1 **Comparative evaluation of the action of two**
2 **different endoglucanases. Part I: On a fully**
3 **bleached, commercial acid sulfite dissolving pulp**

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17 **Abstract** A fully bleached commercial acid dissolving pulp was treated with two
18 endoglucanases, one obtained from *Paenibacillus barcinonensis* (B) and the other one produced
19 from *Cerrena unicolor* (F) with the intention to improve cellulose reactivity and processability in
20 the viscose process. B cellulase was tested under 120 U/g odp (oven dry pulp) and the F cellulase
21 under two conditions, 12U/g odp and 60 U/g odp. In addition, a purification stage, consisting in a
22 cold caustic extraction (CCE) of 9% w/v NaOH, was applied before or after the enzymatic
23 treatment in order to reduce the amount of hemicellulose and improve the action of enzymes. The
24 treated pulps were evaluated in terms of brightness, viscosity, water retention value (WRV), fibre
25 morphology, carbohydrate composition, Fock solubility and NMR. In general, results revealed that
26 both endoglucanases improved cellulose reactivity, albeit in a different way; thus, B caused no
27 scissions in the cellulose chain and no significant reduction in fibre length, whereas F strongly
28 decreased viscosity, shortened fibre length and increased considerably the amount of fines. The
29 result of applying two different doses of F cellulase was reflected on Fock solubility and fibre
30 morphology. F60 treatment was found to give the highest value of Fock solubility and the biggest
31 reduction of fibre length. The effect of both endoglucanases on Fock solubility was increased by
32 introducing an earlier CCE stage. Finally, a CCE_B120 pulp with 3% of hemicellulose and 69% of
33 Fock solubility was obtained.

34 **Keywords** *cellulose; biobleached pulp; dissolving pulp; endoglucanase; oligosaccharides; Fock*
35 *solubility*
36

37 **Abbreviations**

38 B120 > B endoglucanase at 120U/g

39 B120_CCE > B endoglucanase at 120U/g + cold caustic extraction

40 CCE > Cold caustic extraction

41 CCE_B120 > cold caustic extraction + B endoglucanase at 120U/g

42 CCE_F12 > Cold caustic extraction + F endoglucanase at 12U/g

43 CS > Chain scission

44 DP > Degree of Polymerization

45 EG > Endoglucanase

46 F60 > F endoglucanase at 60U/g

47 F12 > F endoglucanase at 12U/g

48 K > Control treatment

49 K_CCE > Control treatment + cold caustic extraction

50 PO > Hydrogen peroxide reinforced with pressurized oxygen

51 TCF > Totally chlorine free

52 n.d. > Not determined

53 odp > Oven dried pulp

54 WRV > Water retention value

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

57

58

59 **Introduction**

60 At present, pulps for chemical processing (so-called “dissolving pulps”) constitute
61 only a small share of the global pulp production (about 3%); however, this share is
62 growing and dissolving pulps are playing an increasing important role in the pulp market
63 by effect of ongoing restrictions on cotton cultivation and the growth of regenerated
64 cellulose fibre production, mainly, in Asian countries. Also, the targeted gradual
65 replacement of petroleum-based products with biomass-based equivalents, and the rise of
66 speciality cellulose markets in emerging economies, are further increasing the importance
67 of dissolving pulps (Tappi 2011; Testova et al. 2014). Most wood-based dissolving grade
68 pulp is obtained with the acid sulphite or pre-hydrolysis kraft processes (Sixta 2006);
69 however, the search for new technological procedures for efficient production of
70 dissolving-grade pulps has aroused much interest in recent years.

71 Cellulose presents an extremely compact structure due to its supramolecular
72 structure (viz., its intermolecular and intramolecular hydrogen bonds). The structure of
73 cellulose consists of crystalline and non-crystalline domains (Krässig 1993; Köpcke et al.
74 2008). This non uniform structure is responsible for the low accessibility and reactivity of
75 cellulose, and hence for its limited solubility in some special solvents. The crystalline
76 domain of cellulose is highly ordered and exhibits extensive hydrogen bonding between
77 molecular chains. As a result, cellulose can crystallize into various polymorphs. Natural
78 cellulose (cellulose I) is the form found in nature and occurs in two allomorphs: I_α and I_β
79 (Nishiyama et al. 2002; Nishiyama et al. 2003; Ciolacu et al. 2011). Cellulose II is the
80 crystalline form that emerges after regeneration from different media or mercerization
81 with aqueous sodium hydroxide (Langan et al. 2001; Nishiyama et al. 2003; Langan et al.
82 2005). Cellulose III_I and III_{II} are obtained by treating cellulose I and II, respectively, with
83 liquid ammonia or an organic amine (Wada et al. 2004).

