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Evaluation of feeding strategies in upflow anaerobic sludge bed reactor for hydrogenogenesis at psychrophilic temperature

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24 **ABSTRACT**

26 The present work evaluated the biohydrogen production from a 0.4 L upflow anaerobic
26 sludge blanket reactor type (UASB) operating at psychrophilic temperature (21 ± 2 °C) at
different feeding strategies varying hydraulic retention times (HRT) and sucrose
28 concentration in the feeding. First strategy (24h/31c) fed semi-continuously $31 \text{ g}_{\text{sucrose}} \text{ L}^{-1}$ at
24 h HRT; second strategy (12h/19c) fed semi-continuously $19 \text{ g}_{\text{sucrose}} \text{ L}^{-1}$ at 12 h HRT; third
30 strategy (4h/8c) fed continuously $8.3 \text{ g}_{\text{sucrose}} \text{ L}^{-1}$ at 4 h HRT.

After 70 days of operation, the UASB accumulated 65.44 L H_2 . The average HY for the
32 whole operation during the three strategies was $62.6 \text{ NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$, and average
hydrogen content was 69.04%. In general terms, the best operation strategy was 12h/19c
34 since it presented good set of results, the best HY ($70.6 \text{ NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$) and a comparable
hydrogen production rate (2.6 L (L d)^{-1}) to that obtained in 4h/8c strategy ($3.17 \text{ L (L d)}^{-1}$).
36 The average gross energy potential rate from the 12h/19c strategy was $46.21 \text{ kJ (L d)}^{-1}$,
whereas energy heating losses were circumvented due to operation at psychrophilic regime.
38 Indeed, psychrophilic or room temperatures should be broadly regarded as an effective
alternative towards net energy gains in biohydrogen production.

40

Keywords: bioenergy, dark fermentation, energy balance, room temperature.

42

Evaluation of feeding strategies in upflow anaerobic sludge bed reactor for

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hydrogenogenesis at psychrophilic temperature

46 1. Introduction

The worldwide energy production is mainly derived from fossil fuels such as oil, coal and
48 natural gas, comprising activities such as exploration, exploitation, refining, transportation
and even combustion, which affect negatively the environment and human health. The
50 conventional stocks of fossil fuels will decrement overtime, whereas energy demand will
continue raising as population and consumption habits increase, following urbanization and
52 modernization [1,2]. In order to expand the energy sources and to reduce fossil fuels related
problems (e.g. air pollution and greenhouse gas emissions), renewable alternatives are
54 under development [3] such as solar, geothermal, ocean and biomass energies [4].
Bioenergy is produced from biomass through thermochemical, thermal and biotechnological
56 processes for its use in industry, power, and transport sectors [5].

Biohydrogen may be considered a nitrogen- and sulfur-free biofuel when produced from
58 ideal conditions dark fermentation (DF). In practice, biological CO₂ is produced as
consequence of microbial anaerobic respiration, hydrogen sulfide may be produced by
60 action of sulfate-reducing bacteria (SRB) if sulfate is present in concentrations over 3 g-
SO₃²⁻ L⁻¹ [6], and NO_x emissions increase when hydrogen is combusted along with gasoline,
62 methane or methanol in spark-ignited combustion engines [7]. Still, hydrogen produced from
natural gas, oil, coal gasification and water electrolysis, require high energy consumption,
64 concomitantly causing air pollution and global warming [8].

Various fermentation systems have been developed for hydrogen production, such as
66 the batch, semi-continuous and continuous, for either one or multiple stages. Depending on
substrate characteristics the bioreactor type may be considerably different. For insoluble
68 substrates the suspended biomass bioreactors —continuous stirred tank reactor (CSTR),
upflow anaerobic sludge bioreactor (UASB), and fluidized bed bioreactors— are a preferred
70 option; whereas for soluble substrates the immobilized biomass reactors (fixed bed
bioreactor and biotrickling filter) are better designs. For instance, UASB is appraised to be
72 a good option for treating organic fraction of municipal solid waste and synthetic wastewater,
as demonstrated in the work by Alzate-Gaviria et al. [9] who demonstrated that an UASB
74 bioreactor outperformed a packed bed reactor treating such substrates. When using soluble
substrates (e.g. cheese whey, sucrose), it has been determined that CSTR is not the most
76 appropriate design, these reactors often have washing of microorganisms caused by a cell
growth rate lower than the rate at which the microorganisms leave the digester [10]. Even
78 though biotrickling filters may be used to treat soluble and insoluble matter, insoluble matter
may cause clogging problems [11]. On the other hand, agroindustrial wastes have been
80 treated by anaerobic solid substrate fermentation [12] using the systems designed by Poggi-
Varaldo and Oleszkiewicz [13], which have been also successfully adapted to semi-
82 continuous acidogenic operation for hydrogen production and to intermittently vented batch
operation [14]. Other important alternative for insoluble biomass is the anaerobic sequencing
84 batch reactor (ASBR), since this system is regarded as the batch type of the UASB.

The UASB perform depends on an adequate formation of microbial flocules. If inoculum
86 is not in granular form, promoting its formation can be a difficult and time-consuming task.
Start-up periods of 150-300 d are commonly reported in literature [15]. Liu et al. [16] explored

88 the retention of anaerobic digester sludges using either activated carbon or carbon
nanotubes, finding that the latter prevented cell washout more effectively. However, many
90 researchers prefer to use already formed granules from brewery wastewater treatment
UASB reactors [17,18]. At laboratory scale, hydrogen production in continuous mode is
92 explored to increase stability, organic matter removal, energy efficiency and yields [19,20].
Important variables used to control and to increase the hydrogen yields and production rates
94 are inoculum pretreatment [21], pH influence [22], effect of hydraulic retention time (HRT)
[23], organic loading rate (OLR), carbon and sludge sources [24,25]. Determining the
96 optimal HRT and OLR could maximize the biogas and hydrogen production [26], and thus it
has been of interest to design strategies to evaluate its influence on hydrogen production.
98 For instance, medium and long-term operation works usually begin with 48 or 24 h HRT
[27,28]; thereupon HRT is reduced by a pair-number factor, mostly of 4 or 6 h [23,24,29].
100 Moreover, changes between continuous and discontinues modes have also been evaluated
[30,31]. Final HRT for soluble substrates –such as monosaccharides– are 8-12 h [32],
102 although some improvements have allowed to reduce it to 4 h not without metabolic
unbalance risk [15,23].

