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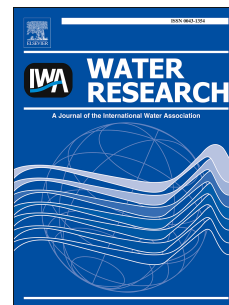
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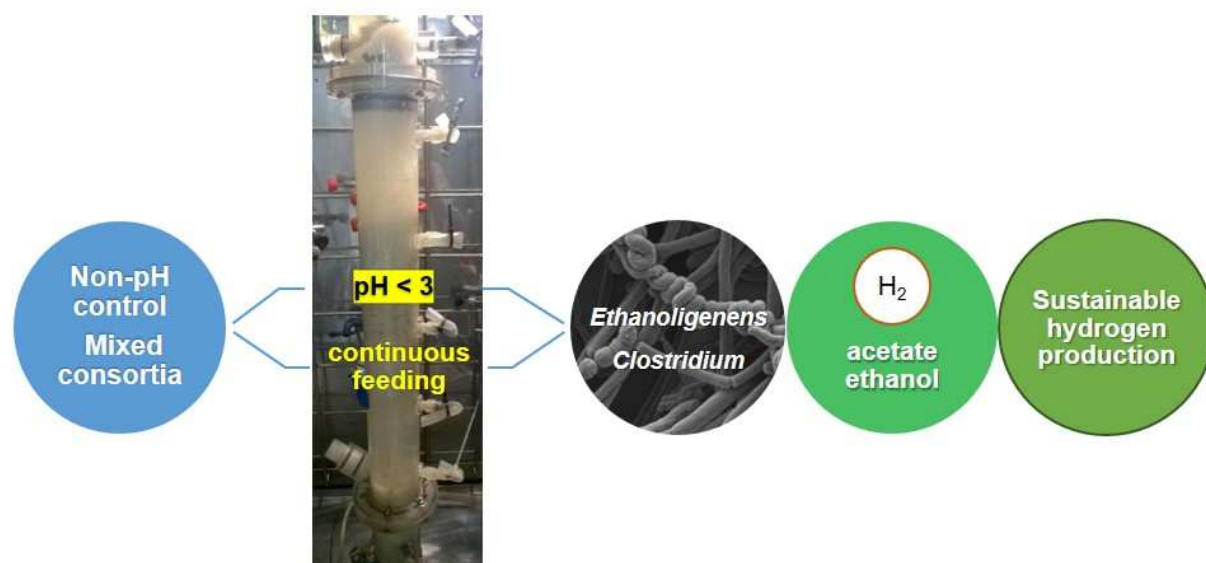


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2

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11

12 Abstract

13

14 Biological hydrogen production was investigated in continuous acidogenic reactors fed with sucrose
15 at 30°C without pH control. In the first experimental phase, three reactors were compared: a
16 structured fixed-bed (FB), a granular UASB (UG) and a flocculent UASB (UF-1). They were run at
17 3.3 h HRT and 33 gCOD L⁻¹d⁻¹ OLR. Hydrogen production occurred throughout the experimental
18 period with an average effluent pH of only 2.8. The FB, UG and UF-1 reactors presented volumetric
19 hydrogen production rates (VHPR) of 95 ± 69, 45 ± 37 and 54 ± 32 mLH₂ L⁻¹h⁻¹, respectively; and H₂
20 yields (HY) of 1.5 ± 0.8, 0.8 ± 0.6 and 1.2 ± 0.7 molH₂ mol⁻¹ sucrose_{consumed}, respectively. The UF-1
21 reactor showed intermediate VHPR and HY, but no declining trend, contrary to what was observed in
22 the FB reactor. Thus, aiming at continuous and long-term H₂ production, a flocculent UASB was
23 applied in the second experimental phase. In this phase, the HRT of the acidogenic reactor, which was
24 named UF-2, was raised to 4.6 h, resulting in an OLR of 25 gCOD L⁻¹d⁻¹. The VHPR and the HY
25 increased considerably to 175 ± 44 mLH₂ L⁻¹h⁻¹ and 3.4 ± 0.7 molH₂ mol⁻¹ sucrose_{consumed},
26 respectively. These improvements were accompanied by greater sucrose removal, higher suspended
27 biomass concentration, less production of lactate and more of acetate, and high ethanol concentration.

28 Contradicting the current published literature data that reports strong inhibition of H₂ production by
29 dark fermentation at pH less than 4.0, the UF-2 reactor presented stable, long-term H₂ production with
30 satisfactory yields at pH 2.7 on average. 16S rDNA sequencing revealed that two sequences assigned
31 as *Ethanoligenens* and *Clostridium* accounted for over 70% of the microbiota in all the reactors. The
32 non-necessity of adding alkalizing agents and the successful H₂ production under very acid
33 conditions, demonstrated in this study, open a new field of investigation in biological hydrogen
34 production by dark fermentation towards a more sustainable and feasible technology.

35

36 **Keywords:** acidogenic reactor; acid-tolerant bacteria; biohydrogen; dark fermentation; hydrogen; pH

37

38 1. Introduction

39

40 In recent years, more attention has been given to the potential for hydrogen production by dark
41 fermentation (DF). Hydrogen is produced concomitantly with volatile fatty acids (VFA) through
42 acidogenesis during anaerobic treatment, and its recovery is a way of extracting additional energy in
43 wastewater treatment plants. The technology is still evolving and stable, long-term H₂ production is
44 challenging due to changes in bacterial metabolic pathways and the concomitant existence of H₂-
45 producing and H₂-consuming microorganisms inside the acidogenic reactors. Current efforts are
46 towards optimization of the operating parameters (e.g. reactor designs, environmental conditions,
47 bacterial consortia, substrates) in order to achieve a sustainable H₂ net production.

48

49 In the DF processes, no more than 4 mol of H₂ per mol of hexose is attainable due to the production of
50 products other than gas. The foregoing notwithstanding, usual H₂ yields are lower, due to the
51 utilization of the substrate in a variety of pathways that produce less or no H₂ and for biomass growth,
52 also due to microbial H₂ consumption.

53

54 Environmental pH plays a crucial role in hydrogen yields. A neutral pH, besides being onerous to
55 maintain, can favour methanogen growth and be detrimental to the achievement of phase separation.

56 On the other hand, pH values less than 4.5 lead to changes in the metabolic pathways, towards the
57 production of compounds more reduced than the VFA (solvents such as acetone and alcohols, and
58 lactic acid) (Bahl *et al.*, 1982; Lay, 2000; Mizuno *et al.*, 2000; Kim *et al.*, 2004); increased
59 concentrations of undissociated forms of organic acids, which affect microbial growth (Dabrock *et al.*,
60 1992; Yokoi *et al.* 1995; Chen *et al.* 2005; Ruggeri *et al.*, 2015); possible inhibition of hydrogenase
61 activity (Micolucci *et al.*, 2014; Ghimire *et al.*, 2015; Ruggeri *et al.*, 2015; Roy and Das, 2016) as
62 well as ferredoxin's capacity to donate electrons for the protons (Ruggeri *et al.*, 2015). In general, the
63 desirable pH for hydrogen-producing reactors ranges from 4.5 to 6.5. However, even in this pH range,
64 H₂-consuming microorganisms such as homoacetogenic and H₂-oxidizing methanogens can be found
65 (Lee *et al.*, 2010).

66

67 The main drawback to controlling the pH in acidogenic reactors lies in the increased costs. Due to the
68 constant CO₂ and acid production, the addition of alkalis to the reactors is usually needed. Ghimire *et*
69 *al.* (2015) state that the use of an excessive amount of pH regulators can decrease the economics and
70 sustainability of the process, as well as increase the salt concentration of the DF effluents.

71

72 The capacity of acid-tolerant facultative or anaerobic bacteria to produce H₂ under extremely acid
73 conditions (pH<3.5) has not yet been investigated in acidogenic reactors, but only in other
74 environments. In the study by Noguchi *et al.* (2010), it was found that live cultures of *Escherichia coli*
75 survived at external pH values of 2.5 and 2.0 due to the activity of the [NiFe]-hydrogenase Hyd-3.

76 The reduction of H⁺ into H₂ to control the internal pH in extremely acidic environments such as the
77 stomach is a strategy also reported for *Helicobacter pylori* (Bhattacharyya *et al.*, 2000). The capacity to
78 grow in very acid environments has been demonstrated for other H₂-producing bacteria, such as

79 *Sarcina ventriculi* and *Clostridium acidisoli*. *S. ventriculi* is a bacterium found in various

80 environments (soil, mud, rabbit and guinea pig stomach contents, elephant dung, human feces and the
81 surface of cereal seeds) and can grow at pH of 2.0-2.5 (Canale-Parola, 1986). However, Goodwin and

82 Zeikus (1987) found that its metabolism shifted from H₂-acetate to ethanol production when the pH

83 decreased from 7.0 to 3.0. Kuhner *et al.* (2000) first isolated *Clostridium akagii* and *Clostridium*

84 *acidisoli* from acid soils (pH ~3.0) and cultured them at pH 3.7-7.1 and 3.6-6.9, respectively. Their
85 capacity to produce H₂ from carbohydrates was demonstrated at pH 5.5 and 6.8, but it was not
86 assayed for other pH values.

