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### ► To cite this version:

Adèle Lazuka, Cécile Roland, Abdellatif Barakat, Fabienne Guillon, Michael O'Donohue, et al.. Ecofriendly lignocellulose pretreatment to enhance the carboxylate production of a rumen-derived microbial consortium. *Bioresource Technology*, 2017, 236, pp.225-233. 10.1016/j.biortech.2017.03.083 . hal-01607636

**HAL Id: hal-01607636**

**<https://hal.science/hal-01607636v1>**

Submitted on 25 May 2020

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## Accepted Manuscript

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PII: S0960-8524(17)30355-3  
DOI: <http://dx.doi.org/10.1016/j.biortech.2017.03.083>  
Reference: BITE 17787

To appear in: *Bioresource Technology*

Received Date: 18 December 2016  
Revised Date: 11 March 2017  
Accepted Date: 13 March 2017

Please cite this article as: Lazuka, A., Roland, C., Barakat, A., Guillon, F., O'Donohue, M., Hernandez-Raquet, G., Ecofriendly lignocellulose pretreatment to enhance the carboxylate production of a rumen-derived microbial consortium, *Bioresource Technology* (2017), doi: <http://dx.doi.org/10.1016/j.biortech.2017.03.083>

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Comment citer ce document :

Lazuka, A., Roland, C., Barakat, A., Guillon, F., O'Donohue, M., Hernandez Raquet, G. (2017). Ecofriendly lignocellulose pretreatment to enhance the carboxylate production of a rumen-derived microbial consortium. *Bioresource Technology*, 236, 225-233. DOI : 10.1016/j.biortech.2017.03.083

1 **Ecofriendly lignocellulose pretreatment to enhance the carboxylate production of a**  
2 **rumen-derived microbial consortium.**

3 Adèle Lazuka<sup>1,2,3</sup>, Cécile Roland<sup>1,2,3</sup>, Abdellatif Barakat<sup>4</sup>, Fabienne Guillon<sup>5</sup>, Michael

4 O'Donohue<sup>1,2,3</sup>, Guillermina Hernandez-Raquet<sup>1,2,3\*</sup>

5 <sup>1)</sup> Université de Toulouse, INSA, UPS, LISBP, 135 Avenue de Rangueil, F-31077 Toulouse  
6 Cedex 4, France

7 <sup>2)</sup> INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse,  
8 France

9 <sup>3)</sup> CNRS, UMR5504, F-31400 Toulouse, France

10 <sup>4)</sup> UMR IATE, CIRAD, Montpellier SupAgro, INRA, Université de Montpellier, 34060,  
11 Montpellier, France.

12 <sup>5)</sup> Centre de Recherche Agro-alimentaire de Nantes, INRA, Rue de la Géraudière, 71627,  
13 44316, Nantes-03, France

14 "LAZUKA Adèle" lazuka@insa-toulouse.fr

15 "Cécile Roland" roland@insa-toulouse.fr

16 "BARAKAT Abdellatif" barakat@supagro.inra.fr

17 "GUILLON Fabienne" Fabienne.Guillon@nantes.inra.fr

18 "O'DONOHUE Michael" michael.odonohue@insa-toulouse.fr

19 \* Corresponding author: Guillermina Hernandez-Raquet, Laboratoire d'Ingénierie des  
20 Systèmes Biologiques et des Procédés UMR5504, UMR792, CNRS, INRA, INSA, 135  
21 avenue de Rangueil, 31077 Toulouse Cedex 04, France. Tel: + 33 (0) 561 55 99 77. Fax: + 33  
22 561 55 97 60. Email: hernandg@insa-toulouse.fr

23 **Abstract**

24 Innovative dry chemo- and chemo-mechanical pretreatments form an interesting approach for  
25 modifying the native physico-chemical composition of lignocellulose facilitating its microbial  
26 conversion to carboxylates. Here, the impact of four dry-pretreatment conditions on the  
27 microbial transformation of wheat straw was assessed: milling to 2 mm and 100  $\mu\text{m}$ , and  
28 NaOH chemical impregnation at high substrate concentrations combined with milling at 2  
29 mm and 100  $\mu\text{m}$ . Pretreatment effect was assessed in the light of substrate structure and  
30 composition, its impact on the acidogenic potential and the major enzyme activities of a  
31 rumen-derived microbial consortium RWS. Chemo-mechanical pretreatment strongly  
32 modified the substrate macroporosity. The highest carboxylate production rate was reached  
33 after dry chemo-mechanical treatment with NaOH at 100  $\mu\text{m}$ . A positive impact of the dry  
34 chemo-mechanical treatment on xylanase activity was observed also. These results underline  
35 that increasing substrate macroporosity by dry chemo-mechanical pretreatment had a positive  
36 impact on the microbial acidogenic potential.

37 **Keywords:** lignocellulose bioconversion; anaerobic microbial consortium; carboxylate  
38 production; enzymes; dry chemo-mechanical pretreatment.

39 **Introduction**

40 For over three decades intense R&D activity has focused on the development of industrial  
41 processes to convert lignocellulosic (LC) biomass into fuels, energy and value added  
42 chemicals, using various combinations of mechanical, chemical and bioconversion  
43 technologies (Kim and Dale, 2004). Among the manufacturing routes that have been  
44 investigated, the carboxylate platform is rather attractive (Agler et al., 2011). Operating under  
45 non-sterilized, non-aerated conditions, the carboxylate platform exploits the robustness and  
46 large enzymatic potential of mixed microbial communities for the transformation of LC  
47 biomass into carboxylates (Agler et al., 2011). In this platform, microbial communities  
48 hydrolyze the LC biomass producing soluble oligomeric and monomeric compounds that are  
49 further transformed into short chain carboxylates (or volatile fatty acids – VFA) by acidogenic  
50 and acetogenic microorganisms. In this respect, the carboxylate platform is related to the  
51 biogas platform, a two-stage anaerobic digestion system that produces carboxylates as  
52 intermediates that are then broken down by methanogenic archaea into methane. However, in  
53 the carboxylate platform, the products are considered to be building blocks for the production  
54 of added value chemicals and materials (e.g polyhydroxyalcanoate bioplastics), and liquid  
55 biofuels (Agler et al, 2011; Torella et al, 2013), rather than intermediates for low value  
56 renewable energy (i.e. bio-H<sub>2</sub>, and bio-CH<sub>4</sub>), which is inevitably in direct competition with  
57 low-priced fossil resources, such as shale gas.

58 Despite its attractive features, the carboxylate platform is nonetheless limited by the ability to  
59 extract fermentable components from LC biomass, which constitutes a highly recalcitrant raw  
60 material. Indeed, LC biomass is a composite material composed mainly of cellulose,  
61 hemicellulose and lignin. Together, these polymers and other minor components, form a  
62 chemically- and structurally-complex, three dimensional matrix that is particularly resistant to  
63 both abiotic and biotic aggression. The structural and protective role played by LC-based

64 structures in plants prevents their destruction by microorganisms and their enzymes (Mosier  
65 et al., 2005). To overcome LC biomass recalcitrance, various types of strategies involving a  
66 pretreatment step have been investigated (Alvira et al., 2010; Hendriks and Zeeman, 2009). In  
67 this case, chemical and/or physical processes are first used to increase the accessible surface  
68 area and porosity of LC biomass, thus rendering it more amenable to the subsequent action of  
69 biocatalytic agents (Hendriks and Zeeman, 2009; Mosier et al., 2005). Despite the  
70 development of numerous pretreatments, cost effectiveness still has to be achieved, notably  
71 by reducing non-specific effects and energy expenditure and maximizing beneficial effects for  
72 the subsequent biocatalytic processes.

