**Soluble inhibitors generated during hydrothermal pretreatment of oil
palm mesocarp fiber suppressed the catalytic activity of *Acremonium*
cellulase**

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28 **ABSTRACT**

29 Oil palm mesocarp fiber was subjected to hydrothermal pretreatment under
30 isothermal and non-isothermal conditions. The pretreated slurries were separated by
31 filtration, pretreated liquids and solids were characterized. An enzymatic digestibility
32 study was performed for both pretreated slurries and solids to understand the effect of
33 soluble inhibitors generated during the pretreatment process. The highest glucose yield
34 obtained from pretreated slurries was 70.1%, and gradually decreased with higher
35 pretreatment severities. The highest glucose yield obtained in pretreated solids was
36 100%, after pretreatment at 210°C for 20 min. In order to study the inhibitory effects of
37 compounds generated during pretreatment with cellulase, technical grade solutions that
38 mimic the pretreated liquid were prepared and their effect on *Acremonium* cellulase
39 activity was monitored using Avicel. Xylo-oligomers and tannic acid were identified as
40 powerful inhibitors of *Acremonium* cellulase, and the lowest hydrolysis rate of Avicel of

41 0.18 g/g-glucose released/L/h was obtained from tannic acid.

42

43 *Keywords:* Oil palm mesocarp fiber, hydrothermal pretreatment, tannic acid,
44 xylo-oligomers, *Acremonium* cellulase inhibition

45

46 **1. Introduction**

47 Pretreatment of lignocellulosic biomass is necessary to reduce the natural
48 recalcitrance of biomass and to make it amenable to enzymatic hydrolysis. Depending
49 on the type of biomass, fractionation of lignocellulosic biomass into cellulose,
50 hemicellulose, and lignin can be performed by physical, chemical, or thermochemical
51 methods, or a combination thereof. Hydrothermal pretreatment has been studied
52 extensively because of its advantages over other pretreatments, such as the green
53 pretreatment method. Hydrothermal pretreatment (1) uses only water as a catalyst
54 ([Garrote et al., 2007](#); [Vegas et al., 2008](#)), (2) requires no addition of acid or alkali
55 catalysts, eliminating the need for corrosive-free equipment ([Nitsos et al., 2013](#)), and (3)
56 consumes less energy ([Lee et al., 2010](#)). All these features combined contribute to lower
57 investment and processing costs. At the same time, hydrothermal pretreatment
58 efficiently reduces hemicellulose content, and dissolves and removes lignin compounds

59 from the cell wall, creating pores that increase the specific surface area for cellulase
60 attack ([Parajó et al., 2004](#); [Hsu et al., 2010](#); [Pu et al., 2013](#)).

61 By manipulation of the reaction temperature and time, the intimate associations
62 in the polymeric alignment of cellulose, hemicellulose, and lignin can be loosened.
63 Progressive removal of hemicellulose components at extreme pretreatment conditions is
64 always accompanied by the formation of degradation by-products in the pretreatment
65 liquid (PL). Soluble inhibitors generated in the PL can be classified as sugars
66 (monomeric or oligomeric forms) ([Hsieh et al., 2014](#); [Kont et al., 2013](#); [Qing et al.,](#)
67 [2010](#); [Zhang and Viikari, 2012](#)), furan and aldehyde derivatives (furfural, 5-HMF) ([Jing](#)
68 [et al., 2009](#)), acids (acetic, formic, levulinic) ([Palmqvist and Hahn-Hagërdahl, 2000](#)), or
69 phenols (gallic, tannin, vanillin) ([Berlin et al., 2006](#); [Ximenes et al., 2010](#); [Tejirian et al.,](#)
70 [2011](#); [Kim et al., 2011](#)) that may inhibit both the saccharification and fermentation
71 process. The inhibition mechanisms vary from competitive and non-productive binding
72 of inhibitors to cellulase ([Qing et al., 2010](#); [Zhang and Viikari, 2012](#); [Kont et al., 2013](#))
73 and to protein precipitation that may result in retardation and deactivation ([Ximenes et](#)
74 [al., 2010](#); [Ximenes et al., 2011](#); [Tejirian et a., 2011](#); [Kim et al., 2011](#)).

75 In order to overcome the excessive degradation of hemicellulose and the
76 accumulation of degradation by-products, pretreatment conditions need to be optimized.

77 The separation of PL from the pretreated slurry and subsequent washing to neutral pH
78 prior to enzymatic saccharification is widely adopted approaches on laboratory scale.
79 Recently, adsorbents such as XAD-4 and activated carbon have been reported to
80 efficiently remove inhibitors from the PL (Weil et al., 2002; Kim et al., 2011). The
81 present study provides progress of pretreatments developed for oil palm biomass with
82 consideration to obtain high recovery of xylose and glucose by chemical-free
83 approaches. Our previous works showed that glucose conversion yield (from pretreated
84 solid) was correlated well with pretreatment severities. The properties of pretreated
85 liquids were characterized and tannic acid (TA) was recorded in increasing trends
86 towards higher pretreatment severities. Therefore, little attention was given on the effect
87 of soluble inhibitors generated from hydrothermal pretreatment of oil palm biomass on
88 *Acremonium* cellulase activity.

