

Effect of enzyme addition on fermentative hydrogen production from wheat straw

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

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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 fermentative H₂ production from wheat straw was evaluated using mixed-cultures in two 25 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 ranged from 5.18 to 10.52 mL-H₂.g-VS⁻¹. Whatever the experimental enzyme addition 28 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 straw⁻¹. 36

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Keywords: biohydrogen, wheat straw, enzymatic hydrolysis, lignocellulosic material, mixed cultures, dark fermentation

40 **1. Introduction**

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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 energy dense and clean burning, it is also considered to be a promising energy carrier to 45 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 composed of cellulose (40–50%), hemicelluloses (25–35%) and lignin (15–20%). Hydrolysis 67 68 has been shown to be the rate-limiting step in biomass conversion to bioenergy. The pretreatments make the main constituents, cellulose and hemicellulose, more accessible to 69 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, H_2 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_2 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 pretreatment in improving H₂ production from poplar leaves. According to our knowledge,
the different methods of enzyme addition to dark fermentation systems have been poorly
studied.

The aim of this study is to determine the impact of an undefined enzyme mixture (CEP) on fermentative H₂ production from wheat straw. Firstly, the efficiency of the CEP enzyme mixture in improving the conversion of wheat straw cellulosic and hemicellulosic compounds to glucose and xylose was investigated. Next, the impact of two enzyme addition procedures was evaluated in a one-stage system (direct enzyme addition) and a two-stage system (enzymatic hydrolysis prior to dark fermentation). The effect of the enzyme dose on H₂ production was finally assessed.

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101 **2. Materials and methods**

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103 **2.1. Raw materials**

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

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110 **2.2. Enzymatic mixture**

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Among the proprietary enzyme mixtures developed by Protéus and IFPEN, CEP was selected for its ability to degrade lignocellulosic biomass. This enzymatic cocktail is secreted by an engineered *Trichoderma* strain and shows optimal pH and temperature of 4.8 and 50°C,
respectively. The cocktail contains enzymatic system transforming efficiently cellulosic
polymers to glucose as well as hemicellulosic activities.

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118 **2.3. Enzymatic hydrolysis**

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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.

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

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H₂ production experiments were carried out in 500 mL glass bottles under discontinuous batch conditions. Mesophilic anaerobically-digested sludge pretreated by heat-shock treatment (90°C, 10 min) was used as *inoculum*. The bacterial consortium was composed mainly of clostridial species. Two milliliters of the pretreated inoculum (final concentration of 250 mg-COD.L⁻¹) were added to a culture medium containing 40 mM of 2-(Nmorpholino)ethanesulfonic acid (MES) buffer, with a final working volume of 200 mL. 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_2 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.

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147 **2.5. Chemical analysis**

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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 burning samples at 550 °C for 2h. The cellulose and hemicellulose fractions corresponded to a 151 152 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.

Biogas volume was periodically measured using an acidified water displacement method. Biogas composition (CH₄, CO₂, H₂ and N₂) was analyzed using a gas chromatograph (Clarus 280, Perkin Elmer) equipped with a column HayeSep Q and a molecular sieve. Operating conditions were as follows: the carrier gas was argon at a pressure of 102 kPa and a flow rate of 4.5 mL.min⁻¹; temperatures of the injector and the detector were both fixed at 150 °C.

Volatile fatty acid (VFA) composition in the liquid phase, *i.e.* acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids was determined in a gas chromatograph (GC-3900, Varian) equipped with a flame ionization detector. The concentration of sugars (glucose, xylose, arabinose, glucuronic acid and galacturonic acid) and non-VFAs metabolic byproducts, such as organic acids (lactate) or ethanol were measured by HPLC analysis.

In order to determine the parameters of H₂ production, the cumulative H₂ production data of
each test was fitted to a modified Gompertz equation (Equation 1) as previously described by
Quéméneur et al..

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

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185 **3. Results and discussion**

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

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

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At 50 °C, the addition of CEP enzyme mixture to wheat straw improved the glucose yield by 192 a factor of 2.3 (27 \pm 6 mg-glucose.g-VS⁻¹ versus 11.9 \pm 0.4 mg-glucose.g-VS⁻¹ in the 193 194 controls) (Figure 1A). No effect of CEP addition was observed on the xylose yield (Figure 1A). Maximal glucose concentration was reached at 8 hours and then decreased until 30 195 196 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 12.7 \pm 0.2 mg.g-VS^-1 at 8 hours to 17 \pm 1 mg.g-VS^-1 after 24 hours. Meanwhile, the lactate 199 concentration increased from 5.4 \pm 0.4 mg.g-VS⁻¹ to 26 \pm 9 mg.g-VS⁻¹. These results 200 suggested that the sugars released were immediately used as a carbon source by indigenous 201 202 microorganisms of the wheat straw.

