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Julian CARILLO-REYES [a,1], *Eric TRABLY* [b,*], *Nicolas BERNET* [b], *Eric LATRILLE* [b], *Elias RAZO-FLORES* [a]

[a] *División de Ciencias Ambientales, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José 2055, Lomas 4a Sección, C.P. 78216, San Luis Potosí, SLP, Mexico*

[b] *INRA, UR0050, Laboratoire de Biotechnologie de l'Environnement, Avenue des Etangs, Narbonne, F-11100, France*

[1] *Present Address: Laboratory for Research on Advanced Processes for Water Treatment, Unidad Académica Juriquilla, Instituto de Ingeniería, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, Querétaro 76230, México*

**corresponding author:* phone: +33 (0)468 425 151 ; fax: +33(0)468 425 160

E-mail addresses: jcarrillor@iingen.unam.mx (J. Carrillo-Reyes), eric.trably@supagro.inra.fr (E. Trably), nicolas.bernet@supagro.inra.fr (N. Bernet), eric.latrille@supagro.inra.fr (E. Latrille), erazo@ipicyt.edu.mx (E. Razo-Flores)

Abstract

Fixed-biomass reactors present the main advantage of increasing the hydrogen production rates by supporting high organic loading rates. However, the use of complex natural inoculum often precludes a long-term operation through the rapid emergence of methanogens. In this study, an Anaerobic Sequencing Batch Biofilm Reactor (ASBBR) was inoculated with a simplified H_2-producing culture to evaluate its influence on robustness and more particularly on methanogenic activity. An ASBBR was operated over 6 months with unsterile lactose-rich effluent and under Hydraulic Retention Time ranging from 1 to 34 hours. Hydrogen performances in terms of productivity and yields ranged from 0.23 to 519 $mmole_{H2}\,L^{-1}\,d^{-1}$ and 0.01 to 7.11 $mole_{H2}\,mole_{lactose}^{-1}$, respectively. No significant methane production was observed all along the experimental procedure, showing that inoculating with a simplified and highly enriched preculture could increase substantially the robustness of the process. Specific preparation of the inoculum may represent a solution to sustain long-term operation of biofilm-based reactors.

Keywords

Anaerobic sequencing batch biofilm reactor; *Clostridium sp.*; Dark fermentation; pre-enriched consortium

1. Introduction

Hydrogen (H_2) has not only the highest energy content among any fuel but its combustion produces only water and H_2 can be directly used in fuel cells to produce electricity. H_2 can be produced in anaerobic biological process, so-called dark fermentation, from many different types of waste that represent one of the potential alternatives to fossil fuels for energy generation. The possible use of agriculture wastes or wastewaters as substrates makes the dark fermentation bioprocesses neutral in terms of carbon dioxide accumulation and, thus, greenhouse gas effect [1,2].

As model substrate, cheese whey is a by-product of cheese manufacturing that has an important organic matter content due to its high lactose concentration. Recently, cheese whey has been successfully used for bio-H_2 production [3]. The large cheese whey production over the world (around 160 million tons per year) [4], makes this by-product a suitable substrate for bio-H_2 production that could be disseminated in small and medium factories that cannot afford other valorization process [5].

Technologically, dark fermentative biohydrogen production has been reported in several types of reactors, and mostly in continuous stirred tank reactors (CSTR). Although CSTR bioprocesses can improve the gas release through mass transfer optimization, the microbial biomass concentration is limited by the intrinsic biomass yield associated to a maximal organic loading rate (OLR). The biomass can even be washed out at hydraulic retention times (HRT) lower than few hours [6]. Unlike CSTRs, fixed biomass reactors present the advantage of supporting higher organic loading rates (OLR) due to increase in biomass concentration by biofilm formation and, as a consequence, hydrogen is still produced at higher rates [7]. In this sense, Keskin et al. [8] reported a higher robustness of an immobilized reactor compared to a CSTR, in terms of resistance to higher OLR at lower HRT (> 6 h), achieving 5 fold more volumetric hydrogen production.

However, fixed biomass reactors frequently present undesirable methanogenic activity due to the settlement of archaeal methanogens issued from the inoculum or from the medium [9]. Their persistence during reactor operation is favored by long solids retention time and the attachment of methanogens to the biofilm or granules. Meanwhile, methanogens are theoretically washed-out in CSTR because of a low maximum growth rate of approximately 17h and 40h, for 31hydrogenotrophs and acetoclasts, respectively [10]. The aforementioned problem is mainly caused by the use of a complex inoculum, such as anaerobic methanogenic sludge. To eliminate the methanogenic *Archaea*, heat-shock pretreatment of the inoculum is generally applied to select spore-forming hydrogen-producing bacteria, mainly from the genus *Clostridium* sp. [1].

However, methane production has been largely reported, even after heat shock pretreatment of the inoculum, suggesting that some methanogens might resist to heat treatment, likely because of local conditions that are slightly more favorable [11]. In those systems inoculated with complex communities such as anaerobic sludge, once methanogenic activity started it cannot be controlled even after the application of several strategies such as organic shock loads and acidic pH [12,13], showing low resistance to methanogenic contamination.

To increase hydrogen production and avoid methane production, several authors proposed to use inocula with no methanogenic community, such as compost from kitchen wastes [14], pure cultures [15] or co-cultures of different hydrogen-producing microorganisms [16]. According to Zeidan et al. [17], using defined co-cultures or simplified consortium of known H_2 producers may offer better performances than mixed-population enrichments. The robustness of a biofilm based hydrogen producing system in the long term has been proved in the relevant work by Kannaiah Goud et al. [18], where the microbial community enriched from an anaerobic sludge remain even after different operational conditions and substrates, glucose and vegetable extract, were applied. However, in the latter cited work eventual pretreatments were used such as the chemical methanogenic inhibitor BESA (2-bromoethane sulphonic acid) and acidic conditions, in order to recover the H_2 production. Considering the complex community tested in the majority of the biohydrogen producing works, it can be hypothesized that the community structure of a simplified consortium can remain stable without treatments, resisting unfavorable conditions.

