Evaluation of Biohydrogen Production from Cheese whey and Assessment of the Associated Microbial Community

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ABSTRACT

The use of cheese whey as substrate for biological H_2 production could help to the waste management problem. The H_2 production at ambient temperature showed the advantage of energy savings. The study of the microbial community may help to determine it links with the performance of the biological process. The aims of this work were: (*i*) to evaluate the H_2 production in anaerobic fluidized bed reactor at ambient temperature using CW as substrate and (*ii*) to determine the changes in the microbial community. There were 4 stages of operation (with H_2 production), 3 with an organic volumetric load (B_v) of 10 g/(L.day) using: (*i*) sucrose, (*ii*) CW, and (*iii*) CW with 1 g/L of NaCl and one using a B_v of 5 g/(L.day) with 0.5 g/l of NaCl . The main response variables were: H_2 productivity (NmL H_2/L_{bed}.day), H_2 pseudoyield (mmol $H_2/gCOD_{fed}$) , Jaccard similarity index), Poggi's discrete divergence index, and Shannon-Weaver index. In sucrose stage the H_2 productivity was 1 011 NmL H_2/L_{bed}.day. In the CW stage, H_2 productivity was 101 NmL $H_2/(L_{bed}$.day), 10 fold lower than the H_2 productivity observed in the sucrose operation. In the CW-NaCl-1 stage we observed H_2 productivity increased 3 times (316 NmL $H_2/(L_{bed}$. day) compared to the value with only CW. We could conclude that the addition of NaCl had positively effect on H_2 productivity. The IJcw-CWNaCl was 0.2 and the ΔP was 0.02, this indicates that the community profiles are almost completely different. The main phyla detected were *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* and the abundance of the phyla change with the change of substrate and the heat shock treatments. There was detected *Lactobacillus* and was related with low H_2 productivity..In the stage with cheese whey and NaCl, the main genus detected were *Clostridium* and *Bifidobacterium.*

Keywords: bioH_2 production, cheese whey, fluidized bed bioreactor

1. Introduction

Hydrogen has been considered as very attractive biofuel because is versatile, safe and has high energy content per unit mass (122 kJ/g; Das & Veziroglu, 2008). There are several biological processes to H_2 generation, but one important goal is to achieve the sustainability of bioH_2 production. The sustainability depends on several factors; two very relevant factors are (*i*) availability of cheap, renewable substrate and (*ii*) the establishment of fermentation conditions that augment the yield of H_2 production [1].

The feasibility of H_2 production from organic waste has been demonstrated [2-4] and has the advantage that organic wastes are cheap and renewable substrate [4]. Therefore, the use of organic wastes can be used as adjutant of both waste treatment and energy recovery. Cheese whey (CW) is considering a dairy industry residue that needs to be

treated before its discharge in order to avoid pollution. The CW is the liquid that separates from the cheese manufacture, containing a high content of organic matter (mainly lactose) and low bicarbonate alkalinity [5, 6]

One meaningful parameter on bioH_2 producing processes is the operating temperature. The selection of the temperature is very important due to could influence the (*i*) substrate degradation, (*ii*) distribution of aqueous products, (*iii*) the growth rate and (*iv*) the metabolic activity of microorganisms [7,8]. And the heat energy used to maintain higher operational temperatures could diminish the net energy gain of biofuels production [9]. Therefore, the H_2 production at ambient temperature attractive due to the save on processes energy expenses such as heating. Currently, little is known on dark fermentation of CW in the psychrophilic range. Furthermore, H_2 production in anaerobic fluidized bed reactors (AFBR) is still scarce, since other bioreactor configurations have been studied

The knowledge of the microbial community is essential in order to understand the bioreactor processes [10]. The PCR combined with the Denaturing Gradient Gel Electrophoresis (DGGE) provide analytical methods of microbial population's research without prior separation of each type of microorganism [11]. These techniques had been successfully used to analyze the microbial communities of bioH_2 fermentations [10, 12, 13]. The Massive semiconductor sequencing of 16S rDNA library is one of the next-generation sequencing technologies that could monitor millions, and potentially billions, of simultaneous sequencing reactions [14].

Thus, the main goals of this work were *(i)* to evaluate the H_2 production in a lab scale AFBRs at ambient temperature using CW as substrate and (*ii*) to determine the change of the microbial community.

