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1 **Effect of enzyme addition on fermentative hydrogen production from wheat**  
2 **straw**

3

4

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19 **Abstract**

20

21 Wheat straw is an abundant agricultural residue which can be used as raw material to produce  
22 hydrogen (H<sub>2</sub>), a promising alternative energy carrier, at a low cost. Bioconversion of  
23 lignocellulosic biomass to produce H<sub>2</sub> usually involves three main operations: pretreatment,  
24 hydrolysis and fermentation. In this study, the efficiency of exogenous enzyme addition on  
25 fermentative H<sub>2</sub> production from wheat straw was evaluated using mixed-cultures in two  
26 experimental systems: a one-stage system (direct enzyme addition) and a two-stage system  
27 (enzymatic hydrolysis prior to dark fermentation). H<sub>2</sub> production from untreated wheat straw  
28 ranged from 5.18 to 10.52 mL-H<sub>2</sub>.g-VS<sup>-1</sup>. Whatever the experimental enzyme addition  
29 procedure, a two-fold increase in H<sub>2</sub> production yields ranging from 11.06 to 19.63 mL-H<sub>2</sub>.g-  
30 VS<sup>-1</sup> was observed after enzymatic treatment of the wheat straw. The high variability in H<sub>2</sub>  
31 yields in the two step process was explained by the consumption of free sugars by indigenous  
32 wheat straw microorganisms during enzymatic hydrolysis. The direct addition of exogenous  
33 enzymes in the one-stage dark fermentation stage proved to be the best way of significantly  
34 improving H<sub>2</sub> production from lignocellulosic biomass. Finally, the optimal dose of enzyme  
35 mixture added to the wheat straw was evaluated between 1 to 5 mg-protein.g-raw wheat  
36 straw<sup>-1</sup>.

37

38 **Keywords:** biohydrogen, wheat straw, enzymatic hydrolysis, lignocellulosic material, mixed-  
39 cultures, dark fermentation

## 40 **1. Introduction**

41

42 Hydrogen (H<sub>2</sub>) is a key molecule of present and future energetic systems. Currently, H<sub>2</sub> is a  
43 commercially important molecule since large quantities of H<sub>2</sub> are needed in the petroleum and  
44 chemical industries (e.g. processing of fossil fuels, production of ammonia). Because H<sub>2</sub> is  
45 energy dense and clean burning, it is also considered to be a promising energy carrier to  
46 replace fossil fuels. However, H<sub>2</sub> is not readily available in sufficient amounts and its  
47 production cost is still high for transportation purposes. The technical challenges to achieve a  
48 stable H<sub>2</sub> economy include improving process efficiencies, lowering the production cost and  
49 harnessing renewable sources for H<sub>2</sub> production.

50 Biological H<sub>2</sub> production by anaerobic bacteria in dark fermentation systems has gained  
51 increased attention in recent years because of high production rates, process simplicity and the  
52 utilization of low-value waste as feed material. Due to its wide abundance and distribution  
53 throughout the world, lignocellulosic biomass is considered to be one of the most attractive  
54 and low-cost feedstocks in H<sub>2</sub> production. Wheat straw has a great potential, among other  
55 lignocellulosic residues, all over Europe because of wide availability and low cost. Although  
56 it has traditionally been used for pulp and paper making, wheat straw is now one of the most  
57 important feedstocks for biological ethanol production, and by extension it can also be  
58 considered as an adapted feedstock for H<sub>2</sub> production.

59 Usually the bioconversion of lignocellulosic waste to H<sub>2</sub> requires pretreatment and hydrolysis  
60 of the biomass prior to dark fermentation. H<sub>2</sub> yields varied greatly depending not only on the  
61 type of pretreatment/hydrolysis process, but also on the quality of the substrates (e.g. wheat  
62 straw, poplar leaves, corn stover) and the starting inoculum, either bacterial consortia (e.g.  
63 anaerobic digested sludge) or pure cultures of mesophilic (e.g. *Clostridium butyricum*) and  
64 thermophilic bacteria (e.g. *Caldicellulosiruptor saccharolyticus*). Several pretreatment

65 methods have been used to improve hydrolysis yields and H<sub>2</sub> production yields. These  
66 pretreatments are required to alter the complex structure of lignocellulosic material that is  
67 composed of cellulose (40–50%), hemicelluloses (25–35%) and lignin (15–20%). Hydrolysis  
68 has been shown to be the rate-limiting step in biomass conversion to bioenergy. The  
69 pretreatments make the main constituents, cellulose and hemicellulose, more accessible to  
70 enzymes that convert carbohydrate polymers into monosaccharides. They are classified into  
71 four groups: physical (e.g. mechanical disruption), chemical (e.g. alkali, dilute acid), thermal  
72 (e.g. steam explosion) and biological (e.g. lignin degradation by white rot fungi) treatments.  
73 The major drawback of the most-used dilute sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) pretreatment is the  
74 formation of undesirable by-products such as aliphatic acids, furan derivatives or phenol  
75 compounds which inhibit the action of enzymes and/or further reduce anaerobic treatment of  
76 the hydrolyzates [20, 21]. In the case of complex anaerobic consortia, H<sub>2</sub> production by dark  
77 fermentation was shown to be more influenced than methane production [22, 23]. The  
78 concentration and diversity of these toxic compounds vary according to the type of raw  
79 material and to pretreatment conditions.

