

BIOHYDROGEN FROM CHEESE WHEY TREATMENT IN AN AnSBBR: ACHIEVING PROCESS STABILITY

D. M. F. Lima¹, R. K. Inoue¹, J. A. D. Rodrigues^{2*}, S. M. Ratusznei² and M. Zaiat¹

¹Escola de Engenharia de São Carlos, Universidade de São Paulo (USP),
Av. Trabalhador São-Carlense 400, CEP: 13.566-590, São Carlos - SP, Brasil.
²Escola de Engenharia Mauá, Instituto Mauá de Tecnologia, (IMT), Praça Mauá 1,
CEP: 09580-900, São Caetano do Sul - SP, Brasil.
E-mail: rodrigues@maua.br

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Abstract - An AnSBBR (anaerobic sequencing batch reactor containing biomass immobilized on an inert support) with liquid phase recirculation, containing a 3.5 L working volume, treated 1.5 L of cheese whey wastewater in 3 and 4 h cycles at 30 °C to produce biohydrogen. From startup the bioreactor presented process instability. To overcome this problem the following measures were taken, however without success: adaptation of the biomass with uncontaminated easily degradable substrates, pH control at very low levels, and a different form of inoculation (natural fermentation of the feed medium). The problem was solved by cooling the feed medium to 4 °C to prevent acidification in the storage container, by eliminating nutrient supplementation to prevent possible formation of H₂S by sulfate-reducing bacteria and by periodic washing of the support material to improve the food/microorganism ratio. Hence, stable hydrogen production could be achieved with minimal presence of methane (36% H₂; 62% CO₂; 2% CH₄) and the AnSBBR fed with cheese whey (influent concentration of 4070 mgCOD.L⁻¹ and 3240 mgCarbohydrate.L⁻¹ and applied volumetric organic loading of 14.6 gCOD.L⁻¹.d⁻¹) presented improved productivity and yield indicators compared to pure lactose and other reactor configurations, reaching values of 420 NmLH₂.L⁻¹.d⁻¹ and 0.60 molH₂.molCarbohydrate⁻¹ in the steady-state phase (conversions of carbohydrates and COD were 98% and 30%, respectively).

Keywords: Biohydrogen; Cheese whey; AnSBBR; Process stability; Selective pressure.

INTRODUCTION

Fossil fuels are non-renewable sources of energy and are becoming increasingly scarce. In addition, the combustion products contribute to increasing global warming. Therefore, researchers have been looking for alternative sources of energy to replace fossil fuels, including thermonuclear, solar, wind, and geothermal energy. However, hydrogen remains one of the best options due to its unique characteristics compared to other fuels (Veziroglu & Sahin, 2008). Hydrogen is considered to be a clean fuel because

when combusted only water is released as the end product. In addition, in comparison with hydrocarbons, 2.75 times more energy can be released (Sinha & Pandey, 2011).

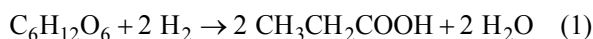
Biological production of hydrogen utilizes organic material present in wastewaters and can be considered to be ecofriendly because no fossil fuels are consumed in the process. (Ren *et al.*, 2007). Typically, two paths are used for obtaining biohydrogen: photosynthesis and fermentation; the latter is technically simpler, with advantages such as a high hydrogen production rate, no direct ATP supply for

*To whom correspondence should be addressed

hydrogenase activity and low costs (Ntaikou *et al.*, 2008).

The use of whey as the source for biohydrogen production was primarily motivated by the large production figures; according to the ADPI (American Dairy Products Institute), the global production of liquid whey from cheese and casein amounted to 186 million metric tons (MT) in 2008. Moreover, even with the technological possibility of using this compound as a byproduct, this may not be economically feasible, especially for small and medium producers, resulting in the disposal of this product; disposal can be a serious problem due to whey's high biodegradability (Mawson, 1994; Calli & Yukselen, 2004).

Several factors can cause instability in hydrogen production. According to Speece (1996), anaerobic digestion is a complex process involving several types of microorganisms and intermediate phases. The complex organic components (i.e., polymers) present in the substrate are hydrolyzed into simpler compounds (monomers). These are metabolized by fermentative bacteria and converted into volatile fatty acids, alcohols, carbon dioxide and hydrogen. The volatile fatty acids with more than two carbons are converted to acetate and hydrogen gas by acetogenic bacteria. Finally, acetate and hydrogen gas are converted into methane by the acetoclastic or hydrogenotrophic methanogenic archaea, respectively. When nitrate and sulfate are present in the environment, sulfate-reducing and nitrate-reducing bacteria are able to use the hydrogen produced as an electron donor to form sulfide and ammonia, respectively (Valdez-Vazquez *et al.*, 2005). Another possibility is the development of microorganisms that produce propionic acid instead of acetic or butyric acid. Vavilin *et al.* (1995) presented a general equation for the propionate production from hexose, showing that this route involves the consumption of hydrogen (Equation (1)):



Lastly, homoacetogenic microorganisms, which are capable of using the Wood-Ljungdahl pathway, may develop (Tanner *et al.*, 1993). Among these *Clostridium ljungdahlii* (Tanner *et al.*, 1993; Köpke *et al.*, 2010) and *Clostridium carboxidivorans* (Liou *et al.*, 2005) should be mentioned, which are capable of growing heterotrophically in sugars (hexoses and pentoses) or autotrophically (Wood-Ljungdahl pathway) in gases (CO or CO₂ + H₂). As previously mentioned, hydrogen is an intermediate product in several biological processes and may be consumed in

the liquid medium, thus requiring inhibition of this consumption.

Anaerobic sequencing batch processes appear to be suitable treatment solution in specific cases, such as the treatment of intermittently generated industrial effluents containing poorly degradable compounds or in the case of very restrictive discharge standards. In these cases, the desired removal can be guaranteed by adjusting the operating parameters of the reactor (Zaiat *et al.*, 2001). There are also other advantages regarding the simple configuration of the reactor, such as improved effluent quality control, the non-existence of hydraulic short circuit, no need for stabilization of the process in view of the discontinuous character of the process, and the absence of an influent distribution system. A typical cycle of the anaerobic reactor operated in batch and fed-batch mode consists of four steps: (i) feeding, which may have a variable filling time, defining the feed strategy as batch and/or fed-batch; (ii) the treatment itself, consisting of biotransforming the wastewater constituents by microorganisms; (iii) sedimentation, when the biomass is in a granular form (ASBR), although when the biomass is immobilized on an inert support (AnSBBR) this step is not necessary; and (iv) discharge, consisting of removal of treated and clarified liquid (Zaiat *et al.*, 2001; Dague *et al.*, 1992; Fernandes *et al.*, 1993).