2. Experimental

Experimental design

The experimental design evaluated the H_2 production at ambient temperature in AFBR using cheese whey as substrate. The stages of operation (with H_2 production) are distinguished according to the type of substrate used; three of them were with a volumetric loading rate (B_v) of 10 g/L_{bed}.day: (*i*) sucrose (S), for bioreactors start-up and hydrogenesis development; (*ii*) cheese whey (CW); and (*iii*) CW with 1 g/L of NaCl (CW-NaCl-1); the fourth was CW with 0.5 g/L of NaCl but with a B_v of 5 g/L_{bed}.day (CW-NaCl-2). The main response variables were: H_2 productivity (NmL H_2/L_{bed}.day) and H_2 pseudoyield Y' (mmol H_2/gCOD_{fed}), Jaccard similarity index (IJ), Poggi's discrete divergence index ($\dot{\Delta}_P$) and Shannon-Weaver index (H'_{10}; H'_2; H'_e).

Inocula and substrate

The inoculum of the AFBR was digestates from methanogenic anaerobic digesters degrading sucrose. Those digesters were operated at mesophilic temperature. Before loading into the reactor, the digestates were pre-treated by heat-shock (90ºC, 1h) in order to select for H_2-producing bacteria.

The start up of the AFBR was with sucrose and a synthetic wastewater: (mg/L; [15, 16]: CH_4N_2O (125); $CaCl_2$*$6H_2O$ (47); $NiSO_4$*$6H_2O$ (1); $FeSO_4$*$7H_20$ (5); $FeCl_3$*$6H_2O$ (0.5); $CoCl_2$*$2H_2O$ (0.08); $NaHCO_3$ (1 g/L); SeO_2 (0.07); KH_2PO_4 (85); K_2HPO_4 (21.7); Na_2HPO_4*$2H_2O$ (33.4).

The cheese was in powder and had the following characteristics: 0.85% fat, pH 6.3, 12.6 % protein and 5 .58% of ash. In the three and four stages NaCl were added in order to reduce the bacteriocin effect to the H_2 producers.

Experimental setup

The AFBR consisted of a glass column of 4.5 cm internal diameter, 185 cm length and 3 L capacity. Granular activated carbon (1 L; 1-2 mm diameter) was used as bed. The hydraulic residence time (HRT) was1 day (fluidized bed volume basis; [17].

Analyses

The H_2 and CH_4 concentration were determined in a Gow-Mac chromatograph (model 350) with a thermal conductivity detector and Molecular Sieve 5A packed column: injector, detector and column temperatures were 25, 100 and 25 ºC, respectively. Argon was the carrier gas [18, 19]. The acids and solvents concentration were

determined in the effluent after filtration through a glass-membrane filter. An aliquot of the filtrate was injected in a gas chromatography Varian Star 3400 equipped with FID. The injector and detector temperatures were set at 250ºC with N_2 as a carrier gas with a 20 mL/min flow rate. The oven temperature was programmed as follows: 60 ºC for 2 min, increasing to 140 ºC at 5ºC/min, and then kept constant at 140ºC for another 6 min. A 50 m 0.32 mm internal diameter fused silica capillary column coated with 0.2 mm CP-Wax 57 CB was used.

A/B ratio and specific energetic potential due to H_2

The ratio of acetic-to-butyric acid (A/B) is a parameter that could indicate the metabolic pathway favored in the fermentative process [17]. The energy evaluation of the process was performed with the analysis of the specific energetic potential ($\hat{E}_H$; [20]).

DNA preparation and PCR- DGGE analysis

Genomic DNA was extracted from bioparticles of the AFBR using the PowerSoil® DNA Isolation Kit of MO BIO Laboratories, Inc. Then, a PCR amplification was made using specific primers for Eubacterial: CGO465 with GC clamp (5'-CTC CTA CGG GAG GCA GCA GCG CCC GCC GCG CGC GGC GGG CGG GGC GGG GGC CCG GGG GG-3') and CGO465R (5´-CTA CCA GGG TAT CTA ATC CTG-3´) ; targeting a region of 332-775 bp of E. coli 16S rRDNA gen. The PCR mixture (25 µL) consisted of: (*i*) 1x buffer 4.0 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.2 µM of forward and reverse primers, 0.025 U/µL of AmpliTaq DNA Polymerase (Fermentas), and 5-7 ng of DNA template. Amplification was performed in an TC-3000 Termocycler (Techne) with cycling as follows: an initial denaturation step at 94ºC for 5 min, followed by 30 cycles of 94 ºC for 30 s, 55.3 ºC for 30 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 7 min.

The PCR products (18 µg) were loaded into an 8% polyacrylamide gel with a denaturing gradient of 25%-70%. Bands of the DGGE gels were visualized by staining them with SYBR Green I (Molecular Probes, Eugene, OR). Gels were digitized and analyzed with the UV transilluminator of electrophoresis documentation and Analysis, Model System 120 (Kodak).