104 It is well known the effect of temperature in hydrogenogenic performance. According to
bacterial metabolism, three temperature intervals are observed even in hydrogen production,
106 psychrophilic (<25 °C), mesophilic (30-40 °C), thermophilic (50-60 °C), among which the
most used have been mesophilic and thermophilic [33]. However, it should be considered
108 that psychrophilic metabolism at 18-25 °C has reduced energy expenses compared to its
counterparts, making it an attractive alternative that if improved could produce net energy
110 gains to the systems [34]. Therefore, the aim of this study was to explore the biohydrogen

production in a bench-scale UASB reactor operating at psychrophilic temperature through
112 different feeding strategies (i.e. varying hydraulic retention times and sucrose concentration
in the feeding); moreover, an energetic evaluation in terms of gross energetic potential rate
114 and energetic heating losses was performed at the best conditions and compared to
literature.

116

2. Materials and Methods

118 2.1. Inoculum

Methanogenic granules were obtained from brewery industry anaerobic sludges (Obregón,
120 Sonora, Mexico) and stored at 4 °C. Before use, granules were rinsed with sterilized isotonic
solution and heat-treated at 90 °C for 1 h in a water bath to inhibit methane producing
122 microflora and to enrich the hydrogen-producing heat-shock resistant microbes [35]. The
inoculum was characterized in terms of pH, humidity, total solids (TS), volatile solids (VS)
124 and ashes (Table 1). Humidity, TS, VS and ashes were carried out in triplicate using
standard techniques [36].

126

PLEASE INSERT TABLE 1 HERE

128

2.2. Experimental system and procedure

130 A plexiglass 0.4 L working volume UASB reactor (0.056 m diameter, 0.23 m height) was fed
with mineral medium containing sucrose as carbon source with a peristaltic pump as shown
132 in Fig. 1. The UASB was installed on a laboratory bench and operated at psychrophilic
temperature (21 ± 2 °C). The initial amounts of inoculum, mineral medium and phosphate

134 buffer solution were 77 g_{wb}, 40 mL and 283 mL, respectively; consequently, the
concentration of the inoculum in terms of VS was 4155 mg L⁻¹. The mineral medium
136 composition was [37] (mg L⁻¹): ZnCl₂, 75; MgCl₂·6H₂O, 100; MnCl₂·4H₂O, 10.77; FeCl₃·6H₂O,
25.97; CuCl₂·2H₂O, 3.41; and NiCl₂·6H₂O, 101.25. A phosphate buffer (pH = 7.28) was
138 prepared using 30 g K₂HPO₄ L⁻¹ and 20 g KH₂PO₄ L⁻¹. The feeding and recirculation
operations were performed through a peristaltic pump (323, Watson Marlow, England).
140 Recirculation was carried out at the lowest attainable speed (30 rpm, 0.1 L h⁻¹).

142 **PLEASE INSERT FIGURE-1 HERE**

144 Three different feeding strategies were applied varying HRT and sucrose concentration to
evaluate biohydrogen production: first strategy (24h/31c) was a 24 h HRT regime fed daily
146 with 400 mL at 31 g_{sucrose} L⁻¹, second strategy (12h/19c) was a 12 h HRT regime fed daily
with 800 mL at 19 g_{sucrose} L⁻¹; third strategy (4h/8c) was a 4 h HRT continuous regime fed
148 with 2400 mL d⁻¹ at 8.3 g_{sucrose} L⁻¹. The corresponding OLR for each strategy were 31, 38
and 49.8 g (L d)⁻¹. For the 24h/31c and 12h/19c strategies, due to specifications of the
150 peristaltic pump, feeding was performed daily during 4 and 8 h of operation, respectively.
The feeding was prepared mixing 40 mL mineral medium and 160 mL phosphate buffer,
152 completing total volume with distilled water if needed. After completing the feeding, the
reactors contents were homogenized by recirculation during 5 min at maximum speed of the
154 peristaltic pump.

156 **2.3. Analytical methods**

The pH was determined with a pH-meter (PC18, Conductronic, Mexico), whereas biogas
158 production was measured by hydraulic displacement (3 L column) method everyday using
NaCl saturated and pH 3.0 acidified (sulfuric acid) solution to inhibit microbial growth and to
160 avoid CO₂ dissolution. Hydrogen composition was measured by absorption in NaOH (4N)
and gas chromatography. The biogas and hydrogen volumes were normalized to 273.15 K
162 and 101.3 KPa. The gas chromatograph (GC-15A, Shimadzu Corporation, Kyoto, Japan)
was equipped with thermal conductivity detector and a Porapak N column. The oven, injector
164 and detector temperatures were 35, 150 and 150 °C, respectively. Helium was used as
carrier gas. The total volatile fatty acids (TVFA) concentration was determined by Anderson
166 et al. [38] titration method. VFA profile of the liquid phase was determined by a gas
chromatograph (Thermo Scientific Trace 1310) equipped with a capillary column (Nukol, 30
168 m x 0.25mm, Fused silica, Supelco, USA) and flame ionized detector (FID). For both the
injector and the detector the temperature was set at 250 °C. The oven was heated at 120 °C
170 for 1 min, followed by heating to 140°C at 20 °C min⁻¹ and kept at this temperature for 1 min.
Finally, the oven was heated from 14 °C to 240 °C at heating rate of 40 °C min⁻¹. Previously,
172 the samples were centrifuged (10,000 rpm, 5 min) and filtrated (0.45 µm). Sucrose
concentration was determined by total sugars method with sulfuric acid and phenol
174 compared against a calibration curve obtained using dextrose [39].

176 **2.4. Calculations**

Concentration of total sugars during feeding for each HRT were correlated through linear
178 regression and expressed through Eq. 1.

$$Y = b_0 + b_1X \quad (1)$$

180 Where Y denotes the total sugars (g L^{-1}), X the time during feeding (h), b_0 and b_1 are the
linear regression coefficients.

