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BIOGAS SEQUESTRATION FROM THE HEADSPACE OF A FERMENTATIVE SYSTEM ENHANCES HYDROGEN PRODUCTION RATE AND YIELD

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Abstract

Total pressure (TP) affects the level of dissolved hydrogen gas in the fermentation medium leading to metabolic shifts in mixed microbial-culture-based systems. In this study, the effect on hydrogen production rate and yield was investigated at different TP of a hydrogen-producing system using a microbial non-sterile culture previously heat-treated. Four continuous stirred-tank reactors (CSTR) were operated in parallel on a mineral salts-molasses medium (21 g-COD. L^{-1}) at 35°C, pH 5.5 and hydraulic retention time (HRT) of 6 h. The TP was set at 80 kPa (R1), 100 kPa (R2), 120 kPa (R3) and 140 kPa (R4) for which reactor performances were estimated at steady-state conditions. As the increase of TP consequently increased the partial pressure of hydrogen (p_{H2}), the hydrogen production rate (HPR) and yield (HY) were consistently negatively influenced. The highest HPR and HY (406.1 ± 36.8 mL-H_2 h^{-1}; 4.51 molH_2 $mol^{-1}_{suc\ eq.}$) were achieved at low pressure conditions (80 kPa). The composition of the microbial community mainly represented by species from *Sporolactobacillus* and *Clostridium* genera, did not change with the increase and /or decrease of the TP, indicating a regulation at cellular but not population level.

Keywords: biohydrogen, dark fermentation, biogas sequestration, ppH_2, sugarcane molasses.

1. INTRODUCTION

The production of biohydrogen ($BioH_2$) from organic waste is a promising biotechnological process with gains at energetic, societal and environmental levels [1]. However, $BioH_2$ production by dark fermentation (DF) is still a technological challenge for being a very sensitive process, requiring careful balancing of the following parameters: pH [2] [3], temperature [4], organic loading rate (OLR) [5] and specific organic loading rate (sOLR) [6].

In fermentative systems using non-sterile mixed cultures, high H2 yields are associated with a mixture of acetate and butyrate fermentation pathways end-products, while low H_2 yields are associated with other reduced end-products such as lactate, solvents (ethanol, butanol and acetone) and alanine. To date, hydrogen yields in fermentative systems are mostly ranging between 1.2 – 2.3 $molH_2.mol^{-1}_{hexose}$ which represent only 30 – 50% of the theoretical maximum hydrogen yield (4 $molH_2.mol^{-1}_{hexose}$, glucose) [7] [8] [9] [10].

Multiple reasons have been associated to low hydrogen yields such as (i) anabolic consumption of the substrate for biomass synthesis [4] (ii) inappropriate fermentative conditions [2] [11] (iii) hydrogenotrophic activity [12] (iv) homoacetogenic activity [13] and (v) inhibition by partial pressure of hydrogen [14].

The partial pressure of hydrogen (p_{H2}) is an extremely important factor especially for continuous BioH2 production [15] [16] [17] [18]. This factor is explained by Le Chatelier's Principle that says "all chemical equilibrium responds to an increase in the pressure, causing the reaction to move in the opposite sense to that, which rises the pressure." In biological multipahses systems, this event is associated to the limitation of the liquid-to-gas mass transfer. The liquid-to-gas mass transfer limitation arises because the gas production rate is higher than the transfer rate to the gas phase [19] [20]. Such a limitation have caused H2 supersaturation in the liquid with concentrations of H2 between 5- and 71/fold higher than the equilibrium value [20] [21]. Thereby, during the fermentation process, as the p_{H2} in bioreactors increases, H_2 synthesis decreases [22]. This also can be explained through

Metabolic pathways shifts are also observed in function of the p_{H2}. According to Hallenbeck [7] in Clostridial-type hydrogen producing fermentation at low p_{H2}, the NADH generated during glycolysis can be reoxidized, probably by a NADH-dependent

[FeFe] hydrogenase. At moderate to high p_{H2}, this reaction is unfavorable, and NADH is reoxidized by the formation of reduced organic compounds (previously mentioned). As a consequence, low hydrogen yields are achieved.

Few methods to control the p_{H2} have been investigated: sparging (*i.e.*, gas flushing to remove other dissolved gas, in this case H2), removing H_2 from the system or reactor operation at low pressure. Mizuno et al [23] evaluated the influence of sparging in a continuous stirred tank (CSTR) fed with a mineral salts-glucose medium (10.7 $gCOD.L^{-1}$). Nitrogen gas was sparged at a flow rate of 15 times the specific hydrogen production rate (sHPR) observed in a control CSTR (i.e., without sparging) that was 1.446 mL H_2. min^{-1}. g^{-1} biomass. An increase of 68% of hydrogen yield was achieved with sparging (1.43 $molH_2.mol^{-1}{}_{glucose}$).

Besides nitrogen, other gases such as internal biogas and only carbon dioxide with different flow rates (100 – 400 $mL.min^{-1}$) were investigated in a CSTR fed with a mineral salts-sucrose medium (20 $gCOD.L^{-1}$) (Kim and co-authors, [24]). The best performances were obtained by CO_2 sparging at 300 $ml.min^{-1}$, resulting in the highest H2 yield of 1.68 $molH_2.mol^{-1}{}_{hexose\ converted}$. Concomitant to the increase of hydrogen production and yield, too much sparging produces dilute gas stream, creating a serious problem with respect to the H2 separation from the sparging gas [25].

Fast collection of biogas was also studied as the p_{H2} control method. Liang et al. [26] investigated the biogas removal using a vacuum pump (31.4 kPa) and membrane purification of H_2 from a fermentation system (Batch reactor; 2.5 $g_{glucose\ added}$). The authors reported that silicone rubber was effective in reducing the p_{H2}, improving the hydrogen production by 10% (2.6 – 3 mmol $H_2.g^{-1}$ VSS. h^{-1}) and the hydrogen yield by 15% (0.84 – 0.92 $molH_2.\ mol^{-1}{}_{glucose}$). Lee et al. [27] investigated the effect of working with reduced pressure in a CSTR, they worked with pressures similar to the ones in Liu and Wang [28], between 0.2 and 0.9 atm, and concluded that H2 production can be improved in fermentative systems with reduced pressure.

Interestingly, no difference regarding H_2 production was observed in pure culture system (*Clostridium butyricum* strain SC-E1) under vacuum (28 kPa) and non-vacuum. Glucose-polypeptone at 0.5 and 1.0% concentration were used as substrate, resulting in maximum hydrogen yields of 1.8 – 2.2 and $molH_2.mol^{-1}{}_{glucose}$ for all condition evaluated [29].

Recently, another strategy to remove BioH2 from the fermentative systems has been tested. Massanet-Nicolau et al. [30] reported a system with electrochemical H2

removal and carbon dioxide absorption as an effective strategy to increase H2 yields and avoid its consumption. Also, membrane systems are suggested as a way to separate and purify H2 [31].

Despite these mentioned studies on this subject, more detailed research on this topic is necessary to enable the production of $BioH_2$ at larger scale and with continuous operation of the fermentation process. In this study, regular collection of biogas from headspace of a fermentative continuous system was carried out aiming to control the p_{H2} in the process and thus, attempt to maintain a high hydrogen productivity. The dynamics of the microbial community was also studied based on the sequencing of the V4 region of 16S rRNA gene for Bacteria using High-Throughput Sequencing (MiSeq Sequencing System - Illumina).

2. MATERIALS AND METHODS

2.1. Seed sludge

The seed sludge was taken from an industry of commercialization of sugarcane and sugar beet plant (UASB-type reactor). The total volatile solids (TVS) concentration of the sludge was 53.7 g/L. Heat-treatment was applied to the sludge at 90°C for 1 h to inactivate hydrogen consumers and to harvest spore-forming anaerobic bacteria such as *Clostridium* sp. [32].

