Carbohydrate Polymers 88 (2012) 830–837

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Paper strength improvement by oxidative modification of sisal cellulose fibers with laccase–TEMPO system: Influence of the process variables

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ARTICLE INFO

Article history:
Received 14 October 2011
Received in revised form 8 December 2011
Accepted 4 January 2012
Available online 16 January 2012

Keywords:
Laccase–TEMPO oxidation
Sisal
Modeling
Carbonyl content
Carboxyl content
Paper strength

ABSTRACT

The oxidation of sisal pulp fibers by the laccase–TEMPO system was investigated and the influence of process variables including the laccase and TEMPO doses, and reaction time, on various properties of the oxidized fibers and of handsheets made from them was for the first time assessed using a three-variable statistical plan. The laccase–TEMPO system was found to oxidatively modify cellulose fibers, largely by introducing aldehyde groups and, to a much lesser extent, by introducing carboxyl groups. Based on the mathematical models used, increasing the TEMPO dose and reaction time increases the aldehyde content of the fibers, thereby also increasing their wet strength by effect of inter-fiber covalent bonding *via* hemiacetal linkages. Although no accurate model for the carboxyl content could be established, this property was found to peak under the specific conditions yielding the highest response in the dry tensile index model. The fact that the oxidative treatment diminished pulp viscosity is indicative of partial depolymerization of cellulose. This was especially marked under the conditions providing the highest contents in aldehyde and carboxyl groups, and the greatest improvements in the dry and wet tensile indices.

1. Introduction

Laccase (EC.1.10.3.2.) is a family of blue multi-copper oxidases produced by microorganisms and plants which catalyze the one-electron oxidation of phenols and aromatic or aliphatic amines to reactive radicals with the concomitant reduction of oxygen to water. The broad substrate range for this enzyme makes it attractive for a number of biotechnological applications (Kunamneni et al., 2008; Riva, 2006). Moreover, the range can be expanded using the enzymes in combination with a chemical mediator enabling the oxidative transformation of compounds with a redox potential higher than that of the enzyme (Bourbonnais & Paice, 1990; Galli & Gentili, 2004). In the last few decades, laccase has played an increasingly important role in pulp and paper research by virtue of its wide applicability along the entire production chain of paper products (Bajpai, 1999; Widsten & Kandelbauer, 2008). Special emphasis has been placed on the potential of laccase and laccase-mediator systems (LMS) for use in biobleaching and mill water treatments (Font, Caminal, Gabarrell, Romero, & Vicent, 2003; Valls & Roncero, 2009). One attractive, fast-growing field of research at present is the enzymatic modification of fibers with a view to improving the chemical or physical properties of fiber products or developing novel alternatives (Chandra & Ragauskas, 2001; Viikari, 2002). One well-known procedure for functionalizing polysaccharides is the catalytic oxidation of primary hydroxyl groups into aldehyde and/or carboxyl groups using the stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) in aqueous media at room temperature (de Nooy, Besemer, & van Bekkum, 1995b). In virtually all studies concerning carbohydrate oxidation, the established NaClO/NaBr system has been used as a primary oxidant (Bragd, Besemer, & Bekkum, 2001; Chang & Robyt, 1996; de Nooy, Besemer, & van Bekkum, 1995a; Isogai & Kato, 1998). In this process, TEMPO and its oxoammonium cation (the actual oxidant) are reduced to an *N*-hydroxyderivative that is continuously reoxidized by sodium hypochlorite, thus affording the use of TEMPO in catalytic amounts. Environmental concerns have shifted research interest toward halide-free oxidative systems. One promising approach for this purpose is the use of oxidative enzymes such as laccases in combination with oxygen as primary oxidants (Arends, Li, Ausan, & Sheldon, 2006; Viikari, Kruus, & Buchert, 1999). Similarly to the NaClO/NaBr system, oxoammonium ion is regenerated *in situ*, so only oxygen is consumed during the reaction. In addition to the environmental benefits associated with the use of an enzyme, this method has the

Abbreviations: BV, borohydride viscosity; Cns, pulp consistency; CS, cellulose chain scission number; DP, degree of polymerization; DTI, dry tensile index; LT, laccase–TEMPO; Odp, oven dried pulp; Rpm, revolutions per minute; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl free radical; TR, pulp treated prior to refining; WTI, wet tensile index.

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doi:10.1016/j.carbpol.2012.01.011

advantage that it operates at near-neutral pH, which reduces the occurrence of β-elimination reactions causing cleavage of polysaccharide chains—a major drawback of the traditional method using pH 10–11 (Isogai & Kato, 1998).

Although the laccase–TEMPO system has been shown to catalyze the oxidation—and consequent functionalization—of various types of polysaccharides (Jetten, Van den Dool, Van Hartingsveldt, & Besemer, 2000; Marzorati, Danieli, Haltrich, & Riva, 2005; Viikari, Niku-Paavola, et al., 1999; Viikari, Kruus, et al., 1999), the influence of the experimental conditions and the characteristics of the resulting products have scarcely been examined. Much research aimed at developing specific applications for cellulose pulp has focused on oxidation reactions mediated by the TEMPO–NaBr–NaClO system on the grounds of its efficiency in functionalizing fiber surfaces with large amounts of aldehyde and/or carboxyl groups. This oxidative modification has been exploited to improve or modulate several physical properties of various types of pulp fibers including inter-fiber bonding capacity—and hence strength-related properties in the resulting paper (Dang, Zhang, & Ragauskas, 2007; Duarte et al., 2006; Lianshan, Kweinam, Deneault, & Brouillette, 2008). Paper from TEMPO-oxidized fibers was recently shown to have an increased wet strength; this was ascribed to the ability of aldehyde groups to form inter-fiber covalent bonds through hemiacetal linkages with hydroxyl groups in adjacent fiber surfaces (Saito & Isogai, 2005, 2006). The potential of laccase for replacing halide-based co-oxidizer systems in the TEMPO-mediated oxidation of cellulose fibers has scarcely been explored to date, however (Viikari, Kruus, et al., 1999). In recent work (Aracri, Vidal, & Ragauskas, 2011), we studied the oxidation of sisal pulp fibers by the laccase–TEMPO system and found it to slightly increase the carboxyl group content of the fibers and to considerably improve the wet strength of the resulting paper, the latter suggesting the formation of a substantial amount of aldehyde groups providing inter-fiber bonding through hemiacetal linkages. In this work, we further investigated the process and examined the influence of the operating conditions (*viz.* the laccase and mediator doses, and treatment time) on the distribution of carboxyl and aldehyde groups in fibers, pulp borohydride viscosity, and dry and wet strength properties of the handsheets for the first time using a three-variable sequential statistical plan.

