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Evaluation of semi-continuous operation to hydrogen and volatile fatty acids production using raw glycerol as substrate

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ABSTRACT

Two fermenters were operated by semi-continuous mode with suspended biomass and glycerol at concentrations of 10 and 50 g/L to evaluate the semi-continuous operational conditions to hydrogen and volatile fatty acids production. A high hydrogen production in the semi-continuous reactors at the experiment was observed, with a drop during the test, mainly in the reactor with higher substrate concentration. The yield median of the semi-continuous reactors was 0.25 and 0.01 mol H_2/mol glycerol, and the volumetric productivity median was 350 and 130 mL H_2/L.d in SCR1 and SCR2 (10 and 50 g/L), respectively. It was possible to associate the high hydrogen and volatile fatty acids production in the semi-continuous reactors with the bacterial genus *Enterobacter*, and the low hydrogen production to the bacterial genus *Clostridium*.

1. Introduction

The energy is fundamental to guarantee modern society's quality of life. However, much of the current energy consumption and production not be sustained in the long term [1]. The alternative sources of energy, called biofuels, are objective of extensive research. The technology involving biohydrogen use seems advantageous [2–6] because its combustion generates only water, and your biologic production can be by renewable sources, as plant biomass and wastes [6,7].

The dark fermentation process produces hydrogen (H_2) and valuable metabolites, as volatile fatty acids (VFA) [8,9]. According to Den Boer et al. [10], the textile, pharmaceutical, food, plastics, and leather industries widely uses the VFAs and their derivatives. These compounds are also potentially renewable carbon sources used in biological nitrogen removal [11], generation of electricity through microbial fuel cells [12], and synthesis of complex polymers [13].

The substrate selected must be from renewable resources, present sufficient volume for fermentation, and low cost to reach the sustainability requirements [14]. Brazil is one of the largest biodiesel producers and consumers in the world [4]. The transesterification process required for biodiesel production generates a lot amount of glycerol, near 10% of the total volume of biodiesel produced [8,9,15].

The textile, chemical, pharmaceutical, and food industries also can use pure glycerol [9]. However, the degree of purity conditions the commercial glycerin use, and purification processes may be required, which would entail high costs. Therefore, the crude glycerol surpluses require new uses [4]. Its conversion to higher-value products by anaerobic fermentation may represent a promising route to achieve economic viability in the biofuel industry [16]. Besides, the energy content of crude glycerol is 25.30 MJ/kg, possibly due to the presence of methanol and biodiesel. Such high-energy content indicates it is the potential to be a useful carbon source for hydrogen [15].

The fermentation to H_2 production requires a higher organic loading rate to carry out an energy-efficient operation. However, initial substrate concentration is also an essential factor to activate the germination process and in the prevention of re-sporulation. Thus, initial substrate concentration within optimum range

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enhances the hydrogen production in the dark fermentation process. The excess of the substrate, on the other hand, may cause unfavorable conditions for the process by causing variation in the pH, the concentration of VFAs, and the hydrogen partial pressure of the reactor. Hence, an optimum range of initial substrate concentration determination is required to minimize the substrate inhibition [17].

The natural conditions of each reactor model interfere in the formation of the final products of hydrogen production by changes in the microbial community. The batch system has a simple operation and control. However, it is more suitable for initial reaction optimization studies [18].

Thus, large-scale operations require other forms of functioning [19]. According to Hallenbeck & Gosh [18], the use of continuous or at least semi-continuous operation may viability the industrial processes. The complete stirring tanks reactors (CSTR) with suspended biomass allows for better mass transfer between microorganisms and substrate. However, it is difficult to maintain a sufficient amount of hydrogen-producing bacteria in the bioreactor under high flow rates or short hydraulic detention times, resulting in cells loading out of the system [20]. On the other hand, the immobilized biomass systems allow the maintenance of high cell concentrations, leading to higher rates of H_2 production. Meantime, the retention and consequent accumulation of biogas are the main problems of these systems [21].

The operation of the successive cycle aims to minimize the adverse effects of continuous systems. The anaerobic sequencing batch reactor (AnSBR) mode has been widely used [22–25], and the subsequent steps are feeding, reaction, decantation, and emptying [22,24]. This condition allows a high biomass retaining, be by decantation [24], or by immobilized biomass [25]. Its additional advantage over other systems includes the operational ease with excellent flexibility to accommodate both batch and continuous operations [24].

According to Borzani [26], the semi-continuous process follows the operation order:

(1) Wait for the established period for fermentation;
(2) After this period, a fraction of the reactor contents removed (with agitation turned on), and a remaining fermented must keep;
(3) Add the substrate to the reactor on the same amount removed in step 2.

The semi-continuous is a distinct technique, in which the operation relates to substrate loading shocks, which may be interesting in certain situations [26], and it allows the solids retention time (SRT) control. The SRT determines the efficiency of substrate utilization, microbial population, and metabolic routes. Even being an important parameter, there are few studies in the literature that report its control for H_2 production. The high SRT leads to the growth of H_2 consuming microorganisms in the production of hydrogen via fermentation. They include methanogenic bacteria and substrate competitors such as no H_2 producing acidogenic bacteria [27,28].

Thus, the sequential batching concept, as described, has as its objective the concentration of biomass in the reactors. In this work, the semi-continuous (SC) operation intends to distinguish from the AnSBR operation. The tests utilized intermittent feeding and emptying, as in sequential reactors, with suspended biomass. As differential, the previous decantation step to fermentation medium remotion not applied. Few studies carried out using the semi-continuous operation, as described above, for hydrogen production, using mixed biomass and crude glycerol.

