Improvement of fermentative biohydrogen production by *Clostridium butyricum* CWBI1009 in sequenced-batch, horizontal fixed bed and biodisc-like anaerobic reactors with biomass retention

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Abstract

A horizontal tubular fixed bed bioreactor (HFBR) and an anaerobic biodisc-like reactor (AnBDR) were designed to both fix *Clostridium* biomass and enable rapid transfer of the hydrogen produced to gas phase in order to decrease the strong effect of H_2 partial pressure and H_2 supersaturation on the performances of *Clostridium* strains. The highest H_2 production rate (703 mL H_2/L.h) and yield (302 mL/g glucose consumed *i.e.* 2.4 mol/mol) with the pure culture were recorded in the AnBDR with 300 mL culture medium (total volume 2.3 L) at pH 5.2 and a glucose loading rate of 2.87 g/L.h. These results are about 2.3 and 1.3-fold higher than those achieved in the same bioreactor with 500 mL liquid medium and with the same glucose consumption rate. Therefore, our experimentations and a short review of the literature reported in this paper emphasize the relevance of performing bioreactors with high L/G transfer.

Keywords: *Clostridium*; hydrogen production; biofilm; bioreactor; pure strain

1. Introduction

The fermentative production of hydrogen has drawn increased attention in recent years. This biological process called "dark fermentation" (DF) offers new opportunities to produce "green" energy from various renewable resources and organic wastes [1-3]. While significant improvements have been made in development of such alternative H_2 production systems, more technical progress and cost reduction needs to occur for them to compete with current large scale technologies e.g. methane-reforming process. By contrast, for local and smaller scale DF and some other opportunities, biohydrogen production processes would be cost competitive since the feedstocks are available almost anywhere and crucial interest is paid for both energy independence and efficient utilization [4, 5]. However, optimization is still needed for DF regarding the bioreactor design, rapid removal and purification of gases, use of cheaper feedstock, genetic and molecular engineering to redirect metabolic pathway [6-10]. Moreover, DF is only likely to be viable as an industrial process if integrated with a process that maximizes energy recovery from the fermentation end-products. The traditional methane-producing anaerobic digestion process is the most promising since about 10 to 30 % more energy could be generated in the two-stage integrated system comparing to a single stage methanogenic process [11]. Besides, very prospective processes to convert acetate from DF spent medium exist such as further biohydrogen production (towards the maximum theoretical yield of 12 mol/mol glucose) by photosynthetic non-sulfur bacteria or direct electricity production in microbial fuel cells [6, 11].

In the past decades, most studies on biohydrogen production processes dealt with suspended culture systems such as the conventional (dis-)continuous stirred tank reactors (CSTR) since they are relatively simple and easy to operate. These investigations, several times reviewed [5, 10, 12, 13], enabled to optimize number of operating parameters such as the inoculum improvement, pH, temperature, hydraulic retention time and other culture conditions.

However, cell washout results in low active biomass in these systems [6, 14, 15]. Considering the low biomass in suspended systems, biofilms or microbial aggregates were used in recent years for fermentative H_2 production since they accommodate higher active biomass and fermentation rate [12, 16-19]. The reactors employing such immobilization systems, e.g. upflow anaerobic sludge blanket and fluidized bed bioreactors, generally show higher and more stable performances as aggregated cells are more resistant to changes in environmental conditions e.g. temperature, pH, hydraulic retention time, substrate load, etc. [9, 16, 20]. Additionally, other physiological factors such as the biochemical pathways and hydrogenase enzyme activity would be positively impacted by these systems comparing to classical mixed bioreactors.

Many bacteria and mixed cultures are known to form biofilms, granules or flocs that were characterized by microscopy, physicochemical or biomolecular techniques [21-25]. Most of them highlighted that Clostridia were the dominant strains in the mixed microbial populations. However, the ability of pure *Clostridium* hydrogen-producing strains to form stable flocs has not been well studied [26]. Zhang et al. [27] tested a *C. acetobutylicum* strain in a trickle bed made of glass beads; Zhu and Yang [28] tested *C. tyrobutyricum* bacteria immobilized in a coton-fibrous bed bioreactor; Mitchell et al [29] tested the same strain immobilized on porous particles (made of polyurethane and activated carbon) in a stirred-tank bioreactor and Jo et al [30] tested another strain of the same species immobilized on a fixed-bed reactor (polyurethane foam matrix).

Since experiments in 250 mL serum bottles without mixing (not reported here) proved floc formation in pure culture of *C. butyricum* CWBI1009 after a few sequences of SBR, the experimentations reported in this paper aimed to study the ability of the pure strain to form microbial aggregates in different bioreactor systems. The investigations were first carried out

in a 5 L bioreactor operated in sequenced-batch (SBR) mode with flocs-retention sieve and without stirring.

In order to further improve the culture conditions and performances specific bioreactors with biomass immobilization and large L/G exchange surface were designed to enable rapid liquid to gas transfer of hydrogen produced since H_2 partial pressure is known to strongly affect hydrogen production rates and yields [7, 31, 32]. A horizontal tubular fixed bed bioreactor (HFBR, 400 mL total volume, 200 mL liquid medium) and an anaerobic biodisc-like reactor (AnBDR, 2.3 L total volume, 0.3 to 1.5 L liquid medium) were experimented. To our knowledge, no biodisc reactor was tested for fermentative H_2 production.

By contrast, a horizontal fixed bed bioreactor was already used for DF by Leite et al. [33]. However, the expanded clay beads used as support strongly reduced the working volume. Therefore, focusing on better industrial perspectives our experiments were carried out with a high void support material with the purpose to retain microbial flocs of *Clostridium* and prevent retention of non biofilm-forming microorganisms. A pure culture was not maintained for the HFBR whereas the culture was pure for the whole experimentation in the AnBDR.

2. Materials and methods

2.1. Experimental procedures and culture conditions

The strain *Clostridium butyricum* CWBI1009 was isolated and identified by the authors [34]. It was maintained in liquid MDT medium [34] containing a lower glucose concentration (2 g/l) in order to promote spore formation after the growth phase.

The first tests were carried out in 5 L glass bottles (Schott) filled with 5 L of MDT culture medium. The bottle containing the medium (except cysteine and glucose) with the pH adjusted to 8.5 with NaOH 5N was autoclaved at 120 °C for 20 minutes. The glucose monohydrate was sterilized separately in aqueous solution in order to prevent Maillard

