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Cellulose oxidation by Laccase-TEMPO treatments

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Abstract

In this work, laccase-TEMPO (Lac-T) treatments were applied to bleached commercial dissolving pulp in order to introduce carbonyl and carboxyl groups, which were found to improve dry and wet strength-related properties. Also the solubility behavior towards xanthate reactions was assessed. The effect of a refining step (R) before the oxidative treatment, the absence or presence of oxygen pressure, TEMPO dose (2 or 8% oven dried pulp) and reaction time (8 or 20h) were thoroughly examined. Treatments conducted in the presence of oxygen pressure exhibited greater amount of functional groups. Introducing a pre-refining treatment resulted in similar functional groups but higher wet strength was achieved. Specifically, a high W/D strength ratio was observed, indicating that wet strength-related property was satisfactorily developed. Besides the fact that all Lac-T treatments caused severe cellulose degradation, no fiber strength loss was detected. In fact, all oxidized samples presented higher wet zero-span tensile strength, mainly in R+ Lac-T (O$_2$) sample, which suggested the formation of hemiacetal linkages between the new introduced aldehyde groups and available free hydroxyl groups resulting from fibrillation.

1. Introduction

Cellulose is a linear homopolymer consisting of anhydro- -D-glucopyranose units (AGU) that are linked together by (1- -4) glycosidic bonds. Every AGU contains three hydroxyl groups in the positions C2, C3 and C6. The hydroxyl group at C6 acts as a primary alcohol whereas the hydroxyl groups at C2 and C3 behave as secondary alcohols (Klemm, Philipp, Heinze, Heinze, & Wagenknecht, 1998). The ability of these hydroxyl groups to form hydrogen bonds plays a major role in the formation of fibrillar and semicrystalline packing, which governs the important physical properties of this highly cohesive material. Importantly, the presence of these reactive hydroxyl groups allows cellulose to be modified and functionalized, by introducing new functional groups to improve its nano-dispersibility or even confer it an added-value for specific applications such as medical (scaffolding material), agricultural, cosmetic and pharmaceutical (Sergiu Coseri et al., 2013; Dias, Peplow, & Teixeira, 2003; T. Isogai, Saito, & Isogai, 2010; Jaušovec, Vogrin i , & Kokol, 2014; Kumar & Banker, 2008). In the paper industry, carbonyl and carboxyl functionalities are known to play a decisive role in the pulping process and hence in the final paper properties. For example, sheets made from the partially oxidized fibers experienced higher wet and dry tensile index, presumably due to an increased opportunity of electrostatic interactions between anionic pulp and cationic polyamideamine-epichlorohydrin, which was added as a wet-strength agent (Kitaoka, Isogai, & Onabe, 1999).

Cellulose oxidation can be induced by very different processes, e.g., radiation, energy impact or the application of oxidizing reagents (Potthast, Kostic, Schiehser, Kosma, & Rosenau, 2007). These chemical oxidants can be divided into non-selective, such as nitrogen oxides (Butrim, Bil'dyukevich, Butrim, & Yurkshtovich, 2007), alkali metal nitrites and nitrates (Painter, n.d.), ozone (Johansson & Lind, 2005) and permanganates (Manhas, Mohammed, & khan, 2007); and

61 selective, such as periodates (Calvini, Gorassini, Luciano, & Franceschi, 2006; Fras et al., 2005;

62 Potthast, Schiehser, Rosenau, & Kostic, 2009) and nitroxyl radicals (Biliuta, Fras, Strnad,

63 Harabagiu, & Coseri, 2010; S Coseri et al., 2009; Sergiu Coseri et al., 2013; de Nooy, Besemer,

64 van Bekkum, van Dijk, & Smit, 1996; A. Isogai & Kato, 1998; Vignon & Tahiri, 2000).

65 Periodates are specific oxidants capable of oxidizing the vicinal hydroxyl groups at carbon atoms

66 2 and 3 in an anhydroglucose unit (AGU) of cellulose, to form two aldehyde groups. As a result,

67 the carbon carbon bond between the carbon atoms 2 and 3 is broken (Sergiu Coseri et al., 2013).

68 Nitroxyl radicals have been widely studied for catalytic and selective oxidation of primary

69 hydroxyl groups of polysaccharides under aqueous conditions (Saito & Isogai, 2004). In

70 particular, TEMPO-mediated oxidation is an efficient method to introduce carboxyl and carbonyl

71 functional groups into cellulose in aqueous suspension (de Nooy et al., 1996; Saito & Isogai,

72 2004). Two TEMPO-mediated oxidation systems have been reported so far:

73 TEMPO/NaBr/NaClO system at pH 10-11 (de Nooy et al., 1996; A. Isogai & Kato, 1998; Saito

74 & Isogai, 2006, 2007) and TEMPO/NaClO/NaClO$_2$ system at pH 4-7 (Saito et al., 2009; Saito,

75 Hirota, Tamura, & Isogai, 2010). NaClO and NaClO$_2$ are used as the primary oxidants in each

76 system. Applying TEMPO/NaBr/NaClO to native cellulose leads to the formation of significant

77 amounts of sodium carboxylate groups and small amounts of aldehyde groups, which is usually

78 accompanied by important depolymerization. On the other hand, the TEMPO/NaClO/NaClO$_2$

79 system avoided the depolymerization of the oxidized cellulose, although the efficiency for the

80 formation of carboxylate groups was somewhat lower (A. Isogai, Saito, & Fukuzumi, 2011). The

81 catalytic conversion of the primary hydroxyl groups to carboxyl via aldehydes has been widely

82 studied over the last two decades, but the mechanism is still under discussion.

