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## Bioresource Technology

journal homepage: [www.elsevier.com/locate/biortech](http://www.elsevier.com/locate/biortech)Towards industrially-feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediatorEsteban D. Babot<sup>a</sup>, Alejandro Rico<sup>a</sup>, Jorge Rencoret<sup>a</sup>, Lisbeth Kalum<sup>b</sup>, Henrik Lund<sup>b</sup>, Javier Romero<sup>c</sup>, José C. del Río<sup>a</sup>, Ángel T. Martínez<sup>d</sup>, Ana Gutiérrez<sup>a,\*</sup><sup>a</sup> Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, P.O. Box 1052, E-41080 Sevilla, Spain<sup>b</sup> Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark<sup>c</sup> ENCE, Marisma de Lourizán s/n, P.O. Box 157, E-36080 Pontevedra, Spain<sup>d</sup> Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

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## ABSTRACT

The ability of two natural phenols to act as mediators of the recombinant *Myceliophthora thermophila* laccase (MtL) in eucalypt-pulp delignification was investigated. After alkaline peroxide extraction, the properties of the enzymatically-treated pulps improved with respect to the control. The pulp brightness increased (3.1 points) after the enzymatic treatment with MtL alone, but the highest improvements were obtained after the MtL treatment using syringaldehyde (4.7 points) and especially methyl syringate (8.3 points) as mediators. Likewise, a decrease in kappa number up to 2.7 points was obtained after the MtL-methyl syringate treatment, followed by decreases of 1.4 and 0.9 points after the treatments with MtL-syringaldehyde and MtL alone, respectively. On the other hand, removal of the main lipophilic extractives present in eucalypt pulp was observed after the above laccase-mediator treatments. Finally, the doses of both MtL and methyl syringate were reduced, and results compatible with industrial implementation were obtained.

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## 1. Introduction

The paper pulp industry is facing an increasing pressure to replace the conventional pulp bleaching techniques, using chlorine-based chemicals, with more environmentally-benign ones. Enzymes offer an environmentally benign and efficient alternative to chemical reagents in many industrial applications, including pulp and paper manufacture (Kenealy and Jeffries, 2003). Lipases (pitch control) and xylanases (bleaching) were introduced in the mill several years ago (Gutiérrez et al., 2001a; Bajpai et al., 2006), yet lignin-degrading oxidoreductases offer additional benefits compared to xylanases for pulp bleaching because they act directly on lignin. Likewise, oxidoreductases also have a high potential for pitch control because they act over a wide range of lipophilic compounds by a recently described oxidative mechanism (Gutiérrez et al., 2009).

Laccases (EC 1.10.3.2) are multicopper oxidases that use molecular oxygen as the final electron acceptor and have been object of high interest for the development of environmentally-benign technologies (Mayer and Staples, 2002). The direct action

of laccases on lignin is in principle restricted to the phenolic units that only represent a small percentage of the total polymer, a fact that limits its biotechnological application for paper pulp delignification. However, the interest on laccases as industrial biocatalysts steadily increased after discovering the effect on laccase activity of some synthetic compounds, including 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Bourbonnais and Paice, 1990) and 1-hydroxybenzotriazole (HBT) (Call, 1994) acting as electron carriers between the enzyme and the final substrate. In this way, the action of laccase is expanded to non-phenolic substrates, which are oxidised by the mediator radical, increasing the potential of the enzyme in degradation of lignin and other aromatic compounds. Since then, a variety of studies have confirmed the potential of the so-called laccase-mediator system for bleaching different pulp types (Bourbonnais and Paice, 1996; Poppius-Levlin et al., 1999; Camarero et al., 2004; Moldes and Vidal, 2008; Fillat and Roncero, 2010). On the other hand, the modification of some lipophilic extractives by laccases has been suggested (Zhang et al., 2005), and recently it was reported the high efficiency of the laccase-mediator system for the removal of lipophilic extractives regardless the pulping process and the raw material used (Gutiérrez et al., 2006a,b; Molina et al., 2008; Valls et al., 2009) and a patent was filed on this application (Gutiérrez et al., 2006c).

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However, the cost, safety and environmental profile of some of the synthetic mediators included in most of the above studies make it difficult to implement laccase–mediator systems in pulp bleaching at industrial scale. Recently, several lignin-related natural phenols, which form stable radicals, have been investigated as laccase mediators for pulp bleaching (Camarero et al., 2007; Fillat et al., 2010) and removal of lipophilic extractives from pulp (Gutiérrez et al., 2007). The present paper provides the first evidence of the feasibility of using a commercial laccase, and a cheap natural phenol with a suitable redox potential (Kulys et al., 2002) as mediator, for removing lignin and lipids from paper pulp. The enzyme used is the thermostable laccase from the ascomycete *Myceliophthora thermophila*, which has been cloned, expressed in *Aspergillus oryzae*, biochemically characterized, and commercialized (Xu et al., 1996; Berka et al., 1997). The results presented make the laccase–mediator treatment economically feasible from an industrial point of view.

## 2. Methods

### 2.1. Pulp

*Eucalyptus globulus* unbleached kraft pulp, with a kappa number of 15.5 (~2.3% lignin content), brightness of 38.5% ISO, and intrinsic viscosity of 1187 mL g<sup>-1</sup> was obtained from the ENCE mill in Pontevedra (Spain) and used in enzyme treatment and control experiments.

