1 **Comparative evaluation of the action of two**
2 **different endoglucanases. Part II: On a biobleached**
3 **acid sulphite pulp**

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18 **Abstract** A TCF sulphite pulp, bleached at the laboratory scale with a laccase–violuric acid
19 system and complemented with a pressurized hydrogen peroxide stage, was treated with two
20 endoglucanases, one obtained from *Paenibacillus barcinonensis* (B) and the other one produced
21 from *Cerrena unicolor* (F) to improve cellulose reactivity. The treated pulps were evaluated in
22 terms of brightness, viscosity, α -cellulose, water retention value (WRV), fibre morphology, Fock
23 solubility, NMR and carbohydrate composition of pulps and liquors. Results revealed that both
24 endoglucanases improved cellulose reactivity, albeit in a different way; thus, B caused no scissions
25 in the cellulose chain and no significant reduction in fibre length, whereas F decreased viscosity
26 and shortened fibre length, leading to lower reactivity value. The liquor composition of soluble
27 carbohydrates released by the enzymatic treatments revealed the B had a processive mode of
28 action since short oligosaccharides, cellobiose and glucose, were obtained. F hydrolysed, from
29 high to low concentration, cellobiose, glucose and cellotriose. Importantly, environmentally
30 friendly dissolving pulp with 90% Fock solubility was obtained, combining two enzymatic
31 treatments: a laccase–mediator system (LMS) and then a cellulase from *Paenibacillus*
32 *barcinonensis* (B). In order to improve the quality of final dissolving pulp, a pulp purification step
33 was introduced before the endoglucanase treatment. The cold caustic extraction lead to reduce the
34 amount of hemicelluloses by 42% with respect to biobleached pulp, but Fock solubility was also
35 reduced. However, complementing the purification step with F treatment, improved Fock
36 solubility by 17%, although some presence of cellulose II was detected by NMR.

37 **Keywords** *cellulose; biobleached pulp; dissolving pulp; endoglucanase; oligosaccharides; Fock*
38 *solubility*
39

40 **Abbreviations**

41 CCE > Cold caustic extraction

42 CS > Chain scission

43 DP > Degree of Polymerization

44 EG > Endoglucanase

45 K_CCE > Control treatment with buffer acetate + cold caustic extraction

46 L > Laccase-mediator treatment

47 L_B120 > Biobleached pulp + B endoglucanase at 120U/g

48 L_CCE > Biobleached pulp + cold caustic extraction

49 L_CCE_B120 > Biobleached pulp + cold caustic extraction + B endoglucanase at 120U/g

50 L_CCE_F12 > Biobleached pulp + cold caustic extraction + F endoglucanase at 12U/g

51 L_F12 > Biobleached pulp + F endoglucanase at 12U/g

52 L_K > Biobleached pulp + control treatment with buffer acetate

53 PO > Hydrogen peroxide reinforced with pressurized oxygen

54 TCF > Totally chlorine free

55 n.d. > Not determined

56 odp > Oven dried pulp

57 VA > Violuric acid

58 WRV > Water retention value

59 ¹³C-CP/MAS NMR > Solid state cross polarization/magic angle spinning carbon 13
60 nuclear magnetic resonance

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64 **Introduction**

65 Finite fossil fuel reserves and their negative impact on the environment require the
66 shift from fossil to bio-renewable carbon feedstock; therefore lignocellulosic biomass is a
67 promising, renewable and globally accessible alternative to petroleum derived
68 compounds. Cellulose, the most abundant polysaccharides in plant biomass, is a linear
69 homopolymer consisting of anhydro- β -D-glucopyranose units (AGU) that are linked
70 together by (1- β -4) glycosidic bonds. Every AGU contains three hydroxyl groups in the
71 positions C2, C3, and C6. All of the three hydroxyl groups are involved in this hydrogen
72 bond network. In addition to the hydrogen bonding, hydrophobic interactions have been
73 suggested to be present in the structures cellulose forms (Lindman et al. 2010; Glasser et
74 al. 2012; Medronho et al. 2012). The molecular structure of cellulose is simple but the
75 most exceptional properties are due to its supramolecular structure. Cellulose chains have
76 an intense tendency to aggregate. During the cell wall biosynthesis, Van der Waals forces
77 and hydrogen bonding between hydroxyl groups and oxygen from adjacent molecules
78 promote parallel stacking of multiple cellulose chains forming elementary fibrils that
79 aggregate into larger structural units named microfibrils (Moon et al. 2011).