84 Strongly alkaline pre-treatments are used in the production of carboxymethyl
85 cellulose and viscose for textile fibres (Mozdyniewicz et al. 2013). Such treatments cause
86 native cellulose (cellulose I) to swell and after washing shrink back to another
87 allomorphs, cellulose II, through Na-cellulose (Klemm et al. 1998; Van de Weyenberg et
88 al. 2006; Pönni et al. 2014). The conversion of cellulose I into Na-cellulose is fast.
89 Cellulose II is unfavourable for the production of cellulose derivatives or regenerated
90 cellulose such as viscose for textile fibres because it has an antiparallel orientation
91 relative to cellulose I and a more entangled and complex hydrogen-bonding network than
92 the natural form (Ibarra et al. 2010a); as a result, cellulose II has higher difficulty of
93 dissolution than cellulose I (Krässig 1993).

94 The presence of cellulose II considerably affects the accessibility of cellulose.
95 Thus, the reactivity of pulp is defined as the accessibility of hydroxyl groups at C6 and

96 C2/C3 of the glucose monomer units to reactants, and dictates the processability of
 97 dissolving pulps for the viscose process (Sixta 2006; Gehmayr and Sixta 2012).

98 Endoglucanase (EG) treatments have become increasingly popular in recent years
 99 to improve pulp reactivity and, as a side effect, precisely adjust pulp viscosity
 100 (Henriksson et al. 2005a; Engström et al. 2006; Köpcke et al. 2008; Ibarra et al. 2010a).

101 Endoglucanases (EG) are typical multi-domain enzymes consisting of a catalytic domain
 102 that randomly cleaves β -1,4-glycosidic bonds in polysaccharide chains. These enzymes
 103 preferentially degrade amorphous cellulose located on the fibre surface and in between
 104 microfibrils, thereby increasing exposed crystalline surfaces, and the swelling ability and
 105 reactivity of pulp (Henriksson et al. 2005a; Gehmayr et al. 2011). Additionally, cellulose
 106 II is attacked by endoglucanases (Rahkamo et al. 1998), which was speculated to play a
 107 role in reactivity increase of the pulp after EG treatment (Henriksson et al. 2005a;
 108 Engström et al. 2006; Kvarnlöf et al. 2006; Gehmayr and Sixta 2012). However,
 109 accessibility is indeed a structural parameter that depends on the nature of the interactions
 110 between reactants and the cellulose substrate, and such interactions are influenced by the
 111 availability of the inner surface and the fibril architecture. The properties of cellulose
 112 depend directly on the moisture regain of the particular fibrous material and on
 113 environmental moisture (Ciolacu et al. 2011). One other key concept in fully
 114 understanding enzymatic degradation of cellulose is the influence of parameters such as
 115 accessibility, crystallinity and supramolecular structure of the substrate.

116 In this work, a commercial dried TCF bleached dissolving pulp was submitted to
 117 endoglucanase treatments in order to bring cellulose activation. Also, the effect of a cold
 118 caustic extraction (CCE) before or after the enzymatic hydrolysis treatment was studied.
 119 Cellulase action was evaluated in terms of Fock solubility, ^{13}C -CP/MAS NMR, water
 120 retention value (WRV), fibre morphology, carbohydrate composition and cellulose
 121 degradation.

123 **Experimental**

124 **Pulp and enzymes**

125 A commercial dried totally chlorine-free (TCF) bleached sulphite dissolving-grade pulp
 126 obtained from a mixture of 60% Norway spruce (*Picea abies*) and 40% Scots pine (*Pinus*
 127 *sylvestris*) was used as a raw material. The pulp was cooked at Domsjö Fabriker mill
 128 (Sweden) and presented the following characteristics: $91.70 \pm 0.15\%$ ISO brightness and
 129 474 ± 0.7 mL/g viscosity with a carbohydrate composition as determined by high-
 130 performance liquid chromatography (HPLC) of $95.1 \pm 0.3\%$ glucan, $2.8 \pm 0.2\%$ manna,

131 0.8 ± 0.0% xylan, 0.2 ± 0.2% ramnan, 0.2 ± 0.2% arabinan, 0.3 ± 0.1% glucuronic acid
132 and 0.2 ± 0.1% acetic acid.

133 Two different endoglucanases were used in the hydrolytic treatments. The B (EC.
134 3.2.1.4) was produced from the newly identified species *Paenibacillus barcinonensis* by
135 Universitat de Barcelona (Spain) (Chiriac et al. 2010). This endoglucanase is a modular
136 enzyme with the structure GH9-CBM3c-Fn3-CBM3b (E1), a truncated derivative of the
137 cellulase with the structure GH9-CBM3c (E2), and a recombinant cellulose binding
138 module CBM3b (CBD) derived from the enzyme. E1 and E2 exhibit cellulase activity as
139 they contain the catalytic module GH9, whereas CBD has no hydrolytic activity on
140 cellulose (Chiriac et al. 2010; Chiriac et al. 2013). B is a family 9 enzyme and shows a
141 processive mode of action. The cellulase activity was 60 CMCase U/mL. The F
142 endoglucanase was supplied by Fungal Bioproducts® (Spain) and was produced from
143 *Cerrena unicolor*. The activity as U/g dry enzyme powder of the cellulase preparation
144 was: 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity on the
145 cellulase, respectively. In both cases, the cellulase activity was determined by measuring
146 the amount of reducing sugars released from carboxymethyl cellulose (CMC, Sigma)
147 according to Somogyi-Nelson method (Spiro 1966). An activity unit (U) is the amount of
148 enzyme capable of converting 1 µmol of substrate per second.

