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1 **BIOGAS SEQUESTRATION FROM THE HEADSPACE OF A**
2 **FERMENTATIVE SYSTEM ENHANCES HYDROGEN PRODUCTION RATE**
3 **AND YIELD**

4

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16 **Abstract**

17 Total pressure (TP) affects the level of dissolved hydrogen gas in the fermentation
18 medium leading to metabolic shifts in mixed microbial-culture-based systems. In this
19 study, the effect on hydrogen production rate and yield was investigated at different TP
20 of a hydrogen-producing system using a microbial non-sterile culture previously heat-
21 treated. Four continuous stirred-tank reactors (CSTR) were operated in parallel on a
22 mineral salts-molasses medium (21 g-COD. L⁻¹) at 35°C, pH 5.5 and hydraulic
23 retention time (HRT) of 6 h. The TP was set at 80 kPa (R1), 100 kPa (R2), 120 kPa
24 (R3) and 140 kPa (R4) for which reactor performances were estimated at steady-state
25 conditions. As the increase of TP consequently increased the partial pressure of
26 hydrogen (p_{H_2}), the hydrogen production rate (HPR) and yield (HY) were consistently
27 negatively influenced. The highest HPR and HY (406.1 ± 36.8 mL-H₂ h⁻¹; 4.51 molH₂
28 mol⁻¹_{suc eq.}) were achieved at low pressure conditions (80 kPa). The composition of the
29 microbial community mainly represented by species from *Sporolactobacillus* and
30 *Clostridium* genera, did not change with the increase and /or decrease of the TP,
31 indicating a regulation at cellular but not population level.

32

33 **Keywords:** biohydrogen, dark fermentation, biogas sequestration, ppH₂, sugarcane
34 molasses.

35

36

37 1. INTRODUCTION

38 The production of biohydrogen (BioH₂) from organic waste is a promising
39 biotechnological process with gains at energetic, societal and environmental levels [1].
40 However, BioH₂ production by dark fermentation (DF) is still a technological challenge
41 for being a very sensitive process, requiring careful balancing of the following
42 parameters: pH [2] [3], temperature [4], organic loading rate (OLR) [5] and specific
43 organic loading rate (sOLR) [6].

44 In fermentative systems using non-sterile mixed cultures, high H₂ yields are
45 associated with a mixture of acetate and butyrate fermentation pathways end-products,
46 while low H₂ yields are associated with other reduced end-products such as lactate,
47 solvents (ethanol, butanol and acetone) and alanine. To date, hydrogen yields in
48 fermentative systems are mostly ranging between 1.2 – 2.3 molH₂.mol⁻¹_{hexose} which
49 represent only 30 – 50% of the theoretical maximum hydrogen yield (4 molH₂.mol⁻¹
50 _{hexose}, glucose) [7] [8] [9] [10].

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

55 The partial pressure of hydrogen (p_{H_2}) is an extremely important factor
56 especially for continuous BioH₂ production [15] [16] [17] [18]. This factor is explained
57 by Le Chatelier's Principle that says "all chemical equilibrium responds to an increase
58 in the pressure, causing the reaction to move in the opposite sense to that, which rises
59 the pressure." In biological multiphases systems, this event is associated to the
60 limitation of the liquid-to-gas mass transfer. The liquid-to-gas mass transfer limitation
61 arises because the gas production rate is higher than the transfer rate to the gas phase
62 [19] [20]. Such a limitation have caused H₂ supersaturation in the liquid with
63 concentrations of H₂ between 5- and 71/fold higher than the equilibrium value [20]
64 [21]. Thereby, during the fermentation process, as the p_{H_2} in bioreactors increases, H₂
65 synthesis decreases [22]. This also can be explained through

66 Metabolic pathways shifts are also observed in function of the p_{H_2} . According to
67 Hallenbeck [7] in Clostridial-type hydrogen producing fermentation at low p_{H_2} , the
68 NADH generated during glycolysis can be reoxidized, probably by a NADH-dependent

69 [FeFe] hydrogenase. At moderate to high p_{H_2} , this reaction is unfavorable, and NADH
70 is reoxidized by the formation of reduced organic compounds (previously mentioned).
71 As a consequence, low hydrogen yields are achieved.

72 Few methods to control the p_{H_2} have been investigated: sparging (*i.e.*, gas
73 flushing to remove other dissolved gas, in this case H₂), removing H₂ from the system
74 or reactor operation at low pressure. Mizuno et al [23] evaluated the influence of
75 sparging in a continuous stirred tank (CSTR) fed with a mineral salts-glucose medium
76 (10.7 gCOD.L⁻¹). Nitrogen gas was sparged at a flow rate of 15 times the specific
77 hydrogen production rate (sHPR) observed in a control CSTR (*i.e.*, without sparging)
78 that was 1.446 mL H₂. min⁻¹. g⁻¹ biomass. An increase of 68% of hydrogen yield was
79 achieved with sparging (1.43 molH₂.mol⁻¹ glucose).

80 Besides nitrogen, other gases such as internal biogas and only carbon dioxide
81 with different flow rates (100 – 400 mL.min⁻¹) were investigated in a CSTR fed with a
82 mineral salts-sucrose medium (20 gCOD.L⁻¹) (Kim and co-authors, [24]). The best
83 performances were obtained by CO₂ sparging at 300 ml.min⁻¹, resulting in the
84 highest H₂ yield of 1.68 molH₂.mol⁻¹ hexose converted. Concomitant to the increase of
85 hydrogen production and yield, too much sparging produces dilute gas stream, creating
86 a serious problem with respect to the H₂ separation from the sparging gas [25].

87 Fast collection of biogas was also studied as the p_{H_2} control method. Liang et al.
88 [26] investigated the biogas removal using a vacuum pump (31.4 kPa) and membrane
89 purification of H₂ from a fermentation system (Batch reactor; 2.5 g glucose added). The
90 authors reported that silicone rubber was effective in reducing the p_{H_2} , improving the
91 hydrogen production by 10% (2.6 – 3 mmol H₂.g⁻¹ VSS. h⁻¹) and the hydrogen yield by
92 15% (0.84 – 0.92 molH₂. mol⁻¹ glucose). Lee et al. [27] investigated the effect of working
93 with reduced pressure in a CSTR, they worked with pressures similar to the ones in Liu
94 and Wang [28], between 0.2 and 0.9 atm, and concluded that H₂ production can be
95 improved in fermentative systems with reduced pressure.

