Enhancement of TCF and ECF Bleaching Processes by Urea and Enzymatic Pretreatments: Optimization of a Laccase-Mediator Pretreatment

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A TCF bleaching sequence consisting of a urea pretreatment stage (U), laccase-mediator system stage (L), alkaline extraction stage (E), and hydrogen peroxide bleaching stage (P) was used to study the effect of five independent variables on the dependent variables pulp properties, hydrogen peroxide consumption, and residual enzyme activity. Results showed that the most influential variable was L stage pulp consistency, followed by mediator and laccase dosages. On the other hand, oxygen pressure did not have a significant effect. The optimal UL partial sequence significantly enhanced the EP bleaching sequence: 49.8% *vs.* 33.4% delignification, up to 65.6% ISO *vs.* 56.3% ISO bleached brightness, and 50.3% *vs.* 89.9% peroxide consumption in the P stage. The ULE partial sequence also improved an ECF bleaching sequence ($ULED_0E_1D_1$): 0.6 *vs.* 1.0 final kappa number; 82.7% ISO *vs.* 76.0% ISO brightness; and 54.1 $N{\cdot}m{\cdot}g^{-1}$ *vs.* 51.9 $N{\cdot}m{\cdot}g^{-1}$ and 3.3 $KPa{\cdot}m^2{\cdot}g^{-1}$ *vs.* 2.7 $KPa{\cdot}m^2{\cdot}g^{-1}$, tensile and burst indexes, respectively, when compared to the control $D_0E_1D_1$ sequence.

Keywords: Pycnoporus sanguineus; Urea; Bleaching variables; Optimization; Eucalyptus globulus; Kraft pulp

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INTRODUCTION

Totally chlorine-free (TCF) processes have been developed as an alternative to elemental chlorine-free (ECF) bleaching to minimize halogenated organic compounds (AOX) emissions and to eliminate chlorinated dioxins and furans. Nevertheless, TCF bleaching has turned out to be less lignin-selective than the chlorine-based or ECF methods (Dence and Reeve 1996).

Biotechnology may be a useful resource to address some of the environmental issues associated with pulp bleaching. There is relevant literature on the application of ligninolytic enzymes, such as laccases, prior to conventional bleaching (Camarero *et al.* 2007; Eugenio *et al.* 2011; Martín-Sampedro *et al.* 2012); pulps produced using ligninolytic enzymes are of comparable quality with conventional pulps with a significant reduction of chemicals needed for bleaching. Using laccase in this field is a sensible choice, based on its capacity to oxidize a large number of lignin derivatives and phenolic compounds; furthermore it requires only the presence of molecular oxygen and certain low molecular weight compounds known as mediators. These mediators, once oxidized by the laccases, become stable radicals that react with other compounds that were not initially the

immediate substrates (Bourbonnais and Paice 1992; Camarero *et al.* 2007; Barneto *et al.* 2012).

This general scheme presents some potential drawbacks, however, such as high cost and/or the toxicity of the synthetic mediators. These drawbacks prevent the application of the laccase mediator system (LMS) in the mill bleaching process at the industrial scale. Consequently, active investigations are ongoing to develop non-toxic mediators and to use laccases with physicochemical features that make them optimal for biobleaching purposes. Nevertheless, these studies often do not comprehensively assess all of the variables pertinent to an enzymatic process, either individually or in combination, and all of the bleached pulp properties.

On a different level, biobleaching may become more effective after particular physical or non-oxidative chemical pretreatments; expectations are high as to how these pretreatments could lead to their industrial application in the bleach plant. Physically, refining modifies the morphology of the fibers in aqueous solution, which results in improved mechanical properties of the paper sheet. Although it has been traditionally a post-bleaching process, refining has been recently applied before biobleaching with promising results (Eugenio and Villar 2012; Lian *et al.* 2012; García-Fuentevilla *et al.* 2013). On the other hand, an example of a non-oxidative chemical option is urea pretreatment, which might disentangle the fiber structure and induce the solution of lignin, hexenuronic acid (HexA), or chromophores. So far only preliminary studies have been carried out in our Centre (Eugenio and Villar 2012; García-Fuentevilla *et al*. 2013) suggesting that the urea pretreatment could enhance a subsequent biobleaching process. To the best of our knowledge, only the study by García-Fuentevilla *et al.* (2013) has examined a biobleaching sequence that includes pretreatment with urea (U), refining (R), and their combination. These authors concluded that optimized U and R, both separately and in combination, boosted the LE biobleaching partial sequence (LMS treatment followed by alkaline extraction). Maximal delignification was achieved by adding urea as the first stage in the partial delignification sequence (ULE and URLE). Compared to the ULE partial sequence, URLE pulps had better mechanical properties (increase of 97%, 149%, and 98% in the tensile, tear, and burst indexes, respectively) but slightly lower brightness (decrease of 2.8% points of ISO brightness). Therefore, the optimal choice of ULE or URLE depends on the final use of the pulp (*e.g.*, whether it is good optical properties or physical strength that are required).

For the present study, optical properties were set as a priority, and the biobleached pulp subjected to further chemical bleaching stages to reach the level of brightness required by the industry. Therefore, before optimizing the enzymatic treatment, a urea pretreatment has been applied under the same optimal conditions as those detailed earlier by García-Fuentevilla *et al.* (2013).

In summary, and taking all of the above points into consideration, the objectives of this study were two-fold: (1) to add a laccase-enriched culture from *Pycnoporus sanguineus* to a urea pretreated eucalyptus kraft pulp, and to optimize this enzymatic step (L stage) by adjusting oxygen pressure, laccase and mediator concentration, pulp consistency, and time; and (2) to study the influence of the optimized enzymatic pretreatment compared to a standard ECF industrial sequence ($D_0E_1D_1$, where D is chlorine dioxide and E is alkaline extraction).

