EVALUATION OF BIOHYDROGEN PRODUCTION FROM SUGARCANE VINASSE IN AN ANAEROBIC FLUIDIZED BED REACTOR WITHOUT PH CONTROL

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***Abstract*— This study aimed to produce biohydrogen from sugarcane vinasse in an anaerobic fluidized bed reactor operated at ambient temperature (26-35 °C) and without influent pH control. Hydraulic retention times (HRT) of 12, 6 and 4 h were applied to increase organic load rate from 17 to 155 kg-COD m^{-3} d^{-1}. The maximums hydrogen yield (2.40 mol-H_2 mol^{-1}-carbohydrate), hydrogen production rate (0.33 L-H_2 h^{-1} L^{-1}-reactor) and percentage of hydrogen in the biogas (33%) were obtained in the HRT of 4 h. The butyric acid fermentative route was favored in the process. The PCR/DGGE analysis showed that the microbial community structure was modified along operational stages with an increase in microbial diversity. The results indicate that biohydrogen production from anaerobic digestion of sugarcane vinasse can be achieved with no alkalization pretreatment.**

***Keywords*— Sugarcane vinasse, biohydrogen production, anaerobic fluidized bed reactor, no-control pH.**

I. INTRODUCTION

Vinasse is the wastewater obtained from the distillation of alcohol, characterized by high chemical oxygen demand (22-45 g-COD L^{-1}), low pH (3.5-4.6), and high nutrient content (Christofoletti *et al*., 2013). The sugar and alcohol sector estimates that each liter of produced ethanol generates about 10 to 15 liters of vinasse. In the 2018/2019 season, Brazilian sugarcane industry produced between 331 and 497 million of cubic meters of vinasse, approximately, correspondent to ethanol production of 33.1 million of cubic meters (Palacios-Bereche *et al.*, 2020).

Vinasse is frequently used in the fertigation approach, in which the raw liquid is disposed of in the plantation area, because of water, nutrients and organic matter are essential substances for growing sugarcane culture (Peiter *et al*., 2019). However, the prolonged application of high volumes of this by-product may result in environmental problems such as contamination of groundwater, salinization of the soil, leaching of metals and sulfates, release of odors, and emission of greenhouse gases (Aparicio *et al*., 2017; Hoarau *et al*., 2018).

Technologies for handling sugarcane vinasse, such as anaerobic digestion, membrane filtration and evaporation, have been investigated to redress the large quantity of the liquid produced worldwide and avoid its excessive use in fertigation. Among the alternatives, anaerobic digestion stands out for efficiency in remove organic matter and for diminishing the polluting potential of vinasse (Christofoletti *et al*., 2013; Peiter *et al*., 2019).

The high concentration of carbohydrates is a common characteristic of agro-industrial effluents, which permits them to be applied in fermentation processes with the consequent generation of bioenergy (Fuess *et al*., 2016). Utilization of vinasse and other agro-industrial wastes for producing biogas has been a promising alternative to supply the world energy demand by relating clean energy production to wastewater treatment.

Biohydrogen production has attracted attention as an alternative to energy source given its high-energy content per unit weight (142 kJ g^{-1}), environmentally friendly combustion and economic feasibility. However, biohydrogen production has not been optimized for industrial-scale yet. Therefore, operational parameters should be studied, aiming to achieve process stability (Sydney *et al*., 2018).

Anaerobic digestion process involves various types of microorganisms. Among them, acidogenic bacteria and methanogenic archaea are the principal generators of gases H_2 and CH_4, respectively. They differ from each other in various physiological aspects, nutritional requirements, and growth kinetics. The difficulty in maintaining the balance between these two groups of microorganisms is the main reason for instability in anaerobic reactors(Volpini *et al*., 2018). Reducing hydraulic retention time and controlling influent pH are strategies to drive the process to the acidogenesis stage and enhance biohydrogen recovery.

