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FEASIBILITY OF BIOHYDROGEN PRODUCTION BY CO-DIGESTION OF VINASSE (SUGARCANE STILLAGE) AND MOLASSES IN AN ANSBBR

Roberta Albanez[1,2], Giovanna Lovato[1,2], Suzana Maria Ratusznei[1], Marcelo Zaiat[2] and José Alberto D. Rodrigues[1*]

[1] Mauá School of Engineering, Mauá Institute of Technology (EEM/IMT), Praça Mauá 1, CEP 09.580-900, São Caetano do Sul, SP, Brazil.
[2] São Carlos School of Engineering, University of São Paulo (EESC/USP), Av. Trabalhador São-Carlense 400, CEP 13.566-590, São Carlos, SP, Brazil.

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Abstract – This work studied the feasibility of biohydrogen production by co-digestion of vinasse/molasses in an AnSBBR operated with mechanical stirring (30°C and 200 rpm). Hydrogen production by co-digestion of vinasse/sucrose was also studied to verify the performance of the process with a known co-substrate with easy degradation. The effects of influent composition (vinasse/sucrose and vinasse/molasses), influent concentration (3000 and 4000 mgCOD.L^{-1}) and cycle time (3 and 4 h) on performance indicators were evaluated using stability, organic matter removal efficiency, molar hydrogen yield, productivity and biogas composition. The condition with vinasse/molasses in the influent that showed the best results was obtained with a 3-hour cycle time, influent concentration of 3000 mgCOD.L^{-1} and composition of 33% vinasse and 67% molasses. The molar productivity in this condition was 3.8 molH_2.m^{-3}.d^{-1} with a hydrogen molar fraction of 16% (and a methane molar fraction of 14%). A first order kinetic model was fitted efficiently to the best conditions.

Keywords: AnSBBR; co-digestion; cycle time; hydrogen; molasses; sugarcane stillage.

INTRODUCTION

Bioethanol is an important alternative fuel, especially considering the increasing demand to reduce carbon dioxide emissions. However, the ethanol production process generates large volumes of effluents, mainly vinasse that is generated in the proportion of 12 to 15 L per liter of ethanol produced. Vinasse is generated in the distillation column at a temperature of 85-90 °C with a low pH, a dark brown color, high ash content and a high percentage of dissolved organic and inorganic matter. The disposal of vinasse in the soil is common and is justified by essential nutrients for the growth of sugar cane. However, this can change the characteristics of the soil, causing soil salinity and contamination of groundwater (Onodera *et al.*, 2013; Santos *et al.*, 2013).

The treatment of vinasse is one of the most challenging problems for the industrial production process of ethanol. Among the potential vinasse treatment options, biological treatment is known as an effective method. The main advantages of the anaerobic treatment of vinasse are the ability to convert a portion of the organic matter into biogas (hydrogen and/or methane), which can be used as a source of energy, and the effluent can be used as fertilizer (Pant and Adholeya, 2007; Moraes *et al.*, 2014). Vinasse can be considered as a substrate for hydrogen production through anaerobic treatment because it has a high chemical oxygen demand (22-45 g.L^{-1}) and macronutrients.

**Corresponding author*: José Alberto D. Rodrigues. E-mail: rodrigues@maua.br

Despite its great potential, there are few studies regarding hydrogen production using this substrate. It is important to mention that hydrogen production by anaerobic treatment of vinasse may present toxicity problems, related to the content of potassium, sulfate, phenolic compounds and melanoidins, and improvement of the biodegradability by the addition of a co-substrate can be a promising performance improvement strategy (Lazaro *et al.*, 2014; Wang *et al.*, 2011). Co-digestion is a feasible option to improve the biodegradability because it dilutes toxic compounds, balances nutrients, encourages synergy between microorganisms and increases the biodegradable organic matter (Mata-Alvarez *et al.*, 2014). The use of molasses as a co-substrate is interesting because it is a by-product of the sugar industry, making it easily available (Soam *et al.* 2015). Molasses contains mostly sucrose (up to 55%), but also other sugars, salts and inorganic compounds (Mironczuk, *et al.*, 2015).

The reason for the choice of the AnSBBR (anaerobic sequencing batch biofilm reactor) is to study the technological options for discontinuous operation as an alternative to continuous operation. The point is to assess the main advantages that are related to better operational control (load-reaction-discharge), flexibility in the feeding mode (different cycle times) because of the different periods of interrupted production, and suitability for the different concentrations of wastewater available for the generation of biogas from relatively small production units. An additional purpose is to evaluate the main limitations of this reactor related to the possibility of overload that leads to consequent microbial inhibition and reduction of overall productivity because of the need to charge-discharge and the inherent transient behavior of discontinuous operations (Lovato *et al.*, 2016).

There are several studies in the literature on the influence of process variables on reactor efficiency and stability when applied to the treatment of various effluents, such as those related to: (i) the type of mixture, which can be implemented by recirculating the liquid phase (Bergamo *et al.*, 2009; Bezerra *et al.*, 2009) or by mechanical agitation (Rodrigues *et al.*, 2003 and 2004; Michelan *et al.*, 2009). (ii) the filling time or feeding strategy (Albanez *et al.*, 2009; Oliveira *et al.*, 2010); and (iii) the organic load (Massé and Masse, 2000; Damasceno *et al.*, 2007; Friedl *et al.*, 2009; Carvalhinha *et al.*, 2010). Currently, in the literature, there is an increase in the potential application of batch and fed-batch reactors to generate bioenergy (methane and hydrogen) in a broad context in which the wastewater should be treated just as raw material and not as a process waste, with the aim of making it possible to obtain energy from the methane/hydrogen generated (Yang *et al.*, 2008; Bezerra *et al.*, 2011; Manssouri *et al.*, 2013; Bravo *et al.*, 2015; Lovato *et al.*, 2012; Lima *et al.*, 2015).

In this context, this study aims to evaluate the feasibility of biohydrogen production by co-digestion of vinasse and molasses in an anaerobic sequencing batch biofilm reactor (AnSBBR) with mechanical agitation. This study evaluated the influence of influent composition/concentration and cycle time on stability, organic matter removal, intermediate metabolites, hydrogen yield and biogas productivity/composition. A first order kinetic model was fitted to the experimental data and its adjustment was evaluated (the best model parameters were determined).

MATERIALS AND METHODS

AnSBBR

The mechanically stirred bioreactor (BIOFLO 110 Bioflo from New Brunswick Scientific Co.) consisted of a glass vessel with a diameter of 20 cm and height of 30 cm, with a total capacity of 6.0 L and working volume of 5.6 L. The inert support was confined in a perforated 316-stainless steel basket with a height of 18 cm and inner/outer diameters of 7 and 17.5 cm, respectively (Figure 1). Stirring was set at 200 rpm, provided by a motor attached to the six-flat-blade Rushton turbine impellers with diameter of 6 cm and installed at 8 and 16 cm from the bottom of the tank.

Feeding and discharge were performed using diaphragm pumps. An automation system, consisting of timers, controlled the on/off switching of the pump and the agitator in order to implement the sequencing batch operation steps: feeding, reaction and discharge. The temperature was set at 30 ± 1°C by circulating water in the jacket of the reactor.

Inoculum and inert support

The inoculum used came from an anaerobic reactor treating effluent from a poultry slaughterhouse. This inoculum presented total volatile solids and total solids concentration of 51 and 62 g L^{-1}, respectively. The support used for biomass immobilization consisted of low-density polyethylene (LDPE) pellets obtained from recycled plastic waste (length of 5 mm and diameter of 3 mm). This inoculum was submitted to heat treatment in which about 50 mL were heated to 90°C for 15 min followed by cooling in an ice bath to 25°C (adapted from Kim *et al.*, 2006).