EXPERIMENTAL

Materials

Raw material

Industrial-grade unbleached *Eucalyptus globulus* kraft pulp was provided by the La Montañanesa pulp mill (Torraspapel-Lecta Group, Spain). This unbleached pulp had a kappa number of 14, brightness of 35% ISO, and intrinsic viscosity of 1166 $mL \cdot g^{-1}$.

Chemicals

All chemicals were reagent-grade purity. Sodium hydroxide, urea, $MgSO_4$, and H_2O_2 were purchased from Panreac Química SAU (Barcelona, Spain), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonate (ABTS) from Roche (Madrid, Spain), diethylene-triamine-pentaacetic acid (DTPA) from Merck (Barcelona, Spain), and acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone) from Sigma-Aldrich (Madrid, Spain). Chlorine dioxide was generated *in situ* from the reaction of sodium chlorite and sulfuric acid; both reagents were purchased from Panreac Química SAU (Barcelona, España). All generated chlorine dioxide was tritrated iodometrically.

Methods

Urea pretreatment

This pretreatment is intended to improve the subsequent biobleaching process, and is based on the report of García-Fuentevilla *et al.* (2013). The urea pretreatment was applied to an unbleached *E. globulus* kraft pulp in polyethylene bags submerged in a thermostatic water bath under the following conditions: 8 M urea concentration, 15% consistency, 8.8 pH, 2 h reaction time, and 80 °C reaction temperature. After this treatment, the pulp was washed with distilled water until the pH of the wash filtrate reached a neutral pH.

Experimental design for optimization of the enzymatic bleaching process, alkaline extraction, and hydrogen peroxide bleaching

Urea pretreated pulp (50 g o.d.) was placed into a reactor and thoroughly mixed with laccase and chemicals before being submerged in a thermostatic water bath. The laccase solution used in this study was the laccase-enriched extracellular liquid obtained from *Pycnoporus sanguineous,* where the laccase activity was 1.9 $U \cdot mL^{-1}$. This was measured with the method of Mansur *et al*. (2003) using ABTS as the substrate. The laccase activity unit, U, was defined as the amount of enzyme needed to produce 1 μmol of product per min at the specific test conditions. Temperature and pH remained invariant during the laccase pre-treatment at 40 °C and pH 3. The choice of temperature and pH was based on previous work (Eugenio *et al.* 2010a,b). Acetosyringone was the natural mediator used in all enzymatic treatments. Also, a few drops of 0.05% Tween 80® solution were added to enhance the interaction between the enzyme and substrate. The independent variables of laccase concentration, mediator concentration, pulp consistency, oxygen dosage, and reaction time were modulated per a statistically designed five-level, three-factor factorial experimental program (*i.e.*, an extended central composite design). In order to be able to establish associations between dependent (detailed below) and independent variables while keeping the number of experiments to a minimum, an orthogonal main-effect design was used, which consisted of a central point (*i.e*., central experiment, in the centre of a cube, duplicated) and 26 additional points (*i.e*., additional experiments lying at the cube vertices) (Table 1). All independent variables were evaluated at five levels: low (-2.28), medium-to-

low (-1), center point or medium (0), medium-to-high (+1), and high level (+2.28). This experimental design enabled the construction of first-order polynomials for the dependent variables as a function of the independent variables, and the detection of statistical significance with the variables (Akhnazarova and Kafarov 1982). The polynomial model used was of the following type,

$$Z = a_0 + \sum_{i=1}^{n} b_i X_{ni} + \sum_{i=1}^{n} c_i X_{ni}^2 + \sum_{i=1;\, j=1}^{n} d_i X_{ni} X_{nj} \quad (\mathrm{i} < \mathrm{j}) \tag{1}$$

where Z and X_{ni} denote dependent and normalized independent variables, respectively, and a_0, b_i, c_i, and d_{ij} are unknown coefficient constants obtained from statistical experimental data. Independent variables were normalized (X_n) (*i.e.*, coded),

$$X_n = \frac{X - X_{mean}}{\left(X_{max} - X_{min}\right)/2} \tag{2}$$

where X is the absolute value of the independent variable concerned, X_{mean} is the average value of the variable, and X_{max} and X_{min} are maximum and minimum values of the variable, respectively.

Table 1. Experimental Design of the Extended Central Composite Design Matrix Used in the Study*****

Experiment	Oxygen	Laccase	Mediator	Consistency	Time
1	1	1	1	-1	-1
2	1	-1	-1	-1	-1
3	0	2.28	0	0	0
4	-1	-1	1	1	1
5	-1	-1	-1	-1	1
6	-2.28	0	0	0	0
7	-1	-1	-1	1	-1
8	0	0	0	0	0
9	0	-2.28	0	0	0
10	0	0	0	0	0
11	1	1	1	1	1
12	0	0	0	0	2.28
13	1	-1	-1	1	1
14	1	-1	1	-1	1
15	0	0	2.28	0	0
16	1	-1	1	1	-1
17	-1	1	1	1	-1
18	-1	1	-1	-1	-1
19	-1	1	1	-1	1
20	0	0	-2.28	0	0
21	1	1	-1	-1	1
22	2.28	0	0	0	0
23	0	0	0	-2.28	0
24	0	0	0	0	-2.28
25	1	1	-1	1	-1
26	-1	1	-1	1	1
27	0	0	0	2.28	0
28	-1	-1	1	-1	-1

***** Values of oxygen, laccase, mediator, pulp consistency, and time are the normalized values

The independent variables used in Eqs. 1 and 2 were those having a statistically significant coefficient (*viz*. those having significance level of 0.05 or less in the Student's *t*-test, and not including zero coefficient in their 95% confidence interval).