149 **Enzymatic hydrolytic treatments and cold caustic extraction (CCE)**

150 Prior to the hydrolytic treatments, the pulp was disintegrated according to ISO 5263-
151 1:2004 and stored at 4 °C until use. Commercial dried TCF-bleached dissolving pulp was
152 subjected to an enzymatic hydrolysis treatment followed by a cold caustic extraction
153 (CCE) treatment or vice versa. Two different endoglucanases, cellulase B and cellulase F,
154 were used at three different doses each, namely: 120, 12 and 60 U/g (odp), corresponding
155 to samples B120, F12 and F60, respectively. The enzyme treatments were conducted in
156 an Easydye Ahiba oscillating individual reactor from Datacolor, at 10% (w/w)
157 consistency in 0.05 M sodium acetate buffer (pH 5.5) at 55 °C for 1 h. The treated pulps
158 were extensively washed with de-ionized water. A control treatment (K) under the same
159 conditions as the enzymatic treatment but in the absence of enzyme was also developed.
160 A cold caustic extraction (CCE) treatment was conducted in an Easydye Ahiba oscillating
161 individual reactor from Datacolor. The treatment was performed at 10 % (w/w)
162 consistency adjusted with 9 % (w/v) NaOH at 25° C for 1 h. Treated pulps were washed
163 with de-ionized water until the filtrate pH was neutral.
164 The treatments were named as follows: CCE, K, K_CCE, B120, CCE_B120, B120_CCE,
165 F12, F12_CCE and F60. After each enzymatic or chemical treatment, an amount of pulp
166 sample was withdrawn for subsequent characterization (see Schema 1).

167 **Analysis of pulp properties**

168 The starting and treated pulp samples were characterized in terms of kappa number,
169 brightness, viscosity and water retention value (WRV) according to ISO 302:2004, ISO
170 2470:2009, ISO 5351:2004 and ISO 23714, respectively. The degree of polymerization
171 (DP) was calculated from intrinsic viscosity values, using the equation of Evans & Wallis
172 (1987) (SCAN-CM 15:88):

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

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

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

177 where DPo is the degree of polymerization of the initial pulp or first treatment and DP
178 that at the end of the completed treatment.

179 The cellulose reactivity of the pulp samples was determined according to slightly
180 modified version of Fock's method (Fock 1959; Ibarra et al. 2010b). This is a micro-scale
181 method simulating the industrial viscose process for manufacturing regenerated cellulose.
182 Prior to analysis, the samples were dried at 50 °C and conditioned in a climate room at
183 23 °C and 50% RH overnight. In the first step, cellulose was soaked in an alkali medium
184 and then converted into cellulose xanthate with CS₂, which makes the cellulose polymer
185 soluble. Then, -CS₂ groups were removed in dilute sulphuric acid to obtain re-
186 precipitated cellulose fibres. Finally, regenerated cellulose was oxidized with K₂Cr₂O₇,
187 refluxed and titrated with Na₂S₂O₃. Fock reactivity was expressed as regenerated
188 cellulose yield,

189 Eq. (3) $R = (100) \cdot 9.62^a \frac{M(V_1C_1 - (V_2C_2 \cdot 100/40^b)/6}{4Y}$

190 where R denotes reacted cellulose (%), Y sample weight (g), M the molecular mass of
191 glucopyranosyl residues (C₆H₁₀O₅, 162 g/mol), V_1 the volume of titrant (Na₂S₂O₃, L), C_1
192 the concentration of K₂Cr₂O₇ (mol/L), C_2 that of Na₂S₂O₃ (mol/L), a the first dilution to
193 100 g and withdrawal of 10 mL (10.4 g = 100/10.4 = 9.62), and b the second dilution of
194 the sample to 100 mL and withdrawal of 40 mL = 100/40. The respective reactivity
195 results were calculated from 10 replications per sample.

196 Carbohydrate composition of TCF-bleached commercial dissolving pulp and the resulted
197 treated pulps were determined using high performance liquid chromatography (HPLC).
198 Samples were studied on a duplicate basis using a modified version of TAPPI 249 cm-09
199 test method. Hydrolysis was carried out in two steps: i) A strong hydrolysis step with
200 concentrated sulfuric acid. Approximately 50 mg of sample with known moisture content