73 The effect of different pretreatments on LC biomass has been extensively studied (Alvira et  
74 al., 2010; Hendriks & Zeeman, 2009; Mosier et al., 2005), revealing that each type of  
75 pretreatment is characterized by advantages and disadvantages. For example, chemical  
76 treatments often generate fermentation inhibitors while mechanical methods are usually  
77 associated with high energy expenditure (Barakat et al., 2013; Jönsson et al., 2013). However,  
78 the absolute need for pretreatment is cleverly illustrated by the truism “the only process more  
79 expensive than pretreatment is no pretreatment” (Wyman, 2007).

80 Among the vast array of LC biomass pretreatments that have been tested, alkaline  
81 pretreatment using sodium hydroxide is one of the most effective and attractive methods. It  
82 presents a high capacity for delignification, disrupts the biomass structure and increases  
83 porosity, thus making cellulose and hemicellulose more accessible, but with low sugar  
84 degradation (Kaar and Holtzaple, 2000; Mathew et al., 2011; Zhao et al., 2008). Some form  
85 of particle size reduction using mechanical methods also usually forms part of pretreatment  
86 technologies (Hendriks and Zeeman, 2009), because it is required to increase biomass surface  
87 area and can improve depolymerization and reduces residual waste (Barakat et al., 2014;  
88 Palmowski and Müller, 2000).

89 Recently, innovative procedures so-called ‘dry chemical’ (DC) and ‘dry chemo-mechanical’  
90 (DCM) pretreatments have been developed to reduce energy consumption (Miao et al., 2011,  
91 Barakat et al., 2013). DC pretreatment consists of moderate chemical treatment using alkali  
92 impregnation of LC biomass at high solids loadings, while dry-chemo-mechanical  
93 pretreatment describes a process in which dry chemical pretreatment is performed  
94 simultaneously with mechanical particle size reduction (Barakat et al., 2014).  
95 Advantageously, these pretreatments reduce the use of chemicals and energy demand,  
96 enhance polysaccharide saccharification when using enzyme cocktails (Barakat et al., 2014)  
97 and, thanks to high solids loadings, permit process intensification and reactor downsizing.  
98 To investigate whether DC and DCM pretreatments can be beneficial for the carboxylate  
99 platform, we have investigated the use of these technologies in combination with anaerobic  
100 conversion of wheat straw using a lignocellulolytic cow rumen-derived microbial consortium  
101 (RWS). The effect of the pretreatments on the subsequent microbial activity has been  
102 investigated, monitoring the kinetics of LC biomass conversion, carboxylate production and  
103 the dynamics of key enzymatic activities. Our findings reveal that DC pretreatment increased  
104 the initial VFA production rate and that this increase was most pronounced when DCM  
105 pretreatment was employed. This increase was accompanied by an increase in early phase  
106 xylanase activity, but CMCase activity was unchanged. Finally, our results indicate a  
107 correlation between lignocellulose macroporosity and its degradability by a hydrolytic  
108 microbial consortium.

## 109 **Materials and methods**

### 110 2.1. Wheat straw

111 A 20 kg batch of wheat straw (Koreli variety grown on an INRA-owned experimental farm,  
112 Boissy-le-Repos, France) was harvested (in August 2011), milled to 2 mm using a knife mill

113 (Retsch SM 100, Germany) and stored at room temperature (20-25°C) until use as the for all  
114 the experiments, hereafter referred to as 2 mm wheat straw (biomass A).

## 115 2.2. Dry chemical pretreatment

116 Sodium hydroxide (NaOH) was dissolved in distilled water (5g in 20 mL). Wheat straw at 2  
117 mm (100g) was impregnated during 5h at ambient conditions (25 °C) with this alkaline  
118 solution using a pulverizing system (5 g of NaOH per 100 g of wheat straw) according to the  
119 procedure described previously (Barakat et al., 20014). The chemically treated wheat straw-  
120 2mm was dried at 105 °C (12h) resulting in a final moisture content of 8-10% (w/w) and  
121 designated biomass B.

## 122 2.3. Mechanical treatment

123 Biomass A and B were comminuted using an impact mill operating at ambient temperature  
124 and 18,000 rpm (Hosokawa-alpine, type UPZ, Augsburg, Germany). Fine particulate fractions  
125 were collected using a 100 µm mesh (the material was milled until it passed through the grid)  
126 and designated as biomass C and D, respectively.

## 127 2.4. Substrate characterization

### 128 2.4.1 Substrate composition

129 For compositional analysis wheat straw (40 mg) was submitted to the sulfuric acid hydrolysis  
130 method described by de Souza et al. (2013), analyzing monosaccharides (glucose, xylose and  
131 arabinose) by high-performance liquid chromatography (HPLC) using an Ultimate 3000  
132 Dionex separation system equipped with a BioRad Aminex HPX 87H affinity column and a  
133 refractive index detector (Thermo Scientific). The protocol used for HPLC analysis was that  
134 described elsewhere (Monlau et al., 2012).

135 Lyophilized wheat straw samples were also analyzed by Fourier transform infrared  
136 spectroscopy (FT-IR) analysis using an attenuated total reflection (ATR) Nicolet 6700 FT-IR  
137 spectrometer (Thermo Fisher), equipped with a deuterated-triglycine- sulfate (DTGS)



138 detector, following the procedure described by Lazuka et al. (2015). For FT-IR spectral  
139 analysis, the peak ratio 1512:1375  $\text{cm}^{-1}$  was considered representative of the  
140 lignin:holocellulose ratio, while peaks at 1430  $\text{cm}^{-1}$  and 898  $\text{cm}^{-1}$  were attributed to crystalline  
141 and amorphous cellulose respectively (Monlau et al., 2012), and the ratio of these peaks was  
142 considered to be the lateral order index (LOI), which represents the ratio of  
143 crystalline:amorphous cellulose.

#### 144 2.4.2 Determination of particle size and energy consumption

145 Particle size was analyzed by laser granulometry using a Mastersizer 2000 (Malvern  
146 Instruments, Orsay, France).

147 The energy consumed during milling was determined in triplicates using a watt meter  
148 following a previously described procedure (Barakat et al., 2014).

#### 149 2.4.3 Determination of macroporosity by suction pressure

150 Macroporosity of the four treated wheat straw samples (A-D) was determined by measuring  
151 the water-absorption kinetics at different osmotic pressures (Robertson and Eastwood,  
152 1981). The water retention capacity (macroporosity profile) was obtained by dialysis of  
153 hydrated wheat straw samples using three solutions of polyethylene glycol (PEG) (MW  
154 10,000) at 10, 75 and 100  $\text{g.L}^{-1}$  inducing a known suction pressure (0.009, 0.112 and  
155 0.206 MPa, respectively). The water retained by the fiber matrix was related to pore size  
156 through suction pressure and surface tension according to equation 1:

$$157 \quad D = \frac{4S}{\Delta P} \text{ (Eq. 1)}$$

158 where D (mm) is the pore diameter, S the solute (water) surface tension,  $\Delta P$  the suction  
159 pressure (MPa) used to measure the ratio water held:pore volume.

