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High hydrogen production rate in a submerged membrane anaerobic bioreactor

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Abstract:

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

1. Introduction

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

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

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



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

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

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

One of the major drawbacks of hydrogen production by DF is the presence of inhibitors that can accumulate during the culture. The first one is the hydrogen itself through the H_2 partial pressure. Indeed, hydrogen production becomes thermodynamically unfavourable when hydrogen partial pressure is higher than 10^{-3} atm [7] resulting in a loss of productivity. To limit the impact of hydrogen partial pressure, conventional techniques like flushing the bioreactor with inert gases (carbon dioxide, nitrogen, argon) [16,17] or increasing the stirring rate can be used [18]. More recently, Gas Separation Membrane

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

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

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

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

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

2. Materials and methods

2.1 Microorganisms

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

2.2 Culture medium

The culture medium was adapted from Gaudet et al. (1992) [25]. The minerals composition was (g.L⁻¹) : (NH₄)₂SO₄ 5.4; FeCl₂.4H₂O 3.75; K₂HPO₄ 1.35; KH₂PO₄ 1.35; NaCl 0.9; ZnCl₂ 0.41 ; H₃BO₃.H₂O 0.3; NiCl₂.6H₂O 0.13 ; Na₂MoO₄.H₂O 0.13 ; CuCl₂.2H₂O 0.075 ; Hemin 10⁻³ ; MgSO₄.7H₂O 2.4.10⁻⁴ ; CaCl₂.2H₂O 2.4.10⁻⁴ ; biotin 2.10⁻⁴; PABA 10⁻⁴; FeSO₄.7H₂O 8.10⁻⁵; MnSO₄.6H₂O 3.10⁻⁵; CoCl₂.6H₂O 3.10⁻⁶. Glucose concentration was 8 and 60 g.L⁻¹ for the precultures and bioreactor medium respectively. After sterilization, medium was flushed with CO₂ and L-cysteine was added to facilitate oxygen elimination. Na₂CO₃ was simultaneously added to fix the pH

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

2.3 Bioreactor culture

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

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

2.4 Characterization of submerged anaerobic membrane bioreactor

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

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

2.5 Analytical methods

2.5.1 Gas phase

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

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

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

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

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

2.5.2 Liquid phase

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

2.5.3 Solid phase (Biomass)

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

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

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

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

The *hydA* gene present in *Clostridium Butyricum* strain DSM10702 was amplified with the following mix: 17.25 µl molecular grade water, 2.5 µl of 10 × pfu turbo buffer, 2 µl of 2.5 mM dNTP mixture, 1 µl of forward primer (500 nM) F234 (ACCGGTGGAGTTATGGAAGC) and reverse primer (500 nM) R273 (CATCCACCTGGGCATGCCAT), 0.25 µl of 2.5 U/µl Stratagene PfuTurbo DNA Polymerase (Stratagene). The PCR conditions were : denaturation for 2 min at 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 elongation for 10 min at 72°C.