201 were treated with 5mL of H₂SO₄ 72 % and kept at 30 °C for 1h with gentle stirring. ii) A
202 mild acid step at high temperature. Tube contents were putted into 250 mL-flasks and
203 diluted to 4 % H₂SO₄. Flasks were putted into an autoclave for 1 h at 103 kPa. Solution
204 was then cooled and passed through a glass filter to remove insoluble lignin. Prior to
205 HPLC analysis samples were filtered using a 0.45µm pore size Whatman membrane.
206 Chromatographic analysis was performed using a 1200 Agilent HPLC instrument
207 furnished with a Biorad Aminex HPX-87H ion-exchange column. Concentrations were
208 calculated by interpolation into calibration curves ran from standards of glucose, xylose,
209 rhamnose and arabinose. In order to resolve xylose, mannose and galactose peaks, the
210 hydrolyzed effluents were neutralized with barium carbonate (BaCO₃), then were filtered
211 through a membrane of 0.45 µm pore size and then were analyzed with a Biorad Aminex
212 HPX-87P column. The chromatographic determination was performed under the
213 following conditions: mobile phase 6 mmol/L (acid samples) or ultrapure water
214 (neutralized samples); flow rate, 0.7 mL/min; column temperature, 60°C (acid sample) or
215 80°C (neutralized sample).

216 ¹³C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument
217 operating at 7.05 T and at 75.5 MHz for ¹³C. Samples were immersed in deionized water
218 for at least 2h. All measurements were performed at 290 ± 1K. The magic angle spinning
219 (MAS) rate was 4 kHz. The cross-polarization contact time was 1 ms and the recycle
220 delay time 2.5 s. Acquisition time was 98.3 ms and sweep-width was 31.2 kHz. The
221 number of scans was 5100. The quantification method was based on the evolution of two
222 independent and isolated signal arising from cellulose I and II, respectively: the decrease
223 of the height of the peak near 65 ppm, assigned to the C6 in the crystalline part of
224 cellulose I and the increase of the peak near 107 ppm, assigned to the C1 in the crystalline
225 part of cellulose II (Janzon et al. 2008b; Arnoul-Jarriault et al. 2014).

226 The morphological properties of the fibres (viz., length, width and curl), and the
227 content in fines of the pulp samples were determined in accordance with TAPPI T 271 on
228 a Metso kajaaniFS300 fibre analyser.

Schema 1. Schema of work applied to dried bleached commercial dissolving pulp and the respective studied properties.

Results and Discussion

During the manufacturing of cellulose derivatives and regenerated cellulose, the accessibility of cellulose is a key parameter to control, since a no homogeneous substitution of the hydroxyl groups of the cellulose chain might lead to the production of low-quality derivatives (Köpcke 2010). In the production of regenerated cellulose, the ideal achievement is to obtain a complete dissolution of the cellulose structure. However, due to its supramolecular structure is difficult to achieve. In addition, the non-uniform and compact structure of cellulose limits its dissolution to some special solvents. On the other hand, the solvents used to dissolve cellulose are usually expensive, highly toxic and have harmful effects on the environment. Hence, the great interest to improve cellulose accessibility and as a result reduce the consumption of solvents.

With the previous observations in mind, the present work deals with the intention to improve pulp reactivity. Therefore, cellulase treatments in combination with strong alkaline treatments were performed. Fock solubility, carbohydrate composition, fibre morphology and WRV of treated pulps were analysed.

1. Fock solubility

The reactivity of pulp was assessed using Fock's method, which measures the amount of pulp that is dissolved in a viscose-like solution (Fock 1959; Ibarra et al. 2010a; Gehmayr

251 and Sixta 2012). As can be seen from Table 1, both enzymes increased Fock solubility:
 252 from 67.3% (starting pulp, Com) to 76.1% with the F12 treatment and 95.8% with the
 253 F60 treatment. The B120 treatment also increased Fock solubility up to 78.7%. The high
 254 reactivity obtained with F60 is comparable to previously reported values of (Henriksson
 255 et al. 2005b; Ibarra et al. 2010a) for similar softwood TCF bleached dissolving pulp.
 256 According to Henriksson et al. (2005), endoglucanase attacks the less ordered cellulose
 257 regions between and on the surface of the fibrils, leading to fibre wall swelling and
 258 therefore an increase in the accessibility to solvents.

259 **Table 1.** Fock solubility value of each treatment

Fock solubility (%)	
Com	67.3 ± 2.1
CCE	49.8 ± 4.4
K	61.2 ± 6.0
K_CCE	48.9 ± 5.0
B120	78.7 ± 1.5
B120_CCE	54.0 ± 0
CCE_B120	69.2 ± 4.2
F60	95.8 ± 0.2
F12	76.1 ± 3.2
CCE_F12	69.1 ± 3.7

