Hydrothermal pretreatment enhanced enzymatic hydrolysis and glucose production from oil palm biomass

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28 **ABSTRACT**

29 The present works investigate hydrothermal pretreatment of oil palm empty fruit bunch
30 and oil palm frond fiber in a batch tube reactor system with temperature and time range
31 from 170 to 250 °C and 10 to 20 min, respectively. The behavior of soluble sugars,
32 acids, furans, and phenols dramatically changed over treatment severities as determined
33 by HPLC. The cellulose-rich treated solids were analyzed by SEM, WAXD, and BET
34 surface area. Enzymatic hydrolysis was performed from both pretreated slurries and
35 washed solid, and data obtained suggested that tannic acid derived from lignin
36 degradation was a potential cellulase inhibitor. Both partial removal of hemicellulose
37 and migration of lignin during hydrothermal pretreatment caused structural changes on
38 the cellulose-hemicellulose-lignin matrix, resulting in the opening and expansion of
39 specific surface area and pore volume. The current results provided important factors
40 that maximize conversion of cellulose to glucose from oil palm biomass by
41 hydrothermal process.

42

43 **Keywords:** Oil palm biomass, hydrothermal pretreatment, tannic acid, specific surface
44 area, enzymatic hydrolysis

45

46 **1. Introduction**

47 Oil palm frond fiber (OPFF) and oil palm empty fruit bunch (OPEFB) have
48 been reported as suitable biomass for biochemicals and biomaterials production since
49 these materials are rich in carbohydrate and lignin, available throughout a year,
50 renewable and sustainable (Ho et al., 2014; Zahari et al., 2014). Better management and
51 maximal utilization of these biomass will increase the value of the by-products
52 generated and promote sustainability to the oil palm industry. However, the natural
53 recalcitrance of lignocellulosic biomass has hindered its potential applications such as
54 biochemical and biofuel production if no pretreatment is performed. Pretreatment is
55 necessary to remove barriers such as hemicellulose and lignin that limit the penetration
56 of enzyme to cellulose. Hydrothermal pretreatment has advantages over other
57 pretreatments since the system only use water and the hydronium ion from water
58 ionization act as catalyst in the reaction medium (Möller et al., 2011; Sabiha-Hanim et
59 al., 2011). It was reported that the breakdown and loosening of the lignocellulosic
60 structure in biomass was achieved by hydrothermal pretreatment and dilute acid at
61 chosen treatment conditions. The removal of hemicellulose and migration of lignin
62 loosened the intact structures of cellulose-hemicellulose-lignin matrix, thus improved
63 adsorption of cellulase on cellulose-rich solids (Hsu et al., 2010; Pu et al., 2013; Xiao et

64 [al., 2013](#)). Successful fractionation of lignocellulosic biomass and the production of
65 soluble sugar derived-carbohydrate and phenol-derived from lignin by hydrothermal
66 pretreatments were greatly influenced by the combined reaction temperature and time
67 normally expressed as treatment severity, log *Ro* ([Overend and Chornet, 1989](#)).

68 Under hydrothermal pretreatment process, several soluble inhibitors were
69 produced, which hampered the efficiency of enzymatic hydrolysis and fermentation.
70 Soluble sugars both in oligomeric and monomeric forms were produced from
71 degradation of hemicellulose and cellulose components. Xylooligomers (XOS) and
72 xylose were the most detectable soluble sugars generated by hydrothermal pretreatment
73 and studies have found that XOS were strong cellulase inhibitors ([Qing et al., 2010](#);
74 [Kont et al., 2013](#)). Acetyl groups in hemicellulose will degrade into acetic acid while
75 pentose and hexose sugars will degrade into furfural and 5-HMF. Both furfural and 5-
76 HMF can be further degraded into formic acids under acidic condition when higher
77 treatment severities is applied ([Möller et al., 2011](#); [Nitsos et al., 2013](#)). Phenolic
78 compounds which originate from degradation of lignin and from phenolic ester groups
79 linked with hemicellulose showed inhibition and deactivation of β -glucosidase from
80 *Trichoderma reesei* and *Aspergillus niger* ([Ximenes et al., 2010](#); [Ximenes et al., 2011](#)).
81 Recently, [Kim and co-workers \(2011\)](#) found that both XOS and phenolic compounds

82 were the most important factors caused decreased cellulase activity. Total phenolic
83 compounds as low as 1.3 g/L which strongly inhibited cellulase by precipitation and
84 deactivation of β -glucosidase. The removal of soluble inhibitors prior enzymatic
85 hydrolysis is necessary in order to maximize enzyme activity and reduction of enzyme
86 dosage, **in order to** promote low cost cellulose to bioethanol production (Ximenes et al.,
87 2010; Kim et al., 2011; Kim et al., 2013).

88 The cellulose-rich solids are more amenable to enzymatic attack since both
89 hemicellulose and lignin had been partially solubilized and removed during the
90 separation and washing steps. Physical and morphological changes of treated solids can
91 be clearly distinguished from untreated sample with severe damages on the biomass
92 surface, creation of pores/holes and fibrillation of fibers/woods. Removal of
93 hemicellulose and lignin also resulted in increased crystallinity index (CrI) of cellulose,
94 specific surface area (SSA) and pore volume (PV), thus improved the enzymatic
95 digestibility of cellulose to glucose (Hsu et al., 2010; Xiao et al., 2013; Pu et al., 2013).