Ranges of values for the independent variables used in the experimental design (time (min), oxygen (KPa), pulp consistency (%), laccase ($U \cdot g^{-1}$) and mediator ($mmol \cdot g^{-1}$)) are shown in Table 2.

Table 2. Ranges of Values of the Independent Variables Used in the Experimental Design Considered in this Study

Normalized Value of the L stage	2.28	1	0	-1	-2.28
O_2 (KPa)	816.9	588.4	408.0	228.5	0.00
Laccase ($U \cdot g^{-1}$)	4.80	3.46	2.4	1.34	0.00
Mediator ($mmol \cdot g^{-1}$)	0.100	0.072	0.050	0.028	0.00
Consistency (%)	13.1	10.0	7.55	5.1	2.0
Time (min)	105.0	79.8	60.0	40.2	15.0

After the enzymatic stage (L stage) of each experiment, the effluent was recovered and the residual laccase activity measured using the same method mentioned earlier (Mansur *et al*. 2003). All pulps were washed with distilled water until the wash filtrate was neutral in pH. Then, alkaline extraction (E) was carried out with 1.5% NaOH at 90 °C for 120 min with a 5% pulp consistency. Subsequently, the extracted pulps were first washed, and secondly, subjected to a hydrogen peroxide bleaching (P) stage with the goal of evaluating the effect of all enzymatic treatments on a standard chemical bleaching (P). Operational conditions of the peroxide stage were: 3% H_2O_2, 1.5% NaOH, 1% DTPA, 0.2% $MgSO_4$, and 5% pulp consistency at 90 °C for 90 min. Residual hydrogen peroxide was measured in the bleaching stage filtrate by standard iodometric titration. Finally, all treated pulps were analyzed in terms of their kappa numbers, brightness levels, and intrinsic viscosities in accordance to the ISO 302 (2004), ISO 2470-1 (2009), and ISO 5351-2 (1981) standards, respectively. Therefore, the properties of the dependent process variables for each model are: kappa number, brightness (% ISO) and intrinsic viscosity ($mL \cdot g^{-1}$) after L, E, and P stages; residual enzyme activity (%) after the L stage; and amount of peroxide consumed (%) after the P stage.

Industrial ECF bleaching

After selecting the optimal operational conditions for the enzymatic treatment according to the experimental design, we examine the benefits of this pretreatment process with an industrial ECF sequence.

Table 3. Operational Conditions of $D_0E_1D_1$ Treatments

Stage Condition	D_o	E_1	D_1
Consistency (%)	10	10	10
Time (min)	40	120	180
Temperature (°C)	55	70	80
pH	2.5 to 3	—	5 to 5.5
Active Chlorine (%)	3 to 3.5	—	0.9 to 1.2
NaOH (%)	—	2.1	—

The ECF sequence consisted of two chlorine dioxide stages in series with an intermediate alkaline extraction stage ($D_0E_1D_1$); this partial sequence was used in place of the P stage. Thus, the studied biobleaching sequence was $ULED_0E_1D_1$, which consisted of a urea pretreatment (U), an optimized enzymatic treatment (LE), and an ECF bleach sequence mentioned earlier. The same sequence without the urea and enzymatic treatment (*i.e.*, $D_0E_1D_1$ only) was tested as the control. The operational conditions of the $D_0E_1D_1$ sequence are shown in Table 3.

Handsheet characterization

Handsheets were formed from the bleached pulps obtained from the $D_0E_1D_1$ and $ULED_0E_1D_1$ bleaching sequences using the ISO 5269-2 (2004) standard before and after refining in a PFI mill (2000 revolutions), which was performed in accordance to the ISO 5264-2 (2011) standard. Kappa number, brightness, and physical strength properties (*i.e.*, tensile, tear, and burst indexes) of the handsheets were determined in accordance to ISO 302 (2004), ISO 2470-1 (2009), and ISO 5270 (2012) standards, respectively.

RESULTS AND DISCUSSION

Enzymatic Bleaching Process Optimization

Table 4. Model Equations for Each Dependent Variable as a Function of the Independent Variables

Polynomial Model Equations	r^2	*df*	*F*
BL = 36.79 + 0.28 O -0.19 L -0.48 M -0.36 C -0.19 T -0.30 O^2 + 0.17 L^2	0.81	7.2	15.6
BE = 39.38 -0.18 M -0.17 T -0.28 OC +0.19 OT +0.16 LC -0.23 MC	0.63	5.96	6.23
BP = 64.64 + 0.17 O + 0.58 M + 0.95 C -0.25 M^2 -0.66 MC	0.65	5.22	8.23
KL = 11.95 -0.06 O^2 -0.10 C^2 -0.14 LT +0.14 MC	0.52	4.23	5.51
KE = 10.24 -0.05 O -0.06 M -0.08 C -0.08 T +0.08 M^2 +0.09 C^2 -0.09 OL -0.11 LM -0.09 CT	0.86	9.18	8.42
KP = 7.01 -0.20 C +0.11 O^2 +0.09 L^2 +0.20 M^2 +0.13 C^2 +0.15 T^2 -0.17 OL	0.79	7.20	6.64
VL = 1100.86 +10.34 O -4.82 L -3.78 C -9.61 T +5.37 L^2-5.42 M^2 +7.24 C^2 +4.68 OT -7.89 LM +13.06 LC +4.79 LT-3.66 CT	0.94	12.15	31.5
VE = 1113.60 -13.74 T +6.75 C^2 +11.88 OT -10.88 LM +7.03 LC +7.98 CT	0.71	6.21	7.92
VP = 1007.78 +7.84 O -14.07 L -9.88 M -18.37 T -8. 80 L^2 +8.29 OT -13.34 LM +14.73 LC +9.08 LT +12.60 CT	0.84	10.17	9.20
HPC = 55.69 + 1.39 M^2 + 3.64 OC -2.26 LC -5.69 CT	0.56	4.23	7.40
REA = 82.18 +12.31 L + 4.68 M +4.92 C -2.43 O^2 -5.46 L^2 -2.00 M^2 +6.75 C^2 -11.24 OT -9.98 LM -4.24 LC	0.96	10.17	34.55