This work aims to evaluate the H_2 and VFA production in reactors operated semi-continuously, using two different crude glycerol concentrations (10 and 50 g/L) and mixed biomass. It was also evaluated the microbial diversity in this operation, in high and low hydrogen production.

2. Methodology

2.1. Experimental procedure

The tests temperature was 35 ± 2 °C. The initial pH was 5.5–6.0, according to Mohan et al. [29], to avoid the growth of methanogenic bacteria. No pH adjustment performed during the fermentation process [30]. The glycerol used in the experiment came from a biodiesel industry located in southern Brazil. The glycerol concentrations used were 10 and 50 g/L, according to previous studies [31,32].

The hydraulic retention time (HRT) was 24 h, considered the time between two substrate insertion steps. The SRT was 1.5 days, similar to the ideal found [33], of 1.4 d.

The inoculum came from an upflow anaerobic sludge blanket reactor (UASB) from an effluent treatment plant of a soybean processing company. The microorganisms received a thermal pretreatment at 100 °C for 15 min, as described by Rossi et al. [34].

The applied medium nutritional formulation was according to Lin and Lay [35]: 40 mg/L of $MgCl_2.6H_2O$; 0,5 mg/L of $CoCl_2.6H_2O$; 1000 mg/L of $CaCl_2.2H_2O$; 0,1 mg/L of $NiCl_2.6H_2O$; 2,5 mg/L of $MnCl_2.6H_2O$; 0,5 mg/L of KI; 50 mg/L of NH_4Cl; 10 mg/L of NaCl; 0,5 mg/L of ZnCl; 5 mg/L of $FeSO_4.7H_2O$; 0,5 mg/L of $MnSO_4.4H_2O$; 5 mg/L of $CuSO_4.5H_2O$; 0,1 mg/L of $Na_2MoO_4.2H_2O$. Additionally, a phosphorus source also inserted, K_2HPO_4 125 mg/L, as described by the same authors.

2.2. Semi-continuous reactors

Two fermenters (New Brunswick Fermenter, BioFlo 110) were employed. Each have a total and useful volume of 7.5 L and 5 L, respectively. The reactor mixture was kept constant at 250 rpm, and the flow rate of produced gas performed by MilliGascounter® gas meters (Ritter model MGC-1). The substrate insertion and effluent removal performed using Masterflex® Easy Load Model 7518-10 pumps at the maximum flow rate of 5.5 mL/s.

The semi-continuous reactors denominated SCR1, and SCR2 had the same operational conditions except by the initial glycerol concentration added, 10 and, 50 g/L, respectively. The crude glycerol diluted in the nutritional medium used in the reactor feed, considering the tank volume in the two proposed concentrations to meet the initial COD. Both the reactors inoculated with total volatile solids (TVS) of 2.5 g/L. It was inserted the same inoculum volume into both reactors, near to 0,8 L each, to achieve the proposed conditions.

The semi-continuous operation follows these steps: (1) the fermentation time was 1 day. (2) After this period, it was removed 3.3 L of the reaction medium (66% of content), with stirring (SRT maintenance of 1,5 days). The remaining 1.7 L (34%) of the reactor content keep as inoculum, (3) glycerol, and nutritional medium insertion, at the same volume removed. These systems operated for 132 days.

2.3. Monitoring analysis

The chemical oxygen demand (COD), pH and VFA analysis performed three times a week, and TVS performed once a week, according to procedures described by APHA [36]. The glycerol analysis realized following the methodology described by Englis & Wollerman [37]. This assay involves a mixture of 3.2 mL of sample

Table 1
Physical and chemical characterization of glycerol and inoculum used in the tests.

Inoculum	
Total solids (mg/L)	58,206
Total volatile solids (mg/L)	15,872
Total fixed solids (mg/L)	42,333
Glycerol	
COD (g/L)	1057
% Ash content	3.2
% Glycerol	80.1
pH	6.1

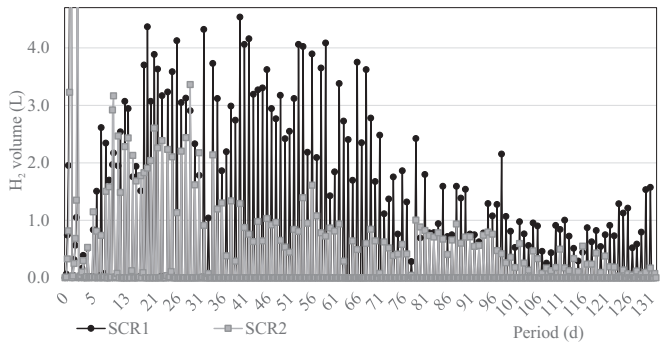

Fig. 1. H_2 volume (L) produced daily on the operation of semi-continuous reactors.

reactor with 12 mL $K_2Cr_2O_7$ solution and 10 mL of concentrated H_2SO_4 in a glass flask. The flask was submitted to a water bath with boiling water for 20 min. After this time, at room temperature, it proceeded to read in a Spectrophotometer UV/VIS UV-1600 Pró-Toolsat 587 nm.

The biogas quality was verified every day, using a gas chromatograph Dani GC 1000 with thermal conductivity detector (TCD) and Molecular Sieve column 80/100. The chromatographic conditions followed the described by Morimoto et al. [38]: injector temperature 50 °C; detector temperature 50 °C; column temperature 40 °C; helium gas for dragging, the flux of 25 mL/min and sample injected with 1 mL volume.

