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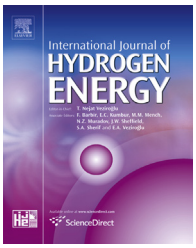

Selective adaptation of an anaerobic microbial community: Biohydrogen production by co-digestion of cheese whey and vegetables fruit waste

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ABSTRACT

The co-digestion process of crude cheese whey (CCW) with fruit vegetable waste (FVW) for biohydrogen production was investigated in this study. Five different C/N ratios (7, 17, 21, 31, and 46) were tested in 2 L batch systems at a pH of 5.5 and 37 °C. The highest specific biohydrogen production rate of 10.68 mmol H_2/Lh and biohydrogen yield of 449.84 mL H_2/g COD were determined at a C/N ratio of 21. A pyrosequencing analysis showed that the main microbial population at the initial stage of the co-digestion consisted of *Bifidobacterium*, with 85.4% of predominance. Hydrogen producing bacteria such as *Klebsiella* (9.1%), *Lactobacillus* (0.97%), *Citrobacter* (0.21%), *Enterobacter* (0.27%), *and Clostridium* (0.18%) were less abundant at this culture period. The microbial population structure was correlated with the lactate, acetate, and butyrate profiles obtained. Results demonstrated that the co-digestion of CCW with FVW improves biohydrogen production due to a better nutrient balance and improvement of the system's buffering capacity.

Introduction

The problems of global warming and fossil fuel exhaustion caused an urgent need for the development of clean and renewable energies. Hydrogen has been proposed as a clean energy carrier and as potential replacement to fossil fuels, since it has the highest energy content and generates no other products than water when burned [1]. For hydrogen to be considered a sustainable alternative fuel, it should be generated from cheap and readily available feedstocks that are renewable or potentially renewable [2,3].

Biohydrogen production by dark fermentation has received broad attention, since this process can utilize renewable feedstock sources (e.g., complex wastewaters, agro-industrial wastes) [4]. Most of the studies have been conducted utilizing

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mono-digestion processes of food wastes [5], vinasses [6], water hyacinth, soybean oil extraction residues, mushroom wastes [7], and wastewater from cheese processing [8]. Data obtained in these studies shows that the biohydrogen production could be reduced or even inhibit due to some specific characteristics of the wastewater or organic wastes, such as deficient buffer capacity, nutrient imbalance, and presence of microbial populations that may consume hydrogen or produce methane [8]. One strategy to overcome these problems is the use of co-digestion processes due to the positive synergistic effects of the mixed materials with complementary characteristics and the supply of missing nutrients by the co-substrate (adequate carbon/nitrogen (C/N) ratio, and the macro- and micro-nutrients concentration). A balanced C/N ratio allows enhancing the buffer capacity of the system. Additionally, the co-digestion process also reduces the possibility of inhibitory effects, which, in turn, increases biohydrogen production [9,10]. Few novel anaerobic co-digestion processes have been developed for biohydrogen production. In such studies, organic wastes such as municipal foods waste-kitchen wastewater [11], waste glycerol-sludge [12], cow manure-waste milk [13], rice straw-sewage sludge [14], food waste-sewage sludge [15–17], cassava stillage-sludge [10], and pressed mud-sewage [18] have been utilized. However, as far as we know, there is no report in the literature regarding biohydrogen production from co-digestion of crude cheese whey (CCW) with fruit vegetable waste (FVW). CCW is a liquid waste generated after manufacturing cheese with high content of lactose, proteins, and other compounds. It has been reported that around 1 million metric tons are generated annually in Mexico [19,20]. On the other hand, fruits and vegetables waste are massively available and they represent a form of highly degradable feedstock. These residues are largely produced by marketplaces, food industry sectors, and the central food distribution market in Mexico City. In 2008, Mexico produced 20 milliard tons of organic solid wastes, also referred to as the organic fraction of municipal solid waste (OFMSW) [21]. Because of their complementary characteristics, their higher organic composition, easy biodegradable nature, the FVW and CCW can be used as good substrates for the co-digestion process to produce biohydrogen. Additionally, the large amounts of these residues make them an available and low cost feedstock.

The use of non-sterilized organic wastes during fermentation processes will modify the inoculated mixed microflora. Recent studies revealed that, in the co-digestion process, the addition of small amounts of co-substrate altered the bacterial communities of the system [22]. More basic research is needed to determine the effects of co-substrates in co-digestion process to better understand and elucidate the mechanisms to enhance biohydrogen production. The biohydrogen-producing communities' structure identification is a critical step toward microbial communities' optimization and improvement of biohydrogen production [23].

The aim of this study was to investigate the potential of biohydrogen production from CCW with FVW in co-digestion processes by: (1) evaluating the effect of previous acclimation on the microbial distribution of the inoculum, (2) investigating the effect of different C/N ratios obtained by mixing different amounts of CCW and FVW, on hydrogen production, and (3) investigating the effect of co-substrates in the co-digestion process on the metabolic profile evolution and over the buffer capacity of the CCW and FVW mixtures.

Materials and methods

Inoculum and substrates

The inoculum was obtained from a 30 L anaerobic digester regularly fed with FVW [24]. Since the microbial population was mainly adapted to the FVW, an adaptation period for the new substrate (CCW) was necessary. The inoculum was previously adapted to use lactose as only carbon source (up to 30 g/L). The adaptation process was performed during two months. Fresh media with increasing lactose concentrations was sequentially fed to the culture to promote predominance of the microbial population suitable for lactose consumption. The new microbial population was preserved at 10 °C. Pre-inoculums with 24 h of culture, with a ratio of 10% v/v were utilized for the next experiments.

