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1 **Innovative CO₂ pretreatment for enhancing biohydrogen production from the Organic**
2 **Fraction of Municipal Solid Waste (OFMSW)**

3

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20

21 **Abstract**

22 A series of batch tests was conducted to investigate the effect of a CO₂ sparging pretreatment
23 on hydrogen production from fermentation of the organic fraction of municipal solid waste.
24 Tests were carried out in a stirred bioreactor without adding inoculum or supplementation
25 solution, at 35°C and with a pH regulated at 5.5. The influence of CO₂ flowrate (30, 100 and
26 500 mL/min) was evaluated. Results showed a decrease in biological hydrogen production for
27 a sparging flowrate of 500 mL_{CO2}/min, similar performances for the control experiments and
28 for a sparging flowrate of 100 mL_{CO2}/min and an increase in biohydrogen production for a
29 sparging flowrate of 30 mL_{CO2}/min. For this latter operating condition, a substantial
30 improvement in hydrogen production performances was obtained with an increase in the
31 hydrogen yield by more than 20% and a decrease in the chemicals consumption used to
32 maintain the pH by more than 20%. No shift in the metabolic pathways and in the bacterial
33 community structure was observed but positive bacterial interactions occurred and enhanced
34 the hydrogen production. Therefore, this study suggested that the growth of hydrogen
35 producing bacteria could be either enhanced or slowed down by a CO₂ sparging pretreatment
36 depending on the level of CO₂ flowrate, the shift being close to 100 mL_{CO2}/min.

37

38 **Keywords**

39 Acidogenesis; Bacterial communities dynamics; Bioreactor; Dark fermentation; Domestic
40 waste; Hydrogen.

41

42 **1. Introduction**

43 Development of renewable energy sources and sustainable management of organic waste are
44 major environmental and economic issues. Energy production from municipal solid waste or
45 industrial by-products is one way to respond to this double challenge [1]. Classic methods for
46 producing energy from organic-rich waste are incineration and more recently, anaerobic
47 digestion. In last years, hydrogen production from waste has arisen as an interesting pathway,
48 due to the high energy content of the hydrogen (122 kJ/g) and its low pollution potential since
49 it only produces water by combustion [2].

50 Among the various hydrogen production processes, biological technologies are attractive
51 since they are carried out at relatively low temperature and ambient pressure, and are less
52 energy-intensive than thermal or electrochemical processes [3-5]. Biological hydrogen
53 production by fermentative microorganisms can be broadly classified into two main
54 categories: photofermentation and dark fermentation. In photofermentative processes, small-

55 chain organic acids are used by bacteria as electron donors to produce hydrogen using light
56 energy [2,6,7]. Even though this conversion theoretically gives high conversion yields, this
57 process has many shortcomings such as low production rates, difficulties in designing the
58 reactors to maintain light penetration into a highly turbid bioreactor, low conversion
59 efficiencies of light energy, and photo inhibition at high solar light intensities [2,8,9]. In dark
60 fermentative processes, organic substrates, usually carbohydrates, are directly converted into
61 H₂, CO₂, and organic acids without any external energy or electron acceptors [10]. Major
62 drawbacks include lower hydrogen yields with only 10–20% of the substrate energy
63 recovered as H₂ due to the limited metabolic energy of hydrogen fermentation; and instability
64 of fermentative H₂ reactors [11-13]. However, dark fermentation is considered as the most
65 favourable hydrogen-generating process due to a high hydrogen production rate, simple
66 operation and design, and no requirement of additional light energy [12,14-16].

67 The production of hydrogen by dark fermentation from municipal solid waste and more
68 especially from the organic fraction of municipal solid waste (OFMSW) is a relatively new
69 process that has been studied by several authors [17-23]. Fermentative hydrogen production is
70 a very complex process influenced by many factors such as the operating conditions (pH,
71 temperature, agitation speed, hydrogen partial pressure, reactor type, hydraulic retention
72 time), the substrate characteristics (type of substrate, organic acids, metal ions concentrations)
73 and the composition of the microbial populations. In particular, mixed cultures appeared to be
74 the most appropriate for producing biohydrogen from solid organic waste [24-26]. Indeed, the
75 diversity of bacterial communities allows adaptation to different substrate composition and
76 operating conditions either by fluctuation in community composition and/or metabolic
77 adaptation [27,28]. However, some limitations regarding process development still remain to
78 be solved, such as the suppression of hydrogen-consuming bacteria [17]. Previous studies
79 reported several pretreatment methods to inhibit or kill hydrogen-consuming bacteria such as
80 thermal, alkaline, acidification, ultrasonic or chemical pretreatment [10,17,29-32]. Another
81 way to increase hydrogen production consists in decreasing the hydrogen partial pressure in
82 the bioreactor with continuous gas sparging. Researches on gas sparging were mainly related
83 to continuous sparging of gas such as N₂, CO₂, CH₄ during the fermentative degradation of
84 simple substrates [3,12,33-35]. However, no clear relationship between the sparging
85 conditions and the changes in H₂ yield was identified [3]. For example, Willquist et al. [35]
86 investigated the influence of CO₂ sparging on batch fermentation of glucose and sucrose by
87 *Caldicellulosiruptor saccharolyticus* at thermophilic conditions and showed that sparging
88 with CO₂ had a negative influence on growth rate and hydrogen production rate but had little

89 influence on hydrogen yield. However, in a study performed by Kim et al. [12] on the effects
90 of CO₂ sparging on continuous fermentative H₂ production from sucrose with seed sludge at
91 mesophilic conditions, it was found that the H₂ yield increased noticeably with CO₂ sparging
92 in comparison to unsparged conditions. Therefore, sparging with CO₂ may have various
93 effects on hydrogen production. The objective of this study was to investigate and to bring
94 new insight in the influence of a pretreatment step with CO₂ sparging on batch fermentative
95 hydrogen production from a mixed culture, the OFMSW.

