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Abstract

A series of batch tests was conducted to investigate the effect of a CO_2 sparging pretreatment on hydrogen production from fermentation of the organic fraction of municipal solid waste. Tests were carried out in a stirred bioreactor without adding inoculum or supplementation solution, at 35°C and with a pH regulated at 5.5. The influence of CO_2 flowrate (30, 100 and 500 mL/min) was evaluated. Results showed a decrease in biological hydrogen production for a sparging flowrate of 500 mL_{CO2}/min, similar performances for the control experiments and for a sparging flowrate of 100 mL_{CO2}/min and an increase in biohydrogen production for a sparging flowrate of 30 mL_{CO2}/min. For this latter operating condition, a substantial improvement in hydrogen production performances was obtained with an increase in the hydrogen yield by more than 20% and a decrease in the chemicals consumption used to maintain the pH by more than 20%. No shift in the metabolic pathways and in the bacterial community structure was observed but positive bacterial interactions occurred and enhanced the hydrogen production. Therefore, this study suggested that the growth of hydrogen producing bacteria could be either enhanced or slowed down by a CO_2 sparging pretreatment depending on the level of CO_2 flowrate, the shift being close to 100 mL_{CO2}/min.

Keywords

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

1. Introduction

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

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

chain organic acids are used by bacteria as electron donors to produce hydrogen using light energy [2,6,7]. Even though this conversion theoretically gives high conversion yields, this process has many shortcomings such as low production rates, difficulties in designing the reactors to maintain light penetration into a highly turbid bioreactor, low conversion efficiencies of light energy, and photo inhibition at high solar light intensities [2,8,9]. In dark fermentative processes, organic substrates, usually carbohydrates, are directly converted into H_2, CO_2, and organic acids without any external energy or electron acceptors [10]. Major drawbacks include lower hydrogen yields with only 10–20% of the substrate energy recovered as H_2 due to the limited metabolic energy of hydrogen fermentation; and instability of fermentative H_2 reactors [11-13]. However, dark fermentation is considered as the most favourable hydrogen-generating process due to a high hydrogen production rate, simple operation and design, and no requirement of additional light energy [12,14-16].

The production of hydrogen by dark fermentation from municipal solid waste and more especially from the organic fraction of municipal solid waste (OFMSW) is a relatively new process that has been studied by several authors [17-23]. Fermentative hydrogen production is a very complex process influenced by many factors such as the operating conditions (pH, temperature, agitation speed, hydrogen partial pressure, reactor type, hydraulic retention time), the substrate characteristics (type of substrate, organic acids, metal ions concentrations) and the composition of the microbial populations. In particular, mixed cultures appeared to be the most appropriate for producing biohydrogen from solid organic waste [24-26]. Indeed, the diversity of bacterial communities allows adaptation to different substrate composition and operating conditions either by fluctuation in community composition and/or metabolic adaptation [27,28]. However, some limitations regarding process development still remain to be solved, such as the suppression of hydrogen-consuming bacteria [17]. Previous studies reported several pretreatment methods to inhibit or kill hydrogen-consuming bacteria such as thermal, alkaline, acidification, ultrasonic or chemical pretreatment [10,17,29-32]. Another way to increase hydrogen production consists in decreasing the hydrogen partial pressure in the bioreactor with continuous gas sparging. Researches on gas sparging were mainly related to continuous sparging of gas such as N_2, CO_2, CH_4 during the fermentative degradation of simple substrates [3,12,33-35]. However, no clear relationship between the sparging conditions and the changes in H_2 yield was identified [3]. For example, Willquist et al. [35] investigated the influence of CO_2 sparging on batch fermentation of glucose and sucrose by *Caldicellulosiruptor saccharolyticus* at thermophilic conditions and showed that sparging with CO_2 had a negative influence on growth rate and hydrogen production rate but had little

influence on hydrogen yield. However, in a study performed by Kim et al. [12] on the effects of CO_2 sparging on continuous fermentative H_2 production from sucrose with seed sludge at mesophilic conditions, it was found that the H_2 yield increased noticeably with CO_2 sparging in comparison to unsparged conditions. Therefore, sparging with CO_2 may have various effects on hydrogen production. The objective of this study was to investigate and to bring new insight in the influence of a pretreatment step with CO_2 sparging on batch fermentative hydrogen production from a mixed culture, the OFMSW.

