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► To cite this version:

Alexandre Noblecourt, Gwendoline Christophe, Christian Larroche, Gaëlle Santa-Catalina, Eric Trably, et al.. High hydrogen production rate in a submerged membrane anaerobic bioreactor. International Journal of Hydrogen Energy, 2017, 42 (39), pp.24656 - 24666. 10.1016/j.ijhydene.2017.08.037 . hal-01657830

HAL Id: hal-01657830

<https://uca.hal.science/hal-01657830>

Submitted on 8 Sep 2023

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1 High hydrogen production rate in a submerged membrane anaerobic bioreactor

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10

11 Abstract:

12 Batch cultivation of an anaerobic consortium fed with glucose as sole carbon source
13 showed a sharp decrease of the hydrogen productivity when volatile fatty acids (VFA)
14 concentration exceeded 12.5g.L⁻¹. To avoid VFA accumulation, fermentative batch
15 cultures were thereafter carried out with a submerged membrane anaerobic bioreactor to
16 continuously remove hydrogen fermentation co-products, while retaining the biomass.
17 The membrane made it possible to separate the residence times of bacterial biomass and
18 hydraulic part. With this technology, average and maximal productivities reached 0.75
19 and 2.46 L_{H2}.L⁻¹.h⁻¹, corresponding to an increase of 44 and 51% in comparison to the
20 control, respectively. By removing the VFAs from the cultivation medium, H₂-
21 producing pathways were favored, confirming the metabolic inhibitory effects of co-

22 product accumulation in fermentation medium. Such hydrogen productivity is one of
23 the highest values encountered in the literature. Readily implementable, such
24 technology offers a good opportunity for further developing high rate hydrogen
25 fermentation bioprocesses.

26

27 Biohydrogen; Dark fermentation; Filtration; Volatile fatty acids; Gompertz model;

28 SSCP

29

30 1. Introduction

31 Over the past decade, progressive exhaustion of fossil fuels and raising awareness on
32 the ecological risks associated to their use have resulted in numerous scientific studies
33 aiming to find alternative solutions. Hydrogen production has been particularly studied.
34 Indeed, H₂ molecule has a high calorific value (142 MJ/kg), twice the energetic
35 potential of natural gas [1]. Moreover, water is the only residue released by combustion.
36 Hydrogen demand should therefore increase in the coming years [2,3] following the
37 wide development of fuel cells in many domains such as transportation, multimedia,
38 aerospace and civil engineering [4]. All these characteristics feature hydrogen as a
39 serious alternative to fossil fuels.

40 Nowadays, 95% of the hydrogen comes from fossil fuels [5], making essential to find
41 alternative methods.

42 Dark fermentation is a great process to produce biohydrogen. The principle is similar to
43 biogas production by anaerobic digestion as the DF is based on a chain of successive
44 reactions. First, some bacteria such as *Clostridia* [6] have the ability to hydrolyze
45 organic material and release simple sugars and amino acids. These products are then
46 converted into volatile fatty acids and alcohols through various metabolic pathways, so-
47 called acidogenesis. Only two metabolic pathways are inducing hydrogen production,
48 the acetate and butyrate pathways [7]. In mixed culture where different metabolic
49 pathways could occur, an average reaction was previously proposed [8]:



51 Hydrogen is produced with hydrogenase [Fe-S] which catalyses NADH oxidation and
52 reduction of two H⁺ into H₂. The main advantage of performing DF with mixed culture

53 is the ability of anaerobic bacteria to degrade many complex substrates such as
54 agricultural residues, lignocellulosic biomass, wastes, industrial and domestic effluents
55 [9]. Moreover, the VFAs co-produced during the culture correspond to high added value
56 platforms molecules that have many industrial applications [10] or potential substrate to
57 lipid production [11].

58 In mixed culture, culture conditions are crucial and have to be optimized first to select
59 only hydrogen producing bacteria (HPB) and further improve hydrogen yields and
60 productivities. In particular, the pH is of utmost importance and should be maintained
61 between 5.2 and 7. Outside this range, hydrogen production decreases because
62 competing pathways are favoured [12]. For a pH below 5.2 and above 7, propionate and
63 lactate can accumulate or solventogenesis reactions can occur inducing the
64 accumulation of acetone, butanol and ethanol (ABE fermentation). Overall, the
65 metabolites produced at that pH can also vary according to the microorganisms, the
66 substrates and the operating conditions. Culture medium composition has also to be
67 optimized to ensure sufficient quantity of macro and micro-elements, and adequate C/N
68 and C/P ratio [13]. Iron is also a key factor to hydrogen production since it triggers the
69 activation of the hydrogenases [14,15].

70 One of the major drawbacks of hydrogen production by DF is the presence of inhibitors
71 that can accumulate during the culture. The first one is the hydrogen itself through the
72 H₂ partial pressure. Indeed, hydrogen production becomes thermodynamically unfavour
73 when hydrogen partial pressure is higher than 10⁻³ atm [7] resulting in a loss of
74 productivity. To limit the impact of hydrogen partial pressure, conventional techniques
75 like flushing the bioreactor with inert gases (carbon dioxide, nitrogen, argon) [16,17] or
76 increasing the stirring rate can be used [18]. More recently, Gas Separation Membrane

77 Bioreactor was designed to improve hydrogen production capacity by stripping the
78 CO₂-concentrated fraction of the membrane separation unit to decrease hydrogen partial
79 pressure [19].

80 In addition, VFAs, as co-products, can accumulate in the liquid phase during the
81 reaction and have a detrimental effect on H₂ production. VFAs do not have the same
82 impact depending on their dissociation state. At pH 6 as commonly used, VFAs are in
83 majority dissociated since the pK_a of these compounds is close to 4.8. Thereby their
84 presence in the extracellular medium increases the osmotic pressure which in turn can
85 lead to cellular lysis of hydrogen-producing microorganisms [20].

86 Although VFAs toxicity has been clearly established in the literature, precisely
87 measuring the inhibition threshold for hydrogen production remains challenging,
88 because it is strongly correlated to the culture conditions as well as the microbial
89 community. To prevent accumulation of VFAs in the medium, a classical Completely
90 Stirred Tank Reactors (CSTR) conducted in a continuous mode allow to control the
91 concentration of VFAs. However, maintaining the concentration below the toxicity
92 threshold of VFAs also implies a limitation of the biomass concentration and hence the
93 productivity of H₂.

94 Using Anaerobic Membrane Bioreactors (AnMBR) can be an interesting technology to
95 limit the accumulation of VFAs while maintaining the biomass in the medium. Several
96 configurations (internal or external immersed membrane and non-immersed membrane),
97 design (tubular, plate and frame, rotary disk or hollow fiber), materials (organic,
98 inorganic, and metallic) and pore size are available [10,21,22].

99 The objective of the present study is to determine thanks to this technology the VFAs
100 toxicity threshold and propose a technical concept to improve hydrogen production.
101 Here, the novelty is to use a submerged anaerobic membrane bioreactor which allows to
102 withdraw continuously the reaction medium containing hydrogen fermentation co-
103 products. The biomass can accumulate into the bioreactor since the membrane cut-off is
104 below microorganism size. In anaerobic culture, cell recycling remains an interesting
105 approach mainly because the quantity of biomass produced as well as the growth rate
106 are generally low. Finally the performances of hydrogen production with and without
107 this technology are compared. 2. Materials and methods

108 2.1 Microorganisms

109 The microbial consortium was derived from sewage treatment plant sludge. A culture
110 was first carried out in complete medium, 2-bromoethanesulfonate (10mM) was added
111 to remove Archaea responsible for methane production [24] and then several
112 subcultures (10% v/v) were performed to stabilize and store the consortium.