96

97 **2. Materials and methods**

98

99 2.1. Feedstock

100 The substrate used in this study was the Organic Fraction of Municipal Solid Waste
101 (OFMSW) collected in Rouen (France). It was composed of fermentescible waste (57.0%),
102 papers (24.6%) and cardboards (18.4%) and its Total Solids (TS) content was 51.4%. The
103 Volatile Solid (VS) content was 136 mg/g of TS. The mixture was ground to a particle size
104 below than 16mm. The BOD was 83.2 mg/g of TS and the COD was 149.6 mg/g of TS.

105

106 2.2. Experimental setup and operation

107 Batch experiments were carried out in stirred reactors with a working volume of 3.5 L. The
108 feedstock was fed with OFMSW at a VS content of 3.4 g/L. Neither exogenous inoculum nor
109 mineral medium was added. After sealing, a pretreatment was performed in two steps at
110 ambient temperature and under a mechanical stirring of 300 rpm. For the control experiments,
111 the first step consisted in adjusting the pH to 5.5 with HCl and the second step consisted in
112 sparging nitrogen during 15 minutes to reach anaerobic conditions through removal of
113 dissolved oxygen. For experiments with CO₂ sparging pretreatment, the pretreatment
114 consisted in firstly sparging the bioreactor with a fixed CO₂ flowrate during 20 minutes and
115 secondly in sparging nitrogen during 15 minutes. The objective of these tests was to evaluate
116 the effect a CO₂ sparging pretreatment on fermentative hydrogen production according to the
117 CO₂ flowrate (30, 100 and 500 mL_{CO2}/min).

118 After pretreatment, the reactors were mixed by mechanical stirring at 300 rpm and the
119 temperature was controlled at 35°C by hot water circulation inside the water jacket of the
120 reactors. They were equipped with temperature and pH probes (Heito) and with a liquid
121 sampling port for microbial and metabolite analyses. The pH was controlled, during the
122 fermentation process, at 5.5 by adding NaOH (1M) or HCl (1M) with a peristaltic pump. The

123 gas produced during the fermentation was cooled to remove the water and then monitored by
124 a volumetric gas flowmeter (milligascounter®, Ritter). A schematic diagram of the
125 experimental set-up is shown in Fig. 1.

126

127 2.3. Analytical method

128 Biogas composition was analysed on-line by using a portable gas chromatograph (Hewlett
129 Packard, serie P200) equipped with a thermal conductivity detector and two columns. Argon
130 was used as a carrier gas. Carbon dioxide content was determined using a 6-meter Poraplot U
131 capillary column working at a temperature of 80°C. Hydrogen content was measured with a
132 12-meters MS-5A capillary column which the operational temperature was kept at 40°C.
133 Methane was analysed in both columns.

134 Liquid samples for analyses of the fermentation end-products were taken at regular intervals
135 in the reactors. They were centrifuged at 11500 g for 15 min before analyses. The acidogenic
136 end-products, i.e. volatile fatty acids such as acetate, propionate, butyrate, isobutyrate,
137 valerate, isovalerate and caproate, were quantified by gas chromatography coupled to flame
138 ionization detection (GC-FID) in a Varian GC 3900, as described elsewhere [36]. The elution
139 was carried out in a semi-capillary column FFAP of 15 m and 0.52 mm in diameter (Phase
140 ECTM 1000). The conditions were as follows: carrier gas nitrogen at 20psi; injector
141 temperature: 210°C; detector temperature: 280°C; gas flow rate: 6 mL/min; oven temperature
142 ramp: 80 to 120 ° C with a ramp rate of 10 ° C per minute after 1 min of elution. The
143 quantification limit was about 40 mg/L. Other fermentation end-products, non-VFAs, such as
144 lactate and ethanol were quantified by high performance liquid chromatography (HPLC)
145 coupled to refractometric detection (Waters 2414). The separation column corresponded to an
146 AMINEX® HPX-87H column (supplier BIORAD) thermostated at 35°C. The separation of
147 the metabolic products was performed under isochratic elution of H₂SO₄ (0.008N) at a flow
148 rate of 0.4 mL.min⁻¹. The refractometer temperature was fixed at 40°C. The quantification
149 limit was about 100 mg/L each.

150

151 2.4. Bacterial community analyses

152 For molecular analyses of the bacterial community dynamics and diversity, about 2 mL of
153 liquid samples were taken before and just after the pretreatment, then at regular intervals in
154 the reactors. Samples were immediately centrifuged (20 min., 14000g) and pellets were stored
155 at -20°C. Genomic DNAs were extracted from frozen pellets with the FastDNA Spin Kit for
156 Soil (Bio101).

157 Dynamics of bacterial community structures were investigated by a fingerprinting technique
158 of CE-SSCP (Capillary Electrophoresis-Single Strand Conformational Polymorphism). For
159 this, about 200 bp of the V3 region (*E. coli* position 331 to 533) of bacterial 16S rRNA genes
160 was PCR amplified (25 cycles, hybridation at 61°C) from genomic DNAs with the universal
161 reverse primer w34 (5'-TTACCGCGGCTGCTGGCAC-3') and the eubacterial forward
162 primer w49 (5'-ACGGTCCAGACTCCTACGGG-3') 5' end-labelled with the fluorescent dye
163 FAM. After heat denaturation (5 min at 95°C) in deionized formamide and immediate cooling
164 on ice, single-stranded PCR products of same length but different sequence were separated by
165 CE-SSCP on an ABI Prism 310 genetic analyzer using a 47 cm long capillary and the non-
166 denaturing 5.6% GeneScan polymer, and sample injection at 15kV, electrophoresis at 12kV,
167 and run at 32°C. The internal standard GeneScan 600 LIZ was used to correct any change in
168 the electrophoretic mobility between runs. Raw CE-SSCP profiles were analyzed using the
169 GeneScan software. The CE-SSCP technique was also applied for monitoring the H₂-
170 producing clostridial population using the functional [Fe-Fe]-hydrogenase (*hydA*) genes
171 amplified with the nondegenerated primers hydAClosF (5'-
172 ACCGGTGGAGTTATGGAAGC-3', *Clostridium pasteurianum* position F1258) and
173 hydAClosR (5'-CATCCACCTGGACATGCCAT-3', *C. pasteurianum* position R1508), as
174 described by Quéméneur et al. [27]. The *hydA* CE-SSCP profiles were aligned with the
175 internal standard GeneScan 400 ROX and the sum of the peak areas were normalized to unit
176 before statistical analysis using the StatFingerprints library [37] from R version 2.9.2 [38].
177 The complexity of the H₂-producing clostridial population was estimated using Simpson's
178 diversity index D from a *hydA* CE-SSCP profile, by considering the number of OTU (number
179 of peaks) as well as their relative abundance (area under each peak) [39].