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

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

3. Results and Discussion

3.1 Determination of VFA inhibition threshold value

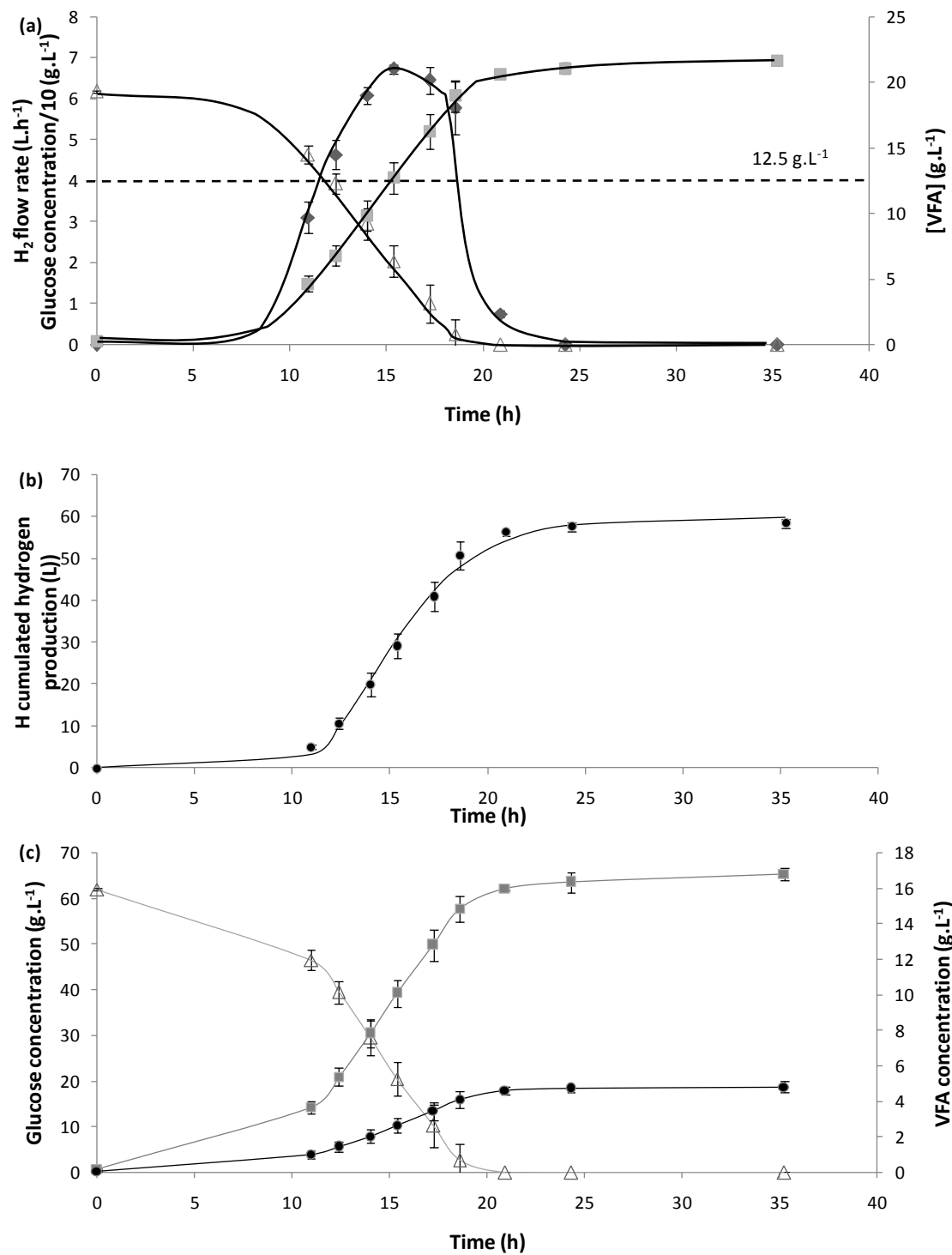
Here, the objective was to determine the minimal VFAs concentration inhibitory for hydrogen production. For that purpose, limitations by carbon source or mineral medium were avoided all along the culture and the hydrogen partial pressure was kept constant because reaction occurs at atmospheric pressure 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.L⁻¹, 35°C, pH6 constant, 100 rpm
 243 with medium described in part 2.2. (a) Kinetic of hydrogen flow rate, glucose

degradation and volatile fatty acids produced during culture. ♦ H_2 flow rate ($L.h^{-1}$) Δ glucose concentration/10 ($g.L^{-1}$) ■ VFA concentrations ($g.L^{-1}$). (b) modelization of H_2 production by Gompertz (described in part 2.5.1) • experimental values. Parameters determined by the model are latency $\lambda=11.21h$, potential hydrogen production $P=59.96 L$ and a hydrogen flow rate max $R_m=7.47 L.h^{-1}$. (c) Kinetic of glucose degradation and acetate, butyrate productions during culture. • Acetate concentration ($g.L^{-1}$) ■ Butyrate concentration ($g.L^{-1}$) Δ Glucose concentration ($g.L^{-1}$).

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

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

Similarly to hydrogen flow rate, the VFAs production started after 10 hours and increased until 20 hours to reach $20 g.L^{-1}$. After this time, the VFA production also slowed down due to glucose exhaustion.

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

In Figure 1b, the kinetic parameters were determined using the Gompertz model which is the reference model used to characterize a biogas production. The experimental data fitted well the model. The kinetic parameters were a lag phase of 11.21 hours, a maximal hydrogen cumulated volume of 60 L and a maximal flow rate of 7.47 L.h⁻¹. The global performance of this batch process was very interesting since the maximal productivity (Hydrogen Production Rate) was one of the highest values found in the literature ($1470 \pm 236 \text{ mL}_{\text{H}_2} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$). The obtained yield ($1.42 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$) was similar to others publications in mixed cultures and batch experiments with a yield between 1-1.8 $\text{mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$ [34,35].

