the most effective for flax pulp delignification. Up to 20% ISO brightness increase with respect to the initial pulp and a decrease of kappa number from 9 to 3 were attained after alkaline extraction of the pulps treated with those LMS. *Pycnoporus cinnabarinus* laccase plus HBT gave also the best selectivity in lignin removal. Analytical pyrolysis of the LMS-treated pulps showed a selective removal of lignin against cellulose and a strong alteration of the residual lignin with preferential removal of the syringyl units. *Pycnoporus cinnabarinus* LMS_{HBT} gave the best results in terms of decrease of lignin/cellulose and syringyl/guaiacyl ratios, in agreement with data from pulp parameters. For the above reasons laccase from *P. cinnabarinus* and HBT were selected for further assays including a peroxide step. Up to 89% relative delignification rate (attaining 1.3 kappa number) and an increase of 44% ISO brightness were attained after LMS_{HBT}-peroxide bleaching. Ongoing studies focus on improvement of a LMS_{HBT}-based totally chlorine free sequence for flax pulp bleaching.

1. INTRODUCTION

Pulp and paper manufacture from non-woody fibers is a widespread reality in Asia, South America and eastern Europe. Beside cereal straw, the leading non-woody fiber and one of the oldest sources of paper pulp, several crops are grown up for their content in long fibers. These textile fibers are mainly used for high added-value products in developed countries. In fact, high-quality pulps for specialty papers (tea bags, filters, cigarettes, bibles, condensers, etc) are manufactured from textile fibers such as flax, hemp, abaca, kenaf, jute or sisal.

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During last years new pulping concepts have arisen to overcome the limitations (i.e. chemical recovery and scaling up) from the application of conventional processes to these type of raw materials [1]. At the same time improvement of pulp quality and environmentally-sound bleaching sequences are searched. In this sense, the use of fungal laccases in the presence of mediators offers the possibility to develop new totally-chlorine free (TCF) sequences to bleach different types of pulps. The potential of these laccase-mediator systems (LMS) must be carefully evaluated since differences in the structure and redox potential of the enzyme as well as reactivity of the oxidized mediator can affect delignification rate and consequently pulp bleaching results [2-5]. Great advances have been achieved during the last decade in the understanding of LMS chemical and enzymatic mechanisms. However, most LMS studies have been carried out onto softwood and hardwood kraft pulps [6;7] and few enzymatic bleaching trials have been performed on non-woody materials.

The present study focuses on bleaching and delignification of a non-woody pulp, namely flax alkaline pulp. We first compared the efficiency of laccases from three white-rot fungi, *Pleurotus eryngii, Trametes versicolor* and *Pycnoporus cinnabarinus*, and two mediators, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT), for lignin removal from flax pulp. In this way, brightness, kappa number and viscosity of the LMS-treated pulps were determined. Moreover, the structure of the residual lignin in pulps was analyzed by pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) [8], a powerful analytical tool which provides information in terms of lignin/carbohydrate and *p*-hydroxyphenyl:guaiacyl:syrinyl (H:G:S) ratios, even when the kappa number is low. Both, pulp properties and pyrolysis evaluation were used for selection of the best LMS to be applied in a TCF sequence for flax pulp bleaching.

2. MATERIAL AND METHODS

2.1. Materials

The initial flax pulp with 36% ISO brightness, 11 kappa number and 900 mL/g viscosity was provided by CELESA mill (Spain). The initial raw material, flax (*Linum usitatissimum*), contained 15% of core fibers (xylem), and the pulps were obtained by soda anthraquinone cooking. ABTS and HBT were purchased from Boehringer and Aldrich respectively.

2.2. Enzyme production and activity determination

Laccases were produced by *P. eryngii* ATCC 90787 (= IJFM A169) grown in glucose-peptone medium [9] with 100 µM $MnSO_4$, and *T. versicolor* IJFM A136 and *P. cinnabarinus* IJFM A720 grown in glucose-ammonia medium [10]. In all cases laccase production was induced by addition of 150 µM $CuSO_4$. Cultures were harvested at the point of maximal laccase activity for the enzymatic bleaching assays. Laccase activity was determined by monitoring the OD_{436} change of 5 mM ABTS oxidation to its cation radical (extinction coefficient at 436 nm 29300 mM^{-1} cm^{-1}) in 100 mM acetate buffer, pH 5. One unit of enzyme activity was defined as the amount of enzyme that transforms 1 µmol of substrate per minute.