FVW were collected from a marketplace. The FVW composition was the same as previously reported [24]. Pre-treatment of FVW consisted in milling the fruits and vegetables in an electrical domestic blender to homogenize the sample. Then, the slurry was sieved through a stainless steel 0.0787-mm sieve (Size No 10).

The CCW used in the experiments was obtained from the Food and Biotechnology Laboratory of the School of Chemistry, UNAM-Mexico. The sample was filtered (filter paper, 8 μm) and refrigerated at 4 °C.

Anaerobic digester setup

The experimental setup of the batch anaerobic digester is shown in Fig. 1. The reactor unit consists of a 2-L Schott Duran bottle with a 1.8 L of working volume. The system was constantly mixed with a magnetic stirrer bar (Cimarec®, Burnstead I Themolyne). The temperature was maintained at 37 °C by using a temperature regulated bath (Poly-Science®, Serial num 109300711), pH was set and maintained at 5.5 by addition of NaOH (10 M) or H_2SO_4 (30% v/v) solutions. Biogas production was measured by a wet gas meter. Gas samples were taken in two ports (head space and wet gas meter) to determine gas composition by chromatography. Liquid samples were taken at regular intervals from a sample port for the analysis of protein, substrate, material degradation, and products concentrations.

Biohydrogen production by a co-digestion process in batch systems

Five CCW:FVW ratios, i.e., 100:0, 75:25, 50:50, 25:75, and 0:100 (%v/v) were tested, corresponding to C/N ratios of 7, 17, 21, 31, and 46, respectively. They were evaluated in terms of their effect over the biohydrogen production. 180 mL of the pre-inoculum was added to 1620 mL of the substrate fresh medium. The initial pH was adjusted to 5.5 with 10 M NaOH. The bottle was sealed and air was displaced by flushing N_2 gas to

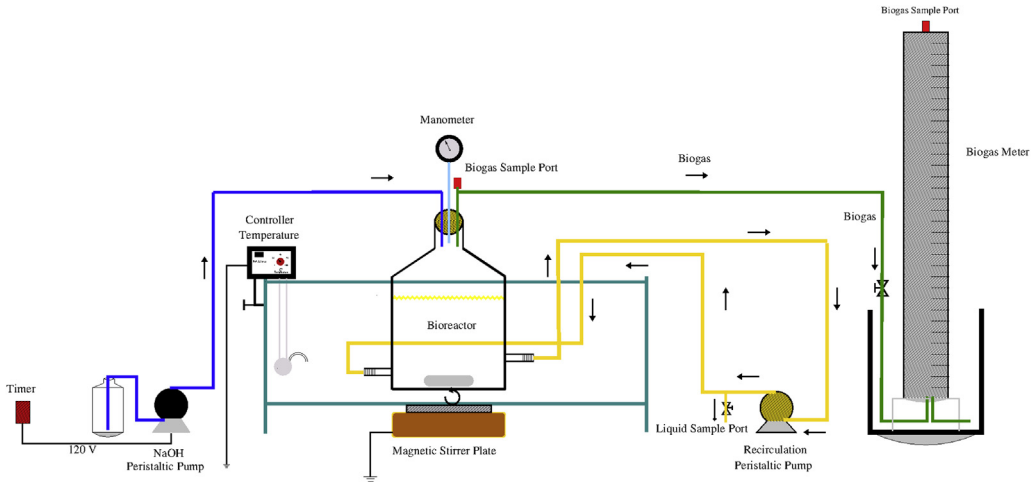

Fig. 1 – Diagram of batch anaerobic digester used in this study 1. Bioreactor, 2. pH control pump, 3. head space sample port, 4. wet gas meter, 5. recirculation line, 6. recirculation pump, 7. magnetic stirrer plate, 8. biogas outlet line, 9. timer.

guarantee anaerobic conditions. Batch experiments were operated for 60–210 h. Liquid samples for biomass, pH and organic acids measurement and gas samples for biohydrogen measurement were taken at regular intervals.

Analytical techniques

The composition of the biogas, including hydrogen and methane, was determined by gas chromatography (Gow-Mac 580®, Bethlehem, PA, USA) equipped with a thermal conductivity detector (TCD, 120 °C). Nitrogen was used as carrier gas at a flow rate of 30 mL/min. The operational column, detector and injector temperatures were 25 °C, 120 °C and 75 °C, respectively.

Fermentation products, such as butyric acid, acetic acid, propionic acid, lactic acid and ethanol, were analyzed by high performance liquid chromatography (HPLC) (Varian ProStar, Model 350) with Rezex ROA organic acid column (Phenomenex®, Torrance, CA, USA), equipped with a UV/Vis, RI detector. A sulfuric acid solution (0.005 N) was used as the mobile phase, and separation was carried out at 65 °C at a flow rate of 0.6 mL/min and pressure at 39 bar. Samples were previously centrifuged at 10,000 rpm (Eppendorf®, Mini Spin) for 10 min, after which the supernatant was filtered (Whatman®, 0.45 μm) prior to analysis. Total sugars (glucose, fructose, and lactose) were analyzed by the di-nitro salicylic acid (DNS) method, while soluble protein was determined by the Bradford method with bovine serum albumin as standard. COD was measured using Hach Standard Method (Kit-Hach, 20 to 1500 ppm). The pH, total solids (TS), volatile suspended solids (VSS), ammonium, and moisture were measured according to standard methods [25]. Liquid samples for metal analysis were filtered (Millipore, Sigma–Aldrich, 0.45 μm) and metals analyzed were iron and nickel with atomic absorption spectroscopy (Perkin Elmer). Carbon, nitrogen, oxygen, phosphorus, sulfur, and metals such as sodium, magnesium, potassium, and calcium analyses were undertaken using energy-dispersive X-ray spectroscopy technique by elemental analysis (X-Ray Bruker D8 Advance).