87
88 Bearing in mind that the application of DF for H₂ recovery is only feasible if the environmental
89 balance is beneficial and the economic costs are kept to a minimum, and, that there is a potential for
90 H₂ production by acid-tolerant bacteria, the acidogenic reactors were run without addition of pH
91 regulators in the present study. As reactor design and the biomass retention mechanism (biofilm, flocs
92 or granules) affect the biological dynamics, and thus net hydrogen production, different
93 configurations of reactors were evaluated.

94

95 **2. Material and methods**

96

97 2.1. Reactor configurations and inoculum

98

99 An up-flow structured fixed-bed reactor, a granular UASB reactor and a flocculent UASB reactor
100 were used. The reactors were made of acrylic, having internal diameters of 6.3 cm, and with total and
101 working volumes of approx. 2.5 and 2.2 L, respectively (Figure 1). The source of inoculum was
102 granular sludge from a single stage UASB reactor treating poultry slaughterhouse wastewater
103 (Pereiras, São Paulo, Brazil). The granules were completely disrupted with a blender prior to
104 inoculating the structured fixed-bed and flocculent UASB reactors. The structured fixed-bed reactor
105 design (Picanço *et al.*, 2001), as an alternative to the packed-bed reactor, prevents channelling and
106 clogging. Polyethylene cylinders were chosen as the support material in the structured fixed-bed
107 reactor (porosity = 82%), as Ferraz Júnior *et al.* (2015) found that the reactor filled with polyethylene
108 obtained higher H₂ production and yield, also greater abundance of H₂-producing bacteria, as
109 compared to the reactors filled with expanded clay, coal and porous ceramics.

110

111 **Figure 1** – Schematic diagram of the acidogenic reactors. 1: distribution chamber, 2: reactional zone, 3:
112 headspace, 4: biogas sampling, 5: biogas outlet

113
114 The initial concentration of total volatile solids (TVS) was 15 g/l. No sludge pretreatment was used.
115 This allows the survival of non-spore forming H₂-producers and makes the inoculation more practical
116 and viable.

117

118 2.2. Substrate

119

120 The reactors were fed with sucrose-based wastewater composed of demerara sugar (Native®) and a
121 nutrient's solution in the following concentrations (mg L⁻¹): demerara sugar (4450), NH₄Cl (170),
122 CaCl₂·2H₂O (8), KH₂PO₄ (37), MgSO₄·4H₂O (9), FeCl₃·4H₂O (2), CoCl₂·6H₂O (2), MnCl₂·4H₂O
123 (0.5), CuCl₂·2H₂O (0.03), ZnCl₂ (0.05), H₃BO₃ (0.05), (NH₄)₆Mo₇O₂₄·4H₂O (0.09), Na₂SeO₃·5H₂O
124 (0.1), NiCl₂·6H₂O (0.05), EDTA (1), HCl 36% (1 μL L⁻¹).

125

126 2.3. Operating conditions

127

128 In the first experimental phase, in which different reactors were evaluated (Table 1), the mean
129 hydraulic retention time (HRT) was 3.3 h. This corresponded to an organic loading rate (OLR) of 33.1
130 gCOD L⁻¹d⁻¹.

131

132 One configuration was chosen to be applied in the next experimental phase, in order to keep the
133 investigation on continuous hydrogen production. In this phase, a different start-up was applied: after
134 inoculation, the reactor was operated at HRT in the 2.8-6.1 h range for 80 days. It was verified that
135 higher hydrogen production was obtained at HRT between 4 and 5 h (data not shown). Thereafter, the
136 HRT was adjusted to 4.6 h in Phase 2. This corresponded to an OLR of 25.0 gCOD L⁻¹d⁻¹.

137

138 According to the design and/or inoculum structure and to the experimental phase, the reactors were
 139 named as follows: (i) structured fixed-bed reactor: FB; (ii) granular UASB reactor: UG; (iii)
 140 flocculent UASB reactors applied in experimental phases 1 and 2: UF-1 and UF-2, respectively (Table
 141 1).

142

143 **Table 1** – Reactor configurations and operating conditions

Reactor	Reactor design	Inoculum structure	Biomass retention	Experimental phase	HRT - h	OLR - gCOD L ⁻¹ d ⁻¹
FB	Structured fixed-bed	Disaggregated granules	Biofilm and flocs	1	3.3	33.1
UG	UASB	Intact granules	Granules	1	3.3	33.1
UF-1	UASB	Disaggregated granules	Flocs	1	3.3	33.1
UF-2	UASB	Disaggregated granules	Flocs	2	4.6	25.0

144

145 The reactors were fed continuously and the temperature was maintained at 30±2 °C. The influent pH
 146 was naturally neutral, 6.5 on average, and the pH in the reactors was not controlled.

147

148 2.4. Analyses

149

150 The biogas flow rate was measured using Milligas counter gas meters (Ritter®). The composition, in
 151 terms of H₂, CH₄ and CO₂, was analysed using Shimadzu GC-2010 gas chromatograph with the
 152 following specifications: thermal conductivity detector; argon as carrier gas; Carboxen 1010 capillary
 153 column; initial detector and injector temperatures of 200 and 230 °C, respectively; oven temperature
 154 of 130-135 °C; flow rate of 12 mL min⁻¹; and, sample volume of 300 µL.

155

156 Sucrose (glucose and fructose) and organic acids (lactic, formic, acetic, propionic, isobutyric, butyric,
 157 isovaleric, valeric) were determined using Shimadzu System UV/DAD (210 nm) high performance
 158 liquid chromatography (HPLC) with Refractive Index (in series) detectors, Aminex HPX-87H
 159 column, 0.005M H₂SO₄ solution as eluent, flow of 0.5 mL min⁻¹, oven temperature of 43 °C, and 100

160 μL of sample injection. Ethanol was determined using Shimadzu GC-2010 gas chromatograph with a
161 flame ionization detector (FID), flow of 1.5 mL min^{-1} with ultra-pure hydrogen as the carrier gas,
162 injector and detector temperature of $250 \text{ }^\circ\text{C}$ and $280 \text{ }^\circ\text{C}$, respectively.

163
164 Total COD of the affluent, soluble COD of the effluent (filtered in $1.2 \mu\text{m}$ membrane) and volatile
165 suspended solids (VSS) concentration in the effluent were analysed according to APHA *et al.* (2005).
166 The pH was measured using a pHmeter (Hach equipment).

167
168 Statistical analyses were done using Statistica 13 software. Normal distribution of the results was
169 checked using the Shapiro-Wilk test before applying the other tests. The 95% confidence level was
170 adopted for all tests.

171
172 2.5. Theoretical calculations of the percentage of acidified sucrose and of hydrogen yield, by the
173 different metabolic routes

174
175 The simplified stoichiometric equations (Equations 1, 2, 3, 4, 5) were used to calculate the molar ratio
176 between sucrose consumed and acids produced ($[\text{sucrose}]/[\text{acid}]$) and between hydrogen gas and acids
177 produced ($[\text{H}_2]/[\text{acid}]$). These equations show calculated acidified sucrose (in mmol L^{-1}) = S (in mmol
178 $\text{sucrose mmol}^{-1} \text{ acid}$) x acid concentration (in mmol acid). It is shown that $S = 0.25$ via lactate, 0.25
179 via acetate and/or formate, 0.25 via propionate, 0.50 via butyrate, and 0.50 via valerate. The
180 percentage of acidified sucrose for each acid is its respective calculated acidified sucrose divided by
181 the total calculated acidified sucrose.

182
183 To determine the HY percentage, the maximum yield or consumption by each route was calculated, as
184 follows: theoretical HY (in $\text{mmol H}_2 \text{ mmol}^{-1} \text{ sucrose}_{\text{consumed}}$) = H (in $\text{mmol H}_2 \text{ mmol}^{-1} \text{ acid}$) x acid
185 yield (in $\text{mmol acid mmol}^{-1} \text{ sucrose}_{\text{consumed}}$). It is shown that $H = 0$ via lactate, 2 via acetate and/or
186 formate, -1 via propionate, 2 via butyrate, and -1 via valerate. The HY percentage is the theoretical

187 HY from each acid divided by the sum of the theoretical HY from acetate and/or formate and
188 butyrate.

189

190 Via lactate: $1 \text{ C}_{12}\text{H}_{22}\text{O}_{11} + 1 \text{ H}_2\text{O} = 4 \text{ CH}_3\text{CH}(\text{OH})\text{COO}^- + 4 \text{ H}^+$ (Eq. 1)

191

192 Via acetate and/or formate*: $1 \text{ C}_{12}\text{H}_{22}\text{O}_{11} + 5 \text{ H}_2\text{O} = 4 \text{ CH}_3\text{COO}^- + 4 \text{ H}^+ + 4 \text{ CO}_2 + 8 \text{ H}_2$ (Eq. 2)

193 *In the mixed-acid fermentation, Equation 2 derives from the sum of the reaction of acetate and
194 formate formation ($1 \text{ C}_{12}\text{H}_{22}\text{O}_{11} + 5 \text{ H}_2\text{O} = 4 \text{ CHOO}^- + 4 \text{ CH}_3\text{COO}^- + 8 \text{ H}^+ + 4 \text{ H}_2$) followed by the
195 reaction of formate cleavage ($4 \text{ CHOO}^- + 4 \text{ H}^+ = 4 \text{ CO}_2 + 4 \text{ H}_2$). Since [formate] ~ 0 , only [acetate]
196 was included in the calculations.