89 In the present study, the efficiency of the pretreatment process was evaluated
90 by enzymatic hydrolysis of both pretreatment slurries and solids. The soluble inhibitors
91 generated in the pretreated liquids were identified and their effects on enzymatic
92 digestibility of Avicel were assessed. The removal efficiency of tannic acid by activated
93 carbon from OPMF was also evaluated.

94

95 **2. Materials and methods**

96 *2.1. Raw materials and component analysis*

97 Oil palm mesocarp fiber (OPMF) was one of the biomass generated from oil
98 palm processing to get crude palm oil (Zakaria et al., 2014). The OPMF was collected at
99 the Seriting Hilir Palm Oil Mill, Jempol, Negeri Sembilan, Malaysia and sun dried prior
100 to analysis. The samples were ground to 2 mm size particles by milling cutters and were
101 dried *in vacuo* at 40°C prior to use. Glucose, xylose and Klason lignin levels in OPMF
102 were determined following the modified NREL Laboratory Analytical Procedure
103 (Technical Report NREL/TP-510-42618) (Sluiter et al., 2008), which involves two-step
104 hydrolysis. Approximately 0.05 g dried OPMF was placed into a glass vial and
105 hydrolyzed in 72% (w/w) H₂SO₄ at 30°C for 90 min and the slurry was further
106 hydrolyzed in diluted 4% (w/w) H₂SO₄ at 120°C for 60 min. The produced sugars were
107 filtered using a Dionex OnGuard™ II A cartridge filter (Thermo Scientific, USA) to
108 remove contaminants, and analyzed by HPLC as described in section 2.5.

109

110 *2.2. Hydrothermal pretreatment*

111 Hydrothermal pretreatment of OPMF was conducted in a 35 mL stainless steel tube
112 reactor, as reported earlier (Zakaria et al., 2014). In this study, ground OPMF samples

113 with a size 2 mm were used instead of samples with the original fiber length (Zakaria et
114 al., 2014) to achieve a better heat transfer and smaller samples size had improved the
115 pretreatment efficiency (Buaban et al., 2010). The OPMF samples were pretreated at
116 150°C-220°C for 20-240 min at a solid to liquid (S:L) ratio of 1:10. Pretreatments were
117 performed under both isothermal and non-isothermal conditions, and the properties of
118 treated liquids and solids were thoroughly compared. The treated samples (slurries)
119 were filtered using filter paper No. 2 (Advantec, Japan) and washed with distilled water
120 until the pH was neutral. The neutralized solid was oven dried at 90°C for 24 h prior to
121 enzymatic hydrolysis. The intensity of the hydrothermal treatment was expressed in
122 terms of severity factor ($\log Ro$). The severity parameters corresponding to different
123 hydrothermal pretreatment conditions are calculated as in Eq. (1), in which t is the
124 reaction time (min) and T is the hydrolysis temperature (°C) (Overend and Chornet,
125 1989).

$$126 \quad Ro = t \exp [(T-100)/14.75] \quad (1)$$

127

128 2.3. Enzymatic hydrolysis

129 *Pretreated slurries and pretreated liquid (PL):* Unless otherwise stated,
130 enzymatic hydrolysis was performed using an enzyme cocktail constituting 40 FPU/mL

131 *Acremonium* cellulase (Meiji Seika Co, Japan) and 10% Optimash BG (Genencor
132 International, California, USA) (Zakaria et al., 2014). In a standard assay, 0.75 mL
133 (10 FPU/g substrate) *Acremonium* cellulase, 2.5 mL 1.0 M sodium acetate buffer pH 5.0,
134 and 0.6 mL 10% Optimash BG were added to the treated samples (3 g of dry weight) in
135 a 65-mL tube (NEG, Japan). Distilled water was added to the reaction mixture to obtain
136 a total volume of 50 mL. Upon completion of the hydrolysis, the pretreated liquid
137 hydrolyzate (PLH) was boiled for 30 min to deactivate enzymes. All the hydrolysis runs
138 were performed in triplicate.

139 *Untreated and pretreated washed solids:* Approximately 0.05 g of dried
140 pretreated washed solids and 1 mL of total hydrolysis mixture (enzyme and buffer) were
141 placed in 2 mL Eppendorf tubes. The enzymatic hydrolysis was performed at 50°C for
142 72 h with stirring/shaking. The experiment was performed in triplicate and the results
143 presented as the average value. All the hydrolysis runs were performed in triplicate. The
144 enzymatic digestibility was represented by the obtained sugars (g sugars/ g materials) or
145 in terms of sugar yield, calculated as in Eq. (2):

146

147 *Sugar yield (%)* = [weight of monomeric sugars after enzymatic hydrolysis/weight of
148 potential total monomeric sugars after hydrolysis using H₂SO₄] × 100 (2)

149 *Avicel*[®] *microcrystalline cellulose*: *Avicel*[®] microcrystalline cellulose (Merck,
150 Germany) was used to test the effect of potential inhibitors generated in hydrothermal
151 PLs and PLHs of OPMF. *The Avicel was used to avoid interference from pseudo-lignin*
152 *and lignin deposited onto the surface of pretreated solids during enzymolysis.* One
153 percent (w/w) of substrate was hydrolyzed using *Acremonium* cellulase at 10 FPU/g
154 substrate. Control solutions were prepared with buffer solution only, designated as BA.
155 Other inhibitor solutions such as furfural, 5-HMF, acetic acid, and xylose are analytical
156 grade and were prepared at concentrations mimicking the actual levels of soluble
157 inhibitors generated during the hydrothermal pretreatment of OPMF. Xylo-oligomers
158 (XOS) were hydrothermally prepared from birch wood xylan (Sigma-Aldrich,
159 Germany) at 180°C for 20 min with a S:L ratio of 1:10, using a tube reactor, as
160 described above. The concentration of the XOS from hydrothermally treated xylan was
161 determined by dilute 4% H₂SO₄ (w/w) analysis. Details regarding the inhibitors tested
162 in the *Avicel* study are presented in [Table 2](#).