2.2. Feeding solution

A mineral salts-sugarcane molasses solution of 21 g COD L^{-1} was used as carbon source in a feeding medium composed by the following macro- and micro-nutrients (mg L^{-1}): $NiSO_4.6H_2O$, 0.5; $FeSO_4.7H_2O$, 2.5; $FeCl_3.6H_2O$, 0.25; $CoCl_2.2H_2O$, 0.04; $CaCl_2.2H_2O$, 2.06; SeO_2, 0.036; HCl, 0.25, according to Del Nery [33]. The C/N ratio of molasses was 52.7.

2.2. Reactor design and operational conditions

Experiments were carried out in four continuous stirred reactors of 4 L with a working volume of 2 L (Figure 1). Each reactor was equipped with a stirring system made of a Rushton turbine and a marine propeller to ensure a homogeneous mixture. A revolution counter was connected to access to the measurement of the stirring velocity which was 250 rpm. The gas flow rate was measured with a peristaltic pump calibrated

at each different levels of pressure. Pressure was regulated with a control device combining a pressure sensor and a peristaltic pump following a two-band control law. A combined sensor was connected to the reactor for measuring the redox potential and pH (4010/120/Pt100, Mettler Toledo). The pH and redox meter (M300 – Mettler Toledo) was connected to a computer for on-line data acquisition (home-made software Odin in collaboration with INRIA teams). The pH was set and controlled at 5.5 by adding NaOH (2 M) with a peristaltic pump. Temperature in the reactor was also controlled using a platinum probe Pt100 and a heating electric resistance. The temperature was maintained constant at 37 ± 0.5 °C. The hydraulic retention time (HRT) was 6 h, resulting in an organic loading rate (OLR) of 84.2 $gCOD.L^{-1}.\ d^{-1}$, as suggested in [5]. The total pressure tested is presented in Table 1.

[Figure 1 – Please here]

[Table 1 – Please here]

The experimental setup was based on the following assumptions: (i) the second column of Table 1 presents the initial TP of the four independent conditions (R1 to R4), to evaluate the real influence of total pressure on hydrogen producing-system. This also represents the condition where the microbial community had the same operating history; (ii) other conditions (the third and fourth columns) were performed to evaluate how the microbial community responds to a variation of total pressure to evaluate whether hydrogen production was inhibited or if such inhibition is irreversible or reversible; (iii) the steady-state for each operating condition was considered when the coefficient of variance of the hydrogen production rate (HPR) was less than 10% based on its mean value from the ten last HRT of each operating phase; (iv) considering the steady-state of hydrogen-producing systems, the p_{H2} of the headspace and the concentration of dissolved hydrogen in the liquid medium ($[H_2]_{Liq.}$) were estimated by Dalton's and Henry's Law, respectively; (v) Experiments without pressure control was not carried out. Thereby, the condition at 100 kPa was set as control both to compare data between conditions and reactors, and microbial community response to the total pressure variation (also controlled). (vi) The inspected pressure range was chosen for be near atmospheric pressure and to evaluate the sensibility of the process.

2.3. Chemical analysis

Biogas composition was analyzed as previously described in [34]. Reduced sugars and fermentation end-products were quantified using high performance liquid chromatography (HPLC; HPX 87 column - Biorad) coupled to a refractometer (Waters R410). The eluent used was a H_2SO_4 solution (0.222 µl L^{-1}). The operating conditions were: elution flow, 0.4 mL min^{-1}; temperature of column, 35 °C; temperature of refractometer, 40 °C. Microbial cells (biomass) concentration was determined as volatile suspended solids (VSS) by filtration at 1.2 µm, according to [35].

2.4. DNA extraction, PCR amplification and High-Throughput Sequencing of hydrogen-producing systems samples

At the end of each operating condition, microbial cells were collected after centrifugation (12000 × *g*; 15 min) of 2 mL of culture. Genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega). The V3-4 region of the 16S rRNA gene was amplified with the forward primer CTTTCCCTACACGACGCTCTTCCGATCTTACGGRAGGCAGCAG and the reverse primer GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAA TCCT plus the respective linkers over 30 amplification cycles at 65° C (annealing temperature). An index sequence was added using the primers AATGA-TACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC and CAAGCAGAAGACGGCATACGAGAT-index-TGACTGGAGTTCAGACGTGT (PCR – 12 cycles). The PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions for sequencing (paired-end; 250 bp reads) which was performed at the GeT PlaGe sequencing center of the genotoul life science network in Toulouse, France (get.genotoul.fr). Quality checking was made using a slightly modified version of the Standard Operation Procedure by Kozich et al. [36] in Mothur version 1.33.0. Alignment and taxonomic outline was made using release information: SILVA 102, as provided by Schloss et al. [37]. The software PAUP* (version 4.0b10) was used to infer a phylogeny - criterion of maximum parsimony [38]. Bootstrap support was calculated using 1500 repetitions. SumTrees (version 3.3.1) of the DendroPy package (version 3.12.0) was used to map bootstrap values to the best phylogeny [39]. Sequences of most abundant operational taxonomic unit (OTU) found in the biofilm were deposited in the NCBI Genbank database under the following accession name SUB5433515 (MK765997 - MK766231).

2.5. Calculations

Hydrogen Production Rate (HPR, mL-H_2 h^{-1}) and Hydrogen Yield (HY, mol-H_2 $mol^{-1}_{suc\ eq.}$) were calculated using Equations 1 and 2 – 4, respectively.

$$HPR = Q_g \cdot \%H_2 \quad (1)$$

$$HY = ((Q_g \cdot nH_2)/V) / ((Q.(C_{S0} - C_{SF}))/MM_S) \quad (2)$$

$$nH_2 = \%H_2 \cdot n \quad (3)$$

$$n = (P.V) / (R.T) \quad (4)$$

where, Q_g is the biogas flow, $\%H_2$ is the hydrogen content in biogas, nH_2 is the number of mol of hydrogen, V is the volume of gas of the sample, Q is the liquid flow in the reactor, C_{S0} is the influent substrate concentration, C_{SF} is the effluent substrate concentration, and MM_S is the sucrose molar mass, n value corresponds to the total number of moles of sample (i.e., $\%H_2$, $\%CO_2$ and $\%CH_4$), P is the gas pressure, R is the universal ideal gas constant, and T is the absolute temperature.

The theoretical expected hydrogen production and the acetate produced from homoacetogenesis were calculated using Equations (5) and (6) as proposed by Luo et al. [3] and Ferraz Júnior et al [25]:

$$H_{2\ theoretical} = 2[A] + 2[B] - [P] \quad (5)$$

$$Acetate_{\ homoacetogenesis} = (2[A] + 2[B] - [P] - [H_2])/6 \quad (6)$$

where [A], [B], [P] and [H_2] are the measured acetic, butyric and propionic acids; and the hydrogen concentrations in mM, respectively.

The COD balance expressed as COD recovery (Equation 7) of the fermentative process was calculated as follows:

$$COD_{\ recovery}\ \% = (COD\ final/COD_0) * 100 \quad (7)$$

where COD_0 is the COD of molasses fed and COD final is the sum of the mass, expressed as g-COD, of every outlet component of the fermentative system, as proposed by Ferraz Júnior et al [5].

Principal component analysis (PCA) was performed using STATISCA 10. Primarily, a factor analysis was performed to identify the number of independent factors [20]. The Kaiser criterion was used to decide the factors that could be retained for interpretation [41]. The factors cut off was identified through the point of wherein the eigenvalue level drop off continuously based on Catell [42].