96 Interestingly, no difference regarding H₂ production was observed in pure
97 culture system (*Clostridium butyricum* strain SC-E1) under vacuum (28 kPa) and non-
98 vacuum. Glucose-polypeptone at 0.5 and 1.0% concentration were used as substrate,
99 resulting in maximum hydrogen yields of 1.8 – 2.2 and molH₂.mol⁻¹ glucose for all
100 condition evaluated [29].

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

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

106 Despite these mentioned studies on this subject, more detailed research on this
107 topic is necessary to enable the production of BioH₂ at larger scale and with continuous
108 operation of the fermentation process. In this study, regular collection of biogas from
109 headspace of a fermentative continuous system was carried out aiming to control the
110 p_{H_2} in the process and thus, attempt to maintain a high hydrogen productivity. The
111 dynamics of the microbial community was also studied based on the sequencing of the
112 V4 region of 16S rRNA gene for Bacteria using High-Throughput Sequencing (MiSeq
113 Sequencing System - Illumina).

114

115 **2. MATERIALS AND METHODS**

116

117 **2.1. Seed sludge**

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

123

124 **2.2. Feeding solution**

125 A mineral salts-sugarcane molasses solution of 21 g COD L⁻¹ was used as
126 carbon source in a feeding medium composed by the following macro- and micro-
127 nutrients (mg L⁻¹): NiSO₄·6H₂O, 0.5; FeSO₄·7H₂O, 2.5; FeCl₃·6H₂O, 0.25; CoCl₂·2H₂O,
128 0.04; CaCl₂·2H₂O, 2.06; SeO₂, 0.036; HCl, 0.25, according to Del Nery [33]. The C/N
129 ratio of molasses was 52.7.

130

131 **2.2. Reactor design and operational conditions**

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

137 at each different levels of pressure. Pressure was regulated with a control device
138 combining a pressure sensor and a peristaltic pump following a two-band control law. A
139 combined sensor was connected to the reactor for measuring the redox potential and pH
140 (4010/120/Pt100, Mettler Toledo). The pH and redox meter (M300 – Mettler Toledo)
141 was connected to a computer for on-line data acquisition (home-made software Odin in
142 collaboration with INRIA teams). The pH was set and controlled at 5.5 by adding
143 NaOH (2 M) with a peristaltic pump. Temperature in the reactor was also controlled
144 using a platinum probe Pt100 and a heating electric resistance. The temperature was
145 maintained constant at 37 ± 0.5 °C. The hydraulic retention time (HRT) was 6 h,
146 resulting in an organic loading rate (OLR) of $84.2 \text{ gCOD.L}^{-1} \cdot \text{d}^{-1}$, as suggested in [5].
147 The total pressure tested is presented in Table 1.

148 **[Figure 1 – Please here]**

149
150 **[Table 1 – Please here]**

151

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

169

170 **2.3. Chemical analysis**

171 Biogas composition was analyzed as previously described in [34]. Reduced
172 sugars and fermentation end-products were quantified using high performance liquid
173 chromatography (HPLC; HPX 87 column - Biorad) coupled to a refractometer (Waters
174 R410). The eluent used was a H₂SO₄ solution (0.222 μl L⁻¹). The operating conditions
175 were: elution flow, 0.4 mL min⁻¹; temperature of column, 35 °C; temperature of
176 refractometer, 40 °C. Microbial cells (biomass) concentration was determined as
177 volatile suspended solids (VSS) by filtration at 1.2 μm, according to [35].

178 **2.4. DNA extraction, PCR amplification and High-Throughput Sequencing of** 179 **hydrogen-producing systems samples**

180 At the end of each operating condition, microbial cells were collected after
181 centrifugation (12000 × g; 15 min) of 2 mL of culture. Genomic DNA was extracted
182 using the Wizard Genomic DNA Purification kit (Promega). The V3-4 region of the
183 16S rRNA gene was amplified with the forward primer
184 CTTTCCCTACACGACGCTCTTCCGATCTTACGGRAGGCAGCAG and the reverse
185 primer GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAA TCCT
186 plus the respective linkers over 30 amplification cycles at 65° C (annealing
187 temperature). An index sequence was added using the primers AATGA-
188 TACGGCGACCACCGAGATCTACACTCTTCCCTACACGAC and
189 CAAGCAGAAGACGGCATAACGAGAT-index-TGACTGGAGTTCAGACGTGT
190 (PCR – 12 cycles). The PCR products were purified and loaded onto the Illumina
191 MiSeq cartridge according to the manufacturer's instructions for sequencing (paired-
192 end; 250 bp reads) which was performed at the GeT PlaGe sequencing center of the
193 genotoul life science network in Toulouse, France (get.genotoul.fr). Quality checking
194 was made using a slightly modified version of the Standard Operation Procedure by
195 Kozich et al. [36] in Mothur version 1.33.0. Alignment and taxonomic outline was made
196 using release information: SILVA 102, as provided by Schloss et al. [37]. The software
197 PAUP* (version 4.0b10) was used to infer a phylogeny - criterion of maximum
198 parsimony [38]. Bootstrap support was calculated using 1500 repetitions. SumTrees
199 (version 3.3.1) of the DendroPy package (version 3.12.0) was used to map bootstrap
200 values to the best phylogeny [39]. Sequences of most abundant operational taxonomic
201 unit (OTU) found in the biofilm were deposited in the NCBI Genbank database under
202 the following accession name SUB5433515 (MK765997 - MK766231).

203

204 2.5. Calculations

205 Hydrogen Production Rate (HPR, mL-H₂ h⁻¹) and Hydrogen Yield
206 (HY, mol-H₂ mol⁻¹_{suc eq.}) were calculated using Equations 1 and 2 – 4, respectively.

$$207 \text{ HPR} = Q_g \cdot \%H_2 \quad (1)$$

$$208 \text{ HY} = ((Q_g \cdot nH_2)/V) / ((Q \cdot (C_{S0} - C_{SF}))/MM_S) \quad (2)$$

$$209 nH_2 = \%H_2 \cdot n \quad (3)$$

$$210 n = (P \cdot V) / (R \cdot T) \quad (4)$$

211 where, Q_g is the biogas flow, %H₂ is the hydrogen content in biogas, nH₂ is the number
212 of mol of hydrogen, V is the volume of gas of the sample, Q is the liquid flow in the
213 reactor, C_{S0} is the influent substrate concentration, C_{SF} is the effluent substrate
214 concentration, and MM_S is the sucrose molar mass, n value corresponds to the total
215 number of moles of sample (i.e., %H₂, %CO₂ and %CH₄), P is the gas pressure, R is the
216 universal ideal gas constant, and T is the absolute temperature.