180 An inventory of *Bacteria* was performed based on gene libraries constructed (TOPO-TA
181 cloning Kit for Sequencing, Invitrogen) from PCR products amplified with primers 8F and
182 1406R [40]. Clones carrying a correct-length insert (about 1400 bp) were screened by CE-
183 SSCP in order to group the clones according to their migration profile. One clone
184 representative of each group was purified and the carried insert was sequenced (Beckman
185 Coulter Cogenics) with plasmid-specific primers. Consensus nucleotide sequences of about
186 1400 nucleotides were submitted to a Blast search (www3.ncbi.nlm.nih.gov/BLAST) for
187 identification of closest bacterial relatives. Nucleotide sequences retrieved in this study have
188 been deposited under Genbank accession numbers JQ362799 to JQ362803.

189

190 2.5. Kinetic analysis of cumulative hydrogen production

191 The kinetic parameters of the cumulative H₂ production were assessed with a modified
192 Gompertz equation. This equation was extensively used to describe the kinetics of hydrogen
193 production from fermentation [8,19,41]. It considers the following expression:

$$194 \quad H(t) = P \cdot \exp\left\{-\exp\left[-\frac{R_m \cdot e}{P} \cdot (\lambda - t) + 1\right]\right\}$$

195 where H(t) = cumulative H₂ production at fermentation time t (mL); P = maximum potential
196 H₂ production (mL); R_m = maximum H₂ production rate (mL/h); λ = lag phase time (h) and e
197 = Euler's number (2.718281828).

198

199 **3. Results and discussion**

200

201 3.1. Gaseous production

202 Fig. 2 shows the time course of cumulative biogas and hydrogen production in control
203 experiments. Duplicates were performed and showed a good reproducibility of the results. As
204 shown in Fig. 2, the biogas was produced in two steps. The first step occurred just after the
205 beginning of the experiment and the biogas produced was only composed of CO₂. The second
206 step was related to hydrogen production. This second phase started after a lag time of 22h -
207 25h with a maximum rate of 63.9 mL_{H₂}/h and the final hydrogen production reached 795.4
208 mL_{H₂} which corresponded to a hydrogen yield of 56.7 mL_{H₂}/g_{V_S} and a hydrogen content in
209 the second phase biogas of 37.4% (mean values – the standard deviations for the maximum
210 H₂ production rate, the final H₂ production, the H₂ yield and the H₂ content being 7 mL_{H₂}/h,
211 10.2 mL_{H₂}, 0.8 mL_{H₂}/g_{V_S} and 2.7% respectively). These results demonstrate the feasibility to
212 produce hydrogen from the organic fraction of municipal waste without inoculum addition or
213 nutritional supplements.

214

215 Most studies on hydrogen production by dark fermentation have been dealing with soluble or
216 well defined substrates [17]. Experimental works on OFMSW or food waste are scarce and
217 they generally include the addition of inoculum and nutrients to enhance hydrogen
218 production. Some results using OFMSW or food waste for biological hydrogen production in
219 batch systems are exhibited in Table 1. The hydrogen yield obtained in this study (56.7
220 mL_{H₂}/g_{V_S}) is lower to that reported by Lay et al. [19] who used OFMSW but the source of the
221 OFMSW used in these two studies was different and Lay et al. [19] also added two types of
222 seed organisms (digested sludge and hydrogen producing bacteria) and a mineral medium to
223 the substrate. Nevertheless, our hydrogen yield is highly consistent to values obtained by

224 Elbeshbishy et al. [32] in the fermentation of household waste with no inoculum or
225 supplementation minerals.

226

227 The effect of a CO₂ sparging pretreatment on H₂ production was evaluated for three CO₂
228 flowrates (30, 100 and 500 mL_{CO2}/min). Results of these experiments are presented in Table
229 2, including the kinetics parameters of the cumulative hydrogen production evaluated with the
230 modified Gompertz equation (Eq. 1). All correlation coefficients of non-linear analysis were
231 over 0.99, suggesting that the modified Gompertz equation was able to adequately describe
232 the progress of cumulative hydrogen production in all batch tests. Methane was not detected
233 whatever the operating conditions. Table 2 shows that the hydrogen yield was improved at a
234 CO₂ flowrate of 30 mL_{CO2}/min with a mean value of 68.6 mL_{H2}/g_{VS} (standard deviation of 1.9
235 mL_{H2}/g_{VS}) with regards to a yield of 56.7 mL_{H2}/g_{VS} (standard deviation of 0.8 mL_{H2}/g_{VS})
236 obtained in the control experiments. This result corresponds to an increase of about 20% of
237 the hydrogen yield. However, a slight decrease in the hydrogen content was observed
238 meaning that the CO₂ sparging pretreatment also enhanced CO₂ production during the
239 fermentation course. Regarding the influence of the CO₂ flowrate on hydrogen production, a
240 shift was observed for a sparging flowrate close to 100 mL_{CO2}/min: the hydrogen production
241 was improved by about 20% (mean value) for a sparging flowrate of 30 mL_{CO2}/min, it was
242 similar for the control experiments and for a sparging flowrate of 100 mL_{CO2}/min, and it was
243 reduced by nearly 12% for a sparging flowrate of 500 mL_{CO2}/min. Moreover, Table 2 shows
244 that an increase in the CO₂ sparging flowrate had a negative impact on the other kinetics
245 parameters: the hydrogen content decreased from about 34% (mean value) to 24% and the lag
246 phase increased from about 18 hours (mean value) to 26.5 hours when the CO₂ flowrate
247 increased from 30 to 500 mL_{CO2}/min. These results suggest that the growth of hydrogen
248 producing bacteria could be either enhanced or slowed down by a CO₂ sparging pretreatment
249 depending on the level of CO₂ flowrate.