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

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

At 50 °C, the addition of the enzyme mixture to the sterile wheat straw improved the glucose yield by a factor of 6 after 30 hours of hydrolysis (41 ± 7 mg-glucose.g-VS⁻¹ versus 7 ± 1 mgglucose.g-VS⁻¹ in the controls) (Figure 2A). As previously observed in non-sterile wheat straw, no significant effect of the enzyme mixture was observed on the xylose yield. However, no decrease in glucose and xylose concentrations, as well as no variation in acetate and lactate concentrations were detected during the hydrolysis of the wheat straw aftersterilization (Figure 2B).

215 At 37°C, the addition of CEP enzyme mixture significantly increased the glucose yield by a factor of 3.4 after 24 hours of hydrolysis (31.4 \pm 5.9 mg-glucose.g-VS⁻¹ instead of 9.2 \pm 4.0 216 mg-glucose.g-VS⁻¹ in controls) (Figure 3). As expected, the decrease in temperature from 217 218 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 was observed in presence of the CEP enzyme mixture $(34 \pm 5 \text{ mg-xylose.g-VS}^{-1} \text{ instead of } 31)$ 221 ± 2 mg-xylose.g-VS⁻¹ in controls). 222

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.

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3.2. Effect of the method for enzyme addition on hydrogen production from wheat straw
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237 **3.2.1. Hydrogen production from wheat straw by mixed-cultures**

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The biogas produced from wheat straw by anaerobic mixed-cultures contained only H_2 and CO_2 , with no detectable CH_4 , indicating that the heat shock pretreatment had effectively removed the methanogens initially present in the anaerobic inoculum. Figure 4 shows the

cumulative H_2 production over 10 days of incubation. The data presented in Figure 4 fitted well to the Gompertz equation (1), with determination coefficients R^2 over 0.98 on average (Table 2).

When no enzyme was added to the wheat straw, the H₂ yields ranged from 5.18 to 10.52 mL-H₂.g-VS⁻¹ (Table 2). These H₂ yields were greater or equal to those reported in the literature from batch tests using untreated wheat straw as substrate, i.e. from 1 to 6.4 mL-H₂.g-VS⁻¹. This result indicates that the heat-treated mesophilic microbial inoculum was able to produce H₂ from raw lignocellulosic material.

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

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The effect of the addition of the CEP enzyme mixture (at 5 mg-protein.g-wheat straw⁻¹) on fermentative H_2 production from sterile and non-sterile wheat straw was tested under mesophilic conditions in two systems: (i) in one-stage dark fermentation batch tests where CEP enzyme mixture was directly added and (ii) in two stage tests where an enzymatic hydrolysis of 24 hours was performed prior to the dark fermentation batch tests.

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

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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-

1

VS⁻¹ in the controls with no enzyme addition) (Table 2). Thus, the tested enzyme mixture effectively enhanced the H_2 yield from wheat straw, probably through the release of additional carbohydrate units, such as glucose, in the medium. This result is consistant with the observations of Cui et al. who reported a significant increase in H_2 yield from poplar leaves with enzymatic pretreatment using 2% Vicozyme L (Novozyme).

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

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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 271 272 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_2 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 glucose (i.e. 160 mL-H₂.g-glucose⁻¹) (data not shown), a first approximation of the two-stage 277 278 H₂ potential can be assessed from the amount of glucose and xylose released during enzymatic hydrolysis (1st stage). It was estimated at 5.5 mL-H₂ g-VS⁻¹ in sterile wheat straw 279 controls from 6.8 mg-glucose.g-VS⁻¹ and 27.0 mg-xylose.g-VS⁻¹ (Figure 2A). In contrast, it 280 was estimated at 1.92 mL-H₂ g-VS⁻¹ in non-sterile wheat straw controls (from 1.0 mg-281 glucose.g-VS⁻¹ and 13.0 mg-xylose.g-VS⁻¹ (Figure 1A). The difference between these two 282 estimated values (3.58 mL-H₂.g-VS⁻¹) is in agreement with the measured H₂ yield difference 283 obtained between sterile and non-sterile wheat straw (3.63 mL-H₂.g-VS⁻¹). The decrease in 284 285 H2 yields from sterile to non-sterile wheat straw in the two-stage systems was therefore explained by a direct and almost total consumption of the sugars released during the 286 287 enzymatic hydrolysis step by indigenous microorganisms. In fact, the consumption of sugars

simultaneously increased the acetate and lactate levels which cannot be further reused in
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 \pm 1.21 mL-H₂.g-293 VS⁻¹) and enzymatic pretreatment of sterile wheat straw ($18.13 \pm 1.57 \text{ mL-H}_2\text{.g-VS}^{-1}$) (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

As expected, the H₂ production yields were strongly related to acetate (Pearson's productmoment correlation r = 0.93, p<0.01) and butyrate accumulation (Pearson's r = 0.89, p<0.05). Moreover, the highest H₂ yield matched with the highest yields of acetate and butyrate (Table 2). The abundance of butyrate as well as acetate is a typical characteristic of acid fermentation of carbohydrate-rich substrates [4]. Similarly, Fan et al. reported that acetate and butyrate are 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).

