Mesophilic biohydrogen production by *Clostridium butyricum* CWBI1009 in trickling biofilter reactor.

Rathnasiri, P.G[a,b], Laurent Beckers[a], Frank Delvigne[a], Alina S. Grigorescu[a], PhilippeThonart[a], Serge Hiligsmann[a]*

[a] Walloon Centre of Industrial Biology (CWBI), Bd du Rectorat, 29, B.40 – P.70 B-4000 Liège, BELGIUM

[b] Department of Chemical and Process Engineering, University of Moratuwa, Sri Lanka

*Corresponding author: s.hiligsmann@ulg.ac.be, rathnasirip@gmail.com;

Tel.: +32 (0) 4 366 28 61

Fax: +32 (0) 4 366 28 62

Abstract

This study investigates the mesophilic biohydrogen production from glucose using a strictly anaerobic strain, *Clostridium butyricum* CWBI1009, immobilized in a trickling bed sequenced batch reactor (TBSBR) packed with a Lantec HD Q-PAC® packing material (132 ft^2/ft^3 specific surface). The reactor was operated for 62 days. The main parameters measured here were hydrogen composition, hydrogen production rate and soluble metabolic products. pH, temperature, recirculation flow rate and inlet glucose concentration at 10 g/l were the controlled parameters. The maximum specific hydrogen production rate and the hydrogen yield found from this study were 146 mmol H_2/L.d and 1.67 mol H_2/mol glucose. The maximum hydrogen composition was 83%. Following a thermal treatment, the culture was active without adding fresh inoculum in the subsequent feeding and both the hydrogen yield and the hydrogen

production rate were improved. For all sequences, the soluble metabolites were dominated by the presence of butyric and acetic acids compared to other volatile fatty acids. The results from the standard biohydrogen production (BHP) test which was conducted using samples from TBSBR as inoculum confirmed that the culture generated more biogas and hydrogen compared to the pure strain of *Clostridium butyricum* CWBI1009. The effect of biofilm activity was studied by completely removing (100%) the mixed liquid and by adding fresh medium with glucose. For three subsequent sequences, similar results were recorded as in the previous sequences with 40% removal of spent medium. The TBSBR biofilm density varied from top to bottom in the packing bed and the highest biofilm density was found at the bottom plates. Moreover, no clogging was evidenced in this packing material, which is characterized by a relatively high specific surface area. Following a PCA test, contaminants of the *Bacillus* genus were isolated and a standard BHP test was conducted, resulting in no hydrogen production.

Keywords : Mesophilic; biohydrogen; trickling biofilter; immobilization; *Clostridium butyricum*

1. Introduction

Biohydrogen production by microorganisms has attracted increasing global attention, owing to its potential to be used as an inexhaustible, low-cost and renewable source of clean energy [1]. Among the biological processes, the anaerobic hydrogen fermentation called dark fermentation seems to be more favorable, since hydrogen is yielded at a high rate and various organic wastes or wastewaters enriched with carbohydrates could be used as substrates, thus reducing production costs [2]. The dark fermentation can be conducted in either suspended or immobilized systems. Previous studies on immobilization were conducted using pure cultures, mixed cultures, different modes of operation, different packing materials and different operating conditions. Biohydrogen production in sequenced batch reactors with microbial biofilm has been studied by

Bhaskar et al. [3] and Venkata Mohan et al.[4]. The immobilization of *Clostridium* species, *i.e.* *C. tyrobutyricum* ATCC 25755 [5] and *C. tyrobutyricum* JM1 [6], was studied to optimize continuous biohydrogen production under various hydraulic retention times and inlet glucose concentrations. Different immobilization techniques [7-12] were investigated in order to improve the biofilm formation, the biohydrogen production rate and the hydrogen yield and composition.

The effect of the hydraulic retention time (HRT) and glucose concentration on hydrogen production in a mesophilic anaerobic fluidized bed reactor (AFBR) was studied by Zhang et al. [13]. They achieved a maximum yield of 1.7 mol H_2/mol glucose at HRT of 0.25 h, pH 5.5 and a glucose concentration of 10 g/L. They used a Continuous Stirred Tank Reactor (CSTR) and an AFBR to study the effect of different inocula on biohydrogen production. A 20-fold increase of the biohydrogen production rate was recorded in the AFBR compared to the CSTR that used a suspended culture for reactor operation. One of the problems associated with AFBR is the washout of biomass from the reactor. An anaerobic fixed bed sequenced batch reactor [14] was operated for 1435 days using synthetic wastewater and vegetable wastewater under different time periods. The reactor produced hydrogen without inhibition and microbial community analysis confirmed the presence of four species among which *Bacillus* sp. and *Clostridium* sp. were dominant in the biofilm. Among the biofilm reactors, the Trickle Bed Reactor (TBR) offers advantages such as high mass transfer rate between the gas–liquid interface, an easy control of pH in the circulating liquid phase and low liquid hold up [15]. The first continuous thermophilic TBR study was conducted using glucose as substrate and a mixed culture grown on a fibrous support matrix [15]. The optimal pH, temperature and hydrogen yield were 5.5, 60°C and 1.11 mol H_2/mol glucose respectively. The same TBR was further studied for continuous biohydrogen production and a microbial analysis confirmed the presence of *Clostridia* and *Bacillus* as

dominant species [16]. More importantly, it was found that the biomass concentration in the TBR gradually decreased as the reactor bed height increased.

Glucose fermentation was conducted using a pure culture of *Clostridium acetobutylicum* ATCC 824 grown on glass beads in TBR [17]. The reactor was tested for various glucose concentrations and the head-space average hydrogen composition was 74% (v/v). The major drawback of this study was the clogging of beads due to biomass formation after 72 h. Two bioreactor systems, *i.e.* trickle bed reactor and fluidized bed reactor, were compared [18] for thermophilic biohydrogen production and the TBR showed yield of 3 mol H_2/mol glucose. However, to achieve this yield, nitrogen gas had to be stripped throughout the experiment. A TBR was packed with perlite and fed with oat straw hydrolysate [19]. By varying HRT and inlet OLR, Arriaga et al. [19] obtained a maximum specific hydrogen production rate of 3.3 mmol $H_2/L_{reactor}$.h and a hydrogen yield of 2.9 mol H_2/mol hexose. The maximum hydrogen composition was 45%. (v/v), the rest being CO_2. Globally the major drawback of many of these studies was the clogging of the trickling filter bed with biomass [17, 19].

It is usually not recommended to use pure cultures in non-sterile conditions due to contamination risks, which can generate deterioration of reactor performances. However thermophilic biohydrogen production was conducted in a 400L non-sterile trickling bed reactor starting with a pure culture of *Caldicellulosiruptor saccharolyticus* using sucrose as major substrate [20]. It was found that contaminants were outcompeted by the pure culture and a hydrogen yield of 2.8 mol H_2/mol hexose could be achieved.

At CWBI, extensive research studies had been conducted using *Clostridium butyricum* CWBI 1009 to improve biohydrogen production in batch, sequenced batch and continuous mode under various operating conditions and using different substrates. Fermentative hydrogen production was conducted using a co-culture of pure *Clostridium butyricum* and *Citrobacter freundii* with five different carbon sources [21]. To investigate the optimal culture conditions for production of hydrogen using *Clostridium butyricum*, batch and sequenced batch experiments were conducted using glucose and starch as substrates [22]. For glucose degradation, it was found that the maximum hydrogen yield could be obtained when pH was controlled at 5.2. In order to characterize the biohydrogen potential of different strains and sludge inocula growing on glucose, a series of experiments using serum bottles was conducted [23], showing that the pure *Clostridium butyricum* strains achieved the highest hydrogen yield. To further improve the performances of *Clostridium butyricum*, experiments were conducted using horizontal tubular fixed bed and biodisc-like anaerobic reactors [24]. The major objective was to improve biofilm formation by simultaneously enhancing liquid to gas mass transfer. For the anaerobic biodisc-like reactor, when the reactor bulk volume was reduced from 500 mL to 300 mL, both hydrogen production rate and yields were improved significantly. Experiments conducted in a 20 L fixed bed SBR [25] using polyurethane as a support material and an artificial co-culture, composed initially of *Clostridium butyricum* CWBI1009 and *Clostridium pasteurianum* DSM525, achieved maximum hydrogen yields when a mixed substrate was used in this reactor. Drawbacks found in this reactor set up were the poor hydrodynamics and susceptibility for clogging due to biomass build up.