3. RESULTS AND DISCUSSION

3.1. Hydrogen production (HPR) and yield (HY)

Four similar stirred reactors were operated in parallel with the same conditions of pH, temperature, stirring, initial concentration of substrate and HRT. However, different initial total pressures (TP) were applied (R1 – 80 kPa; R2 – 100 kPa; R3 – 120 kPa; and R4 – 140 kPa). The steady-state was reached after approximately 60 HRT from the time when the TP of R1, R2 and R3 was increased to 100, 120 and 140 kPa, respectively (Phase II). The R4 was disassembled, according to the experimental design. A second steady-state was achieved within the same period as Phase I for the remaining reactors. Then, R1 and R2 had their total pressure increased to 120 and 140 kPa, respectively, and R3 decreased to 100 kPa (Phase III). The steady-state Phase III was also achieved after 15 days.

Hydrogen content of biogas was around 47 – 54% for all reactors and conditions. Methane was not observed in the biogas suggesting that the inoculum heat-treated, and the operating conditions inhibited the methanogenesis and favored the hydrogen-producing process. The partial pressure of hydrogen (p_{H2}) was determined by Dalton's law and the values ranged between 41-70 kPa. These values are slightly higher than [26] and [29].

The different total pressure (TP) showed a strong influence on hydrogen production rate (HRP, mL-H_2 h^{-1}) (Figure 1). The highest HPR (406.1±36.8 mL-H_2 h^{-1}) was achieved in R1 with the lowest TP of 80 kPa. At atmospheric pressure (100 kPa), the HRP decreased 25% in relation to Phase I. The HPR decreased even more (52%) with the increase of TP to 120 kPa.

The same behavior was observed in R2 and R3. The increase of the TP from 100 to 140 kPa; and from 120 to 140 kPa, was reflected in HPR decrease of 70% (R2) and 17% (R3), respectively (Phase I). When the TP was alleviated to 100 kPa in R3, the HPR increased by 90% in Phase I. In addition, the highest TP (140 kPa) as applied to R4 resulted in the lowest value of HRP (61.6±5.8 mL-H_2 h^{-1}) (Table 2). These findings show that gas removal had a positive effect on HPR. The increase of the TP with a consequent increase of the p_{H2} influenced negatively the Bio-H_2 production.

Hydrogen yield (HY) followed the same trend as HPR, being the maximum and the minimum values achieved of 4.51 and 0.56 mol-H_2 $mol^{-1}_{suc\ eq.}$ when the TP was 80 and 140 kPa, respectively (Table 2). The HY at initial TP of 80 KPa represented an increase of 61.6% and 705% comparing to the controlled atmospheric pressure (100 kPa) and to the highest TP evaluated (140 kPa), respectively, reaffirming the high influence of TP on biological hydrogen production process.

[Figure 2 – Please here]

[Table 2 – Please here]

Based on the p_{H2} of the headspace, the concentration of dissolved hydrogen in the liquid medium ($[H_2]_{Liq.}$) was estimated by Henry's Law. The correlation between $[H_2]_{Liq.}$ and; HPR and HY indicated a linear coefficient of 0.979 and 0.968, respectively (Figure 3).

[Figure 3 – Please here]

By applying linear regression analysis on the experimental results, the equations (4) and (5) were obtained to describe the influence of $[H_2]_{Liq.}$ on HPR and HY, respectively. The $[H_2]_{Liq.}$ of 0.57 mg. L^{-1} resulted in maximum values of HPR and HY while the $[H_2]_{Liq.}$ of 0.99 mg. L^{-1} resulted in the lower values of the respective variables indicating that during fermentation process, as the p_{H2} in bioreactors decreases, BioH_2 synthesis increases (vice versa).

$$HPR = -821.15*[H_2]_{Liq.} + 890.1 \qquad R^2 = 0.979 \qquad (4)$$

$$HY = -9.2954*[H_2]_{Liq.} + 9.6264 \qquad R^2 = 0.968 \qquad (5)$$

3.2. Intermediates products from molasses fermentation

The conversion of sucrose, the main carbon source presented in the mineral salts-sugarcane molasses, was higher than 99% for all reactors and conditions. However, reducing sugars such as glucose and fructose remained in the acidogenic reactors liquid outlet in percentage between 29% and 37.4% (Figure S1). These findings are similar to the ones reported by [3] who evaluated different configurations of reactor to produce hydrogen from sucrose.

In addition to the molasses fermentation products, the organic acids were quantified to investigate the main metabolic pathways in the hydrogen-producing systems. Table 3 shows that the main intermediates products were acetate (49.1 – 22 mM) followed by lactate (16.7 – 27.8 mM), ethanol (10.9 – 33.2 mM) and butyrate (16.1 – 24.1 mM). Traces of propionate (0.1 mM) were detected in conditions with TP higher than 100 kPa (Table 3).

At steady state, the metabolite yields were 0.4 – 0.9 mol $_{acetate.}$ mol^{-1} $_{suc.\ eq.}$; 0.3 – 0.5 mol $_{lactate}$ mol^{-1} $_{suc.\ eq.}$; 0.2 – 0.6 mol $_{ethanol}$ mol^{-1} $_{suc.\ eq.}$; and 0.3 – 0.4 mol $_{butyrate}$ mol^{-1} $_{suc.\ eq}$. Similar values of organic acids yields have been reported by Palomo-Briones et al. [43] who studied the influence of OLR on hydrogen production using a cheese whey-fed CSTR. Despite the carbon source being different from this study, heat pretreatment of the sludge and operating conditions of pH, temperature, stirring, OLR and HRT were analogous.

The theoretical hydrogen production was also estimated for each TP evaluated according to the organic acids concentrations detected, mainly acetate, butyrate and propionate. The measured hydrogen ranged between 9.9% and 44.2% of the theoretical hydrogen estimated (Table 3), suggesting the homoacetogenesis pathway especially at a TP of 140 kPa. Ferraz Júnior et al. [6] and Corona & Razo-Flores [44] reported similar values for the measured H_2 and theoretical H_2 ratio. Finally, it is worth mentioning that biomass, residual sugars, organic acids and H_2 represented between and 87.4 and 105.2% of the COD fed to the fermentation systems (Figure S1). In this study, the COD fed drive to hydrogen production increased from 2.31% to 18% as the TP decreased from 140 kPa to 80 kPa.

3.3. Microbial community analysis

16S ribosomal DNA gene sequences at steady states were analyzed by Illumina MiSeq technology to characterize the microbial community structure and reveal the

total pressure-associated changes. Microbial composition of all TP evaluated is depicted in Figure 4A.

More than 400 thousand partial 16S ribosomal DNA gene sequences were obtained out of which 94 – 98% were assigned to the domain *Bacteria* more specifically phylum *Firmicutes*. No sequence was assigned to the domain *Archaea* (9.6% of inoculum) by the end of reactors operation, indicating that the sludge pretreatment added to the operating conditions inhibited successfully methanogenesis.

More precisely, the most abundant microorganism harbored in all reactor and conditions were *Sporolactobacillus* (57 – 82%) followed by species of genus *Clostridium* (14 – 31%) and *Ethanoligenens* (1.2 – 4.6%) (Figure 4A). This low microbial diversity is considered as a common characteristic in Bio-H_2 producing systems [11] and apparently, the strong pressure of selection becomes accentuated in reactor with suspended biomass [45]. Remarkably, no drastic change in the microbial community was observed at different TP, suggesting that both HPR and HY were directly affected by the mass transfer process (Liquid-Gas) or even by inhibition of synthesis/consumption of hydrogen at the cellular level rather than microbial composition (Figure 4B). However, specific studies must be carried out to validate such a statement.

Both *Sporolactobacillus* and *Clostridium* have been reported as obligate anaerobes capable of producing endospores [32]. Therefore, these two genera are strongly associated to the heat-treatment of seed sludge that is able to inactivate hydrogen consumers, primely methanogenic archaea, and induce the formation of spore-forming anaerobic bacteria [1]. However, *Sporolactobacillus* is described as homofermentative, lactic acid-producing organisms [46] [47] while species within the *Clostridium* genus have been well proved to possess a high ability to produce hydrogen independently of the reactor configuration [3], organic loading rate [32], immobilization [4] or in suspension [12] [48]. With less dominance, in this study, but not less important, the genus *Ethanoligenens* harbors the most promising hydrogen-producing organisms due to their capability to generate hydrogen at high rates and efficiency [49].

