# OPTIMIZING DELIGNIFICATION AND PITCH REMOVAL BY TREATING EUCALYPT PULP WITH *Myceliophthora* LACCASE AND A PHENOLIC MEDIATOR

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## ABSTRACT

This work shows the ability of two natural phenols, namely syringaldehyde and methyl syringate, to act as mediators of recombinant *Myceliophthora thermophila* laccase (MtL) in eucalypt-pulp delignification. After alkaline peroxide extraction, the properties of the enzymatically-treated pulps improved with respect to the control. Likewise, removal of the main lipophilic extractives present in eucalypt pulp, such as free and conjugated sterols, was observed after the above laccase-mediator treatments. In the same study, the doses of both MtL and methyl syringate were reduced, and results compatible with industrial implementation were obtained, taking also advantage from the high-yield production of the recombinant enzyme that enables its commercial availability.

## I. INTRODUCTION

Lipases (pitch control) and xylanases (bleaching) were introduced in the paper pulp mill several years ago, yet lignin-degrading oxidoreductases offer additional benefits compared to xylanases for pulp bleaching because they act directly on lignin, and also have a high potential for pitch control because they act over a wide range of lipophilic compounds (Gutiérrez et al. 2009). Laccases (phenoloxidases, EC 1.10.3.2) use molecular oxygen as the final electron acceptor and have been object of high interest for the development of environmentally-benign technologies. The direct action of laccases on lignin is in principle restricted to the phenolic units, a fact that limits its biotechnological application for pulp delignification. However, the interest on laccases as industrial biocatalysts steadily increased after discovering the effect on laccase of some synthetic compounds 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 oxidized by the mediator radical, increasing the potential of laccases in lignin degradation. Since then, a variety of studies have confirmed the potential of the so-called laccase-mediator system for bleaching different pulp types and removing lipophilic extractives (Gutiérrez et al. 2009). However, the cost of the synthetic mediators 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 and removal of lipophilic extractives from pulp (Camarero et al. 2007; Gutiérrez et al. 2007). The present paper provides evidence of the feasibility of using a commercial laccase, and a cheap natural phenol as mediator, for removing lignin and lipids from pulp. The results presented make the laccase-mediator treatment economically feasible from an industrial point of view.

## II. EXPERIMENTAL

*Pulp. Eucalyptus globulus* unbleached kraft pulp, with a kappa number of 15.5, brightness of 38.5% ISO, and intrinsic viscosity of 1187 $mL \cdot g^{-1}$ was obtained from the ENCE mill in Pontevedra (Spain) and used in enzyme treatment and control experiments.

*Fungal laccase and mediators.* A commercial fungal laccase from the ascomycete *Myceliophthora thermophila* (MtL) provided by Novozymes (Bagsvaerd, Denmark) was used. Laccase activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical ($\varepsilon_{436}$ 29300 $M^{-1} \cdot cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24ºC. 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. Syringaldehyde and methyl syringate were assayed as mediators.

*Laccase-mediator treatments of eucalypt pulp.* Pulp treatments with 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 $U \cdot g^{-1}$ pulp and concentration of mediators in the reaction was kept at 6.75 mM. The treatments were carried out in 500-mL flasks with $O_2$ bubbling, placed in a thermostatic shaker at 170 $rev \cdot min^{-1}$ and 50 ºC, for 12 h. Some enzymatic treatments 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). In a subsequent step, pulps at 5% consistency (w:w) were submitted to an alkaline peroxide extraction (Ep) using 3% (w:w) $H_2O_2$ and 1.5% (w:w) NaOH, both referred to pulp dry weight, at 90 ºC for 2 h. Controls including laccase without mediator were also performed.

*Pulp lipid extraction and chromatographic analysis.* Treated pulps and controls were air dried and samples were Soxhlet-extracted with acetone for 8 h. 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) as previously described (Gutiérrez et al. 1998).

*Papermaking evaluation of eucalypt pulp.* Pulp brightness, kappa number (before and after acetone extraction) and intrinsic viscosity were analyzed following ISO 3688:1999, ISO 302:1981 and ISO 5351/1:1981 standard methods, respectively (International Organisation 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.

## III. RESULTS AND DISCUSSION

The ability of recombinant MtL to delignify eucalypt kraft pulp, and simultaneously remove lipophilic extractives (causing pitch deposits), in the presence of two simple syringyl (S)-type phenols (syringaldehyde and methyl syringate) was tested. The commercially available MtL retains activity through a wide pH range and has higher thermostability than the different basidiomycete laccases investigated for biotechnological application (Xu et al., 1996). More importantly, MtL can be heterologously expressed in industrial hosts 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.

