Evaluation of the Enzyme Manganese Peroxidase in an Industrial Sequence for the Lignin Oxidation and Bleaching of Eucalyptus Kraft Pulp

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ABSTRACT: Manganese peroxidase produced by the white-rot fungus *Bjerkandera* sp. strain BOS55 was used for lignin oxidation and bleaching of eucalyptus oxygen-delignified kraft pulp. The optimization of the enzymatic stage and its implementation into an industrial chemical bleaching sequence were performed for the purpose of defining a new bleaching sequence. Parameters related to the selection and concentration of a chelating organic acid, Mn^{2+}, and hydrogen peroxide concentrations were optimized and applied to evaluate the implementation of an enzymatic stage into a chemical sequence composed of chelator and hydrogen peroxide or hydrogen peroxide with oxygen pressure stages. The brightness, reduction of the κ number, and remaining manganese peroxidase activity were assessed in conventional and enzyme-based bleaching sequences. High International Organization for Standardization (ISO) brightness (83%) and parallel κ number reduction (5.5 points) were obtained with an enzymatic stage/chelator stage/hydrogen peroxide with oxygen pressure stage sequence under the best operational conditions: 33 μ*M* Mn^{2+}, oxalic acid, and 41.7 μ*M* H_2O_2 added in pulses every 5 min for the enzymatic stage and a 2-h hydrogen peroxide with oxygen pressure stage at 588.4 kPa of oxygen pressure. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 1319–1327, 2008

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INTRODUCTION

The production of bright paper makes necessary the inclusion of bleaching processes based on the utilization of chemical reagents in certain combined sequences. One of the substances that has traditionally been used as a bleaching agent is elemental chlorine. From its use, some of the most important environmental contaminants caused by the pulp and paper industry, organochlorines (AOX), are derived. The response of the pulp and paper industry to the imposition of emission standards for AOX was the modification of the bleaching process. Oxygen, ozone, and hydrogen peroxide are currently used in bleaching, reducing the demand for chlorine derivatives. The kraft process, dominant worldwide, is characterized by the removal of most of the lignin content (typically 90–95%). However, residual lignin is highly oxidized, providing a dark appearance to the pulp, and further treatments must be applied to obtain an acceptable brightness level. In many cases, pulp is subjected to an oxygen treatment that produces eucalyptus oxygen-delignified kraft pulp (ODKP), and this is followed by chelation and hydrogen peroxide stages.

Enzymes have been suggested as an alternative to reduce the use of chemicals,[1] and their implementation is intended to be adjusted to any traditional or modern bleaching sequences without significant investment in existing plants. So far, two approaches based on the use of hemicellulases or ligninolytic enzymes have been evaluated. The latter attack lignin directly, and hence they are more effective. Secreted by fungi in response to low levels of key nutrients such as carbon, nitrogen, and sulfur, the most relevant enzymes are lignin peroxidase (LiP),[2–4] manganese peroxidase (MnP),[5,6] versatile peroxidase (VP),[7,8] laccases,[9–11] and H_2O_2-generating oxidases.[12–14]

Among the different enzymes, MnP is detected in active biobleaching cultures of different strains[15] and has been correlated with the biobleaching ability of different white-rot fungi. The main function of MnP is the oxidation of Mn^{2+} to Mn^{3+}, for which it requires H_2O_2. The Mn^{3+} ion is a strong oxidant (1.54 V) and can act on a great variety of phenolic compounds as a diffusible oxidant through the

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lignin irregular matrix. Increased stability of Mn^{3+} is achieved by the formation of complexes with dicarboxylic acids (malonate, oxalate, etc.) or other acids such as lactate.[16,17] The Mn^{3+} complex can reach certain parts of lignin in which access to the enzyme is hindered and oxidize, in a nonspecific manner, aromatic structures. Kondo et al.[18] proved the bleaching ability of purified MnP in the presence of Mn^{2+}, Tween 80, malonate, and H_2O_2. Moreover, the use of MnP prevents operational problems related to the need of a mediator as in the case of laccase-based bleaching, with the large associated benefits from economic and environmental points of view. However, the application of MnP on a higher scale is still lacking, and this would be the first step in its potential use.

The main aim of this work is to introduce an MnP-based enzymatic stage in a bleaching sequence and analyze its effect on the bleaching and delignification of ODKP of *Eucalyptus globulus* to evaluate the possibility of a subsequent scale-up of the process to a pilot scale. The effect on the physical properties of the hand sheets made after bleaching is also considered.

