



Delignification of eucalypt kraft pulp with manganese-substituted polyoxometalate assisted by fungal versatile peroxidase

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ABSTRACT

Oxidation of the manganese-substituted polyoxometalate $[\text{SiW}_{11}\text{Mn}^{\text{II}}(\text{H}_2\text{O})\text{O}_{39}]^{6-}$ ($\text{SiW}_{11}\text{Mn}^{\text{II}}$) to $[\text{SiW}_{11}\text{Mn}^{\text{III}}(\text{H}_2\text{O})\text{O}_{39}]^{5-}$ ($\text{SiW}_{11}\text{Mn}^{\text{III}}$), one of the most selective polyoxometalates for the kraft pulp delignification, by versatile peroxidase (VP) was studied. First, $\text{SiW}_{11}\text{Mn}^{\text{II}}$ was demonstrated to be quickly oxidized by VP at room temperature in the presence of H_2O_2 ($K_m = 6.4 \pm 0.7$ mM and $k_{\text{cat}} = 47 \pm 2$ s⁻¹). Second, the filtrate from eucalypt pulp delignification containing reduced polyoxometalate was treated with VP/ H_2O_2 , and 95–100% reoxidation was attained. In this way, it was possible to reuse the liquor from a first $\text{SiW}_{11}\text{Mn}^{\text{III}}$ stage for further delignification, in a sequence constituted by two polyoxometalate stages, and a short intermediate step consisting of the addition of VP/ H_2O_2 to the filtrate for $\text{SiW}_{11}\text{Mn}^{\text{II}}$ reoxidation. When the first ClO_2 stage of a conventional bleaching sequence was substituted by the two-stage delignification with polyoxometalate (assisted by VP) a 50% saving in ClO_2 was obtained for similar mechanical strength of the final pulp.

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1. Introduction

The residual lignin remaining after wood pulping is the target of the bleaching process to produce high quality pulp for the paper-making. This plant polymer, which is responsible for the undesirable dark color and photoyellowing of pulp, must be selectively removed without destroying the polysaccharides to preserve the physical properties of the bleached pulp.

In the middle of the 1990's, polyoxometalates (POM) were proposed as an environmentally-friendly alternative to the chlorine-based bleaching reagents, as well as to conventional alkaline oxygen pre-bleaching (Evtuguin and Neto, 1997; Weinstock et al., 1997). POM have been evaluated for the bleaching/delignification of pulps both as reagents under anaerobic conditions (in this case a second stage is required for POM reoxidation and reuse) or as catalysts under aerobic conditions (Gamelas et al., 2008; Gaspar et al., 2007; Weinstock et al., 1997). Applied as catalysts, POM oxidizes the residual lignin in pulp, and the reduced form of POM is reoxidized by molecular oxygen in the same stage. Therefore, it is possible to reuse the POM solutions in a closed system. The thermodynamic conditions required for lignin oxidation and reoxidation of the POM are related to the corresponding redox

potentials as follows: $E(\text{Lignin}) < E(\text{POM}) < E(\text{O}_2) = 1.22 - 0.059$ pH.

Several POM types, mostly with the Keggin-type structure (Fig. 1), have been considered for kraft pulp delignification, such as $[\text{SiW}_{11}\text{VO}_{40}]^{5-}$, $[\text{SiW}_{10}\text{V}_2\text{O}_{40}]^{6-}$, “ $\text{SiW}_{10.1}\text{Mo}_{1.0}\text{V}_{0.9}\text{O}_{40}$ ”, and $[\text{SiW}_{11}\text{Mn}(\text{H}_2\text{O})\text{O}_{39}]^{5-}$ (SiW_{11}Mn) (Gamelas et al., 2005a; Gamelas et al., 2008; Gaspar et al., 2003; Gaspar et al., 2007; Gaspar et al., 2009; Weinstock et al., 1997; Weinstock et al., 2001). However, some of them possessing high $M^{(n+1)/n}$ redox potentials ($E = 0.7$ – 0.8 V), although lower than molecular oxygen redox potential, are hardly reoxidized even at extreme conditions of oxygen pressure and temperature (Gamelas et al., 2005a; Gaspar et al., 2003; Weinstock et al., 1997), thus, limiting their reuse for pulp delignification.

In particular, SiW_{11}Mn has been found to be highly selective in pulp delignification (Gamelas et al., 2005a; Gaspar et al., 2003). The $\text{SiW}_{11}\text{Mn}/\text{O}_2$ catalytic system has been compared to the conventional alkaline oxygen process already used by the pulp industry. In addition to lignin removal, an important advantage of the SiW_{11}Mn -based process, when applied to eucalypt kraft pulps, is the higher reduction of kappa number than in the alkaline oxygen process, due to the degradation of hexenuronic acids at the low pH used in these reactions (Gamelas et al., 2005a). However, $\text{SiW}_{11}\text{Mn}^{\text{II}}$ is very slowly reoxidized under these conditions, limiting its practical application.