250

251 3.2. Production of the main metabolites

252 The formation of hydrogen is accompanied by volatile fatty acids and alcohols production
253 during the anaerobic digestion process. Monitoring their concentration is thus useful to
254 explain metabolic pathways and to predict hydrogen production. The main metabolic products
255 detected during the experiments were lactate, acetate and butyrate. Fig. 3 shows the variation
256 of these metabolites over fermentation time for the control experiments. Lactate and acetate
257 were detected at the beginning of the experiments, i.e. before fermentation, with

258 concentrations of 13.9 mmol/L and 4.6 mmol/L respectively. During the first hours of
259 fermentation it was observed an increase in lactate content. After about 20 hours, the lactate
260 content decreased while butyrate was produced, this time corresponding to the beginning of
261 the hydrogen production, as shown in Fig. 2. Similar trends of metabolite dynamics were
262 observed for all the experiments even with CO₂ sparging pretreatments meaning that a CO₂
263 sparging pretreatment did not induce shifts in the metabolic pathways. These results are in
264 accordance with the statement of Willquist et al. [35] who showed that sparging with CO₂ did
265 not change the pathways to acetate and hydrogen.

266

267 Metabolite concentrations and the Butyrate to Acetate (B/A) ratio calculated at the end of the
268 experiments are given in Table 3. Table 3 shows a higher lactate concentration at the end of
269 the experiments with CO₂ sparging pretreatment, the value of lactate content increasing with
270 the CO₂ sparging flowrate from 0.2 mmol/L at 30 mL_{CO₂}/min to 2.3 mmol/L at 500
271 mL_{CO₂}/min. Moreover, a higher butyrate concentration was observed for the two CO₂ flowrate
272 conditions leading to an increase in hydrogen production, i.e. at 30 mL/min and 100 mL/min,
273 the butyrate concentration increasing from 11.4 mmol/L for the control experiments to 18.5
274 mmol/L and 17.2 mmol/L respectively. The experiments with a CO₂ flowrate of 100 mL/min
275 also induced an increase in acetate concentration whereas no such change was observed in
276 tests with a CO₂ flowrate of 30 mL/min. With a CO₂ flowrate of 500 mL/min associated to a
277 deterioration of hydrogen production performances, the acetate concentration was lower than
278 in the control experiments. Therefore, no clear relation was found between pretreatment
279 conditions and metabolite concentrations at the end of the experiments, in agreement with
280 findings from previous study [36]. Regarding the B/A ratio, this ratio was always higher than
281 2.0 even in control experiments meaning the butyrate pathway was predominant during
282 fermentation.

283

284 3.3. Chemicals requirement

285 Chemicals requirement to maintain the pH is an economic burden that has to be considered
286 for the evaluation of the process feasibility. Assessments of chemicals consumption are
287 presented in Table 4, where total chemicals consumption corresponds to the sum of the total
288 number of moles of acid and base used throughout the experiment. The pH after pretreatment
289 ranged from 5.4 to 5.6 in all experiments. Table 4 shows that total chemicals consumption
290 decreased significantly with a CO₂ sparging pretreatment at 30 mL/min. The total chemicals
291 consumption was reduced by more than 20% (mean value), mainly due to a decrease of the

292 acid consumption. This can be linked to the dissolution of carbon dioxide which forms
293 carbonic acid (H_2CO_3) and exists in the form of bicarbonate ions (HCO_3^-) and protons (H^+)
294 for pH values less than 8 ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+$); carbonic acid and
295 bicarbonate ions maintaining the equilibrium and helping to resist to pH changes [34,45,46].
296 However, with a CO_2 flowrate higher than 30 mL/min, the total chemicals consumption
297 increased, reaching 48.0 mmol/L for a CO_2 flowrate of 500 mL/min against a chemicals
298 consumption of about 22.0 mmol/L in control experiments i.e. an increase of nearly 120%.
299 This increase was mainly due to a strong increase in the base consumption which occurred at
300 the beginning of the experiment. This could be explained by an accumulation of bicarbonate
301 in the liquid due to a higher partial CO_2 pressure which induced a higher consumption of base
302 to maintain the pH. Bicarbonate content at the end of the pretreatment was assessed using the
303 chemicals consumption over the first 30 minutes to maintain the pH to 5.5 (data not shown).
304 The values were 1.2 mmol of HCl/L in the control experiment, 0.6 mmol of NaOH/L after
305 sparging with 30 mL CO_2 /min, 0.8 mmol of NaOH/L after sparging with 100 mL CO_2 /min and
306 1.5 mmol of NaOH/L after sparging with 500 mL CO_2 /min. These chemicals consumptions
307 were representative of the bicarbonate content, and showed that the bicarbonate content after
308 pretreatment increased with the sparging CO_2 flowrate. This phenomenon was previously
309 reported by Willquist et al. [35] who studied the influence of sparging of N_2 or CO_2 on
310 hydrogen production by *Caldicellulosiruptor saccharolyticus*.

311

312 3.4. Bacterial community structure

313 The dynamics of the bacterial community structure was monitored for the two pretreatment
314 conditions leading to an improved or a similar H_2 production with regard to the control
315 experiments, i.e. with a CO_2 sparging of 20 minutes at 30 and 100 mL/min both at a stirring
316 speed of 300 rpm.