2. Experimental

2.1. Chemicals, enzyme and pulp

All chemicals were purchased from Aldrich and used as received. Laccase from *Trametes villosa* was supplied by Novozymes (Bagsvaerd, Denmark). One activity unit was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical ($\varepsilon_{436\,nm} = 29{,}300\ M^{-1}\ cm^{-1}$) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. Sisal (*Agave sisalana*) pulp (1% lignin content) from a soda–anthraquinone cooking process was supplied by CELESA (Tortosa, Spain). Following conditioning at 2% consistency at pH 4 (H_2SO_4) under stirring for 30 min, the pulp was filtered and extensively washed with de-ionized water. This step was needed to remove contaminants and metals, and also to bring the pulp to a suitable pH for the enzyme treatment. The main properties of the initial pulp were as follows: 108 ± 5 μmol/g carboxyl groups content, 1 ± 0 aldehyde groups content μmol/g and 736 ± 0 ml/g borohydride viscosity.

2.2. Pulp treatments

A series of preliminary tests was performed using an amount of 30 g of pulp in a 5 L reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5 in the presence of 60 U/g odp laccase and 8% odp TEMPO, and variable conditions of time, temperature, applied oxygen pressure and consistency (Table 1). Pulp samples treated in the absence of TEMPO, or both laccase and TEMPO, at room temperature and 1% consistency under an oxygen pressure of 0.6 MPa for 18 h were used as controls.

Table 1
Operating conditions used in the laccase–TEMPO (LT) treatments of the preliminary study.

Sample ID	Time (h)	Temperature	Applied O_2 pressure (MPa)	Pulp consistency (% odp)
LT – 18 h	18	Room	0.6	1
LT – 30 h	30	Room	0.6	1
LT – 50 °C – 30 h	30	50 °C	0.6	1
LT – no P_{O_2} – 30 h	30	Room	–	1
LT – 4 h	4	Room	0.6	1
LT – cns 5% – 30 h	30	Room	0.6	5

Subsequently, laccase–TEMPO treatments were performed according to the established experimental design in plastic containers, using a jar testing apparatus at a stirring speed of 60 rpm, 15 g of pulp at 1% consistency in 50 mM acetate buffer at pH 5 at room temperature under oxygen bubbling. The operating variables (factors) studied were the laccase dose, mediator dose and reaction time. After treatment, each pulp was filtered and washed with de-ionized water until a colorless, neutral filtrate was obtained.

2.3. Experimental design

Laccase–TEMPO treatments were conducted in accordance with a 2^3 experimental design involving three variables at two levels each and three replicates at the central point, which required a total of 11 tests. The ranges examined for the independent variables were 20–100 U/g odp (laccase dose, x_1), 2–8% odp (TEMPO dose, x_2) and 8–20 h (reaction time, x_3), and the results were coded as −1 or +1, both for direct comparison of coefficients and for easier understanding of the effect of the variables on the responses. The independent variables were zeroed at the central point. The results of the three replicates at the central point, and their variance, were used in combination with the variance of the saturated model to calculate Snedecor's *F*-value in order to determine whether the variance was homogeneous or heterogeneous. The variance was homogeneous in all cases, so a linear model was constructed, its significant terms identified and its potential curvature detected. Linear multiple regression was applied by means of an Excel spreadsheet in order to implement the stepwise backward regression method and discard all terms with a probability $p < 0.05$.

2.4. Analysis of pulp properties

Pulp viscosity was determined in accordance with ISO 5351/1. Borohydride viscosity was measured after treatment with 2% $NaBH_4$, at 5% consistency at room temperature for 30 min (Roncero, Queral, Colom, & Vidal, 2003). The bulk acid group content was determined by conductimetric titration as described elsewhere (Aracri et al., 2011). TEMPO-oxidized pulp samples were further oxidized with $NaClO_2$ for selective conversion of aldehyde groups into carboxyl groups at room temperature for 48 h. The carboxyl content was determined with the above-described conductimetric titration method. The carboxyl groups formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde groups originally present in the pulp (Saito & Isogai, 2005).

2.5. Paper testing

Prior to oxidative treatment, each pulp was disintegrated for 30,000 revolutions and then refined for 4500 revolutions according

Table 2
Carboxyl and aldehyde group bulk contents of the control pulp, and of samples treated with laccase alone or the laccase–TEMPO system (LT) under variable operating conditions, and physical properties of the resulting handsheets. TR denotes pulp treated prior to refining.

