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Comparing the efficiency of the laccase–NHA and laccase–HBT systems in eucalyptus pulp bleaching

Cristina Valls[a], José F. Colom[a], Carole Baffert[c], Isabelle Gimbert[b], M. Blanca Roncero[a,*], Jean-Claude Sigoillot[b]

[a] Textile and Paper Engineering Department, ETSEIAT, Universitat Politècnica de Catalunya, Colom 11, E-08222 Terrassa, Spain
[b] Laboratoire de Biotechnologie des Champignons Filamenteux, INRA, Avenue de Luminy, Case Postale 925, 13288 Marseille Cedex 09, France
[c] Laboratoire de Bioénergétique et Ingénierie des Protéines, BIP-CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex, France

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ABSTRACT

Laccase–mediator systems have the disadvantage that the mediator is expensive and potentially toxic. In this work, we used N-hydroxyacetanilide (NHA) in combination with laccase for the first time to bleach eucalypt pulp and found it to be a very promising, advantageous alternative to 1-hydroxybenzotriazole (HBT) as mediator. Thus, NHA is efficiently oxidized by laccase to a radical that absorbs light at 350 nm. Also, NHA is a better substrate for laccase than is HBT. An innovative result is that the enzyme is inactivated to a similar extent by both mediators under the typical treatment conditions of the bleaching step (L). This adverse effect, however, is strongly reduced in the presence of pulp. Moreover, the laccase–NHA system is as efficient as the laccase–HBT system in reducing the kappa number of eucalyptus pulp. Using a xylanase pretreatment or unbleached pulp boosts kappa number reduction and bleaching with the laccase–mediator system. Based on the results of cyclic voltammetry tests, NHA has a slightly lower redox potential than HBT, which further supports use of the former; also, unlike HBT, NHA is oxidized in a reversible, pH-dependent manner. Interestingly, the laccase–NHA system provides more efficient bleaching of eucalyptus pulp at pH 5 than it does at pH 4.

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1. Introduction

Biotechnology has aroused much interest and provided very promising results in the bleaching of paper pulp in recent years. The use of enzymes such as xylanases for this purpose constitutes a very important technological improvement since it boosts the bleaching effect of chemical agents, thereby affording substantial savings and, more important, considerably reducing the production of contaminants during the bleaching process [1–3]. The use of laccases in the form of laccase–mediator systems is an advantageous alternative to that of xylanases by virtue of the former acting directly on pulp lignin. Xylanases are simple and economic to use on an industrial scale; by contrast, laccases require some fine-tuning for this purpose and have been the subject of several pilot scale trials towards this goal.

Laccase–mediator systems facilitate the development of totally chlorine free (TCF) bleaching sequences and the replacement of ozone and oxygen based delignification steps, thereby providing substantial savings in reagents, and reductions in kappa number and sterol contents [4–8]. The mediator is usually a compound of low molecular weight capable of diffusing into cellulose fibres.

The efficiency of a laccase–mediator system in degrading lignin depends on the properties of the laccase, the mediator and their combination [9,10]. The radical formed by oxidation of mediators containing an N-hydroxyl function by laccase, NO•, is responsible for the high rates of lignin oxidation obtained with their combinations [11]. By virtue of its nature, however, the radical causes stability problems and restricts the use of these systems to catalytic delignification. 1-Hydroxybenzotriazole (HBT) is known to degrade in a rapid manner, and also to attack laccase and diminish its enzyme activity as a result in some cases [12–14]. The extent to which laccase is inactivated by the mediator oxidized form depends on the particular mediator [9].

One other shortcoming of laccase–mediator systems is the high cost, limited biodegradability and potential toxicity of the mediator. This has fostered a search for new, less expensive and environmentally aggressive mediators. 2,2′-Azino-*bis*(3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS) was the first mediator to prove efficient in delignifying pulp in combination with laccase [15]. A number of additional mediators have so far been studied the most efficient for pulp delignification among which are

* Corresponding author. Tel.: +34 937398210; fax: +34 937398101.
E-mail address: roncero@etp.upc.edu (M.B. Roncero).

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seemingly those containing an N–OH functional group [16], and, more specifically, HBT [10,17–19].

N-hydroxyacetanilide (NHA) is a recently synthesized, more affordable mediator than HBT. Like HBT, NHA contains an N-hydroxyl function (N–OH) that has proved effective towards delignifying some types of pulp [20–26]. However, it has never to date been applied to eucalyptus pulp, and nor has its effect on laccase activity under the typical operating conditions of pulp bleaching been examined.

Cyclic voltammetry has been used by several authors to assess the performance of various mediators for laccase [16,23,27,28]. The redox potential of the mediator has a strong influence on its efficiency [11] by effect of its oxidation rate depending on the difference between the redox potential of the N–OH substrate and the laccase. Thus, an N–OH substrate with a low redox potential will have an increased oxidation rate [16].

In this work, we assessed the ability of the laccase–NHA system to delignify and bleach eucalyptus kraft pulp, and compared it with that of the laccase–HBT system. To this end, we compared the kinetic constants of the laccase–NHA system with those of the laccase combinations with HBT and ABTS, as well as the enzyme stability against NHA and HBT. Also, we examined the effects of NHA and HBT on the delignification and bleaching of eucalyptus pulp, and analysed both mediators by cyclic voltammetry.

2. Experimental

2.1. Raw material

Two types of *Eucalyptus globulus* kraft pulp were used, namely: brown (unbleached) pulp (B) produced at the ENCE mill in Pontevedra (Spain) (11.8 ± 0.0 kappa number, 41.3% ISO brightness) and oxygen delignified pulp (O) produced at the Torraspapel S.A. mill in Zaragoza (Spain) (7.7 ± 0.1 kappa number, 51.3% ISO brightness).