182 For the energy evaluation two variables were defined: energy heating losses (EHL) and
gross energetic potential rate (EPR_{gross}). According to Eq. 2, EHL –expressed as kJ (L d)^{-1} –
184 1 – is an intensive property equivalent to the energy consumed for heating a liter of influent
to the continuous reactor during one average day of operation, C_p is the specific heat at
186 constant pressure for the medium considered, which for practical reasons was stated to be
the same as water ($4.184 \text{ kJ (kg } ^\circ\text{C)}^{-1}$), ΔT is the difference of temperature between
188 operation temperature and a reference temperature ($21 \text{ } ^\circ\text{C}$), HRT is the hydraulic retention
time expressed in days, ρ is the density of the influent (considered the same as water at 20
190 $^\circ\text{C}$, 0.9982 kg L^{-1}).

$$EHL = \frac{C_p \Delta T}{HRT} * \rho \quad (2)$$

192 According to Eq. 3, EPR_{gross} –expressed as kJ (L d)^{-1} – is the energy potential of hydrogen
produced from a liter of operation volume of the continuous reactor in one average day
194 [25,40]. Eq. 3 was calculated as the product of the hydrogen production rate (HPR , in NL H_2
(L d^{-1}), the higher heating value of hydrogen ($HHV_{H_2} = 282.8 \text{ kJ mol}^{-1}$) and the factor for
196 converting NL H_2 to mol H_2 (1 mol H_2 is equivalent to 22.4 L H_2 according to ideal gas law
for 273.15 K and 1 atm).

$$198 \quad EPR = HPR * HHV_{H_2} * \frac{1 \text{ mol}_{H_2}}{22.4 \text{ L}} \quad (3)$$

200 3. Results and discussion

The UASB reactor was operated for 70 days at room temperature (21 ± 2 °C) at different
202 HRT of 24, 12 and 4 h, and sugar concentrations of 12.4, 15.2 and 19.92 $\text{g}_{\text{sucrose}} \text{d}^{-1}$,
respectively. The best operation conditions were 12h/19c in terms of hydrogen yield (HY,
204 $70.6 \pm 15.31 \text{ NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$) and 4h/8c in terms of hydrogen production ($\text{HPR}_{\text{avg}} = 1270$
 $\pm 177 \text{ NmL H}_2 \text{ d}^{-1}$, $\text{HPR}_{\text{max}} = 1552 \text{ NmL H}_2 \text{ d}^{-1}$), whereas the lowest results corresponded to
206 strategy 24h/31c. The intervals of yield in each strategy were 0-35.5 $\text{NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$, 36.0-
97.8 $\text{NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$ and 50.3-77.6 $\text{NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$ for 24h/31c, 12h/19c and 4h/8c,
208 respectively. The average yield during the whole operation was $62.6 \pm 20.5 \text{ NmL H}_2 \text{ g}_{\text{sucrose}}^{-1}$
($0.88 \text{ mol H}_2 \text{ mol}_{\text{sucrose}}^{-1}$) and average hydrogen content was $69.04 \pm 16.65\%$. The total
210 volume of accumulated biogas after the 70 days operation was 100.83 NL (65.44 NL H_2).

In Fig. 2a the performance of the UASB reactor in terms of daily hydrogen production is
212 observed, whereas the pH and TVFA variations during the operation of the UASB are found
in Fig. 2b. The pH in the reactor was recorded twice a day: the first previous to its daily
214 feeding (pH_r) and the second after it was completed and its contents were homogenized
(pH_{adj}).

216 The first strategy (24h/31c) began after a heat-shock pretreatment of the microbial
granules, which were previously acclimated to sucrose during 20 d [24]. It can be seen that
218 despite previous acclimatization to the substrate, the reactor presented a three-day lag
phase, most likely due to germination of the spore-forming microbes, which may take from
220 60 minutes when fermentation occurs at proper pH, temperature, nutrients condition, to
several days when operation is performed at not optimal germination conditions [32,41].

222 Another possibility is the adaptation phenomena of biomass to psychrophilic temperature.
Anaerobic bacteria may adapt easily to low temperatures, and high-rate anaerobic treatment
224 has been achieved at psychrophilic conditions [42]. Afterwards, the hydrogen production
during 24h/31c raised up to 382.8 NmL H₂ d⁻¹ at the end of this strategy. It is noteworthy that
226 pH varied before and after feedings (Fig. 2): pH_r was 5.8-5.3 whereas pH_{adj} was 6.5-6.3.
This was indicative of a fermentative activity that was in accordance to high TVFAs contents;
228 the TVFAs exhaust the buffering capacity of the phosphates buffer (alkalinity), which is 6.71-
7.71 according to Hendersson-Hasselbach equation. Indeed, adequate pH for hydrogen
230 production has been reported in the range 4.5 to 7, depending on substrate, operation
temperature, inoculum and reactor type [15]. The TVFA concentration showed slight
232 variations according to the lag-phase presented in the beginning of the operation; the
minimum TVFA concentration was 5800 mg L⁻¹ at day 6, and from that day on the
234 concentration of VFA increased continuously. The variations in hydrogen production during
days 5 to 11 were lower than 15%, indicating that the reactor achieved the pseudo steady-
236 state. According to Sivagurunathan et al. [43] such variations in time are considered to be
acceptable before attempting a change in feed strategy, or a change in HRT and sucrose
238 concentration as in our case. Hereinafter the second strategy 12h/19c was applied.