Many researchers have analyzed the biohydrogen production from vinasse using different types of bioreactors, such as packed-beds, fluidized-beds and sequencing batch systems (Fuess *et al*., 2019; Albanez *et al*., 2018;

Ferraz Júnior *et al*., 2014; Ramos and Silva, 2018; Santos *et al*., 2014; Siqueira *et al.*, 2013). Among them, anaerobic fluidized bed reactor (AFBR) is a favorable configuration for this purpose because it provides a suitable biomass adhesion in support materials, allowing high organic loading rates (OLR) and low hydraulic retention time (HRT) (Barros and Silva, 2012). Besides the reactor type, a set of other parameters can affect the hydrogen yield, including inoculum, substrate, nitrogen, phosphate, metal ion, temperature, and pH (Wang and Wan, 2009).

The pH has been identified as a determinant factor in fermentative biohydrogen. Variation in pH may affect various characteristics of the microscopic organisms, such as metabolic routes, morphology, the structure of the cells and growth rates (Penniston and Gueguim Kana, 2018; Saady, 2013). In general, the pH limit for biohydrogen production ranges from 4.0 to 7.0. However, several authors reported that it is possible to achieve maximum biohydrogen production by dark fermentation, using vinasse as a substrate, under different operational pH values. Sydney *et al*. (2014) attained a maximum production of biohydrogen at pH 7.0 when vinasse was supplemented with sugarcane juice. Ferraz Júnior *et al*. (2015) stated that the mean pH of 5.5 was characteristic in anaerobic up-flow packed-bed reactors. In a study with anaerobic sequencing batch biofilm reactor for co-digestion of vinasse/molasses, Albanez *et al*. (2018) produced hydrogen at pH of 4.5 ± 0.2. As can be noticed, there exists disagreement on the optimal pH regarding hydrogen production, which varies with the sort of substrate, reactor configuration, inoculum, and initial pH.

The automatic pH control is usually indicated as an approach to avoid inhibition conditions and improve the fermentation process. However, since vinasse is an acid liquid with significant compositional variability, pH regulation can be expensive to implement because of the use of chemicals (Shah, 2017). Thus, it is interesting to investigate the viability of biohydrogen production under natural non-linear vinasse pH. Therefore, this work evaluated an AFBR filled with expanded clay as support material, using sugarcane vinasse for producing biohydrogen. The objective was to observe the interference of control parameters (OLR and HRT) and process performance when treating vinasse, with no pH regulation.

II. METHODS

A. AFBR configuration and operation

Table 1 presents the equipment and methods used to analyze the wastewater.

Initially, 450 L of vinasse was collected in a sugarcane industry at Maceió, State of Alagoas, in Brazil. Vinasse was reserved in plastic recipients of 20 and 35 mL at -15 °C. The material utilized as inoculum was a sludge from a UASB (Upflow Anaerobic Sludge Blanket) reactor of a sewage treatment plant. It suffered a heat pretreatment (90 °C) during 10 minutes to deactivate the H_2 consuming microorganisms (Maintinguer *et al*., 2008), and subsequent cooling in an ice bath until it reaches 25 °C.

The AFBR was constructed using acrylic, with section of 5 cm internal diameter, 11 cm external diameter, 115 cm height, total volume of 1.2 L and operational volume of 908.4 cm^3.

An initial adaptation phase promoted the biomass adhesion of microorganisms to the expanded clay used as support material. The particle size was approximately 2.8-3.3 mm, with a real density of 1.50 g cm^{-3}, apparent density of 1.06 g cm^{-3} and average pore diameter of 0.19 μm.

Table 1. Equipment and method used in analysis.

Analysis	Equipment or method	Reference
pH, COD, VSS, Alkalinity, Phosphorus, Total Nitrogen	Standard methods for the examination of water and wastewater	(APHA, 2017)
Carbohydrate	Colorimetric Method for Determination of Sugars and Related Substances	(Dubois *et al*., 1956)
Volatile fatty acids and alcohol concentration	GC-2010, Shimadzu, Tokyo, Japan[a]	(Adorno *et al*., 2014)
Biogas composition	GC-2010, Shimadzu, Japan[b]	(Maintinguer *et al.*, 2008)
PCR-DGGE	DNA Thermal Cycler GeneAmp PCR System 2400" - Perkin Elmer Cetus, Norwalk, CT[c]	(Nübel *et al.*, 1996)

[a] equipped with a flame-ionization detector (FID), a COMBI-PAL headspace auto-sampler (AOC model 5000) and an INNOWAX column (30 m high × 0.25 mm i.d. × 0.25 μm film thickness)

[b] with a thermal conductivity detector (TCD) and argon as the carrier gas. The column was packed with Supelco Carboxen 1010 Plot (30 m×0.53 mm i.d.). Injector, detector and column temperatures were maintained at 30 °C, 200 °C and 230 °C, respectively.