### 2.2. Fungal laccase and mediators

A commercial fungal laccase from the ascomycete *M. thermophila*, provided by Novozymes (Bagsvaerd, Denmark) was used in this study. Laccase activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical ( $\epsilon_{436}$  29,300 M<sup>-1</sup> cm<sup>-1</sup>) in 0.1 M sodium acetate (pH 5) at 24 °C. The laccase activity of the enzyme preparation was 945 U/ml. One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per min. A fungal laccase preparation (45 U/ml) obtained from a laccase-hyperproducing strain of the basidiomycete *Pycnoporus cinnabarinus* that was provided by Beldem (Andenne, Belgium) was also used for comparative studies.

Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde; Cat.: S760-2, Lot.: S16872-326) from Sigma–Aldrich (Steinheim, Germany) and methyl syringate (methyl 4-hydroxy-3,5-dimethoxybenzoate; Cat.: A18054/L05417, Lot.: 10110113) from Alfa Aesar (Karlsruhe, Germany) were assayed as mediators.

### 2.3. Laccase–mediator treatments of eucalypt pulp

First, pulp treatments with *M. thermophila* laccase (MtL)–mediator were carried out in duplicate using 10 g (dry weight) of pulp at 3% consistency (w:w) in 50 mM sodium dihydrogen phosphate buffer (pH 6.5). Laccase loading was 20 U g<sup>-1</sup> pulp and concentration of mediators (syringaldehyde and methyl syringate) in the reaction was kept at 6.75 mM. The treatments were carried out in 500 mL flasks with O<sub>2</sub> bubbling, placed in a thermostatic shaker at 170 rev min<sup>-1</sup> and 50 °C, for 12 h. Finally, some enzymatic treatments (with MtL) were performed in 200 mL bioreactors (Labomat, Mathis) under conditions more similar to those of industrial application (including 10% pulp consistency and 4 bar oxygen pressure).

Pulp treatments were repeated in the presence of Tween 20 (Cat.: TW00200250, Lot.: 10926401) or Tween 80 (Cat.: TW00800250, Lot.: 10628801) from Scharlau (0.05% w:v) and with lower doses of laccase (from 20 U g<sup>-1</sup> to 1 U g<sup>-1</sup> pulp) and mediator (from 6.75 mM to 0.6 mM).

In a subsequent step, pulps at 5% consistency (w:w) were submitted to an alkaline peroxide extraction (Ep) using 3% (w:w) H<sub>2</sub>O<sub>2</sub> and 1.5% (w:w) NaOH, both referred to pulp dry weight, at 90 °C for 2 h. Controls including laccase without mediator were also performed.

Pulp treatments with *P. cinnabarinus* laccase were performed as described above for *M. thermophila* ones, but using 50 mM sodium tartrate (pH 4) as a buffer.

### 2.4. Pulp lipid extraction and chromatographic analysis

Treated pulps and controls were air dried and samples were Soxhlet-extracted with acetone for 8 h (Gutiérrez et al., 2001b). All extracts were evaporated to dryness and redissolved in chloroform for analysis of the lipophilic fraction by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS).

The GC analyses of lipids were performed in an Agilent 6890N Network GC system using a short fused-silica DB-5HT capillary column (5 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific, enabling simultaneous elution of the different lipid classes (Gutiérrez et al., 1998). The temperature program started at 100 °C with 1 min hold, then raised to 350 °C at 15 °C min<sup>-1</sup>, and held for 3 min. The injector and flame-ionization detector (FID) temperatures were set at 300 °C and 350 °C, respectively. Helium (5 mL min<sup>-1</sup>) was used as carrier gas, and the injection was performed in splitless mode. Peaks were quantified by area.

The GC–MS analyses were performed with a Varian 3800 chromatograph coupled to an ion-trap detector (Varian 4000) using a medium-length (12 m) capillary column of the same characteristics described above for GC using FID. The oven was heated from 120 °C (1 min) to 380 °C at 10 °C min<sup>-1</sup>, and held for 5 min. The transfer line was kept at 300 °C, the injector was programmed from 120 °C (0.1 min) to 380 °C at 200 °C min<sup>-1</sup> and held until the end of the analysis, and helium was used as carrier gas at a rate of 2 mL min<sup>-1</sup>. Compounds were identified by mass fragmentography, and by comparing their mass spectra with those of the Wiley and NIST libraries, and standards.

### 2.5. Pyrolysis–GC–MS analysis of pulp lignin

Pyrolysis of pulps (approximately 1 mg) after Soxhlet extraction with acetone, was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC–MS system equipped with a DB-5MS (Agilent J&W) fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 °C. The oven temperature was programmed from 40 °C (1 min) to 300 °C at 6 °C min<sup>-1</sup> (10 min). Helium was the carrier gas (1 mL min<sup>-1</sup>). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries.

For pulp lignin analysis in terms of its *p*-hydroxyphenyl: guaiacyl:syringyl (H:G:S) composition, single-ion pyrolysis–GCms (Py–GC–MS) traces were obtained using the molecular ions of the following selected markers (del Río et al., 2001): *m/z* 108 (4-methylphenol), 122 (4-ethylphenol), 120 (4-vinylphenol) and 134 (4-*t*-propenylphenol) for H-lignin units; *m/z* 138 (4-methylguaiacol), 152 (4-ethylguaiacol), 150 (4-vinylguaiacol) and 164 (4-*t*-propenylguaiacol) for G-lignin units; and *m/z* 168 (4-methylsyringol), 182 (4-ethylsyringol), 180 (4-vinylsyringol), and 194 (4-*t*-propenylsyringol) for S-lignin units. Phenol, guaiacol and syringol were not selected as lignin markers, to prevent interferences due to eventual formation of syringol from residual amounts of the S-type mediators used.