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

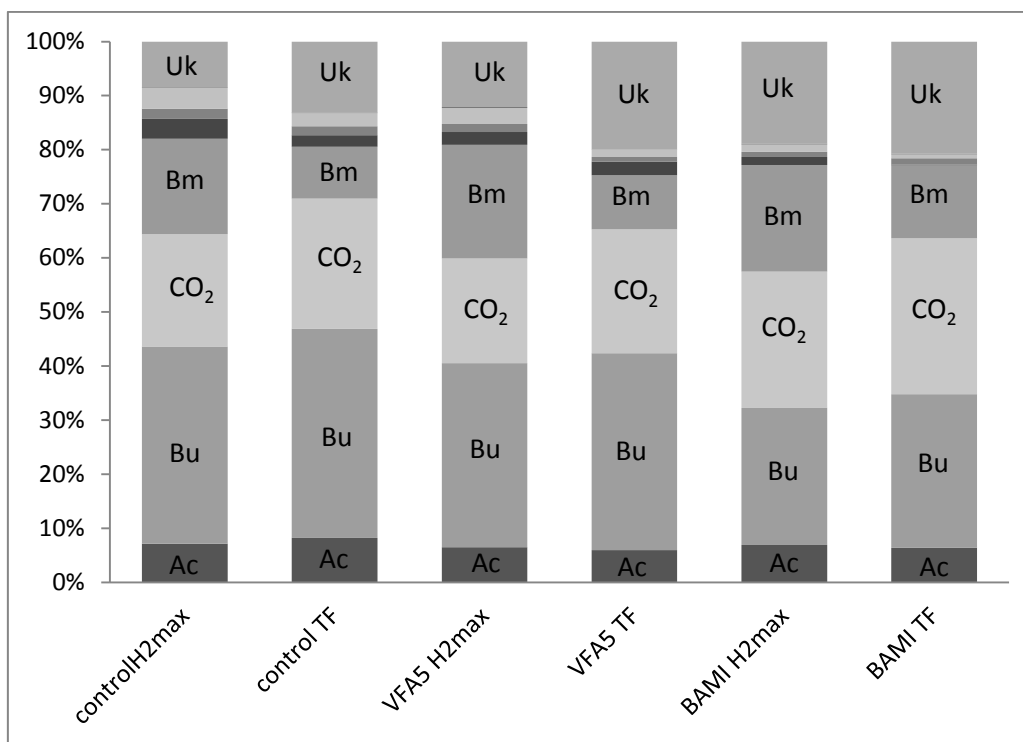


Figure 2: Carbon footprint of three cultures conditions: Control, VFA5 (Batch with 5 g.L⁻¹ of VFA adding in the culture medium initially), BAMI (culture in semi-continuous with submerged anaerobic bioreactor). The products were in ascending order: acetate, butyrate, CO₂, biomass, lactate, ethanol, glycerol, propionate and unknown metabolites. In this graphic, propionate was present in very small quantities in the three cultures, consequently it is not visible. The main metabolites were identified by abbreviation. Ac: acetate, Bu: butyrate, CO₂, Bm: biomass, Uk: unknow.

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

3.2 Impact of VFAs addition on hydrogen production

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

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

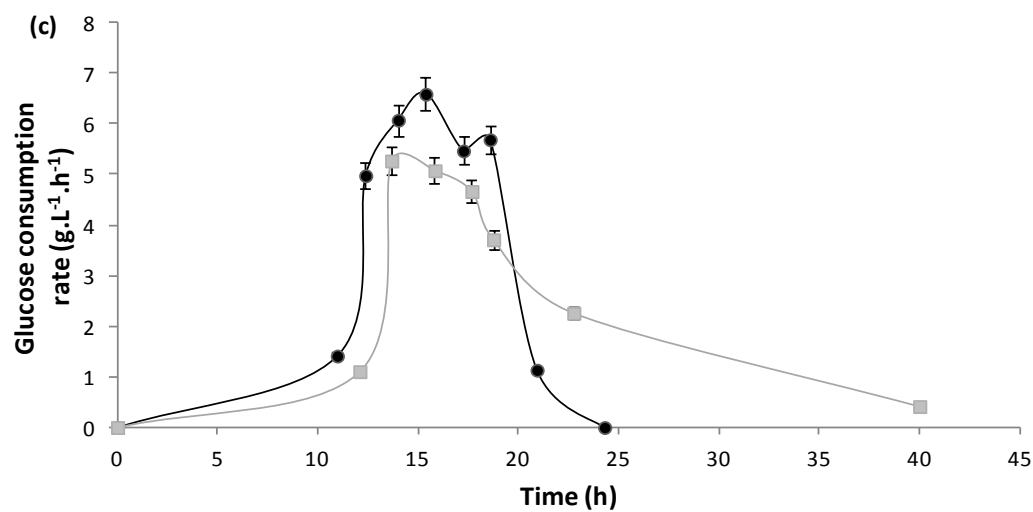
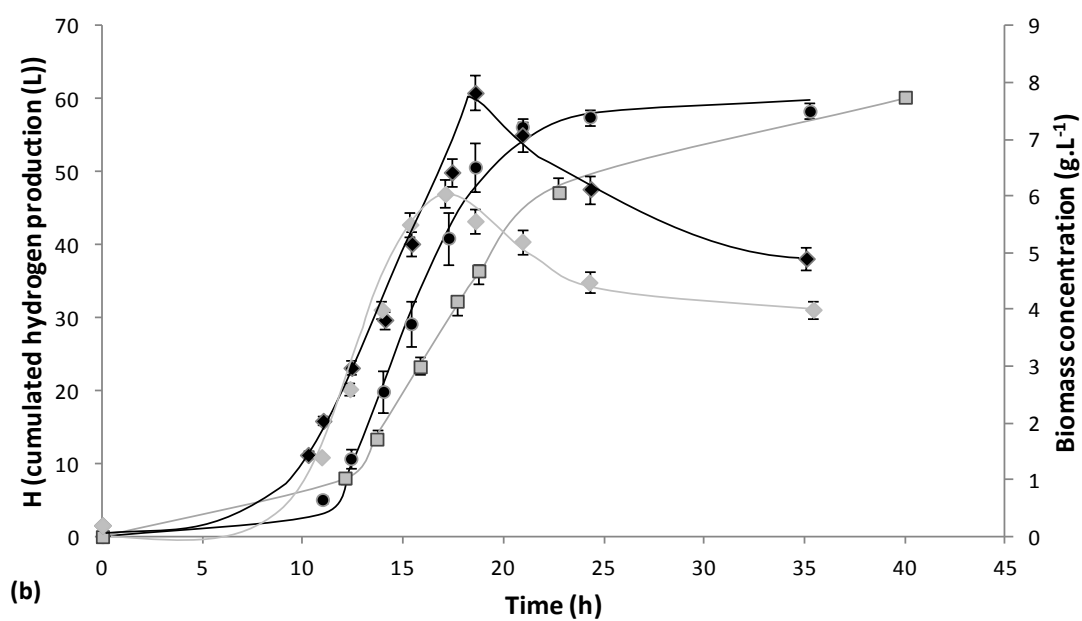
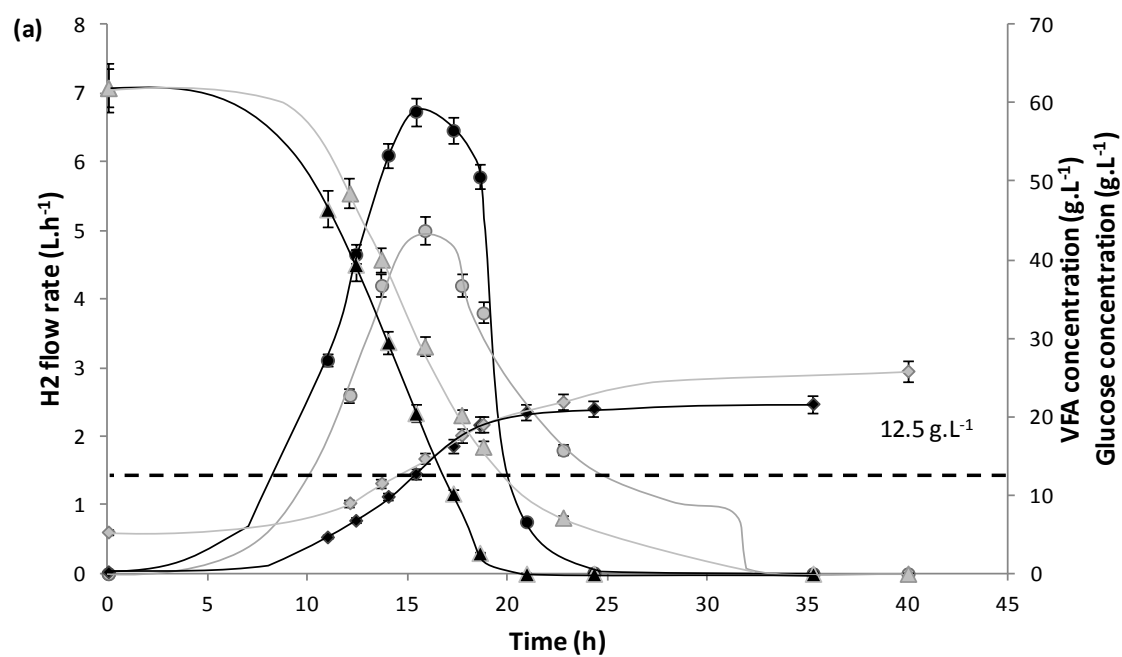