80 Most cellulolytic microorganisms produce a battery of cellulases which act
81 synergistically to solubilize crystalline cellulose (Lynd et al. 2002; Chiriac et al. 2010;
82 Chiriac et al. 2013). Hydrolysis of cellulose (i.e. cleavage of the (1- β -4) glycosidic bonds)
83 depends on three classes of enzymes: cellobiohydrolases (CBHs; EC 3.2.1.91),
84 endoglucanases (EGs, EC 3.2.1.4) and β -glucosidases (EC 3.2.1.21). Cellobiohydrolases
85 remove cellobiose from either the non-reducing or the reducing ends of chain with higher
86 apparent activity on crystalline cellulose, and as a result, a rapid production of soluble
87 sugars but slow decrease of the degree of polymerization (DP) is observed.
88 Endoglucanases are typical multi-domain enzymes consisting of a catalytic domain that
89 randomly cleaves β -1,4-glycosidic bonds in the middle of the polysaccharide chains and
90 typically act on more irregular physical structures of the substrates, giving a rapid
91 reduction of the DP but slow release of soluble sugars from crystalline cellulose (Ek et al.
92 2009). The degradation of amorphous cellulose located on the fibre surface and in
93 between microfibrils, resulted in increasing exposed crystalline surfaces, and improving
94 the swelling ability and reactivity of pulp (Henriksson et al. 2005; Gehmayr et al. 2011).
95 Beta-glucosidases (β -D-glucopyranoside glucosylhydrolases) are enzymes that hydrolyze
96 glycosidic bonds to release nonreducing terminal glucosyl residues from glycosides and
97 oligosaccharides (Cairns and Esen 2010).

98 The bioconversion process uses enzymes, such as cellulase, to break down cellulose
99 into sugars that can be later fermented into ethanol. Therefore, following the biorefinery
100 concept, endoglucanases are also used for upgrading paper-grade pulps to dissolving-

101 grade pulp, and obtain hemicellulose free dissolving pulp as a primary product, but also
102 lignin, bioethanol and a number of complementary products. The removal of
103 hemicelluloses from paper kraft pulp is the main challenge to convert it into dissolving
104 pulp. Cold caustic extraction (CCE), commonly used as post treatment to PHK pulp for
105 acetate grade dissolving pulp production can dissolve selectively hemicelluloses (Sixta
106 2006; Arnoul-Jarriault et al. 2014). The dissolution of hemicelluloses during CCE is
107 related to structural change of cellulose pulps when they are subjected to concentrated
108 sodium hydroxide solution (> 8% w/v).

109 According to FAO (2012), nowadays, pulps for chemical processing, or dissolving
110 pulps constitute only a small share of the global pulp production (around 3% or $4.22 \cdot 10^6$
111 ton,) but this share is increasing and prospective consumer markets indicate that will
112 continue in subsequent decades. Interestingly, EG treatments have become more and
113 more popular in recent years to improve pulp reactivity and, as a side effect, precisely
114 adjust pulp viscosity (Henriksson et al. 2005; Engström et al. 2006; Köpcke et al. 2008;
115 Ibarra et al. 2010a). Additionally, cellulose II is attacked by endoglucanases (Rahkamo et
116 al. 1998), which was speculated to play a role in reactivity increase of the pulp after EG
117 treatment (Henriksson et al. 2005; Engström et al. 2006; Kvarnlöf et al. 2006; Gehmayr
118 and Sixta 2012). However, cellulose II is unfavourable for the production of cellulose
119 derivatives or regenerated cellulose such as viscose for textile fibres because it has an
120 antiparallel orientation relative to cellulose I and a more entangled and complex
121 hydrogen-bonding network than the natural form (Ibarra et al. 2010a); as a result, the
122 presence of cellulose II considerably affects the accessibility of cellulose.

123 On the other side, the introduction of new technologies to bleach dissolving pulps
124 using enzymatic treatments and complemented with treatments based on oxygen-derived
125 compound can be an alternative to traditional bleaching processes, such as elemental
126 chlorine free (ECF) and totally chlorine free (TCF) processes. In a previous work, a
127 laccase–mediator system (LMS) proved to be effective to bleach never-dried softwood
128 sulphite pulp (Quintana et al. 2013). The proposed L_{VA}QPO sequence provided
129 acceptable results in terms of dissolving pulp requirements (Quintana et al. 2015) and had
130 a positive contribution from an environmentally point of view. However, in the direction
131 of improving pulp reactivity and the quality of the final product (i.e. reduce the amount of
132 hemicelluloses) further treatments were required. The remarkable results obtained from
133 commercial dissolving pulp and endoglucanase treatments approved the idea to conduct a
134 similar study but on biobleached dissolving pulp (see *Comparative evaluation of the*
135 *action of two different endoglucanases. Part I: On a fully bleached, commercial acid*
136 *sulfite dissolving pulp*, submitted). Therefore, in this work, cellulose activation by two
137 endoglucanase enzymes one obtained from *Paenibacillus barcinonensis* (B) and the other