2. Materials and methods

2.1. Feedstock

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

2.2. Experimental setup and operation

Batch experiments were carried out in stirred reactors with a working volume of 3.5 L. The feedstock was fed with OFMSW at a VS content of 3.4 g/L. Neither exogenous inoculum nor mineral medium was added. After sealing, a pretreatment was performed in two steps at ambient temperature and under a mechanical stirring of 300 rpm. For the control experiments, the first step consisted in adjusting the pH to 5.5 with HCl and the second step consisted in sparging nitrogen during 15 minutes to reach anaerobic conditions through removal of dissolved oxygen. For experiments with CO_2 sparging pretreatment, the pretreatment consisted in firstly sparging the bioreactor with a fixed CO_2 flowrate during 20 minutes and secondly in sparging nitrogen during 15 minutes. The objective of these tests was to evaluate the effect a CO_2 sparging pretreatment on fermentative hydrogen production according to the CO_2 flowrate (30, 100 and 500 mL_{CO2}/min).

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

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

2.3. Analytical method

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

Liquid samples for analyses of the fermentation end-products were taken at regular intervals in the reactors. They were centrifuged at 11500 g for 15 min before analyses. The acidogenic end-products, i.e. volatile fatty acids such as acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and caproate, were quantified by gas chromatography coupled to flame ionization detection (GC-FID) in a Varian GC 3900, as described elsewhere [36]. The elution was carried out in a semi-capillary column FFAP of 15 m and 0.52 mm in diameter (Phase ECTM 1000). The conditions were as follows: carrier gas nitrogen at 20psi; injector temperature: 210°C; detector temperature: 280°C; gas flow rate: 6 mL/min; oven temperature ramp: 80 to 120 ° C with a ramp rate of 10 °C per minute after 1 min of elution. The quantification limit was about 40 mg/L. Other fermentation end-products, non-VFAs, such as lactate and ethanol were quantified by high performance liquid chromatography (HPLC) coupled to refractometric detection (Waters 2414). The separation column corresponded to an AMINEX® HPX-87H column (supplier BIORAD) thermostated at 35°C. The separation of the metabolic products was performed under isochratic elution of H_2SO_4 (0.008N) at a flow rate of 0.4 $mL.min^{-1}$. The refractometer temperature was fixed at 40°C. The quantification limit was about 100 mg/L each.

2.4. Bacterial community analyses

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

Dynamics of bacterial community structures were investigated by a fingerprinting technique of CE-SSCP (Capillary Electrophoresis-Single Strand Conformational Polymorphism). For this, about 200 bp of the V3 region (*E. coli* position 331 to 533) of bacterial 16S rRNA genes was PCR amplified (25 cycles, hybridation at 61°C) from genomic DNAs with the universal reverse primer w34 (5'-TTACCGCGGCTGCTGGCAC-3') and the eubacterial forward primer w49 (5'-ACGGTCCAGACTCCTACGGG-3') 5' end-labelled with the fluorescent dye FAM. After heat denaturation (5 min at 95°C) in deionized formamide and immediate cooling on ice, single-stranded PCR products of same length but different sequence were separated by CE-SSCP on an ABI Prism 310 genetic analyzer using a 47 cm long capillary and the non-denaturating 5.6% GeneScan polymer, and sample injection at 15kV, electrophoresis at 12kV, and run at 32°C. The internal standard GeneScan 600 LIZ was used to correct any change in the electrophoretic mobility between runs. Raw CE-SSCP profiles were analyzed using the GeneScan software. The CE-SSCP technique was also applied for monitoring the H_2-producing clostridial population using the functional [Fe-Fe]-hydrogenase (*hydA*) genes amplified with the nondegenerated primers hydAClosF (5'-ACCGGTGGAGTTATGGAAGC-3', *Clostridium pasteurianum* position F1258) and hydAClosR (5'-CATCCACCTGGACATGCCAT-3', *C. pasteurianum* position R1508), as described by Quéméneur et al. [27]. The *hydA* CE-SSCP profiles were aligned with the internal standard GeneScan 400 ROX and the sum of the peak areas were normalized to unit before statistical analysis using the StatFingerprints library [37] from R version 2.9.2 [38]. The complexity of the H_2-producing clostridial population was estimated using Simpson's diversity index D from a *hydA* CE-SSCP profile, by considering the number of OTU (number of peaks) as well as their relative abundance (area under each peak) [39].