## 2.6. Papermaking evaluation of eucalypt pulps

Pulp brightness, kappa number (before and after acetone extraction, as described in Section 2.4) and intrinsic viscosity were analyzed following ISO 3688:1999, ISO 302:1981 and ISO 5351/1:1981 standard methods, respectively (International Organization for Standardization Documentation and Information (ISO), 2003). Data from replicates were averaged. In all cases the standard deviations were below 2% of the mean values.

## 3. Results and discussion

In this paper, the ability of recombinant MtL to delignify eucalypt kraft pulp, and simultaneously remove lipophilic extractives causing pitch deposits, in the presence of two simple S-type phenols of plant origin (syringaldehyde and methyl syringate) was tested. The commercially available MtL retains activity through a wide pH range (from pH 4 to pH 9) and has higher thermostability (being active up to 70 °C) than the different basidiomycete laccases investigated for biotechnological application (Xu et al., 1996). More importantly, MtL can be heterologously expressed in industrial hosts (such as *Aspergillus* species) with high yields (Berka et al., 1999), compared with the basidiomycete laccases whose heterologous expression results in low yields currently limiting their large-scale commercialization in pulp bleaching.

### 3.1. Pulp delignification with MtL and S-type mediators

Unbleached eucalypt kraft pulp was treated with MtL in the presence and absence of syringaldehyde and methyl syringate, and subsequently extracted with alkaline peroxide. The delignification degree was evaluated before and after the alkaline peroxide extraction, by determining the kappa number (an estimation of lignin content in pulp) and the ISO brightness. Intrinsic viscosity (an estimation of cellulose integrity) was also determined. The results obtained are shown in Table 1, compared with the control pulp.

The positive effects of the enzymatic treatments on pulp properties, especially on brightness, were only evidenced after the alkaline extraction, revealing the need of an alkaline peroxide stage after the treatment with laccase and natural mediators. In the absence of this extraction, the pulp brightness decreased with the enzymatic treatment. Likewise, the kappa number only decreased 0.4 and 1.1 points with the MtL alone and in the presence of methyl syringate, respectively, and increased 1.2 points with the MtL–syringaldehyde treatment (due to partial retention of the mediator on the non-extracted pulp). After the peroxide extraction, the properties of the treated pulps improved with respect to the control. The brightness of eucalypt pulp increased up to 3.1, 4.7 and 8.3 points after the treatments with MtL alone, MtL–syringaldehyde, and MtL–methyl syringate, respectively. On the other hand, a decrease in kappa number up to 2.7 points was obtained after the MtL–methyl syringate treatment followed by a decrease of 1.4 points in the MtL–syringaldehyde treatment and by 0.9 points decrease in the

treatment with MtL alone. The increased delignification (up to 25%) and brightness (up to 15%) obtained using methyl syringate as mediator are very promising results since they provide the first evidence on pulp properties improvement using a commercial laccase and a cost-effective natural mediator.

### 3.2. Comparison with other laccases and mediators

For comparative purposes, the (non-commercial) laccase from the basidiomycete *Pycnoporus cinnabarinus* (PcL) was also applied to eucalypt pulp in the presence of syringaldehyde and methyl syringate as mediators (data not shown). In spite of PcL being a high-redox potential laccase, less reactivity was shown towards lignin (compared with MtL) since worse results in terms of brightness increase (only 0.5 points) and kappa decrease (only 0.1 points) were obtained in the treatments with laccase alone. In the PcL treatments carried out in the presence of syringaldehyde and methyl syringate, similar increases in brightness (about 5 points) and decreases in kappa number (about 1 point) were obtained with both mediators. These results are inferior to those described above using the MtL and methyl syringate as mediator.

The above improvements of eucalypt pulp brightness are lower than reported using the synthetic mediator HBT in combination with high redox-potential laccases from basidiomycetes (Camarero et al., 2007), although the latter treatment has not been industrially implemented due to the high cost and environmental issues associated to this and other synthetic mediators. However, the low redox-potential laccase from the ascomycete *M. thermophila* can be heterologously expressed in *A. oryzae*, in contrast to the high redox-potential basidiomycete laccases. Therefore, MtL has the advantage of being a commercially-available cheap laccase, which will enable the up-scaling of the enzymatic treatment of pulp. Although low redox-potential laccases cannot oxidize mediators such as HBT (Ibarra et al., 2006), this is not a problem here since the mediators used (syringaldehyde and methyl syringate) are phenolic mediators of low redox-potential. These phenolic mediators can be easily obtained from natural sources and are also present in eucalypt pulping liquor (Gutiérrez et al., 2007). In addition, the decrease in pulp viscosity reported here (5%) is lower than that produced using HBT (18%) (Camarero et al., 2007).