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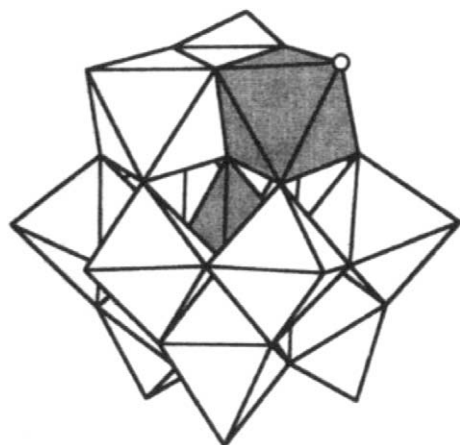


Fig. 1. Structural representation of the Mn-substituted polyoxoanion, α -[SiW₁₁Mn^{III}(H₂O)O₃₉]⁵⁻. The dark octahedron represents the Mn^{III}O₅(H₂O) group with Mn at the centre of the octahedron.

Enzymatic catalysis is a promising approach to regenerate some of the POM species that are difficult to be reoxidized by O₂ and other chemical oxidizers. In this context, fungal laccase (from *Trametes versicolor*) has been assayed for the reoxidation of [SiW₁₁V^{IV}O₄₀]⁶⁻ and [SiW₁₁Mn^{II}(H₂O)O₃₉]⁶⁻ (SiW₁₁Mn^{II}) (Gamelas et al., 2005b; Tavares et al., 2004). Although laccase easily oxidized V^{IV} to V^V in the former POM, the corresponding oxidation of Mn^{II} to Mn^{III} in the manganese-based POM was slow, with less than 50% reoxidation after 4 h at 45 °C, and under 0.3 bar oxygen pressure (Gamelas et al., 2005b). This urged the search for alternative faster methods for oxidation of Mn^{II}-substituted POM.

In contrast to laccase, versatile peroxidase (VP) produced by fungi of the genera *Pleurotus* and *Bjerkandera* is a high redox potential enzyme able to oxidize a variety of substrates, including free Mn^{II}, due to the presence of different catalytic sites in its molecular architecture (Ruiz-Dueñas et al., 2009). VP is activated by H₂O₂ in a two-electron reaction yielding highly reactive intermediate states. Activated VP can oxidize two molecules of substrate consecutively in two successive one-electron reactions. It has been demonstrated that Mn^{III}, resulting from Mn^{II} oxidation by VP or related manganese peroxidase (Ruiz-Dueñas et al., 2007), is stabilized in solution by the chelation of dicarboxylic acids of small size produced by ligninolytic fungi. The manganic cation can, then, act as an oxidizer of lignin contributing to wood delignification in nature (Wariishi et al., 1992).

In the present work, reoxidation of the Mn^{II}-containing POM, SiW₁₁Mn^{II}, by the VP/H₂O₂ system, was studied for the first time. Based on the easy oxidation of Mn^{II} (as a free ion or in POM complexes) by the enzyme a novel approach for the delignification catalysis was developed. Reduced POM in the liquor from a first eucalypt pulp delignification stage was reoxidized by VP, and the resultant liquor mixed with the partially delignified pulp for a further delignification stage in a simple POM–VP–POM trial. In addition, delignification of eucalypt pulp by POM in a VP-assisted process was tested as a pre-bleaching stage to substitute the first ClO₂ stage in a conventional elemental chlorine free (ECF) bleaching sequence.

2. Methods

2.1. Pulp samples and POM synthesis

The delignification assays were carried out with *Eucalyptus globulus* unbleached kraft pulp supplied by ENCE pulp mill (Spain).

The pulp had a kappa number of 13.7, and an intrinsic viscosity of 1180 cm³/g.

For the kinetic studies of SiW₁₁Mn^{II} oxidation by VP/H₂O₂, the potassium salt of the Mn^{II}-containing POM, K₆[SiW₁₁Mn^{II}(-H₂O)O₃₉]·10H₂O, was prepared (Tourné et al., 1970). For the delignification experiments, a solution containing 2.8 ± 0.1 mmol/L of [SiW₁₁Mn^{III}(H₂O)O₃₉]⁵⁻ (SiW₁₁Mn^{III}) was prepared as previously reported (Galli et al., 2007).

2.2. VP expression, in vitro activation and purification

Recombinant VP was obtained from *Escherichia coli* W3110 transformed with the pFLAG-VPL2 expression vector as previously described (Pérez-Boada et al., 2002). The enzyme was activated *in vitro* after solubilization of inclusion bodies in 8 M urea. The folding conditions included 0.15 M urea, 5 mM Ca²⁺, 20 μM hemin, a 4:1 oxidized-glutathione/reduced-glutathione ratio and 0.1 mg/mL protein at pH 9.5. The active enzyme was purified in a single chromatographic step (Resource-Q column, Pharmacia-Biotech) using a 0–0.3 M NaCl gradient (2 mL/min) in 10 mM sodium tartrate (pH 5.5) containing 1 mM CaCl₂. The concentration of the enzyme was determined by spectrophotometry (ϵ_{407} 150 000 M⁻¹ cm⁻¹) (Ruiz-Dueñas et al., 1999).