160 Water absorption at different suction pressures (used to explore different pore diameters) was  
161 determined by presoaking wheat straw samples overnight at 4°C, and transferring the  
162 equivalent of 0.2-0.3 g dry wheat straw as hydrated fibers into dialysis bags (6-8 kDa cutoff,

163 32 mm diameter, Visking R dialysis bag, PolyLabo, Strasbourg, France). The dialysis bags  
164 were then sealed and placed in a 100 mL PEG solution and shaken overnight at 37°C  
165 (100 cycles.min<sup>-1</sup>). The content of the dialysis bags was weighed accurately then dried  
166 overnight (100°C) to determine dry weight and water content. Each wheat straw sample at  
167 each PEG concentration was tested in triplicate. Results (g water g<sup>-1</sup> dry sample) represented  
168 the difference between the total pore volume (estimated at 10 g/L PEG) and the volume of 1  
169 μm-diameter pores (estimated at 100 g/L PEG), which constitute the pore volume with  
170 diameter >1μm likely to be accessible to bacteria. After presoaking, the liquid fraction was  
171 collected and the neutral sugar content was evaluated using a Skalar autoanalyzer (Skalar,  
172 Breda, Netherlands), employing the sulfuric orcinol method (Tollier and Robin, 1979).

#### 173 2.4.4 Saccharification with commercial enzymes

174 Saccharification of the four treated wheat straw samples, A to D, was evaluated using a  
175 commercial enzymatic mixture (Celluclast 1.5L Novozyme). Dry wheat straw (1 %, w/w) was  
176 incubated for 72h at 50°C with 20 FPU.g<sup>-1</sup> dw cellulase supplemented with 81 U.mL<sup>-1</sup> β-  
177 glucosidase (Novozyme 188) in 50 mM sodium acetate buffer (pH 5.0) containing 0.5 g.L<sup>-1</sup>  
178 sodium azide. Following centrifugation (7197 g, 10 min at 4°C), the reducing sugar content in  
179 the supernatant was quantified using the di-nitro-salicylic acid method (DNS).

#### 180 2.5. Lignocellulose bioconversion by RWS in anaerobic reactors

181 Microbial bioconversion of LC substrate by a cow-rumen derived consortium (RWS) that  
182 displays a good ability to degrade wheat straw was carried out in anaerobic batch reactors (2L  
183 BIostat<sup>®</sup> A+, Sartorius, Germany). Bioreactors containing wheat straw samples (A to D) as  
184 the sole carbon source (20g.L<sup>-1</sup>) suspended in mineral medium (Lazuka et al., 2015). The  
185 bioreactors were operated under agitation (300 rpm) at a mesophilic temperature (35°C) and  
186 pH (6.15), which was adjusted by the appropriate addition of H<sub>3</sub>PO<sub>4</sub> at the beginning of the  
187 experiment (before inoculation) and thereafter regulated by the automated addition of 1 M

188 NaOH, according to the protocol established by Lazuka et al., (2015). Each microbial  
189 bioconversion reactor was performed in biological duplicates over a 15-day period. Wheat  
190 straw removal, residual substrate compositional analysis, total organic carbon (TOC), VFA  
191 production and enzymatic activities were monitored throughout the incubation period.

## 192 2.6. Chemical analyses

193 To quantify total solids (TS) 10 mL samples were removed, centrifuged (7197 x g, 10 min,  
194 4°C), rinsed twice with distilled water and dried 24h at 105°C. For the mineral fraction (MF),  
195 mineralization was performed at 500°C for 2h, and volatile solids (VS) were estimated from  
196 the difference between TS and MF. VS degradation was expressed as weight/weight  
197 percentages.

198 The composition of the residual substrate in the reactor was characterized as described above  
199 for substrate characterization (section 2.4.1).

200 VFA production was monitored using a Varian 3900 gas chromatograph as described by  
201 Cavaillé et al. (2013). The total organic carbon (TOC) content of the liquid fraction was  
202 measured using a TOC analyzer (TOC-V<sub>CSN</sub>, Shimadzu Co., Japan). Gas composition was  
203 analyzed using an HP 5890 gas chromatograph equipped with a conductivity detector and a  
204 HAYSEP D column. All chemical measurements were done in technical duplicates.

205 All the macro-kinetic parameters are expressed as average values obtained from duplicate  
206 biological reactors (biological duplicates). Smoothed data and derivatives were obtained after  
207 polynomial regression on the raw data. The statistical significance of differences between the  
208 types of pretreatment was evaluated by one-way ANOVA (P<0.05) for characterization  
209 parameters (LOI, macroporosity, saccharification, composition).

## 210 2.6. Enzyme activity assays

211 To measure enzyme activity, triplicate samples (5 mL) were withdrawn at regular intervals  
212 and centrifuged (7197 x g, 10 min, 4°C) yielding a supernatant and a sonicated solid pellet

213 fraction as described by Lazuka et al. (2015). These two fractions were considered to be  
214 representative of extracellular (supernatant), and sum of intracellular and cell-bound  
215 (sonicated pellet) localizations respectively. For each reactor and each sampling time, end-  
216 point enzymatic activities were measured in technical duplicates in both the extracellular and  
217 the intracellular and cell-bound fractions. All enzymatic activities were expressed as averages  
218 of the two values obtained from the duplicate reactors.

219 Xylanase and endoglucanase (CMCase) activity were measured using 1% w/v xylan  
220 beechwood (Sigma) and 1% w/v carboxymethyl cellulose (CMC) (Sigma) respectively  
221 according to the previously described protocol (Lazuka et al., 2015). One unit of CMCase or 1  
222 unit of xylanase activity (UA, unit of activity) was defined as the amount of enzyme that  
223 produces 1  $\mu\text{mol}$  of reducing sugars per minute.

## 224 **Results and discussion**

225 The impact of dry pretreatment on the kinetics of wheat straw degradation and carboxylate  
226 production by a microbial consortium RWS was assessed using four different pretreated  
227 wheat straws. Samples A and C were milled to 2 mm, B and D to 100  $\mu\text{m}$ , and C and D were  
228 submitted to 5% (w/w) NaOH impregnation at high LC solids. These conditions facilitated the  
229 assessment of a possible synergy between dry milling and chemical pretreatment. Moreover,  
230 it is important to underline that D was first chemically pretreated and then milled, because this  
231 sequence is expected to reduce the energy demand associated with milling and increase  
232 biomass component extractability (Barakat et al., 2014).

### 233 3.1. Characterization of the pretreated wheat straw

234 The energy consumption associated with the production of A was  $223.3 \text{ kJ.kg}^{-1}$ , while the  
235 production of B required 2.8 times more energy (Table1). The energy demand associated with  
236 the production of C was the same as that for A, which is logical since chemical impregnation  
237 was carried out after milling. However, energy consumption associated with the production of

238 D (100  $\mu\text{m}$ ) was almost half that required to produce B, which clearly demonstrates that prior  
239 chemical pretreatment was beneficial. It is likely that during NaOH impregnation, the  
240 lignocellulosic matrix is weakened and disintegrated into finer components, thus increasing  
241 overall energetic efficiency (Kaar and Holtzapfle, 2000; Mathew et al., 2011; Zhao et al.,  
242 2008; Barakat et al., 2014).