260

261 The Fock solubility increase was calculated with respect to the value obtained from the
 262 previous stage of each treatment. As can be seen from Figure 1, a cold caustic extraction
 263 by itself or the combination of a cold caustic extraction after an enzymatic treatment
 264 diminished the Fock solubility of the final pulp due to the hornification phenomena,
 265 which is an aggregation of microfibrils. Gehmayr et al. (2011) previously found drying or
 266 strong dewatering of pulp after a treatment at high alkalinity induce the formation of
 267 molecular aggregates via inter- and intra fibrillar hydrogen bonding of the cellulose
 268 microfibrils. In other words, these high values of Fock solubility obtained by F60 and F12
 269 treatments can be expressed as a 42% and 13% of reactivity increase with respect to the
 270 starting pulp. Fock solubility was also considerably improved by alkaline extraction and
 271 then followed by enzymatic treatment. Although the increase in reactivity was similar
 272 with CCE_F12 and F60, the Fock solubility level obtained was 69% with the former and
 273 96% with the latter. In the case of B120, the hydrolytic treatment caused a 17% reactivity
 274 increase with respect to commercial pulp, and led to a reactivity value of 79%. Using a
 275 subsequent alkaline stage had an adverse effect, but inserting it prior to the hydrolytic
 276 treatment increased Fock solubility by a further 39% reaching a 69% value. These results

277 can be explained by the fact that the cold caustic treatment (concentration > 8% wt)
 278 transforms cellulose I into cellulose II and endoglucanases have greater affinity for the
 279 latter form (Engström et al. 2006; Köpcke et al. 2008; Gehmayr et al. 2011).

280

281 **Figure 1.** Reactivity increase according to Fock method calculated with respect to the
 282 reactivity value of the previous stage for each enzymatic and control treatment.
 283

284 In Figure 2 is shown the relation of Fock solubility increase and viscosity results. The
 285 cold caustic extraction, the control treatment and the control treatment followed by a cold
 286 caustic extraction had the same range of viscosities but slightly different reduction of
 287 Fock solubility. Comparing K and K_CCE samples, the latter suffered a higher reduction
 288 of Fock solubility, but with respect to the CCE treatment a similar solubility loss was
 289 found. This result indicates that the conditions of the control treatment had less influence
 290 on Fock solubility than the cold caustic treatment. The action of F endoglucanase can be
 291 evaluated comparing F60 and F12 samples. As can be seen, both treatments exhibited
 292 comparable viscosities, but at higher enzyme dose (F60) the Fock solubility improvement
 293 was 26% higher with respect to the lower enzyme dose treatment (F12). This result
 294 confirms that the effect on Fock solubility was due to the enzyme action. Nevertheless,
 295 introducing a cold caustic extraction treatment before the F12 treatment enabled to reach
 296 practically the same Fock solubility improvement as F60 treatment, with no differences
 297 on viscosity. Therefore, the endoglucanase treatment was responsible for the Fock
 298 solubility improvement since a strong alkaline treatment provided a negative effect on
 299 that property. It is known that endoglucanases have greater affinity to cellulose II. From
 300 B endoglucanase treatment interesting conclusions can be drawn. Firstly, the B treatment
 301 and the B_CCE treatment showed the same viscosity values but a big difference on Fock
 302 solubility increase was found. This variation was caused by the strong alkaline

303 concentration which is responsible for the transformation of cellulose I to cellulose II.
 304 The cellulose II polymorph has a closer and denser structure and as a result present lower
 305 reactivity (Sixta 2006). On the other hand, when the caustic extraction was introduced
 306 before the hydrolytic treatment (CCE_B120) a Fock solubility increase was observed
 307 with respect to the enzymatic treatment alone. However, since respective treatments did
 308 not exhibit identical viscosity values, the Fock solubility improvement cannot only be
 309 related to the enzyme action.

310

311 **Figure 2.** Fock solubility increase, calculated with respect to the previous treatment, and
 312 viscosity values for each treatment.

313 2. Carbohydrate composition of treated pulps

314

315 Carbohydrate composition was determined by HPLC, with special attention on the
 316 hemicelluloses content (Figure 3). The control treatment presented the following
 317 carbohydrate composition: 0.5% xylan, 0.2% rahmnan, 0.6% galactan, 2.8% mannan and
 318 0.2% arabinan. As can be seen from Figure 3a, with respect to control treatment, all
 319 treatments reduced the amount of xylans with exception of B120 treatment. A comparison
 320 of control treatment (K) and control treatment followed by strong alkaline treatment
 321 (K_CCE) let to distinguish the action resulted from the own alkali treatment. With
 322 K_CCE treatment the amount of xylan and mannan was reduced by 57% and 25%,
 323 respectively. The B endoglucanase treatment did not diminish the global amount of
 324 hemicelluloses, however, with the introduction of a cold caustic extraction a variation
 325 was found. Interestingly, no differences were detected with the combination of an
 326 alkaline extraction before (CCE_B120) or after the enzymatic treatment (B120_CCE) and
 327 the final amount of hemicelluloses was 3.1% in both cases. The action of F

328 endoglucanase on mannan fraction was higher than the one produced by B. The
 329 CCE_F12 treatment reduced the amount of hemicelluloses to a final content of 3.4%,
 330 affecting principally the xylan fraction. Working at higher enzyme dose (F60 treatment)
 331 did not result in higher reduction of hemicelluloses; in fact, the final amount of
 332 hemicelluloses was the same as the control treatment.