### *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 an alkaline peroxide extraction, by determining the kappa number and the ISO brightness. Intrinsic viscosity 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. After the peroxide extraction, the properties of the treated pulps improved with respect to the control. 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.

Table 1. Pulp papermaking properties, kappa number, brightness (% ISO), and viscosity ($mL \cdot g^{-1}$) of eucalypt pulp treated with MtL in the absence and presence of syringaldehyde (SA) or methyl syringate (MS), and control without enzyme, before (initial) and after an alkaline peroxide extraction (Ep) (adapted from Babot et al. 2011).

| | 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 | 43.5 | 57.0 | 41.5 | 60.1 | 35.6 | 61.7 | 36.5 | 65.3 |
| Intrinsic viscosity | 1230 | 1030 | 1240 | 1020 | 1220 | 972 | 1240 | 978 |

Table 2. Removal (percentage of reduction) of the main lipophilic extractives (sterols) from eucalypt pulp after treatment with MtL in the absence and presence of syringaldehyde (SA) or methyl syringate (MS) before and after an alkaline peroxide extraction (Ep) (adapted from Babot et al. 2011).

| | 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 |

*Pitch removal with MtL and S-type mediators*

The main lipophilic compounds present in eucalypt pulp include sterols (predominantly sitosterol) in free and esterified form, and minor amounts of sterol glycosides. These compounds have been shown to be the main responsible for pitch problems during manufacturing of eucalypt pulp (Gutiérrez et al. 2009). The removal of lipophilic extractives by eucalypt pulp treatments with MtL and syringadehyde and methyl syringate as mediators, was evaluated before and after the alkaline peroxide extraction by GC and GC-MS analyses. The results obtained are shown in Table 2.

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. Some of them were already present in the control although in low amounts. Among them, the main compound identified was 7-ketositosterol followed by 7β-hydroxysitosterol with only traces of 7α-hydroxysitosterol. Other products found in the treated samples were triols, such as sitostanetriol. Further oxidation of the above oxidation products leads to dehydration and subsequent abstractions of the hydroxy group on $C_3$, which generates conjugated dienes and trienes such as stigmasta-3,5-diene and stigmasta-3,5,7-triene, which increased in the enzymatically treated pulps. Other oxidized derivatives, which appeared with the enzymatic treatment, included 7-ketositosteryl 3β-D-glucopyranoside formed by the oxidation of sitosteryl 3β-D-glucopyranoside and the oxidation products of sterol esters tentatively assigned to sitosterol ester core aldehyde.

*Optimization of 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 and 20 U per gram of pulp together with mediator doses from 0.6 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.

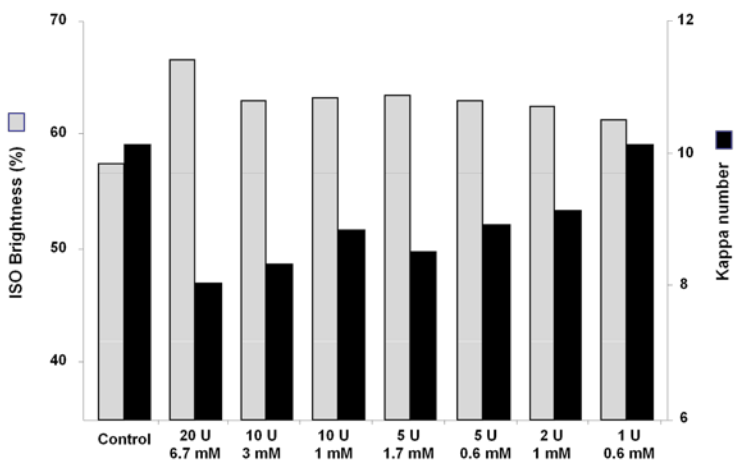


Figure 1. Brightness and kappa number of eucalypt pulp after treatment with different MtL doses (from 1 to 20 $U \cdot g^{-1}$) and methyl syringate (from 0.6 mM to 6.7 mM) followed by an alkaline peroxide extraction, compared with control (without both laccase and mediator) (adapted from Babot et al. 2011)

It is noteworthy that no differences were observed among the treatments using 10, 5 or 2 $U \cdot g^{-1}$ of laccase. Likewise, similar brightness results were obtained with methyl syringate doses from 0.6 to 3 mM. 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 $U \cdot g^{-1}$) and mediator (0.6-1.0 mM).

## IV. 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.

## V. ACKNOWLEDGEMENT

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