Microbiological analysis

DNA extraction

Liquid samples (~1.5 mL) were taken for microbial analysis and further DNA extraction from the already adapted inoculum. DNA was extracted by using the CTAB protocol [26]. Cells from 1.5 mL of the broth were harvested in a tube by centrifugation at 14,000 ×*g* for 5 min followed by decantation of the supernatant. The pellet was re-suspended in 0.5 mL of a pre-heated CTAB extraction buffer, the mixture was incubated during 1 h at 65 °C. Once the samples were at room temperature, a chloroform-octanol solution (24:1) was added and then centrifuged at 1813 ×*g* during 10 min. The aqueous supernatant was transferred into a sterile tube (1.5 mL) and 30 μL of RNase solution (10 mg/mL) were added. Samples were incubated during 50 min at room temperature. The DNA pellet was obtained by adding one volume of iso-propanol and centrifugation at 2414 ×*g* for 10 min. The DNA pellet was re-suspended in TE buffer (1 M Tris pH 8 and 0.5 M EDTA) and incubated overnight at room temperature. Then, 1 mL phenol-chloroform solution (1:1) was added and the mixture was centrifuged at 1813 ×*g* during 10 min. Afterwards, the total nucleic acid was precipitated with NaCl-ethanol solution and centrifuged at 9659 ×*g* and 4 °C for 1 min. The purified DNA was eluted with 40 μL of miliQ water and kept at −20 °C before using it as template DNA for pyrosequencing analysis. The quantity and quality of the extracted DNA were checked by measuring its absorbance at 260 and 280 nm.

454-Pyrosequencing

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed at the Research and Testing Laboratory (Lubbock, TX, USA). Bacterial primers 104F and 530R were used to amplify the V2 and V3 hyper-variable regions of the 16S rRNA gene spanned nucleotides 137-242 and 433-497 base pair, respectively (numbering based on the *Escherichia coli* 16S rRNA gene). Amplicons were sequenced with the FLX-Titanium System Genome Sequencer. QIIME 1.7.0 pipeline was used to process raw sequences. Sequences with at least one of the

following characteristics were omitted for the downstream analysis: shorter than 200 bps, quality score of 25 or below, any primer or barcode mismatches, more than 6 homopolymers. From the sequences that passed the quality filtering, OTUs were picked based on 97% sequence similarity, using uClust algorithm as described in Edgar [27].

Data analysis

Cumulative biohydrogen production curves were obtained over the course of the batch experiment and analyzed using the modified Gompertz Equation (1) [28].

$$H = P \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

Where, H is the cumulative volume of biohydrogen produced (mL), R_m is the maximum biohydrogen production rate (mL H_2/L*h), λ is the lag-phase time (h), t is the culture time (h), P is the biohydrogen production potential (mL H_2), and e is 2.718. Parameters P, R_m, and λ were determined by best fitting the biohydrogen production data, using the Gretl software (version 1.9.7). Origin software (version 8.6) was used for graphical analysis.

Results and discussion

Changes in microbial structure due to adaption to the new substrate (lactose)

The total bacterial community structure after the adaptation period was identified by a pyrosequencing analysis. The adapted microbial population at phylum level was mainly composed of *Actinobacteria* (87.3%), low percentages of *Proteobacteria* (11.5%) and *Firmicutes* (1.13) phylum were found. These data showed a microbial change compared with the one determined for the original inoculum, mainly composed of fermentative bacteria from the *Firmicutes* phylum (89.9%), and *Actinobacteria* (6.9%) and *Bacteroidetes* (2.3%) [24].

Fig. 2 shows the comparison between the bacterial community obtained at the initial phase of the co-digestion process, after the adaptation period, and the microbial population of the inoculum at genus level. In the original inoculum, *Lactobacillus* (72%) was the predominant bacteria at genus level (Fig. 2a). Other less abundant species as *Bifidobacterium* (6%), *Clostridium* (2.5%) and others were also present. After the adaptation period, the main microbial population at genus level was *Bifidobacterium*, representing 84.5% of the total sequences analyzed (Fig. 2b), followed by those related to the genus *Klebsiella* (9.1%), *Actinomyces* (1.9%) and *Lactobacillus* (0.97%). The predominance of *Bifidobacterium* in the lactose-adapted inoculum suggested that lactate might be the product of an initial fermentation of the total carbohydrates (glucose, fructose, and lactose) at the initial phase of the co-digestion process.

The presence of species such *Clostridium*, *Lactobacillus*, *Klebsiella*, and *Enterobacter*, known as hydrogen producers [29–32], suggests a symbiotic relation with *Bifidobacterium* sp.