80 In addition, enzymatic hydrolysis presents several advantages over chemical hydrolysis, such  
81 as lower equipment costs, higher monosaccharide conversion yields and lower release of  
82 inhibitory compounds. Cellulases and hemicellulases play an important role in the hydrolysis  
83 of cellulose and hemicellulose, respectively. The efficiency of such enzymatic hydrolysis has  
84 been shown to depend on various factors, such as substrate, environmental conditions (e.g.  
85 temperature, pH) and biomass physico-chemical pretreatments. Furthermore, commercial  
86 enzyme cocktails are often not effective when used alone. The impact of the addition of  
87 exogenous enzyme on fermentative H<sub>2</sub> production has not received much investigation,  
88 despite being widely studied regarding bioethanol production or anaerobic digestion.  
89 Nevertheless, enzymatic pretreatment has been recently found to be more effective than acid

90 pretreatment in improving H<sub>2</sub> production from poplar leaves. According to our knowledge,  
91 the different methods of enzyme addition to dark fermentation systems have been poorly  
92 studied.

93 The aim of this study is to determine the impact of an undefined enzyme mixture (CEP) on  
94 fermentative H<sub>2</sub> production from wheat straw. Firstly, the efficiency of the CEP enzyme  
95 mixture in improving the conversion of wheat straw cellulosic and hemicellulosic compounds  
96 to glucose and xylose was investigated. Next, the impact of two enzyme addition procedures  
97 was evaluated in a one-stage system (direct enzyme addition) and a two-stage system  
98 (enzymatic hydrolysis prior to dark fermentation). The effect of the enzyme dose on H<sub>2</sub>  
99 production was finally assessed.

100

## 101 **2. Materials and methods**

102

### 103 **2.1. Raw materials**

104

105 Wheat straw (*Triticum aestivum*) was harvested in France (Ardissan) during summer 2010,  
106 milled to reach a particle size of between 5 and 10 mm, and then stored at room temperature  
107 in a dry place. The basic chemical composition of the wheat straw is given in Table 1. Sterile  
108 (120°C, 20 min) or non-sterile wheat straw was used for enzymatic hydrolysis.

109

### 110 **2.2. Enzymatic mixture**

111

112 Among the proprietary enzyme mixtures developed by Protéus and IFPEN, CEP was selected  
113 for its ability to degrade lignocellulosic biomass. This enzymatic cocktail is secreted by an

114 engineered *Trichoderma* strain and shows optimal pH and temperature of 4.8 and 50°C,  
115 respectively. The cocktail contains enzymatic system transforming efficiently cellulosic  
116 polymers to glucose as well as hemicellulosic activities.

117

### 118 **2.3. Enzymatic hydrolysis**

119

120 Enzymatic hydrolysis was performed in 100-mL erlenmeyer flasks containing 5 mg of  
121 proteins and 1 g of wheat straw in a total liquid volume of 20 mL of distilled water at pH 5.5.  
122 The flasks were shaken at 100 rpm and incubated at 37°C (the optimal temperature for H<sub>2</sub>  
123 production using the selected mesophilic microbial inoculum) or 50°C (the optimal  
124 temperature for CEP enzyme mixture activity – data not shown), for 24-50 hours. Hydrolysis  
125 efficiency was assessed by measuring the total sugar content in the liquid phase. For this,  
126 samples of 1 mL were periodically collected, centrifuged, filtered through 0.2 µm filters and  
127 kept at -20°C before chemical analysis. Control tests were carried out under the same  
128 conditions with no enzyme addition.

129

### 130 **2.4. Hydrogen production in batch tests**

131

132 H<sub>2</sub> production experiments were carried out in 500 mL glass bottles under discontinuous  
133 batch conditions. Mesophilic anaerobically-digested sludge pretreated by heat-shock  
134 treatment (90°C, 10 min) was used as *inoculum*. The bacterial consortium was composed  
135 mainly of clostridial species. Two milliliters of the pretreated inoculum (final concentration of  
136 250 mg-COD.L<sup>-1</sup>) were added to a culture medium containing 40 mM of 2-(N-  
137 morpholino)ethanesulfonic acid (MES) buffer, with a final working volume of 200 mL.  
138 Sterile or non-sterile wheat straw hydrolyzates (20 mL) as well as 1 g of non-sterile

139 unhydrolyzed wheat straw were used as substrate. The initial pH was adjusted to 5.5 (pH  
140 previously determined as optimum for H<sub>2</sub> production). All batch tests were performed in  
141 triplicate. After inoculation, each bottle was flushed for 5 minutes with nitrogen gas to  
142 establish anaerobic conditions. The bottles were then capped with a rubber stopper and  
143 incubated at 37°C for 10 days. Two milliliters of the mixed cultures were periodically  
144 collected and then centrifuged (20,000 g, 10 min). The supernatants were stored at -20°C for  
145 further chemical analysis.