243 In order to identify the biomass features that affect microbial bioconversion, samples A to D  
244 were characterized with regard to four parameters: (i) the LOI which gives insight into  
245 substrate crystallinity; (ii) the 1  $\mu\text{m}$ -pore volume or macroporosity, considered as the volume  
246 accessible to bacteria (Guillon et al., 1998); (iii) biochemical composition in terms of  
247 cellulose, hemicellulose and lignin (C, H, L) and (iv) the enzymatic saccharification under  
248 standard conditions. Figure 1A shows that particle size had no significant impact on wheat  
249 straw crystallinity (LOI), irrespective of the substrate pretreatment. However, chemical  
250 pretreatment decreased LOI from  $0.766 \pm 0.079$  (mean of A and B substrates, without  
251 chemical pretreatment) to  $0.572 \pm 0.05$  (mean of C and D substrates, with chemical  
252 pretreatment), irrespective of particle size. Regarding the impact of NaOH pretreatment on  
253 cellulose crystallinity, previous studies have provided contradictory results. For instance,  
254 when applying DCM, soda pretreatment to wheat straw at a particle size below 60  $\mu\text{m}$ ,  
255 Barakat et al. (2014) obtained an 11% increase in crystallinity. This result was attributed to  
256 the solubilization of amorphous polymers and not to an actual increase in crystallinity. In fact,  
257 several parameters can impact cellulose crystallinity, notably the severity of a given  
258 treatment. Bali et al. (2015) showed that the cellulose crystallinity of alkali-pretreated  
259 *Populus* varied as function of severity. The authors showed that shorter pretreatments (lower  
260 severity) reduced cellulose crystallinity, probably through the actual disruption of the  
261 cellulose crystalline structure. However, in the same study, it was reported that crystallinity  
262 increased when the pretreatment was prolonged, this observation being attributed to the

263 solubilization of the amorphous cellulose component. The results obtained in the present  
264 study are consistent with the hypothesis that NaOH pretreatment causes the cellulose to swell  
265 and decreases cellulose crystallinity, as described by Agbor et al. (2011), suggesting that the  
266 severity of the applied treatment did not induce any rise in crystallinity.

267 The comparison of the macroporosity of samples A to D (Fig. 1B) revealed a negative  
268 correlation between macroporosity with fine milling (100  $\mu\text{m}$ ) in the absence of chemical  
269 pretreatment. For sample B, macroporosity was  $1.5 \pm 0.3 \text{ g.g}^{-1}$ , which is lower than that  
270 measured for A,  $2.9 \pm 0.4 \text{ g.g}^{-1}$ . On the other hand, soda pretreatment had a positive effect on  
271 macroporosity, reaching  $4.4 \pm 0.9 \text{ g.g}^{-1}$  and  $4.6 \pm 0.6 \text{ g.g}^{-1}$  for samples C and D, respectively.

272 These results suggest that milling to 100 $\mu\text{m}$  (B) actually reduced accessibility to bacteria,  
273 whereas chemical pretreatment (i.e. samples C and D) increased it. In early studies on  
274 resistant starch dietary fiber, a correlation between macroporosity (generally in the range 1  
275 and 10  $\text{g.g}^{-1}$ ) and substrate fermentability was described (Guillon et al., 1998; Robertson et al.,  
276 2000). Consistently, in the present study, macroporosity was shown to be correlated to the  
277 quantity of fermentable sugars formed by pretreatment, with samples C and D displaying the  
278 highest amounts of soluble neutral sugars ( $1.705 \pm 0.003 \text{ mg.L}^{-1}$  and  $2.394 \pm 0.003 \text{ mg.L}^{-1}$ ,  
279 respectively), while non-chemically treated substrates displayed lower levels ( $0.948 \pm 0.004$   
280  $\text{mg.L}^{-1}$  and  $0.682 \pm 0.003 \text{ mg.L}^{-1}$  for substrates A and B, respectively).

281 The biochemical analysis of the four wheat straw samples revealed no significant  
282 compositional changes after pretreatment, with the average composition being (in % w/w)  
283  $43.6 \pm 3.4 \%$  cellulose,  $23.2 \pm 1.4 \%$  hemicellulose and  $19.7 \pm 1.7 \%$  lignin (Fig. 1C), values  
284 that are similar to those measured in the raw substrate. This compositional stability can be  
285 explained by the fact that no extraction was performed, meaning that solubilized components  
286 remained in the sample, and also indicates that very little biomass loss occurred, consistent  
287 with the findings of Barakat et al. (2014). Carbon loss is often associated with alkaline

288 pretreatments and occurs in the form of carbon dioxide release associated with peeling  
289 reactions. This phenomenon is highly correlated with the pretreatment severity factor and  
290 substrate recalcitrance (Hendriks and Zeeman, 2009; Karp et al., 2015). Therefore, the results  
291 obtained on the biochemical composition of LC before and after pretreatment indicate that the  
292 pretreatments used in this study are relatively mild, ensuring good mass conservation.  
293 Enzymatic saccharification of the four pretreated materials (Fig. 1D) did not reveal any clear  
294 correlations between particle size and substrate accessibility to enzyme hydrolysis. However,  
295 soda treatment clearly enhanced the enzymatic release of reducing sugars, since on the dry  
296 chemo-mechanical pretreatment (D) the release of soluble reducing sugars was 2-fold higher  
297 when compared to A and B (no soda treatment) and approximately 1.2-fold higher than that  
298 obtained with C, consistent with previous findings (Barakat et al. 2014).

299 In conclusion, assuming that cellulose crystallinity, the aptitude towards enzymatic  
300 saccharification and macroporosity are reliable indicators of pretreated substrate accessibility  
301 for microbial bioconversion, it appears reasonable to suggest that samples C and D should be  
302 readily amenable to bioconversion by suitable microbial consortia. In this regard, considering  
303 samples A and B, which had not been submitted to soda treatment, the only clear difference  
304 concerned their macroporosity (lower for B). This makes prediction more difficult, but  
305 suggests that B (100  $\mu\text{m}$  particle size) might be less amenable to microbial bioconversion.

### 306 3.2. Fermentation of the pretreated wheat straw by the microbial consortium RWS

#### 307 3.2.1 Wheat straw degradation and VFA production by RWS

308 Wheat straw A to D were anaerobically fermented by the RWS consortium. The biochemical  
309 analysis of residual wheat straw revealed that only the holocellulose fraction was degraded,  
310 irrespective of the pretreatment applied, meaning that the lignin concentration was unchanged  
311 throughout the experiment (average lignin concentration of  $4.35 \pm 0.55 \text{ g.L}^{-1}$  for all reactors  
312 and sampling times, representing  $21.7 \pm 2.75 \%$  w/w of the initial substrates; data not show).

313 Therefore, subsequently wheat straw degradation was expressed more simply as the  
314 percentage of holocellulose-related carbon (in moles) removal (expressed as percentage of the  
315 initial holocellulose-related carbon content, %  $iCmol_{Holo}$ ).