Massive semiconductor sequencing of 16S rDNA library

The sequencing was performed at Ion Torrent PGM. The primers used for the amplification targeted the V3 region of the 6S rDNA. The forward primer was V3-341F with 12bp Golay barcode [21] and adapters for massive sequencing; the antisense primer V3-518R containing A and Truncated P1 adapters [22].

The PCR mixture consisted of: (*i*) 1X Buffer, (*ii*) 2 mM $MgCl_2$, (*iii)* 0.2 mM dNTP´s, (*iv*) 0.025U/µL Taq DNA, (*v*) 0.2µM of each primer, and (*iv*) 10 ng of DNA template. Amplification was performed in a GeneAmp PCR System 2700 Thermocycler (Applied Biosystems) with an initial denaturation (95°C for 5 min) followed by 30 cycles of denaturation (94°C for 15 s), annealing (62°C for 15 s) and extension (72°C for 15 s) and final extension (72°C for 10 min).

The PCR products were purified by electrophoretic separation on 2% agarose gel and using Wizard SV Gen PCR Clean-Up System (Promega). The concentration of PCR amplicons was measured by NanoDrop spectrophotometer (ThermoScientific).After purification, the samples were sent out for the barcoded library preparation and sequencing on an Ion Torrent PGM with 316 chip using the Ion Sequencing 200 bp kit (Life Technologies) according to the standard protocol (Ion Xpress_ Plus gDNA and Amplicon Library Preparation, Life Technologies). The sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences; and sequences matching the 3'-adapter were automatically trimmed and filtered.

The demultiplex of the sequenced data was performed using a Sequenced data-Microbiota analysis Ion torrent PGM software, Torrent_Suite v 4.0.2. It was based on their barcodes and poor quality reads were eliminated from the datasets, i.e. quality score <20, containing homopolymers >6, length <200 nt, and containing errors in primers and barcodes. The analysis of the demultiplexed sequencing data was made with the program QIIME version 1.8.0 software pipeline. Closed reference Operational taxonomic units (OTU) were determined at 97% similarity level with UCLUST Algorithm [23].

It was used the Chimera Slayer in order to detect and removed chimeras from the datasets [24]. Sequence alignments were done against the Greengenes core set [25]. Statistical analysis of the OTU table Microbial diversity had been assessed through both alpha and beta diversity. Using rarefied OTU tables, alpha diversities were calculated using various matrixes like Shannon, PD whole tree, chao1, observed species.

The beta diversity analysis was calculated using UniFrac analysis [26], by a phylogenetic tree computed with FastTree and a rarefiled biom table as inputs. Abundance of the bacterial groups at different taxonomic levels (phylum, order, and genus) was separately explored with a Principal Component Analysis (PCA) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) Clustering.

Richness and diversity estimation

The Jaccard similarity index (IJ) was used for estimation of the microbial richness variation intra-stage and between stages, in the DGGE. The IJ ranges from 0 to 1 when the community profiles are completely similar and 0 when the profiles are completely different [27]:

$$IJ = \frac{n_{AB}}{(n_A + n_B - n_{AB})} \qquad (1)$$

Where:
n_{AB}= number of bands that are present both in lane A and lane B
n_A= total number of bands of lane A
n_B= total number of bands of lane B

To compare the richness of a community with respect to time it was used the Poggi's divergence index ($\dot{\Delta}_P$; [28]):

$$\dot{\Delta}_P = \frac{d\Delta P}{dt} \qquad (2)$$

$$\Delta_P = \frac{(\dot{n\prime}_A + n\prime_B)}{(n_A - n_B)} \qquad (3)$$

Where:
n'_A= number of bands of lane A absent in lane B
n'_B= number of bands of lane B absent in lane A
n_A= total number of bands of lane A
n_B= total number of bands of lane B

The $\dot{\Delta}_P$ index indicates that the microbial community is relatively stable with respect to time. For instance, analysis of a microbial community in attached-growth bioreactors with this tool has shown very low values of $\dot{\Delta}_P$. On the other hand, higher values of $\dot{\Delta}_P$ are related to dynamic changes in community composition such as those that occur in suspended-growth bioreactors.

The results of the massive semiconductor sequencing of 16S rDNA library were analyzed using the Shannon-Weaver diversity index [29], defined as:

$$H' = \sum_{i:1}^{S} \dot{(p_i log p_i)} \qquad (4)$$

where:

p_i : is often the proportion of individuals belonging to the i species in the dataset of interest,
S: is the total number of species.