138 from *Cerrena unicolor* (F) was examined on biobleached sulphite pulp. Cellulase action
139 was evaluated in terms of Fock solubility, ^{13}C -CP/MAS NMR, water retention value
140 (WRV), fibre morphology, cellulose degradation and carbohydrate composition of pulp.
141 Another important contribution of the present study was to examine the composition of
142 dissolved carbohydrate present in the liquors. Understanding the mode of action of the
143 respective cellulases, in terms of pulp activation and cellulose modification, provided
144 interesting information in view of a potential industrial application. The work was
145 completed with a pulp purification step, with 9% w/v NaOH, at 25°C, before the
146 endoglucanase treatments. The combination of a cold caustic extraction followed by a
147 hydrolytic treatment was evaluated in terms of Fock solubility, hemicellulose content and
148 ^{13}C -CP/MAS NMR.

150 **Experimental**

151 **Pulp and enzymes**

152 Unbleached sulphite cellulose obtained from a mixture of 60% Norway spruce (*Picea*
153 *abies*) and 40% Scots pine (*Pinus sylvestris*) was used as a raw material. The pulp was
154 cooked at Domsjö Fabriker mill (Sweden) and the characteristics were as follows: $4.2 \pm$
155 0.2 kappa number, $61.25 \pm 0.6\%$ ISO brightness and 511 ± 11 mL/g viscosity, and a
156 carbohydrate content as determined by HPLC of $90.2 \pm 0.38\%$ glucan, $4.3 \pm 0.1\%$
157 mannan, $2.1 \pm 0.0\%$ xylan, $0.8 \pm 0.0\%$ glucuronic acid, $0.2 \pm 0.0\%$ arabinan and $0.04 \pm$
158 0.01% acetyl groups. This pulp was biobleached using a laccase treatment (an L
159 sequence) before the hydrolytic treatment with endoglucanases.

160 The biobleaching treatment involved laccase from *Trametes villosa* (TvL), which
161 was supplied by Novozymes® (Denmark) with a laccase activity of 746 U/mL. Its
162 activity was measured as the extent of oxidation of 5 mM 2,2'-azinobis(3-
163 ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical ($\epsilon_{436} = 29\,300\text{ M}^{-1}\text{ cm}^{-1}$)
164 in 0.1 M sodium acetate buffer (pH 5) at 24 °C. One activity unit (U) was defined as the
165 amount of enzyme converting 1 μmol of ABTS per min. Violuric acid (VA), the mediator
166 used for the bleaching treatment, was purchased from Sigma–Aldrich and used as
167 received.

168 Two different endoglucanases were used in the hydrolytic treatments. The B (EC.
169 3.2.1.4) was produced from the newly identified species *Paenibacillus barcinonensis* by
170 Universitat de Barcelona (Spain) (Chiriac et al. 2010). This endoglucanase is a modular
171 enzyme with the structure GH9-CBM3c-Fn3-CBM3b (E1), a truncated derivative of the
172 cellulase with the structure GH9-CBM3c (E2), and a recombinant cellulose binding
173 module CBM3b (CBD) derived from the enzyme. E1 and E2 exhibit cellulase activity as

174 they contain the catalytic module GH9, whereas CBD has no hydrolytic activity on
175 cellulose (Chiriac et al. 2010; Chiriac et al. 2013). B is a family 9 enzyme and shows a
176 processive mode of action. A cellulase activity of 60 CMCase U/mL was determined by
177 measuring the amount of reducing sugars released from carboxymethyl cellulose (CMC,
178 Sigma) according to Somogyi-Nelson method (Spiro 1966). An activity unit (U) is the
179 amount of enzyme capable of converting 1 μ mol of substrate per second. The F
180 endoglucanase was supplied by Fungal Bioproducts® (Spain) and was produced from
181 *Cerrena unicolor*. The activity as U/g dry enzyme powder of the cellulase preparation
182 was: 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity on the
183 cellulase, respectively. The activity was also determined in our laboratory using the
184 Somogyi-Nelson method.

185 **Biobleaching sequence (L) for unbleached sulphite pulp**

186 Prior to the bleaching treatment, unbleached sulphite fibres were conditioned at
187 pH 4 adjusted with H₂SO₄, stirred at 2% pulp consistency for 30 min and washed with de-
188 ionized water in a glass filter funnel. This step was needed to remove contaminants and
189 metals, and also to bring the pulp to the pH required for the enzymatic treatment.