[c] PCR Amplification was performed, programmed for pre-denaturation at 94 °C for 7 minutes and followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 56 °C for 45 seconds, extension at 72 °C for 1 minute, final extension at 72 °C for 10 minutes, and cooling to 4 °C.

Table 2. Operational conditions established in AFBR

HRT (h)	12	6	4
OLR (kg-COD m^{-3} d^{-1})	17 ± 6.4	75 ± 12.86	155 ± 29
Influent COD (mg L^{-1})	8443 ± 3201	18623 ± 2588	25871± 4895
Influent carbohydrate concentration (mg L^{-1})	7174 ± 1243	5194 ± 653	5003 ± 391
Theoretical flowrate (mL.h^{-1})	80	160	240

Table 3. Characteristics of analyzed raw vinasse

Characteristic	Average
pH	4.42 ± 0.16
Total suspended solids (g L^{-1})	20.37 ± 0.11
Volatile solids (g L^{-1})	20.26 ± 0.06
Fixed suspended solids (g L^{-1})	0.11 ± 0.04
Total carbohydrates (g L^{-1})	12.93 ± 2.40
Chemical oxygen demand (g L^{-1})	37.88 ± 5.84
Volatile acids (g L^{-1})	3.15 ± 0.47
Phosphorus (g L^{-1})	0.078 ± 0.002
Total nitrogen (g L^{-1})	0.076 ± 0.047

The reactor operation occurred for 220 days, at ambient temperature (26-35 ºC). The operational conditions are displayed in Table 2.

The fluidization velocity of expanded clay was 1.24 cm s^{-1} (Amorim *et al*., 2009). The total liquid flow rate was 30% superior to the minimum fluidization speed of the material support.

Inoculation phase, in which H_2-producing sludge is activated, was conducted in batch mode for 48 h. Then, AFBR operation started in continuous mode employing the HRT of 12 h, and subsequently, 6 and 4 h, in which a variation of 5-10% in constant volumetric H_2-production was achieved.

Alkalization pretreatment was not adopted as a strategy to reduce the experimental costs and to approach a more realist condition, as sugarcane vinasse is naturally acid wastewater.

B. Experimental analysis

The experimental analyses (Tab.1) were daily carried out for pH and biogas composition, while VSS, COD, alkalinity, and carbohydrates were measured twice a week. The volumetric H_2 flow measurement procedure was performed by quantifying the gas volume using the Milligas-Counter meter for 1 min, for 10 consecutive times (Peixoto *et al*., 2012).

The species diversity in each band obtained from PGR-DGGE was determined using the Shannon-Wiener Index, where the levels of biological organization were presented in different scales.

III. RESULTS

Table 3 presents the characterization of vinasse used in experiments. The results also encompasses the behavior for carbohydrate consumption, COD, metabolites produced, hydrogen produced influence of pH and microbial community dynamic.

A. Carbohydrate and COD conversion

The HRT reduction caused a drop in the percentage of converted carbohydrates, ranging from 37 ± 0.07% at HRT of 12 h to 25 ± 0.03% at 4 h (Tab.4). Since lower HRTs entail higher OLR, the result discloses that elevated substrate concentrations inhibit the capacity of microorganisms to consume carbohydrates because of the kinetic limitations (Amorim *et al*., 2009).