In this sense, a simplified consortium operated under sterile conditions, constituted from hydrogen producers as dominant bacteria, showed a high hydrogen productivity of 217.7 $mmole_{H2}\ L^{-1}\ d^{-1}$, reaching a yield of 1.92 $mole_{H2}\ mole_{glucose}^{-1}$ at 40 g glucose $L^{-1}\ d^{-1}$ [19]. However, as soon as the considered effluent is unsterile, these systems could be exposed to a contamination by methanogens or other hydrogen-consuming microorganisms, promoting complex and uncontrolled population dynamics in the reactor according to the operational parameters such as HRT, OLR or pH and solids retention time [20].

In the case of fixed biomass reactors, the differential ability of microorganisms to form a biofilm on a carrier can be used to select proper microorganisms according to the type of support [7,21].

Previous studies showed that hydrogen-producing microorganisms adhere preferentially in glass supports [15] than methanogens in comparison with plastic supports [22].

In this work, the robustness of a simplified microbial consortium highly enriched in hydrogen-producing bacteria was investigated in an anaerobic sequencing batch biofilm reactor (ASBBR), evaluating the stability of the community structure. In this system, a long-term hydrogen production from unsterile reconstituted lactose effluents was evaluated under a wide range of HRTs (1 to 34 h) and OLRs (10 to 120 $g_{COD}\ L^{-1}\ d^{-1}$).

2. Materials and methods

2.1 Origin of the inoculum

The inoculum was obtained after anaerobic sludge pretreatment and reaching stable conditions of hydrogen production in a CSTR fed with glucose (10 g/L), as previously described [19]. In brief, a heat treated anaerobic sludge was inoculated in the CSTR and operated at 6 h of HRT, pH 5.5, 37°C, agitation at 300 rpm and under sterile conditions during more than 4 months. The inoculum developed corresponded to a simplified microbial consortium highly enriched in hydrogen-producing bacteria, composed by *Clostridium pasteurianum* and *Clostridium beijerinckii* as dominant bacteria, and several types of low dominant species such as *Escherichia coli, Enterobacter cloacae* or *Lactobacillus* sp, characterized by CE-SSCP and clone libraries [19].

The inoculum was initially added to the ASBBR reactor to reach a concentration of 4.5 g VS/L.

2.2 Reactor configuration

The reactor configuration and mode of operation corresponded to an ASBBR with a working volume of 0.5 L. Initially, continuous biogas recirculation ensured the mixing during the first two phases, with no carrier. After carrier addition, and to avoid its lost during effluent pumping, the biogas recirculation worked in cycles as follows: each 30 minutes the gas recirculation was stopped letting the support to float for 2 minutes, then the sensor level that controlled the effluent pump was activated adjusting the reactor volume to 0.5 L. The influent pump and the pH control worked continuously. The carrier used was floating glass spheres with an average diameter of 172 μm [23].

2.3 Experimental set-up

The reactor temperature was controlled in a water bath chiller at 37 °C. The pH was monitored and controlled at 5.5 automatically by adding NaOH (5 N), consistently with the simplified inoculum development [19]. After inoculation, the reactor was flushed with N_2 for 5 minutes to reach anaerobic conditions.

The experimental conditions of the ASBBR operation is shown in Table 1, the strategy was to adapt first the inoculum to lactose during 11 days at an initial HRT of 8 hours and under sterile conditions (phase A). Thereafter, the reactor was fed with unsterile substrate medium (phase B). At day 17, end of phase B, the carrier was added in a relation of 10% (v v^{-1}) with respect to the working volume. To accelerate the selection of bacteria able to form a biofilm on the carrier, a low HRT of 3 h was used in phase C. Such very short HRT washed out planktonic bacteria and therefore favored the biofilm development from adhered bacteria [24]. A lower HRT of 1 h was then evaluated following the same purpose (phase D). Finally, the effect of longer hydraulic retention times ranging from 6 to 34 h were evaluated in order to elucidate the biofilm resistance to slow growing microorganisms that could impact the overall reactor performances (phases E to H, Table 1).

2.4 Mineral medium and substrate

The mineral content of the feed solution was modified from [19] with the following composition (mg L^{-1}): K_2HPO_4, 500; NH_4Cl, 2000; yeast extract, 200; $MgCl_2{\cdot}6H_2O$, 100; $(FeSO_4)_2$ $(NH_4)_2{\cdot}6H_2O$; 17.6; $ZnCl_2$, 1; $MnCl_2{\cdot}4H_2O$, 17.56; $CuSO_4{\cdot}5H_2O$, 5; $CoSO_4{\cdot}H_2O$, 2.16; BO_3H_3, 0.1; $Mo_7O_{24}(NH_4)_6{\cdot}4H_2O$, 9.12; $Cl_2{\cdot}6H_2O$, 0.08; Na_2SeO_4, 0.01. The lactose concentration is expressed as COD in Table 1.

Table 1. Operational conditions evaluated and duration of each phase.