190 The bleaching process was conducted at the laboratory scale. A TCF
191 biobleaching sequence including a laccase–mediator treatment was applied to unbleached
192 sulphite cellulose. The sequence was designated L_{VA}PO, where L denotes the enzymatic
193 treatment and PO a hydrogen peroxide stage reinforced with oxygen. The enzymatic stage
194 (L) was carried out with the laccase–violuric acid system in an oxygen pressurized reactor
195 (0.6 MPa) at stirring rate of 30 rpm, using 50 mM sodium tartrate buffer (pH 4) to adjust
196 5% (w/w) pulp consistency, at 50 °C for 4 h. The enzyme dose was 20 U/g odp (oven dry
197 weight of pulp) of laccase and 1.5% odp of violuric acid (Quintana et al. 2013). The
198 enzymatic treatment was followed by a chemical bleaching stage involving alkaline
199 hydrogen peroxide reinforced with oxygen. PO was carried out at 5% (w/w) consistency
200 in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm under the
201 following conditions: 10% odp H₂O₂, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp
202 MgSO₄, at 90°C for 1h. Subsequently, the treated pulps were extensively washed with de-
203 ionized water and stored until use.

204 **Enzymatic hydrolytic treatments and cold caustic extraction**

205 The resulting biobleached pulp (L_{VA}PO) was subjected to enzymatic hydrolysis
206 with the two endoglucanases, B and F. The former enzyme was used at 120 U/g odp
207 (L_B120 treatment) and the latter at 12 U/g odp (L_F12 treatment). An amount of 20 g of
208 dry pulp at 10% consistency in 0.05 M sodium acetate buffer at pH 5.5 at 55 °C was

209 treated with each enzyme for 1 h in each run (Cadena et al. 2010). The enzymatic
 210 treatments were performed in polyethylene bags that were placed in a laboratory water
 211 bath. The samples were periodically kneaded. The reaction was stopped by washing the
 212 pulp with de-ionized water in a porous glass filter funnel of porosity grade 2. A control
 213 treatment (L_K) was also performed under the same conditions but in the absence of
 214 enzyme. In parallel, the biobleached pulp (L_{VA}PO) was also treated with a cold caustic
 215 extraction in an Easydye Ahiba oscillating individual reactor from Datacolor. The
 216 treatment was performed at 10% (w/w) consistency adjusted with 9% (w/v) NaOH at
 217 25°C for 1h. Then the hydrolytic treatments with B or F enzymes were conducted,
 218 following the same conditions described earlier but using an Easydye Ahiba oscillating
 219 individual reactor (see Schema 1).

220 **Analysis of pulp properties**

221 The starting and treated pulp samples were characterized in terms of kappa number,
 222 brightness, viscosity, α -cellulose and water retention value (WRV) according to ISO
 223 302:2004, ISO 2470:2009, ISO 5351:2004, TAPPI method T-203 cm-99 and ISO 23714,
 224 respectively. The bleaching process was also characterized in terms of chroma (C^*),
 225 which is the perpendicular distance of a point from the lightness axis [$C^* = (a^{*2} + b^{*2})^{1/2}$]
 226 and represents the amount of colour of a sample. The degree of polymerization (DP) was
 227 calculated from intrinsic viscosity values, using the equation of Evans & Wallis (1987)
 228 (SCAN-CM 15:88):

$$229 \text{ Eq. (1) } DP^{0.85} = 1.1 \cdot [\eta]$$

230 Pulp degradation can also be assessed via the number of scissions in the cellulose chain
 231 (CS), which is defined mathematically as (Bouchard et al. 2000):

$$232 \text{ Eq. (2) } CS = \frac{DPo - DP}{DP}$$

233 where DPo is the degree of polymerization of the initial pulp or previous stage and DP
 234 that at the end of any chemical or enzymatic treatment.

235 The cellulose reactivity of the pulp samples was determined according to slightly
 236 modified version of Fock's method (Fock 1959; Ibarra et al. 2010b). This is a micro-scale
 237 method simulating the industrial viscose process for manufacturing regenerated cellulose.
 238 Prior to analysis, the samples were dried at 50 °C and conditioned in a climate room at
 239 23 °C and 50% RH overnight.

240 Carbohydrate composition of initial (TCF-bleached dissolving pulp and unbleached
 241 sulphite pulp) and treated pulps was determined using high performance liquid
 242 chromatography (HPLC). Samples were studied on a duplicate basis using a modified
 243 version of TAPPI 249 cm-09 test method. Hydrolysis was carried out in two steps: i) A
 244 strong hydrolysis step with concentrated sulfuric acid. Approximately 50 mg of sample