EXPERIMENTAL

Kraft pulp and property determinations

The pulp used in this study was eucalyptus ODKP. This pulp was provided by a kraft mill (ENCE, Pontevedra, Spain) and was characterized by a κ number of 10 and brightness of 63.2% International Organization for Standardization (ISO). All the experiments were performed at 1% pulp consistency. The κ number was determined by oxidation of potassium permanganate[19] (TAPPI T236 method). Brightness (% ISO) was measured in 8-cm-diameter handmade sheets with a Minolta (Tokyo, Japan) CM-500 spectrophotometer[19] (TAPPI T452 method).

MnP production

MnP was produced from *Bjerkandera* sp. strain BOS55 (ATTC 90940) as described by Feijoo et al.[20] The preinoculum was obtained by the addition of five agar plugs (from 7-day-old colonized plates) to a 1-L Fersnbach flask containing 100 mL of an N-limited culture medium with the following composition: 10 g/L glucose, 2 g/L ammonium tartrate, 500 μ*M* Mn^{2+}, and 100 mL of BII mineral salts[21] in a 20 m*M* sodium acetate buffer. Then, the preinoculum was incubated in a culture oven at 30°C for 5 days as a static culture. The preinoculum (10% v/v blended and homogenized mycelium) was added to 250-mL Erlenmeyer flasks containing 90 mL of the culture medium. The flasks were incubated in an orbital shaker (Inova 4000, New Brunswick Scientific, Edison, NJ) at 30°C. Pellets were transferred to a 10-L Biostat-E bioreactor (Braun Biotech, Melsungen, Germany) in a proportion of approximately 10%. Skimmed cheese whey (25 g/L) obtained from a milk factory (Abiasa SA, Pontevedra, Spain) was used as a carbon source in the fermenter instead of glucose. The extracellular culture broth was collected at the point of highest MnP activity (typically after 4–6 days) and filtered through a Whatman (Maidstone, England) no. 1 paper filter. Thereafter, the filtered culture broth containing the MnP (45 kDa) was concentrated by ultrafiltration (Filtron Minisette System, Pall Corporation, Hauppauge, NY; 10-kDa cutoff). MnP was kept at −21°C until it was used in the delignification assays. In the experiments as indicated, a previous dialysis of the enzymatic crude against acetic acid (0.1*M*, pH 4.5) was performed.

Enzyme activity determination

The activity of the enzyme was determined spectrophotometrically with a Hitachi (Tokyo, Japan) U-2000 spectrophotometer at 30°C and 468 nm. At this wavelength, the oxidation of 1 m*M* 2,6-dimethoxyphenol could be followed in a 50 m*M* sodium malonate buffer (pH 4.5) with 1 m*M* $MnSO_4$. The reaction started with the addition of 0.4 m*M* H_2O_2.[22] The amount of enzyme that converted 1 μ*M* substrate/min was considered 1 unit of MnP activity.

Experimental bleaching procedures

Enzymatic stages

The experimental assays were performed with a 20-mL pulp solution in 100-mL flasks in triplicate. The flasks contained 0.2 g of pulp in a total volume of 20 mL (1% consistency) with different concentrations of organic acid, Mn^{2+}, and hydrogen peroxide. Just before the beginning of the experiment, 60 U of MnP/g of dry pulp was added. The necessary volume of H_2O_2 was added in pulses every 5 min. The total amount of peroxide added to the flasks was 0.5 m*M*, unless stated otherwise. To reach this concentration, pulses of 100 μL from a 8.34 m*M* H_2O_2 stock solution were added every 5 min. The assays were incubated in a thermostated orbital shaker at 100 rpm and 40°C. The contact time was 1 h in all cases. The optimization of the enzymatic stages involved three sets of experimental assays studying the effects of the organic acid, manganese, and hydrogen peroxide.

The selection of the organic acid and its optimal concentration was determined with experiments using 0, 0.5, 1, 5, 10, and 50 m*M* concentrations of the following organic acids: oxalic, malonic, and glycolic acids. In the experiments in which the man-