reactions between carbohydrates and amino acids. The aqueous cysteine solution was sterilized in hermetically stoppered tubes to prevent its oxidation by ambient air. After the medium had been prepared, sterilized and cooled down, the glucose and cysteine solutions were added sterilely and the medium was inoculated with 500 mL of inoculum. The bottle was capped tightly with a lid equipped with tubings for liquid or gas transfer and gas collection through gas filter (Sartorius Midisart 0.2 um). One tubing was equipped with flocs-retention sieve (section 1 mm²) used to maintain cell agglomerates inside the bioreactor during the removal of culture medium at the end of each sequence. The bioreactor was flushed after inoculation with sterile nitrogen and then incubated at a temperature of 30°C. Purity check of *Clostridium* cultures was realized by spreading a 100 µL sample on a PCA Petri dish before incubation at 30°C for 24 to 48 h.
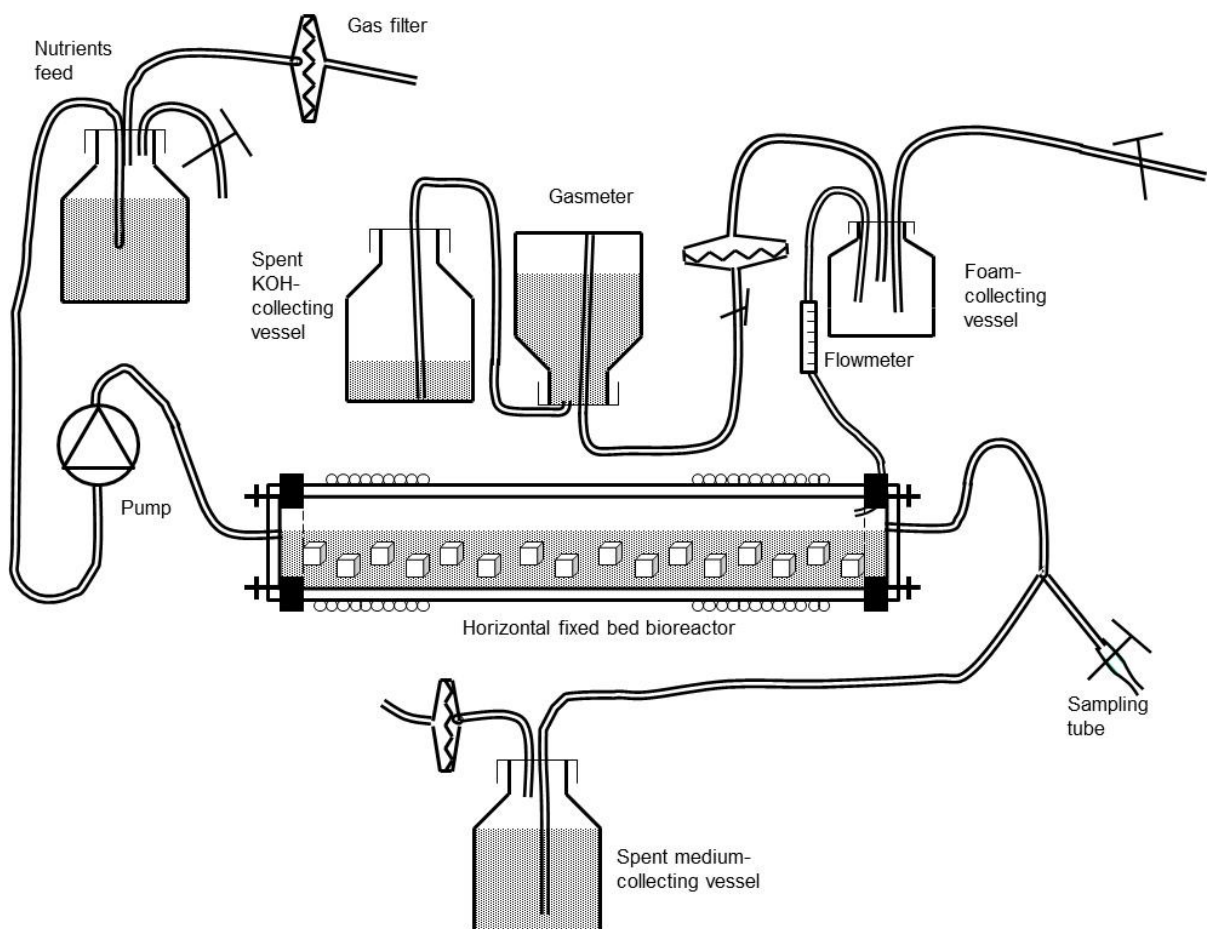

Figure 1. Setup of the horizontal fixed-bed reactor (400 mL total volume and 200 mL liquid medium).

The horizontal fixed bed bioreactor (Fig. 1) was self-made with a glass cylinder (400 mL total volume, 37.5 cm length, 3.7 cm inside diameter) and silicone tubings for liquid or gas transfer and gas collection through gas filter (Sartorius Midisart 0.2 um). Reticulated polyurethane cubes (1.5 cm x 1.5 cm size; specific surface area +/- 1800m²/m³, Type Filtren TM30, Recticel, Belgium) forming a 3D sieve of 2 mm² section were used as immobilization support for microorganisms. The liquid volume in the bioreactor was maintained at 200 mL in order to attain both a larger exchange surface and a head-space volume for rapid gas transfer. The bioreactor was flushed after inoculation with sterile nitrogen and then incubated at a temperature of 30°C. The nutrients was pumped from the nutrients feed vessel to the bioreactor. The spent medium was collected separately from the biogas that flowed through a bubble-soap flowmeter and a foam-collecting vessel before being collected in a water replacement equipment filled with KOH 9N (gasmeter).

The anaerobic biodisc reactor (Fig. 2 A) was composed of a 2.3 L glass vessel with double envelope (Bibby Quickfit JRV2L, UK; 30.9 cm length, 10 cm inside diameter) and a stainless-steel lid with septum, shaft, pH probe (465-35-SC-P-K9/320, Mettler Toledo), gas filters (Sartorius Midisart 0.2 um) and silicone tubings for gas outlet and medium removal or addition. The shaft was equipped with a tubular-rotating support (60 RPM; Fig. 2 B) to enable floc immobilization. The medium removal tubing was placed at the specific overflow level to maintain the required liquid volume inside the bioreactor (varying from 0.3 to 1.5 L) before being collected in the spent medium vessel. The nutrients were pumped from the nutrients feedstock to the bioreactor using a peristaltic pump. In order to prevent impact of liquid pumping on the gas measurement, the biogas flowed through a foam-collecting vessel and the other vessels containing nutrients and spent medium before being measured in a gas-flowmeter (Ritter, D).

A

Biogas outlet
Medium addition

Medium removal

B

PP sieve (section 1 mm²)

Cotton fibers

PP support (section 1 cm²)

PP sieve-blade

C

Gas-flowmeter

Oxygen remover

Water   Water

Biogas sampling system

N₂

Spent medium-collecting vessel

H₃PO₄ 1.5 N

Nutrients feed

Peristaltic pump

Liquid-sampling tube

Foam-collecting vessel

Water bath for bioreactor temperature control
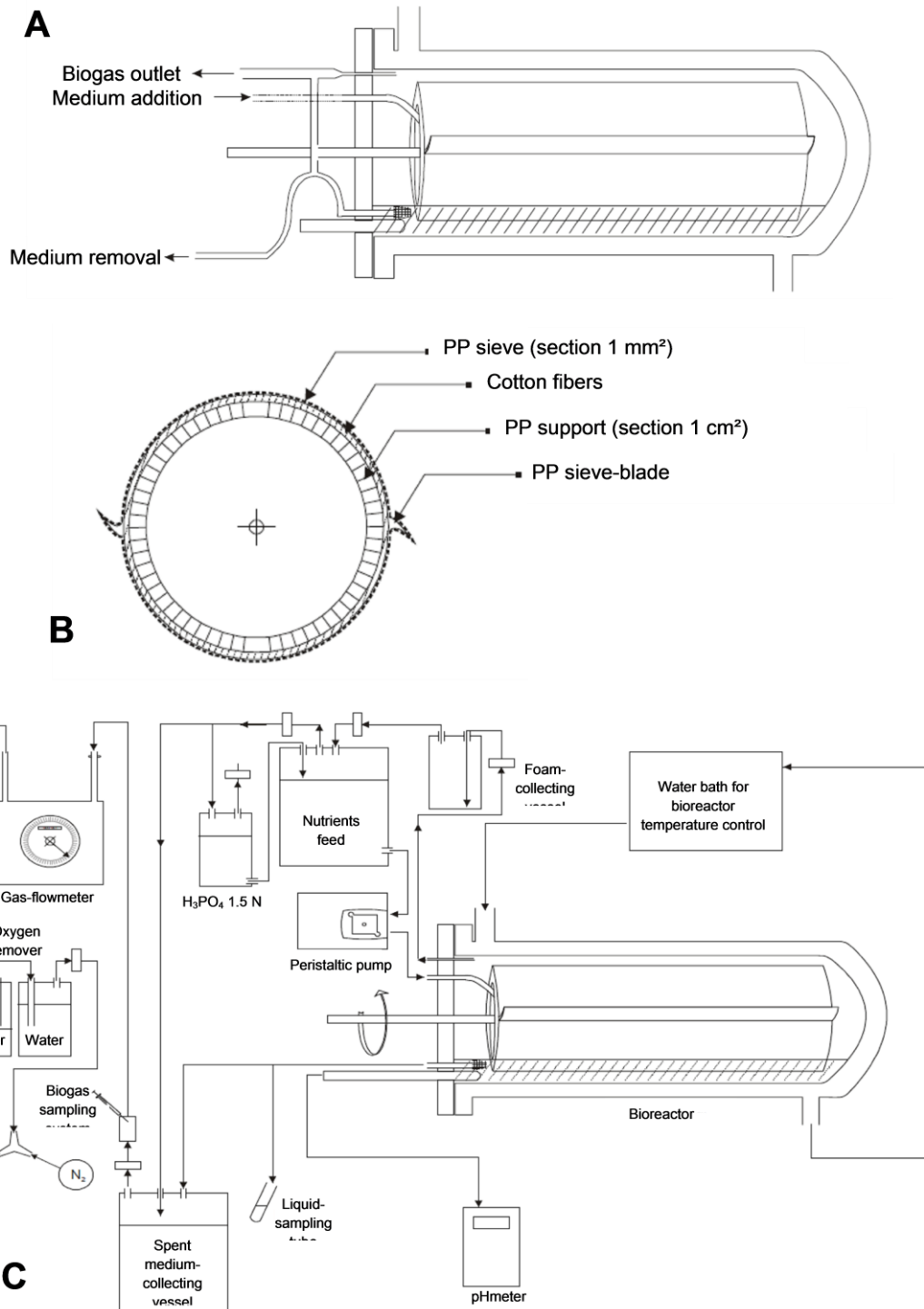
Bioreactor

pHmeter

Figure 2. Setup of the anaerobic biodisc-like reactor (2.3 L total volume and 0.3 to 1.5 L liquid medium) : cross-section view (A), section view (B) and complete setup (C).