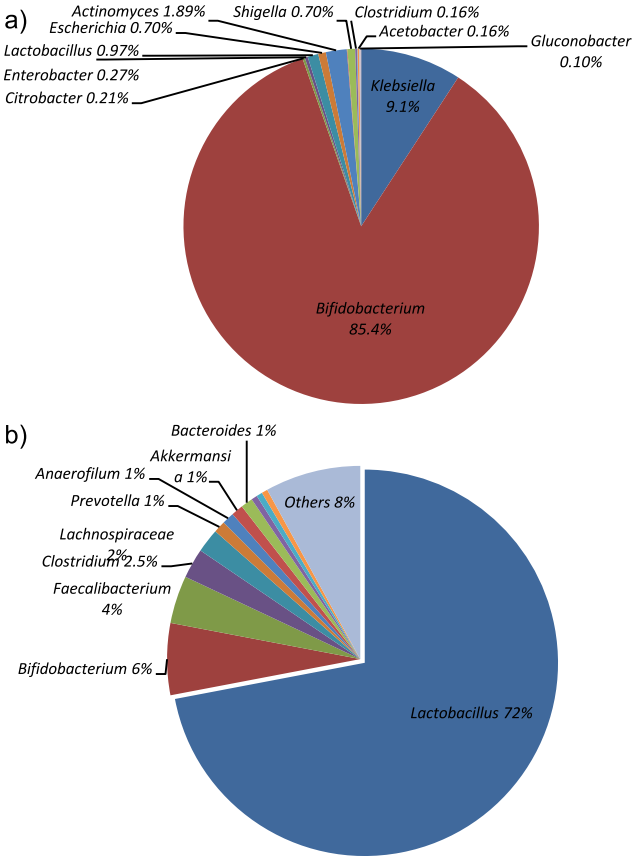

Fig. 2 – Genus level distribution of the bacterial population in the a) Original inoculum from the anaerobic digester system [20] and b) Lactose-adapted inoculum used for the co-digestion process.

Thus, their presence in the analysis supports the observed production of biohydrogen in the present study.

Similar behaviors were found in starch hydrolysis [33], where *Bifidobacterium* sp. represented 40–60% of the total microbial population, whereas *Clostridium* sp. constituted only around 40%. The authors have suggested a symbiotic relation: *Bifidobacterium* sp. initially breaks down the starch into small molecules which then are utilized by the *Clostridium* species during biohydrogen production [33]. Doi et al. [34] concluded that *Clostridium* sp. might generate biohydrogen not only directly from hexoses, but also from acetic acid and lactic acid produced by rhizosphere-originated lactobacilli, *Bifidobacterium* sp.

As regards with changes in the microbial population present in both, the initial inoculum (adapted to the organic wastes) and the CCW adapted microbial population, it has been reported that changes in culture conditions can modify bacterial diversity [8]. Moreover, factors such as inoculum pretreatment and substrate composition may act as an enhancer for certain population within the culture. For example, *Kim* et al. [35] reported that heat treatment causes a change in the microbial community composition of a fresh culture. Most of the species found in the fresh sludge were affiliated to *Lactobacillus* sp. and *Bifidobacterium* sp. In contrast, *Clostridium perfringens* was found in the heat-treated sludge. More recently,

Table 1 – Physical and chemical characterization of CCW and FVW.

Parameter	CCW	FVW
Soluble protein (μg/mL)	739.46 ± 4.6	371.43 ± 2.24
Total carbohydrates (g/L)	28.88 ± 0.392	0.64 ± 0.03
Chemical oxygen demand (mg/L)	67,880 ± 737	144,133 ± 5292
Total nitrogen Kjeldalh (g/L)	4.18 ± 0.68[wb]	1.43 ± 0.23 (g/kg)[wb]
Total organic carbon (%w/w)	2.95 ± 0.17	15 ± 2.5
C/N ratio	7	104
Lactate (g/L)	6.18 ± 0.59	0.142 ± 0.014
Acetate (g/L)	1.21 ± 0.15	BDL
Ethanol (g/L)	1.46 ± 0.04	BDL
Density (g/mL)	1.005 ± 0.013	0.919 ± 0.19
Total solids (g/L)	45.5 ± 0.99	64.9 ± 1.55
pH	4.5	5.27
C% (w/w)	52.77 ± 3.38	53.4 ± 9.5
N% (w/w)	4.59 ± 0.23	2.13 ± 0.03
O% (w/w)	30.93 ± 2.68	39.7 ± 8.61
P% (w/w)	0.60 ± 0.13	0.18 ± 0.04
S% (w/w)	0.16 ± 0.01	0.16 ± 0.03
Na% (w/w)	2.27 ± 0.19	0.09 ± 0.01
Mg% (w/w)	0.11 ± 0.00	0.09 ± 0.03
K% (w/w)	2.20 ± 0.29	2.17 ±0 .02
Ca% (w/w)	0.45 ± 0.10	0.12 ±0 .02
Fe (g/L)	BDL	0.752
Zn (g/L)	2.15	0.40

[wb] Wet basis.
BDL: below detection limit.

other studies on the co-digestion process of cassava stillage with organic wastes indicated that the addition of small amounts of the co-substrate alters the bacterial community in the system, with the consequent growth and enrichment of hydrogen-producing bacteria such as *Clostridium cellulosi* and *Thermoanaerobacterium thermosaccharolyticum* [22].

Feedstock characterization

Results from physical and chemical characterization of the FVW and CCW are listed in Table 1. Total solids in FVW showed higher total solids (64 g/L), 2.2 times higher than that in the CCW (28.6 g/L). Density and pH values were similar for both substrates. The chemical oxygen demand (COD) and the total organic carbon (TOC) concentrations for the FVW were 144,133 ± 5292 mg/L and 15 ± 2.5% w/w, respectively. Those values were 2.1 and 2.5 times higher than the ones obtained for the CCW (67,880 ± 737 g/L and 2.95 ± 0.17% w/w, respectively). On the other hand, the nitrogen content was quantified as total nitrogen Kjeldalh (TNK) and soluble protein. TNK was 34.21% lower in FVW compared to the TNK obtained for the CCW. Similarly, the CCW soluble protein was higher than the one determined for the FVW. These properties resulted in very different C/N ratios of 7 and 104 for CCW and FVW, respectively. These C/N values are not adequate for the co-digestion process, since optimum C/N vales are between 15 and 20 according to the values cited in the literature [36].