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

$$220 H_{2 \text{ theoretical}} = 2[A] + 2[B] - [P] \quad (5)$$

$$221 \text{ Acetate}_{\text{ homoacetogenesis}} = (2[A] + 2[B] - [P] - [H_2])/6 \quad (6)$$

222 where [A], [B], [P] and [H₂] are the measured acetic, butyric and propionic acids; and
223 the hydrogen concentrations in mM, respectively.

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

$$226 \text{ COD}_{\text{ recovery}} \% = (\text{COD}_{\text{ final}}/\text{COD}_0) * 100 \quad (7)$$

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

230 Principal component analysis (PCA) was performed using STATISCA 10.
231 Primarily, a factor analysis was performed to identify the number of independent factors
232 [20]. The Kaiser criterion was used to decide the factors that could be retained for
233 interpretation [41]. The factors cut off was identified through the point of wherein the
234 eigenvalue level drop off continuously based on Catell [42].

235

236 **3. RESULTS AND DISCUSSION**

237

238 **3.1. Hydrogen production (HPR) and yield (HY)**

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

249 Hydrogen content of biogas was around 47 – 54% for all reactors and
250 conditions. Methane was not observed in the biogas suggesting that the inoculum heat-
251 treated, and the operating conditions inhibited the methanogenesis and favored the
252 hydrogen-producing process. The partial pressure of hydrogen (p_{H_2}) was determined by
253 Dalton's law and the values ranged between 41-70 kPa. These values are slightly higher
254 than [26] and [29].

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

260 The same behavior was observed in R2 and R3. The increase of the TP from 100
261 to 140 kPa; and from 120 to 140 kPa, was reflected in HPR decrease of 70% (R2) and
262 17% (R3), respectively (Phase I). When the TP was alleviated to 100 kPa in R3, the
263 HPR increased by 90% in Phase I. In addition, the highest TP (140 kPa) as applied to
264 R4 resulted in the lowest value of HRP ($61.6 \pm 5.8 \text{ mL-H}_2 \text{ h}^{-1}$) (Table 2). These findings
265 show that gas removal had a positive effect on HPR. The increase of the TP with a
266 consequent increase of the p_{H_2} influenced negatively the Bio-H₂ production.

267 Hydrogen yield (HY) followed the same trend as HPR, being the maximum and
268 the minimum values achieved of 4.51 and 0.56 mol-H₂ mol⁻¹_{suc eq.} when the TP was 80
269 and 140 kPa, respectively (Table 2). The HY at initial TP of 80 KPa represented an
270 increase of 61.6% and 705% comparing to the controlled atmospheric pressure (100
271 kPa) and to the highest TP evaluated (140 kPa), respectively, reaffirming the high
272 influence of TP on biological hydrogen production process.

273

274 **[Figure 2 – Please here]**

275

276 **[Table 2 – Please here]**

277

278 Based on the p_{H_2} of the headspace, the concentration of dissolved hydrogen in
279 the liquid medium ($[\text{H}_2]_{\text{Liq.}}$) was estimated by Henry's Law. The correlation between
280 $[\text{H}_2]_{\text{Liq.}}$ and; HPR and HY indicated a linear coefficient of 0.979 and 0.968, respectively
281 (Figure 3).

282

[Figure 3 – Please here]

283

284 By applying linear regression analysis on the experimental results, the equations
285 (4) and (5) were obtained to describe the influence of $[\text{H}_2]_{\text{Liq.}}$ on HPR and HY,
286 respectively. The $[\text{H}_2]_{\text{Liq.}}$ of 0.57 mg. L⁻¹ resulted in maximum values of HPR and HY
287 while the $[\text{H}_2]_{\text{Liq.}}$ of 0.99 mg. L⁻¹ resulted in the lower values of the respective variables
288 indicating that during fermentation process, as the p_{H_2} in bioreactors decreases, BioH₂
289 synthesis increases (vice versa).

290
$$\text{HPR} = -821.15 * [\text{H}_2]_{\text{Liq.}} + 890.1 \quad R^2 = 0.979 \quad (4)$$

291
$$\text{HY} = -9.2954 * [\text{H}_2]_{\text{Liq.}} + 9.6264 \quad R^2 = 0.968 \quad (5)$$

292

293 **3.2. Intermediates products from molasses fermentation**

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

300 In addition to the molasses fermentation products, the organic acids were
301 quantified to investigate the main metabolic pathways in the hydrogen-producing
302 systems. Table 3 shows that the main intermediates products were acetate (49.1 – 22
303 mM) followed by lactate (16.7 – 27.8 mM), ethanol (10.9 – 33.2 mM) and butyrate
304 (16.1 – 24.1 mM). Traces of propionate (0.1 mM) were detected in conditions with TP
305 higher than 100 kPa (Table 3).

306 At steady state, the metabolite yields were $0.4 - 0.9 \text{ mol}_{\text{acetate}} \text{ mol}^{-1}_{\text{suc. eq.}}$; $0.3 -$
307 $0.5 \text{ mol}_{\text{lactate}} \text{ mol}^{-1}_{\text{suc. eq.}}$; $0.2 - 0.6 \text{ mol}_{\text{ethanol}} \text{ mol}^{-1}_{\text{suc. eq.}}$; and $0.3 - 0.4 \text{ mol}_{\text{butyrate}} \text{ mol}^{-1}_{\text{suc. eq.}}$. Similar values of organic acids yields have been reported by Palomo-Briones et al.
308 [43] who studied the influence of OLR on hydrogen production using a cheese whey-
309 fed CSTR. Despite the carbon source being different from this study, heat pretreatment
310 of the sludge and operating conditions of pH, temperature, stirring, OLR and HRT were
311 analogous.
312

313 The theoretical hydrogen production was also estimated for each TP evaluated
314 according to the organic acids concentrations detected, mainly acetate, butyrate and
315 propionate. The measured hydrogen ranged between 9.9% and 44.2% of the theoretical
316 hydrogen estimated (Table 3), suggesting the homoacetogenesis pathway especially at a
317 TP of 140 kPa. Ferraz Júnior et al. [6] and Corona & Razo-Flores [44] reported similar
318 values for the measured H_2 and theoretical H_2 ratio. Finally, it is worth mentioning that
319 biomass, residual sugars, organic acids and H_2 represented between and 87.4 and
320 105.2% of the COD fed to the fermentation systems (Figure S1). In this study, the COD
321 fed drive to hydrogen production increased from 2.31% to 18% as the TP decreased
322 from 140 kPa to 80 kPa.