2.3. Laccase-mediator treatments

The laccase-mediator treatments (L-stage) were carried out in duplicate with 10 g of flax pulp at 2% consistency in 50 mM tartrate buffer, pH 4. Enzyme and mediators dosages were 10-20 U of laccase (the whole liquid cultures being used) and 20 mg of ABTS or HBT per gram of pulp. Tween 80 (0.05% w/v) was also added as surfactant. Flasks were kept under O_2

atmosphere for 24 h, at 160 rev/min and 30ºC. In subsequent experiments, L-stage was performed with 20 U/g of *P. cinnabarinus* partially purified laccase (ultrafiltered by 3 kd cut off) and 4% (w/w) of HBT relative to pulp. As controls, pulps were treated under identical conditions but without enzyme and mediator.

2.4. Post LMS stages

LMS-treatments were followed by an alkaline extraction of the pulps (E-stage consisting of 1.5% NaOH treatment for 1 h at 60°C). Subsequent bleaching with hydrogen peroxide (P-stage) consisted of 3% H_2O_2 in 1.5% NaOH, for 2 h at 90°C. Pressurized peroxide bleaching (P_O-stage) was carried out with 3% H_2O_2 in 1.5% NaOH, for 2 h at 90°C in the presence of 5 bar O_2). A reductive step (R-stage) was applied under the following conditions: 2% $NaBH_4$, 30 min at 20ºC. All post-LMS treatments were carried out at 5% pulp consistency. Brightness, kappa number and viscosity were measured at the different stages according to ISO 302, ISO 5351/1 and ISO 3688 standards, respectively.

2.5. Analytical Pyrolysis

Pyrolysis was performed in duplicate with a Curie-point pyrolyzer coupled to a Varian Saturn 2000 GC/MS equipment, using a 30 m x 0.25 mm DB-5 column (film thickness 0.25 μm). Approximately 1 mg of sample was deposited on a ferromagnetic wire, then inserted into the glass liner and immediately placed in the pyrolyzer. The pyrolysis was carried out at 610°C for 3.5 seconds. The chromatograph was programmed from 40°C (1 min) to 300°C at a rate of 6°C/min. The final temperature was held for 20 min. The injector, equipped with a liquid carbon dioxide cryogenic unit, was programmed from -30°C (1 min) to 300°C at 200°C/min, while the gas chromatography-mass spectrometry (GC-MS) interface was kept at 300°C. Pyrolysis products were identified by comparison with those reported in the literature and in the Wiley and Nist computer libraries.

Since some of the lignin-derived compounds are minor peaks in most pyrograms, their areas were integrated in single-ion chromatographic traces corresponding to their molecular ions. Furthermore, for relative estimation of polysaccharide and lignin removal, selected compounds were quantified as representative markers for cellulose and the different lignin units. Then, the decrease of lignin/cellulose ratio and the changes in lignin S/G ratio were calculated from the peak areas of 4-hydroxy-5,6-dihydro(2*H*)-pyran-2-one (*m/z* 114) as cellulose marker, compared with the following lignin markers: 4-methylguaiacol (*m/z* 138), 4-ethylguaiacol (*m/z* 152), 4-vinylguaiacol (*m/z* 150) and *trans*-4-propenylguaicol (*m/z* 164) as guaiacyl 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 syringyl markers.

3. RESULTS AND DISCUSSION

3.1. Selection of the best LMS for flax pulp bleaching

Table 1 shows that the laccases from *P. cinnabarinus* and *T. versicolor* were the most effective for flax pulp delignification, in all cases the best results being obtained with HBT as mediator. An increase of 10-15% ISO brightness with respect to the control pulp, which represents 20% ISO brightness increase with respect to the initial pulp, was attained after alkaline extraction of the pulps treated with *P. cinnabarinus* and *T. versicolor* laccases in the presence of HBT. At the same time a significant decrease of kappa number occurred (from 8.3 of the control to values around 3). *Pycnoporus cinnabarinus* laccase plus HBT gave also the

best selectivity in lignin removal measured as the ratio between delignification efficiency and reduction of pulp viscosity (3.2 compared with 2.2 of *T. versicolor* LMS_{HBT}).