Wastewater

The wastewater used was formulated based on vinasse and molasses that were from a sugar/alcohol plant located in São Paulo, Brazil. The vinasse had approximately 25 g $COD.L^{-1}$ and molasses 1100 g $COD.L^{-1}$. To examine the system behavior using a known and easily biodegradable substrate, sucrose-based wastewater was used. Therefore, different based wastewaters were used: sucrose; vinasse/sucrose; molasses; and vinasse/molasses.

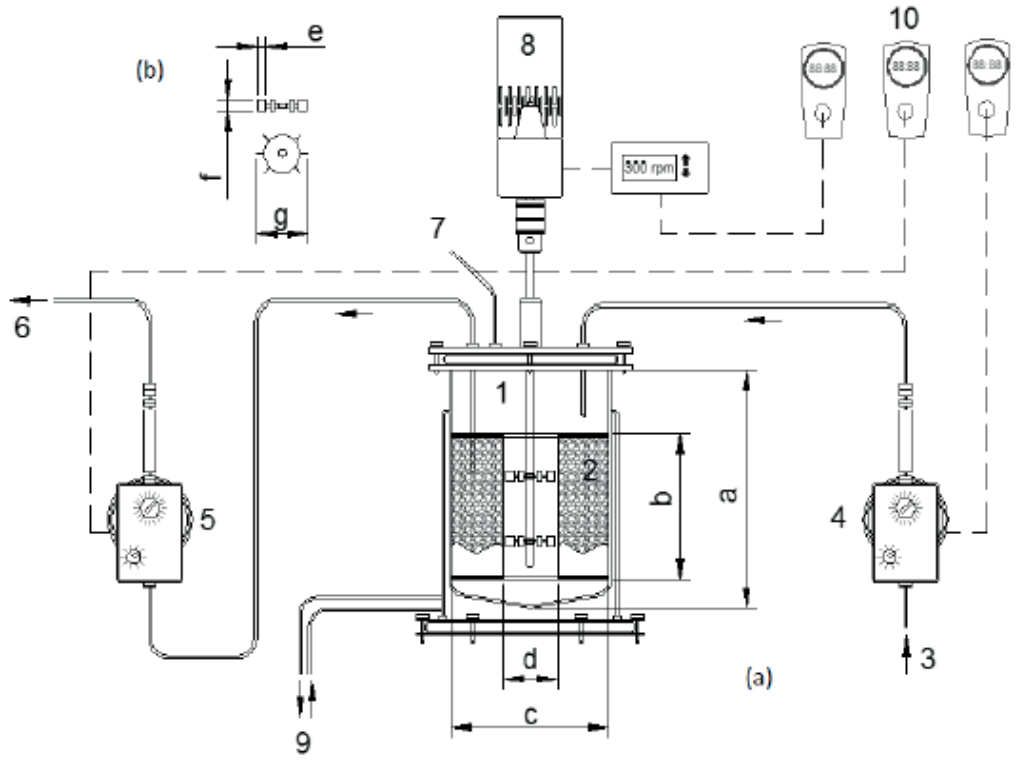


Figure 1. Scheme of the AnSBBR: [(a) Reactor 1 – Bioreactor BIOFLO III (New Brunswick Scientific.); 2 – Basket containing support material for the biomass; 3 – Influent; 4 – Feed pump; 5 –Discharge pump; 6 – Effluent; 7 –Biogas outlet; 8 – Agitation system; 9 – Temperature control system; 10 – Automation system; (b) Details of the six-flat-blade turbine impellers].

The salt solution added had the composition: urea/ CH_4N_2O 11.5 mg.L^{-1}, $NiSO_4.6H_2O$ 0.5 mg.L^{-1}, $FeSO_4.7H_2O$ 2.5 mg.L^{-1}, $FeCl_3.6H_2O$ 0.25 mg.L^{-1}, $CoCl_2.2H_2O$ 0.04 mg.L^{-1}, $CaCl_2.6H_2O$ 2.06 mg.L^{-1}, SeO_2 0.036 mg.L^{-1}, KH_2PO_4 5.36 mg.L^{-1}, K_2HPO_4 1.3 mg.L^{-1}, and $Na_2HPO_4.H_2O$ 2.7 mg.L^{-1}.

Physical-chemical analyses

Reactor monitoring was carried out by measuring influent and effluent samples, unfiltered (C_{CT}) and filtered (C_{CF}) organic matter concentration as chemical oxygen demand (COD) and as total carbohydrates for determining unfiltered (C_{ST}) and filtered (C_{SF}), total alkalinity (TA), total volatile acids (TVA), total solids (TS), total volatile solids (TVS), total suspended solids (TSS), volatile suspended solids (VSS), pH and volume fed/discharged per cycle (Standard Methods for the Examination of Water and Wastewater, 1995; Dubois *et al.*, 1956).

The intermediate compounds of the anaerobic metabolism (acetone, methanol, ethanol, n-butanol, acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acid) were analyzed by an Agilent Technologies 7890 gas chromatograph equipped with a flame ionization detector, 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 flow rate of 1.56 mL.min^{-1}. Injector temperature was 250 °C, injection volume was 400 µL, and split ratio 10. Oven temperature was programmed as follows: from 35 to 38 °C at 2 °C min^{-1}, from 38 to 75 °C at 10 °C min^{-1}, from 75 to 120 °C at 35 °C min^{-1}, at 120 °C for 1 min, from 120 to 170 °C at 10 °C min^{-1}, and at 170 °C for 2 min. 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).

Composition of the biogas generated via anaerobic degradation was analyzed by gas chromatography using an Agilent Technologies 7890 gas chromatograph equipped with thermal conductivity detector and GS-Carbonplot column (30 m×0.53 mm×3.0 μm). Argon was used as carrier gas at 3.67 mL min^{-1}, the injector temperature was 185 °C, injection volume 200 μL, and split ratio 10. Oven temperature was programmed as follows: 40 °C isotherm for 5 min. Detector temperature was 150 °C, with makeup (argon) flow rate of 8.33 mL.min^{-1} (Manssouri *et al.* 2013). Volumetric biogas production was measured with a Ritter Milligas counter gas meter.

Theoretical – performance indicator parameters

The organic matter removal efficiency for unfiltered (ε_{CT}) and filtered (ε_{CF}) samples (COD basis) was calculated by Equation (1). The organic matter removal efficiency based on carbohydrates for unfiltered (ε_{ST} – $C_{ST,I}$ and C_{ST}) and filtered (ε_{SF} – $C_{ST,I}$ and C_{SF}) samples was calculated in a similar way (Equation 2).

$$\varepsilon_{CT}(\%) = \frac{C_{CT,I} - C_{CT}}{C_{CT,I}} .100 \quad \varepsilon_{CF}(\%) = \frac{C_{CT,I} - C_{CF}}{C_{CT,I}} .100 \quad (1)$$

$$\varepsilon_{ST}(\%) = \frac{C_{ST,I} - C_{ST}}{C_{ST,I}} .100 \quad \varepsilon_{SF}(\%) = \frac{C_{ST,I} - C_{SF}}{C_{ST,I}} .100 \quad (2)$$

The applied volumetric organic load ($AVOL_{ST}$ or $AVOL_{CT}$ – gCarbohydrate.L^{-1}.day^{-1} or gCOD L^{-1}.day^{-1}) was calculated by Equation (3).