2.2. Enzymes and mediators

The laccase used was from *Pycnoporus cinnabarinus* [27] and obtained by INRA (Marseille, France) from the monokaryotic hyper producing strain ss3, with an activity of 1.95 U mg^{-1}. Laccase activity was determined by monitoring the oxidation of 5 mM ABTS buffered with 50 mM sodium tartrate at pH 4 at 30 °C for 30 s. Formation of the ABTS cation radical was monitored at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount transforming 1 μmol of substrate per minute.

Feruloyl esterase and xylanase were used to pretreat the samples for delignification. The former was produced by INRA (Marseille, France) from a recombinant strain of *Aspergillus niger* [29] (activity, 38.76 U mL^{-1}, one unit being the amount of enzyme catalysing the release of 1 μmol of feruloyl acid per minute). Xylanase (Pulpzyme HC) was supplied by NOVOZYMES® (activity, 332 U mL^{-1}, one unit being the amount of enzyme releasing 1 μmol of reducing sugar, measured as xylose equivalents, per minute at pH 5 at 50 °C).

The mediators ABTS, HBT and NHA were all synthetic and supplied by Sigma–Aldrich. On the other hand, NHA was prepared by the authors according to Oxley et al. [30].

2.3. Kinetic parameters for the reactions of laccase with the mediators

The optimum wavelength for detecting NHA radical was identified by examining the absorbance spectra over the range 250–450 nm for laccase alone, NHA alone and the two in combination—after 0, 5 and 15 min reaction in the last case. Quartz tubes, 0.1 M sodium tartrate buffer at pH 4, and a laccase and NHA concentration of 0.6 U mL^{-1} and 16.5 mM, respectively, were used for this purpose.

The kinetic constants of the reactions of *P. cinnabarinus ss3* laccase with the mediators ABTS, HBT and NHA were determined by monitoring the formation of their respective radicals. The ABTS radical was monitored at 420 nm (A_{420}, ε_{420} = 36 mM^{-1} cm^{-1} [31]); and the HBT radical at 408 nm (A_{408}) [32], its molar extinction coefficient (ε_{408}) being calculated by applying the Beer–Lambert law upon complete oxidation of the substrate by the enzyme. The maximum absorbance of the NHA radical (A_{NHA}) and its $\varepsilon_{\lambda NHA}$ value were also determined. The laccase concentration used was 3.7×10^{-4} μM with ABTS, and 3.7×10^{-2} μM with both HBT and NHA. The mediator concentration ranged from 0.01 to 0.1 mM for ABTS, 1 to 14.7 mM for HBT and 0.5 to 16.5 mM for NHA. The reaction time was 1 min for ABTS and 15 min for both HBT and NHA. The Michaelis constant (K_m) and the maximum rate (V_{max}) were calculated from a Lineweaver–Burk plot and used to determine the catalytic constant (K_{cat}) and catalytic efficiency (E), using Eqs. (1)–(3); the laccase specific activity (A_s) was 234 U mg^{-1} and the molecular weight (MW) of the enzyme 70 000 mg mmol^{-1}. Methacrylate tubes and sodium tartrate buffer at pH 4 at 30 °C were used in all tests. Spectra were recorded on a PerkinElmer UV/Vis Lambda 12 instrument.

$$K_{cat} = \frac{V_{max}}{[\text{laccase}]_{mM}} \quad (1)$$

$$E = \frac{K_{cat}}{K_m} \quad (2)$$

$$[\text{laccase}]_{mM} = [\text{laccase}]_{U l^{-1}} \times \frac{1}{A_s \text{ laccase (U mg}^{-1})} \times \frac{1}{\text{MW laccase (mg mmol}^{-1})} \quad (3)$$

2.4. Stability of laccase under the treatment conditions

Laccase activity in the presence and absence of HBT and NHA as mediators on the one hand, and pulp on the other, was monitored over a period of 4 h. Treatments were carried out in 250 mL Erlenmeyer flasks at 50 °C, using sodium tartrate buffer at pH 4 and agitation at 190 rpm. A sample aliquot was withdrawn from the reaction mixture at hourly intervals to measure laccase activity. Six different treatments were performed, using (1) laccase alone, (2) laccase with HBT, (3) laccase with NHA, (4) laccase with pulp, (5) laccase with pulp and HBT, and (6) laccase with pulp and NHA. The laccase and mediator concentrations used were chosen in accordance with current recommendations [21,33]. Thus, the laccase concentration was 1 U mL^{-1} (or 20 U g^{-1} odp, oven-dried pulp), and the mediator concentration 9.9 mM (3% odp) for HBT and 3.3 mM (1% odp) for NHA. The treatments conducted in the presence of pulp involved the use of 5 g odp of brown pulp (B) at 5% consistency. The oxidation of HBT and NHA by laccase was much slower than that of ABTS; also, the former two required substantially higher substrate concentrations to saturate the enzyme, consistent with the respective K_m values [9]. For this reason, the presence of HBT or NHA should not interfere with the measurement of laccase activity in the presence of ABTS as substrate.

The evolution of the radicals was monitored via A_{408} for HBT and the previously calculated $A_{\lambda NHA}$ values for NHA.

2.5. Enzyme treatment of eucalypt pulp

The laccase–NHA and laccase–HBT treatments were compared in terms of efficiency in reducing kappa number and facilitating

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