ganese concentration was evaluated, assays were performed with 0, 10, 33, 78, and 150 μ*M* Mn^{2+}. The concentrated MnP was stored with 0.06 μmol of Mn^{2+}/U of MnP to preserve the enzyme from inactivation. The addition of nondialyzed MnP would supply 33 μ*M* Mn^{2+}. For this reason, the experiments with the three lower concentrations were performed with an acetate-dialyzed enzyme, and the other two were performed with a nondialyzed one. In the case of the three higher Mn^{2+} concentrations (78, 150, and 550 μ*M* Mn^{2+}), the quantity of Mn^{2+} to be added was calculated on the basis of the content of Mn^{2+} present in the enzyme. The effect of the hydrogen peroxide concentration was studied in experiments in which H_2O_2 was added in pulses every 5 min to reach final concentrations of 0.25, 0.5, 1, 5, 10, and 15 m*M*. During the course of the experiment, 12 pulses of 200 μL from a H_2O_2 stock solution were added. For instance, the peroxide concentration of the stock solution in the experiment of 0.5 m*M* was 8.34 m*M*. The H_2O_2 added in each pulse was 0.83 μmol, which represents 41.7 μ*M* H_2O_2 in the total volume (20 mL). After the last pulse, a total of 10 μmol of H_2O_2 was added, representing 0.5 m*M* H_2O_2. For the other experimental points, the volume of the pulse was maintained, whereas the concentration of the stock solution was modified. This stock solution was prepared just before the beginning of the experiments because of its very low stability (ca. 2 h). After each experiment, residual MnP activity was measured, and the paper pulp was washed thoroughly with deionized water and finally dried overnight at 35°C.

The enzymatic stages before the chemical sequences were performed in 250-mL Erlenmeyer flasks with a 100-mL pulp solution at 1% consistency.

Chemical stages

The chelator (Q) stage was performed in sealed polypropylene bags immersed into a temperature-controlled glycerin bath at 85°C for 1 h. The use of these bags avoided the presence of air bubbles. The pulp was used with 10% consistency by the addition of a 0.4% diethylenetriamine pentaacetic acid solution. The pH of the solution was adjusted to 6 with sulfuric acid (1*M*).

The hydrogen peroxide (P) stage was conducted under the same conditions described for the Q stage, but without the chelator and with a temperature of 98°C. Additionally, at the beginning of the P stage, H_2O_2 was added in a single pulse to reach a final concentration of 1.5% (H_2O_2/pulp w/w), which represents a peroxide concentration of 4.41 m*M*. The reaction mixture also included $MgSO_4$ (0.1%), NaOH (1.5%), and sodium silicate (0.25%).

The hydrogen peroxide with oxygen saturation (PO) stage was carried out in a similar way to the P stage with saturation of the headspace of an internally Teflon-covered steel reactor of 500 mL by oxygen (C-40, 99.99%; Carburos Metálicos SA, Barcelona, Spain) to reach an internal pressure of 588.4 KPa and temperature of 110°C. In both cases, a reciprocating shaker kept the reaction mixtures agitated. The length of the P and PO stages was typically 1 h unless otherwise stated. When the effect of the time period of peroxide and pressurized peroxide is evaluated, the number after P and PO indicates the hours of treatment.

Paper pulp was washed with deionized water between stages. The remaining MnP activity, brightness, and κ number were measured at the end of the sequence.

RESULTS

The objective of the optimization of the enzymatic step was the study of the effect of the most important variables in the delignification of kraft pulp by the enzyme MnP. The analyzed variables were the selection and optimal concentration of organic acid, manganese, and hydrogen peroxide.

Optimization of the enzymatic step

The first part of this work was the optimization of the enzymatic step in pulp bleaching. All the experiments during the optimization were carried out with discontinuous addition of hydrogen peroxide. Each parameter was studied independently because in previous works the interaction between these factors was observed to be negligible when the enzymatic system was applied to the degradation of other contaminants: industrial dyes and polycyclic aromatic hydrocarbons.[23,24]

Effect of the organic acid

The selection of the organic acid and its optimal concentration was determined in experiments with the addition of oxalic, malonic, and glycolic acids in concentrations ranging from 0 to 50 m*M*. The results of these experiments are shown in Table I. A statistical analysis was conducted with the software program SSPS for Windows, version 13.0.1 (Lead Technologies, Inc., Charlotte, NC), to determine the best organic acid and its concentration as a function of the reduction of the κ number and the level of remaining MnP activity after the enzymatic treatment. First, an analysis of variance (ANOVA) was carried out to determine if the values obtained at the different organic acid concentrations were significantly different. Then, if the ANOVA confirmed the difference