Within this context, the present study strived to achieve, through implementation of several operational strategies, stable production of hydrogen from cheese whey wastewater, as well as to minimize methane production, using an anaerobic sequencing batch reactor containing biomass immobilized on an inert support (AnSBBR). Mixing of the liquid medium (substrate) and the biomass (microorganisms) was accomplished by recirculating the liquid phase. The different operating strategies used were: addition of hydrochloric acid in the feed medium; adaptation of the biomass with sucrose and lactose, pure and highly biodegradable carbohydrates; different forms of inoculation; use of skimmed milk to assess the presence of contaminants in the cheese whey; refrigeration of the feed medium; removal of micronutrients and urea from the feed medium; and periodic washing of the biomass support material.

The bioreactor and the experimental protocol adopted were chosen to investigate the technological options of discontinuous operation as an alternative to the commonly used continuous operation, to assess the main advantages related to: better operational control (load–reaction–discharge); flexibility in feeding strategy; and suitability to different concentrations of wastewater available for biogas

generation in “small scale” production units. An additional goal was to assess the main limitations of this reactor related to: the possibility of over-load and resulting microbial inhibition; lower productivity due to sequencing charge–discharge operations; and the transient behavior inherent in batch operation.

MATERIAL AND METHODS

AnSBBR with Recirculation of the Liquid Phase and Immobilized Biomass

Figure 1 shows the experimental system proposed for the production of biohydrogen from the anaerobic treatment of whey. The reactor consisted of a cylindrical acrylic flask with the following dimensions: height of 540 mm, external diameter of 100 mm and wall thickness of 3.5 mm. The lower part of the reactor consisted of a compartment with height of 40 mm designed to facilitate wastewater distribution. At the top of the reactor a compartment with a height of 60 mm was used as biogas collector (H_2 , CH_4 and CO_2). The total reactor volume was 4.3 L (empty volume).

The support for biomass immobilization was placed between stainless steel (grade 314) screens,

covered at the top by PVC screens, dividing the reactor into five compartments to prevent compaction of the bed and block passage of particles between the support compartments.

The recirculation system was composed of: (i) a lateral reservoir, which contained a volume of 2.9 L (empty volume), consisting of a cylindrical acrylic flask with the following dimensions: height of 430 mm, external diameter of 100 mm and wall thickness of 3.5 mm; and (ii) a peristaltic pump with adjustable flow, brand Ismatec, MCP model with maximum flow of $52.3 \text{ L}\cdot\text{h}^{-1}$. A 100 mL graduated cylinder was connected to the recirculation system to measure the recirculation flow. Hence, the total volume, resulting from the sum of the reactor volume (4.3 L) and the reservoir volume (2.9 L), was 7.2 L.

A control unit was used to automate feeding, discharge and recirculation operations. Feeding and discharge were carried out by diaphragm pumps (Prominent[®], model Beta/5 with a capacity of up to $30 \text{ L}\cdot\text{h}^{-1}$ and Concept/0223 with a capacity of up to $23 \text{ L}\cdot\text{h}^{-1}$, respectively), aided by an automation system composed by timers (Coel[®], model RTST/20).

To maintain a constant operation temperature ($30 \pm 1 \text{ }^\circ\text{C}$), the system was kept in a chamber provided with resistances, coolers, fans, temperature sensor and controller (Lovato *et al.*, 2012).

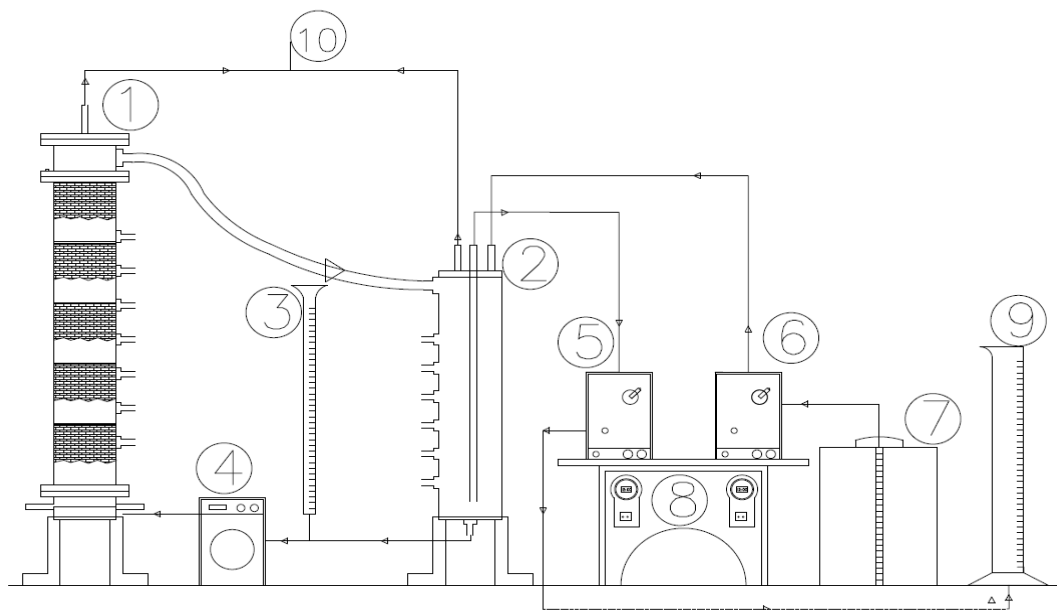


Figure 1: Scheme of the AnSBBR with recirculation used in the assays: 1) reactor containing immobilized biomass; 2) lateral reservoir; 3) flow meter; 4) recirculation pump; 5) discharge pump; 6) feeding pump; 7) storage container for the wastewater; 8) control unit; 9) measuring cylinder to collect the effluent of the reactor; 10) biogas exit.

Inoculation

The inoculum was obtained from previous hydrogen production studies (Manssour *et al.*, 2013; Santos *et al.*, 2014). The support material, along with the biomass grown in these reactors, were stored in a refrigerator and used in the present work. Only in one condition was a different inoculation used. This inoculum was obtained from natural fermentation of the feed medium, prepared with tap water and kept for three days in an open container. This process favored fermentation of the medium by microorganisms present in the atmosphere and from the tap water used (Leite *et al.*, 2008).

Support for Anaerobic Biomass Immobilization

Low-density polyethylene (LDPE) pellets, obtained from plastic waste recycling, were used as support for biomass immobilization. The particles were approximately 5 mm long with a diameter of 3 mm.

Apparent and real densities of the support material were calculated by immersing a known mass of this material in a measuring cylinder with known volume of water. An apparent density of 470 g.L⁻¹ and a real density of 825 g.L⁻¹ were encountered, allowing calculation of a bed porosity of 43% (Manssour *et al.*, 2013; Santos *et al.*, 2014).

Wastewater

The wastewater used in most conditions was formulated based on cheese whey (approximately 900 mg.L⁻¹ of cheese whey). Commercial dehydrated cheese whey powder was used to prepare the wastewater and, after dissolution with tap water, contained the following composition: carbohydrates (73.1%), proteins (11.2%), total fat (2.0%), fibers (0.0%), calcium (0.4%), sodium (0.7%), other minerals (7.6%), moisture (5.0%) and an energy value of 3540 kcal.kg⁻¹ (15040 kJ.kg⁻¹).