3. Results and discussion

3.1. Operation with sucrose

The fluidized bed reactor was operated for 30 days with a B_v of 10 g sucrose/L_{bed}.day. The average H_2 productivity obtained was 1011 NmL H_2/L_{bed}.day with a H_2 pseudoyield of 4.51 mmol H_2/$gCOD_{fed}$ (Table 1) that is 1.13 times lower than the obtained in the same system using 8 g sucrose/L_{bed}.day [17]. This could be due to the high concentration of solvents in the effluent with ratio φ (defined as the sum of volatile organic acid divided the sum of solvent products) 5.8 times lower than the ratio with Bv=8 g/L_{bed}.day (3.3. and 19, respectively). The low value of φ in our work may indicate that the fermentation was being diverted to a solvent production. Indeed, ethanol was the main solvent in the effluent (Table 1) and it is known that the ethanolic fermentation is related to low production of H_2 [30].

Table 1. Average performance of anaerobic fluidized bed reactor at ambient temperature using cheese whey as substrate

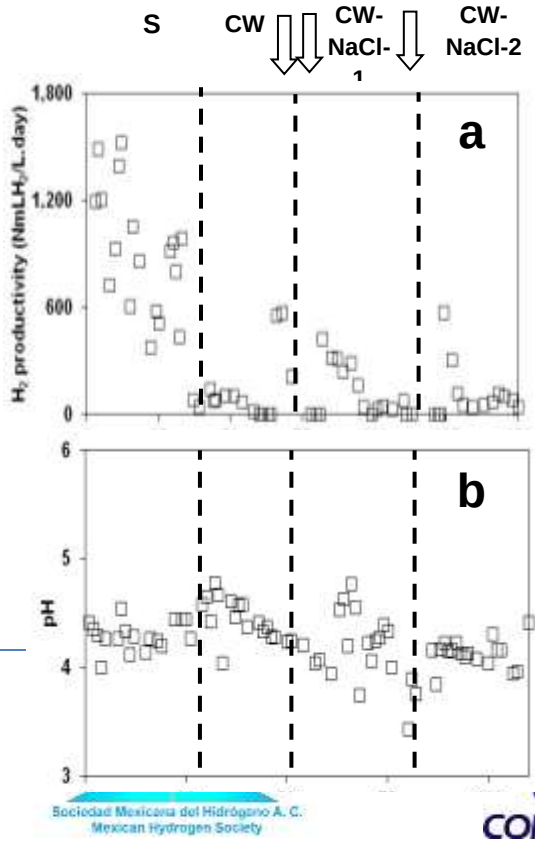
Parameter	Sucrose	Cheese whey		
	(S) 10 g/L_{bed}.day	**Without NaCl (CW) 10 g/L_{bed}.day**	**With NaCl (CW-NaCl-1) 10 g/L_{bed}.day**	**With NaCl (CW-NaCl-2) 5 g/L_{bed}.day**
pH	4.27 ± 0.12	4.31 ± 0.07	4.1 ± 0.2	4.3 ± 0.2
H_2 concentration (%)	39.4 ± 8.5	11.8 ± 1.6	32.1 ± 6.9	14.5 ± 3.1
H_2 productivity				
(NmL H_2/L_{bed} day)	1 011 ± 339	101 ± 25	316 ± 67	92.12 ± 20
(mmol H_2/gCOD day)	4.2	0.4	1.4	0.8
Soluble microbial products (mg COD/L)				

Acetic acid	885 ± 362	289 ± 149	720 ± 259	682 ± ---
Propionic acid	282 ± 140	14.2 ± 13.7	273 ± 4	106 ± ---
Butiric acid	4 392 ± 1 570	299 ± 28	942 ± 194	1864 ± 1580
Lactic acid	ND	0.07 ± 0.02	0.012 ± 0.07	ND
Acetone	ND	ND	ND	ND
Methanol	ND	ND	ND	ND
Ethanol	1193 ± 822	117 ± 67	ND	263 ± 79
Butanol	477 ± 98	77 ± 12	ND	76.7 ± ---
EtOH/SMP (%)	17.1 ± 10.8	14.0 ± 4.6	0 ± 0	15.7 ± 14.9
BuOH/SMP (%)	6.9 ± 1.8	9.8 ± 1.1	0 ± 0	6.1 ± ---
HAc/SMP (%)	12.0 ± 2.5	35.1 ± 9.3	36.6 ± 4.9	8.6 ± ---
HPr/SMP (%)	3.9 ± 1.7	1.6 ± 1.3	14.5 ± 3.6	1.3 ± ---
HBu/SMP (%)	60.1 ± 6.4	39.4 ± 14.1	48.8 ± 1.3	68.2 ± 9.4
A/B	0.20 ± 0.02	1.0 ± 0.6	0.8 ± 0.1	0.11 ± ---
TVOA (mg COD/L)	5 559± 2027	602 ± 135	1 935 ± 449	2258 ± 2139
SMP (mg COD/L)	7 229 ± 2 320	796. ± 215	1 935 ± 449	2598 ± 1959
ρ, TVOA/SOLV	3.9 ± 2.6	3.2 ± 0.6	---	9.65 ± ---