Furthermore, the same acetate and butyrate yields were found in the case of direct enzyme addition to non-sterile wheat straw as well as after enzymatic pretreatment of sterile wheat straw: an acetate yield of $32.2 \text{ mg-acetate.g-VS}^{-1}$ was measured for sterile wheat straw treated by CEP in a two-stage system, and a similar value of $31.5 \text{ mg-acetate.g-VS}^{-1}$ was obtained after direct enzyme addition (Table 2). This result confirms that the separated hydrolysis step and/or sterilization had no beneficial effect on H₂ production yields when compared to the one-stage dark fermentation system.

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

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The impact of enzyme dose on cumulative H_2 production performances was also evaluated in additional one-stage fermentation batch tests with a gradient of CEP enzyme mixture concentration: 0, 1, 5 and 10 mg-protein.g-wheat straw⁻¹.

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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_2 production (Figure 5). The Gompertz model provided a good fit with the cumulated H_2 production data as shown in Figure 5, with determination coefficients R^2 over 0.98 (Table 2).

339 The higher the enzyme dose, the greater was the H_2 production rate. Indeed, the highest H_2 production rates $(17.91 \pm 0.91 \text{ mL-H}_2.\text{day}^{-1})$ were obtained for 10 mg-protein.g-wheat straw⁻¹. 340 However, with a value of 6.51 ± 2.04 mL-H₂.day⁻¹, a dose of 1 mg of enzyme did not 341 342 significantly increase the H₂ production rate compared to the controls (8.30 \pm 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 between H₂ yield and the enzyme mixture concentration (Pearson's r = 0.89, p>0.05). The 346 347 increments in H₂ production rate were lower when enzyme concentration exceeded 5 mgprotein.g-wheat straw⁻¹ (Figure 6). Indeed, H_2 production differences ranged from 4.94 to 348 349 0.33 mL-H₂.mg-additional protein⁻¹ while enzyme mixture concentrations increased respectively from 1 to 10 mg-protein.g-wheat straw⁻¹. 350

351

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

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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 increased from 12.8 to 37.9 mg.g-VS⁻¹ as enzyme mixture concentration increased. Butyrate 356 357 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 straw⁻¹ was related to the highest H₂ yield. Butyrate level was 3.3 times greater than in the 359 360 control for this amount of added enzyme. However, no linear correlation was observed between the H₂ and the butyrate/acetate ratio (Bu/Ac) (Pearson's r = 0.41, p>0.05) (Table 2). 361

Furthermore, no significant difference in Bu/Ac ratios, ranging between 1.27 and 1.56, were detected according to the enzyme load (Table 2), suggesting that no metabolic switches 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 H_2 376 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.

References

390

Figure 1. Amounts of sugars released (A) from enzymatic hydrolysis of non-sterile wheat straw (WS^{raw}) at 50°C using CEP enzyme mixture, and microbial metabolite accumulation (B). Values correspond to means of three replicated independent values \pm confidence intervals (error bars).

395

Figure 2. Amounts of sugars released (A) from enzymatic hydrolysis of sterile wheat straw
(WS^{ster}) at 50°C using CEP enzyme mixture, and microbial metabolite accumulation (B).
Values correspond to means of three replicated independent values ± confidence intervals
(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

Figure 4. Cumulative hydrogen production at 37°C from sterile (WS^{ster}) and non-sterile wheat straw (WS^{raw}) in a one-stage system, 1-SS (with direct CEP enzyme addition), and in a twostage system, 2-SS (with 24h enzymatic hydrolysis prior to dark fermentation). Values correspond to means of three replicated independent values \pm confidence intervals (error bars).

410

Figure 5. Cumulative hydrogen production from non-sterile wheat straw (WS^{raw}) in a onestage 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 \pm
- 418 confidence intervals (error bars).

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

Substrate	Methods of enzyme addition	Enzyme mixture	Modified Gompertz equation parameter values					Butvrate	Butvrate/	
		concentration (mg-protein.g-raw wheat straw ⁻¹)	$\begin{array}{c} R_{m} \\ (mL-H_{2}.day^{-1}) \end{array}$	P (mL-H ₂)	λ (days)	R ² (range)	H_2 yield (mL.g-VS ⁻¹)	Acetate yield (mg.g-VS ⁻¹)	yield (mg.g-VS ⁻¹)	acetate ratio
	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)	$1.07 (\pm 0.08)$	0.983 - 0.989	15.52 (± 0.58)	19.1 (± 2.5)	29.9 (± 2.4)	1.56
Unsterile		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
wheat straw		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
	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)	$1.20 (\pm 0.03)$	0.949 - 0.999	11.06 (± 1.40)	24.7 (± 4.0)	39.7 (± 4.6)	1.61
Sterile		0 (Control)	3.86 (± 0.34)	7.71 (± 0.77)	$2.66 (\pm 0.60)$	0.968 - 1	$8.81 (\pm 0.88)$	14.1 (± 12.4)	16.8 (± 4.1)	1.19
wheat straw		5	14.45 (± 1.58)	15.86 (± 1.37)	$1.19 (\pm 0.04)$	0.982 - 0.994	18.13 (± 1.57)	$32.2 (\pm 0.7)$	46.3 (± 3.1)	1.43

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

Figure 1

Α.



Β.



Figure 3



Figure 4





