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Enhancement of corn stover conversion to carboxylates by extrusion and biotic triggers in solid state fermentation

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Abstract

Solid-state fermentation is a potential technology for developing lignocellulosic biomass-based biorefineries.

This work dealt with solid-state fermentation for carboxylates production from corn stover, as building blocks for a lignocellulosic feedstock based biorefinery. The effect of extrusion pretreatment, together with the action of a microbial consortia and hydrolytic enzymes as biotic triggers, was investigated on corn stover conversion, microbial metabolic pathways and populations.

25 The extrusion caused changes in the physical and morphological characteristics, without altering the biochemical
26 composition of the corn stover. Extrusion also led to remarkable differences in the composition of the indigenous
27 microbial population of the substrate. Consequently, it affected the structure of community developed after
28 fermentation and the substrate conversion yield, which increased by 118% (from 23 ± 4 gCOD/kgVSi obtained with
29 raw substrate to 51 ± 1 gCOD/kgVSi with extruded corn stover) with regards to self-fermentation experiments.

30 The use of activated sludge as inoculum further increased the total substrate conversion into carboxylates, up to $60 \pm$
31 2 gCOD/kgVSi, and shaped the microbial communities (mainly composed of bacteria from the *Clostridia* and
32 *Bacteroidia* classes) with subsequent homogenization of the fermentation pathways. The addition of hydrolytic
33 enzymes into the reactors, further increased the corn stover conversion, leading to a maximum yield of 142 ± 1
34 gCOD/kgVSi. Thus, extrusion pretreatment combined with the use of an inoculum and enzyme addition increased by
35 506% corn stover conversion into carboxylates. Beside biomass pretreatment, the results of this study indicated that
36 biotic factor greatly impacted solid-state fermentation by shaping the microbial communities and related metabolic
37 pathways.

38
39 **Keywords:** Solid-State Fermentation; mixed culture; pretreatment; lignocellulosic biomass; carboxylates;
40 environmental biorefinery

41

42 **Introduction**

43 Lignocellulosic biomass is the most abundantly available organic raw material on Earth and it is considered to be one
44 of the most promising resource for biofuels and biomolecules production at industrial scale (Adekunle and Okolie
45 2015). While lignocellulosic biomass cultures dedicated to energy production have been widely criticized for the
46 competition with food dedicated cultures, agricultural residues, such as corn stover, do not compete with food
47 production. In addition, the constantly increasing quantity of crop residues issued from the agricultural activities
48 constitutes an opportunity for developing new valuable processes to produce bioenergy or biomolecules. Corn is
49 currently the world largest crop, with production of about 885 million MT of grain (FAO 2013). Two tons of
50 lignocellulosic residues are produced for each ton of grain, making corn stover (CS) one of the most abundant
51 lignocellulosic resources issued from agricultural residues. Currently, this resource is poorly valorized since the stoves
52 are incorporated into soil (buried in the fields or not) and/or used for animal farming as litter.

53 An interesting alternative is to use CS as raw material in biorefineries to maximize its exploitation and produce value
54 added products and energy by fermentation and anaerobic digestion (AD). The remaining nutrients could here be
55 returned into the soils in form of digestate as bio fertilizer. Biorefinery pathways to produce higher value products
56 from heterogeneous low value feedstock could be based on the conversion by anaerobic fermentation of the organic
57 matter into short chain organic acids (mainly acetate, propionate, butyrate) and alcohols, but also lactate: these
58 compounds are all referred to carboxylates (Agler et al. 2011). Compared to bio-H₂ and bio-CH₄ that can be produced
59 as well by anaerobic microbial consortia, carboxylates are not in competition with low price energy sources, such as
60 natural gas (~0.5 \$US·m⁻³), as they can be further converted into value-added products, such as polyhydroxyalkanoate
61 bioplastics. Carboxylates may also be used as intermediate building blocks for the production of commodity chemicals
62 and biofuels (e.g via chain elongation processes) notably in integrated bioconversion steps (Agler et al. 2011).

63 However, due to their large content in lignocellulose, fermentative degradation of agricultural residues remains a
64 technological challenge. Indeed, pretreatment is considered to be the most important and expensive step for obtaining
65 an efficient conversion of biomass to value-added product (Mussatto and Dragone 2016). Several physical, chemical
66 and biological pretreatment methods are described which promote the accessibility of polysaccharides in a
67 lignocellulose complex for enzymatic and microbial hydrolysis (Mussatto and Dragone 2016). Mechanical
68 pretreatment of lignocellulosic biomass is an essential prerequisite to improve particle distribution and densification,
69 enzymatic accessibility and bioconversion efficiency (Bhutto et al. 2017).

70 Compared to other pretreatment methods, which are mainly performed in batch mode, extrusion has the advantage to
71 be a continuous process, with a short residence time and high solid content (50-80%), which makes it easy to be scaled-
72 up and with great adaptability to process modification (Muthukumarappan and Karunanithy 2016). Moreover, the total
73 energy requirement of extrusion process has been estimated as 0,22-0,69 kWh/ t TS (Lamsal et al. 2010), which is
74 quite low compared to the energy required for other pre-treatments. Indeed, Menardo et al. (2012) reported that the
75 energy required for straw bale breaker and shredder was 15 kWh/ t TS while the energy required for thermo-alkaline
76 pre-treatment has been estimated around 42 kWh/ t TS (Monlau et al. 2015).

77 Extrusion process consists in making chipped material passing between twin-screws and the compression of the screw
78 causes a deconstruction of the organic material. Advantages of this process over others is that the compression causes
79 a size reduction and a defibrillation of the material. Since the process is carried out at moderate temperature, the
80 formation of degradation and oxidation products of lignin and polysaccharides is prevented (Vrije et al. 2002).

81 Originally used in the plastic industry and later adapted to the food industry, extrusion process is a new promising
82 physical pretreatment method for biomass conversion to sugars platform for biorefinery purposes (Zheng and Rehmann
83 2014; Muthukumarappan and Karunanithy 2016). In the context of environmental biorefinery, extrusion pre-treatment
84 has been evaluated to increase the biogas yield in anaerobic digestion (Vrije et al. 2002; Panepinto and Genon 2016)
85 and to increase sugar recovery by enzymatic hydrolysis (Karunanithy and Muthukumarappan 2011).

86 After having pretreated the lignocellulosic biomass by extrusion, solid-state (high-solids) fermentation (SSF) processes
87 present several operational and technological advantages compared to the conventional “liquid” bioconversions: (i) a
88 reduction of water requirements, (ii) a technical simplicity of extraction of metabolic by-products because of their
89 higher concentrations in the digestate, (iii) a smaller reactor volume, (iv) less energy requirements to heat the reactor
90 and (v) easier handling of the digestate residues (Kothari et al. 2014).

91 Usually, the digestate or leachate of SS-Anaerobic Digestion is used to inoculate the fresh feedstock but some studies
92 reported an increase in the production of VFAs from lignocellulosic feedstock when rumen fluid was used as inoculum
93 in Li et al. (2011).

94 However, a simple mechanical pretreatment alone is insufficient for attaining economically feasible biomass bio-
95 conversion yields (Vidal et al. 2011). Thus, a combination of mechanical pretreatment with enzymatic or chemical
96 treatment is required to significantly improve the conversion of organic material by reduction in particle size and
97 enhancement of the biological hydrolysis and fermentation processes (Vrije et al. 2002).

98 To the best of our knowledge, there is a lack of investigation concerning the use of extrusion pre-treatment to enhance
99 carboxylates production from lignocellulosic biomass by mixed-culture solid-state (SS-)fermentation.

100 The present work aims to evaluate CS deconstruction and bioconversion by combining a pretreatment by extrusion
101 with the action of a microbial consortium and enzymes to produce carboxylates in SSF process. The effects of the
102 extrusion pretreatment on CS conversion, microbial metabolic pathways and populations, in mesophilic SS-
103 fermentation alone (self-fermentation) and in combination with the use of biotic triggers such inoculation and
104 enzymatic addition were investigated.