Figure 3: comparison of control culture and VFA5 culture (same culture with addition of 5g.L⁻¹ VFA with ratio B/A of 4.5 before starting culture). **(a)** Monitoring of hydrogen flow rate in control and VFA5 cultures • control ● VFA5, monitoring VFA productions ◆ control ◇ VFA5 and glucose concentration ▲ control △ VFA5. **(b)** Monitoring of cumulate hydrogen production modeled by Gompertz model and biomass concentration. Parameters determined was respectively for control and VFA5 cultures: latency 11.21 and 10.67h, potential hydrogen production (P) 59.96 and 60.38 L, hydrogen maximal flow rate (R_m) 7.47 and 4.55 L.h⁻¹. • Control experimental values ■ VFA5 experimental values ◆ control biomass concentration ◇ VFA5 biomass concentration. **(c)** Monitoring of glucose consumption rate in control and VFA5 culture, control ● VFA5 ■.

In the presence of initial VFAs, the lag phase was one hour longer compared to the control. The toxicity of VFAs led to adjustment to the biomass before starting to produce Hydrogen. A maximal flow rate of 5 L.h⁻¹ was observed after 15 hours in presence of VFAs while a maximum of 7 L.h⁻¹ was reached in the control culture (figure 3a). The flow rate slowed down when the VFAs concentration reached 12.5 and 13.5 g.L⁻¹ and glucose exhaustion occurred at 20 and 32 hours in the control and the batch with VFAs, respectively. Consequently, the average hydrogen production rate as determined with the Gompertz model (figure 3b) was lower (360mL_{H2}.L_{medium}⁻¹.h⁻¹) with VFAs than in the control culture (524 mL_{H2}.L_{medium}⁻¹.h⁻¹). The main difference between the two cultures was the glucose consumption rate (figure 3c). In the two cultures the glucose consumption rate increased until 6.6 and 5.3 g_{glucose}.L⁻¹.h⁻¹ in the control and VFA5 cultures, respectively. When a VFAs concentration of 12.5 g.L⁻¹ was reached, glucose consumption rate decreased progressively until glucose exhaustion.