197

198 Via propionate: $1 \text{ C}_{12}\text{H}_{22}\text{O}_{11} + 4 \text{ H}_2 = 4 \text{ CH}_3\text{CH}_2\text{COO}^- + 4 \text{ H}^+ + 3 \text{ H}_2\text{O}$ (Eq. 3)

199

200 Via butyrate: $1 \text{ C}_{12}\text{H}_{22}\text{O}_{11} + \text{H}_2\text{O} = 2 \text{ CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2 \text{ H}^+ + 4 \text{ CO}_2 + 4 \text{ H}_2$ (Eq. 4)

201

202 Via valerate: $1 \text{ C}_{12}\text{H}_{22}\text{O}_{11} + 2 \text{ H}_2 = 2 \text{ CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}^- + 2 \text{ H}^+ + 2 \text{ CO}_2 + 3 \text{ H}_2\text{O}$ (Eq. 5)

203

204 2.6 Molecular analysis

205 Biomass were collected from different heights from the FB, UG, UF-1 and UF-2 reactors by the end
206 of operation. Cells were separated by centrifugation (6000 g, 10 min, 4 °C). Genomic DNA was
207 extracted and purified using the protocol of Griffiths et al. (2000). The amount and purity of DNA in
208 the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan). The extracted
209 DNA was stored at -20 °C until further use. The 16S rDNA gene V4-5 region was amplified with the
210 forward primer CTTTCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA and the
211 reverse primer GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTRAGT plus
212 the respective linkers over 30 amplification cycles at an annealing temperature of 65 °C. In a second
213 PCR reactor of 12 cycles, an index sequence was added. The resulting PCR products were purified
214 and loaded onto the Illumina MiSeq cartridge for sequencing of paired 375-380 bp reads. Sequencing-

215 related work was done at the GeT PlaGe sequencing center of the genotoul life science network in
216 Toulouse, France (get.genotoul.fr). Forward and reverse sequences were retained after assembly and
217 quality checking using a slightly modified version of the Standard Operation Procedure for MiSeq
218 data by Kozich et al. (2013) in Mothur version 1.35.0 (Schloss et al., 2009). SILVA SSU Ref NR 99,
219 release 128, was used for alignment and as taxonomic outline (Pruesse et al., 2012). The sequences
220 found in this study were submitted to the GenBank (accession numbers MF612196-MF613645). For
221 the construction of a phylogenetic tree, the most abundant sequences found in the reactors were then
222 compared with the available sequences in the GenBank database using the BLAST program (Altschul
223 et al., 1990). Phylogenetic analyses of the sequences were performed using the Molecular
224 Evolutionary Genetic Analysis (MEGA7) software (Kumar et al., 2016). Evolutionary distances were
225 based on the Kimura model (Kimura, 1980) and tree reconstruction on the Neighbor-Joining method
226 with bootstrap values calculated from 500 replicate runs.

227

228 **3. Results and Discussion**

229

230 3.1. Volumetric hydrogen production rate and biogas composition

231

232 Figure 2 shows the volumetric hydrogen production rate (VHPR) and effluent pH of the FB, UG and
233 UF-1 reactors. The mean effluent pH values were 2.8, 2.8 and 2.9 in the FB, UG and UF-1 reactors,
234 respectively. As no buffers, acids or bases were added, pH reduction resulted from the production of
235 organic acids and carbon dioxide. Despite the low pH, hydrogen production occurred throughout the
236 experimental period.

237

238 **Figure 2** – Volumetric hydrogen production rate and pH in the first experimental phase: (a) FB reactor, (b) UG
239 reactor, (c) UF-1 reactor.

240

241 Punctual increases in pH, accompanied by reduction of sucrose removal, organic acid and H₂
242 production, were observed. This was more noticeable in the UF-1 reactor, when pH values were

243 above 3.4 on days 30, 80 and 133 (Figure 2). The lowest organic acid concentrations in effluent were
244 also reported on these days and sucrose removal efficiency was null on days 80 and 133. On days 23,
245 29, 59, 60, 79 and 130, there were feeding problems in the UF-1 reactor due to clogging of tubes. It
246 was likely that feeding reduction or interruption led to biomass decay, as could be inferred by the
247 lower visible turbidity of the medium and reduction in effluent VSS concentrations after these events.
248 Nevertheless, pH above 3.0 accompanied by a drastically reduced sucrose conversion, was also
249 observed in the UG reactor notably on days 32, 85 and 136, and in the FB reactor on day 85.
250 Consequently, effluent pH was increased due to dilution of medium with non-consumed affluent.

251
252 The VHPR were equivalent to: $95 \pm 69 \text{ mLH}_2 \text{ L}^{-1}\text{h}^{-1}$ in the FB reactor, $45 \pm 37 \text{ mLH}_2 \text{ L}^{-1}\text{h}^{-1}$ in the UG
253 reactor, and $54 \pm 32 \text{ mLH}_2 \text{ L}^{-1}\text{h}^{-1}$ in the UF-1 reactor. The non-parametric Kruskal-Wallis ANOVA
254 by Ranks test showed statistically significant differences regarding H_2 production (p -value = 0.006).
255 Further analysis of multiple comparisons of mean ranks for all groups showed that H_2 production in
256 reactors UG and UF-1 was not significantly different (p -value = 0.575). As shown in Figure 2,
257 although the FB reactor achieved the highest VHPR at the beginning of the operation, it tended to
258 decrease during the experimental period. This did not occur in the UG and UF-1 reactors. The UF-1
259 reactor showed superior stability during the entire period of operation, as indicated by its VHPR data.
260 These were the only data that presented normal distribution.

261
262 A possible explanation for the higher initial VHPR in the FB reactor could be the lower biomass
263 wash-out, owing to the presence of the support material. Biomass was observed to be "trapped" in the
264 polyethylene cylinders, although it did not form a thick biofilm. The flocs formed in the sludge bed at
265 the bottom of the FB reactor were visually larger than those from the UF-1 reactor. This was probably
266 due to the shear stress and physical selection caused by the support material, which retained larger
267 particles, while the smaller ones passed easily through the pores. Low interspecies distances are a key
268 point of efficient interspecies hydrogen transfer between acetogenic bacteria and hydrogenotrophic
269 methanogenic archaea in anaerobic aggregates, biofilms and granules (MacLeod et al., 1990; Davey
270 and O'toole, 2000; Hulshoff Pol et al., 2004; Felchner-Zwirello et al. 2013). As stated by Dinamarca

271 et al. (2011), this mechanism also can play a relevant role in non-methanogenic mixed cultures,
272 through hydrogen transfer between hydrogen producers and consumers, limiting sustainable hydrogen
273 production due to homoacetogenesis. Therefore, in the present study, the biomass agglutination in
274 larger flocs, granules and biofilm could have had an adverse effect on long-term H₂. Acetate
275 formation was observed to increase from day 77 in the FB reactor. However, this was not followed by
276 an increase in hydrogen production, which can be an indicator of homoacetogenic activity. Penteado
277 et al. (2013) studied seven structured fixed-bed reactors having different sources of inoculum, fed
278 with sucrose. VHPR decreased over time in all reactors, and it was observed that HY decreased as the
279 percentage of acetic acid produced by homoacetogenesis increased. In addition, the filling in the FB
280 and UG reactors with support material and granules, respectively, may have hindered the escape of
281 the produced biogas, increasing the H₂ partial pressure in the medium, which inhibits its own
282 production (Sikora *et al.*, 2013).

283
284 The granules inside the UG reactor were originally dark colored with an average diameter of 2.1 mm.
285 They became whitened and smaller, with an average diameter of 1.5 mm by the end of operation.
286 Floc formation and suspended biomass growth were also observed. On the 135th day of operation, the
287 UG reactor lost most of its biomass due to a remarkable wash-out of the granules. The granule
288 flotation likely occurred due to the adherence of gas bubbles to their surfaces, and reduction of their
289 inner densities. This assumption is based on the fact that the environmental conditions were not
290 favourable for the maintenance of the methanogenic microorganisms, leading to biomass decay in the
291 inner layers of the granules. Then, in the outer layers, the granules became most colonized by
292 acidogenic bacteria that survived in the acid environment. The increasing substitution of mixed-
293 consortia granules by specific acidogenic bacteria inside the UG reactor probably had a positive effect
294 on H₂ production, as indicated by the increased VHPR at the end of operation (Figure 2).

295
296 In the UF-1 reactor, it is probable that faster biomass decay and washing-out occurred at the
297 beginning of the operation due to the larger contact surface of the biomass with the medium and the
298 initial absence of a biomass retention mechanism. This may be the reason that H₂ production started

299 later in this reactor and with less intensity. Reyes *et al.* (2012) also observed a delay in H₂ production
300 in the reactors inoculated with disintegrated granules. This production started after about 40-70 hours
301 of continuous operation, compared to the reactors inoculated with intact granules, in which H₂
302 production started within the first 12 hours. On the other hand, the selection of bacteria resistant to
303 the adverse conditions (low pH and high organic acids concentration) as well as the increasing
304 biomass concentration due to the self-flocculation phenomenon provided a superior stability to the
305 UF-1 reactor. The higher selectivity of the desired bacteria from the disaggregated granules was
306 verified by Reyes *et al.* (2012), who found that this form of inoculation resulted in greater specific
307 hydrogenogenic activity compared to that from intact granules.