### 3.3. Enzymatic modification of pulp lignin as shown by analytical pyrolysis

With the aim of investigating how the enzymatic treatment affects the composition of residual lignin, the lignin in the enzymatically-treated pulps was analyzed by pyrolysis coupled to GC–MS. Py–GC–MS allows for *in situ* analysis of lignin by chromatographic separation and mass-spectrometric identification of the compounds released after the pyrolytic breakdown of whole pulp. Unfortunately, the lignin content in chemical pulps is often too low for direct detection of these breakdown products. However, their relative abundances can be analyzed in single-ion chromatographic traces

**Table 1**

Pulp papermaking properties (kappa number, brightness, and viscosity) and lignin S/G molar ratio (from selected Py–GC–MS markers, see SD Fig. S1) of eucalypt pulp treated with *M. thermophila* laccase (MtL) in the absence and presence of syringaldehyde (SA) or methyl syringate (MS), and control without enzyme, before and after an alkaline peroxide extraction (Ep).

	Control		MtL		MtL–SA		MtL–MS	
	Before Ep	After Ep	Before Ep	After Ep	Before Ep	After Ep	Before Ep	After Ep
Kappa number	13.3	10.7	12.9	9.8	14.5	9.3	12.2	8.0
Brightness (% ISO)	43.5	57.0	41.5	60.1	35.6	61.7	36.5	65.3
Intrinsic viscosity (mL g <sup>-1</sup> )	1230	1030	1240	1020	1220	972	1240	978
S/G ratio	1.45	0.92	1.10	0.61	1.19	0.55	0.73	0.26

corresponding to selected marker compounds, as described by del Río et al. (2001).

Although the Py-GC-MS analyses were performed after pulp extraction with acetone (and kappa numbers only slightly varied before and after the extraction) the presence of residual syringaldehyde and methyl syringate was detected in chromatographic traces corresponding to their molecular ions ( $m/z$  182 and 212, respectively). With the aim of preventing an eventual interference of these compounds, syringol was excluded from the selected markers used to analyze pulp residual lignin, together with phenol and guaiacol (see Supplementary Data, SD, Fig. S1). From the traces obtained (that did not show H-type lignin markers), the relative lignin composition in the enzymatically-treated eucalypt pulps and controls were calculated, and shown in Table 1 as S/G molar ratios. A slight decrease in the S/G ratio was already observed after the enzymatic treatment with MtL alone, which was the largest after MtL treatment in the presence of methyl syringate followed by the alkaline peroxide stage, indicating a preferential removal of lignin S-units by this laccase-mediator system. This result is in agreement with a more linear structure of S lignin with a predominance of  $\beta$ -O-4' inter-unit linkages (Ibarra et al., 2007), while G lignin is more condensed (branched) due to additional linkages involving the C5 position at the benzenic ring (which is blocked by a second methoxy group in S-units) making it more resistant towards degradation.

### 3.4. Pitch removal with MtL and S-type mediators

The removal of lipophilic extractives by eucalypt pulp treatments with MtL and syringaldehyde and methyl syringate as mediators, was evaluated before and after the alkaline peroxide extraction by GC and GC-MS analyzes. The results obtained are shown in Table 2. The main lipophilic compounds present in eucalypt pulp (see SD, Fig. S2A) included sterols (predominantly sitosterol) in free (c) and esterified (j) form. Minor amounts of sterol glycosides (g) were also present. The detailed composition of lipophilic extractives from eucalypt pulp has been published elsewhere (Gutiérrez and del Río, 2001b). These compounds have been shown to be the main responsible for pitch problems during manufacturing of eucalypt pulp (Gutiérrez et al., 2009).

It was observed (Table 2) that the enzymatic treatment (without the subsequent alkaline peroxide extraction) using laccase alone was able to partially remove the free sterols (about 25%) and sterol esters (about 41%). When the laccase treatment was performed in the presence of syringaldehyde, a decrease of free sterols (up to 73%), sterol glycosides (up to 91%) and sterol ester (about 89%) contents, was produced (see SD, Fig. S2B). A similar removal of sterol esters (up to 92%) seems to be attained in the laccase treatments using methyl syringate as mediator (see SD, Fig. S2C), whereas a lower decrease of free sterols (up to 48%) was observed. A decrease on the sterol glycoside content was not observed with the latter mediator. Similar values of lipophilic extractives removal were obtained in the enzymatic treatments followed by the alkaline peroxide extraction (Table 2).

**Table 2**  
Removal (percentage of reduction) of the main lipophilic extractives (steroids) from eucalypt pulp after treatment with *M. thermophila* laccase (MtL) in the absence and presence of syringaldehyde (SA) or methyl syringate (MS) before and after an alkaline peroxide extraction (Ep).

	MtL		MtL-SA		MtL-MS	
	Before Ep	After Ep	Before Ep	After Ep	Before Ep	After Ep
Free sterols	25	21	73	69	48	40
Sterol glycosides	0	7	91	86	24	24
Sterol esters	41	41	89	82	92	80

Several oxidation products of steroids were observed in the pulps as a result of enzymatic treatment that were especially evident in the treatments with syringaldehyde (see SD, Fig. S2B). Some of them were already present in the control although in low amounts. Among them, the main compound identified was 7-ketositosterol (f) followed by 7 $\beta$ -hydroxysitosterol (d) with only traces of 7 $\alpha$ -hydroxysitosterol. Other products found in the treated samples were triols, such as sitostanetriol (e). By observing the oxysterol pattern, it could be inferred that oxidation would start with the abstraction of a reactive allylic hydrogen at C<sub>7</sub> followed by oxygen attack to form the peroxy radical. Hydrogen addition to this radical gives the 7-hydroperoxide, whose degradation forms the stable 7 $\alpha$ - and 7 $\beta$ -hydroxysitosterol and 7-ketositosterol (Johnsson, 2004). On the other hand, sitostanetriol may be formed after hydration of the epoxides, although the latter compounds were not detected in the pulp samples. Further oxidation of the above oxidation products leads to dehydration and subsequent abstractions of the hydroxy group on C<sub>3</sub>, which generates conjugated dienes and trienes such as stigmasta-3,5-diene (b) and stigmasta-3,5,7-triene (a), which increased in the enzymatically-treated pulps. Other oxidized derivatives, which appeared with the enzymatic treatment, included 7-ketositosteryl 3 $\beta$ -D-glucopyranoside (i) formed by the oxidation of sitosteryl 3 $\beta$ -D-glucopyranoside and the oxidation products of sterol esters tentatively assigned to sitosterol ester core aldehyde (n).