In some of the conditions investigated the substrate was changed in order to regain hydrogen production and inhibit methane production. The substrates used were sucrose and lactose (893 mg.L⁻¹ sucrose/lactose per mgCOD.L⁻¹).

In addition, in one of the experimental conditions sterilized skimmed milk was used, with the following composition (per 100 mL): 66 kcal of energy value; 9.9 g carbohydrate, 6.1 g protein; 263 mg sodium and 337 mg calcium (3600 mgCOD.L⁻¹ was equivalent to 1430 mgCarbohydrates.L⁻¹).

In some assays nitrogen, phosphorus and salts were supplemented to the wastewater (nitrogen at a

C/N ratio of 140 and phosphorus at a COD/P ratio of less than 500). This supplementation contained the following composition (Fontes Lima & Zaiat, 2012): urea/CH₄N₂O (11.5 mg.L⁻¹), NiSO₄.6H₂O (0.5 mg.L⁻¹), FeSO₄.7H₂O (2.5 mg.L⁻¹), FeCl₃.6H₂O (0.25 mg.L⁻¹), CoCl₂.2H₂O (0.04 mg.L⁻¹), CaCl₂.6H₂O (2.06 mg.L⁻¹), SeO₂ (0.036 mg.L⁻¹), KH₂PO₄ (5.36 mg.L⁻¹), K₂HPO₄ (1.3 mg.L⁻¹), Na₂HPO₄.H₂O (2.7 mg.L⁻¹) and NaHCO₃ (500 mg.L⁻¹).

Physical-Chemical Analysis

Reactor monitoring was performed by measuring the following in both influent and effluent samples: chemical oxygen demand (COD), total solids (TS), total volatile solids (TVS), total suspended solids (TSS), volatile suspended solids (VSS) and pH (APHA, 1998); partial alkalinity (PA), intermediate alkalinity (IA), total alkalinity (TA), bicarbonate alkalinity (BA) and total volatile acids (TVA) (Ripley *et al.*, 1986); and total carbohydrates (Dubois *et al.*, 1956).

The intermediate compounds of anaerobic metabolism (solvents: acetone, methanol, ethanol, n-butanol; volatile acids: acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids) were analyzed with an Agilent Technologies 7890 gas chromatograph equipped with a flame ionization detector (FID), automatic injection (head space), GC Sampler 80, and an HP-Innowax column (30 m × 0.25 mm × 0.25 μm). Hydrogen was used as carrier gas with a flow rate of 1.56 mL.min⁻¹. The injector temperature was 250 °C, injection volume 400 μL and split ratio 10. The oven temperature was programmed as follows: from 35 °C to 38 °C at 2 °C.min⁻¹, from 38 °C to 75 °C at 10 °C.min⁻¹, from 75 °C to 120 °C at 35 °C.min⁻¹, at 120 °C for 1 min, from 120 °C to 170 °C at 10 °C.min⁻¹ and at 170 °C for 2 min. The detector temperature was 280 °C with hydrogen flow (fuel) of 30 mL.min⁻¹, synthetic air flow (oxidant) of 300 mL.min⁻¹ and make up (nitrogen) flow of 30 mL.min⁻¹. The head space method was employed in these analyses, using as internal standard crotonic acid (for volatile acids determination) and isobutanol (for determination of acetone and alcohols).

The biogas composition generated via anaerobic degradation was analyzed by gas chromatography using an Agilent Technologies® 7890 gas chromatograph equipped with thermal conductivity detector (TCD) and GS-Carbonplot column (30 m × 0.53 mm × 3.0 μm). The carrier gas used was argon at 3.67 mL.min⁻¹, the injector temperature was 185 °C, the injection volume 200 μL and the split ratio 10. The oven temperature was programmed as follows: 40 °C

isotherm for 5 min. The detector temperature was 150 °C, with a makeup (argon) flow rate of 8.33 mL.min⁻¹.

Volumetric biogas production (V_G) was measured with a Ritter Milligascounter gas meter.

At the end of each experimental condition the biomass inside the reactor was quantified. Initially, the liquid inside the reactor was drained and this volume was measured (V_R). Next, all inert support containing biomass was removed from the reactor and measured (M_{T-IS+B}), this material was homogenized for a sample of inert support and biomass (M_{S-IS+B}). The pellets of this sample were “washed”, resulting in two parts, a solid phase of pellets and a liquid phase containing biomass from the reactor. In the solid phase the TS was calculated and in the liquid phase the TS and TVS. From the TS of the pellets (M_{S-TS}) and the TVS of the liquid phase (M_{S-TVS}) the biomass in the reactor was estimated; this information was presented in three different ways:

(i) The total amount of biomass in the reactor (M_{TVS} - Equation (2)): measures the ability of the reactor to retain biomass; this variable was also used in other indicators, such as the applied specific organic load (ASOL) and the daily specific molar productivity of hydrogen (SMPr).

$$M_{TVS} = \frac{M_{S-TVS} \cdot M_{T-IS+B}}{M_{S-IS+B}} \quad (2)$$

(ii) Relation between the amount of biomass and the volume of liquid medium in the reactor (C_X - Equation (3)): the biomass concentration in the reactor and the liquid volume treated per cycle enable the measurement of the ratio between the amount of liquid medium available for treatment and the biomass involved in the biotransformations inherent to the biological process under investigation (C_X).

$$C_X = \frac{M_{TVS}}{V_R} \quad (3)$$

(iii) Relation between the amount of biomass and the amount of support material in the reactor (C_X' - Equation (4)): the biomass concentration and the mass of inert support in the reactor enable the measurement of the ratio between the quantity of inert support available for immobilization/retention of the biomass and the biomass involved in the biotransformations inherent to the biological process under investigation (C_X').

$$C_X' = \frac{M_{S-TVS}}{M_{S-TS}} \quad (4)$$

Experimental Procedure

The assays performed in the AnSBBR with recirculation are summarized in Table 1. After adding the inert support and inoculum to the reactor, the operation was carried out as follows: in the first cycle the system was fed with 3.5 L (2.0 L in the reactor, and 1.5 L in the reservoir) in 20 min. Next, flow recirculation was started (20 Lh⁻¹ - recirculation rate of 0.2 cm.s⁻¹). At the end of a cycle, the recirculation flow was turned off and 1.5 L of the medium was discharged (in 10 min) and 2.0 L was maintained in the reactor (called residual volume), i.e., the total volume of the liquid medium in the system was 3.5 L. Immediately after this discharge, a new cycle began with feeding (with a constant flow of 1.5 L of medium) in 10 min, followed by recirculation of the liquid phase. At the end of the cycle, recirculation was again discontinued and the reactor discharged. Thus, the cycle was repeated, characterizing a sequencing batch.

Table 1: Characteristics of each condition (selective pressures) tested to achieve process stability.