316 In this respect, the results obtained for holocellulose removal (%  $iCmol_{Holo}$ ) on samples A, C  
317 and D at the end of the experiment were similar with removal values of  $64.2 \pm 1.0$ ,  $68.8 \pm 3.4$   
318 and  $58.2 \pm 5.4$  %, respectively (Fig. 2A). As predicted by the macroporosity analysis, despite  
319 the larger surface area available in B, this sample was less apt for bioconversion by the RWS  
320 consortium ( $37.0 \pm 1.1$  % of holocellulose removed at the end of the experiment). Likewise,  
321 measurement of specific VFA production (expressed as moles of carbon of VFA produced per  
322 mole of carbon of the initial holocellulose,  $Cmol_{VFA} \cdot iCmol_{Holo}^{-1}$ ) revealed a similar trend.  
323 Maximal VFA production levels (approximately  $0.45 Cmol_{VFA} \cdot iCmol_{Holo}^{-1}$ ) were obtained  
324 with A, C and D at the end of the incubation, whereas significantly lower VFA production  
325 was recorded for B ( $0.31 \pm 0.02 Cmol_{VFA} \cdot iCmol_{Holo}^{-1}$ ) (Fig. 2B). Accordingly, the  
326 holocellulose degradation and VFA production rates (Fig. 2C and D) displayed similar trend  
327 for each substrate but the maximal value and time needed to reach such maximum value  
328 varied importantly in function of the pretreatment applied. Comparing these specific rates for  
329 the different pretreated substrates, it is noteworthy that the highest rates for holocellulose  
330 degradation and VFA production were obtained after DCM pretreatment (sample D), DC  
331 pretreatment being the next best option (sample C). Indeed, with these samples, holocellulose  
332 degradation reached a maximum at an earlier stage in the experiment (i.e. 12 and 14.6 %  
333  $iCmol_{Holo}^{-1} \cdot day^{-1}$  after 3.5 and 2.8 days for C and D, respectively) whereas maximum  
334 degradation rate was lower and occurred later for A and even more for B (i.e. 7.6 and 4%  
335  $iCmol_{Holo}^{-1} \cdot day^{-1}$  after 4.8 and 5.2 days for A and B, respectively). Likewise, the highest VFA  
336 production rates were also measured for C and D ( $0.066$  and  $0.094 Cmol_{VFA} \cdot iCmol_{Holo}^{-1} \cdot day^{-1}$ ,  
337 respectively), with maximum production being reached after 2.5 days. In contrast, with



338 samples A and B, VFA production peaked after 5 days, with rates reaching only 0.045 and  
339  $0.028 \text{ Cmol}_{\text{VFA}} \cdot \text{iCmol}^{-1}_{\text{Holo}} \cdot \text{day}^{-1}$ , respectively. Overall, these results underline the beneficial  
340 effect of the soda treatment, particularly when it was coupled to mechanical milling to  
341  $100 \mu\text{m}$ .

342 Compared to the fermentation of 2mm wheat straw (sample A), the VFA,  $\text{CO}_2$  and  $\text{H}_2$  yields  
343 increased when chemically-pretreated substrates were used (Table 2). The highest yields were  
344 systematically obtained after DCM pretreatment (sample D), while the fermentation of  
345 samples B and C procured intermediate values.

346 The composition of the carboxylates produced at the end of the experiment (Table 2), was for  
347 all samples mostly acetic, propionic and butyric acids, with minor quantities of valeric and  
348 hexanoic acids. Quantification of TOC level in the supernatant corresponded to the VFA  
349 concentration (data not shown) indicated that no other metabolites were produced. The main  
350 difference was observed for chemically-treated samples C and D. The fermentation of these  
351 substrates procured a higher amount of butyric acid, which was associated with a lower level  
352 of acetic acid. It is known that members of the *Clostridia* genus are able to ferment hexoses  
353 into acetate, and xylose into butyrate and dihydrogen (Jaros et al., 2013; Liu and Yang, 2006;  
354 Raganati et al., 2014). Furthermore, it is known that alkaline pretreatment efficiently removes  
355 hemicellulose from LC biomass (Hendriks and Zeeman, 2009). Since *Clostridia* is one of the  
356 main microbial groups found in RWS (Lazuka et al., 2015), it is possible that a metabolic shift  
357 from acetate (from glucose) to butyrate (from xylose) pathway in this microbial group  
358 induced a higher proportion of butyrate in soda treatment conditions. Nevertheless, to confirm  
359 such a hypothesis, further characterization of the microbial community's composition during  
360 incubation time will be needed.

361 Regarding particle size, it is often assumed that size reduction facilitates substrate  
362 accessibility to enzymes and microorganisms. However, herein we show that extremely fine

363 milling (i.e. to 100 $\mu$ m) has an adverse effect on biodegradation and VFA production. This  
364 result could be attributed to the liberation of inhibitory compounds or to an increase in  
365 recalcitrance due to pretreatment severity. However, as discussed above, LOI results revealed  
366 that crystallinity did not increase. Moreover, it is unlikely that mechanical milling would  
367 generate inhibitors of the type associated with chemical pretreatments (Kumar et al., 2009). In  
368 contrast, measurements showed that milling decreased macroporosity level, a phenomenon  
369 that was no doubt responsible for the lowered fiber degradation and thus fermentability of  
370 sample B.

371 Previous studies comparing the degradability of lignocellulosic substrates showed a positive  
372 effect of pretreatment on the degradation performance of different microbial consortia. For  
373 instance, Wongwilaiwalin et al. (2013) reported an increase in the degradation of alkali-  
374 pretreated rice straw, sugarcane bagasse and corn stover by two selected thermophilic  
375 consortia BGC-1 and CRC-1 (50°C). Compared to the raw substrate, Guo et al., (2011) also  
376 reported a 3.3-fold increase in the degradation of NaOH-pretreated rice straw by the MC1  
377 thermophilic consortium (50°C) and the production of 2.72 g.L<sup>-1</sup> organic acids after 3 days.  
378 Similarly, Zhao et al. (2014) reported 75% degradation of alkali-pretreated rice straw by the  
379 BMC-9 consortium, which accumulated a maximum of 3.3 mg VFA.L<sup>-1</sup> after 12 days  
380 incubation at 60°C. Although it is difficult to compare the bioconversion efficiency reported  
381 in studies applying different pretreatment protocols (different NaOH concentration, solids  
382 loadings, incubation period) and culture conditions (inocula, culture media, incubation  
383 temperature), it is clear that alkali-pretreatment enhances the accessibility of lignocellulose  
384 towards bacteria. In this respect, the results reported herein are consistent with previous data,  
385 although VFA production levels were much higher, probably because of the strict anaerobic  
386 conditions that were applied (static or anoxic conditions were used in previous studies).  
387 Indeed, strict anaerobic conditions prevent microbial VFA consumption.

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388 3.2.2. Enzymatic activity profiles and biomass composition during wheat straw degradation  
389 by RWS

390 Xylanase and CMCase activities were measured regularly over the 15-day incubation period  
391 (Figure 3). For xylanase activity, two distinct profiles can be distinguished. Fermentation of  
392 the dry milled samples, A and B, procured maximum activities of  $0.61 \pm 0.14 \text{ UA.mL}^{-1}$  and  
393  $0.81 \pm 0.11 \text{ UA.mL}^{-1}$ , respectively, after 5 and 7 days of incubation. Thereafter, the activities  
394 remained stable. In contrast, the dry milled, chemically-pretreated samples, C and D, procured  
395 maximum enzyme activities after just 3 days of incubation, with the two activities attaining  
396 approximately  $1.2 \text{ UA.mL}^{-1}$ . Thereafter, the activities decreased slightly (Figure 3A).