2.3. SiW₁₁Mn^{II} oxidation by VP

Oxidation of the Mn^{II}-substituted POM was followed at 20 °C in a quartz cuvette (1 cm optical path) under stirring: 3.0 mL of 0.1 M acetate solution (pH 4.5) containing 2.7 mM K₆[SiW₁₁Mn^{II}(-H₂O)O₃₉]·10H₂O, 0.56–1.26 μM VP and 0.57–2.24 mM H₂O₂ were mixed inside the cell. The increase of absorbance at 490 nm was followed during 1 min intervals, until the reaction reached a plateau. The oxidation degree was estimated using the molar extinction coefficients of the oxidized and reduced POM (SiW₁₁Mn^{III} ϵ_{490} 325 M⁻¹ cm⁻¹; and SiW₁₁Mn^{II} ϵ_{490} 22 M⁻¹ cm⁻¹) (Tourné et al., 1970). For the assays with the delignification liquor, the H₂O₂ amount varied between 0 and 2.06 mM, with the amount of enzyme kept at 1.20 μM (41.4 mg/L). The total absorbance was corrected for the liquor contribution. Typically, each assay was run in duplicate and the results of the replicates were similar (with deviations lower than 2%).

Steady-state kinetic constants were calculated during VP oxidation of increasing SiW₁₁Mn^{II} concentrations in 0.1 M sodium tartrate, pH 5, containing 0.1 mM H₂O₂. The enzymatic activity at 20 °C was measured as the initial velocity, taking linear increments. Mean values and standard errors for the apparent affinity constant (Michaelis constant, K_m) and maximal enzyme turnover (catalytic constant, k_{cat}) were obtained by non-linear least-squares fitting of the experimental measurements to the Michaelis–Menten model. Fitting of these constants to the normalized equation $v = (k_{cat}/K_m)[S]/(1 + [S]/K_m)$ yielded the efficiency value (k_{cat}/K_m) with its corresponding standard error.

2.4. Pulp delignification experiments

Pulp delignification under oxygen was carried out in a PARR reactor, model 4843 (0.25 L) equipped with an automatic temperature control system, pressure control (pO₂ of 0.5 MPa) and mechanical stirring (220 rpm). 7.5 g of pulp (dry weight), 67 mL of 0.2 M sodium acetate (pH 4.5), 13 mL of 28 mM POM (SiW₁₁Mn^{III}) solution, and water to make a final volume of 132 mL were put inside the reactor. The final concentration of POM was 2.7 mM. At the end of the reactions, the reactor was quickly cooled with water and degasified.

In the two-stage experiments, including intermediate POM reoxidation with VP (POM–VP–POM_{reox}), the pulp from the first

stage was filtered and pressed, the required amounts of enzyme and H_2O_2 previously optimized to attain near 100% POM reoxidation (POM/VP and H_2O_2 /POM molar ratios of 2200 and 0.4, respectively) were added to the delignification liquor, and the solution was stirred at 20–25 °C for 10 min. The liquor containing the reoxidized POM (verified by visible absorption spectrophotometry) was mixed again with the filtered pulp and a second delignification stage was applied under the same experimental conditions of the first stage. A two-stage experiment not including the reoxidation step of POM by VP/ H_2O_2 was also performed by adding fresh POM ($\text{SiW}_{11}\text{Mn}^{\text{III}}$), acetate buffer and water to the washed pulp obtained after the first stage.

After each delignification sequence the pulps were filtered and washed with water until neutral filtrate. Alkaline extraction of pulps was carried out at 70 °C during 1 h and NaOH charge of 2% w/w dry pulp.

The results of the delignification experiments carried out in duplicate were similar, as measured by the kappa number and intrinsic viscosity of the pulps obtained (deviations did not exceed 4%).

2.5. Modified ECF bleaching sequence

Bleaching with ClO_2 was performed on untreated kraft pulp and with pulp delignified with POM, at 10% pulp consistency, in plastic bags in a Grant model Y28 thermostatic bath. Two bleaching sequences, D–Ep–D–Ep–D and POM–VP–POM_{reox}–E–D–Ep–D, were studied (D refers to ClO_2 stage; Ep to peroxide-reinforced alkaline extraction; POM–VP–POM_{reox} corresponds to VP-assisted two stage [2 h + 2 h] POM treatment; and E to alkaline extraction).

The bleaching conditions in the D–Ep–D–Ep–D sequence were as follows: first D stage at 50 °C for 1 h; second D stage at 70 °C for 2 h; third D stage at 70 °C for 2.5 h; first Ep stage at 70 °C for 1 h, using 2.0% NaOH and 0.2% H_2O_2 ; second Ep stage at 70 °C for 1 h, using 1.5% NaOH and 0.1% H_2O_2 .