96 The aim of the current study was to **investigate the chemical and physical changes**
97 **behavior of OPFF and OPEFB in the process of hydrothermal pretreatment. Factors**
98 **affecting maximal conversion of cellulose to glucose was highlighted and discussed.**

99

100 **2. Materials and methods**

101 **2.1 Raw materials and componential analysis**

102 The OPEFB was collected from Seri Ulu Langat Palm Oil Mill, Dengkil,
103 Selangor, Malaysia, and the OPFF was obtained from the oil palm plantation at
104 Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The samples were prepared as
105 discussed earlier ([Zakaria et al., 2014a](#)) prior to component analysis. The oil palm
106 biomass components were determined using a method recommended by [Teramoto et al.](#)
107 ([2008](#)).

109 **2.2 Hydrothermal pretreatment**

110 Unless otherwise stated, hydrothermal pretreatment of OPFF and OPEFB were
111 conducted in a [35 mL stainless steel tube reactor](#) as reported earlier ([Zakaria et al.,](#)
112 [2014b](#)). 3 g (oven dried) of lignocellulosic biomass (OPFF and OPEFB) and 30 mL of
113 distilled water was used to fill the reactor (equivalent to 1:10, solid: liquid ratio). The
114 working volume employed in this study was 30 mL. The tightened reactor filled with
115 biomass sample and water was then carefully emerged into a sand bath, which was
116 maintained at temperature range from 170 - 250°C governed by automatic temperature
117 controller. The reactor was agitated at 60 rpm in order to provide homogenize mixing of

the sample in the tube reactor. After 10-20 min of residence time, the reactor was transferred from the sand bath into water reservoir and cooled down to 30°C. The pH values of the raw materials and hydrothermally treated samples were measured using a digital pH meter (D-53, Horiba, Japan). The treated samples were filtered using filter paper No. 2 with a pore size of 5.0 µm (Advantec, Japan) under gravity flow and washed with distilled water until neutral pH. The neutralized solid was oven dried at 90°C for 24 h prior to enzymatic hydrolysis. The intensity of the hydrothermal treatment was expressed as severity factor (log R_0). The severity parameters corresponding to different hydrothermal pretreatment conditions are calculated as in Eq. (1).

$$R_0 = t \exp [(T-100)/14.75] \quad (1)$$

In which t is the reaction time (min), and T is the hydrolysis temperature (°C) (Overend and Chornet, 1989).

2.3 Enzymatic hydrolysis

Unless otherwise stated, enzymatic hydrolysis was performed using an enzyme cocktail constituting 40 FPU/ mL *Acremonium* cellulase (Meiji Seika Co, Japan), and 10% Optimash BG (Genencor International, California, USA). In a standard assay, 0.75

136 mL (10 FPU/g substrate) of *Acremonium* cellulase, 2.5 mL of 1.0 M acetate buffer, pH
 137 5.0, and 0.6 mL of 10% Optimash BG were added to treated samples (3g of dry weight)
 138 in 65 mL tube (NEG, Japan). The reaction mixture was added with distilled water to a
 139 total volume of 50 mL. In a smaller scale reaction, about 0.05 g of treated solids with
 140 total hydrolysis mixtures (1 mL) were placed in 2 mL Eppendorf tubes. The enzymatic
 141 hydrolysis was performed at 50°C for 72 h with stirring/shaking. The experiment was
 142 performed in triplicate and the results are presented as the average values. The
 143 enzymatic digestibility was represented by the sugars obtained (g sugars/ g materials) or
 144 sugar yield as calculated as in Eq. (2):

145

$$146 \text{ Sugar yield (\%)} = [\text{weight of monomeric sugars after enzymatic hydrolysis} / \text{weight of} \\ 147 \text{ potential total monomeric sugars after hydrolysis using H}_2\text{SO}_4] \times 100 \quad (2)$$

148

149 **2.4 Analytical procedures**

150 The untreated and treated OPEFB and OPFF samples that were lyophilized
 151 with t-butyl alcohol were further dried at 105°C for 6 h with degassing (BELPREP; Bel
 152 Japan, Japan) before setting up the measuring device. The measurement was performed
 153 using BELSORP-max (Bel Japan, Japan) at a temperature of -196°C. The specific

154 surface area of the sample was determined from the Brunauer-Emmett-Teller plot of
155 nitrogen adsorption-desorption isotherms (Ishiguro and Endo, 2014). The total pore
156 volume was determined at At $P/P_0 = 0.99$.

157 Detection of hydrolyzed sugars, acetic acid, furfural, 5-HMF, and formic acid
158 from HPT and enzymatic hydrolysis were performed using high-performance liquid
159 chromatography (HPLC) according to the analytical method (NREL/TP-510-42623)
160 (Sluiter et al., 2008). Tannic acid and gallic acid were determined by HPLC equipped
161 with a UV detector (UV-2070 Plus, Jasco, Japan) using an Aminex 87H column (7.8
162 mm I.D. $\times$ 30 cm, BioRad, USA) with a Carbo-P micro-guard cartridge. The column
163 oven was set at 60°C and samples were eluted at 0.60 mL/min with 8.0 mM H₂SO₄.