Phase	Days	Duration (d)	HRT[1]	Substrate concentration [2]	OLR[3]
A	1-11	11	8	6.6	20.0
B	12-17	6	8	5.0	15.0
C	18-28	11	3	5.0	40.0
D	29-58	30	1	5.0	120.0
E	59-76	18	6	5.0	20.0
F	77-141	65	6	5.0	20.0
G	142-153	12	12	5.0	10.0
H	154-201	48	34	17.9	12.6

[1] Hydraulic retention time, h; [2] g_{COD} L^{-1}; [3]Organic loading rate, g_{COD} L^{-1} d^{-1}

2.5 Analytical methods

Every three hours, the biogas composition (H_2, CO_2 and CH_4 content) was analyzed using an automatic R-300 micro-gas chromatograph (μGC-TCD, SRA instrument, Marcy l'Etoile, France), details are described elsewhere [25]. The biogas production rate was measured with a homemade volumetric gas counter, based in water displacement and calibrated to emit an electrical pulse every 2 mL. The pulse was recorded by a computer with a homemade data acquisition software. The gas counter was calibrated and each electrical pulse represented to 2 mL of gas. and registered in real time by the data acquisition system. The volumetric hydrogen production (VHP) rate was assessed under standard conditions (0°C, 1 atm). The volatile fatty acids (VFA) was determined periodically by gas chromatography (GC-FID, GC 8000, Fisons Instruments), as reported elsewhere [19]. Other fermentation end-products, ie. lactate, ethanol, as well as residual lactose were measured using high performance liquid chromatography (HPLC) coupled to a refractometer (Waters R410). The chromatograph was equipped with an HPX 87 column (Biorad) and the eluent corresponded to a H_2SO_4 solution (0.222 ml L^{-1}) operated under isocratic elution at 0.4 mL min^{-1}. The operating conditions were: temperature of column, 35 °C; temperature of refractometer, 40 °C. COD and VS were analyzed according to the standard methods [26].

2.5 Microbial community characterization

Microbial community structure of the attached and suspended bacteria and *Archaea* was characterized after DNA extraction and PCR amplification by Capillary Electrophoresis-Single Strand Conformation Polymorphism (CE-SSCP), as described elsewhere [27,28]. Bacterial *16s rRNA* genes were amplified using Pfu Turbo DNA polymerase (Stratagene) and universal primers W49 (5'-ACGGTCCAGACTCCTACGGG-3') and 5'-fluorescein phosphoramidite-labeled W104 (5'-TTACCGCGGCTGCTGGCAC-3'), targeting the V3 region. Meanwhile, the archaeal *16s rRNA* were amplified using universal primers W274 (5'-CCCTACGGGGCGCAGCAG-3') and 5'-fluorescein phosphoramidite-labeled W275 (5'- TTACCGCGGCGGCTG-3'). Details of the PCR programs and CE-SSCP analysis for bacterial and archaeal community characterization were already described elsewhere by Quéméneur et al., [28] and Abbassi-Guendouz et al. [27], respectively. The CE-SSCP profiles were aligned with the internal standard to take into account inter-sample electrophoretic variability. The CE-SSCP profiles were normalized and a

dendrogram showing the similarity between the bacterial communities was constructed using the Pearson method and clustered by the nearest neighbor algorithm [29].

A multivariate analysis by Principal Components was carried out in order to correlate the community fingerprints of the CE-SSCP profiles to metabolic pathways at every operational condition [30]. Besides the ordination led by the principal components analysis (PCA), the dominant species inferred as discriminating peaks of the CE-SSCP profiles were fit onto the PCA, looking for correlations of this abundant *16s* fragments to certain operational conditions or metabolic pathways. All statistical analyses were performed in R environment [31]. The CE-SSCP profiles and their similarity were processed using the "StatFingerprints" package [32], whereas the PCA were run using the "Vegan" package [33].

In order to elucidate the taxonomy of the microorganisms, a PCR-based 454-pyrosequencing was carried out on samples at different times of the experiment from phases D, E and H. The PCR for 454 pyrosequencing was targeting the V4-V5 regions, using the universal primers 515-532F and 909-928R, according to Wang & Qian [34]. The downstream sequences processing involved a chimeric analysis to validated the quality of the recovered sequences, a random subsampling (8288 recovered sequences of each sample) and search of their taxonomic affiliation. These sequence data have been submitted to the BioSample database of NCBI 18under accession numbers **SAMN03761425**, **SAMN03761428**, **SAMN03761430** and **SAMN03761431**.

3. Results and Discussion

3.1 Reactor performances

The ASBBR was successfully operated over 201 days from phase A to H. Six different HRT and seven OLR were evaluated according to Table 1. The purpose of phase A run at 8 h of HRT with no carrier was the adaptation of the initial simplified consortium to lactose under sterile conditions. The corresponding OLR was 20 $g_{COD}\ L^{-1}\ d^{-1}$ with an average molar hydrogen production rate (MHPR) of 203± 53 $mmole_{H2}\ L^{-1}\ d^{-1}$ (Figure 1). After 11 days of operation, unsterile substrate was fed with an OLR of 15 $g_{COD}\ L^{-1}\ d^{-1}$ (phase B). The slight change in OLR and the presence of unsterile substrate caused a diminution of the hydrogen yield (Figure 1). A similar example in the literature was reported by Xing et al, [35] who observed that hydrogen

production of a pure culture of *Ethanoligenens harbinense* decreased rapidly when the reactor was operated under unsterile conditions.

At the end of phase B, the support was added to the reactor and the HRT was diminished to 3 h (phase C). The average MHPR increased to 287.2 ± 47 $mmole_{H2}\ L^{-1}\ d^{-1}$, in accordance with the OLR increment to 40 $g_{COD}\ L^{-1}\ d^{-1}$ and a subsequent increase in hydrogen yield to 2.78 $mole_{H2}\ mole_{lactose}^{-1}$. The higher hydrogen production per mol of substrate consumed when compared to phase B (Figure 1) could be explained by the biomass adhesion to the support, as shown in Figure 2. Although biomass concentration increased in the reactor, the overall metabolic routes, ie. acetate-butyrate pathways, remained dominant in both phases (B and C). It has to be pointed out that the sequencing reactor operation regime, its discontinuous mixing and the precipitation of the detached biomass, implied a strong selection pressure on the biomass fostering flocs development. Coexistence of a biofilm and self-aggregated biomass has already been observed in a biofilm-based hydrogen producing system [7], where the biofilm detachment from the support favored granules development. Unlike the previous cited work where the support was eventually washed-out, in the present study flocs and biofilms were both present all along the reactor operation.