The volatile fatty acids concentrations analysis (acetic, butyric and propionic acids) was performed by gas chromatograph Dani GC 1000, with flame ionization detector (FID) and capillary column Nukol™ Supelco® (30m × 0.25 mm × 0.25 μm). The methodology followed the described by Cuetos et al. [39], with adaptations: injector and detector temperature of 220 °C and 250 °C, respectively. The oven temperature was 60–180 °C, with two ramps: 60 °C until 170 °C, to 10 °C/min; 170 °C–180 °C, to 50 °C/min. The dragging gas was helium, with a flow of 1 mL/min and the injection volume of 1 μL. The samples subjected to the chromatography filtered on a glass fiber filter, with 0.45 μm porosity, and stored under refrigeration, using formic acid for preservation.

2.4. Microbial DNA analysis

Four microbial samples of the semi-continuous reactors were analyzed - two in each reactor: one at the highest (at 26th day of operation) and another one at the smaller (at 111th day) gas production condition.

The DNA extraction followed the methodology described by Krsek et al. [40], with the modifications described by Soares et al. [41], with successive washes with phenol and chloroform to DNA cleaning. The Polymerase Chain Reaction (PCR) used to amplify a partial segment of 16S rRNA gene (region V4) using the F515 (5' -GTGCCAGCMGCCGCGGTAA – 3′) and R806 (5' – GGACTACVSGGGTATCTAAT – 3′) primers [42]. The amplification reactions contained approximately 5.0 ng of DNA, 0.1 mM MgCl2,

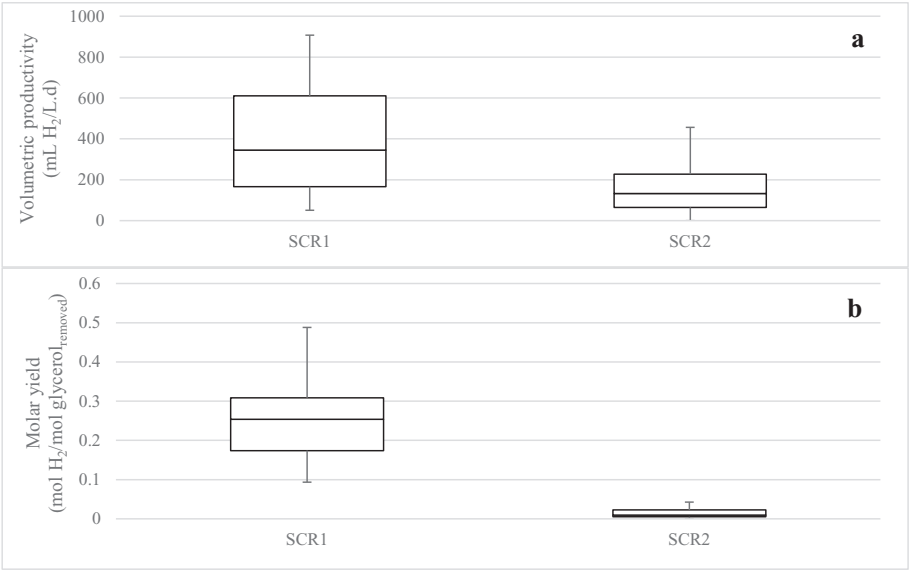

Fig. 2. Parameters of evaluation of hydrogen production in semi-continuous reactors: (a) volumetric productivity and (b) molar yield.

Table 2
Comparison of the results found in the bibliography using glycerol (pure and crude) as substrate in continuous systems, with the data obtained in this work.

Authors	Inoculum	Reactor model	HRT and temperature	Glycerol concentration	Higher H_2 production	Higher molar yield
Moncayo Bravo et al. [58]	Anaerobic sludge	AnSBBR	4 h 30 °C	5 g/L Raw and pure	1512 mL H_2/L.d (pure) 200 mL H_2/L.d (raw)	Not informed
Ito et al. [53]	*Enterobacter aerogenes* HU-101	Continuous with fixed biomass	10 h 37 °C	10 g/L Raw and pure	46272 mL H_2/L.d (pure) 36439 mL H_2/L.d (raw)	1.12 mol H_2/mol (raw)
Temudo et al. [54]	Anaerobic mixed culture	CSTR	10 h 30 °C	4–24 g/L Glycerol + glucose	Not informed	0.05 mol H_2/mol glycerol
Chookaew, O-Thong & Prasertsan [56]	*Klebsiella sp. TR17 with no sterile conditions*	UASB	4 h 40°C	30 g/L Raw	5835 mL H_2/L.d	Not informed
Lo et al. [30]	*Clostridium pasteurianum* CH4	CSTR	12 h 35 °C	10 g/L Raw and pure	2474 mL H_2/L.d (pure) 3984 mL H_2/L.d (raw)	0.50 molH_2/mol (pure) 0.77 mol H_2/mol (raw)
Reungsang, Sittijunda & O-Thong [32]	*Enterobacter aerogenes* ATCC 13048	UASB	24 h 37 °C	50 g/L.d Raw and pure	4824 mL H_2/L.d (pure) 3333 mL H_2/L.d (raw)	0.41 mol H_2/mol (pure) 0.32 mol H_2/mol (raw)
Lovato et al. [25]	Anaerobic sludge	AnSBBR	3 and 4 h 30 °C	4 g/L (pure) 5 g/L (raw)	2258 mL H_2/L.d (pure) 217 mL H_2/L.d (raw)	1.19 mol H_2/mol (pure) 0.10 mol H_2/mol (raw)
Sarma et al. [6]	*Enterobacter aerogenes* NRRL B-407	Semi-continuous	40 and 64 h Not informed	60 and 120 g/L Raw	4800 e 5180 mL H_2/L.d (60 and 120 g/L, respectively)	4.06 and 4.19 mols H_2/mol glycerol (60 and 120 g/L, respectively)
Jitrwung & Yargeau [60]	*Enterobacter aerogenes* ATCC 35029	CSTR	±52 h 37 °C	18,5 g/L Raw	Not informed	0.86 mol H_2/mol glycerol
Silva-Illanes et al. [61]	Actived sludge	CSTR	12 h Not informed	10 g/L Raw	Not informed	0.41 mol H_2/mol raw glycerol
This work	Anaerobic sludge	Semi-continuous	24 h 35 °C	10 and 50 g/L Raw	Median of 350 and 130 mL H_2/L.d (10 and 50 g/L respectively)	Median of 0.25 and 0.01 mol H_2/mol glycerol (10 and 50 g/L, respectively)