The purpose of the current study was to further investigate the biohydrogen production by developing a new reactor configuration such as TBR for improving biofilm formation and high

L/G transfer. In this study, a 20 L fermenter was converted into Trickle Bed Sequenced Batch Reactor (TBSBR) to produce hydrogen using Lantec HD Q-PAC® as packing material with growing *Clostridium butyricum* CWBI1009 utilizing glucose as main substrate. Lantec HD Q-PAC had already been applied in bio trickling filters used for odor removal from waste air streams [26]. To the best of our knowledge, this is the first study that applies Lantec HD Q-PAC material in trickling biofilter for biohydrogen production. The reactor performances were evaluated based on biogas production rate, hydrogen yield, soluble metabolites and biomass. To overcome contamination, a new thermal pretreatment strategy was developed. To evaluate the hydrogen production potential of the final mixed culture from the TBSBR, the standard biochemical hydrogen potential was conducted and compared with the performance of the pure *Clostridium butyricum* strain. TBSBR was operated by removal and addition of 40% from the bulk liquid volume. To investigate the effect of biofilm activity towards hydrogen production, three subsequent sequences were conducted by 100% removal of the mixed liquid and adding the same amount of fresh medium.

2. Materials and methods

2.1. Inoculum and culture medium

The bacterial strain used in this study was *Clostridium butyricum* CWBI1009, which was previously isolated at CWBI [22]. This strain was maintained at 30°C by transferring 1 mL from a hermetically sealed 25 mL culture tube into a new tube filled with sterile MDT medium. The MDT growth medium contained per liter of deionized water: glucose monohydrate (5 g), casein peptone (5 g), yeast extract (0.5 g), KH_2PO_4 (2 g), $MgSO_4.7H_2O$ (0.5 g) and L-cysteine hydrochloride (0.5 g). All the chemicals used were of analytical or extra pure quality and were supplied by Merck, UCB and Sigma. Casein peptone and yeast extract were supplied by

Organotechnie (La Courneuve, France). The inoculum for the trickling biofilter reactor was prepared in a 2 L bottle equipped with silicone tubings and air filters needed for sterile liquid transfer. A 2 L bottle containing 1600 mL of MDT medium (without glucose and L-cysteine), a 300 mL aqueous solution containing glucose monohydrate and a 50 mL L-cysteine solution were sterilized separately at 121°C for 20 min to prevent Maillard reactions between amino acids and carbohydrates. After cooling down to room temperature, two 25 mL culture tubes, 300 mL glucose monohydrate and 50 mL L-cysteine solutions were transferred under sterile conditions into the 2 L bottle containing 1600 mL MDT medium. After purging nitrogen gas to remove oxygen from the 2 L bottle head-space, it was incubated at 30°C. In order to increase active biomass in growth phase, three experimentation sequences were carried out by removing 40% of the bulk liquid and adding an equal volume of fresh MDT medium containing glucose as formerly experimented < to reach 5g/l of glucose in the whole liquid medium and avoid inhibitory effect of VFA accumulation.

Figure 1. Lantec HD Q-PAC bed and rotating liquid distributer inside 20 L TBSBR.

2.2. Reactor set up and operations

Biohydrogen production was conducted in a 20 L fermenter (Solvay manufacture) which is operated and controlled by a PLC system. This reactor consists of double envelope in stainless steel, shaft with impeller, lid provided with tubing for gas and liquid transfer and butyl septum. The packing material used inside the fermenter was Lantec HD Q–PAC (Agoura Hills, CA, USA), which is available in standard module size of 12 x 12 x 12". The smallest grid opening is 0.16"x 0.16" and specific surface area, bulk density and void fraction are 132 ft^2/ft^3, 7.5 lb/ft^3 and 87.8% respectively. Using this material, cylindrical packed bed with diameter D = 21 cm and Height H= 30 cm was made and placed inside the fermenter (Fig. 1). A liquid distributer was fixed on the shaft in order to uniformly trickle the liquid medium over packing material. When

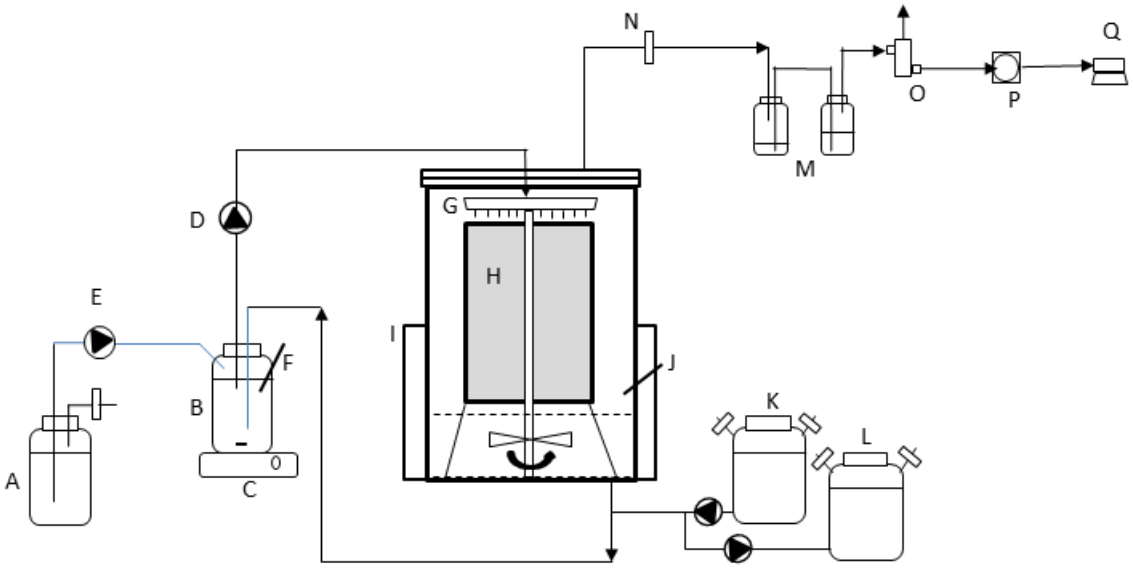


Figure 2. Schematic diagram of the trickling biofilter system developed in this study : (A) 3N KOH solution bottle; (B) 1 L bottle with pH probe immersed in mixed liquid; (C) Magnetic stirrer; (D) Peristaltic pump (WATSON MARLOW) for recirculation; (E) Peristaltic pump (GILSON minipuls 2); (F) pH probe (Hamilton®); (G) Rotating liquid distributer; (H) Trickling filter bed (Lantec HD Q-PAC); (I) Steam jacket; (J) Temperature probe; (K) Feeding bottle; (L) Mixed liquid removal bottle; (M) Liquid trap; (N) Air filter; (O) Gas sampling device; (P) Flow meter; (Q) Computer for data acquisition.

the reactor was operated, liquid from the bottom was pumped through a 1 L bottle containing a pH probe for automatic pH control by injecting sterile 3N KOH solution via a needle placed through the butyl septum (Fig. 2). Before starting up the reactor, a pressure test was conducted by filling the reactor with air until it reached 1 bar gauge pressure and monitoring any pressure reduction during 30 min. The reactor was next filled with 10 L of water and sterilized at 120°C for 20 min. After filling the head-space with nitrogen, the reactor was allowed to cool down to 30°C and liquid water was discharged under sterile conditions. Before inoculating, glucose monohydrate and L-Cysteine solutions were autoclaved separately to prevent Maillard reactions. The reactor was then inoculated by adding 2 L of culture incubated at 30°C, 4 L of sterilized MDT medium and 1L of glucose and cysteine solution sterilized separately to make the final working volume of 7 L To enhance the formation of biofilm on the packing material, the reactor working volume was increased up to 11 L at the beginning and brought down to 4 L and maintained at this value until the reactor operation was stopped (Fig. 3A). The recirculation flow rate was 146 mL/min. Since the reactor was operated in sequence batch mode with daily removal and addition of 40% of medium, the hydraulic retention time was 2.5 d. Medium addition and mixed liquor removal were performed using tubing connected to the recirculation line before the 1L bottle. To avoid oxygen entering into the reactor head-space, a liquid trap was installed in the gas outlet tubing consisting of two 250 mL bottles containing yeast and glucose solution. This method with low pressure drop was efficient to maintain suitable conditions for *Clostridium* metabolism since yeast degrades glucose with oxygen consumption. During mixed liquid removal and addition of medium, a minimum amount of nitrogen gas was supplied via the liquid trap to prevent the entering of oxygen into the reactor. The reactor was operated at 30°C and the impeller speed was 90 rpm. Following 60 days of reactor operation, the effect of biofilm formation on hydrogen production was investigated by completely removing the mixed liquid

and replacing it with MDT medium and glucose solution. This procedure was followed for three consecutive sequences.

2.3. Analytical methods

The flow rate of the biogas produced in the bioreactor head space was continuously measured with a wet flow meter (Ritter Gas meter MGC-10) connected to a computer running the Rigamo software (V1.30-K1) for data acquisition. The proportion of hydrogen gas was determined using the method described elsewhere [27]. Mixed liquid samples collected during and at the end of each sequence were centrifuged at 13000 g for 10 min. The supernatants were then filtered through a 0.2 µm cellulose acetate membrane (Midisart Sartorius) and analyzed by HPLC as previously described by Masset et al. [22]. Glucose concentration in the liquid samples was also rapidly measured by the RTU kit method (BioMerieux, France) and spectrophotometer.