Condition	Characteristics
1	Operated with an $AVOL_{CT}$ of 14.6 gCOD.L ⁻¹ .d ⁻¹ , t_c of 3 hours and addition of hydrochloric acid to the feed medium.
2	Operated with an $AVOL_{CT}$ of 10.9 gCOD.L ⁻¹ .d ⁻¹ and t_c of 4 hours, adaptation of the biomass was tested with sucrose, a pure and highly biodegradable carbohydrate.
3	Also operated with an $AVOL_{CT}$ of 10.9 gCOD.L ⁻¹ .d ⁻¹ and t_c of 4 hours, but adaptation of the biomass was tested with lactose instead of sucrose.
4	Operated with an $AVOL_{CT}$ of 14.6 gCOD.L ⁻¹ .d ⁻¹ and t_c of 3 hours, and a different form of inoculation was tested.
5	Operated with an $AVOL_{CT}$ of 14.6 gCOD.L ⁻¹ .d ⁻¹ , t_c of 3 hours, using skimmed milk as substrate and evaluating the presence of contaminants in the cheese whey.
6	Also operated with an $AVOL_{CT}$ of 14.6 gCOD.L ⁻¹ .d ⁻¹ and t_c of 3 hours; showed that refrigeration of the feed medium to avoid contamination of the influent in the storage container had no positive effect when performed alone.
7	Operated with an $AVOL_{CT}$ of 14.6 gCOD.L ⁻¹ .d ⁻¹ , t_c of 3 hours and with a set of strategies (refrigeration of the feed medium, removal of micronutrients and urea in the feed medium, plus periodic washing of the support material containing biomass) which enabled achievement of process stability.

In some experiments, profiles were taken of the following: filtered organic matter concentration (carbohydrates and COD), bicarbonate alkalinity (BA), total volatile acids (TVA), intermediate metabolites (volatile acids and solvents), and biogas (composition and production), as well as pH. This enabled a better understanding of the metabolic pathways throughout a cycle. The samples for the profile were collected at 30 min intervals throughout the cycle. The total volume of the withdrawn samples did not exceed 350 mL, or less than 10% of the volume of the reaction medium.

Theoretical

The efficiencies of organic matter removal in the forms of COD and carbohydrates in unfiltered samples (ε_{ST} and ε_{CT}) were calculated by Equations (5) and (6), in which C_{CT} and C_{ST} are the organic matter concentrations in the forms of COD and carbohydrates of the unfiltered influent samples, $C_{CT,E}$ and $C_{ST,E}$ are the effluent organic matter concentrations in the form of COD and carbohydrates of unfiltered samples.

$$\varepsilon_{CT}(\%) = \frac{C_{CT} - C_{CT,E}}{C_{CT}} \cdot 100 \quad (5)$$

$$\varepsilon_{ST}(\%) = \frac{C_{ST} - C_{ST,E}}{C_{ST}} \cdot 100 \quad (6)$$

The efficiencies of organic matter removal in the forms of COD and carbohydrates in filtered samples (ε_{CF} and ε_{SF}) were calculated in a similar way by Equations (7) and (8).

$$\varepsilon_{CF}(\%) = \frac{C_{CT} - C_{CF,E}}{C_{CT}} \cdot 100 \quad (7)$$

$$\varepsilon_{SF}(\%) = \frac{C_{ST} - C_{SF,E}}{C_{ST}} \cdot 100 \quad (8)$$

The applied volumetric organic loads ($AVOL_{CT}$ or $AVOL_{ST}$), expressed in $\text{kgCOD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ and $\text{kgCarbohydrate} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, are defined as the amount of organic matter in the form of COD or carbohydrate applied to the reactor per unit time and per reactor medium. For batch reactors, they are calculated by Equations (9) and (10), in which V_F is the volume of wastewater fed to the reactor per cycle, N is the number of cycles per day and V_R is the total volume of wastewater contained in the reactor per cycle.

$$AVOL_{CT} = \frac{(V_F \cdot N) \cdot C_{CT}}{V_R} \quad (9)$$

$$AVOL_{ST} = \frac{(V_F \cdot N) \cdot C_{ST}}{V_R} \quad (10)$$

The applied specific organic loads ($ASOL_{CT}$ or $ASOL_{ST}$), expressed in $\text{kgCOD} \cdot \text{gSVT} \cdot \text{d}^{-1}$ and $\text{kgCarbohydrate} \cdot \text{gSVT} \cdot \text{d}^{-1}$, are defined as the amount of organic matter in the form of COD or carbohydrates applied to the reactor per unit time and per mass of total volatile solids in the reactor ($\text{gCOD} \cdot \text{gTVS}^{-1} \cdot \text{d}^{-1}$ or $\text{gCarbohydrate} \cdot \text{gTVS}^{-1} \cdot \text{d}^{-1}$). For batch reactors they are calculated by Equations (11) and (12), in which M_{TVS} is the mass of total volatile solids inside the reactor.

$$ASOL_{CT} = \frac{(V_F \cdot N) \cdot C_{CT}}{M_{TVS}} \quad (11)$$

$$ASOL_{ST} = \frac{(V_F \cdot N) \cdot C_{ST}}{M_{TVS}} \quad (12)$$

The daily molar productivity of hydrogen (MPr), expressed in $\text{molH}_2 \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, is defined by the ratio between the hydrogen produced per unit time and per volume of medium. It is calculated by Equation (13), in which n_{H_2} represents the daily molar production of hydrogen and V_R is the volume of medium in the reactor.

The daily specific molar productivity of hydrogen ($SMPr$), expressed in $\text{molH}_2 \cdot \text{kgSVT}^{-1} \cdot \text{d}^{-1}$, is defined by the ratio between the hydrogen produced per unit time and per mass of total volatile solids inside the reactor. It is calculated by Equation (14).

$$MPr = \frac{n_{H_2}}{V_R} \quad (13)$$

$$SMPr = \frac{n_{H_2}}{M_{TVS}} \quad (14)$$

The molar yields per applied load, defined by the ratio between the hydrogen produced per organic matter applied in the form of COD ($MYAL_{C,m}$) in $\text{molH}_2 \cdot \text{kgCOD}^{-1}$ and in the form of carbohydrate ($MYAL_{S,m}$) in $\text{molH}_2 \cdot \text{kgCarbohydrate}^{-1}$, are calculated by Equations (15) and (16), respectively.

$$MYAL_{C,m} = \frac{n_{H_2}}{N \cdot V_F \cdot C_{CT}} \quad (15)$$

$$MYAL_{S,m} = \frac{n_{H_2}}{N.V_F.C_{ST}} \quad (16)$$

The molar yields per removed load, defined by the ratio between hydrogen produced per organic matter removed in the form of COD ($MYRL_{C,m}$) in $\text{molH}_2.\text{kgCOD}^{-1}$ and in the form of carbohydrate ($MYRL_{S,m}$) in $\text{molH}_2.\text{kgCarbohydrate}^{-1}$, are calculated by Equations (17) and (18), respectively.

$$MYRL_{C,m} = \frac{n_{H_2}}{N.V_F.(C_{CT}-C_{CF,E})} \quad (17)$$

$$MYRL_{S,m} = \frac{n_{H_2}}{N.V_F.(C_{ST}-C_{SF,E})} \quad (18)$$

Washing Procedure of the Support Material During Reactor Operation

During Condition 7, systematic washing of the support material was performed for a better control of the amount of biomass present in the reactor to make up for the cell growth due to metabolic characteristics of the acidogenic microorganisms and the high organic applied/removed load (growth kinetics). Therefore, the aim was to improve stability and efficiency of biohydrogen production.