164 SEM and WAXD patterns analysis of the untreated and hydrothermally treated
165 oil palm biomass samples were determined as reported earlier (Zakaria et al., 2014a).
166 The crystallinity index (CrI) was calculated using Eq. (3) based on the method of Segal
167 et al. (1959).

$$168 \text{ Crystallinity index (\%)} = [(I_{002} - I_{am})/I_{002}] \times 100 \quad (3)$$

169 I_{002} : The intensity at about $2\theta = 22.2^\circ$

170 I_{am} : The intensity at $2\theta = 17.6^\circ$

171

172 **3. Results and discussion**

173 **3.1 Chemical compositions and properties of oil palm biomass**

174 As shown in [Table 1](#) the major component of untreated OPFF and OPEFB is
175 cellulose, followed by hemicellulose and lignin. The chemical compositions of oil palm
176 biomass reported in this study were in the ranges of earlier reports ([Sabiha-Hanim et al.,](#)
177 [2011](#); [Goh et al., 2012](#); [Hong et al., 2013](#); [Ho et al., 2014](#)). OPFF and OPEFB were
178 pretreated by hydrothermal process at 170-250°C for 10-20 min. Solid yield, pH of the
179 treated slurries and chemical compositions changed over treatment severities. The
180 dissolution of soluble components of oil palm biomass occurred and correlated well
181 with treatment severities resulting in decreased solid recovery yield. About 52.2-52.7%
182 of solids yield were obtained from OPFF and OPEFB samples, at the highest treatment
183 severity. pH decreased from neutral to acidic towards higher treatment severities due to
184 accumulation of acetic acid from de-acetylation of hemicellulose degradation ([Sabiha-](#)
185 [Hanim et al., 2011](#), [Ho et al., 2014](#)).

186 The chemical composition of oil palm biomass was greatly affected by the
187 hydrothermal pretreatment especially hemicellulose which decreased at higher reaction
188 temperature. However, only partial degradation was obtained from OPFF instead of
189 complete removal of hemicellulose component by dilute acid and hydrothermal

190 pretreatment observed from rice straw and *Tamarix ramosissima*, respectively (Hsu et
191 al., 2010; Xiao et al., 2013). This might be due to moderate treatment severities and
192 types of biomass used. About 2-fold increase of Klason lignin content was recorded
193 towards higher treatment severities from both OPFF and OPEFB. This can be explained
194 by the fact that most of the lignin was solubilized at selected treatment temperature
195 range and re-condensation of this material took place upon cooling process. The current
196 finding is in agreement with other studies which reported accumulation of Klason lignin
197 under acidic environments (Goh et al., 2010; Sabiha-Hanim et al., 2011; Pu et al., 2013).
198 The dissolution of hemicellulose and relocation or migration of lignin resulted in higher
199 cellulose content, with the highest at 58.7% and 52.7% from OPFF and OPEFB when
200 hydrothermally treated at $\log Ro = 3.94$ and 4.83, respectively. Reduction of cellulose
201 content was recorded when oil palm biomass was pretreated beyond these values
202 indicates `over cooking` and degradation of hexose sugars. The removal of amorphous
203 hemicellulose has increased the crystallinity index (CrI) of cellulose towards higher
204 treatment condition, and the increase in CrI of cellulose indirectly showed higher
205 exposure of cellulose components amenable to cellulase attack.

206 Untreated and hydrothermally pretreated samples were subjected to SEM and
207 BET analysis for their morphological structure, specific surface area (SSA) and pore

208 volume (PV) properties to understand the effect of hydrothermal pretreatment on oil
209 palm biomass at structural level. Fig. S1 presents the morphological changes of OPFF
210 and OPEFB samples over hydrothermal pretreatment at different treatment severities. At
211 $\log Ro = 3.06$ to 4.24 , OPFF had fibrillated (Fig. S1b-f) and can be distinguished clearly
212 from its untreated form (Fig. S1a). Meanwhile, deposition of spherical Klason lignin
213 droplets were obviously observed on the surface of OPEFB-treated solids (Fig. S1i-l) at
214 $\log Ro = 4.83$ to 5.72 , indicating dissolution and degradation of hemicellulose taking
215 place compared to untreated OPEFB with solid and intact surface (Fig. S1g).