2.3. Monitoring and analytical methods

Both a 5 L replacement equipment (filled with KOH 9 N) or a Ritter gasmeter (TG-01) and a ABB catharometric gas analyser (EL1020 calibrated with a 80% H_2 – 20% CO_2 gas mixture, Air Liquide, B) were used to monitor the biogas production and composition during the experimentations. The composition of biogas was validated using a gas chromatographer fitted with a thermal conductivity detector as described elsewhere [35].

Culture samples were centrifuged at 13000 g for 10 min and the supernatants were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The glucose, ethanol, formate, acetate, propionate, butyrate, and lactate were analyzed using a HPLC equipped with a differential refraction index detector as described formerly [34]. The data on the concentrations of glucose and metabolites present in the culture medium were used to calculate the mass balance (MB) of glucose conversion into the major soluble metabolites using the method reported elsewhere [35].

3. Results and discussion

3.1. Hydrogen production in a 5 L bioreactor: effect of mixing and floc formation

Study of H_2 production yields

The fermentative hydrogen production by a pure strain of *Clostridium butyricum* CWBI1009 was monitored in a 5 L bioreactor with glucose substrate. The culture conditions were similar to that used in 250 mL BHP tests described elsewhere [36] except for the total gas pressure. In the experiments reported here, a constant atmospheric pressure was maintained in the vessel during the 8 days of culture due to continuous removal of the biogas and accumulation in a water replacement system (filled with 9N KOH for carbon dioxide capture).

After 24 h of batch culture and complete depletion of the glucose, the hydrogen production decreased and stopped. A hydrogen production yield of 84 ml/g of glucose consumed was achieved *i.e.* 0.68 mol H_2/mol. This result is 26% lower than that obtained with the same strain in 250 mL serum bottles [36]. This could be related to the different partial pressure and H_2 supersaturation in the culture medium as reported by Kraemer and Bagley [37]. A sequenced-batch reactor (SBR) mode was further performed as investigated before [34] with removal-addition of 40 % of the culture medium at each sequence and addition of glucose monohydrate to 5 g / L. The removal of the culture medium was carried out through tubing equipped with floc retention sieve (section 1 mm²). Indeed experiments not reported here showed floc formation of pure *C. butyricum* CWBI1009 cultures after a few sequences of SBR in 250 mL serum bottles without mixing. Zhang et al. [24] and many authors already mentioned floc formation containing *Clostridium* hydrogen-producing strains. The experiments reported here confirm these observations for the pure *C. butyricum* CWBI1009 strain and that the sieve used at each sequence for the removal of spent culture medium was suitable to keep large flocs inside the bioreactor. It is to notice that floc formation occurred in the bioreactor without any acid treatment of the inoculum as mentioned by Zhang et al. [38]. A mean H_2 yield of 0.62 ± 0.005 mol/mol glucose consumed was achieved for a series of three sequences carried out in these conditions. The low standard deviation indicates a relative stability of the process that is promising for further developments at higher scale. However, the yield was 8% lower than during the batch culture. A second series of 4 similar sequences was carried out with gentle mixing (60 RPM performed by a magnetic stirrer bar). A H_2 production yield of 1.17 +/- 0.02 mol/mol glucose was achieved. This result is 73% higher than the yield reported for the batch culture and is in accordance with Lamed et al. [39] who achieved 1.5 to 3 fold higher H_2 yields in stirred vessels compared to unstirred cultures. Furthermore, the results reported here should be related to the 2,3 mol H_2/mol glucose

recorded by Masset et al [34] in a 2,3 L SBR completely mixed with the same strain. However, their pH was controlled at the optimal value of 5.2 whereas in the 5 L bioreactor the pH varied from 6.9 ± 0.1 to 5 ± 0.1, respectively at the beginning and end of sequences. Therefore pH control at the optimal pH enabled to achieve H_2 yield about twice higher than without pH control.

Study of soluble metabolite yields

Regarding the soluble metabolites produced from glucose, the Table 1A shows that the SBR mode with mixing leads to a lower production of lactate and formate which is balanced by a higher production of butyrate than in the SBR without mixing. These results are in accordance

Table 1. Metabolite analysis of *Clostridium butyricum* CWBI1009 glucose fermentation in a 5 L SBR with floc retention. Metabolite concentration at the end of each sequence (A) and carbon mass balance (B) for the batch sequence and average balance for the sequences 1 to 3 and sequences 4 to 7. Sequences "batch" and 1 to 3 were performed without mixing and sequences 4 to 7 were performed with gentle mixing (60 RPM).

A	Metabolite concentration (mM)				
Sequence	Lactate	Formate	Acetate	Ethanol	Butyrate
batch	8,42	25,26	8,95	0,53	17,89
1	11,58	30,00	8,42	1,05	11,58
2	12,63	30,00	7,89	1,05	12,11
3	7,89	30,79	10,00	0,95	13,68
4	5,00	23,16	10,00	1,05	18,95
5	2,89	22,11	12,11	2,89	20,00
6	4,21	23,95	11,05	1,84	19,74
7	3,16	25,00	12,89	1,05	18,42

B	Carbon converted from glucose (%)				
	Lactate	Formate	Acetate	Ethanol	Butyrate
Batch sequence	13,90	13,11	9,17	0,17	39,32
Mean for seq. 1-3	8,27	8,72	4,91	0,65	10,93
Mean for seq. 4-7	1,62	5,64	6,65	0,92	22,23

with those mentioned by other authors [34, 40]. More precisely, taking into account the carbon mass balance of glucose conversion to metabolites (Table 1B), the percentages may be compared to those recorded by Masset et al [34] in a 2.3 L SBR with pH control. Particularly the formate yield (about 14%) achieved in the 5 L experiments is about the average yield recorded in the 2.3 L bioreactor whatever the culture conditions. Indeed, the formate yield in the 2.3 L bioreactor was of 19.1 and 8.1 % for batch cultures at pH 7.3 and 5.2 respectively (these pH values correspond to the limits of the pH range in uncontrolled pH culture in the 5 L bioreactor). Moreover, SBR mode in the 5 L bioreactor did not enable to considerably decrease the formate yield as achieved in 2.3 L SBR at optimal pH (with a formate yield of 0.06 %). This suggests that the uncontrolled pH is not the sole parameter involved in the high formate and low butyrate and acetate production as well as their associated H_2 production [13]. As highlighted by many authors [31, 32, 37], the effect of H_2 partial pressure and gas-liquid transfer coefficient should be considered as major improvement parameters to investigate. It is easily understandable that both these parameters are less favourable for DF in the 5 L bioreactor than in the 2.3 L bioreactor and furthermore in the 250 mL serum bottles. On the biochemical point of view it could be assumed that a reduced hydrogenase activity in the 5 L bioreactor would result from a H_2 excess in the liquid phase [2, 43, 44]

Study of floc formation

Regarding the biomass, floc formation was observed already at the end of the batch culture and was amplified during the further sequences. The phase-contrast microscope observations of the flocs collected at the end of the experiment showed dense microorganisms agglomeration as reported by Van Ginkel and Logan [41]. A 3 mL culture sample (containing flocs and suspended cells) and another 3 mL of suspended cells (after flocs removal through the 1 mm² section sieve) were collected separately and used as inoculum for 250 mL BHP

tests in order to compare the viability and performances of the microorganisms. A 8-fold higher H_2 production (*i.e.* 60 mL compared to 7 mL, respectively) was achieved after 18h of culture in the bottles inoculated with the whole culture sample containing flocs. The BHP tests inoculated with the sole suspended cells evolved classically as reported elsewhere [36]. This suggests that flocs contained a high concentration of efficient H_2-producing microorganisms. These results confirm the high potential of cell retention to improve H_2 production performances due to biofilm formation with the pure *C. butyricum* CWBI1009 strain.