The first step of the procedure consisted of discharging the entire liquid medium contained in the reactor and measuring the volume, total solids and total volatile solids (m_{BL1}). After removing "inert support + biomass" contained in the reactor the total mass was measured. A small sample was collected to calculate the values of C_x and C_x' (relation between the amount of biomass and the volume of the liquid medium and the amount of support material in the reactor, respectively) in accordance with previously described methodology.

The next step was to carry out the washing of the "inert support + biomass" by adding 1 liter of tap water and separating the washing water using a sieve. A 50 mL sample was withdrawn from this washing water for analysis of total solids and total volatile solids to calculate the biomass removed from the system (m_{BL2}), in the same way as for the liquid discharged in the first step.

The amount of biomass removed by washing (in grams of TVS) was the sum of m_{BL1} (calculated by the concentration of the total volatile solids divided by the volume of the liquid discharged from the reactor) and m_{BL2} (calculated by the concentration of the total volatile solids divided by the volume of water

used for washing the system "biomass + inert support," i.e., one liter). From the values of C_x and C_x' before and after washing it was possible to estimate cell growth during the experimental condition.

RESULTS AND DISCUSSION

The operation of the AnSBBR with recirculation of the liquid phase (3.5 ± 0.1 L of working volume and treating 1.5 ± 0.1 L per cycle at 30 ± 1 °C) using cheese whey as substrate (4070 ± 390 mgCOD.L^{-1} and 3240 ± 310 $\text{mgCarbohydrate.L}^{-1}$) experienced, from the very beginning, problems to produce biohydrogen. In order to obtain stable production of H_2 , as well as to minimize the production of methane, seven conditions were performed with different operating strategies.

Under all conditions the efficiency of carbohydrate conversion was always high ($98 \pm 2\%$) and that of organic matter was always low ($30 \pm 3\%$). Among the intermediate products, the highest concentrations were acetic acid, butyric acid and ethanol.

Figure 2 shows the volume of total biogas produced and the daily molar production of each component. Thus, given that the operation with cheese whey for hydrogen production presented problems from the very beginning, several selective pressures were tested to achieve process stability (Table 1).

The reason for adding hydrochloric acid in Condition 1 was to create extreme pH conditions where the spore-forming hydrogen producing microorganisms would survive, while the non-spore forming methane-producing microorganisms would be eliminated (Kraemer & Bagley, 2007). However, this strategy had no effect and the production of CH_4 occurred even at very low pH values, contradicting findings in the literature (Luo *et al.*, 2011). It should be mentioned that the presence of methane, even at pH below 5.0, has been reported in other studies that used cheese whey in acidogenic reactors (Castelló *et al.*, 2009; Carrillo-Reyes *et al.*, 2012).

The strategies in which the biomass was acclimated with pure carbohydrates (sucrose and lactose - Conditions 2 and 3, respectively) also had no effect. It should be mentioned that during the period in which the reactor was fed with pure carbohydrate, there was stable production of H_2 without the presence of CH_4 . Acclimation of the biomass was performed by changing reactor feeding from pure carbohydrate to cheese whey straightforwardly.

In Condition 4 a different form of inoculation was used, consisting of the natural fermentation of the feed medium. In this strategy, following the

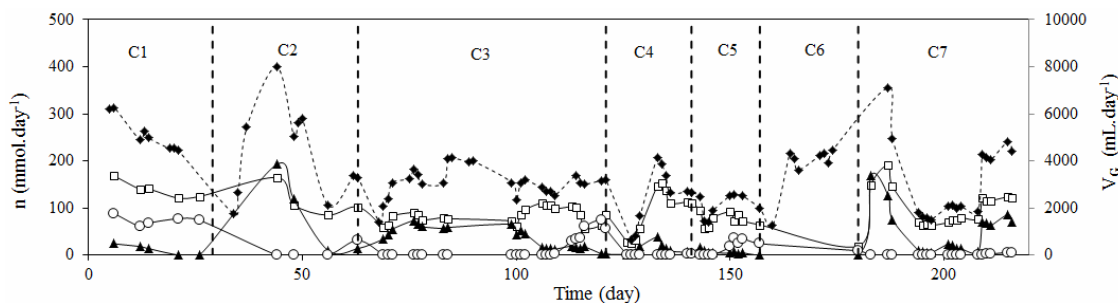


Figure 2: Volume of total biogas produced (---◆---) and daily molar production of each component of this biogas (□ – CO₂; ▲ – H₂; ○ – CH₄).

methodology proposed by Leite *et al.* (2008), four liters of feed medium prepared with 4250 mgCOD.L⁻¹ were left open in the storage container for 3 days, and then introduced into the reactor in batch mode, remaining in recirculation for an additional 3 days. Justification for this procedure was to favor cultivation of spore forming microorganisms and minimize the development of non-spore forming methanogenic microorganisms. In this context, Penteadó *et al.* (2013) studied the influence of different inoculum pretreatment methods for the production of biohydrogen from synthetic sucrose-based wastewater and also managed to prevent the production of methane by using natural fermentation of the feed medium. However, in the present work, the elimination of methane production with this form of inoculation was not accomplished, indicating that this strategy is not totally guaranteed, and methanogens may survive the adverse conditions imposed by this type of inoculation, even without spore formation.

The use of commercial sterilized skimmed milk in Condition 5 was performed to test whether the problem in the hydrogen production with cheese

whey as substrate was associated with any possible contamination of the raw material used (cheese whey powder). However, this condition also resulted in zero H₂ at the end of the operation, indicating that the instability in hydrogen production with cheese whey was not caused by contamination.

Storage of the feed medium in a refrigerator at 4 °C was the first positive step towards stability of hydrogen production (Condition 6), probably because of contamination of the cheese whey in the storage container, due to its high biodegradability (Castelló *et al.*, 2009; Prazeres *et al.*, 2012; Perna *et al.*, 2013). The AnSBBR was thus fed with significant amounts of volatile acids, turning it into a methanogenic reactor of a combined acidogenic-methanogenic process. Table 2 contains results of some analyses carried out with the "non-refrigerated and 24 h stored" feed medium, showing that degradation occurred in the storage container. Table 3 contains some analyses with the "refrigerated and 24 h stored" feed medium showing that its characteristics were maintained, without degradation of the substrate or increase in the production of volatile fatty acids.

