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Lignocellulose pretreatment technologies affect the level of enzymatic cellulose oxidation by LPMO

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Abstract

Sugarcane bagasse, corn stover, and wheat straw are among the most available resources for production of cellulosic ethanol. For these biomasses we study the influence of pre-treatment methods on the chemical composition, as well as on the subsequent reactions of enzymatic hydrolysis and oxidation of cellulose. The applied pre-treatment methods are organosolv, hydrothermal, and alkaline. Hydrothermally pretreated wheat straw gave the highest cellulose conversion with 80% glucose yield and 0.8% oxidized cellulose products. Recent studies have shown that lignin is able to boost the activity of the cellulose oxidizing enzyme lytic polysaccharide monooxygenase (LPMO). The highest activity of LPMO was observed for the hydrothermally pretreated biomasses, which also contained the highest level of lignin. All hydrolysis were done at high dry matter levels, using a commercial enzyme preparation containing hydrolytic and oxidative enzymes.

28

1. INTRODUCTION

Agricultural residues such as sugarcane bagasse, corn stover, and wheat straw are the most available sources of lignocellulosic biomass in South America, North America, and Europe, and are therefore the materials of highest interest for cellulosic ethanol production.¹

33 The basic process for cellulosic ethanol is a biochemical conversion of plant biomass by the use of enzymes
34 (cellulases, hemicellulases, and accessory enzymes) to degrade the structural polysaccharides into monomeric
35 sugars, the so-called sugar platform for further microbial transformation into the desired final products.^{2;3}
36 Prior to the enzymatic conversion, the cellulose chains arranged in highly ordered and tightly packed regions
37 of microfibrils, ⁴ needs to be loosened in the cell wall matrix in order to make the crystalline structure more
38 accessible to cellulose-degrading enzymes. ⁵ Therefore the first step that the biomass undergo is a physico-
39 chemical pretreatment,⁶ which alters the chemical and physical structure of the hemicellulose and lignin
40 matrix, providing a more accessible cellulose component. Numerous pre-treatment strategies have been
41 developed over the past decades, showing that no single universal method can be successfully applied to all
42 types of biomass, reflecting the wide range of biomass structures and inherent recalcitrance.^{6;7}

43 In search of industrially relevant scenarios for the enzymatic breakdown of cellulose, a common approach is
44 to operate the process at high dry matter (DM).^{8;9} Such conditions are advantageous from a techno-economic
45 perspective, ¹⁰ but working at high DM e.g. above 20%, can be problematic due to impaired performance of
46 the cellulolytic enzymes. A new generation of commercial cellulolytic cocktails offers several improvements
47 compared to earlier products, one of the most important is a greater tolerance to high DM conditions by the
48 use of improved β -glucosidases.¹¹ Another development is the introduction of a new class of cellulose
49 oxidizing enzymes, known as lytic polysaccharide monoxygenases (LPMOs), classified today as AA9,
50 AA10, and AA11.^{9;12;13}

51 Conventional mechanistic models of the cellulolytic machinery composed of exo- and endo-cellulases does
52 not include the action of the LPMO enzymes. ¹⁴ The LPMOs will oxidatively cleave the cellulose chains and
53 act synergistically with the hydrolytic enzymes. ¹⁵ The product is oxicelluloses with a normal non-reducing
54 end and a C1-oxidized end, or native reducing end and an oxidation of the C4 at the non-reducing terminal.
55 ^{16;17} The products of the subsequent action of exo-cellulases and β -glucosidases are the monomeric forms of
56 the carbohydrates, including the oxidized forms of glucose: gluconic acid and gemdiol 4-ketoaldose, C1 and
57 C4 oxidation, respectively. ¹⁸ This was shown by the seminal studies of Vaje-Kolstad *et al.*, Harris *et al.* and
58 Beeson *et al* 2011.^{12;17;19} Nowadays the interest within the scientific community is focused towards the
59 structure and functional mechanism of LPMOs, mostly to elucidate how the electrons are transferred via
60 LPMO during the redox action.¹⁵

61 It has also been shown that pivotal is the role of cofactors for the redox activity: LPMO is boosted by adding
62 electron donors such as ascorbic acid, gallic acid, and reduced glutathione, which are not found in plant cell
63 walls. ^{19;20} Moreover also enzymes can donate electrons and this is the case of CDH.¹⁵ Since LPMOs have
64 been found to oxidize lignocellulosic substrates without the addition of an external electron donor, lignin has
65 been speculated to be the electron supplier for the activity of LPMOs.^{19;21;22} Moreover, to the best of our
66 knowledge, few studies ^{9;22} have focused on the action of these enzymes in the complex system of a real
67 lignocellulosic substrate and within current commercial cellulase cocktails, under industrially relevant
68 conditions. The role of lignin acting as electron donor for LPMOs, used together with cellulase/LPMO

69 formulations, is thus especially relevant in relation to those pretreatment technologies that target lignin
70 degradation and solubilization (organosolv and alkali pretreatments).

71 Organosolv pretreatment separates cellulose and lignin by extraction of the lignin from lignocellulosic
72 feedstock using an organic solvent.²⁴ In this work, ethanol was employed as a solvent, due to its relatively
73 low cost and proven capacity for delignification and hemicellulose solubilization.^{25,26}

74 Soda treatment is a classical alkaline pulping process that is mainly used industrially to digest wood pulps. Its
75 advantages are the high levels of cellulose remaining, short processing times, and the absence of formation of
76 sulfur by-products.²⁷

77 Another option is the acidic hydrothermal pretreatment frequently used for agricultural residues.²⁸ It
78 selectively removes the hemicellulose fraction, increasing the overall digestibility of the residual cellulose. In
79 this case most of the LCC-bonds (lignin-carbohydrates complex) are cleaved and the phenolic structures in
80 lignin are reorganized but not removed.²⁹

81 This aim of the present work was to obtain a better understanding of the relationship between the lignin
82 remaining in the biomass after pretreatment and the oxidative reactions of LPMO enzymes. Different
83 pretreatment methods (organosolv, hydrothermal, and alkaline) were applied to sugarcane bagasse in order to
84 obtain a residual fraction with different levels and quality of lignin. Hydrothermal pretreatment was then used
85 for three different types of biomass (corn stover, sugarcane bagasse, and wheat straw) in order to determine
86 whether different sources of lignin affected the LPMO catalyzed oxidation of cellulose.