317 16S rRNA gene CE-SSCP fingerprints were similar before and after CO_2 exposure (data not
318 shown). The CO_2 pretreatment had thus no immediate effect on the bacterial community
319 structure. A good reproducibility of the diversity profiles detected at initial time was obtained
320 from one reactor to another (Fig. 4) which means that the three reactors were inoculated with
321 very similar communities of bacteria originated from the OFMSW. Regarding the global
322 evolution of the bacterial community structure, the behaviour was similar for the three
323 reactors. The diversity became more complex with time, as evidence by the increasing ratio of
324 Group A peaks which were undetectable initially. This result shows that a specific microflora
325 grew over the fermentation time. However, if same major peaks were detected, their ratio

326 varied from one reactor to another. When looking at the diversity retrieved at the end of the
327 H₂ production phase (around 45h), it was seen that the abundance of the peaks related to this
328 specific microflora was higher in the experiments with CO₂ pretreatment. This observation is
329 correlated with an increase in H₂ production, the highest peaks abundance corresponding to
330 the best hydrogen production.

331
332 In addition, functional *hydA* gene CE-SSCP fingerprinting was used to specifically compare
333 the diversity and the structure of H₂-producing clostridial population at different CO₂ flow
334 rates over time (Fig.5). Similar *hydA* CE-SSCP fingerprints were obtained at the beginning of
335 the experiments, demonstrating comparable inoculations of H₂-producing populations from
336 OFMSW, in accordance with 16S rRNA gene CE-SSCP observations. Whatever the CO₂
337 flowrate, the highest changes in dominant peaks of *hydA* CE-SSCP profiles were observed
338 during the first hours of the experiments, indicating the appearance of specific H₂ producers
339 from OMSFW during the first biogas production step. Higher *hydA* diversity was observed
340 when the maximum hydrogen production rate occurred. At this time, the highest *hydA*
341 diversity was observed at a CO₂ flow rate of 30 mL/min (Simpson's diversity indice D =
342 3.34) related to the highest hydrogen yield (mean value of 68.6 mL/g_{VS}), while the control
343 experiments were related to the lowest diversity (D = 1.1) and lower hydrogen yield (mean
344 value of 56.7 mL/g_{VS}). These results indicate that a higher *hydA* diversity was directly related
345 to higher H₂ yield, suggesting the establishment of positive bacterial interactions (*e.g.* co-
346 metabolism or synergy). After hydrogen production, it was observed a simplification of the
347 *hydA* CE-SSCP patterns. A simplification followed by a stabilization of the H₂-producing
348 community structure was previously reported in H₂-producing batch tests using synthetic
349 substrates due to competition/cooperation processes [47,48]. At the end of the experiments,
350 the highly similar *hydA* CE-SSCP profiles were composed of only one major peak and several
351 minor peaks (diversity ranging from 1.3 to 1.8). These results were in agreement with
352 previous studies showing that the H₂ production potential was associated with low bacterial
353 diversity and more generally that changes in H₂ production performances are related to shift
354 of the *hydA* structure over time [47-50].

355
356 Some of the bacteria that developed in the 30 mL_{CO2}/min pretreated reactor were identified
357 based on retrieved 16S rRNA gene sequences. The bacterial community was dominated by
358 members of the *Clostridium* genus (43% of the sequences) such as *C. saccharolyticum*, *C.*
359 *beijerinckii*, *C. acetobutylicum* and *C. diolis*. Blast similarities varied between 93% and 97%

360 which means that these affiliations to known *Clostridium* species could not be clearly defined
361 since similarity percentages with reference sequences were below the 97% acknowledge for
362 species definition [51]. All the *Clostridium*-related sequences were positioned among the
363 Group A peaks on the reactor 16S rRNA gene CE-SSCP profiles. Many *Clostridium* species
364 are well known H₂ producing bacteria. Thus their concomitant development when H₂ was
365 produced strongly suggests that *Clostridium* spp. are main actors of H₂ production in such
366 systems. Besides, their role in hydrogen production was confirmed by the detection of
367 clostridial *hydA* genes. The lactic bacterium *Lactobacillus sakei* was also detected (30% of the
368 sequences with a blast similarity of 99%). Its migration position on the diversity profile
369 corresponds to the peaks detected at the beginning of the experiments (Group B peaks). This
370 supports the role of such bacterium in the initial fermentative processes of the OFMSW.
371 These results are somewhat contradictory with previous statements of Kim et al. [12] who
372 showed that high CO₂ partial pressure had little effect on H₂-producing bacteria but inhibitory
373 effect on other microorganisms such as acetogens and lactic acid bacteria which were
374 competitive with H₂-producing bacteria. However, their study was performed with a
375 continuous sparging of CO₂ or N₂ during fermentation of sucrose therefore impacts of CO₂
376 partial pressure on bacterial communities may be stronger than in our works.

377
378 Results obtained in this study allow understanding the mechanisms of hydrogen production
379 from anaerobic digestion of OFMSW. This substrate initially contained a large population of
380 lactic acid producing bacteria such as *Lactobacillus sakei* which induced production of lactate
381 at the beginning of the experiments. During the fermentation course, the hydrogen producers,
382 which include members of the *Clostridium* genus, grew over time leading to a shift in the
383 metabolic pathway from lactic acid fermentation to hydrogen production. In particular, it was
384 shown that *C. beijerinckii*, *C. acetobutylicum* and *C. diolis* are related to conversion of lactate
385 to butyrate, CO₂ and H₂ in presence of acetate [52-54] and Matsumoto et al. [53] suggested
386 that this ability may be widely conserved in *Clostridium* genus. Hydrogen was then mainly
387 produced through the butyrate pathway. However, as shown in Table 5, hydrogen yields
388 obtained in this study were lower than 2 mol H₂/mol butyrate which is the theoretical
389 maximum yield for a butyrate pathway i.e. when only butyrate is produced during dark
390 fermentation. This low level could be attributed to other metabolic pathways and to the
391 presence of lactic acid producing bacteria since it was reported by several authors that lactic
392 acid producing bacteria can excrete other metabolites such as bacteriocins which inhibit the
393 activity of hydrogen producing bacteria and then lower hydrogen yields [44,55]. When a CO₂