308

309 The effluent VSS concentrations were (in mg L⁻¹): 89.9 ± 68.4 , 101.7 ± 95.9 and 90.9 ± 63.4 in the FB,
310 UG and UF-1 reactors, respectively. In each reactor, the effluent VSS concentration correlated
311 positively with the VHPR according to the Spearman non-parametric test ($\alpha = 5\%$). The values of the
312 R correlation coefficients were 0.45 ($p = 0.0063$), 0.56 ($p = 0.0014$) and 0.40 ($p = 0.0198$) for the FB,
313 UG and UF-1 reactors, respectively.

314

315 For the application in the second experimental phase, the UG reactor was considered less
316 advantageous as it had the lowest VHPR and HY. The FB reactor, however, showed the highest
317 VHPR and HY mean values, although with a tendency toward performance decrease over time. The
318 UF-1 reactor showed intermediate VHPR and HY values, but no declining trend was observed. From
319 the 80th day of operation, the H₂ yield in the UF-1 reactor also showed progressive improvement,
320 contrasted to the FB reactor. Thus, aiming at continuous and long-term H₂ production, this
321 configuration seemed to be the most adequate among those studied. It is also pertinent that the
322 flocculated UASB reactor design has the greatest potential to use the entire reactor volume to be
323 filled with active biomass, thus maximizing the reactor space utility and virtually increasing cell
324 density in the reactor, without formation of close microbial associations such as biofilms and
325 granules. For these reasons, this configuration was chosen for the second experimental phase.

326

327 The flocculated UASB applied in Phase 2 was identified as the UF-2 reactor and was operated at a
328 higher HRT (4.6 h) and lower OLR (25.0 gCOD L⁻¹d⁻¹). It obtained constant and stable H₂
329 production, and achieved significant improvement over the previous experimental phase (Figure 3).
330 The VHPR was very satisfactory, corresponding to 175 ± 44 mLH₂ L⁻¹h⁻¹. Continuous acid and CO₂
331 production in the reactor led to strong acidification of the effluent, and pH was self-adjusted to values
332 consistently less than 3.0, with an average value of 2.7 (Figure 3). The H₂ production in the UF-2
333 reactor presented normal distribution and was statistically higher, according to Kolmogorov-Smirnov
334 test (p-value < 0.001), compared to the other reactors.

335

336 **Figure 3** – Volumetric hydrogen production rate and pH in the second experimental phase: UF-2 reactor

337

338 The growth of suspended biomass was much more noticeable than in the reactors during Phase 1,
339 achieving an effluent concentration of 295 ± 275 mgVSS L⁻¹. There was also a significant correlation
340 of VSS with VHPR at the 5% significance level (Spearman R = 0.33).

341

342 The biogas of the FB, UG, UF-1 and UF-2 reactors presented a H₂ content equal to (%): 59.6 ± 11.0,
343 62.1 ± 10.8, 62.2 ± 7.1 and 59.8 ± 5.9, respectively. The percentage of hydrogen in the biogas was not
344 significantly different, according to the Kruskal-Wallis ANOVA (FB, UG, UF-1 reactors) and the
345 Kolmogorov-Smirnov tests (UF-2 reactor vs. FB, UG, UF-1 reactors). Methane was not detected in
346 the biogas in any of the reactors. This leads to the assumption that the environmental conditions
347 established by the pH self-adjustment and low HRT were sufficient to completely inhibit
348 methanogenesis. Operating with extreme pH values seems to be an efficient strategy for avoiding
349 methanogenic activity, as verified by Wang et al. (2015), studying hydrogen production in waste
350 activated sludge at pH 10.

351

352 3.2. Hydrogen yield and sucrose removal

353

354 The results of sucrose removal and HY are plotted in box and whisker graphics (Figures 4 and 5), that
355 show the distribution of data into quartiles, highlighting the mean (X). The lines extending vertically
356 indicate variability outside the upper and lower quartiles.

357

358 **Figure 4** – Sucrose removal

359

360 **Figure 5** – H₂ yield

361

362 The mean sucrose removal in the UF-2 reactor was 81% while, in the FB, UG and UF-1 reactors, it
363 was 64, 67 and 56%, respectively (Figure 4). However, as the OLR applied in the UF-2 reactor was
364 less than in the other reactors, the mean volumetric sucrose removal rate was in the same range as the
365 other reactors: 2.22, 2.38, 1.91 and 2.16 mmol sucrose_{consumed} L⁻¹h⁻¹ in the FB, UG, UF-1 and UF-2
366 reactors, respectively. Thus, the substantial increase in VHPR obtained in the UF-2 reactor was
367 mainly due to the improvement in the H₂ yield. The mean HY of 1.50, 0.76 and 1.19 mol H₂ mol⁻¹
368 sucrose_{consumed} obtained in the FB, UG and UF-1 reactors, respectively, was surpassed by a level of
369 3.35 mol H₂ mol⁻¹ sucrose_{consumed} obtained in the UF-2 reactor (Figure 5). Statistical analyses revealed
370 that H₂ yield in Phase 1 differed significantly among reactors (Kruskall-Wallis ANOVA, p-value =
371 0.002); however, the difference between the FB and UF-1 reactors was not significant (multiple
372 comparisons of mean ranks, p-value = 0.413). Nevertheless, H₂ yield obtained in the UF-2 reactor was
373 statistically higher than that obtained in the FB, UG and UF-1 reactors (Kolmogorov-Smirnov test, p-
374 value<0.001).

375

376 Table 2 shows the organic acid concentrations, and the percentages of respective acidified sucrose and
377 H₂ yield. Comparing the effluent organic acid composition of the UF-2 reactor to the other reactors, it
378 was concluded that there was a shift from less lactate to more acetate production, thus accounting for
379 UF-2 reactor superior performance. Lactate production involves the consumption of NADH and
380 pyruvate, reducing the potential production of H₂ by both the NADH-pathway and, mainly, substrate
381 competition due to pyruvate consumption. On the other hand, the acetate production represented by

382 the reactions in Equation 2 is desired for both Clostridial- and Enterobacterial-type fermentation
 383 (mixed acid fermentation), as the acetate route provides the highest H₂ yield in Clostridial-type
 384 fermentation and acetate is produced along with formate in Enterobacterial-type fermentation. Since
 385 H₂ can be produced from formate cleavage, acetate indicates that the formate route took place in the
 386 mixed acid fermentation. Also, the concentrations of propionate and valerate, which are produced at
 387 the expense of H₂ consumption, were lower in the UF-2 reactor.

388

389 **Table 2** – Organic acid concentrations and the respective percentages of acidified sucrose and H₂ yield

Parameter	Reactor	lactate	formate	acetate	propionate	butyrate	Valerate
mean (sd) - mmol L⁻¹	FB	5.8 (±5.5)	0.2 (±0.2)	3.9 (±3.9)	1.8 (±3.0)	1.0 (±1.6)	0.6 (±0.5)
	UG	8.0 (±7.5)	0.1 (±0.1)	3.7 (±2.0)	4.3 (±5.7)	1.7 (±2.0)	0.5 (±0.4)
	UF-1	7.0 (±6.8)	0.1 (±0.1)	3.2 (±1.9)	2.2 (±3.2)	0.7 (±1.0)	0.6 (±0.4)
	UF-2	3.6 (±1.3)	0.2 (±0.1)	7.8 (±2.7)	1.1 (±0.6)	1.1 (±0.6)	0.4 (±0.2)
calculated acidified sucrose (sd) - %	FB	42 (±33)	26 (±20)	11 (±17)	14 (±20)	7 (±5)	
	UG	44 (±33)	20 (±10)	18 (±21)	14 (±12)	5 (±3)	
	UF-1	44 (±32)	24 (±14)	16 (±22)	9 (±13)	7 (±4)	
	UF-2	25 (±8)	50 (±7)	7 (±3)	14 (±4)	5 (±2)	
calculated HY (sd) - %	FB	0 (±0)	74 (±26)	-13 (±28)	26 (±26)	-6 (±4)	
	UG	0 (±0)	75 (±19)	-27 (±33)	25 (±19)	-5 (±3)	
	UF-1	0 (±0)	81 (±19)	-25 (±38)	19 (±19)	-8 (±5)	
	UF-2	0 (±0)	88 (±3)	-6 (±3)	12 (±3)	-2 (±1)	

390

391 The reduced OLR and possible higher biomass concentration (indicated by higher VSS
 392 concentrations) in the UF-2 reactor resulted in lower specific organic loading (food/ microorganism
 393 ratio). Thus, the efficiency of the substrate conversion was increased, which was verified by the
 394 greater sucrose removal.

395

396 The overloading in Phase 1 seemed to be the main factor accounting for reduced hydrogen yields.
 397 According to Cohen et al. (1984), lactate pathway is energetically less favourable and its formation in
 398 acid digestion could be associated with an imbalance between electron donating and electron

399 accepting reactions, in conditions of high accessibility of the substrate, such as low HRT and shock
400 loading. Apart from the influence of organic loading on metabolic routes, the *Lactobacillus* genus was
401 found in greater relative abundance in the reactors of Phase 1 (section 3.3).