83   The presence of carboxylic groups can promote re-wetting and re-swelling of fibers, and

84   contribute to the negative charge of fibers, which favorably influences fiber flexibility, pulp

85   refining, and dry-strength of paper (Hubbe, Venditti, & Rojas, 2007). Carbonyls in cellulose are

86   "hot spots" along the carbohydrate chain acting as localized sites of increased chemical

87   instability where cleavage will primarily occur. Oxidized groups in cellulose are chiefly

88   responsible for strength loss and decreased performance parameters in pulp, paper, textiles and

89   other cellulosic materials. They also account for general ageing of cellulosics (Lewin, 1997), as

90   well as thermal and light-induced yellowing process (Potthast, Rosenau, Kosma, Saariaho, &

91   Vuorinen, 2005). However, works using TEMPO-oxidized mechanism have been found to

92   considerably improve wet tensile strength. Development of this property was ascribed to the

93   formation of substantial amounts of aldehyde groups on the surfaces of cellulose fibers as

94   intermediate structures during the course of TEMPO-mediated oxidation. Once formed, the

95   aldehyde groups seemingly established covalent interfiber bonds through hemiacetal linkages

96   with sterically close hydroxyl groups in cellulose (de Nooy et al., 1996; T. Isogai et al., 2010; T.

97   Isogai, Saito, & Isogai, 2011; Okita, Saito, & Isogai, 2010; Saito & Isogai, 2006, 2007). As a

98   considering point, the carbonyl or carboxyl groups introduced may also be contemplated as

99   reactive "chemical hooks" for further chemical modification or graft co-polymerization (Potthast

100   et al., 2007).

101   In this line, but in view of introducing biotechnology concept, some recent works

102   demonstrated the use of laccase as a cocatalyst in stoichiometric oxidation. Laccase, having a

103   redox potential in the range of 0.7–0.9 V, can easily oxidize the stable oxyl- radical form of

104   TEMPO to oxoammonium ion ($E°$ 0.2 V). This ion is the actual oxidant, while laccase would

105   regenerate TEMPO from the generated hydroxyl-amine. Then, either acid-induced

106 disproportionation of TEMPO, or further oxidation of it by laccase, would form the

107 oxoammonium ion once again (Fabbrini, Galli, Gentili, & Macchitella, 2001). Some authors

108 demonstrated the good performance of a chemoenzymatic modification using laccase as

109 biocatalyst and TEMPO as enhancer (Aracri, Valls, & Vidal, 2012; Aracri, Vidal, & Ragauskas,

110 2011; Aracri & Vidal, 2012; Bragd, Besemer, & Bekkum, 2001; Jaušovec et al., 2014; Patel,

111 Ludwig, Haltrich, Rosenau, & Potthast, 2011; Viikari, Kruus, & Buchert, 1999; Xu, Song, &

112 Qian, 2013). The interest of cellulosic modification and functionalization have been explored

113 using various methods including hydrophobization with laccase-lauryl gallate (Cusola, Roncero,

114 Vidal, & Rojas, 2014; Cusola, Valls, Vidal, & Roncero, 2013, 2014; Garcia-Ubasart et al., 2012;

115 Garcia-Ubasart, Vidal, Torres, & Rojas, 2013) or antimicrobial properties with laccase-natural

116 phenols (Fillat et al., 2012) and antioxidant capacity (Cusola, Valls, Vidal, & Roncero, 2015).

117   Based on the foregoing, the purpose of this work was to study laccase-TEMPO oxidation of a

118 commercial dissolving pulp, to evaluate the dissolution behavior towards Fock solubility, and the

119 degree of functionalization towards wet strength improvement. Therefore, different experimental

120 conditions, such as TEMPO dose (2% or 8% oven dried pulp (odp)), reaction time (8h or 20h),

121 presence or absence of oxygen pressure and a refining pretreatment were studied.

122 ## 2.   Experimental

123 ### 2.1 Pulp, enzymes and reagents

124   A totally chlorine-free (TCF), bleached dried commercial dissolving-grade pulp was used as a

125 starting pulp and presented a 91.70 ± 0.15% ISO brightness and 522 ± 4 mL/g viscosity. The

126 carbohydrate composition, as determined by high-performance liquid chromatography (HPLC),

127 was as follows: 95.6 ± 0.03% glucan, 3.8 ± 0.16% xylan, 0.6 ± 0.14% glucuronic acid and 0.03 ±

128 0.01% arabinan.

6

129   A laccase from *Trametes villosa* (TvL) with an activity of 746 U/mL supplied by

130   Novozymes® (Denmark), was used for the oxidative treatments. The enzyme activity was

131   measured as the oxidation of 5 mM 2,2-azinobis(3-thylbenzothiazoline-6-sulfonic acid) (ABTS)

132   to the cation radical (ε_{436}=29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 24 °C. One

133   activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per min.

134   2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) purchased from Sigma–Aldrich was used as

135   mediator for the oxidative treatments. Characteristics of TEMPO reagent was: 98% assay, CAS

136   number 2564-83-2 and molecular weight (Mw) of 156.24532 g/mol.

137   **2.2 Pulp refining and oxidative treatments**

138   Prior to oxidative treatments, a portion of the starting pulp was refined (R) at 5000 rev in a PFI

139   mill according to ISO 5264, and the remained was used as received with no refining step.

140   Laccase–TEMPO oxidation treatments were performed at room temperature, at 5%

141   consistency in a 5 L reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5 and 20 U/g

142   odp laccase (Lac). Different experimental conditions such as, TEMPO (T) dose, reaction time

143   and presence or absence of oxygen pressure (O_2) were tested in order to provide the greatest

144   increases in carboxyl and aldehyde groups, and hence improve wet and dry tensile strength. The

145   specific conditions used for each treatment are described in Table 1 and were taken from the

146   study conducted by Aracri et al., (2012) and considering the highest formation of aldehyde and

147   carboxyl groups. Pulp samples treated under identical conditions as in the chemoenzymatic

148   treatment but in the absence of laccase and TEMPO (Cont-Buffer), or only TEMPO (Cont-Lac),

149   were used as controls. After treatment, each pulp was filtered and washed with de-ionized water

150   until a colourless, neutral filtrate was obtained.