BL = brightness with laccase treatment (% ISO), BE = brightness with extraction treatment (% ISO), BP = brightness with hydrogen peroxide treatment (% ISO), KL = kappa number with laccase treatment, KE = kappa number with extraction treatment, KP = kappa number with hydrogen peroxide treatment, VL = intrinsic viscosity with laccase treatment ($mL \cdot g^{-1}$), VE = intrinsic viscosity with extraction treatment ($mL \cdot g^{-1}$), VP = intrinsic viscosity with hydrogen peroxide treatment ($mL \cdot g^{-1}$), HPC = hydrogen peroxide consumption (%), and REA = residual enzymatic activity (%), as dependent variables
O, L, M, C, and T denote the normalized value of the oxygen, laccase, mediator, pulp consistency and time, respectively, as independent variables.
df denotes the degrees of freedom and *F* denotes the computed *F*-distribution statistic.

The normalized values of the independent variables and the properties of the pulp obtained with the proposed experimental design (Table 1) were correlated. The value shown for each of these properties resulted from averaging three experimental results. Deviations of these parameters from their respective means were all less than 10%. The polynomial models generated for all the dependent variables are shown in Table 4. The differences between the experimental values and the estimations made with these models never exceeded 10% of the former (20% for kappa number after laccase treatment, brightness after extraction and hydrogen peroxide treatments, and hydrogen peroxide consumption).

Identifying the independent variables that have the most and least influence on the dependent variables of the polynomial models shown in Table 4 may be challenging because of the interactions between two independent variables. For this reason, a Pareto chart, also known as Pareto distribution diagram, was used to determine those independent variables ($p > 0.05$) that had the greatest cumulative effect in this study. Figure 1 shows a plot of each dependent variable (compound) and its Pareto chart of the standardized effects (as percentages) based on the independent variables.

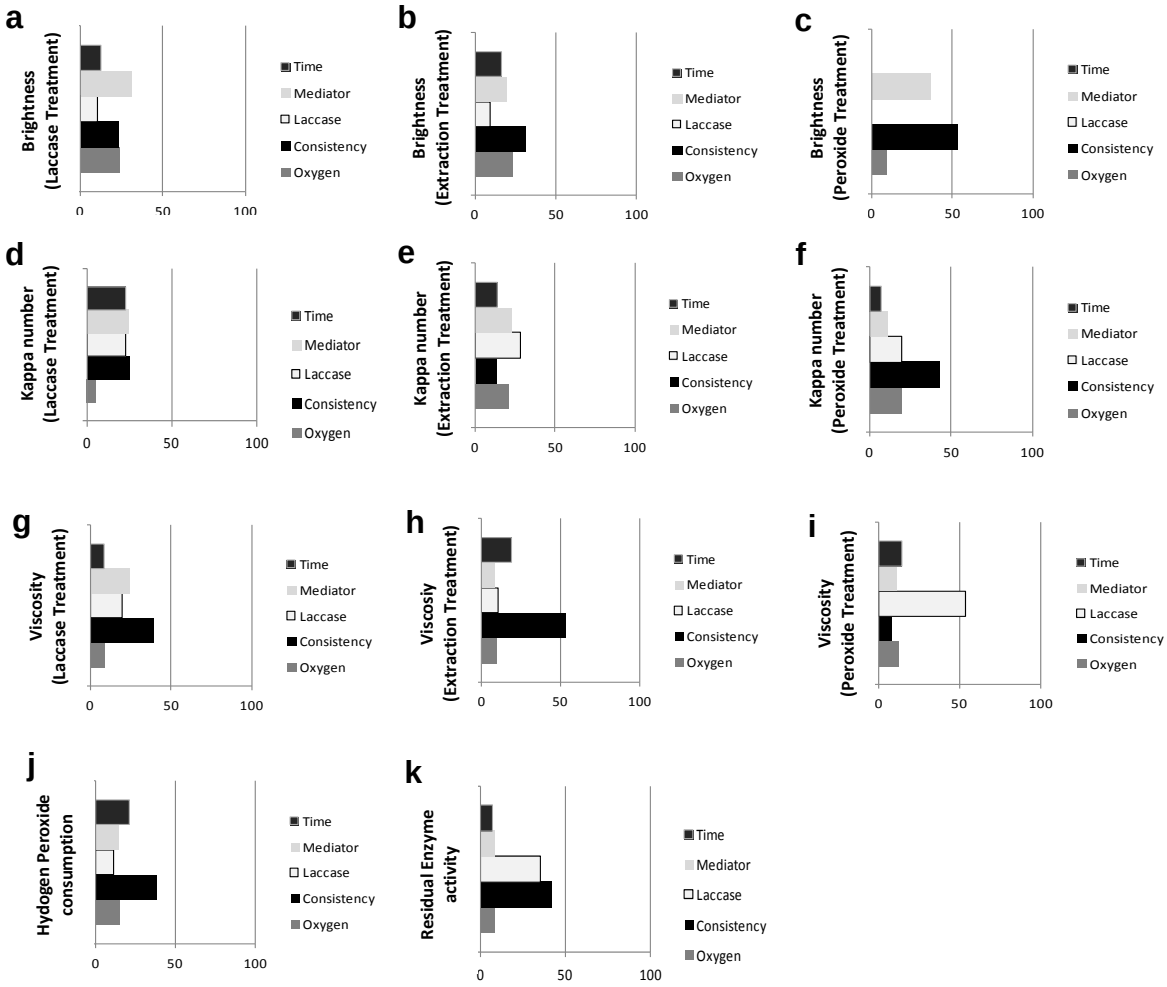

Fig. 1. Pareto charts of standardized effects (as percentages) based on independent variables, for each dependent variable