At the end of each daily sequence, the Oxidation Reduction Potential (ORP) of the mixed liquid was measured using an ORP probe (Sentix ORP, WTW). The growth of contaminant strains was observed by spreading 100 µL of sample on a PCA Petri dish and incubating at 30°C for 48h. This test was also used as purity check for the pure anaerobic strain. The PCA medium contained per liter of deionized water: glucose monohydrate (1 g), casein peptone (5 g), yeast extract (2.5 g), agar (15 g). The cell density of *C. butyricum* was determined by microscopic observations on a Bürker counting chamber.

The packed bed with attached biofilm was finally removed from the fermenter and the top, middle and bottom plates were carefully dismantled. Each plate was thoroughly washed with distilled water and the resultant suspension was centrifuged at 13000 g for 10 min. The weight of dry biomass attached to individual plates was measured as total solids (APHA, 1995).

2.4. Biomolecular methods

2.4.1. Identification of the contaminant strain

Total DNA was extracted from freshly grown biomass using the Wizard® Genomic DNA purification Kit (Promega, Madison, WI, USA). For the identification of the isolate, the 16S rRNA gene was PCR-amplified using the universal primers 16SP0 (5'-GAA GAG TTT GAT CCT GGC TCA G-3') and 16SP6 (5'-CTA CGG CTA CCT TGT TAC GA-3') [28]. The PCR reactions contained 1x ReadyMix Taq PCR Reagent Mix (Sigma-Aldrich, St. Louis, MO, USA), 0.5 µM of each primer and ~ 50 ng of genomic DNA as template. The PCR program included a 5-min initial denaturation step at 95°C, followed by 26 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, and a final extension for 10 min at 72°C. The presence and size of amplified products were checked by migration on 1% agarose gel stained with ethidium bromide. The O'GeneRuler 1kb DNA Ladder (Fermentas) was used as molecular size marker.

The PCR product was purified using the GFX PCR DNA and Gel Band Kit (GE Healthcare, Buckinghamshire, UK), then sequenced using the Big Dye v3.1 Kit and an ABI 3730 DNA Analyser (Applied Biosystems/Life Technology, Carlsbad, CA, USA) at the GIGA Center at the University of Liege. The primers used for sequencing were 338F: 5'-ACT CCT ACG GGA GGC AGC AG-3' and 907R: 5'-CCG TCA ATT CCT TTR AGT TT-3' [29]. The obtained sequences were then assembled by using the program CodonCode Aligner (version 4.2.7, CodonCode Corporation, Centerville, MA, USA). The resultant 16S sequence was compared with those in the GenBank database by using the BLASTN program [30], in order to identify the closest organism match.

2.4.2. Metagenomic analysis

A biofilm sample was sent to Progenus SA (Gembloux, Belgium) for community metagenome sequencing. In short, the sample was first incubated overnight at 56°C with T1 buffer and ProtK (Macherey-Nagel), then DNA was extracted using the Nucleomag 96 Trace kit (Macherey-Nagel) and the KingFisher 96 system (Thermo Scientific), according to the manufacturer's instructions. The V3 region of the 16S rRNA gene was PCR-amplified using tagged universal bacterial primers (i.e. 337F and 533R) in order to conduct a metagenomic analysis. The PCR program included a 2-min initial denaturation step at 94°C, followed by 29 cycles of 94°C for 30 sec, 48°C for 30 sec and 68°C for 2 min, and a final extension for 35 min at 68°C. PCR products were first verified on a 2% agarose gel and then purified with a High Pure PCR Product Purification kit (Roche) and quantified using a Qubit kit (Life Technologies). An ion torrent library was constructed using the Short Amplicon Prep Ion Plus Fragment Library Kit (Life Technologies) according to the manufacturer's instructions and quantified using the Ion Library Quantitation Kit (Life Technologies). The library was further prepared using the Ion PGM template OT2 200 Kit and finally sequenced on an Ion PGM machine using the Ion PGM Sequencing 200 Kit v2 and a 316 micro-chip.

The raw reads obtained from the high-throughput sequencing step were processed through two different filters in order to retain only the reads with the highest quality, i.e. reads with a low rate of sequencing error. The reads lacking a valid tag sequence were discarded. The tag sequences were then removed from the reads and the reads shorter than 150 bp were eliminated from the analysis, since the expected PCR products were about 200 bp, based on the *E. coli* numbering system. The reads were then assigned with the RDP Classifier program [31]. The number of sequences corresponding to each identified rank was divided by the total number of sequences retained in the sample after filtering and multiplied by 100 to yield a relative abundance expressed as a percentage.

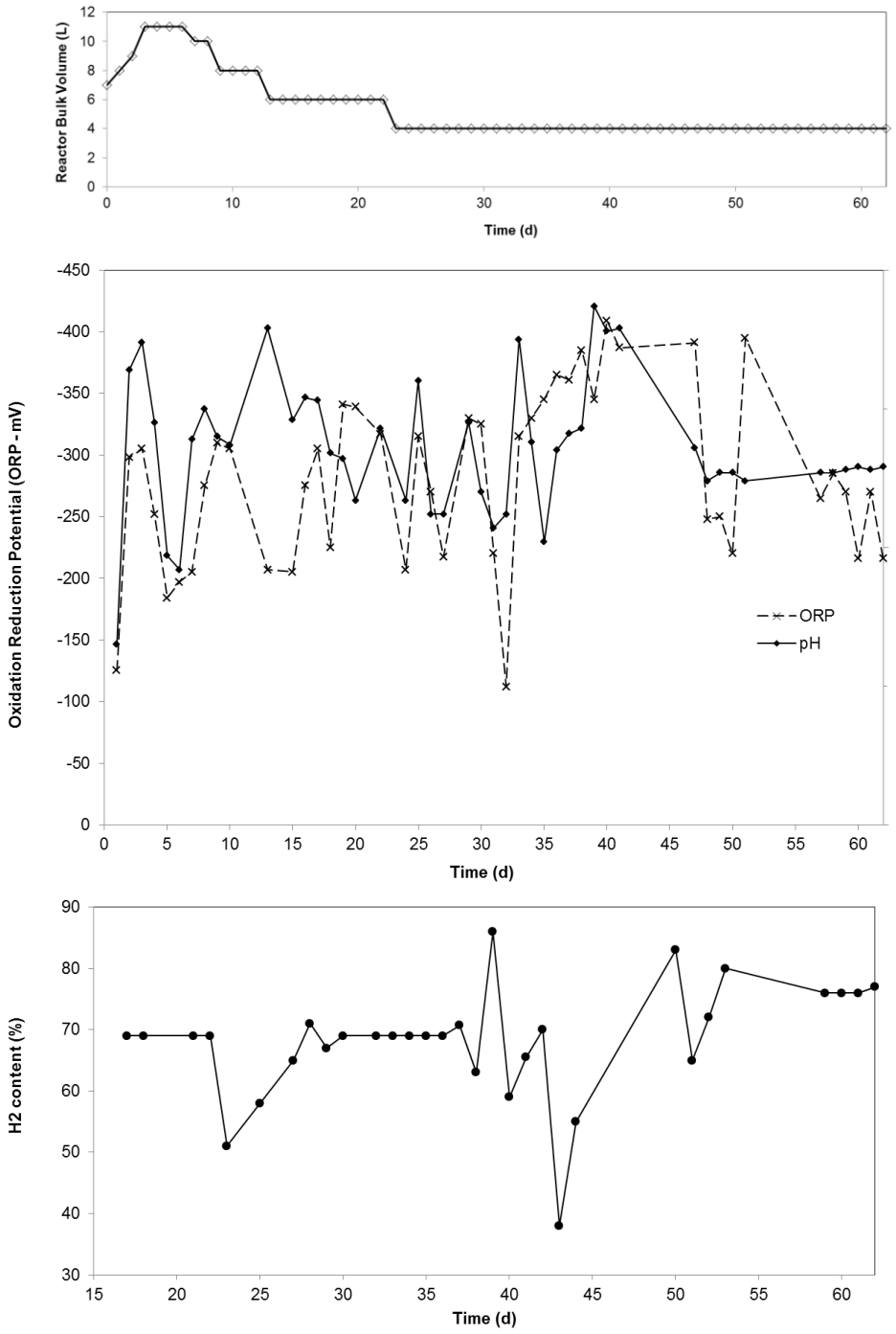


Figure 3. Evolution of (A) liquid volume, (B) pH and ORP in bioreactor liquid phase and (C) hydrogen content in bioreactor head-space at the end of each sequence in 20 L TBSBR inoculated with *C. butyricum* CWBI1009.

277 **Table 1**. Details of Trickling Bed Sequencing Batch Reactor operated for 62 days.