### 3.5. Effect of Tween (20 and 80) on enzymatic delignification and pitch removal

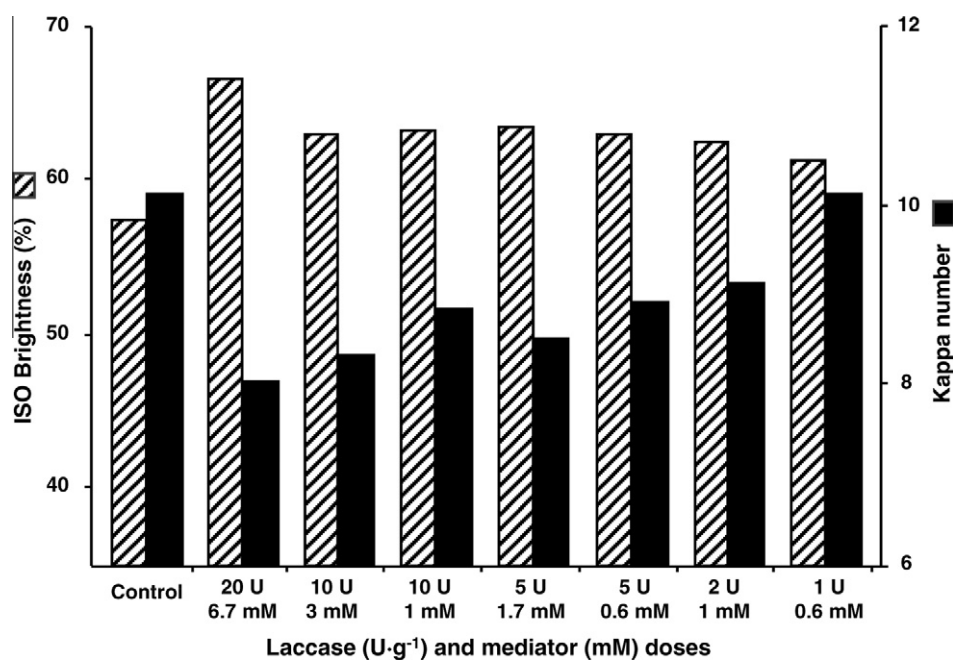
The above results on pulp delignification and pitch removal were obtained in laccase-mediator treatments without addition of any surfactant. Since most of the published studies on laccase-mediator treatment of paper pulps have been performed in the presence of Tween 80 (Moldes and Vidal, 2008; Fillat et al., 2010), the effect of surfactant addition was also studied here. Taking into account that Tween 80 (due to its content of unsaturated fatty acids) can promote peroxidation reactions (Jensen et al., 1996) in addition to its surfactant effect, enzymatic treatments in the presence of Tween 20 (which contains only saturated fatty acids) were also carried out. Table 3 shows the effect of the addition of Tween 20 and Tween 80 to the pulp treatments with laccase alone, as well as in the presence of syringaldehyde or methyl syringate, on the pulp properties. Concerning kappa number, no differences could be observed among the enzymatic treatments performed in the absence and presence of Tween. In contrast, lower brightness values were obtained, especially in the presence of Tween 80. Only the viscosity values show an improvement in the treatment with Tween 80.

The effect of adding Tween on the enzymatic removal of lipophilic extractives from eucalypt pulp was also evaluated. Generally, the removal of lipophilic extractives (data not shown) did not improve with respect to those without addition of Tween (Table 2). Only the removal of sterol glycosides was increased by the addition of Tween 20 and Tween 80 in the treatments with

**Table 3**

Effect of the addition of Tween 20 (T20) or Tween 80 (T80) on the modification of eucalypt pulp properties (kappa number, ISO brightness and intrinsic viscosity) by *M. thermophila* laccase (MtL) treatment in the absence and presence of syringaldehyde (SA) or methyl syringate (MS), and control without Tween (Con), followed by an alkaline peroxide stage.

	MtL			MtL-SA			MtL-MS		
	Con	T20	T80	Con	T20	T80	Con	T20	T80
Kappa number decrease	0.9	0.8	0.4	1.4	1.4	0.8	2.7	2.4	2
Brightness increase	3.1	2.8	1.1	4.7	4.3	2	8.3	5.9	5
Viscosity decrease	8	13	-38	59	55	-1	53	50	11



**Fig. 1.** Brightness and kappa number of eucalypt pulp after treatment with different doses of *M. thermophila* laccase (from 1 U g<sup>-1</sup> to 20 U g<sup>-1</sup>) and methyl syringate (from 0.6 mM to 6.7 mM) followed by an alkaline peroxide extraction, compared with control (without both laccase and mediator).