216 The removal of hemicellulose, migration and re-deposition of lignin upon
217 cooling process on the surface of treated solids has been reported to increase the SSA
218 and PV respectively thus substantially improved adsorption of cellulase to cellulose
219 (Hsu et al., 2010; Pu et al., 2013; Xiao et al., 2013). The SSA and PV of untreated OPFF
220 and OPEFB were recorded at $0.98 \text{ m}^2/\text{g}$, $0.0072 \text{ cm}^3/\text{g}$ and $0.95 \text{ m}^2/\text{g}$, $0.0056 \text{ cm}^3/\text{g}$,
221 respectively (Table 1). Increments of SSA and PV were observed when the oil palm
222 biomass was imposed to higher treatment severities. The highest SSA and PV obtained
223 were $7.2 \text{ m}^2/\text{g}$, $0.045 \text{ cm}^3/\text{g}$ and $5.5 \text{ m}^2/\text{g}$, $0.042 \text{ cm}^3/\text{g}$, respectively from OPFF and
224 OPEFB at the harshest treatment condition. Inconsistent values of SSA and PV from
225 OPEFB samples at higher treatment severities may be due to severe disruption of

226 biomass particles and adherent of spherical lignin droplets on the surface of treated
227 OPEFB solids, as supported by SEM image. Similar result was reported when *Tamarix*
228 *ramosissima* was imposed at severe treatment condition which caused the lignocellulose
229 damage and resulted in the reduction of SSA (Xiao et al., 2013). Nano pore size of
230 treated beech wood obtained by hydrothermal treatment and expansion in SSA had
231 improved interactions between biomass and enzymes leading to saccharification
232 enhancement (Nitsos et al., 2013). Recently, Ishiguro and Endo (2014) found that
233 fibrillation of *Eucalyptus* chips by addition of 20% NaOH in hydrothermal-
234 mechanochemical treatment improved the SSA by 76% (232 m²/g) and resulted in
235 100% glucose yield. The impact of hydrothermal pretreatment on glucose conversion
236 yield from OPFF and OPEFB will be discussed in a later section.

237

238 **3.2 Composition of the liquid fraction from hydrothermal pretreatment**

239 [Table 2](#) presents the formation of total and monomeric sugars from liquid
240 fraction of OPFF and OPEFB as functions of pretreatment severities. The oligomeric
241 sugars can be estimated by the difference between the total and monomeric sugar
242 concentrations. Xylooligosaccharides (XOS) and glucooligosaccharides (GlcOS) were
243 the major products obtained by hydrothermal pretreatment of OPFF and OPEFB with

244 maximum production recorded at treatment severity, $\log R_o = 3.65$ and 5.42 ,
245 respectively. OPFF has shown higher production of XOS and GlcOS with
246 concentrations of 97.9 mg/g and 54.2 mg/g , respectively at optimal pretreatment
247 condition, $\log R_o = 3.65$ compared to OPEFB. This can be explained by higher
248 composition of hemicellulose in OPFF compared to OPEFB (Table 1). Other oligomeric
249 and monomeric galactose, arabinose and mannose were detected in lesser amounts and
250 concentration of sugars were decreased at higher treatment severities, suggesting further
251 degradation of pentose and hexose sugars into fermentation inhibitors. Sabiha-Hanim et
252 al., (2011) reported that about 4.3% of xylose was detected in the hydrolysate of treated
253 OPFF samples when autohydrolyzed at $\log R_o = 2.40$ (121°C , 60 min). On the other
254 hand, low XOS and xylose was obtained from OPEFB in this study was due to the high
255 treatment severity applied in the hydrothermal pretreatment process that further
256 converted those sugars into degradation by-products. Recently, a higher XOS
257 concentration of 17.6 g/L was reported from solubilisation of 63 g/100 g xylan at 210°C
258 ($\log R_o = 3.91$) from OPEFB samples (Ho et al., 2014). This indicates that reaction
259 temperature and time are crucial parameters in decomposition of hemicellulose
260 component and selective generation of oligomeric and monomeric sugars.

261 In general acetyl groups in hemicellulose will degrade into acetic acid while

262 pentose and hexose sugars will degrade into furfural and 5-HMF. Fig. 1 shows
263 degradation by-products produced from OPFF and OPEFB hydrolysate as a function of
264 treatment severities. Acetic acid, furfural, 5-HMF and formic acid are the four major by-
265 products produced. The concentrations increased at increasing treatment severities and
266 reached the maximum at the harshest treatment condition, $\log Ro = 4.24$, with
267 production of 71.7 mg/g (acetic), 18.4 mg/g (5-HMF), 37.4 mg/g (furfural) and 48.2
268 mg/g (formic acid) from OPFF samples (Fig. 1a). Meanwhile, OPEFB showed lower
269 concentration and different profiles of degradation by-products formation than OPFF. 5-
270 HMF produced from OPFF and OPEFB samples were detected at moderate
271 concentrations, indicating minimal impact on hexose-base polysaccharides, suggesting
272 that both oil palm biomass were pretreated in the suitable reaction temperatures and
273 time range. Formic acid production may contributed from further degradation of furfural
274 and 5-HMF at severe treatment condition (Nitsos et al., 2013). The highest formic acid
275 accumulation from hydrothermally treated OPEFB was 68.6 mg/g at $\log Ro = 5.1$, and
276 decreased towards higher treatment severities. The reduction of formic acid at $\log Ro =$
277 5.42-5.72 probably due to either decomposition of formic acid through decarbonylation
278 and decarboxylation in a water-gas-shift (WGS) reaction (Yasaka et al., 2006). Yoshida
279 and co-workers (2004) reported that the reversibility of decarbonylation has been

280 evidenced by the direct conversion of carbon monoxide to form formic acid in hot
281 water. Overall, the generation of acetic acid, furfural, 5-HMF and formic acid from both
282 OPFF and OPEFB samples were below the threshold limit of fermentation inhibitory
283 level (Gong et al., 1999).