3.2. Hydrogen production in a 400 mL horizontal fixed bed bioreactor

In further experimentations, specific bioreactors were designed to enable cell immobilisation and increase the L/G exchange surface in order to improve H_2 gas transfer from liquid to gas phase since a H_2 partial pressure higher than 60 Pa has a negative impact on hydrogen yields [31].

In a first step, a horizontal fixed bed bioreactor was used to study the effect of these conditions on H_2 production from glucose and to investigate how cell immobilisation may enable to maintain pure culture. The L/G surface exchange in this bioreactor (140 cm² of clear area without taking the presence of the immobilisation support into account) is similar to that of the 2.3 L bioreactor (95 cm²) whereas the maximum liquid depth was about 13 times lower (1.8 cm and 24 cm respectively). The bioreactor (non sterile) was filled with 200 mL of sterile liquid medium and inoculated with 100 mL of pure *C. butyricum* CWBI1009 (grown in BHP test conditions). No other care was taken to maintain the pure culture inside the bioreactor. After a few days of culture and along the whole experiment a relatively homogenous population of about 10^8 cells of *bacillus*-like bacteria was regularly measured in the culture

medium sampled from the bioreactor. The pH of these samples also stabilized at 5.9+/-0.2 during the experimentation.
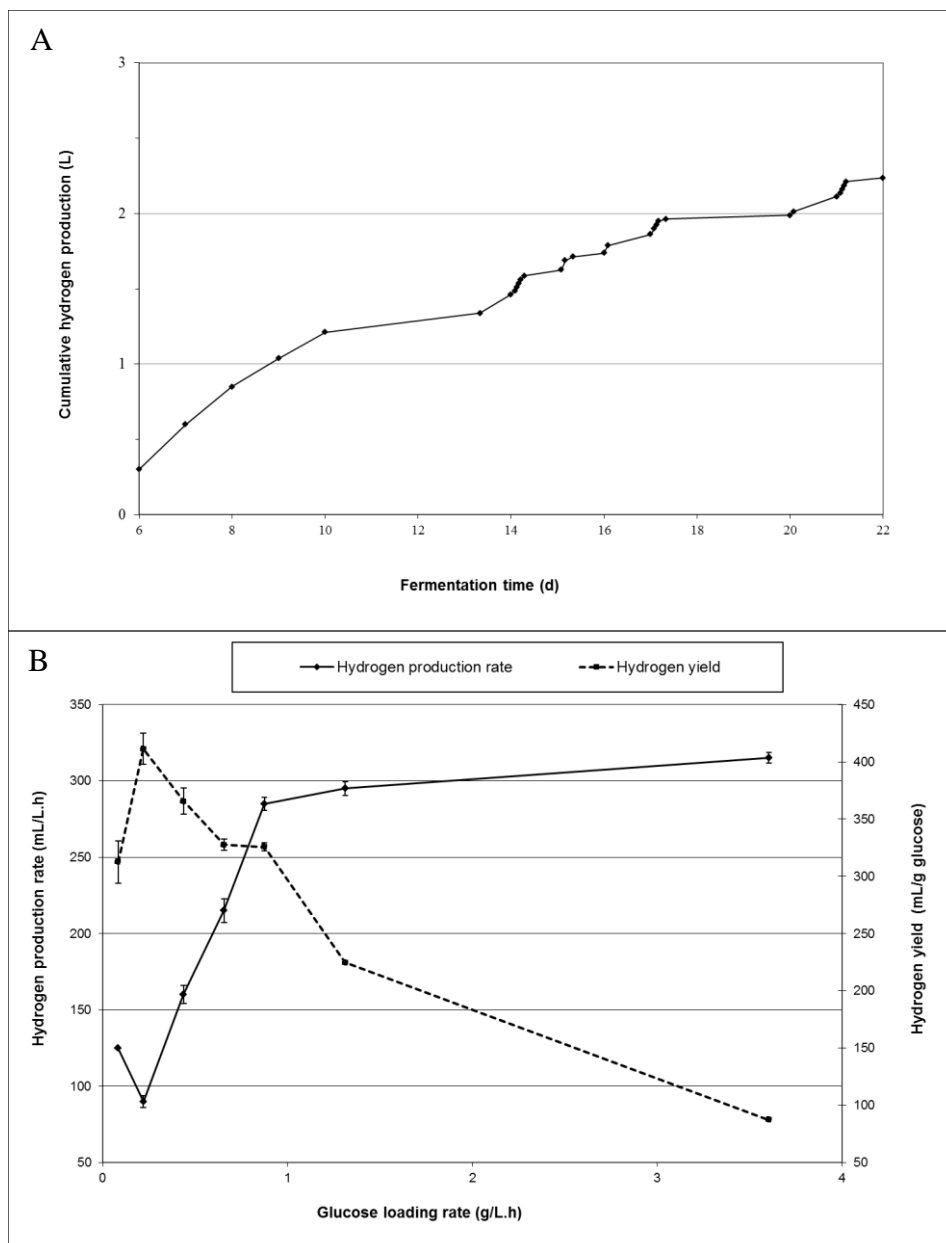

Figure 3. Hydrogen production from glucose in a 400 mL horizontal fixed-bed reactor inoculated with the pure strain *C. butyricum* CWBI1009. (A) Cumulative hydrogen production during the experimentations in SBR mode with a daily removal-addition of 80 mL of culture medium (*i.e.* glucose loading rate of 0.083 g/L.h). (B) Evolution of hydrogen production rate per litre of culture medium and yield versus glucose loading rate.

Table 2 Successive operating conditions investigated in a 400 mL horizontal fixed-bed reactor inoculated with the pure strain *C. butyricum* CWBI1009.

Pseudo hydraulic retention time (h)	60	23	11	8	6	4	1.4
Number of sequences per day	1	7	14	21	28	42	continuous
Liquid volume removal/addition per sequence (mL)	80	30	30	30	30	30	continuous
Glucose load per hour (g)	0.017	0.04	0.09	0.13	0.18	0.26	0.72
Glucose loading rate (g/L.h)	0.083	0.22	0.44	0.66	0.88	1.31	3.60
Experimental period with the related mode (days)	1-22	22-34	34-41	41-45	48-50	50-52	52-53

After the batch culture, a first series of experiments in SBR mode involved 20 sequences of removal-addition of 80 mL of culture medium (*i.e.* 40% of total liquid volume as performed with 2.3 L and 5 L bioreactors) containing 5 g/l glucose. The measures before day 6 are not shown due to inefficient gas collection.

Study of H_2 production yields and HPR

Figure 3A shows progressive and regular increase of cumulative H_2 production except between day 10 and 13 and between day 18 and 20 corresponding to a longer sequence duration respectively with and without removal-addition of fresh culture medium. After 13 days a relatively stable process was achieved with a mean production rate of 125 mL H_2/day. The corresponding H_2 yield reached 312 mL/g glucose consumed *i.e.* 2.5 mol H_2/mole glucose. This result is similar to the 2.3 mol H_2/mol glucose recorded with the 2.3 L SBR. H_2 production starts rapidly after substrate addition and for sequences at day 14, 17 and 21, it is shown that the majority of the H_2 cumulated volume was released during an average of 6 h (Fig. 3A). This suggests that sequences shorter in time might be performed in the next experimentations. The further series of experimentation in this bioreactor were carried out in pseudo continuous mode with removal/addition of 30 mL liquid volume at higher frequency than in the former SBR mode. Finally the continuous mode was investigated. Table 2 describes the different operating parameters used for the 400 mL HFBR.

Analysis of the removed medium between each sequence showed that glucose was completely metabolised. The biogas flowrate was measured several times per day using a bubble soap flowmeter connected to the bioreactor preceding the gasmeter equipment. The hydrogen content in the biogas was regularly measured at an average of 80±2 % H_2 and 20 ±2 % CO_2 by gas chromatography. High H_2 concentration of 70 to 79% in gas phase was also reported by Zhang et al. [27] in an unsaturated flow reactor. However the low CO_2 content would also be related to CO_2 absorption in the unstirred liquid medium and in the replacement equipment [32].

The H_2 production rate was calculated as the product of the biogas flowrate and the H_2 content. The results were in accordance with the average H_2 production rate measured with the replacement equipment (with KOH) during the period of time between two addition/removal of culture medium. Indeed, during that time the measurement of the cumulative H_2 production was relevant and not affected by any other flow (such as pump flow) since the H_2 flow was the sole flow exhausting from the bioreactor.