20 μM each dNTP, 0.3 μM each primer and 1U of Platinum Taq DNA polymerase (Invitrogen) in a final volume of 25 μL. PCR Express Temperature Cycling System (Thermo Hybrid) was used for the amplification, and performed: (1) an initial denaturation step at 95 °C for 3 min followed by 25 cycles at 95 °C for 30 s; (2) 52 °C for 1 min; (3) 72 °C for 1 min and one cycle at 72 °C for 7 min for final elongation. The analyze of PCR products were done by electrophoresis in 1% agarose gels with ethidium bromide in TBE buffer and visualized by UV light.

The fragments generated by PCR were subjected to high throughput sequencing using an Ion PGM sequencer (Thermo Fisher). The Ion Plus Fragment Library kit was employed to the libraries' construction, for short amplicons (≤350 pb), from 100 ng of PCR product of each amplification. Sequencing was conducted on an Ion PGM System (Thermo Fisher) using an Ion 316 chip, following the manufacturer's instructions.

The 16S rRNA reads submitted to quality control that retained sequences with a minimum length of 100 bp and trimmed to remove low-quality bases for the minimum Phred score of 30 and to remove any sequence with ambiguous bases and homopolymers (PRINSEQ) [43]. Before the global clustering, the remaining sequences were dereplicated, sorted by decreasing read abundance and then filtered to exclude singletons (734,564 singletons, corresponding to 51.2% of the sequences at this step) using USEARCH v7.0.1090 [44]. After, 222 putative chimera sequences removed (which corresponds to 3.4% of the sequences at this step) using the RDP reference database [45] in USEARCH. The taxonomic assignment obtained using QIIME v1.7 [46]. Operational taxonomic units (OTUs) were selected based on a 97% sequence similarity, and taxonomic data through the classification algorithm using the 97% OTUs version of GreenGenes 13.8 [47].

3. Results and discussion

3.1. Initial inoculum and glycerol characterization

The physical and chemical characterization of glycerol, based on COD, pH, glycerol percentage, and ash content, was the initial activity of experimental studies, which also it evaluated the inoculum TVS concentration. Table 1 shows the obtained values.

The data in Table 1 show the high concentration of organic matter in glycerol, represented by the COD. Also, the glycerol content of 80% highlights the presence of other compounds in the raw effluent.

3.2. Hydrogen volume, hydrogen molar yield and volumetric productivity

Fig. 1 shows the maximum H_2 volume results verified in the SCR1 and SCR2. The obtained values from the first two days of semi-continuous reactors were high and, therefore, cannot be visualized in the graph scale. On the first day of operation, the production was 11.7 L in SCR1, and 14.6 L in SCR2, and SCR1 of 6.6 L and SCR2 with 9.5 L on the second day.

Fig. 1 also exhibits an H_2 higher volume in semi-continuous reactors on the 70 first days. It shows a decreasing tendency after this period in both reactors. In general, the SCR1 had a higher H_2 production during the whole operational period.

The high hydrogen production observed (Fig. 1) in the first two days may have been due to the natural conversion of the substrate. The fast-growing H_2 producing microorganisms produce more hydrogen gas because the carbon source is surplus in these situations. The hydrogen accumulation occurs due to H_2 transfer limitations from the producing to the consumer's microorganisms [48]. However, the increase of partial hydrogen pressure in the headspace can result in an H_2 production decrease in subsequent days of operation. This effect can interfere the fermentation reactions and result in a drop in hydrogen yield [23,49]. It seems this effect occurred until day 10 when it reached an equilibrium, and gas production started again.

Fig. 2a shows the volumetric productivity data, and Fig. 2b exhibits the obtained molar yield (mol H_2/$mol_{glycerolremov}$) of semi-continuous reactors.

The maximum values of volumetric production (Fig. 4) were

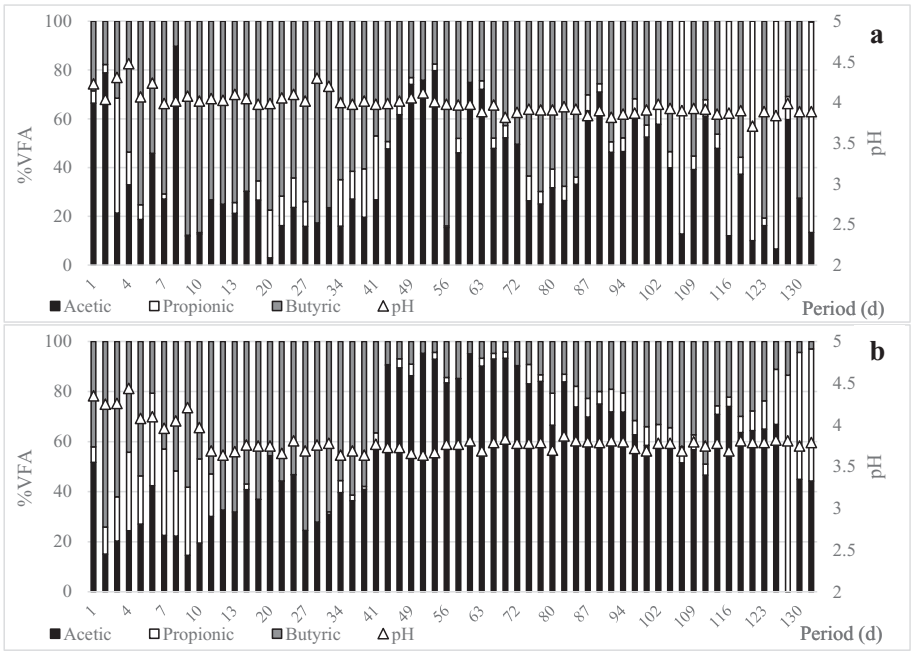

Fig. 3. VFA massic distribution related to pH of semi-continuous reactors 1 (SCR1 – a) and 2 (SCR2 – b), respectively, during the experimental period.