284 An attempt to characterize phenolic compounds from hydrothermally-treated
285 oil palm biomass was performed in the present study, since little or no information
286 regarding these compounds are available in the literature. Phenolic compound identified
287 as tannic acid (TA) was characterized from pretreated oil palm biomass's slurries and
288 the profiles of TA over treatment severities are depicted in Fig. 1c. Tannic acid
289 concentration was detected in the range from 0.0 to 0.47 g/L from OPFF and 0.19 to
290 0.28 g/L from OPEFB at $\log Ro = 3.06$ to 4.24 and 4.54 to 5.72 , respectively. Recently,
291 total phenolic compounds were detected about 3.42 g/L from hydrothermally treated
292 OPEFB samples under non-isothermal process at $\log Ro = 4.22$ (Ho et al., 2014).

293 Several researchers extensively reviewed the inhibitory level of phenolic compounds
294 derived from liquid fraction of pretreated biomass by decreasing the rate and extent of
295 cellulose hydrolysis (Kim et al., 2011; Ximenes et al., 2011; Tejirian and Xu, 2011). Full
296 enzyme activity was observed after removal of those compounds (Kim et al., 2011). In
297 another study, TA was reported as the single and the most damaging aromatic compound

298 compared to gallic acid, hydroxyl-cinnamix, 4-hydroxybenzoic acids, and vanillin,
299 which caused 20-80% deactivation and loss of cellulase and or β -glucosidase activities
300 (Ximenes et al., 2011). Furthermore, Tejirian and Xu (2011) found that TA as low as 1
301 mM caused substantial inhibition with 70-80% decrease in cellulose hydrolysis and
302 initial hydrolysis rate. Thus removal of these compounds prior to enzymatic hydrolysis
303 is necessary in order to avoid cellulase inhibition and to obtain maximum conversion of
304 sugars.

305

306 **3.3 Enzymatic hydrolysis**

307 [Fig. 2](#) shows glucose yield obtained from OPFF and OPEFB samples at
308 different treatment severities. Both treated slurries (solid and liquid) and washed treated
309 solids were subjected to enzymatic hydrolysis for 72 h at 50°C. It was clearly shown
310 that cellulose rich-solids from OPFF and OPEFB achieved greater glucose conversion
311 yield compared to pretreated slurries. This was probably due to removal of inhibitory
312 compounds in the separation and washing steps. Glucose yield increased with treatment
313 severities, and the highest glucose yield obtained was about 87.1% and 100% from
314 OPFF and OPEFB, respectively at log R_o = 4.54 and 5.13. On the other hand,
315 enzymatic hydrolysis of pretreated OPFF and OPEFB slurries achieved maximum at

316 61.5% and 73.6%, respectively at moderate treatment severities and decreased towards
317 higher treatment severities ($\log Ro = > 3.94$ and $\log Ro = > 5.13$ for OPFF and OPEFB).
318 This indicated higher concentrations of inhibitory compounds were released, as shown
319 in [Fig. 1](#) resulting in more detrimental effects to cellulase and other enzyme activities.
320 Several studies have shown that oligomers such as XOS, xylose ([Qing et al., 2010](#); [Kont](#)
321 [et al., 2013](#)) and phenolic compounds ([Kim et al., 2011](#); [Ximenes et al., 2011](#); [Tejirian](#)
322 [and Xu, 2011](#)) were potential inhibitors to cellulase and fermentative microorganisms.
323 Since moderate concentration of XOS and xylose resulted in reduction of enzyme
324 activities towards higher treatment severities ([Table 2](#)), we postulate that the inhibition
325 of cellulase and enzymatic hydrolysis from treated slurries may be probably derived
326 from phenolic compounds, especially TA that was recorded about 0.47 g/L at $\log Ro =$
327 4.24. This value was high enough to deactivate and destroy cellulase activities as
328 compared to 1mM ([Tejirian and Xu, 2011](#); [Ximenes et al., 2011](#)). Even though factors of
329 inhibition may vary from types of enzymes and phenolic compounds present, further
330 investigation in reducing and removal of phenolic compounds in reducing and removal
331 of phenolic compounds will be carried out in the near future.

332 Maximal glucose conversion yield can be obtained from cellulose- rich solids
333 with removal of hemicellulose and phenolic compounds that inhibited cellulase activity.

334 [Hsu and co-workers \(2010\)](#) reported that, even though nearly complete removal of
335 xylan was achieved at higher treatment severity, only 70% of glucose yield was
336 obtained and it was found that the cellulose structure was wrapped by the lignin.
337 Interestingly, in contrast, hemicellulose was not completely removed in our study even
338 at the harshest treatment severity, therefore resulting in 87-100% of glucose conversion
339 from OPFF and OPEFB samples. Hence, we suggest that the hydrothermal pretreatment
340 of OPFF and OPEFB at selected treatment severities efficiently dissolved and partially
341 removed the hemicellulose. The migration and re-condensation of acid insoluble lignin
342 component onto OPFF and OPEFB-treated solids during hydrothermal pretreatment did
343 not give any substantial effect on the enzymatic hydrolysis. Furthermore there is no
344 direct correlation of CrI of cellulose with enzymatic digestibility and these studies are in
345 agreement with other studies ([Hsu et al., 2010](#); [Xiao et al., 2013](#)). The present results
346 suggest that both partial removal of hemicellulose and migration of Klason lignin
347 during hydrothermal pretreatment gave substantial effects on the structural changes of
348 cellulose-hemicellulose-lignin matrix opening and expansion of SSA and PV. Thus we
349 conclude that the most important factor for obtaining maximal enzymatic digestibility
350 from oil palm biomass is the expansion of the SSA and PV and this is the first report
351 elucidating factors limiting enzymatic hydrolysis of oil palm biomass highlighting

352 phenolic compounds, SSA and PV.