TABLE I
Effect of Organic Acid Addition on κ Number Reduction

Concentration (m*M*)	Residual activity (U of MnP/g of pulp)[a]	Δκ	Δκ/U of MnP × 100[b]
No acid			
0	8.2 ± 1.1	0.95 ± 0.33	1.86 ± 1.96
Oxalic acid			
0.5	25.3 ± 0.9	0.77 ± 0.43	2.22 ± 2.90
1	35.1 ± 1.6	1.07 ± 0.23	4.30 ± 4.76
5	49.4 ± 0.4	0.51 ± 0.60	4.82 ± 9.33
10	47.4 ± 1.0	0.41 ± 0.71	3.25 ± 9.16
50	28.7 ± 1.7	0.00 ± 0.95	0.00 ± 2.49
Malonic acid			
0.5	10.2 ± 1.1	0.43 ± 0.19	0.86 ± 3.55
1	12.7 ± 1.6	0.53 ± 0.29	1.12 ± 3.12
5	17.8 ± 1.4	0.15 ± 0.11	0.36 ± 4.36
10	33.2 ± 1.0	0.13 ± 0.18	0.48 ± 4.09
50	25.0 ± 1.3	−0.01 ± 0.14	−0.03 ± 2.86
Glycolic acid			
0.5	19.5 ± 2.3	0.70 ± 0.25	1.73 ± 2.32
1	26.5 ± 2.0	0.73 ± 0.34	2.18 ± 3.04
5	29.9 ± 1.6	0.55 ± 0.25	1.83 ± 2.08
10	16.8 ± 3.1	0.13 ± 0.68	0.30 ± 0.79
50	36.0 ± 4.5	0.37 ± 0.30	1.54 ± 3.59

[a] The initial MnP activity in all of the experiments was 60 U of MnP/g of pulp.
[b] Reduction of κ as a function of consumed units of MnP.

between the mean values, a post hoc analysis (Tukey HSD) was used to determine between which values that difference was significant. From this analysis, the best concentration was selected for each organic acid. When no differences could be determined, a median of the results at all the concentrations was calculated. Finally, the control experiment with no acid was compared to the best result obtained for each organic acid or the mean value. This procedure was applied for the two measured parameters, κ number reduction and remaining MnP activity. In all cases, a level of significance of 0.05 was considered. A summary of the results obtained with this statistical analysis is presented in Table II.

According to data, no significant differences were observed between the different concentrations of oxalic and glycolic acids ($p = 0.414$ and 0.400, respectively). In the case of malonic acid, a significant difference was found ($p = 0.023$). The post hoc analysis showed significant differences only between concentrations of 1 and 50 m*M* ($p = 0.031$). On the basis of these results, the lower concentration (1 m*M*), which attained the greatest reduction in the κ number, was selected to be compared with the control and the mean values of the other two acids by the performance of a new ANOVA. The result of this analysis demonstrated no statistically significant differences at the 5% level. Thus, there is no effect of either the organic acid or its concentration on the reduction of the κ number during the enzymatic treatment.

A similar analysis scheme was applied to the remaining MnP activity. In this case, the ANOVA reported significant differences between the acid concentrations, with p values lower than 0.05 in the three cases. By the application of the post hoc analysis to each organic acid, the best results were found at 5, 10, and 5 m*M* for oxalic, malonic, and glycolic acids, respectively. Subsequently, the best results of each acid were compared to the control experiment. The application of a Tukey HSD analysis demonstrated that there was no significant difference between the malonic and glycolic acids ($p = 0.451$), whereas the other two pairwise comparisons were statistically different. This permitted us to conclude that the best results as a function of the remaining MnP activity were obtained with oxalic acid at 5 m*M*.

The statistical analysis confirmed that the remaining MnP activity was positively affected by the organic acid. Thus, when no organic acid was added, the remaining activity after 1 h was approximately 12% of the initial activity. By the addition of a 0.5 m*M* concentration of any of the organic acids, the remaining activity increased up to a mean value of 27%. The extent of this protective effect depended on the organic acid used; thus, when 5 m*M* oxalic acid was used, the remaining activity was 82% of the initial value. To relate the effect on the pulp with the efficient use of the enzyme, the consumed MnP activity and κ number reduction were combined in a parameter, which is denoted Δκ/U of MnP and is pre-

TABLE II
Statistical Analysis of the Effects of Organic Acids on the Enzymatic Treatment

Statistical analysis	Organic acid	Concentration (m*M*)	*p*
κ reduction			
ANOVA	Oxalic	0.5–50	0.414
ANOVA	Malonic	0.5–50	0.023[a]
Tukey HSD	Malonic	1–50	0.031[a,b]
	Malonic	Other pairwise combination	>0.071
ANOVA	Glycolic	0.5–50	0.400
ANOVA	Control	0	
	Oxalic	Mean value	
	Malonic	1	
	Glycolic	Mean value	0.653
Remaining MnP activity			
ANOVA	Oxalic	0.5–50	0.000[a]
Tukey HSD	Oxalic	0.5–50	0.144
	Oxalic	5–10	0.310[c]
	Oxalic	Other pairwise combination	0.000[a]
ANOVA	Malonic	0.5–50	0.000[a]
Tukey HSD	Malonic	0.5–50	0.268
	Malonic	Other pairwise combination	0.000[a]
ANOVA	Glycolic	0.5–50	0.000[a]
Tukey HSD	Glycolic	0.5–1, 0.5–10, 1–5	>0.098
	Glycolic	5–50	0.296[c]
	Glycolic	Other pairwise combination	<0.016[a]
ANOVA	Control	0	
	Oxalic	5	
	Malonic	10	
	Glycolic	5	0.000[a]
Tukey HSD	Malonic–glycolic	10–5	0.451
	Other pairwise combination		0.000[a]