At the beginning of 12h/19c the biogas production was constantly increasing showing a
240 six cycles wave-like performance (at 19, 25, 32, 40, 45 and 55 h) reaching a highest of 1466
NmL H₂ d⁻¹ at day 47. At this point HY reached its maximum value: 97.8 Nm H₂ g_{sucrose}⁻¹.
242 Afterwards, production diminished to a minimum of 920 NmL H₂ d⁻¹ (day 53), following a
new oscillation. This reduction in hydrogen production was corresponded by a reduction in
244 the pH_{adj}, which fell from 6.5 to 5.5. TVFA profile presented a slightly increasingly oscillations

during this strategy. Indeed, 89% of the operation in 12h/19c production fluctuated between
246 800-1470 NmL H₂ d⁻¹. The average molar hydrogen yield in this strategy was 0.685 mol H₂
mol_{hexose}⁻¹. which was a value much below than the theoretical maximum from concomitant
248 acetic and butyric fermentation of 3 mol H₂ mol_{hexose}⁻¹, or 6 mol H₂ mol_{sucrose}⁻¹, considering
that half a mol of hexose undergoes acetic fermentation whereas the other half mol
250 undergoes butyric fermentation. When low hydrogen yields are obtained it could be ascribed
to part of the substrate being used to microbial growth and maintenance [44], or more likely
252 to a fermentative deviation [14]; for instance, at lower pH values the abundance of lactic acid
bacteria is acknowledged, which compete with the hydrogenogenic microorganisms for the
254 carbon source and even alter the redox balance. In our case it is noteworthy that the most
abundant metabolite was butyric acid (5.25-7.03 g L⁻¹), which was in accordance with lower
256 hydrogen yields (theoretical HY is 2 mol H₂ mol_{hexose}⁻¹ in butyric fermentation, against 4 mol
H₂ mol_{hexose}⁻¹ in acetic fermentation). Indeed, our HY was in the interval of reported In Table
258 3 (0.5-1.47 mol H₂ mol_{hexose}⁻¹).

In strategy 4h/8c, biogas production ranged from 1006 to 1550 NmL H₂ d⁻¹, the highest
260 production attained during the whole operation of the UASB reactor, which occurred at day
65. Due to the continuous operation followed in this strategy, no difference could be
262 represented as pH_r and pH_{adj} in Fig. 2, therefore only one measurement of the pH was
performed. The pH presented decreasing values from 5.5 down to a minimum of 4.9;
264 concomitantly, TVFA increased up to the maximum for the whole operation (7500 mg L⁻¹).
Chang and Lin [24] suggested this effect to be expectable considering that as HRT is
266 reduced, acid fermentation is fostered causing an accumulation in VFA that consequently

decreases alkalinity and pH. In their work, this led to TVFA of 1,083 mg L⁻¹, yield of 100 mL
268 H₂ (g_{sucrose} d)⁻¹ and hydrogen composition of ca. 20% at a HRT of 4 h.

The pH effects in hydrogen production are undeniable yet there is no consensus on
270 establishing an optimal fixed value. Our highest HPR 3625 and 3750 NL H₂ (L d)⁻¹ were found
at days 45 and 65, with corresponding pH of 5.15 and 5.1. In contrast, Mu et al. [45] studied
272 the role of pH in the fermentative H₂ production from an acidogenic granule-based reactor,
finding that at pH 4.2 their HPR_{max} and HY_{max} were 3.48 L H₂ (L d)⁻¹ and 1.61 mol H₂ mol_{hexose}⁻¹
274 ¹, respectively. However, operating at low pH is risky since iron containing hydrogenases
may be affected [46].

276

PLEASE INSERT FIGURE-2 HERE

278

Some authors have assayed low HRT, such as Chang and Lin [24] or Si et al. [23].
280 Chang and Lin [24] in a UASB fed with sucrose (20 g L⁻¹) found that hydrogen production
depended greatly on the HRT changes evaluated from 24 to 4 h. They reported that at 24
282 and 4 h HRT the hydrogen production was drastically affected, whereas at 8 h HRT they
obtained their HPR rate (6.84 L (L d)⁻¹) with an average hydrogen content of 32% (v/v). Si
284 et al. [23] in a UASB reactor using glucose as carbon source and carbon nanotubes for
supporting the biomass, also found low hydrogen production at 24 h HRT, whereas at 8 and
286 4 h they achieved the highest productions (4.38 L (L d)⁻¹).

In Fig. 3 it is shown the total sugars consumption at HRT 24, 12, and 4 h during their
288 first 10 h after feeding as a depiction of microbial activity. For 24 and 12 h HRT the sucrose
concentration was increasing over time pointing to an accumulation substrate. For 4 h HRT,
290 the profile shown in this figure was obtained after beginning the strategy 4h/8c, showing that

sucrose concentration continuously decreased (a deficit of substrate), an indicative of a
292 higher consumption rate of the sugars. According to Eker et al. [47] at low initial sugars
concentration (3.84 to 18.9 g L⁻¹) its consumption is faster. The initial sugar concentration at
294 4, 12 and 24 h HRT was 8.3, 19 and 37 g_{sucrose} L⁻¹. The profiles of each strategy were fitted
to a linear regression model, showing that 24 and 4 h HRT had a characteristic linear
296 performance ($R^2 = 0.96$ and 0.87 , respectively), whereas at 12 h HRT deviated from linear
behavior ($R^2 = 0.6$).

298 **PLEASE INSERT FIGURE 3 HERE**

Our average results in terms of HY, molar hydrogen yield (MHY), HPR and metabolites
300 found are listed in Table 2; the best results for HY and MHY were at 12h/19c. Even though
the 4h/8c strategy had the best average HPR, the average of the strategy 12h/19c included
302 a period of 10 days where the HPR was beginning to rise after the low results from 24h/31c,
which lowered the average value.

304 In Table 3, the best results from our 12h/19c strategy are shown along with selected
results from literature works using continuous reactors, mainly UASB, and soluble
306 substrates such as sucrose, glucose or cheese whey as substrate. The best HPR were
reported to be either at 6 or 8 h HRT, whereas at 12 h HRT also good results were obtained.
308 For instance, Si et al. [23] reported that at 12 and 4 h HRT their HPR were 2.88 and 5.28
NL H₂ (L d)⁻¹, respectively. The highest HPR from 12h/19c (3.66 NL H₂ (L d)⁻¹) was
310 comparable to those from literature (2.35 – 4 NL H₂ (L d)⁻¹) at similar HRT than ours.
Regarding HY, it varied in the range 0.5 – 1.47 mol H₂ mol_{hexose}⁻¹ in mesophilic, thermophilic
312 and hyperthermophilic regimes (35, 38, 55 or 70 °C). Our HY (0.685 mol H₂ mol_{hexose}⁻¹)
obtained at psychrophilic temperature was close to the minimal value of the precedent

314 ranges and no real stabilization of HY was obtained during the experimental sequence.
Differences may be ascribed mainly to operation temperature and inoculum type.