397 Correlating this data with the dynamics of hemicellulose degradation (Supplementary data  
398 and Figure 3C) revealed that the highest hemicellulose degradation rates (Figure 3C)  
399 coincided with increasing xylanase activity. For chemically-treated samples, measurements  
400 performed at day 1 revealed that xylanase levels were quite low, although hemicellulose  
401 degradation had already started, suggesting that the chemical pretreatment had already  
402 degraded the xylans, producing xylo-oligosaccharides. This observation is consistent with the  
403 release of free sugars observed after 24h presoaking during macroporosity measurements.

404 Comparing xylanase activity profiles and hemicellulose degradation with those of CMCase  
405 and cellulose degradation revealed quite different trends (Figure 3B and 3D). CMCase  
406 activity remained low (approximately  $0.05 \text{ UA.mL}^{-1}$ ) in all of the experiments, irrespective of  
407 pretreatment. Moreover, no reliable correlations between CMCase activity and cellulose  
408 degradation rates were evidenced, CMCase activity did appear to reach maximum levels  
409 earlier (around day 4) in the case of samples C and D, compared to A and B (peaking after  
410 day 5). Correspondingly, cellulose degradation rates were also reached earlier (around day 3)  
411 for samples C and D ( $12.7$  and  $16.0 \text{ %mCmol.day}^{-1}$  respectively), whereas maximum rates in  
412 the case of A and B ( $7.5$  and  $5 \text{ %mCmol.day}^{-1}$  respectively) were achieved latter (about 5

413 days). Taken together, our results indicate that hemicellulose and cellulose were degraded  
414 simultaneously. However, in the case of the chemically-pretreated samples the hemicellulose  
415 was probably solubilized and partially degraded, meaning that xylose was more readily  
416 available at early stage for bioconversion

417 To understand whether the different CMC<sub>ase</sub> activity profiles reflect differential attack of the  
418 amorphous and crystalline fractions of cellulose, the evolution of crystallinity was  
419 investigated throughout the bioconversion of the four wheat straw samples. Figure 4 shows  
420 that crystallinity (LOI) evolved differently for each substrate. Since LOI is the ratio of  
421 crystalline versus amorphous cellulose, increases in LOI correlate with increased degradation  
422 of amorphous cellulose relative to the crystalline fraction. The microbial bioconversion of  
423 sample A was characterized by increasing LOI (from  $0.73 \pm 0.04$  to  $0.98 \pm 0.11$ ), whereas  
424 bioconversion sample B produced an opposite trend (decreased LOI, Fig. 4). However, in the  
425 chemically-pretreated samples (C and D) LOI remained mostly unchanged, suggesting that  
426 amorphous cellulose was preferentially degraded during substrate A fermentation, while the  
427 attack of the crystalline fraction of substrate B appeared to be facilitated by milling  
428 pretreatment. It is known that endocellulases mainly act on amorphous cellulose regions,  
429 while exocellulases act on crystalline regions (Lynd et al., 2002). Hence, simply measuring  
430 CMC<sub>ase</sub> did not provide a complete view of cellulose degradation during RWS-mediated  
431 bioconversion. Nevertheless, our results indicate that RWS did possess a full cellulose  
432 arsenal, allowing it to degrade both amorphous and crystalline cellulose. Caution should  
433 nonetheless apply, because determining LOI using FT-IR only procures surface-related  
434 information, thus it is unclear whether FT-IR provides information on intra-fiber crystallinity.

435 Further analyses at the metaproteomics will no doubt be needed to demonstrate whether RWS  
436 can adapt its enzymatic pool to a substrate structure.

437 **Conclusion**

438 Dry milling combined with NaOH pretreatment enhanced wheat straw enzymatic hydrolysis  
439 and bioconversion using a microbial consortium RWS, leading to increased xylanase activity  
440 and VFA production rate. Compared to raw wheat straw, the optimal pretreatment was dry  
441 milling to 100  $\mu\text{m}$ , combined to alkaline impregnation, which procured a greater than two-  
442 fold increase in VFA production rate. Acetic, propionic and butyric acids were the main VFA  
443 produced by RWS, irrespective of the pretreatment method. An increase in butyric acid  
444 production was observed with chemically-pretreated substrates. Macroporosity appeared as  
445 the parameter that best predicts the biological acidogenic potential of RWS.

446 **Acknowledgments.** This research was supported by the French National Agency for Energy  
447 and the Environment (ADEME), the CEPIA department of INRA and the PROBIOS project  
448 funded by the Carnot Institute 3BCAR. We would like to acknowledge M. Abadie's important  
449 contribution in enzymatic analysis. M. Bounouba and E. Mangelle are also thanked for their  
450 technical support.

451

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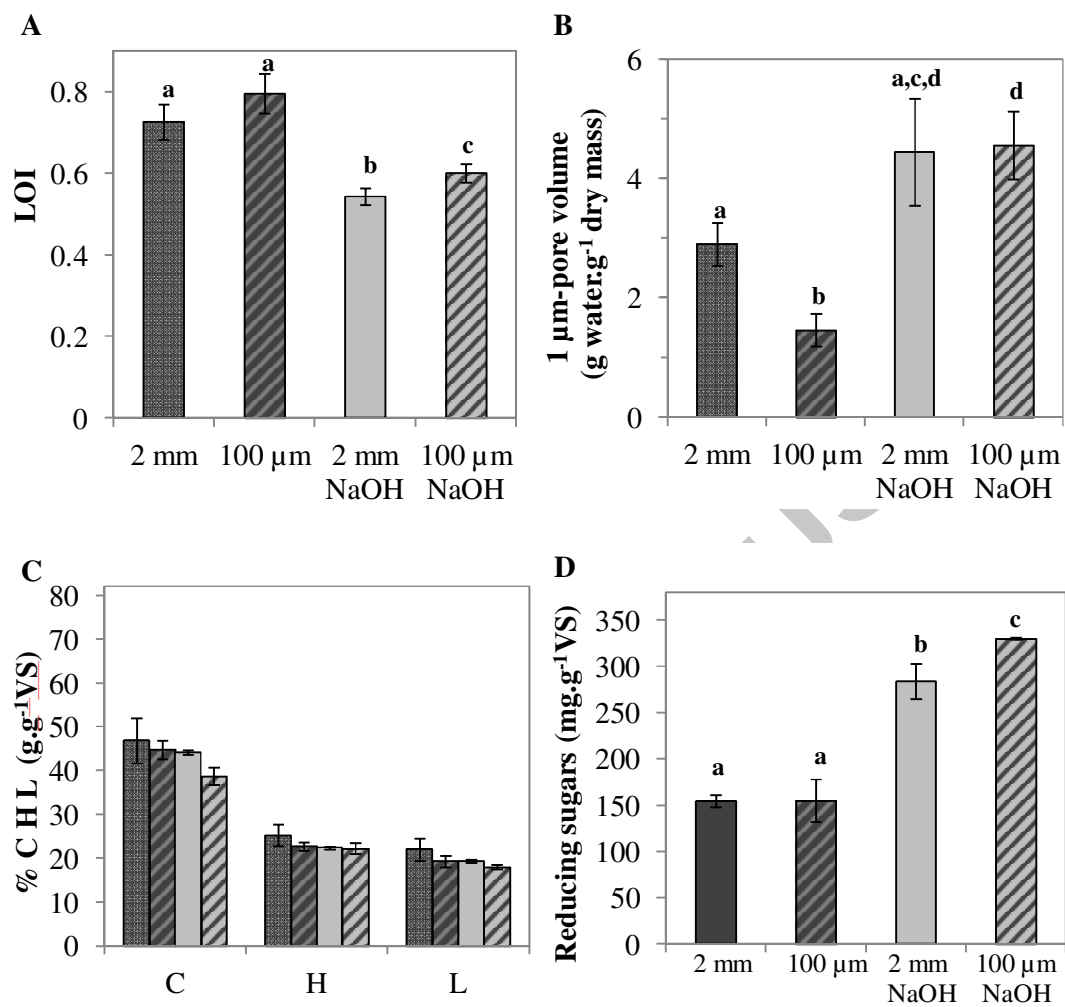
543 **Figure captions**

