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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 (H₂), a promising alternative energy carrier, at a low cost. Bioconversion of
23 lignocellulosic biomass to produce H₂ usually involves three main operations: pretreatment,
24 hydrolysis and fermentation. In this study, the efficiency of exogenous enzyme addition on
25 fermentative H₂ 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). H₂ production from untreated wheat straw
28 ranged from 5.18 to 10.52 mL-H₂.g-VS⁻¹. Whatever the experimental enzyme addition
29 procedure, a two-fold increase in H₂ production yields ranging from 11.06 to 19.63 mL-H₂.g-
30 VS⁻¹ was observed after enzymatic treatment of the wheat straw. The high variability in H₂
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 H₂ 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⁻¹.

37

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

40 **1. Introduction**

41

42 Hydrogen (H₂) is a key molecule of present and future energetic systems. Currently, H₂ is a
43 commercially important molecule since large quantities of H₂ are needed in the petroleum and
44 chemical industries (e.g. processing of fossil fuels, production of ammonia). Because H₂ is
45 energy dense and clean burning, it is also considered to be a promising energy carrier to
46 replace fossil fuels. However, H₂ 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 H₂ economy include improving process efficiencies, lowering the production cost and
49 harnessing renewable sources for H₂ production.

50 Biological H₂ 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 H₂ 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 H₂ production.

59 Usually the bioconversion of lignocellulosic waste to H₂ requires pretreatment and hydrolysis
60 of the biomass prior to dark fermentation. H₂ 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 H₂ 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 (H₂SO₄) 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, H₂ 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 H₂ 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 H₂ 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 H₂ 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 H₂
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 H₂
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 H₂ 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⁻¹) 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 H₂ 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 H₂SO₄ 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 H₂SO₄. The column
159 temperature was maintained at 35°C, and the flow rate at 0.4 mL.min⁻¹. 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 (CH₄, CO₂, H₂ and N₂) 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⁻¹; 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 H₂ production, the cumulative H₂ 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 H₂ production (mL), R_m is the maximum H₂ production
180 rate (mL-H₂.day⁻¹), λ is lag-phase time (day), t is the incubation time (day) and e is exp(1).
181 The values of P , R_m and λ were estimated using a non-linear regression algorithm developed
182 in Matlab (version 6.5, MathWorks®). The H₂ production yield was calculated by dividing
183 the maximal H₂ 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 ± 6 mg-glucose.g-VS⁻¹ versus 11.9 ± 0.4 mg-glucose.g-VS⁻¹ 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 ± 0.2 mg.g-VS⁻¹ at 8 hours to 17 ± 1 mg.g-VS⁻¹ after 24 hours. Meanwhile, the lactate
200 concentration increased from 5.4 ± 0.4 mg.g-VS⁻¹ to 26 ± 9 mg.g-VS⁻¹. 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 ± 7 mg-glucose.g-VS⁻¹ versus 7 ± 1 mg-
210 glucose.g-VS⁻¹ 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 ± 5.9 mg-glucose.g-VS⁻¹ instead of 9.2 ± 4.0
217 mg-glucose.g-VS⁻¹ 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 ± 5 mg-xylose.g-VS⁻¹ instead of 31
222 ± 2 mg-xylose.g-VS⁻¹ 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 H₂ and
240 CO₂, with no detectable CH₄, indicating that the heat shock pretreatment had effectively
241 removed the methanogens initially present in the anaerobic inoculum. Figure 4 shows the
242 cumulative H₂ production over 10 days of incubation. The data presented in Figure 4 fitted
243 well to the Gompertz equation (1), with determination coefficients R² over 0.98 on average
244 (Table 2).