105

106 **Material and methods**

107 **Substrate**

108 The substrate corresponded to a raw CS. Samples were chopped to a particle size of approximately 10-20cm using a
109 vegetable milling machine. The chopped samples were then shredded using knife milling (Retsch SM 100, Germany)
110 with a screen size of 4mm. The grinded biomass was then impregnated with water using a spray system to reach 400g/L
111 of total solid then treated using twin screw extruder (Fig. 1). Impregnated CS was extruded at 200 rpm and 37°C in an
112 automatic weight feeder extrusion (Thermo scientific Eurolab 16). The substrate was dried and kept at room
113 temperature in a dry and dark room.

114

115 **Substrate characterization**

116 **Physical state**

117 Images of particles observed in bulk were acquired using a macrovision system including a CCD color video camera
118 (JVC KY F55B, Alliance Vision, Montélimar) equipped with a 50 mm Nikon lens (AF Nikkor, 1/1.8) (Devaux et al.
119 2008). Two neon tubes (2 X9 W) were used for illumination. A specific lighting box was built in order to illuminate
120 samples with diffuse light. Samples were put into a petri dish and inserted in the box using a drawer that closed the
121 box. The height of the camera and the lens focus were manually adjusted to observe a 3.5 cm x 2.6 cm area. The size
122 of pixel was 8.21 μm .

123 To observe the disintegration of particles on hydration, a torus reactor equipped for visualization of particles was used
124 (Devaux et al. 2006). 300 mg of powder were suspended in water and introduced into the reactor. The volume was
125 completed to 200 ml with water. The particles were maintained under stirring with a propeller and continuously moved

126 within the reactor. Images (10 per time) constituted of 768 x 576 pixel, each pixel being of size 17.3 μm x 17.3 μm
127 were acquired every ten min per hour. The grey levels were coded on 256 values starting from 0 (black) up to 255
128 (white).

129 Quantitative information concerning particle size was extracted by image analysis using grey level granulometry
130 through line opening as structural element (Devaux et al. 2006). The method can be considered as a sieving of the
131 objects in images which provides granulometric curves that can be interpreted as usual granulometric curves.

132

133 **Composition**

134 Extractive-free samples were ground 3 min in nitrogen liquid (SPEX 6700 freezer Mill).

135 ***Infrared spectroscopy***

136 FT-IR spectra were recorded from KBr pellets made of 2 mg extractive-free samples mixed with 100 mg of KBr. The
137 spectra were collected in transmission mode between 4000 and 400 cm^{-1} at 4 cm^{-1} interval (Bruker vector 22
138 spectrometer). The infrared spectra resulted from the co-addition of 200 interferograms. Eight spectra per sample were
139 collected. The infrared spectra in the 2000-700 cm^{-1} region were baseline corrected and unit vector normalized using
140 the OPUS software (version 6.5).

141 ***Cellulose crystallinity***

142 The crystallinity percentage of cellulose was determined by ^{13}C solid state NMR as previously described (Timpano et
143 al. 2015).

144 ***Hydration properties***

145 Water soluble sugars were determined as follows. Samples (360mg) were suspended in distilled water (30 ml) and
146 gently stirred overnight at 4°C using a rotating shaker. Each suspension was centrifuged at 20°C -3000g for 20 min.
147 The supernatant was recovered, filtered on G2 sintered glass and analyzed for neutral sugars by the automated orcinol
148 method (Tollier and Robin, 1979). Swelling, ‘volume occupied by a known weight of particles under the conditions
149 used’ and water retention capacity ‘the amount of water retained by by a known weight of particles under the conditions
150 used’ was determined as described by Robertson et al. (Robertson et al. 2000). Swelling was expressed as volume/g
151 original substrate weight and water retention capacity as the amount of water retained by the insoluble material (g/g
152 dry weight).

153

154 Fermentation tests

155 Inoculum

156 The microbial inoculum corresponded to dewatered Activated Sludge (AS) collected from a municipal wastewater
157 treatment plant (Narbonne, France). The choice of this inoculum was dictated by the necessity of operating the
158 fermentation experiment at high solid load. The initial total (TS) and volatile (VS) solids of the AS were 17.3% and
159 13.9% respectively. Preliminary tests were carried out to determine the optimal storage condition. Storage at at -20°C
160 was chosen among the use of fresh AS, heat pre-treated AS (90°C, 30'), storage at 4°C and storage at -80°C, since it
161 ensured the highest conversion yield and reproducibility of results (data not shown). The inoculum was placed at 4°C
162 one day before the start-up of the reactors, before inoculating the reactors, an inoculum solution (20 gVS/L) was
163 prepared by suspending the inoculum into demineralized water and mixing for 30 min.

164 Batch tests

165 Fermentation experiments were carried out in 120 mL flask. Each reactor contained: 4 g of substrate; 1 mL of NH₄Cl
166 solution 16 %(m/v); 0.2 mL oligo-element solution (1.5 g.L⁻¹ of FeCl₂.H₂O; 62 mg.L⁻¹ of H₃BO₃.H₂O; 117 mg.L⁻¹ of
167 MnSO₄.H₂O; 26 mg.L⁻¹ of CoCl₂.6H₂O; 120 mg.L⁻¹ of ZnCl₂; 28 mg.L⁻¹ of NiCl₂.6H₂O; 38 mg.L⁻¹ of CuCl₂.2H₂O;
168 31.8 mg.L⁻¹ of NaMoO₄.2H₂O; and 4.4 mL of HCl 37%); phosphate buffer (0.05M, pK = 6.4); in case of inoculated
169 reactors, inoculum at substrate/Inoculum ratio of 18 (g_{vs}/g_{vs}) and distilled water. Enzymatic addition, with the
170 objective of increasing the accessibility of the cellulose, was performed by directly adding a mix of enzymes (cellulase
171 and endo-1,4-xylanase from *Trichoderma longibrachiatum*, SIGMA-ALDRICH®), at 20 U/gTS each, into the
172 fermentation reactors following the procedure proposed by Sambusiti et al. (Sambusiti et al. 2015). Demineralized
173 water was added according to a mass balance on the TS contents (including CS, inoculum, buffer, and solution
174 addition) to obtain a final TS concentration of 15%. To ensure the homogeneity, a medium solution containing all the
175 above mentioned components was prepared and the pH was adjusted at 6.4 with KCl or NaOH solutions (1N) before
176 to be added into the reactors. After the solution was added, the reactors were mixed by hands with spatula to obtain a
177 homogenous composition. Then, the reactors were closed with rubber septa and plastic plugs and the headspace was
178 flushed with N₂ for 5 minutes to ensure anaerobic conditions. The batch tests were then incubated at 37°C for 9 days.
179 The experiments were stopped at 9 days since no significant increase of conversion yield were observed during longer
180 operation (up to 15 days) (data not shown).

181 The effect of the extrusion pretreatment on CS fermentation was evaluated in three different conditions (Table 2): first,
182 the effect of extrusion pretreatment was assessed by fermenting the substrate without the use of biotic triggers (self-
183 fermented); second, the use of AS as inoculum was evaluated (inoculated); third, the increase of hydrolysis with
184 enzymes was carried out (enzymes addition). Experiments were performed in duplicates on CS Ext-CS.

185

186 **Analytical methods**

187 Total solids (TS) and volatile solids (VS) content were measured according to the standard Methods (APHA 2005).
188 Biogas production was periodically monitored during fermentation by measuring the pressure in headspace (100 mL)
189 using a digital manometer (LEO 2, Keller), and sampling biogas with a 0.2 mL syringe for analysis. If pressure of one
190 reactor was superior to 1.75 bars, after gas sampling, all reactors were degased to reach 1.25 bar. Biogas composition
191 was determined using a gas chromatograph (Perkin Elmer Clarus 580), equipped with two capillary columns: a R-Q-
192 bond column for CO₂ determination (30 m x 0.32 mm) and an Rt-Molsieve 5 Å (30 m x 0.32 mm) column for O₂, N₂,
193 CH₄ and H₂. Argon (350 kPa at 34 mL min⁻¹) was used as carrier gas to transport gas sample from the injector (250°C)
194 to the thermal conductivity detector (150°C).