323 **3.3. Microbial community analysis**

324 16S ribosomal DNA gene sequences at steady states were analyzed by Illumina
325 MiSeq technology to characterize the microbial community structure and reveal the

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

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

333 More precisely, the most abundant microorganism harbored in all reactor and
334 conditions were *Sporolactobacillus* (57 – 82%) followed by species of genus
335 *Clostridium* (14 – 31%) and *Ethanoligenens* (1.2 – 4.6%) (Figure 4A). This low
336 microbial diversity is considered as a common characteristic in Bio-H₂ producing
337 systems [11] and apparently, the strong pressure of selection becomes accentuated in
338 reactor with suspended biomass [45]. Remarkably, no drastic change in the microbial
339 community was observed at different TP, suggesting that both HPR and HY were
340 directly affected by the mass transfer process (Liquid-Gas) or even by inhibition of
341 synthesis/consumption of hydrogen at the cellular level rather than microbial
342 composition (Figure 4B). However, specific studies must be carried out to validate such
343 a statement.

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

356
357

[Table 3 – Please here]

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

370

371 **[Figure 4 – Please here]**

372

373 **[Figure 5 – Please here]**

374

375 **3.4. Highly efficient Bio-H₂ condition with regard to the literature**

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

381 **[Table 4 – Please here]**

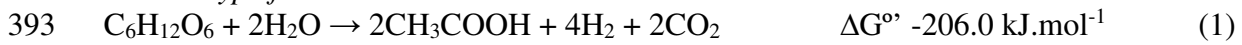
382 **References linked to Table 4:** [24] [23] [26] [29] [50] [10] [44]

383

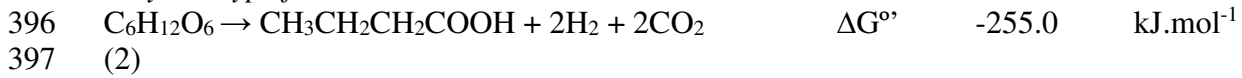
384 As previously presented, the microbial community composition was clearly
385 dominated by *Sporolactobacillus*, *Clostridium* and *Ethanoligenens* genera which
386 catalyzed/ regulated the Bio-H₂ production in the dark fermentation process,
387 independently of the TP imposed (Figure 4A). Theoretically, 8 moles of H₂ per mole of
388 sucrose (4 moles of H₂ from glucose and other 4 moles of H₂ from fructose) can be
389 produced if acetate is obtained as the only fermentation product (Reaction 1). If butyrate

390 or ethanol are the fermentation products, 4 moles of H₂ per mole of sucrose are rather
 391 obtained (Reaction 2 and 3) [51] [52] [53].

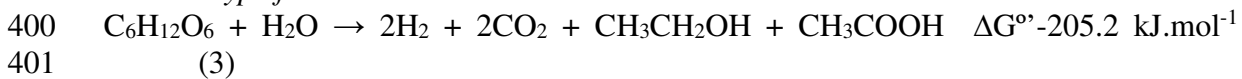
392 *Acetate-type fermentation*



395 *Butyrate-type fermentation*

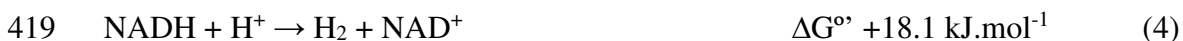


398 *Ethanol-type fermentation*

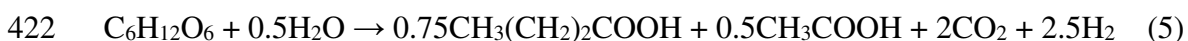


403 For acetate-type fermentation (glucose-model), the breakdown of pyruvate
 404 yields (2 moles of H₂ per mole of glucose), and an additional 2 moles of H₂ per mole of
 405 glucose is derived through Reaction 4 [54]. The reduction of hydrogenase by NADH is
 406 energetically unfavorable under standard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

417 *NADH:ferredoxin oxireductase activity*



421 *Acetate and butyrate-type fermentation using mixed culture*



424 In addition, Procentese *et al.* [58] reported that species of *Clostridium*
 425 *acetobutylicum* could be inhibited by the accumulation of acetate (26 Mm) and butyrate
 426 (34 Mm). In the present study, acetate and butyrate were in the range of the inhibitory

427 concentrations (Table 3). In fermentative systems, these acids normally accumulate in
 428 the growth medium as dead-end metabolites, since the conversion of these acids into
 429 additional H₂ is thermodynamically unfavorable. Consequently, a redirection of the
 430 cellular metabolic pathways towards solvent production is often taken. As an
 431 illustration, *Clostridium beijerinckii* strains have been reported to reconsume the
 432 produced acids at low pH, converting them into ethanol, isopropanol and butanol [59]
 433 [60]. Considering the low abundance of *Ethanoligenens* (1.2 – 4.6%), ethanol
 434 concentrations detected in the acidogenic reactors liquid outlet was attributed to
 435 solventogenesis rather than the ethanol-type fermentation thus, not being accounted in
 436 the theoretical hydrogen production (Equation 1).

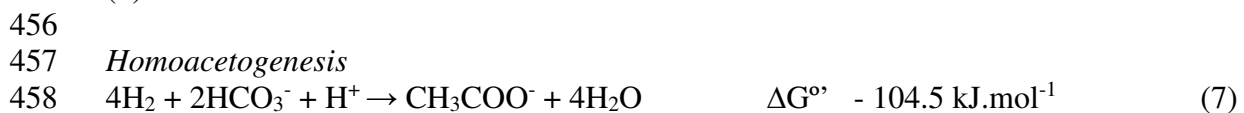
437

438 **3.5. Homoacetogenesis still occurred at low pressure**

439 In the anaerobic digestion process, hydrogenotrophic methanogenesis (Reaction
 440 6) is thermodynamically more favorable than homoacetogenesis (Reaction 7) in
 441 standard conditions [61]. Acetate evolution as sole metabolite in the liquid phase can
 442 only occur at p_{H_2} below 0.06 kPa. Homoacetogenesis is also a possible pathway that
 443 consumes hydrogen and generates acetate in anaerobic digestion, but the p_{H_2} threshold
 444 for acetate production through this pathway is 0.25 kPa at 35°C, which is high when
 445 compared to the thresholds of 0.06 kPa (hydrogenotrophic methanogenesis pathway)
 446 [14][62].