MtL alone (about 40% and 50% degradation, respectively) and in the presence of methyl syringate (about 50% and 45%, respectively). No differences are observed between the addition of Tween 20 and Tween 80 revealing that only the surfactant effect seems to play a role in this removal.

### 3.6. Laccase and mediator doses for pulp delignification and pitch removal

Taking into account that the main obstacles that prevent the use of laccase–mediator systems in the pulp mill are the high costs arisen from the high doses of enzymes and mediators usually reported, therefore, lower enzyme and mediator (methyl syringate) doses have been evaluated here. Laccase dosages between 1 U g<sup>-1</sup> and 20 U g<sup>-1</sup> of pulp together with mediator doses from 0.6 mM to 6.7 mM were tested. Fig. 1 shows the brightness and kappa values (after the alkaline peroxide extraction) obtained using the different doses of laccase and mediator. It is noteworthy that no substantial differences were observed among the treatments using 10 U g<sup>-1</sup>, 5 U g<sup>-1</sup> or 2 U g<sup>-1</sup> of laccase. Likewise, similar brightness results were obtained with methyl syringate doses from 0.6 mM to 3 mM. The ability of methyl syringate to mediate the oxidation of non-phenolic lignin model compound at low mediator concentration levels has recently been reported (Nousiainen et al., 2009). Although the best brightness and kappa number results were obtained with the highest doses of laccase and mediator, promising results were observed with very low

doses of laccase and mediator. Interestingly, when the pulp treatments were performed under oxygen pressure (Labomat experiments under conditions more similar to those of industrial application) the treatment duration could be reduced over 50% to attain similar kappa and brightness improvements, even with the lowest enzyme and mediator doses.

The removal of lipophilic extractives from these pulp samples was also evaluated (data not shown). Generally, no differences in the removal of the sterol esters and free sterols were observed among the different treatments and even better results in terms of sterol glycosides removal (54–74% removal) were attained in treatments with low doses of enzyme (1–2 U g<sup>-1</sup>) and mediator (0.6–1.0 mM).

The combination of substantial bleaching benefits and effective control of pulp extractives giving less down-time may provide sufficient advantages for the enzymatic process to be a cost-effective alternative to current bleaching and extractive control programs.

## 4. Conclusions

In the work reported here, which includes the use of commercial MtL in the presence of lignin-derived phenolic mediators, an important increase of eucalypt pulp brightness and decrease of kappa number were attained using methyl syringate as mediator, together with high removal of pitch-forming lipophilic extractives. Moreover, by careful selection of the laccase–mediator couple, low enzyme–mediator doses are able to catalyze pulp delignification

and lipid removal thus paving the way for an industrially-feasible cost-effective bleaching stage. This optimized enzymatic stage has potential for substituting oxygen delignification to achieve more sustainable TCF sequences, as suggested by pilot-scale trials currently in course.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.03.100.

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**Supplementary data 1.** Fig. S1. Single-ion chromatographic traces of markers from Py–GC/MS of paper pulp (control, before alkaline peroxide extraction) used to estimate the lignin S/G ratio. G-lignin markers (A) included: 4-Methylguaiacol ( $m/z$  138), 4-ethylguaiacol ( $m/z$  152), 4-vinylguaiacol ( $m/z$  150) and 4-*t*-propenylguaiacol ( $m/z$  164). S-lignin markers (B) included: 4-Methylsyringol ( $m/z$  168), 4-ethylsyringol ( $m/z$  182), 4-vinylsyringol ( $m/z$  180) and 4-*t*-propenylsyringol ( $m/z$  194). Same vertical scale was used in A and B chromatographic traces. Fig. S2. GC–MS analysis (total-ion chromatograms) and chemical structures of the main lipophilic compounds identified in eucalypt kraft pulp (without alkaline peroxide extraction) before (A) and after treatment with laccase *M. thermophila* using syringaldehyde (B) and methyl syringate (C) as mediators. Peak identification: (a) stigmasta-3,5,7-triene; (b) stigmasta-3,5-diene; (c) sitosterol; (d) 7-hydroxysitosterol; (e) sitostanetriol; (f) 7-ketositosterol; (g) sitosteryl 3 $\beta$ -d-glucopyranoside; (h) sitosteryl 9-oxononanoate; (i) 7-ketositosteryl 3 $\beta$ -d-glucopyranoside; and (j) sitosterol esters. Same vertical scale was used in the three chromatograms.