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

2.5. Kinetic analysis of cumulative hydrogen production

The kinetic parameters of the cumulative H_2 production were assessed with a modified Gompertz equation. This equation was extensively used to describe the kinetics of hydrogen production from fermentation [8,19,41]. It considers the following expression:

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

where H(t) = cumulative H_2 production at fermentation time t (mL); P = maximum potential H_2 production (mL); Rm = maximum H_2 production rate (mL/h); λ = lag phase time (h) and e = Euler's number (2.718281828).

3. Results and discussion

3.1. Gaseous production

Fig. 2 shows the time course of cumulative biogas and hydrogen production in control experiments. Duplicates were performed and showed a good reproducibility of the results. As shown in Fig. 2, the biogas was produced in two steps. The first step occurred just after the beginning of the experiment and the biogas produced was only composed of CO_2. The second step was related to hydrogen production. This second phase started after a lag time of 22h - 25h with a maximum rate of 63.9 mL_{H2}/h and the final hydrogen production reached 795.4 mL_{H2} which corresponded to a hydrogen yield of 56.7 mL_{H2}/g_{VS} and a hydrogen content in the second phase biogas of 37.4% (mean values – the standard deviations for the maximum H_2 production rate, the final H_2 production, the H_2 yield and the H_2 content being 7 mL_{H2}/h, 10.2 mL_{H2}, 0.8 mL_{H2}/g_{VS} and 2.7% respectively). These results demonstrate the feasibility to produce hydrogen from the organic fraction of municipal waste without inoculum addition or nutritional supplements.

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

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

The effect of a CO_2 sparging pretreatment on H_2 production was evaluated for three CO_2 flowrates (30, 100 and 500 mL_{CO2}/min). Results of these experiments are presented in Table 2, including the kinetics parameters of the cumulative hydrogen production evaluated with the modified Gompertz equation (Eq. 1). All correlation coefficients of non-linear analysis were over 0.99, suggesting that the modified Gompertz equation was able to adequately describe the progress of cumulative hydrogen production in all batch tests. Methane was not detected whatever the operating conditions. Table 2 shows that the hydrogen yield was improved at a CO_2 flowrate of 30 mL_{CO2}/min with a mean value of 68.6 mL_{H2}/g_{VS} (standard deviation of 1.9 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}) obtained in the control experiments. This result corresponds to an increase of about 20% of the hydrogen yield. However, a slight decrease in the hydrogen content was observed meaning that the CO_2 sparging pretreatment also enhanced CO_2 production during the fermentation course. Regarding the influence of the CO_2 flowrate on hydrogen production, a shift was observed for a sparging flowrate close to 100 mL_{CO2}/min: the hydrogen production was improved by about 20% (mean value) for a sparging flowrate of 30 mL_{CO2}/min, it was similar for the control experiments and for a sparging flowrate of 100 mL_{CO2}/min, and it was reduced by nearly 12% for a sparging flowrate of 500 mL_{CO2}/min. Moreover, Table 2 shows that an increase in the CO_2 sparging flowrate had a negative impact on the other kinetics parameters: the hydrogen content decreased from about 34% (mean value) to 24% and the lag phase increased from about 18 hours (mean value) to 26.5 hours when the CO_2 flowrate increased from 30 to 500 mL_{CO2}/min. These results suggest that the growth of hydrogen producing bacteria could be either enhanced or slowed down by a CO_2 sparging pretreatment depending on the level of CO_2 flowrate.

3.2. Production of the main metabolites

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

concentrations of 13.9 mmol/L and 4.6 mmol/L respectively. During the first hours of fermentation it was observed an increase in lactate content. After about 20 hours, the lactate content decreased while butyrate was produced, this time corresponding to the beginning of the hydrogen production, as shown in Fig. 2. Similar trends of metabolite dynamics were observed for all the experiments even with CO_2 sparging pretreatments meaning that a CO_2 sparging pretreatment did not induce shifts in the metabolic pathways. These results are in accordance with the statement of Willquist et al. [35] who showed that sparging with CO_2 did not change the pathways to acetate and hydrogen.