394 sparging pretreatment was performed, no shift in metabolic pathways was observed as
395 confirmed by the similar bacterial community structure. Moreover, operating conditions of
396 CO₂ sparging pretreatment greatly influenced performances of the hydrogen production
397 system. In particular, a pretreatment with a CO₂ flowrate of 30 mL/min decreased lag phase
398 and increased the diversity of H₂ producing bacteria leading to an improvement of the
399 hydrogen yield by about 20%. In contrast, a pretreatment with CO₂ flowrate of 500 mL/min
400 increased lag phase and decreased the hydrogen yield by about 12%. This phenomenon was
401 accompanied with an increase in NaOH requirements to maintain the pH to its regulated value
402 and could then be explained by an enhanced osmotic pressure which could slowed down the
403 growth of some hydrogen producing microorganisms.

404

405 **4. Conclusions**

406 This study demonstrated the feasibility to produce hydrogen through fermentation of the
407 organic fraction of municipal solid waste (OFMSW) without adding an inoculum or any
408 nutrient solution. The hydrogen yield reached 56.7 mL_{H₂}/g_{VS}. The influence of an innovative
409 CO₂ sparging pretreatment on hydrogen production was investigated and the effects of the
410 CO₂ flowrate were evaluated. It was shown that hydrogen production decreased for a sparging
411 flowrate of 500 mL_{CO₂}/min, was not changed for a sparging flowrate of 100 mL_{CO₂}/min and
412 was increased for a sparging flowrate of 30 mL_{CO₂}/min. For this latter operating condition, a
413 substantial improvement in hydrogen production performances was obtained with an increase
414 in the hydrogen yield by more than 20%. A shift in chemicals consumption was also observed
415 with a reduction of more than 20% with a CO₂ flowrate of 30 mL/min whereas chemicals
416 required for maintaining the pH increased for all other operating conditions. Regarding the
417 metabolites production and consumption, similar trends were observed for all the experiments
418 even with CO₂ sparging pretreatments meaning that a CO₂ sparging pretreatment did not
419 induce shifts in the metabolic pathways. Finally, analyses of the bacterial community
420 structure showed that profile dynamics was similar in all cases but it was shown that high 16S
421 rRNA gene CE-SSCP fingerprints and *hydA* diversity were related to higher H₂ yield which
422 suggested the establishment of positive bacterial interactions (*e.g.* co-metabolism or synergy)
423 in mixed cultures producing biohydrogen. Therefore, an optimized CO₂ sparging pretreatment
424 can improve performances of the fermentative hydrogen production process by enhancing the
425 buffer capacity of the system and the development of hydrogen producing bacteria.
426 Furthermore, CO₂ could also be used as an acidifying agent for lowering the pH to a favorable
427 value for fermentative hydrogen production which could allow a more important reduction in

428 chemicals consumption. Since the fermentation process generates a gas containing CO₂ and
429 H₂ and that separation of CO₂ is needed for producing a H₂ gas which can be used in fuel cells
430 for electricity production, it could then constitute an innovative approach to reduce the global
431 carbon footprint of this technology.

432

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434

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441

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586 **Figures legends**

587

588 Fig. 1 : Schematic diagram of the anaerobic reactor used for the hydrogen production from OFMSW

589 Fig. 2 : Cumulative biogas and H₂ production for the control experiments

590 Fig. 3 : Evolution of acetate, butyrate and lactate concentrations for the control experiments (mean
591 values)

592 Fig. 4 : CE-SSCP profiles of 16S rRNA gene fragments retrieved from H₂-producing experiments
593 conducted on OFMSW at CO₂ flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

594 Fig. 5 : CE-SSCP profiles of hydA gene fragments retrieved from H₂-producing experiments
595 conducted on OFMSW at CO₂ flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

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598

599 Table 1 : Hydrogen production in batch processes fed with OFMSW and food waste

Feedstock	Inoculum	Reactor configuration	Supplementation	pH/Temperature	Pretreatment	H ₂ yield	Reference
<u>OFMSW from a French municipal waste collection</u>	No	Fermenters, 3.5L	No	pH: 5.5 T: 35°C	None	56.7 mL/gvs	This study
<u>OFMSW from a dining hall</u>	Digested sludge and H ₂ producing bacteria	Glass bottles, 120mL	Yes	pH: no regulation T: 37°C	None	180 mL/gvs	[19]
Food waste from a cafeteria	No	Fermenters, 200mL	No	pH: 5.0 T: 35°C	None Optimal: heat treatment at 90°C for 20 min	4.4 mL/gvs 96.9 mL/gvs	[29]
Food waste from households	No	Serum bottles, 200mL	No	pH : 5.5 initially, no regulation T : 37°C	None Optimal: ultrasonic pretreatment then acid pretreatment (pH = 3 for 24h)	40 mL/ gvs 118 mL/ gvs	[32]
Food waste from households	Seed sludge	Reactors, 500mL	No	pH : no regulation T : 35°C	None	Max: 39 mL/gvs	[42]
Food waste from a cafeteria	Seed sludge	Digester, 250mL	Yes	pH: various initial pH values then no regulation T: 35°C	Yes for initial pH adjustment	Max: 120 mL/gvs	[43]
Food waste from a cafeteria	Seed sludge	Serum bottles, 120mL	Yes	No pH regulation T: 30°C	None	Max: 104.8 mL/gvs	[44]