113 2.2 Culture medium

114 The culture medium was adapted from Gaudet et al. (1992) [25]. The minerals
115 composition was (g.L^{-1}) : $(\text{NH}_4)_2\text{SO}_4$ 5.4; $\text{FeCl}_2,4\text{H}_2\text{O}$ 3.75; K_2HPO_4 1.35; KH_2PO_4
116 1.35; NaCl 0.9; ZnCl_2 0.41 ; $\text{H}_3\text{BO}_3, \text{H}_2\text{O}$ 0.3; $\text{NiCl}_2,6\text{H}_2\text{O}$ 0.13 ; $\text{Na}_2\text{MoO}_4, \text{H}_2\text{O}$ 0.13 ;
117 $\text{CuCl}_2,2\text{H}_2\text{O}$ 0.075 ; Hemin 10^{-3} ; $\text{MgSO}_4,7\text{H}_2\text{O}$ $2.4 \cdot 10^{-4}$; $\text{CaCl}_2,2\text{H}_2\text{O}$ $2.4 \cdot 10^{-4}$; biotin
118 $2 \cdot 10^{-4}$; PABA 10^{-4} ; $\text{FeSO}_4,7\text{H}_2\text{O}$ $8 \cdot 10^{-5}$; $\text{MnSO}_4,6\text{H}_2\text{O}$ $3 \cdot 10^{-5}$; $\text{CoCl}_2,6\text{H}_2\text{O}$ $3 \cdot 10^{-6}$.
119 Glucose concentration was 8 and 60 g.L^{-1} for the precultures and bioreactor medium
120 respectively. After sterilization, medium was flushed with CO_2 and L-cysteine was
121 added to facilitate oxygen elimination. Na_2CO_3 was simultaneously added to fix the pH

122 at 6 and 7 for the bioreactor and precultures medium, respectively. The pH was higher
123 in preculture to avoid solventogenesis, occurring when pH decreases along with VFAs
124 accumulation [26].

125 2.3 Bioreactor culture

126 The culture was carried out in a submerged anaerobic membrane bioreactor of 7L
127 (GPC-Bio) with 5L of working volume. The 500 mL inoculum represented 10% (v/v) of
128 the bioreactor. The culture parameters were as follows: temperature 35°C, stirring rate
129 100 rpm, pH regulated at 6 by KOH (5 mol.L⁻¹) and/or H₂SO₄ (4 mol.L⁻¹) addition.

130 Two types of culture were performed: in batch (without membrane) or in sequential
131 continuous mode with the use of a submerged membrane bioreactor (BAMI culture). In
132 this second operation, one liter of liquid reaction medium containing VFAs was
133 withdrawn after 11; 12.5; 14.4; 16.3; 18.7 hours of culture and was replaced by fresh
134 medium.

135 2.4 Characterization of submerged anaerobic membrane bioreactor

136 The submerged membrane was previously described and characterized by Trad et al.
137 (2015) [27]. It was composed of 142 polymeric hollow fibers in PolyVinylidene
138 Fluoride (PVDF) with 0.2 µm cut-off. The membrane length was 32.5 cm and the
139 exchange area was 0.155 m². The external loop volume was 0.5 L. The reaction medium
140 was continuously circulated in the membrane module with a recirculation pump
141 (Watson Marlow Qdos 30 Universal+) at the constant flow rate of 395.3 mL.min⁻¹. The
142 fibers of the membrane module were connected to a withdrawal pump (Ismatec 1076A)
143 for medium culture extraction at flow rate of 40 mL.min⁻¹. Analysis of the permeate

144 indicate a VFAs recovery yield of 97.2% [11]. During withdrawal of the supernatant,
145 the membrane was clogged by the biomass present on the fibers surface. To maintain a
146 constant flow rate, cleaning method was performed according the model developed in
147 our laboratory by Trad et al. (2015) [27] . The membrane was periodically flushed for
148 five minutes under a CO₂ flow rate of 0.2 to 0.3 L.min⁻¹, depending on the degree of
149 clogging. During the first two minutes of this operation, backwashing with extracted
150 supernatant was performed at the same time for more efficient unclogging. These
151 maintenance operations were renewed every 15 minutes.

152 2.5 Analytical methods

153 2.5.1 Gas phase

154 Biogas volume and flow rate were measured with a drum-type gas meter (Ritter TG05).
155 RIGAMO[®] software was used for analysis and real-time monitoring. Biogas
156 composition analysis was carried out with a micro-GC (Agilent 3000) equipped by two
157 channels and a thermal conductivity detector (TCD). The composition in hydrogen,
158 oxygen, nitrogen and methane was determined from the first channel with a molecular
159 sieve column (5Å). Argon was used as a vector gas at a pressure of 30 psi, the injector
160 and oven temperatures were respectively 80 and 90°C. Carbon dioxide and hydrogen
161 sulfur were determined by the other channel with a Pora plot U (PPU) column.
162 Hydrogen was the vector gas at a pressure of 30 psi, the temperatures of the injector and
163 of the column were 80 and 60°C, respectively. The software SOPRANE[®] provided to
164 SRA instruments was used to set parameters of gas chromatograph and analyze results
165 of gas composition.

166 The hydrogen production curves were modelled by the modified Gompertz model
167 primarily designed for pure batch cultures [26,28]. The model was described by the
168 following equation:

$$170 \quad H = P \exp \left\{ -\exp \left[\frac{R_m \cdot \exp(1)}{P} (\lambda - t) + 1 \right] \right\} \quad (\text{eq. 2})$$

171 where H represented the cumulated hydrogen production (L), P the potential hydrogen
172 production (L), R_m the maximal flow rate of hydrogen production ($\text{L}\cdot\text{h}^{-1}$), λ the lag
173 phase duration (h) and t the time (h).

174 The parameters P, R_m and λ were estimated by the solver function of Microsoft Office
175 Excel 2007 software. This function used the Newton algorithm calibrated with 100
176 iterations, 10^{-6} precision, 10^{-4} convergence and 5% tolerance to fit with experimental
177 values.

178 2.5.2 Liquid phase

179 The glucose and VFAs concentrations were determined in filtered aliquots of the culture
180 by HPLC. The HPLC equipment (Agilent 100, Agilent Technology) was composed of
181 two columns (Rezex ROA 300x7.8 nm, Phenomenex, USA) mounted in serial in an
182 oven (50°C), and coupled to a refractometer as detector. The mobile phase consisted of
183 2 mM sulfuric acid in ultra-pure water pumped at $0.7 \text{ mL}\cdot\text{min}^{-1}$ and 70 bars (7000 kPa).
184 For the analysis, 2 mL of sample were mixed with 250 μL of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ (0.3 M)
185 and 250 μL of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (5% w/v). The mixture was centrifuged at 10 000 g for 5
186 minutes. The supernatant was filtered through a 0.2 μm cellulose acetate filter and
187 injected in the HPLC.

188 2.5.3 Solid phase (Biomass)

189 During culture, the biomass was estimated by centrifugation of 5 mL of sample at
190 10000 g during 10 minutes. Cell dry weight was obtained by drying the sample in pre-
191 weighed tubes in an oven at 110°C until a constant weight was reached.

192 Six experimental times were selected for Single Strand Conformation Polymorphism
193 (SSCP) and quantitative PCR (qPCR) analysis: inoculation, start of gas production,
194 maximal gas production rate, stationary phase, beginning of the decrease in gas
195 production rate and end of gas production.

196 DNA extraction was carried out with Fast DNA SPIN Kit for Soil (MP Bio) from one
197 milliliter sample. DNA quantity and purity were assessed by spectrophotometry
198 (Infinite NanoQuant M200, Tecan).