Figure 3B depicts the H_2 production rate and yield versus glucose loading rate (GLR) including the maximum HPR recorded for the first series of experiment at a GLR of 0.083 g/L.h. For better comparison with the results of section 3.3 the HPR is reported to a 1 L liquid volume. It increases constantly from 90 mL H_2/L.h to 285 mL H_2/L.h with the GLR increasing from 0.22 to 0.88 g/L.h. By contrast the H_2 yield slightly decreases from 411 mL/g (3.3 mol H_2/mol glucose) to 326 mL/g (2.6 mol/mol) respectively. This yield is similar to that recorded for SBR mode (Fig. 3A) with a pseudo HRT of 60 h *i.e.* at a GLR of 0.083 g/L.h. The HPR and yields reported at a GLR of 0.22 g/L.h are consistent with the results reported by Van Ginkel and Logan [41] (at a HRT of 10 h) and Mitchell et al. [29] (at a HRT of 16.7 h) in similar loading and physico-chemical conditions. At a GLR higher than 0.88 g/L.h, only

Table 3. Carbon mass balance at the end of the sequences related to the different GLR in a 400 mL horizontal fixed-bed reactor inoculated with the pure strain *C. butyricum* CWBI1009. For a GLR of 0.083 g/L.h, two periods were considered : the sequences of days 1 to 10 whereas the pure strain was dominant and those of days 13 to 21.

GLR (g/L.h)	Lactate	Formate	Acetate	Propionate	Ethanol	Butyrate
0.083 *(1-10)*	1.8	4.7	15.3	1.2	0.4	51.2
0.083 *(13-21)*	0.1	3.2	16.7	15.5	4.1	40.6
0.22	2.0	3.2	17.0	20.3	11.4	26.4
0.44	5.3	2.9	19.8	29.4	3.2	37.3
0.66	17.0	0.0	9.1	14.7	7.5	39.8
0.88	1.8	3.8	21.2	20.8	10.1	32.4
1.31	13.0	0.0	16.7	21.8	5.9	26.1
3.6	29.6	0.0	12.8	23.2	1.4	16.3

a marginal increase of HPR could be observed. By contrast, the H_2 yield dramatically decreased to 225 mL/g and 87 mL/g (0.7 mol/mol) respectively at a GLR of 1.31 and 3.6 g/L.h. Therefore an important amount of glucose was consumed for other metabolic pathways than H_2 production. These results suggest that the HRT of 6h and the organic loading rate of 0.88 g/L.h are the maximum affordable conditions for H_2 production by the *C. butyricum* CWBI1009 in mixed culture. They are confirmed by the study of soluble metabolites.

Study of soluble metabolite yields

Relatively specific metabolites distributions were recorded according to the different HRT as depicted in Table 3 (and Additional file : Fig A1). During the first 10 days of culture in our bioreactor the major metabolites *i.e.* butyrate and acetate were associated with *Clostridium*

strain activity since the related yields from glucose (Table 3) were similar to that recorded by Masset et al [34] in sequencing-batch conditions. After each change of operating mode, a stable distribution of metabolites was achieved within a period of about 6 times the HRT as also reported by Jo et al. [30]. Their experimentations were carried out with a pure *C. tyrobutyricum* strain cultured in similar medium conditions in a fixed bed column packed with a high void support as in the 400 mL HFBR. Comparing to the metabolites profile (Additional file Fig A1), Jo et al. [30] at HRT higher than 2h, measured a 2-fold higher butyrate concentration. By contrast their HPR and both acetate and H_2 yields were 2-fold lower than ours. These different metabolite distributions should be related to the different *Clostridium* species involved in our and their experiments since high butyrate yields and low acetate yields were also reported by Mitchell et al [29] with another *C. tyrobutyricum* strain.

Comparing to the results reported in section 3.1 the distribution of the metabolites detected in the 400 mL HFBR is in favour of H_2 production. Indeed the formate yield was about 2 to 4-fold lower than in the 5 L bioreactor and about 3-fold lower than that recorded in the 2.3 L batch bioreactor at pH 5.85 [34]. Therefore the decreasing of formate yield and increasing of acetate, butyrate and H_2 yields should be related to increasing H_2-L/G transfer [31] and not only to stirring or pH control in the culture medium.

A relatively low production of propionate (not exceeding 3 % of carbon converted from glucose) is recorded during some of the sequences performed during the first 10 days of culture. This is in accordance with the presence of other microorganisms than the *C. butyricum* CWBI1009 strain, already observed in the first sequence. After day 13 and until the experimentation was set in continuous mode (at day 52 and at a GLR of 3.6 g/L.h) a higher propionate concentration was measured in the culture medium with a related carbon recovery from glucose of about 15 to 30 %. However at a GLR of 0.66 g/L.h the propionate yields and also the whole fermentation profile seemed quite different than those for other GLR values.

Therefore, while those conditions led to a similar H_2 yield than for a GLR of 0.88 g/L.h, they should be considered as unfavourable for the process as well as those with the highest GLR in continuous mode. Indeed, in these conditions other microorganisms or the environmental parameters, especially those regarding H_2 concentration in culture medium, would progressively outcompete *C. butyricum* CWBI1009 and decrease H_2 yields. As a consequence a HRT of 6 h and a GLR of 0.88 g/L.h can be considered as the optimal condition for H_2 production rate and yield but also for metabolite distribution since a high yield is recorded for cumulated acetate and butyrate (53.6% of carbon converted from glucose). Moreover higher H_2 production performances would be expected at this GLR with pure culture since no or relatively low amount of propionate, ethanol and formate would be produced.

Study of biomass

Regarding biomass a biofilm developed on the immobilisation support (Additional file A2) in the first quarter of the bioreactor length. The other part of the bioreactor volume was filled by biogas bubbles. They confirm the relevance of a bioreactor with biomass immobilisation and

Table 4. Successive operating conditions investigated in an anaerobic biodisc-like reactor with the pure strain *C. butyricum* CWBI1009.

HRT (h)	10.9	6.6	10.9	6.6	3.9
Volume of liquid phase (mL)	500	500	500	500	300
Liquid volume removal/addition per hour (mL)	46	76	46	76	76
Glucose load per hour (g)	0.21	0.34	0.52	0.86	0.86
Glucose loading rate (g/L h)	0.42	0.68	1.04	1.72	2.87
Experiment duration with the related HRT (days)	2	1	1	1	9

large surface exchange for efficient L/G transfer. The experimentation reported in this section also demonstrated the need for mixing or for intermediate sampling along the bioreactor length in order to investigate the distribution of metabolites in the bioreactor. This system would be considered as a piston-like reactor regarding the liquid medium flow.

3.3. Hydrogen production in a 2.3 L biodisc-like reactor

A third type of bioreactor was designed to investigate H_2 production in optimal conditions of mixing, homogeneity, L/G transfer and biomass immobilisation. The anaerobic biodisc-like reactor (AnBDR, Fig. 2) enables to distribute substrate equally on the biofilm, to collect homogeneous liquid samples, to prevent H_2 supersaturation in the liquid phase and to limit substrate consumption for biomass growth. In order to maximize the control of purity, the bioreactor was sterilised empty and inoculated under a nitrogen gas flow by transferring (at a flowrate of 76 mL/h) a 5 L culture carried out in a SBR (see section 3.1). The liquid volume exceeding 1.5 L was removed continuously and flocs of biomass were let to settle inside the tubular-rotating support. Following that operation the bioreactor was operated for 14 days with continuous addition of fresh culture medium and removal of spent medium exceeding 500 mL in the first step and 300 mL in the second step.

The biogas production rate was measured with a gas-flowmeter and H_2/CO_2 composition determined by a gas analyser. During the experimentation the H_2 content in the biogas stabilized at 64 +/- 3 %. Four different glucose loading rates (GLR) ranging from 0.42 to 2.87 g/L.h were tested as mentioned in table 4 with the related HRT.