Table 2: Analysis of the "non-refrigerated and 24 h stored" feed medium.

Temperature (°C)	Concentration of the feed medium at preparation (mgCarbohydrate.L ⁻¹)	Concentration 24 h after preparation of the feed medium (mgCarbohydrate.L ⁻¹)	Biodegradation (%)	"Fresh" feed medium		Feed medium "after 24 h"	
				pH (u)	TVA (Total Volatile Acids) (mgHAc.L ⁻¹)	pH (u)	TVA (Total Volatile Acids) (mgHAc.L ⁻¹)
35	3822	680	82	7.59	112	4.11	823
29	3679	2937	20	5.09	139	4.05	379
26	3795	1996	47	5.44	118	4.05	362
29	3795	2099	45	5.68	111	4.05	198

Table 3: Analysis of the "refrigerated and 24 h stored" feed medium.

Temperature (°C)	Concentration of the feed medium at preparation (mgCarbohydrate.L ⁻¹)	Concentration 24 h after preparation of the feed medium (mgCarbohydrate.L ⁻¹)	Biodegradation (%)	"Fresh" feed medium		Feed medium "after 24 h"	
				pH (u)	TVA (Total Volatile Acids) (mgHAc.L ⁻¹)	pH (u)	TVA (Total Volatile Acids) (mgHAc.L ⁻¹)
4	3482	3439	1.2	7.69	103	7.92	108
4	3736	3696	1.1	7.62	109	7.73	109
4	3673	3521	4.1	7.54	114	7.94	116
4	3732	3647	2.3	6.28	109	6.28	109
4	3538	3445	2.6	7.68	111	7.91	112

Refrigeration of the feed medium in the storage container, on the other hand, apparently resulted in H₂S production; there was a characteristic odor in the reactor and formation of white substance covering the exterior of the supply hoses. This probably occurred due to the action of sulfate-reducing bacteria (SRB); however, specific analyses for the detection of sulfur compound have not been carried out. This would explain the decrease in hydrogen production without concomitant increase of methane. So, in order to prevent this apparent H₂S production, it was decided, in Condition 7, to feed the reactor only with cheese whey and sodium bicarbonate, eliminating supplementation of urea and nutrients, because the nutrient solution used contained approximately 13 mgSO₄.L⁻¹ (from nickel sulfate and ferrous sulfate), a considerably high amount for a nutrient that is required in small amounts for cell growth.

Hence, the operational strategy of Condition 7 was able to eliminate the action of sulfate-reducing bacteria (SRB), which were probably consuming hydrogen in the presence of sulfate according to the reaction $\text{SO}_4^{2-} + 4\text{H}_2 \rightarrow \text{H}_2\text{S} + 2\text{H}_2\text{O} + 2\text{OH}^-$ ($\Delta G = -154$ kJ). Castelló *et al.* (2009) highlight in their work the importance of specific studies related to nutrient supplementation of cheese whey for biohydrogen production. In the specific case of the AnSBBR, supplementation was inadequate for the process and its removal resulted in a significant increase in hydrogen production.

The production of biogas and hydrogen, however, began to show a gradual decrease, even without production of H₂S or CH₄. So, it was decided to perform systematic washings of the support material in order to minimize the concentration of biomass in the reactor. This procedure was performed to control cell growth by maintaining the concentrations of biomass (C_x and C_{x'}) at low values, as can be seen in Table 4. As a result, there was stable production of biohydrogen (Condition 7) with minimal methane formation

(Figure 2); the success of this strategy was attributed to a probable increase in the applied specific organic load by decreasing the amount of biomass in the reactor, creating more favorable conditions for the growth of acidogenic bacteria, which have a higher growth rate than the methanogenic microorganisms (Lettinga & Haandel, 1993).

The operating strategy (Condition 7) that proved to be effective for stable production of biohydrogen in an AnSBBR treating cheese whey was the removal of the nutrient solution and urea from the feed medium, along with refrigeration of this medium during the storage period, as well as the periodic washing of the support material. Table 5 contains the results of biohydrogen production indicators obtained under the conditions of process stability: beginning of Condition 2, with sucrose feeding; beginning of Condition 3, with lactose feeding; and the end of Condition 7.

The condition with sucrose as substrate showed the best values of the biohydrogen production indicators in relation to the condition with lactose as substrate. This result was expected due to the biodegradability characteristics of these carbohydrates. However, the condition in which cheese whey was used as substrate also showed the best values of biohydrogen production indicators in relation to pure lactose. This behavior may be attributed to the balanced composition of the cheese whey, in nutritional terms, for the acidogenic microorganisms.

A more important comparison can be made with different reactor configurations (Table 6). There are no other studies in the specialized literature that use an AnSBBR fed with cheese whey. In addition, the reactor configuration used in the present work showed excellent results compared with traditional configurations like the UASBr or the CSTR. Another important point that should be highlighted is the stability reached by the AnSBBR in the steady-state phase, while most of the other configurations mentioned increased instability, with sharp peaks and valleys.

Table 4: Biomass in the reactor related to the washing procedure of the support material.

Time (day)	Amount of biomass removed from the system (gTVS)	C _x before washing (gTVS.L ⁻¹)	C _{x'} before washing (mgTVS.g-support ⁻¹)
212	41.2	8.9	17.9
219*	17.5	-	-
223	33.7	10.4	22.1
229	23.3	6.6	14.2

*On day 219 only liquid in the side reactor was removed, without washing the support material, seeking a simpler operational alternative. This procedure, however, did not have the same effect of the washing on biomass control.

Table 5: Biohydrogen production indicators under the conditions with sucrose, lactose and cheese whey.

Substrate	MYRL _{S,n} ($\frac{\text{molH}_2}{\text{kgCarbohydrate}}$)	nH ₂ ($\frac{\text{molH}_2}{\text{d}}$)	MPr ($\frac{\text{molH}_2}{\text{m}^3 \cdot \text{d}}$)	SMPPr ($\frac{\text{molH}_2}{\text{kgTVS} \cdot \text{d}}$)	MYAL _{C,m} ($\frac{\text{molH}_2}{\text{kgCOD}}$)	MYAL _{S,m} ($\frac{\text{molH}_2}{\text{kgCarbohydrate}}$)	MYRL _{C,m} ($\frac{\text{molH}_2}{\text{kgCOD}}$)	MYRL _{S,m} ($\frac{\text{molH}_2}{\text{kgCarbohydrate}}$)
Sucrose	1.54	0.11	31.1	-	3.32	3.71	16.4	4.45
Lactose	0.48	0.04	10.5	2.08	1.19	1.31	6.92	1.41
Cheese whey	0.60	0.07	18.1	4.32	1.37	1.72	4.50	1.74
Intermediate product	Metabolic pathway						Theoretical MYRL_{S,n}	Theoretical MYAL_{S,m}
Acetic acid	$\text{C}_{12}\text{H}_{22}\text{O}_{11} + 5 \text{H}_2\text{O} \rightarrow 4 \text{CH}_3\text{COOH} + 4 \text{CO}_2 + 8 \text{H}_2$						8	23.4
Butyric acid	$\text{C}_{12}\text{H}_{22}\text{O}_{11} + 2 \text{H}_2\text{O} \rightarrow 2 \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 4 \text{CO}_2 + 4 \text{H}_2$						4	11.7

Influent concentration of $4070 \pm 390 \text{ mgCOD} \cdot \text{L}^{-1}$ and $3240 \pm 310 \text{ mgCarbohydrate} \cdot \text{L}^{-1}$.