These charts indicated that pulp consistency in the L stage was the most important parameter of the biobleaching process because it was the variable that had the greatest influence on brightness after extraction (E) and hydrogen peroxide (P) stages, kappa number after laccase (L) and P stages, intrinsic viscosity after L and E stages, consumption of hydrogen peroxide, and residual enzymatic activity. The mediator dosage was the second most influential variable on brightness, while the laccase dosage showed a strong influence on kappa number, intrinsic viscosity after P stage, and residual enzymatic activity.

Each dependent variable was plotted against the three most influential independent variables. The two least influential independent variables were included in the calculation as constants (mean). These graphs were used to determine what values of these most influential independent variables optimized the dependent variables (Figs. 2, 3, 4, and 5).

Figure 2 shows the response surface for brightness. As expected, brightness in the laccase treatment (Fig. 2a) and in the extraction treatment (Fig. 2b) increased when the oxygen pressure was high. However, oxygen pressure was found to have little statistical influence on brightness after the hydrogen peroxide treatment (Fig. 2c). Other authors have also reported the lack of association between oxygen pressure and brightness after an LEP sequence where the L stage was shorter than 3 h (Fillat and Roncero 2009; 2010a; Martín-Sampedro *et al.* 2011; 2014). Brightness after the laccase treatment primarily decreased with increasing mediator dosages, as opposed to what was observed in the extraction and hydrogen peroxide treatment, especially at the lower consistencies. Barneto *et al*. (2012) reported that when the mediator structure is based on a syringyl unit (*i.e.*, with both methoxyl groups *ortho* to the phenolic group), such as is the case of acetosyringone, the laccase-induced reactions reduced the pulp's brightness significantly and shifted the CIE *L*a*b** color coordinates after the L stage.

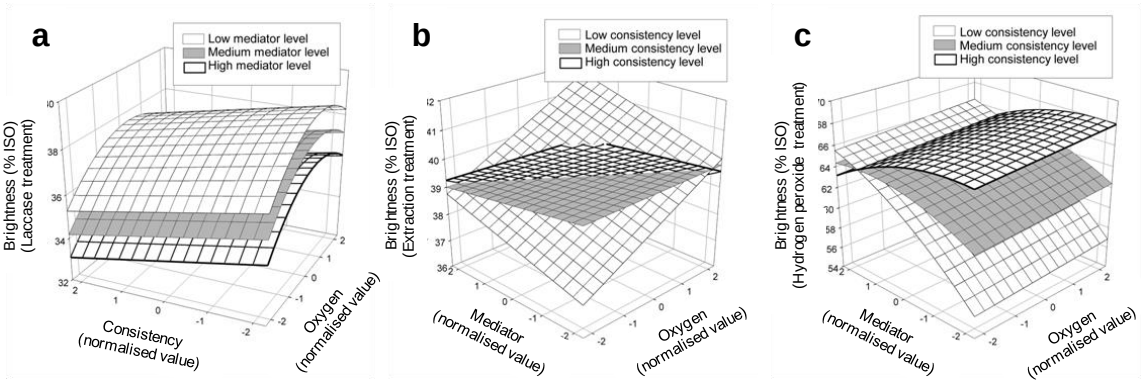

Fig. 2. Response surfaces for brightness after (a) laccase pretreatment, (b) alkaline extraction, and (c) hydrogen peroxide bleaching

The color shift suggested the formation of substantial amounts of chromophoric groups as a result of the enzymatic polymerization of the syringyl derivates (Barneto *et al*. 2012). Therefore, it is reasonable to conclude that the higher the dose of mediator, the more numerous the chromophoric groups formed, and consequently, the lower the brightness after the L stage. However, during the E and P stage treatments, these chromophoric groups are oxidized and dissolved (Aracri *et al.* 2012), which results in an increase of brightness. Furthermore, these natural mediators are simultaneously involved in lignin oxidation reactions (Aracri *et al*. 2012; Barneto *et al*. 2012), which would also increase the brightness

after the E and P stage (*i.e.*, the lignin degradation products formed in the L stage are dissolved and removed). In contrast to our observations with low and medium pulp consistencies, when pulp consistency was high, brightness after the P treatment increased when the mediator dose decreased from 0.1 to 0.05 mmol·g^{-1}, and remained almost unaltered with further reduction of the mediator dose. The reason for this effect could be the formation of such a great amount of chromophoric groups and lignin-degradation products in the L treatment (as a result of the high concentration of mediator and pulp) that cannot be completely removed in subsequent E and P treatments.