163

164 2.4. Removal of inhibitors by activated carbon

165 *Activated carbon* (Merck, Germany) and activated carbon from OPMF
166 (AC-OPMF) provided by Biomass Technology Center, University Putra Malaysia, were

167 used to remove potential inhibitory compounds in the pretreatment liquid hydrolyzate
168 (PLH). Activated carbon purchased from Merck, Germany was used as control. The
169 efficiencies of both activated carbons were compared. The adsorbent was washed
170 several times with deionized water and dried *in vacuo* at 40°C, prior to specific surface
171 area (SSA) determination. The SSA of activated carbon from Merck and OPMF-AC
172 were determined to be 627 m² g⁻¹ and 545 m² g⁻¹, respectively. The adsorbents were
173 added to the PLHs at 10% (w/v) and mixed for 1 h at 200 rpm in a shaking incubator at
174 25°C, followed by centrifugation at 10,000 ×g for 5 min. The pH of PLHs was recorded
175 in the range from 4.8 to 5.0. The supernatant was collected and stored at 4°C until
176 analysis.

177 In separate experiments, activated carbon from OPMF (AC-OPMF) was tested
178 at different activated carbon loading and pure tannic acid concentrations to test its
179 adsorption capacity. The AC-OPMF loadings were between 0.2 to 10% (wt.vol) and
180 tannic acid concentrations between 1.0 to 10 g/L at 1 to 2 h reaction time. The initial pH
181 was recorded between pH 5.5 to pH 6.5 depending on the TA concentrations. All the
182 adsorption tests were performed in triplicate.

183

184 2.5. Analytical procedures

185 The activated carbon, untreated, and hydrothermally treated OPMF samples
186 that were lyophilized with *t*-butyl alcohol were further dried at 105°C for 6 h with
187 degassing (BELPREP; Bel Japan, Japan) before setting up the measuring device. The
188 measurement was performed using BELSORP-max (Bel Japan, Japan) at a temperature
189 of −196°C, as reported earlier ([Ishiguro and Endo, 2014](#)).

190 Monomeric and oligomeric sugars, acetic acid, furfural, 5-HMF, and formic
191 acid from pretreated liquids and enzymatic hydrolysis were detected using
192 high-performance liquid chromatography (HPLC) according to the analytical method
193 reported in Technical Report NREL/TP-510-42618 ([Sluiter et al., 2008](#)). Tannic acid
194 concentration from pretreatment liquid was measured using the Folin–Ciocalteu method
195 ([Makkar et al., 2004](#)).

196

197 **3. Results and discussion**

198 *3.1. Properties of pretreated solids and liquids*

199 OPMF samples were pretreated in both isothermal and non-isothermal
200 conditions with varying reaction temperatures and times, in order to optimize the
201 reaction conditions for the recovery of soluble sugars in pretreated liquids and
202 subsequent enzymatic hydrolysis of pretreated solids. In addition, it **was** important to

203 minimize the formation of soluble degradation by-products ([Garrote et al., 2007](#); [Vegas](#)
204 [et al., 2008](#); [Nitsos et al., 2013](#)). The properties of untreated and pretreated OPMF
205 samples as function of pretreatment severity **were** shown in [Table 1](#). In general,
206 hemicellulose was degraded gradually towards higher treatment severities. Partial
207 dissolution of hemicellulose (56%) was recorded at pretreatment severity $\log Ro = 3.85$,
208 and degraded completely (0.0%) at treatment severity $\log Ro = 4.54$ (210°C, 20 min). It
209 was obvious that the dissolution of hemicellulose was more pronounced at higher
210 reaction temperatures than at longer reaction times with lower reaction temperatures. No
211 substantial increase in cellulose content was observed at moderate reaction temperatures,
212 with a pretreatment severity $\log Ro$ between 3.55 and 3.85. This might be explained by
213 the partial removal of hemicellulose compared to the complete dissolution of
214 hemicellulose at higher treatment severities. Similar trends were observed for cellulose
215 and Klason lignin content at non-isothermal conditions, with 42.3% and 58.1%,
216 respectively, at the harshest pretreatment severity, $\log Ro = 4.83$, in response to
217 complete degradation of hemicellulose ([Table 1](#)).