The pulp delignified with POM (2 h)–VP–POM_{reox} (2 h) and extracted with NaOH was subjected to D–Ep–D bleaching (POM–VP–POM_{reox}–E–D–Ep–D sequence). The conditions of the last stages in this sequence were as follows: first D stage at 50 °C for 1 h; second D stage at 70 °C for 2.5 h; Ep stage at 70 °C for 1 h, using 1.5% NaOH and 0.2% H_2O_2 . The loads of ClO_2 for each stage in both sequences are discussed in the text.

2.6. Pulp characterization

The treated pulps were characterized according to TAPPI T236 cm-99 standard for the kappa number (Tappi, 2006), and the SCAN-CM 15:88 standard for the intrinsic viscosity (Scandinavian Pulp Paper and Board Committee, 1994). Hexenuronic acid content was determined by acid hydrolysis in sodium formate (pH 3.0) followed by spectrophotometric (245 nm) quantitation of the furan derivatives formed (Vuorinen et al., 1999). The acid hydrolysis pulp treatments were carried out in the same PARR reactor mentioned above (see Section 2.4). The strength properties, brightness, and opacity of the bleached pulps were determined according to ISO (International Organisation for Standardization Documentation and Information, 2003) and TAPPI (Tappi, 2006) standards.

3. Results and discussion

3.1. Kinetics of $\text{SiW}_{11}\text{Mn}^{\text{II}}$ oxidation by VP

The ability of VP, a peroxidase acting on free Mn^{II} and other substrates, to oxidize this metal ion in Mn-substituted POM is

demonstrated here for the first time. The steady-state kinetic constants for $\text{SiW}_{11}\text{Mn}^{\text{II}}$ oxidation, obtained by non-linear fitting of initial velocities vs substrate concentration (Fig. 2), revealed high VP turnover on $\text{SiW}_{11}\text{Mn}^{\text{II}}$, with a k_{cat} of $47 \pm 2 \text{ s}^{-1}$, and a moderate affinity for this compound, with a K_{m} of $6.4 \pm 0.7 \text{ mM}$. This VP activity was lower than on free Mn^{II} , with a reported k_{cat} value near 300 s^{-1} , but the main difference between both substrates concerned K_{m} that was around 0.19 mM for free Mn^{II} , revealing over 30-fold higher affinity of VP for the free metal ion (Ruiz-Dueñas et al., 2007). As a result, the global catalytic efficiency of VP oxidizing $\text{SiW}_{11}\text{Mn}^{\text{II}}$ ($7.36 \pm 0.6 \text{ mM}^{-1} \text{ s}^{-1}$) was around 200-fold lower than that for oxidation of free Mn^{II} ($1600 \pm 100 \text{ mM}^{-1} \text{ s}^{-1}$).

The high affinity of VP for free Mn^{II} is due to the existence of a specific catalytic site in this enzyme constituted by three acidic residues forming a small channel on the internal heme propionate, where the free metal cation is oxidized (Ruiz-Dueñas et al., 2007). The lower efficiency observed for $\text{SiW}_{11}\text{Mn}^{\text{II}}$ oxidation by VP was in the order of those reported both for veratryl alcohol oxidation taking place at a tryptophan residue exposed to the solvent (Pérez-Boada et al., 2005), and for oxidation of phenols at the edge of the main heme access channel (Ruiz-Dueñas et al., 2008). This suggested that $\text{SiW}_{11}\text{Mn}^{\text{II}}$ could be oxidized in one of these two easily accessible catalytic sites, and not in the narrow channel described for free Mn^{II} that most probably provides a limited access to the bulky $\text{SiW}_{11}\text{Mn}^{\text{II}}$. A detailed kinetic study of different VP variants mutated at the three catalytic sites mentioned above would be necessary to definitively identify the $\text{SiW}_{11}\text{Mn}^{\text{II}}$ oxidation site in VP.

3.2. Optimization of $\text{SiW}_{11}\text{Mn}^{\text{II}}$ oxidation by VP

A set of assays was carried out aimed at optimizing the oxidation of $\text{SiW}_{11}\text{Mn}^{\text{II}}$ to $\text{SiW}_{11}\text{Mn}^{\text{III}}$ by VP (in the presence of H_2O_2), either by using an aqueous solution of $\text{SiW}_{11}\text{Mn}^{\text{II}}$ buffered at pH 4.5, and the liquor from previous eucalypt kraft pulp delignification with POM (Table 1). The assays were performed at 20 °C, with 2.7 mM POM concentration, and varying the H_2O_2 /POM and POM/VP molar ratios.