151   Table 1. Detailed conditions of the performed treatments

Sample ID	Pulp refined at 5000 rev	TEMPO dose (% odp)	O_2 Applied pressure (MPa)	Time (h)
Lac-T 2%, no O_2, 8h	-	2	-	8
Lac-T 8%, no O_2, 8h	-	8	-	8
Lac-T 8%, 8h	-	8	0.6	8
R+Lac-T 8%, 8h	✓	8	0.6	8
R+Lac-T 8%, 20h	✓	8	0.6	20
R+Cont-Lac, 20h	✓	-	0.6	20
R+Cont-Buffer, 20h	✓	-	0.6	20

2.3 Analysis of pulp properties

153   Pulp brightness, and Fock solubility were assessed according to ISO 3688 and Fock (1959),

154   respectively. Pulp viscosity (as intrinsic viscosity for a sample of cellulose dissolved in a dilute

155   solution of cupriethylenediamine) was determined in accordance with ISO 5351:2010. Because

156   viscosity measurement is conducted using alkaline Cuen solution, carbonyl groups derived from

157   chemoenzymatic treatment can suffer -elimination reaction and final viscosity results are

158   misunderstood (Roncero, Queral, Colom, & Vidal, 2003). This problem was avoided by treating

159   the TEMPO-oxidized samples with 2% $NaBH_4$ at 5% consistency at room temperature for 30

160   min in order to reduce carbonyl groups to hydroxyl groups. After this post-treatment, viscosity

161   was determined again using Cuen solution. The degree of polymerization (DP) was calculated

162   from the intrinsic viscosity values, using the equation of Evans & Wallis (1987) (SCAN-CM

163   15:88):

$$DP^{0.85} = 1.1 \cdot [\eta]$$

164   Eq. 1

165 Pulp degradation can also be assessed via the number of scissions in the cellulose chain (CS),

166 which is defined mathematically as (Bouchard, Morelli, & Berry, 2000):

$$CS = \frac{DPo - DP}{DP}$$

167
<center>Eq. 2</center>

168   where DP_0 is the degree of polymerization of the original pulp or previous stage and DP that at

169   the end of any chemical or enzymatic treatment.

170   The bulk acid group content was determined by conductimetric titration as described elsewhere

171   (Aracri et al., 2011). In brief, an amount of 1.50 g o.d. pulp was stirred in 300 mL of 0.10 M HCl

172   for 1 h, followed by rinsing with de-ionized water in a finely fritted funnel. The sample was then

173   resuspended in 250 mL of 1.00 mM NaCl, spiked with 1.5 mL of 0.10 M HCl and titrated against

174   0.05 M NaOH in 0.25 mL increments, with conductivity measurement after each addition.

175   Titration data were plotted in conductivity vs. volume graphs in order to determine the

176   milliequivalents of acid groups present in each gram of pulp. All reported results are the averages

177   of two measurements.

178   TEMPO-oxidized pulp samples were further oxidized with $NaClO_2$ for selective conversion of

179   aldehyde groups into carboxyl groups at room temperature for 48 h. The carboxyl content was

180   determined with the above-described conductimetric titration method. The carboxyl groups

181   formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde groups originally

182   present in the pulp (Aracri & Vidal, 2012; Saito & Isogai, 2005).

183   Fiber length and fines content (%) of samples were measured using Kajaani fiber analyzer

184   (FS300, Metso automation, Finland) according to TAPPI T 271. Measurement is based on the

185   ability of these fibers to change the direction of polarized light. Sample preparation was as

186   follows: fibers were soaked in water for at least 4 h and disintegrated in the standard

187   disintegrator (TAPPI T 205) until no fiber bundles remain. Then a stock suspension of fibers of

188   approximately 0.025% consistency was prepared. Samples were further diluted so that an

189 average of 20,000 fibers was counted in the 500 mL of the analyzed suspension. Duplicate

190 measurements of at least two different stock suspensions were performed for each sample.

191 Water retention value (WRV) was determined according to ISO 23714 and surface SEM

192 images of the handsheets were taken on a JEOL JSM-6400 microscope. Samples were placed on

193 the SEM sample holding stub with the aid of conductive double side sticky carbon film and

194 coated with Au/Pd alloy prior to analysis. Zeta potential of fibers was determined according to

195 Cadena et al. (2009), using Mütek zeta potential equipment (SZP-06, Mütek, Germany).

196 Once treated, the samples were used to prepare handsheets on a Rapid-Köthen laboratory

197 former according to ISO 5269-2. The handsheets were then conditioned at 23 °C at 50% relative

198 humidity for at least 24 h before physical testing. Mechanical properties were assessed in

199 accordance with the standards in brackets as follows: dry tensile strength (ISO 1924-3:2005), wet

200 tensile strength (ISO 3781), tearing resistance (ISO 1974:1990), air permeance (ISO 5636-

201 3:2003) and bulk density. For mechanical properties evaluation, ten handsheets were prepared

202 and triplicate measurements were conducted for each handsheet. A wet-to-dry (W/D) strength

203 ratio of more than 15% should be considered wet-strength paper (Scott, 1996). The Wet Zero

204 Span Tensile Strength (WZSTS) was measured according to T-273 pm-95 and ISO 15361:2000,

205 on a Zero-Span 1000 Pulmac tester, using strips previously soaked in de-ionized water for 5 s.