218 The lowest specific surface area (SSA) and pore volume (PV) values were
219 recorded for untreated samples at 1.1 m²/g and 0.009 cm³/g, respectively. The highest
220 SSA and PV were recorded at 44.2 m²/g and 0.184 cm³/g, respectively, for the harshest

221 treatment condition ($\log R_o = 4.83$). The expansion of SSA and increase in PV values
222 are in line with an increase in pretreatment severity, indicating the dissolution of
223 hemicellulose and migration of lignin (Hsu et al., 2010; Pu et al., 2013). Glucose,
224 xylose, and arabinose are the dominant monomeric sugars detected in PLs (Table 1).
225 Hydrothermal pretreatment was reported to efficiently remove hemicellulose at selected
226 pretreatment severities and degrade it to sugar monomers and oligomers (Parajó et al.,
227 2004; Kont et al., 2013). Xylo-oligomers (XOS) were the dominant oligomeric sugars in
228 PLs and were detected at concentrations ranging from 0.5 g/L to 7.0 g/L, at treatment
229 severity $\log R_o = 3.25$ –4.83. The highest XOS level (7.0 g/L) was obtained upon
230 pretreatment at 190°C for 20 min. This amount of XOS was equivalent to 11.5% XOS
231 from raw OPMF and constitutes 44.7% of total xylan. The XOS yield was higher than
232 that of barley husk and corncobs, which only yielded about 27.1% and 24.8% XOS,
233 respectively (Parajó et al., 2004). To the best of our knowledge, this the first report on
234 XOS generation by hydrothermal pretreatment of OPMF.

235 The reduction of XOS values after this point was probably due to the
236 conversion of xylose to furfural at higher treatment temperatures. Other degradation
237 by-products such as furfural, 5-HMF, and acetic and formic acids were also detected in
238 the PLs (Table 1). Therefore, the formation patterns are almost similar to those reported

239 previously ([Zakaria et al., 2014](#)). The formation of soluble inhibitors during the
240 hydrothermal process has been reported previously, and they can be grouped in several
241 categories such as sugars (xylose, XOS), furan derivatives (furfural, 5-HMF), organic
242 acids (acetic, formic, levulinic) and lignin derivatives (poly and mono-phenolic
243 compounds) ([Palmqvist and Hahn-Hagërdahl, 2000](#); [Ximenes et al., 2011](#); [Kim et al.,](#)
244 [2013](#)).

245 An important degradation inhibitor formed during hydrothermal pretreatment
246 in the present study **was** tannic acid (TA). TA **was** a polyphenolic compound derived
247 from lignin degradation in a soluble form during the hydrothermal process ([Tejirian et](#)
248 [al., 2011](#); [Ximenes et al., 2011](#); [Kim et al., 2011](#)). Most of the lignins adhere onto the
249 surface of pretreated solids upon cooling ([Pu et al., 2013](#); [Li et al., 2014](#)) and increase in
250 percentage with rising pretreatment severities ([Table 1](#)). Both lignin and
251 phenolic-derived lignin degradation affected cellulose hydrolysis by enzymolysis
252 ([Berlin et al., 2006](#); [Li et al., 2014](#); [Ximenes et al., 2010, 2011](#); [Kim et al., 2011, 2013](#)).

253 TA accumulation in PLs was quantified and its production profile as a function of the
254 pretreatment severity **was** shown in **Table 1**. TA production increases towards higher
255 pretreatment severities and was recorded the highest at 1.97 and 4.5 g/L at log $R_o = 3.85$
256 and log R_o 4.83 (150°C for 240 min and 220°C for 20 min), the most severe conditions

257 for isothermal and non-isothermal pre-treatment, respectively. The present data **was** in
258 agreement with a previous study showed that TA production **was** affected by
259 pretreatment severity ([Zakaria et al., 2015a](#)). Even though all potential enzyme and
260 fermentation inhibitors were detected in the hydrothermal liquid of oil palm biomass,
261 the specific factors that affected *Acremonium* cellulase inhibition were not identified
262 ([Zakaria et al., 2015a](#)). The effect on enzymatic digestibility of each potential soluble
263 inhibitor present in the pretreated liquid was evaluated and discussed in detail in section
264 3.2.2.

265

266 3.2. Enzymatic hydrolysis

267 3.2.1. Pretreated slurries versus washed solids

268 Previous hydrothermal pretreatments showed that high conversions of xylose
269 (63.2%) and glucose (97.3%) from pretreated solids were achieved when OPMF was
270 impregnated with 1.5% NaOH at 200°C for 20 min with subsequent ball milling (BM)
271 for 120 min ([Zakaria et al., 2014](#)). The pretreatment of OPMF could be improved by a
272 chemical-free approach in which pretreatment with hot compressed water (HCW) at
273 180°C for 20 min was combined with wet disk milling (WDM) for nine milling cycles.
274 This approach yielded 90% and 86% of xylose and glucose, respectively, from liquid

streams (Zakaria et al., 2015b). However, the previous studies were focused only on pretreated solids with little attention for the generation of PL and its effect on the enzymolysis process. In the present study, an attempt has been made to obtain biosugars from pretreated slurries, especially oligomeric sugars, without separation of pretreated solids by saccharification. Therefore, isothermal and non-isothermal pretreatment conditions were optimized to minimize the formation of hemicellulose degradation by-products that later retard downstream processing. Furthermore, maximal utilization of biomass (pentose and hexose sugars) was required to improve the economic viability of the process.