199 ARNr 16s of V3 region was amplified with *Escherichia coli* position F331
200 (ACGGTCCAGACTCCTACGGG) and R500 (TTACCGCGGCTGCTGGCAC)
201 primers. The mix PCR was described by Saur et al. (2015) [29] and PCR conditions by
202 Milferstedt et al. (2013) [30].

203 The *hydA* gene present in *Clostridium Butyricum* strain DSM10702 was amplified with
204 the following mix: 17.25 µl molecular grade water, 2.5 µl of 10 × pfu turbo buffer, 2 µl
205 of 2.5 mM dNTP mixture, 1 µl of forward primer (500 nM) F234
206 (ACCGGTGGAGTTATGGAAGC) and reverse primer (500 nM)
207 R273 (CATCCACCTGGGCATGCCAT), 0.25 µl of 2.5 U/µl Stratagene PfuTurbo
208 DNA Polymerase (Stratagene). The PCR conditions were : denaturation for 2 min at
209 94°C ; 35 cycles of 3 steps, 30 sec at 94°C, 30 sec at 57°C and 30 sec at 72°C ; a final
210 elongation for 10 min at 72°C.

211 PCR products were analyzed by Capillary Electrophoresis – Single Strand
212 Conformation Polymorphism (CE–SSCP) in an ABI 3130 genetic analyzer (Applied
213 Biosystems) as described by (Rochex et al., 2008) [31], yielding a bacterial community
214 fingerprint. The obtained community profiles were aligned based on the ROX internal
215 size standards and normalized with the package Statfingerprints [32] in R 2.12 (R
216 Development Core Team, 2010).

217 Quantitative PCR samples were prepared on 96-well PCR plate (Eppendorf, Hamburg,
218 Germany) and analyzed with Mastercycler ep gradient S (Eppendorf, Hamburg,
219 Germany). The conditions for ARNr 16S were the same that Braun et al. 2011 [33]. For
220 *hydA* amplification by qPCR, the mix was composed of: 5.5 µl of molecular grade
221 water, 12.5 µl of SsoAdvanced™ Universal SYBR® Green Supermix, 1 µl of primers
222 [250nM] F234 (ACCGGTGGAGTTATGGAAGC) and R233
223 (CATCCACCTGGGCATGCCAT), 5 µl of DNA with two dilutions (1 and 0.1 ng). The
224 PCR cycle was composed of an enzyme activation step (2 min 98°C) followed by 40
225 cycles (15 sec 98°C and 1 min 60°C) with monitoring of the melting curve.

226

227 3. Results and Discussion

228 3.1 Determination of VFA inhibition threshold value

229 Here, the objective was to determine the minimal VFAs concentration inhibitory for
230 hydrogen production. For that purpose, limitations by carbon source or mineral medium
231 were avoided all along the culture and the hydrogen partial pressure was kept constant
232 because reaction occurs at atmospheric pressure and the hydrogen concentration in

233 biogas was constant at 50%. The partial pressure was estimated at 0.565 bar by the

234 Dalton's law:

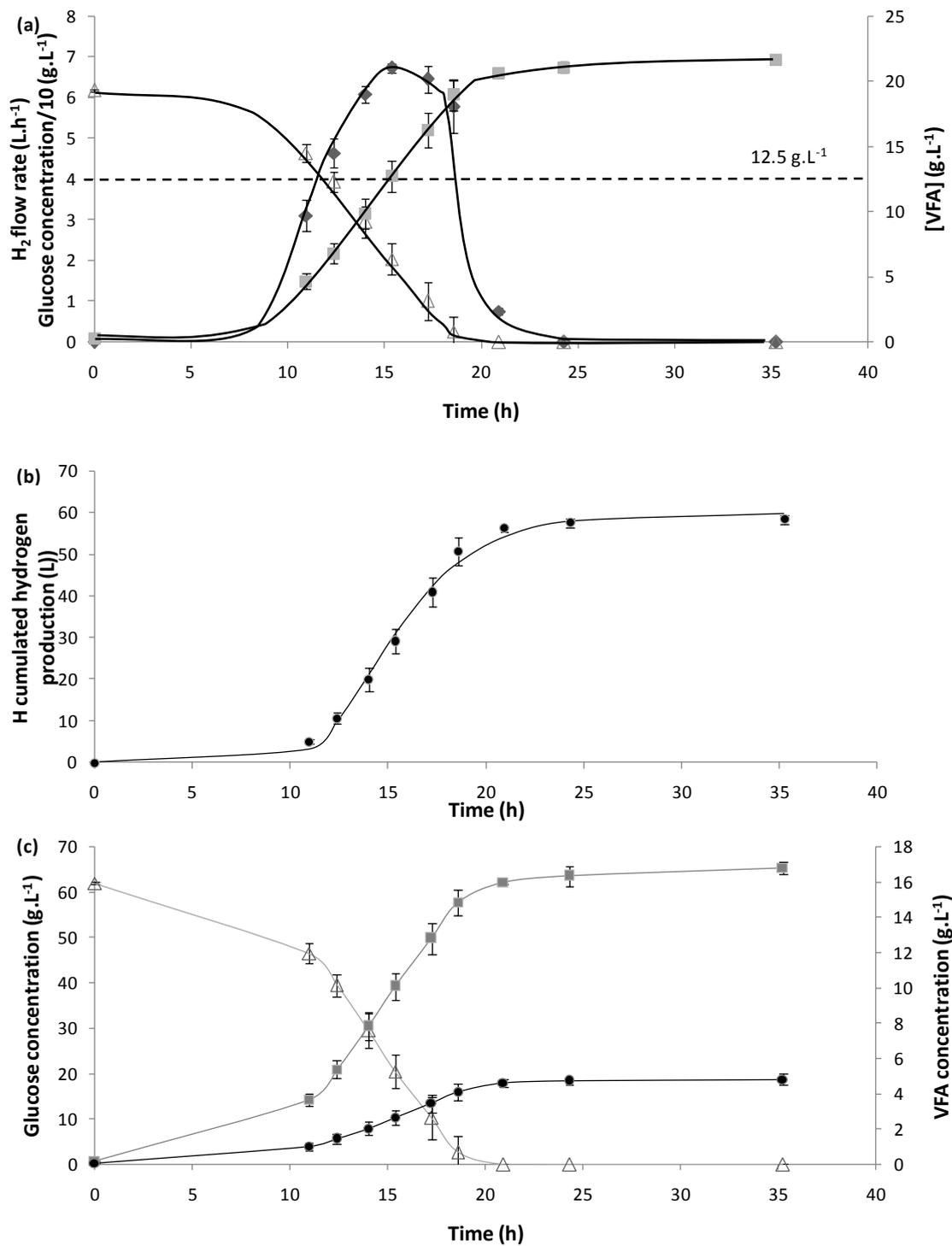
235
$$P_{H_2} = P_{tot} * Y_i \text{ (eq. 3)}$$

236 where P_{H_2} is the hydrogen partial pressure, P_{tot} the total pressure and Y_i the hydrogen

237 mole fraction.

238 Figure 1 shows the kinetics of the hydrogen flow rate, the modeling of cumulative

239 hydrogen production and the VFAs production over time.