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

Table 1: Comparative table of VFA concentration impact on hydrogen production. (1) Butyrate/Acetate ratio (2) Productivity expressed in $\text{mL.g}_{\text{vss}}^{-1}.\text{h}^{-1}$.

inoculum	substrate	inhibitor	[VF A] (g.L^{-1})	Y_{H_2} ($\text{mol}_{\text{H}_2}.\text{mol}_{\text{glucose}}^{-1}$)	HPR ($\text{mL}_{\text{H}_2}.\text{L}_{\text{medium}}^{-1}.\text{h}^{-1}$)	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 Bifermentans</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	-	

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

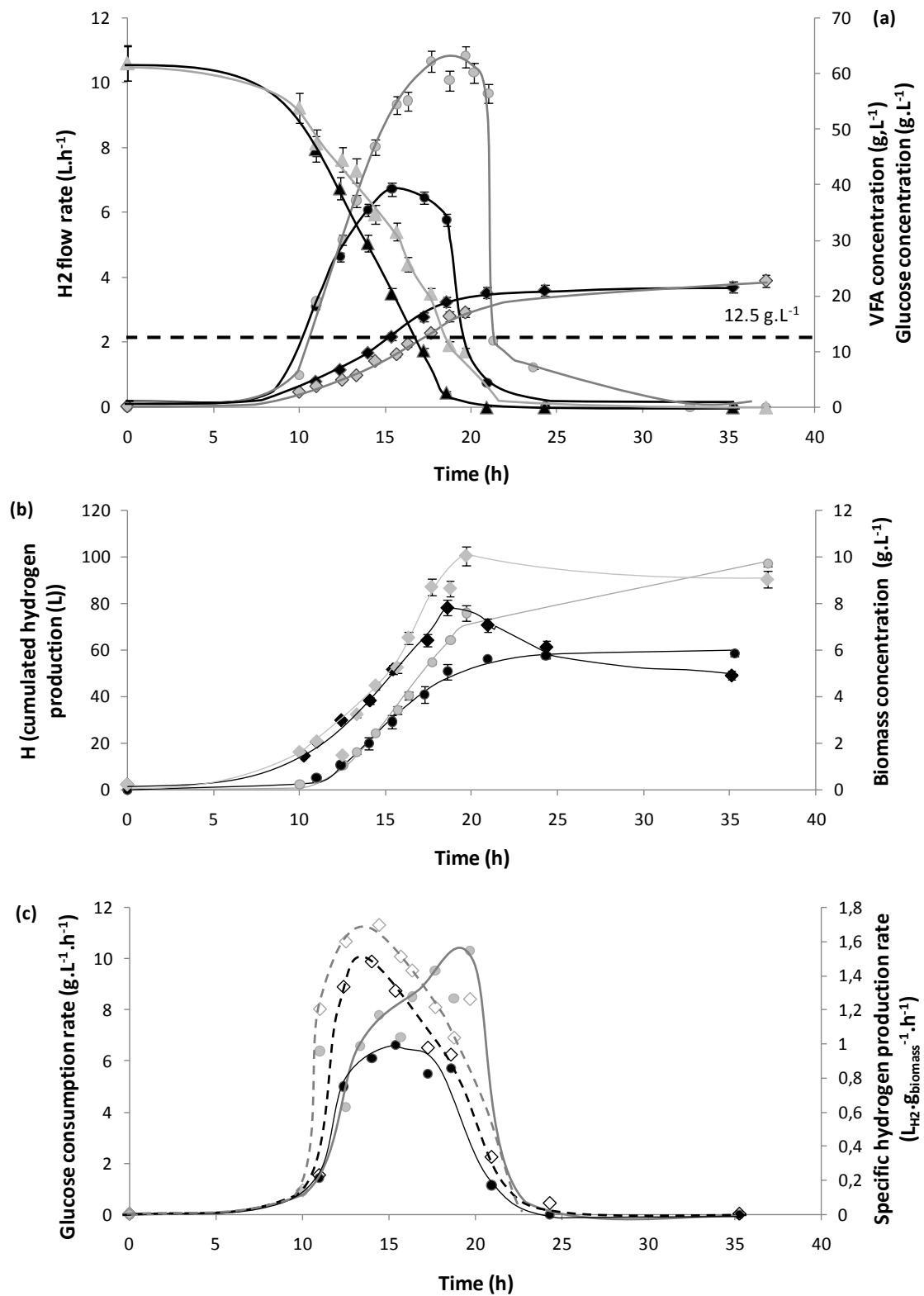
3.3 Sequential continuous operation with submerged anaerobic membrane bioreactor

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

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

3.3.1 Hydrogen production versus Volatile fatty acids accumulation

Cumulated hydrogen production, H₂ flow rate, VFAs production and glucose 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