206 Since high variability was detected, more than 20 repetitions were conducted.

3. Results and Discussion

208 In previous work (Quintana, Valls, Vidal, & Roncero, 2015), bleached commercial dissolving

209 pulp was fully characterized in terms of carbohydrate composition, ^{13}C-CP/MAS NMR, Fock

210 solubility, ISO brightness, fiber morphology, viscosity and WRV. Then commercial dissolving

211 pulp was further purified with chemoenzymatic treatments (specifically with combinations of

212 cold caustic extraction (CCE) and endoglucanase treatments). That cellulose modification led to

213 Fock solubility improvement and the formation of cellulose II. In the same line of

214 chemoenzymatic modification of cellulosic pulps, oxidative reactions in presence of Laccase-

215 TEMPO system were conducted in this work.

216 3.1 Relating functional groups to dry and wet strength properties

217 The amount of aldehyde (CHO) and carboxyl (COOH) groups introduced in cellulose structure

218 after Lac-T oxidation were quantified by titration. Importantly, this specific functional groups are

219 known to improve strength-related properties (Aracri et al., 2011; Saito & Isogai, 2005, 2006).

220 Although, not relevant for dissolving pulps, testing dry and wet tensile strength is a way to

221 indirectly assess the efficiency of cellulose functionalization.

222 As shown in Table 1, two control treatments (R+Cont-Lac, 20 h and R+Cont-Buffer, 20 h)

223 were performed in order to find any contribution from laccase and buffer parameters. No

224 changes in carboxyl/aldehyde content, wet/dry tensile strength, fiber morphology or WRV were

225 found, with respect to starting and refined pulp (results not shown). Consequently, the results of

226 these treatments are not further discussed from here on.

227 In Figure 1 is shown the content of carboxyl (COOH) and aldehyde (CHO) functional groups;

228 and dry/wet tensile index. As can be seen from Figure 1a, the original pulp presented 35.6

229 μmol/g of carboxyl group and no presence of aldehyde groups. In general, Laccase-TEMPO

230 treatments with no oxygen pressure and no refining step, increased carboxyl group moderately

231 with respect to the starting pulp. Two doses of TEMPO (2 and 8% odp) were studied, failing to

232 show significant differences in terms of carboxylic group content. Specifically, a 32% and 46%

233 of improvement was detected for Lac-T 2% and Lac-T 8%, with respect to original pulp.

234 Carboxyl groups are known to contribute to dry strength in paper (Hubbe et al., 2007); but results

235 showed that the low content in carboxylic acids led to low dry tensile strength values with no

236 differences between samples (Figure 1c). On the other side, the use of 2% odp TEMPO provided

237 an amount of 55.6 μmol/g of aldehyde groups, whereas the use of 8% odp TEMPO only 6.9. It is

238 known that, aldehyde groups once formed in the native cellulose fibers are able to form

239 hemiacetals with hydroxyl groups of cellulose sterically close to each other. However, as

240 suggested by some authors (Saito & Isogai, 2006), not all aldehydes groups formed in the

241 TEMPO-oxidized cellulose fibers necessarily contribute to the development of the wet strength

242 of paper. Pulp samples treated with Lac-T 2% and Lac-T 8% exhibited a wet tensile index of 0.7

243 and 0.8 Nm/g, which correspond to a 32 and 60% of improvement, with respect to original pulp.

244 In other words, the low amount of aldehyde groups introduced was not enough to develop good

245 wet strength property since an only wet-to-dry (W/D) strength ratio of 11 and 14% was achieved,

246 respectively. Moreover, Lac-T 2% and Lac-T 8% treatments suffered a laccase inhibition of 31

247 and 80%, respectively (results not shown). These results suggest that the higher of TEMPO dose

248 and the absence of additional oxygen pressure could inactivate the enzyme and therefore

249 hindering the oxidation of C6 primary hydroxyl groups.

250   On the other hand, applying oxygen pressure in the system (Lac-T 8%, O_2), let to introduce a

251 greater amount of aldehyde and carboxyl groups, 182 and 243 μmol/g, respectively; as compared

252 to only 35.5 μmol/g of carboxyl group in the original pulp (Figure 1b). It is known that

253 dissolving pulps exhibit poor strength properties and low bonding availability due to low content

254 in hemicelluloses. However, the laccase-TEMPO treatment using oxygen pressure (Lac-T 8%,

255 O_2) increased dry and wet strength to 9.3 and 1.7 N·m/g (Figure 1d), from 5.2 and 0.5 N·m/g,

256 respectively, in the original pulp. The Lac-T 8% (O_2) sample had a W/D ratio of 18% indicating

257 that wet strength was well developed.

258   The effect of a refining treatment was also studied (Figure 1b, d). As observed for original

259   pulp, the refined pulp presented carboxylic groups (47.6 μmol/g) but no aldehyde groups. The

260   fibrillation effect resulted from the refining treatment provided in turn a positive effect in

261   mechanical properties. Thus, the greater interfiber bonds allowed to improve dry and wet

262   strength with respect to the original pulp. The combined treatment, R+Lac-T 8% (O_2), provided

263   an amount of carboxyl and aldehyde groups of 290.7 and 210.9 μmol/, respectively. The content

264   of these new functional groups was slightly higher than Lac-T 8% (O_2), but the former showed a

265   markedly improvement in dry and wet strength, with a W/D strength ratio of 26%. These results

266   demonstrated that the refining treatment did not contribute to introduce more functional groups

267   (no synergy effect between pre-refining and Lac-T oxidation) but bettered strength-related

268   properties. The marked improvement observed was due to the combined effect of the large

269   amounts of functional (carboxyl and aldehyde) groups and new available free hydroxyl groups

270   resulting from the fibrillation, which led to the formation of hemiacetal linkages. It was observed

271   that a near-unity aldehyde/carboxyl ratio ensured good wet strength property. Therefore, the poor

272   bonding capability of dissolving pulp due to low content in hemicelluloses justified the refining

273   pretreatment in terms of strength developed property.