402
403 Propionate concentration was higher in the UG reactor (Table 2). Butyrate production was similar
404 among the reactors, suggesting that activity of the butyrate-producers, was not severely affected by
405 the different conditions. Since propionate production is not likely to occur under very acid conditions
406 (Wang et al., 2006), it was likely that the bacteria arrangement in the granules kept the medium pH in
407 microcolonies higher than in the external environment, allowing the activity of propionate-producing
408 microorganisms.

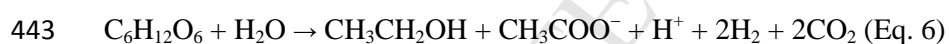
409
410 The results presented in Table 2 are only for comparison, based on the equations shown in section 2.5.
411 Many other pathways could have taken place in the reactors. The calculated acidified sucrose
412 corresponded to 55%, 66%, 64% and 39% of the consumed sucrose in the FB, UG, UF-1 and UF-2
413 reactors, respectively. Naturally, part of the sucrose could have been used for cellular growth.
414 Moreover, it is likely that other pathways leading to hydrogen formation were also present. The mean
415 calculated HY in the FB, UG, UF-1 and UF-2 reactors was equivalent to 1.00, 0.78, 0.85 and 1.62
416 $\text{mmolH}_2 \text{ mmol}^{-1}\text{sucrose}_{\text{consumed}}$, respectively, which corresponded to 67%, 103%, 71% and 48% of the
417 measured HY, respectively.

418
419 Ethanol was measured in the effluent from the UF-2 reactor. Unfortunately, this measurement was not
420 performed in the other reactors, due to technical problems. The average concentration was 11 mmol L^{-1} ,
421 which accounted for 27% of total soluble COD effluent, while COD from organic acids and sucrose
422 were 33% and 24%, respectively. In the FB, UG and UF-1 reactors COD from organic acids and
423 sucrose accounted for a greater proportion, being respectively: 33% and 41% (FB), 45% and 37%
424 (UG), 32% and 53% (UF-1) of total soluble COD effluent. From COD balance analysis, it was
425 inferred that ethanol concentrations in the reactors of Phase 1 did not reach such high levels as were
426 reached in the UF-2 reactor.

427

428 The high concentrations of ethanol in the UF-2 reactor is contrary to what was first expected, because
429 ethanol is a more reduced compound than organic acids and its formation is usually associated with
430 HY reduction. Nevertheless, some pathways are proposed for ethanol formation along with hydrogen
431 (Xu et al., 2008; Lee et al., 2009). Equation 6 shows the reaction proposed by Hwang et al. (2004) for
432 bacterial conversion of glucose into ethanol, acetate and hydrogen. Although the ethanol-acetate
433 pathway yields less hydrogen than the acetate-pathway (Equation 2), the hydrogen yield could be 4.0
434 mol of H₂ per mol of sucrose consumed (which is in the range achieved in the UF-2 reactor),
435 considering sucrose as substrate. Since the hydrogen yield per mol of acetate produced is the same of
436 as shown in Equation 2 (H = 2 mmol H₂ mmol⁻¹ acetate), the assumption of this reaction would not
437 change the HY percentage values depicted in Table 2, whereas the acidified sucrose percentage would
438 be higher from acetate (S = 0.50 mmol sucrose mmol⁻¹ acetate). However, as ethanol was not analysed
439 in all effluents, however, it was not possible to account for it in the estimations presented in Table 2.
440 Ethanol formation is in agreement with the findings of sequencing analyses (section 3.3), that
441 revealed an abundance of microorganisms affiliated with *Ethanoligenens harbinense*.

442



444

445 3.3 Structure and composition of the microbial community in the FB, UG, UF-1 and UF-2 reactors

446

447 There were 123838 partial 16S ribosomal DNA gene sequences obtained from the microbial
448 sequencing, of which 94-99% were assigned to the phylum Firmicutes in the reactors versus 17% in
449 the inoculum. Sequences assigned to the domain Archaea were 9.6% of the inoculum and less than
450 0.1% of the reactors, indicating that the conditions applied in this study dispensed with an inoculum
451 pretreatment. Based on the operational taxonomic units (OTU), the Shannon-diversity index was
452 reduced from 4.0 in the inoculum to 1.2, 1.3, 1.4 and 0.7 in the FB, UG, UF-1 and UF-2 reactors,
453 respectively, by the end of operation. The self-established harsh environment likely played a key role
454 in the reduction of biomass diversity. An annotated abundance relative description is given in Table 3.

455 Representative sequences (abundance of more or equal to 1.0%) were selected from the acidogenic
456 reactors to infer a phylogenetic tree (Figure 6).

457

458

459 According to the results of 16 rDNA sequencing, the main emerging classes were related to Bacilli
460 and Clostridia, which represented approximately 23% and 74%, respectively, of the total sequences in
461 the reactors of Phase 1, and 1% and 99% of the total sequences in the UF-2 reactor. Only two
462 sequences, represented by OTU0002 and OTU0003, accounted for more than 70% of the total
463 bacteria (Table 3). The alignment of the sequence of OTU0002 (*Ethanoligenes*) in BLAST revealed
464 an identity of 99% to the *Ethanoligenens harbinense* strain YUAN-3. The same procedure applied to
465 OTU0003 revealed it is 98% affiliated with *Clostridium acidisoli* (Figure 6). These results are in
466 agreement with the literature that reports the ability of both *Ethanoligenens harbinense* and
467 *Clostridium acidisoli* to grow and produce hydrogen under very acid conditions; specifically, pH
468 below 4.0 (Kuhner et al. 2000; Xing et al., 2008; Carosia et al., 2017; Zhao et al., 2017). However,
469 this has never been demonstrated for pH below 3.0.

470

471 Although the microbial structure is very similar among the reactors of Phase 1, it is not possible to
472 conclude that biomass retention mechanism does not affect microbial composition, because the
473 samples were only analyzed by the end of operation. As discussed in section 3.1, considerable
474 suspended biomass grew in the FB and UG reactors, the latter as a consequence of granule wash-out
475 and disruption. In the UF-2 reactor, the relative abundance of sequences affiliated with *E. harbinense*
476 was the highest, corresponding to 81%. From these results, it is inferred that *E. harbinense* played the
477 most relevant role in the reactor performance. However, the differences in terms of relative abundance
478 should be interpreted with caution, considering that the 16S sequencing technique is subjected to
479 errors in terms of quantification (Haas et al., 2011), the efficiency of DNA extraction can interfere
480 with the results, and the microorganisms found were not necessarily active. While most of
481 *Clostridium*, including *C. acidisoli* are able to sporulate (Kuhner et al., 2000), *Ethanoligenens* is not
482 (Xing et al., 2006). Therefore, the high relative abundance of sequences related to *Clostridium* does

483 not mean that they were active in the same proportion. Also, the absolute abundance of each
484 microorganism is very relevant to the performance of the reactors, since the efficiency of sucrose
485 consumption was associated with increased production and yield of hydrogen (section 3.2).

486

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487 **Table 3** – Comparative study of 16S rDNA sequencing (V4-5 region) using SINA (v1.2.11). Relative abundance > 1% is shown for the FB, UG, UF-1 and UF-
 488 2 reactors; and > 5% for the inoculum

Domain	OTU	Phylum	Class	Order	Family	Genus	Inoculum*	FB	UG	UF-1	UF-2
	OTU0002	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ethanoligenens</i>	0%	40%	43%	41%	81%
	OTU0003	Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	<i>unclassified</i>	0%	31%	31%	35%	15%
	OTU0009	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	0%	27%	20%	13%	0%
	OTU0023	Firmicutes	Bacilli	Bacillales	Sporolactobacillaceae	<i>Sporolactobacillus</i>	0%	0%	3%	2%	1%
Bacteria	OTU0001	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>unclassified</i>	0%	0%	0%	7%	0%
	OTU0171	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	<i>Pectinatus</i>	0%	0%	0%	0%	1%
	OTU0008	Bacteroidetes	vadinHA17	unclassified	unclassified	<i>unclassified</i>	16%	0%	0%	0%	0%
	OTU0014	Bacteroidetes	vadinHA17	unclassified	unclassified	<i>unclassified</i>	6%	0%	0%	0%	0%
	OTU0146	Firmicutes	Clostridia	Clostridiales	Family XI	<i>Tissierella</i>	5%	0%	0%	0%	0%
Archaea	Otu002	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	<i>Methanosaeta</i>	85%	0%	0%	0%	0%
	Otu004	Euryarchaeota	Methanobacteriales	Methanobacteriaceae	Methanobacterium	<i>Methanobacterium</i>	5%	0%	0%	0%	0%

489 * Domain *Bacteria* and *Archaea* represented 90.4% and 9.6% of total sequences, respectively, in the inoculum.

490

491

492 **Figure 6** – Consensus phylogenetic tree based on 16S rDNA for bacteria domain obtained from the highly
493 abundant OTUs found in the reactors. The tree is drawn to scale, with branch lengths in the same units as those
494 of the evolutionary distances used to infer the phylogenetic tree. There was a total of 373 positions in the final
495 dataset. Outgroup: *Methanosarcina acetivorans*.