[a] The difference between mean values was significant at a 0.05 level.
[b] The concentration with the greatest κ reduction (1 m*M*) was chosen as the best value.
[c] The concentration with the greatest remaining MnP activity was chosen as the best value.

sented in Table I. On the basis of this parameter, the best result was oxalic acid at 5 m*M*, which agreed with the result obtained from the statistical analysis.

Effect of the Mn^{2+} concentration

Figure 1 shows the influence of adding different exogenous manganese concentrations in a series of experiments with a total addition of 0.5 m*M* H_2O_2 added in 12 pulses as previously described. Lower manganese concentrations were obtained by dialysis of the enzymatic crude because dialysis removed the metal content present in the pulp. It was observed that κ number reduction did not depend on the Mn^{2+} concentration when this parameter was in the range of 0–150 μ*M*. On the other hand, values as high as 550 μ*M* negatively affected lignin oxidation, which was expressed in terms of κ number reduction. For this reason, the Mn^{2+} concentration of 33 μ*M* was selected as the standard value of the enzymatic stage for avoiding the dialysis step and a subsequent addition of more Mn^{2+}.

Effect of the H_2O_2 concentration

Hydrogen peroxide is essential for the activation of the MnP catalytic cycle. On the other hand, an excess

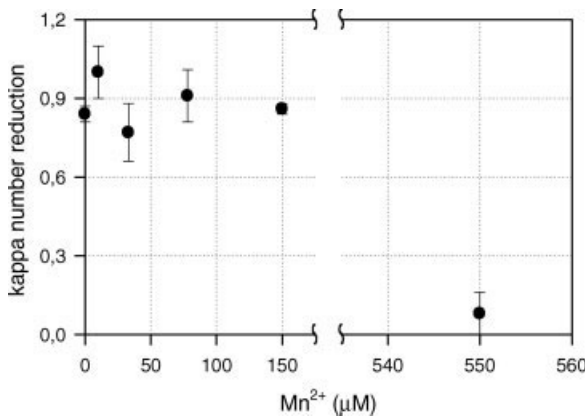

Figure 1 Effect of the manganese concentration on κ number reduction. The Mn^{2+} experimental range was 0–550 μ*M*. The other reaction conditions are described in Table III.

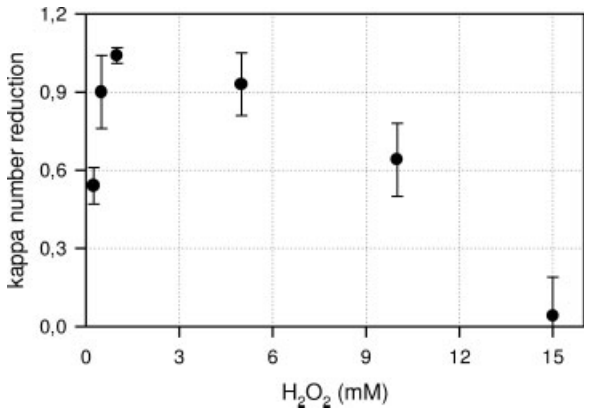

Figure 2 Effect of the hydrogen peroxide concentration on κ number reduction. The experimental range of the H_2O_2 total addition was 0.25–15 m*M*. The other reaction conditions are described in Table III.

of H_2O_2 can produce enzyme inactivation. Figure 2 shows the effect of the hydrogen concentration on the κ number reduction. The κ number reduction quickly reached a maximum at 1 m*M* and then decreased until delignification was not significant at 15 m*M*. At this optimal point, κ number reduction of 1.05 was obtained. However, for subsequent enzymatic stages, a total addition of 0.5 m*M* peroxide was selected. Although reduction of the κ number was slightly lower for 0.5 m*M*, added H_2O_2 was reduced by half.

Bleaching sequences

After optimization of the enzymatic stage, its implementation into industrial biobleaching sequences was studied. In these experiments, the conditions of the enzymatic stages were fixed according to the previously optimized parameters shown in Table III. The enzymatic stage was performed before the chemical stages. The use of single or double chemical sequences and the optimal period and pressure of the P stage were evaluated.