Both hydrothermally pretreated OPMF slurries and washed solids were subjected to enzymatic hydrolysis for 72 h at 50°C, and the glucose yields obtained from both treatments were compared as shown in Fig. 1. At mild pretreatment severities (150°C for 60-120 min), the glucose conversion from pretreated slurries was higher than in pretreated solids and this may be attributed to the free glucose released upon hemicellulose degradation. Both the pretreated slurries and solids shared similar glucose yields at 180°C for 20 min, indicating that the soluble inhibitory compounds generated in this condition do not have a negative impact on cellulase activity. At this stage potential inhibitory compounds were recorded such as xylo-oligomers, acetic acid,

293 5-HMF, furfural, formic acid and tannic acid at concentrations of 6.6 g/L, 1.7 g/L, 0.1
294 g/L, 0.2 g/L, 0.9 g/L and 1.9 g/L, respectively (Table 1). The highest glucose yield from
295 pretreated slurries was recorded for pretreatment conditions of 190°C for 20 min, and it
296 decreased with higher treatment severities, indicating higher levels of accumulation of
297 soluble cellulase inhibitors especially 5-HMF, furfural and tannic acid.

298 In contrast, the glucose yields from washed pretreated solids increased with
299 higher pretreatment severities and reached a yield of 100% at pretreatment conditions of
300 210°C for 20 min, a yield that is almost double that of pretreated slurries. The
301 successful or maximal conversion of cellulose to glucose from washed pretreated solids
302 can be explained by the progressive removal of hemicellulose during the hydrothermal
303 pretreatment (Table 1). Dissolution of hemicellulose and migration of lignin loosens the
304 cellulose-hemicellulose-lignin network and creates porous structures that expose
305 cellulose components, thus enhancing penetration of cellulase and enzymatic
306 digestibility (Lee et al., 2010; Hsu et al., 2010; Pu et al., 2013). Therefore, a correlation
307 between SSA and hemicellulose removal against glucose yield was plotted as illustrated
308 in Fig. 2. Moreover, removal of degradation by-products by filtration and washing with
309 distilled water eliminates inhibitory compounds and improves the saccharification
310 process.

311

312 3.2.2 *Acremonium cellulase inhibition study*

313 Although it was obvious that pretreated slurries contain inhibitors, there **was** no
314 conclusive answer as to what compounds suppress the activity of *Acremonium* cellulase
315 and Optimash BG since a mix of soluble compounds were present in the PL. It is vital
316 to identify the factors that limit or inactivate cellulase activity from OPMF slurries to
317 obtain the optimal pretreatment conditions that recover high pentose and hexose sugars
318 with a low generation of inhibitors. The details of the experimental design were shown
319 in [Table 2](#). The pretreatment liquid (PL) was prepared from hydrothermal pretreatment
320 of OPMF at 150°C for 240 min and its inhibitory capacity was tested on Avicel using
321 *Acremonium* cellulase. Since Avicel is lignin-free, microcrystalline cellulose, it was
322 selected as a substrate instead of pretreated OPMF solids to avoid interference from
323 pseudo-lignin and lignin deposited onto the surface of pretreated solids during
324 enzymolysis ([Kumar and Wyman, 2009](#); [Zhang and Viikari, 2012](#); [Kim et al., 2013](#); [Li](#)
325 [et al., 2014](#)). The inhibitor concentrations derived from hydrothermally pretreated
326 OPMF liquid were identified by HPLC ([Sluiter et al., 2008](#)) and colorimetric methods
327 ([Makkar et al., 2004](#)), and subsequently, technical grade solutions were prepared to
328 monitor the effect of each inhibitor on *Acremonium* cellulase digestion of Avicel.

329 A variety of potential soluble inhibitors were identified in OPMF PL at the
330 following concentrations: XOS (11.3 g/L), xylose (0.5g/L), TA (1.7 g/L), acetic acid
331 (3.3 g/L), HMF and furfural (0.4 g/L). PL was enzymatically hydrolyzed with Optimash
332 BG for 72 h at 50°C and pretreatment liquid hydrolyzate (PLH) was formed. The PLH
333 was quantified by HPLC and the XOS contained in the PL was hydrolyzed to xylose
334 monomers (11.7 g/L). Optimash BG was used since it contains an enzyme cocktail of
335 β -glucosidase, xylanase, and β -xylosidase that promotes further degradation of glucan
336 and xylan into glucose and xylose, respectively ([Inoue et al., 2008](#); [Buaban et al., 2010](#)).
337 [Qing et al. \(2010\)](#) supplemented xylanase and β -glucosidase together with cellulase to
338 boost the conversion of both cellulose and hemicellulose through the degradation of
339 xylan and other oligomers to the less inhibitory xylose. Because of the inhibiting power
340 of XOS, supplementation of xylanase and β -xylosidase was necessary ([Kumar and](#)
341 [Wyman, 2009](#); [Zhang and Viikari, 2012](#)). Acetic acid concentration was doubled from
342 3.3 g/L to 7.3 g/L due to the degradation of acetylated xylan components ([Jing et al.,](#)
343 [2009](#)). Meanwhile, TA, HMF and furfural concentration remains unchanged.