[Table 3 – Please here]

Aiming to better understand the interaction among the indicators of reactor performances, a principal component analysis (PCA) was performed (Figure 5). Two principal components accounted for nearly 74% of the dataset variance. The results showed two well-defined axes or principal components (PC): PC 1 which represents the main effect of HPR, HY, acetate yield, TP 80 kPA and TP 100 kPa opposing TP 140 kPa; and PC II which represents TP 120 kPa, lactate, butyrate and ethanol yields opposing TP 140 kPa. PC I reaffirms that the higher values of HPR and HY were archived at the lowest TP evaluated while PC II indicates a direct effect of high pressures on byturate, lactate and ethanol yields. The results also showed an inverse relationship between ethanol yield and TP 140 kPa. It should be noticed that the microbial community was not computed in the PCA analysis due to its quite low variability (Figure 4B).

[Figure 4 – Please here]

[Figure 5 – Please here]

3.4. Highly efficient Bio-H2 condition with regard to the literature

The operation of the dark fermentative high-rate CSTR fed with a mineral salts-sugarcane molasses solution at low total pressure (i.e., TP and p_{H2} of 80 kPa and 41 kPa, respectively) was found to favor successfully the Bio-H_2 production. The observed Bio-H_2 yields were even slightly one of the highest value when compared to other reports (Table 4).

[Table 4 – Please here]

References linked to Table 4: [24] [23] [26] [29] [50] [10] [44]

As previously presented, the microbial community composition was clearly dominated by *Sporolactobacillus*, *Clostridium* and *Ethanoligenens* genera which catalyzed/ regulated the Bio-H_2 production in the dark fermentation process, independently of the TP imposed (Figure 4A). Theoretically, 8 moles of H_2 per mole of sucrose (4 moles of H_2 from glucose and other 4 moles of H_2 from fructose) can be produced if acetate is obtained as the only fermentation product (Reaction 1). If butyrate

or ethanol are the fermentation products, 4 moles of H_2 per mole of sucrose are rather obtained (Reaction 2 and 3) [51] [52] [53].

Acetate-type fermentation

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$ $\Delta G^{o'}$ -206.0 kJ.mol^{-1} (1)

Butyrate-type fermentation

$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$ $\Delta G^{o'}$ -255.0 kJ.mol^{-1} (2)

Ethanol-type fermentation

$C_6H_{12}O_6 + H_2O \rightarrow 2H_2 + 2CO_2 + CH_3CH_2OH + CH_3COOH$ $\Delta G^{o'}$-205.2 kJ.mol^{-1} (3)

For acetate-type fermentation (glucose-model), the breakdown of pyruvate yields (2 moles of H_2 per mole of glucose), and an additional 2 moles of H_2 per mole of glucose is derived through Reaction 4 [54]. The reduction of hydrogenase by NADH is energetically unfavorable under standard conditions unless at extremely low p_{H2} (< 0.1 kPa)[55]. Based on the Gibb's free energy change, butyrate-type fermentation is more energetically favorable and thus NAD is often used in butyrate-type fermentation. In this sense, the combination of acetate and butyrate-type fermentation might occur simultaneously during H_2 production using mixed cultures; and therefore, the maximum hydrogen yield may never exceed 2.5 moles of H_2 per mole of glucose (i.e., 62.5% of its maximum theoretical yield) (Reaction 5) [56] [57] [18]. In the case of sucrose as carbon source, this value is equivalent to 5 moles of H_2. Based on this assumption, the low pressure applied in this study achieved a HY of 4.51 mol-H_2 mol$^{-1}{}_{suc\ eq.}$, which represents 95% of the maximum hydrogen yield through mixed biological path (Reaction 5).

NADH:ferrodoxin oxireductase activity

$NADH + H^+ \rightarrow H_2 + NAD^+$ $\Delta G^{o'}$ +18.1 kJ.mol^{-1} (4)

Acetate and butyrate-type fermentation using mixed culture

$C_6H_{12}O_6 + 0.5H_2O \rightarrow 0.75CH_3(CH_2)_2COOH + 0.5CH_3COOH + 2CO_2 + 2.5H_2$ (5)

In addition, Procentese *et al.* [58] reported that species of *Clostridium acetobutylicum* could be inhibited by the accumulation of acetate (26 Mm) and butyrate (34 Mm). In the present study, acetate and butyrate were in the range of the inhibitory

concentrations (Table 3). In fermentative systems, these acids normally accumulate in the growth medium as dead-end metabolites, since the conversion of these acids into additional H_2 is thermodynamically unfavorable. Consequently, a redirection of the cellular metabolic pathways towards solvent production is often taken. As an illustration, *Clostridium beijerinckii* strains have been reported to reconsume the produced acids at low pH, converting them into ethanol, isopropanol and butanol [59] [60]. Considering the low abundance of *Ethanoligenens* (1.2 – 4.6%), ethanol concentrations detected in the acidogenic reactors liquid outlet was attributed to solventogenesis rather than the ethanol-type fermentation thus, not being accounted in the theoretical hydrogen production (Equation 1).

3.5. Homoacetogenesis still occurred at low pressure

In the anaerobic digestion process, hydrogenotrophic methanogenesis (Reaction 6) is thermodynamically more favorable than homoacetogenesis (Reaction 7) in standard conditions [61]. Acetate evolution as sole metabolite in the liquid phase can only occur at p_{H2} below 0.06 kPa. Homoacetogenesis is also a possible pathway that consumes hydrogen and generates acetate in anaerobic digestion, but the p_{H2} threshold for acetate production through this pathway is 0.25 kPa at 35°C, which is high when compared to the thresholds of 0.06 kPa (hydrogenotrophic methanogenesisis pathway) [14][62].

In this study, the p_{H2} value at low-pressure was still 160 times higher than the p_{H2} threshold for acetate production by homoacetogenesis, indicating that even if methanogenesis was prevented by heat-pretreatment of sludge and operating condition, homoacetogenesis could have occured. The steady-state operation, low pH (5.5) and HRT of 6 h might also favor such reaction 7.

Hydrogenotrophic methanogenesis

$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ $\Delta G°'$ - 135.5 kJ.mol^{-1} (6)

Homoacetogenesis

$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$ $\Delta G°'$ - 104.5 kJ.mol^{-1} (7)

In an experiment at atmospheric pressure performed by Corona and Razo-Flores [44], the increase in the agitation speed from 150 to 300 rpm was implemented as a strategy to collect the hydrogen gas from the liquid phase and avoid its consumption by

463 homoacetogens. The authors reported that values between 30% and 38% of the
464 measured acetate came from homoacetogenesis, being the lower value of the acetate
465 estimated from homoacetogenesis was achieved at the highest stirring condition. This
466 finding is in accordance with the values obtained in this study at TP condition of 80 kPa
467 and 100 kPa (i.e., p_{H2} of 41 kPa and 49 kPa, respectively). Consistently, when TP of
468 140 kPa (p_{H2} of 70 kPa) was applied, acetate issued from homoacetogenesis reached
469 values up to 56.6% due to a higher availability of hydrogen in the liquid medium,
470 resulting in the worst condition for Bio-H_2 production even with agitation speed set at
471 250 rpm (operating condition – *subhead 2.2.*).

472 **3.6. Lactate-type fermentation might comprise an additional pathway to produce**
473 **Bio-H_2**

474 Lactic acid bacteria (LAB) are often detected in mesophilic hydrogen producing
475 consortia as bacteria that accompany hydrogen producers [47]. However, the real role of
476 LAB in hydrogen-producing systems and their influence on hydrogen producers are still
477 unclear.