353

354 **4. Conclusion**

355 Hydrothermal pretreatment and enzymatic hydrolysis of oil palm biomass demonstrated
356 high conversion of cellulose to glucose. Partial removal of hemicellulose and migration
357 of lignin of treated solids has substantial effect on expanding the surface area and
358 creation of pores for cellulase adsorption. Tannic acid production increased at increasing
359 treatment severities and identified as potential inhibitor to enzymatic hydrolysis. There
360 is no direct correlation between CrI of cellulose and lignin accumulation on the
361 enzymatic digestibility. The most critical factor for the highest adsorption of cellulase
362 on cellulose is specific surface area and pore volume.

363

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370 **References**

371

- 372 1. Goh, C.S., Lee, K.T., Bhatia, S., 2010. Hot compressed water pretreatment of oil
373 palm fronds to enhance glucose recovery for production of second generation
374 bio-ethanol *Bioresour. Technol.* 101, 7362-7367.
- 375 2. Goh, C.S., Tan, H.T., Lee, K.T., 2012. Pretreatment of oil palm frond using hot
376 compressed water: An evaluation of compositional changes and pulp
377 digestibility using severity factors. *Bioresour. Technol.* 110, 662-669.
- 378 3. Gong, C.S., Cao, N.J., Du, J., Tsao, G.T., 1999. Ethanol production from
379 renewable resources. *Adv. Biochem. Eng. Biotechnol.* 65, 207-241.
- 380 4. Ho, A.L., Carnevalheiro, F., Duarte, L.C., Roseiro, L.B., Charalampopoulos, D.
381 Rastall, R.A., 2014. Production and purification of xylooligosaccharides from
382 oil palm empty fruit bunch fibre by a non-isothermal process. *Bioresour.*
383 *Technol.* 152, 526-529.
- 384 5. Hong, J.Y., Kim, Y.S., Oh, K.K., 2013. Fractionation and delignification of
385 empty fruit bunches with low reaction severity for high sugar recovery.
386 *Bioresour. Technol.* 146, 176-183.
- 387 6. Hsu, T.C., Guo, G.L., Chen, W.H., Hwang, W.S., 2010. Effect of dilute acid

388 pretreatment of rice straw on structural properties and enzymatic hydrolysis.
389 Bioresour. Technol. 101, 4907-4913.

390 7. Ishiguro, M., Endo, T., 2014. Addition of alkali to the hydrothermal-
391 mechanochemical treatment of *Eucalyptus* enhances its enzymatic
392 saccharification. Bioresour. Technol. 153, 322-326.

393 8. Kim, Y., Ximenes, E., Mosier, N.S., Ladisch, M.R., 2011. Soluble
394 inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass.
395 Enzyme. Microbial. Technol. 48, 408-415.

396 9. Kim, Y., Kreke, T., Hendrickson, R., Parenti, J., Ladisch, M.R., 2013.
397 Fractionation of cellulase and fermentation inhibitors from steam pretreated
398 mixed hardwood. Bioresour. Technol. 135, 30-38.

399 10. Kont, R., Kurašin, M., Teugjas, H., Våljamäe, P., 2013. Strong cellulase
400 inhibitors from the hydrothermal pretreatment of wheat straw. Biotechnol.
401 Biofuels. 6, 135.

402 11. Möller, M., Nilges, P., Harnisch, F., Schröder, U., 2011. Subcritical water as
403 reaction environment: fundamentals of hydrothermal biomass transformation.
404 Chem. Sus. Chem. 4, 566-579.

405 12. Nitsos, C.K., Matis, K.A., Triantafyllidis, K.S., 2013. Optimization of

406 hydrothermal pretreatment of lignocellulosic biomass in the bioethanol
 407 production process. Chem. Sus. Chem. 6, 110-122.

408 13. Overend, R.P., Chornet, E., 1989. Steam and aqueous pretreatments: Are they a
 409 prehydrolyses? In: Wood Processing and Utilization. Elsevier, New York. 395-
 410 400.

411 14. Pu, Y., Hu, F., Huang, F., Davison, B.H., Ragauskas, A.J., 2013. Assessing the
 412 molecular structure basis for biomass recalcitrance during dilute acid and
 413 hydrothermal pretreatments. Biotechnol. Biofuels. 6, 15.

414 15. Qing Q., Yang, B., Wyman, C.E., 2010. Xylooligomers are strong inhibitors of
 415 cellulose hydrolysis by enzymes. Bioresour. Technol. 101, 9624-9630.