316 When comparing the results of 12h/19c against reports at psychrophilic and ambient
temperatures (Table 3), it is worth noticing that our HPR was higher. The closest to ours
318 was that obtained by Moureira et al. [48] in a packed bed reactor using 2 g COD L⁻¹ sucrose
as substrate. In contrast, the highest HY (0.918 mol H₂ mol_{hexose}⁻¹) in similar experiments
320 was that reported by Muñoz-Páez et al. [25] using an anaerobic fluidized bed reactor,
compared the hydrogen production from sucrose at 8 g (L d)⁻¹ using anaerobic fluidized bed
322 reactor (AFBR) at ambient temperature (<30 °C). When comparing their own results against
mesophilic operation, they found that HPR at ambient temperature was nearly 2.2-fold (920
324 NmL H₂ (l_{bed} d)⁻¹) than mesophilic operation. Other substrate that has shown good results
was cheese whey at 20 g COD (L d)⁻¹, that Carrillo-Reyes et al. [49] used in UASB reactors,
326 obtaining HPR 1.41 NL H₂ (L d)⁻¹ and HY of 0.56 mol H₂ mol_{hexose}⁻¹ at 24 h HRT. Among the
reasons of our highest results may dwell in the differences of sludge pretreatment, HRT
328 used and reactor configuration [9,50]. For instance, Carrillo-Reyes et al. [49] disaggregated
and heat-treated an anaerobic granular sludge; according to Show et al. [51], hydrogen
330 production by granular sludge exhibits very high HPR at low HRT (up to ten-fold) than
disaggregated sludge.

332 Selected results from Table 3 were used for calculating the *EHL* (Eq. 2) and the *EPR_{gross}*
(Eq. 3); the results are shown in Fig. 4. It was observed that all the works using temperature
334 for maintaining mesophilic, thermophilic or hyperthermophilic regimes presented higher *EHL*
than *EPR_{gross}*, except for those reports using psychrophilic or room temperature regimes
336 despite not having the highest *EPR_{gross}*. According to Eq. 3, the *EHL* rises as temperatures

of operation increases and HRT decreases, which was particularly evident for the works of
338 Tähti et al. [52], Braga et al. [53] and Yu and Mu. [54], who applied high temperatures (70,
55 and 38 °C, respectively) and short HRT (5, 2 and 6 h, respectively). This remarks the
340 importance that if biohydrogen production systems are to be applied at industrial scale, it is
not only important to maximize the yields and production rates, but also to decrease energy
342 expenses. In this sense, the psychrophilic systems may have a real opportunity of
development despite the lower hydrogen production when compared to mesophilic,
344 thermophilic or hyperthermophilic systems. Perera et al. [55] arrived to similar observations
than ours, arguing that the improvement in hydrogen production at temperatures above
346 25 °C is not defensible as net energy gains were negative and declining as temperature
increased. Moreover, other strategies to increase the energetic gains of psychrophilic
348 biohydrogen production would be by its coupling to other bioenergy processes such as
microbial fuel cells, photofermentation and anaerobic digestion, among others [56–59].

350

PLEASE INSERT TABLE 2 HERE

352

PLEASE INSERT TABLE 3 HERE

354

PLEASE INSERT FIGURE 4 HERE

356

4. Conclusion

358 Semi-continuous and continuous feeding of sucrose in an UASB reactor operated at
psychrophilic temperature achieved considerably hydrogen generation and accumulation of
360 TVFAs. The best operation strategy was 12h/19c since it presented comparable results to
those in literature that were obtained at mesophilic temperatures and similar HRT. Using
362 HRT of 24 h gave low hydrogen production, whereas 4 h HRT presented higher production

at the expense of lower hydrogen yields, which is undesirable when pure substances such
364 as sucrose or glucose are used as substrate.

It is noteworthy that biohydrogen systems operating at psychrophilic or ambient
366 temperature would not require energy expenditures for the heating of the influents, which
would thus present an opportunity for achieving net energy gains.

368

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374

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574 Fig. 1. Schematic diagram of the experimental apparatus.

576 Fig. 2. UASB reactor performance (A) HRT and normalized hydrogen production versus time, (B) pH and VFAs versus time.

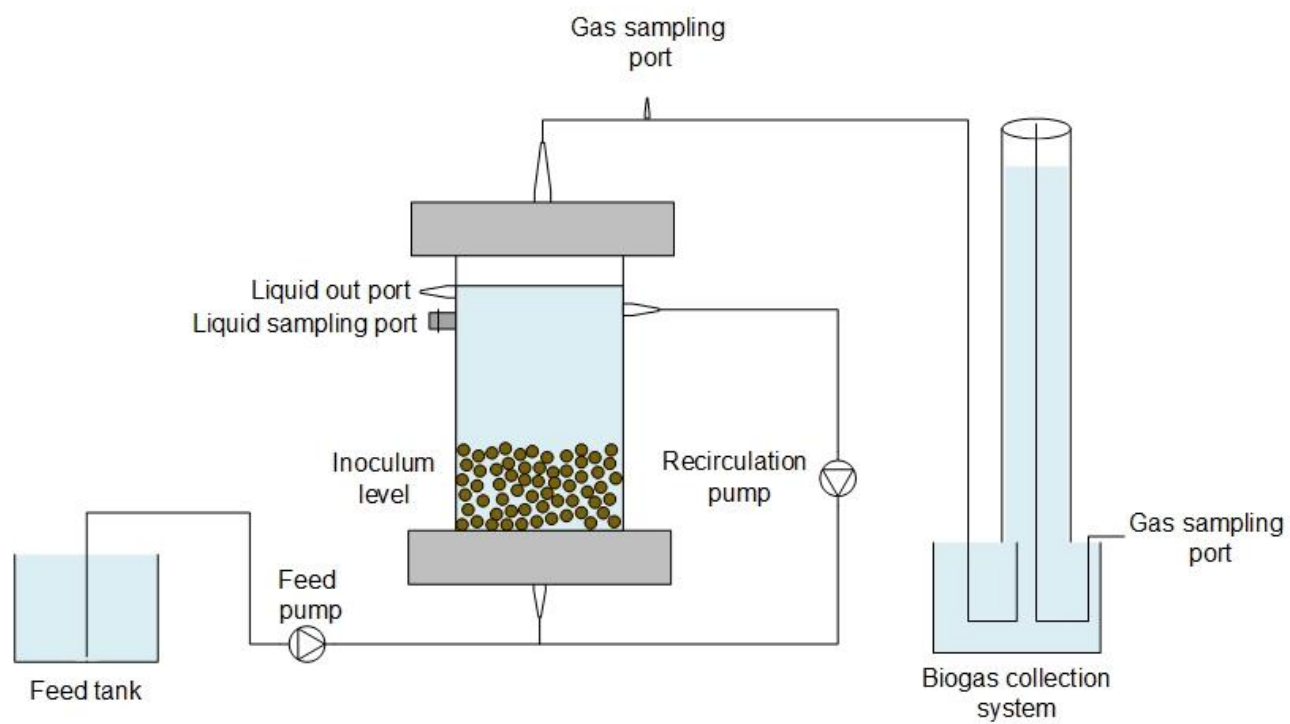
578 Fig. 3. Influence of HRT on total sugar concentration in function on time.