447 In this study, the p_{H_2} value at low-pressure was still 160 times higher than the
 448 p_{H_2} threshold for acetate production by homoacetogenesis, indicating that even if
 449 methanogenesis was prevented by heat-pretreatment of sludge and operating condition,
 450 homoacetogenesis could have occurred. The steady-state operation, low pH (5.5) and
 451 HRT of 6 h might also favor such reaction 7.

452



460 In an experiment at atmospheric pressure performed by Corona and Razo-Flores
 461 [44], the increase in the agitation speed from 150 to 300 rpm was implemented as a
 462 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_{H_2} of 41 kPa and 49 kPa, respectively). Consistently, when TP of
468 140 kPa (p_{H_2} 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**

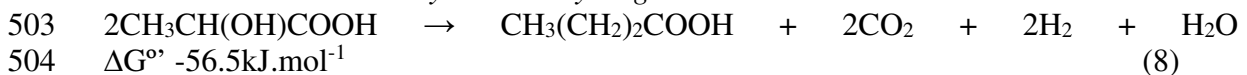
474 Lactic acid bacteria (LAB) are often detected in mesophilic hydrogen producing
475 consortia as bacteria that accompany hydrogen producers [47]. However, the real role of
476 LAB in hydrogen-producing systems and their influence on hydrogen producers are still
477 unclear.

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.

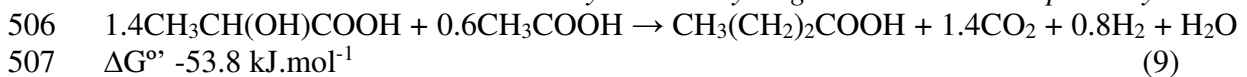
488 Corroborating to the positive role of LAB in dark fermentation process, several
489 clostridia have also demonstrated the ability to ferment lactate. *Clostridium*
490 *propionicum* uses the acrylate pathway to metabolize lactate, as a sole carbon and
491 energy source [68]. *Clostridium acetobutylicum* cultures metabolize lactate in corn steep
492 liquor [69]. *Clostridium beijerinckii* [70] and *Clostridium tyrobutyricum* [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.

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

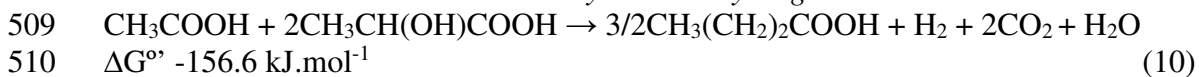
502 *Lactate conversion to butyrate and hydrogen*



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



508 *Lactate and acetate conversion to butyrate and hydrogen*



511 More recently, hydrogen and butyrate were produced from a mixture of acetate
 512 (50.8 mM) and lactate (33.3 mM) using *Clostridium diolis* JPCC H-3. A molar ratio of
 513 consumption of acetate to lactate was 1:2 and the very favorable Gibbs free energy of
 514 the reaction (Reaction 10) strongly suggests that this reaction would have proceeded
 515 [74]. Interestingly, in this study, *Sporolactobacillus* species were the most abundant
 516 microorganisms in all reactors and conditions while lactate was the second most
 517 abundant organic acids detected in the acidogenic reactor liquid outlet. As previously
 518 mentioned, a moderate relationship was found between butyrate and lactate yields
 519 indicating a direct interaction within these two metabolic intermediates (Figure 5).

520 Considering the actual concentrations of intermediates, butyrate and H₂
 521 synthesis from lactate and acetate is favorable (Table 5). Therefore, the consumption of
 522 lactate using acetate as co-substrate was suggested to be an additional pathway to
 523 produce H₂ under the evaluated conditions.

524

525 **[Table 5 – Please here]**
 526 **References linked to Table 5: [75]**

527

528 **4. Conclusions**

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

531 H₂ . mol⁻¹_{suc eq.}) achieved was obtained under a low total pressure of 80 kPa.
532 Interestingly, the composition of the microbial community did not change with the
533 increase and /or decrease of the total pressure. Acetate from homoacetogenesis was
534 accounted even at low pressure conditions. In addition, observations suggest that
535 lactate-type fermentation might play a key role in dark fermentation and might be more
536 considered as additional pathway to produce hydrogen.

537

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539

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547

548 **Compliance with ethical standards**

549

550 *Conflict of interest*

551 *The authors declare that they have no conflict of interest.*

552

553 *Ethical statement*

554 *The authors confirm that the article does not contain any studies with human*
555 *participants or animals.*

556

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827 **Figure captions**

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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 H₂ 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-H₂ h⁻¹) and concentration
843 of hydrogen dissolved in the liquid medium ([H₂]_{Liq.}) ratio. B. Hydrogen yield (HY),
844 mol-H₂ mol⁻¹_{suc eq.} and concentration of hydrogen dissolved in the liquid medium ([H₂]
845 Liq.) ratio.

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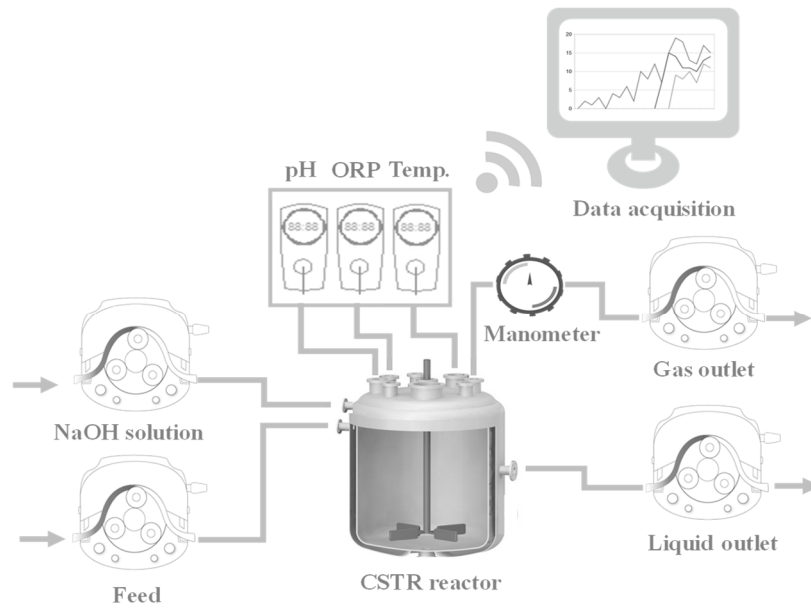
847 **Figure 4.** A. Composition of microbial community of hydrogen-producing systems at
848 different total pressure (TP, kPa) fed with a mineral salts-sugarcane molasses solution.
849 B. Correlation between *Clostridium*, *Ethanoligenens* and *Sporolactobacillus* species
850 within TP (kPa).