Finally, as Fig. 1 illustrated, the independent variable that influenced brightness the most (especially after the P treatment) was the pulp consistency in the L stage. This means that higher L stage consistencies resulted in higher brightness after the LEP sequence (except for when the mediator dose was the highest). Higher L stage consistencies can be expected to afford better contact of the fibers with laccase-oxidized mediator, as well as to contribute to laccase stability (Ibarra *et al.* 2006; Moldes and Vidal 2008). However, Moldes and Vidal (2008) reported no significant changes in brightness after LEP bleaching of eucalyptus kraft pulp (using a commercial laccase from *Trametes villosa* and HBT (1-hydroxybenzotriazole) as a mediator) when the pulp's consistency in the L stage increased from 5% to 10%. Nevertheless, these authors selected the highest L stage consistency as optimum condition since it substantially reduced the water consumption and it was a more realistic condition for mill-scale implementation. When taking into consideration the influence of all independent variables on the final brightness at the end of the biobleaching, only high pulp consistency (13.1 to 10 %) and low or medium mediator dosage (0 to 0.05 mmol g^{-1}) can be chosen as the optimum conditions; all the other independent variables did not significantly affect the brightness after the P stage.

Figure 3 illustrates the relationship between the kappa number after each stage and the independent variables. Similar to what was observed with brightness after each stage, increased delignification (*i.e.*, lower kappa numbers) were associated with higher L stage consistency; likewise, this can also be attributed to better contact between laccase-oxidized mediator and fibers, as well as laccase stabilization, when utilizing a higher L stage consistency.

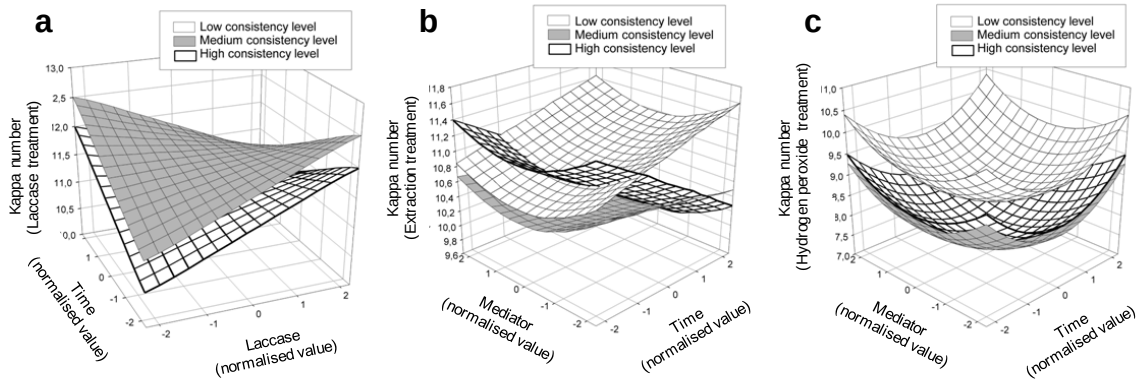

Fig. 3. Response surfaces for kappa number after (a) laccase pretreatment, (b) alkaline extraction, and (c) hydrogen peroxide bleaching

After the L stage, lower kappa numbers were found in two different situations: low laccase dosages and short treatment times, or high laccase doses and long treatment times. These results suggested that when the laccase dosage is higher, a longer treatment time is

needed to give the laccase sufficient reaction time. Accordingly, Fillat and Roncero (2009) reported that using the highest laccase dosage in combination with the longest L stage reaction time resulted in the lowest kappa number. On the other hand, the use of a low laccase dosage and a long reaction time probably favors the polymerization and grafting of the mediator onto the fibers versus lignin oxidation, due to a high mediator/laccase ratio, causing an increase of the kappa number.

After the E stage, a long enzymatic treatment was needed to lower the kappa numbers of the higher L stage consistencies, whereas low L stage consistencies also yielded low kappa numbers at shorter reaction times. In both the E and P stages, the laccase dosage did not significantly influence the kappa number; thus, an intermediate mediator dosage (0.05 mmol·g^{-1}) was judged to be optimum for pulp delignification. Fillat and Roncero (2010b) also found that medium mediator doses provide lower kappa numbers after the P stage. Finally, the lowest kappa numbers after the P stage were obtained when a medium or high L stage consistency (7.5 to 10%) and an intermediate L stage reaction time (60 min) were employed. Moldes and Vidal (2008) also reported that increasing the L stage reaction time seemingly resulted in increased delignification and brightness after the L stage; however, the differences did not carry through the LEP partial sequence. These authors concluded that longer L stage reaction times were not justified for the negligible differences observed after the LEP partial sequence.

The response surfaces for intrinsic viscosity are shown in Fig. 4. Intrinsic viscosity is an indirect indicator of the length of the cellulosic chains in the pulp. Thus, a low intrinsic viscosity value indicates the degradation of the cellulose during the biobleaching sequence. In general, the differences in intrinsic viscosities for the various experimental conditions were low. After the L and E stages, the reduction in intrinsic pulp viscosity were at the level of 1 to 8%; this may be attributed to the acid hydrolysis in the L stage (buffer at pH 3). As expected, intrinsic viscosity was maximally reduced after the P stage (reductions between 9 and 29%, compared to the unbleached pulp).

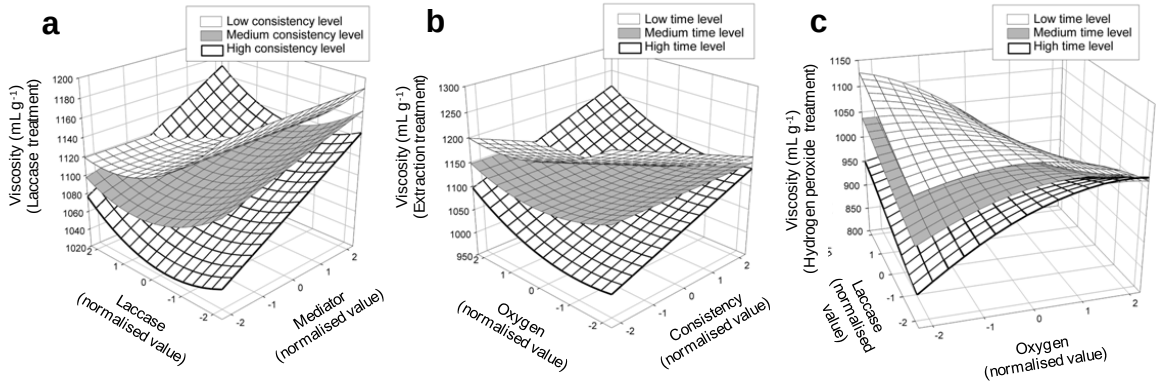

Fig. 4. Response surfaces for intrinsic viscosity after (a) laccase pretreatment, (b) alkaline extraction, and (c) hydrogen peroxide bleaching