$$AVOL_{ST} = \frac{(V_F.N).C_{ST,I}}{V_R} \quad AVOL_{CT} = \frac{(V_F.N).C_{CT,I}}{V_R} \quad (3)$$

The applied specific organic load ($ASOL_{ST}$ or $ASOL_{CT}$ – gCarbohydrate.$gTVS^{-1}$.day^{-1} or gCOD.$gTVS^{-1}$.day^{-1}) was calculated by Equation (4).

$$ASOL_{ST} = \frac{(V_F.N).C_{ST,I}}{M_{TVS}} \quad ASOL_{CT} = \frac{(V_F.N).C_{CT,I}}{M_{TVS}} \quad (4)$$

The removed volumetric organic load ($RVOL_{SF}$ or $RVOL_{CF}$ – gCarbohyadrate.L^{-1}.day^{-1} or gCOD.L^{-1}.day^{-1}) was calculated by Equation (5).

$$RVOL_{SF} = \frac{(V_F.N).(C_{ST,I} - C_{SF})}{V_R} \quad RVOL_{CF} = \frac{(V_F.N).(C_{CT,I} - C_{CF})}{V_R} \quad (5)$$

The removed specific organic load ($RSOL_{SF}$ or $RSOL_{CF}$ – gCarbohydrate.$gTVS^{-1}$.day^{-1} or gCOD.$gTVS^{-1}$.day^{-1}) was calculated by Equation (6).

$$RSOL_{SF} = \frac{(V_F.N).(C_{ST,I} - C_{SF})}{M_{TVS}} \quad RSOL_{CF} = \frac{(V_F.N).(C_{CT,I} - C_{CF})}{M_{TVS}} \quad (6)$$

The daily molar productivity of hydrogen (MPr – $molH_2$.L^{-1}.d^{-1}) was calculated by Equation (7), and the daily specific molar productivity of hydrogen (SMPr – $molH_2$.$gTVS^{-1}$.d^{-1}) was calculated by Equation (8).

$$MPr = \frac{n_{H2}}{V_R} \quad (7)$$

$$SMPr = \frac{n_{H2}}{M_{TVS}} \quad (8)$$

The molar yield per applied load ($MYAL_{S,m}$ or $MYAL_{C,m}$ – $mmolH_2$.gCarbohydrate^{-1} or $mmolH_2$.$gCOD^{-1}$) was calculated by Equation (9).

$$MYAL_{S,m} = \frac{n_{H2}}{(N.V_F).C_{ST,I}} \quad MYAL_{C,m} = \frac{n_{H2}}{(N.V_F).C_{CT,I}} \quad (9)$$

The molar yield per removed load ($MYRL_{S,m}$ or $MYRL_{C,m}$ – $mmolH_2$.gCarbohydrate^{-1} or $mmolH_2$.$gCOD^{-1}$) was calculated by Equation (10).

$$MYRL_{S,m} = \frac{n_{H2}}{(N.V_F).(C_{ST,I} - C_{SF})} \quad MYRL_{C,m} = \frac{n_{H2}}{(N.V_F).(C_{CT,I} - C_{CF})} \quad (10)$$

Theoretical - kinetic model of the metabolic route

The kinetic model used in this work was adapted from the one developed by Rodrigues *et al.* (2004) based on the model of Bagley and Brodkorb (1999) and from Lovato *et al.* (2016), which used it for a methanogenic process. In the adapted kinetic model developed for the anaerobic sequencing batch biofilm reactor, one alcohol (ethanol) and three volatile acids (acetic, propionic, butyric acids) were considered.

The anaerobic process of organic matter degradation was simplified into eight steps (Equations 11 to 18). In the first seven parallel steps, the substrate (S, which is considered as sucrose) is converted to acetic acid (HAc), propionic acid (HPr), butyric acid (HBu) and ethanol (EtOH). Then, there is consumption of the propionic and butyric acids to form acetate and hydrogen. Finally, there are the acetoclastic and hydrogenotrophic routes to produce methane if methanogenesis is not completely inhibited. In all stages, the conversion reactions were considered as being first-order.

$$C_{12}H_{22}O_{11} + 5\ H_2O \xrightarrow{k_1} 4\ CH_3COOH + 4\ CO_2 + 8\ H_2 \quad (11)$$

$$C_{12}H_{22}O_{11} + 4\ H_2 \xrightarrow{k_2} 4\ CH_3CH_2COOH + 3\ H_2O \quad (12)$$

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{k_3} 2\ CH_3CH_2CH_2COOH + 4\ CO_2 + 4\ H_2 \quad (13)$$

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{k_4} 4\ CH_3CH_2OH + 4\ CO_2 \quad (14)$$

$$CH_3CH_2COOH + 2\ H_2O \xrightarrow{k_5} CH_3COOH + CO_2 + 3\ H_2 \quad (15)$$

$$CH_3CH_2CH_2COOH + 4\ H_2O \xrightarrow{k_6} CH_3COOH + 2\ CO_2 + 6\ H_2 \quad (16)$$

$$CH_3COOH \xrightarrow{k_7} CH_4 + CO_2 \quad (17)$$

$$4\ H_2 + CO_2 \xrightarrow{k_8} CH_4 + 2\ H_2O \quad (18)$$

Equations 19 to 25 present the mass balance of the reactor components in batch mode according to the kinetic model (substrate, volatile acids, alcohol, hydrogen and methane). These equations were used to determine the kinetic parameters of the model. The reaction rates are represented in the equations 19 to 25 as substrate consumption (r_S), formation and consumption of acetic acid (r_{HAc}), propionic acid (r_{HPr}), butyric acid (r_{HBu}), ethanol (r_{EtOH}), hydrogen (r_H) and methane (r_M). The parameters k'_{1S}, k_{1HAc}, k_{5HAc}, k_{6HAc}, k_{7HAc}, k_{2HPr}, k_{5HPr}, k_{3HBu}, k_{6HBu}, k_{4EtOH}, k_{1H}, k_{2H}, k_{3H}, k_{5H}, k_{6H}, k_{8H}, k_{7M}, k_{8M} are the same apparent kinetic parameters associated with substrate consumption, volatile acids formation and consumption, alcohol, hydrogen and methane formation, respectively.

The kinetic parameter "k" is related to the reaction rate, indicating a relation to the time that is necessary for the concentration of the reagent to reach a value in accordance with the hypothesis of the kinetic model. Subscripts "1, 2, 3, 4, 5, 6, 7 and 8" are related to the reactions. Subscripts

$$\frac{dC_S}{dt} = r_S = -(k_{1S} + k_{2S} + k_{3S} + k_{4S}) \cdot C_S = -k'_{1S} \cdot C_S = \quad (19)$$

$$\frac{dC_{HAc}}{dt} = r_{HAc} = k_{1HAc} \cdot C_S + k_{5HAc} \cdot C_{HPr} + k_{6HAc} \cdot C_{HBu} - k_{7HAc} \cdot C_{HAc} \quad (20)$$

$$\frac{dC_{HPr}}{dt} = r_{HPr} = k_{2HPr} \cdot C_S - k_{5HPr} \cdot C_{HPr} \quad (21)$$

$$\frac{dC_{HBu}}{dt} = r_{HBu} = k_{3HBu} \cdot C_S - k_{6HBu} \cdot C_{HBu} \quad (22)$$

$$\frac{dC_{EtOH}}{dt} = r_{EtOH} = k_{4EtOH} \cdot C_S \quad (23)$$

$$\frac{dC_H}{dt} = r_H = k_{1H} \cdot C_S - k_{2H} \cdot C_S + k_{3H} \cdot C_S + k_{5H} \cdot C_{HPr} + k_{6H} \cdot C_{HBu} - k_{8H} \cdot C_H \quad (24)$$

$$\frac{dC_M}{dt} = r_M = k_{7M} \cdot C_{HAc} + k_{8M} \cdot C_S \quad (25)$$

"S, HAc, HPr, HBu, EtOH, H and M" are related to the components' experimental values (daily monitoring) used to calculate the parameters.