Micronutrients and trace elements were also determined in the FVW and CCW samples (Table 1). Both substrates showed high concentrations of macro-nutrients (Mg, Na, K, and Ca) in values between 900 and 227,000 mg/kg. Similar concentrations of Mg (0.11 ± 0.00 and 0.09 ± 0.03% w/w) were determined in the CCW and FVW samples, respectively. The high content of Mg in the CCW was due to high molecular substrates (e.g., protein and vitamins). Soluble inorganic salts as sodium (Na), potassium (K), and calcium (Ca) dissolved in the liquid fraction were also higher in the CCW sample (2.27 ± 0.19, 2.20 ± 0.29, and 0.45 ± 0.10% w/w, respectively). Only potassium was found at a higher concentration in the FVW (2.17 ± 0.02% w/w) when compared to the CCW sample. In particular, most inorganic elements were higher in CCW than in the FVW. However, trace elements such as iron (Fe) were not detected in CCW, whereas, in the FVW, Fe had a value of 0.752 g/L. Iron is an important micronutrient for biohydrogen fermentation, as it is a constituent of hydrogenase enzymes [37]. Iron limitation negatively affects the hydrogenase activity, which could cause a deviation in the fermentation pathways towards the production of more reduced end products, and reducing the biohydrogen production [38]. Macronutrients and trace elements play important roles in biohydrogen production although they are required in minimum quantities by several enzymes and co-enzymes for their function [36].

Both macro and micronutrients are essential for the anaerobic digestion process and their deficiencies have been shown to cause problems in the microbial degradation chain [39]. Poor conversion efficiency and even process failure have been reported as due to deficiency in nutrient supply to anaerobic digestion processes [40]. Reports on co-digestion studies (food waste with cow manure) have shown that supplementation with trace elements increases the anaerobic digestion activity.

The results indicate that CCW has high nitrogen content whereas FVW has high carbon content, thus, their complementary characteristics could provide an adequate combination in a co-digestion process with optimal C/N ratio, which in turn could improve the biohydrogen production.

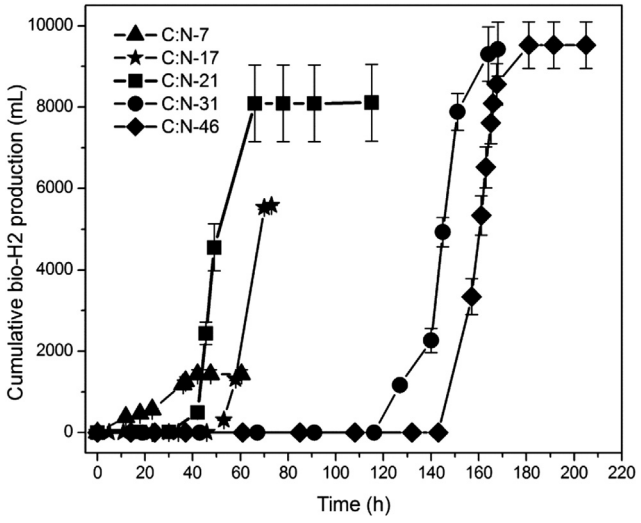

Fig. 3 – Cumulative biohydrogen production in batch experiment at different C/N ratios of CCW and FVW mixed for the co-digestion system: C/N 7 (▲), C/N 17 (★), C/N 21 (■), C/N 31 (●), and C/N 46 (◆).

Table 2 – Gompertz equation coefficients for the biohydrogen production at the different C/N ratios evaluated.

Ratio C/N	$P_{H_2,max}$ (mL)	R_m (mL h^{-1})	λ (h)	R^2
7	3832.06	248.30	23.94	0.99
17	6060.87	475.13	55.17	0.99
21	8121.93	663.74	41.94	0.99
31	9561.35	614.35	136.55	0.99
46	9622.05	682.00	152.44	0.99

Effect of different C/N ratios when using CCW and FVW for biohydrogen production

Fig. 3 shows the effect of the C/N ratio on the cumulative biohydrogen production during co-digestion of CCW and FVW in the mesophilic batch reactor. Experiments with CCW as only substrate showed that biohydrogen production was low when C/N = 7, i.e., 3832.06 mL. The biohydrogen production behavior for the C/N = 7 ratio treatment showed a second lag-phase. Other studies have observed also this second lag-phase and authors mention that it is due to the rapidly changing environment after the initial biohydrogen production phase [41,42]. The increment in acid concentration in the medium promotes the production of undiscounted weak acid that could pass through the cell affecting the glycolytic enzymes and reducing growth. Thus the microorganism should activate a response mechanism to facilitate the reestablishment of cell growth and biohydrogen production [41,42]. It could be explained also by the low buffer capacity of cheese whey, which leads to rapid acidification and inhibition of activity [31,43]. Some studies have demonstrated that the use of sludge, as the only substrate, produces significant low biohydrogen concentrations due to the high proteinaceous composition of this substrate [17]. The low biohydrogen production obtained with the CCW revealed the need of using a co-digestion process. When the CCW system was supplemented with FVW, an improvement in overall process efficiency was observed. On the other hand, when only FVW was used as single substrate (C/N ratio = 46) the biohydrogen production was initiated many days after those obtained when CCW and FVW were combined. This fact suggests a lack in proteinaceous nutrients in the FVW, which are essential for biohydrogen production. Additionally, the behavior of the system when using higher C/N ratios could be partially explained by the low buffer capacity or low amounts of alkalinity that frequently lead to acidification and inhibition of activity when using vegetable wastes [44].

Fig. 3 shows that the cumulative biohydrogen production was increased from 3832 to 9266 mL when FVW in the mixture increased from 25 to 75% (v/v) (C/N ratios of 17, 21, 31, and 46, respectively). These values were 1.5, 2.1, 2.9, and 2.5 times higher compared with the one obtained with the only CCW system (C/N ratio = 7). These data are similar to other reported studies performed in co-digestion process of cow manure and milk waste and swine manure and vegetable waste [13,45]. CH_4 was not detected in any of the treatments during the whole fermentation period.