Effluent concentration of $2830 \pm 170 \text{ mgCOD} \cdot \text{L}^{-1}$ and $51 \pm 4 \text{ mgCarbohydrate} \cdot \text{L}^{-1}$.

Conversion of COD and carbohydrates of $30 \pm 3\%$ and $98 \pm 3\%$, respectively.

Influent pH of 7.7 ± 0.2 and Effluent pH of 4.3 ± 0.3 .

Temperature of $30 \pm 1 \text{ }^\circ\text{C}$.

Working volume of $3.5 \pm 0.1 \text{ L}$ and treating $1.5 \pm 0.1 \text{ L}$ in 3 h cycle.

Table 6: Comparison between different reactor configurations using cheese whey as substrate.

Reactor configuration	HRT / t _c (h)	Temperature (°C)	Working volume (L)	AVOL _{CT} (gCOD.L ⁻¹ .d ⁻¹)	VHPR (NmLH ₂ .L ⁻¹ .d ⁻¹)	Methane presence	Reference
UASBr	6	Ambient temperature	1.3	20	360	Yes	Carrillo-Reyes <i>et al.</i> (2012)
CSTR	24	55 ± 1	2.0	10	800	Yes	Azbar <i>et al.</i> (2009)
CSTR	24	35 ± 1	1.5	10	350	Yes	Yang <i>et al.</i> (2007)
UASBr	12	30 ± 1	4.6	20	122	Yes	Castelló <i>et al.</i> (2009)
Upflow anaerobic fixed-bed reactor	24	30 ± 1	2.5	37	1000	No	Perna <i>et al.</i> (2013)
AnSBBR with recirculation of the liquid phase	3	30 ± 1	3.5	14.6	420	Yes	current work

CONCLUSIONS

The use of cheese whey for the production of biohydrogen in an AnSBBR (3.5 L of working volume and treating 1.5 L in 3 or 4 h cycles at 30 °C) was studied by analyzing the influence of the following selective pressures on process stability: acclimation of the biomass with pure carbohydrates (sucrose and lactose), pH control at very low values, supplementation of nutrients, a different form of inoculation (natural fermentation of the feed medium), refrigeration of the feed medium at 4 °C during storage and systematic washing of the support material. Favorable operating conditions for achieving stable hydrogen production with a very small amount of methane (36% H₂; 62% CO₂; 2% CH₄) were: the refrigeration strategy, preventing substrate degradation in the storage recipient and consequent acidification of the influent, eliminating nutrient supplementation to prevent the action of sulfate-reducing bacteria and periodic washing of the support material to increase the specific ratio of feeding and minimize the action of

homoacetogenic microorganisms. The best results for biohydrogen production were obtained using cheese whey as a substrate with an applied volumetric organic loading of 14.6 gCOD.L⁻¹.d⁻¹ (3 h cycle and influent concentration of 4070 mgCOD.L⁻¹ and 3240 mgCarbohydrate.L⁻¹) in relation to pure lactose (0.60 and 0.48 molH₂.molCarbohydrate⁻¹, respectively), demonstrating the potential of this wastewater. Furthermore, the AnSBBR fed with cheese whey showed excellent biohydrogen production results of 420 NmLH₂.L⁻¹.d⁻¹ in the steady state (conversions of carbohydrates and COD were 98% and 30%, respectively), compared with other classic reactor configurations using the same substrate.

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NOMENCLATURE

AnSBBR	Anaerobic Sequencing Batch Biofilm Reactor	M_{T-IS+B}	carbohydrates for filtered samples (%) Total mass of the inert support and the biomass in the reactor (g)
TS	Total solids concentration (mgTS.L ⁻¹)	M_{S-IS+B}	Sample mass of the inert support and the biomass in the reactor (g)
TVS	Total volatile solids concentration (mgTVS.L ⁻¹)	M_{S-TS}	Total solids mass (TS) of the biomass sample (g)
TSS	Total suspended solids concentration (mgTSS.L ⁻¹)	M_{S-TVS}	Total volatile solids mass (TVS) of the biomass sample (g)
VSS	Volatile suspended solids concentration (mgVSS.L ⁻¹)	M_{TVS}	Total biomass in the reactor in total volatile solids (gTVS)
pH	Hydrogen ion potential (u)	m_{BL1}	Concentration of total volatile solids divided by the volume of the liquid discharged from the reactor (gTVS)
C_{CT}	Concentration based on organic matter for unfiltered samples in the influent (mgCOD.L ⁻¹)	m_{BL2}	Concentration of total volatile solids divided by the volume of water used for washing the system "biomass + inert support," i.e., one liter (gTVS)
C_{ST}	Concentration based on carbohydrates for unfiltered samples in the influent (mgCarbohydrate.L ⁻¹)	N	Number of cycles per day
$C_{CT,E}$	Concentration based on organic matter for unfiltered samples in the effluent (mgCOD.L ⁻¹)	n_{CH4}	Daily molar production of methane (mmolH ₂ .d ⁻¹)
$C_{CF,E}$	Concentration based on organic matter for filtered samples in the effluent (mgCOD.L ⁻¹)	n_{CO2}	Daily molar production of carbon dioxide (mmolH ₂ .d ⁻¹)
$C_{ST,E}$	Concentration based on carbohydrates in the influent for unfiltered samples (mgCarbohydrate.L ⁻¹)	n_{H2}	Daily molar production of hydrogen (mmolH ₂ .d ⁻¹)
$C_{SF,E}$	Concentration based on carbohydrates for filtered samples in the effluent (mgCarbohydrate.L ⁻¹)	MPr	Daily molar productivity of hydrogen (molH ₂ .m ⁻³ .d ⁻¹)
AVOL _{CT}	Applied volumetric organic load based on organic matter (gCOD.L ⁻¹ .d ⁻¹)	SMPr	Daily specific molar productivity of hydrogen (molH ₂ .kgTVS ⁻¹ .d ⁻¹)
AVOL _{ST}	Applied volumetric organic load based on carbohydrate (gCarbohydrate.L ⁻¹ .d ⁻¹)	MYRL _{S,n}	Molar yield per removed load based on carbohydrates, expressed as moles (molH ₂ .molCarbohydrate ⁻¹)
ASOL _{CT}	Applied specific organic load based on organic matter (gCOD.gSVT ⁻¹ .d ⁻¹)	MYAL _{S,m}	Molar yield per applied load based on carbohydrates expressed as kg (molH ₂ .kgCarbohydrate ⁻¹)
ASOL _{ST}	Applied specific organic load based on carbohydrate (gSAC.gSVT ⁻¹ .d ⁻¹)	MYRL _{S,m}	Molar yield per removed load based on carbohydrates expressed as kg (molH ₂ .kgCarbohydrate ⁻¹)
C_X	Relation between the amount of biomass and the volume of liquid medium in the reactor (gTVS.L ⁻¹)	MYAL _{C,m}	Molar yield per applied load based on organic matter expressed as kg (molH ₂ .kgCOD ⁻¹)
C_X'	Relation between the amount of biomass and the amount of support material present in the reactor (mgTVS.g-support ⁻¹)	MYRL _{C,m}	Molar yield per removed load based on organic matter expressed as kg (molH ₂ .kgCOD ⁻¹)
ϵ_{CT}	Removal efficiency based on organic matter (COD) for unfiltered samples (%)	UASBr	Up-flow Anaerobic Sludge Blanket Reactor
ϵ_{CF}	Removal efficiency based on organic matter (COD) for filtered samples (%)	t_C	Cycle length (h.Cycle ⁻¹)
ϵ_{ST}	Removal efficiency based on carbohydrates for unfiltered samples (%)	V_F	Volume of wastewater fed during the cycle (L.Cycle ⁻¹)
ϵ_{SF}	Removal efficiency based on	V_R	Volume of liquid medium in the reactor (L)
		V_G	Volume of total biogas produced in the TP (NmL.d ⁻¹)
		VHPR	Volumetric hydrogen production rate (NmLH ₂ .L ⁻¹ .d ⁻¹)