Operation period (d)	Biogas production rate (L/d)	Hydrogen production rate (L/d)	Substrate degradation efficiency (%)	Hydrogen yield (mole-H2/Mole-Glucose	Remarks
0			0		Sterlization
1 -12			75 ±14		Gas leak from bottle
13					**Thermal treatment**
14 -15			87 ±1.62		Gas leak from bottle
16	10.7	7,38	71,00	1,08	
17	13.8	9,55	72,00	1,23	
18-19					No temperature regulation
20	11.59	7,99	92,70	0,93	
21	5.19	3,58	80,27	0,52	
22	3.80	2,62	77,16	0,27	
23					**Thermal treatment**
24	9.34	5,41	85,21	0,48	
25					No feeding
26	9.12	5,92	91,08	1,18	
27	5.30	3,76	71,00	0,75	
28	10.06	6,74	100,00	1,10	
29 -30					pH bottle replaced
31	10.5	7,24	76,47	1,09	
32	3.2	2,25	92,21	0,32	
33	3.05	2,1	82,42	0,42	
34					**Thermal treatment**
35	11,47	7,92	90,71	1,20	
36	6,7	4,74	72,70	1,11	
37	2,18	1,65	76,1	0,25	Initial pH adjusted
38	8,91	7,66	88,58	1,19	
39	9,52	5,62	100,00	0,87	
40	6,76	4,42	90,00	0,94	
41	5,98	4,186	100,00	0,76	
42	6,94	2,637	100,00	0,49	
43	6,9	3,795	100,00	0,72	
44-47					Impeller stopped
48					**Thermal treatment**
49	15,76	13,08	90,00	1,67	
50	7,37	4,79	90,91	0,97	
51	7,49	5,39	100,00	0,99	
52	7,91	6,328	100,00	1,22	
53	5,94	4,455	100,00	0,90	
54-57					No feeding
58					100% removal/addition
59	5,78	4,3928	90,00	0,92	100% removal/addition
60	7,65	5,814	100,00	0,90	100% removal/addition
61	5,86	4,4536	100,00	0,85	100% removal/addition
62	3,13	2,41	90,00	0,40	Sampling for daily sequence

278

3. Results and discussion

3.1. Startup of the reactor

To enhance the formation of biofilm on the packing material, the reactor working volume was increased progressively from 7 L to 11 L (Fig. 3A). Between day 6 and day 23 it was decreased progressively down to 4 L (below the packing level, in the stirred compartment) and maintained at this value until the reactor operation was stopped .

3.2. Glucose conversion and hydrogen production in TBSBR.

Fermentation was conducted with glucose monohydrate as substrate at a controlled pH of 5.2. Sequenced batch reactor was followed by removal/addition of 40% from the bulk liquid volume. Following each sequence, pH and ORP were measured and glucose concentration was determined. Using this value, the inlet glucose concentration was adjusted for subsequent sequence and thereby glucose conversion efficiency during each sequence was calculated. Detailed TBSBR operation is given in Table 1. Starting from day 1 and up to day 15, glucose conversion efficiency increased from 50 to 87 %. During this period, no biogas production was recorded by the gas flow meter due to gas leak from the liquid trap bottles. For each daily sequence, the mean biogas production rate and the hydrogen yields were calculated based on the active gas production time during the sequence.

The final pH and ORP measured from samples collected at the end of a daily sequence are given in Fig. 3B. For the entire experimental period, ORP varied between -125 and -409 mV while pH varied between 4.65 and 5.87. Masset et al. [22] found that pH 5.2 was optimal for the conversion of glucose to hydrogen by *C. butyricum.* Though the pH was set at 5.2 in this study, the pH variation found here was due to varying ORP. According to the results from Fig. 3B,

when pH is around 5.2, ORP was most frequently (deviations due to the influence of environmental conditions at the measurement on collected samples and due to pH probe calibration in aerobic conditions) lower than a threshold of -200 mV suitable for dark fermentation..

3.3. The effect of thermal treatment on hydrogen production

When pH varied between 5.2 and 5.4, the daily hydrogen composition was above 70% (Fig. 3C). When the daily biogas production rate decreased or the hydrogen yield decreased at days 13, 23, 34 and 48, the reactor was thermally treated to minimize contamination. The tested temperature profile for thermal treatment is shown in Fig. 4.

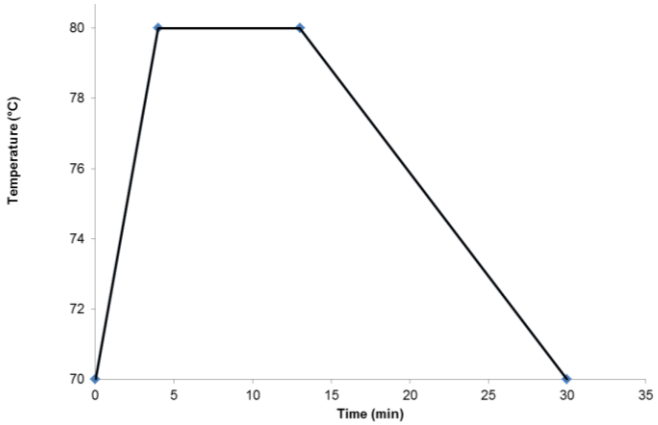
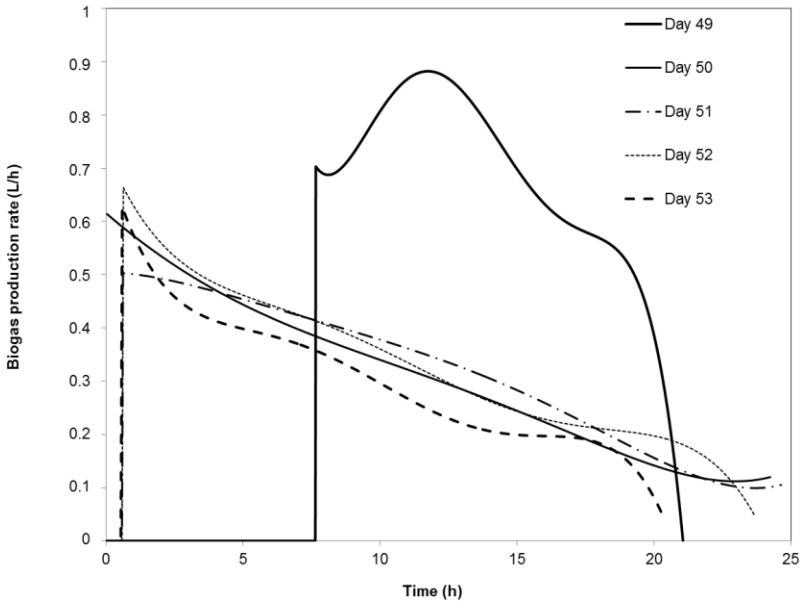


Figure 4. Temperature profile applied for thermal treatment

During the whole operation or running period of TBSBR, the thermal treatment was done four times according to a similar procedure as further described (Fig. 5) for the thermal treatment conducted on 48th day. At day 48 the reactor was thermally treated due to contamination and following thermal treatment no fresh inoculum was added to reactor. After cooling down the reactor to 30°C and removal/addition of fresh medium, it took about 8 h time period to start biogas production since bacterial spores had to reactivate after thermal stress (non spore-forming microorganisms would not survive after this thermal treatment). The production rate peaked at

0.9 L/h of biogas (Fig.5A). Biogas production started more rapidly (about 0.5 h) after the following sequences with removal/addition of culture medium since the whole bacteria population was involved and in lack of substrate. The production kinetic decreased progressively until 20 h and stopped after substrate depletion. From day 50, the activation period after fresh