The kinetic parameters were obtained from experimental data of each evaluated system by using the Gompertz model (Equation (1)). The biohydrogen production behavior for all treatments was adequately described by the model with correlation coefficients (R^2) of around 0.99 (see Table 2). The lag time ranged from 23 to 152 h, depending on the C/N ratio. By increasing the FVW concentration in the mixture, an increase in the phase lag of biohydrogen production was observed (C/N ratios = 31 and 46). The higher lag phase was determined at C/N ratios of 31 and 46, which might be attributed to the presence of cellulosic material. The cellulosic material should be initially hydrolyzed to be used as substrate. Supporting this fact, Lee et al. [29], reported that the hydrolysis of cellulose becomes the limiting step in some anaerobic digestion processes.

The different C/N ratios had also an effect on biohydrogen production rates. The highest volumetric biohydrogen production (VHPR) was obtained at a C/N ratio = 21 (10.68 mmol H_2/L*h) (see Table 3). This result could be explained by the positive synergism established with the mixture of CCW and FVW, which allowed an adequate nutrient balance and a natural control of the pH [13,45,46].

The maximum VHPR value obtained in the present study was higher than those reported in previous works where CCW was used as only substrate in anaerobic digestion processes. Ferchichi et al. [42] used diluted crude cheese whey in batch experiments obtaining a VHPR of 9.4 mmol H_2/L*h. In another study, Davila-Vazquez et al. [47] performed batch experiments using cheese whey powder (CWP) and reported a VHPR of 8.1 mmol H_2/L*h. Recently, Perna et al. [31] obtained a maximum VHPR of 1.3 mmol H_2/L*h using CWP. Some authors have found an enhancement in VHPR or H_2 yield in co-digestion processes, employing different feedstocks as substrates. Zhu et al. [17] found that a mixture of municipal food waste with sewage sludge improved biohydrogen production as compared with the digestion of the single components wastes. Tenca et al. [45] reported a maximum VHPR of 4.9 mmol H_2/L*h using a proportion of 35% FVW with 65% swine manure (SM). These results are explained by an optimal balance in the mixture between carbohydrates from FVW and

Table 3 – Summary of experimental results of volumetric production hydrogen rate (VPHR), total productivity, biohydrogen yields, and organic matter removal percentage at different C/N ratios.

Ratio C/N	VPHR max. (mmol H_2 L^{-1} h^{-1})	Total productivity (mmol H_2 L^{-1} h^{-1})	Biohydrogen yield (mL H_2 g COD^{-1})	Organic matter removal (%)
7	1.32	0.60	144.49	8.31
17	6.21	1.36	325.80	6.68
21	10.68	2.16	449.84	16.36
31	9.40	0.99	341.04	22.74
46	10.45	0.93	330.13	21.29

the alkali/nutrients supply from SM at this mixing ratio. Authors demonstrated that the alkalinity and the ratio of total VFA and the total alkalinity ($gCaCO_3/kg$) showed a close correlation with the substrate proportion and with the higher biohydrogen production when the organic acids production was successfully equilibrated by alkaline species.

The product yields and total productivities obtained for each C/N ratio varied significantly (Table 3). Lower productivities were obtained for C/N ratios of 7 and 19. These results could be explained by the higher amount of protein compounds present in the culture medium. At the higher C/N ratios (31 and 46), the cumulative biohydrogen production was higher than that obtained at lower C/N ratios; however, lower productivities were obtained due to longer fermentation times (ratio 31 and 46). These results indicate that the high cellulosic material content needs to be previously hydrolyzed and then consumed for biogas generation. At the optimal C/N ratio for biohydrogen production of 21, the overall productivity was 2.16 mmol H_2/L*h.

Biohydrogen yield and organic matter removal

The biohydrogen yield increased from 144 to 449 mL H_2/g COD by increasing the C/N ratios from 7 to 21. For C/N ratios from 31 to 46, the biohydrogen yields were of 441.04 and 330.13 mL H_2/g COD, respectively. The biohydrogen yield increment suggested that the mixture of CCW with FVW has higher biohydrogen production potential as compared with those obtained with these substrates separately as only feedstock. The lower biohydrogen yield at higher C/N ratios (31 and 46) can be explained mainly as due to the metabolites accumulation or over-load of the system.

The highest biohydrogen yield (449.84 mL/g COD) was obtained at C/N ratio = 21. These results suggest that biohydrogen yield is a function of the C/N ratio, i.e., the proportion of both substrates (CCW and FVW) in the mixture. When comparing the maximum biohydrogen yield obtained at the 21C/N ratio with previous studies of co-digestion for different substrates, in batch experiments, this is higher than that reported by Tawfik and El-Qelish [11] who found a biohydrogen yield of 245 ± 131 mL H_2/COD_{total} for a co-digestion system of municipal food waste and kitchen wastewater. Radjaram and Saravanane [18] reported a maximum biohydrogen yield of 40.6 ml H_2/COD in a co-digestion process of pressed mud with sewage in a UASB reactor system. The differences in biohydrogen yield obtained in the present work and previous studies may be attributed to several factors such as: 1) characteristics of feedstock, 2) microbial communities, 3) different pH conditions, 4) inoculum source and pretreatment, and 5) the conditions under which the anaerobic co-digestion were performed in each study.

Regarding organic matter removal, it was observed that the higher the C/N ratio, the higher the removal percentage (up to 22.7%). These values agreed with the previously reported range (17–40%) [45].