87

88 2. MATERIALS AND METHODS

89 2.1 Biomasses

90 Sugarcane bagasse was kindly donated by the Center for Sugarcane Technology (CTC, Piracicaba, São Paulo,
91 Brazil). Instead Danish wheat straw (*Triticum aestivum* L.) and corn stover (*Zea mays* L.) were from an
92 internal collection at University of Copenhagen (Denmark). All the biomasses were dried until ~10% moisture
93 content, milled to a particle size of <1 mm using a Wiley mill, and stored at -5 °C during the study. An
94 overnight extraction with hot water was performed in order to remove non-structural material from the
95 biomasses.

96 2.2 Enzymes

97 Cellic[®] CTec2 was kindly donated by Novozymes A/S (Bagsvaerd, Denmark). The protein content of the
98 enzymatic preparation was 141.6 mg protein/g, as determined by the bicinchoninic acid (BCA) assay,
99 performed according to the instructions of the supplier (Pierce, Rockford, IL). The cellulase activity was
100 measured by the filter paper assay giving a value of 126 FPU/g of preparation. Cellic[®] CTec2 was stored at 4
101 °C until needed for hydrolysis of the pretreated biomasses.

102 2.3 *Pretreatments*

103 Two different sets of pretreatment were carried out. The first evaluated the effect of different pretreatment
104 techniques (alkaline, hydrothermal, and organosolv) on the same biomass (sugarcane bagasse) at 10% DM.
105 The second instead was designed to test the effects the hydrothermal technique on three different biomasses
106 (sugarcane bagasse, wheat straw, and corn stover) at high dry matter contents (20% DM).

107 In the hydrothermal pretreatment, 10 grams of each biomass were placed separately in 250 mL blue cap
108 bottles. Distilled water was added to adjust the solids content to 20% DM. The bottle was placed in a closed
109 metal cylinder and immersed in a high temperature silicone oil bath. The heat-up time was 35-45 min, and the
110 temperature was recorded by a probe in the metal cylinder. After 10 min at 190 °C, the metal cylinder was
111 removed from the oil bath and cooled in air. All samples were washed with distilled water under vacuum
112 filtration until around pH 5, and stored in a fridge (at 5 °C) prior to enzymatic hydrolysis.

113 The alkaline pretreatment was performed in an autoclave for 30 min at 121 °C. The loading of NaOH was 4%
114 w/w (based on dry bagasse) and the total dry matter loading was 10%.

115 For the ethanol organosolv treatment, 25 g (dry basis) of sugarcane bagasse was mixed with aqueous ethanol
116 (50%) to give a final DM loading of 10%. The mixture was treated in a 5.0 L Parr pressure reactor equipped
117 with temperature controller (Parr Instrument Company, Moline, IL). The reaction mixture was heated at 190
118 °C for 90 min, with continuous stirring.

119 All the pretreated materials were extensively washed with distilled water to eliminate the soluble molecules
120 generated during the pretreatments. In the case of the organosolv pretreatment, the pulp was first washed with
121 ethanol solution at 50% (v/v) and then with hot water (60 °C).

122 2.4 *Hydrolysis*

123 Hydrolysis of the pretreated biomasses (75 mg, dry basis) was performed in 2 mL Eppendorf tubes, with
124 mixing in a tumbler reactor system for 96 h at 48 °C and an enzyme loading of 10 FPU/g glucan. The water
125 content was adjusted with a buffer solution of 50 mM sodium acetate to obtain a final loading of 15% DM.
126 Samples were removed from the reactor at 24 hours intervals and boiled for 10 min. at 105 °C to stop the
127 enzymatic reaction. The supernatant was separated by centrifugation for further analysis of glucan conversion
128 and quantification of oxidized products.

129 2.5 *Biomass compositional analysis and sugar analysis*

130 The total solids, structural carbohydrate, and lignin contents of the raw and pretreated biomasses were
131 analyzed using standard laboratory analytical procedures (LAP) developed by the National Renewable Energy
132 Laboratory (NREL).³⁰ For the structural carbohydrate determination, sugar analysis was performed with a
133 Dionex ICS5000 HPAEC system (Dionex, Sunnyvale, CA, USA) equipped with a pulsed amperometric
134 detector (PAD), a CarboPac-PA1 2x250 mm analytical column, and a CarboPac PA1 2x50 mm guard column.
135 The columns were maintained at 30 °C. Pure water was used as the main eluent for 32 min at 0.250 mL/min,

136 followed by a washing step with 0.25 M NaOH for 10 min, after which the initial conditions were restored for
137 15 min, prior to a new injection. For the post-column eluent, a solution of 0.2 M NaOH was added at 0.1
138 mL/min during each step.

139 The ash content of the solid fraction was determined by incineration of 0.5 g of dried sample at 550 °C for 3 h.

140 The glucose released in the enzymatic hydrolysis experiments was measured using an UltiMate 3000 HPLC
141 (Dionex, Germering, Germany) equipped with a refractive index detector (Shodex, Japan). The separation was
142 performed with a Phenomenex Rezex ROA column, kept at 80 °C, with 5 mM H₂SO₄ as eluent at a flow rate
143 of 0.6 mL/min. The results were analyzed using Chromeleon software (Dionex).