478 Noike et al. [63]; Ren et al. [49] and Gomes et al. [64] reported inhibition of
479 hydrogen producers by LAB due to substrate competition (replacement of hydrogen
480 fermentation by lactic acid fermentation) and excretion of bacteriocins. In contrast, a
481 positive role of LAB in dark fermentation process has also been reported [65].
482 Fluorescence In Situ Hybridization (FISH) images from a high-rate fermentative
483 hydrogen system suggested that *Streptococcus* cells acted as seeds for granule formation
484 [66]. It is particularly important in CSTR, since this may help increasing biomass
485 concentration into the reactor leading it to higher Bio-H_2 production [65]. Yang et al.
486 [67] even declared the isolation of *Lactobacillus* bacteria capable of hydrogen
487 production during lactose fermentation.

488 Corroborating to the positive role of LAB in dark fermentation process, several
489 clostridia have also demonstrated the ability to ferment lactate. *Clostridium*
490 *propionicurn* uses the acrylate pathway to metabolize lactate, as a sole carbon and
491 energy source [68]. *Clostridium acetobutylicum* cultures metabolize lactate in corn steep
492 liquor [69]. *Clostridium beijerinckii* [70] and *Clostridium tyrobutryicum* [71] require
493 acetate as co-substrate to utilize lactate but the role of acetate and the pathway of lactate
494 metabolism have not been defined.

One of the first reports of lactate conversion to butyrate and hydrogen was the study made by Thauer et al. [72] (Reaction 8). However, this reaction does not include acetate reduction. Later, in experiments made with *Clostridium acetobutylicum* strain P262, acetate was included in the equation and the Gibbs free energy was estimated at approximately -53.8 kJ.mol^{-1} (Reaction 9) [73]. These last authors added that lactate utilization was catabolized by an inducible NAD-independent lactate dehydrogenase (iLDH) with the Michaelis constant of enzyme reaction (K_m) of 3.2 mM for D-lactate.

Lactate conversion to butyrate and hydrogen

$$2CH_3CH(OH)COOH \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2 + H_2O$$

$\Delta G^{o\prime}$ -56.5kJ.mol^{-1} (8)

Lactate and acetate conversion to butyrate and hydrogen via NAD-iLDH pathway

$$1.4CH_3CH(OH)COOH + 0.6CH_3COOH \rightarrow CH_3(CH_2)_2COOH + 1.4CO_2 + 0.8H_2 + H_2O$$

$\Delta G^{o\prime}$ -53.8 kJ.mol^{-1} (9)

Lactate and acetate conversion to butyrate and hydrogen

$$CH_3COOH + 2CH_3CH(OH)COOH \rightarrow 3/2CH_3(CH_2)_2COOH + H_2 + 2CO_2 + H_2O$$

$\Delta G^{o\prime}$ -156.6 kJ.mol^{-1} (10)

More recently, hydrogen and butyrate were produced from a mixture of acetate (50.8 mM) and lactate (33.3 mM) using *Clostridium diolis* JPCC H-3. A molar ratio of consumption of acetate to lactate was 1:2 and the very favorable Gibbs free energy of the reaction (Reaction 10) strongly suggests that this reaction would have proceeded [74]. Interestingly, in this study, *Sporolactobacillus* species were the most abundant microorganisms in all reactors and conditions while lactate was the second most abundant organic acids detected in the acidogenic reactor liquid outlet. As previously mentioned, a moderate relationship was found between butyrate and lactate yields indicating a direct interaction within these two metabolic intermediates (Figure 5).

Considering the actual concentrations of intermediates, butyrate and H_2 synthesis from lactate and acetate is favorable (Table 5). Therefore, the consumption of lactate using acetate as co-substrate was suggested to be an additional pathway to produce H_2 under the evaluated conditions.

[Table 5 – Please here]

References linked to Table 5: [75]

4. Conclusions

In this study, it was shown that the sequestration of biogas from bioreactor headspace enhanced the hydrogen production rate and yield. The higher hydrogen yield (4.51 mol-

H_2 . $mol^{-1}_{suc\ eq.}$) achieved was obtained under a low total pressure of 80 kPa. Interestingly, the composition of the microbial community did not change with the increase and /or decrease of the total pressure. Acetate from homoacetogenesis was accounted even at low pressure conditions. In addition, observations suggest that lactate-type fermentation might play a key role in dark fermentation and might be more considered as additional pathway to produce hydrogen.

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Compliance with ethical standards

Conflict of interest
The authors declare that they have no conflict of interest.

Ethical statement
The authors confirm that the article does not contain any studies with human participants or animals.

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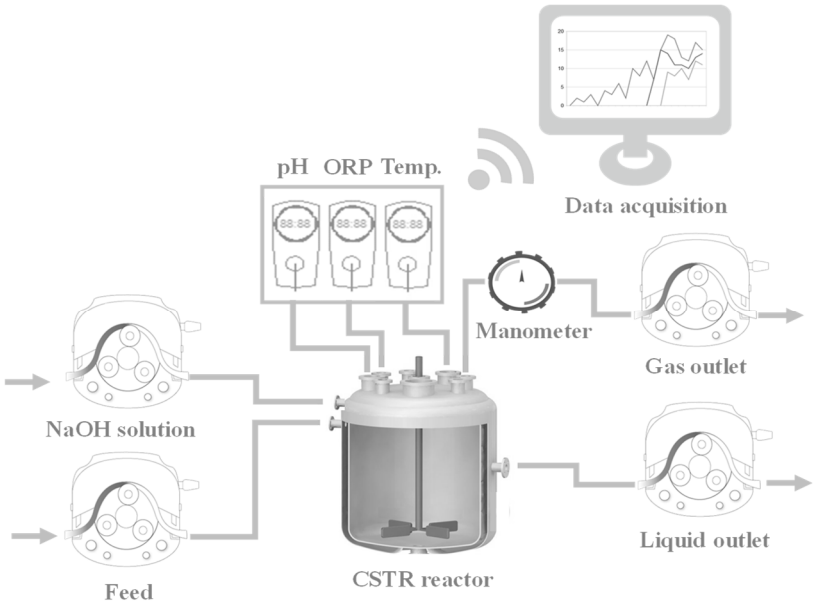
827 **Figure captions**
828
829
830 **Figure 1.** Schematic of experimental apparatus to control the total pressure of
831 headspace of the hydrogen-producing systems fed with a mineral salts-sugarcane
832 molasses solution. CSTR reactor image taken from
833 http://enacademic.com/pictures/enwiki/66/Batch_reactor.2.jpg
834
835 **Figure 2.** Influence of total pressure (TP, kPa) on hydrogen production rate (HRP, mL-
836 H_2 h^{-1}) of fermentative systems fed with a mineral salts-sugarcane molasses solution. A.
837 Reactor 1 (R1) - 80 – 120 kPa, B. Reactor 2 (R2) - 100 – 140 kPa, C. Reactor 3 (R3) -
838 120 – 100 kPa. D. Reactor (R4) - 140 kPa.
839
840 **Figure 3.** Linear fit of the experimental data obtained from monitoring of fermentative
841 systems fed with a mineral salts-sugarcane molasses solution with different total
842 pressure (TP, kPa). A. Hydrogen production rate (HPR, mL-H_2 h^{-1}) and concentration
843 of hydrogen dissolved in the liquid medium ($[H_2]_{Liq.}$) ratio. B. Hydrogen yield (HY),
844 mol-H_2 $mol^{-1}_{suc\ eq.}$) and concentration of hydrogen dissolved in the liquid medium ($[H_2]$
845 $_{Liq.}$) ratio.
846
847 **Figure 4.** A. Composition of microbial community of hydrogen-producing systems at
848 different total pressure (TP, kPa) fed with a mineral salts-sugarcane molasses solution.
849 B. Correlation between *Clostridium*, *Ethanoligenens* and *Sporolactobacillus* species
850 within TP (kPa).
851
852 **Figure 5.** Principal components analysis of hydrogen-producing systems at different
853 total pressure (TP, kPa) fed with a mineral salts-sugarcane molasses solution.
854
855