To address the differential equations, the Euler numerical integration method (Excel software) was used. These parameters were calculated using an objective function in the optimization procedure (function Solver in Excel software) for the minimum square error between experimental and model data, accordingly to Lovato *et al.* (2016).

Experimental procedure

The fed/discharged volume (V_F) was 1.0 L per cycle,

Table 1. Studied experimental conditions.

Conditions	Concentration (mgCOD.L^{-1})	Cycle time (h)	AVOL (gCOD.L^{-1}.d^{-1})	Influent Composition		
				%S	%M	%V
1	3000	4	5.1	100	0	0
2	4000	4	6.9	100	0	0
3	3000	4	5.1	67	0	33
4	3000	4	5.1	0	100	0
5	4000	4	6.9	0	100	0
6	3000	4	5.1	0	67	33
7	3000	3	6.9	0	67	33
8	3000	3	6.9	0	25	75

%S – sucrose; %M – molasses; %V – vinasse.

the liquid medium volume maintained in the reactor (V_{RES}) was 2.5 L, so the total volume of liquid medium (V_R) was 3.5 L, and the volume of biomass+support (V_{S+B}) was 2.1 L, and the total useful reactor volume (V_T) was 5.6 L.

The reactor (AnSBBR) operation was carried out as follows: in the first cycle, 3.5 L of influent was fed to the reactor in 20 min, and the reactor was previously prepared by placing an inert support (low-density polyethylene, LDPE) containing inoculum. After the influent was fed into the reactor, an agitation of 200 rpm was implemented. At the end of the cycle (duration of the cycle depended on experimental conditions), agitation was turned off and 1.0 L of wastewater was discharged (with 2.5 L of residual volume) in 10 min. After this discharging, a new cycle was started with the feeding of 1.0 L of wastewater (in 10 min) with agitation. At the end of the cycle, agitation was stopped again and discharge was performed so that the cycle could be repeated, featuring the sequencing batch mode.

After verifying stable values (variability less than 10%) of the monitored variables, profiles along the cycle were obtained for: filtered organic matter and carbohydrate concentrations, bicarbonate alkalinity, total volatile acids, intermediate metabolites, pH and biogas (production/composition). To obtain these profiles, samples were taken in time slots of 30 to 60 min. The volume collected did not exceed 300 mL (9% of the total volume). These profiles allowed a better understanding of metabolic routes, and, therefore, allowed the adjustment of the kinetic model of the proposed metabolic route (item 2.6).

The experimental conditions were performed by modifying the volumetric organic load as a function of substrate concentration (3000 and 4000 mgCOD.L^{-1}) and cycle time (4 and 3 h-cycles), and influent composition (sucrose, vinasse/sucrose, molasses and vinasse/molasses). Table 1 summarizes the conditions performed (Conditions 1 to 8: 21 days each).

In conditions with vinasse/molasses (6/7/8) it was necessary to perform a systematic cleaning of the reactor (once a week) to better control the amount of biomass. In this way, about 40% of the total biomass was removed each week to compensate cell growth due to the metabolic characteristics of acidogenic microorganisms. The objective was to improve stability and efficiency of biohydrogen production. The procedure consisted of discharging the liquid medium in the reactor and washing the glass reactor vessel. The "inert support + biomass" was kept inside the perforated steel basket.

The effects of influent composition and concentration, and cycle time on performance indicators were evaluated using stability, organic matter removal efficiency, molar hydrogen yield, productivity and biogas composition. In Conditions 1-2-3, the influence of increasing influent concentration was evaluated with sucrose based wastewater and with co-digestion of vinasse and sucrose. In Conditions 4-5-6, the increase in influent concentration was evaluated with molasses-based wastewater and with co-digestion of vinasse and molasses. With Conditions 6 and 7, it was possible to verify the influence of cycle time and in Conditions 7 and 8 the influence of increasing the vinasse percentage in the influent composition was verified.

RESULTS AND DISCUSSION

Monitored variables and performance indicator parameters

Hydrogen production from vinasse in co-digestion with sucrose or molasses in AnSBBR performed in this work was stable. This stability was in relation to the stable production of biogas and its composition during the monitoring of the conditions studied (except Condition 8, in which it was not possible to obtain hydrogen production).

The system showed in all conditions (Table 2) low organic matter removal in the form of COD (approximately 20% for filtered samples) and a high removal of organic matter in the form of carbohydrates (approximately 90% for filtered samples). It was also noticed that by adding vinasse in the influent there was a decrease in pH and an increase in total volatile acids, which was expected because vinasse is characterized as acidified with a low pH (Table 2).

In all conditions there was a generation of total volatile acids, which is expected in systems that aim for hydrogen production (acidogenic phase). The average value of the effluent pH was 4.5 ± 0.2, indicating stability. It is worth mentioning that studies have shown that a suitable pH range to obtain hydrogen is between 4.0 and 7.0 (Wan and Wang, 2009). Lay *et al.* (1999) emphasized that a pH below 4.5 is unfavorable for the production of hydrogen, due to inhibition of the dehydrogenase activity and other enzymes, with a consequent alteration of the metabolic routes. However, studies have shown different values that can be attributed to substrate, inoculum and initial pH. According to Khanal *et al.* (2003) the maximum hydrogen yield was obtained at pH 4.5; to Fang and Liu (2002) the optimum pH for hydrogen production is in the range between 5.0 and 6.5; to Mu *et al.* (2006) the optimum pH was 5.5 and to Chen *et al.* (2009) 4.9.

Table 3 shows the values of pH, total alkalinity (TA) and total volatile acids (VTA) for all conditions. Table 4 shows the concentration of intermediate compounds in influent and effluent, with a predominance of acetic acid in all conditions with molasses and/or vinasse in the influent composition (Conditions 3-4-5-6-7-8), which theoretically is the best route for hydrogen production, since a high HAc/HBu ratio is a good parameter for hydrogen production in acidogenic systems (Das and Veziroglu, 2001). However, in Lovato *et al.* (2015) the highest productivities for hydrogen were obtained under conditions that produced more butyric acid than acetic acid, which shows that this

Table 2 – Monitored substrate consumption in all conditions.