Cysteine was used as reducing agent only for the first 48 h of culture. While this compound was not added in the following operations, the anaerobiosis was maintained in the bioreactor without affecting the pure culture performances. The purity checks performed regularly did
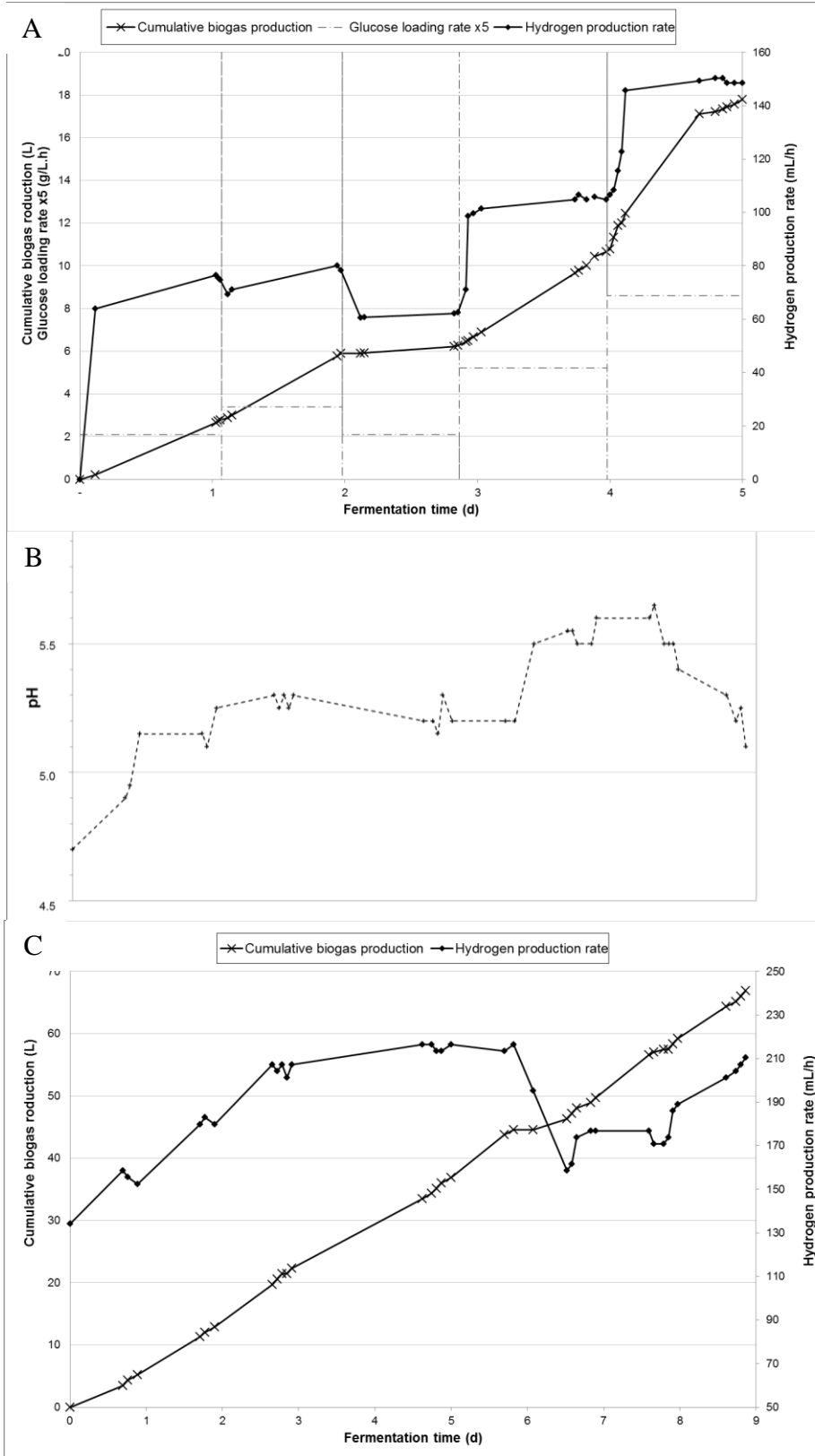
Figure 4. Hydrogen production by a pure culture of *C. butyricum* CWBI1009 in a 2.3 L anaerobic biodisc-like reactor (AnBDR). Cumulative biogas production and HPR in the AnBDR containing 500 mL (A) or 300 mL (B-C with pH evolution) of culture medium.

not reveal contamination. A second test at a GLR of 0.42 g/L.h was carried out after the operation at 0.68 g/L.h since the first one was affected by residual glucose from the pre-culture and a pH of 5.5±0.2, slightly higher than the pH recorded for the other operation *i.e.* 5.1±0.3.

Study of the performances of the AnBDR with 500 mL culture medium

The evolution of cumulative biogas production and HPR are presented in Fig. 4A for the first step of the AnBDR operation. It shows that the HPR stabilized about 5 h after the changing of operating parameters *i.e.* faster than in the 400 mL HFBR due to mixing in the AnBDR. The HPR increases with increasing GLR up to 1.04 g/L.h without glucose accumulation. At a GLR of 1.72 g/L.h a residual glucose concentration of 2.3 g/L was measured in the spent medium. This suggests that the culture would operate without any limitation (glucose accumulation) at a maximum GLR of 1.36 g/L.h. However, as shown on Fig. 6B reporting the mean HPR and yields versus GLR, the H_2 yield decreases of 23 % (*i.e.* from 286 mL/g to 220 mL/g, respectively or 2.3 to 1.8 mol H_2/mol glucose consumed) with increasing GLR from 0.42 to 1.72 g/L.h (only the results of the second test at a GLR of 0.42 g/L.h are reported in this Figure). It is to notice that the yield of 286 mL/g recorded at a GLR of 0.42 g/L.h and a load of 0.21 g/h glucose added to the culture medium is consistent with the H_2 yield estimated for the same glucose load in the 400 mL HFBR (*i.e.* 273 mL/g at a GLR of 1.05 g/L.h). The results are further discussed in section 3.4.

Study of the performances of the AnBDR with 300 mL culture medium

In the second step the bioreactor was operated with the same glucose load of 0.86 g/h but the volume of liquid culture medium in the bioreactor was reduced from 500 to 300 mL. Therefore the GLR was of 2.87 g/L.h. Figure 4B-C shows the evolution of pH, cumulative

biogas production and H_2 production rate during the 9 days of operation. Continuous H_2 production was recorded following the activity of the former step. However the pH (4.8) measured in the spent culture medium was lower than the optimum value of 5.2 and the glucose residual reached 4 g/L. Therefore the pH in the feed was progressively adapted with KOH 1.5 N in order to reach optimum and stable conditions of pH for H_2 production at day 3 (the mean pH at day 2 was of 5.1±0.3). As a consequence the HPR increased progressively to stabilize at 211 mL H_2/h between day 4 and 6. Moreover the glucose residual concentration decreased to 2 g/L leading to a H_2 yield of 302 ml/g glucose (2.4 mol/mol) *i.e.* about 30 % higher than in the first step with 500 mL culture medium. It should be mentioned that a similar glucose consumption rate of about 0.7 g/h was measured in both conditions suggesting a stable maximum population in the bioreactor. Such a maximum affordable glucose consumption corresponding to a GLR of 2.34 g/L.h was confirmed during days 6 to 8 where the pH was regulated at pH 5.6±0.05 aiming to test the effect of pH on H_2 production in these bioreactor and culture conditions by comparison to the effect discussed by Masset et al [34]. As shown on Fig. 4C the H_2 production rate decreased to 190±10 mL/h and the H_2 yield decreased to 270 mL/g glucose consumed (2.2 mol/mol).

These results confirm that the optimum pH for H_2 production from glucose by the strain *C. butyricum* CWBI1009 is about 5.2 [34]. Compared to that study the H_2 yield recorded in this work is similar to the 2.3 mol/mol reported by Masset et al. [34] in the 2.3L SBR at pH 5.2. In addition, they are about twice higher than the results achieved at similar GLR in the 400 mL HFBR. Before being discussed further in section 3.4, these results should be related to soluble metabolites distribution.

Table 5. Average carbon mass balance related to different hydraulic retention times in an anaerobic biodisc-like reactor with a pure culture of *C. butyricum* CWBI1009.