496

497 *C. acidisoli* was isolated from acidic peat-bog soil and was grown at pH 3.6-6.9, with no distinct
498 optimum between pH 3.6-6.6, in a temperature range of 5-37 °C, with an optimum of 25-30 °C
499 (Kuhner et al., 2000). At pH 4.0, 5.5 and 6.5, glucose fermentation yielded lactate, acetate, butyrate,
500 H₂ and CO₂ as end-products. At pH 5.5, the molar ratio of H₂ to lactate, acetate and butyrate produced
501 was 6.4, 4.1 and 3.6, respectively, and the HY was 1.8 mmolH₂ mmol⁻¹ glucose_{consumed}. Lee et al.
502 (2009) found great abundance of a species affiliated with *Clostridium* sp. HPB-16, which is
503 phylogenetically close to *C. acidisoli*, during batch fermentation with hydrogen production at final pH
504 of 3.5. Acetate and butyrate were the dominant organic products. These authors assumed that
505 hydrogen was formed by the pyruvate decarboxylation-ferredoxin-hydrogenase pathway, which is the
506 common mechanism for H₂ formation by the *Clostridium* and *Ethanoligenes* species.

507

508 Xing et al. (2006) isolated *E. harbinense* YUAN-3 from anaerobic activated sludge of molasses
509 wastewater. They found that it grows in the pH range 3.5-9.0 at 20-44 °C, and the optima for growing
510 were pH 4.5-5.0 and 35 °C. Acetate, ethanol, hydrogen and carbon dioxide were formed as end
511 products of glucose fermentation. At 35 °C a hydrogen yield up to 2.8 molH₂ mol⁻¹ glucose was
512 achieved, along with production of 1.1 mol ethanol and 0.7 mol acetate per mol of glucose. In the UF-
513 2 reactor, the mean production was 1.1 mol ethanol per mol of sucrose (= 0.6 per mol of hexose) and
514 0.8 mol acetate per mol of sucrose (= 0.4 mol per mol of hexose). The differences in the yields of
515 ethanol and acetate were expected because the fermentation in the UF-2 reactor was carried out by a
516 microbial consortium, which means that many more pathways were possible, and relatively high
517 amounts of lactate were formed (section 3.2). Nevertheless, the molar proportion of ethanol and
518 acetate is similar between the UF-2 reactor (= 1.4 ethanol: acetate) and that reported by Xing et al.

519 (2006) (= 1.6 ethanol: acetate). Since the maximum achieved hydrogen yield with the pure culture of
520 *E. harbinense* (Xing et al. 2006) was higher than the theoretical yield depicted in Equation 6 (section
521 3.2), it is probable that this bacterium is able to produce hydrogen and ethanol by pathways other than
522 ethanol-acetate fermentation. Xu et al. (2008) also found an HY higher than 2 molH₂ mol⁻¹ glucose
523 with the *Ethanoligenens harbinense* B49 strain. They suggested oxidative decarboxylation of
524 pyruvate as the possible route for the hydrogen production observed, in accordance with Lee et al.
525 (2009). However, the ethanol-type hydrogen production mechanism by *E. harbinense* is still unclear
526 (Zhao et al., 2017).

527

528 *Lactobacillus* sp. ranged from 20% to 27% in the reactors of Phase 1 and was less than 1% in the UF-
529 2 reactor. The most representative sequence of *Lactobacillus* was affiliated with *L. nagelii* (Figure 6),
530 which is characterized as producing lactic acid from glucose without gas formation (Edwards *et al.*,
531 2000). Then, it is probable that the presence of *Lactobacillus* in the FB, UG and UF-1 reactors
532 contributed to higher lactic acid formation and less hydrogen yield, due to the reduction of pyruvate
533 availability for the H₂-producing pathways (section 3.2). The excretion of extracellular polymeric
534 substances (EPS) by lactic acid bacteria protects them against hostile environments and favors the
535 formation of flocs and biofilm (Rafrafi et al. 2013), which may have implied competitive advantages
536 at higher OLR.

537

538 The presence of *Pectinatus* sp. (OTU0171) in the UF-2 reactor probably is associated with alcoholic
539 fermentation, because this genus is usually found in beer spoilage (Chihib and Toloza, 1999).

540

541 3.4. Interaction among performance evaluation parameters

542

543 Table 4 shows the overall results obtained, indicating the minimums, maximums, means, standard
544 deviations (SD) and coefficients of variation (CV).

545

546 **Table 4** – Performance evaluation parameters of all reactors

Parameter	Reactor	Minimum	Maximum	Mean	SD	CV
VHPR - mLH₂ L⁻¹h⁻¹	FB	4.9	259.3	94.9	68.6	72%
	UG	4.0	171.4	44.7	37.5	84%
	UF-1	0.4	114.0	53.7	32.2	60%
	UF-2	92.3	300.8	175.2	43.9	25%
H₂ in biogas - %	FB	40.5	85.4	59.6	11.0	18%
	UG	38.6	82.1	62.1	10.8	17%
	UF-1	47.5	77.5	62.2	7.1	11%
	UF-2	48.4	75.9	59.8	5.9	10%
HY - molH₂ mol⁻¹ sucrose_{consumed}	FB	0.10	3.16	1.50	0.83	55%
	UG	0.06	2.47	0.76	0.56	74%
	UF-1	0.11	3.05	1.19	0.71	60%
	UF-2	1.63	4.94	3.35	0.68	20%
Sucrose removal - %	FB	0.0	100.0	64.3	23.0	36%
	UG	18.7	95.7	66.8	21.4	32%
	UF-1	0.0	90.8	53.1	19.1	36%
	UF-2	56.1	99.7	80.3	9.9	12%

547

548 The VHPR and HY improvements in the UF-2 reactor are noteworthy, with respect to the others.

549 These improvements were attributed mainly to the HRT increasing from 3.3 to 4.6 h and, therefore,
 550 the OLR decreasing from 33.1 to 25.0 gCOD L⁻¹d⁻¹, since these were the only operational parameters
 551 changed intentionally. Several operating indicators accompanied the UF-2 improvement in H₂
 552 production. These indicators include: increased VSS concentration; higher sucrose removal; less
 553 production of lactate and more of acetate, and high production of ethanol; pH always below 3.0; and,
 554 longer chains of rods. Figure 7 presents a proposed model of the relationship among these parameters
 555 that led to higher H₂ production in the UF-2 reactor.

556

557 **Figure 7** – Proposed model to explain changes in the UF-2 reactor that led to increased H₂ production

558

559 Based on the proposed model, we suggest that the increased HRT led to greater removal of sucrose,
 560 due to the longer contact time between the substrate and the biomass, and to a higher VSS

561 concentration (biomass) that resulted from the lower wash-out and longer time for bacterial growth.
562 The higher HRT allowed the formation of long chains of bacteria, increasing their adaptability to the
563 harsh environmental conditions (low pH) and contributing to the increased biomass in the reactor. The
564 growth of acid tolerant bacteria such as *Ethanoligenens* was favoured and the competitive advantage
565 of *Lactobacillus* was reduced. The higher VSS and HRT resulted in a lower specific organic loading
566 rate, which enhanced the sucrose removal efficiency. The increased sucrose removal resulted in
567 higher concentrations of fermentation products, such as acids, CO₂ and H₂. This latter directly
568 reflected in higher VHPR. The high levels of acids and CO₂/ carbonic acid caused a reduction in the
569 pH of the medium. The maintenance of a very acid environment and less relative abundance of
570 *Lactobacillus* resulted in reduced lactate formation. The increased pyruvate availability to other H₂-
571 producing pathways, such as acetate and ethanol formation, thus increased hydrogen yield and
572 production.

573
574 On the other hand, increasing HRT over the suitable values is not recommended as it leads to OLR
575 reduction. In addition to increasing reactor volume requirements, this can reduce volumetric substrate
576 removal rates, reducing the attainable VHPR. Very low OLR can also lead to cellular decay, reducing
577 the biomass concentration. In addition, reduced HRT values can increase the pH (through the
578 consumption and release of CO₂, H⁺ and acids) and the H₂ in the medium, due to the mass transfer
579 reduction caused the less turbulence. Increasing the pH and H₂ in the liquid medium then favours the
580 growth of H₂-consuming bacteria; and, it can reduce the competitive advantage of the H₂-producing
581 bacteria tolerant to very acid conditions.