Condition	$C_{CT,I}$ (mgCOD.L^{-1})	e_{CT} (%)	e_{CF} (%)	$AVOL_{CT}$ (gCOD.L^{-1}.d^{-1})	$ASOL_{CT}$ (gCOD.TVS^{-1}.d^{-1})
1	2960 ± 276	16 ± 9	21 ± 9	5.1	5.3
2	4073 ± 216	17 ± 3	22 ± 5	7.1	7.4
3	3038 ± 103	17 ± 3	20 ± 2	5.6	3.0
4	3172 ± 137	22 ± 5	24 ± 3	5.5	5.9
5	4084 ± 141	24 ± 2	27 ± 2	7.1	7.7
6	3075 ± 92	19 ± 4	22 ± 3	5.4	1.0
7	3124 ± 194	19 ± 4	21 ± 4	7.3	2.6
8	3374 ± 321	15 ± 5	20 ± 6	7.8	3.5
Condition	**$C_{ST,I}$ (mgCarbohydrate.L^{-1})**	**e_{ST} (%)**	**e_{SF} (%)**	**$AVOL_{ST}$ (gCarbohydrate.L^{-1}.d^{-1})**	**$ASOL_{ST}$ (gCarbohydrate. TVS^{-1}.d^{-1})**
1	2986 ± 78	98 ± 1	99 ± 2	5.1	5.3
2	3397 ± 115	97 ± 1	97 ± 1	5.9	6.2
3	2129 ± 448	96 ± 1	97 ± 1	3.9	2.1
4	2423 ± 226	96 ± 1	96 ± 1	4.2	4.5
5	3198 ± 367	96 ± 1	97 ± 1	5.6	6.1
6	1501 ± 141	94 ± 1	95 ± 1	2.6	0.5
7	1433 ± 104	92 ± 2	94 ± 1	3.4	1.2
8	1326 ± 100	83 ± 2	84 ± 2	3.1	1.4

The number of samples analyzed in each condition was 13 for the analysis of $C_{CT,}$ $\varepsilon_{C,}$ $C_{ST,I}$, ε_S, volume, pH, TA and TVA; 1 for Cx. Fed volume (L): 1.02 ± 0.09; Cx (gTVS.L^{-1}): Conditions 1, 2, 4 and 5: 0.94 ± 0.02; Conditions 3 and 6: 4.1 ± 1.2; Conditions 7 and 8: 2.4 ± 0.7;

is not a general rule. Table 5 shows the values obtained for the performance indicators related to biogas production.

Influent concentration and composition (vinasse/sucrose) – Conditions 1, 2 and 3

Conditions 1-2 were used to compare the results obtained in conditions with more complex substrates (molasses and/or vinasse) since sucrose is an easily biodegradable substrate. Condition 3 aimed to verify the effects of vinasse/sucrose co-digestion on hydrogen production.

The molar productivity (Table 5) increased from 18.1 to 37.7 molH_2.m^{-3}.d^{-1} from Condition 1 to 2 and decreased to 8.3 molH_2.m^{-3}.d^{-1} in Condition 3. The molar yield increased from 17.0 to 23.7 mmolH_2.g COD^{-1} from Condition 1 to 2 and decreased to 7.4 mmolH_2.gCOD^{-1} in Condition 3. These results show that the increase in influent concentration caused an increase in hydrogen productivity/yield and the insertion of vinasse caused a decrease in hydrogen productivity/yield, indicating that is difficult for the microorganisms to assimilate a complex substrate such as vinasse for hydrogen production. Vinasse also decreased the biogas composition. Conditions 1-2 did not show methane in the biogas, but Condition 3 did (4% CH_4).

Manssouri *et al.* (2013) and Inoue *et al.* (2014) worked with sucrose-based wastewater in AnSBBR, the first in batch and the second in fed-batch mode. Manssouri *et al.* (2013) obtained in the best condition (3600 mgCOD.L^{-1} and cycle time of 4 h) a hydrogen molar fraction of 20%, molar productivity of 19.4 molH_2.m^{-3}.d^{-1} and molar yield of 9.54 mmolH_2.gCOD^{-1}. Inoue *et al.* (2014) obtained in the best condition (3500 mgCOD.L^{-1}, feeding time of 2 h and cycle time of 4 h) a hydrogen molar fraction of 32%, molar productivity of 24.5 molH_2.m^{-3}.d^{-1} and molar yield of 14.1 mmolH_2.gCOD^{-1}. The results obtained in the works of Manssouri *et al.* (2013) and Inoue *et al.* (2014) for hydrogen production were inferior to the results obtained in the present work (condition 2: hydrogen molar fraction of 39%, molar productivity of 37.7 molH_2.m^{-3}.d^{-1} and molar yield of 23.7 mmolH_2.gCOD^{-1}). Thus, the results obtained under conditions 1 and 2 are consistent with the previous studies analyzed and these results can be used to compare the performance of conditions 1 and 2 (simple substrate – sucrose) with the conditions realized with more complex substrates. Reis *et al.* (2015) studied the co-digestion of vinasse/glucose (influent vinasse composition: 0%, 25%, 75% and 100%) for hydrogen production and noted that the vinasse decreased hydrogen production, as was noted in the present work. The best result was with 25%-vinasse/75%-glucose, which gave a molar yield of 14.7 mmolH_2.gCOD^{-1} with the presence of methane in the biogas.

Table 4 shows a decrease of acetic acid and butyric acid concentration in the effluent of Condition 3 compared to Conditions 1-2, which may indicate a change in the

Table 3 – Values of pH, total volatile acids (TVA) and bicarbonate alkalinity (TA) in all conditions.

Condition	pH		TA ($mgCaCO_3.L^{-1}$)		TVA ($mgHAc.L^{-1}$)	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
1	8.1± 0.1	4.8 ± 0.3	382 ± 30	161 ± 33	21 ± 9	977 ± 108
2	8.1± 0.1	4.6 ± 0.1	427 ± 8	149 ± 33	16 ± 1	979 ± 34
3	5.8 ± 0.1	4.4 ± 0.1	170 ± 16	62 ± 11	352 ± 50	945 ± 35
4	7.3 ± 0.1	4.5 ± 0.2	154 ± 31	60 ± 12	76 ± 21	718 ± 47
5	7.1 ± 0.2	4.3 ± 0.1	168 ± 17	3 ± 1	85 ± 7	882 ± 74
6	5.6 ± 0.2	4.6 ± 0.1	181 ± 21	103 ± 36	421 ± 21	888 ± 73
7	5.3 ± 0.3	4.5 ± 0.1	151 ± 24	67 ± 31	413 ± 17	816 ± 32
8	5.6 ± 0.2	4.9 ± 0.1	191 ± 21	280 ± 40	559 ± 109	1162 ± 108

The number of samples analyzed in each condition was 13 for the analysis of volume, pH, TA and TVA.

metabolic pathway that may have reduced hydrogen production. Furthermore, also observed was a higher amount of solids in the reactor (M_{TVS} – Conditions 1-2 was 3.4 g and Condition 3 was 6.7 g) that can be due to the solids present in the vinasse.

Influent concentration and composition (vinasse/molasses) – Conditions 4, 5 and 6.

The aim of conditions 4, 5 and 6 was to verify the effect of molasses and co-digestion of vinasse/molasses on hydrogen production. This replacement of sucrose by molasses is interesting because molasses is a by-product of the sugarcane and alcohol industry.

The molar productivity (Table 5) increased from 3.2 in Condition 4 to 7.9 $molH_2.m^{-3}.d^{-1}$ in Condition 5 and the molar yield increased from 2.4 to 4.1 $mmolH_2.g\ COD^{-1}$. In Condition 6 the molar productivity was 1.7 $molH_2.m^{-3}.d^{-1}$ and molar yield was 1.4 $mmolH_2.gCOD^{-1}$. These values are 80% lower than those obtained in Conditions 1-2. Furthermore, the biogas composition was: 16% of hydrogen in Condition 4, 23% in Condition 5 and 11% in Condition 6, with the presence of methane (12-14%) in Conditions 4-5-6. The hydrogen decreases and the increase in methane production may be explained by the sucrose characteristics (pure substrate that is easily biodegraded) and molasses characteristics (complex substrate). This was also noticed by Lovato *et al.* (2015) and Bravo *et al.* (2015) in hydrogen production by glycerin-based wastewater.

Table 4 shows a decrease in acetic acid and butyric acid concentration in the effluent of Condition 4-5-6 compared to Conditions 1-2-3, which may indicate a change in the metabolic pathway that may have reduced hydrogen production.

Cycle time and influent composition (vinasse/molasses) – Conditions 6, 7 and 8.