146

## 147 **2.5. Chemical analysis**

148

149 The total solid (TS) content was measured by weighing samples in triplicate before and after  
150 drying at 105 °C for 12 h in an oven. The volatile solid (VS) content was determined after  
151 burning samples at 550 °C for 2h. The cellulose and hemicellulose fractions corresponded to a  
152 chromatographic quantification of the sugars contained in the hydrolyzate after acid  
153 hydrolysis of the raw material. Samples (300 mg) were hydrolyzed with 12 M H<sub>2</sub>SO<sub>4</sub> acid for  
154 2 h at room temperature, then diluted to reach a final acid concentration of 1.5 M, and kept at  
155 100°C for 3 h. All samples were then centrifuged (20,000 g, 10 min) and filtered (0.20 µm)  
156 prior to their analysis by high performance liquid chromatography (HPLC) and refractometric  
157 detection (Waters R410). The components were separated in an Aminex HPX-87H, 300 x 7.8  
158 mm column (Biorad) with an isocratic elution solution of 0.005 M H<sub>2</sub>SO<sub>4</sub>. The column  
159 temperature was maintained at 35°C, and the flow rate at 0.4 mL.min<sup>-1</sup>. From this, cellulose  
160 concentration was expressed in glucose equivalent and hemicellulose concentration  
161 corresponded to the sum of xylose, arabinose, glucuronic acid and galacturonic acid contents.  
162 Acid-insoluble lignin was measured by weighing the hydrolysis residues after overnight  
163 drying at 105°C.



164 Biogas volume was periodically measured using an acidified water displacement method.  
165 Biogas composition (CH<sub>4</sub>, CO<sub>2</sub>, H<sub>2</sub> and N<sub>2</sub>) was analyzed using a gas chromatograph (Clarus  
166 280, Perkin Elmer) equipped with a column HayeSep Q and a molecular sieve. Operating  
167 conditions were as follows: the carrier gas was argon at a pressure of 102 kPa and a flow rate  
168 of 4.5 mL.min<sup>-1</sup>; temperatures of the injector and the detector were both fixed at 150 °C.  
169 Volatile fatty acid (VFA) composition in the liquid phase, *i.e.* acetic (C2), propionic (C3),  
170 butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6)  
171 acids was determined in a gas chromatograph (GC-3900, Varian) equipped with a flame  
172 ionization detector. The concentration of sugars (glucose, xylose, arabinose, glucuronic acid  
173 and galacturonic acid) and non-VFAs metabolic byproducts, such as organic acids (lactate) or  
174 ethanol were measured by HPLC analysis.

175 In order to determine the parameters of H<sub>2</sub> production, the cumulative H<sub>2</sub> production data of  
176 each test was fitted to a modified Gompertz equation (Equation 1) as previously described by  
177 Quéméneur et al..

$$178 \quad H(t) = P \cdot \exp \left\{ -\exp \left[ \frac{R_m \cdot e}{P} (\lambda - t) + 1 \right] \right\} \quad (1)$$

179 Where  $P$  is the maximal cumulated H<sub>2</sub> production (mL),  $R_m$  is the maximum H<sub>2</sub> production  
180 rate (mL-H<sub>2</sub>.day<sup>-1</sup>),  $\lambda$  is lag-phase time (day),  $t$  is the incubation time (day) and  $e$  is exp(1).  
181 The values of  $P$ ,  $R_m$  and  $\lambda$  were estimated using a non-linear regression algorithm developed  
182 in Matlab (version 6.5, MathWorks®). The H<sub>2</sub> production yield was calculated by dividing  
183 the maximal H<sub>2</sub> production ( $P$ ) by the weight of the substrate sample in gram of VS.

184

### 185 **3. Results and discussion**

186

#### 187 **3.1. Characterization of the CEP enzymatic activity on the saccharification process of** 188 **wheat straw**

189

### 190 **3.1.1. Enzymatic saccharification of non-sterile wheat straw**

191

192 At 50 °C, the addition of CEP enzyme mixture to wheat straw improved the glucose yield by  
193 a factor of 2.3 ( $27 \pm 6$  mg-glucose.g-VS<sup>-1</sup> versus  $11.9 \pm 0.4$  mg-glucose.g-VS<sup>-1</sup> in the  
194 controls) (Figure 1A). No effect of CEP addition was observed on the xylose yield (Figure  
195 1A). Maximal glucose concentration was reached at 8 hours and then decreased until 30  
196 hours concomitantly with an increase in acetate and lactate concentrations (Figure 1B).  
197 Interestingly, no significant differences in acetate and lactate contents were observed with or  
198 without addition of the enzyme mixture. On average, the acetate concentration increased from  
199  $12.7 \pm 0.2$  mg.g-VS<sup>-1</sup> at 8 hours to  $17 \pm 1$  mg.g-VS<sup>-1</sup> after 24 hours. Meanwhile, the lactate  
200 concentration increased from  $5.4 \pm 0.4$  mg.g-VS<sup>-1</sup> to  $26 \pm 9$  mg.g-VS<sup>-1</sup>. These results  
201 suggested that the sugars released were immediately used as a carbon source by indigenous  
202 microorganisms of the wheat straw.

203

### 204 **3.1.2. Enzymatic saccharification of sterile wheat straw**

205

206 To avoid contamination by indigenous microorganisms, an enzymatic hydrolysis test was then  
207 performed after sterilization of the wheat straw.

208 At 50 °C, the addition of the enzyme mixture to the sterile wheat straw improved the glucose  
209 yield by a factor of 6 after 30 hours of hydrolysis ( $41 \pm 7$  mg-glucose.g-VS<sup>-1</sup> versus  $7 \pm 1$  mg-  
210 glucose.g-VS<sup>-1</sup> in the controls) (Figure 2A). As previously observed in non-sterile wheat  
211 straw, no significant effect of the enzyme mixture was observed on the xylose yield.  
212 However, no decrease in glucose and xylose concentrations, as well as no variation in acetate

213 and lactate concentrations were detected during the hydrolysis of the wheat straw after  
214 sterilization (Figure 2B).