580 Fig. 4. Gross energetic potential rate and energy heating loss expenditures of selected references.

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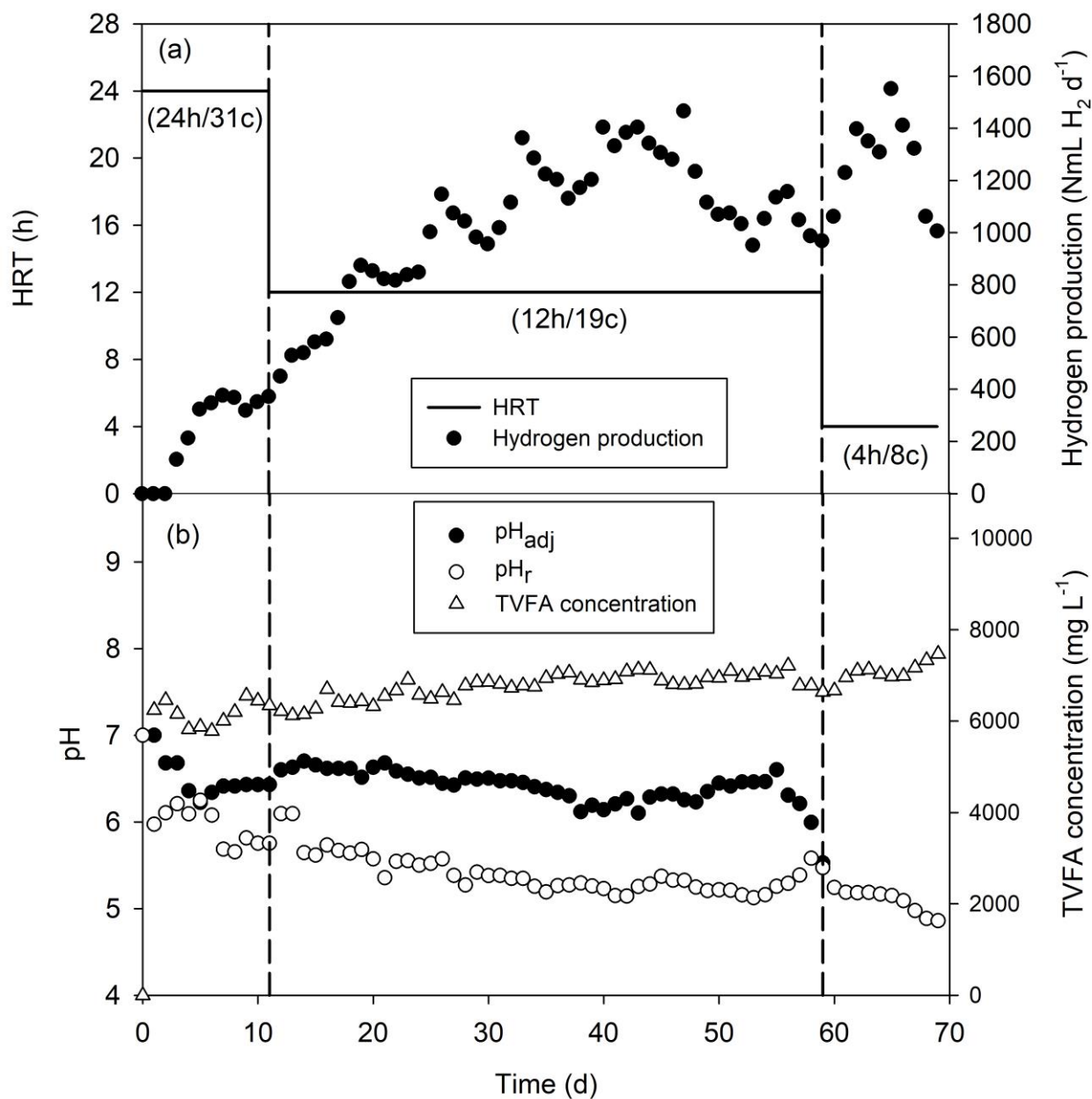
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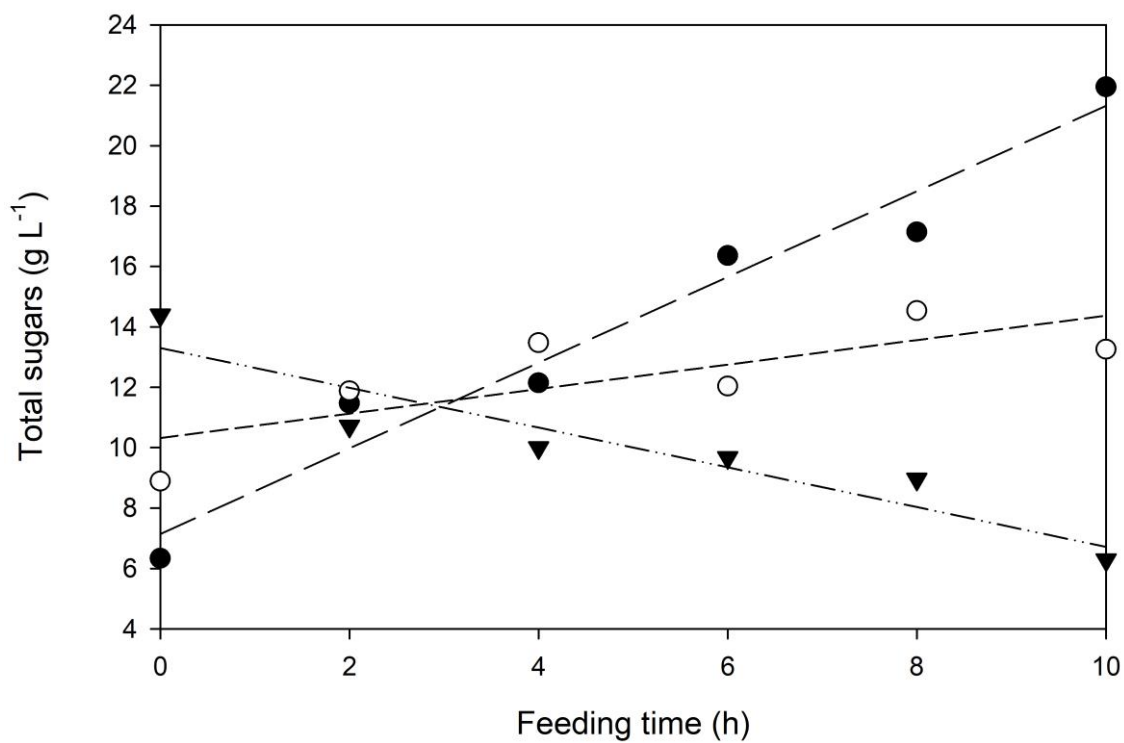
Fig. 1. Schematic diagram of the experimental apparatus.