544 **Figure 1.** Characterization of the pretreated wheat straw substrates. (A) Lateral order index  
545 (LOI). (B) Macroporosity estimated at 1  $\mu\text{m}$ -pore volume determined as the difference  
546 between total and inferior-to-1  $\mu\text{m}$  pore volume. (C) Biochemical composition of wheat straw  
547 (Cellulose -C, hemicellulose -H and lignin -L). The types of pretreatment are indicated by the  
548 bar colors as in A. (D) Reducing sugars liberated from wheat straw by the saccharification test  
549 performed under standard conditions (Cellulase, 20 FPU.L<sup>-1</sup>,  $\beta$ -glucosidase 81U.mL<sup>-1</sup>, 72h,  
550 50°C, pH 5). Error bars indicate the standard deviation of the mean of three technical  
551 replicates. Different lowercase letters indicate differences within a treatment (one-way  
552 ANOVA, P<0.05).

553 **Figure 2.** Wheat straw transformation kinetics by RWS incubated with the four pretreated  
554 wheat straws. (A) Holocellulose degradation and (C) Holocellulose degradation rate. (B)  
555 Specific VFA production and (D) Specific VFA production rate. Experimental points and  
556 smoothed curves are presented with error bars corresponding to the standard deviation of the  
557 mean of two biological replicates.

558 **Figure 3.** Kinetics of enzymatic activity and polysaccharide degradation throughout the  
559 fermentation of pretreated-wheat straw by a RWS consortium. Xylanase (A) and CMCase (B)  
560 activity and cellulose (C) and hemicellulose (D) degradation rates. Experimental points and  
561 smoothed curves are presented with error bars corresponding to the standard deviation of two  
562 biological replicates.

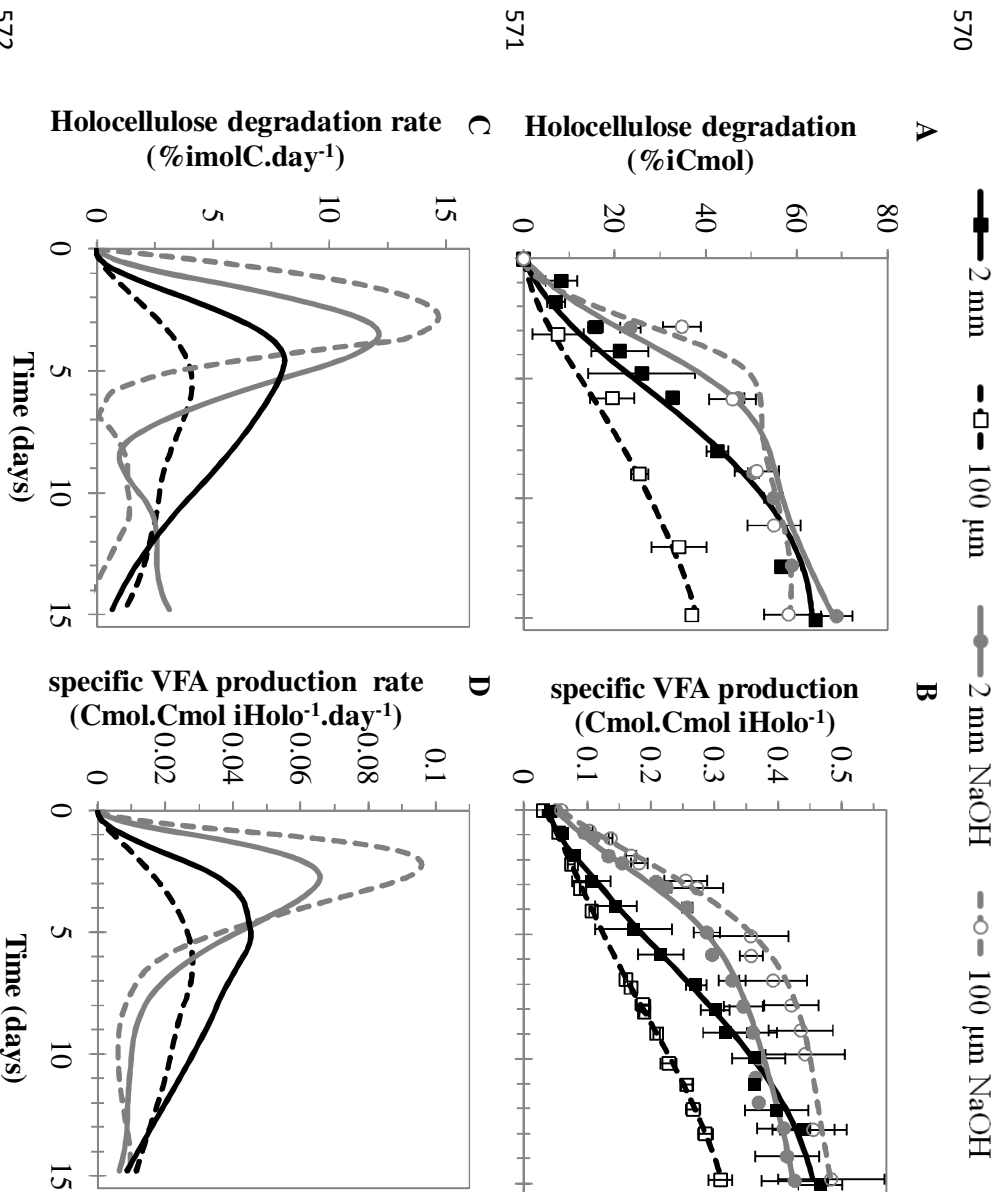
563 **Figure 4.** Lateral order index (LOI) profiles during fermentation of pretreated wheat straw by  
564 a RWS consortium. Error bars correspond to the standard deviation of 2 biological duplicates.