245 When no enzyme was added to the wheat straw, the H₂ yields ranged from 5.18 to 10.52 mL-
246 H₂.g-VS⁻¹ (Table 2). These H₂ 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-H₂.g-VS⁻¹.
248 This result indicates that the heat-treated mesophilic microbial inoculum was able to produce
249 H₂ 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⁻¹) on
254 fermentative H₂ 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 H₂ production yields by a factor of 2 (19.63 mL-H₂.g-VS⁻¹ vs 10.52 mL-H₂.g-

263 VS⁻¹ in the controls with no enzyme addition) (Table 2). Thus, the tested enzyme mixture
264 effectively enhanced the H₂ 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 H₂ 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 H₂ production yields (11.06 mL-H₂.g-VS⁻¹ vs 5.18 mL-H₂.g-VS⁻¹ 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 H₂ yields were
274 significantly lower than those obtained under the same conditions using sterile wheat straw.
275 Interestingly, these H₂ yields were statistically similar to the controls performed in one-stage
276 dark fermentation (Table 2). Assuming that the H₂ potential of xylose is about the same of
277 glucose (i.e. 160 mL-H₂.g-glucose⁻¹) (data not shown), a first approximation of the two-stage
278 H₂ 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-H₂ g-VS⁻¹ in sterile wheat straw
280 controls from 6.8 mg-glucose.g-VS⁻¹ and 27.0 mg-xylose.g-VS⁻¹ (Figure 2A). In contrast, it
281 was estimated at 1.92 mL-H₂ g-VS⁻¹ in non-sterile wheat straw controls (from 1.0 mg-
282 glucose.g-VS⁻¹ and 13.0 mg-xylose.g-VS⁻¹ (Figure 1A). The difference between these two
283 estimated values (3.58 mL-H₂.g-VS⁻¹) is in agreement with the measured H₂ yield difference
284 obtained between sterile and non-sterile wheat straw (3.63 mL-H₂.g-VS⁻¹). The decrease in
285 H₂ 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 H₂ production, as shown previously (Figure 1B).

290 To summarize, both methods of enzyme addition increased the H₂ production yield by a
291 factor of 2 (Table 2). However, no significant difference in the H₂ production yields was
292 obtained between direct enzyme addition to non-sterile wheat straw (19.63 ± 1.21 mL-H₂.g-
293 VS⁻¹) and enzymatic pretreatment of sterile wheat straw (18.13 ± 1.57 mL-H₂.g-VS⁻¹) (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 H₂ 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 H₂
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 H₂ 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 H₂ 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 H₂ 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 H₂ production in
316 dark fermentation processes (Han and Shin, 2004). However, in our study, no correlation was
317 observed between the H₂ 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⁻¹ 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⁻¹ 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 H₂ 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 H₂ 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⁻¹.

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 H₂ production (Figure 5). The Gompertz model provided a good fit with the

337 cumulated H₂ production data as shown in Figure 5, with determination coefficients R² over
338 0.98 (Table 2).

339 The higher the enzyme dose, the greater was the H₂ production rate. Indeed, the highest H₂
340 production rates (17.91 ± 0.91 mL-H₂.day⁻¹) were obtained for 10 mg-protein.g-wheat straw⁻¹.
341 However, with a value of 6.51 ± 2.04 mL-H₂.day⁻¹, a dose of 1 mg of enzyme did not
342 significantly increase the H₂ production rate compared to the controls (8.30 ± 0.40 mL-
343 H₂.day⁻¹). The H₂ production potential increased gradually from 9.21 to 18.91 mL-H₂ with an
344 increase in enzyme concentration (Table 2). Although positive effects of enzyme mixture
345 loading were observed on H₂ yield (Figure 6), no significant linear correlation was obtained
346 between H₂ yield and the enzyme mixture concentration (Pearson's r = 0.89, p>0.05). The
347 increments in H₂ production rate were lower when enzyme concentration exceeded 5 mg-
348 protein.g-wheat straw⁻¹ (Figure 6). Indeed, H₂ production differences ranged from 4.94 to
349 0.33 mL-H₂.mg-additional protein⁻¹ while enzyme mixture concentrations increased
350 respectively from 1 to 10 mg-protein.g-wheat straw⁻¹.