274   Patel et al. (2011) observed that in Lac-T system carboxyls were mainly formed in later phases

275   of the reaction when the carbonyl generation became slower or even leveled off. However, the

276   mechanism behind the formation of carboxyl is still under study. Some authors proposed the

277   formation of carboxyls mainly as a result of aldehyde subsequent autoxidation under oxygen-

278   saturated medium or a direct participation of the oxoammonium ion in the reaction (Patel et al.,

279   2011). It is described in the literature that Lac-T system is able to modify cellulose by

280   introducing predominantly carbonyl groups and, to a much lesser extent carboxyl groups (Aracri

281 et al., 2011; Aracri & Vidal, 2012; Jaušovec et al., 2014; Patel et al., 2011). However, this trend

282 was not observed with our samples, since somewhat higher content of carboxyl than aldehyde

283 groups was found in all treatments with exception of Lac-T 2% (no O_2), which exhibited same

284 concentration of both functional groups. These results suggested that raw material composition

285 influences the reaction pattern of TEMPO-oxidation.
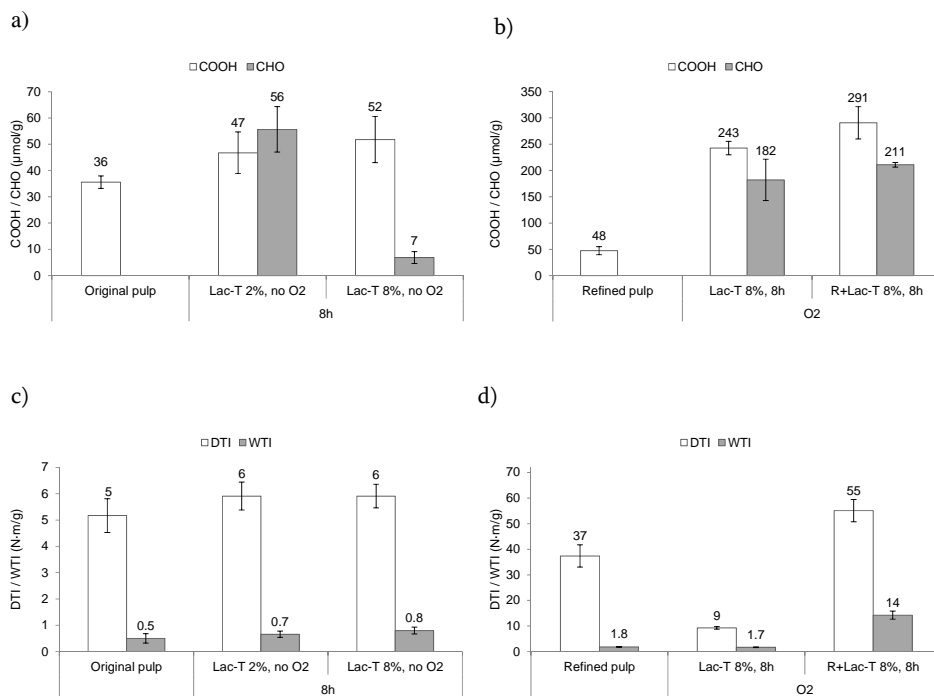
286

287 Figure 1 Carboxylic (COOH)/ aldehyde (CHO) group content (± standard deviation), dry / wet tensile strength (±

288 standard deviation) for original pulp, refined pulp at 5000 rev and respective enzymatic oxidative treatments.

289

290   The water retention value (WRV) is a measure of the water absorption capacity of fibers and

291 provides information about the degree of swelling of TEMPO-oxidized samples (Gehmayr,

292 Schild, & Sixta, 2011). WRV is influenced by the content and distribution of hydrophilic

293 carboxyl groups introduced and specific surface areas or degrees of fibrillation of cellulose

294 (Saito, Kimura, Nishiyama, & Isogai, 2007). Moreover, it is known that carboxyl groups increase

295   hydrophilic character in a major degree compared to hydroxyl groups. Therefore, a good

296   correlation between WRV and carboxylic acids was found, with exception of Lac-T 8%

297   treatment performed under oxygen pressure, which did not result in higher WRV (Figure 2).
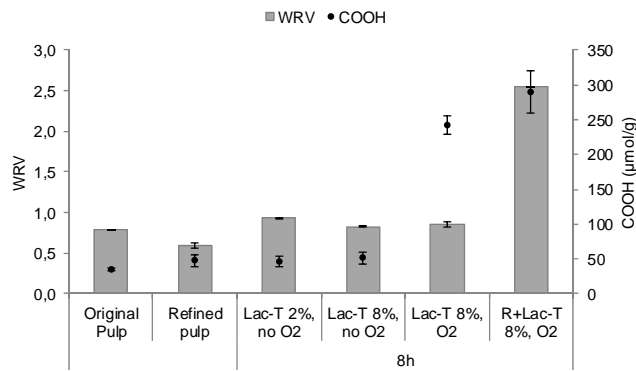
298

299   Figure 2 Water Retention Value (WRV) (± standard deviation) and content of carboxylic acids (± standard

300   deviation).