During phase C, a stable hydrogen production was observed, achieving an average yield of 2.78 ± 0.4 $mole_{H2}\ mole_{lactose}^{-1}$ or 7.1 $mmole_{H2}\ g_{COD}^{-1}$, comparable to other systems fed with similar substrate. Consistently, Davila-Vazquez et al. [3] reported a very similar yield of 2.84 $mole_{H2}\ mole_{lactose}^{-1}$ or 7.3 $mmole_{H2}\ g_{COD}^{-1}$ and Azbar et al. [36] observed about 3 $mmole_{H2}\ g_{COD}^{-1}$ under thermophilic conditions. In Azbar et al. [36], a similar OLR (47 $g_{COD}\ L^{-1}\ d^{-1}$) than phase C was applied, but hydrogen production rates ranged from 0.5 to 3.5 $L\ L^{-1}\ d^{-1}$, that were significantly lower than in this study where the average value reached 6.43 $L_{H2}\ L^{-1}\ d^{-1}$. During a long term evaluation, similar reactor configuration and OLR (24.44 - 44.25 $g_{COD}\ L^{-1}\ d^{-1}$), Kannaiah Goud et al. [18] obtained a considerable lower MHPR from 10.87 to 15.84 $mmole_{H2}\ L^{-1}\ d^{-1}$, comparing to our average value in condition C (287.2 $mmole_{H2}\ L^{-1}\ d^{-1}$). This fact can be explained by the higher HRT at 24 h, proving that HRT is determinant despite the microbial community attachment to the biofilm.

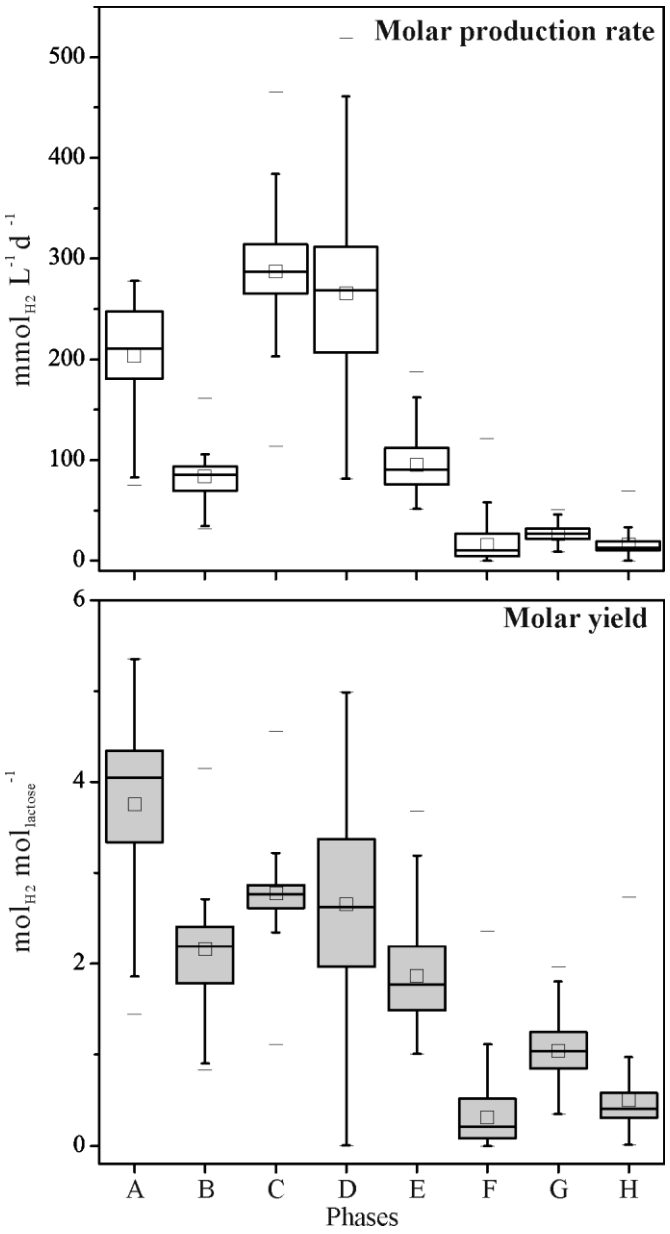

Figure 1. Box plots of the reactor performance in the different operational phases, A and B, C, D, E and F, G and H, correspond to 8, 6, 1, 3, 12 and 34 h of HRT, respectively. The box plots show the mean, median, maximum, minimum values and percentiles. The mean value is represented by a square inside the box plot.

The low HRT of 3 h applied in phase C and the associated yields indicated that such biofilm- based reactor constitutes a solution to avoid the potential wash out of suspended biomass at HRT lower than 6 h and subsequent process instability, as reported by Davila-Vazquez [3]. In phase D, a lower HRT of 1 hour was evaluated to further increase the selection pressure. In this case,

MHPR and hydrogen yield showed instability and higher variation with values ranging from 81 to 518 $mmole_{H2}\ L^{-1}\ d^{-1}$ and 0.1 to 4.99 $mole_{H2}$ mole $_{lactose}^{-1}$, respectively (Figure1). Such variability only observed at 1 hour of HRT was likely due to rapid and substantial changes in the bacterial community. As expected, the increase in OLR from phase C to D produced higher MHPR, reaching a maximal value of 518 $mmole_{H2}\ L^{-1}\ d^{-1}$ (Figure 1). This production rate is similar to reactor performances (874 $mmole_{H2}\ L^{-1}\ d^{-1}$) previously observed using cheese whey in a CSTR operated at 6 hour of HRT and a slightly higher OLR of 129.36 $g_{COD}\ L^{-1}\ d^{-1}$ (115.5 $g_{lactose}\ L^{-1}\ d^{-1}$) [3].