Metabolite concentrations and the Butyrate to Acetate (B/A) ratio calculated at the end of the experiments are given in Table 3. Table 3 shows a higher lactate concentration at the end of the experiments with CO_2 sparging pretreatment, the value of lactate content increasing with the CO_2 sparging flowrate from 0.2 mmol/L at 30 mL_{CO2}/min to 2.3 mmol/L at 500 mL_{CO2}/min. Moreover, a higher butyrate concentration was observed for the two CO_2 flowrate conditions leading to an increase in hydrogen production, i.e. at 30 mL/min and 100 mL/min, the butyrate concentration increasing from 11.4 mmol/L for the control experiments to 18.5 mmol/L and 17.2 mmol/L respectively. The experiments with a CO_2 flowrate of 100 mL/min also induced an increase in acetate concentration whereas no such change was observed in tests with a CO_2 flowrate of 30 mL/min. With a CO_2 flowrate of 500 mL/min associated to a deterioration of hydrogen production performances, the acetate concentration was lower than in the control experiments. Therefore, no clear relation was found between pretreatment conditions and metabolite concentrations at the end of the experiments, in agreement with findings from previous study [36]. Regarding the B/A ratio, this ratio was always higher than 2.0 even in control experiments meaning the butyrate pathway was predominant during fermentation.

3.3. Chemicals requirement

Chemicals requirement to maintain the pH is an economic burden that has to be considered for the evaluation of the process feasibility. Assessments of chemicals consumption are presented in Table 4, where total chemicals consumption corresponds to the sum of the total number of moles of acid and base used throughout the experiment. The pH after pretreatment ranged from 5.4 to 5.6 in all experiments. Table 4 shows that total chemicals consumption decreased significantly with a CO_2 sparging pretreatment at 30 mL/min. The total chemicals 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 ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$); 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_{CO2}/min, 0.8 mmol of NaOH/L after sparging with 100 mL_{CO2}/min and
306 1.5 mmol of NaOH/L after sparging with 500 mL_{CO2}/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

varied from one reactor to another. When looking at the diversity retrieved at the end of the H_2 production phase (around 45h), it was seen that the abundance of the peaks related to this specific microflora was higher in the experiments with CO_2 pretreatment. This observation is correlated with an increase in H_2 production, the highest peaks abundance corresponding to the best hydrogen production.

In addition, functional *hydA* gene CE-SSCP fingerprinting was used to specifically compare the diversity and the structure of H_2-producing clostridial population at different CO_2 flow rates over time (Fig.5). Similar *hydA* CE-SSCP fingerprints were obtained at the beginning of the experiments, demonstrating comparable inoculations of H_2-producing populations from OFMSW, in accordance with 16S rRNA gene CE-SSCP observations. Whatever the CO_2 flowrate, the highest changes in dominant peaks of *hydA* CE-SSCP profiles were observed during the first hours of the experiments, indicating the appearance of specific H_2 producers from OMSFW during the first biogas production step. Higher *hydA* diversity was observed when the maximum hydrogen production rate occurred. At this time, the highest *hydA* diversity was observed at a CO_2 flow rate of 30 mL/min (Simpson's diversity indice D = 3.34) related to the highest hydrogen yield (mean value of 68.6 mL/g_{VS}), while the control experiments were related to the lowest diversity (D = 1.1) and lower hydrogen yield (mean value of 56.7 mL/g_{VS}). These results indicate that a higher *hydA* diversity was directly related to higher H_2 yield, suggesting the establishment of positive bacterial interactions (*e.g.* co-metabolism or synergy). After hydrogen production, it was observed a simplification of the *hydA* CE-SSCP patterns. A simplification followed by a stabilization of the H_2-producing community structure was previously reported in H_2-producing batch tests using synthetic substrates due to competition/cooperation processes [47,48]. At the end of the experiments, the highly similar *hydA* CE-SSCP profiles were composed of only one major peak and several minor peaks (diversity ranging from 1.3 to 1.8). These results were in agreement with previous studies showing that the H_2 production potential was associated with low bacterial diversity and more generally that changes in H_2 production performances are related to shift of the *hydA* structure over time [47-50].