240

241 Figure 1: Results obtained in liquid and gaseous phase in control culture with the follow
 242 conditions: batch 5L, glucose concentration of $60 g \cdot L^{-1}$, $35^\circ C$, pH6 constant, 100 rpm
 243 with medium described in part 2.2. (a) Kinetic of hydrogen flow rate, glucose

244 degradation and volatile fatty acids produced during culture. ◆ H₂ flow rate (L.h⁻¹) Δ
245 glucose concentration/10 (g.L⁻¹) ■ VFA concentrations (g.L⁻¹). (b) modelization of H₂
246 production by Gompertz (described in part 2.5.1) • experimental values. Parameters
247 determined by the model are latency λ=11.21h, potential hydrogen production P=59.96
248 L and a hydrogen flow rate max R_m=7.47 L.h⁻¹. (c) Kinetic of glucose degradation and
249 acetate, butyrate productions during culture. • Acetate concentration (g.L⁻¹) ■ Butyrate
250 concentration (g.L⁻¹) Δ Glucose concentration (g.L⁻¹).

251 In Figure 1a, the hydrogen flow rate increased until a maximum of 7 L.h⁻¹ at 15 hours
252 and after a lag period of 10 hours. Then it slowly decreased between 15 and 20 hours
253 while glucose concentration was not yet limiting (20.48 g.L⁻¹ at 15 hours). No
254 improvement of hydrogen flow rate was observed by adding glucose at this time of the
255 process indicating that glucose concentration was not limiting (data not shown). Only a
256 gradual decrease of hydrogen flow rate was observed with initially VFAs addition in
257 figure 3b.

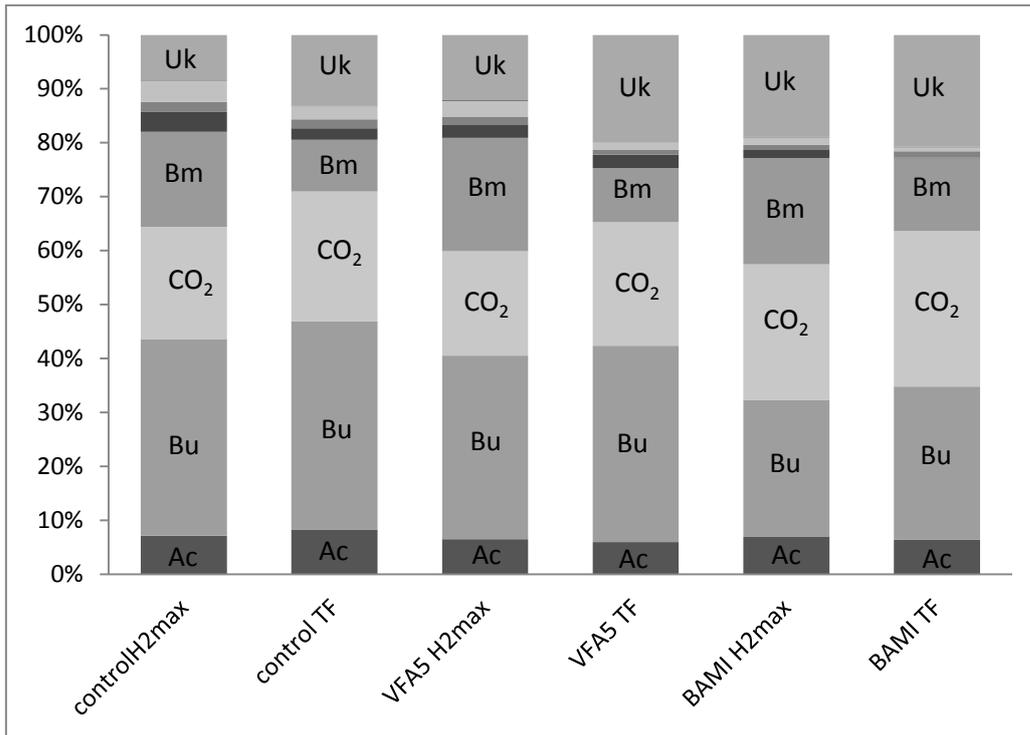
258 Finally, hydrogen production stopped after 24 hours, in response to glucose exhaustion.

259 Similarly to hydrogen flow rate, the VFAs production started after 10 hours and
260 increased until 20 hours to reach 20 g.L⁻¹. After this time, the VFA production also
261 slowed down due to glucose exhaustion.

262 Interestingly, when VFAs concentration reached 12.5 g.L⁻¹ with a B/A ratio of 3.8, the
263 hydrogen flow rate started to decrease from 7 to 6 L.h⁻¹. Under the assumption of no
264 limitation by mineral or carbon and a stable hydrogen partial pressure, such kinetic
265 slowdown was most likely due to an excessive accumulation of volatile fatty acids.

266 In Figure 1b, the kinetic parameters were determined using the Gompertz model which
267 is the reference model used to characterize a biogas production. The experimental data
268 fitted well the model. The kinetic parameters were a lag phase of 11.21 hours, a
269 maximal hydrogen cumulated volume of 60 L and a maximal flow rate of 7.47 L.h⁻¹.
270 The global performance of this batch process was very interesting since the maximal
271 productivity (Hydrogen Production Rate) was one of the highest values found in the
272 literature (1470 ± 236 mL_{H2}.L⁻¹.h⁻¹). The obtained yield (1.42 mol_{H2}.mol_{glucose}⁻¹) was
273 similar to others publications in mixed cultures and batch experiments with a yield
274 between 1-1.8 mol_{H2}.mol_{glucose}⁻¹ [34,35].

275 The metabolite distributions are presented in figure 1c. The main metabolites were
276 butyrate and acetate. The final concentration of acetate and butyrate reached
277 respectively 4.81 and 16.81 g.L⁻¹ for a B/A ratio of 3.5. Here, with the selected
278 microbial consortium and under these operating conditions, the butyrate pathway was
279 favoured with regard to the acetate pathway. Hydrogen production is generally
280 uncorrelated to the acetate pathway because of possible homoacetogenesis reactions, but
281 the butyrate pathway remains a good indicator [36]. With these concentrations, 2 mole
282 of hydrogen should be produced for one mole of cumulated acetate and butyrate. The
283 homoacetogenesis ratio of 0.86 indicates that only small quantity of butyrate and
284 hydrogen was converted into acetate. Other metabolites produced by competing
285 pathways of hydrogen production were detected in only very small quantity. The final
286 concentrations of propionate, lactate, ethanol and glycerol were respectively 0.06, 1.28,
287 0.76 and 1.46 g.L⁻¹. These metabolites represented only 6.3% of the carbon consumed.
288 The global carbon recovery for this batch was 87% of total carbon (figure 2).