pH ORP Temp.
Data acquisition
Manometer
Gas outlet
NaOH solution
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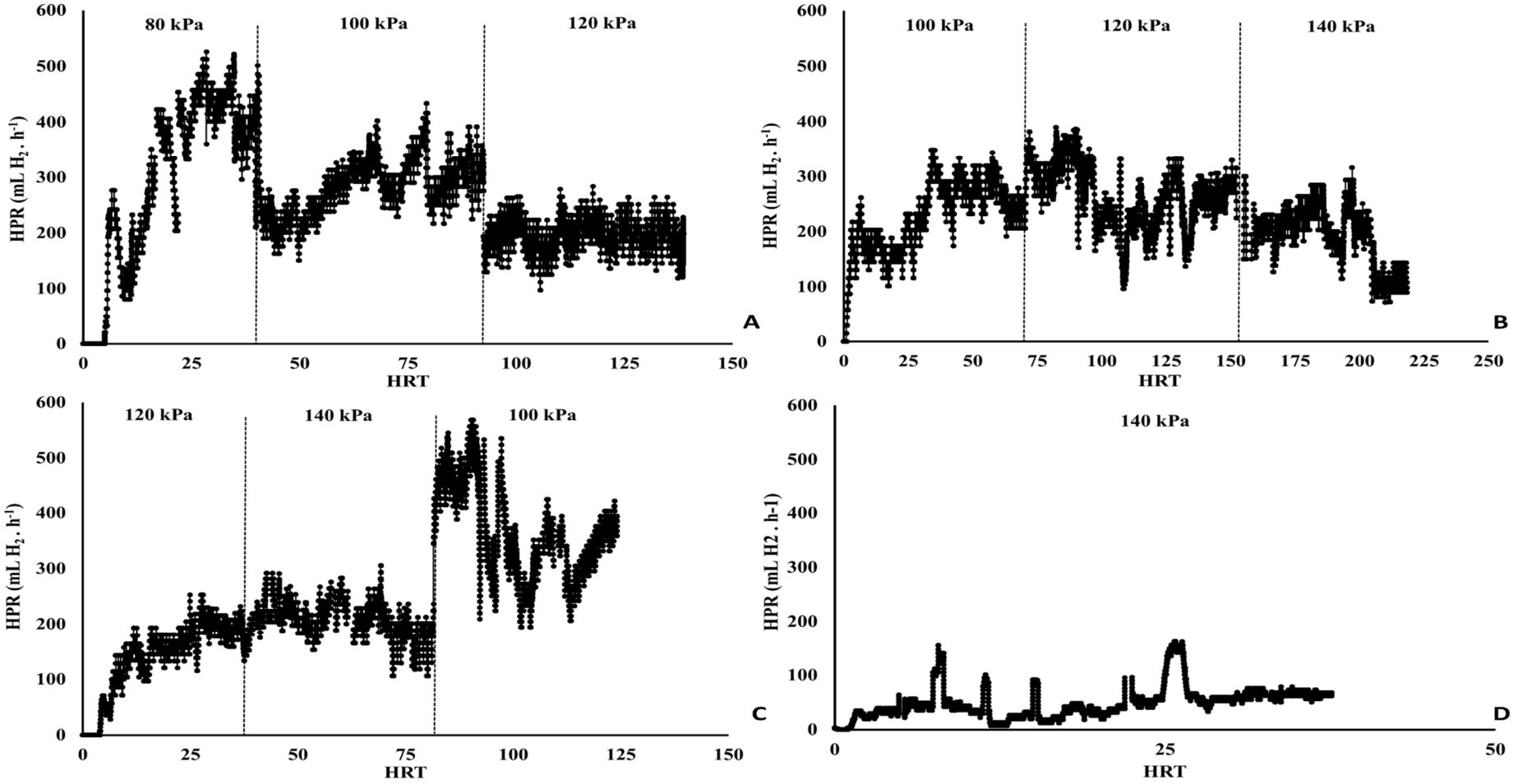
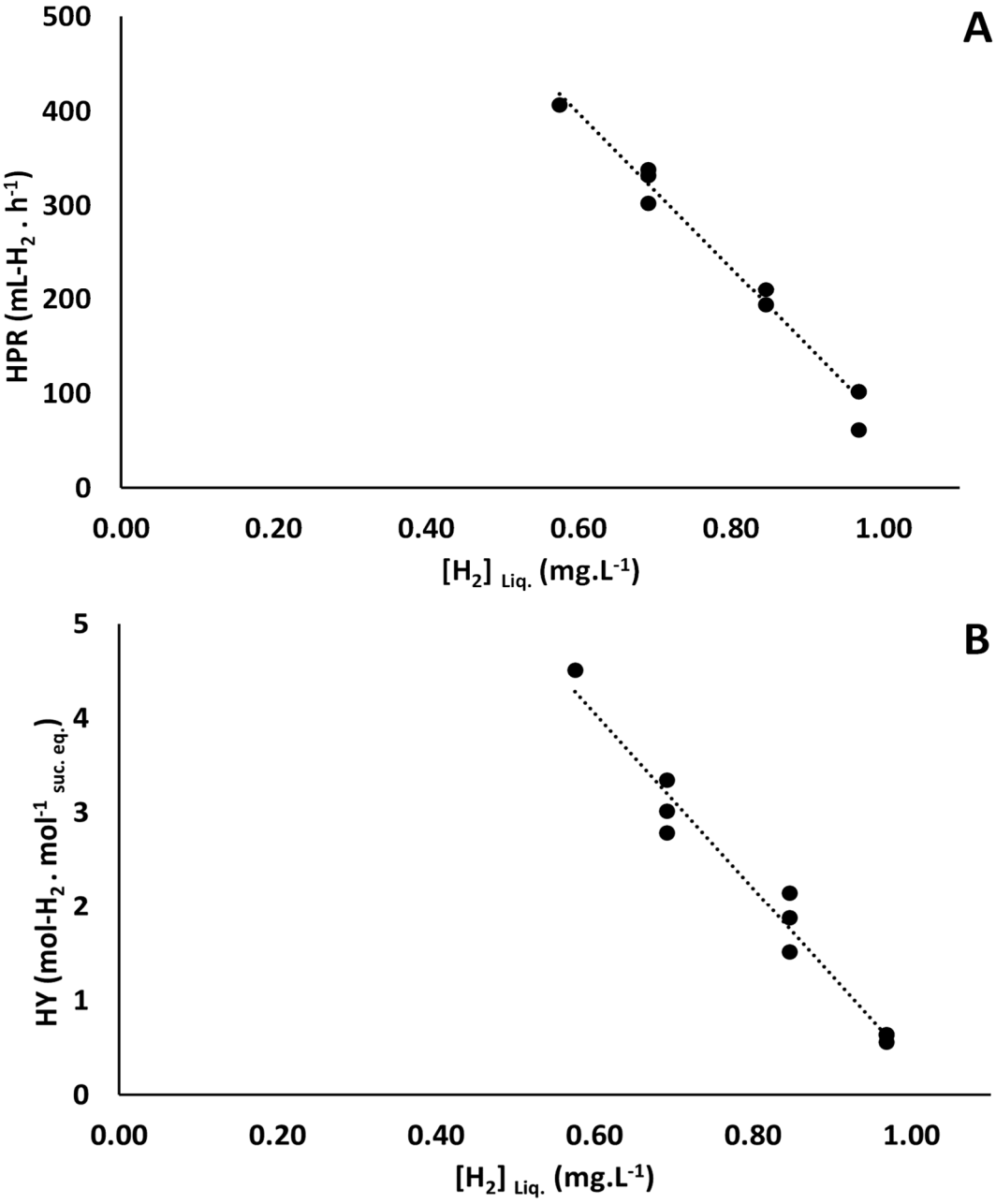
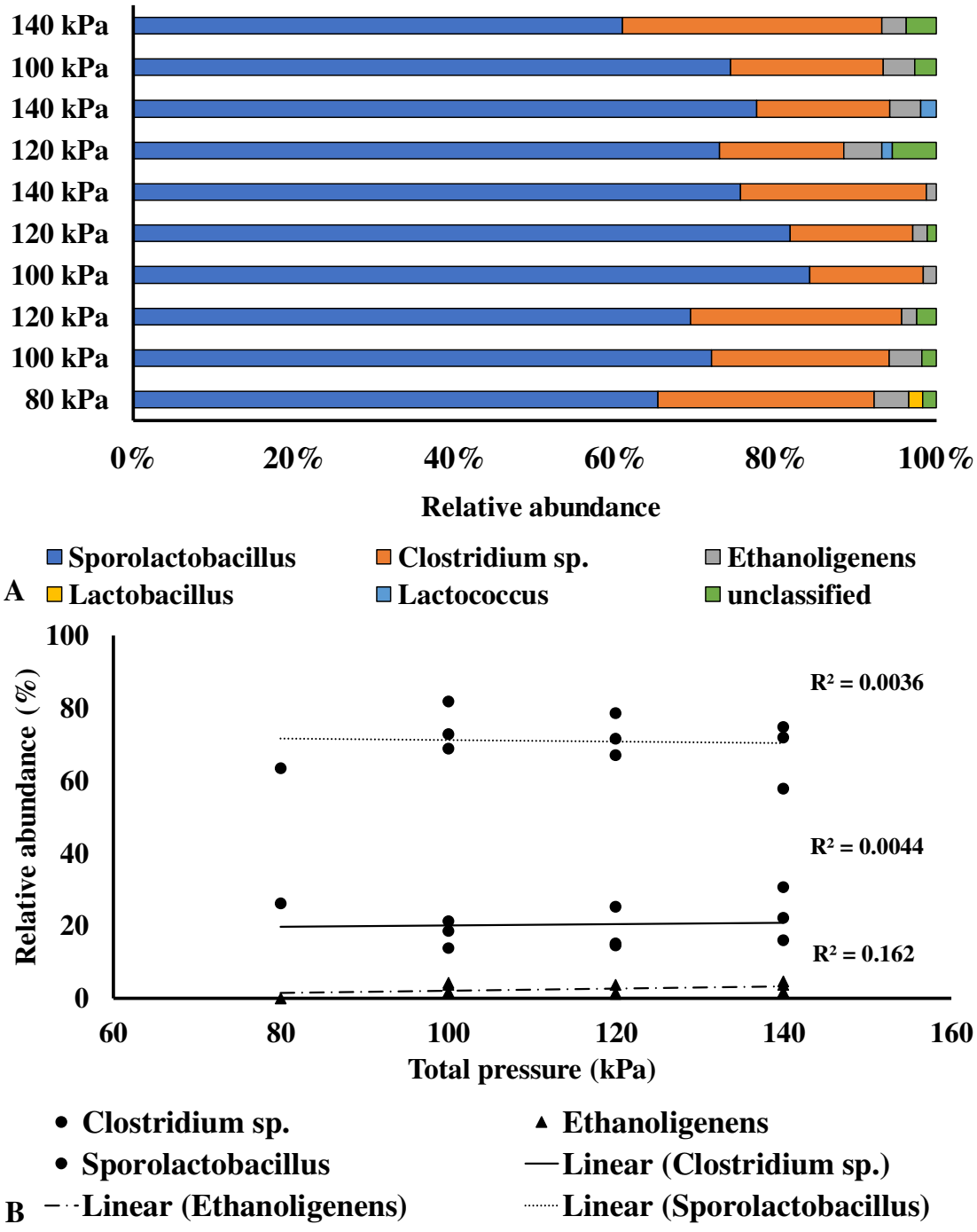
CSTR reactor