Table 1
Pulp properties and Py-GC-MS analysis after flax pulp treatment with different LMS followed by alkaline extraction (treatments were carried out by adding the pulp to fungal cultures with high laccase activity, 10-20 U/g pulp)

	Pulp properties			Pyrolysis analysis	
	Brightness (%)	Kappa number	Viscosity (mL/g)	Lignin/Cellulose decrease (%)	S/G ratio
Initial pulp	40.0	9.0	750	0	0.23
Control pulp	45.3	8.3	710	0	0.17
T. versicolor laccase + ABTS	48.4	6.3	434	15	0.07
T. versicolor laccase + HBT	60.2	3.3	600	56	0.03
P. cinnabarinus laccase + ABTS	34.2	8.5	565	0	0.12
P. cinnabarinus laccase + HBT	55.3	2.9	678	69	0.02
P. eryngii laccase + ABTS	33.4	9.8	586	18	0.11
P. eryngii laccase + HBT	35.8	8.0	607	30	0.11

Lignin removal as well as changes produced in the residual lignin of alkaline pulps after the LMS treatments were examined by Py-GC-MS. This degradative technique permits the identification of a series of products as derived from either cellulose or lignin polymers. In all cases, the main peaks corresponded to carbohydrate-derived compounds whereas the lignin-derived peaks were much lower. However, by monitoring individual ions corresponding to the M_w of selected lignin markers (representative compounds derived from the three types of lignin units) more information on lignin content and composition could be obtained (Fig. 1). This fact is specially interesting for pulps with very low kappa number (e.g. those enzymatically treated where kappa number has strongly decreased). By using this methodology, the presence of a G-rich GS-type residual lignin in flax pulps was revealed. The analytical pyrolysis of the pulps treated with the different LMS showed a selective removal of lignin against cellulose (Table 1). Moreover, a strong alteration of the residual lignin with preferential removal of S-units was observed. The use of laccases from *P. cinnabarinus* and *T. versicolor* and HBT as mediator resulted in higher delignification rates compared with ABTS utilization. *Pycnoporus cinnabarinus* LMS_{HBT} gave the best results in terms of lignin removal and modification of S/G ratio. Figure 2 illustrates the preferential removal of two selected lignin markers against cellulose marker with *P. cinnabarinus* LMS_{HBT} compared with LMS_{ABTS} and control. The lowest lignin/cellulose ratio given by *P. cinnabarinus* LMS_{HBT} after Py-GC-MS analyses coincides with the strong brightness increase and the highest (and most selective) lignin removal showed by kappa number and viscosity data.

In the light of these results, it appears that the capability of the above enzymatic systems to degrade lignin in flax pulp fibers and to modify the composition of the residual polymer is proved. Moreover, a correlation between some pulp properties and pyrolysis data could be established, showing the potential of this technique for lignin analysis when small pulp samples are used in bleaching optimization studies. Furthermore, by sample permethylation

before Py-GC-MS analyses [11;12] more information on the structure of residual lignin could be obtained, similar to that provided by other techniques used for pulp analysis [13;14].

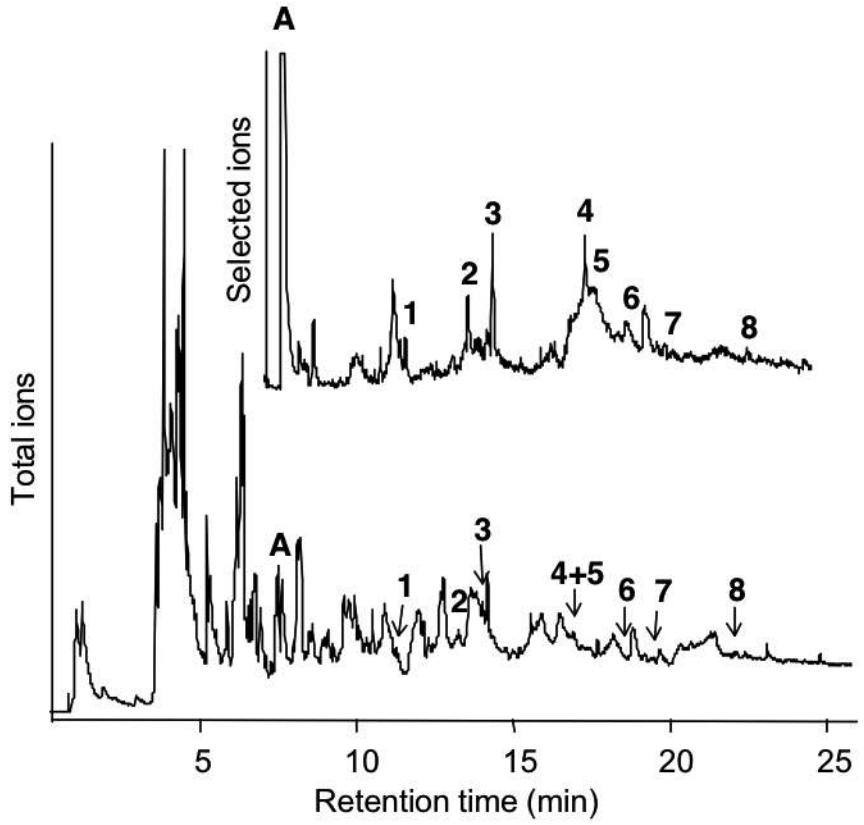

Figure 1. Total-ion Py-GC-MS chromatogram of initial flax pulp (11 kappa number) showing all carbohydrate and lignin-derived compounds released upon pyrolysis. Inset is the reconstructed ion-chromatogram for the selected marker for cellulose (A, 4-hydroxy-5,6-dihydro(2*H*)-pyran-2-one; *m/z* 114) compared with the following lignin markers: 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); and 8, *trans*-4-propenylsyringol (*m/z* 194) (1-4 as guaiacyl markers, and 5-8 as syringyl markers).