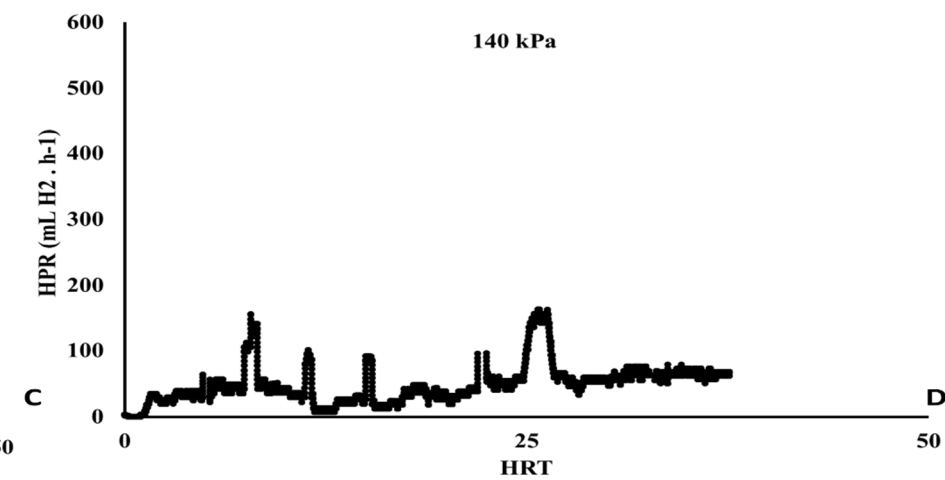
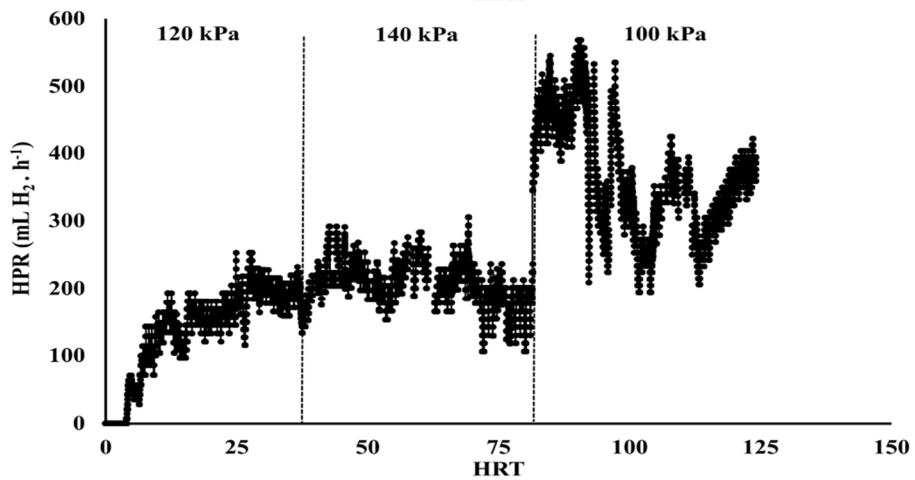
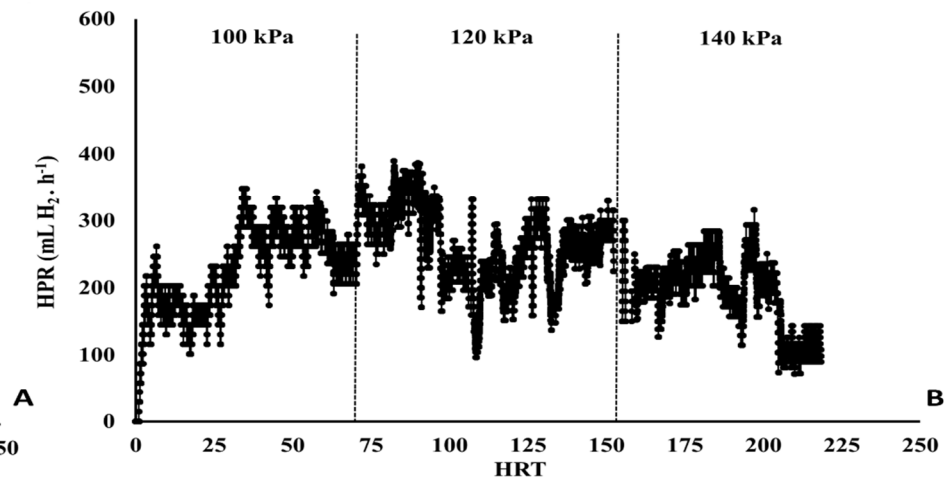
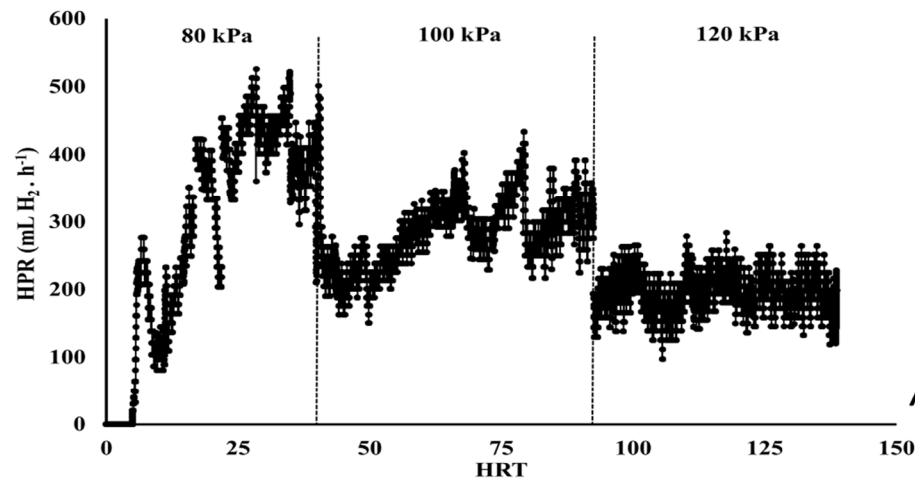
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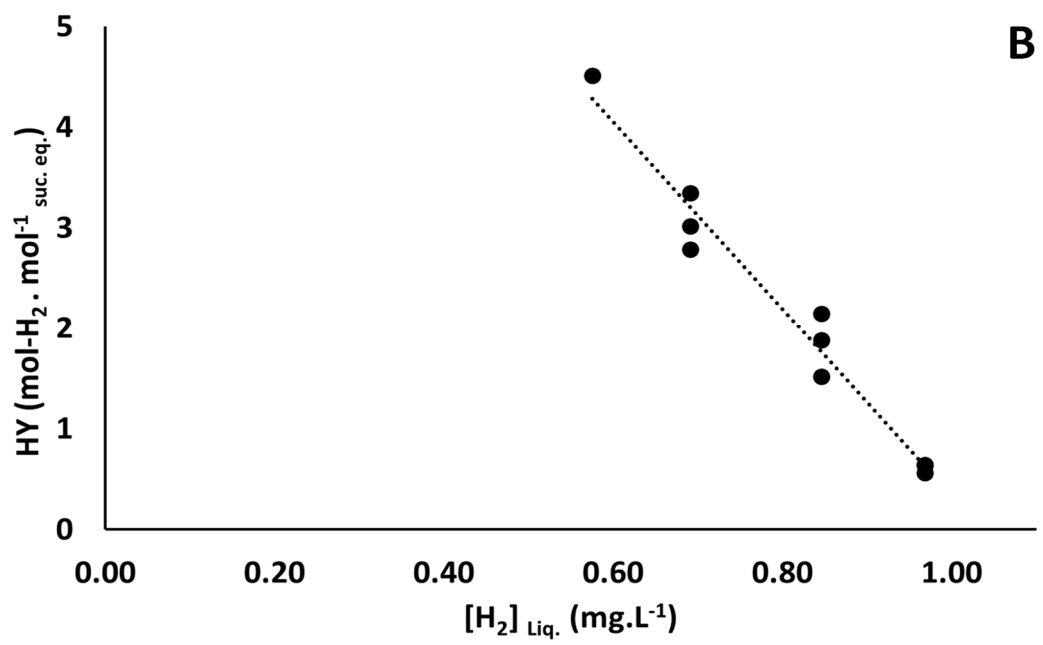