Silva *et al*. (2019) treated dairy wastewater using an AFBR and also observed a direct association between the amount of carbohydrate conversion (54 ± 9.1, 40 ± 15.1 and 36.6 ± 16.2%) and HRT (8, 6 and 4 h), with pH effluent of 3.6-3.9. The authors indicated that organic matter overcapacity affects carbohydrate removal. Similarly, Santos *et al*. (2014) analyzed AFBRs fed with diluted and raw vinasse, which reduced the carbohydrate conversions by decreasing HRT from 4 to 1 h, with pH effluent of 4.44-4.47.

In an analysis of co-fermentation of vinasse and cheese whey, employing AFBR reactors, Ramos and Silva (2018) showed that COD conversion ranged between 12.5 ± 3.3 and 7.8 ± 2.4 % for HRT from 6 to 1 h, with pH between 4.26 ± 0.21 and 4.95 ± 0.10. The reduction in COD removal was credited to the augmentation in the volatile fatty acids concentration to levels probably toxic to the microbiota.

Since propionic and butyric acids (Table 5) significantly increased at HRT of 4 h in the present research, it may have interfered in the COD conversion efficiency, as stated by Ramos and Silva (2018).

B. Hydrogen production

HRT diminution from 12 to 4 h caused an elevation in hydrogen production rate (HPR) from 0.10 ± 0.035 to 0.33 ± 0.13 L-H_2 h^{-1} L^{-1}-reactor (Fig. 1). Commonly, HPR is favored at lower HRT (or higher OLR), when substrate availability increases.

This direct correlation between OLR and HPR was reported in previous research using sugarcane vinasse for

Table 5. Production of soluble metabolites.

HRT (h)	12	6	4
Methanol (mmol.L^{-1})	13.2	23.4	7.5
Ethanol (mmol.L^{-1})	7.7	11.5	10.1
Caproic acid (mmol.L^{-1})	0.77	0.32	0.77
Acetic acid (mmol.L^{-1})	7.3	6.6	5.3
Propionic acid (mmol.L^{-1})	9.7	8.3	15.3
Isobutyric acid (mmol.L^{-1})	1.5	1.2	1.3
Butyric acid (mmol.L^{-1})	18.8	11.0	25.2
Valeric acid (mmol.L^{-1})	1.03	0.88	0.74

Table 4. Main parameters analyzed during the operational period of the AFBR

HRT (h)	12	6	4
pH influent	4.17 ± 0.07	4.20 ± 0.12	4.00 ± 0.13
pH effluent	4.52 ± 0.03	4.53 ± 0.10	4.46 ± 0.11
Average carbohydrate conversion (%)	37 ± 0.07	30 ± 0.06	25 ± 0.03
Average COD removal (%)	40 ± 0.07	32 ± 0.09	24 ± 0.02
H_2 content in biogas (v/v %)	15 ± 0.04	20 ± 0.13	32 ± 0.10
CO_2 content in biogas (v/v %)	76 ± 0.15	65 ± 0.23	48 ± 0.11
CH_4 content in biogas (v/v %)	9 ± 0.12	15 ± 0.09	20± 0.09

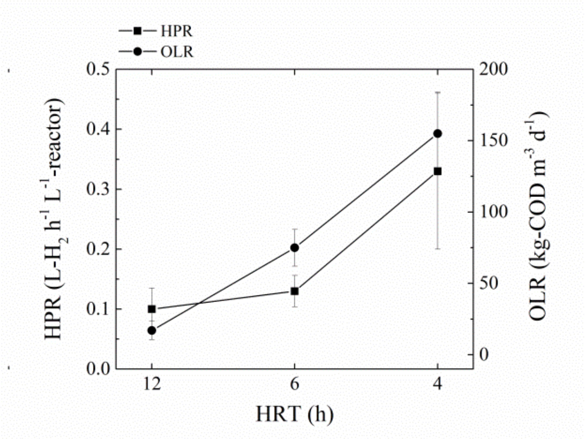

Figure 1: Effect of hydraulic retention time on the hydrogen production rate.