289

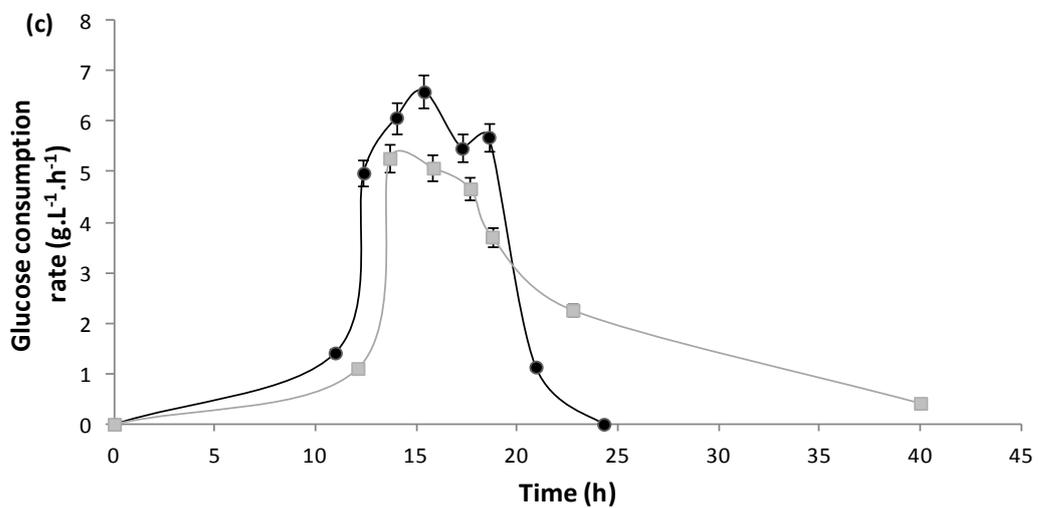
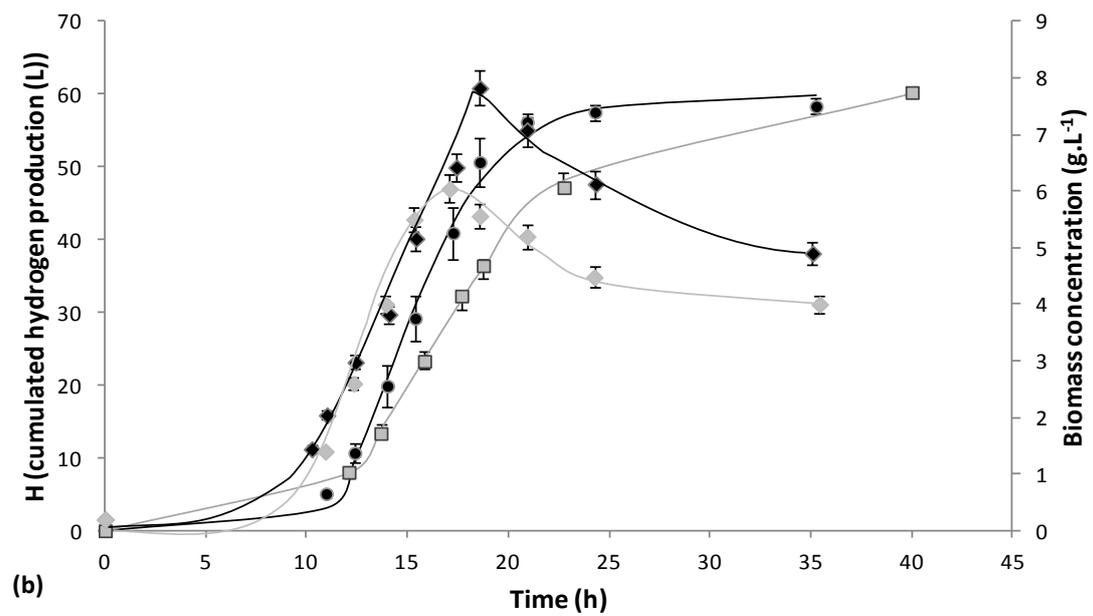
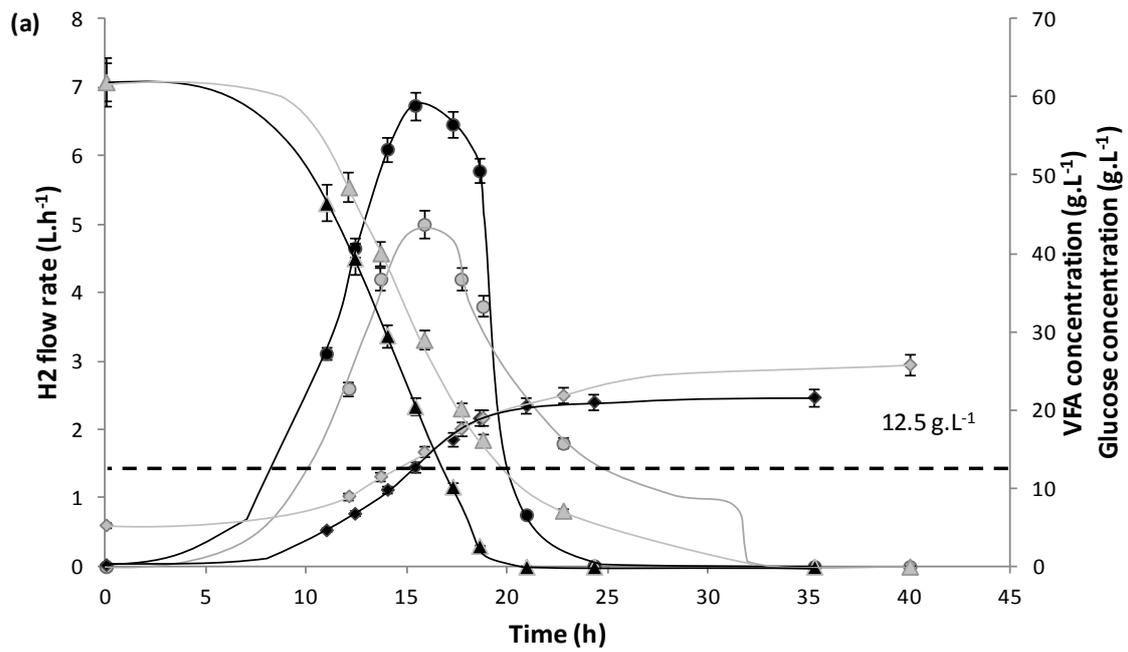
290 Figure 2: Carbon footprint of three cultures conditions: Control, VFA5 (Batch with 5
 291 g.L^{-1} of VFA adding in the culture medium initially), BAMI (culture in semi-continuous
 292 with submerged anaerobic bioreactor). The products were in ascending order: acetate,
 293 butyrate, CO_2 , biomass, lactate, ethanol, glycerol, propionate and unknown metabolites.
 294 In this graphic, propionate was present in very small quantities in the three cultures,
 295 consequently it is not visible. The main metabolites were identified by abbreviation. Ac:
 296 acetate, Bu: butyrate, CO_2 , Bm: biomass, Uk: unknow.

297 The toxicity of VFAs is clearly demonstrated in literature but the operating condition
 298 significantly differs. Several cultures were carried out with many initial VFAs
 299 concentration and generally the cumulative hydrogen decreased from VFAs
 300 concentration was closed to 5 g.L^{-1} .

301 3.2 Impact of VFAs addition on hydrogen production

302 The results obtained in this first batch experiment showed interesting results and more
303 especially for Hydrogen Production Rate, suggesting the presence of highly efficient
304 microorganisms. The results also indicated that above a certain concentration of VFAs
305 (around 12.5 g.L^{-1}) the flow rate can slow down rapidly. To confirm these results, and
306 compare with other publications, a set of experiments were performed by adding VFAs
307 in the culture medium. A special focus was made on acetate and butyrate since they
308 were mostly present in the culture with regard to the other metabolites such as lactate,
309 propionate and ethanol [28,37].

310 A culture was carried out with an initial addition of 5 g.L^{-1} VFAs (ratio B/A = 4.5) in
311 the initial culture medium and was compared with the control culture (no VFAs added).
312 The results of these two experiments are presented in Figure 3.



314 Figure 3: comparison of control culture and VFA5 culture (same culture with addition
 315 of $5\text{g}\cdot\text{L}^{-1}$ VFA with ratio B/A of 4.5 before starting culture). (a) Monitoring of hydrogen
 316 flow rate in control and VFA5 cultures • control ● VFA5, monitoring VFA productions
 317 ◆ control ◆ VFA5 and glucose concentration ▲ control ▲ VFA5. (b) Monitoring of
 318 cumulate hydrogen production modeled by Gompertz model and biomass
 319 concentration. Parameters determined was respectively for control and VFA5 cultures:
 320 latency 11.21 and 10.67h, potential hydrogen production (P) 59.96 and 60.38 L,
 321 hydrogen maximal flow rate (R_m) 7.47 and $4.55\text{ L}\cdot\text{h}^{-1}$. • Control experimental values ■
 322 VFA5 experimental values ◆ control biomass concentration ◆ VFA5 biomass
 323 concentration. (c) Monitoring of glucose consumption rate in control and VFA5 culture,
 324 control ● VFA5 ■.