144 2.6 Analysis of oxidized products

145 HPAEC was conducted using an ICS5000 system (Dionex, Sunnivale, CA, USA) equipped with a gold
146 electrode PAD. Samples (2, 5, or 10 µL in 50 mM NaOH) were injected onto a column system comprising a
147 CarboPac PA1 2x250 mm analytical column (Dionex, Sunnivale, CA, USA) and a CarboPac PAC1 2x50 mm
148 guard column, maintained at 30 °C. The gradient elution method used has been described in detail
149 previously.¹⁸

150 2.7 FT-IR Spectroscopy

151 The resulting lignocellulosic materials after the pretreatments were analyzed by a Thermo Nicolet 6700 FT-IR
152 spectrometer equipped with a Golden Gate (diamond) ATR accessory and DTGS (KBr) detector. For the
153 analysis of lignin fractions the crystal temperature of the detector was set at 25°C. A background of 150 scans
154 was acquired, and the spectrum of each sample is reported as the average of three spectra. The maximum
155 absorbance peak at 1025 cm⁻¹ typically associated with cellulose, was chosen for normalization such that
156 $A(1025\text{ cm}^{-1}) = 1$.

157

158

159 3. RESULTS AND DISCUSSION

160 The pretreatment methods and biomasses utilized are shown in the experimental plan (Figure 1), in which the
161 two main series of experiments are highlighted. In the first, different pretreatments (organosolv, hydrothermal,
162 and alkali) were applied to sugarcane bagasse. In the second set of experiments, the hydrothermal
163 pretreatment was applied to the different biomasses (corn stover, sugarcane bagasse, and wheat straw). The
164 two series of pretreatments were conducted with different dry matter (DM) contents: 10% and 20%,
165 respectively. The structural carbohydrates (cellulose and hemicellulose), lignin, and ash contents were
166 measured before and after the pretreatments (Tables 1 and 2). All the pretreated biomasses were hydrolyzed
167 with the Cellic Ctec2 cellulolytic cocktail containing a LPMO enzyme.

168

169 3.1 Sugarcane bagasse as a feedstock to study LPMOs

170 3.1.1 Different pretreatments of sugarcane bagasse

171 Sugarcane bagasse was pretreated using the organosolv, hydrothermal, and alkaline techniques. All the
172 pretreatments were carried out at 10% (w/w) DM loading, and the chemical composition of the solid fraction
173 was determined before and after the pretreatments (Table 1). As expected, each pretreatment had a different
174 effect in terms of the main structural components of the biomass, but overall the greatest changes occurred for
175 xylan and lignin. Total xylan was reduced by 87% after the hydrothermal pretreatment, while reductions of
176 26% and 46% were obtained for the organosolv and alkaline pretreatments, respectively. After the organosolv
177 and alkali pretreatments, the cellulose content increased by more than 60%, compared to the raw material,
178 while an increase of only 5% was obtained for the hydrothermal pretreatment (Table 1, and Figure 2:
179 qualitative data from FT-IR spectra region 900-1200 cm^{-1}).

180 Lignin was affected differently by the pretreatment methods applied. An overview of the pretreatment effects
181 is given by the FT-IR spectra which were collected for all the pretreated materials and compared with the raw
182 sugarcane bagasse spectra (Figure 2). Mainly the peak at 1510 cm^{-1} was analyzed as indicative for lignin and a
183 correlation with the chemical composition analysis has been found: organosolv and alkaline pretreatment
184 spectra stand out with a limited (if not) absorbance, whereas hydrothermal pretreatment spectra gained
185 absorbance when compared to the raw material. In overall the last two spectra have a coherent overlap, whereas
186 the organosolv and alkali induced such radical changes that the spectra are not comparable to the raw material,
187 qualitatively and semi-quantitatively. Organosolv pretreatment, using an aqueous/organic solvent mixture at
188 pH 3.6, removed 61% of the lignin, due to cleavage of hemicellulose-lignin bonds or the hydrolysis of
189 glycosidic bonds in hemicelluloses releasing hemicellulose-lignin fragments, and the cleavage of α -aryl and β -
190 aryl ethers in the native lignin.³¹ Alkaline treatment provided the greatest lignin removal of 70%. The alkaline
191 process also cleaves the α -ether and ester linkages in the phenolic polymer and between lignin and
192 polysaccharides. In general, both organosolv and alkaline delignification act on the β -O-4' alkyl-aryl ether
193 linkages, which are the most common intra-molecular linkages in the lignin. A complete delignification is
194 harder to achieve, because of the carbon-carbon linkages in the residual lignin.

195 The effect of the hydrothermal treatment on lignin did not achieve any quantitative removal. Lignin was not
196 reduced in the residual substrate, although it has been found that spatial reorganization can occur, with
197 droplets appearing on the surface of the fibers.²⁹ These droplets are the result of
198 depolymerization/repolymerization after the transition of lignin from a glassy state to a rubbery state, followed
199 by coalescence and migration from the cell wall.^{28,32}

200 3.1.2 Hydrolysis of sugarcane bagasse pretreated using organosolv, hydrothermal, and alkali methods

201 The solid fractions of pretreated sugarcane bagasse were enzymatically hydrolyzed for 96 h, at 15% DM
202 loading. The glucan and xylan conversion profiles are shown in Figure 3. The highest cellulose conversions
203 were observed for the hydrothermal and alkaline pretreatments, despite their different levels of residual lignin

204 (38 and 8% w/w, respectively). Hence, the presence of lignin did not appear to have any important impact on
205 the hydrolytic action of the enzyme. The recalcitrance of the pretreated bagasse therefore appeared to be
206 associated with the amount of residual hemicellulose, which was higher for the organosolv pretreated bagasse,
207 with a consequently lower hydrolysis efficiency of 60 % glucan conversion, as well as physical characteristics
208 of the cellulose. Besides the overall larger removal of lignin, the alkaline pretreated substrates also showed
209 high cellulose digestibility, with a conversion yield of 94 %. Structural effects that have been reported after
210 soda processes include swelling of the remaining cellulose fibers, together with decreased crystallinity and
211 lower degree of polymerization.^{33,34} In the case of the hydrothermally pretreated bagasse, hydrolysis of the
212 cellulose fraction reached 87 %.