Based on the response surfaces, if excessive cellulose degradation at the end of the LEP sequence is to be minimized, an L stage should be operated at a short reaction time with a medium laccase and mediator dosage level. These observations were consistent with the findings previously reported by Fillat and Roncero (2009), who reported a decrease in pulp viscosity with longer L stage reaction times and higher reagent dosages with LMS treatment of flax pulp. Other authors have observed that LMS pretreatment has a minimal

impact on carbohydrate degradation during the biobleaching sequence, and this effect accounts for the high selectivity of biodelignification without lowering pulp viscosity (Oudia *et al.* 2008; Eugenio *et al*. 2010a).

Figure 5 shows the response surfaces for hydrogen peroxide consumption during the P stage (Fig. 5a) and for residual enzyme activity after the L stage (Fig. 5b). Pulp consistency and treatment time in the L stage clearly affected the hydrogen peroxide consumption. When the L stage consistency was low, more hydrogen peroxide was consumed in the P stage as the L stage reaction time increased. However, when higher L stage consistencies were used, the effect of longer L stage reaction time afforded the opposite results. When medium consistencies in the L stage were used, there were no observed effects on peroxide consumption as the L stage reaction time was increased. The effect on the kappa number after the E stage was similar (Fig. 3b): the enzymatic treatment resulted in the highest kappa numbers after alkaline extraction (E) when pulp-consistency was the highest and the treatment time shortest, and *vice versa*, when pulp consistency was lowest and treatment time longest. This is evidence of the presence of a significant amount of lignin in the pulp that would consume more bleaching reagent in the subsequent hydrogen peroxide treatment.

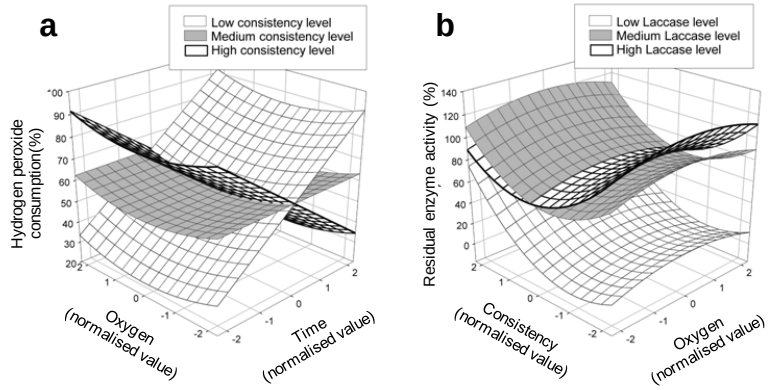

Fig. 5. Response surfaces for (a) hydrogen peroxide consumption and (b) residual enzyme activity

Regarding the residual enzyme activity after the L stage (Fig. 5b), the most influential variables were L stage consistency and laccase dosage. Unsurprisingly, a higher laccase dosage resulted in higher post L stage residual enzyme activity. Additionally, and as previously reported by others (Ibarra *et al*. 2006; Moldes and Vidal 2008), L stage consistency contributed to laccase stabilization (*i.e*., high residual enzyme activity). And a more stable laccase is also more effective, which partly explains why pulp consistency has a significant influence in brightness and kappa numbers, as it was discussed above.

In summary, the optimum conditions for the enzymatic treatment (L stage) would be those that provided the highest delignification and brightness improvement at the end of the bleaching sequence without an excessive reduction of pulp intrinsic viscosity while maintaining low hydrogen peroxide consumption and high residual enzyme activity. Accordingly, these L stage conditions where selected as optimum: 10% pulp consistency, 3.46 $U \cdot g^{-1}$ laccase dosage, 0.05 $mmol \cdot g^{-1}$ mediator dosage, 60 min treatment time, and 408.0 KPa oxygen pressure. Taking also into account the urea pretreatment, this optimal sequence afforded a total delignification of 49.8% and an overall increase in brightness of 30.1% ISO units; these optimized conditions caused the pulp intrinsic viscosity to decrease

by 14.3%, and caused the P stage to consume 50.3% of the applied hydrogen peroxide. Furthermore, the residual enzyme activity remained almost intact when the optimal conditions were employed (*i.e.*, 96.5% residual activity), which indicated that it could be recycled back into the L stage to lower the enzymatic operational cost. A control sequence without urea and enzymatic treatments (*i.e.*, EP sequence) resulted in much lower delignification (33.4%) and overall brightness increase (21.3% ISO units); the control sequence caused a similar decrease in intrinsic viscosity (11.8%) while consuming significantly more of the applied hydrogen peroxide (89.9%) in the P stage.