215 At 37°C, the addition of CEP enzyme mixture significantly increased the glucose yield by a  
216 factor of 3.4 after 24 hours of hydrolysis ( $31.4 \pm 5.9$  mg-glucose.g-VS<sup>-1</sup> instead of  $9.2 \pm 4.0$   
217 mg-glucose.g-VS<sup>-1</sup> in controls) (Figure 3). As expected, the decrease in temperature from  
218 50°C to 37°C reduced the enzymatic hydrolysis yield. Contrary to the observations made at  
219 50°C, the glucose yield reached a steady state level within a time period of 24 to 50 hours  
220 (Figure 3). Consistently with the results obtained at 50°C, no improvement of the xylose yield  
221 was observed in presence of the CEP enzyme mixture ( $34 \pm 5$  mg-xylose.g-VS<sup>-1</sup> instead of  $31$   
222  $\pm 2$  mg-xylose.g-VS<sup>-1</sup> in controls).

223 To summarize, whatever the conditions, the CEP enzyme mixture used in this study exhibited  
224 a mainly cellulosic activity with significant glucose release from wheat straw. However, a  
225 sterilization step was required to avoid immediate glucose and xylose degradation by  
226 indigenous bacteria. Despite a significant increase in glucose conservation after the enzymatic  
227 attack, this wheat straw sterilization step is energy consuming and therefore not economically  
228 feasible for industrial purposes. Nevertheless, taking into account the efficient glucose yield  
229 obtained at 37°C, our results suggested that a direct addition of the CEP enzyme mixture to  
230 non-sterile wheat straw in a one-stage dark fermentation system could be recommended for  
231 concomitant release and degradation of the monomeric units. Such an approach was also  
232 described as being more attractive and having a higher simplicity for implementation than two  
233 step processes.

234

## 235 **3.2. Effect of the method for enzyme addition on hydrogen production from wheat straw**

236

### 237 **3.2.1. Hydrogen production from wheat straw by mixed-cultures**

238

239 The biogas produced from wheat straw by anaerobic mixed-cultures contained only H<sub>2</sub> and  
240 CO<sub>2</sub>, with no detectable CH<sub>4</sub>, indicating that the heat shock pretreatment had effectively  
241 removed the methanogens initially present in the anaerobic inoculum. Figure 4 shows the  
242 cumulative H<sub>2</sub> production over 10 days of incubation. The data presented in Figure 4 fitted  
243 well to the Gompertz equation (1), with determination coefficients R<sup>2</sup> over 0.98 on average  
244 (Table 2).

245 When no enzyme was added to the wheat straw, the H<sub>2</sub> yields ranged from 5.18 to 10.52 mL-  
246 H<sub>2</sub>.g-VS<sup>-1</sup> (Table 2). These H<sub>2</sub> yields were greater or equal to those reported in the literature  
247 from batch tests using untreated wheat straw as substrate, i.e. from 1 to 6.4 mL-H<sub>2</sub>.g-VS<sup>-1</sup>.  
248 This result indicates that the heat-treated mesophilic microbial inoculum was able to produce  
249 H<sub>2</sub> from raw lignocellulosic material.

250

### 251 **3.2.2. One-stage versus two-stage dark fermentation systems**

252

253 The effect of the addition of the CEP enzyme mixture (at 5 mg-protein.g-wheat straw<sup>-1</sup>) on  
254 fermentative H<sub>2</sub> production from sterile and non-sterile wheat straw was tested under  
255 mesophilic conditions in two systems: (i) in one-stage dark fermentation batch tests where  
256 CEP enzyme mixture was directly added and (ii) in two stage tests where an enzymatic  
257 hydrolysis of 24 hours was performed prior to the dark fermentation batch tests.

258

#### 259 **3.2.2.1. Hydrogen production from wheat straw in one-stage systems**

260

261 Direct CEP enzyme addition to non-sterile wheat straw (one-stage system) significantly  
262 increased the H<sub>2</sub> production yields by a factor of 2 (19.63 mL-H<sub>2</sub>.g-VS<sup>-1</sup> vs 10.52 mL-H<sub>2</sub>.g-

263 VS<sup>-1</sup> in the controls with no enzyme addition) (Table 2). Thus, the tested enzyme mixture  
264 effectively enhanced the H<sub>2</sub> yield from wheat straw, probably through the release of  
265 additional carbohydrate units, such as glucose, in the medium. This result is consistent with  
266 the observations of Cui et al. who reported a significant increase in H<sub>2</sub> yield from poplar  
267 leaves with enzymatic pretreatment using 2% Vicozyme L (Novozyme).