344 Activated carbon (AC), either purchased from Merck (AC-Merck) or produced
345 from OPMF (AC-OPMF), was used in this study to remove potential inhibitors. The
346 AC-treated PLH products were designated as PLH-ACA and PLH-ACB, respectively

347 (Table 2). Both adsorbents totally removed TA, furfural and HMF, while xylose and
348 acetic acids were adsorbed 16%-20% and 20%-32%, respectively. The AC-Merck
349 adsorbent showed more efficient adsorption of the tested inhibitors because it has a
350 larger SSA, as mentioned earlier (section 2.4). Based on the data obtained, technical
351 grade individual inhibitors were prepared and mixed in enzyme-reaction buffer together
352 with Avicel at the beginning of the experiment and designated as BA, BB, BC, BD, BE,
353 and BF as shown in Table 2. The Buffer-only solution (BA) was used as a control and
354 XOS (BD) was prepared from technical grade birch wood xylan (Sigma-Aldrich,
355 Germany) under hydrothermal conditions at 180°C for 20 min and diluted to 11.3 g/L,
356 mimicking the concentration of XOS produced from hydrothermally treated OPMF. The
357 concentrations of the individual inhibitors were diluted to the concentration based on
358 the data obtained from OPMF PL and PLH. The reaction mixtures were incubated at
359 50°C for 72 h and the samples were collected at certain time intervals to facilitate the
360 hydrolysis rate of Avicel.

361 Fig. 3 shows the time course of glucose yields from microcrystalline cellulose
362 (Avicel) upon incubation with the selected inhibitors. Fig. 3a shows that the PL affected
363 the glucose yield (66.1%), showing a 23.8% reduction compared to the control sample,
364 86.7% (BA). The glucose yield (15.6%) was improved when PL was first enzymatically

365 hydrolyzed by Optimash BG to form PLH, and then treated by activated carbon from
366 OPMF (PLH-ACB), which efficiently removed furfural, HMF, and TA (Table 2).
367 Similar findings were reported by Kim et al. (2011), who showed that activated carbon
368 removed 98% of the phenols and furan aldehydes. The morphology of the activated
369 carbon produced from OPMF (AC-OPMF) was analyzed, and its efficiency in removal
370 of TA at different concentrations and adsorbent loadings were shown in Fig. S1 and Fig.
371 S2, respectively. Factors affecting enzymatic digestibility of Avicel cannot be
372 anticipated since the mixture of inhibitors present in PL may contribute to complex
373 reactions with *Acremonium* cellulase. Therefore, individual inhibitors were tested on
374 Avicel and the results are presented in Fig. 3b.

375 The level of inhibition varied (6.2%-61.6%) depending on the type of inhibitor
376 tested. Xylose monomers showed a limited inhibitory effect (6.2%) on cellulose, with a
377 glucose yield of 81.3%. A reduction in glucose yield of approximately 29.1% (61.5%)
378 was recorded for XOS, and the level of inhibition was doubled (61.6%) when the
379 mixture was supplemented with TA, which showed a glucose yield of only 33.3%. An
380 approximately 19.8% improvement in glucose yield was achieved in BF samples when
381 XOS was completely hydrolyzed by Optimash BG into monomeric xylose. The results
382 presented here were consistent with previously published data which suggested that

383 hydrolysis of XOS to xylose reduced cellulase inhibition (Qing et al., 2010; Kim et al.,
384 2011; Zhang and Viikari, 2012; Kont et al., 2013). Addition of hemicellulase several
385 hours before the addition of cellulase was suggested since it has a higher adsorption
386 affinity than cellulase and xylanase for xylan and glucan (Qing et al., 2010).

387 In order to understand the level of inhibition that each inhibitor imposed on
388 cellulase, a graph of the hydrolysis rate over each of the potential inhibitors tested was
389 plotted as illustrated in Fig. 3c. After 24 h of incubation, the hydrolysis rate of Avicel to
390 glucose from control samples was 0.48 g-glucose release/L.h. A higher hydrolysis rate
391 was observed for PLH-ACA and PLH-ACB, when compared to the control (BA),
392 probably due to the free glucose present in the PL after hydrothermal pretreatment.
393 Based on the error bars (0.48–0.5), there was no significant difference between them.
394 PL and PLH showed a similar level of inhibition of cellulase with 0.39 g-glucose
395 release/L.h. The lowest hydrolysis rate was observed for TA (BE) (0.18 g-glucose
396 release/L.h), which indicates that TA was the most severe inhibitor compound and that it
397 affected cellulase activity at an early stage in the hydrolysis process.

398 Tannic acid was followed by XOS (0.35 g-glucose release/L.h) and xylose
399 (0.44 g-glucose release/L.h). These three inhibitors were intensively discussed in the
400 literature. Inhibition of cellulase by XOS can be explained by competitive and

401 non-specific binding mechanisms (Qing et al., 2010; Zhang and Viikari, 2012; Kont et
402 al., 2013). Kont et al. (2013) reported that XOS mimics the structure of the cellulose
403 chain and binds the active site of cellulase Trce17A. In another report, Qing et al.
404 (2010) found that XOS was hydrolyzed by Spezyme CP, indicating its competitive and
405 non-productive binding to cellulase.