195 To extract the liquid fractions of the reactors, a gravimetric dilution to 5% TS was performed. The liquid phase was
196 then extracted after filtration with a microfiber filter GF/D (2.7 µm Whatman, Kent, UK). The pH was immediately
197 measured after dilution. Sugars and metabolites produced during fermentation were analyzed by High Performance
198 Liquid Chromatography (HPLC) equipped with an Aminex HPX 87H column (Biorad) at 35°C, with a 0.005 M H₂SO₄
199 water solution (0.4 mL min⁻¹) as eluent. The concentration of each metabolite (g/L) provided by the HPLC was
200 expressed in gram of Chemical Oxygen Demand (gCOD) and was referred to the VS initial amount (kg) of substrate.
201 Total substrate conversion was used to assess fermentation performances, it was defined as the sum of metabolites
202 measured in the reactor at the end of fermentation: succinate, acetate, propionate, butyrate, valerate, isovalerate,
203 caproate, ethanol and lactate.

204

205 **Microbial community analysis**

206 Genomic DNA was extracted and purified as reported elsewhere (Pierra et al. 2014). The V3 region of 16S rRNA
207 genes was amplified using universal primers for bacteria (Moscoviz et al. 2016). The resulting PCR products were
208 purified and sequenced, using the MiSeq v3 chemistry (Illumina) at the GenoToul platform (www.genotoul.fr).

209 Sequences (32393 ± 4295 in average per sample) were retrieved after demultiplexing, cleaning, and affiliating
210 sequences using mother (Schloss et al. 2009). They were grouped into operational taxonomic units (OTUs) at a 97%
211 similarity and most abundant sequences were affiliated to their closest relatives in the NCBI database (Table 3).
212 Sequences were submitted to GenBank, with accession No. MG578513 - MG579773. Shannon (H) and Simpson (1-
213 D) diversity indices were calculated by using R package 'vegan'. Quantitative PCR (qPCR) of 16S rDNA was
214 performed as described elsewhere (Moscoviz et al. 2016).

215

216 **Calculation**

217 Hydrogen and carbon dioxide daily yields (in NmL/g_{VS}) were calculated according to eq.1:

$$218 \quad Y_{biogas(j+1)} = \frac{(\%Biogas_{j+1} \times P_{j+1}) - (\%Biogas_j \times P_j) \times T_0 \times V_{HS}}{P_0 \times T_{reactor} \times m_{VS}} \quad (\text{eq.1})$$

219 Where %*Biogas*_{*j*} is the percentage (mol/mol) of H₂ or CO₂ at day *j*, *P*_{*j*} is the pressure of reactor headspace (bar) at day
220 *j*, *T*_{*reactor*} is the Temperature of the reactor, i.e. 37°C, *T*₀ and *P*₀ the temperature and pressure at Normal conditions (*T*₀
221 = 273 K, *P*₀=1.000 bar), *V*_{*HS*} the volume of the headspace in mL and *m*_{*VS*} the mass of volatile solids in g_{VS}.

222

223 **Results**

224 **Substrate characterization**

225 *Particle morphology*

226 Raw biomass was composed of particles with different sizes and shapes. Elongated particles corresponded to fibre
227 enriched fragments while large particles originated mainly from pith parenchyma. In extruded biomass, small
228 fragments and larger particles with a rounded shape and coarse surface could be observed, suggesting that extrusion
229 led to particle fragmentation and aggregation. Images of the raw and extruded particles in bulk acquired with the
230 macro-imaging device are shown in Supplementary Materials. To quantify more precisely the particle size distribution
231 and its evolution when hydrating the samples, particles were imaged by the torus reactor. Quantitative information was
232 extracted from the images by applying image texture analysis. The curves resulted from an averaging of the individual
233 curves calculated for the images recorded at different times of sample hydration. The position of the peak reveals the
234 particle size for which the major grey level variations occurred. At *t*₀, large distributions were observed for both the
235 raw and extruded samples indicating a heterogeneity in particle size as previously viewed in images of particles in bulk

236 (Fig. 2). After one-hour hydration in water, no evolution of the particle size was observed in the raw samples. In
237 contrast, for the extruded sample, the initial peak clearly shifted to lower values. Grey level mean sizes were assessed
238 from the granulometric curves. For the raw substrate, the grey level mean size was $1178 \pm 87 \mu\text{m}$. For the extruded
239 sample, the grey level mean size decreased from $683 \pm 50 \mu\text{m}$ at t_0 to 411 ± 48 after 1h under stirring in water,
240 suggesting a fragmentation of the aggregates.

241

242 *Cell wall composition and properties*

243 To investigate the changes in composition induced by the extrusion pretreatment, the cell wall composition was
244 analysed by Fourier-transformed infrared spectroscopy (FTIR spectra are shown in Supplementary materials). The
245 spectra from extruded and raw biomass revealed differences in the intensity of the bands at 1730 , 1513 and 1250 cm^{-1}
246 which could be assigned to ester linked acetyl groups and hydroxycinnamic acids. There is no major change in cellulose
247 crystallinity. (Table 1).

248 The ability to swell in presence of water, to retain water and the amount of soluble sugars are factors that can influence
249 the rate and extent of fermentation. Extrusion decreased the swelling and water retention capacity (Table 1). The
250 amount of water soluble sugars was low (less than 3% of the dry matter) and in the same range for raw and extruded
251 substrates.

252

253 **Solid State Fermentation**

254 The effect of the extrusion step on substrate conversion during SSF (15%TS) was here evaluated under the same
255 operating conditions for Ext-CS and CS. The effect of the use of biotic triggers, such as inoculation and the addition
256 of hydrolytic enzymes, to improve substrate conversion was also investigated. The three different conditions performed
257 are summarized in Table 2. The total conversion of CS and Ext-CS into metabolites of the three conditions is presented
258 in Fig. 3 while soluble COD (SCOD) composition (gCOD/KgVS_i) and pH are reported in Table 2.

259 Substrate conversion highly varied between the different tests, but the replicates were highly reproducible (Fig. 3). The
260 total conversion into fermentation metabolic products of extruded substrate was $50 \pm 2 \text{ gCOD/kgVS}_i$ for self-
261 fermented, $60 \pm 02 \text{ gCOD/kgVS}_i$ for inoculated and $142 \pm 1 \text{ gCOD/kgVS}_i$ for enzymatic/inoculated test. The total
262 conversion significantly increased after extrusion independently of the tested conditions, by 118%, 59% and 56% when
263 compared to self-fermented, inoculated and enzymatic augmented/inoculated unextruded tests, respectively. This also

264 led to lower pH values measured at the end of fermentation for Ext-CS compared to CS in all the tested conditions
265 (Table 2).

266 Inoculation with AS led to an increase of the total conversion for both substrates. This was particularly noticeable for
267 CS which had a substrate conversion yield of self-fermentation of 23.4 ± 3.7 gCOD/kgVS_i, while inoculated CS
268 yielded 37.6 ± 0.5 gCOD/kgVS_i. As a consequence, inoculum addition led to an increase of metabolites production of
269 more than 61%. Comparatively, Ext-CS conversion increased by only 17% (i.e. from 50.9 ± 0.5 to 59.8 ± 2.0
270 gCOD/kgVS_i).

271 A significant improvement of the total conversion was observed after addition of the enzymes. The total substrate
272 conversion was four times higher than the one obtained by self-fermentation for CS and almost three times for Ext-
273 CS, reaching yields of 90.8 ± 1.9 gCOD/KgVS_i and 141.9 ± 0.9 gCOD/KgVS_i with CS and Ext-CS, respectively.
274 Consecutively, with the enzymatic addition the final pH values were the lowest for both substrates.

275 Experimental conditions highly affected the metabolites composition since at the end of fermentation different
276 metabolic behaviors were observed. Lactate was the main product of CS self-fermentation. Acetate, propionate and
277 other metabolites (mainly succinate, data not shown) represented only 5% of the COD. In contrast, lactate was
278 produced in a lesser extent during self-fermentation of Ext-CS. Here, the main metabolite was butyrate, followed by
279 acetate, ethanol and propionate (Table 2).

280 Interestingly, with no inoculum, fermentation was incomplete for both substrates, with soluble sugars detected at the
281 end of the self-fermentation. The use of AS as inoculum led to a total consumption of soluble sugars during the course
282 of fermentation of CS and Ext-CS, independently of the tested conditions and enzyme addition. After inoculation,
283 metabolites distribution was mostly similar for the two inoculated substrates, while, compared to self-fermentation
284 different metabolites distribution were observed, indicating different metabolic pathways. Acetate was the main
285 product, followed by propionate and butyrate (Table 2). Ethanol represented a minor part of the metabolites produced
286 and no lactate was detected. In inoculated tests, medium chain carboxylates, *i.e.* valerate and other metabolites (mainly
287 iso-butyrate and iso-valerate) were observed after fermentation of both substrates. Caproate was also produced in
288 fermentation of Ext-CS.