found on the first day of operation, equal to 2340 mL H_2/L.d in SCR1 and 2928 mL H_2/L in SCR2. The Boxplot data distribution shows median values in SCR1 of 350 and SCR2 of 130 mL H_2/L.d. Thus, the comparative molar yield results (Fig. 4) show the highest molar yield values on the first day of operation were 3.58 and 0.24 mol H_2/mol, and median values of 0.25 and less than 0.05 mol H_2/mol glycerol for SCR1 and SCR2, respectively.

The analysis of the parameters indicates the semi-continuous reactors present satisfactory volumetric productivity results. Nonetheless, low H_2 yield when compared to the data found in the literature (Table 2) related to other reactors configurations but using continuous or semi-continuous systems. The proposed model of operation is related to the high volumetric productivity values, and according to Borzani [26], is based on load shock. There is a VFA accumulation under organic or hydraulic load shock conditions. Therefore, the acidogenic step is favored to the detriment of the methanogenic phase. However, if the reactor is overloaded, their performance is drastically reduced [49], because the substrate or the products formed may have an inhibitory effect when the ideal limit of the volumetric organic load is exceeded [50]. It may explain the decrease in H_2 production during the experiment, as shown in Fig. 1.

The molar yield, on the other hand, relates the glycerol amount effectively converted to hydrogen. The used glycerol concentrations (10 and 50 g/L) and the applied HRT (24h) may contribute to the low yield values. In semi-continuous reactors, these two factors seem to contribute together to direct the metabolic pathway to the production of intermediates that decrease or consume not only H_2 but also the formed VFA. According to several studies [33,53–55], the increase in glycerol concentration and the accumulation of fermentation products, such as acetic and butyric acids [51,52], results in a decrease in H_2 yield.

The low yield may also due to the HRT employed. Chookaew, O-Thong & Prasertsan [56] and Zhang et al. [57] found that low HRT values favor the production of lower ethanol concentrations, increasing H_2 production. The low HRT (values below 24h) may increase H_2 production [56] because it can eliminate the competitor generation [5].

Another reason may be associated with the inhibition caused by the substrate or the adverse effects caused by the raw glycerol constituents. Moncayo Bravo et al. [58] mention that impurities in crude glycerol can harm H_2 production, as well as hamper biomass growth. According to Chi et al. [59], the impurities mainly present in glycerol were soap, free fatty acids, methanol, and unreacted glycerides.

Due to the intrinsic operation of the semi-continuous, with the

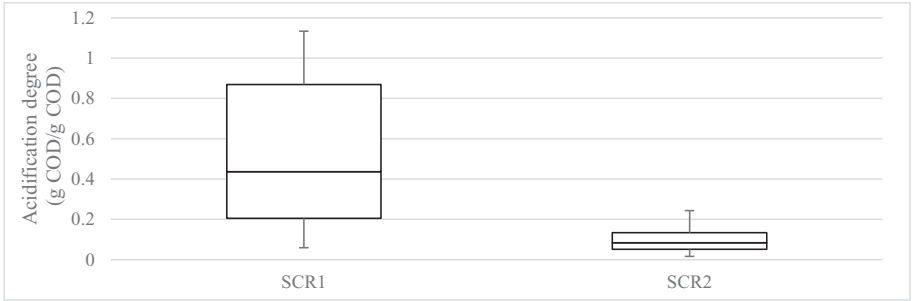

Fig. 4. Boxplot graph of acidification degree obtained on semi-continuous reactors.

remotion of 66% of the reactor content, and 34% maintained, the dilution rate (flow rate applied by reactor volume) was approximately 0.6 d^{-1}. Thus, the constant concentration of these substances may also contribute to this result, mainly at SCR2, operated at high organic load. This effect can occur even remotion part of the liquid medium to attend the proposed initial operating condition.

According to Table 2, high yields and volumetric productivity were verified using pure culture systems using glycerol as a substrate in continuous systems. However, pure cultures do not represent real situations; for example, the use of industrial effluent [4]. A previous stage of substrate sterilization is required to maintain the chosen pure culture in the reactor, which represents an additional cost and time-consuming, as well as the use of high temperatures that may make the process economically unfeasible. Thus, the development of research, optimizing the parameters for higher the efficiency of H_2 production, employing mixed cultures, and mesophilic environments are essential, in terms of costs and time. There are other works in the literature reporting high yields using batch systems but not considered due to the difficulty of their application on a larger scale.

Table 2 shows a comparison of values found in the literature using other operational models with the results of this work. It is important to emphasize that the evaluation of volumetric productivity by the authors, in general, utilizes the maximum values found of this parameter. So, the results of volumetric productivity when mixed cultures and mesophilic systems obtained in this work are considered satisfactory. However, the main disadvantage observed of semi-continuous mode, in the employed conditions of glycerol concentration and HRT, was the low yield of H_2.