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

which means that these affiliations to known *Clostridium* species could not be clearly defined since similarity percentages with reference sequences were below the 97% acknowledge for species definition [51]. All the *Clostridium*-related sequences were positioned among the Group A peaks on the reactor 16S rRNA gene CE-SSCP profiles. Many *Clostridium* species are well known H_2 producing bacteria. Thus their concomitant development when H_2 was produced strongly suggests that *Clostridium* spp. are main actors of H_2 production in such systems. Besides, their role in hydrogen production was confirmed by the detection of clostridial *hydA* genes. The lactic bacterium *Lactobacillus sakei* was also detected (30% of the sequences with a blast similarity of 99%). Its migration position on the diversity profile corresponds to the peaks detected at the beginning of the experiments (Group B peaks). This supports the role of such bacterium in the initial fermentative processes of the OFMSW. These results are somewhat contradictory with previous statements of Kim et al. [12] who showed that high CO_2 partial pressure had little effect on H_2-producing bacteria but inhibitory effect on other microorganisms such as acetogens and lactic acid bacteria which were competitive with H_2-producing bacteria. However, their study was performed with a continuous sparging of CO_2 or N_2 during fermentation of sucrose therefore impacts of CO_2 partial pressure on bacterial communities may be stronger than in our works.

Results obtained in this study allow understanding the mechanisms of hydrogen production from anaerobic digestion of OFMSW. This substrate initially contained a large population of lactic acid producing bacteria such as *Lactobacillus sakei* which induced production of lactate at the beginning of the experiments. During the fermentation course, the hydrogen producers, which include members of the *Clostridium* genus, grew over time leading to a shift in the metabolic pathway from lactic acid fermentation to hydrogen production. In particular, it was shown that *C. beijerinckii, C. acetobutylicum* and *C. diolis* are related to conversion of lactate to butyrate, CO_2 and H_2 in presence of acetate [52-54] and Matsumoto et al. [53] suggested that this ability may be widely conserved in *Clostridium* genus. Hydrogen was then mainly produced through the butyrate pathway. However, as shown in Table 5, hydrogen yields obtained in this study were lower than 2 mol H_2/mol butyrate which is the theoretical maximum yield for a butyrate pathway i.e. when only butyrate is produced during dark fermentation. This low level could be attributed to other metabolic pathways and to the presence of lactic acid producing bacteria since it was reported by several authors that lactic acid producing bacteria can excrete other metabolites such as bacteriocins which inhibit the activity of hydrogen producing bacteria and then lower hydrogen yields [44,55]. When a CO_2

sparging pretreatment was performed, no shift in metabolic pathways was observed as confirmed by the similar bacterial community structure. Moreover, operating conditions of CO_2 sparging pretreatment greatly influenced performances of the hydrogen production system. In particular, a pretreatment with a CO_2 flowrate of 30 mL/min decreased lag phase and increased the diversity of H_2 producing bacteria leading to an improvement of the hydrogen yield by about 20%. In contrast, a pretreatment with CO_2 flowrate of 500 mL/min increased lag phase and decreased the hydrogen yield by about 12%. This phenomenon was accompanied with an increase in NaOH requirements to maintain the pH to its regulated value and could then be explained by an enhanced osmotic pressure which could slowed down the growth of some hydrogen producing microorganisms.

4. Conclusions

This study demonstrated the feasibility to produce hydrogen through fermentation of the organic fraction of municipal solid waste (OFMSW) without adding an inoculum or any nutrient solution. The hydrogen yield reached 56.7 mL_{H2}/g_{VS}. The influence of an innovative CO_2 sparging pretreatment on hydrogen production was investigated and the effects of the CO_2 flowrate were evaluated. It was shown that hydrogen production decreased for a sparging flowrate of 500 mL_{CO2}/min, was not changed for a sparging flowrate of 100 mL_{CO2}/min and was increased for a sparging flowrate of 30 mL_{CO2}/min. For this latter operating condition, a substantial improvement in hydrogen production performances was obtained with an increase in the hydrogen yield by more than 20%. A shift in chemicals consumption was also observed with a reduction of more than 20% with a CO_2 flowrate of 30 mL/min whereas chemicals required for maintaining the pH increased for all other operating conditions. Regarding the metabolites production and consumption, similar trends were observed for all the experiments even with CO_2 sparging pretreatments meaning that a CO_2 sparging pretreatment did not induce shifts in the metabolic pathways. Finally, analyses of the bacterial community structure showed that profile dynamics was similar in all cases but it was shown that high 16S rRNA gene CE-SSCP fingerprints and *hydA* diversity were related to higher H_2 yield which suggested the establishment of positive bacterial interactions (*e.g.* co-metabolism or synergy) in mixed cultures producing biohydrogen. Therefore, an optimized CO_2 sparging pretreatment can improve performances of the fermentative hydrogen production process by enhancing the buffer capacity of the system and the development of hydrogen producing bacteria. Furthermore, CO_2 could also be used as an acidifying agent for lowering the pH to a favorable value for fermentative hydrogen production which could allow a more important reduction in