416 16. Sabiha-Hanim, S., Mohd Noor, M.A., Rosma, A., 2011. Effect of autohydrolysis
 417 and enzymatic treatment on oil palm (*Elaeis guineensis Jacq.*) frond fibres for
 418 xylose and xylooligosaccharides production. Bioresour. Technol. 102, 1234-
 419 1239.

420 17. Segal, L., Creely, J.J., Martin, A.E., Conrad, C.M., 1959. An empirical method
 421 for estimating the degree of crystallinity of native cellulose using the X-ray
 422 diffractometer. Text. Res. 29, 786-794.

423 18. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008.

424 Determination of sugars, byproducts, and degradation products in liquid fraction
 425 process samples. NREL/TP-510-42623, Laboratory Analytical Procedure
 426 (LAPs). National Renewable Energy Laboratory, Golden, CO.

427 19. Tejirian, A., Xu, F., 2011. Inhibition of enzymatic cellulolysis by phenolic
 428 compounds. *Enzyme. Microbial. Technol.* 48, 239-247.

429 20. Teramoto, Y., Tanaka, N., Lee, S-H., Endo, T., 2008. Pretreatment of eucalyptus
 430 wood chips for enzymatic saccharification using combined sulfuric acid-free
 431 ethanol cooking and ball milling. *Biotechnol. Bioeng.* 99, 75-85.

432 21. Xiao, L.P., Shi, Z.J., Xu, F., Sun, R.C., 2013. Hydrothermal treatment and
 433 enzymatic hydrolysis of *Tamarix ramosissima*: Evaluation of the process as a
 434 conversion method in a biorefinery concept. *Bioresour. Technol.* 135, 73-81.

435 22. Ximenes, E., Kim, Y., Mosiera, N., Diend, B., Ladisch, M., 2010. Inhibition of
 436 cellulases by phenols. *Enzyme. Microbial. Technol.* 46, 170-176.

437 23. Ximenes, E., Kim, Y., Mosiera, N., Dien, B., Ladisch M., 2011. Deactivation of
 438 cellulases by phenols. *Enzyme. Microbial Technol.* 48, 54-60.

439 24. Yasaka, Y., Yoshida, K., Wakai, C., Matubayasi, N., Nakahara, M., 2006.
 440 Kinetic and equilibrium study on formic acid decomposition in relation to the
 441 water-gas-shift reaction. *J. Phys. Chem. A.* 110, 11082-11090.

- 442 25. Yoshida, K., Wakai, C., Matubayasi, N., Nakahara, M., 2004. NMR
443 spectroscopic evidence for an intermediate of formic acid in the water-gas-shift
444 reaction. *J. Phys. Chem. A*. 108, 7479-7482.
- 445 26. Zahari, M.A.K.M., Abdullah, S.S.S., Roslan, A.M., Ariffin, H., Shirai, Y.,
446 Hassan, M.A., 2014. Efficient utilization of oil palm frond for bio-based
447 products and biorefinery. *J. Clean. Prod.* 65, 252-260.
- 448 27. Zakaria, M.R., Fujimoto, S., Hirata, S., Hassan M.A., 2014a. Ball milling
449 pretreatment of oil palm biomass for enhancing enzymatic hydrolysis.
450 *Appl. Biochem. Biotechnol.* 173, 1778-1789.
- 451 28. Zakaria, M.R., Hirata, S., Hassan, M.A. 2014b. Combined pretreatment using
452 alkaline hydrothermal and ball milling to enhance enzymatic hydrolysis of oil
453 palm mesocarp fiber. *Bioresour. Technol.* 169, 236-243.

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456 LIST OF TABLES

457

458 **Table 1** Solid yield, pH, chemical compositions and properties of untreated and
459 hydrothermally treated OPFF and OPEFB.

460

461 **Table 2** Formation of hydrolyzed sugars from OPFF and OPEFB at different treatment
462 severities, log *Ro*.

463 ^aOligomeric sugar concentrations can be estimated by the difference between the total (T)
464 and monomeric (M) sugar concentrations determined by HPLC.

465

466 **FIGURE CAPTIONS**

467 **Fig. 1** Formation of major degradation by-products from hydrothermal pretreatment of

468 a) OPEFB and b) OPFF at different treatment severities, log *Ro*. Symbols represent
469 acetic acid (triangle), HMF (circle), furfural (square) and formic acid (diamond). c)

470 Generation of phenolic compounds, tannic acid from OPFF and OPEFB as functions of
471 treatment severities, log *Ro*.

472

473 **Fig. 2** Yield of glucose from a) OPEFB and b) OPFF at different treatment severities. The
474 samples (5%) were enzymatically hydrolyzed at cellulase loading 10 FPU/g-substrate,
475 incubate at 50°C for 72 h.

476

Table 1 Solid yield, pH, chemical compositions and properties of untreated and hydrothermally treated OPFF and OPEFB.