Optimum Enzymatic Pretreatment Applied to an Industrial ECF Bleaching Sequence

After selecting the optimal conditions of the enzymatic treatment, the P stage was replaced by an ECF sequence consisting of two chloride dioxide stages in series with an intermediate alkaline extraction stage ($D_0E_1D_1$). The goal was to study what effect the enzymatic treatment would have on an industrial bleaching sequence, in which final brightness and degree of delignification were higher to meet the requirements demanded of commercially bleached pulp. As it was performed in the experimental design, before the enzymatic treatment, a urea pretreatment was carried out based on results reported by García-Fuentevilla *et al*. (2013). Thus, the final bleaching sequence was $ULED_0E_1D_1$, which was compared to a $D_0E_1D_1$ control sequence. Handsheets were made before and after PFI refining; kappa number, brightness, and mechanical properties of the obtained handsheets are listed in Table 5.

Table 5. Kappa Number, Brightness, and Mechanical Properties of Handsheets from $D_0E_1D_1$ and $ULED_0E_1D_1$ Bleached Pulps Before and After PFI Refining

	Before PFI Refining		After PFI Refining	
	$D_0E_1D_1$	$ULED_0E_1D_1$	$D_0E_1D_1$	$ULED_0E_1D_1$
Kappa number	1.0	0.6	-	-
Brightness	81.3	86.8	76.0	82.7
Schopper Riegler (ºSR)	14	16	24.0	33.0
Tensile Index ($N \cdot m \cdot g^{-1}$)	19.0	20.2	51.9	54.1
Tear Index ($mN \cdot m^2 \cdot g^{-1}$)	2.7	2.5	9.1	8.9
Burst Index ($KPa \cdot m^2 \cdot g^{-1}$)	-	-	2.7	3.3

The urea and enzymatic treatments (*i.e.*, ULE partial sequence) increased the delignification during the bleaching sequence from 93% in the control sequence to 96%. An increase in brightness of 6.7% ISO units was also observed in refined pulps when the pretreatment was carried out when compared with the control sequence. Kandioller and Christov (2012) found a similar increase in brightness (between 2.9 and 12.2% ISO units, depending on the pulp type) associated with an LMS pretreatment prior to DED bleaching. These authors reported that this increase in brightness also means a reduction of 40 to 60% in chlorine dioxide consumption, which is consistent with results of Bajpai *et al*. (2007), who reported a 45.6% reduction of chlorine dioxide requirements when an LMS treatment was applied prior to DED bleaching. Madlala *et al*. (2001) also reported an increase in brightness of 5% ISO units or a 30% reduction of chlorine dioxide usage when applying a xylanase pretreatment before ECF bleaching.

As expected, refining of the pulps increased the mechanical properties but reduced brightness. Refining is known to cause fibrillation, a process in which fibrils are generated

on the surface of fibers and become intertwined with other fibrils from adjoining fibers. This situation improves the bonding of the fibers with one another, thus improving the mechanical properties of the handsheets. Darkening of the pulps after refining has been explained by Du *et al*. (2013), who by means of a thioacidolysis-SEC analysis observed an increase in chromophores in the refined pulps when compared to the unrefined control pulp.

The tear index, decreases in which are related to fiber degradation, was found to be similar in pretreated and control pulps. This result indicated that the ULE pretreatments did not cause significant damage to the cellulose fibers. With regards to the tensile and burst indexes, pretreated pulps showed higher values than control pulps. These indexes are related to the inter-fiber bonding capacity, which would be increased by the urea pretreatment due to increased bonding between the swollen fibers (García-Fuentevilla *et al*. 2013). Furthermore, the enzymatic treatment also contributed to improved mechanical properties of the bleached pulps, as different authors have previously observed (Herpoël *et al.* 2002; You *et al.* 2008; Moldes *et al.* 2010; Martín-Sampedro *et al*. 2012). This improvement is probably caused by the increased flexibility of the enzyme-treated pulps fibers as a result of increased delignification and delamination of the fiber walls.

CONCLUSIONS

1. According to the design of experiments employed in this study, L stage consistency is the variable that has the most influence on pulp properties, P stage peroxide consumption, and residual enzyme activity. Nevertheless, laccase and mediator dosage also influence the bleaching process, while oxygen pressure was found to be the least influential variable.
2. The optimum conditions of the L stage were: 10% pulp consistency, 3.46 $U \cdot g^{-1}$ laccase dosage, 0.05 $mmol \cdot g^{-1}$ mediator dosage, 60 min treatment time, and 408.0 KPa oxygen pressure.
3. This optimal ULEP sequence significantly improved the EP control sequence: 49.8% *vs.* 33.4% delignification, up to 65.6% ISO *vs.* 56.3% ISO increase in brightness, and 50.3% *vs.* 89.9% consumption of hydrogen peroxide.
4. The enzyme after the L stage can be reused since it retains almost all of its original activity (*i.e.*, 96.5% residual enzyme activity); reuse of the laccase can reduce the cost of the enzymatic treatment.
5. The urea pretreatment and the optimal enzymatic treatment improved the ECF sequence, achieving increases in delignification (kappa number of 0.6 *vs.* 1.0 for $ULED_0E_1D_1$ and $D_0E_1D_1$, respectively), brightness (82.7% ISO *vs.* 76.0% ISO), tensile (54.1 $N \cdot m \cdot g^{-1}$ *vs.* 51.9 $N \cdot m \cdot g^{-1}$), and burst (3.3 $KPa \cdot m^2 \cdot g^{-1}$ *vs.* 2.7 $KPa \cdot m^2 \cdot g^{-1}$) indexes.

ACKNOWLEDGMENTS

The authors wish to thank the Spanish MINECO for funding this study *via* Projects CTQ 2009-11268, CTQ2011-28503-C02-01 and CTQ2013-46804-C2-1-R, and Program PTA 2011-4857-I.

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Article submitted: November 5, 2014; Peer review completed: February 5, 2015; Revised version received and accepted: February 9, 2015; Published: February 20, 2015.
DOI: 10.15376/biores.10.2.2289-2304