Sample ID	Carboxylic groups (μmol/g)	Aldehyde groups (μmol/g)	Dry tensile index (N m/g)	Wet tensile index (N m/g)	Burst index (kPa m²/g)	Tear index (mN m²/g)
Control – 18 h	99 ± 2	1 ± 0	55.6 ± 4.0	1.4 ± 0.2	4.16 ± 0.44	24.4 ± 0.5
Laccase – 18 h	94 ± 2	1 ± 1	56.7 ± 4.6	1.5 ± 0.1	3.83 ± 0.46	21.4 ± 0.7
LT – 18 h	129 ± 2	73 ± 6	61.4 ± 5.7	7.6 ± 1.3	3.82 ± 0.52	11.9 ± 0.7
LT – 30 h	126 ± 2	107 ± 13	61.7 ± 4.3	9.9 ± 1.4	3.75 ± 0.59	11.4 ± 0.3
TR LT – 30 h	106 ± 6	86 ± 15	58.7 ± 4.1	6.6 ± 0.7	3.16 ± 0.49	11.0 ± 0.7
LT – 50 °C – 30 h	93 ± 3	49 ± 3	54.5 ± 5.7	3.6 ± 0.7	2.87 ± 0.62	11.7 ± 0.5
LT – no P_{O_2} – 30 h	96 ± 11	53 ± 10	54.4 ± 4.5	3.6 ± 0.6	3.23 ± 0.46	11.2 ± 0.7
LT – 4 h	93 ± 5	17 ± 4	58.7 ± 4.9	2.6 ± 0.2	4.04 ± 0.46	15.5 ± 0.5
LT – cns 5% – 30 h	312 ± 11	159 ± 11	68.7 ± 4.0	13.1 ± 1.6	4.40 ± 0.29	9.1 ± 0.5

to ISO 5263. Once treated, the samples were disintegrated for 10,000 revolutions and used to prepare handsheets on a Rapid-Köthen laboratory former according to ISO 5269-2. The handsheets were then conditioned at 23 °C at 50% relative humidity for at least 24 h before physical testing. Dry tensile strength and wet tensile strength were determined according to ISO 1924-3 and ISO 3781, respectively, and wet tensile index was measured in 15 mm wide specimen strips soaked in de-ionized water for 5 s.

3. Results and discussion

3.1. Preliminary pulp treatments

Preliminary tests were conducted using 60 U/g odp laccase and 8% odp TEMPO under different operating conditions (reaction time, temperature, applied oxygen pressure and pulp consistency). In treatment LT – 30 h, refining was applied both before and after treatment. As can be seen from Table 2, all LT treatments introduced aldehyde groups, especially at long treatment times and high consistency levels. The wet tensile index increased with increasing aldehyde content, which confirmed their mutual correlation. Both raising the temperature to 50 °C and removing applied O_2 pressure resulted in low yields of aldehyde and carboxyl groups relative to the initial treatment applied for 30 h. This can be ascribed to thermal decomposition of oxoammonium ion *via* a ring-opening mechanism (Ma, Loyns, Price, & Chechik, 2011) and to the enzyme requiring O_2 to regenerate TEMPO from its reduced form. Application of treatment LT – 30 h to unrefined pulp resulted in lower yields of aldehyde and carboxyl groups relative to refined pulp; this was probably the result of an increased fiber surface area available for the oxidant after refining. The most salient effect was that obtained by increasing pulp consistency, which increased the amount of carboxyl and aldehyde groups by 150% and 50%, respectively, with respect to the same treatment at 1% consistency by effect of the improved oxidant–fiber interactions. Treatment 4 h provided only a slight increase in aldehyde groups, and hence a modest improvement in wet tensile index, thus reflecting the significant influence of the reaction time on these properties. As a result of enhanced inter-fiber hydrogen bonding (Barzyk, Page, & Ragauskas, 1997), the pulp samples with an increased carboxyl content exhibited an also increased dry tensile index. The tear index of the LT-treated samples was very low in relation to the control samples, especially at high consistency levels and, to a lesser extent, short reaction times; this was ascribed to the loss of fiber strength as a consequence of depolymerization of cellulose chains during the oxidative treatment. Finally, the burst index was seemingly influenced by both the carboxyl group content and the fiber strength.

3.2. Experimental design

3.2.1. Modeling

Experimental data (Table 3) were fitted to a second-order polynomial equation with wet tensile index (Y_{WTI}), dry tensile index (Y_{DTI}), aldehyde content (Y_{CHO}) and borohydride viscosity (Y_{BV}) as response variables. Available data allowed no accurate model for the carboxyl content to be constructed from the process variables. A preliminary test with the models for aldehyde content, dry tensile index and borohydride viscosity revealed that the quadratic term was significant ($p < 0.05$). Two additional tests were therefore required in order to identify the specific variables possessing a significant term and ensure their accurate discrimination. A second analysis of the modeling equations provided responses where all significant terms had $p < 0.05$. The quadratic term in the model for wet tensile index was not significant.

$$\text{Wet tensile index model}: \quad Y_{WTI} = 3.3 + 1.1 \times_2 + 0.7 \times_3 \qquad (1)$$

with $R^2 = 0.79$

$$\text{Dry tensile index model}: \quad Y_{DTI} = 50.3 - 0.7 \times 1 - 1.1 \times_1 x_3 - 1.1 \times_1 x_2 \times 3 + 3.7 \times_1^2 + 2.1 \times_2^2 \qquad (2)$$

Table 3
Operating conditions of the LT treatments and results of wet tensile index, dry tensile index, carboxyl content, aldehyde content and borohydride viscosity.

Exp.	Laccase dose (U/g)	TEMPO dose (%)	Time (h)	WTI (N m/g)	DTI (N m/g)	COOH (μmol/g)	CHO (μmol/g)	Borohydride viscosity (ml/g)
1	20	2	8	1.8	57.3	105	6	536
2	100	2	8	1.9	55.4	118	7	527
3	20	8	8	2.9	53.8	101	31	370
4	100	8	8	3.7	57.4	110	60	400
5	20	2	20	2.5	56.9	119	21	491
6	100	2	20	2.5	55.1	114	15	495
7	20	8	20	6.6	59.0	137	83	389
8	100	8	20	4.6	53.5	113	57	353
9	60	5	14	2.9	50.6	110	43	402
10	60	5	14	3.6	49.6	99	43	403
11	60	5	14	3.0	50.4	110	41	404
12	100	5	14	2.5	53.5	126	18	341
13	60	2	14	1.9	52.5	121	8	498

with $R^2 = 0.98$

$$\text{Aldehyde content model}: \quad Y_{CHO} = 42 + 23 \times_2 + 9 \times 3 - 8 \times_1 x_3 + 3 \times_2 x_3 - 6 \times_1 x_2 \times 3 - 24 \times_1^2 - 12 \times_2^2 + 29 \times_3^2 \quad (3)$$