Notes: Average results were obtained under steady-state conditions: S: day 8 to 22; CW: and CW-NaCl: day 15 to 22. A/B: acetic to butyric acid ratio. EtOH: ethanol; BuOH: butanol; HAc: acetate; HPr: propionate; HBu: butyrate; TVOA: total volatile organic acids= HAc+HPr+HBu; SMP: soluble microbial products=TVOA+EtOH+BuOH. Based in COD/L.

3.2. Operation with cheese whey

Afterwards, CW was used as substrate (stage two) and the productivity decreased to 101 ca. NmL H_2/L_{bed}.day (Figure 1 a), that was almost 10 times lower than the H_2 productivity observed in the operation with sucrose (Table 1). The Figure 1b shows the pH in the effluent of the AFBR, the values were between 4-5, that low values of pH could be related to a high accumulation of organic acids. However, in this case, the organic acid accumulation diminished in the same way as pH.



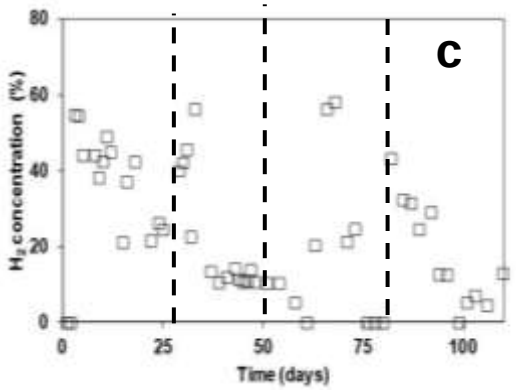


FIGURE 1. Time courses of: (a) H_2 productivity.; (b) pH and (c) H_2 concentration. Arrows indicate a heat shock treatment.

We detected lactic acid in the effluent, and its presence could indicate the presence of lactic acid bacteria (LAB, [31]. This type of bacteria is related to low H_2 production [31, 32] as a combination of H_2 sink when hexoses are fermented to lactic acid as well as inhibitory effects of LAB on H_2-producing bacteria.

There are three known mechanisms that can explain the antimicrobial activities of LAB: (*i*) increased amount of organic acids that can cause rapid acidification of the medium [33], (*ii*) nutrient competition [31], and (*iii*) generation of antimicrobial compounds [31, 34, 35] investigated the effect of LAB on H_2 fermentation of organic waste and suggested that the inhibitory effect on H_2 production could be explained by the excretion of bacteriocins. Bacteriocins are proteins that have activity towards gram-positive bacteria, frequently include organisms capable of sporulation such as *Clostridium* and/ or food pathogens [35-38].

In this work, the inocula had a shock treatment in order to eliminate the microorganisms that are the main competitors of the H_2-producers, such as methanogenic archeae and LAB's that cannot sporulate [31,39]. It seems that the shock treatment was not sufficient to eradicate these microorganisms; the detection of methane during the operation with sucrose [18] could support that affirmation. Therefore, the first strategy implemented was to repeat the heat shock treatment, but now for more hours (arrows in Figure 1) and after that the AFBR-A was operated with CW but no H_2 production was detected (data no shown). We realized another shock treatment and start up with sucrose but no H_2 production was produced (data not shown).

Table 2. Hydrogen production using cheese whey as substrate.

Inoculum	Bioreactor, fermentations conditions, and volumetric organic load (gCOD/(L.day))	Y', Hydrogen pseudoyield * ($mmolH_2/gCOD_{fed}$)	$\hat{E}_{H2}$ (J/gCOD)	Ref.
Sludge Heat shock treatment	CSTR ; HRT: 1, 2 y 3.5 d T = 55ºC ; pH = 5.5 ; 47	0.91	257.5	[39]
Digestates of acidogenic reactor	UASB ; HRT=12 h T=30°C; pH=5 ; 20	0.18	50.9	[40]
Microorganisms in wastewater	CSTR ; HRT: 24 h T = 35°C; pH = 5.2; 30	1.5	424.5	[41]
Anaerobic mixed microflora Heat shock treatment	CSTR ; HRT:24 h T = 55ºC; pH=5.5; 30	3.5	990.5	[42]
Methanogenic sludge Heat shock treatment	AFBR ; HRT:24 h T = 25ºC; pH=4.1; 10	1.4	396.2	This study

Notes: HRT, hydraulic retention time; CSTR, continuously stirred tank reactor; UASB, upflow anaerobic sludge blanket reactor; AFBR, anaerobic fluidized bed reactor.