325 In the presence of initial VFAs, the lag phase was one hour longer compared to the
 326 control. The toxicity of VFAs led to adjustment to the biomass before starting to
 327 produce Hydrogen. A maximal flow rate of $5\text{ L}\cdot\text{h}^{-1}$ was observed after 15 hours in
 328 presence of VFAs while a maximum of $7\text{ L}\cdot\text{h}^{-1}$ was reached in the control culture (figure
 329 3a). The flow rate slowed down when the VFAs concentration reached 12.5 and 13.5
 330 $\text{g}\cdot\text{L}^{-1}$ and glucose exhaustion occurred at 20 and 32 hours in the control and the batch
 331 with VFAs, respectively. Consequently, the average hydrogen production rate as
 332 determined with the Gompertz model (figure 3b) was lower ($360\text{mL}_{\text{H}_2}\cdot\text{L}_{\text{medium}}^{-1}\cdot\text{h}^{-1}$)
 333 with VFAs than in the control culture ($524\text{ mL}_{\text{H}_2}\cdot\text{L}_{\text{medium}}^{-1}\cdot\text{h}^{-1}$). The main difference
 334 between the two cultures was the glucose consumption rate (figure 3c). In the two
 335 cultures the glucose consumption rate increased until 6.6 and $5.3\text{ g}_{\text{glucose}}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ in the
 336 control and VFA5 cultures, respectively. When a VFAs concentration of $12.5\text{ g}\cdot\text{L}^{-1}$ was
 337 reached, glucose consumption rate decreased progressively until glucose exhaustion.

338 According to metabolite distribution (figure 2), no significant redirection of the
 339 metabolic pathways was observed although the presence of an initial VFAs
 340 concentration of $5\text{g}\cdot\text{L}^{-1}$ but solventogenesis reaction occurred at higher VFAs
 341 concentration of $8\text{g}\cdot\text{L}^{-1}$, and the hydrogen yield and productivity significantly decreased
 342 (table 1). All these results suggest that the flow rate decrease in the VFA5 culture was
 343 due to a slowing down of the metabolism flux (figure 3c) to resist to a high VFAs
 344 concentration in the medium. During the culture with VFAs addition, the inhibitory
 345 threshold was reached earlier, that's why less biomass was produced (figure 3b) and
 346 consequently hydrogen productivity was reduced.

347

348 Table 1: Comparative table of VFA concentration impact on hydrogen production. (1)

349 Butyrate/Acetate ratio (2) Productivity expressed in $\text{mL}\cdot\text{g}_{\text{VSS}}^{-1}\cdot\text{h}^{-1}$.

inoculum	substrate	inhibitor	[VF A] ($\text{g}\cdot\text{L}^{-1}$)	Y_{H_2} ($\text{mol}_{\text{H}_2}\cdot\text{mol}_{\text{glucose}}^{-1}$)	HPR ($\text{mL}_{\text{H}_2}\cdot\text{L}_{\text{medium}}^{-1}\cdot\text{h}^{-1}$)	H2 cumulated (mL)	Ref
sludge	glucose	-	0	1.42	524	59960	This study
		Bu/Ac = 4.5 ⁽¹⁾	5	1.48	360	60237	
		Bu/Ac = 4.2	8	1.3	250	52225	
		BAMI (semi- continuous)	0	1.58	751	-	
seed sludge	glucose	-	0	1.75	61.8	212	[38]
		butyrate	4.18	1.85	54.5	225	
<i>Clostridium</i> <i>Bifermans</i>	glucose	-	0	-	57 ⁽²⁾	33,2	[39]
		acetate	4.72	-	51 ⁽²⁾	24	
		butyrate	4.72	-	31 ⁽²⁾	23	
seed sludge	sucrose	-	0	1.06	74.2	-	[40]
		acetate	5	1.04	52.6	-	

350

351 These results are similar to those obtained by Zheng and Yu, 2005 [38], who worked
352 with an initial butyrate concentration of 5 g.L⁻¹ (table 1), and observed that the
353 productivity decreased but the hydrogen production yield remained the same. Some
354 results from another experiment performed in the laboratory showed that yield and
355 productivity decline when VFAs concentration is higher than 5 g.L⁻¹ similarly to Y.
356 Wang et al., 2008 [40]; Zhang et al., 2012 [39] results. During this experiment (table 1),
357 solventogenesis reaction produced lactate in largest quantity and consequently the
358 hydrogen yield decreased. Butyrate has a negative effect on hydrogen production
359 comparable to acetate, with a higher toxicity on bacterial cells [39,41].

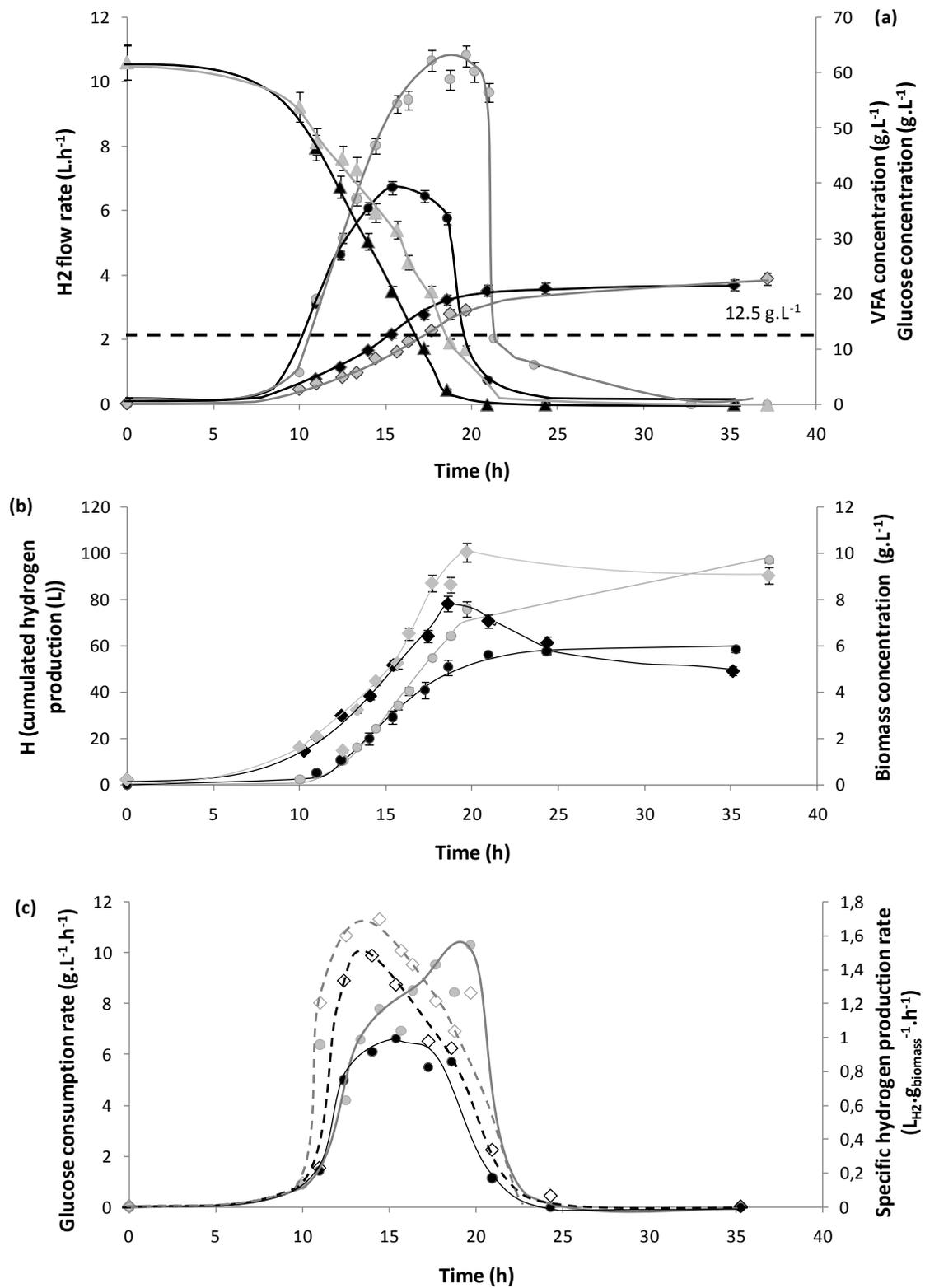
360 3.3 Sequential continuous operation with submerged anaerobic membrane 361 bioreactor