chemicals consumption. Since the fermentation process generates a gas containing CO_2 and H_2 and that separation of CO_2 is needed for producing a H_2 gas which can be used in fuel cells for electricity production, it could then constitute an innovative approach to reduce the global carbon footprint of this technology.

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Figures legends

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

Fig. 2 : Cumulative biogas and H_2 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 H_2-producing experiments conducted on OFMSW at CO_2 flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

Fig. 5 : CE-SSCP profiles of hydA gene fragments retrieved from H_2-producing experiments conducted on OFMSW at CO_2 flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

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
OFMSW from a French municipal waste collection	**No**	**Fermenters, 3.5L**	**No**	**pH: 5.5 T: 35°C**	**None**	**56.7 mL/g_{VS}**	**This study**
OFMSW from a dining hall	Digested sludge and H_2 producing bacteria	Glass bottles, 120mL	Yes	pH: no regulation T: 37°C	None	180 mL/g_{VS}	[19]
Food waste from a cafeteria	No	Fermenters, 200mL	No	pH: 5.0 T: 35°C	None	4.4 mL/g_{VS}	[29]
					Optimal: heat treatment at 90°C for 20 min	96.9 mL/g_{VS}	
Food waste from households	No	Serum bottles, 200mL	No	pH : 5.5 initially, no regulation T : 37°C	None	40 mL/ g_{VS}	[32]
					Optimal: ultrasonic pretreatment then acid pretreatment (pH = 3 for 24h)	118 mL/ g_{VS}	
Food waste from households	Seed sludge	Reactors, 500mL	No	pH : no regulation T : 35°C	None	Max: 39 mL/g_{VS}	[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/g_{VS}	[43]
Food waste from a cafeteria	Seed sludge	Serum bottles, 120mL	Yes	No pH regulation T: 30°C	None	Max: 104.8 mL/ g_{VS}	[44]

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602 Table 2 : Effect of pretreatment conditions on H_2 fermentation

Pretreatment conditions			Results						
CO_2 flowrate (mL/min)	Stirring speed (rpm)	Time (min)	P (mL_{H2})	ΔP (% of the control exp.)	Yield (mL_{H2}/g_{VS})	Rm (mL_{H2}/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 H_2 production; ΔP: variation in H_2 production potential compared to the control
604 experiments; Rm: maximum H_2 production rate; λ: lag phase time; $\%H_2$: 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

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611

612 Table 4 : Influence of CO_2 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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Table 5 : Ratio H_2/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