289 The enzymatic addition induced a change in the proportions of each metabolite when compared to the simple
290 inoculation, but not in the overall spectrum of metabolites produced. With enzymatic addition, acetate, despite still
291 representing the major end-product, decreased at the expense of butyrate and ethanol produced from the fermentation

292 of both substrates (Table 2). Propionate and valerate production did not significantly increase, while in this condition,
293 caproate was produced from the fermentation of both substrates. Interestingly, metabolic distribution was very similar
294 for the self-fermentation and the enzymatic augmented fermentation of Ext-CS with the exception of lactate (produced
295 during self-fermentation) and medium chain carboxylates (produced only with enzymatic augmented fermentation).
296 To further investigate the fermentation dynamics, the produced biogas over the fermentation time was daily analyzed
297 and quantified. CH₄ was never detected in reactors headspace, while CO₂ production followed a similar trend in all the
298 tested conditions: CO₂ production rate increased during the first three days of fermentation and afterward remained
299 almost constant. The volume of CO₂ produced was linearly correlated ($R^2= 0.87$) with the accumulated amount of
300 metabolites (data not shown). Regarding H₂ gas production, different behaviors between self-fermented and inoculated
301 experiments were observed (Fig. 4). Almost no H₂ was produced during self-fermentation of CS, while H₂ was
302 continuously produced during self-fermentation of extruded CS, reaching a concentration of 34% in the headspace,
303 that was equivalent to a cumulative volume of 15.4 ± 0.7 mL H₂ (4.4 mL H₂/gVS_i) after nine days of fermentation. In
304 all the experiments inoculated with AS, independently of enzymes addition, H₂ production followed a similar trend: a
305 sharp increase of H₂ concentration was observed in the headspace of the reactors during the first three days and then
306 H₂ was completely consumed between days three and six. The maximum cumulative H₂ was observed in the test with
307 enzymatic addition for both CS and Ext-CS, reaching at three days of fermentation respectively 12.2 ± 0.2 and $17.0 \pm$
308 0.1 mL.

309 **Microbial community analysis**

310 The microbial communities were analyzed by Illumina-based 16S sequencing after nine days of fermentation of the
311 CS and Ext-CS substrates, in all the tested conditions, as well as the ones from the initial inoculum (AS) and the
312 indigenous populations of the substrates. Table 3 reports the relative abundances (%) of the key microbial groups (at
313 the genus level) and the alpha diversity indices (Shannon and Simpson) calculated for each microbial community.

314 Taxonomic affiliation of metagenomic data showed that sequences were mainly affiliated to the phyla *Firmicutes*
315 (54.5%), *Proteobacteria* (21.4%), *Bacteroidetes* (13.1%) and *Actinobacteria* (9.0%). Other phyla such as *Nitrospirae*,
316 *Chlorobi*, *Acidobacteria*, *Acidobacteria* and *Chloroflexi* were present but at very low abundances (<2%). Sequences
317 with more than 3% of similarity were grouped into single Operational Taxonomic Unit (OTU). By excluding AS
318 inoculum and CS substrate, only sixteen OTUs explained the overall diversity (> 92%) found. Taxonomic affiliation
319 is shown in Table 3.

320 The *Proteobacteria* phylum dominated in the AS (49%) and the indigenous community of CS substrate (63%), with
321 bacteria representative of the three classes of *Gammaproteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*.
322 Meanwhile, the phylum *Firmicutes*, with bacteria belonging to the classes of *Bacilli* and *Clostridia*, strongly dominated
323 the indigenous microbial communities of Ext-CS substrate and the community found in all the reactors, independently
324 of the fermentation condition. Thus, surprisingly, extrusion pretreatment had a large impact on the microbial
325 community. Indeed, wide difference was found in the indigenous populations of the two substrates. Whereas the
326 number of 16S gene copies found in Extr-CS was similar to the ones found in the inoculum and the inoculated samples
327 (in the order of 10¹⁰ copies 16S/g VS), the number of 16S gene copies found in CS (5.68E+08 copies 16S/g VS) was
328 two orders of magnitude lower. Despite the lower bacterial abundance, the microbial community of CS showed high
329 level of diversity, showing Shannon index (4.34) and 1-Simpson index (0.98) comparable to AS values, which, as
330 expected, showed the highest diversity values among all samples (i.e. 5.42 and 0.99 for Shannon and 1- Simpson
331 indices respectively). Conversely, Ext-CS was characterized by a low value of Simpson diversity (0.58), indicating a
332 clear dominance of only few species, since the Simpson Index gives more weight to the most abundant species. The
333 microbial community of the Ext-CS substrate was mainly composed of bacteria from the *Bacillaceae* family. Only two
334 OTUs were dominant, from *Bacillus* and *Lysinibacillus* genera respectively, representing 71 % and 18 % of the total
335 bacterial community. Nucleotide sequence analyses of their 16S rRNA genes revealed respectively 99 and 100 % of
336 sequence homology with *Bacillus licheniformis* and *Lysinibacillus halotolerans*. Both are known to be facultative
337 anaerobic species, commonly found in the soil (Kong et al. 2014), which is consistent with the origin of the substrate.
338 The presence of these two OTUs represented a sort of signature of the Ext-CS, since they were found, even in a minor
339 proportion (<10%), in all the microbial communities developed during fermentation of Ext-CS while they were never
340 found in samples of CS substrate.

341 After nine days of self-fermentation an inverse trend was observed where the self-fermented CS showed a lower
342 diversity (and higher dominance) when compared to Ext-CS (Table 3). The microbial community of self-fermented
343 CS was mainly composed of bacteria from the class of *Bacilli* (63%), with an OTU affiliated to *Enterococcus* genus
344 representing 57% of sequences, and more particularly 100% of homology with *Enterococcus hirae*. This OTU was
345 also detected in the community of CS substrate but at very low abundance (1.4%). Apparently the fermentation
346 condition imposed in the reactor (O₂ depletion, 37°C, initial pH 6.4, 15%TS) favored its development. This is
347 confirmed by the fact that sequences from this OTU represented 19% of the sequences found in the self-fermentative

348 community of Ext-CS substrate, while they represented only 0.3% of the sequences of the community of Ext-CS. The
349 remaining part of the microbial community of self-fermented CS was mainly composed of bacteria belonging to the
350 family of *Enterobacteriaceae*. Specifically, two OTUs represented 36% of the total number of sequences. Nucleotide
351 sequence analyses of their 16S rRNA genes revealed resp. 99 and 100% of sequence homology respectively with
352 *Cronobacter sakazakii* (24%) and *Enterobacter cloacae* (12%), which are known to be facultative anaerobic
353 fermentative species. The OTU affiliated with *Enterobacter cloacae* seems to be characteristic of CS substrate, since
354 after fermentation, independently of the conditions, it explained a non-negligible part of the sequences (6-14%).
355 Self-fermenting Ext-CS community, a part of the bacteria belonging to the class of *Bacilli* (35%), was composed of
356 bacteria of the *Clostridia* (39%) and *Coriobacteria* (17%) classes. Among *Clostridia*, the *Ruminococcaceae* family
357 was predominant and the genus *Caproiciproducens* was mostly represented (22%), being the sequences of the two
358 most abundant OTUs homologues of *Caproiciproducens galactitolivorans* (showing respectively 98 and 94% of
359 identity). As indicated by the name, this species is known for the ability to produce caproic acid (Jeon et al. 2010), but
360 this metabolite was not detected during self-fermentation of Ext-CS.