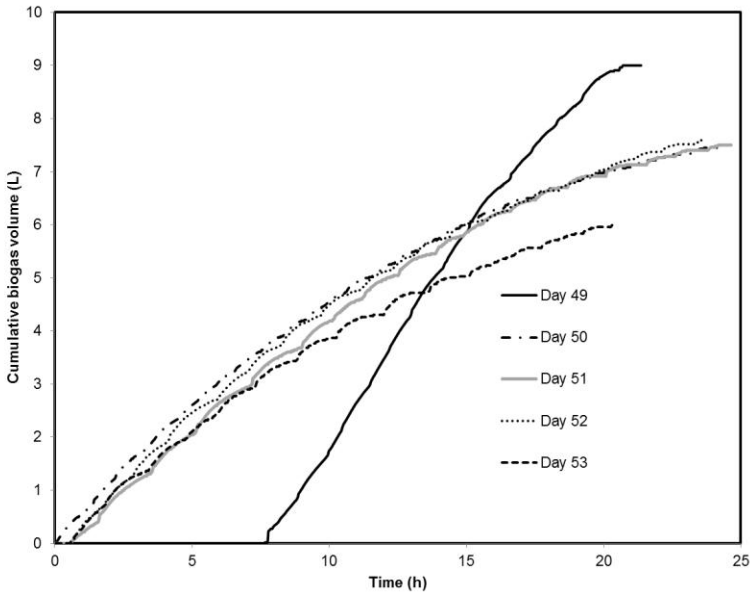
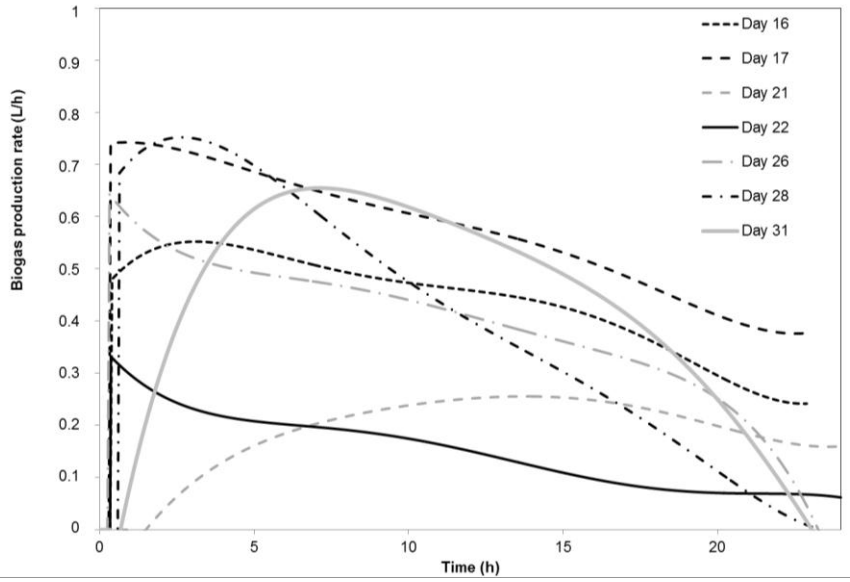
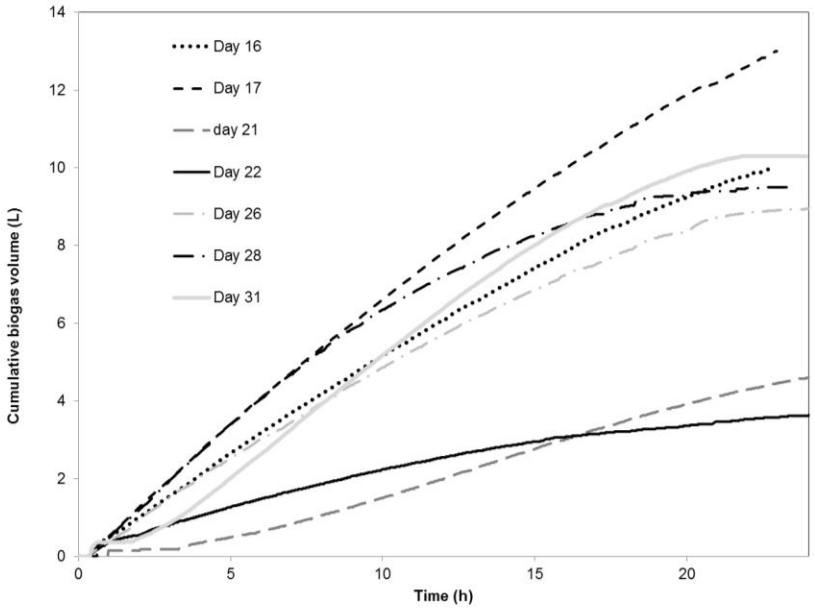


Figure 5. Effect of thermal treatment carried out at day 48 on cumulative biogas production rate (A) and yield (B)



329



330

331 **Figure 6.** Biogas production rate (A) and cumulative biogas production (B) from day 16 to day
332 31 after thermal treatment carried out at days 13 and 23 respectively

333

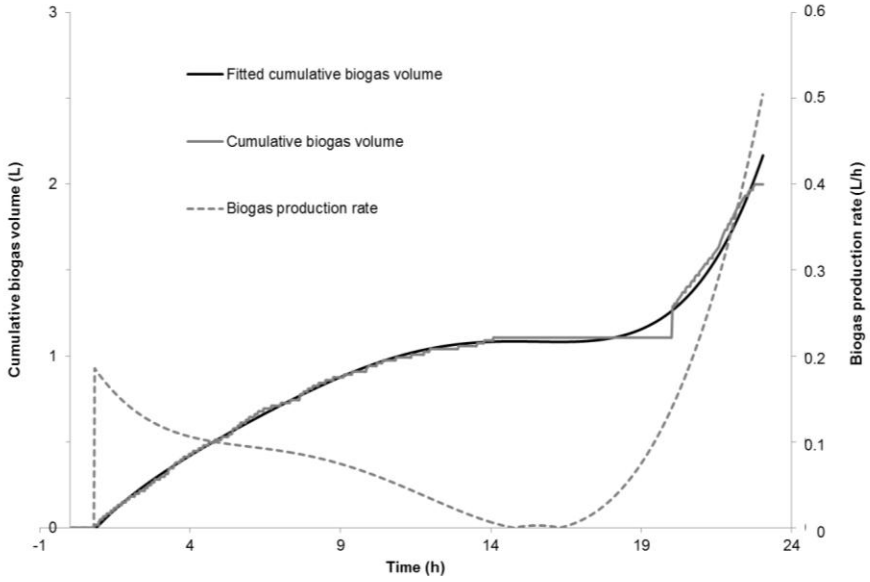
334 medium addition decreased to 0.5h but maximum biogas production decreased by 33%. The
335 hydrogen yield also decreased from 1.67 to 0.9 mol H_2/mol glucose. A similar trend was also
336 observed after other operations. Following the first thermal treatment at day 13, the gas
337 production rate increased up to 0.74 L/h at day 17. However, at days 21 and 22, (Fig. 6A) the

maximum biogas production rate decreased by 66%. Sequences after thermal treatment at day 23 maintained efficient performances until day 31.

At day 37, only 1.1 L of cumulative biogas was produced in 19 h. An increase of the bulk liquid pH up to 6.2 enabled the restart of biogas production and an increase in the production rate (Fig. 7). Following this observation, at the end of each subsequent sequence, the initial pH after feeding was adjusted to 6.2 and let to decrease naturally down to the set point of 5.2 for further regulation. From day 37 to day 43, the daily mean biogas production rate was consistent at 7.5 ±1.38 L/d.

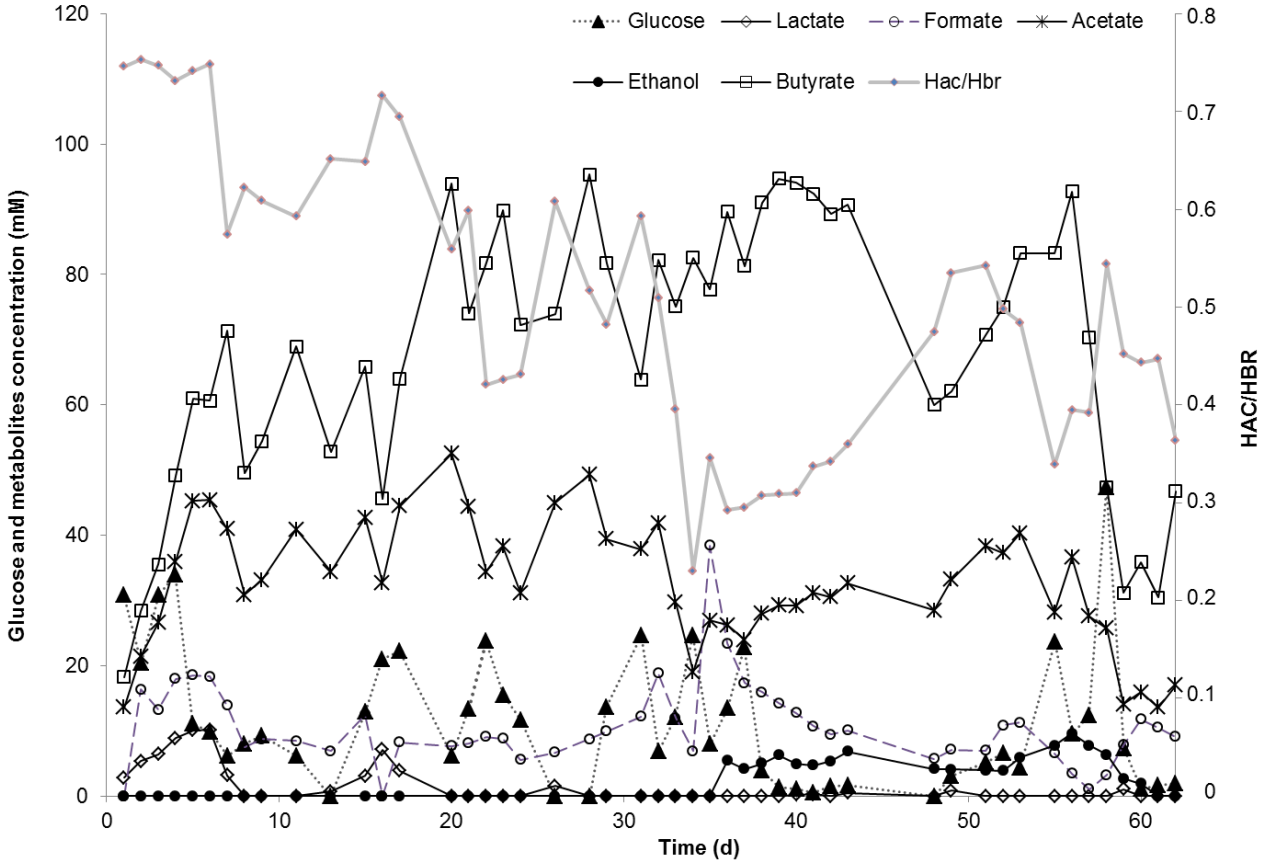
3.4. The effect of soluble metabolite production on hydrogen production