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

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

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

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

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

3.3.2 Study of consortium by SSCP and quantitative PCR analysis

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

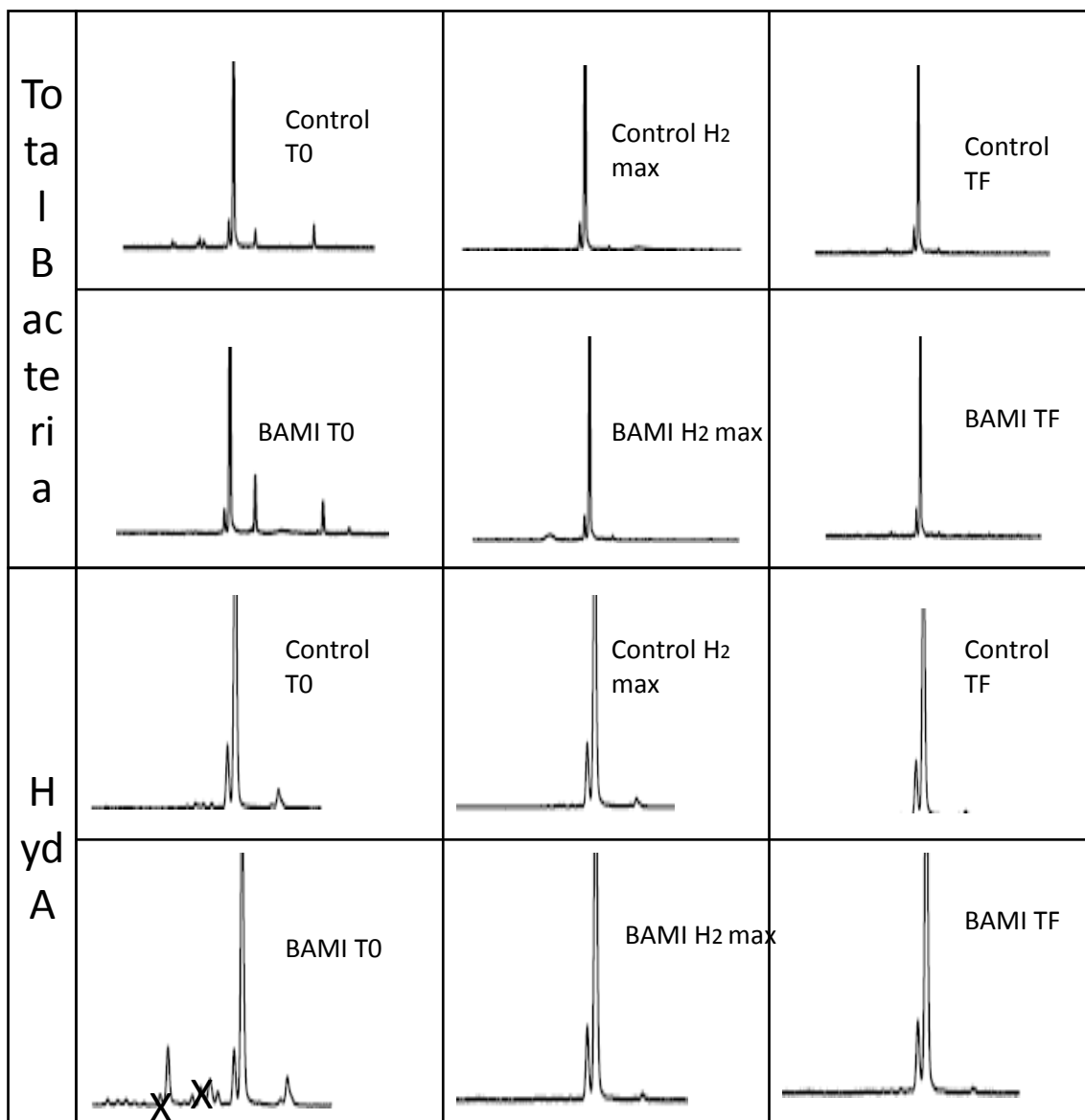


Figure 5: Qualitative and quantitative analyzes of microorganisms realized on samples during culture. SSCP profiles with specific primers of the V3 region DNA 16S representative of the total bacteria and primers of hydA to analyze sample biodiversity after inoculation (T_0), maximal hydrogen flow rate (H_2 max) and final time (T_F) for control and BAMi cultures.

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

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

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

4. Conclusion

With the bacterial consortium used in this study, the toxicity threshold of VFAs was determined. A decrease of the hydrogen production rate was observed when the concentration of hydrogen fermentation co-products reached 12.5 g.L^{-1} . It was demonstrated that using Anaerobic Membrane Bioreactors (AnMBR) represent an interesting technology to limit the accumulation of VFAs while maintaining the biomass in the medium. In response, maximal hydrogen production rate was increased of 51% 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. Even if clogging represents a risk with this technology and can lead to technical difficulties, the improvement in hydrogen production remains significant. The challenge now is to see how the system behaves during a longer operating time by increasing the withdrawal flow rate and limiting the accumulation of VFAs. The valorization of the permeate enriched in VFAs in an associated process represent also an interesting perspective of this work.