213 Improved xylan hydrolysis has been achieved by the inclusion of accessory enzymes such as endo-xylanases
214 and xyloglucanase in the latest commercial enzyme cocktails, contributing to the conversion of the
215 hemicellulose fraction. The xylan conversion trends observed here were generally similar to the corresponding
216 glucan conversions for each pretreatment method (Figures 3a and 3b). Interestingly, the relative hydrolysis of
217 xylan around 65% was similar for the bagasse samples pretreated by the hydrothermal and alkaline methods.
218 However, the absolute amount of xylan converted was higher for the alkaline pretreated bagasse, which
219 contained four times more hemicellulose, compared to the hydrothermally pretreated material.

220 The role of lignin as a physical barrier to cellulolytic enzymes during hydrolysis has been much discussed.³⁵
221 The organosolv pretreatment reduced the lignin fraction of the bagasse to a small percentage (~10%) of the
222 remaining solids fraction, while the glucan hydrolysis yield was only 60%. In contrast, despite a greater
223 quantity of residual lignin, use of the hydrothermal pretreatment method resulted in 87% glucan conversion. A
224 question therefore arises concerning the role of lignin as a barrier to enzymatic hydrolysis, and the possibility
225 that specific chemical compositions or structural architectures of lignin might affect the enzymes or the
226 recalcitrance of the lignocellulose. The selection of pretreatment technique might be an important factor
227 affecting the lignin pool, due to the occurrence of different chemical or structural modifications. For example,
228 organosolv treatment leads to the phenomenon of covalent repolymerization of lignin derivatives on the
229 cellulose fiber surface, hence restricting access of the cellulases. Lignin deconstruction and repolymerization
230 reactions involving the formation of new carbohydrate-phenolic complexes (by means of β - β , β -1, and β -5
231 linkages) have been reported.³¹ The residual hemicellulose and repolymerized lignin evident in organosolv
232 pretreated biomasses might function as a barrier and reduce the cellulase activity, hence explaining the lower
233 enzymatic conversion.³⁶

234 Hydrothermal pretreatment results in lignin relocation in the form of droplets physically adsorbed to the
235 cellulose surface. Since these clustered lignin structures are adsorbed, they might be displaced from the
236 cellulose surface by the progressive action of exo-cellulases (cellobiohydrolases), as proposed by Li *et al.*,³⁷ so
237 that only partial restriction of enzymatic activity would occur. Moreover, together with an almost complete
238 removal of xylan, very high overall cellulose hydrolysis values (~95%) have been reported for wheat straw
239 that was hydrothermally pretreated at a DM content of 30%.⁹

240 3.1.3 Effect of pretreatment technique on LPMOs activity

241 The activity of LPMOs during the hydrolysis of bagasse was monitored in order to identify the influence of
242 the type of pretreatment technique applied. The concentration of gluconic acid was used as a marker of
243 cellulose oxidation occurring at the C1 position of the pyranose rings constituting the cellulose fibers. It has
244 been already demonstrated that this oxidative activity can be attributed to the LPMOs contained in the Cellic
245 Ctec2 cellulolytic cocktail used in this work.²¹ The results are reported as the level of cellulose oxidation,
246 calculated as the percentage of hydrolyzed cellulose, measuring the concentration of gluconic acid relative to
247 the concentration of glucose, at each time point (Figures 3 and 5). Using this procedure, only the hydrolysable
248 cellulose is measured, rather than the total available cellulose, which is preferable because LPMOs only act on
249 the surface of the cellulose substrate. It is important to note that the enzymatic hydrolysis was carried out
250 without addition of any external electron donor, such as the ascorbic acid used in previous studies.^{12,38} The
251 intention was to determine whether lignin may act as a native electron donor for LPMOs, and whether the type
252 of pretreatment technique affected this characteristic of the lignin applied.

253 The highest gluconic acid concentration after 96 h of hydrolysis was obtained using hydrothermally pretreated
254 bagasse, with 0.43% cellulose oxidation and hydrolysis of 87% of the cellulose. The organosolv pretreated
255 bagasse showed only 0.12% cellulose oxidation, and there were no detectable oxidized products for the
256 bagasse submitted to alkaline pretreatment (the analytical sensitivity of the instrument was 5 ppm). These
257 results correlate with the different quantities of lignin remaining after the pretreatment processes, being the
258 hydrothermal having the highest amount.

259 If in one hand the amount of remaining lignin seems to be the mandating factor for a highest level of cellulose
260 oxidation, in the other the physicochemical properties of the remaining lignin could also differ, and thus to be
261 considered for further speculations. Similar concentrations of lignin were found in the materials submitted to
262 alkaline and organosolv pretreatments, but their capacities to act as electron donors for the LPMOs were not
263 the same. Previous work has shown that lower depolymerization of lignin is induced by organic solvents,
264 compared to alkaline depolymerization.³⁹ It is possible that the phenolic fractions in the organosolv pulps
265 might have higher molecular weight and greater thermal stability than those released by the alkaline process,
266 and these characteristics could affect a possible electron donor capacity. According to Trajano *et al.*,²⁸ lignin
267 can be dissolved in hot liquid water environments, but its high reactivity causes the fast recondensation of the
268 moieties, which reprecipitate on the pretreated fibers. Using optical techniques, Coletta *et al.*³² observed that
269 under acid conditions, that solubilized lignin molecules react with monomers and oligomers to form larger
270 molecules. The native lignin structure can therefore be affected by these processes and undergo changes in its
271 stable or metastable conformation at the nanoscale.^{32,37}