Liquid phase volume (ml)	Duration (d)	Lactate	Formiate	Acetate	Ethanol	Butyrate
500	1	14	4	26	2	38
500	2	0	2	30	0	45
500	3	0	1	27	0	46
500	4	3	0	26	1	38
500	5	3	0	28	1	47
300	6	5	0	30	0	44
300	7	4	0	30	1	43
300	8	2	1	29	1	39
300	9	2	0	28	1	41
300	10	2	0	29	1	40
300	11	5	0	28	1	41
300	12	4	1	31	1	40
300	13	3	0	30	1	41
300	14	4	0	34	1	47

Study of soluble metabolites yields

The analysis of the carbon mass balance (Table 5) shows that the highest H_2 yield is correlated with high acetate and butyrate production. According to Table 5 the metabolite yields are stable with average acetate and butyrate yields of 29±2 and 42±3 calculated all over the experimentation excluding the first day. These yields are respectively about 50 % higher and 20 % lower than those achieved in 2.3 L SBR in similar physico-chemical conditions except for L/G transfer parameters. The improved H_2 transfer conditions from liquid to gas phase experimented in the AnBDR enabled to achieve a HPR (reported to the liquid volume in the bioreactor) about 5.5 fold higher (703 mL H_2/L h) compared to the 126 mL H_2/L h for the 2.3 L SBR.
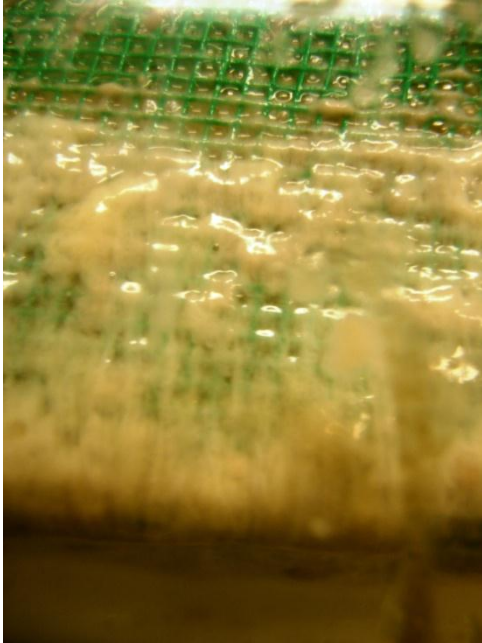
Figure 5. Biofilm growth of *C. butyricum* CWBI1009 on the polypropylene immobilisation support (1 mm²
sieve at the back front) in a 2.3 L anaerobic biodisc-like reactor (AnBDR).

No propionate was detected in the spent culture medium. This confirms that pure culture of
Clostridium butyricum was maintained in the bioreactor. Biofilm growth was observed on the
immobilisation support (Fig. 5). It evidences the ability of a pure *Clostridium butyricum* strain
to form biofilm in bioreactor without specific treatment as reported by many authors.
Additionally, the results of these experimentations show the feasibility of a biodisc reactor for
H_2 production since, to our knowledge, it was not formerly tested.

**3.4. General discussion about hydrogen production performances in (pseudo-)
continuous bioreactors**

Basically, when comparing both horizontal bioreactors used in this study the AnBDR system
enabled to achieve relatively favorable and homogenous environmental conditions and to
collect relatively homogenous liquid samples whereas a lack of information was observed
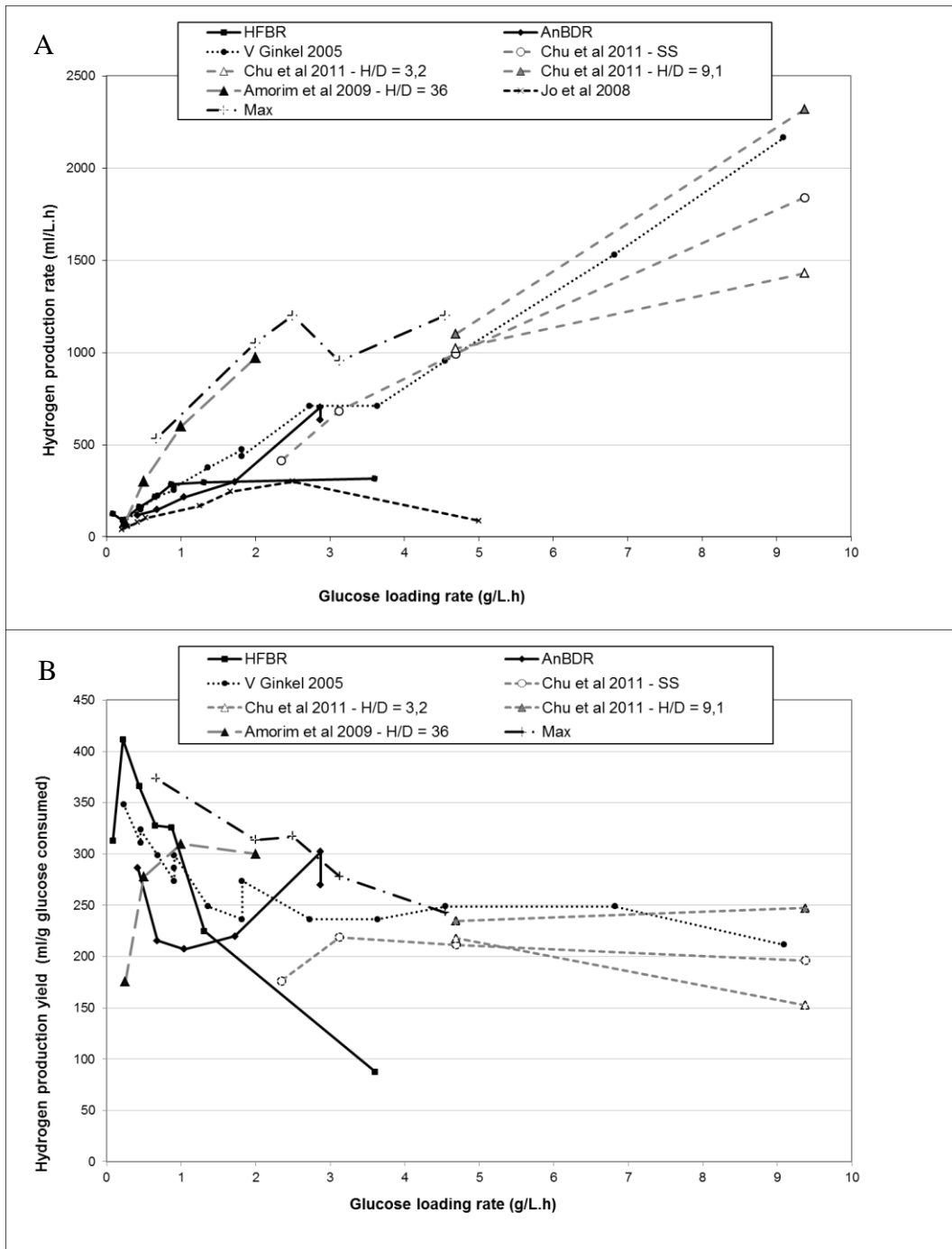about the distribution of substrate and metabolites along the HFBR. The pH was also less

Figure 6. Evolution of HPR (A) and yields (B) versus GLR : review of the results achieved in the HFBR and

AnBDR (this study; the data at a GLR of 2.87 g/L.h for the AnBDR are related to the experimentation step with

a liquid volume of 300 mL at pH 5.2 and 5.6) and some of the highest or most relevant performances reported in

the literature by Van Ginkel and Logan [41]; Jo et al. [30]; de Amorim et al. [42] (in a bioreactor with a height to

diameter ratio H/D of 36); Chu et al. [16] (in 3 different bioreactors with suspended sludge SS or with a H/D

ratio of 3.2 or 9.1) and for the curve "Max" : Peintner et al. [18]; Barros et al. [43]; Mitchell et al. [29] and Lee et

al. [44] at a GLR of 0.67; 2.00; 3.13 and 4.55 respectively.

controlled with probably a huge impact on bacteria performances. For bench-scale investigations the AnBDR has therefore many advantages. By contrast regarding industrial applications the HFBR is more suitable since it needs less engineering and energy during operation (e.g. no energy consumption for support rotation). Industrial perspectives will be discussed later. The different HPR and H_2 yields recorded versus GLR in both bioreactors tested here and some of the highest performances reported in the literature are reviewed in Fig. 6. At low GLR up to about 1 g/L.h, *i.e.* while a significant *C. butyricum* activity was expected in the HFBR from the glucose substrate, the yields and also the HPR recorded in the AnBDR were 30% lower than in the HFBR. These higher results in the HFBR with a specific transfer area (area of exchange surface between liquid and gaseous phase reported to the liquid phase volume) being about 40% higher (*i.e.* 0.7 m²/m³ against 0.5 m²/m³) would be related to the higher L/G transfer conditions that can be assumed in this system compared to the AnBDR containing 500 mL of liquid medium even with the rotating immobilization support that was immerged in culture medium for 40 % of time. Additionally, a similar decrease in yields was evidenced in the HFBR and the AnBDR with GLR increasing from 0.22 to 1.04 g/L.h. A same trend was reported by Van Ginkel and Logan [41] with H_2 yields decreasing from 348 to 236 mL/g with GLR increasing from 0.23 to 1.82 g/L.h. At higher GLR, up to 9.1 g/L.h, their yields stabilized at 246.8 +/- 14.5 mL/g. Recently Chu et al. [16] confirmed the trend for GLR varying from 2.34 to 18.75 in three different bioreactors with suspended sludge (SS) or granules in two configurations (height to diameter ratios H/D of 3.2 and 9.1). De Amorim et al. [42] observed a quite different trend with increasing GLR. However, other parameters might be strongly influenced considering the high H/D ratio of 36 of their reactor.