361 Inoculation led to the development of fermentative communities showing similar diversity values for the two substrates
362 but the observed diversity was impacted by the fermentation conditions: the enzyme addition led to more diverse
363 microbial communities with respect to simple fermentation. Strong similitudes were found in the community structure
364 of the fermentative communities developed after inoculation, independently of the conditions (with or without
365 enzymes). Few differences were observed, mostly linked to the substrate. Indeed, the four communities (Inoculated
366 and enzymatic augmented CS and Ext-CS) were all mainly composed of bacteria from the *Clostridia* and *Bacteroidia*
367 classes (representing respectively 44 and 22 % of sequences in average). Bacteria belonging to the class of *Clostridia*
368 were mostly affiliated to the family of *Ruminococcaceae*, a family that contains a number of cellulolytic and amylolytic
369 species isolated from several types of anaerobic environments, including AD reactors (Nelson et al. 2011). Specifically,
370 two OTUs were affiliated to the *Caproiciproducens* genus. As above mentioned, these species are capable of producing
371 caproic acid (Jeon et al. 2010), which is consistent with macroscopic observations.

372 The class of *Bacteroidia* was mostly represented by a unique OTU belonging to the *Prevotella* genus from the family
373 of *Prevotellaceae*. This OTU represented 1% of the sequences found in the microbial community of AS.
374 Microorganisms from this family are saccharolytic bacteria, which have already been found in fermentative
375 communities developed from AS from wastewater treatment plant (Xing et al. 2008). *Prevotella* species showed

376 potential to produce propionate via the randomizing (succinate) pathway, which consume H₂, through the fermentation
377 of sugars and lactate (Purushe et al. 2010). Thus, the presence of these bacteria could explain the H₂ consumption
378 during fermentation observed into the reactors which were inoculated.

379

380 **Discussion**

381 **Effect of extrusion pretreatment on SSF**

382 During extrusion process, biomass undergoes physical and chemical changes via shearing, mixing, heating. In this
383 study, extrusion results in particle size decrease, changes in morphology, surface aspect, swelling and water retention
384 capacity. No drastic changes were observed in the biochemical characteristics of biomass (apart from a slight decrease
385 in ester-linked compounds) or on the crystallinity of the cellulose. Consistently, Chen et al. (Chen et al. 2014) observed
386 that extrusion-pretreated rice straw showed significant particle size reduction, physical properties improvement and
387 volume expansion, but no changes in the chemical composition as compared to the milling-pretreatment and
388 unpretreated rice straw. The particle size decrease and thus increase in specific surface area are the well-known effects
389 of extrusion. They result from the shear friction between particles during the extrusion process. These phenomena are
390 further enhanced by low moisture content and large particle size of biomass (Karunanithy and Muthukumarappan
391 2011).

392 Some authors reported an increase in water soluble fractions in extruded plant biomass (Ralet et al. 1990; Ning et al.
393 1991; Ralet et al. 1993; Myat and Ryu 2014). The water solubility has been shown to be proportional to specific
394 mechanical energy up to a certain value over which solubility did not further increase (Ralet et al. 1990; Ralet et al.
395 1993). In our study, the severity of extrusion was probably too soft to induce solubilization of the cell wall components.
396 However, only few data are available on the impact of extrusion on water hydration properties. Our results are in
397 agreement with those reported by Ning et al. (Ning et al. 1991) and Ralet et al (Ralet et al. 1993). The lower values
398 obtained for hydration properties could be explained by the fact that extrusion caused particle disruption and
399 aggregation thus reducing interstitial spaces between particles that can participate in retaining water. The impact of
400 extrusion on cellulose crystallinity is more controversial. Zhang et al. (Zhang et al. 2012) reported no drastic change
401 in cellulose crystallinity of CS submitted to twin-screw extrusion. The authors noticed that extrusion could result in
402 the appearance of cellulose II structure under certain conditions. Similarly, no significant difference in crystallinity
403 index between raw and extruded CS fiber was reported by Myat et al. (Myat and Ryu 2014). Zheng et al. (Zheng et al.

404 2015) noticed only a slight increase of the cellulose crystallinity index in extruded CS with no significant effect of
405 screw configurations while Yoo et al. (Yoo et al. 2011) found an increase of cellulose crystallinity by 82 % with no
406 change in sugar composition in extruded compared to untreated soybean hull. Variations in the biomass moisture
407 content and extrusion operation condition may account for these discrepancies.

408 The increase in total conversion observed for of Extr-CS compared to CS was probably due to a higher specific surface
409 of the substrate (Vidal et al. 2011) that enabled a better access to enzymatic hydrolysis (Chen et al. 2014). When testing
410 hydrolysis efficiencies of enzymes on extruded or 1 mm ground *Miscanthus* in addition with NaOH, Vrije et al. (Vrije
411 et al. 2002) improved glucan hydrolysis by 34% and total biomass conversion by 15%. Similarly, Motte et al. (2015b)
412 found an increase of 22% conversion of wheat straw into ethanol when substrate was pretreated with ball-milling
413 (particle size of $0.3600 \pm 0.004\text{mm}$) instead of traditional milling (particle size of $1.03 \pm 0.01 \text{ mm}$). Results presented
414 in the present study showed the effectiveness of extrusion pretreatment for SS- fermentative conversion of CS. In
415 addition, the increase in total conversion was always linked with a decrease in final pH. Probably, a higher substrate
416 hydrolysis occurred with Extr-CS, followed by higher acidogenesis, consequently decreasing the pH due to organic
417 acid accumulation (Zoetemeyer et al. 1982). Extrusion decreased the swelling and water retention capacity, meaning
418 that more water was available for microorganisms during fermentation of Ext-CS compared to CS. García-Bernet et
419 al. (García-Bernet et al. 2011) showed that the critical water content (corresponding to the amount of water under
420 which there is no free water in the medium, only capillary and bound water) is proportional to the TS content; at 15%
421 content of TS it can have a considerable effect on the effective diffusion volume available. Moreover, Bollon et al.
422 (2013) reported that the transfer limitation has a threshold limit at 15% of TS. Therefore, the observed difference of
423 water retention capacity of untreated CS and Ext-CS could have had a great impact on fermentation performances at
424 15% TS. The higher swelling of ground CS indicates that mass transfer may be lower compared to extruded CS,
425 resulting in a more heterogeneous medium for ground CS. Low water content during the hydrolysis of CS implies a
426 larger viscosity, low mass transfer of intermediates and end products, having consequent effect on limited enzymes
427 diffusivity and fermentation performances (Pimenova and Hanley 2004; Felby et al. 2008; Hodge et al. 2009).

428

429 **Effect of biotic triggers on SSF**

430 Beside biomass pretreatment, the results of this study indicated that biotic factor greatly impacted SSF. According to
431 previous researches (Marone et al. 2012; Marone et al. 2014), it was shown that the naturally-occurring microbes

432 hosted by the substrate can perform the biodegradation via so-called self-fermentation. However, the impact of
433 inoculation with AS was very high, not only because it increased the total amounts of recovered products, but especially
434 because it seemed to drive the fermentation, by stabilizing the community and consequently the spectra of produced
435 metabolites.

436 During self-fermentation of CS, lactate accounted for almost half of the conversion products, which was consistent
437 with the fact that microbial community was dominated by lactic acid bacteria (*Enterococcus* sp.). Since lactic
438 fermentation was the major metabolic pathway carried out by endogenous bacteria, accumulation of lactate might have
439 inhibited the fermentation process (Cabrol et al. 2017), which could explain a lower total conversion and the presence
440 of remaining sugars at the end of fermentation. Moreover, the strong dominance of lactic acid bacteria could also
441 explain the absence of H₂ production, since they are able to secrete bacteriocins which inhibit H₂ production (Noike et
442 al. 2002).

443 Ext-CS showed a different behavior during self-fermentation, probably explained by the different microbial
444 community found in this substrate. Indeed, the high amount of bacteria (4.52E+10) found on the Ext substrate, together
445 with high dominance of few air tolerant strains, suggested that extrusion pretreatment favored the proliferation of these
446 bacteria. This different initial community, together with the highest accessibility due to the higher specific surface of
447 the substrate, led to the development of a more efficient fermentative community during self-fermentation of Ext-CS,
448 which produced different spectra of metabolites. A possible explanation could be that the small particle size of Ext-CS
449 allowed a faster hydrolysis rate, making soluble sugars rapidly available. Thus, *Clostridia*, which have fast growth rate
450 and are known to be efficient fermenting bacteria, gained the competition over other microorganisms (Cabrol et al.
451 2017).