According to Gavala, Skiadas & Ahring [62], there is a distinct trend between technical and economic H_2 production efficiency. The first considered on yield and the second on volumetric productivity. The semi-continuous model showed promising economic efficiency, according to the results obtained in the present work. However, the technical efficiency might also be improved.

3.3. pH and volatile fatty acids

The semi-continuous operation performance was evaluated regarding volatile fatty acids production by their massic distribution (%) as well as by the acidification degree. The acidification degree relates to the produced VFA concentrations and substrate influent, both in COD form.

Fig. 3a and b shows the VFA massic distribution related to the pH of the SCR1 and SCR2, respectively. Fig. 3 exhibits the acetic and butyric acids predominance during the experiment, except for the last operation days, with a higher proportion of propionic acid.

The obtained results shown in Fig. 1 highlights two pH-related findings. The first is a decrease in their values in the reactor operated with lower substrate concentration (SCR1) after 60 days. In SCR2, the lowest pH values were reached in a shorter time, close to 15 days. The second finding is related to the pH value with the substrate concentration. The higher glycerol concentration leads to a lower pH value.

The proposed metabolic reactions for glycerol fermentation may follow two possibilities. The glycerol is converted to dihydroxyacetone by the enzyme glycerol dehydrogenase (dhaD) at first oxidative route. Subsequently, it is phosphorylated by adenosine triphosphate (ATP) in the presence of dihydroxyacetone kinase (dhaK). Dihydroxyacetone-P is subjected to glycolysis to form pyruvate. The pyruvate formed can be converted into different products, such as lactic, acetic, and butyric acids, carbon dioxide, and H_2 [63,64]. The glycerol is reduced to 3-hydroxypropionaldehyde by the enzyme glycerol dehydratase (dhaB) in the second route and then converted by an enzyme

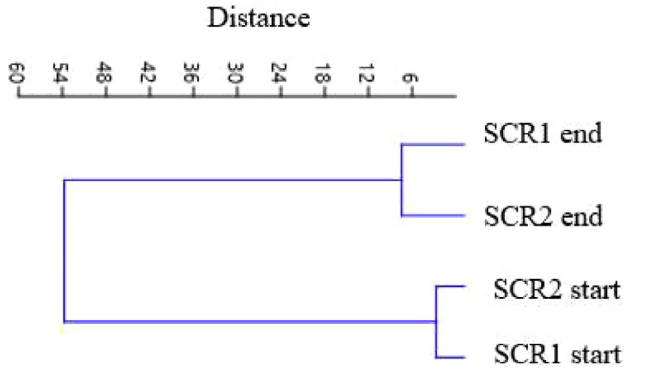

Fig. 5. Classic hierarchical grouping based on the present microorganism relative abundance of each reactor.

grouped to $NADH_2$ to 1,3-propanediol (1,3-PD) and excreted from the cells [63].

The H_2 theoretical maximum production is related to the formation of acetic acid (3 mol H_2/mol glycerol – Eq. (1)), followed by butyric acid (2 mol H_2/mol glycerol – Eq. (2)) [11]. The production 1,3-propanediol (1,3-PD – Eq. (5)) and propionic acid does not generate H_2 (Eq. (6)) [65].

$$C_3H_8O_3 + H_2O \rightarrow \underbrace{CH_3COOH}_{Acetic\ acid} + CO_2 + 3H_2 \quad (1)$$

$$2C_3H_8O_3 \rightarrow \underbrace{C_4H_8O_2}_{Butyric\ acid} + 2CO_2 + 4H_2 \quad (2)$$

$$2C_3H_8O_3 \rightarrow \underbrace{C_4H_{10}O}_{Butanol} + 2CO_2 + 2H_2 + H_2O \quad (3)$$

$$C_3H_8O_3 \rightarrow \underbrace{C_2H_6O}_{Etanol} + CO_2 + H_2 \quad (4)$$

$$2C_3H_8O_3 + H_2 \rightarrow \underbrace{CH_3COOH}_{1,3-PD} + H_2O \quad (5)$$

$$C_3H_8O_3 \rightarrow \underbrace{C_3H_6O_2}_{Propionic\ acid} + H_2O \quad (6)$$

The H_2 generation by fermentation also produces organic acids. There is high hydrogen producing when the byproducts formed are acetic and butyric acids, but the H_2 generation decreases with alcohol and 1,3-PD production.

The change in the VFA production route verified in the last operating days may be related to the cellular energy generation. Wang, Zhou & Li [66] relates the accumulation of propionic acid often occurred in the condition of the high yield of NADH. This effect may due to a proper ratio of NADH/NAD + maintained inside the cell, and propionic-type fermentation can produce more NAD + than butyric-type fermentation.

Thus, under the condition of high yield NADH, propionic acid fermentation replaces the butyric acid fermentation spontaneously for maintaining a proper ratio of NADH/NAD+.

According to Bengtsson et al. [67], the acidification degree relates to the amount of the initial substrate inserted was converted to VFA (acetic, propionic, and butyric acids). In the form g COD/g COD, it is calculated by individually converting each VFA to COD units to give the CODVFA. A sum of these values is made, which is

Table 3
The relative abundance of microorganisms found in semi-continuous reactors. SCR1 and SCR2 at high H_2 production (26 days) and low H_2 production final point (110 days). *R.A. = relative abundance.