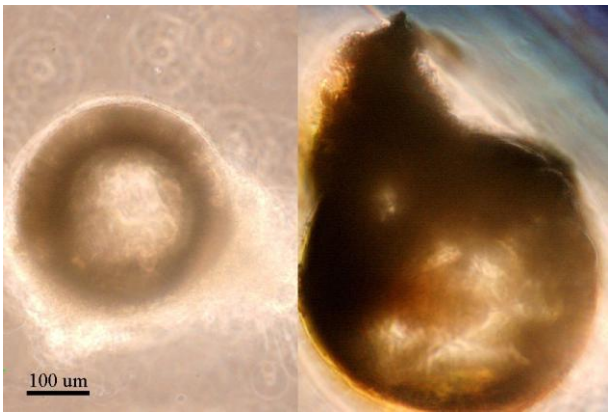

Figure 2. Microscopic observation of colonized carrier at day 17, end of phase B (HRT, 8 h), 40x magnifications.

After phase D, an HRT of 6 h was applied during 83 days (phase E and F, table 1) to explore the capability of microorganisms with lower growth rates than fermentative bacteria, eg. methanogens, to adhere into the reactor. After 18 days of reactor operation at phase E, low methane amount started to be produced (phase F). The OLR diminution at 20 $g_{COD}\ L^{-1}\ d^{-1}$ corresponded to an average MHPR of 95.4 $mmole_{H2}\ L^{-1}\ d^{-1}$ equivalent to 2.14 $L_{H2}\ L^{-1}\ d^{-1}$. Using a CSTR under thermophilic conditions, a similar volumetric hydrogen production of approximately 1.5 $L_{H2}\ L^{-1}\ d^{-1}$ was achieved [36]. High-rate reactors such as mesophilic UASB (up-flow anaerobic sludge blanket) reached average productions of around 1 $L_{H2}\ L^{-1}\ d^{-1}$ [14] and 1.12 ± 0.19 $L_{H2}\ L^{-1}\ d^{-1}$ [11].

In phase F, as mentioned above, methane started to be produced decreasing the MHPR to an average value of only 16.14 $mmole_{H2}\ L^{-1}\ d^{-1}$ (Figure 1). Methane occurrence in such fermentative systems is likely due to hydrogen consumption in a relation of 4 moles of hydrogen per mol of methane. Methane production was not inhibited by the acidic conditions in which some

hydrogenotrophic methanogens can survive [12], implying that the hydrogen production potential was reduced down to 2.92 $mmole_{H2}\,L^{-1}\,d^{-1}$ with an average methane production of 0.73 $mmole_{CH4}\,L^{-1}\,d^{-1}$. Despite the heat-shock treatment of the inoculum, the methanogenic activity was promoted by longer solid retention time as it was observed in a previous UASB reactor reported with kitchen compost as inoculum and fed with cheese whey [14]. During phase G, a HRT of 12 hours was evaluated keeping the same substrate concentration. Although the OLR was lowered if compared to phase E, there was a slight increase in the average MHPR reaching 28.7 $mmole_{H2}\,L^{-1}\,d^{-1}$, and a similar average methane production of 0.62 $mmole_{CH4}\,L^{-1}\,d^{-1}$. The increment in the hydrogen production is explained by the higher yield observed in phase G, up to 1.99 $mole_{H2}\,mole_{lactose}^{-1}$. The methanogenic activity was transitory and rapidly repressed in the next phase. The effect of a higher substrate concentration with a similar OLR was evaluated in phase H (Table 1). Methane production was completely stopped likely because of the organic shock load [12]. In other long solid retention systems reported in literature when inoculated with pre-treated anaerobic sludge, methane production could not be stopped once it started [9,13,37], unless a second biomass heat treatment is applied [37]. Therefore, it was assumed that the initial inoculation with a simplified pre-enriched inoculum provided a higher robustness of the system to methanogenic contamination and facilitated its recovering when environmental conditions are unfavorable to methanogens. This might be explained by a carrier effect and the capacity of the microbial consortium to avoid methanogen attachment and as well as the lower capacity of methanogenic archaea to adhere on glass compared to bacteria, as shown by Habouzit et al. [22]. This provides higher robustness of the process to environmental variation.

In the last step, a longer HRT of 34 hours was applied and the hydrogen production rate lowered down to 16.3 $mmole_{H2}\,L^{-1}\,d^{-1}$ (Figure 1), caused by a change in the metabolic pathway producing higher proportions of ethanol than previous phase, with no methanogenic activity. Here, the applied HRT was not suitable as already pointed out in others works using disaccharides as substrate, and reporting optimal HRTs between 8 and 13.7 h [6].

3.2 Community Analysis

The simplified pre-enriched hydrogen-producing culture used as inoculum was mainly composed by *Clostridium spp*. (around 70 % of the total abundance), with sub dominant species such as *Escherichia coli, Enterobacter cloacae* or *Lactobacillus* sp. representing all less than 19% of the total abundance [19]. During the 201 days of reactor operation, the biofilm bacterial community had changed to adapt to different conditions. For instance, in phases B, E and F (HRTs of 8 and 6h), a high similarity of the bacterial community composition was observed according to the clustering of their CE-SSCP profiles and the similarity index (Figure 3), even though lower HRTs were evaluated during 40 days (phase C and D, 3 and 1 h) between phase B and E. This fact corroborates that bacterial community distribution was reproducible along reactor operation depending on HRT, and this stability was probably prompted by the simplified inoculum composition.

The CE-SSCP profiles of phases E and F confirm that the MHPR drop observed in the latter phases, was only caused by the methanogenic activity and not by changes in the bacterial community, considering that samples of days 68, 103 and 138 were clustered together. The archaeal community was also analyzed by CE-SSCP at days 82 and 138 (phase F) identifying only two major peaks (profiles do not shown). Such low methanogen richness is consistent to previous results where only five methanogenic species were identified related to the *Methanobacteriaceae* family [12]. However, in the aforementioned work, an anaerobic sludge was used as seed sludge, which could increase the methanogenic richness despite inoculum pretreatment.