with known moisture content were treated with 5mL of H₂SO₄ 72 % and kept at 30 °C for 1h with gentle stirring. ii) A mild acid step at high temperature. Tube contents were putted into 250 mL-flasks and diluted to 4 % H₂SO₄. Flasks were putted into an autoclave for 1 h at 103 kPa. Solution was then cooled and passed through a glass filter to remove insoluble lignin. Prior to HPLC analysis samples were filtered using a 0.45µm pore size Whatman membrane. Chromatographic analysis was performed using a 1200 Agilent HPLC instrument furnished with a Biorad Aminex HPX-87H ion-exchange column. Concentrations were calculated by interpolation into calibration curves ran from standards of glucose, xylose, rhamnose and arabinose. In order to resolve xylose, mannose and galactose peaks, the hydrolyzed effluents were neutralized with barium carbonate (BaCO₃), then were filtered through a membrane of 0.45 µm pore size and then were analyzed with a Biorad Aminex HPX-87P column. The chromatographic determination was performed under the following conditions: mobile phase 6 mmol/L (acid samples) or ultrapure water (neutralized samples); flow rate, 0.7 mL/min; column temperature, 60°C (acid samples) or 85°C (neutralized samples). ¹³C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument operating at 7.05 T and at 75.5 MHz for ¹³C. Samples were immersed in deionized water for at least 2h. All measurements were performed at 290 ± 1K. The magic angle spinning (MAS) rate was 4 kHz. The cross-polarization contact time was 1 ms and the recycle delay time 2.5 s. Acquisition time was 98.3 ms and sweep-width was 31.2 kHz. The number of scans was 5100. The quantification method was based on the evolution of two independent and isolated signal arising from cellulose I and II, respectively: the decrease of the height of the peak near 65 ppm, assigned to the C6 in the crystalline part of cellulose I and the increase of the peak near 107 ppm, assigned to the C1 in the crystalline part of cellulose II (Janzon et al. 2008b; Arnoul-Jarriault et al. 2014).

The morphological properties of the fibres (viz., length, width and curl), and the content in fines of the pulp samples were determined in accordance with TAPPI T 271 on a Metso kajaaniFS300 fibre analyser. All samples were analysed in duplicate. Surface SEM images of the handsheets were taken on a JEOL JSM-6400 microscope. Samples were placed on the SEM sample holding stub with the aid of conductive double side sticky carbon film and coated with Au/Pd alloy prior to analysis.

Effluent properties

Dissolved carbohydrates present in the liquors released from the cellulase treatments of biobleached dissolving pulps were quantified by HPLC (Agilent 1200 HPLC instrument), using a Biorad Aminex HPX-87H ion-exchange resin column that affords the separation of glucose, xylose and arabinose. Prior to analysis, the liquors were subjected to acid

281 hydrolysis with 4% H₂SO₄ and the flasks placed in an autoclave at 103 ± 7 kPa for 20 min
 282 in order to convert oligomers into monomers for easier on-column separation. Then, the
 283 hydrolyzed solutions were filtered through Whatman membranes of 0.45 µm pore size
 284 and the chromatographic determination was performed under the following conditions:
 285 mobile phase, 6 mmol/L sulphuric acid; flow rate, 0.7 mL/min; column temperature, 60
 286 °C.

287 The concentration of oligosaccharides present in the treatment liquors was also
 288 determined by HPLC, using a Biorad Aminex HPX-42A ion-exchange column (Garcia-
 289 Ubasart et al. 2013). Effluents were filtered using a 0.45 µm pore size Whatman
 290 membrane and their pH was neutralized (to pH 7) using HCl or NaOH. The determination
 291 was performed under the following conditions: mobile phase, ultrapure water; flow rate,
 292 0.35 mL/min; column temperature, 65°C. Identification and quantification of compounds
 293 was done by interpolation into calibration curves run from standards.

294 **Schema 1.** Schema of work applied to the never-dried unbleached sulphite pulp and the
 295 respective studied properties.
 296

297

298 Results and Discussion

299 In previous works (Quintana et al. 2013; Quintana et al. 2015), sulphite pulp was
 300 satisfactorily biobleached by a combination of a laccase-violuric acid treatment, chelant
 301 stage and a pressurized hydrogen peroxide treatment. These biobleached pulps (L_{VA}QPO)
 302 were characterized in terms of Fock solubility, viscosity, ISO brightness, alkali resistance,

303 α -cellulose, metal ion content and thermal degradation by TGA. The pulps exhibited
 304 acceptable market dissolving pulp characteristics but in the direction of improving pulp
 305 reactivity and the quality of the final product (i.e. lower amount of hemicelluloses) further
 306 treatments were required. Therefore, this part of the work want to elucidate the effect of
 307 two endoglucanases, one from *Paenibacillus barcinonensis* (B) and the other one from
 308 *Cerrena unicolor* (F), on biobleached sulphite pulps (L_{VA}PO). Furthermore, the
 309 outstanding results obtained from a bleached commercial dissolving pulp, cold caustic
 310 extraction and the respective endoglucanase treatments, supported the idea to conduct the
 311 same investigation with L_{VA}PO pulp. Therefore, the cellulase treatments were compared
 312 with those where a cold caustic extraction with 9% (w/v) of NaOH was introduced before
 313 the hydrolytic treatment. Based on the previous results, the K_CCE treatment was
 314 eliminated since no differences were observed with respect to control treatment (K). As a
 315 novelty, the carbohydrate composition of liquors was analyzed and leads to understand
 316 the mechanism of action of each enzyme.