TABLE III
Optimized Parameters for the Enzymatic Stage

Consistency of pulp	1.0% [g of dry pulp/total volume (mL) × 100]
Dose of nondialyzed enzyme	60 U of MnP/g of pulp
Agitation	100 rpm
pH	6.0
Temperature	40°C
Mn^{2+}	33 μ*M*
Organic acid	Oxalic acid
H_2O_2	41.7 μ*M*, 5-min pulses, stock solution concentration of 45 m*M*

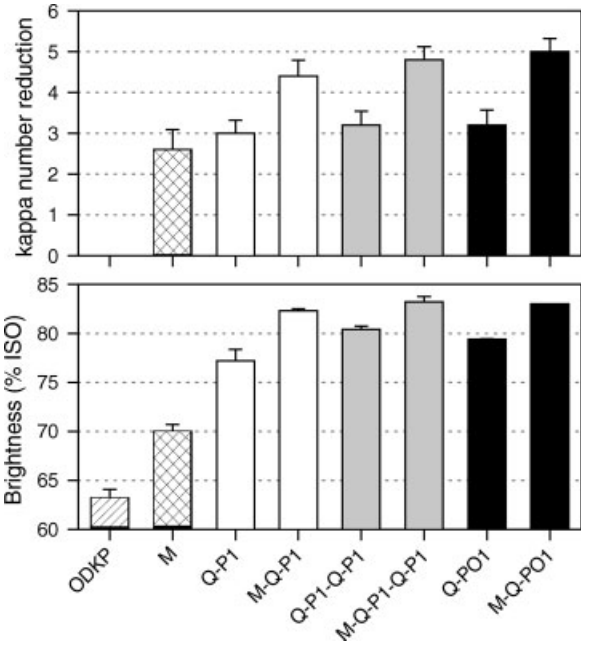

Figure 3 Brightness of ODKP after M, Q–P1, M–Q–P1, Q–P1–Q–P1, M–Q–P1–Q–P1, Q–PO1, and M–Q–PO1. The sequences steps M, Q, P, and PO refer to the enzymatic, chelator, hydrogen peroxide, and hydrogen peroxide with oxygen saturation stages, respectively.

Figure 3 shows the brightness and the reduction of the κ number of pulp paper after M, Q–P1, M–Q–P1, M–Q–P1–Q–P1, Q–PO1, and M–Q–PO1 sequences (where M represents the enzymatic stage). The values of the untreated pulp are also presented. In comparison with the control, pulp bleaching with a single optimized enzymatic stage increased its brightness by 6.8%, whereas a κ number reduction of 2.5 was derived. Moreover, in all the sequences, the introduction of an enzymatic stage improved the final brightness of the pulp and decreased the κ number. Thus, brightness was increased by 5.0, 2.8, and 3.6% ISO for the Q–P1, Q–P1–Q–P1, and Q–PO1 sequences, respectively. For the same sequences, the κ number reduction was increased by 1.4, 1.6, and 1.8, respectively.

The effect of the time period of the peroxide and pressurized peroxide stages on the κ number and brightness can be observed in Figure 4. In chemical sequences with the P stage [Fig. 4(A)], κ number reduction and brightness increased linearly with the duration of that stage. This effect was also observed when M–Q–P1 and M–Q–P2 sequences were compared (82% ISO brightness was obtained with the latter sequence). However, when time was increased from 2 to 3 h, no further improvement was observed.

No significant differences in brightness were observed by a comparison of M–Q–P and M–Q–PO sequences of the same peroxide period [Fig. 4(A,B)].

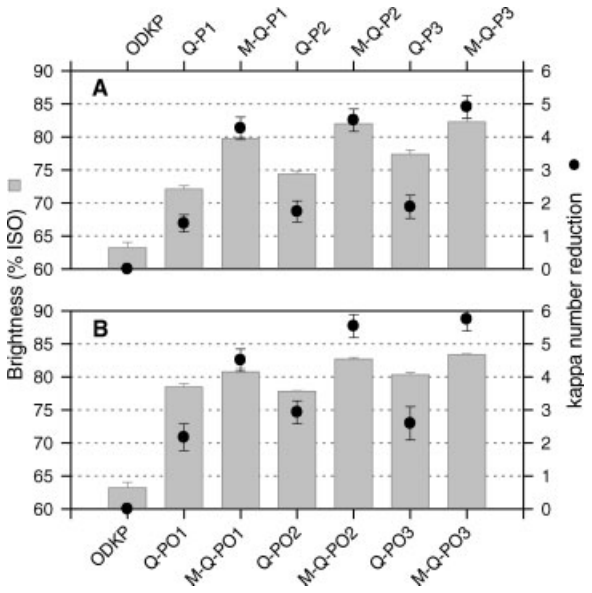

Figure 4 Brightness and κ number reduction at (A) atmospheric and (B) pressurized peroxide stages of 1, 2, and 3 h. The sequences steps M, Q, O, and PO refer to the enzymatic, chelator, hydrogen peroxide, and hydrogen peroxide with oxygen saturation stages, respectively. The index of stages P and PO indicates the duration in hours.