272 It has been reported that the deconstruction of the lignocellulose matrix by LPMOs follows a redox
273 mechanism, with participation of the synergistic action of the fungal cellulases. A new synergy will result
274 after the oxidative disruption of the crystalline cellulose chain, where the pyranose ring undergoes

275 transformation after the introduction of a charged carboxylic end, in the case of C1 oxidation.¹⁵ The
276 disruption of the crystalline packing due to LPMOs will therefore increase the accessibility of the substrate to
277 hydrolytic cellulases, and this synergy is enhanced by the presence of an electron donor. The results indicate
278 the capability of pretreated lignin to act as a reducing agent by supplying the electrons needed for the
279 oxidation step, confirming earlier findings by Hu *et al.*^{21,22,23}

280 3.2. Enhancement of LPMOs activity using hydrothermal pretreatment

281 3.2.1 Hydrothermal pretreatment of different biomasses

282 Three biomasses (sugarcane bagasse, wheat straw, and corn stover) were selected to study the effect of
283 different compositions and structures of the lignocellulose when hydrolyzed with cocktail containing LPMOs.
284 Hydrothermal pretreatment was chosen because the resulting lignin was more reactive for the redox activity of
285 the LPMOs. Table 2 shows the compositions of the biomasses before and after the hydrothermal treatment
286 carried out at 190 °C for 10 minutes, using 20% (w/w) DM content.

287 At elevated temperatures and pressures (180-230 °C and 2.4-2.8 MPa) liquid water behaves like an acid, and
288 the pH values of water at 190 and 220 °C are around 5.8 and 5.5, respectively.³³ Acetic acid, produced from
289 deacetylation of the hemicellulose, also enhances the acid-catalyzed reactions. As a result, hemicellulose is
290 hydrolyzed to soluble oligomers and monomers, and glucans are affected because the liquid fraction contains
291 a certain amount of monomeric glucose. Lignin and glucans presented different ratios in the residual solids
292 fraction of the pretreated biomasses, with wheat straw being more enriched in cellulose (~20%), compared to
293 corn stover (~10%) and sugarcane bagasse (~8%). Due to the solubilization of the hemicellulose, there was an
294 increase in the relative level of Klason lignin of about 50% for all the pretreated biomasses.

295 3.2.2 Hydrolysis of hydrothermally pretreated biomasses

296 Different to the earlier experiments (section 3.1.1), the pretreatments here were conducted using 20% DM
297 loadings, and 15% DM loading was maintained for the subsequent enzymatic hydrolyses. Wheat straw
298 lignocellulose showed the highest digestibility, with production of 80% of the maximum theoretical glucose
299 yield. The overall hydrolysis yield for corn stover, where both glucan and xylan were converted at 50% of
300 their maximum yields, was slightly lower than reported elsewhere for corn stover.³⁴ The yield for bagasse
301 decreased when the pretreatment was done at a higher level of solids (Figure 4). The chemical compositions
302 of bagasse hydrothermally pretreated using DM loadings of 10% and 20% were similar, but the latter yielded
303 20% lower conversion after 96 h of enzymatic hydrolysis. This indicates that despite similar contents of
304 cellulose and lignin (Table 2), the structural and morphological characteristics of the two materials were
305 different.

306 Figure 5 shows the LPMOs activity, calculated as the amount of gluconic acid produced relative to the
307 production of glucose during the enzymatic hydrolysis. The table included in Figure 5 summarizes the global
308 production of gluconic acid at the end of the hydrolysis, compared with the lignin content. All the

309 hydrothermally pretreated biomasses led to higher amounts of oxidized cellulose, indicative of higher LPMO
310 activity, as compared to the organosolv and alkaline methods. It could therefore be inferred that there were no
311 substantial differences between the lignins of the three different biomasses used in this work, in relation to
312 their capacity to react with the LPMOs. It is notable that the amount of gluconic acid detected increased in
313 line with the solids loading used in the pretreatment (from 10% to 20% of DM for the hydrothermal
314 pretreatment of sugarcane bagasse). This was even more evident when the relative oxidation as a percentage
315 of the glucose released was taken into account. The lower release of glucose for the bagasse pretreated at 20%
316 DM resulted in cellulose oxidation values approximately 50% higher than for the bagasse pretreated at 10%
317 DM.

318

319 4 CONCLUSIONS

320 The results obtained in this study are significant in terms of selection of the pretreatment method applied to
321 lignocellulosic biomass prior to enzymatic hydrolysis in biorefineries, with the aim of maximizing the
322 oxidative activity of the LPMO enzymes. The production of gluconic acid was monitored during the
323 enzymatic hydrolysis, as a marker for C1 oxidation of the glucose monomers composing the cellulose fibers,
324 resulting from LPMO catalysis. The residual lignin present in the solid fraction of the pretreated biomass
325 influenced the oxidative activity of the LPMOs, and hydrothermal pretreatment was identified as resulting in
326 better preservation of the reactivity of the lignin, compared to organosolv and alkaline techniques. The benefit
327 of hydrothermal pretreatment was observed for all the agricultural residues studied (corn stover, sugarcane
328 bagasse, and wheat straw), showing that the origin of the lignin did not affect its capacity to potentially act as
329 an electron donor for LPMOs.