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 H₂ yields, the acetate yields
356 increased from 12.8 to 37.9 mg.g-VS⁻¹ 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⁻¹ was related to the highest H₂ 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 H₂ 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⁻¹, no significant VFA production increment was observed for concentrations ranging
369 from 5 to 10 mg-protein.g-wheat straw⁻¹. These results reveal that the optimal dose of
370 enzymes in terms of cost effectiveness for H₂ production ranged from 1 to 5 mg-protein.g-
371 wheat straw⁻¹.

372

373 **4. Conclusions**

374

375 The present study shows that enzyme addition substantially enhanced fermentative H₂
376 production from wheat straw. A two-fold increase in H₂ production yields was obtained with
377 enzymatic treated wheat straw compared to no treatment. The impact of the enzyme mixture
378 on the H₂ 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 H₂ 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, H₂ 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 H₂ 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^{raw}) 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^{ster}) 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^{ster}) 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^{ster}) and non-sterile wheat
406 straw (WS^{raw}) 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^{raw}) 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⁻¹) 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).

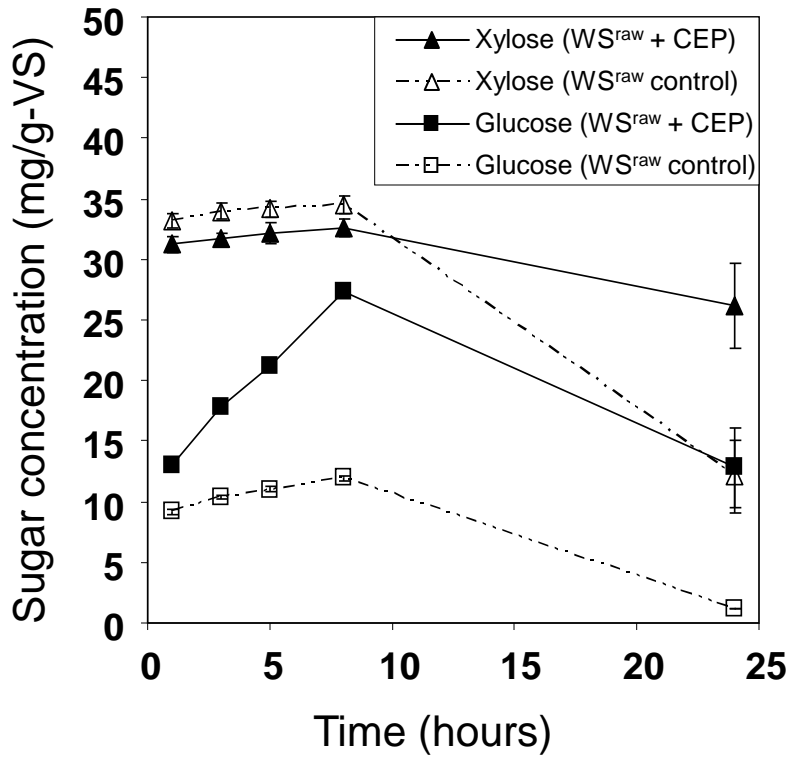
Table 1. Composition of the untreated wheat straw used as substrate.

Component (unit)	Value
Total solids – TS (%)	94.1 ± 0.6
Volatile solids - VS (%)	87.5 ± 0.9
Cellulose (g.g-VS ⁻¹)	0.29 ± 0.03
Hemicellulose (g.g-VS ⁻¹)	0.24 ± 0.03
Lignin (g.g-VS ⁻¹)	0.28 ± 0.01
Glucose (g.g-VS ⁻¹)	0.29 ± 0.03
Xylose (g.g-VS ⁻¹)	0.22 ± 0.025
Arabinose (g.g-VS ⁻¹)	0.016 ± 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 ⁻¹)	Modified Gompertz equation parameter values				H ₂ yield (mL.g-VS ⁻¹)	Acetate yield (mg.g-VS ⁻¹)	Butyrate yield (mg.g-VS ⁻¹)	Butyrate/acetate ratio
			R _m (mL-H ₂ .day ⁻¹)	P (mL-H ₂)	λ (days)	R ² (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

A.



B.