317 1. Fock Solubility

318 Table 1 shows the Fock solubility results. The initial pulp had a Fock solubility
 319 value of 76.6%, while L_F12 treatment had an 88% and the L_B120 treatment reached a
 320 Fock solubility value of 91%.

321 **Table 1.** Fock solubility values obtained for each sample

Fock solubility (%)	
L _{VA} PO (=L)	76.6 ± 3.7
L_K	76.3 ± 5.5
L_CCE	69.7 ± 3.6
L_B120	90.9 ± 1.9
L_CCE_B120	70.8 ± 3.7
L_F12	88.1 ± 1.1
L_CCE_F12	82.4 ± 2.4

322

323 In Figure 1 is shown the relation of Fock solubility increase and chain scission values
 324 (CS), calculated from the viscosity results. As expected, the control cellulase treatment
 325 did not undergo Fock solubility improvement, although the viscosity value was slightly
 326 different from biobleached pulp. With respect to biobleached pulp, the L_B120 improved
 327 Fock solubility by 18.7% with no variation on viscosity. Therefore, this change was due
 328 to the action of the own endoglucanase treatment. The F12 treatment also suffered a Fock

solubility increase but a viscosity loss with respect to initial pulp was found. Therefore, the improvement on Fock solubility cannot only be related to the enzyme action. The relationship between pulp solubility according to Fock method and chain scission values provided useful information to understand the action that each enzyme caused on biobleached cellulose fibres. Special attention had the B endoglucanase which provided the highest increase in Fock solubility (18.7 % respect to L_{VA}PO) even though CS was virtually zero, suggesting that this enzyme produced a fibrillated effect rather than cutting the fibres, as previously demonstrated by some authors (Cadena et al. 2010; Garcia-Ubasart et al. 2013). With F endoglucanase, the L_F12 improved Fock solubility by 13.5 % and exhibited slightly greater CS number of 0.16 with respect to starting pulp, which is in agreement with its slightly greater viscosity loss. As can be seen from Table 2 and Figure 1, the buffer solution used in the control treatment (L_K) did not contribute to improve Fock solubility or shorten fibres and CS value remained 0.

Figure 1. Fock solubility increase (calculated with respect to the previous treatment) and number of chain scission for each treatment.

Carbohydrate composition was determined by HPLC, with special interest on the hemicelluloses content (Figure 2a). The hemicellulose composition of biobleached pulp (L_{VA}PO) was as followed: 2.4% of xylan, 0.3% of mannan, 3.0% galactan and 6.0% mannan. As can be seen, B and F endoglucanases exhibited the same pattern of action for rhamnan and galactan but acted differently on mannan and xylan fraction. With B endoglucanase treatment, the hemicellulose content was diminished by 37% and the final amount of hemicelluloses was 7.4%. As can be seen, the highest reduction was observed with xylan fraction (77%), then galactan (47%) and finally mannan (19%), with respect to initial pulp. The amount of rhamnan was not modified with enzymatic treatment. F

355 endoglucanase decreased the amount of hemicelluloses by 39% which corresponds a
356 7.1% of final hemicelluloses. The xylan fraction suffered a reduction of 53%, the galactan
357 a 47% and the mannan a 30%. In both treatments, the xylan removal was superior to
358 mannan. Gehmayr and Sixta (2012) described that during a CCE80 (80g/L of NaOH) at
359 30°C, mannan was resistant to physical dissolution due to its highly ordered structure but
360 xylan was removed because of its good solubility under these conditions. As the results of
361 commercial pulp and endoglucanases treatments revealed (see *Comparative evaluation of*
362 *the action of two different endoglucanases. Part I: On a fully bleached, commercial acid*
363 *sulfite dissolving pulp*, submitted), the action caused on mannan fraction was higher with
364 F than with B endoglucanase. In addition, as observed with B, the rhamnan fraction was
365 not modified with F treatment.

366 In terms of pulp purification step, the strong alkaline treatment before the hydrolytic
367 treatment contributed to reduce the total amount of hemicelluloses (Figure 2b). The final
368 amount of hemicelluloses for L_CCE_B120 and L_CCE_F12 was 6.2% and 5.8%,
369 respectively. As previously observed, the B endoglucanase had slightly higher action on
370 xylan fraction than F. In particular, B reduced the amount of xylans by 79% while the F a
371 70%. With the hydrolytic treatments alone some changes in the mannan fraction were
372 observed. However, incorporating a cold caustic extraction and then the hydrolytic
373 treatments, the mannan fraction was not affected, although the respective amounts were
374 in the range of L_B120 and L_F12 samples. These results may indicate that the remaining
375 fraction of mannan is not susceptible to alkali treatment.