During the last five days of operation at 1 hour HRT (Day 53 to 58), the MHPR showed a more stable performance (241 ± 53 $mmole_{H2}\,L^{-1}\,d^{-1}$) compared to the entire phase D (Figure 1). The community at the end of phase D (day 58), was clustered beside samples of 8 and 6 h of HRT (Figure 2), where peaks at 200 of retention time (CE-SSCP profile) increased, whereas those at 600 of retention time disappeared, in comparison to previous samples of the same phase (days 31 and 50). This transition between CE-SSCP profiles of phase D suggests that at the end of the phase, the lower adhesion capability of some members of the bacterial community played an important role in the stability of hydrogen production. The relevance of members at 200 retention time of the CE-SSCP profiles is discussed below.

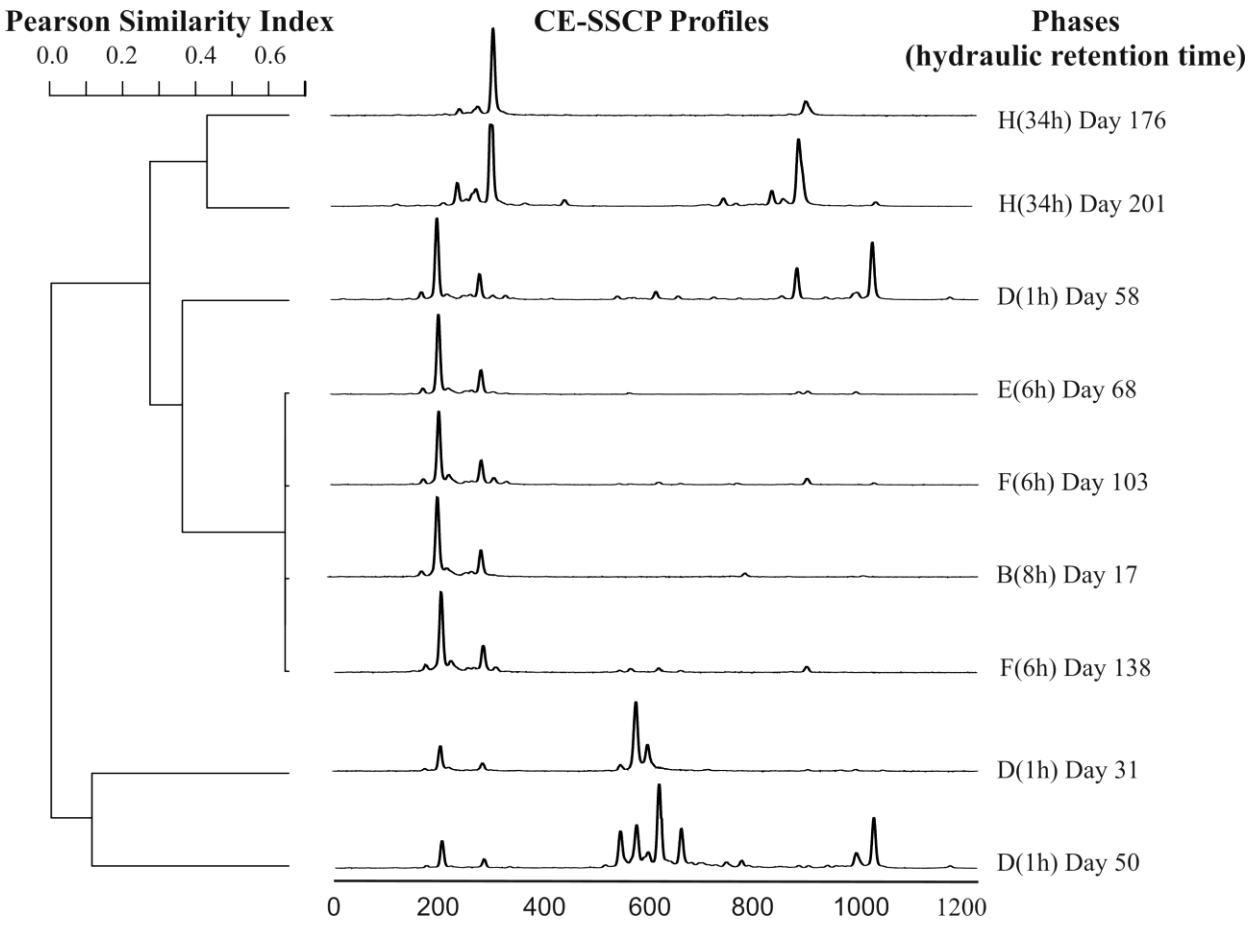

Figure 3. Dendrogram of the CE-SSCP bacterial community profiles at different phases and days between brackets, the hydraulic retention times and the similarity index are also pointed.

At 34 h of HRT a similar bacterial composition was observed (Figure 3). The consistence in the bacterial community and the higher abundance of peaks around 300 of retention time clarify the stable but lower hydrogen production, in comparison of previous conditions evaluated even the similar OLR. Overall, the number of abundant species was uniform along the 201 days of reactor operation, and no more than 10 dominant and sub-dominants peaks were identified per CE-SSCP sample profile.

The most common strategy for inoculating fermentative hydrogen-producing reactors is the anaerobic sludge after heat shock treatment. However, the number of species generally corresponds to more than 20 bacterial species, mainly composed of hydrogen producers, other microorganisms with competitive metabolic pathways such as propionate or lactate, and even hydrogen consumers [12]. Another inoculum enrichment strategy was reported in the literature

and corresponded to anaerobic sludge batch growth in biofilm-based reactor operated with BESA (2-bromoethanesulfonic acid) as methanogenic inhibitor. After 50 days of continuous operation, the authors concluded that a mesophilic biofilm was not suitable for H_2 production due to the attachment of hydrogen consumers that decrease the system performances [20].