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496

497

- 498 [1] Darkrim-Lamari F, Malbrunot P. Combustible hydrogène - utilisation 2013.
499
- 500 [2] Dayhim M, Jafari MA, Mazurek M. Planning sustainable hydrogen supply chain
501 infrastructure with uncertain demand. *Int J Hydrog Energy* 2014;39:6789–801.
502
- 503 [3] Ma T, Ji J, Chen M. Study on the hydrogen demand in China based on system
504 dynamics model. *Int J Hydrog Energy* 2010;35:3114–9.
505
- 506 [4] Steele BCH, Heinzl A. Materials for fuel-cell technologies. *Nature* 2001;414:345–
507 52.
508
- 509 [5] Sherif SA, Barbir F, Veziroglu TN. Wind energy and the hydrogen economy—
510 review of the technology. *Sol Energy* 2005;78:647–60.
511
- 512 [6] Desvaux M, Guedon E, Petitdemange H. Cellulose Catabolism by *Clostridium*
513 *cellulolyticum* Growing in Batch Culture on Defined Medium. *Appl Environ*
514 *Microbiol* 2000;66:2461–70.
515
- 516 [7] Guo XM, Trably E, Latrille E, Carrère H, Steyer J-P. Hydrogen production from
517 agricultural waste by dark fermentation: A review. *Int J Hydrog Energy*
518 2010;35:10660–73.
519
- 520 [8] Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark
521 fermentative hydrogen production by mesophilic microflora: Principles and
522 progress. *Int J Hydrog Energy* 2007;32:172–84.
523
- 524 [9] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. *Enzyme*
525 *Microb Technol* 2006;38:569–82. doi:10.1016/j.enzmictec.2005.09.015.
526
- 527 [10] Singhania RR, Christophe G, Perchet G, Troquet J, Larroche C. Immersed
528 membrane bioreactors: An overview with special emphasis on anaerobic
529 bioprocesses. *Bioresour Technol* 2012;122:171–80.
530
- 531 [11] Bélignon V, Noblecourt A, Christophe G, Lebert A, Larroche C, Fontanille P.
532 Proof of concept for biorefinery approach aiming at two bioenergy production
533 compartments, hydrogen and biodiesel, coupled by an external membrane. *Biofuels*
534 2016;0:1–12.
535

- [12] Wong YM, Wu TY, Juan JC. A review of sustainable hydrogen production using seed sludge via dark fermentation. *Renew Sustain Energy Rev* 2014;34:471–82.
- [13] Argun H, Kargi F, Kapdan IK, Oztekin R. Biohydrogen production by dark fermentation of wheat powder solution: Effects of C/N and C/P ratio on hydrogen yield and formation rate. *Int J Hydrog Energy* 2008;33:1813–9.
- [14] Bao MD, Su HJ, Tan TW. Dark fermentative bio-hydrogen production: Effects of substrate pre-treatment and addition of metal ions or L-cysteine. *Fuel* 2013;112:38–44.
- [15] Boni MR, Sbaiffoni S, Tuccinardi L. The influence of iron concentration on biohydrogen production from organic waste via anaerobic fermentation. *Environ Technol* 2014;35:3000–10.
- [16] Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. Enhancement of hydrogen production from glucose by nitrogen gas sparging. *Bioresour Technol* 2000;73:59–65.
- [17] Kim D-H, Han S-K, Kim S-H, Shin H-S. Effect of gas sparging on continuous fermentative hydrogen production. *Int J Hydrog Energy* 2006;31:2158–69.
- [18] Chou C-H, Wang C-W, Huang C-C, Lay J-J. Pilot study of the influence of stirring and pH on anaerobes converting high-solid organic wastes to hydrogen. *Int J Hydrog Energy* 2008;33:1550–8.
- [19] Bakonyi P, Buitrón G, Valdez-Vazquez I, Nemestóthy N, Bélafi-Bakó K. A novel gas separation integrated membrane bioreactor to evaluate the impact of self-generated biogas recycling on continuous hydrogen fermentation. *Appl Energy* 2017;190:813–23.
- [20] Bundhoo MAZ, Mohee R. Inhibition of dark fermentative bio-hydrogen production: A review. *Int J Hydrog Energy* 2016;41:6713–33.
- [21] Bakonyi P, Nemestóthy N, Simon V, Bélafi-Bakó K. Fermentative hydrogen production in anaerobic membrane bioreactors: A review. *Bioresour Technol* 2014;156:357–63.

574 [22] Bakonyi P, Nemestóthy N, Lankó J, Rivera I, Buitrón G, Bélafi-Bakó K.
575 Simultaneous biohydrogen production and purification in a double-membrane
576 bioreactor system. *Int J Hydrog Energy* 2015;40:1690–7.
577

578 [23] Jung K-W, Kim D-H, Kim S-H, Shin H-S. Bioreactor design for continuous dark
579 fermentative hydrogen production. *Bioresour Technol* 2011;102:8612–20.
580

581 [24] Bullock JD, Kristiansen, B. *Basic Biotechnol Acad Press*.
582

583 [25] Gaudet G, Forano E, Dauphin G, Delort A-M. Futile cycling of glycogen in
584 *Fibrobacter succinogenes* as shown by in situ ¹H-NMR and ¹³C-NMR investigation.
585 *Eur J Biochem* 1992;207:155–62.
586