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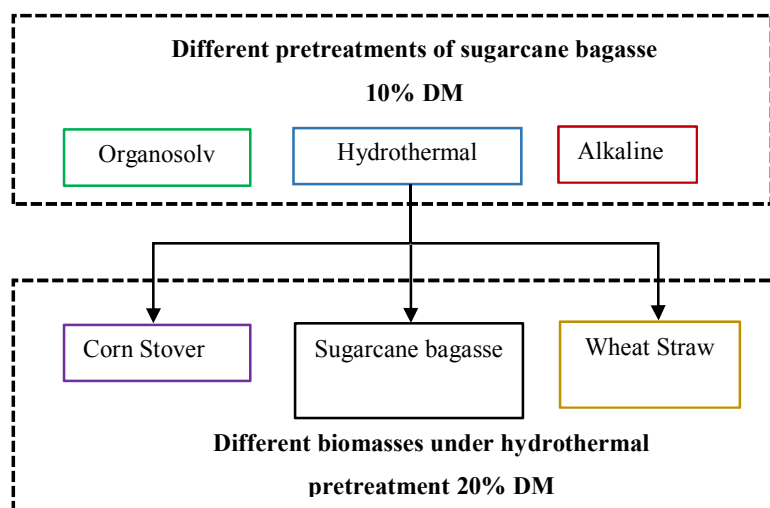
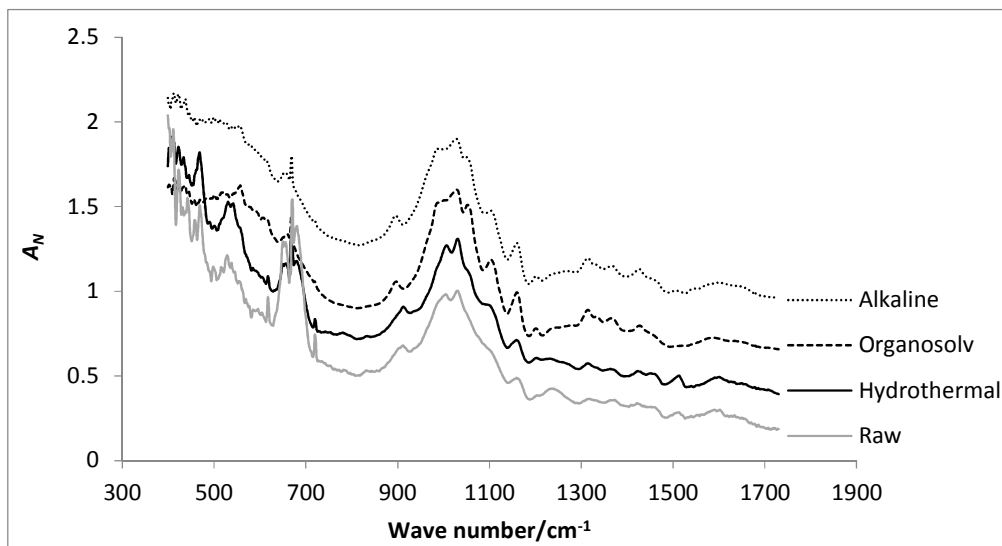


Figure 1 Methodology flow sheet: first raw sugarcane bagasse pretreated with various methods at 10% dry matter; second raw hydrothermal pretreatment applied at various biomasses at 20% dry matter.



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458 **Figure 2.** FT-IR spectra of raw sugar cane bagasse (raw) and after three different pretreatment technology:
459 hydrothermal, organosolv and alkaline. The region at 1510 cm^{-1} indicates the presence of lignin. Instead the
460 region between 950 and 1100 cm^{-1} is typically associated with cellulosic component of the material.
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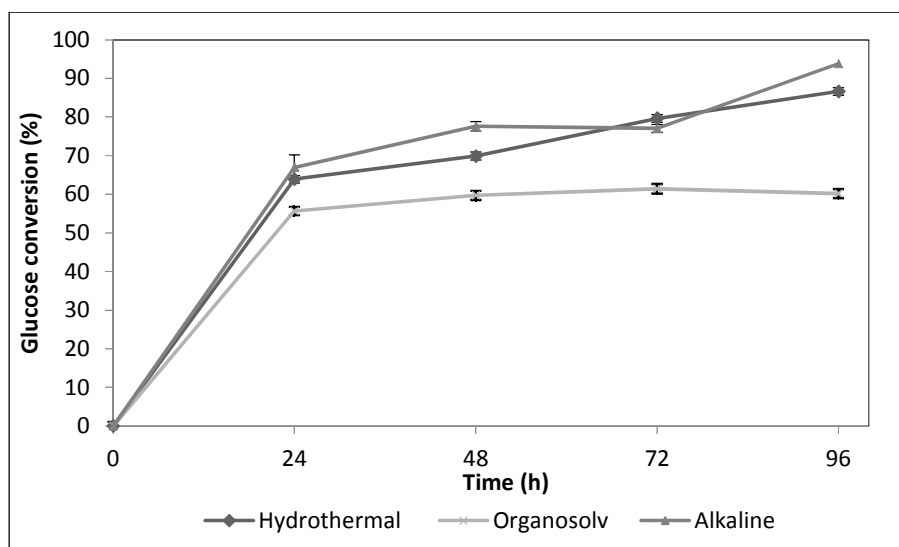
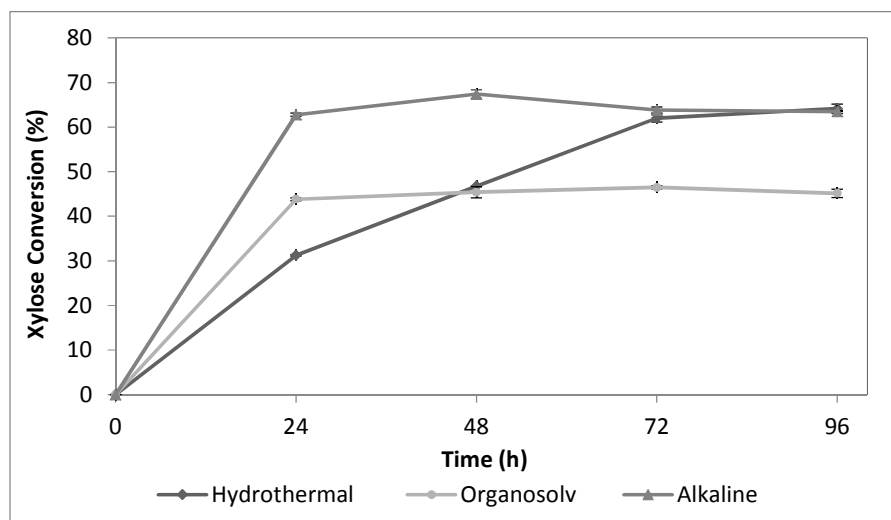
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Figure 3. Enzymatic hydrolysis of sugarcane bagasse pretreated with different technologies: hydrothermal (rhombus), alkaline (triangle) and organosolv (circle). Experimental conditions: Cellic Ctec2 was used for the enzymatic hydrolysis with a loading of 10 FPU/g_{dry cellulose}, 96 hours at 48°C, 15% (w/w) dry matter load. The conversion is given as percentage based on the theoretical maximum glucose (upper plot) and xylose (bottom plot) yields from the pretreated materials.