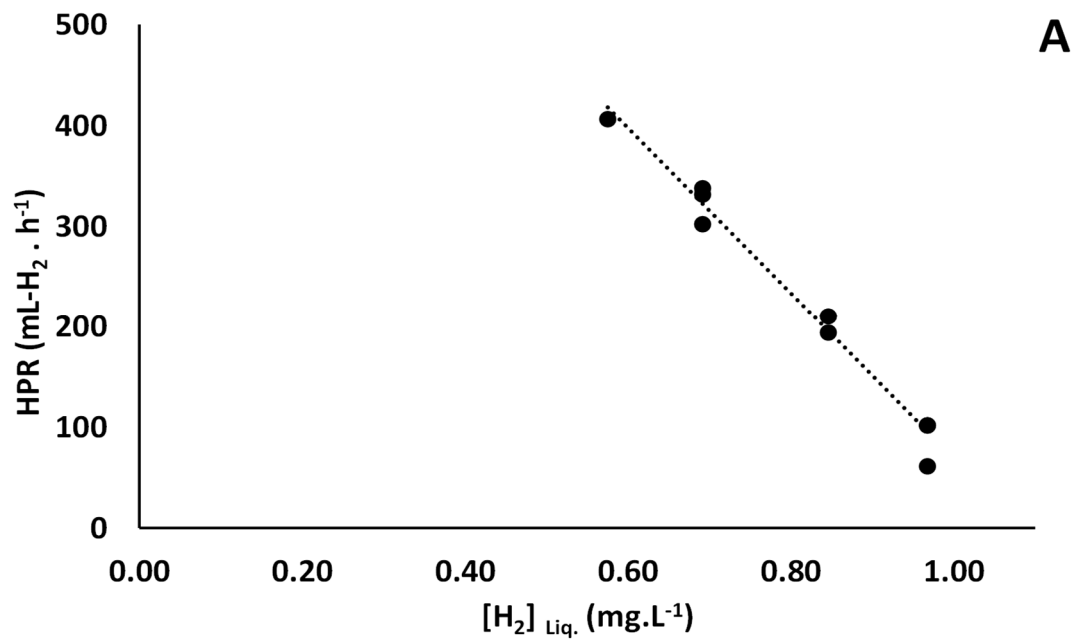
852 **Figure 5.** Principal components analysis of hydrogen-producing systems at different
853 total pressure (TP, kPa) fed with a mineral salts-sugarcane molasses solution.