In our experiments with a *C. butyricum* CWBI1009 pure strain a stable H_2 yield was already achieved in the AnBDR for a GLR varying from 0.68 to 1.72 whereas in the HFBR with

mixed culture it dramatically decreased at GLR higher than 0.88 g/L.h.This confirms that in

the HFBR other microorganisms (non H_2 producers) were progressively competing with the

Clostridium strain for the glucose substrate or that environmental conditions prevented further

increase of HPR due to inhibiting H_2 concentration in liquid phase. This inhibition would

focus on H_2 dehydrogenase activity as supported by many authors [2, 43, 44].

Therefore taking into account the HPR stabilization at a GLR higher than 0.88 g/L.h it can be

assumed that the *Clostridium* strain had achieved its maximum activity and contributed to

glucose consumption for H_2 production at a yield of about 325 mL/g (*i.e.* that obtained at a

GLR of 0.66 and 0.88 g/L.h). A yield of that order was reached by the pure culture in the

AnBDR with 300 mL liquid medium (GLR of 2.87 g/L.h). It is about 30% higher than that

achieved in the same bioreactor with 500 mL liquid medium and the same glucose

consumption rate. Chu et al. [16] also mentioned H_2 yields higher in a bioreactor with a high

H/D ratio than with a lower ratio. Few papers mention H_2 yields of the order of 370 mL/g *i.e.*

3 mol/mol hexose [18] or H_2 yields higher than 280 mL/g (2.25 mol/mol) at GLR higher than

2 g/L.h [29, 45, 46]. The results of these authors are reported in Fig. 6B in the curve "Max".

These relevant results would be related to the high L/G transfer of H_2 occurring in the

bioreactors tested that were trickle bed systems or characterized by a high superficial liquid

and gas velocity e.g. $7 \ 10^{-3}$ and $7.4 \ 10^{-2}$ cm/s in the bioreactor with H/D ratio of 9.1 used by

Chu et al. [16] at a GLR of 9.4 g/L.h (*i.e.* a HRT of 2 h). By comparison, these velocities were

1.8 and 2.7-fold higher respectively than those reported at the same GLR in the bioreactor

with H/D ration of 3.2.

The evolution of HPR versus GLR in both HFBR and AnBDR reactors are consistent with

those reported by Van Ginkel and Logan [41] and other authors (Fig. 6A), except in the

HFBR at GLR higher than 0.88 g/L.h suggesting that such an organic loading rate and a HRT

of 6 h are the optimum conditions leading to efficient use of glucose substrate by the mixed

culture containing *C. butyricum* CWBI1009 strain. Jo et al. [30] already reported a HRT or GLR for maximum HPR. The general performances of their mixed culture were however lower than that experimented here. The best results regarding HPR are depicted by the curve "Max" (Fig. 6A) and those recorded by de Amorim et al. [42] at a high liquid velocity. It is to mention that the HPR reported at a GLR higher than 3 g/L.h are closer to those mentioned by other authors.

As a consequence, for large-scale applications trickle bed reactors would be promising since their technological and energy requirements would be lower than in other systems.

4.   Conclusions

The ability of a pure *Clostridium butyricum* strain to form microbial aggregates was tested in bioreactor systems designed to both fix biomass and enable rapid liquid to gas transfer of hydrogen produced since H_2 partial pressure is known as hardly affecting hydrogen production rates and yields. The highest performances recorded in the anaerobic biodisc-like reactor were about 5.5-fold higher (703 mL H_2/L h) than in a 2.3 L stirred bioreactor. These results, amongst the most relevant results reported in the literature, confirm the relevance of performing bioreactors with high L/G transfer. The tested HFBR and AnBDR systems are obviously promising since they originally enabled to highlight some crucial phenomenon in comparison to the classical stirred tank bioreactor. For industrial perspectives, they would compete with trickle bed systems that would probably be more suitable but still need further investigations.

5.   Acknowledgements

6.   References

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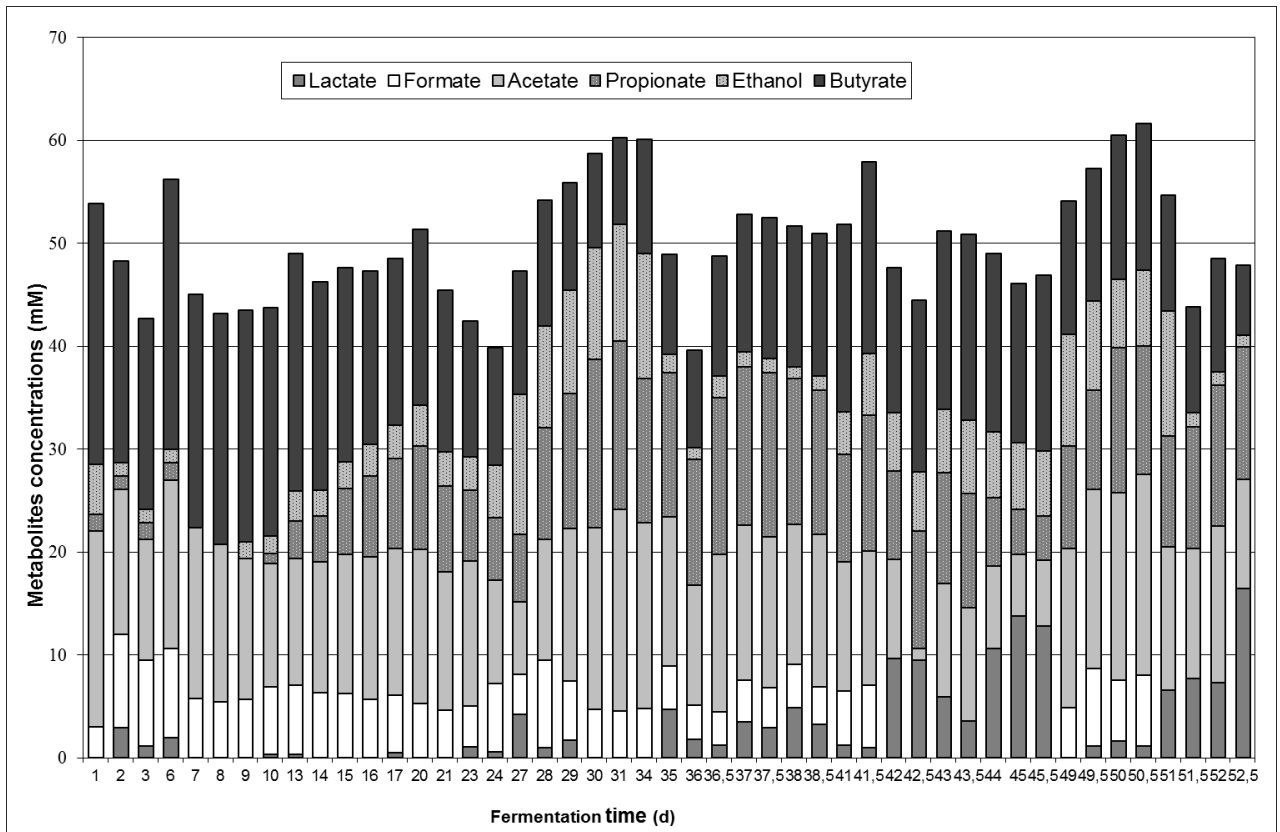
Figure A1. Metabolite analysis in the culture medium at the end of the sequences related to the different GLR in a 400-mL horizontal fixed-bed reactor inoculated with the pure strain *C. butyricum* CWBI1009.

Figure A2. Biofilm (A) and biogas bubbles (B) development on reticulated polyurethane support (green at the back front) in a 400 mL horizontal fixed-bed reactor inoculated with the pure strain *C. butyricum* CWBI1009.