452 Inoculum addition shaped substantially the metabolite composition of SS- Fermentation. When adding inoculum, a
453 different metabolic pathway was observed: no lactate was produced, no sugars remained after 9 days of fermentation
454 and the two major metabolites were acetate and butyrate. As metabolites composition was significantly different from
455 self-fermentation, it was concluded that bacteria from the inoculum won the competition over endogenous bacteria and
456 imposed their metabolism as main metabolic pathway. That was confirmed by the analysis of microbial community
457 structures after fermentation. The profiles of the different microbial communities developed during the inoculated
458 fermentation were highly similar, independently of the conditions (with or without enzymes). Few differences were
459 observed, mostly linked to the substrate. Although the strict conditions of fermentation imposed a strong selective

460 pressure leading to the establishment of a specific microbial community developed from the inoculum, the indigenous
461 community brought by the substrate played a role in shaping the final structure of fermentative community. Indeed, a
462 sort of substrate signature was identified only by the presence of specific strains or groups of bacteria, which were
463 characteristic for each substrate.

464 Interestingly, some amounts of medium chain carboxylates were produced only after inoculation even though the
465 bacteria responsible of chain elongation pathways (i.e. *Caproiciproducens* spp.) were found also in the self-
466 fermentative community in Ext-CS. The inoculation led likely to a faster hydrolysis confirmed by the observation of a
467 faster H₂ production rate, followed by a faster fermentation. This difference in hydrolysis kinetics affected the types
468 of metabolites produced, making the substrates (i.e. ethanol, propionate, butyrate) (Angenent et al. 2016) for chain
469 elongation sooner available.

470 In addition, differences in H₂ metabolism were observed between self-fermenting Ext-CS and inoculated reactors,
471 which might be explained by the absence of hydrogen consuming bacteria in the self-fermenting Ext-CS. Indeed, H₂
472 consuming bacteria of *Prevotella* genus (Purushe et al. 2010) were found only in the fermenting communities
473 developed after inoculation with AS.

474 Finally, the best result of 141.9 ± 0.9 gCOD/KgVS_i, which corresponded to a yield of 21 g COD/L of total metabolites,
475 was obtained after enzymes addition, which allowed the simultaneous saccharification and fermentation into the
476 reactors. This result is comparable with CS conversion yields obtained by applying different kinds of pre-treatment
477 and bio-conversion processes. Darvekar et al. (Darvekar and Holtzapple 2016) obtained similar results (around 20 g/L
478 acetic acid equivalent) from CS fermentation at 10% of TS, but adopting a time consuming procedure: after applying
479 submerged lime pre-treatment, conducted at 50 °C for 28 days with lime loading of 0.15 g Ca(OH)₂/g dry biomass,
480 followed or not by shock (a mechanical process that uses a shockwave to alter the biomass structure) the fermentation
481 lasted 35 days. A slightly higher conversion yield, equal to 25 g/L of total metabolites, was obtained after 12 days of
482 anaerobic digestion at 22% of TS of grinded CS pre-treated with 5.0% NaOH (Zhu et al. 2010). Beside the different
483 pre-treatment adopted, this better result it may be due to the use of a previously activated inoculum, i.e. the effluent of
484 liquid anaerobic digestion. In our study, the microbial inoculum corresponded to dewatered AS, the dewatering
485 procedures (the use of flocculants coupled with centrifugation) may have somehow made less active the
486 microorganisms, probably the usage of more active microbiome, would gave different results.

487 However, accordingly with the present study, the combination of mechanical treatment with chemical or enzymatic
488 treatment was shown to be the key step to increase the bioconversion of lignocellulosic biomass (Motte et al. 2015a).
489 These authors used SSF as both a pretreatment process and a metabolite production process. After 64 days of
490 fermentation at 23.5% TS with an initial pH of 6.8 of 10 mm ground wheat straw, a substrate conversion into
491 metabolites of 170.7 ± 6.5 gCOD/kgVS was observed, which is a little bit higher than the one obtained in this study
492 (141.9 ± 0.9 gCOD/kgVS) with a different substrate and in a much shorter fermentation (9 days). It could be supposed
493 that, in the present study, extrusion greatly decreased the reaction time. Similarly, Chen et al. (Chen et al. 2014)
494 reported that extrusion of rice straw accelerated the hemicellulose and cellulose hydrolysis and led to higher cellulose
495 and hemicellulose degradation efficiencies, thus contributing to the enhancement of substrate conversion (i.e. into
496 methane in that case). Therefore, the increased production of metabolites, such as ethanol and butyrate (with a
497 concomitant decreased in produced acetate), observed in this study after enzymatic addition could be due to a rapid
498 decrease in pH. Due to the rapid production of acids and the low homogeneity due to the solid state environment, the
499 pH could locally reach very low values, causing a shift in bacteria metabolism to the production of metabolites with
500 no charge (ethanol) or higher carbon content per unit (butyrate).

501
502 In conclusion, a significant effect of an extrusion pretreatment combined with the action of microbial consortia and
503 hydrolytic enzymes was shown in favour to the production of carboxylates from lignocellulosic biomass by SSF. The
504 extrusion, while it did not alter the biochemical composition of CS, caused changes in the physical and morphological
505 characteristics. These changes, such as the increase in surface area and the decrease of water retention capacity, enabled
506 a better action of microbial consortia and a better access to enzymatic hydrolysis, leading to an increase up to 506% of
507 CS conversion into carboxylates. Biotic factors greatly impacted SSF: the use of AS as inoculum, beside increased the
508 total amounts of recovered products, drove the fermentation pathways by stabilizing the community and consequently
509 the spectra of produced metabolites. Nevertheless, it was possible to identify a sort of substrate signature in the
510 communities characterized at the end of fermentation process, by the presence of specific strains or groups of bacteria
511 which were characteristic for each substrate. Further studies are required to clarify the effective impact of the microbial
512 community present on the substrate itself on fermentation at high organic load. Beyond, such extrusion pretreatment
513 could be potentially used for enhancing carboxylates production from solid-state fermentation of CS biomass; however,
514 the choice of an appropriate inoculum and effective hydrolytic enzymes should be taken into account and optimized.

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531

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662

663 **FIGURE CAPTIONS**

664

665 **Fig.1** Pretreatment of corn stover using extrusion.

666

667 **Fig. 2** Granulometry of raw and extruded samples. a) example of images of particles acquired at t0 and
668 after 1h under stirring in water; b) average granulometric curves at different residence time in the reactor
669 assessed using grey level granulometry .

670

671 **Fig. 3** Substrate conversion (gCOD/kgVS_i) after 9 days of SS-fermentation of raw (CS) and extruded (Ext-
672 CS) corn stover in the different conditions tested: self-fermented, inoculated and enzymatic bio-
673 augmented. Only metabolic products of fermentation are presented, unfermented sugars are not taken
674 into account. Average data and standard deviations of replicates are reported.

675

676 **Fig. 4** Time course profiling of cumulative hydrogen (H₂) production during 9 days of SS-fermentation of
677 ground (CS, plain lines) and extruded (Ext CS, dashed lines) corn stover in the differet conditions tested:
678 self-fermented (diamonds), inoculated (triangles) and enzymatic bio-augmented (circles).

679

Table 1: Characteristics of raw and extruded corn stover (CS).