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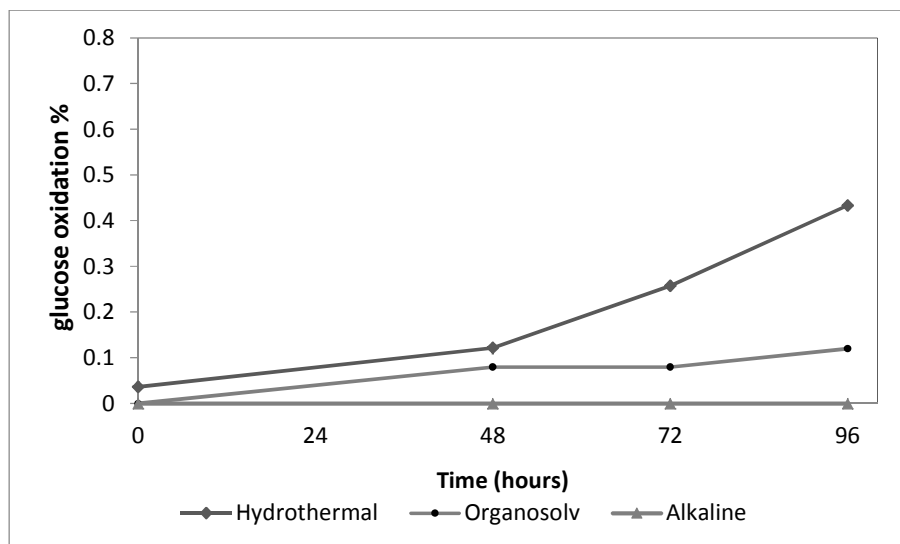
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Figure 4. Glucose oxidation during the enzymatic hydrolysis of sugarcane bagasse pretreated with three different technologies: hydrothermal (rhombus), alkaline (triangle) and organosolv (circle). The glucose oxidation is calculated as percentage of amount of gluconic acid over amount of glucose hydrolyzed from cellulose.

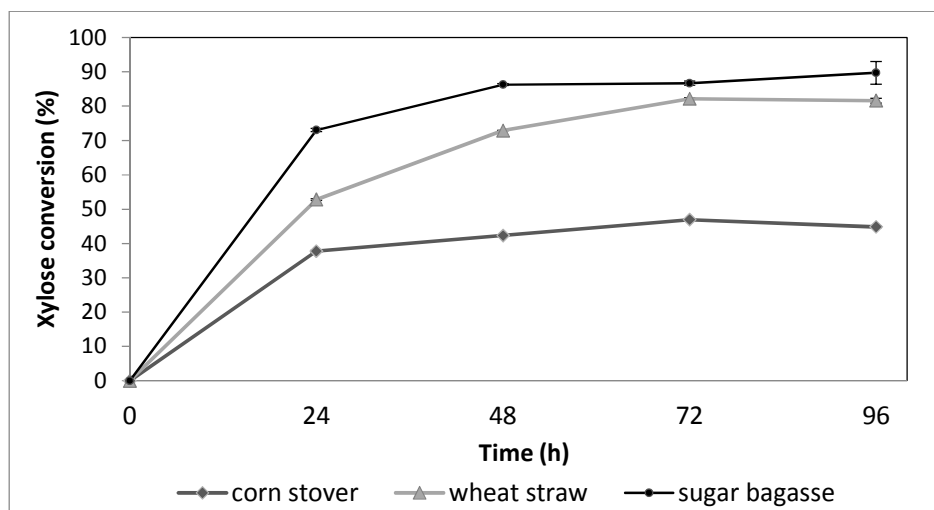
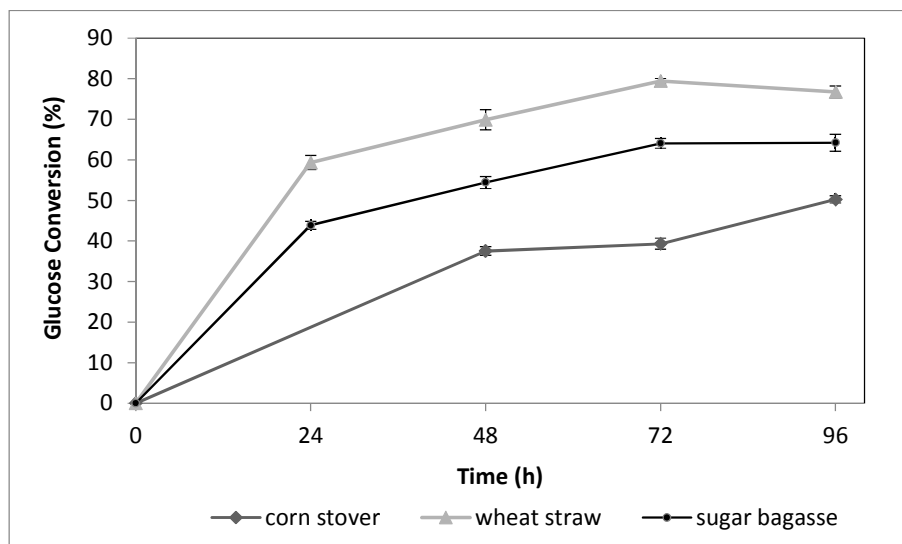