The aim of conditions 6, 7 and 8 was to verify the effects of cycle time and the influent composition (vinasse/molasses) on hydrogen production.

The hydrogen molar fraction increased when the cycle time decreased from 11% in Condition 6 to 16% in Condition 7, both with 14% of methane. The molar productivities and yield (Table 5) increased from 1.7 (Condition 6) to 3.8 $molH_2.m^{-3}.d^{-1}$ (Condition 7) and from 1.4 (Condition 6) to 2.5 $mmolH_2.COD^{-1}$ (Condition 7). Therefore, 3-hour cycle time resulted in better molar productivity and yield but did not eliminate the methanogenesis. An increase in hydrogen production when the cycle time decreased was

Table 4. Monitored intermediate compounds in all conditions.

Condition	Ethanol	Acetic acid	Propionic acid	Butyric acid
	($mmol.L^{-1}$)	($mmol.L^{-1}$)	($mmol.L^{-1}$)	($mmol.L^{-1}$)
1	17.3	10.5	0.9	2.6
2	18.8	12.9	1.0	2.8
3	5.1	9.0	1.4	2.1
4	1.1	6.3	0.6	1.3
5	2.1	7.2	0.8	2.0
6	0.4	5.6	1.0	1.2
7	0.3	7.5	1.1	1.4
8	1.5	6.5	4.1	1.5

Table 5 – Performance indicators related to biogas production in all conditions.

Condition	n_{H2}	MPr	SMPr	$MYAL_{S,m}$	$MYAL_{C,m}$	$MYRL_{S,m}$	$MYRL_{C,m}$	V_G	$\%H_2$	$\%CH_4$	$\%CO_2$
1	63.3	18.1	18.8	3.6	3.6	17.0	3.6	624	38	0	62
2	133.1	37.7	39.5	5.3	5.5	23.7	5.5	1286	39	0	61
3	29.5	8.3	4.5	1.5	2.1	7.4	2.2	361	31	4	65
4	10.8	3.2	3.4	0.6	0.9	2.4	0.9	293	16	13	71
5	27.5	7.9	8.7	1.1	1.6	4.1	1.7	445	23	12	65
6	5.8	1.7	0.3	0.3	0.7	1.4	0.7	198	11	14	75
7	13.0	3.8	1.4	0.5	1.2	2.5	1.3	233	16	14	70
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	131	0	0	100

Notation: n_{H2} – ($molH_2.d^{-1}$), MPr – ($molH_2.m^{-3}.d^{-1}$), SMPr – ($molH_2.kgTVS^{-1}.d^{-1}$), $MYAL_{S,m}$ – ($mmolH_2.gCOD^{-1}$), $MYAL_{C,m}$ – ($mmolH_2.gCarbohydrate^{-1}$), $MYRL_{S,m}$ – ($mmolH_2.gCOD^{-1}$), $MYRL_{C,m}$ – ($mmolH_2.gCarbohydrate^{-1}$), V_G – ($NmL.cycle^{-1}$)

also observed in other studies with ASBR treating different wastewater (Chen *et al.*, 2009; Buitrón and Carvajal, 2010; Cheong *et al.*, 2007; Arooj *et al.*, 2008).

Table 4 shows an increase of acetic acid and butyric acid concentration in the effluent of Condition 7 compared to Condition 6, which may indicate a change in the metabolic pathway that improved hydrogen production.

Condition 8 (highest concentration of vinasse among all conditions) did not reach stability and hydrogen production decreased until it was no longer detected in biogas. This result shows the difficulty of hydrogen production by vinasse anaerobic treatment, which indicates the importance of the co-digestion study, because an easier biodegradable substrate is necessary to enable a stable system that produces bioenergy. The co-digestion is a feasible option to overcome the drawbacks of monodigestion and to improve the economic feasibility of the process, because it dilutes toxic compounds, balances nutrients, encourages synergy between microorganisms and increases the biodegradable organic matter (Mata-Alvarez *et al.*, 2014; Bouallagui *et al.*, 2009; Wang *et al.*, 2012).

Some authors have shown an improvement in hydrogen production when working with co-digestion, Wang *et al.* (2011) showed that anaerobic co-digestion offers the best condition of pH and carbon / nitrogen ratio for hydrogen production using water of cassava processing and sewage sludge (in thermophilic conditions) to yield an increase in hydrogen production with the mixture of substrate. Hydrogen production in the optimum condition was 17% higher than the condition that only treated water from the processing of cassava. Xia *et al.* (2012) have shown the influence of three different co-substrates (glucose, xylose, and starch) on the conversion of cellulose; experiments were carried out in sequential batch in serum bottles of 50 ml with a work volume of 30 mL. Microcrystalline cellulose was used as substrate (concentration 4.0 $g.L^{-1}$) and glucose, xylose and soluble starch were individually measured as co-substrate (0.4 $g.L^{-1}$). In conclusion, the use of xylose as co-substrate resulted in higher conversion and hydrogen production (reaching a yield for hydrogen of 180 $mL.L^{-1}$).

Reis *et al.* (2015) worked with AFBR reactors and studied the co-digestion of vinasse and glucose to produce hydrogen (studied percentages of vinasse: 0%, 25%, 75% and 100%). They concluded that the increase in the percentage of vinasse in the influent damaged the production of hydrogen and methane production was detected in the condition with 100% of vinasse. In the case of Reis *et al.* (2015), fixing HRT of 6 hours, the maximum percentage of vinasse in the influent which allows hydrogen production without the presence of methane in the biogas was 75%, which also shows the importance of studying co-digestion of vinasse with an easier degraded substrate.

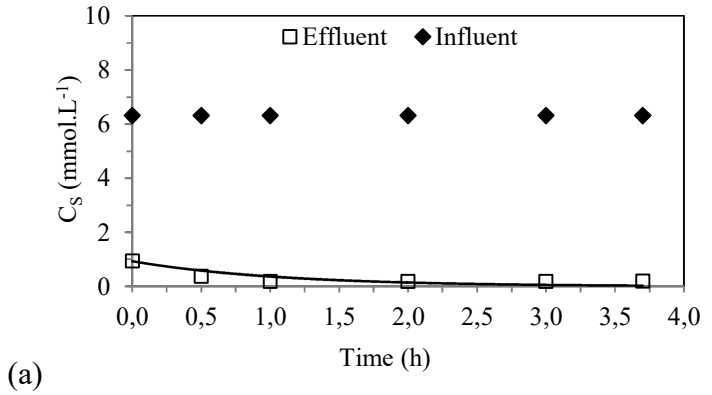
Profiles and kinetic model of the metabolic route

Figures 2, 3 and 4 show the values obtained experimentally (markers) and the values obtained by the adjusted kinetic model (lines), along the cycle for the main variables that are monitored and that are related to the understanding of the acidogenic metabolism in conditions 3, 6 and 7. Substrate is considered as being the sucrose measured by the Dubois method.

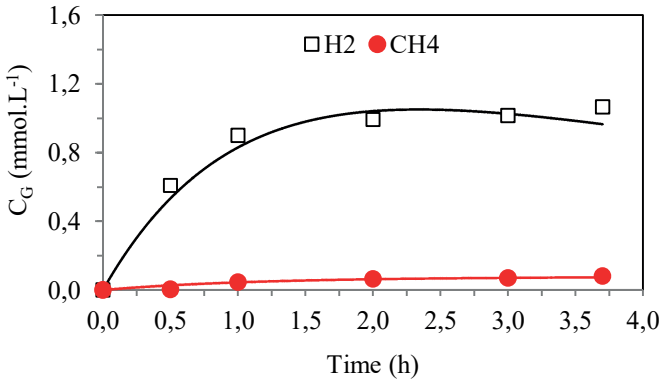
From these figures, it is possible to notice that the model was effective in predicting the experimental data regarding the substrate, acetic acid, propionic acid, butyric acid, ethanol, hydrogen and methane concentrations, validating the interpretation of the kinetic parameters in the experimental conditions.