80 kPa
100 kPa
120 kPa
HPR (mL H2 . h-1)
HRT
A
100 kPa
120 kPa
140 kPa
HPR (mL H2 . h-1)
HRT
B
120 kPa
140 kPa
100 kPa
HPR (mL H2 . h-1)
HRT
C
140 kPa
HPR (mL H2 . h-1)
HRT
D


A
500
400
300
200
100
0
HPR (mL-H2 . h-1)
0.00
0.20
0.40
0.60
0.80
1.00
[H2] Liq. (mg.L-1)
B
5
4
3
2
1
0
HY (mol-H2 . mol-1 suc. eq.)
0.00
0.20
0.40
0.60
0.80
1.00
[H2] Liq. (mg.L-1)


140 kPa
100 kPa
140 kPa
120 kPa
140 kPa
120 kPa
100 kPa
120 kPa
100 kPa
80 kPa
0%
20%
40%
60%
80%
100%
Relative abundance
Sporolactobacillus
Clostridium sp.
Ethanoligenens
Lactobacillus
Lactococcus
unclassified
A
Relative abundance (%)
100
80
60
40
20
0
R² = 0.0036
R² = 0.0044
R² = 0.162
60
80
100
120
140
160
Total pressure (kPa)
Clostridium sp.
Ethanoligenens
Sporolactobacillus
Linear (Clostridium sp.)
Linear (Ethanoligenens)
Linear (Sporolactobacillus)
B