333

334

335 **Figure 3.** Hemicellulose composition of treated pulps determined by HPLC. (a) B
 336 endoglucanase treatments and (b) F endoglucanase treatments. The sum of total
 337 hemicelluloses is indicated next to each label.

338 3. Cellulase effects

339 Once already detected that Fock solubility was significantly increased by the
 340 endoglucanases treatments, and a diminution of the hemicellulose content was observed

341 by CCE treatment; the ISO brightness and viscosity properties were also measured after
342 each enzymatic and chemical treatment (Table 2).

343 The results showed that both enzymes lead to substantially reduced brightness at
344 the end of the process. Thus, the B120 and F60 treatments decreased brightness by 9.21%
345 and 3.32%, respectively, from the initial pulp (Com); by contrast, the control treatment
346 (K) decreased it by only 1.1%. Interestingly, the increase of the enzyme dose from F12 to
347 F60 resulted in no significantly greater brightness reduction. The brightness loss observed
348 is thought to be caused by the enzyme solution (i.e., the mixture where the enzymes were
349 prepared) since inserting a treatment at high sodium hydroxide concentration after the
350 enzymatic treatment (B120_CCE) raised brightness to virtually the same level as in the
351 starting pulp. Also, B and F cellulases caused a marked increase in colour saturation (C*);
352 however, removal of adsorbed enzyme products by subsequent cold caustic treatment
353 decreased chroma, which suggests that cellulose chains were not damaged or modified. In
354 order to clarify these results, the enzymes were denatured at a high temperature (> 90 °C)
355 before use and then the enzymatic treatments were performed following the same
356 conditions. The results revealed a significant brightness loss and hence that the enzymes
357 itself did not contribute to the adverse effect on brightness.

358 In general, F cellulases degraded cellulose chains and decreased viscosity, but
359 using a high enzyme dose of F (F60 sample) resulted in no further viscosity loss with
360 respect to F12. According to Gurnagul et al. (1992), a heterogeneous attack of cellulase
361 on fibre wall can weaken fibres without significantly diminishing cellulose degree of
362 polymerization or viscosity, which is consistent with our results at high enzyme doses.
363 The B120 treatment did not cause an adverse effect on viscosity (Cadena et al. 2010;
364 Garcia-Ubasart et al. 2013), even with the combination of a cold caustic extraction and a
365 posterior enzymatic treatment. The treatment with buffer solution alone (K), and the cold
366 caustic extraction applied after the buffer treatment (K_CCE), resulted in no cellulose
367 degradation with respect to starting pulp. The gain of viscosity observed after a CCE with
368 respect to initial pulp can be explained by the alkaline pH used, which is responsible for
369 dissolving degraded cellulose and low molecular weight hemicelluloses.

Table 2. Results of ISO brightness ($\pm$ confidence intervals), viscosity ($\pm$ confidence intervals), Chroma coordinate and Fock solubility ($\pm$ confidence intervals) for treated pulps. CCE: cold caustic extraction, K: control treatment with no presence of enzyme, B and F cellulases.

	ISO Brightness (%)	C*	Viscosity (mL/g)
Com	91.70 $\pm$ 0.2	1.62	476 $\pm$ 1
CCE	91.90 $\pm$ 0.2	1.75	536 $\pm$ 29
B120	83.25 $\pm$ 1.6	4.76	495 $\pm$ 67
B120_CCE	91.10 $\pm$ 0.5	1.94	487 $\pm$ 77
CCE_B120	84.00 $\pm$ 0.9	5.16	415 $\pm$ 47
F60	88.65 $\pm$ 0.9	1.89	390 $\pm$ 6
F12	88.40 $\pm$ 1.7	2.32	404 $\pm$ 11
CCE_F12	89.10 $\pm$ 0.7	2.15	406 $\pm$ 126
K	90.60 $\pm$ 0.7	2.30	539 $\pm$ 104
K_CCE	90.85 $\pm$ 0.3	1.59	474 $\pm$ 5
Inactivated B120 enzyme	74.80 $\pm$ 10	7.94	Nm
Inactivated F60 enzyme	87.80 $\pm$ 0.1	3.13	Nm

*Nm: Not measured

Intrinsic viscosity data allowed us to calculate the number of cellulose chain scissions (CS) caused by the enzymatic treatments and cold caustic extraction. CS represents the number of chain cleavage steps per initial cellulose chain. As can be seen from Figure 4a, B cellulase-treated pulp exhibited none chain scission value while F cellulase had a chain scission value around 0.2. These results indicated that B and F endoglucanases must have acted differently on cellulose chains. Cold caustic extraction (CCE) gave a negative CS value that was assumed to be zero; however, a subsequent enzymatic treatment (CCE_B120) increased CS by 35%. This behaviour was not observed when the caustic treatment was applied after the enzymatic treatment, B120_CCE, which failed to raise the number of scissions of CCE_B120. Also, in the case of CCE_F12 a higher CS was obtained compared to the simple F12 enzymatic treatment. From these results can be concluded, that the presence of cellulose II promoted during the cold caustic extraction facilitated the action of the enzymes. On the other hand, the combined treatment CCE_F12 led to more scissions than F60 at a high enzyme dose, which further underlines the favourable effect of cold caustic extraction. (Janzon et al. 2008a) reported that the conversion of cellulose I into cellulose II starts at a NaOH concentration about 6–7 wt% in wood pulps and above 10 wt% in cotton linters. Also, cellulose II has proved especially susceptible to hydrolysis by endoglucanases (Atalla 1979; Rahkamo et al. 1998). Similarly to us, (Gehmayr and Sixta 2012) stated that pulps