Thus, from the adjusted kinetic parameters (Table 6), the hydrogen production in Conditions 3 (k_{1H} 0.76 h^{-1} and k_{3H} 0.78 h^{-1}), 6 (k_{1H} 0.32 h^{-1} and k_{3H} 0.34 h^{-1}) and 7 (k_{1H} 0.84 h^{-1} and k_{3H} 0.76 h^{-1}) mainly occurs via the production of acetic and butyric acids, since the parameters associated with the production of hydrogen by the formation of these acids (k_{1H} and k_{3H}) are higher than the ones associated with the production of hydrogen by acetate formation (Equations 15 and 16).

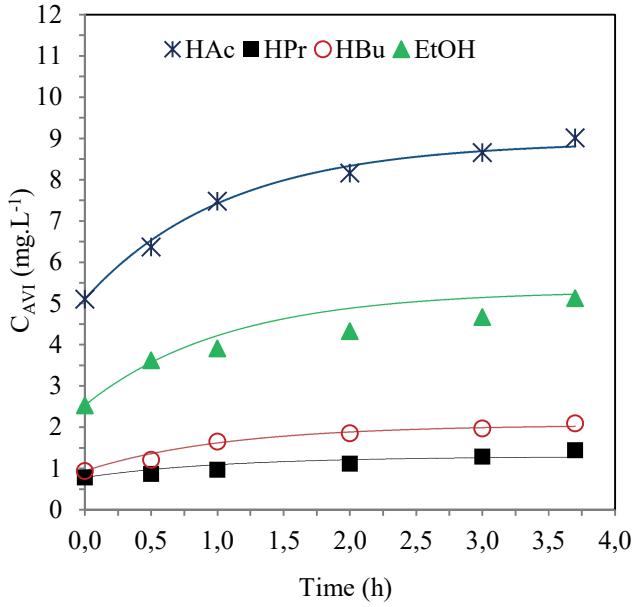
It can also be noted that in conditions 6 and 7 the parameters associated with hydrogen consumption (k_{2H})



(a)



(b)



(c)

Figure 2. Profiles of the experimental data (symbols) and of the kinetic model (lines) in Condition 3.

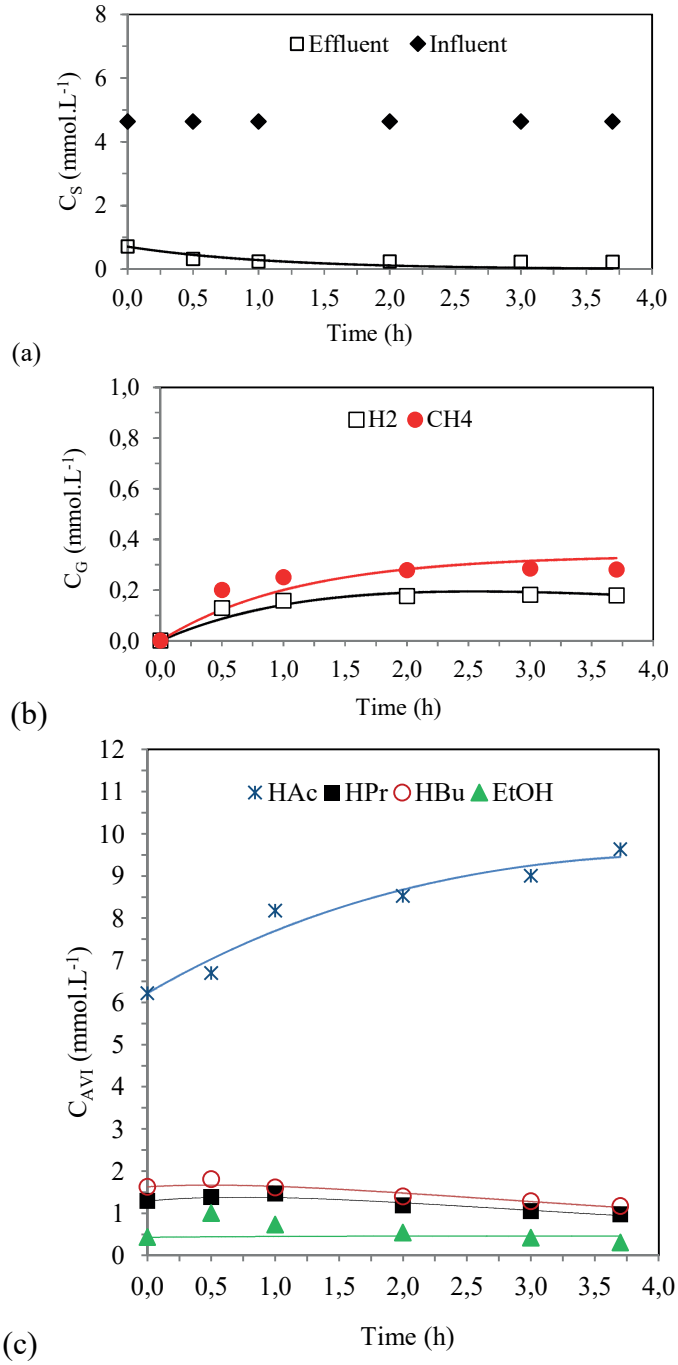


Figure 3. Profiles of the experimental data (symbols) and of the kinetic model (lines) in Condition 6.

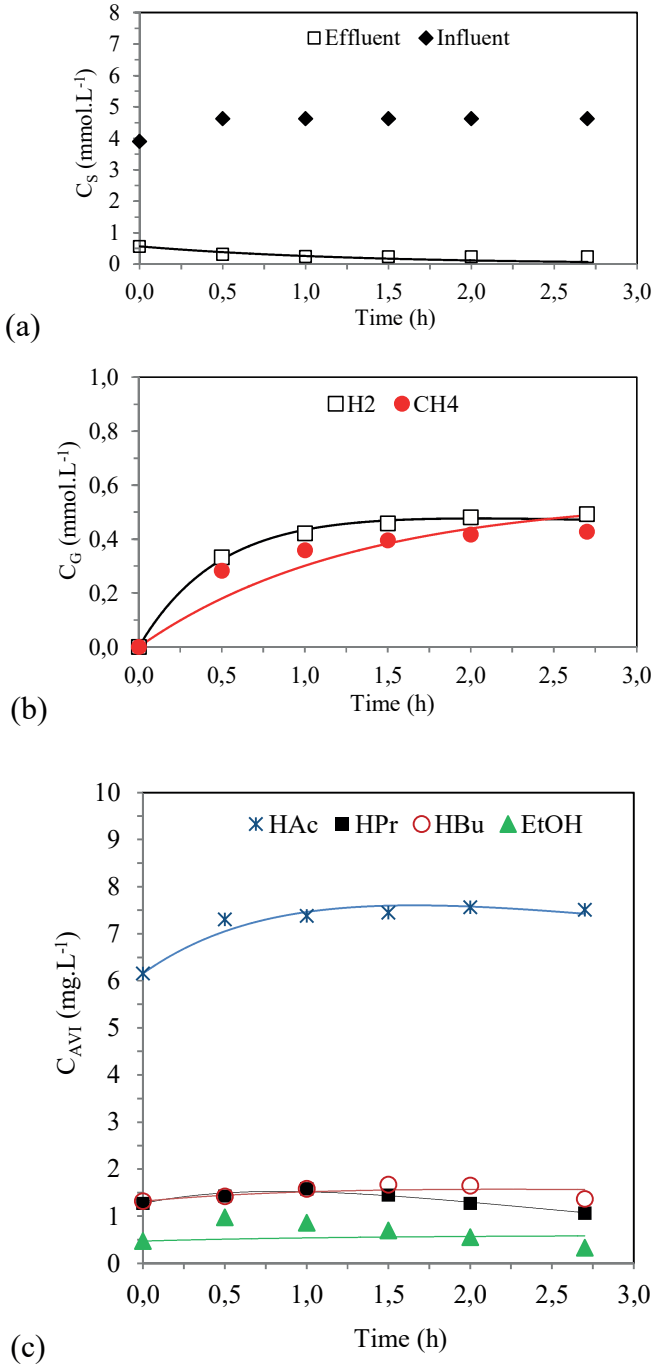


Figure 4. Profiles of the experimental data (symbols) and of the kinetic model (lines) in Condition 7.