301   Table 2 summarizes the morphologycal and mechanical properties of treated pulp samples. In

302   terms of fiber morphology, the refining treatment caused an important reduction of fiber length,

303   from 1.5 ± 0.01 mm to 0.8 ± 0.02 mm, and increased the content of fines, with respect to the

304   original pulp. Interestingly, the presence or absence of oxygen during the Lac-T treatment led to

305   a different yield in functional groups but had no effect on fiber morphology. The oxidation of

306   primary hydroxyl groups occurs only on the surfaces of fibrils in cellulose fibers, and this

307   regioselective oxidation probably resulted in such specific water-absorption behavior without

308   any significant changes in the fibrous morphology (Saito et al., 2007). Introducing a refining step

309   before the enzymatic treatment decreased fiber length to the same extent as the refining treatment

310   alone. The smaller fiber length was directly associated to the refining treatment rather than to

311   oxidative modification.

312   Regarding mechanical properties, the refining step provided an important improvement in tear

313   strength with respect to the original pulp. The Lac-T treatments also increased tear strength,

15

314 which suggests that higher energy was required to tear the fiber network. This improvement was

315 due to increased inter-fiber hydrogen bonding provided by new introduced carboxyl groups

316 (Barzyk, Page, & Ragauskas, 1997).

317   The refining treatment markedly reduced the air permeance (i.e. closer structure) and was

318 accompanied by a density increase. This effect was not observed when oxidative treatments were

319 performed alone and same air permeance as original pulp was found. By its side, R+Lac-T 8%

320 (O_2) sample offered higher air permeance relative to refined pulp (R), although exhibited same

321 bulk density. The difference in air permeance can be explained by the presence of hemiacetal

322 linkages found for R+Lac-T 8% (O_2) sample.

323   The surface charge of fibers can be measured by zeta potential. Due to the presence of

324 carboxylic groups (weak acid group) on the surface, the zeta potential values were negative.

325 Original pulp exhibited a zeta potential of -342 mV, and a notable charge decrease was obtained

326 for all Lac-T oxidized pulp samples. Consistent with the presence of ionized carboxylic acids at

327 the surface of fibers, similar zeta potential values were obtained for Lac-T 2%, Lac-T 8% and

328 Lac-T 8% (O_2). The substantial difference in zeta potential of R+Lac-T 8% sample was thought

329 to be caused by a modification of ionic distribution and repulsive forces between fibers and fines.

330 Fiber hydrophilicity and swelling, which are highly relevant on fiber-fiber bonding, are also

331 affected by fiber charge.

332 Table 2 Physical and chemical properties of Lac-T treated pulps

	Air Permeance (μm/Pa·s)	Density (g/cm^3)	L (l) (mm)	Fines (%)	Tear strength Index (mN·m^2/g)	Z potential (mV)
Original pulp	639 ± 10	0.42 ± 0.01	1.53 ± 0.01	38.77± 0.01	4.01 ± 0.52	-342 ± 18

Refined pulp	40 ± 2	0.72 ± 0.01	0.82 ± 0.02	49.72 ± 0.0	6.71 ± 0.23	-
Lac-T 2%, no O_2, 8h	621 ± 8	0.41 ± 0.01	1.6 ± 0.02	35.3 ± 1.3	5.94 ± 0.83	-133 ± 0
Lac-T 8%, no O_2. 8h	593 ± 40	0.4 ± 0.01	1.6 ± 0.03	40.2 ± 1.1	5.51± 0.66	-144 ± 29
Lac-T 8%, O_2, 8h	622 ± 7	0.41 ± 0.01	1.6 ± 0.02	39.4 ± 1.6	5.72 ± 0.77	-148 ± 3
R+Lac-T 8%, O_2, 8h	292 ± 37	0.7 ± 0.0	0.8 ± 0.0	57.7 ± 0.2	3.23 ± 0.22	-42.15 ± 3

333

3.2 Influence of the treatment conditions on fiber properties

The Lac-TEMPO pulp samples were subjected to a post-reduced treatment with $NaBH_4$ in

order to avoid further degradation during the viscosity measurements since Cuen-solution

(cupriethylenediamine) is used. It is known that under alkaline conditions aldehyde groups at C-6

and keto groups at C-2/C3 undergo -elimination reactions that cause the cleavage of the

cellulose chain (Patel et al., 2011). As can be seen from Figure 3, all oxidative treatments caused

a considerably degradation of pulp and this depolymerization was more pronounced when the

oxidative treatment was performed under oxygen pressure, showing about 85% of degree of

polymerization loss, with respect to initial pulp. Patel et al. (2011) also suggested that

degradation processes of the enzymatic approach are most probably homolytic (radical) and can

also account for cellulose degradation.

Interestingly, same viscosity values determined directly after Lac-T treatment or after a post-

reduced treatment with $NaBH_4$ were found (Table 3). According to Potthast et al. (2005), the

formation of carbonyl hydrates and/or hemiketals protects pulp from all reactions attributable to

the reactive carbonyl moieties. In addition, double-bonded carbonyl groups are base-sensitive but

relatively stable in acidic media, whereas hydrates and hemiacetals are stable in basic media, but

350 are very labile under acidic conditions. These postulations are consistent with viscosity results

351 where no depolymerization due to carbonyl groups was observed, which are suggested to be

352 mostly as hemiacetals and only to a small extent as aldehydes or aldehydes hydrates. In

353 accordance to some authors described (Jaušovec et al., 2014), chemical oxidation of carbonyl to

354 carboxyl form by $NaClO_2$ was inefficient due to the precedent formation of highly-stable

355 covalent hemiacetal linkages, between primarily C-6 aldehyde groups and surrounding hydroxyls

356 groups in cellulose.