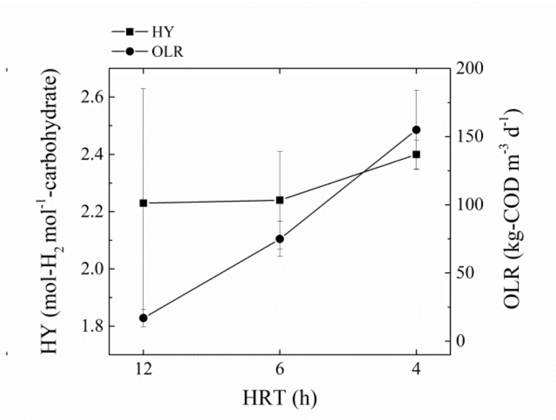

Figure 2: Effect of hydraulic retention time on hydrogen yield.

biohydrogen production. Santos *et al*. (2014) analyzed an AFBR treating vinasse and reported that HPR augmented from 0.32 to 0.81 L-H_2 h^{-1} L^{-1}-reactor when OLR varied between 90 and 360 kg-COD m^{-3} d^{-1}.

Hydrogen yield (HY) increased from 2,23 ± 0.40 (HRT of 12 h) to 2.40 ± 0.05 mol-H_2 mol^{-1}-carbohydrate (HRT of 4 h), along with OLR augmentation. The maximum hydrogen yield occurred at HRT of 4 h, as showed in Fig. 2.

Ferraz Júnior *et al*. (2015) achieved the same HY value when applied an HRT of 12 h with an average pH of 5.5. In that case, the decrease of HRT from 12 to 8 h resulted in the decline of HY to 1.4 mol-H_2 mol^{-1}-carbohydrate. It infers that the AFBR may have a greater capacity of hydrogen production for greater OLR when compared to fixed bed configurations.

Fuess *et al*. (2016), operating a continuous acidogenesis packed-bed reactor in a pH range from 5.1 to 5.2, achieved HY of 3 mol-H_2 mol^{-1}-carbohydrate and HPR of 1.11 L-H_2 L^{-1} d^{-1} in their best operational condition (temperature of 55 °C and HRT of 7.5 h). Sydney *et al.* (2018) achieved a maximum HY of 2.1 mol-H_2 mol^{-1}-carbohydrate from the anaerobic digestion of the vinasse, with pH of 4.52, using a specific bacterial consortium.

The results imply that hydrogen production for the investigated operational conditions was equivalent-to-superior compared to similar studies. Furthermore, multiple fermentative routes identified in the process through soluble metabolite analysis may justify the maximum HY.

C. Metabolites production

Among the diverse anaerobic reactions and their by-products, acetate and butyrate are prominent as the two pathways producing hydrogen, resulting respectively in 4 and 2 mol-H_2 mol^{-1}-carbohydrate (Sydney *et al*., 2014). Therefore, the higher amount of butyrate (11.0-25.2 mmol L^{-1}), showed in Table 5, indicates that butyrate type fermentation, which is characterized by the production of butyrate, acetate, CO_2, and H_2, prevailed over other fermentative routes (Ren *et al*., 2007).

This factor feasibly favored the maximum HY of 2.4 ± 0.05 mol-H_2 mol^{-1}-carbohydrate at HRT of 4 h, as in previous research. Ottaviano *et al.* (2017) also disclosed a rise in HY (from 2.36 ± 0.39 to 3.10 ± 0.44 mol-H_2 mol^{-1}-lactose) when butyric acid augmented (from 485 ± 89 to 776 ± 31 mg L^{-1}), with a pH effluent of 4.3 ± 0.1. Sydney *et al*. (2014) achieved maximum HY of 2.97 mol-H_2 mol^{-1}-glucose (microorganisms' consortia from a sample of fruit bat feces) and 1.41 mol-H_2 mol^{-1}-glucose (microorganisms' consortia from a lake of a dairy farm) accompanied by considerable amounts of butyrate production.

Acetic acid (5.3-7.3 mmol L^{-1}) had small amounts in comparison with other metabolites, such as ethanol (7.5-11.5 mmol L^{-1}), methanol (7.5-23.4 mmol L^{-1}), propionate (8.3-15.3 mmol L^{-1}) and butyrate. This fact can be referred to as the shifting of metabolism from acetate to more reduced by-products such as propionate, butyrate, or from VFAs to alcohol production.