	Raw CS	Extruded CS
Amount of water extractible sugars (mg.g ⁻¹ TS)	22.0 ± 1.7	25.5 ± 0.3
Swelling (ml.g ⁻¹ TS)	17.5 ± 0.5	9.8 ± 0.2
Water retention capacity (g H ₂ O.g ⁻¹ TS)	15.5 ± 0.1	6.8 ± 0.0
Cellulose crystallinity (%)	48.0 ± 3.7	51.2 ± 3.4
Total Solid (TS%)	95.4 ± 0.1	91.2 ± 0.3
Volatile Solid (VS%)	91.2 ± 0.3	87.4 ± 0.5

Values are means ± SD

Table 3. Fermentative microbial communities developed after 9 days of SSF of raw (CS) and extruded (Ext CS) corn stover in the different conditions tested, as well as the ones from inoculum (AS) and substrates: self-fermented, inoculated and enzymatic bio-augmented

CLASS <i>Family, Genus</i>	AS	Substrate		Self-fermented		Inoculated		Enzymatic augmented	
		CS	Ext-CS	CS	Ext-CS	CS	Ext-CS	CS	Ext-CS
BACILLI	1.3%	8.7%	93.2%	62.6%	35.2% ^a	13.4%	10.2%	13.8%	19.0%
<i>Bacillaceae, Bacillus</i>	0.2%	4.1%	71.4%	0.1%	8.9%	0.0%	2.8%	0.0%	3.8%
<i>Enterococcaceae, Enterococcus</i>	0.3%	1.4%	0.3%	57.2%	18.9% ^a	10.8%	4.8%	9.4%	10.4%
<i>Bacillaceae, Lysinibacillus</i>	0.1%	0.0%	18.5% ⁰	0.0%	6.1%	0.0%	2.1%	0.0%	2.2%
<i>Lactobacillaceae, Lactobacillus</i>	0.3%	0.0%	0.0%	5.1%	0.4%	1.0%	0.0%	1.0%	1.6%
<i>other Bacilli</i>	0.4%	3.2%	3.1%	0.1%	0.9%	1.6%	0.4%	3.3%	1.1%
GAMMAPROTEOBACTERIA	12.5%	17.1%	6.7%	35.9%	0.4%	10.7%	3.4%	20.3%	1.6%
<i>Enterobacteriaceae, Cronobacter</i>	0.0%	4.0%	5.2%	24.1%	0.0%	0.0%	0.0%	0.1%	0.0%
<i>Enterobacteriaceae, Enterobacter</i>	0.3%	3.5%	1.5%	11.5%	0.4%	6.4%	1.7%	14.1%	1.4%
<i>Enterobacteriaceae, Escherichia-Shigella</i>	0.1%	0.0%	0.0%	0.0%	0.0%	3.8%	1.5%	5.6%	0.1%
<i>others Gammaproteobacteria</i>	12.2%	9.5%	0.0%	0.2%	0.0%	0.5%	0.2%	0.5%	0.1%
CLOSTRIDIA	3.2%	0.4%	0.1%	0.2%	39.1%	49.9%	55.1%	37.9%	33.8%
<i>Ruminococcaceae, Caproiciproducens</i>	0.8%	0.0%	0.0%	0.1%	22.0%	20.7%	51.3%	31.2%	18.5%
<i>Ruminococcaceae, Ruminiclostridium</i>	0.0%	0.0%	0.0%	0.0%	0.0%	25.7%	0.0%	0.0%	0.0%
<i>Clostridiaceae, Clostridium</i>	0.3%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	10.9%
<i>Lachnospiraceae</i>	0.0%	0.0%	0.0%	0.0%	7.4%	0.2%	0.1%	0.0%	0.0%
<i>other Clostridia</i>	2.1%	0.4%	0.1%	0.0%	9.8%	3.2%	3.7%	6.7%	4.5%
BACTEROIDIA	1.7%	0.0%	0.0%	0.1%	0.0%	20.0%	25.4%	19.4%	22.1%
<i>Prevotellaceae, Prevotella</i>	1.2%	0.0%	0.0%	0.1%	0.0%	8.0%	19.6%	17.8%	22.1%
<i>other Bacteroidia</i>	0.6%	0.0%	0.0%	0.0%	0.0%	12.0%	5.8%	1.6%	0.0%
CORIOBACTERIA	0.9%	0.0%	0.0%	0.1%	17.0%	0.5%	0.4%	0.7%	15.3%
<i>Coriobacteriaceae, Collinsella</i>	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	12.9%
<i>Coriobacteriaceae, Atopobium</i>	0.1%	0.0%	0.0%	0.0%	16.3%	0.4%	0.1%	0.2%	0.4%
<i>others Coriobacteriia</i>	0.1%	0.0%	0.0%	0.0%	0.6%	0.1%	0.3%	0.3%	2.0%
ALPHAPROTEOBACTERIA	20.5%	38.7%	0.0%	0.9%	0.6%	1.3%	0.9%	1.2%	0.5%
ACTINOBACTERIA	13.4%	13.8%	0.0%	0.1%	7.6%	1.6%	1.7%	1.7%	5.7%
BETAPROTEOBACTERIA	12.8%	6.6%	0.0%	0.1%	0.0%	0.9%	0.4%	0.5%	0.2%
ACIDIMICROBIA	11.1%	0.4%	0.0%	0.0%	0.0%	0.7%	0.9%	1.0%	0.6%
SPHINGOBACTERIA	6.2%	6.3%	0.0%	0.1%	0.0%	1.1%	0.4%	0.1%	0.1%
<i>other bacteria classes</i>	16.4%	8.0%	0.0%	0.0%	0.0%	0.0%	1.3%	3.4%	1.0%
N° copies 16S/g VS	1.74E+10	5.68E+08	4.52E+10	2.14E+09	1.12E+10	7.69E+10	8.27E+10	4.51E+10	6.45E+10
Shannon Diversity (H)	5.42	4.34	1.36	1.22	2.34	2.96	2.51	3.23	3.02
Simpson Diversity (1-D)	0.99	0.98	0.58	0.60	0.88	0.89	0.79	0.89	0.92

Bacterial classes are represented in bold characters, families are in normal characters and genera are in italic. Only the species with relative abundance higher than 5% in at least one sample are presented. **Bold text** is used for the species with abundance higher than 10% and **bold emphasized text** for the species with abundance higher than 50%.

Table 2. COD composition and pH after 9 days of SSF of raw and extruded corn stover.

CONDITION	self-fermented		inoculated		enzymes addition	
	no		yes		yes	
enzymes	no		no		yes	
Substrate	CS	Ext CS	CS	Ext CS	CS	Ext CS
Soluble Sugars	2.9 ± 0.1	1.8 ± 0.2	0	0	0	0
Lactate	10.3 ± 0.1	2.7 ± 0.1	0	0	0	0
Acetate	7.0 ± 0.9	14.4 ± 0.6	16.9 ± 0.8	28.2 ± 0.7	35.6 ± 0.0	47.2 ± 0.7
Propionate	2.0 ± 0.2	5.0 ± 0.2	8.2 ± 0.5	8.7 ± 0.2	10.2 ± 0.0	10.6 ± 0.1
Ethanol	0	10.4 ± 0.4	1.2 ± 1.7	2.6 ± 0.1	8.6 ± 0.2	31.6 ± 0.3
Butyrate	0	16.6 ± 0.4	6.9 ± 0.4	11.5 ± 0.6	27.4 ± 0.6	43.7 ± 0.2
Valerate	0	0	2.4 ± 0.2	3.3 ± 0.2	3.4 ± 0.2	3.3 ± 0.2
Caproate	0	0	0	2.7 ± 0.1	4.1 ± 0.4	4.9 ± 0.6
Other Metabolites*	1.2 ± 0.1	0	2.0 ± 0.7	2.8 ± 0.1	1.5 ± 0.8	0
Total SCOD	23.4 ± 3.7	50.9 ± 0.5	37.6 ± 0.5	59.8 ± 2.0	90.8 ± 1.9	141.9 ± 0.9
pH _f	5.4 ± 0.1	4.9 ± 0.0	5.4 ± 0.0	4.8 ± 0.0	5.0 ± 0.0	4.5 ± 0.2

Soluble COD (SCOD) composition (gCOD/kgVS_i) and pH after 9 days of SSF of raw (CS) and extruded (Ext CS) corn stover in the different conditions tested: self-fermented, inoculated and enzymatic addition. Values are means ± Standard deviation. *sum of succinate + iso-butyrate + iso-valerate