3.2. LEP bleaching sequence using laccase from *P. cinnabarinus* and HBT

Because of the results described above, *P. cinnabarinus* laccase was selected for further assays using HBT as mediator. Then, the LMS_{HBT} performance on flax pulp was followed by alkaline extraction and peroxide bleaching, and final values of 80% ISO brightness and kappa number around 1 were reached after the LEP sequence (Table 2). It is worth saying that the flax pulp used in this study is very resistant against bleaching in part because of the presence of core fibers in the raw material, and 80% ISO brightness is a value difficult to be reached in chemical TCF sequences. For this reason, the brightness obtained does not correspond with that expected from the very low kappa value attained (using other

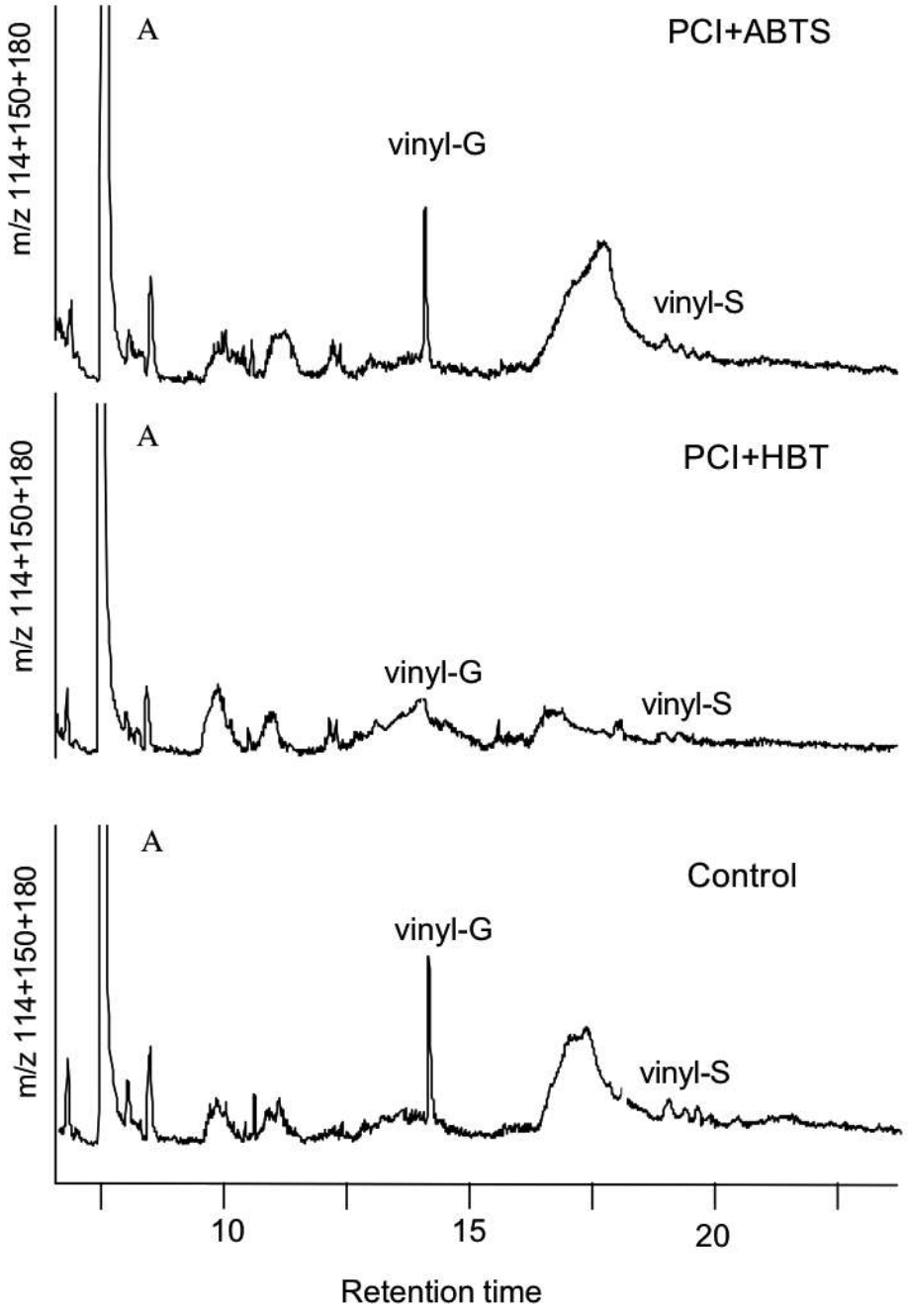

Figure 2. Py-GC-MS chromatograms of pulps treated with *Pycnoporus cinnabarinus* laccase in the presence of ABTS and HBT compared with the control. The sum of three single ions corresponding to the molecular ions of the cellulose marker (A, 4-hydroxy-5,6-dihydro(2*H*)-pyran-2-one, 114 mz), and two main lignin markers, 4-vinylguaiacol (*m/z* 150) and 4-vinylsyringol (*m/z* 180), is represented.

raw materials, e.g. hardwoods, up to 90%. ISO brightness would be attained for this kappa value [15]). The high delignification rate (89%) and the strong increase of brightness (from 36% to 81% ISO) obtained after LEP, showed that L-stage improves the poor capacity of hydrogen peroxide as delignifying agent, increasing also its bleaching properties. At the same time the P-stage significantly enhances bleaching results obtained after LE stages.

Table 2
Brightness, kappa number and viscosity of flax pulp treated with *P. cinnabarinus* laccase (20 U/g) in the presence of HBT followed by alkaline extraction (LE) and a peroxide bleaching stage (LEP)

	LE			LEP		
	Brightness (%)	Kappa number	Viscosity (mL/g)	Brightness (%)	Kappa number	Viscosity (mL/g)
Initial pulp	36.7	10.2	855	57.1	6.9	630
Control pulp	41.8	8.9	920	61.3	5.5	683
Laccase + HBT	59.4	2.8	613	80.6	1.3	470