582

583 3.5. Comparative studies

584

585 Hydrogen production in extremely acidic environments, average pH of 2.8 in the FB, UG, and UF-1
586 reactors, and of 2.7 in the UF-2 reactor, was unexpected. Extensive data in the literature indicate
587 drastic reduction or cessation of hydrogen production by dark fermentation at pH values below 4.5-
588 4.0 (Yokoi *et al.*, 1995; Lay, 2000; Mizuno *et al.*, 2000; Lee *et al.*, 2002; Kim *et al.*, 2004; Liu and

589 Shen, 2004; Hwang *et al.*, 2004; Chen *et al.*, 2005; Liu *et al.*, 2006; Chojnacka *et al.*, 2011; Ruggeri
 590 *et al.*, 2015). It is only in specific cases, in continuous acidogenic reactors, that H₂ production at pH
 591 values below 4.0 is reported (Xing *et al.*, 2008; Tähti *et al.*, 2013, Carosia *et al.*, 2017). The capacity
 592 of *Ethanoligenens harbinense* strain YUAN-3 to produce H₂ was evaluated by Xing *et al.* (2008) in a
 593 continuous stirred reactor at 35 °C for 21 days. The pH value was kept above 3.5 by a pH controller
 594 and they observed that H₂ production was not severely affected when the pH reached the minimum
 595 values (i.e., around 3.6), obtaining HY of approx. 1.5 molH₂ mol⁻¹glucose. Carosia *et al.* (2017) found
 596 bacteria similar to *Ethanoligenens harbinense* to be dominant bacteria in H₂-producing anaerobic
 597 fluidized bed reactors, inoculated with heat-treated sludge. Although buffers (hydrochloric acid and
 598 sodium bicarbonate) were added, effluent pH was approximately 3.7, and the optimum HY obtained
 599 was 0.76 molH₂ mol⁻¹glucose. Tähti *et al.* (2013) used an extreme thermophilic (70 °C) UASB reactor
 600 for H₂ production from glucose by mixed culture. However, a low HY was obtained, equivalent to
 601 0.73 mol mol⁻¹glucose_{added}, which was accompanied by a decrease in pH to around 3.7. In the present
 602 study, despite the lowest pH values already being reported, the HY and VHPR obtained are in the
 603 highest-range. For comparison purposes, Table 5 shows the results obtained in the UF-2 reactor with
 604 results from other studies applying continuous hydrogen-producing reactors fed with sucrose-based
 605 wastewater, in the mesophilic range.

607 **Table 5** – Comparison of hydrogen production in continuous acidogenic reactors using sucrose as substrate

Reactor type	OLR - gCOD L ⁻¹ d ⁻¹	Effluent pH	Temp - °C	H ₂ in biogas - %	VHPR* - mL H ₂ L ⁻¹ h ⁻¹	HY - mol H ₂ mol ⁻¹ sucrose	Ref.
stirred tank	48.6	5.5	26	63	542	3.9	Fang <i>et al.</i> (2002)
stirred tank	80	5.25	35	55	506	2.3	Kyazze <i>et al.</i> (2006)
granular UASB	7.1 - 37.4/ 8.5 - 128	4.4	38	57 - 37/ 44 - 42	50 - 190/ 33 - 202	2.9 - 2.0/ 1.6 - 1.0	Yu and Mu (2006)
granular UASB	4.4 - 30	4.0	30	26 - 50	4 - 122	0.5 - 3.3	Zhao <i>et al.</i> (2008)
UASB	12	4 - 4.5	35	45 (approx.)	12 (approx.)	0.3	Wang and Li (2010)
fixed-bed	24	4.4	25	46 - 56	73 - 125	0.9 - 1.4	Lima and Zaiat

							(2012)
fixed-bed	24	4.8	25	54 - 62	15.1 – 61.6	0.7 – 2.1	Penteado <i>et al.</i> (2013)
granular UASB	21.6	4.0	36	40 (approx.)	92 (approx.)	1.6 (approx.)	Ning <i>et al.</i> (2013)
structured fixed-bed	24.0	6.5	25	70	12 - 25	0.4 - 0.6	Anzola-Rojas and Zaiat, (2016)
flocculent UASB (UF-2)	25.0	2.7	30	60	175	3.4	This study

608 *The reference conditions adopted were 25 °C and 1 atm.

609

610 These results indicate that the formation of a very acidic environment allowed the growth of acid-
611 tolerant bacteria that were able to produce H₂ under very acid conditions, especially *Clostridium* sp.
612 and *Ethanoligenens* sp.

613

614 4. Conclusions

615

616 This study stands out as the first to demonstrate the real possibility for continuous, long-term, stable
617 H₂ production at pH below 3.0, with a mean yield of 3.4 mols of H₂ per mol of sucrose consumed.
618 Proper HRT and OLR were crucial for enhancing hydrogen production. This was associated with
619 increased sucrose consumption, reduced lactate formation, high acetate and ethanol concentrations,
620 reduction of relative abundance of *Lactobacillus* sp. and increase of *Ethanoligenens* sp.

621

622 The operating requirements were keep at minimum and the non-pH control, along with the production
623 of H₂ in extremely acid environments, presents several operating and economic advantages, including:
624 the non-addition of alkalizing agents, which contributes to reduction of the costs; elimination of the
625 demand for sludge pretreatment, due to the naturally acid environment; and, the non-necessity of
626 constant sludge removal, since higher biomass concentration leads to enhanced H₂ production. These
627 results open a new field of investigation in biological hydrogen production by dark fermentation
628 towards a more sustainable and feasible technology.

629

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631

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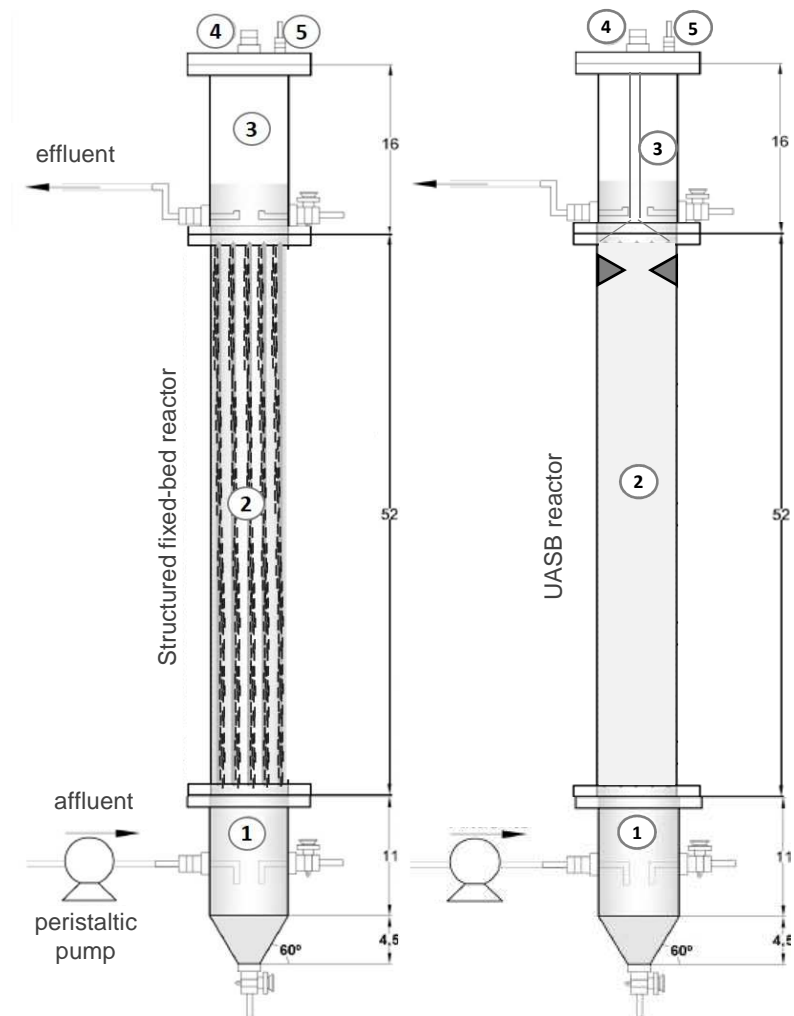


Figure 1 – Schematic diagram of the acidogenic reactors. 1: distribution chamber, 2: reactional zone, 3: headspace, 4: biogas sampling, 5: biogas outlet

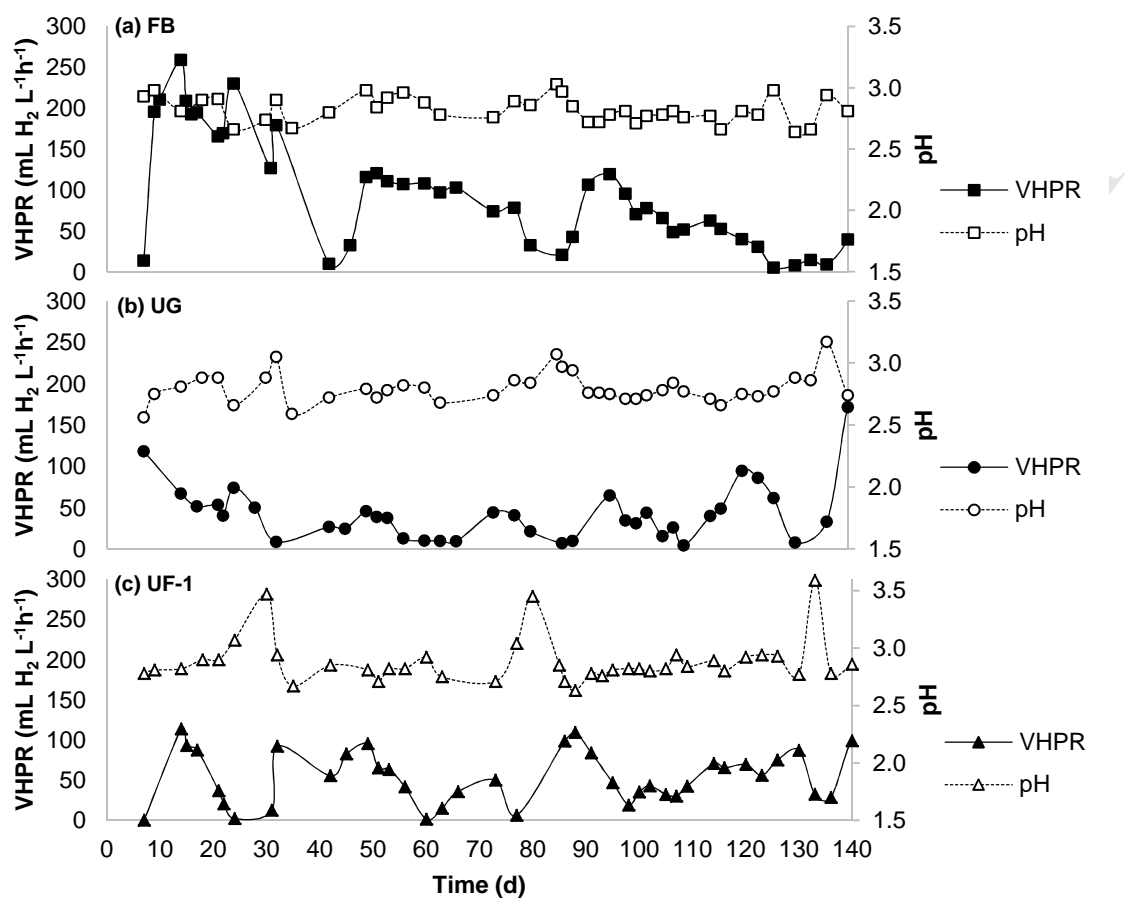


Figure 2 – Volumetric hydrogen production rate and pH in the first experimental phase: (a) FB reactor, (b) UG reactor, (c) UF-1 reactor.