357 A low viscosity was associated to an increase in the number of scissions in the cellulose chain.

358 As mentioned earlier, cellulose depolymerization was due to the chemoenzymatic oxidative

359 treatment itself since no degradation due to carbonyl groups was detected. The treatments

360 conducted with no oxygen pressure exhibited about 2.4 chain scissions (CS), whereas the

361 addition of oxygen pressure caused a greater viscosity loss and gave a CS value of 5.2. The

362 increased CS values obtained in the presence of oxygen pressure, suggested that additional

363 aldehydes might be present at C-2 or/and C-3 due to the cleavage of the connecting carbon-

364 carbon bond. These new aldehydes groups were not the result of the Lac-T oxidation of primary

365 hydroxyl groups. The refining treatment followed by the oxidative treatment (R+Lac-T 8%)

366 increased the CS up to 6.1 and also showed the biggest Fock solubility improvement, although

367 final Fock solubility was slightly lower than Lac-T 8% (O_2) sample (Table 3). This is consistent

368 with the results of other authors (Beltramino, Valls, Vidal, & Roncero, 2015) who also observed

369 that higher chain scissions corresponded to higher pulp solubility.

370 Table 3 Viscosity values (± standard deviation) determined directly with Cuen solution or after applying a reduced
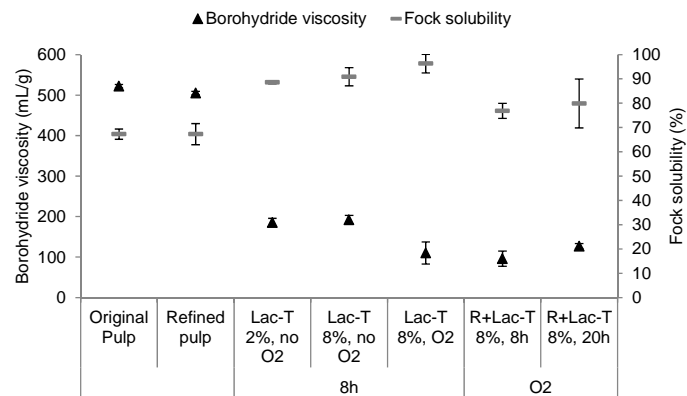371 treatment with $NaBH_4$

	Original pulp	Refined pulp	Lac-T 2%, no O_2, 8h	Lac-T 8%, no O_2, 8h	Lac-T 8%, O_2, 8h	R+Lac-T 8%, O_2,

						8h
Viscosity (mL/g)	476 ± 1	474 ± 1	189 ± 16	197 ± 2	87 ± 13	99 ± 13
NaBH$_4$ viscosity (mL/g)	522 ± 4	505 ± 5	185 ± 10	192 ± 11	110 ± 27	96 ± 19
Chain Scission (CS)	-	-	2.4	2.2	5.2	6.1

372

373 Figure 3 illustrates degree of polymerization after NaBH$_4$ treatment and the Fock solubility

374 results. Fock solubility increased with decreasing pulp viscosity. However, because Fock

375 solubility is highly dependent on the degree of polymerization, deep analysis must be conducted

376 in order to draw accurate conclusions. The Lac-T 2% and Lac-T 8% treatments, which used no

377 oxygen pressure, exhibited 89 and 91% Fock solubility, respectively. An identical increase in

378 Fock solubility can be assumed since both pulp samples had a similar viscosity. The presence of

379 oxygen pressure increased the amounts of carboxyl and aldehyde groups, and as a result higher

380 value of Fock solubility was obtained. The new carboxyl groups in the glucose monomer shows

381 increased hydrophilic character compared to the hydroxyl group and may act as a kind of spacer

382 in-between the cellulose microfibrils, reducing the aggregation tendency (Gehmayr, Potthast, &

383 Sixta, 2012). It is interesting to remark that refined pulp (R) suffered a notable Fock solubility

384 loss, but the treatment R+Lac-T let to retrieve Fock solubility value. Importantly, after the

385 refining treatment the effect of Lac-T treatment was more marked than the ones observed for the

386 other treatments. As noted earlier, the refining effect helped to improve strength properties, but

387 the treatment R+Lac-T 8% (O$_2$) led to a lower final Fock solubility value than Lac-T 8% (O$_2$).

388 The difference in Fock solubility can be explained by the presence of hemiacetal linkages that

389 hindered cellulose dissolution. In addition, Gehmayr et al. (2012) suggested that low Fock

390   solubility performance can be attributed to the partially oxidized C6 carbons that are not

391   available for xanthation reactions with CS_2.

392

393   Figure 3 Degree of polymerization (DP) determined after subjecting pulps to a reduced treatment with $NaBH_4$

394   (±standard deviation) and Fock solubility, as a % of reacted cellulose, (± standard deviation) for original pulp,

395   refined pulp at 5000 rev and respective enzymatic oxidative treatments.

396

397   Figure 4 shows Wet-Zero Span Tensile Index (WZSTI) against viscosity values. Wet-Zero

398   Span test measures the intrinsic strength of a single fiber independently of fiber network

399   (Hagglund, 2004), and indirectly providing information about the effect caused by the treatment

400   to the fiber structure. As observed earlier, cellulose underwent considerable degradation after

401   oxidative treatments but intrinsic fiber strength was not affected. The treatments conducted with

402   no presence of oxygen pressure led to no differences between the resulting pulps, which in turn

403   exhibited a higher Wet Zero-Span Strength than the original pulp. The addition of oxygen

404   pressure to the system increased cellulose degradation but no variation in fiber strength was

405   observed, with respect to the original pulp. The combination of a refining step followed by the

406   oxidative treatment (R+Lac-T 8%, O_2) resulted in no further degradation with respect to Lac-T

407   8% (O_2) but in much higher intrinsic fiber strength. This result can be ascribed to the formation

408   of hemiacetal linkages which difficulted fiber individualization and interfered in the wet zero-

409 span tensile strength measurement. In addition, the R+Lac-T 8% (O_2) sample was found to