A sample of bioparticles of the reactor was cultivated in MRS agar (agar formulation designed to make evident the growth *Lactobacilli* and other LAB's) and growth of colonies was observed. We concluded that the heat treatment did not eliminate the LAB. This could be due to the microorganisms present in the biofilm protected from environmental conditions by the particle. Hence, it is possible that the temperature of the inner biofilm layers (where it seems to be the place where anaerobic microorganisms concentrate, [43, 44] is lower than that at the surface of the biofilm. Regarding the effect of LAB on hydrogen producer microorganisms, we look for other alternative besides the heat treatment. There are reports that supplementation of cultures with salts, such as NaCl, could reduce the

bacteriocin effect. So, we tested a second strategy that consisted of new heat shock treatment and the addition of 1 g/L of NaCl to the influent to the AFBR.

3.3. Operation with cheese whey and NaCl

The AFBR was operated with sucrose (B_v=5 g/(L_{bed}.day) and at day 75 we changed to CW (B_v=10 g/(L.day)). Afterwards, no H_2 was detected in 7 days and the highest concentration of lactic acid was observed the highest concentration of lactic acid. After this, the supplementation of CW with NaCl started; almost immediately the H_2 production resumed, with an H_2 concentration of almost 20% and a noticeable increase in the pH (Figure 1 b).

BioH_2 productivity increased to up to 316 NmL H_2/(L_{bed}. day), i.e., 3 times compared to the value obtained without the presence of NaCl. A reduction in the concentration of lactic acid was also observed. The effect of the NaCl is unclear but some works suggested that there exists a decrease in bacteriocin production in the presence of salt because of the interference of NaCl binding to bacteriocin induction factor, which is essential for bacteriocin production. Other authors indicated that in the case of the bacteriocin of *L. sakei CTC 494*, however, it appears that the water binding effect of salt is the major factor responsible for the decrease in specific bacteriocin production [45].

The Y' with CW-NaCl-1 was 1.4 mmol H_2/gCOD_{fed} and the specific energetic potential was ca. 400 J/g COD; these values are in the middle of the range of reported H_2 productions from CW in others works where the bioreactors were operated at higher temperatures than our AFBR (0.18-3.5 mmol H_2/g COD_{fed} ; Table 2) .

3.4. Operation with cheese whey and NaCl with Bv= 5 g/L_{bed}.day

The H_2 production last one month, after that, almost no H_2 was detected. The third strategy was to repeat the shock treatment and the feed with CW-NaCl but now start with a low Bv (5 g/L_{bed}.day) and increase slowly until reach the 10 g/L_{bed}.day. The average H_2 productivity achieved was 0.8 mmol H_2/g COD_{fed} that is almost 2 fold to that obtained with 10 gS/L_{bed}.day. The H_2 production decreased after 30 days and no more H_2 was detected.

4. PCR- DGGE analysis of microbial communities harbored in the bioreactors

Figure 2 shows the DGGE of bioparticles samples of the different stages of operation. Regarding the meaning of the Jaccard Index, a value near to 1 indicates that the profiles are similar and when the value is near to 0 the profiles are completely different. It can observe (Table 3) that the profiles with sucrose operation and the CW are moderately different (IJ=0.4), that could be due to the change of a more complex substrate (cheese whey), this could restrict the growing of some microorganisms.

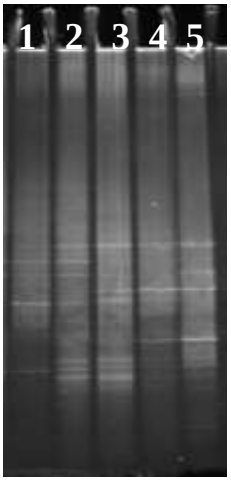


Table 3. Jaccard similarity index (IJ) and Poggi's divergence index ($\dot{\Delta}_P$)

Lane	Stage	IJ					$\dot{\Delta}_P$				
		1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
1	Sucrose	1	0.4	0.2	0.1	0.1	1	0.01	0.02	0.02	0.01
2	CW		1	0.5	0.2	0.2		1	0.01	0.02	0.01
3	CW-1hst			1	0.2	0.2			1	0.02	0.01
4	CW -2hst				1	0.4				1	---
5	CW-NaCl-1					1					1

FIGURE 2. DGGE of different stages of operation in a fluidized bed bioreactor.