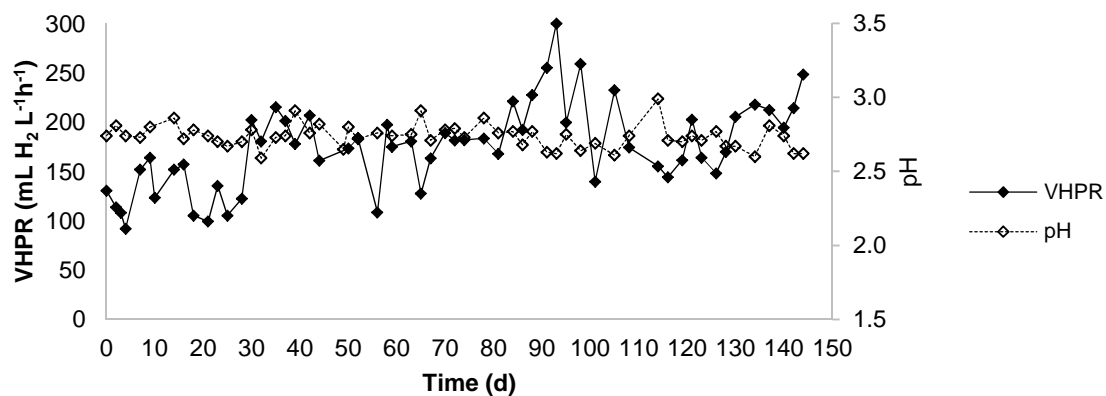


Figure 3 – Volumetric hydrogen production rate and pH in the second experimental phase: UF-2 reactor

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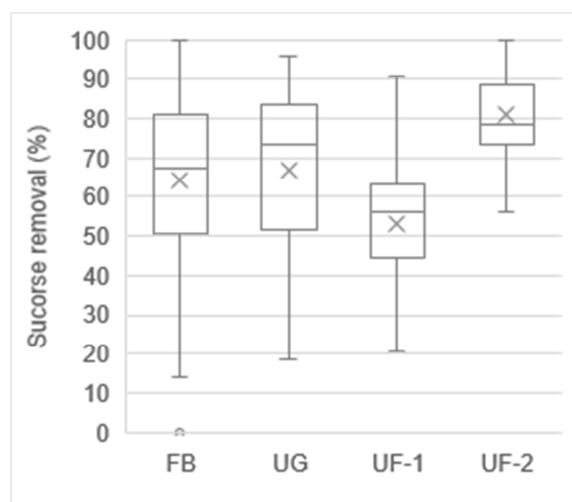


Figure 4 – Sucrose removal

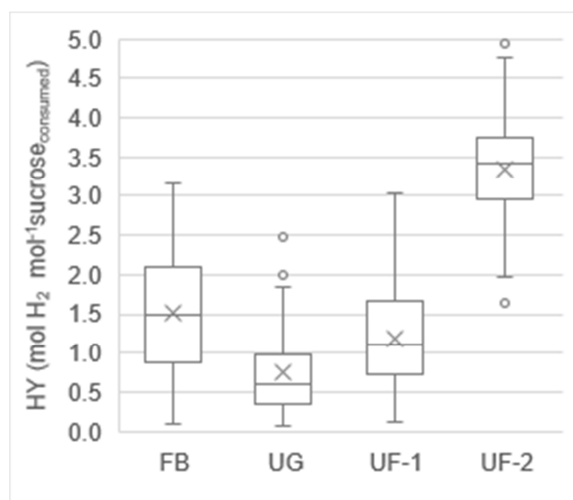


Figure 5 – H₂ yield

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NR 113819.1 *Lactobacillus parakefiri* strain NBRC 15890NR 112757.1 *Lactobacillus parakefiri* strain JCM 8573NR 041659.1 *Lactobacillus rapi* strain YIT 11204

OTU0009 this study

NR 041007.1 *Lactobacillus nagelii* strain NRIC 0559NR 119275.1 *Lactobacillus nagelii* strain LuE10NR 112754.1 *Lactobacillus nagelii* strain JCM 12492

OTU0023 this study

NR 112774.1 *Sporolactobacillus putidus* strain QC81-06NR 134815.1 *Sporolactobacillus shoreae* strain BK92NR 134816.1 *Sporolactobacillus spathodeae* strain BK117-1

OTU0002 this study

NR 115307.1 *Ethanoligenens harbinense* strain YUAN-3NR 042828.1 *Ethanoligenens harbinense* strain YUAN-3NR 074333.1 *Ethanoligenens harbinense* strain YUAN-3

OTU0003 this study

NR 028898.1 *Clostridium acidisoli* strain CK74NR 104822.1 *Clostridium pasteurianum* strain DSM 525NR 113023.1 *Clostridium pasteurianum* strain JCM 1408

Otu0171 this study

NR 117702.1 *Pectinatus frisingensis* strain CCM 6217NR 042900.1 *Pectinatus portalensis* strain B6NR 043658.1 *Pectinatus haikarae*NR 074110.1 *Methanosarcina acetivorans* strain C2A

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Mota, V. T., Ferraz-Júnior, A. D. N., Trably, E., Zaiat, M. (2018). Biohydrogen production at

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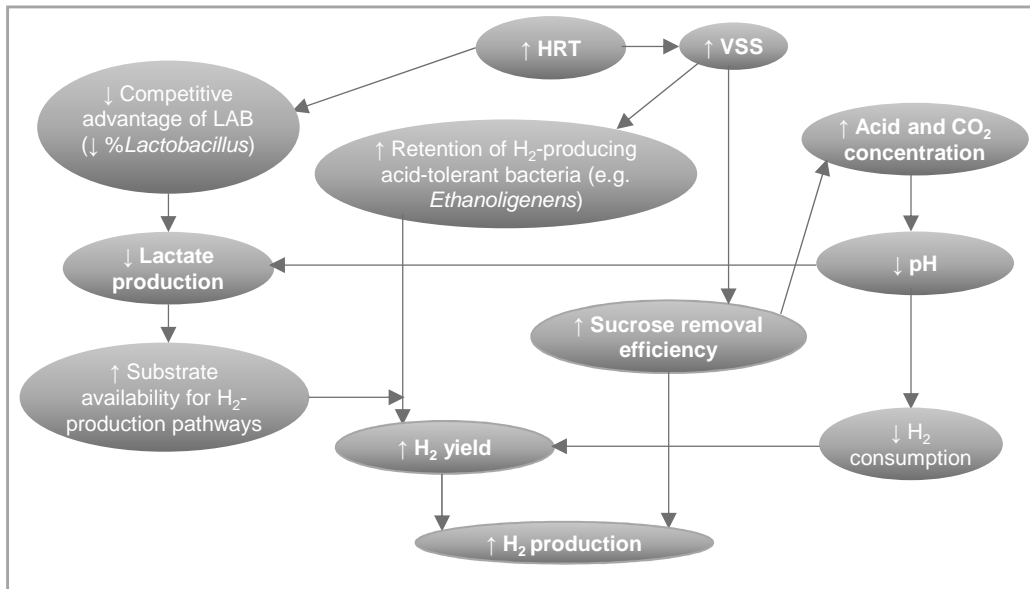


Figure 7 – Proposed model to explain changes in the UF-2 reactor that led to increased H₂ production

Highlights

Acidogenic reactors were fed with sucrose (4.7 gCOD L^{-1}) without pH regulator.

Flocculent UASB showed stability superior to granular UASB and fixed bed reactors.

Acetate production replaced lactate when the HRT increased from 3.3 to 4.6 h.

Continuous long-term and stable H_2 production was achieved at pH always below 3.0.

H_2 production of $175 \text{ mLH}_2 \text{ L}^{-1}\text{h}^{-1}$ and yield of $3.4 \text{ molH}_2 \text{ mol}^{-1}\text{sucrose}$ were obtained.

Bacteria affiliated with *Ethanoligenens harbinense* were predominant.

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