268

### 269 **3.2.2.2. Hydrogen production from wheat straw in two-stage systems**

270

271 A two-fold increase in the H<sub>2</sub> production yields (11.06 mL-H<sub>2</sub>.g-VS<sup>-1</sup> vs 5.18 mL-H<sub>2</sub>.g-VS<sup>-1</sup> in  
272 controls) was also observed in the two-stage systems where an enzymatic hydrolysis of 24  
273 hours was performed on non-sterile wheat straw (Table 2). However, these H<sub>2</sub> yields were  
274 significantly lower than those obtained under the same conditions using sterile wheat straw.  
275 Interestingly, these H<sub>2</sub> yields were statistically similar to the controls performed in one-stage  
276 dark fermentation (Table 2). Assuming that the H<sub>2</sub> potential of xylose is about the same of  
277 glucose (i.e. 160 mL-H<sub>2</sub>.g-glucose<sup>-1</sup>) (data not shown), a first approximation of the two-stage  
278 H<sub>2</sub> potential can be assessed from the amount of glucose and xylose released during  
279 enzymatic hydrolysis (1st stage). It was estimated at 5.5 mL-H<sub>2</sub> g-VS<sup>-1</sup> in sterile wheat straw  
280 controls from 6.8 mg-glucose.g-VS<sup>-1</sup> and 27.0 mg-xylose.g-VS<sup>-1</sup> (Figure 2A). In contrast, it  
281 was estimated at 1.92 mL-H<sub>2</sub> g-VS<sup>-1</sup> in non-sterile wheat straw controls (from 1.0 mg-  
282 glucose.g-VS<sup>-1</sup> and 13.0 mg-xylose.g-VS<sup>-1</sup> (Figure 1A). The difference between these two  
283 estimated values (3.58 mL-H<sub>2</sub>.g-VS<sup>-1</sup>) is in agreement with the measured H<sub>2</sub> yield difference  
284 obtained between sterile and non-sterile wheat straw (3.63 mL-H<sub>2</sub>.g-VS<sup>-1</sup>). The decrease in  
285 H<sub>2</sub> yields from sterile to non-sterile wheat straw in the two-stage systems was therefore  
286 explained by a direct and almost total consumption of the sugars released during the  
287 enzymatic hydrolysis step by indigenous microorganisms. In fact, the consumption of sugars

288 simultaneously increased the acetate and lactate levels which cannot be further reused in  
289 fermentative pathways for H<sub>2</sub> production, as shown previously (Figure 1B).

290 To summarize, both methods of enzyme addition increased the H<sub>2</sub> production yield by a  
291 factor of 2 (Table 2). However, no significant difference in the H<sub>2</sub> production yields was  
292 obtained between direct enzyme addition to non-sterile wheat straw ( $19.63 \pm 1.21$  mL-H<sub>2</sub>.g-  
293 VS<sup>-1</sup>) and enzymatic pretreatment of sterile wheat straw ( $18.13 \pm 1.57$  mL-H<sub>2</sub>.g-VS<sup>-1</sup>) (Figure  
294 4). These results showed that wheat straw sterilization and the separated hydrolysis step (two-  
295 stage system) before dark fermentation is not advantageous in comparison to direct addition  
296 of the CEP enzyme mixture. In contrast, Lo et al. reported that a similar two-stage system  
297 consisting of a hydrolytic step of xylan followed by a dark fermentation stage with *C.*  
298 *butyricum* CGS5 exhibited higher H<sub>2</sub> production efficiency than a one-stage system with  
299 simultaneous saccharification and dark fermentation. The authors attributed the differences in  
300 performance to the operation temperature (37 °C) used in the one-stage system which was not  
301 optimal for xylan hydrolysis. Despite the same temperature constraints, our results reveal that  
302 the one-stage system is more favourable than the two-stage system due to higher H<sub>2</sub>  
303 production efficiency, higher simplicity, and lower operational cost.

304

### 305 **3.2.3. Main metabolic pathways in both one- and two- stage dark fermentation systems**

306

307 As expected, the H<sub>2</sub> production yields were strongly related to acetate (Pearson's product-  
308 moment correlation  $r = 0.93$ ,  $p < 0.01$ ) and butyrate accumulation (Pearson's  $r = 0.89$ ,  $p < 0.05$ ).

309 Moreover, the highest H<sub>2</sub> yield matched with the highest yields of acetate and butyrate (Table  
310 2). The abundance of butyrate as well as acetate is a typical characteristic of acid fermentation  
311 of carbohydrate-rich substrates [4]. Similarly, Fan et al. reported that acetate and butyrate are  
312 dominant VFAs when wheat straw is used as a substrate for mesophilic H<sub>2</sub> production. The

313 production of butyrate and acetate as main fermentation end-products also suggested that  
314 clostridial species were the dominant bacteria in the mixed cultures. In addition, the  
315 butyrate/acetate ratio has been suggested as a useful indicator for monitoring H<sub>2</sub> production in  
316 dark fermentation processes (Han and Shin, 2004). However, in our study, no correlation was  
317 observed between the H<sub>2</sub> yields and the butyrate/acetate ratios (Pearson's  $r = 0.47$ ,  $p > 0.05$ )  
318 (Table 2).

319 Furthermore, the same acetate and butyrate yields were found in the case of direct enzyme  
320 addition to non-sterile wheat straw as well as after enzymatic pretreatment of sterile wheat  
321 straw: an acetate yield of 32.2 mg-acetate.g-VS<sup>-1</sup> was measured for sterile wheat straw treated  
322 by CEP in a two-stage system, and a similar value of 31.5 mg-acetate.g-VS<sup>-1</sup> was obtained  
323 after direct enzyme addition (Table 2). This result confirms that the separated hydrolysis step  
324 and/or sterilization had no beneficial effect on H<sub>2</sub> production yields when compared to the  
325 one-stage dark fermentation system.

326

### 327 **3.3. Effect of enzyme dose on hydrogen production performances from wheat straw**

328

329 The impact of enzyme dose on cumulative H<sub>2</sub> production performances was also evaluated in  
330 additional one-stage fermentation batch tests with a gradient of CEP enzyme mixture  
331 concentration: 0, 1, 5 and 10 mg-protein.g-wheat straw<sup>-1</sup>.

332

#### 333 **3.3.1. Effect of enzyme dose on hydrogen production**

334

335 Whatever the tested concentrations, CEP enzyme addition led to a significant increase in  
336 cumulated H<sub>2</sub> production (Figure 5). The Gompertz model provided a good fit with the

337 cumulated H<sub>2</sub> production data as shown in Figure 5, with determination coefficients R<sup>2</sup> over  
338 0.98 (Table 2).