The HPLC analysis of the liquid samples collected from the bioreactor showed that the major soluble metabolites from glucose fermentation were formate, lactate, acetate, butyrate, and ethanol. Up to day 7, lactate was found in the reactor but in the subsequent sequences no lactate or a very small amount of it was detected (Fig. 8). For the entire operational period, butyric and acetic acids were the major volatile fatty acids measured inside the reactor. No ethanol was detected up to day 35 and after this day ethanol was detected in the mixed liquid at a concentration not exceeding 10 mM. By comparison, acetate and butyrate reached concentrations about 4 to 9-fold higher respectively. Both the production of alcohols such as ethanol and of reduced acids such as lactate are related to sub optimal conditions for hydrogen production e.g. ethanol is particularly related to *Clostridium* stationary growth phase and spore formation [32]. The drop of the acetic/butyric ratio observed after 31 days and its low level up to day 49 should be linked to ethanol formation. It can also be observed that an increase of acetic/butyric ratio occurred after each thermal treatment operation.



361

362 **Figure 7**. Effect of pH adjustment after 19h of operation at day 37 on cumulative and biogas
363 production rate.



364

365 **Figure 8**. Evolution of soluble metabolites concentration and acetic/butyric ratio during the
366 operation of TBSBR over 61 days

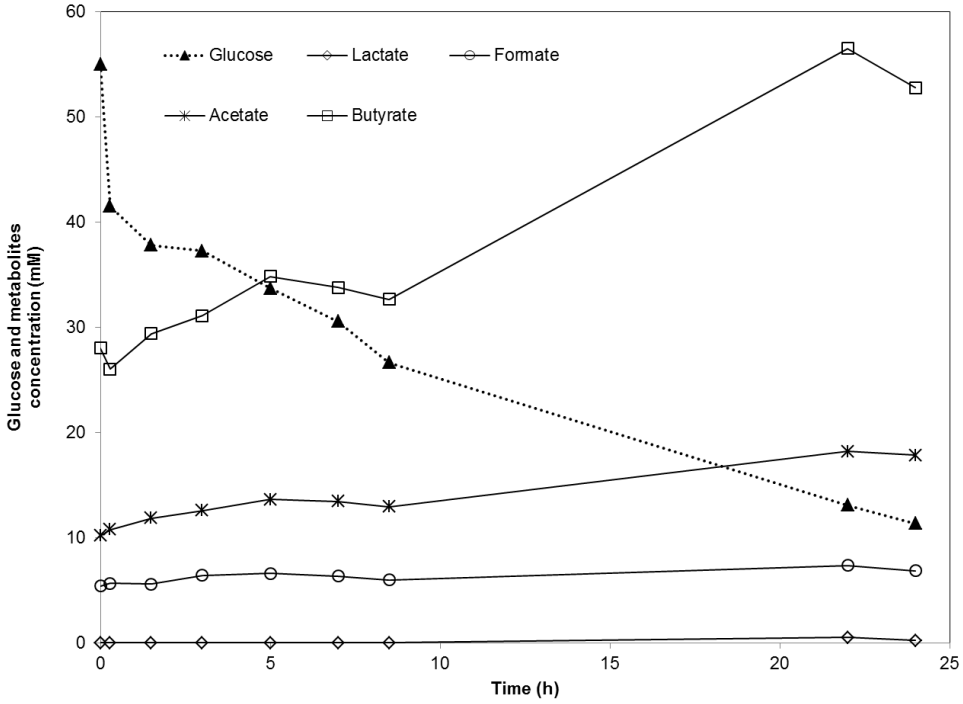


Figure 9. Glucose and soluble metabolites concentration variation during daily sequence

On day 62, samples were collected regularly from the reactor for analysis. The results for consumed glucose and produced metabolites are shown in Fig. 9. During this sequence, the hydrogen composition was also analyzed. Due to the large head space volume, the measured hydrogen composition did not show any significant variation and averaged to 73±6 %. A 90% glucose conversion was achieved and no lactate was found in the liquid phase.

3.5. The effect of biofilm on hydrogen production

Hydrogen production from TBSBR is due to the activity of biofilm grown on the packing material and the biomass present in the bulk liquid phase. To assess the sole biofilm activity, three consecutive sequences were operated with 100% removal/addition of fresh culture medium *i.e.* at day 59, 60 and 61. Cumulative biogas volume and biogas production rates are shown in Fig. 10. Biogas production rates for these consecutive sequences were 7.9, 8.5, 6.4 (L/d)

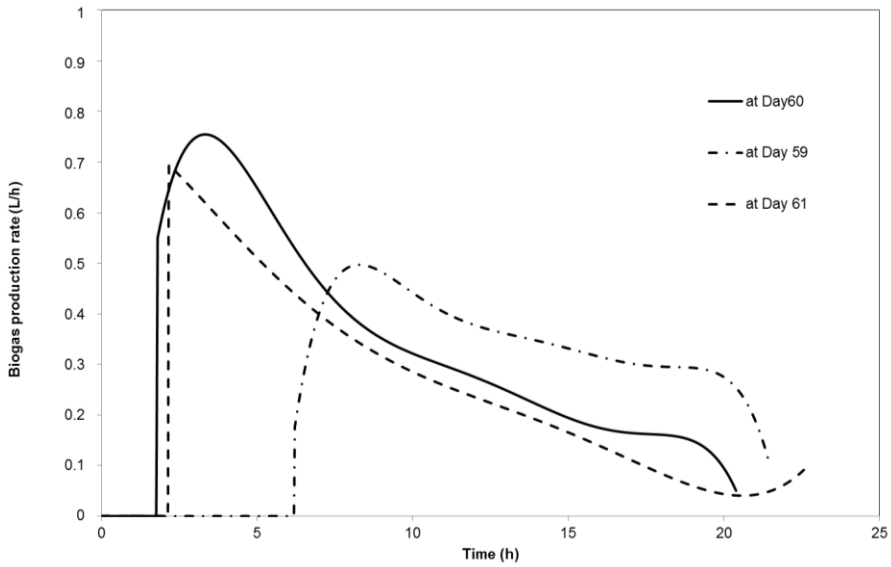
respectively. Both the maximum biogas production rate i.e. 17.75 (L/d) and the average biogas production rate of 8.5 L/d were found at day 60. They were similar at day 61.

3.6. The culture activity in TBSBR after 62 days.

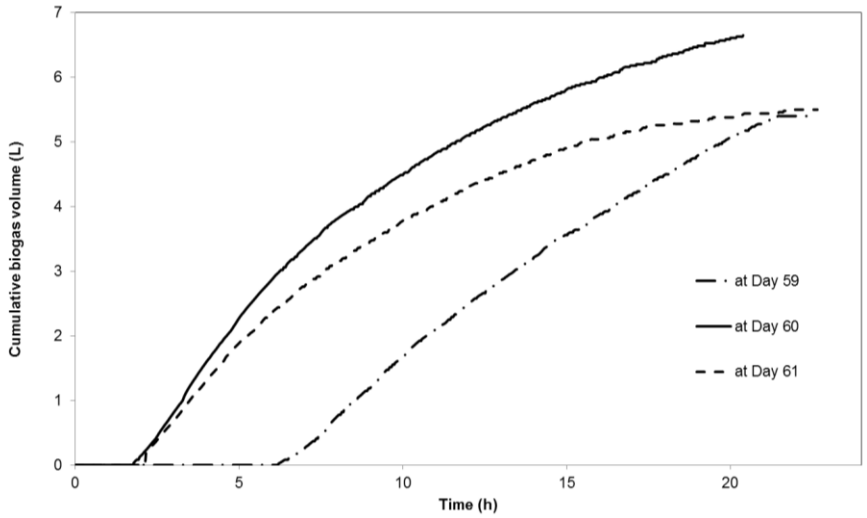
At day 62, a mixed liquid sample was collected into a 15 mL vial under sterile conditions. Cell count on the Bürker counting chamber indicated a total microbial concentration of 4.8 $x10^8$ cells/mL. For the same culture sample and a 10-fold dilution, a purity check was conducted using the PCA method. Growth of contaminants lead to a cell count of 5.5 $x10^3$ cells/mL of mixed liquid sample indicating a relatively low and homogenous level of contamination. A colony of the isolated contaminants was identified as belonging to the genus *Bacillus* by 16S rRNA gene analysis.