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601

602 Table 2 : Effect of pretreatment conditions on H₂ fermentation

Pretreatment conditions			Results						
CO ₂ flowrate (mL/min)	Stirring speed (rpm)	Time (min)	P (mLH ₂)	ΔP (% of the control exp.)	Yield (mLH ₂ /gvs)	Rm (mLH ₂ /h)	λ (h)	R ²	%H ₂
Control 1			802.7		57.3	68.8	22.0	0.9996	39.3
Control 2			788.2		56.1	59.0	25.0	0.9998	35.5
30	300	20	983.5	23.2	69.9	92.5	20.1	0.9969	33.0
30	300	20	944.4	18.6	67.3	72.9	16.5	0.9993	35.7
100	300	20	809.5	1.2	57.4	79.9	22.0	0.9994	29.6
500	300	20	712.2	-11.9	50.0	69.5	26.5	0.9982	24.0

603 P: maximum potential H₂ production; ΔP: variation in H₂ production potential compared to the control
 604 experiments; Rm: maximum H₂ production rate; λ: lag phase time; %H₂: hydrogen content of the
 605 biogas produced during the second step

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609 Table 3 : Metabolites at the end of the experiments (mean values are given for duplicates)

Pretreatment conditions			Final metabolite concentration (mmol/L)			Final B/A
CO ₂ flowrate (mL/min)	Stirring speed (rpm)	Time (min)	Acetate	Butyrate	Lactate	
Control			5.3	11.4	0.2	2.2
30	300	20	5.8	18.5	1.0	3.2
100	300	20	8.4	17.2	1.9	2.0
500	300	20	4.1	11.6	2.3	2.8

610

611

612 Table 4 : Influence of CO₂ pre-treatment on chemicals consumption (mean values are given for
 613 duplicates)

Pretreatment conditions			Results				
CO ₂ flowrate (mL/min)	Stirring speed (rpm)	Time (min)	pH after pretreatment	Acid consumption (HCl - mmol/L)	Base consumption (NaOH - mmol/L)	Total chemicals consumption (nacid + nbase) (mmol/L)	Δ Total chemicals consumption (% of the control exp.)
Control			5.6	16.4	5.5	21.9	
30	300	20	5.4	10.2	7.0	17.2	-21.4
100	300	20	5.4	15.6	12.0	27.6	25.6
500	300	20	5.4	13.3	34.7	48.0	118.5

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615

616 Table 5 : Ratio H₂/Butyrate at the end of the experiments (mean values are given for duplicates)

Pretreatment conditions			Final H ₂ /Butyrate (mol _{H₂} /mol _{butyrate})
CO ₂ flowrate (mL/min)	Stirring speed (rpm)	Time (min)	
Control			0.9
30	300	20	0.6
100	300	20	0.6
500	300	20	0.7

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Fig. 1 : Schematic diagram of the anaerobic reactor used for the hydrogen production from OFMSW

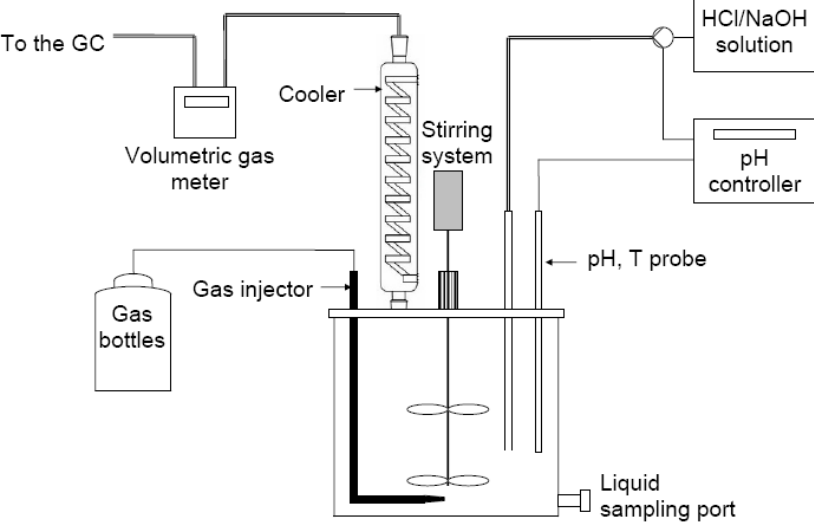


Fig. 2 : Cumulative biogas and H₂ production for the control experiments

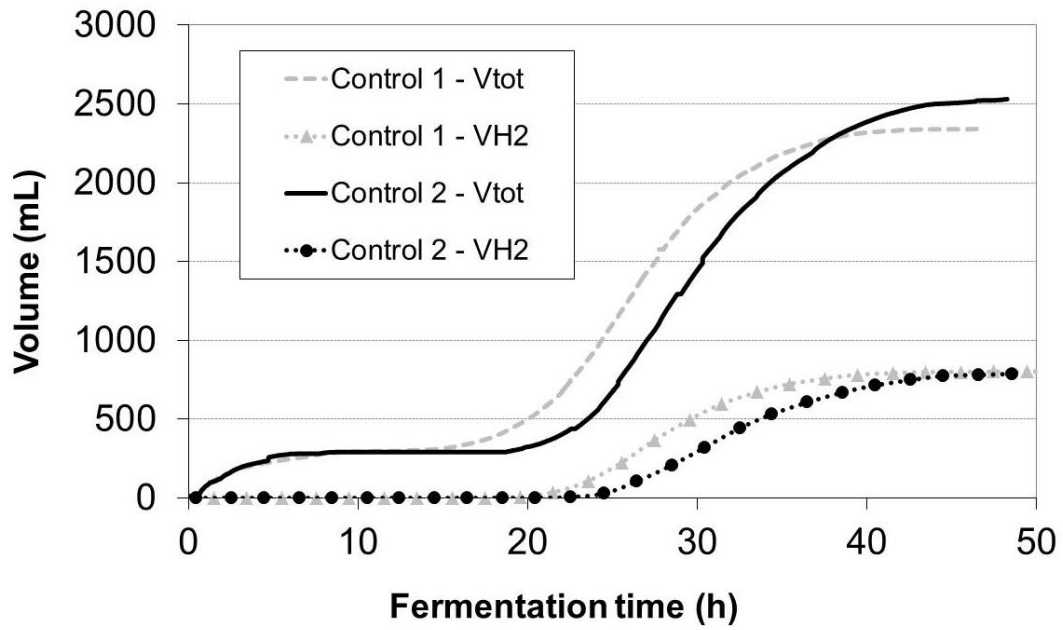


Fig. 3 : Evolution of acetate, butyrate and lactate concentrations for the control experiments (mean values)