339 The higher the enzyme dose, the greater was the H<sub>2</sub> production rate. Indeed, the highest H<sub>2</sub>  
340 production rates (17.91 ± 0.91 mL-H<sub>2</sub>.day<sup>-1</sup>) were obtained for 10 mg-protein.g-wheat straw<sup>-1</sup>.  
341 However, with a value of 6.51 ± 2.04 mL-H<sub>2</sub>.day<sup>-1</sup>, a dose of 1 mg of enzyme did not  
342 significantly increase the H<sub>2</sub> production rate compared to the controls (8.30 ± 0.40 mL-  
343 H<sub>2</sub>.day<sup>-1</sup>). The H<sub>2</sub> production potential increased gradually from 9.21 to 18.91 mL-H<sub>2</sub> with an  
344 increase in enzyme concentration (Table 2). Although positive effects of enzyme mixture  
345 loading were observed on H<sub>2</sub> yield (Figure 6), no significant linear correlation was obtained  
346 between H<sub>2</sub> yield and the enzyme mixture concentration (Pearson's r = 0.89, p>0.05). The  
347 increments in H<sub>2</sub> production rate were lower when enzyme concentration exceeded 5 mg-  
348 protein.g-wheat straw<sup>-1</sup> (Figure 6). Indeed, H<sub>2</sub> production differences ranged from 4.94 to  
349 0.33 mL-H<sub>2</sub>.mg-additional protein<sup>-1</sup> while enzyme mixture concentrations increased  
350 respectively from 1 to 10 mg-protein.g-wheat straw<sup>-1</sup>.

351

### 352 **3.3.2. Effect of enzyme dose on metabolites**

353

354 Whatever the enzyme dose, the predominant VFA present in the one-stage systems was  
355 butyrate, followed by acetate (Table 2). As observed in the H<sub>2</sub> yields, the acetate yields  
356 increased from 12.8 to 37.9 mg.g-VS<sup>-1</sup> as enzyme mixture concentration increased. Butyrate  
357 levels followed a similar pattern to that of acetate: the higher the dose, the greater was the  
358 butyrate concentration. The highest butyrate yield observed with 10 mg-protein.g-wheat  
359 straw<sup>-1</sup> was related to the highest H<sub>2</sub> yield. Butyrate level was 3.3 times greater than in the  
360 control for this amount of added enzyme. However, no linear correlation was observed  
361 between the H<sub>2</sub> and the butyrate/acetate ratio (Bu/Ac) (Pearson's r = 0.41, p>0.05) (Table 2).



362 Furthermore, no significant difference in Bu/Ac ratios, ranging between 1.27 and 1.56, were  
363 detected according to the enzyme load (Table 2), suggesting that no metabolic switches  
364 occurred depending only on changes in enzyme mixture concentration.

365 Correlation analysis highlighted a strong positive correlation between VFA production and  
366 enzyme concentrations (Pearson's  $r = 0.96-0.98$ ,  $p < 0.05$ ). Despite the significant increase in  
367 VFA concentration with increasing enzyme concentrations from 1 to 5 mg-protein.g-wheat  
368 straw<sup>-1</sup>, no significant VFA production increment was observed for concentrations ranging  
369 from 5 to 10 mg-protein.g-wheat straw<sup>-1</sup>. These results reveal that the optimal dose of  
370 enzymes in terms of cost effectiveness for H<sub>2</sub> production ranged from 1 to 5 mg-protein.g-  
371 wheat straw<sup>-1</sup>.

372

#### 373 **4. Conclusions**

374

375 The present study shows that enzyme addition substantially enhanced fermentative H<sub>2</sub>  
376 production from wheat straw. A two-fold increase in H<sub>2</sub> production yields was obtained with  
377 enzymatic treated wheat straw compared to no treatment. The impact of the enzyme mixture  
378 on the H<sub>2</sub> production performances was comparable using enzyme addition to one-stage  
379 fermentation or enzyme pretreatment of 24 hours prior to fermentation. When using the  
380 separate hydrolysis step, the sterilization of wheat straw is an important requirement in order  
381 to maintain the benefit of the action of the enzymes. Indeed, a decrease in H<sub>2</sub> production  
382 yields was observed in non-sterile wheat straw and was associated with the reconsumption of  
383 sugar released during enzymatic hydrolysis. Using one-stage dark fermentation, H<sub>2</sub> yield can  
384 also be improved with increasing amounts of CEP enzyme mixture. Taking this into account,  
385 direct enzyme addition to a one-stage dark fermentation system was found finally to be the  
386 most suitable experimental design to improve H<sub>2</sub> production performances from wheat straw.

387 **References**

388

389 **Figures captions**

390

391 **Figure 1.** Amounts of sugars released (A) from enzymatic hydrolysis of non-sterile wheat  
392 straw (WS<sup>raw</sup>) at 50°C using CEP enzyme mixture, and microbial metabolite accumulation  
393 (B). Values correspond to means of three replicated independent values  $\pm$  confidence intervals  
394 (error bars).

395

396 **Figure 2.** Amounts of sugars released (A) from enzymatic hydrolysis of sterile wheat straw  
397 (WS<sup>ster</sup>) at 50°C using CEP enzyme mixture, and microbial metabolite accumulation (B).  
398 Values correspond to means of three replicated independent values  $\pm$  confidence intervals  
399 (error bars).