Pyrolysis of the LMS_{HBT}-treated pulp before and after P-stage showed that pulp delignification was mainly due to laccase-mediator action since only a small increase of lignin removal was produced when a P-stage was added to the LE sequence (Table 3). The same could be said for modification of the lignin remaining in pulp. Py-GC-MS results evidenced that the main modification of residual lignin composition, as shown by S/G values, was due to the enzymatic treatment (Table 3). The S/G ratio decreased from 0.35 (control pulp) to 0.03 after LMS-treatment followed by alkaline extraction. Peroxide bleaching of the LE sample resulted in the completely disappearance of S units.

Table 3
Relative removal of lignin from Py-GC-MS analyses (with respect to lignin/cellulose ratio in the control pulp before peroxide) and changes of S/G ratio of flax pulp treated with *P. cinnabarinus* laccase (20 U/g) in the presence of HBT followed by alkaline extraction (LE) and a peroxide bleaching stage (LEP)

	LE		LEP	
	Lignin/Cellulose decrease (%)	S/G ratio	Lignin/Cellulose decrease (%)	S/G ratio
Control pulp	0	0.35	16	0.29
Laccase + HBT	85	0.03	92	0

The rate of lignin removal obtained by pyrolysis after LE sequence was very similar to delignification rate given by kappa number determination. Moreover, correlation between kappa values and pyrolysis data of the LMS_{HBT}-treated pulps before and after P bleaching was established with regression coefficient $R^2 = 0.90$ (Fig 3).

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treatment of flax pulp, are currently being developed. Therefore, pulp properties would be predicted as a function of the three main variables of the LMS treatment.

Table 4
Comparison of brightness, kappa number and viscosity of flax pulps treated with LMS_{HBT} after different post-LMS treatments: alkaline extraction (E), alkaline extraction followed by peroxide stage (EP), peroxide stage (P), pressurized peroxide stage (Po) and reductive treatment with $NaBH_4$ (R)

	Brightness (% ISO)	Kappa number	Viscosity (mL/g)
Initial pulp	37.3	10.11	1025
Initial control	38.0	13.7	1065
Initial LMS_{HBT}	41.6	9.5	835
Control E	39.1	9.4	1000
LMS_{HBT} E	49.3	5.0	780
Control EP	62.0	5.5	530
LMS_{HBT} EP	76.4	1.4	465
Control P	63.2	5.4	685
LMS_{HBT} P	79.8	1.6	725
Initial control R	42.5	9.1	1070
Initial LMS_{HBT} R	50.7	6.5	1010
Control Po	65.4	4.6	610
LMS_{HBT} Po	81.5	1.3	640

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