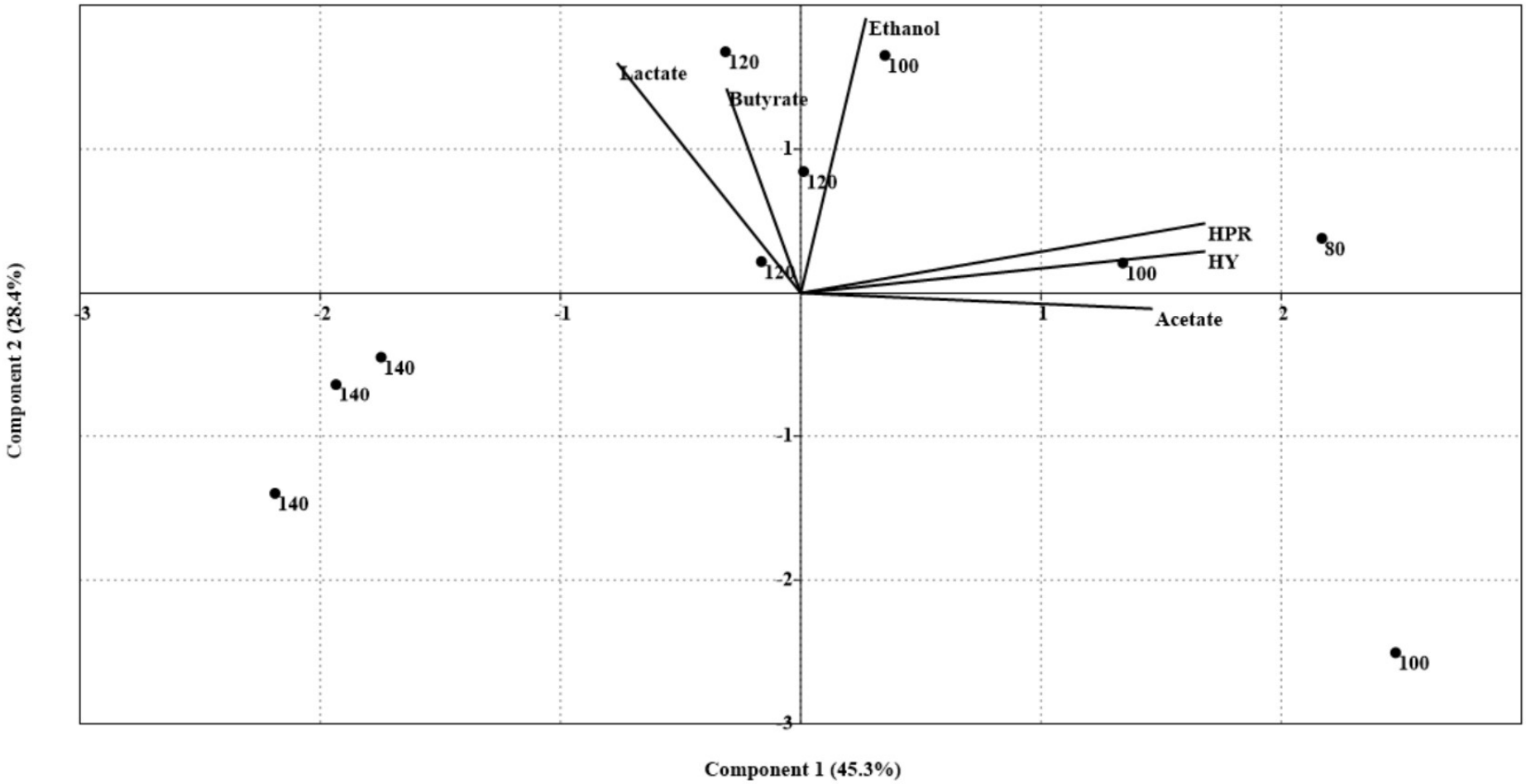

Ethanol
Lactate
120
100
Butyrate
1
120
HPR
HY
80
120
100
-3
-2
-1
1
2
Acetate
140
140
-1
140
-2
100
-3
Component 2 (28.4%)
Component 1 (45.3%)

Table 1. Experimental design and different total pressure (kPa) applied to the hydrogen-producing systems fed with a mineral salts-sugarcane molasses solution.

Reactor	Total pressure (kPa)		
	Initial TP and independent condition [a]	Condition of TP Increment/Decrement [a]	
R1	80	100	120
R2	100	120	140
R3	120	140	100
R4	140	-	-

a. The steady-state of the reactor was adopted as criterion of condition change.

Table 2. Hydrogen production rate (HPR, mL-H_2 h^{-1}) and hydrogen yield ((HY), mol-H_2 $mol^{-1}_{suc\ eq.}$) of fermentative systems fed with a mineral salts-sugarcane molasses solution.

Reactor	Total pressure (TP; kPa) / Partial pressure of hydrogen (p_{H2}; kPa)			
	80 / 41	100 / 49	120 / 61	140 / 70
R1	406 (4.51)	302 (3.02)	194 (2.15)	-
R2	-	332 (2.79)	210 (1.88)	102 (0.64)
R3	-	338(3.35)	210 (1.52)	102 (0.63)
R4	-	-	-	62 (0.56)

Table 3. Intermediates of hydrogen-producing systems at different total pressure (TP, kPa) and fed with a mineral salts-sugarcane molasses solution.

Reactor	Conditions	Acetate [a] (mM)	Butyrate [a] (mM)	Propionate[a] (mM)	Ethanol [a] (mM)	Lactate [a] (mM)	Experimental H_2 (mM)	Theoretical H_2 [b] (mM)	Exp. H_2/ Theoretical H_2 (%)	Acetate from homoacetogenesis/ Total acetate [c] (%)	COD_{rec} [d] (%)
	80 kPa	37.3 ± 1.7	24.1 ± 1.1	0.0 ± 0.0	19.5 ± 2.2	18.9 ± 0.0	54.2 ± 4.9	122.7	44.2	30.7	99.1
R1	100 kPa	39 ± 1.4	18.4 ± 2.3	0.1 ± 0.0	30.4 ± 4.3	21.1 ± 2.2	40.3 ± 3.4	114.5	35.2	31.8	98.2
	120 kPa	35.6 ± 1.2	24.1 ± 1.2	0.1 ± 0.0	19.5 ± 2.1	21.1 ± 1.1	25.8 ± 2.6	119.2	21.7	43.8	100.2
	100 kPa	33.2 ± 0.3	21.8 ± 0.9	0.0 ± 0.0	26.2 ± 3.5	27.8 ± 1.7	44.3 ± 3.1	110	40.2	33	100.6
R2	120 kPa	35.2 ± 3.1	21.8 ± 1.1	0.1 ± 0.0	33.2 ± 0.5	25.7 ± 1.4	28 ± 2.5	114	24.6	40.7	104.8
	140 kPa	29.5 ± 1.6	19.5 ± 1.0	0.1 ± 0.0	13 ± 2.2	26.1 ± 1.9	13.6 ± 1.0	97.8	13.9	47.6	87.4
	120 kPa	44 ± 1.7	20.7 ± 1.1	0.1 ± 0.0	26.1 ± 6.5	26.1 ± 3.3	28 ± 2.8	129.3	21.7	38.3	105
R3	140 kPa	28.8 ± 1.0	21.8 ± 0.7	0.1 ± 0.0	17.4 ± 3.2	22.2 ± 1.1	13.6 ± 2.2	101.1	13.5	50.6	89.6
	100 kPa	49.1 ± 3.4	16.1 ± 0.6	0.0 ± 0.0	10.9 ± 2.2	16.7 ± 1.2	45.1 ± 4.4	130.4	34.6	28.9	96.7
R4	140 kPa	22 ± 0.2	19.5 ± 1.2	0.0 ± 0.0	15.2 ± 4.3	20 ± 6.7	8.2 ± 0.9	83.1	9.9	56.6	90.8

a. Mean value ± standard deviation; n=6.
b. Theoretical hydrogen production and yield are based on the acetate, butyrate and propionate produced according to Ferraz Júnior et al. (2014b).
c. Acetate from homoacetogenesis was calculated according to Luo et al. [27].
d. Calculated according to Ferraz Júnior et al. [18].

Table 4. Maximum hydrogen yield reported from different methods of controlling the partial pressure of hydrogen (p_{H2}).

Controlling method of p_{H2}	Reactor	Sludge	Substrate / OLR ($gCOD.L^{-1}.d^{-1}$)	HY ($mol\ H_2.\ mol^{-1}_{substrate}$)	Reference
CO_2 and N_2 sparging	CSTR	Mixed	Sucrose / 40	1.68	[14]
N_2 sparging	CSTR	Mixed	Glucose / 27.02	1.43	[13]
Membrane separation	Batch	Mixed	Glucose / 2.5[a]	0.92	[16]
Collection of biogas	CCS [b]	Pure [c]	Glucose-polypeptone / 5.4 - 30	2.3	[17]
CO_2 sequestration	IBRCS [d]	Mixed	Glucose / 25.7	2.96	[40]
Increase of temperature	APBR [e]	Mixed	Sugarcane vinasse / 84.2	3.7	[41]
Stirring	CSTR	Mixed	Agave bagasse / 44	44.6%* [f]	[32]
Biogas collection	CSTR	Mixed	Molasse / 84.2	4.51	This study

a. Food / Microorganisms ratio equal to 23.8 (2.5 g of sucrose added);
b. Continuous culture system;
c. *Clostridium butyricum strain SC-E1*;
d. Integrated biohydrogen reactor clarifier systems;
e. Anaerobic packed-bed reactor;
f. Value obtained from the ration of hydrogen measured and the estimated hydrogen produced via acetic and butyric pathways. The authors do not express HY in $mol.mol^{-1}$ probably due to the lignocellulose hydrolysates be composed by glucose, xylose, arabinose, cellobiose, lignin fragments, among others.

Table 5. Gibb's energy of lactate and acetate conversion into butyrate and hydrogen reaction.

Reactor	Total pressure (TP; kPa) / Gibb's energy (ΔG^{o}; $kJ.mol^{-1}$)			
R1	80 / -80.2	100 / -79.5	120 / -79.5	-
R2	-	100 / - 77.8	120 / -78.3	140 / -78.2
R3	-	100 / -81.0	120 / -78.2	140 / -78.2
R4	-	-	-	140 / -79.8

$\Delta G°$ were calculated at 25 °C and standard concentrations. ΔG were calculated at pH 5.5, 37 °C and the intermediates concentrations as shown in Table 3. Gibbs' energy values were computed in accordance with Kleerebezem and Van Loosdrecht [67].