From a community point of view, our system was stable assuming that the pre-enrichment inoculum strategy generated a lower species richness within the biofilm when compared to other inocula [12,18,20,38]. Moreover the mixing conditions and the mode of operation in ASBBR allowed operating the reactor at low HRT with no undesirable microorganism attachment and biomass washout. At higher HRT of 6 and 12 h at phases F and G respectively, a methanogenic activity was favored. By an organic loading shock the methanogenic activity was completely inhibited, even the higher HRT applied in phase H. Comparing our results with those obtained by other authors using an ASBBR [18] indicates that the longer HRT (16 and 24 h) they applied during all reactor operation, may be determinant to obtain lower MHPR and higher species richness than in our work.

3.3 Statistical Analysis and community taxonomy

The bacterial community composition (CE-SSCP profiles) correlated well with the metabolic patterns as shown with a multivariate statistical analysis of principal components (Figure 4). The discriminating peaks correspond to the community members that were determinant in the ordination of the data. Furthermore, the most abundant CE-SSCP peaks were related with the 454-pyrosequencing results in order to elucidate the taxonomy of dominant and sub-dominant bacterial species during the different phases of the reactor.

Otherwise, the peak at the position 569 correlated with samples having a high variable hydrogen production (1 h of HRT) associated to lactic acid accumulation. Therefore, it was concluded that this peak represent a putative lactic acid producer. It can be assumed that a competition between H_2-producing microorganisms and lactic acid bacteria during phase D caused the hydrogen variation.

From the pyrosequencing analysis of the microbial community sampled at day 31 (Figure 5), the two most abundant strains represented 28% and 20% of the 8288 sequences and were affiliated to the *Enterobacteriaceae* family (unknown species). Many members of this family are known to ferment lactose to lactate [39]. It has to be pointed out that other lactic acid bacteria were

detected such as *Lactoccocus* and *Lactobacillus* sp. in phase E and H, respectively. Their relative abundance of less than 6 % of the recovered sequences suggested that they could contribute to the low lactate concentration observed during the last steps of the experiment.

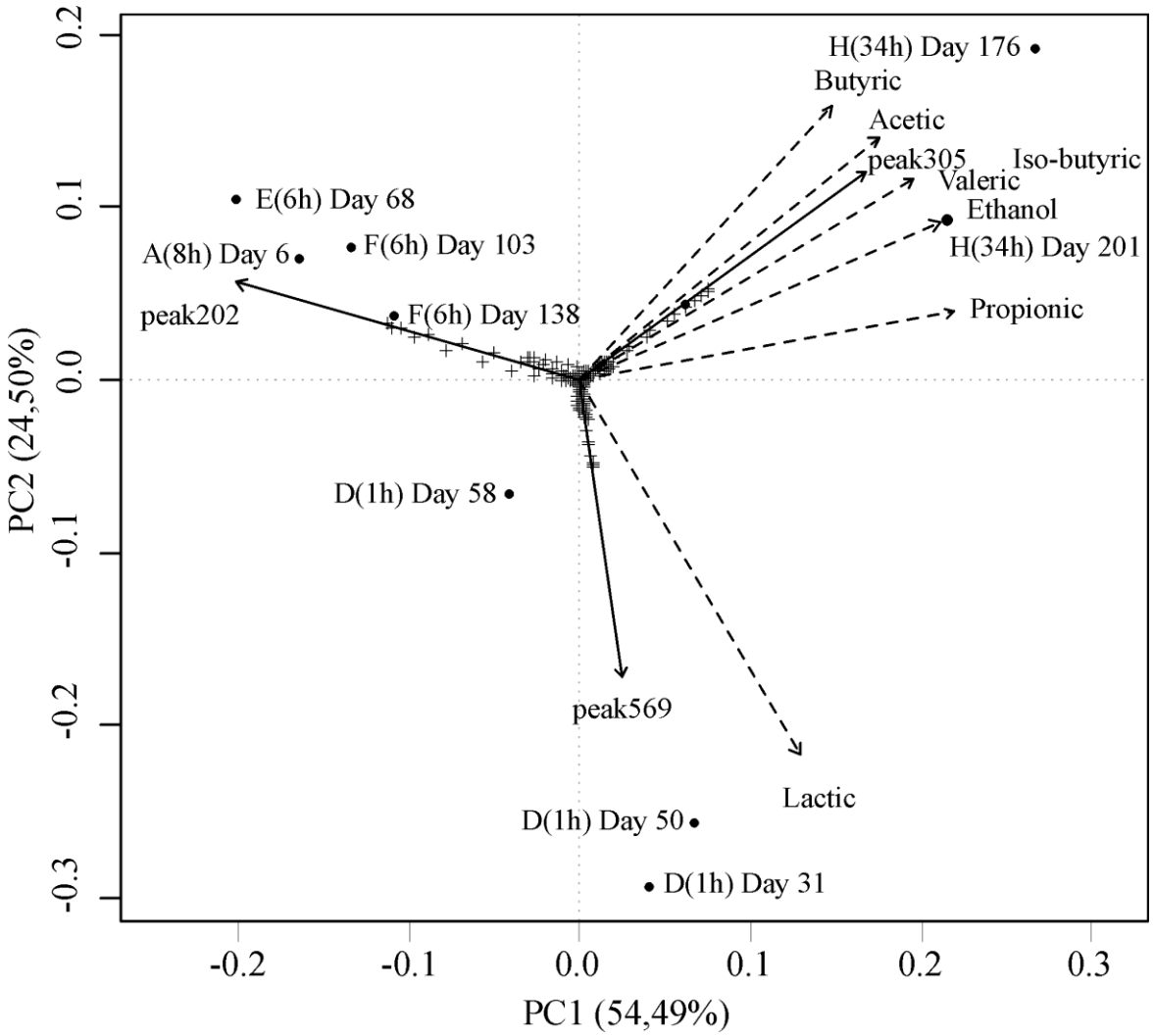

Figure 4. Principal Components Analysis of the correlations between the CE-SSCP profiles and the metabolites produced in the reactor. Cross symbols correspond to different species (peaks); black circles show the different operational conditions distribution. Continuous and dash arrows represent vectors from discriminating peaks and metabolites, respectively.