Semi-continuous 1 (High H_2 production)		Semi-continuous 1 (Low H_2 production)		Semi-continuous 2 (High H_2 production)		Semi-continuous 2 (Low H_2 production)	
Family	***R. A.**** ***(%)***	***Family***	***R. A.**** ***(%)***	***Family***	***R. A.**** ***(%)***	***Family***	***R. A.**** ***(%)***
Enterobacteriaceae	89.07	*Clostridiaceae*	62.60	*Enterobacteriaceae*	92.80	*Clostridiaceae*	53.00
Enterobacter	46.5	*Clostridium*	31.3	*Enterobacter*	49.00	*Clostridium*	26.6
Erwinia	0.55	unknown OTU	31.3	*Erwinia*	0.77	unknown OTU	26.4
Citrobacter	0.12			*Citrobacter*	0.15		
unknown OTU	41.9			unknown OTU	42.70		
Clostridiaceae	4.00	*Enterobacteriaceae*	33.10	*Pseudomonadaceae*	0.22	*Enterobacteriaceae*	40.20
Clostridium	2.1	*Enterobacter*	17.2			*Enterobacter*	20.5
unknown OTU	1.9	*Erwinia*	0.34			*Erwinia*	0.36
		unknown OTU	15.6			unknown OTU	19.4
Porphyromonadaceae	0.12	*Veillonellaceae*	0.34	*Shewanellaceae*	0.18	*Veillonellaceae*	0.20
Shewanellaceae	0.10	Others	3.58	Others	6.56	*Shewanellaceae*	0.77
Others	6.47					Others	4.83

divided by the influent COD. Fig. 4 shows the obtained values in the Boxplot form.

The SCR1 presented the highest acidification (referring to the analyzed acids) with a median of 45%. On the other hand, SCR2 showed a median of approximately 10%. The acidification degree relates to the COD of the VFA generated (acetic, propionic, and butyric acids) with the COD of influent. This value in SCR2 was close to 0.1 in the whole operational period, or only 10% of the initial substrate converted to VFA.

The acid production accompanied by H_2 production. There was a high generation in the first month, and a falling tendency at the experiment end. This fact may explain the high variability found in the degree of acidification, mainly in SCR1.

The glycerol concentration showed direct interference on the reaction medium pH [18] and fermentation products. In the absence of excess available carbonaceous source, the acids produced are consumed, avoiding a sudden drop in pH. Higher concentrations of glycerol favored the formation of other fermentation products than VFA production, which also results in a lower yield of H_2, corroborating the data already shown. As described by Keskin et al. [49], the metabolic pathway during H_2 production can shift to solvent production by acidogenic fermentation under stressful environmental conditions. After this shift, acetone, ethanol, and butanol production can occur in the reactor, and these solvents can cause a decrease or cessation of biohydrogen production.

According to Huang & Gong [68] and Rossi et al. [63], to the physiological function of the transformation of glycerol into 1,3-PD is probably due to the need for the oxidation of equivalent reduced

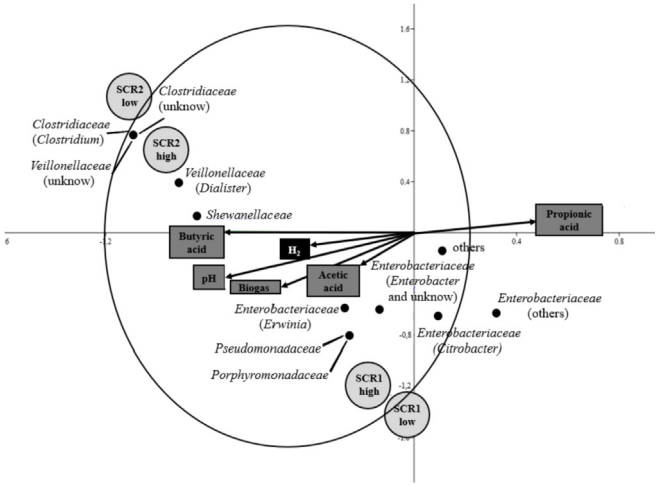

Fig. 6. Canonical correlation analysis realized between microorganisms, chemical parameters, biogas, and hydrogen volume. The graph shows the correlation proximity between the evaluated variables.

forms, NAD, to be used in energy-producing routes from glycerol degradation [63]. That is, the production of 1,3-PD is a way to regenerate NAD, used for biomass production, and other products from glycerol [69].

The higher acid production, analyzed by acidification degree

values in the semi-continuous reactors, is related to the lower applied glycerol concentration (SCR1). The increase of glycerol concentration may reduce its conversion, resulting in intermediates not related or related to a low yield of H_2 [36], as 1,3-PD (Eq. (5)) or alcohol (Eqs. (3) and (4)) [36]. Reungsang, Sittijunda & O-Thong [32], using *Enterobacter aerogenes* ATCC 13048 immobilized as inoculum in the UASB reactor operated with 37.5; 50 and 62.5 g/L showed higher production of 1,3-PD and ethanol as final products from crude glycerol fermentation. Sarma et al. [31] related the production of acetone, butanol, and ethanol when used crude glycerol at high concentration (above 60 g/L). The authors also concluded that substrate concentration is the parameter that determines the amount and type of intermediates during the fermentative production of H_2.

As described by Khanal et al. [70], the reactions shift from a hydrogen/acid production phase to a solvent production phase when the microbial population reaches the stationary growth phase when the pH drops to 4.5 or below. The build-up of volatile fatty acids and hydrogen during the exponential growth phase can induce this shift. Therefore, the fermentation in the SCR2 reactor may favor solvent formation for most of the operational period, which justifies its lower H_2 production, the pH decrease in a shorter period, and the lower degree of acidification obtained in SCR2.

3.4. Microorganisms community analysis

There were two microbial communities associated with the SCR1 and SCR2, determined from high throughput DNA sequencing. As shown in Fig. 1, it is possible to differentiate two conditions from the 70th day of operation. Thus, the collection of microbial samples occurred at two times: on the 26th day to evaluate the high H_2 production and at low production at the 111th day. The 26th-day analysis represents a microbial diversity at the high H_2 production, considering the adaptation condition of inoculum to the substrate employed.