The occurrence of propionate-type fermentation probably resulted in the formation of propionic acid and some valerate (0.74-1.03 mmol L^{-1}) (Saady, 2013). Generally, this route is unfavorable to biohydrogen production.

The incidence of caproate (0.32-0.77 mmol L^{-1}) probably concerns the route in which butyrate and ethanol are converted to caproic acid (Barker *et al*., 1945).

The high methanol concentration at HRTs of 12 and 6 h may have caused the lower HYs at those phases. García and Cammarota (2019) reported that methanol-based solutions and methanol-rich wastewater are not suitable for biohydrogen production, but favor CH_4 formation. Although methanol can be directly converted into methane by methanogens, under specific conditions it can also be converted into acetate and butyrate by acetogens. Therefore, methanol conversion routes at HRT of 4 h could also influence high levels of butyrate.

D. Biogas composition

The presence of H_2 in biogas (15-32%) increased with HRT reduction from 12 to 4 h (Tab.4). Ramos and Silva (2018) obtained the maximum H_2 of 47.0 ± 3.5% at the HRT of 4h, correspondent to an OLR of 40 kg-COD m^{-3} d^{-1}. In that study, a thermophilic AFBR fed with vinasse was operated, with the pH of the reactors maintained between 4.26 ± 0.21 to 4.95 ± 0.10 by the addition of 6 mol-NaOH L^{-1} in the effluent. By decreasing the HRT from 8

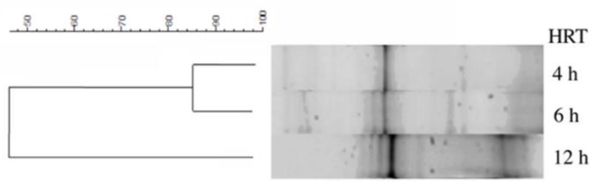

Figure 3: Profiles obtained by PCR-DGGE analysis.

h to 1 h in the AFBR, the %H_2 varied between 25.9 ± 3.2 and 47.0 ± 3.5%. Santos *et al*. (2014) achieved the highest H_2 content of 57.1% at HRT of 4 h, using diluted vinasse under thermophilic conditions and pH of 4.39 ± 0.10. Therefore, the low content of H_2, in comparison with the previous research, may have occurred because of concomitant methanogenic and thermodynamically unfavorable acetogenic reactions, which produces propionate, butyrate, and ethanol from acetate and H_2 consumption (Saady, 2013).

The occurrence of methane in the biogas (9-20%) was detected in all the AFBR operational phases (Tab.4). In this case, the heat treatment was probably inefficient to inhibit completely the methanogenic microbial community in the inoculum. It also can be explained by the detrition of the expanded clay as HRT decreased and flow rate increased, causing zones of stagnation where methanogenic microorganisms could have been developed. Likewise, Rosa *et al*. (2014) carried out a heat pretreatment of the inoculum to produce biohydrogen from cheese whey wastewater and observed the formation of methane. In their study, pH was controlled within the range of 4.0–4.5. Albanez *et al*. (2018), utilizing vinasse in sequential batches, obtained 14% of CH_4 and 15% of H_2 at pH of 4.5, and 23% of CH_4 and 9% of H_2 at pH of 4.7. Peixoto *et al*. (2012) reported that both the influence of the HRT and the vinasse composition can propitiate the development of hydrogen-consuming microorganisms (methanogenic archaea and sulfate-reducing bacteria).

The percentage of CO_2 in the biogas reduced from 76 to 48% with HRT decrease (Tab. 4). This diminution may have occurred because CO_2 is a reagent in many intermediate reactions of dark fermentation process (Saady, 2013).

E. Influence of pH

The influent pH (4.00 ± 0.13 - 4.17 ± 0.07) was lower than the effluent pH (4.46 ± 0.11 - 4.52 ± 0.03). Previous studies reported that the effluent recirculation tends to lessen the influence of acidification in the reactor, which may have elevated the pH, with consequent generation of methane (López-López *et al*., 2015). This statement can be attributed to possible interference of inoculum, a sludge from a UASB reactor, with elevated pH (approximately 8) (Steinmetz *et al*., 2016).