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594 Fig. 2. UASB reactor performance (A) HRT and normalized hydrogen production versus time,
 (B) pH and TVFAs versus time. 24h/31c operated at 24 h HRT fed with 31 g_{sucrose} L⁻¹; 12h/19c
 596 operated at 12 h HRT fed with 19 g_{sucrose} L⁻¹; 4h/8c operated at 4 h HRT fed with 8.3 g_{sucrose}
 L⁻¹.



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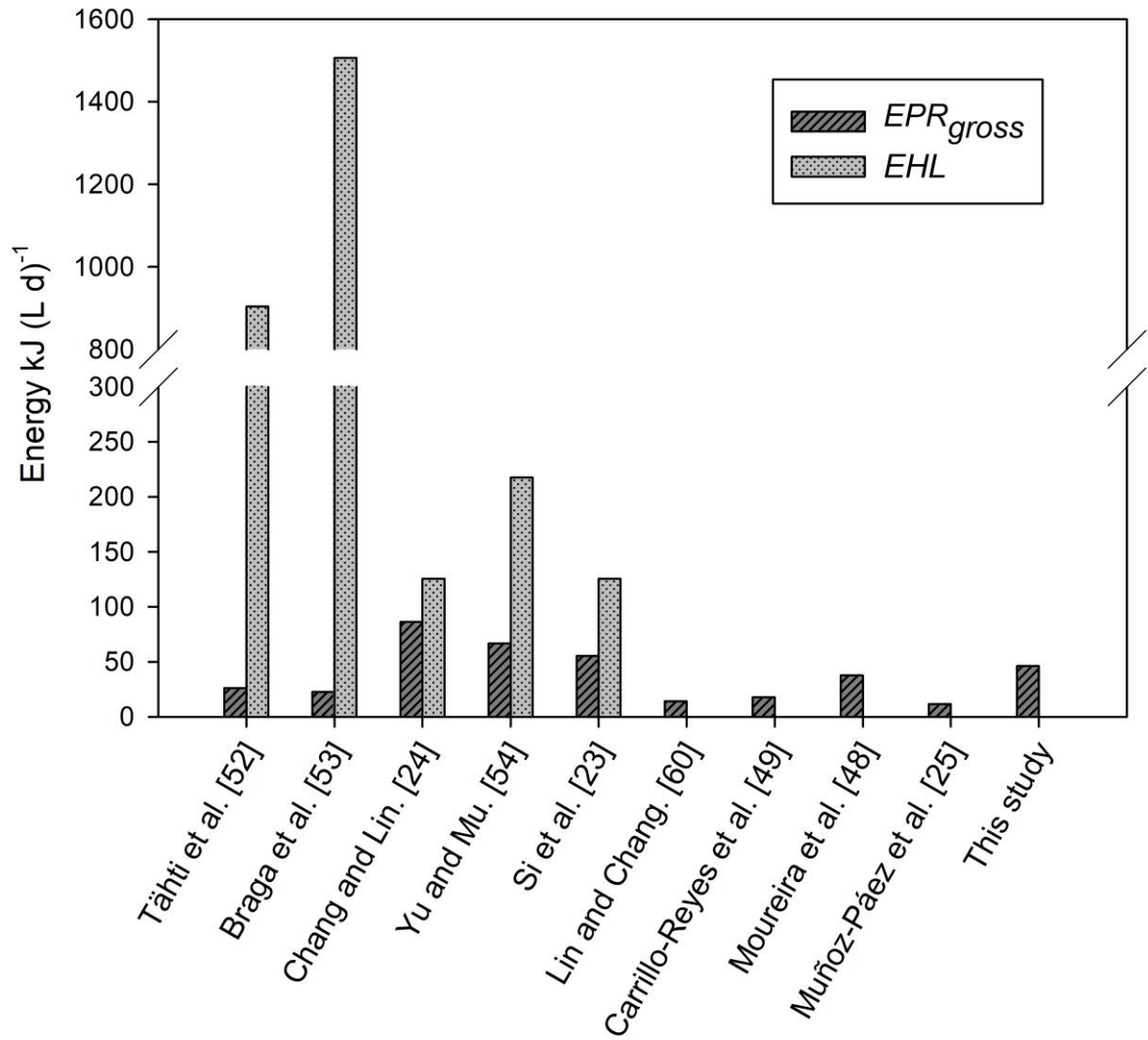
602 Fig. 3. Influence of HRT on total sugar concentration in function on time. Linear regression
604 curves were represented by dashed lines. (●) 24h/31c, $Y = 7.14 + 1.41X$, $R^2 = 0.96$; (○)
604 12h/19c, $Y = 10.32 + 0.40X$, $R^2 = 0.60$; (▼) 4h/8c, $Y = 13.29 - 0.66X$, $R^2 = 0.87$.