The profiles with the sucrose to the operation with CW-NaCl-1 are very different (IJ=0.1: Table 3), it is important to remember that the stage with cheese whey and NaCl was after two shock heat treatment, this could explain the change to the profiles. Huang *et al*. [46] observed that the heat shock treatment could result in the elimination of non-spore-forming bacteria with the consequence reducing to the consume oxygen system and the decreased conversion of the substrate into H_2. The values of Poggi's divergence index(ΔP) are low and could be related with attached-growth bioreactors.

3.6. Massive semiconductor sequencing of 16srDNA library

The phyla of the sequences detected with the massive semiconductor sequencing are displayed in Figure 3. The main phyla observed in all stages are *Firmicute*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria.* In the sucrose stage, the abundance of the phyla was: 70% *Firmicute*, 6%, *Proteobacteria*, 21% *Bacteroidetes* and 3% *Actinobacteria.* These percentages vary with the change of substrate, there were and increment in *Firmicutes* (91%) and *Actinobacteria* (5%) and a substantial decrease of *Proteobacteria* (2%) and *Bacteroidetes* (2%).

It is important to recall that the H_2 productivity decreased 10 fold with the change to CW. This tendency continued after the first heat shock treatment and no H_2 production was detected. With the second heat shock treatment, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* incremented to 14%, 61% and 15%, respectively. The *Firmicutes* reduced to 11%; but still no H_2 was observed. With a third heat shock treatment and the addition of NaCl (CW, 5 g/L) *Firmicutes* and *Actinobacteria* incremented to 26 and 54%, respectively, the *Proteobacteria* reduced to 6% and the *Bacteroidetes* remained to 13%. In this last stage the H_2 productivity incremented 2 fold with respect to that obtained with only CW.

Table 4 shows the genus of some microorganisms (> 1%) obtained at the different stages of operation. The *Clostridium* is known as H_2 producer and its abundance increased 2 fold (20.9%) when the substrate to bioreactors was changed to CW but less H_2 was detected. Possibly there were present a few non-clostridia bacteria, that despite there are not H_2 producers, might have contributed to the degradation of carbon substrates [47]. Furthermore, not all *Clostridia* species in the dark fermentation system are capable or responsible for H_2 production [48,49].

In the two stages with shock heat treatment, the abundance of *Clostridium* diminished to 11.2 and 0.6 %, respectively; no H_2 was observed. In the stage CW-NaCl-2 the H_2 productivity was 2 fold of the obtained with only CW, whereas the abundance of *Clostridium* was 7.7%.

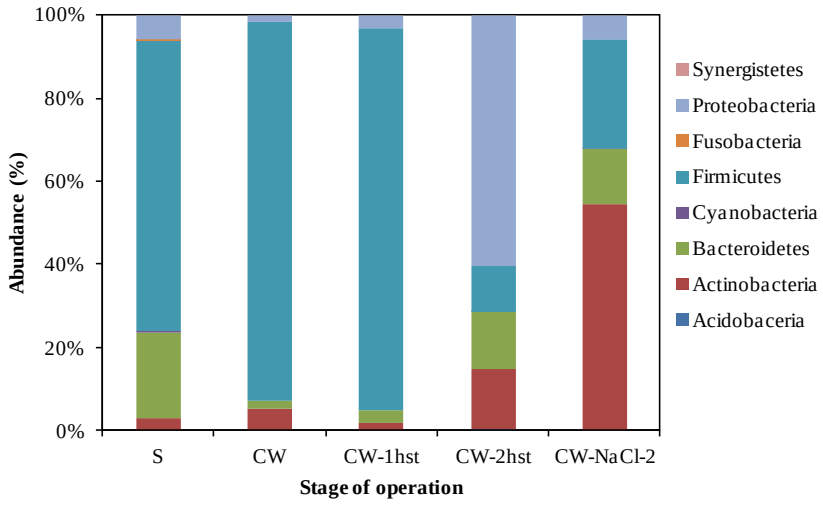


FIGURE 3. Phylum level identification of all sequences.

Table 4. Microorganisms profiles (phylum, class, order and genus) at different stages of operation