Metabolic profiles obtained in different experimental systems

The total carbohydrates concentration profiles (lactose, glucose, and fructose), as well as the soluble protein in the bulk volume, were determined for all the co-digestion systems at different C/N ratios (7, 17, 21, 31, and 46) (Fig. 4a and b). These easily degradable-carbohydrates were rapidly consumed during the first 40 h of culture time, as shown in Fig. 4. In all reactors, the total carbohydrates consumption (lactose, glucose, and fructose) was almost completed in a range of 96–100% at different culture periods between 18 and 22 h for the lowest C/N ratios and around 40 h for the highest C/N ratios. These results are in agreement with other studies using ethanol fermentation residues, where maltose was rapidly consumed during the first 20 h and there was no biohydrogen production until 18 h of experiment [30]. During the first hours of culture, corresponding to the initial carbohydrates consumption stage, no biohydrogen was produced but in the lowest C/N ratio tested (C/N = 7). Data suggest that the initial consumption of the easily degraded substrates (mainly lactose, glucose or fructose) allowed an initial metabolic activity then the hydrolysis of more complex substrate will support the biohydrogen production.

The soluble-protein measured in the bulk liquid of all treatments is shown in Fig. 4b. In the first hours of the co-digestion process there was a decrease of the soluble-protein concentration. This was associated with the consumption of easy assimilation carbohydrates, but after at 20 h of culture time, there was an increment of soluble-protein concentration in the liquid medium. The high concentration of soluble-protein in the bulk liquid could be correlated with hydrolytic activity. Similar behavior has been reported with vegetable and flower wastes where a maximum hydrolytic activity of cell-free extracellular enzymes (i.e., amylase, protease) occurred at 48 h of culture time [4,15,48]. In another study in which organic solid wastes were utilized, hydrolytic activity was also observed after 12–14 h culture [49]. Microorganisms produce and excrete hydrolytic enzymes such as amylases, cellulases, proteases, and lipases to break down and solubilize the macromolecular structures into soluble matter such as sugars, amino acids, and glycerol, as well as long-chain fatty acids to facilitate transport through the cell membrane [50].

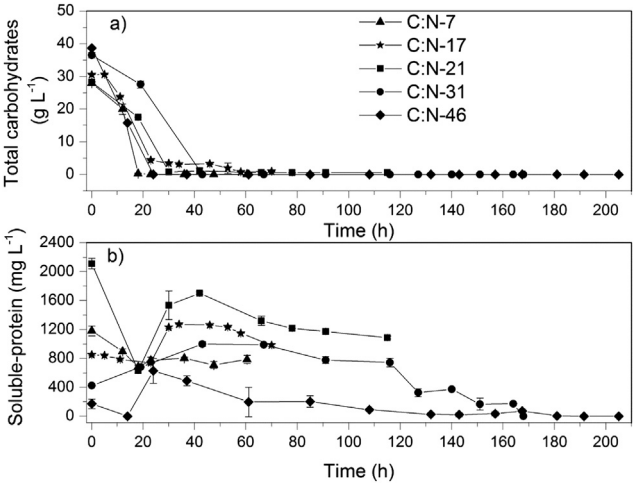

Fig. 4 – Behavior of total carbohydrates and soluble-protein in the co-digestion system.

Fig. 5a, b, and c show the results of the volatile fatty acids (VFA) determined for all treatments. The lactate concentration was increased at the early stage in a range from 1.5 to 2.5 g/L for all the C/N ratios evaluated (Fig. 5a). A similar behavior was obtained for acetate concentration during the first hours of operation. Then, the lactate and acetate concentrations decreased, whereas the concentration of butyric acid increased in a range from 1.8 to 2.5 g/L during the last operation period of the different treatments. We could postulate that during the first 50 h the consumption of lactose from CCW generates lactic acid as an end fermentation product. Simultaneously, fermenters used the FVW to produce acetate. Then, acetate and lactate are consumed by *Clostridium*. Under such conditions, *Clostridium* triggers its metabolism for growth using the most energetically favorable pathway, which implies production of butyrate and biohydrogen.

The overall results suggest that the initial assimilation of easily degradable carbohydrates allows cell growth with the consequent production of acetate and lactate, since both organic acids are produced by cells in order to keep redox balance (lactate) and ATP production (acetate-butyrate). The simultaneous consumption of lactate, acetate and more complex substrates (from FVW and CCW) finally support the highest biohydrogen production, at different cultures time which is a function of the content of FWV or CCW.

The highest biohydrogen production rate occurred at the maximum rates for acetate and butyrate production and lactate consumption. Such behaviors have been explained through substrate competition by interactions of hydrogen-producing bacteria with lactic acid bacteria [51]. Azbar et al. [52] used cheese wastewater for the biohydrogen production. In this work lactose (83% of the cheese whey) was converted to lactic acid by the mixed culture rather than acetic formation. Lactate is a key intermediate of lactose fermentation. On other hand, Lee et al. [29] found that fruit kitchen wastes were fermented to lactate and reached a lactate concentration at about 4 g/L. In addition, it has been shown that lactose is degraded to lactate by *Clostridium thermolacticum* [53]. Similar results were observed for lactate production, which was produced and consumed afterwards [41]. Other studies have reported that lactate and acetate can be consumed by *Clostridium tyrobutyricum*, *Lactobacillus bifermentans* [29,31,32,54]. Thus, increasing levels of butyrate and generation of biohydrogen is associated with the consumption of both lactate and acetate [30]. Likewise, Grause et al. [55] showed that biohydrogen production from lactic acid required the presence of acetate. In the absence of acetate, cells have to provide first acetate by other metabolic processes.