Besides that, the presence of methane, associated with pH higher than 4.3, can indicate the occurrence of alkalinity in the reactor. Therefore, another possibility is the occurrence of the reaction in which CO_2 reacts with water to form carbonic acid (H_2CO_3) that will dissociate, first, to ion bicarbonate (HCO^{-3}) and then, to carbonate (CO_3^{-2}). This reaction characterizes a buffer system, where the formation of bicarbonate may be responsible to control the pH (López-López *et al*., 2015).

In general, not adjusting the inlet pH to optimum values may have affected the biohydrogen production and the conversion of organic compounds. Low pH can inactivate the hydrogenase enzyme, reducing hydrogen production, while neutral pH may be a favor to methanogenic microorganisms (Nualsri *et al*., 2016). Moreover, the acetate route, which produces the highest quantity of hydrogen per mol of generated acid, is favored at pH in the range of 5 to 6.

The butyrate route has predominated at pH lower than 4.5. Previous studies with vinasse presented the occurrence of other intermediate products under different pH. Fuess *et al*. (2019) observed an anaerobic structured-bed reactor and identified three major metabolic pathways according to the pH: lactate-producing (pH<5.0), biohydrogen-/butyrate-producing (pH=5.0–5.5) and biohydrogen-producing/sulfate-reducing (pH>6.0) systems. Albanez *et al*. (2018) obtained the highest HY at pH of 4.5 with the predominance of acetic acid. Ramos and Silva (2018) achieved the highest HY with a high concentration of butyric acid route and pH of 4.26-4.95. In opposite, Santos *et al*. (2014) displayed that H_2 production was inhibited by elevated concentrations of butyric and acetic acids.

The influent pH of the operational period possibly was not favorable to the acetate route. The multiple soluble produced metabolites suggest that the process was conducted through several mutually competing fermentation routes, causing a reduction in biohydrogen yield in comparison to the stoichiometric production. However, the butyric acid pathway was favored under lower pH as this metabolite presented the highest concentrations, mainly at the HRT of 4 h. Furthermore, the butyrate has been reported as the principal soluble metabolite in the anaerobic processes treating vinasse aiming for obtaining biohydrogen (Santos et al., 2014).

F. Microbial communities

Comparing the band profiles of each community in Fig.3, changes in microbial community structure were observed through the intensification, emergence and/or disappearance of bands. The similarities among the profiles were verified by Pearson's coefficient of similarity. The dendrogram analysis infers that the similarity index was higher (85%) between the shorter HRTs (6 and 4 h). Microbial diversity increased with increasing OLR and promoted higher HY at lower HRT. The average diversity indexes of Shannon-Wiener were 1.956 for 12 h, 2.344 for 6 h and 2.581 for 4 h. Arreola-Vargas *et al*. (2018) similarly stated a diversification in the bacterial miscellany associated with high OLRs. Despite the methane generated in all the phases, the Archaea domain was not detected in the analysis.

IV. CONCLUSIONS

The use of AFBR to treat sugarcane vinasse without pH control resulted in maximums hydrogen yield of 2.40 ±

0.05 mol-H_2 mol^{-1}-carbohydrate) and a hydrogen production rate of 0.33 L-H_2 h^{-1} L^{-1}-reactor at HRT of 4 h, with OLR of 155 kg-COD m^{-3} d^{-1}. H_2 content in biogas (15–32%) was small in comparison with previous studies. Methane was detected in biogas composition. The concomitant possible inefficacy of inoculum pretreatment to adopted conditions and the consequence of effluent recirculation, which can elevate the medium alkalinity, could be the causes for the occurrence of CH_4. Natural low vinasse pH, below the optimum range, favored the butyric acid fermentation route, which is thermodynamically suitable for biohydrogen formation. Thus, further studies should be carried out to explore the interference of the inoculum pretreatment and recirculation rate in biohydrogen production. The molecular biology analysis suggested that operational HRT reduction favors the upsurge of microbial diversity. Therefore, AFBR configuration is proper for biohydrogen obtaining from sugarcane vinasse given its high effectiveness at low HRT.

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