with $R^2 = 0.99$

$$\text{Borohydride viscosity model}: \quad Y_{BV} = 403 - 1 \times_1 - 67 \times_2 - 13 \times_3 - 6 \times_1 x_3 + 6 \times_2 x_3 - 10 \times_1 x_2 \times_3 - 61 \times_1^2 + 27 \times_2^2 + 76 \times_3^2 \quad (4)$$

with $R^2 = 0.99$

3.2.2. Wet tensile index model

The model for wet tensile index [Eq. (1) and Fig. 1] predicted responses from 1.4 to 5.1 N m/g. Based on it, the specific variables affecting this property were x_2 (TEMPO dose) and x_3 (reaction time), both in a linear manner. By contrast, the laccase dose had no effect on the wet tensile index over the studied range. This suggests that the lowest enzyme dose used sufficed to effect the conversion of primary hydroxyl groups to aldehyde groups promoting the formation of hemiacetal linkages between fibers, and hence the development of wet strength in the resulting handsheets.

3.2.3. Dry tensile index model

The model relating dry tensile index and process variables conformed to Eq. (2) and predicted property responses from 50.1 to 59.0 N m/g. Based on it, the laccase dose (x_2) had a weak linear influence on the dry tensile index, with a coefficient of −0.7. In addition, a quadratic influence on the response was exhibited by the laccase dose, ${x_1}^2$, and TEMPO dose, ${x_2}^2$. As can be seen from Fig. 2, the dry tensile index exhibited a constant value of 56.8 N m/g at a low laccase dose ($x_1 = -1$) and an also low TEMPO dose ($x_2 = -1$), throughout the time range studied. Increasing the laccase dose from 20 to 52 U/g at a low TEMPO dose reduced the index to 52.7 N m/g; also, further increasing the enzyme dose increased it up to 55.4 N m/g irrespective of the particular reaction time. Using a low laccase dose ($x_1 = -1$) in combination with a medium ($x_2 = 0$) or high ($x_2 = 1$) TEMPO dose caused the dry tensile index to increase linearly with time (from 53.6 to 55.8 N m/g and from 54.6 to 59.0 N m/g, respectively). The opposite trend was observed at a high laccase dose ($x_1 = 1$), with dry tensile index decreasing from 54.4 to 52.2 N m/g at a medium TEMPO dose and from 57.6 to 53.2 N m/g at a high TEMPO dose. The index value obtained at a medium laccase dose was lower than those at high and low laccase doses throughout the TEMPO dose and reaction time ranges studied (see Fig. 2).

3.2.4. Aldehyde content model

Fig. 3 shows the surface graphs for the aldehyde content model, based on Eq. (3). As can be seen, the model predicted aldehyde contents from −17 to 96 μmol/g. The negative predictions were taken to be 0 μmol/g. Based on the model, the variables influencing the aldehyde content were the TEMPO dose (x_2) and reaction time (x_3). Similarly to the model for the wet tensile index, the laccase dose (x_1) had no influence on the aldehyde content and the TEMPO dose was the variable most strongly influencing this property, with a coefficient of 23. All variables exerted a quadratic influence on the response. With high ($x_1 = +1$) and low ($x_1 = -1$) laccase doses combined with a low TEMPO dose ($x_2 = -1$), the model predicted a decrease in aldehyde content from *ca.* 10 to −17 μmol/g between the eighth and twelfth hour of reaction, and an increase up to *ca.* 20 μmol/g after the twelfth. The decrease during the former time may have resulted from the oxidation rate of aldehyde groups to carboxyl groups exceeding that of primary alcohols to aldehyde groups. With a medium laccase dose and a low TEMPO dose, the aldehyde response decreased from 30 to 18 μmol/g as the reaction time was increased from 8 to 17 h, respectively, after which the response increased up to 42 μmol/g. Whichever the laccase dose, raising the TEMPO dose increased the aldehyde content—especially from the low mediator level to its medium level. Based on the model, the highest aldehyde content can be expected from the use of a high TEMPO dose in combination with a medium laccase dose and a high reaction time.

3.2.5. Borohydride viscosity model

Borohydride viscosity measurements were performed to assess changes in the degree of cellulose polymerization during the laccase–TEMPO treatments. Prior to measurement, oxidized pulp samples were treated with sodium borohydride to inactivate carbonyl groups (by reduction to hydroxyl groups) and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium (Aracri et al., 2011). The model for borohydride viscosity [Eq. (4) and Fig. 4] predicted property responses from 330 to 593 ml/g. Based on it, all process variables influenced borohydride viscosity, the laccase dose (x_1) exhibiting a weak effect (a coefficient of 1) and the mediator dose (x_2) the strongest influence (a coefficient of 67). In addition, all variables exerted a quadratic influence on the response. As can be seen from Fig. 4, borohydride viscosity was decreased as the time was increased from 8 to 14, 16.5 and 17.5 h with a low, medium and high TEMPO dose, respectively, used in combination with low laccase dose. Although, based on the mathematical model, increasing the time beyond these points resulted in slightly increased viscosity, the increase was experimentally unlikely and the property was instead assumed to remain constant over the second time range. Borohydride viscosity dropped as the TEMPO dose was raised, especially from a low dose ($x_2 = -1$) to medium one ($x_2 = 0$). The response peaked as the laccase dose was raised from 20 to 60, 79 and 72 U/g at a low, medium and high TEMPO dose, respectively, and decreased slightly to values similar near those obtained at a low laccase dose as the time was increased to 20 h. The highest borohydride viscosity in the studied plan (593 ml/g) was obtained at a low reaction time and an also low TEMPO dose in combination with a medium laccase dose, which however provided low degree of aldehyde and carboxyl functionalization and almost no improvement of dry and wet tensile index.