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614 Fig. 4. Gross energetic potential rate and energy heating loss expenditures of selected
 616 references.

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626 temperatures: psychrophilic (< 25°C), mesophilic, thermophilic and hyperthermophilic
conditions.

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Table 1. Characteristics of inoculum.

Parameter	
pH	6.66 ± 0.10
Humidity (% _{wb})	93.80 ± 0.07
Total solids (% _{wb})	6.20 ± 0.07
Volatile solids (% _{db})	34.82 ± 3.59
Ash (% _{db})	65.18 ± 3.59

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Notes: wb, wet basis; db, dry basis

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Table 2. Average results and soluble metabolites produced by different strategies.

Strategies	HY (NmL H ₂ g _{sucrose} ⁻¹)	MHY (mol H ₂ mol _{sucrose} ⁻¹)	HPR (NL H ₂ (L d) ⁻¹)	Soluble metabolites (g L ⁻¹)					
				EtOH	HAc	HPr	HBu	SM	TVFA
24h/31c	23.9	0.18	0.67	0.47	1.65	0.02	4.89	7.03	6.18
12h/19c	69.1	0.51	2.60	0.13	1.41	0.03	3.68	5.25	6.81
4h/8c	63.5	0.48	3.17	0.26	1.55	0.06	4.67	6.54	7.08

640 **Notes:** HY, hydrogen yield; MHY, molar hydrogen yield; HPR, hydrogen production rate; EtOH, ethanol;
642 HAc, acetic acid; HPr, propionic acid; HBu, butyric acid; SM, soluble metabolites
(EtOH+HAc+HPr+HBu).

Table 3. Biohydrogen production in continuous reactors operating at different temperatures: psychrophilic (< 25°C), mesophilic, thermophilic and hyperthermophilic conditions.

Substrate (concentration/OLR)	Inoculum	Reactor type	HRT/ T _{op}	HY/ [H ₂] _{avg} in biogas	HPR	Ref
Glucose (5 g COD L ⁻¹ / 25.1 g COD (L d) ⁻¹)	Granular sludge	UASB	5 h 70 °C	0.73 mol H ₂ mol _{hexose} ⁻¹ 51%	2.07 NL H ₂ (L d) ⁻¹	[52]
Sucrose (1.8 g COD L ⁻¹ / 24 g COD (L d) ⁻¹)	Granular sludge	UASB	2 h 55 °C	0.865 mol H ₂ mol _{hexose} ⁻¹ 51.49%	1.8 NL H ₂ (L d) ⁻¹	[53]
Sucrose (20 g COD L ⁻¹ / 60 g COD (L d) ⁻¹)	Sewage sludge	UASB	8 h 35 °C	0.75 mol H ₂ mol _{hexose} ⁻¹ 42.4%	6.84 NL H ₂ (L d) ⁻¹	[24]
(20 g COD L ⁻¹ / 40 g COD (L d) ⁻¹)			12 h 35 °C	0.81 mol H ₂ mol _{hexose} ⁻¹ NR	4 NL H ₂ (L d) ⁻¹	
Sucrose (10.67 g COD L ⁻¹ / 42.68 g COD (L d) ⁻¹)	Anaerobic sludge	UASB	6 h 38 °C	0.5 mol H ₂ mol _{hexose} ⁻¹ NR	5.28 NL H ₂ (L d) ⁻¹	[54]
(10.67 g COD L ⁻¹ / 25.61 g COD (L d) ⁻¹)			10 h 38 °C	0.9 mol H ₂ mol _{hexose} ⁻¹ NR	2.88 NL H ₂ (L d) ⁻¹	
Glucose (8 g L ⁻¹ / 24 g (L d) ⁻¹)	Anaerobic sludge supported on carbon nanotubes	UASB	8 h 35 °C	1.47 mol H ₂ mol _{hexose} ⁻¹ 46%	4.38 NL H ₂ (L d) ⁻¹	[23]
(8 g L ⁻¹ / 16 g (L d) ⁻¹)			12 h 35 °C	1.45 mol H ₂ mol _{hexose} ⁻¹ 38%	3 NL H ₂ (L d) ⁻¹	
Glucose (NR/ 12.46 g (L d) ⁻¹)	Sewage sludge	Chemostat- type anaerobic reactor	1.5 ^a 24-28 °C	0.68 mol H ₂ mol _{hexose} ⁻¹ 45.1%	1.13 NL H ₂ (L d) ⁻¹	[60]
Cheese whey (NR/ 20 g COD (L d) ⁻¹)	Anaerobic granular sludge	UASB	24 h 22-25 °C	0.56 ^b mol H ₂ mol _{hexose} ⁻¹ 76%	1.41 NL H ₂ (L d) ⁻¹	[49]

Sucrose (2 g COD L ⁻¹ / NR)	Natural fermentation inoculation	PBR ^c	2 h 25 °C	0.7 mol H ₂ mol _{hexose} ⁻¹ 75.6%	2.99 NL H ₂ (L d) ⁻¹	[48]
Sucrose (8 g L ⁻¹ / NR)	Methanogenic digestates	AFBR ^d	24 h 30 °C	0.918 mol H ₂ mol _{hexose} ⁻¹ 56%	0.92 NL H ₂ (L d) ⁻¹	[25]
Sucrose (19 g L ⁻¹ / 38 g (L d) ⁻¹)	Brewery industry anaerobic sludge	UASB	12 h 21 ± 2 °C	0.685 mol H ₂ mol _{hexose} ⁻¹ 69.04%	3.66 NL H ₂ (L d) ⁻¹	This study

646 Notes: ^asolid retention time (HRT equivalent 28.8 h); ^b calculated as hexose equivalent; PBR, packed bed reactor; AFBR,
648 anaerobic fluidized bed reactors; [H₂]_{avg}, hydrogen content in biogas; COD, chemical oxygen demand; HPR, hydrogen
production rate; HRT, hydraulic retention time; HY, hydrogen yield; NR, not reported; Top, temperature of operation.

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