However, a clear positive effect on κ number reduction was obtained with an increase in the pressure of the P stage from atmospheric conditions to 588.4 kPa. Thus, differences in κ number reduction of 1.0 and 0.8 were obtained between M–Q–P and M–Q–PO sequences with 2- and 3-h peroxide periods, respectively. The extent of this positive effect was more important in the case of chemical sequences without an enzymatic stage. Thus, for the sequences with an enzymatic stage, changing from atmospheric pressure to 588.4 kPa increased the reduction of the κ number by a mean value of 14.4%, whereas it reached up to 54.8% for the chemical sequences.

DISCUSSION

The use of ligninolytic peroxidases in the delignification and bleaching of kraft pulp is hampered by the limited action of the enzymes, which results in a poor yield for the delignification process. Therefore, the optimization of the biobleaching process and the enhancement of its production are necessary before their use on a higher scale is considered. In this work, the efficient use of MnP from *Bjerkandera* sp. in the treatment of kraft pulp through the optimization of three parameters related to the activity and stability of the enzyme was attempted. Moreover, the enzymatic process was implemented into a complete industrial bleaching sequence.

The catalytic cycle of MnP implies oxidation of Mn^{2+} to Mn^{3+} by a complex of the enzyme oxidized by H_2O_2.[25] Mn^{3+} is the catalytic agent that causes lignin degradation because it is a high-oxidizing compound that can degrade nonphenolic compounds of this polymer. To avoid the dismutation of Mn^{3+} to MnO_2 and Mn^{2+}, any dicarboxylic or α-hydroxylic acids must be added as Mn^{3+} chelators.[17,26] Malonic acid has been the compound more frequently used as a manganese chelator.[18,27–30] The effect of the mediator used with MnP in kraft pulp biodegradation was previously studied by Bermek et al.[31] They selected the best MnP mediator from different compounds at a fixed concentration, including dicarboxylic acids, unsaturated fatty acids, thiol-containing compounds, and laccase mediators. However, its concentration for being used in pulp bleaching enzymatic stages was not optimized.

In this work, we have selected the best mediator from a list of organic dicarboxylic acids, found its optimal concentration, and determined its relation with MnP stability during the delignification process. Our results indicate that oxalic acid preserves the residual MnP activity (maintaining >80% of the initial activity) after 1 h of enzymatic degradation. Reduction of the κ number is similar in the experiments with all the evaluated acids. When the enzyme stability was taken into account, the optimal results were obtained with 5 m*M* oxalic acid. This acid has been previously proposed to be the physiological chelator for Mn^{2+}.[32] On the other hand, Boe et al.[26] selected lactate, from a list of six organic acids, as the best chelating agent and found that it enhanced the bleaching of kraft pulps.

The manganese concentration is also very important for pulp delignification. It is necessary for the MnP catalytic cycle, but it can also have deleterious effects on subsequent chemical stages. For instance, it is known that high manganese concentrations can reduce the effect of the peroxide stage,[33] and it may also produce darkening of pulps at high temperatures and pH values.[34] In this work, an optimal value of 33 μ*M* was found. Our result is in agreement with a previous work, in which a similar concentration was found to be optimal for the biodegradation of an industrial dye using the same ligninolytic enzyme.[35] However, Moreira et al.,[36] working with the same enzyme and kraft pulp but using malonic acid as the chelator, found an optimal Mn^{2+} concentration of 100–500 μ*M*. It must be observed that very low concentrations of Mn^{2+} decreased κ number reduction to 80% of the maximum. No exogenous manganese was added to the experimental point noted as 0 μ*M* Mn^{2+}, but traces of Mn^{2+} from the pulp or enzymatic crude may have been present. This experiment was performed with acetate-dialyzed enzyme. Thus, the only source of Mn^{2+} was the paper pulp itself. To understand this behavior, the catalytic characteristics of MnP from *Bjerkandera*

TABLE IV
Comparison of the Performances of Different Bleaching Sequences that Included an Enzymatic Step in the Bleaching of the Kraft Pulps