In the assays performed with the $\text{SiW}_{11}\text{Mn}^{\text{II}}$ solution (Table 1A), the extent of POM oxidation (for a fixed amount of enzyme) increased with the H_2O_2 /POM molar ratio until a 0.5–0.6 ratio, and then decreased at higher ratios. Using this H_2O_2 /POM ratio (0.5–0.6), 95–100% POM oxidation was obtained in less than 5 min, with a POM/VP ratio ~ 2200 . These values were in agreement with the stoichiometry of the overall enzymatic reaction, which predicts that 0.5 mol of H_2O_2 will be needed to oxidize 1 mol of $\text{SiW}_{11}\text{Mn}^{\text{II}}$. For the H_2O_2 /POM ratio of 0.8, only 57% oxidation of $\text{SiW}_{11}\text{Mn}^{\text{II}}$ was obtained, indicating enzyme inactivation by the excess of H_2O_2

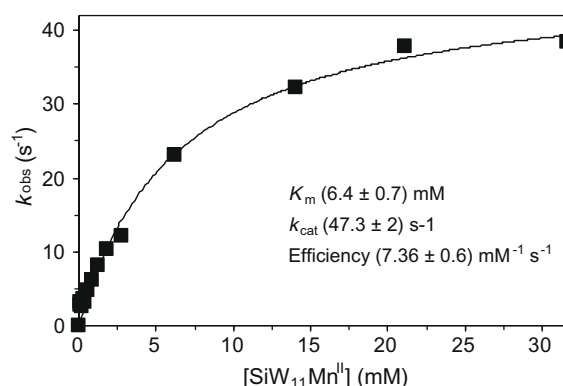


Fig. 2. Michaelis–Menten kinetics of $\text{SiW}_{11}\text{Mn}^{\text{II}}$ oxidation by VP. The K_{m} , k_{cat} and efficiency values (means and standard errors) are shown inside the plot.

Table 1Oxidation of SiW₁₁Mn^{II} by VP/H₂O₂ in two distinct reaction media using different H₂O₂, POM and VP molar ratios^a.

H ₂ O ₂ /POM	H ₂ O ₂ /VP	POM/VP	Oxidation (%)	Time (min)
<i>(A) Oxidation of SiW₁₁Mn^{II} in aqueous solution buffered at pH 4.5</i>				
0.19	446	2382	40	2
0.40	883	2226	78	2
0.50	1117	2226	95	5
0.61	1351	2226	100	5
0.81	1793	2226	57	22
0.50	2495	5009	55	29
0.50 ^b	2495	5009	94	10
<i>(B) Oxidation of SiW₁₁Mn^{II} in the filtrate from one-stage delignification with POM</i>				
0	0	2227	18	1
0.21	472	2227	57	2
0.40	896	2227	93	6
0.50	1116	2230	97	2
0.59	1321	2227	74	27
0.79	1769	2227	44	18

^a The assays were performed at 20 °C, with 2.6–2.8 mM POM concentration.^b H₂O₂ was added in five portions each of them including 20% of the total volume required.

(Valderrama et al., 2002). If the amount of enzyme was reduced to about 50%, keeping the H₂O₂/POM ratio of 0.5, the oxidation extent also decreased (to only 55%) due to the increased H₂O₂/VP ratio. However, when the later assay was carried out by adding the H₂O₂ in several steps, without exceeding a 500-fold molar excess of H₂O₂ in each addition, the extent of oxidation (94%) was similar to that attained using a higher amount of enzyme. These data confirmed VP inactivation by H₂O₂ (even in the presence of enough amount of SiW₁₁Mn^{II} to consume all the H₂O₂) and showed that the enzyme dose can be reduced by stepwise addition of H₂O₂ (to prevent VP inactivation). The POM oxidation was not observed only with H₂O₂ or only with the enzyme.

In the assays with the liquor from POM delignification of eucalypt kraft pulp (Table 1B), which were performed using a POM/VP ratio ~2200, and varying the H₂O₂/POM ratio between 0 and 1, the highest POM oxidation degrees (over 90%) were obtained at the H₂O₂/POM ratios of 0.4–0.5. In the absence of H₂O₂, some POM oxidation occurred suggesting that some substances present in the delignification liquor may act as the enzyme oxidizing agents. In fact, for all the assays performed with a H₂O₂/POM ratio up to 0.5, the oxidation of POM was higher when the reactions were conducted in the delignification liquor than those in the SiW₁₁Mn^{II} aqueous solutions. Again, the use of H₂O₂/POM ratios ≥0.6 did not improve POM reoxidation in the delignification liquor, and lower rates were obtained. It was concluded that a H₂O₂/POM ratio around 0.5 and a POM/VP ratio of 2000–3000 should be used to obtain near complete oxidation of the manganese-substituted POM.