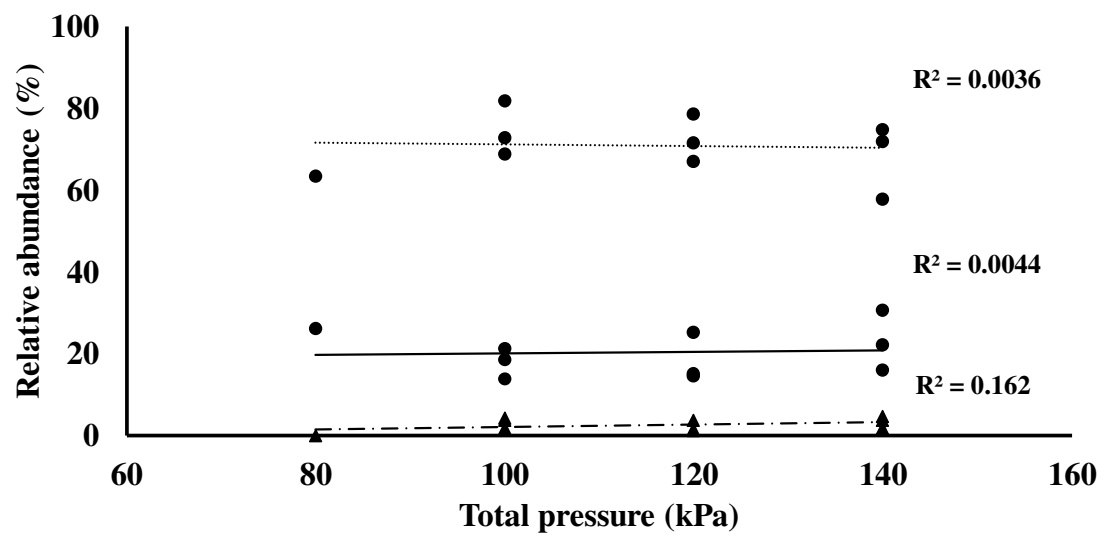
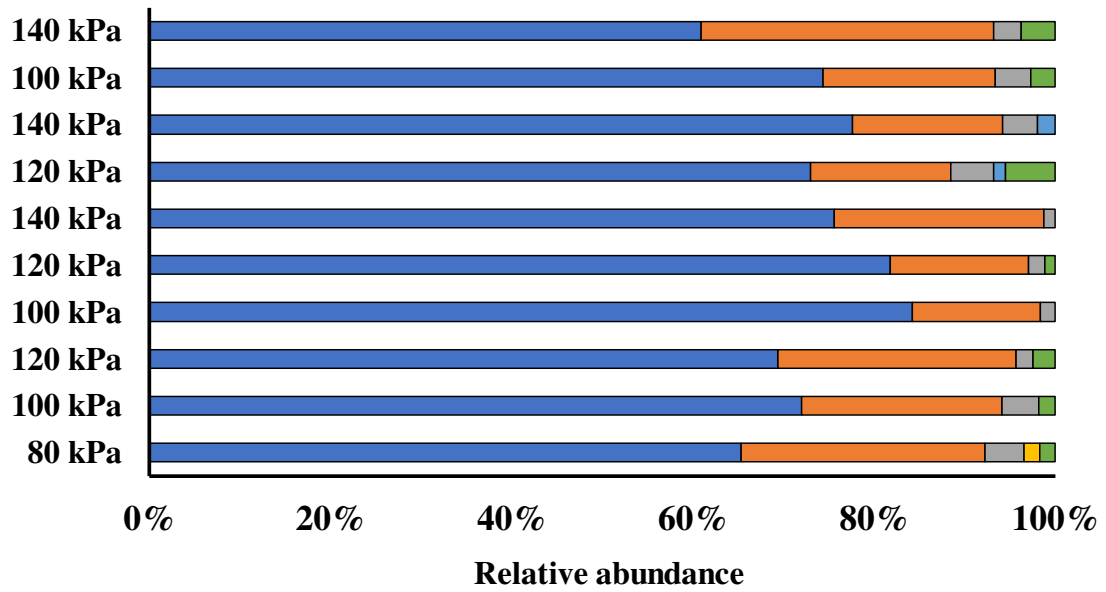
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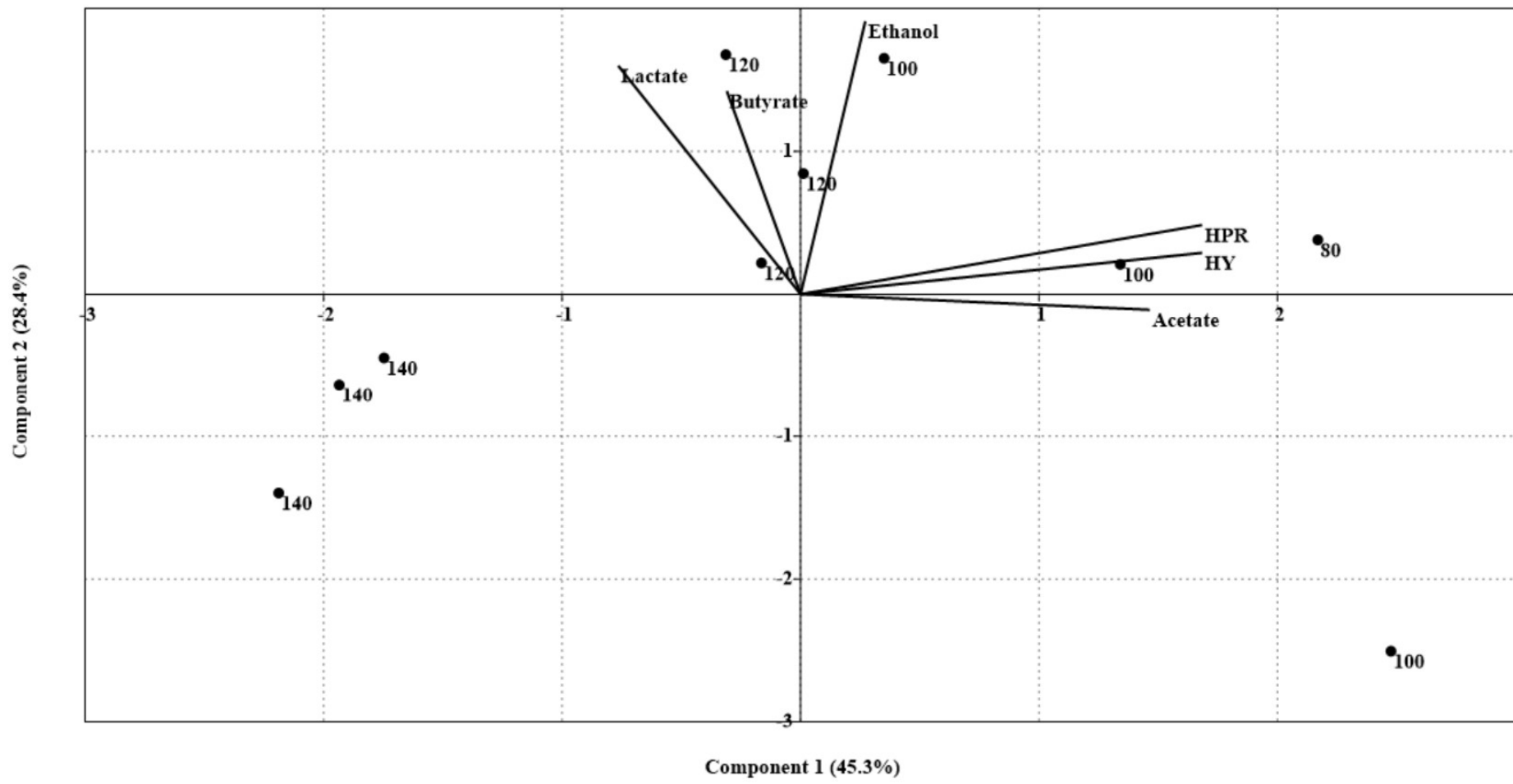


Table 1. Experimental design and different total pressure (kPa) applied to the hydrogen-producing systems fed with a mineral salts-sugarcane molasses solution.

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-H₂ h⁻¹) and hydrogen yield ((HY), mol-H₂ 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_{H_2} ; 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 (%)
R1	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
	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
R2	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
	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
R3	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
	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_{H_2}).

Controlling method of p_{H_2}	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° ; 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

ΔG° 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].