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

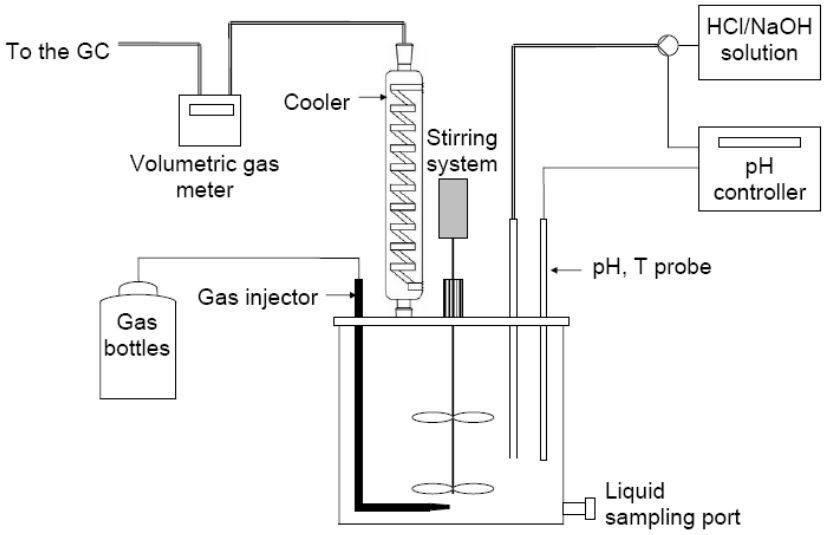

Fig. 2 : Cumulative biogas and H_2 production for the control experiments

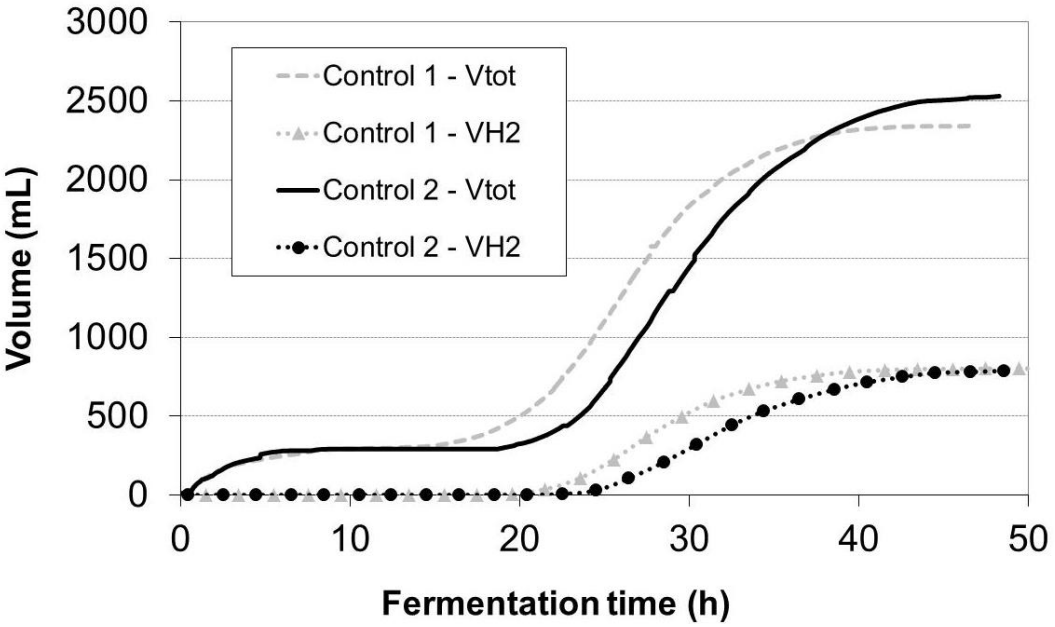

Fig. 3 : Evolution of acetate, butyrate and lactate concentrations for the control experiments (mean values)