3.2.6. Statistical analysis of carboxyl groups

The 2^3 factorial design used allowed us no accurate model for predicting the carboxyl content of the fibers to be constructed. In fact, the points in the corresponding semi-zeta graph for the saturated model obtained clustered in no definite manner (results not shown). This may have been the result of (a) heteroscedasticity (*i.e.* a non-constant variance), (b) the factor having little influence on the response or (c) excessive experimental noise. Although the carboxyl content could be fitted to no specific model, we reported the results of the experiments prescribed by the statistical plan in graphs such as those shown in Fig. 5 in order to assess the influence of changing a variable between its minimum and maximum levels at four different combinations of maximum and minimum levels of the other two variables. Fig. 5a illustrates the effect of the laccase dose on the carboxyl content. The most marked changes as the laccase dose was increased from 20 to 100 U/g were observed with both the mediator dose (x_2) and the treatment time (x_3) at their lowest (A) or highest levels (D), with an increase from 105 to 118 μmol/g in the former case, and a decrease from 137 to 113 μmol/g in the latter. Based on the graph of Fig. 5b, increasing the TEMPO dose from 2% to 8% odp had significant, opposite effects when a high laccase dose was used in combination with a low reaction time (B) (a decrease from 118 to 110 μmol/g) or a low enzyme dose was used together with a high reaction time (C) (an increase from 118 to 137 μmol/g). As can be seen from Fig. 5c, increasing the

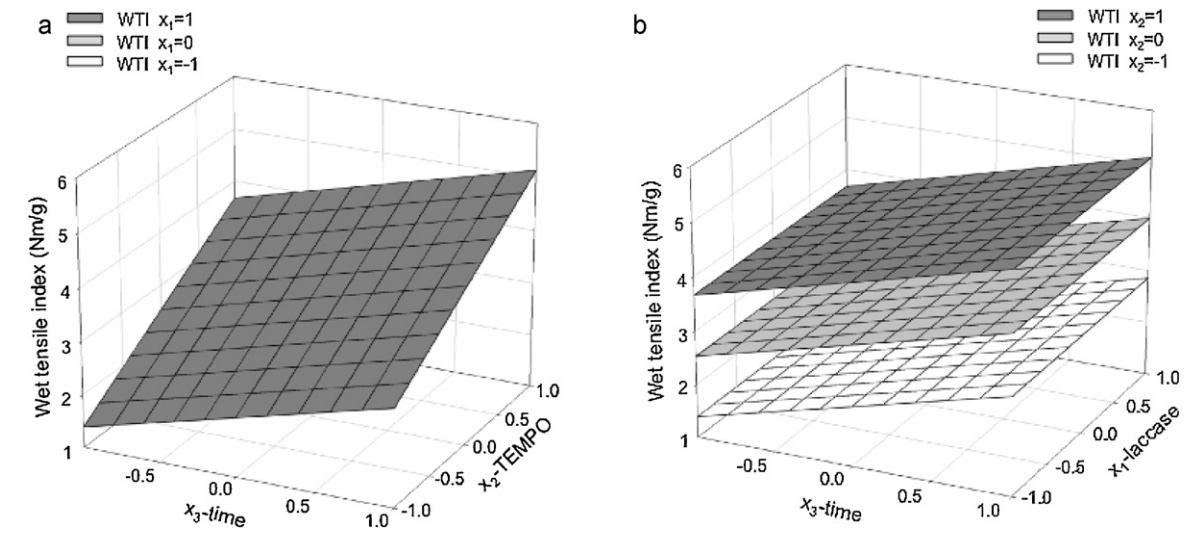

Fig. 1. Variation of the wet tensile index as a function of the factors of the statistical plan, with the laccase dose, x_1 (a) and TEMPO dose, x_2 (b) at low, medium and high levels.

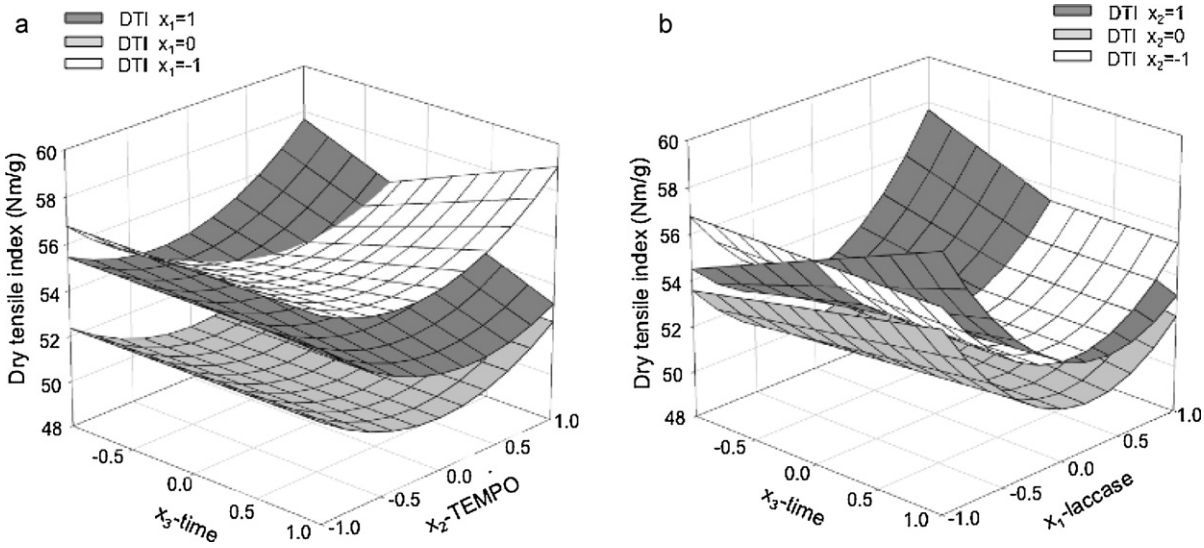

Fig. 2. Variation of the dry tensile index as a function of the factors of the statistical plan, with the laccase dose, x_1 (a) and TEMPO dose, x_2 (b) at low, medium or high levels.