Figure 2. Hemicellulose composition of treated pulps determined by HPLC. (a) Biobleached pulp and the respective endoglucanase treatments (b) biobleached pulp followed by cold caustic extraction and then the respective endoglucanase treatments. The sum of total hemicelluloses is indicated next to each label.

2. Brightness, chromatic coordinate, viscosity, α -cellulose and fibre morphology results

The B and F treatments led to decrease brightness in the biobleached sulphite pulp, mainly through adsorption of products present in the enzyme solution (see part 1). Also, the viscosity results manifested that the enzymatic treatments had no effect on cellulose integrity, although L_F12 seemed to present a slightly lower viscosity. On the other hand, α -cellulose was adversely affected by the enzymatic treatments, particularly with B. It should be noted that viscosity represents the average molecular

weight of all cellulose chains, whereas α -cellulose indicates the content of undegraded, higher molecular weight cellulose in pulp. Therefore, although no damage of cellulose chains was perceived, the content in high-molecular weight cellulose (α -cellulose) was considerably altered by B enzyme. Regarding the proportion of fines, enzymatic treatments increased the amount of fines by up to 19% with L_B120 and 34% with L_F12, relative to L_{VA}PO sample. Likewise, fibre length was reduced by 19% and 41%, respectively. The latter results suggest that both endoglucanases act on cellulose chains but have a different effect on fibre length, which is also suggestive of a different mechanism of action.

It can also be appreciated in table 2 that the viscosity, the α -cellulose and the contents in fines, of the treated control pulp was practically identical with those of the starting pulp.

Table 2. Results ($\pm$ confidence intervals) of brightness, chroma coordinate (C*), viscosity and α -cellulose. The content of fines and fibre length were determined according to TAPPI standard and calculated as an arithmetic mean for the different treated pulps.

	ISO Brightness (%)	C*	Viscosity (mL/g)	α -cellulose	Fines (%)	L (n) (mm)
L _{VA} PO	88.45 $\pm$ 0.11	3.07	395 $\pm$ 6	87.3 $\pm$ 0.39	30.98 $\pm$ 1.04	0.85 $\pm$ 0.02
L_K	88.95 $\pm$ 0.11	3.00	453 $\pm$ 28	89.5 $\pm$ 0.71	30.96 $\pm$ 1.04	0.86 $\pm$ 0.01
L_B120	82.90 $\pm$ 0.27	4.14	384 $\pm$ 88	80.4 $\pm$ 0.02	38.32 $\pm$ 1.12	0.69 $\pm$ 0.02
L_F12	85.75 $\pm$ 0.60	3.20	348 $\pm$ 43	83.4 $\pm$ 0.61	47.30 $\pm$ 0.52	0.50 $\pm$ 0.01

The biobleached pulp treated with B increased the WRV by 17% but fibre length was only reduced by 19% and the content of fines increased by 19%, with respect to L_{VA}PO pulp (Figure 3). By contrast, L_F12 treatment did not modify the WRV (4% increase) but raised the content in fines by 53% and reduced fibre length by 42%, regarding biobleached pulp. The respective results suggest that B enzyme might alter fibre surface via external fibrillation (Cadena et al. 2010; Garcia-Ubasart et al. 2013) while F enzyme showed a preferable action to cut the fibres.

The differences found between fibre length, fine content, WRV and CS pointed out that B and F endoglucanases had different performing mechanism.

416

417 **Figure 3.** Relation between fibre lengths determined according to TAPPI standard and
 418 calculated as an arithmetic mean and water retention value (WRV) for biobleached
 419 sulphite pulps treated with B and F enzyme.

420 ¹³C-CP/MAS NMR

421 The solid state ¹³C-NMR spectra of biobleached pulp, the enzymatic treated pulps and 9%
 422 NaOH extracted pulps followed by endoglucanase treatments are illustrated in Figure 4.
 423 The spectra of L_CCE_B120 and L_CCE_F12 samples exhibited different polymorphic
 424 form than samples submitted to an endoglucanase treatment alone. The introduction of a
 425 9% NaOH extraction converted cellulose I to cellulose II, since the C-6 signal at 64 ppm
 426 increased, obtaining two peaks with nearly identical heights at 66 ppm and 64 ppm.
 427 Nevertheless, the C-1 signal did not exhibit a shoulder at 108 ppm, which is characteristic
 428 of cellulose II (Janzon et al. 2008a). The greater proportion of cellulose II of
 429 L_CCE_B120 and L_CCE_F12 samples can be related to the low Fock solubility values,
 430 in comparison to L_B120 and L_F12 (Krässig 1993; Janzon et al. 2008a).

Figure 4. ^{13}C -CP/MAS NMR spectra of biobleached pulp (L) compared to samples treated with B and F endoglucanases alone or after an extraction with 9% NaOH.