390 with an increasing proportion of cellulose II experienced an increasing extent of maximal
391 chain scission and this effect may be attributed to enhanced accessibility of the cellulose
392 II modification.

393 Plotting the proportion of fines as a function of the number of chain scissions
394 with F treatments (Figure 4b) afforded some interesting conclusions. As somehow
395 suggested by the viscosity results, F cellulase reduced fibre size and as a result the
396 amount of fines (viz., fibres with a particle size between 0.0–0.2 mm) relative to the
397 starting pulp increased significantly, whereas the control treatment (K) did not (results not
398 shown). The higher the cellulase dose used was, the higher was the content in fines,
399 which suggests increased degradation of cellulose chains. The F60 treatment increased
400 the amount of fines by 42% and 18% relative to initial and F12 treatment. Again,
401 introducing a cold caustic extraction before the enzymatic treatment increased the content
402 in fines by up to 9% and the number of chain scissions by 46% in CCE_F12 treated pulps
403 relative to F12. However, a cold caustic extraction (CCE) by itself did not modify the
404 number of chain scissions. In addition, the application of a strong alkaline treatment after
405 the buffer treatment neither caused greater scissions than control treatment alone. In terms
406 of fibre morphology, the fibre length and the proportion of fines of K_CCE were identical
407 to the values for K. However, the content in fines of control treatment was increased by
408 13.5% with respect to the starting pulp although no degradation of cellulose fibres was
409 apparent from viscosity results. This result can be explained by the oscillating agitation
410 used during the treatment that helped to release small particles. On the other hand, the
411 combination of an enzymatic treatment and a cold caustic extraction had a significant
412 effect: the chain scission increased and the content in fines became slightly greater. These
413 results suggest that the high sodium hydroxide concentration contributed to fibre swollen
414 and opened-up the cellulose structure, thereby facilitating the action of the posterior
415 enzyme.

Figure 4. (a) Chain scission number for the different studied samples. (b) Proportion of fines obtained from Metso Kajaani FS300 against the number of chain scission for F treatments and their respective control treatments.

4. ^{13}C -CP/MAS NMR

The solid state ^{13}C -NMR spectra of commercial dissolving pulp, the enzymatic treated pulps and the combination of 9% NaOH extracted pulps before or after endoglucanase treatments are shown in Figure 5. The C-6 signal of commercial dissolving pulp consisted of two peaks nearly identical heights at 64 and 66 ppm. This spectrum suggests a small presence of cellulose II and this cellulose polymorph is more susceptible to enzymatic hydrolysis by endoglucanases (Krässig 1993; Teleman 2001). Therefore, B endoglucanase treatment was dominated by cellulose I signal at 66 ppm while the F12 treatment increased the signal intensity but in less extent. The 9% NaOH extraction

431 presented different polymorphic form from commercial dissolving pulp, but no signals of
 432 cellulose II. The most pronounced structural changes were detected with CCE_F12
 433 sample. A high presence of cellulose II was apparent by a perceptible shoulder at 108
 434 ppm of C-1 signal and an increased C-6 signal intensity at 64 ppm.

435
 436 **Figure 5.** ^{13}C -CP/MAS NMR spectra of commercial dissolving pulp compared to
 437 samples treated with 9% NaOH before or after a hydrolytic treatment.

439 5. Fiber morphology

440 The outcomes of the F cellulase treatments were examined in greater detail
 441 (Table 3). The water retention value (WRV) provides information about the integral pore
 442 volume of a pulp and the content of hydrophilic groups (Gehmayr et al. 2011). Fines are
 443 thought to increase WRV improvement since these particles have more accessible –OH
 444 groups and plays a major role in dewatering. Treating commercial dissolving pulp with
 445 the lowest dose of F cellulase (F12 treatment), reduced fibre length and substantially
 446 increased the proportion of fines, and as a result of that the WRV rose to 1.1 %. By
 447 contrast, a high enzyme dose (F60 treatment) had virtually no effect on WRV even
 448 though fibre length was shortened by 57%, and the amount of fines increased to 41%,
 449 with respect to commercial dissolving pulp. The conditions used in the control treatment,
 450 i.e. oscillating agitation and the buffer solution had slight effect on fibre morphology.
 451 Introducing a cold caustic extraction (K_CCE) stage resulted in no change in fibre
 452 morphology or WRV relative to the control treatment (K). The greatest change in WRV
 453 was produced by CCE_F12, which suggests increased hydrogen bonding; fibres were
 454

shortened by 43% and the content in fines grew by up to 34%, with respect to starting pulp. In terms of fibre width, a slight increase was observed with F12 treatment, but a substantial greater size was found with F60 treatment, with respect to commercial pulp. Interestingly, K_CCE, CCE_F12 and F60 presented the same value of fibre width. A comparison of K with K_CCE on the one hand, and F12 with CCE_F12 on the other hand, revealed that the incorporation of an alkaline step into the process enhanced the fibre width.