3.3. Two-stage POM delignification of pulp assisted by VP

As a continuation of the studies above, a novel approach for the paper pulp delignification was developed. A first delignification stage using chemically-prepared SiW₁₁Mn^{III} and O₂ was followed by pulp filtration, and a short intermediate step consisting of the addition of VP and H₂O₂ to the filtrate. Fig. 3 shows the UV–Vis absorption spectra of the filtrate before (a) and after the enzymatic treatment (b), together with those of the initial SiW₁₁Mn^{III} aqueous solution (c) and the enzyme (d), for comparison. It indicates a complete reoxidation of the previously reduced POM (when comparing the spectrum of the filtrate after the addition of VP/H₂O₂ (b) with that of the initial SiW₁₁Mn^{III} aqueous solution (c), some slight differences are observed, specially in the region of 380–450 nm, which are due to the additional presence of the enzyme and of the dissolved oxidized lignin substructures in the filtrate solution). After the POM reoxidation, the filtrate with reoxidized POM was

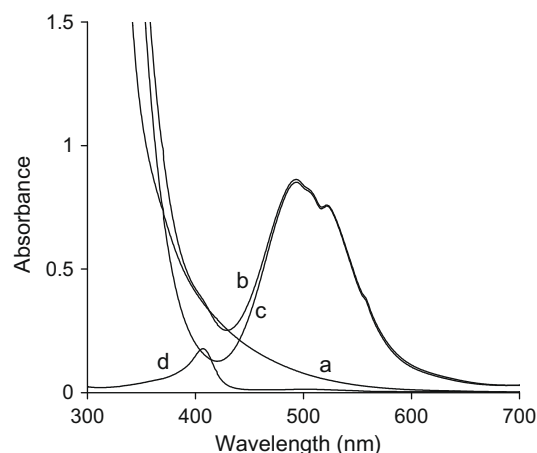


Fig. 3. UV–Vis spectra of the filtrate after 2 h treatment of eucalypt kraft pulp with POM/O₂ (a), and after short incubation of this delignification liquor with VP and H₂O₂ at 20–25 °C (b), together with those of the initial SiW₁₁Mn^{III} aqueous solution (c) and the enzyme (1.20 μM, d).

mixed again with the pulp, and a second delignification stage (under the same conditions of the first one) was applied. The results were compared with those obtained when the second delignification stage was performed by adding chemically-prepared SiW₁₁Mn^{III}, as well as when only one-stage POM delignification was performed (Table 2).

After one-stage POM delignification at 110 °C, decreases of kappa number (a rough measure of the lignin content in pulp) of 40% and 50%, with viscosity losses of only 3% and 6%, were obtained after 1-h and 2-h reaction, respectively (Table 2). Besides residual lignin, hexenuronic acids contribute significantly to the kappa number in *E. globulus* kraft pulps and to the consumption of bleaching reagents (Furtado et al., 2001). In fact, a significant removal of hexenuronic acids (up to 70% after 2 h) was detected after the POM treatment. It is noteworthy that the POM/O₂ system was highly selective for delignification when compared with the oxygen-delignification control, which showed a viscosity loss of 28% (near 5-fold higher than that obtained with POM delignification) (Gamelas et al., 2005a).

After two-stage (2-h each) POM delignification including the intermediate reoxidation step with VP and H₂O₂, kappa number was reduced by 62% and the viscosity dropped by 11% (Table 2). Interestingly, this treatment also degraded almost 90% of the

Table 2

Delignification of eucalypt kraft pulp with $\text{SiW}_{11}\text{Mn}^{\text{III}}/\text{O}_2$ assisted by $\text{VP}/\text{H}_2\text{O}_2$ (effect of different treatments on pulp kappa number, intrinsic viscosity and hexenuronic acid content (HexA))^a.

	Kappa number	Viscosity (cm^3/g)	Kappa decrease (%) ^d	Viscosity loss (%)	HexA (mmol/kg)
Initial kraft pulp	13.6	1215	–	–	61.2
O_2 (without POM, 2 h)	7.3	875	46 (33)	28	15.7
POM (1 h)	8.2	1180	40 (33)	3	28.5
POM (2 h)	6.8	1140	50 (40)	6	18.4
POM (1 h)–VP–(1 h) ^b	6.5	1130	52 (42)	7	16.8
POM (2 h)–VP–(2 h) ^b	5.2	1080	62 (51)	11	9.3
POM (2 h)–POM (2 h) ^c	5.2	1085	62 (50)	11	8.2

^a Pulp consistency of 5.4%; 2.7 mM POM; pH 4.5; $p\text{O}_2$ of 0.5 MPa; 110 °C; and 220 rpm.

^b The pulp after the first stage was filtered, and the POM in the filtrate reoxidized by $\text{VP}/\text{H}_2\text{O}_2$.

^c The pulp after the first stage was washed, and fresh POM ($\text{SiW}_{11}\text{Mn}^{\text{III}}$) was added.

^d Kappa number reduction corrected for HexA ($\text{kappa}_{\text{cor}} = \text{kappa} - 0.073 \times [\text{HexA}]$) in parentheses.

Table 3

ClO_2 consumption and oxidation equivalents (OXE) for eucalypt pulp bleaching in a conventional ECF sequence and after substituting the first D-stage by VP-assisted two-stage POM delignification (89% ISO final brightness).

	D–Ep–D–Ep–D	POM–VP–POM _{reox} –E–D–Ep–D
ClO_2 consumption ^a	25 + 9 + 6	15 + 5
OXE ^b	90	134

^a As active chlorine in each D stage (kg/ton).