	Reference			
	Bajpai et al.[44]	Wong et al.[45]	Arias et al.[46]	This study
Kraft pulp origin	Eucalyptus	*Pinus radiata* (κ = 69.6)	Eucalyptus	Eucalyptus (κ = 10)
Bleaching sequence	CEH[a]	M–Q–P[b]	Enzymatic–alkaline extraction	M–Q–P2[c]
κ reduction	Not determined	5.1	2.3	2.8
Change in brightness (% ISO)	4.9	Not determined	2.2	7.6

[a] Chlorination, extraction, and treatment with calcium hypochlorite.
[b] Enzymatic, chelating, and hydrogen peroxide treatment.
[c] Enzymatic, chelating, and 2-h hydrogen peroxide treatment.

sp. must be considered. It is known that this enzyme can also act as a VP without manganese.[8,37] As a VP, it could directly oxidize hydroquinones and substituted phenols that are not efficiently oxidized by LiP or MnP in the absence of veratryl alcohol or Mn^{2+}, respectively. This behavior has been extensively described by Martinez.[38]

Another important parameter to be optimized is the H_2O_2 concentration. The optimal hydrogen peroxide concentration depends greatly on the peroxidase origin. It is important to know the optimal peroxide concentration of each ligninolytic peroxidase and the alternative approaches to overcome this limitation. In general, fungal peroxidases become inactivated by concentrations greater than 100 μ*M*.[39,40] In this work, we have found that the most useful alternative is the addition of pulses, from a 45 m*M* solution, every 5 min to reach a maximal H_2O_2 concentration of 41.7 μ*M* in the reaction mixture, which represents a total addition of 0.5 m*M* after 1 h of reaction. In this way, the peaks of peroxide that could inactivate the enzyme are avoided, and MnP activity is preserved longer. The hydrogen peroxide additions are expressed as the concentrations reached after application of the pulse because this is the relevant parameter to be considered in the inactivation of MnP and is the value commonly reported in the inactivation studies. The total addition is useful for comparing the inactivation effect if all the peroxide is added at the beginning of the reaction. It has been previously demonstrated that the inactivation of MnP can be considerably reduced by the use of this strategy with the same enzyme in the decoloration of industrial dyes.[41]

Biobleaching sequences

In this work, not only was an enzymatic stage introduced into a chemical biobleaching sequence, but the combined resulting process was also improved. The three types of sequences studied in this work show that the introduction of an enzymatic MnP stage increases by 2.8–5.1% ISO points the brightness of the pulp in comparison with the chemical treatment (Fig. 3).

The Q–P1 sequence shows a lower result for brightness than the Q–P1–Q–P1 and Q–PO1 ones. However, when an M stage was inserted, the results were similar in the three cases. Therefore, the Q–P1–Q–P1 sequence can be ruled out because no improvement was obtained by the doubling of the chemical sequence. More interesting were the experiments with a long contact time in the P and PO stages [Fig. 4(A,B)]. From these results, we can conclude that it is beneficial to increase the P stage duration to 2 h. In this way, a 7.6% ISO brightness increase and 2.8-point κ number reduction were obtained.

A similar conclusion was obtained from the sequences with a pressurized P stage, for which the 2-h stage seems to be optimal. The best absolute results (83% ISO brightness and 5.5 κ number reduction) were obtained in the latter case. This is a slight improvement of the PO stage against P, perhaps due to the higher stability of peroxide radicals responsible of the ligninolytic effect of these processes. This result is in agreement with the work of Kadla et al.,[42] who applied a P stage at 138 kPa (20 psig) of oxygen pressure and 90°C to the treatment of pine kraft pulp. The use of oxygen pressure during a P stage for the delignification of chemical pulp is a process currently used on an industrial scale,[43] so its application in an enzymatic–chemical process will be straightforward.

The results presented in this work, in terms of brightness and κ number reduction, are similar to those obtained with other commercially available enzymes such as xylanases and laccases (see Table IV). For example, an increase of 4.9% ISO brightness was obtained by Bajpai et al.[44] using commercial xylanases (Cartazyme HS-10, Sandoz Chemicals Ltd., Leeds, UK) in the treatment of eucalyptus kraft pulp with the sequence C–E–H (chlorination, extraction, and treatment with calcium hypochlorite). The application of the laccase-mediator system with commercial (ML-100, Novo Nordisk, Bagsvaerd, Denmark)

and nonfungal (from *Streptomyces cyaneus*) laccases to kraft pulps yielded reductions in the κ number, after peroxide extraction, of 5.1 and 2.3, respectively.[45,46] Considering the promising results presented here, we are planning to test the optimized bleaching sequence in a pulp paper factory on a higher scale.

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