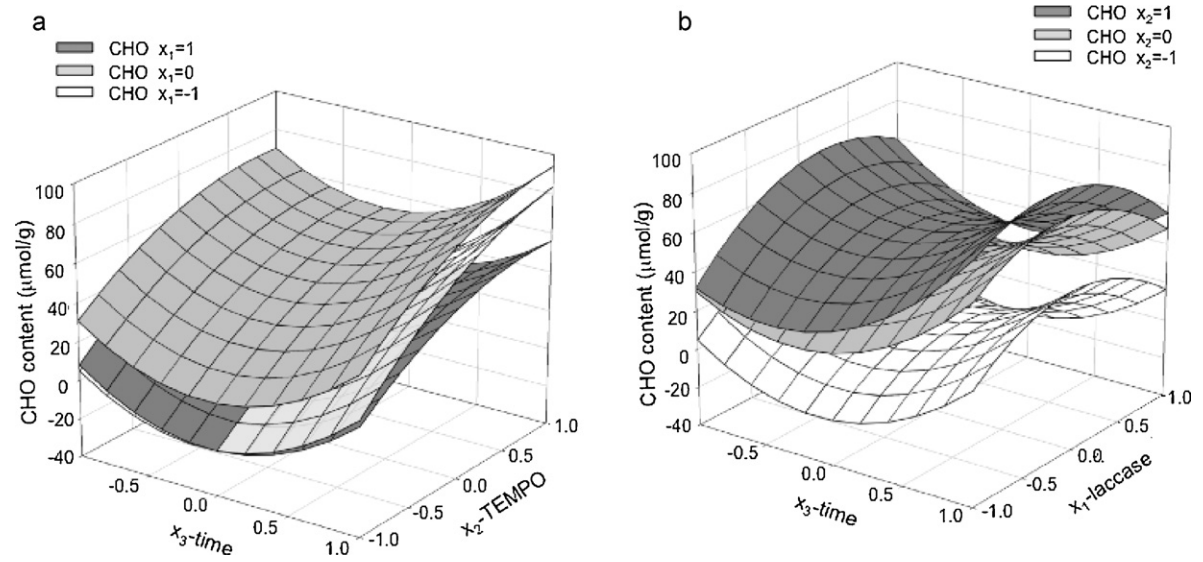

Fig. 3. Variation of the aldehyde content as a function of the factors of the statistical plan, with the laccase dose, x_1 (a) and TEMPO dose, x_2 (b) at low, medium or high levels.

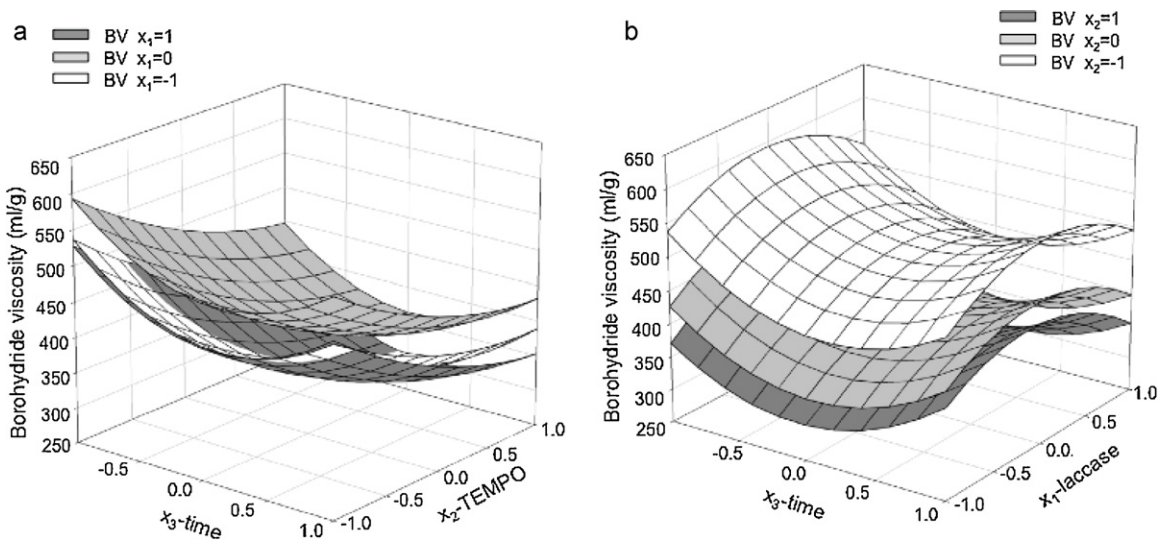

Fig. 4. Variation of the borohydride viscosity as a function of the factors of the statistical plan, with the laccase dose, x_1 (a) and TEMPO dose, x_2 (b) at low, medium or high levels.

reaction time increased the carboxyl content in all cases except B (where x_1 was at the highest level and x_2 at the lowest), especially with a low laccase rate and a high TEMPO rate.

An increased concentration of acid groups in pulp fibers is known to increase the dry tensile strength of the resulting paper by facilitating inter-fiber hydrogen bonding. In a recent study (Patel, Ludwig, Haltrich, Rosenau, & Potthast, 2011) reporting the effect of the laccase–TEMPO system on cotton linter pulp, the oxidative modification was found to involve the introduction of carbonyl groups and, to a much lesser extent, that of carboxyl groups. The latter result was ascribed to aldehyde autoxidation promoted by the oxygen-saturated medium since both isolated laccase and the TEMPO-derived oxoammonium ion proved unable to oxidize aldehyde groups to the corresponding carboxyl groups. In this study, we could not establish the exact influence of process variables on the carboxyl content; also, the mechanism by which aldehyde groups are further oxidized to carboxyl groups by the laccase–TEMPO system remains unclear. This may explain why the model for the dry tensile index was more complex than that for the wet tensile index—development of which depends mainly on the formation of inter-fiber covalent bonds such as those provided by hemiacetal linkages involving aldehyde groups. However, the highest carboxyl content was obtained under those conditions providing the highest response of dry tensile index in the model for this property, which confirms the contribution of carboxyl groups to improving the dry tensile index in handsheets from sisal fibers.