362 Previous results show that VFAs accumulation was harmful to hydrogen production,
363 and the toxicity threshold value was estimated at 12.5g/L. In order to maintain a VFAs
364 concentration below this toxicity level, the use of a membrane was evaluated, which
365 may allow the extraction of the culture medium containing VFAs while recycling the
366 microbial biomass. At the same time, the reactor volume can be kept constant by
367 addition of culture medium and dilution of residual VFAs.

368 The results obtained with the submerged anaerobic membrane bioreactor were
369 compared to the control culture in batch.

370 3.3.1 Hydrogen production versus Volatile fatty acids accumulation

371 Cumulated hydrogen production, H₂ flow rate, VFAs production and glucose
372 consumption rate as well as specific hydrogen production rate are presented in figure 4.



373

374 Figure 4: comparison of control culture and BAMI culture in submerged membrane

375 anaerobic bioreactor. During BAMI culture, five withdrawal of one liter medium was

376 realized with membrane at 11, 12.5, 14.4, 16.3 and 18.7 hours of culture and replaced
377 by standard medium. **(a)** Monitoring of hydrogen flow rate and VFA concentrations in
378 control and BAMI cultures • Control H₂ flow rate • BAMI H₂ flow rate, ◆ control VFA
379 concentration ◆ BAMI VFA concentration. **(b)** Monitoring of cumulate hydrogen
380 production modeled by Gompertz model and biomass concentration. Parameters
381 determined was respectively for control and BAMI cultures: latency 11.21 and 12.02h,
382 potential hydrogen production (P) 59.96 and 98.4 L, hydrogen maximal flow rate (R_m)
383 7.47 and 9.99 L.h⁻¹ • control experimental values ■ BAMI experimental values ◆
384 control biomass concentration ◆ BAMI biomass concentration. **(c)** Monitoring of
385 glucose consumption rate in control and BAMI cultures, • control • BAMI; and specific
386 hydrogen production rate, ◇ control and ◇ BAMI.

387 The Hydrogen flow rate was similar to the control during the ten first hours of culture.
388 A significant difference was observed from the time when VFAs began to be extracted
389 thanks to the membrane technology. The reduction of fermentative co-product
390 accumulation in the medium induced an increase of the maximum H₂ flow rate. This
391 latter reached 10 L.h⁻¹ at 17 hours of culture compared to 7 L.h⁻¹ in the control (figure
392 4a). This value was maintained close to 10 L.h⁻¹ during 4 hours and then dropped after
393 21 hours of culture because of glucose exhaustion. This maximal flow rate was same
394 that the value determined by Gompertz model in the figure 4b (9.99 L.h⁻¹). Stopping the
395 increased Hydrogen flow rate could be correlated to a VFAs concentration close to
396 13g.L⁻¹. This value was very close to the toxicity threshold determined for the control
397 culture in our previous experiment. Therefore, the use of a submerged membrane
398 significantly improved the average performance of the anaerobic process (table 1). The
399 average and maximal hydrogen production rates reached 0.75 and 2.47 L_{H₂}.L⁻¹.h⁻¹

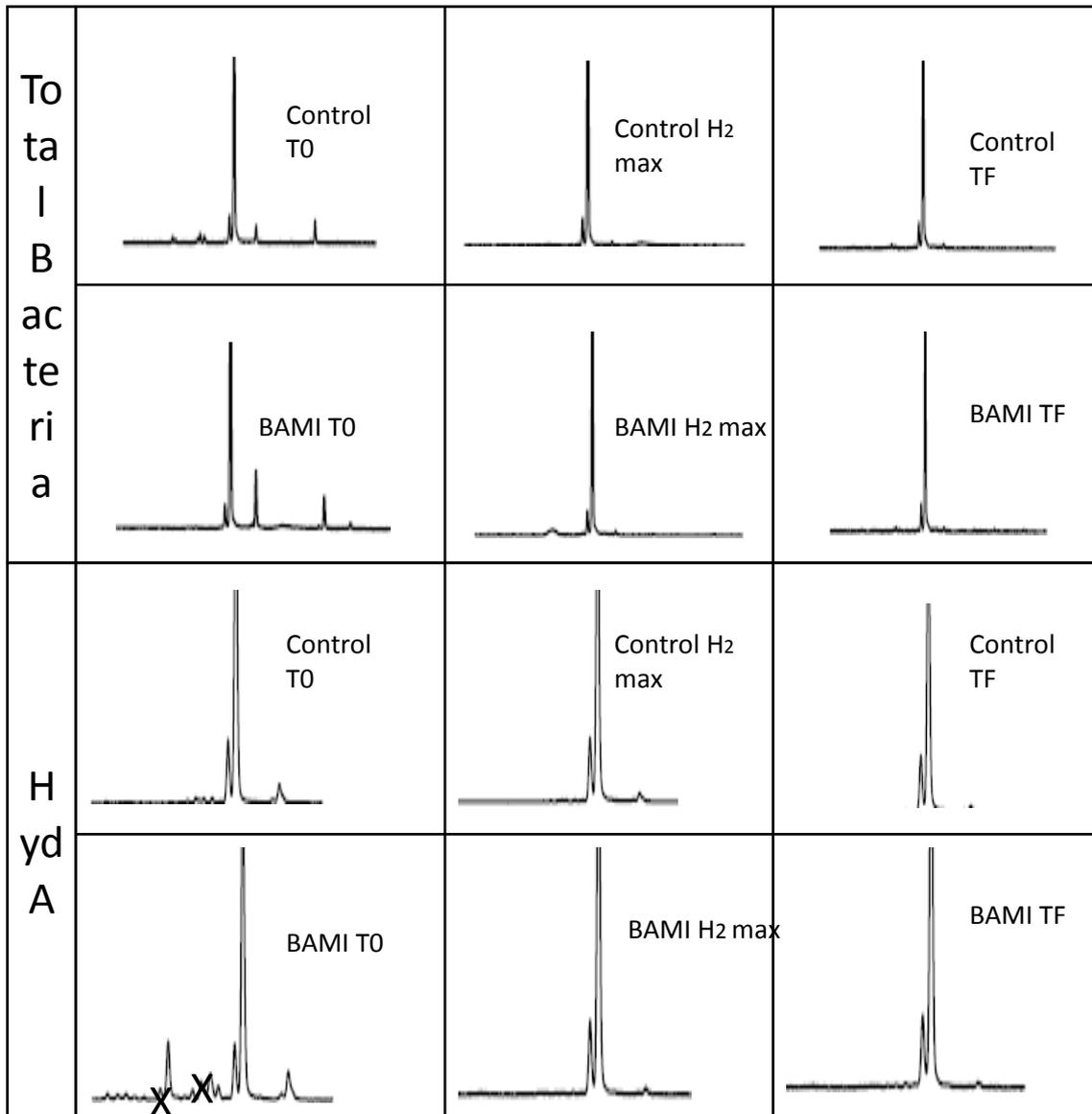
400 against 0.52 and 1.64 in the control respectively because more biomass was produced
401 (figure 4b) thanks to VFAs extraction which delays the attainment/ reaching of the
402 inhibitory threshold. The hydrogen yield ($1.58 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$) was also high when
403 compared to the control ($1.42 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$). The obtained yield was higher than
404 Lee et al. (2008) [42] with $1.27 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$. No recent publication investigated
405 hydrogen production with anaerobic membrane bioreactor performing in semi-
406 continuous mode as this study, but the maximum hydrogen production rate ($2.47 \text{ L}_{\text{H}_2} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)
407 was greater than others process operated in continuous mode as Lee et al. (2008)
408 ($1.48 \text{ L}_{\text{H}_2} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$) [42] and Oh et al. (2004) ($0.64 \text{ L}_{\text{H}_2} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$) [43]. It can suppose that the
409 performance will be close in continuous mode. The membrane technology applied in
410 these experiments offered great performance in comparison with others technology used
411 to concentrate biomass and improve hydrogen production. For example, Ishikawa et al.,
412 2008 [44] developed a compact stacked flatbed reactor performed in continuous mode
413 and obtained $1.2 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$ and $0.8 \text{ L}_{\text{H}_2} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$).