Table 6. Parameters of the first order kinetic model.

Parameter	3	6	7
k'_{1S} (h^{-1})	0.93	0.90	0.78
k_{1HAC} (h^{-1})	3.83	1.03	5.17
k_{5HAC} (h^{-1})	0.00	0.55	0.00
k_{6HAC} (h^{-1})	0.00	0.63	3.70
k_{7HAC} (h^{-1})	0.00	0.11	0.87
k_{2HPR} (h^{-1})	0.51	0.77	2.28
k_{5HPR} (h^{-1})	0.00	0.21	0.44
k_{3HBU} (h^{-1})	1.12	0.67	0.66
k_{6HBU} (h^{-1})	0.00	0.18	0.04
k_{4ETOH} (h^{-1})	2.79	0.04	0.17
k_{1H} (h^{-1})	0.76	0.32	0.84
k_{2H} (h^{-1})	0.05	0.68	1.16
k_{3H} (h^{-1})	0.78	0.34	0.76
k_{5H} (h^{-1})	0.00	0.03	0.00
k_{6H} (h^{-1})	0.00	0.12	0.61
k_{8H} (h^{-1})	0.15	0.98	2.10
k_{7M} (h^{-1})	0.00	0.00	0.00
k_{8M} (h^{-1})	0.07	0.43	0.77

and propionic acid formation (k_{2HPR}) are higher than the ones obtained in condition 3, which agrees with the values obtained in the experimental data: condition 3 has the highest values of hydrogen production and the lowest values of propionic acid concentration.

Regarding the methane production, the model indicates that the methane formation is via the hydrogenotrophic route for all conditions (the k_{7M} values – kinetic constant for the acetoclastic route – are zero), which is a coherent result, since it is an acidogenic reactor and, theoretically, it would not produce CH_4. Observing k_{8M}, condition 3 has the parameter with the lowest value and condition 7 has the highest, which also agrees with the results obtained in the experimental monitoring.

The kinetic parameter for ethanol (k_{4ETOH}) also agrees with the production obtained in the monitoring: condition 3 has the constant with the highest value (2.79 h^{-1}).

CONCLUSIONS

Hydrogen production by co-digestion of vinasse/sucrose and vinasse/molasses proved feasible in an AnSBBR. In the co-digestion of vinasse/sucrose (33-67%; 5.6 $gCOD.L^{-1}.d^{-1}$; 4-hour cycle time) a molar productivity of 8.3 $molH_2.m^{-3}.d^{-1}$ was obtained (31% hydrogen and 4% methane) and in the co-digestion of vinasse/molasses (33-67%; 6.9 $gCOD.L^{-1}.d^{-1}$; 3-hour cycle time) 3.8 $molH_2.m^{-3}.d^{-1}$ was obtained (16% hydrogen and 14% methane). The use of molasses as a co-substrate has economic advantages over sucrose. Increasing the percentage of vinasse (75-25% vinasse/molasses) was unfavorable and made the system unstable. Moreover, the decrease in cycle time (from 4 to 3 hours) favored the production of biohydrogen, but did not eliminate methanogenesis. A fist order kinetic model was fitted properly to the obtained data.

NOTATION

$\%CH_4$ Molar fraction of methane (%)
$\%CO_2$ Molar fraction of carbon dioxide (%)
$\%H_2$ Molar fraction of hydrogen (%)
AnSBBR Anaerobic Sequencing Batch Biofilm Reactor
$ASOL_{CT}$ Applied specific organic load based on organic matter ($gCOD.gTVS^{-1}.d^{-1}$)
$ASOL_{ST}$ Applied specific organic load based on carbohydrate ($gSAC.gTVS^{-1}.d^{-1}$)
$AVOL_{CT}$ Applied volumetric organic load based on organic matter ($gCOD.L^{-1}.d^{-1}$)
$AVOL_{ST}$ Applied volumetric organic load based on carbohydrate ($gCarbohydrate.\ L^{-1}.d^{-1}$)
$C_{CT,I}$ Concentration based on organic matter for unfiltered samples in the influent ($mgCOD.L^{-1}$)
$C_{ST,I}$ Concentration based on carbohydrates for unfiltered samples in the influent ($mgCarbohydrate.L^{-1}$)
C_X Relation between the amount of biomass and the volume of liquid medium in the reactor ($gTVS.L^{-1}$)
HST Heat Shock Treatment
LDPE Low-density polyethylene
MPr Daily molar productivity of hydrogen ($molH_2.m^{-3}.d^{-1}$)
M_{TVS} Total biomass in the reactor in total volatile solids (gTVS)
$MYAL_{C,m}$ Molar yield per applied load based on organic matter expressed as kg ($mmolH_2.gCOD^{-1}$)
$MYAL_{S,m}$ Molar yield per applied load based on carbohydrates expressed as kg ($mmolH_2.gCarbohydrate^{-1}$)
$MYRL_{C,m}$ Molar yield per removed load based on organic matter expressed as kg ($mmolH_2.gCOD^{-1}$)
$MYRL_{S,m}$ Molar yield per removed load based on carbohydrates expressed as kg ($mmolH_2.gCarbohydrate^{-1}$)
N Number of cycles per day
n_{H2} Daily molar production of hydrogen ($mmolH_2.d^{-1}$)
pH Hydrogen ion potential (u)
SMPr Daily specific molar productivity of hydrogen ($molH_2.kgTVS^{-1}.d^{-1}$)
TA total alkalinity
t_C Cycle length ($h.Cycle^{-1}$)
TS Total solids concentration ($mgTS.L^{-1}$)
TSS Total suspended solids concentration ($mgTSS.L^{-1}$)
TVA total volatile acids
TVS Total volatile solids concentration ($mgTVS.L^{-1}$)
V_{B+S} Volume of biomass+support (L)
V_F Volume of wastewater fed/discharge during the

cycle (L)
V_G Volume of total biogas produced in the STP ($NmL.d^{-1}$)
V_R Volume of liquid medium in the reactor (L)
V_{RES} Volume of liquid medium maintained in the reactor (L)
VSS Volatile suspended solids concentration ($mgVSS.L^{-1}$)
V_T Total useful reactor volume (L)
e_{CF} Removal efficiency based on organic matter (COD) for filtered samples (%)
e_{CT} Removal efficiency based on organic matter (COD) for unfiltered samples (%)
e_{SF} Removal efficiency based on carbohydrates for filtered samples (%)
e_{ST} Removal efficiency based on carbohydrates for unfiltered samples (%)

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