A 5 mL volume of this sample was used as inoculum in the standard BHP test carried out in 250 mL bottles at 30°C with glucose as substrate. The results are compared (Table 2) with those of the control BHP test with the pure strain of *C.butyricum* CWBI 1009. They are consistent with those reported by Hiligsmann et al. [23].

Only a slight growth of the aerobic contaminants was measured in the BHP test performed with the sample from TBSBR: from an initial cell density of $1.34x10^2$ cells/mL to a final cell density of $6.4x10^2$cells/mL at the end of culture. In addition, BHP tests carried out with the contaminants did not generate biogas. By contrast, no contaminants were evidenced for the pure culture BHP test via the PCA purity check. The culture from the TBSBR showed better BHP results with a total volume of biogas production about 10% higher than with the pure culture. This improved activity could be due to a higher initial biomass because bacterial flocks were evidenced in this inoculum.

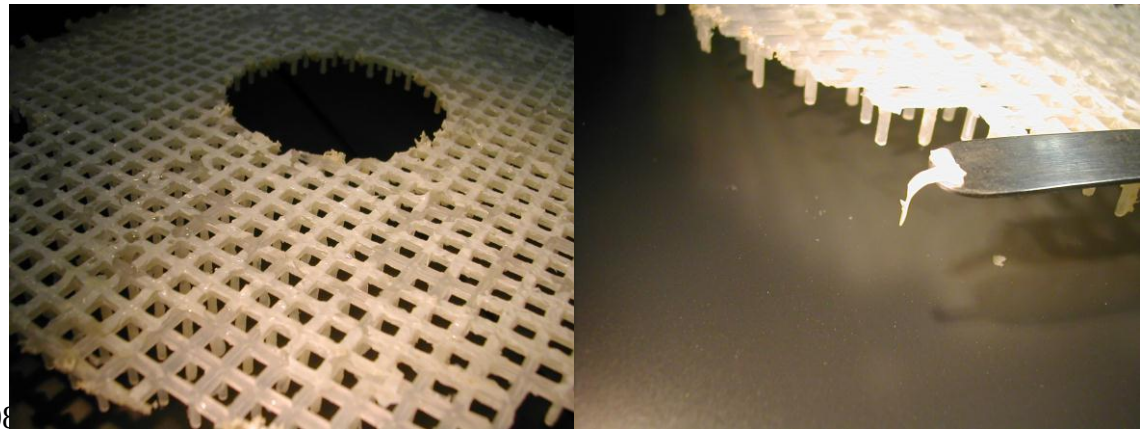


404



405

406 **Figure 10**. Biogas production rate (A) and cumulative biogas production (B) for the sequences
407 with 100% removal /addition of mixed liquid medium



408

409 **Figure 11**. *Clostridium butyricum* biofilm (A) growing on Lantec packing material and enlarged
410 view of biofilm (B).

411 **Table 2**. Biogas and CO_2 production and soluble metabolites analysis in standard BHP test with
412 5 mL inoculum collected from TBSBR or the pure *Clostridium butyricum* strain (control)

Sample name	Biogas vol. (mL)	CO_2 vol. (mL)	Lactate (mM)	Formiate (mM)	Acetate (mM)	Ethanol (mM)	Butyrate (mM)
Pure *Cl. but.* CWBI 1009	165	18	2.2	23.4	8.2	0.0	14.9
Clostridium from TBSBR (this study)	183	29	0.0	22.0	6.0	1.3	21.3

413

414 Regarding the soluble metabolite concentrations at the end of BHP tests Table 2 shows that the
415 VFA profiles are similar except the low ethanol and no lactate production for the TBSBR
416 sample. This would be related to the larger inoculum preventing some metabolites production
417 associated to cell growth. After the experiments, the packing bed was removed from the
418 fermenter and three dismantled plates from top, middle and bottom were further investigated.
419 The thin biofilm evidenced on the plates (Fig. 11) was thoroughly washed with distilled water,
420 centrifuged and dried at 105°C to determine the dry weight of the biomass. It was found that
421 biomass increases from top to middle and finally bottom plate from 0.1098 g to 0.4193 g and
422 1.0676 g respectively. This biofilm was mainly composed of bacteria belonging to the class
423 *Clostridia* (92.71 %), as indicated by the metagenomic analysis of a biofilm sample.

424

425 ## 4. Discussion

426 In this study, a TBSBR was operated for 62 days for mesophilic fermentative biohydrogen
427 production. The reactor system of 20 L total volume was steam sterilized, inoculated with a pure
428 culture of *C. butyricum* CWBI1009 and operated for about two months with daily
429 removal/addition of fresh culture medium. During the first 10 days of operation no gas
430 production could be recorded on the Rigamo software (V1.30-K1) due to a gas leak from the
431 liquid trap bottles located before the gas meter. However, according to the soluble metabolites

analyzed in the spent medium, the metabolism of *C.butyricum* was effective with a VFA pattern similar to those reported in stirred SBR [22]. After 12 days of operation, the bioreactor became contaminated and the hydrogen yield decreased. A thermal treatment technique was successfully tested in order to reduce the contamination of the culture. Globally the hydrogen production rate averaged at about 8 L/d up to day 20

Similar to our study, Goud et al. [14] conducted biohydrogen production experiments with upflow packed bed reactor (1.4 L) with SBR mode for 1400 days using synthetic and vegetable waste extract as substrates under diverse operating conditions. When the hydrogen production activity deteriorated, they conducted pretreatment for 24 h using 2-bromoethane sulphonic acid (BESA) and hydrogen production improved to 12.56 mmol H_2/d. In the TBSBR reactor, after a thermal treatment carried out for less than one hour, the hydrogen production increased to 583 mmol H_2/d within about 6 hours without fresh inoculum addition. Goud et al. [14] also reported the presence of *Bacilli* contaminants and *Clostridia* in the biofilm confirming the ability of hydrogen production under contamination conditions. Non sterile thermophilic biohydrogen production was also carried out successfully by van Groenestijn et.al [20] in a 400 L trickle bed bioreactor. When compared to our maximum H_2 yield of 1.67 mol H_2/mol glucose converted, their higher hydrogen yield of 2.8 mol H_2/mol hexose should be related to the thermophilic environmental conditions (with advantageous lower hydrogen solubility) that also outcompeted non H_2-producing contaminants.

A trickling bed bioreactor packed with perlite beads was used by Arriaga et al. [19] to produce biohydrogen from oat straw acid hydrolysate at 30°C. By varying HRT between 24h and 6h, the specific hydrogen production rate reached a maximum of 3.3 mmol/($L_{reactor}$.h). Biomass clogging inside the packing bed was the major problem reported in their reactor operation. By contrast, reactor clogging due to biomass built up on the packing material was not observed during our

experiments because Lantec packing has excellent hydrodynamic and self-sloughing characteristics. In addition, a maximum specific hydrogen production rate of 6.1 mmol/(L_{medium}.h) was achieved after thermal pretreatment. Moreover the about 0.7 L/h global biogas production rate reached in this TBSBR with only 4 L of MDT medium should be compared with the 0.4 L/h produced by the pure *C. butyricum* in the same bioreactor vessel containing 18L stirred MDT medium [33]. These biogas production rates should also be compared, when rescaled to one liter of culture medium (*i.e.* 0.175 and 0.02 L/L_{medium}.h respectively), to the 1.1 L/L_{medium}.h achieved with the same pure bacteria strain in the biodisc-like reactor [24]. The 5-fold higher H_2 production rate (31.4 mmol H_2/L_{medium}.h) and 40 % higher H_2 yield (2.4 mol/mol) achieved in this bioreactor would be related to the absence of contaminants (consuming glucose without hydrogen production), to a higher L/G transfer efficiency and to more stable environmental conditions (pH, ORP, substrate dispersion, etc.). Indeed, our results showed lower performances when the whole culture medium was removed at the beginning of each sequence at day 59 to 61. This would highlight that the bacteria suspended in the liquid medium contributed at a non-negligible extent to the global biogas production.