410 develop good wet strength property which may have influenced on fiber strength.
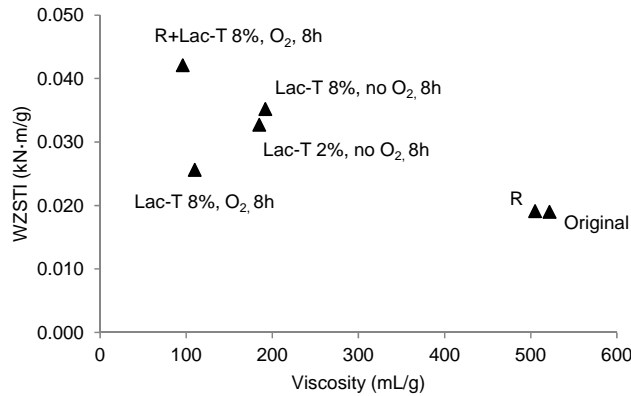
411

412 Figure 4 Wet Zero-Span Tensile Index (WZSTI) against viscosity results determined from pulps subjected to a post-
413 reduced treatment with $NaBH_4$.

414

415   Figure 5 shows a cross sectional view of handsheets made from Lac-T 2% (no O_2, 8 h), Lac-T

416 8% (no O_2, 8 h), Lac-T 8% (O_2, 8 h), refined pulp (R) and R+Lac-T 8% (O_2, 8h). In agreement

417 with fibre length and bulk density results, no differences between non-refined chemoenzymatic

418 oxidative treatments were found. The refining treatment increased the bonding ability and as a

419 result a highly compacted handsheet was observed. Interestingly, the combined treatment

420 (R+Lac-T 8%) revealed lower adhesion and sheet compaction relative to the refined sample.

421 Both samples had the same fibre length and bulk density, but differed in air permeance. Thus, the

422 R+Lac-T sample showed more pore structures, which explain the higher air permeance value.

423

Lac-T 2%, no O₂, 8h   Lac-T 8%, no O₂, 8h   Lac-T 8%, O₂, 8h

Refined pulp (R)   R+ Lac-T 8%, O₂, 8h
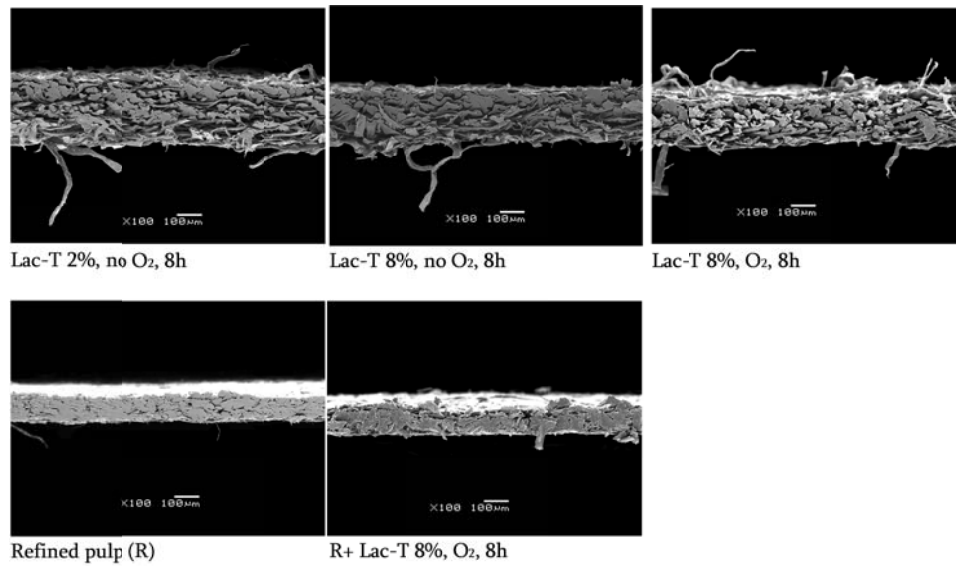
424

425   Figure 5 Cross section of laccase-TEMPO oxidized samples by Scanning Electron Microscope (SEM).

426   ## 4. Conclusions

427   In the present work, chemo-enzymatic modification of commercial bleached dissolving pulp

428   using laccase as biocatalyst and TEMPO as enhancer was investigated. Different treatment

429   conditions, such as the presence or absence of oxygen pressure, TEMPO dose, reaction time and

430   the introduction of a refining step before the oxidative treatment, were studied in order to

431   maximize the content of carboxyl and aldehyde groups, which were found to contribute

432   positively on dry and wet strength properties. From all results, the treatment Lac-T which used

433   no oxygen pressure provided low carboxyl and aldehyde contents and therefore no wet strength

434   was developed, even though it increased Fock solubility by 35% with respect to the original

435   pulp. Importantly, using oxygen pressure brought higher amount of carboxyl and aldehyde

436   groups, strength-related properties were improved but further cellulose degradation was

437   observed. The treatment Lac-T 8% (O₂) improved Fock solubility by 43%, regarding original

438   pulp, and led to an acceptable wet strength ratio (W/D=18%). On the other hand, R+Lac-T 8%

439   (O₂) caused slightly increase in carboxyl and aldehyde groups with respect to Lac-T 8% (O₂)

440 sample, but improved the wet strength property significantly, with a W/D strength ratio of 26%.

441 These results demonstrated that the fibrillation effect resulted from the refining treatment let to

442 form hemiacetal covalent linkages with aldehyde groups derived from Lac-TEMPO treatment

443 and available intra- hydroxyl groups. Specifically, the combined effect between fibrillation and a

444 favorable aldehyde/carboxyl ratio led to improve wet strength property. As a relevant conclusion,

445 the refining treatment exhibited a Fock solubility value similar to that of non-refined treated

446 pulp, but improved bonding capability and as a consequence strength-related properties.

453 **Conflicts of Interest** The authors declare no conflict of interest.

454

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