^b As moles of active chlorine per ton of dry pulp and kappa unit.

hexenuronic acids present in the pulp. The delignification degree corrected for the hexenuronic acids content was 51%. Similar results in terms of pulp kappa number, viscosity and hexenuronic acids degradation were obtained in parallel assays with freshly-prepared $\text{SiW}_{11}\text{Mn}^{\text{III}}$ added after the first POM stage, revealing that the presence of the enzyme did not exert a negative effect on the performance and selectivity of the $\text{SiW}_{11}\text{Mn}^{\text{III}}/\text{O}_2$ system.

3.4. Modified ECF bleaching including a VP-assisted POM stage

Pulp treatment with the above VP-assisted two-stage (2-h each) POM delignification followed by an alkaline extraction (POM–VP–POM_{reox}–E) was investigated to substitute the first ClO_2 stage in a conventional D–Ep–D–Ep–D ECF bleaching sequence for eucalypt kraft pulp. Results from the conventional D–Ep–D–Ep–D bleaching sequence (see Methods) and the sequence including VP-assisted two-stage POM delignification, (POM–VP–POM_{reox}–E–D–Ep–D), were compared in terms of ClO_2 savings for the same final brightness (~89% ISO). Pulp bleached by the sequence including VP and POM showed a ClO_2 consumption 50% lower than the conventional ECF sequence (Table 3). The ClO_2 oxidation equivalents (OXE) per

Table 4

Physical properties of unbeaten bleached pulps (89% ISO, and 65 g/m²) from a conventional ECF sequence and after substituting the first ClO_2 stage by VP-assisted two-stage POM delignification.

	D–Ep–D–Ep–D	POM–VP–POM _{reox} –E–D–Ep–D
Beating degree (°SR)	19	20
Bulk density (g/cm^3)	0.56	0.57
Burst index ($\text{kPa m}^2/\text{g}$)	1.39	1.45
Tensile strength ($\text{N m}/\text{g}$)	30.4	28.9
Tear index ($\text{mN m}^2/\text{g}$)	5.2	6.0
Elongation (%)	2.2	2.1
Stiffness (kN/m)	409	396
Opacity (%)	75.8	77.2
Internal bonds (Scott test, J/m^2)	106	114
Air resistance (Curley-100 mL, s)	0.8	0.8

kappa number unit in the modified sequence were higher than in the conventional sequence. Moreover, the main strength properties of the unbeaten pulps after the two bleaching sequences were very similar (Table 4). The results obtained suggest that VP-assisted continuous reutilization of $\text{SiW}_{11}\text{Mn}^{\text{III}}$ in a two-reactor system (Gamelas et al., 2007) may be implemented in future industrial ECF sequences, with no apparent deterioration of the pulp strength properties, while significantly reducing the ClO_2 consumption, and consequently lowering the environmental impact of the bleaching process.

4. Conclusions

In this work, we demonstrate that the reduced Mn-substituted POM, $\text{SiW}_{11}\text{Mn}^{\text{II}}$, can be oxidized by VP (in the presence of H_2O_2) following Michaelis–Menten kinetics. This POM, whose oxidized form is highly selective for delignification, was fully oxidized by $\text{VP}/\text{H}_2\text{O}_2$ at 20–25 °C in less than 10 min. In this way, a two-stage POM delignification process, including a fast intermediate step consisting of the addition of VP (and H_2O_2) to the delignification filtrate for POM reoxidation, was performed, resulting in 62% reduction of the pulp kappa number and a viscosity loss of only 10%. The substitution of the first ClO_2 stage by a POM–VP–POM_{reox} treatment in a conventional ECF bleaching sequence allowed 50% ClO_2 saving without decreasing the pulp strength properties. Hence, the continuous reutilization of $\text{SiW}_{11}\text{Mn}^{\text{III}}$ in a two-reactor system may be implemented in the future.

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References

- Evtuguin, D.V., Neto, C.P., 1997. New polyoxometalate promoted method of oxygen delignification. *Holzforschung* 51, 338–342.
- Furtado, F.P., Evtuguin, D.V., Gomes, T.M., 2001. Effect of the acid stage in ECF bleaching on *Eucalyptus globulus* kraft pulp bleachability and strength. *Pulp Pap. Can.* 102, 89–92.
- Galli, C., Gentili, P., Pontes, A.S.N., Gamelas, J.A.F., Evtuguin, D.V., 2007. Oxidation of phenols employing polyoxometalates as biomimetic models of the activity of phenoloxidase enzymes. *New J. Chem.* 31, 1461–1467.
- Gamelas, J.A.F., Gaspar, A.R., Evtuguin, D.V., Neto, C.P., 2005a. Transition metal substituted polyoxotungstates for the oxygen delignification of kraft pulp. *Appl. Catal. A: General* 295, 134–141.
- Gamelas, J.A.F., Tavares, A.P.M., Evtuguin, D.V., Xavier, A.M.B., 2005b. Oxygen bleaching of kraft pulp with polyoxometalates and laccase applying a novel multi-stage process. *J. Mol. Catal. B Enzym.* 33, 57–64.