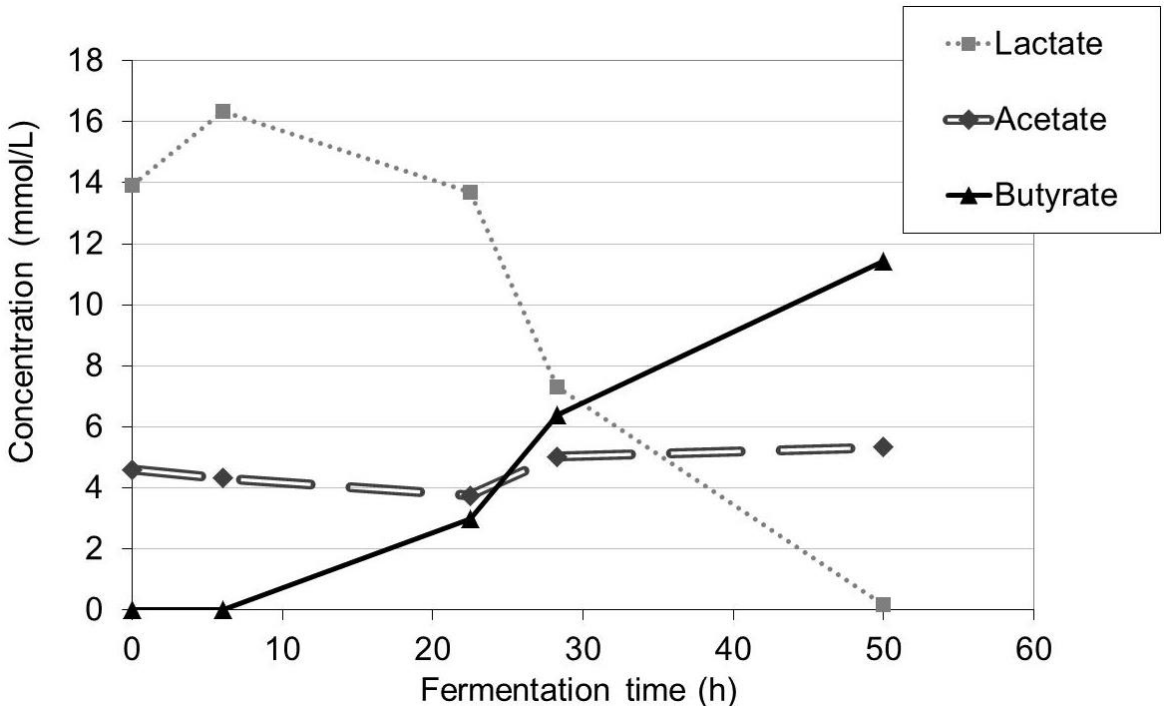

Fig. 4 : CE-SSCP profiles of 16S rRNA gene fragments retrieved from H_2-producing experiments conducted on OFMSW at CO_2 flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.

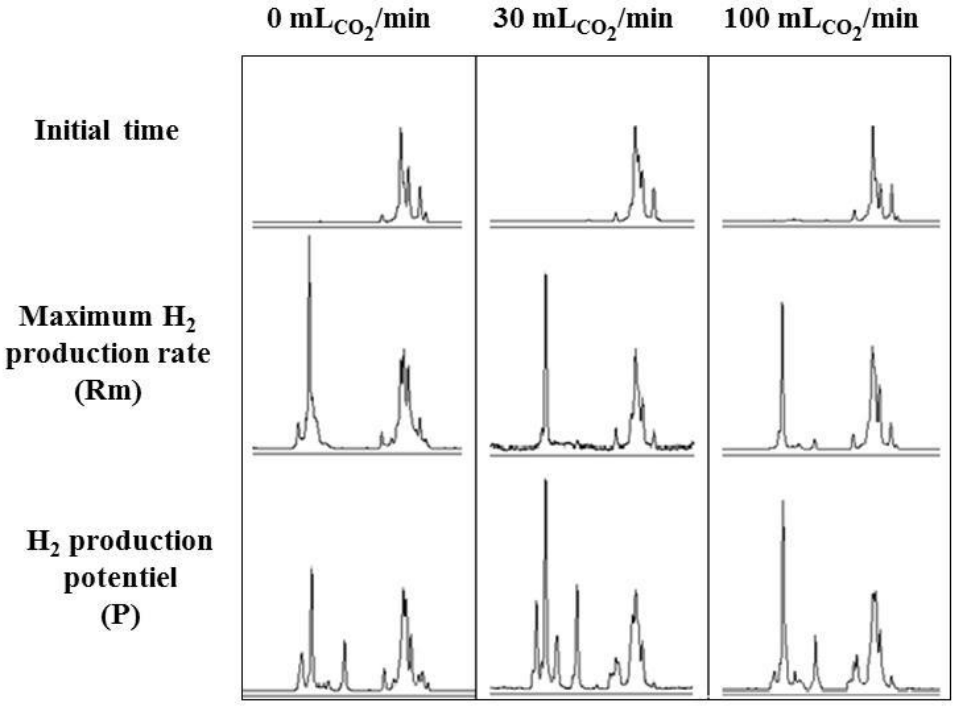

Fig. 5 : CE-SSCP profiles of hydA gene fragments retrieved from H_2-producing experiments conducted on OFMSW at CO_2 flow rates of 0 (control experiments), 30 mL/min and 100 mL/min.