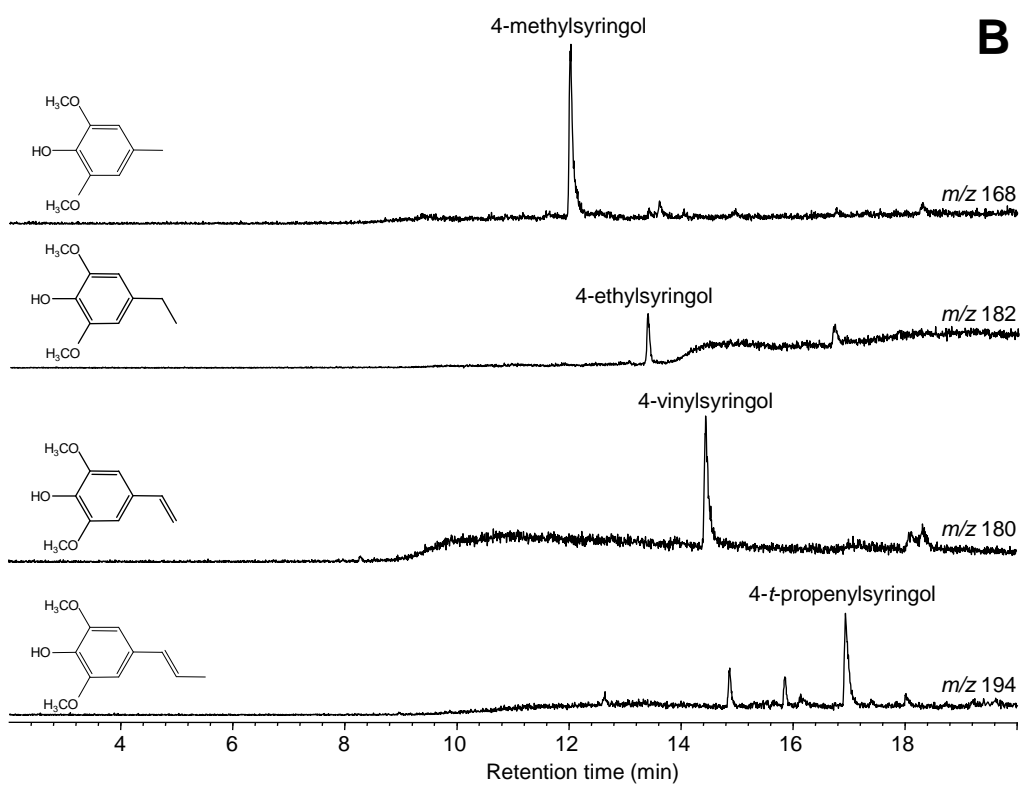
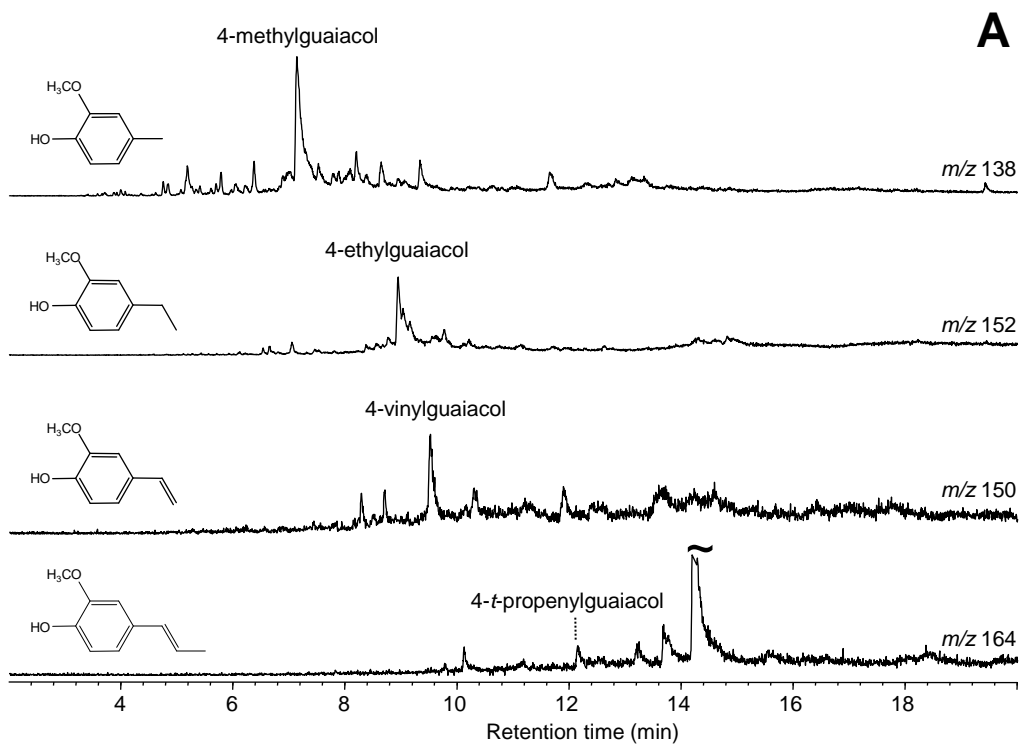