REFERENCES

- APHA, Standard Methods for the Examination of Water and Wastewater. New York: American Public Health Association (1998).
- Azbar, N., Dokgöz, F. T. Ç., Keskin, T., Korkmaz, K. S., Syed, H. M., Continuous fermentative hydrogen production from cheese whey wastewater under thermophilic anaerobic conditions. *International Journal of Hydrogen Energy*, 34, 7441-7447 (2009).
- Calli, B., Yukselen, M. A., Anaerobic treatment by a hybrid reactor. *Environmental Energy Science*, 19, 143-150 (2004).
- Carrillo-Reyes, J., Celis, L. B., Alatraste-Mondragón, F., Razo-Flores, E., Different start-up strategies to enhance biohydrogen production from cheese whey in UASB reactor. *International Journal of Hydrogen Energy*, 37, 5591-5601 (2012).
- Castelló, E., Garcia Santos, C., Iglesias, T., Paolino, G., Wenzel, J., Borzacconi, L., Etchebehere, C., Feasibility of biohydrogen production from cheese whey using a UASB reactor: Links between microbial community and reactor performance. *International Journal of Hydrogen Energy*, 34, 5674-5682 (2009).
- Dague, R. R., Habben, C. E., Pidaparti, S. R., Initial studies on the anaerobic sequencing batch reactor. *Water Science Technology*, 26, 2429-2432 (1992).
- Dubois, S. M., Gilles, K. A., Hamilton, J. L., Rebers, P. A., Smith, F., Colorimetric methods for determination of sugar and related substance. *Analytical Chemistry*, 28, 13-21 (1956).
- Fernandes, L., Kennedy, K. J., Ning, Z., Dynamic modeling of substrate degradation in sequencing batch anaerobic reactors (SBAR). *Water Research*, 27, 1619-1628 (1993).
- Ghaly, A. E., Ramkumar, D. R., Sadaka, S. S., Rochon, J. D., Effect of reseed and pH control on the performance of a twostage mesophilic anaerobic digester operating on acid cheese whey. *Canadian Agricultural Engineering*, 42, 173-183 (2000).
- Köpke, M., Held, C., Hujer, S., Liesegang, H., Wiezer, A., Wollherr, A., Ehrenreich, A., Liebl, W., Gottschalk, G., Dürre P., *Clostridium ljungdahlii* represents a microbial production platform based on syngas. *PNAS*, 107, 13087-13092 (2010).
- Lima, D. M. F., Zaiat, M., The influence of the degree of back-mixing on hydrogen production in an anaerobic fixed-bed reactor. *International Journal of Hydrogen Energy*, 37, 9630-9635 (2012).
- Kraemer, J. T., Bagley, D. M., Improving the yield from fermentative hydrogen production. *Biotechnology Letters*, 29, 685-695 (2007).
- Leite, J. A. C., Fernandes, B. S., Pozzi, E., Barboza, M., Zaiat M., Application of an anaerobic packed-bed bioreactor for the production of hydrogen and organic acids. *International Journal of Hydrogen Energy*, 33, 579-586 (2008).
- Lettinga, G., Haandel, A. C., Anaerobic Digestion for Energy Production and Environmental Protection. In: *Renewable Energy - Sources for Fuels and Electricity*, T. B. Johansson, *et al.* (Editors). Island Press, London, 817-839 (1993).
- Liou, J., Balkwill, D. L., Drake, G. R., Tanner, R. S., *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. *International Journal of System and Evolution Microbiology*, 55, 2085-2091 (2005).
- Luo, G., Karakashev, D., Xie, L., Zhou, Q., Angelidaki, I., Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnology and Bioengineering*, 108, 1816-1827 (2011).
- Lovato, G., Bezerra, R. A., Rodrigues, J. A. D., Ratusznei, S. M., Zaiat, M., Effect of feed strategy on methane production and performance of an AnSBBR treating effluent from biodiesel production. *Applied Biochemical and Biotechnology*, 166, 2007-2029 (2012).
- Manssouri, M., Rodrigues, J. A. D., Ratusznei, S. M., Zaiat, M., Effects of organic loading, influent concentration and feed time on biohydrogen production in a mechanically stirred AnSBBR treating sucrose based wastewater. *Applied Biochemical and Biotechnology*, 171, 1832-1854 (2013).
- Mawson, A. J., Bioconversion for whey utilisation and waste abatement. *Bioresource Technology*, 47, 195-203 (1994).
- Ntaikou, I., Gavala, H. N., Kornaros, M., Lyberatos, G., Hydrogen production of sugars and sweet sorghum biomass using *Ruminococcus albus*. *International Journal of Hydrogen Energy*, 33, 1221-1231 (2008).
- Penteado, E. D., Lazaro, C. Z., Sakamoto, I. K., Zaiat, M., Influence of seed sludge and pretreatment method on hydrogen production in packed-bed anaerobic reactors. *International Journal of Hydrogen Energy*, 38, 6137-6145 (2013).
- Perna, V., Castelló, E., Wenzel, J., Lazaro, C. Z., Lima, D. M. F., Borzacconi, L., Varesche, M. B. A., Zaiat, M., Etchebehere, C., Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. *International Journal of Hydrogen Energy*, 38, 54-62 (2013).