Based on the DNA classical hierarchical grouping, by the UPGMA method, samples submitted to high throughput sequencing and grouped accord to the experimental model (Fig. 5). It found similar microorganisms present in both reactors, in each initial glycerol concentration. They were also like each other at the low H_2 production condition, but different from the initially collected sample.

Thus, the cluster showed in Fig. 5 revealed two groups: the first related to the high H_2 generation and the second to the lower production of H_2. The differences between the two groups may occur due to the substrate concentration influence on the selection of the microorganisms.

The predominance on the SCR1 was from the bacterial family *Enterobacteriaceae*, with 89.07% of the total sequences, according to Table 3. Some modifications to the relative abundance of the microorganisms in SCR1 occurred at low H_2 production, and the family *Clostridiaceae* was predominant at this condition, corresponding to 62.60%.

The same pattern also observed in SCR2 in both operation times. The dominance was of family *Enterobacteriaceae* at better H_2 production, with 92.80% of the total sequences. At the 111th day, the family *Clostridiaceae* was the most abundant, with 53.00% of organisms.

The changes in the metabolic pathway of the microorganisms in SCR at the high and low H_2 production were evident. This modification can be explained by three main reasons: pH, microbial community composition, and the impurities accumulation (already described above).

A falling drop pH in the liquid medium occurred on the SCR1, reaching values below 4.0 after 50 days of operation. On the other hand, the SCR2 presented pH below 3.8 after 15 days. Besides it may have favored the formation of other intermediates, and consequently decrease the H_2 production, the pH also can and cause modification in the microbial population. As related by Khanal et al. [70], the pH depletion could cause a metabolic alteration of the microorganisms involved in hydrogen production, thereby resulting in the shift of intermediates production pathway and a consequent decrease in hydrogen production.

The predominant microorganisms were similar in SCR1 and SCR2 on the 26th day of the experiment. The high H_2 production might be related to the microorganisms from the family *Enterobacteriaceae* (genera *Enterobacter* and *Erwinia*). On the other hand, the association with low H_2 was with the family *Clostridiaceae* (genus *Clostridium* and an unknown OTU).

As related by Maru et al. [71] and Choi et al. [69], the type of carbon source and the initial substrate concentration usually play an essential role in bacterial growth and H_2 yield. One of the primary issues when using crude glycerol for bioconversions is to acquire a microbial host who can tolerate their characteristics. Some species of *Klebsiella*, *Citrobacter*, *Clostridium*, and *Enterobacter* presents oxidative and reductive metabolism of glycerol.

Nakashimada et al. [72] evaluated the H_2 production with *Enterobacter aerogenes* HU-101 in batch cultures using various substrates. The glycerol presented the highest amount of hydrogen. Maru et al. [71] related high yield of H_2 (0.85 mol H_2/mol glycerol) and ethanol fermentation from glycerol by *Enterobacter* spH1. Ito et al. [61] also found satisfactory yield values (Table 2) of H_2 from a glycerol-containing waste of a biodiesel manufacturing process using *Enterobacter aerogenes* HU-101, using a CSTR model. These microorganisms can related to the high-yield production of H_2 and ethanol from biodiesel wastes containing glycerol, as the authors' highlights.

Different strains of *Clostridium* produce different ratios of end-products, thus affecting their hydrogen-producing potential [15]. A higher production of other intermediates decreased the VFA generation (mainly acetic and butyric acids) with *Clostridia* as inoculum and glycerol as substrate [73–75]. *Clostridium propionicum* mainly produce propionate, as well as non-spore-forming [14], and no generation of H_2 occur in this case. Besides, some homoacetogenic bacteria use CO_2 and H_2 to produce acetic acid [76,77]. Some of these bacteria belong to the genus *Clostridium* and are resistant to the pretreatment [78,79]. Thus, this finding corroborates the lower gas production results verified at the end of the experiment, especially in SCR2, with a higher acetic acid proportion, about the other VFA analyzed.

The canonical correlation analysis compares the evaluated parameters and microbial diversity with the H_2 production, aiming the identification and quantification of this association (Fig. 6).

Thus, the microorganisms from the family *Enterobacteriaceae (Enterobacter* and *Erwinia)* are related to the H_2 production, as well as the acetic and butyric acid production and maintenance of optimum pH of the reaction medium.

4. Conclusions

The initial glycerol concentration interfered in the results. The SCR1 performance (10 g/L) was higher than the SCR2 (50 g/L) in all the H_2 production evaluated parameters. The degree of acidification values and the proportion of VFA showed the higher conversion of substrate to VFA related to H_2 production (acetic, butyric, and propionic acids) in SCR1. However, the higher glycerol concentrations in influent may favor the formation of other end products, which also contributes to the lower H_2 yield.

There found a predominance of members from the family *Enterobacteriaceae* (*Enterobacter* and *Erwinia*) at high H_2 production in semi-continuous reactors. On the other hand, the dominant

family in the low production was the *Clostridiaceae* (*Clostridium* and an unknown OTU).

Thus, the semi-continuous operating model has a simple operation and requires a device to remove the reaction medium without the need for an additional decanting step. The obtained results of volumetric H_2 production, mainly SCR1, are satisfactory when compared to the values cited in the literature.

Author contribution

Maria Cristina de Almeida Silva: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision. **Luiz Olinto Monteggia**: Conceptualization, Methodology, Project administration. **José Carlos Alves Barroso Júnior**: Formal analysis, Resources. **Camille Eichelberger Granada**: Formal analysis, Resources. **Adriana Giongo**: Formal analysis, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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