Figure 5. Hydrolysis of the hydrothermally pretreated biomasses: corn stover (rhombus), wheat straw (triangle) and sugarcane bagasse (circle). Experimental conditions: Cellic Ctec2 was used for the enzymatic hydrolysis with a loading of 10 FPU/g_{dry cellulose}, 96 hours at 48°C, 15% (w/w) dry matter load. The conversion is given as percentage based on the theoretical maximum glucose (upper plot) and xylose (bottom plot) yields from the pretreated materials.

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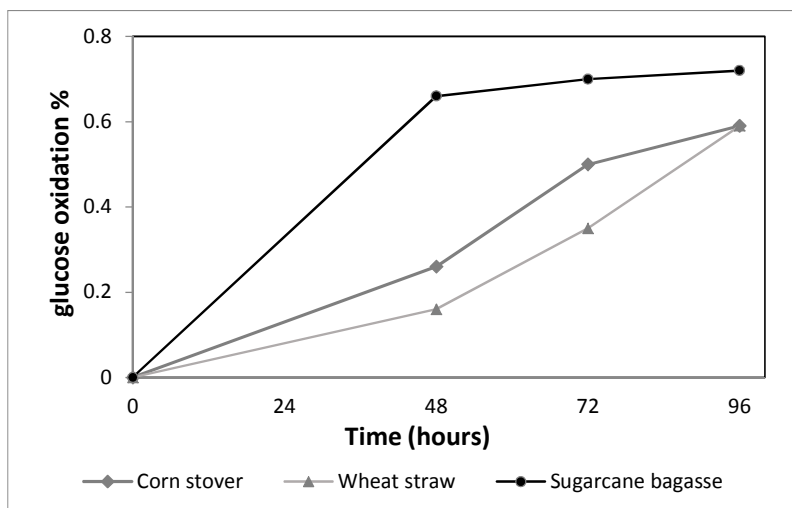


Figure 6. Glucose oxidation during the enzymatic hydrolysis with CellicCtec2 at 15%DM of hydrothermal-pretreated materials: corn stover (rhombus), wheat straw (triangle) and sugarcane bagasse (circle), same conditions as described in figure 4.

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505 **Table** 1
 506 Compositional analysis of Bagasse native biomass, and after hydrothermal, organosolv and alkaline
 507 pretreatment at 10% of solids loading.

	Glucan	Xylan	Mannan	Arabinan	Galactan	Klason Lignin	Ashes
Native	42.9±2.2	19.5±1.6	nd	2.6±0.2	0.5±0.1	23.0±0.3	3.0±0.6
Hydrothermal	45.2±0.9	2.4±0.1	nd	nd	nd	38.2 ± 0.4	4.9 ± 0.8
Organosolv	66.1 ± 2.3	14.5±0.1	0.3±0.1	0.4±0.1	nd	9.6 ± 1.0	3.2 ± 0.8
Alkaline	72.2 ± 3.1	10.2±1.7	nd	0.3±0.1	nd	7.8 ± 0.4	4.3 ± 0.6

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513 **Table** 2
 514 Compositional analysis of wheat straw, corn stover and bagasse of native biomass and after
 515 hydrothermal pretreatment at 20% solids loading.

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	Lignocellulosic fraction in % of dry matter						
	Glucan	Xylan	Mannan	Arabinan	Galactan	Klason Lignin	Ashes
Wheat Straw							
Native	46.0±2.2	21.6±1.8	1.5 ±0.2	2.9±0.2	1.2±0.2	20.6±1.0	2.3±0.3
Hydrothermal pretreat.	54.7±1.6	5.1±0.3	nd	nd	nd	34.1±0.9	3.3±0.6
Corn Stover							
Native	45.9±0.9	18.0±1.0	1.7±0.3	2.7±0.1	1.0±0.1	20.3±0.9	2.6±0.2
Hydrothermal pretreat.	50.5±0.2	2.2±0.1	nd	nd	nd	35.1±0.3	4.5±0.7
Sugarcane bagasse							
Native	42.9±2.2	19.5±1.6	nd	2.6±0.2	0.5±0.1	23.0±0.3	3.0±0.6
Hydrothermal pretreat.	45.2±2.1	4.1±0.1	nd	1.6±0.5	nd	39.2±0.6	5.1±0.8

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520 **Table** **3**
 521 Gluconic acid and cellulose conversion yield after 96 hours of enzymatic hydrolysis compared to
 522 lignin content of the residual fraction after each pretreatment.
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Solid fraction	Gluconic acid (g/kg)	Cellulose conversion yield (%)	Klason Lignin (% DM)
Bagasse			
Hydrothermal	0.38	86.6	38.2
Organosolv	0.07	60.2	9.6
Alkali	nd	93.8	7.8
Hydrothermal			
Corn stover	0.25	50.2	35.1
Wheat straw	0.40	76.8	34.1
Bagasse	0.46	64.2	39.2

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