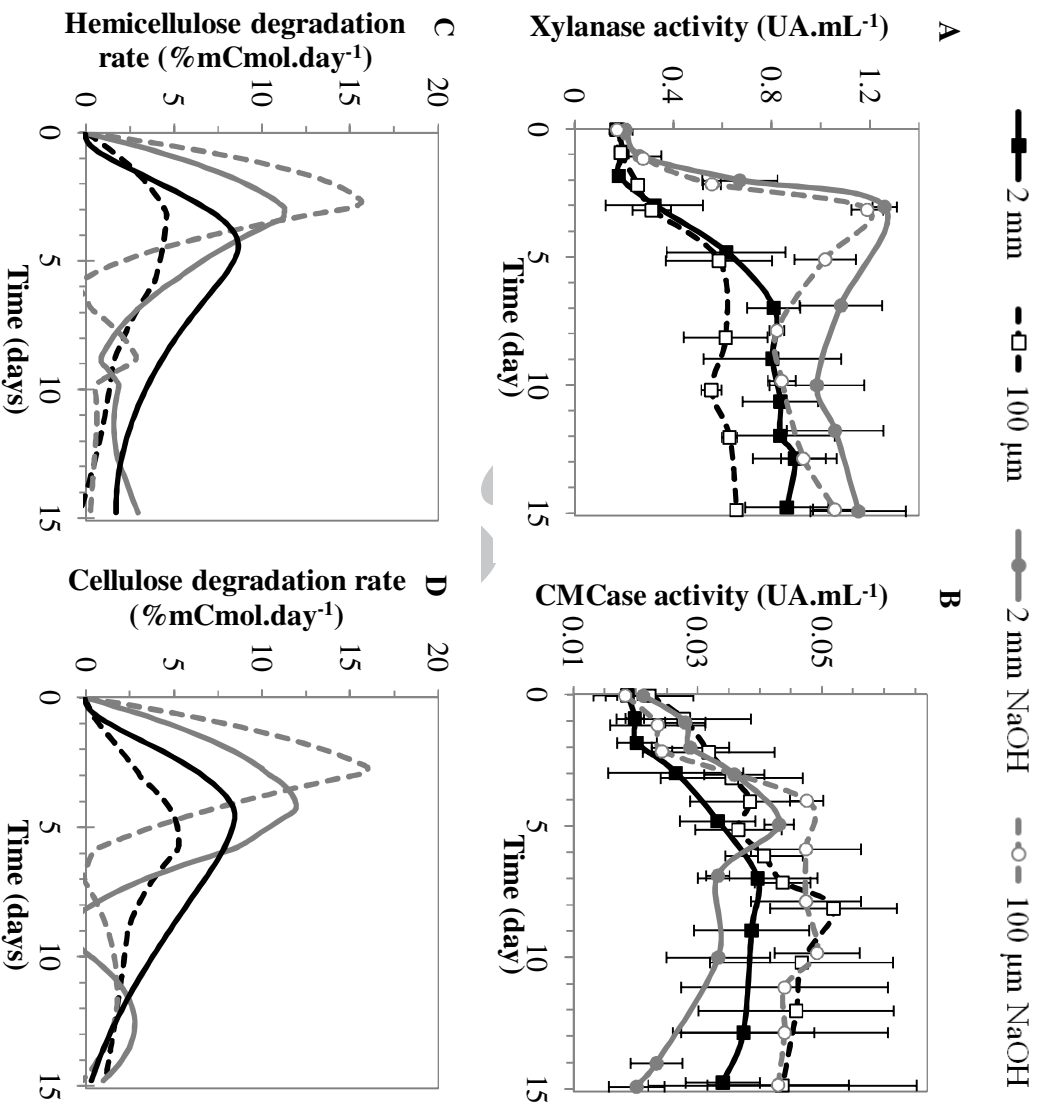
565 **Figure 1**

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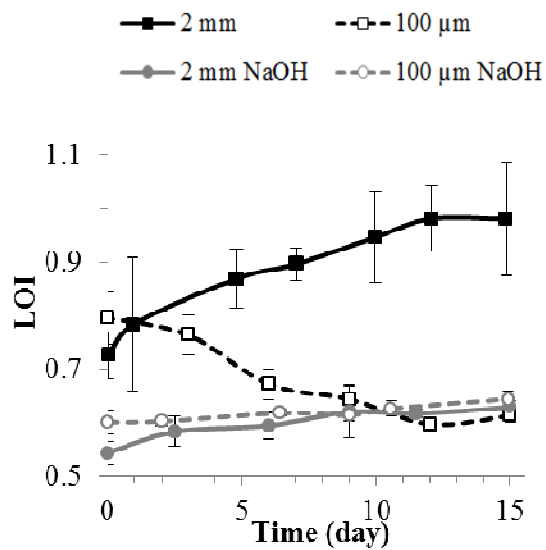
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569 **Figure 2.**

574 **Figure 3.**575  
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577 **Figure 4.**

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581 **Tables**

582 **Table 1:** Size and energy consumption of the four types of wheat straw pretreatment.

	Pretreatment	Size ( $\mu\text{m}$ ), D50	Energy ( $\text{kJ}\cdot\text{kg}^{-1}$ )
A	2 mm	-	223.34
B	100 $\mu\text{m}$	115.87 $\pm$ 3.24	635.23
C	2 mm - NaOH	-	223.34
D	100 $\mu\text{m}$ - NaOH	91.54 $\pm$ 2.24	327.45

583

584



585 **Table 2:** Product concentrations and metabolic yields after a 15-day fermentation of the  
 586 pretreated wheat straw substrates by a RWS microbial consortium.

	(A) 2 mm	(B) 100 $\mu$ m	(C) 2 mm NaOH	(D) 100 $\mu$ m NaOH
VFA yield* (g eq AA.g <sup>-1</sup> )	0.61 $\pm$ 0.07	0.77 $\pm$ 0.03	0.70 $\pm$ 0.07	0.83 $\pm$ 0.14
CO <sub>2</sub> yield* (g CO <sub>2</sub> .g <sup>-1</sup> )	0.26 $\pm$ 0.03	0.47 $\pm$ 0.02	0.41 $\pm$ 0.07	0.58 $\pm$ 0.15
H <sub>2</sub> yield* (mg H <sub>2</sub> .g <sup>-1</sup> )	0.55 $\pm$ 0.19	1.52 $\pm$ 0.20	2.70 $\pm$ 0.47	3.25 $\pm$ 0.31
Time at maximum VFA prod. rate (days)	5.2	6.0	2.9	2.2
Max VFA prod. rate (mCmol.L <sup>-1</sup> .day <sup>-1</sup> )	25.6 $\pm$ 1.0	13.4 $\pm$ 0.14	35.6 $\pm$ 6.0	43.4 $\pm$ 4.1
VFA concentration at max VFA prod. rate (mCmol.L <sup>-1</sup> )	109.0 $\pm$ 20.8	72.2 $\pm$ 7.4	100.1 $\pm$ 4.2	94.2 $\pm$ 4.4
Final VFA prod. (mCmol.L <sup>-1</sup> )	226.5 $\pm$ 21.1	149.0 $\pm$ 3.5	228.3 $\pm$ 22.1	217.1 $\pm$ 20.9
% AA (%Cmol)	65.6 $\pm$ 3.9	50.8 $\pm$ 2.6	38.6 $\pm$ 3.6	34.3 $\pm$ 1.2
% PA (%Cmol)	20.5 $\pm$ 3.4	24.7 $\pm$ 0.4	24.0 $\pm$ 7.6	22.3 $\pm$ 6.1
% BA (%Cmol)	10.0 $\pm$ 2.9	17.8 $\pm$ 2.3	28.0 $\pm$ 2.1	33.1 $\pm$ 6.5
% VA (%Cmol)	3.8 $\pm$ 3.1	4.9 $\pm$ 0.3	5.5 $\pm$ 0.2	6.0 $\pm$ 0.6

587 \* Metabolic yield, against consumed substrate

588

589

590 **Highlights**

- 591 • Dry chemo-mechanical pretreatments impact biomass crystallinity and macroporosity.
- 592 • Dry chemo-mechanical pretreatment increased the carboxylate production rate by
- 593 RWS.
- 594 • Dry chemo-mechanical pretreated biomass enhances the microbial xylanase activity.
- 595 • Initial biomass macroporosity correlated with the biological acidogenic potential.
- 596