406 Tannic acid was a phenolic compound derived from lignin degradation of
407 biomass pretreatment, and its concentration was heavily dependent on the type of
408 biomass and the pretreatment method and conditions (Jing et al., 2009; Ximenes et al.,
409 2010; Ximenes et al., 2011; Kim et al., 2011; Tejirian et al., 2011). Detailed studies of
410 the effects of TA on cellulase enzymes show that TA was the single, most inhibitory
411 aromatic compound causing both precipitation and deactivation of proteins in spite of
412 its low concentration in the PL (Kim et al., 2011; Ximenes et al., 2011; Tejirian et al.,
413 2011). Tejirian et al. (2011) reported a decrease of approximately 70%–80% in the
414 initial hydrolysis rate and final yields after addition of only 1 mM of TA. The 62.5%
415 reduction in hydrolysis rate recorded in this study for TA (BE) suggests that inactivation
416 of *Acremonium* cellulase already occurred, since the TA and enzyme were mixed
417 immediately at the start of the experiment. We conclude that the tested cellulase
418 inhibitors can be classified as TA > XOS > xylose > furfural and HMF > acetic acid,

419 based on their inhibitory action.

420

421 **4. Conclusion**

422 Hydrothermal pretreatment of OPMF under isothermal and non-isothermal
423 conditions was successfully performed. Comparative enzymatic digestibility tests
424 between pretreated slurries and solids showed that soluble inhibitors in the PL attenuate
425 cellulase activity, resulting in lowered glucose yields. The generated PLs were analyzed
426 and tested individually on Avicel. The effect of individual inhibitors on *Acremonium*
427 cellulase showed that the most severe inhibitor interfering with cellulase activity was
428 TA, followed by XOS, xylose, furfural and HMF, and acetic acid. Activated carbon
429 from OPMF could efficiently remove TA, furfural, and HMF, and its adsorption
430 capacity was comparable to that of technical grade activated carbon.

431

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438

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528 cellobiohydrolase I from *Thermoascus aurantiacus*. *Bioresour. Technol.* 117,
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530

531 LIST OF TABLES

532

533 **Table 1** Properties of pretreated solids and liquids of OPMF samples as functions of
534 pretreatment severities log, *Ro*.

535 *Oligomeric sugar concentrations can be estimated by the difference between the total
536 and monomeric sugar concentrations determined by HPLC according to the analytical
537 method reported in Technical Report NREL/TP-510-42618 (Sluiter et al., 2008).

538

539 **Table 2** Inhibitor concentrations derived from hydrothermally treated OPMF at 150°C
540 for 240 min and technical grade solutions to monitor the effect on the *Acremonium*
541 cellulase.

542 ¹Pretreated liquid (PL) was obtained from hydrothermally treated OPMF at 150°C for
543 240 min.
544 ²OPMF pretreated liquid hydrolyzate (PLH) was prepared by hydrolysis of PL with
545 Optimash BG at 50°C for 72h.
546 ³The inhibitors from PLH were removed by both activated carbons purchased from
547 Merck and from OPMF at 10% activated carbon loadings (wt/vol) with initial pH of 5.0
548 and at 25°C.
549 ⁴Xylo-oligomers (XOS) was prepared from birch wood xylan (Sigma-Aldrich,
550 Germany) under hydrothermal condition at 180°C for 20 min in a tube reactor.

551

552 FIGURE CAPTIONS

553

554 **Fig. 1** Glucose yields from pretreated slurries and washed solids at different
555 pretreatment conditions.

556

557 **Fig. 2** Correlation between specific surface area expansion, hemicellulose removal and
558 glucose yield of hydrothermally treated OPMF.

559

560 **Fig. 3** Time course of glucose yields from microcrystalline cellulose Avicel under
561 influenced of selected inhibitors (a, b) and c) hydrolysis rate of glucose release after 24
562 h of hydrolysis at 50°C at cellulase loadings of 10 FPU/g substrate.
563

Fig. 1 Glucose yields from pretreated slurries and washed solids at different pretreatment conditions.

Fig. 2 Correlation between specific surface area expansion, hemicellulose removal and glucose yield of hydrothermally treated OPMF

Fig. 3 Time course of glucose yields from microcrystalline cellulose (Avicel) under influenced of selected inhibitors (a, b) and c) hydrolysis rate of glucose release after 24 h of hydrolysis at 50°C at cellulase loadings of 10 FPU/g substrate.