587 [26] Khanal SK, Chen W-H, Li L, Sung S. Biological hydrogen production: effects of
588 pH and intermediate products. *Int J Hydrog Energy* 2004;29:1123–31.
589

590 [27] Trad Z, Akimbomi J, Vial C, Larroche C, Taherzadeh MJ, Fontaine J-P.
591 Development of a submerged anaerobic membrane bioreactor for concurrent
592 extraction of volatile fatty acids and biohydrogen production. *Bioresour Technol*
593 2015;196:290–300.
594

595 [28] Wang B, Wan W, Wang J. Inhibitory effect of ethanol, acetic acid, propionic
596 acid and butyric acid on fermentative hydrogen production. *Int J Hydrog Energy*
597 2008;33:7013–9.
598

599 [29] Saur T, Escudié R, Santa-Catalina G, Bernet N, Milferstedt K. Conservation of
600 acquired morphology and community structure in aged biofilms after facing
601 environmental stress. *Water Res* 2016;88:164–72.
602

603 [30] Milferstedt K, Santa-Catalina G, Godon J-J, Escudié R, Bernet N. Disturbance
604 Frequency Determines Morphology and Community Development in Multi-Species
605 Biofilm at the Landscape Scale. *PLOS ONE* 2013;8:e80692.
606

607 [31] Rochex A, Godon J-J, Bernet N, Escudié R. Role of shear stress on composition,
608 diversity and dynamics of biofilm bacterial communities. *Water Res* 2008;42:4915–
609 22.
610

611 [32] Michelland RJ, Dejean S, Combes S, Fortun-Lamothe L, Cauquil L.
612 StatFingerprints: a friendly graphical interface program for processing and analysis
613 of microbial fingerprint profiles. *Mol Ecol Resour* 2009;9:1359–63.

614

615 [33] Braun F, Hamelin J, Gévaudan G, Patureau D. Development and application of
616 an enzymatic and cell flotation treatment for the recovery of viable microbial cells
617 from environmental matrices such as anaerobic sludge. *Appl Environ Microbiol*
618 2011;77:8487–93.

619

620 [34] Kawagoshi Y, Hino N, Fujimoto A, Nakao M, Fujita Y, Sugimura S, et al. Effect
621 of inoculum conditioning on hydrogen fermentation and pH effect on bacterial
622 community relevant to hydrogen production. *J Biosci Bioeng* 2005;100:524–30.

623

624 [35] Cheong D-Y, Hansen CL. Acidogenesis characteristics of natural, mixed
625 anaerobes converting carbohydrate-rich synthetic wastewater to hydrogen. *Process*
626 *Biochem* 2006;41:1736–45.

627

628 [36] Guo XM, Trably E, Latrille E, Carrere H, Steyer J-P. Predictive and explicative
629 models of fermentative hydrogen production from solid organic waste: Role of
630 butyrate and lactate pathways. *Int J Hydrog Energy* 2014;39:7476–85.

631

632 [37] Tang J, Yuan Y, Guo W-Q, Ren N-Q. Inhibitory effects of acetate and ethanol
633 on biohydrogen production of *Ethanoligenens harbinense* B49. *Int J Hydrog Energy*
634 2012;37:741–7.

635

636 [38] Zheng X-J, Yu H-Q. Inhibitory effects of butyrate on biological hydrogen
637 production with mixed anaerobic cultures. *J Environ Manage* 2005;74:65–70.

638

639 [39] Zhang S, Kim T-H, Lee Y, Hwang S-J. Effects of VFAs Concentration on Bio-
640 hydrogen Production with *Clostridium Bifermentans* 3AT-ma. *Energy Procedia*
641 2012;14:518–23.

642

643 [40] Wang Y, Zhao Q-B, Mu Y, Yu H-Q, Harada H, Li Y-Y. Biohydrogen
644 production with mixed anaerobic cultures in the presence of high-concentration
645 acetate. *Int J Hydrog Energy* 2008;33:1164–71.

646

647 [41] Van Ginkel S, Logan BE. Inhibition of Biohydrogen Production by
648 Undissociated Acetic and Butyric Acids. *Environ Sci Technol* 2005;39:9351–6.

649

650 [42] Lee K-S, Lin P-J, Fangchiang K, Chang J-S. Continuous hydrogen production
651 by anaerobic mixed microflora using a hollow-fiber microfiltration membrane
652 bioreactor. *Int J Hydrog Energy* 2007;32:950–7.

653

[43] Oh S-E, Iyer P, Bruns MA, Logan BE. Biological hydrogen production using a membrane bioreactor. *Biotechnol Bioeng* 2004;87:119–27.

[44] Ishikawa M, Yamamura S, Ikeda R, Takamura Y, Sode K, Tamiya E, et al. Development of a compact stacked flatbed reactor with immobilized high-density bacteria for hydrogen production. *Int J Hydrog Energy* 2008;33:1593–7.

[45] Gorwa MF, Croux C, Soucaille P. Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824. *J Bacteriol* 1996;178:2668–75.