- Gamelas, J.A.F., Pontes, A.S.N., Evtuguin, D.V., Xavier, A.M.R.B., Esculcas, A.P., 2007. New polyoxometalate-laccase integrated system for kraft pulp delignification. *Biochem. Eng. J.* 33, 141–147.
- Gamelas, J.A.F., Evtuguin, D.V., Gaspar, A.R., 2008. Transition metal complexes in the delignification catalysis. In: Varga, B., Kis, L. (Eds.), *Transition Metal Chemistry: New Research*. Nova Science Publishers, Inc., New York, pp. 15–57.
- Gaspar, A., Evtuguin, D.V., Neto, C.P., 2003. Oxygen bleaching of kraft pulp catalysed by Mn(III)-substituted polyoxometalates. *Appl. Catal. A: General* 239, 157–168.
- Gaspar, A.R., Gamelas, J.A.F., Evtuguin, D.V., Neto, C.P., 2007. Alternatives for lignocellulosic pulp delignification using polyoxometalates and oxygen: a review. *Green Chem.* 9, 717–730.
- Gaspar, A.R., Gamelas, J.A.F., Evtuguin, D.V., Neto, C.P., 2009. Polyoxometalate-catalyzed oxygen delignification process: kinetic studies, delignification sequences and reuse of HPA-5-Mn^{II} aqueous solution. *Chem. Eng. Commun.* 196, 801–811.
- International Organisation for Standardization Documentation and Information, 2003. ISO Standards Collection on CD-ROM. Paper, Board and Pulp. ISO, Geneva.
- Pérez-Boada, M., Doyle, W.A., Ruiz-Dueñas, F.J., Martínez, M.J., Martínez, A.T., Smith, A.T., 2002. Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimisation of in vitro folding. *Enzyme Microb. Technol.* 30, 518–524.
- Pérez-Boada, M., Ruiz-Dueñas, F.J., Pogni, R., Basosi, R., Choinowski, T., Martínez, M.J., Piontek, K., Martínez, A.T., 2005. Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigations of three long-range electron transfer pathways. *J. Mol. Biol.* 354, 385–402.
- Ruiz-Dueñas, F.J., Martínez, M.J., Martínez, A.T., 1999. Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. *Mol. Microbiol.* 31, 223–236.
- Ruiz-Dueñas, F.J., Morales, M., Pérez-Boada, M., Choinowski, T., Martínez, M.J., Piontek, K., Martínez, A.T., 2007. Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: a site-directed mutagenesis, kinetic and crystallographic study. *Biochemistry* 46, 66–77.
- Ruiz-Dueñas, F.J., Morales, M., Rencoret, J., Gutiérrez, A., del Río, J.C., Martínez, M.J., Martínez, A.T., 2008. Improved Peroxidases. Patent P200801292, Spain.
- Ruiz-Dueñas, F.J., Morales, M., García, E., Miki, Y., Martínez, M.J., Martínez, A.T., 2009. Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. *J. Exp. Bot.* 60, 441–452.
- Scandinavian Pulp Paper and Board Committee, 1994. SCAN Test Methods. Sweden.
- Tappi, 2006. 2006–2007 TAPPI Test Methods. TAPPI Press, Norcross, GA 30092, USA.
- Tavares, A.P.M., Gamelas, J.A.F., Gaspar, A.R., Evtuguin, D.V., Xavier, A.M.R.B., 2004. A novel approach for the oxidative catalysis employing polyoxometalate-laccase system: application to the oxygen bleaching of kraft pulp. *Catal. Commun.* 5, 485–489.
- Tourné, C.M., Tourné, G.F., Malik, S.A., Weakley, T.J.R., 1970. Triheteropolyanions containing copper(II), manganese(II), or manganese(III). *J. Inorg. Nucl. Chem.* 32, 3875–3890.
- Valderrama, B., Ayala, M., Vázquez-Duhalt, R., 2002. Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes. *Chem. Biol.* 9, 555–565.
- Vuorinen, T., Fagerstrom, P., Buchert, J., Tenkanen, M., Teleman, A., 1999. Selective hydrolysis of hexenuronic acid groups and its application in ECF and TCF bleaching of kraft pulps. *J. Pulp Pap. Sci.* 25, 155–162.
- Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. *J. Biol. Chem.* 267, 23688–23695.
- Weinstock, I.A., Atalla, R.H., Reiner, R.S., Moen, M.A., Hammel, K.E., Houtman, C.J., Hill, C.L., Harrup, M.K., 1997. A new environmentally benign technology for transforming wood pulp into paper. Engineering polyoxometalates as catalysts for multiple processes. *J. Mol. Catal. A Chem.* 116, 59–84.
- Weinstock, I.A., Barbuzzi, E.M.G., Wemple, M.W., Cowan, J.J., Reiner, R.S., Sonnen, D.M., Heintz, R.A., Bond, J.S., Hill, C.L., 2001. Equilibrating metal-oxide cluster ensembles for oxidation reactions using oxygen in water. *Nature* 414, 191–195.