Figure S1



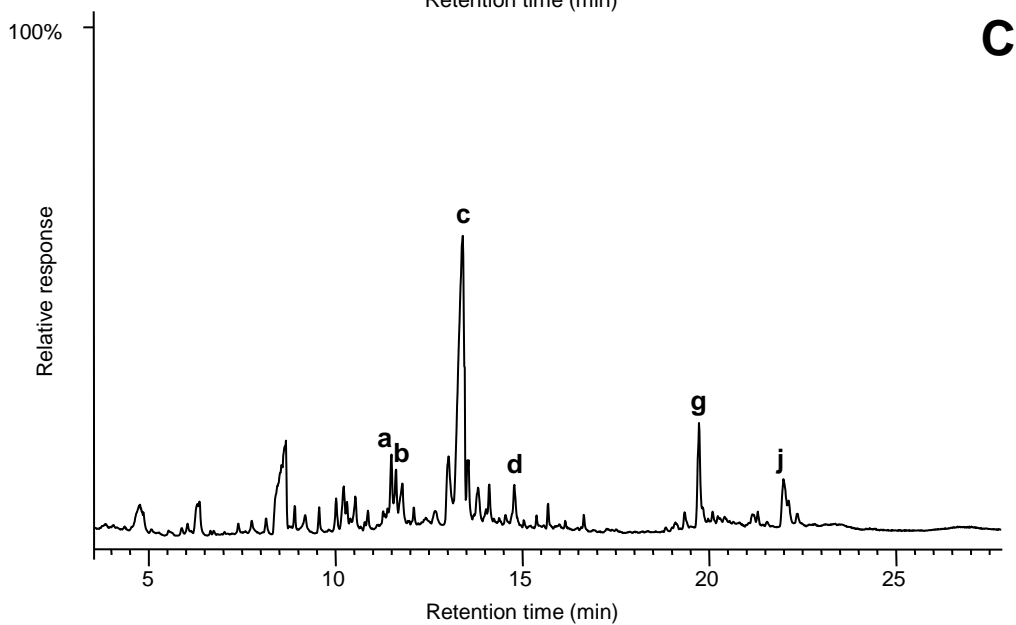
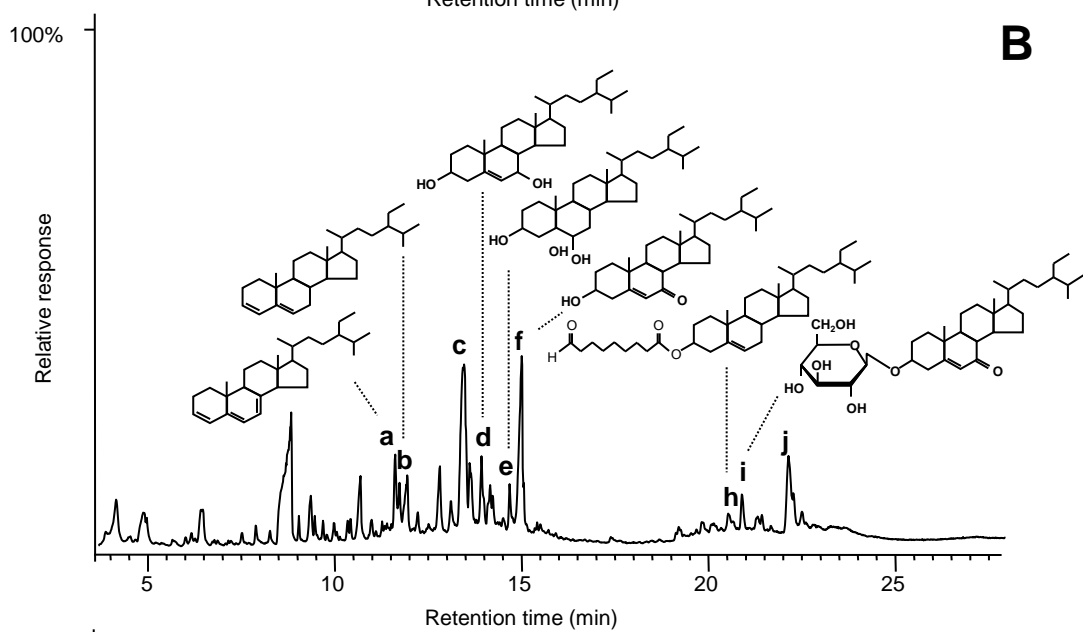
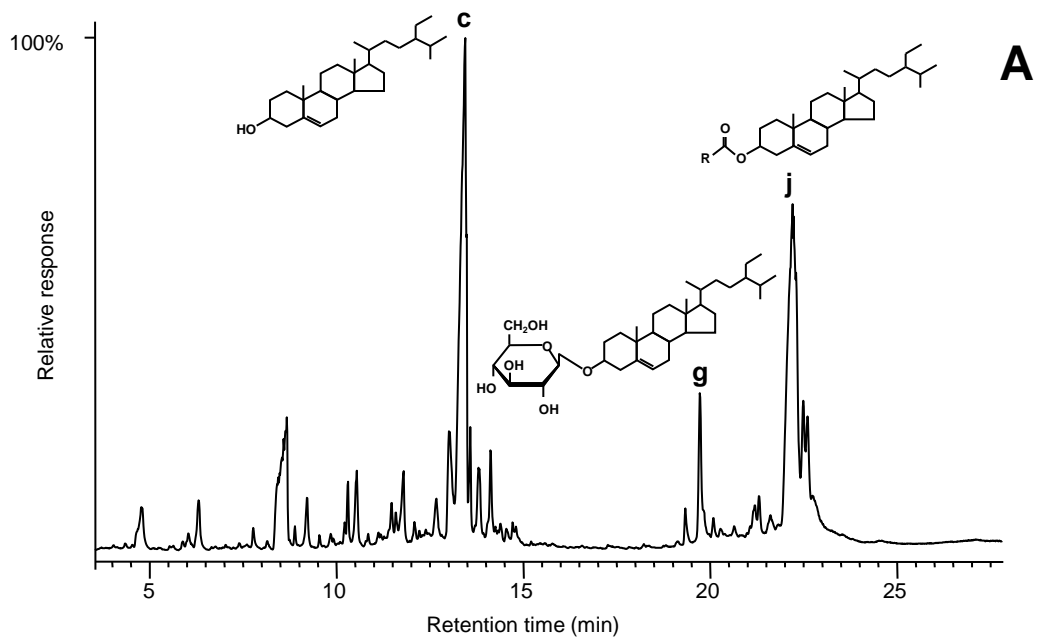


Figure S2