3.3. Assessing the effects of aldehyde groups on pulp properties

3.3.1. Intrinsic viscosity

The effect of the oxidative treatments on the degree of cellulose polymerization in the fibers was assessed from intrinsic viscosity measurements. Because a cupriethylenediamine solution is highly alkaline and depolymerization by β-elimination promoted by carbonyl groups may occur in oxidized fibers during viscosity measurements, the pulp samples were treated with sodium borohydride (borohydride viscosity) in order to inactivate carbonyl groups by reduction to hydroxyl groups (Cadena, Vidal, & Torres, 2010; Roncero, Colom, & Vidal, 2002).

The difference between the viscosity values obtained with and without the reductive treatment provided an indication of the depolymerizing effect of carbonyl groups formed by TEMPO-mediated oxidation.

Oxidized pulp samples exhibited a marked loss in viscosity (up to 53%) compared to reduced samples, the difference increasing with increase in TEMPO dose and reaction time (results not shown).

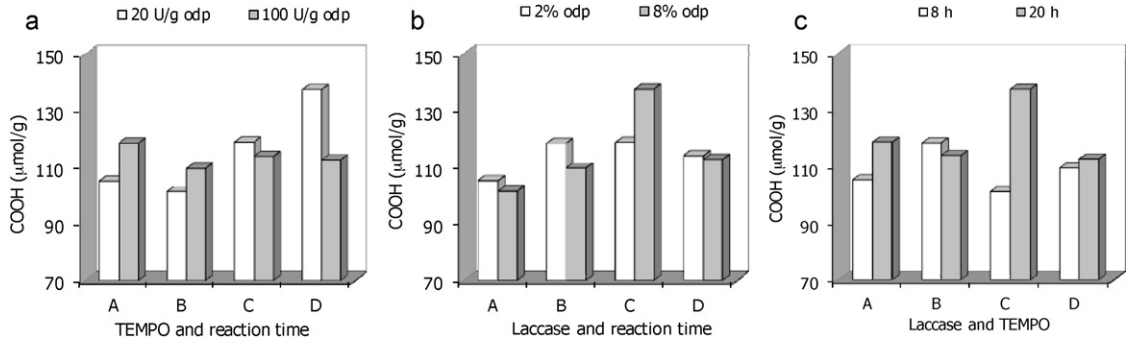

Fig. 5. Effect of increasing the variables from their lowest level to the highest on the content of carboxyl groups. (a) Effect of increasing the laccase dose from 20 to 100 U/g odp: (A) 2% TEMPO, 8 h; (B) 8% TEMPO, 8 h; (C) 2% TEMPO, 20 h; and (D) 8% TEMPO, 20 h. (b) Effect of increasing the TEMPO dose from 2% to 8% odp: (A) 20 U/g laccase, 8 h; (B) 100 U/g laccase, 8 h; (C) 20 U/g laccase, 20 h; and (D) 100 U/g laccase, 20 h. (c) Effect of increasing the reaction time from 8 to 20 h: (A) 20 U/g laccase, 2% TEMPO; (B) 100 U/g laccase, 2% TEMPO; (C) 20 U/g laccase, 8% TEMPO; and (D) 100 U/g laccase, 8% TEMPO.

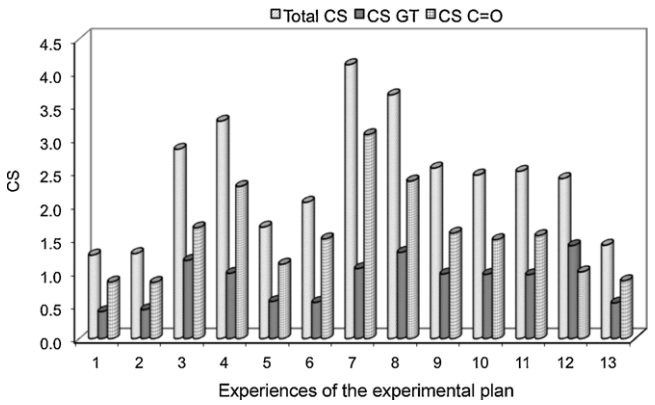

Fig. 6. CS_T, CS_{GT} and $CS_{C=O}$ for each pulp obtained in the experiments of the statistical plan.

Pulp degradation can also be assessed *via* the number of scissions in the cellulose chain (CS), which is defined mathematically as (Bouchard, Morelli, & Berry, 2000):

$$CS = \frac{DP_O - DP}{DP} \quad (5)$$

where DP_O is the degree of polymerization of the initial pulp and DP that after the oxidative treatment. The degree of polymerization is calculated from the intrinsic viscosity [η], using the equation of (Evans & Wallis, 1997) (SCAN-CM 15:88):

$$DP^{0.085} = 1.1 \cdot [\eta] \quad (6)$$

The intrinsic viscosity values obtained in the presence and the absence of a reductive treatment allowed us to calculate the number of scissions due to the oxidative treatment itself (CS_{GT}) and to β-elimination promoted by carbonyl groups ($CS_{C=O}$) (Aracri et al., 2011). Fig. 6 shows CS_{GT}, $CS_{C=O}$ and their combination (CS_T) for each pulp obtained in the experiments of the statistical plan. As can be seen, all oxidative treatments caused cellulose depolymerization and the formation of carbonyl groups responsible for further chain scissions in the alkaline measurement medium. TEMPO-mediated oxidation is highly selective for primary hydroxyl groups, which are converted into aldehydes/(hemi)acetals; however, secondary hydroxyl groups can take part in side reactions leading to the formation of keto groups (Fabbrini, Galli, Gentili, & Macchitella, 2001). We found a linear relationship between the aldehyde content and the number of chain scissions due to carbonyl groups as determined in the experimental plan (Fig. 7a); this is suggestive of preferential conversion of hydroxyl groups into aldehyde groups. The classical oxidation method with alkaline hypohalite as the actual oxidant results in severe molecular weight losses in cellulose that are mainly due to alkali-induced β-alkoxy elimination reactions starting from carbonyl groups (Potthast, Schiehser, Rosenau, & Kostic, 2009). The slightly acidic medium used by the laccase–TEMPO system hinders β-elimination, so cellulose degradation is most probably a result of homolytic processes involving some active radical species formed *in situ* as by-products during the oxidation treatment (Patel et al., 2011; Tamura, Hirota, Saito, & Isogai, 2010). Since cellulose degradation decreases fiber strength, the loss of pulp viscosity is a major drawback in processes intended to have paper develop strength-related properties. The improved dry and wet tensile index values obtained under certain conditions of the experimental plan despite the decreased pulp viscosity can be ascribed to the formation of hydrogen and covalent inter-fiber bonds offsetting the loss of fiber strength.