Figure 1

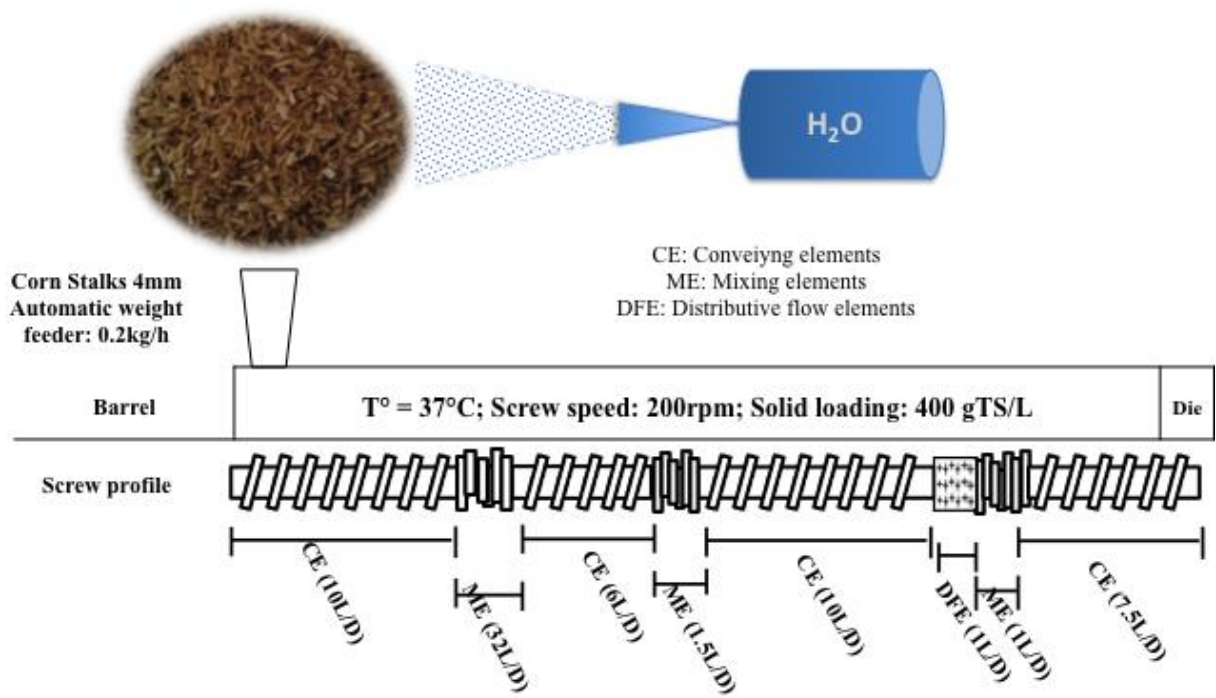


Figure2

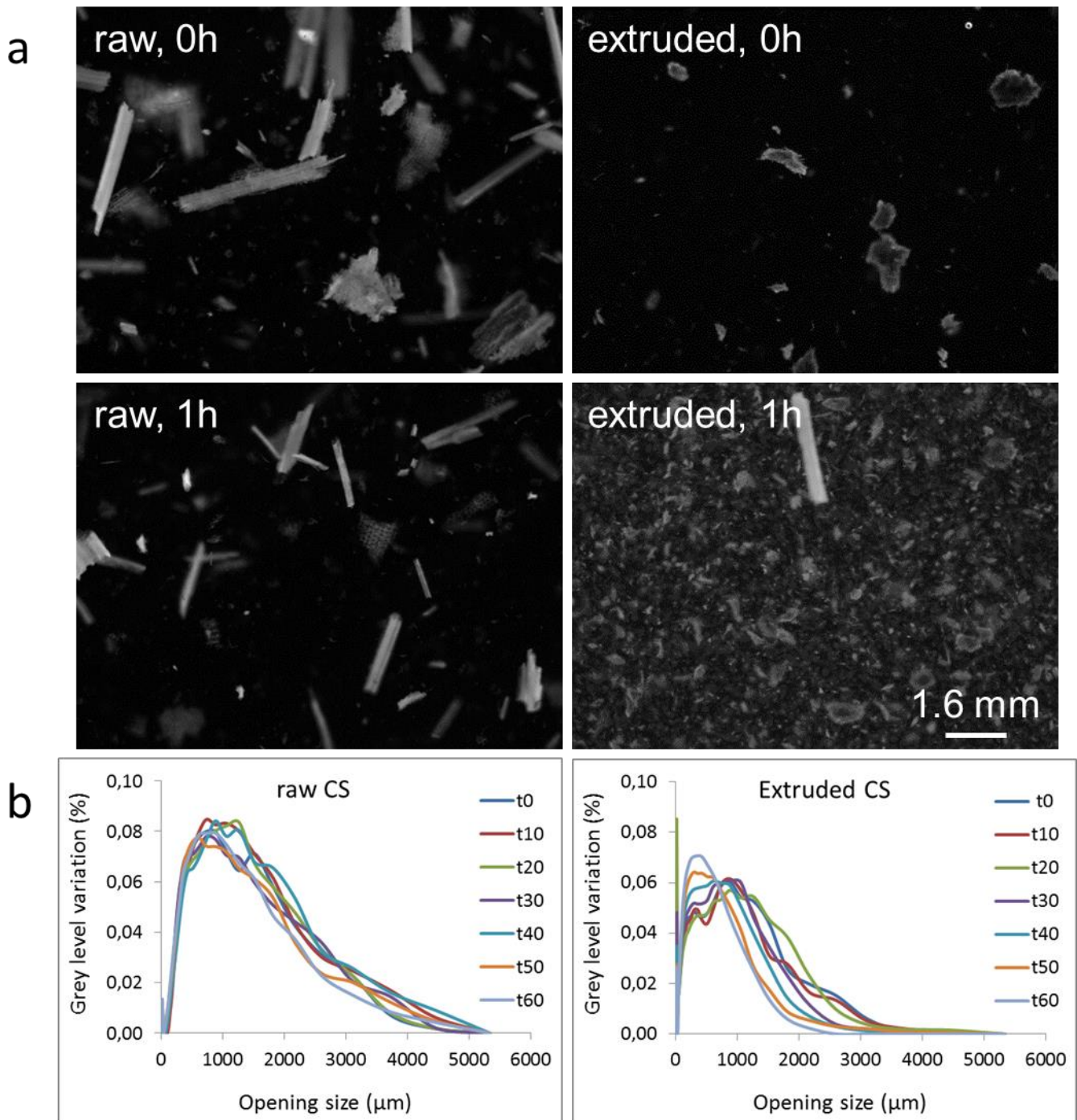


Figure3

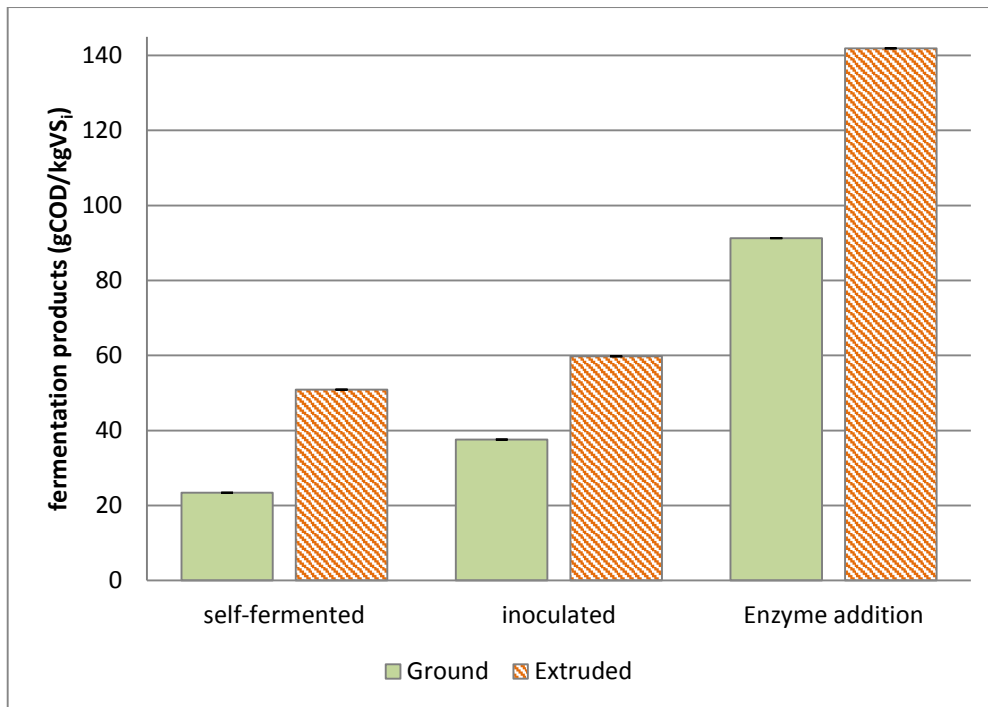


Figure 4