Figure 5 shows SEM images of handsheets made from L_{VAPO} , L_{B120} , L_{F12} and L_{K} treated pulps. Interestingly, the action of F enzyme was apparently observed in increased amount of free particles and disruption of the cellulose wall structure. With the L_{B120} treatment, differences in appearance with respect to control and starting pulp were inappreciable; however, the treated pulp was 19% more reactive, but similar in viscosity and fibre length to the starting pulp.

Figure 5. SEM images of the biobleached treated pulps and the respective hydrolytic treatments

3. Mode of action of B and F endoglucanases

In order to further explain the results, the concentration and composition of dissolved sugars present in the liquors released from the enzymatic treatments were determined by HPLC. Comparing the results for the different treatments required expressing the composition of dissolved monosaccharides relative to the effluent volume resulted from each treatment. As can be seen from Table 3, the L_{VA} treatment produced a greater release of glucose and xylose than did L_{VAPO} . After the biobleaching treatment, pulps were subjected to an endoglucanase treatment with B or F. As also observed with Fock solubility and fibre morphology, the results for the control treatment (L_K) revealed that the buffer solution by itself had no effect on carbohydrate composition, while L_{F12} treatment caused the greatest release of glucose, followed by L_{B120} . These results are consistent with the number of chain scissions caused by each enzyme and with the extent cellulose degradation, expressed as viscosity. As expected, the amount of xylose in the liquors was smaller than that of glucose in all treatments with an exception of control treatment (L_K), which led to very similar amounts of the two monomers. Although F had a xylanase activity of 680 U/g, the liquors contained little xylose monomer—probably due to the low xylan content in the starting pulp, as determined by HPLC.

Table 3. Amount of dissolved monosaccharides (g) found in the liquors of each treatment

465

466 In addition, the low amount of oligosaccharides detected in L_B120 treatment is
467 consistent with the low decrease of pulp viscosity.

468 **Table 4** shows the concentration and composition of oligosaccharides in the effluents as
469 determined by HPLC. Glucose standard oligomers were used as references. The pattern
470 of products released by the L_F12 treatment decreased in the following sequence:
471 cellobiose > glucose > cellotriose. The fact that oligosaccharides of different size and
472 glucose monomer are found is consistent with the mode of action of F, which randomly
473 cleaves glycosidic bonds. With L_B120 treatment, cellobiose in major proportion and
474 glucose in lower were identified in the liquors but no presence of cellotriose or larger
475 oligosaccharides. The contents in hydrolysis products suggest that B is an endoglucanase
476 with an exo type mode of action (Chiriac et al. 2010). According to Chiriac et al. (2010),
477 the absence of long oligosaccharides among the hydrolysis products from crystalline
478 cellulose would indicate a processive mode of action of B, as previously reported for
479 some endoglucanases of family 9 (Irwin et al. 1998; Zverlov 2003). In addition, the low
480 amount of oligosaccharides detected in L_B120 treatment is consistent with the low
481 decrease of pulp viscosity.

482 **Table 4.** Concentration of oligosaccharides (mg/mL) in enzymatic treatments liquors

mg/mL	L_B120	L_F12	L_K
Glucose	0.270	0.829	0.000
Cellobiose	0.592	1.486	0.000
Cellotriose	0.000	0.05	0.000

483

484

485 **Conclusions**

486 Two different cellulases, B and F, were studied in never-dried biobleached sulphite pulp
487 ($L_{VA}PO$) with the intention to increase Fock solubility and bring a satisfactory advantage
488 to the viscose process. The enzymes, however, exhibited a different mode of action. Thus,
489 while B increased Fock solubility strongly but had little effect on fibre length, F increased
490 Fock solubility to a lesser extent but produced a strong reduction in fibre length and
491 increased notably the amount of fines. In terms of the number of chain scission, the F
492 endoglucanase produced higher values than B endoglucanase. The soluble carbohydrate
493 composition present in the liquors as determined by HPLC confirmed that B
494 endoglucanase had an exo type mode of action since cellobiose and glucose in different
495 proportion were detected. On the other hand, the F liquors contained cellobiose, glucose
496 and traces of cellotriose soluble oligomers.

497 The most salient contribution of this study is that the introduction of a
498 biobleaching sequence involving a laccase-mediator system (LMS) provides an
499 environmentally friendly process without detracting the final characteristics of a
500 dissolving pulp, and the subsequent endoglucanase treatment (L_{B120}) reached a 91% of
501 Fock solubility. Moreover, the cold caustic extraction introduced before the hydrolytic
502 treatment could reduce the percentage of hemicellulose although the final Fock solubility
503 was lower due to the formation of cellulose II as observed by ^{13}C -CP/MAS NMR.
504 Importantly, complementing the purification step with F12 treatment, lead to improve
505 Fock solubility and reach a final value of 82.4%.

506

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516

517 **References**

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