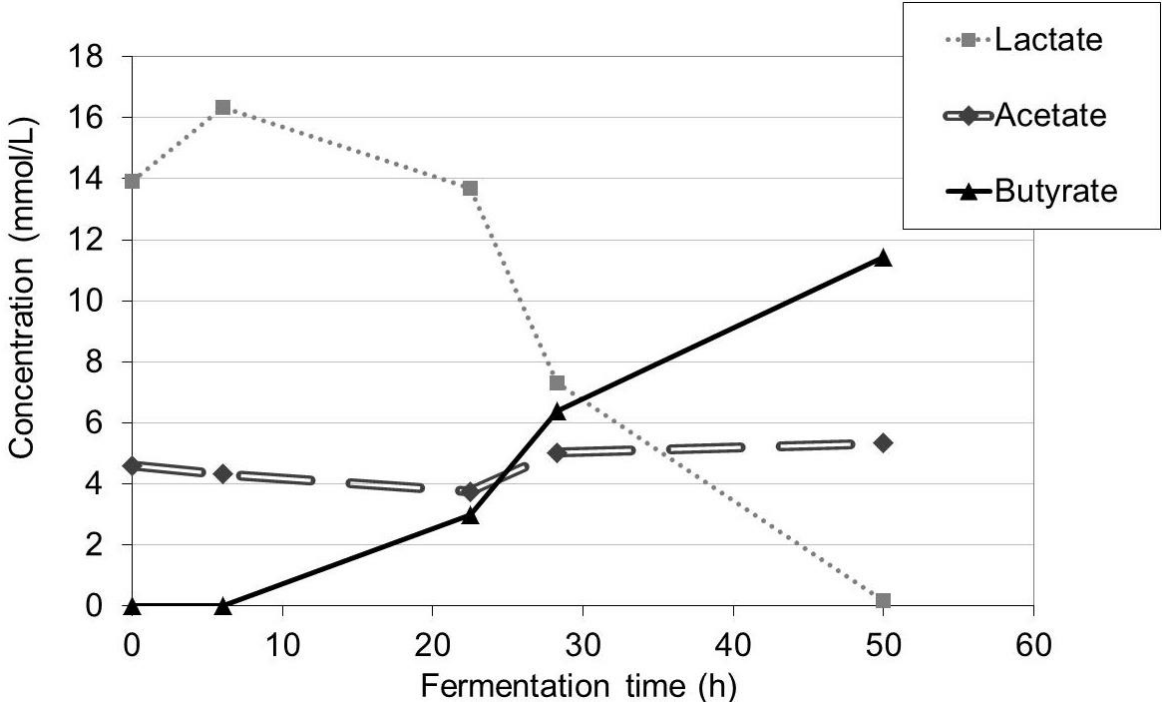


Fig. 4 : CE-SSCP profiles of 16S rRNA gene fragments retrieved from H₂-producing experiments conducted on OFMSW at CO₂ flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

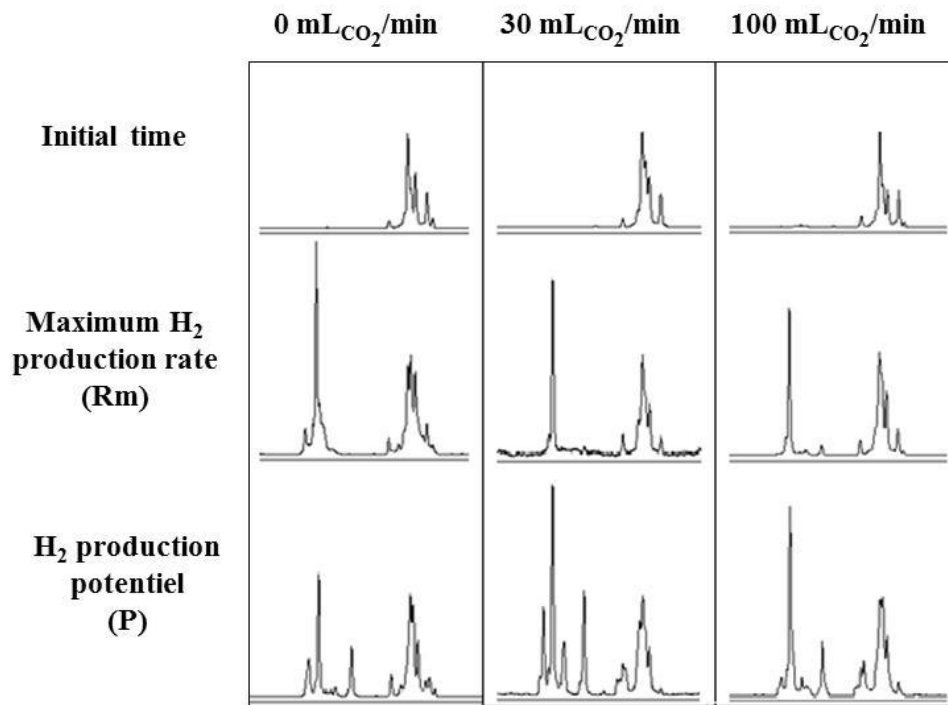


Fig. 5 : CE-SSCP profiles of *hydA* gene fragments retrieved from H₂-producing experiments conducted on OFMSW at CO₂ flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