414 The produced metabolites were similar (figure 2). The B/A ratio was slightly lower
415 (3.3) than in the control (3.7) and no homoacetogenesis reactions was observed (ratio
416 homoacetogenesis reaction was 0.99). These results can explain the higher hydrogen
417 yield because in the acetate pathway, 4 moles of hydrogen are produced from one mole
418 of glucose versus only two in the butyrate pathway. The other metabolites produced by
419 concurrent hydrogen pathway represented only 4% of the total available carbon (figure
420 2). The other metabolites yields were lower than in the control. The global carbon
421 recovery at end of the BAMI culture (Anaerobic Immersed Membrane Bioreactor) was
422 80% but a part of biomass was not considered because it clogged at the surface of fibers
423 membrane.

424 These experiences confirmed the influence of metabolic co-products on hydrogen
425 production, with a inhibitory concentration close to 13 g.L^{-1} . The use of a submerged
426 membrane in the anaerobic bioreactor has delayed the accumulation of this inhibitor in
427 the medium consequently; more biomass was produced, which increases the rate of
428 hydrogen production even if the yield of hydrogen produced remains very close. It also
429 allowed obtaining a solution of fatty acids, without microorganisms, which could be
430 directly used for green chemistry or for the coupling of other processes as
431 photofermentation or yeast culture for single one oil production [11].

432 3.3.2 Study of consortium by SSCP and quantitative PCR analysis

433 To achieve a better understanding of the microbial dynamics during the culture,
434 molecular and quantitative analyzes were performed from the solid phase. SSCP
435 profiles using the V3 region of RNA 16S specific primers were used as fingerprint
436 analysis to provide information on biodiversity present in the culture (figure 5).



437

438 Figure 5: Qualitative and quantitative analyzes of microorganisms realized on samples
 439 during culture. SSCP profiles with specific primers of the V3 region DNA 16S
 440 representative of the total bacteria and primers of hydA to analyze sample biodiversity
 441 after inoculation (T₀), maximal hydrogen flow rate (H₂ max) and final time (T_F) for
 442 control and BAMI cultures.

443 The chromatograms obtained for total bacteria from BAMI and control cultures were
 444 very close at the beginning of the culture suggesting that the initial conditions were

445 similar regarding the microbial community. A low diversity was observed in these
446 samples with mainly one major bacterium and only few other bacteria. During culture at
447 maximal H₂ production and at the end of batch, the minor bacteria were still present but
448 in very small proportions compared to the dominant species. Hydrogen producer
449 biodiversity was also analysed thanks to SSCP profiles targeting the *hydA* gene. The
450 SSCP profiles presented low diversity as for total bacteria. Several bacteria were found
451 but only one was predominantly present in the sample. By cross-checking with RNA
452 16S profiles, it appears that the main dominant species carried also the *hydA* gene. Strict
453 anaerobic fermentative bacteria such as *Clostridium* sp. are well known to carry this
454 gene for hydrogen production [45]. This was confirmed by the sequencing results,
455 where the most abundant species was related to *Clostridium* sp. (94 - 99% in BAMI
456 culture and control culture). The most abundant was *Clostridium butyricum strain CDC-*
457 *51208*. At the end of the culture, the same bacteria were found but the difference in
458 concentration between other species was significant both in the BAMI and the control
459 culture. According to the qPCR study, the difference between the total population and
460 the H₂-producing population remained constant regardless of the conditions tested. Only
461 an increase in the bacterial population was logically observed when membrane was
462 used. During these cultures, no sub-dominant bacteria emerged, and the consortium
463 remained stable, meaning that the initial presence of VFAs did not influence the
464 microbial community structure, but only affected the metabolic pathways. VFAs
465 accumulation increased the osmotic pressure; when it became too large a progressive
466 lysis of microorganisms is observed and hydrogen production can slow down [20]. This
467 phenomenon occurred here when the concentration of volatile fatty acids is higher than
468 12.5 g.L⁻¹. In BAMI culture, the specific hydrogen production rate was slightly higher

469 than in the control (15%) but this difference was not significant because a part of
470 biomass stayed on fibers membrane. The specific hydrogen production rate was closed
471 in the two cultures and the increased hydrogen productivity in BAMI culture was due to
472 the biomass recycling which promoted biomass production that's why the glucose
473 consumption rate was higher in BAMI culture. The use of membrane technology did not
474 cause metabolic perturbation (figure 2) but favored biomass production and
475 consequently improved hydrogen production.

476

477 4. Conclusion

478 With the bacterial consortium used in this study, the toxicity threshold of VFAs was
479 determined. A decrease of the hydrogen production rate was observed when the
480 concentration of hydrogen fermentation co-products reached 12.5 g.L^{-1} . It was
481 demonstrated that using Anaerobic Membrane Bioreactors (AnMBR) represent an
482 interesting technology to limit the accumulation of VFAs while maintaining the biomass
483 in the medium. In response, maximal hydrogen production rate was increased of 51%
484 with respectively 1.64 and $2.47 \text{ L}_{\text{H}_2} \cdot \text{L}_{\text{medium}}^{-1} \cdot \text{h}^{-1}$ for BAMI culture and control culture.
485 Even if clogging represents a risk with this technology and can lead to technical
486 difficulties, the improvement in hydrogen production remains significant. The challenge
487 now is to see how the system behaves during a longer operating time by increasing the
488 withdrawal flow rate and limiting the accumulation of VFAs. The valorization of the
489 permeate enriched in VFAs in an associated process represent also an interesting
490 perspective of this work.

491

492 This work has been sponsored by the French government research program
493 “Investissements d’avenir” through the IMobS3 Laboratory of Excellence (ANR-10-
494 LABX-16-01), by the European Union through the regional program "competitiveness
495 and employment 2014- 200 " (ERDF-Auvergne region) and by the Auvergne region.

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