400

401 **Figure 3.** Amounts of sugars released from enzymatic hydrolysis of sterile wheat straw  
402 (WS<sup>ster</sup>) at 37°C using CEP enzyme mixture. Values correspond to means of three replicated  
403 independent values  $\pm$  confidence intervals (error bars).

404

405 **Figure 4.** Cumulative hydrogen production at 37°C from sterile (WS<sup>ster</sup>) and non-sterile wheat  
406 straw (WS<sup>raw</sup>) in a one-stage system, 1-SS (with direct CEP enzyme addition), and in a two-  
407 stage system, 2-SS (with 24h enzymatic hydrolysis prior to dark fermentation). Values  
408 correspond to means of three replicated independent values  $\pm$  confidence intervals (error  
409 bars).

410

411 **Figure 5.** Cumulative hydrogen production from non-sterile wheat straw (WS<sup>raw</sup>) in a one-  
412 stage system with direct addition of different CEP enzyme mixture concentrations (0, 1, 5 and

413 10 mg-protein.g-wheat straw<sup>-1</sup>) at 37°C. Values correspond to means of three replicated  
414 independent values ± confidence intervals (error bars).

415

416 **Figure 6.** Relationship between the hydrogen production yields and the CEP enzyme mixture  
417 concentrations. Values correspond to means of three replicated independent values ±  
418 confidence intervals (error bars).

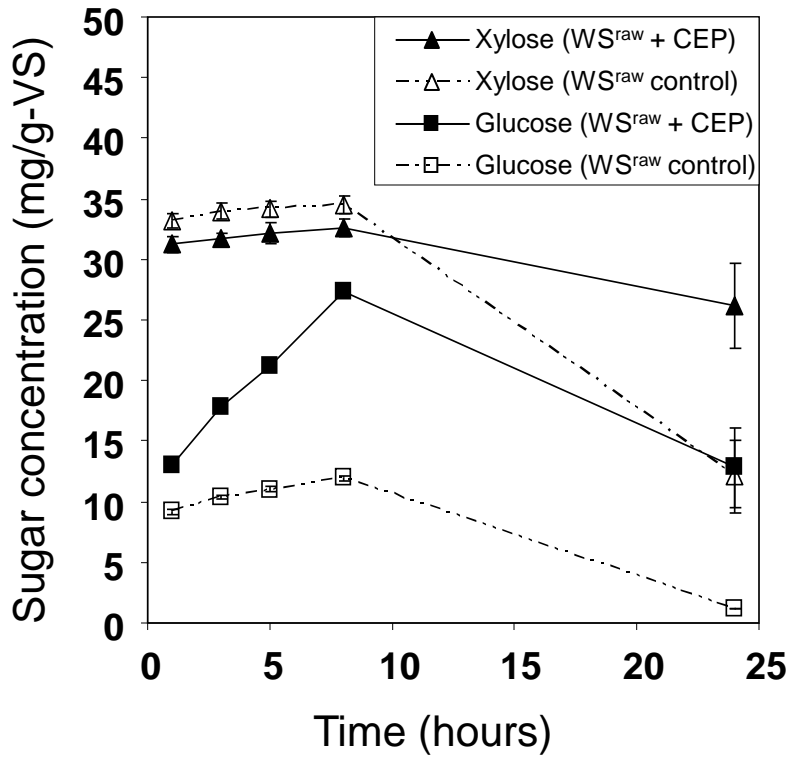
**Table 1.** Composition of the untreated wheat straw used as substrate.

Component (unit)	Value
Total solids – TS (%)	$94.1 \pm 0.6$
Volatile solids - VS (%)	$87.5 \pm 0.9$
Cellulose (g.g-VS <sup>-1</sup> )	$0.29 \pm 0.03$
Hemicellulose (g.g-VS <sup>-1</sup> )	$0.24 \pm 0.03$
Lignin (g.g-VS <sup>-1</sup> )	$0.28 \pm 0.01$
Glucose (g.g-VS <sup>-1</sup> )	$0.29 \pm 0.03$
Xylose (g.g-VS <sup>-1</sup> )	$0.22 \pm 0.025$
Arabinose (g.g-VS <sup>-1</sup> )	$0.016 \pm 0.002$

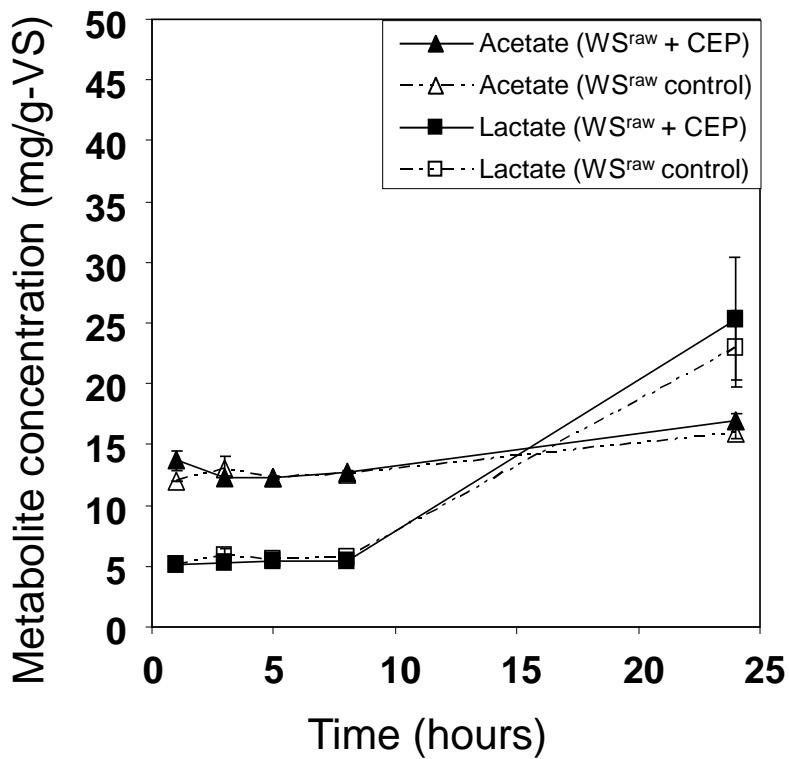
**Table 2.** Fermentative hydrogen production performances from wheat straw in mesophilic conditions