3.3.2. Wet tensile index

In recent years, TEMPO-mediated oxidation in the presence of NaClO/NaBr has proved an efficient method to improve wet strength in cellulose materials including pulp fibers by effect of the introduction of aldehyde functionalities promoting the formation of inter-fiber covalent bonding through hemiacetal linkages with sterically close hydroxyl groups in cellulose (Saito & Isogai, 2005, 2006). In previous work (Aracri et al., 2011), we demonstrated the ability of the enzymatic method (*viz.* the laccase–TEMPO system) to significantly improve the wet strength of sisal pulp fibers; this suggested the formation of a substantial amount of aldehyde groups during the oxidative treatment. In this work, we found the wet tensile index to be influenced similarly to the aldehyde content by process variables; in addition, a graphical comparison of these properties revealed that the wet tensile index and aldehyde content followed a clear-cut trend that fitted a straight line with a coefficient of determination $R^2 = 0.88$ (Fig. 7b).

The formation of hemiacetal linkages is the mechanism by which aldehyde-containing resins develop temporary wet-strength in paper (Chen, Hu, & Pelton, 2002). These resins improve wet strength of paper by forming inter-fiber covalent bonds through hemiacetal/or acetal linkages between hydroxyl groups of cellulose/hemicellulose and the aldehyde groups of the resins. Laccase–TEMPO oxidation generates aldehyde functionalities in cellulose fibers and these are able to form hemiacetal linkages with hydroxyl groups in adjacent fiber surfaces, with the advantages of developing wet strength without the addition of any synthetic resin and providing easily repulpable papers.

The use of laccase for improving wet strength of lignocellulosic pulps has been already reported (Lund & Felby, 2000, 2001), although limited to lignin-rich pulps. In these studies combinations of the enzyme with a lignin-rich extractive or a mediator were applied to high-yield unbleached kraft pulp. The improvements obtained were ascribed to polymerization of lignin in the handsheets, and also to enhanced production of phenoxy-radical increasing cross-linking between fibers. However, the gain in wet strength showed by the authors was not as high as in the present work unless it was combined with a heat treatment.

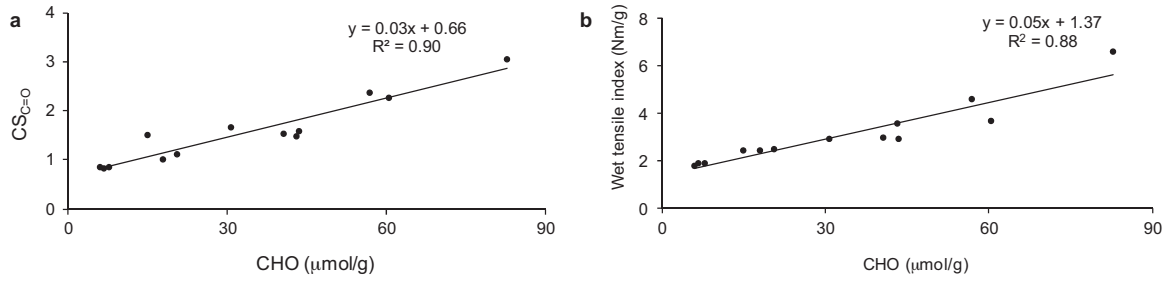

Fig. 7. Variation of the number of chain scissions in the cellulose chain (a) and the wet tensile index (b) with the content in aldehyde groups.

4. Conclusions

The influence of laccase–TEMPO process variables on the properties of a cellulose pulp was for the first time examined using a statistical plan. Preliminary results showed applying oxygen pressure and using room temperature, a long reaction time and, especially, a high consistency, to boost functionalization and improve the wet and dry tensile indices of sisal pulp as a result. Based on the mathematical models derived, using a high TEMPO dose and a long reaction time favors the formation of aldehyde groups and leads to improved wet strength in the resulting handsheets. Based on the model for dry tensile index, the responses can be expected to peak at high TEMPO doses used in combination with either a low laccase dose and long reaction time or a high laccase dose and short reaction time. Although no accurate model for carboxyl content could be established, the improvement peaked under those conditions yielding the highest dry tensile index, which suggests that carboxyl groups promote inter-fiber hydrogen bonding in the resulting handsheets. Besides the environmental benefits associated with the replacement of halide-containing compounds with an enzyme as co-oxidant, laccase–TEMPO oxidation has the advantage over the classical method that it operates at near-neutral pH—which reduces cellulose depolymerization by β-elimination. As shown here, however, the enzyme treatment reduces pulp viscosity somewhat, probably by effect of side reactions involving active radical species formed during the oxidation process. Further study will be required to confirm this assumption and acquire a better understanding of the mechanisms behind cellulose degradation in the enzymatic process with a view to preventing fiber strength losses and maximizing the benefits derived from the oxidative effects in terms of paper strength.

Acknowledgements

The authors are especially grateful to Spain's MICINN for funding this research in the framework of Projects FUNCICEL (CTQ2009-12904) and BIOFIBRECELL (CTQ2010-20238-CO3-01). E. Aracri is additionally grateful to MICINN for award of a Research Trainee Fellowship.

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