5. Conclusions

A trickling bed sequence batch reactor was operated for mesophilic biohydrogen production using glucose as substrate and operated for 62 days. A new thermal treatment strategy was applied to reduce the contamination of *C. butyricum* CWBI1009 with other microbial species. While no inoculum was added to the reactor for activating the culture after thermal treatment, hydrogen production restarted within 6 hours. The Lantec packing material was used here for the first time for biohydrogen production. This new packing material showed excellent properties in

terms of biofilm development, hydrodynamics and liquid to gas mass transfer. No clogging of biomass was observed. The biofilm activity for hydrogen production was assessed by 100% removal/addition of the mixed liquor medium and lead to similar performances as when operating with 40% removal/addition conditions. Regarding the biofilm characteristics, the amount of biomass attached to the packing material increases from the top to bottom of the fixed bed. Biomolecular analysis confirmed the high level of biofilm colonization by *Clostridium* strain and the presence of the major *Bacillus* contaminant. This result is consistent with other published data [14]. The maximum hydrogen composition recorded was 83% (v/v) and no methane was found in the head-space. The maximum specific hydrogen production rate and the hydrogen yield measured from this study were 146 mmol H_2/L.d and 1.67 mol H_2/mol glucose respectively. This study proved the ability of producing hydrogen by *C. butyricum* CWBI1009 even under the presence of contaminants. Thus, the TBSBR can be considered as a promising technology for recovering energy from industrial wastewaters.

6. Acknowledgements

This work received financial support and a postdoctoral fellowship for Dr. P.G.Rathnasiri from the ARC project named "MICROH$_2$" (Concerted Research Action ARC-07/12-04, www.microH2.ulg.ac.be) and from the Walloon Region.

L. Beckers and A. Grigorescu are recipient of a FRS-FNRS fellowship (Fonds de la Recherche Scientifique)

7. References

[1] Show KY, Lee DJ, Chang JS. Bioreactor and process design for biohydrogen production. Bioresour Technol 2011;102:8524-8533.

[2] Lee DJ, Show KY, Sud A. Dark fermentation on biohydrogen production: Pure culture. Bioresour Technol 2011;102:8393-8402.

[3] Bhaskar YV, Venkata Mohan S, Sarma PN. Effect of substrate loading rate of chemical wastewater on fermentative biohydrogen production in biofilm configured sequencing batch reactor. Bioresour Technol 2008;99:6941-6948.

[4] Venkata Mohan S, Mohanakrishna G, Ramanaiah SV, Sarma PN. Simultaneous biohydrogen production and wastewater treatment in biofilm configured anaerobic periodic discontinuous batch reactor using distillery wastewater. Int J Hydrogen Energy 2008;33:550-558.

[5] Mitchell RJ, Kim JS, Jeon BS, Sang BI, Continuous hydrogen and butyric acid fermentation by immobilized *Clostridium tyrobutyricum* ATCC 25755: Effects of the glucose concentration and hydraulic retention time. Bioresour Technol 2009;100:5352-5355.

[6] Jo JH, Lee DS, Park D, Park JM. Biological hydrogen production by immobilized cells of *Clostridium tyrobutyricum* JM1 isolated from a food waste treatment process. Bioresour Technol 2008;99:6666-6672.

[7] Kim JO, Kima YH, Ryua JY, Song BK, Kim IH, Yeom SH. Immobilization methods for continuous hydrogen gas production biofilm formation versus granulation. Process Biochemistry 2005;40:1331-1337.

[8] Chang JS, Lee KS, Lin PJ. Biohydrogen production with fixed-bed bioreactors. Int J Hydrogen Energy 2002;27:1167-1174.

[9] Keskin T, Aksoyek E, Azbar N. Comparative analysis of thermophilic immobilized biohydrogen production using packed materials of ceramic ring and pumice stone. Int J Hydrogen Energy 2011;36:15160-15167.

[10] Leite JAC, Fernandes BS, Pozzi E, Barboza M, Zaiat M. Application of an anaerobic packed-bed bioreactor for the production of hydrogen and organic acids. Int J Hydrogen Energy 2008;33:579-586.

[11] Lo YC, Lee KS , Lin PJ, Chang JS. Bioreactors configured with distributors and carriers enhance the performance of continuous dark hydrogen fermentation. Bioresour Technol 2009;100:4381-4387.

[12] Chu CY, Wu SY, Hsieh PC, Lin CY. Biohydrogen production from immobilized cells and suspended sludge systems with condensed molasses fermentation solubles. Int J Hydrogen Energy 2011;36:14078-14085.

[13] Zhang ZP, Show KY, Tay JH, Liang DT, Lee DJ. Biohydrogen production with anaerobic fluidized bed reactors-A comparison of biofilm-based and granule-based systems. Int J Hydrogen Energy 2008;33:1559-1564.

[14] Goud RK, Veer Raghavulu S, Mohanakrishna G, Naresh K, Venkata Mohan S. Predominance of Bacilli and Clostridia in microbial community of biohydrogen producing biofilm sustained under diverse acidogenic operating conditions. Int J Hydrogen Energy 2012;37:4068-4076.

[15] Oh YK, Kim SH, Kim MS, Park S, Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng 2004;88(6):690-698.

[16] Ahn YH, Park EJ, Oh YK, Park S, Webster G, Weightman AJ. Biofilm microbial community of a thermophilic trickling biofilter used for continuous biohydrogen production. FEMS Microbiology Letters 2005;249:31-38.

[17] Zhang H, Bruns MA,. Logan BE. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. Water Res 2006;40:728-734.

[18] Peintner C, Zeidan AA, Schnitzhofer W. Bioreactor systems for thermophilic fermentative hydrogen production: evaluation and comparison of appropriate systems. J Clean Prod 2010;18:S15-S22.

[19] Arriaga S, Rosas I, Alatriste-Mondrago´n F, Razo-Flores E. Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter. Int J Hydrogen Energy 2011;36:3442-3449.

[20] van Groenestijn JW, Geelhoed JS, Goorissen HP, Meesters KP, Stams AJ, ClaassenPA. Performance and population analysis of a non-sterile trickle bed reactor inoculated with *Caldicellulosiruptor saccharolyticus*, a thermophilic hydrogen producer. Biotechnol Bioeng 2009;102(5):1361-1367.

[21] Beckers L, Hiligsmann S, Hamilton C, Masset J, Thonart P. Fermentative hydrogen production by *Clostridium butyricum* CWBI1009 and *Citrobacterfreundii* CWBI952 in pure and mixed cultures. Biotechnol Agron Soc Environ 2010;14(S2):541-548.

[22] Masset J, Hiligsmann S, Hamilton C, Beckers L, Franck F, Thonart P. Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently

isolated strain of hydrogen-producing *Clostridium butyricum*CWBI1009. Int J Hydrogen Energy 2010;35(8):3371-3378.

[23] Hiligsmann S, Masset J, Hamilton C, Beckers L, Thonart P. Comparative study of biological hydrogen production by pure strains and consortia of facultative and strict anaerobic bacteria. Bioresour Technol 2011;102:3810-3818.

[24] Hiligsmann, S, Beckers, L, Masset, J, Hamilton, C, Thonart, P. Improvement of fermentative biohydrogen production by *Clostridium butyricum* CWBI1009 in sequenced-batch, horizontal fixed bed and biodisc-like anaerobic reactors with biomass retention. Int J Hydrogen Energy 2014;39(13):6899-6911.

[25] Masset J. Study of hydrogen production by anaerobic bacteria chemosynthetic. PhD thesis, University of Liege; 2013.

[26] Wu M. Trickling biofilters for hydrogen sulfide odor control. Agoura Hills, CA, USA: Lantec Products Inc; 1999. (downloaded on http://www.lantecp.com)

[27] Hamilton C, Hiligsmann S, Beckers L , MassetJ, Wilmotte A, Thonart P. Optimization of culture conditions for biological hydrogen production by *Citrobacter freundii* CWBI952 in batch, sequenced-batch and semicontinuous operating mode. Int J Hydrogen Energy 2010;35:1089-1098.

[28] Ventura M, Elli M, Reniero R. & Zink R. Molecular microbial analysis of *Bifidobacterium* isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). FEMS Microbiol Ecol 2001;36:113-121.

[29] Grigorescu AS, Hozalski RM, and La Para TM. Haloacetic acid-degrading bacterial communities in drinking water systems as determined by cultivation and by terminal restriction fragment length polymorphism of PCR-amplified haloaciddehalogenase gene fragments. J Appl Microbiol 2012;112(4):809-822.

[30] Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W. &Lipman D. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 1997;25:3389-3402.

[31] Wang Q, Garrity GM, Tiedje JM and Cole JR. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol 2007;73(16):5261-5267.

626 [32] Hawkes FR, Dinsdale R, Hawkes DL and Hussy I. Sustainable fermentative hydrogen
627 production challenges for process optimization. Int J Hydrogen Energy 2002:27(11-
628 12):1339-1347.

629 [33] Masset, J, Calusinska, M, Hamilton, C, Hiligsmann, S, Joris, B, Wilmotte, A, Thonart, P.
630 Fermentative hydrogen production from glucose and starch using pure strains and artificial
631 co-cultures of *Clostridium* spp. Biotechnology for Biofuels 2012;5(35):1-15

632