T (°C)	t (min)	Severity (log Ro)	Solid yield (%)	pH	Cellulose (%)	Hemicellulose (%)	Klason lignin (%)	Crystallinity index, CrI (%)	Specific surface area (m ² /g)	Pore volume (cm ³ /g)
OPFF										
Untreated	-	-	100	6.85	32.7 ± 4.9	22.5 ± 4.9	15.2 ± 0.6	58.4	0.98	0.007
170	10	3.06	68.4	4.10	42.0 ± 2.9	21.1 ± 0.3	22.6 ± 3.5	63.0	2.17	0.010
180	10	3.36	63.5	3.95	48.3 ± 1.0	20.2 ± 0.8	25.3 ± 4.0	68.1	2.55	0.014
190	10	3.65	57.9	3.81	54.6 ± 2.8	12.0 ± 0.1	27.1 ± 4.4	69.1	3.46	0.019
200	10	3.94	53.5	3.58	58.5 ± 0.3	9.1 ± 1.1	29.9 ± 1.9	72.0	5.29	0.033
210	10	4.24	52.2	3.39	53.2 ± 1.7	7.7 ± 0.9	33.7 ± 1.5	74.4	7.20	0.045
OPEFB										
Untreated	-	-	100	6.85	40.4 ± 2.4	20.2 ± 2.3	23.1 ± 0.5	57.6	0.91	0.006
210	20	4.54	67.8	3.86	50.6 ± 1.9	0.0 ± 0.0	37.5 ± 6.6	68.8	5.02	0.029
220	20	4.83	65.1	3.82	52.7 ± 1.8	0.0 ± 0.0	38.1 ± 3.1	72.9	4.64	0.028
230	20	5.13	62.6	3.80	42.1 ± 0.5	0.0 ± 0.0	43.0 ± 3.8	73.7	5.50	0.033
240	20	5.42	57.7	3.77	39.0 ± 0.5	0.0 ± 0.0	47.0 ± 5.5	74.4	5.14	0.033
250	20	5.72	52.7	3.73	33.6 ± 0.2	0.0 ± 0.0	55.4 ± 6.3	72.1	6.21	0.042

Table 2 Formation of hydrolyzed sugars from OPFF and OPEFB at different treatment severities, log *Ro*.

Sample	Concentration (mg/g)									
	Glucose		Xylose		Galactose		Arabinose		Mannose	
	(T) ^a	(M) ^a	(T) ^a	(M) ^a	(T) ^a	(M) ^a	(T) ^a	(M) ^a	(T) ^a	(M) ^a
OPFF										
170°C, 10 min	76.4±3.3	70.9±2.1	44.0±2.7	3.4±4.9	29.4±7.7	4.7±1.0	50.7±3.7	22.4±1.0	37.2±2.4	11.6±11.9
180°C, 10 min	54.3±2.2	52.1±3.0	66.0±4.8	4.9±0.4	18.6±0.9	5.5±1.1	20.6±24.0	24.6±1.2	11.7±14.5	20.2±2.6
190°C, 10 min	54.2±0.4	31.7±8.9	97.9±5.9	12.3±1.1	24.2±3.2	6.4±0.2	20.3±14.4	20.9±0.9	18.9±15.5	15.8±2.0
200°C, 10 min	82.6±3.4	23.9±3.5	92.7±2.4	20.2±1.6	24.4±10.8	5.5±0.2	25.5±7.9	13.6±0.3	37.6±11.6	4.7±0.2
210°C, 10 min	29.8±1.6	11.7±2.2	41.3±2.0	20.2±2.2	13.3±12.0	3.4±0.9	14.4±14.7	4.6±2.0	27.3±24.0	5.4±1.2
OPEFB										
210°C, 20 min	7.9±3.6	3.1±0.2	8.4±1.9	2.3±2.9	5.4±0.6	0.5±0.7	0.0±0.0	1.0±0.2	30.8±21.8	0.7±0.7
220°C, 20 min	7.5±1.9	4.0±1.6	7.9±1.4	1.7±2.4	4.8±0.7	0.9±1.3	0.0±0.0	1.3±0.5	0.0±0.0	0.8±0.2
230°C, 20 min	5.6±1.8	3.2±1.3	3.2±4.6	1.7±2.4	5.3±1.1	1.0±1.5	0.0±0.0	1.2±0.4	0.0±0.0	0.3±0.2
240°C, 20 min	6.0±3.4	2.5±0.5	4.1±5.7	1.6±1.7	5.0±0.2	1.1±0.1	0.0±0.0	1.5±0.6	0.4±0.5	0.6±0.5
250°C, 20 min	5.7±5.3	1.6±0.6	4.3±6.0	1.8±2.3	13.3±1.4	0.5±0.7	2.6±3.7	1.8±0.1	0.0±0.0	0.9±0.3

^aOligomeric sugar concentrations can be estimated by the difference between the total (T) and monomeric (M) sugar concentrations determined by HPLC.

Fig. 1 Formation of major degradation by-products from hydrothermal pretreatment of
a) OPFF and b) OPEFB at different treatment severities, log R_o . c) Generation of
phenolic compounds, tannic acid from OPFF and OPEFB as functions of treatment
severities, log R_o .

Fig. 2 Yield of glucose from a) OPFF and b) OPEFB at different treatment severities. The samples (5%) were enzymatically hydrolyzed at cellulase loading 10 FPU/g-substrate, incubate at 50°C for 72 h with stirring.