Phylum	Class	Order	Genus	Abundance (%)				
				S	CW	CW-1hst	CW-2hst	CW-NaCl-2
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Lentzea*	0.0	0.0	0.0	5.0	0.2
			Rhodococcus	0.0	0.0	0.0	2.6	0.3
			Propionibacteriaceae (family)*	1.3	0.4	0.1	2.0	5.5
			Streptomyces	0.1	0.0	0.0	1.0	1.2
		Bifidobacteriales	*Bifidobacterium*	0.5	0.0	0.7	2.0	44.3
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroides*	6.9	0.1	0.1	0.7	1.0
			Prevotella	11.1	1.6	2.8	7.0	11.4
	Flavobacteriia	*Flavobacteriales*	*Flavobacterium*	0.0	0.0	0.0	4.8	0.1
Firmicutes	*Bacilli*	*Bacillales*	*Sporolactobacillus*	19.9	1.4	0.2	0.0	0.2
		Lactobacillales	*Lactobacillus*	1.0	6.0	65	0.0	0.0
			Lactococcus	1.2	0.0	0.0	0.0	0.0
	Clostridia	*Clostridiales*	*Clostridiales* (order)*	2.8	0.3	0.3	0.7	1.1
			Clostridiaceae (familiy)*	1.7	10.1	2.6	1.6	0.6
			Clostridium	11.6	20.9	11.2	0.6	7.7
			Lachnospiraceae (family)*	7.2	0.5	0.7	2.1	3.6
			Coprococcus	1.6	0.1	0.1	0.5	0.6
			Lachnospira	1.0	0.1	0.1	0.4	0.6
			Ruminococcaceae (family)*	3.5	9.3	0.7	1.1	1.8
			Anaerofilum	0.1	2.2	0.0	0.0	0.0
			Ethanoligenens	1.2	30.4	3.9	0.0	0.3
			Faecalibacterium	7.0	0.4	0.6	1.9	3.7
			Oscillospira	1.5	0.6	0.1	0.4	1.1
			Ruminococcus	1.6	7.3	4.5	1.2	1.2
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiales* (order)*	0.0	0.0	0.0	1.1	0
			Xanthobacter	0.0	0.0	0.0	5.0	0.1
	Betaproteobacteria	*Burkholderiales*	*Curvibacter*	0.0	0.0	0.0	18.8	0.1
			Oxalobacteraceae (family)*	0.1	0.0	0.0	18.0	0.2

	Gammaproteobacteria	*Aeromonadales*	*Succinivibrio*	1.3	2.1	1.2	0.4	0.6
		Enterobacteriales	*Enterobacteriaceae* (family)*	2.4	0.7	1.6	1.2	0.6
		Pseudomonadales	*Pseudomonas*	0.1	0.0	0.1	1.3	0.1
		Xanthomonadales	*Xanthomonadaceae* (family)*	0.0	0.0	0.0	0.1	1.2

Note: * the genus are not identified yet

As we mentioned before, *Lactobacillus* are microorganisms related to low H_2 production [50]. We have found this type of microorganism in the stage with sucrose and there was and increment of *Lactobacillus* with the change to the CW substrate (1.0 to 6.0), it is important to mention that H_2 productivity decreased 10 folds in this stage.

After the first heat shock treatment, the feed was only CW and the *Lactobacillus* increased to almost 66%. After performing a second heat shock treatment we fed sucrose during a first short subperiod and afterwards, the substrate was changed to CW. In this process, *Lactobacillus* diminished to 0.1% but still no H_2 was detected. In this stage *Clostridium* decreased its abundance (10%).

It is important to remark that in the stage with CW-NaCl-2 the percentage of *Bifidobacterium* was almost 10 fold higher than in the stage with only CW. *Bifidobacterium sp.* is a microorganism that has been found in H_2 fermentation of starch and xylose [47, 51]. In the H_2 production using starch [51], the highest H_2 yield was observed at the HRT of 0.5 h and with *Clostridium sp.* and *Bifidobacterium sp.* as the main microorganisms detected (a cell counts of 40 and 40-60%, respectively).

Table 5. Shannon-Weaver Index of the different stages of operation

Shannon-Weaver Index	**S**	**CW**	**CW-1hst**	**CW-2hst**	**CW-NaCl-2**
H_{10}	0.38	0.16	0.15	0.48	0.49
H_2	1.27	0.54	0.51	1.61	1.62
H_e	0.88	0.38	0.35	1.11	1.12

Table 5 shows the Shannon-Weaver index of the operation stages, it can observe that they decreased to the CW-1hst stage, after that, the index increase in the CW-2hst and remains in the CW-NaCl-2 stage.

4. Summary and perspectives

- The H_2 productivity using CW as substrate was almost 10 fold lower than the obtained using sucrose.
- The use of CW-NaCl allowed the increase of H_2 productivity (almost 3 times) compared to the value obtained without the supplementation of the salt.
- The H_2 pseudoyield was in the middle of the range of H_2 production using CW reported in other works that performed the dark fermentation of CW at higher temperatures.
- The main phyla detected were *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria*. The abundance of the phyla changed with the change of substrate
- Presence of *Lactobacillus* was related with low H_2 productivity; we found *Clostridium* in all the stages of operation
- In the stage with CW and NaCl, the main microbial orders detected were *Clostridium* and *Bifidobacterium*

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