Ammonia production and its effect on pH of the system at different C/N ratios

Biohydrogen dark fermentation systems require strong medium buffer capabilities to resist pH changes caused by the produced organic acids. Fig. 6 shows the pH changes occurring

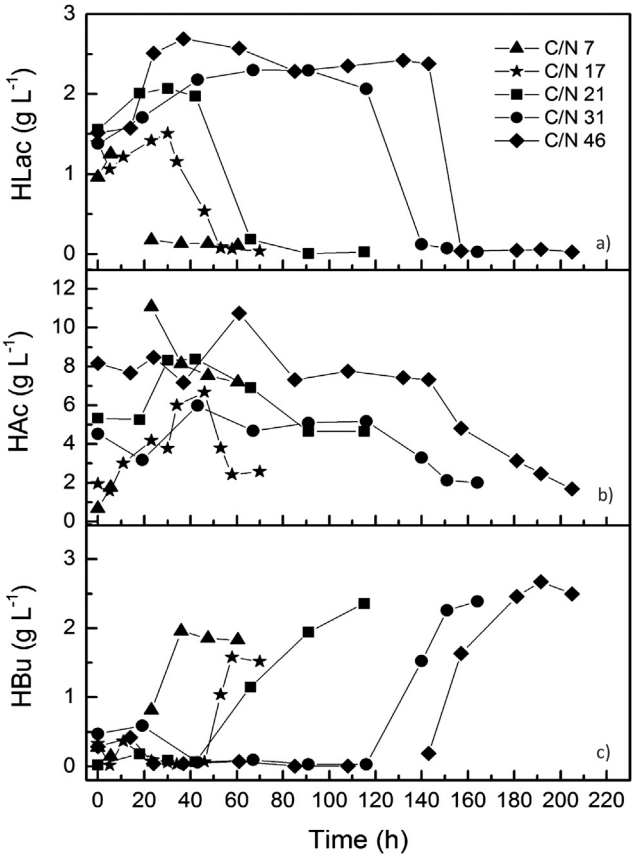

Fig. 5 – a) Lactate, (HLac), b) acetate (HAc), and c) butyrate (HBu) generation during the biohydrogen production process at different C/N ratios.

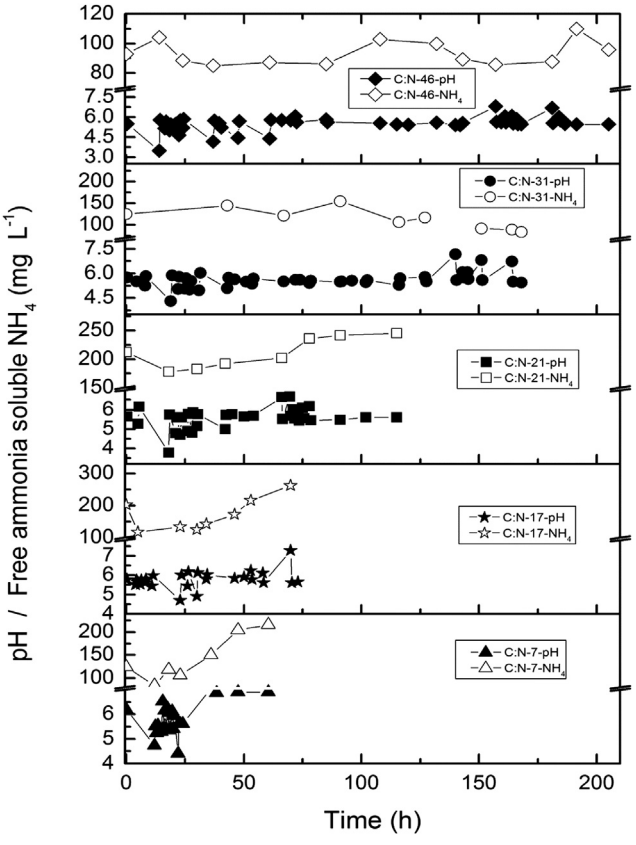

Fig. 6 – pH and free ammonia generated profiles in biohydrogen reactors.

and generation of free ammonia (NH_4) throughout the co-digestion process. During the lag phase (λ) (from 15 to 130 h), the pH drops below 5. Nevertheless, pH was raised between 6 and 7 units at the last co-digestion period. For example, the final pH was similar for 7, 17, and 21C/N ratios. An increase in the amount of free ammonia was observed in a range from 80 to 250, 100 to 300, and 170–275 mg/L for C/N = 7, 17, and 21, respectively. These results could be explained by ammonia (NH_4) production. CCW is rich in proteins that can supply a large amount of ammonia ions when they are hydrolyzed [56]. This ammonia could be utilized to mitigate pH drop and to neutralize acidic materials to reduce the acidification during the fermentation process [57]. In other studies, the buffer capacity was increased when an activated sludge fraction was added to food waste in the mixture [17]. On the other hand, adding sewage into vegetables wastes showed a positive effect on the buffer capacity of the system [44]. Therefore, VFA accumulation and the corresponding pH drop might be other reasons that contributed to a lower biohydrogen production at higher CCW or FVW ratios.

Conclusions

In this work the feasibility of the biohydrogen production by co-digestion process of crude cheese whey with fruit vegetables wastes was determined. The results showed that biohydrogen production was enhanced by the co-digestion. The highest overall productivity of biohydrogen (2.16 mmol H_2/L*h), yield (449.84 mL H_2/g COD), and the maximum volumetric H_2 production rate (10.56 mmol H_2/L*h) were all obtained at a C/N ratio of 21.

The enhanced H_2 production was a result of an optimum C/N ratio adjusted by the adequate CCW and FVW proportion. This mixture showed a buffer effect.

Bacterial 16s rRNA gene analysis showed an important change in microbial populations during the adaptation period. This change positively affects the biohydrogen production. *Bifidobacterium* was predominantly detected at 85.4%. The metabolic profile was correlated with the microbial community distribution. *Bifidobacterium* produce lactic acid, while other microbial population assimilated it to produce acetic and butyric to finally produce biohydrogen.

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