Substrate	Methods of enzyme addition	Enzyme mixture concentration (mg-protein.g-raw wheat straw <sup>-1</sup> )	Modified Gompertz equation parameter values				H <sub>2</sub> yield (mL.g-VS <sup>-1</sup> )	Acetate yield (mg.g-VS <sup>-1</sup> )	Butyrate yield (mg.g-VS <sup>-1</sup> )	Butyrate/acetate ratio
			R <sub>m</sub> (mL-H <sub>2</sub> .day <sup>-1</sup> )	P (mL-H <sub>2</sub> )	λ (days)	R <sup>2</sup> (range)				
Unsterile wheat straw	Direct addition (one-stage system)	0 (Control)	8.30 (± 0.40)	9.21 (± 0.41)	1.47 (± 0.22)	0.999 - 1	10.52 (± 0.46)	12.8 (± 6.7)	16.3 (± 4.4)	1.27
		1	6.51 (± 2.04)	13.59 (± 0.51)	1.07 (± 0.08)	0.983 - 0.989	15.52 (± 0.58)	19.1 (± 2.5)	29.9 (± 2.4)	1.56
		5	17.47 (± 1.73)	17.18 (± 1.06)	1.41 (± 0.22)	0.997 - 0.999	19.63 (± 1.21)	31.5 (± 5.7)	43.4 (± 4.4)	1.38
		10	17.91 (± 0.91)	18.91 (± 0.77)	1.23 (± 0.02)	0.985 - 0.999	21.61 (± 0.88)	37.9 (± 10.3)	53.9 (± 12.4)	1.42
Sterile wheat straw	Enzymatic hydrolysis of 24h (two-stage system)	0 (Control)	5.08 (± 1.16)	4.53 (± 0.12)	0.22 (± 0.03)	0.972 - 0.997	5.18 (± 1.13)	1.2 (± 2.1)	1.5 (± 2.5)	1.19
		5	12.74 (± 3.94)	9.68 (± 1.22)	1.20 (± 0.03)	0.949 - 0.999	11.06 (± 1.40)	24.7 (± 4.0)	39.7 (± 4.6)	1.61
		0 (Control)	3.86 (± 0.34)	7.71 (± 0.77)	2.66 (± 0.60)	0.968 - 1	8.81 (± 0.88)	14.1 (± 12.4)	16.8 (± 4.1)	1.19
		5	14.45 (± 1.58)	15.86 (± 1.37)	1.19 (± 0.04)	0.982 - 0.994	18.13 (± 1.57)	32.2 (± 0.7)	46.3 (± 3.1)	1.43

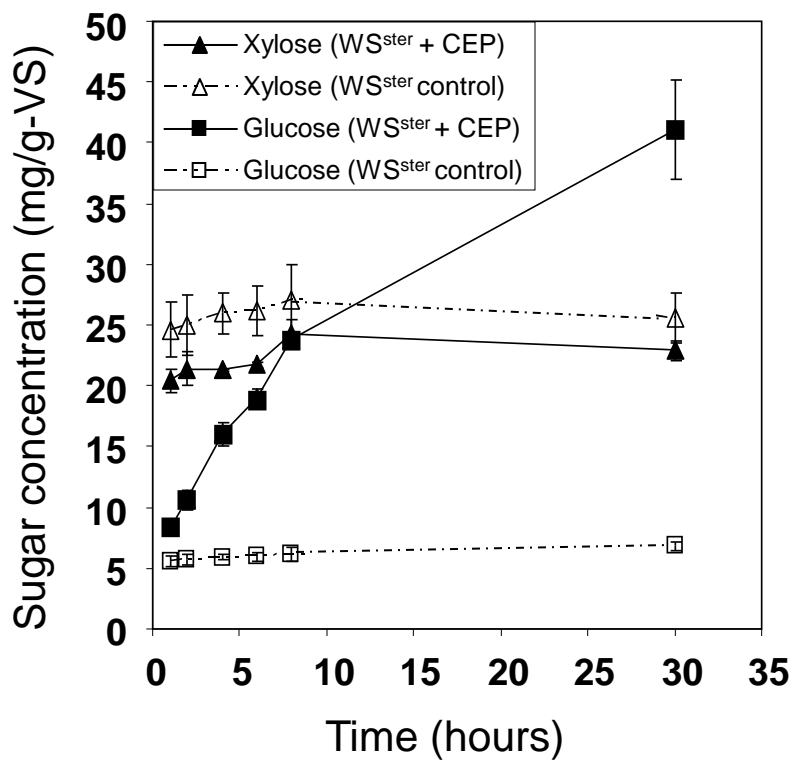
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B.



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B.