Furthermore, peaks around position 305 of retention time were located near to samples at 34 h of HRT in the statistical analysis. From pyrosequencing analyses of communities sampled at days 82 and 176 at which this peak was dominant, the results suggest that it belongs to a *Clostridium* sp. strain counting for 62% and 14% of the 7451 and 5336 sequences analyzed in the 176 and 82

samples respectively. Other members detected by the pyrosequencing in phase H were *Lactobacillus* and *Pseudomonas*, presumable those species were responsible of the low hydrogen production, representing a substrate competitors without hydrogen production and the production of bacteriocins for the former genus [40]. Although the exact role of *Pseudomonas* species has not been well elucidated, several members of *Pseudomonas* genus have already been previously observed in hydrogen producing systems at HRT higher than 4 h (8 and 12 h) [41].

Correlating the CE-SSCP profiles (Figure 3) to the pyrosequencing analysis, it can be assumed that *Clostridium* genus (peak position 200) was dominant in those conditions with a HRT of 8 and 6 hours, and at the end of phase C (1 h or HRT), which as it was mentioned could be related to its adhesion capability at such low HRT. In this regard, after day 50 in phase C *Clostridium* genus could overcome to those species related to *Enterobacteriaceae* family (peak position 569).

At the highest HRT evaluated, other species also related to *Clostridum* genus were dominant, however the MHPR and molar yield were the lowest achieved (Figure 1). Even the Clostridium genus was reported having a good hydrogen production potential, the HRT time is determinant, which could be confirmed comparing our results to a previous ASBBR operated at 24 h of HRT and *Clostridium* as its major genus [18], having both MHPR from 7.56 to 16.3 $mmole_{H2}\,L^{-1}\,d^{-1}$ at similar OLR.

Another outstanding result from the microbial community characterization by pyrosequencing (Figure 5), was that only 7 families represented the most abundant OTUs, corroborating the low diversity of the community. As discussed previously, the high abundance of *Enterobacteriaceae* beside *Clostridiaceae*, could explain the important variation in the hydrogen production in phase D (Figure 1). In phase E, the longer HRT than previous phases (6 h) could promote the development of other bacterial member such as *Streptococcaceae* family, which has been reported widely in hydrogen producing granules, in relation to their fixation capability more than their ability to produce hydrogen [40]. In the present study, during phase F, the hydrogen productivity and yield were lower and methane occurrence was responsible from the emergence of members of *Methanobacterium* genus (Figure 5).

These methanogens represented 2 % of the recovered sequences at day 82, selected by the acidic pH as reported elsewhere [12]. Regarding phase H, the lower MHPR and yield were obtained (Figure 1), despite the high abundance of *Clostridiaceae* members (Figure 5). In that case, the *Lactobacillaceae* bacteria (representing 6.5 %, Figure 5) could be determinant by diverting the

available electrons to reduced compounds such as lactate, that having an adverse effect on the overall hydrogen production [19,40].

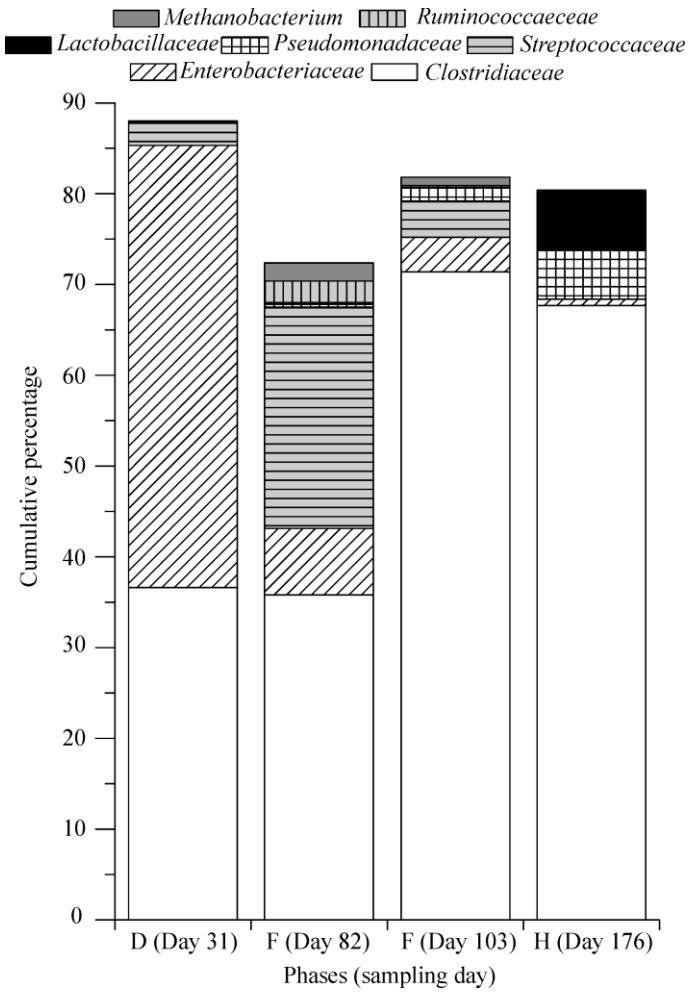

Figure 5. Microbial community distributions at family level of the pyrosequenced samples. Only most abundant OTUs (>2.0 %) are considered.

4. Conclusions

The inoculum based in a simplified microbial consortium, prompted the bacterial community stability and the robustness under methanogenic presence at HRT higher than 3 h, increasing the substrate concentration easily inhibited those methanogens. A compromise between stability and productivity was achieved at 3 h of HRT, favor by the biofilm and flocs formed. The low bacterial richness persisted along the reactor operation, despite unsterile conditions and different HRT and OLR. Once the stability of the simplified inoculum has been proved in a biofilm based

reactor and unsterile conditions, is needed the evaluation with a real substrate which could contain an indigenous microflora such as cheese whey.

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