Table 1

Properties	Untreated		Hydrothermal pretreatments							
Reaction temperature (°C)	-	150°C	150°C	150°C	150°C	180°C	190°C	200°C	210°C	220°C
Reaction time (min)	-	60 min	120 min	180 min	240 min	20 min	20 min	20 min	20 min	20 min
Severity (log Ro)	-	3.25	3.55	3.73	3.85	3.66	3.94	4.25	4.54	4.83
Cellulose (%)	24.1 ± 2.0	27.2 ± 1.0	32.0 ± 1.0	31.5 ± 0.2	31.0 ± 1.2	35.9 ± 2.4	38.5 ± 1.3	39.7 ± 1.5	41.3 ± 0.7	42.3 ± 1.8
Hemicellulose (%)	25.7 ± 0.7	22.4 ± 2.5	20.5 ± 2.8	14.2 ± 0.4	11.3 ± 0.1	11.5 ± 0.8	5.2 ± 0.7	0.8 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
Klason lignin (%)	25.5 ± 1.3	35.9 ± 1.7	37.1 ± 2.1	42.6 ± 0.7	46.0 ± 2.2	44.4 ± 0.8	48.0 ± 0.3	52.8 ± 1.4	56.9 ± 2.1	58.1 ± 0.9
Specific surface area (m ² /g)	1.1	5.8	11.2	19.0	21.7	27.9	31.7	40.2	42.5	44.2
Pore volume (cm ³ /g)	0.009	0.030	0.043	0.064	0.074	0.085	0.101	0.144	0.167	0.184
<i>Soluble sugars from pretreated liquid (g/L)</i>										
Gluco-oligomers *	-	1.7 ± 0.3	2.0 ± 0.1	0.7 ± 0.3	1.2 ± 0.4	0.8 ± 0.0	1.4 ± 0.7	0.9 ± 0.0	1.2 ± 0.3	0.4 ± 0.1
Xylo-oligomers *	-	1.7 ± 0.3	4.6 ± 0.5	5.0 ± 0.8	5.5 ± 0.2	6.6 ± 0.9	7.0 ± 1.0	2.1 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
Ara-oligomers *	-	0.4 ± 0.1	2.1 ± 0.2	1.6 ± 0.3	1.7 ± 0.1	0.3 ± 0.0	0.7 ± 0.2	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
Glucose	-	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	2.5 ± 0.4	2.8 ± 0.1	4.2 ± 0.6	2.6 ± 0.5	2.3 ± 0.4
Xylose	-	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	3.9 ± 0.6	18.0 ± 2.0	17.8 ± 3.2	0.7 ± 0.2	0.5 ± 0.0
Galactose	-	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	4.6 ± 0.3	5.1 ± 0.6	4.9 ± 0.3	2.2 ± 0.4	2.7 ± 0.1
Arabinose	-	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	14.4 ± 1.4	7.9 ± 0.2	3.7 ± 0.5	1.7 ± 0.7	2.3 ± 0.1
Mannose	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 0.9	7.3 ± 0.4	2.9 ± 0.1	4.5 ± 0.2

*Soluble by-products from
pretreated liquid (g/L)*

Acetic acid	-	0.3 ± 0.1	0.8 ± 0.3	1.9 ± 0.5	2.9 ± 0.6	1.7 ± 0.1	3.7 ± 0.4	6.4 ± 0.6	7.4 ± 0.7	7.5 ± 0.4
5-HMF	-	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.6 ± 0.0
Furfural	-	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.0 ± 0.1	3.3 ± 0.2	3.4 ± 0.4	2.5 ± 0.1
Formic acid	-	0.9 ± 0.3	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	0.9 ± 0.2	1.3 ± 0.1	2.4 ± 0.2	2.9 ± 0.2	2.3 ± 0.1
Gallic acid	-	1.2 ± 0.2	1.6 ± 0.1	1.7 ± 0.4	1.9 ± 0.1	1.9 ± 0.0	2.9 ± 0.0	4.0 ± 0.0	4.4 ± 0.0	4.5 ± 0.0

*Oligomeric sugar concentrations can be estimated by the difference between the total and monomeric sugar concentrations determined

by HPLC according to the analytical method reported in Technical Report NREL/TP-510-42618 ([Sluiter et al., 2008](#)).

Table 2 Inhibitor concentrations derived from hydrothermally treated OPMF at 150°C for 240 min and technical grade solutions to monitor the effect on the *Acremonium* cellulase.

Experiments	Concentration (g/L)				
	XOS ⁴	Xylose	Tannic	Acetic	HMF + Furfural
PL: OPMF pretreated liquid ¹	11.3 ± 0.7	0.5 ± 0.2	1.7 ± 0.1	3.3 ± 0.0	0.4 ± 0.1
PLH: OPMF pretreated liquid hydrolyzate ²	0.0 ± 0.0	11.7 ± 1.0	1.7 ± 0.4	7.3 ± 0.4	0.3 ± 0.1
PLH-ACA: OPMF PLH + AC Merck ³	0.0 ± 0.0	9.2 ± 0.1	0.0 ± 0.0	4.9 ± 0.1	0.0 ± 0.0
PLH-ACB: OPMF PLH + AC OPMF ³	0.0 ± 0.0	9.8 ± 0.3	0.0 ± 0.0	5.8 ± 0.3	0.0 ± 0.0
BA: Buffer only	-	-	-	-	-
BB: Buffer + acetic	-	-	-	3.3 ± 0.1	-
BC: Buffer + Furfural + HMF	-	-	-	-	0.4 ± 0.1
BD: Buffer + XOS ¹	11.3 ± 0.5	-	-	-	-
BE: Buffer + tannic acid	-	-	1.7 ± 0.3	-	-
BF: Buffer + xylose	-	11.7 ± 0.5			

¹Pretreated liquid (PL) was obtained from hydrothermally treated OPMF at 150°C for 240 min.

²OPMF pretreated liquid hydrolyzate (PLH) was prepared by hydrolysis of PL with Optimash BG at 50°C for 72h.

³The inhibitors from PLH were removed by both activated carbons purchased from Merck and from OPMF at 10% activated carbon loadings (wt/vol) with initial pH of 5.0 and at 25°C.

⁴Xylo-oligomers (XOS) was prepared from birch wood xylan (Sigma-Aldrich, Germany) under hydrothermal condition at 180°C for 20 min in a tube reactor.