Table 3. Water retention value (WRV) ($\pm$ confidence interval) and fiber length and fiber width ($\pm$ confidence intervals) determined according to TAPPI standard and calculated as an arithmetic mean for the F treatments.

	WRV	Fiber length, Ln (mm)	Fiber Width, Wn (μ m)	Fines (%)
Com	0.79 ± 0.04	0.65 ± 0.01	21.3 ± 0.1	36.48 ± 0.43
K	1.00 ± 0.02	0.60 ± 0.0	22.2 ± 0.0	42.18 ± 0.60
K_CCE	0.99 ± 0.07	0.57 ± 0.0	23.6 ± 0.0	42.30 ± 0.98
F60	0.88 ± 0.18	0.28 ± 0.01	23.5 ± 0.7	62.18 ± 0.49
F12	1.11 ± 0.21	0.47 ± 0.02	22.6 ± 0.2	50.71 ± 0.39
CCE_F12	1.27 ± 0.31	0.37 ± 0.02	23.5 ± 0.1	55.59 ± 1.60

Combining fibre length results with WRV results (Figure 6) some conclusions can be drawn. In general, the lower the fibre length resulted, the higher the WRV became, with the exception of F60 treatment. Focusing the attention on the control treatment followed by a caustic extraction (K_CCE), it failed to improve WRV and to alter cellulose fibre size relative to control treatment (K). The CCE_F12 suffered a slight improvement in WRV but a 10% reduction in fibre length, with respect to F12 treatment; although these differences were more significant with respect to starting pulp.

473

474 **Figure 6.** Relation between fibre lengths determined according to TAPPI standard and
 475 calculated as an arithmetic mean and water retention value (WRV) for biobleached
 476 sulphite pulps treated with B and F enzyme.

477 Figure 7 represents the population fractions determined according to TAPPI standard. As
 478 can be seen, the proportion of fibres in the length range of 0.5–3.2 mm was considerably
 479 diminished when the F60 treatment was performed, and thereby the content of fines
 480 increased by up to 41.3% (length range of 0.0-0.2 mm) with respect to starting pulp.
 481 However, the effect caused by the enzyme was not detected in the length range of 0.2-0.5
 482 mm since the same population as starting pulp was obtained. After F60 treatment, the
 483 increase of fines was followed by CCE_F12 and then F12. The CCE_F12 treatment
 484 increased the amount of fines by up to 35% and the F12 treatment by up to 28%. F12 and
 485 CCE_F12 treatments presented similar proportion of fibres in the length range of 0.2-1.2
 486 mm, but the differences in the long length fraction became more accentuated. As
 487 observed earlier, the control treatment had similar population fractions as starting pulp,
 488 except for slight difference in the content of fines and therefore different proportion of
 489 fibres in the range of 0.2-1.2 mm. These results can be explained due to the oscillating
 490 agitation used in the treatments which facilitated the release of small particles.

Figure 7. Population fractions ($\pm$ confidence intervals) for the FB treatments determined according to TAPPI standard

The microscopy images (Figure 8) provided visual confirmation of the previous results. Thus, the F60 sample exhibited a high content in fines but also in long fibers. In addition, slight difference between F12 and CCE_F12 were detected.

Figure 8. Optic Microscopy images for the F treatments. The pictures were taken with a magnification of 40.

Conclusions

Two different cellulases, named as B and F, were used to increase the reactivity according to Fock method of dried bleached commercial dissolving pulp with the intention to bring a satisfactory advantage to the viscose process. Working at high enzyme dose, F60 treatment, provided a reactivity increase of 42%, which belongs to a 96% Fock solubility. In terms of fibre morphology, the amount of fines increased significantly after F60 treatment, which is in connection with viscosity loss. The F12 treatment provided a 13% Fock solubility improvement. Interestingly, applying a cold caustic extraction (CCE) treatment before the hydrolytic treatment (CCE_F12) resulted in further increased Fock solubility than an enzymatic hydrolysis alone (F12), although the viscosity loss was more pronounced. In terms of WRV, this property was also improved with the combination of a cold caustic extraction and hydrolytic treatment (CCE_F12). The B enzyme did not cause viscosity loss, although the Fock solubility was improved by 17% with respect to commercial pulp, which corresponds to a 79% of Fock solubility. The NMR results revealed that the starting pulp had already an important proportion of cellulose II, but after the CCE_F12 treatment this amount of cellulose II became higher.

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