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2        **Evaluation of feeding strategies in upflow anaerobic sludge bed reactor for**  
4        **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  
28 sludge blanket reactor type (UASB) operating at psychrophilic temperature ( $21 \pm 2$  °C) at  
30 different feeding strategies varying hydraulic retention times (HRT) and sucrose  
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  
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<sub>2</sub>. The average HY for the  
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  
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 ( $2.6 \text{ L (L d)}^{-1}$ ) to that obtained in 4h/8c strategy ( $3.17 \text{ L (L d)}^{-1}$ ).  
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.  
Indeed, psychrophilic or room temperatures should be broadly regarded as an effective  
alternative towards net energy gains in biohydrogen production.

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

42

# Evaluation of feeding strategies in upflow anaerobic sludge bed reactor for

44

## 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 CO<sub>2</sub> 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-  
SO<sub>3</sub><sup>2-</sup> L<sup>-1</sup> [6], and NO<sub>x</sub> 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**

### **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 ( $21 \pm 2$  °C). The initial amounts of inoculum, mineral medium and phosphate

134 buffer solution were 77 g<sub>wb</sub>, 40 mL and 283 mL, respectively; consequently, the  
concentration of the inoculum in terms of VS was 4155 mg L<sup>-1</sup>. The mineral medium  
136 composition was [37] (mg L<sup>-1</sup>): ZnCl<sub>2</sub>, 75; MgCl<sub>2</sub>·6H<sub>2</sub>O, 100; MnCl<sub>2</sub>·4H<sub>2</sub>O, 10.77; FeCl<sub>3</sub>·6H<sub>2</sub>O,  
25.97; CuCl<sub>2</sub>·2H<sub>2</sub>O, 3.41; and NiCl<sub>2</sub>·6H<sub>2</sub>O, 101.25. A phosphate buffer (pH = 7.28) was  
138 prepared using 30 g K<sub>2</sub>HPO<sub>4</sub> L<sup>-1</sup> and 20 g KH<sub>2</sub>PO<sub>4</sub> L<sup>-1</sup>. 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<sup>-1</sup>).

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<sub>sucrose</sub> L<sup>-1</sup>, second strategy (12h/19c) was a 12 h HRT regime fed daily  
with 800 mL at 19 g<sub>sucrose</sub> L<sup>-1</sup>; third strategy (4h/8c) was a 4 h HRT continuous regime fed  
148 with 2400 mL d<sup>-1</sup> at 8.3 g<sub>sucrose</sub> L<sup>-1</sup>. The corresponding OLR for each strategy were 31, 38  
and 49.8 g (L d)<sup>-1</sup>. 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 CO<sub>2</sub> 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<sup>-1</sup> 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<sup>-1</sup>. 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 ( $\text{g L}^{-1}$ ),  $X$  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  $\text{kJ (L d)}^{-1}$ –  
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 ( $4.184 \text{ kJ (kg } ^\circ\text{C)}^{-1}$ ),  $\Delta T$  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,  $\rho$  is the density of the influent (considered the same as water at 20  
190  $^\circ\text{C}$ ,  $0.9982 \text{ kg L}^{-1}$ ).

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

192 According to Eq. 3,  $EPR_{gross}$  –expressed as  $\text{kJ (L d)}^{-1}$ – 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  $\text{NL H}_2$   
( $\text{L d}^{-1}$ ), the higher heating value of hydrogen ( $HHV_{H_2} = 282.8 \text{ kJ mol}^{-1}$ ) and the factor for  
196 converting  $\text{NL H}_2$  to  $\text{mol H}_2$  (1 mol  $\text{H}_2$  is equivalent to 22.4 L  $\text{H}_2$  according to ideal gas law  
for 273.15 K and 1 atm).

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

### 200 3. Results and discussion

The UASB reactor was operated for 70 days at room temperature ( $21 \pm 2$  °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  $\text{H}_2$ ).

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 ( $\text{pH}_r$ ) and the second after it was completed and its contents were homogenized  
( $\text{pH}_{\text{adj}}$ ).

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 H<sub>2</sub> d<sup>-1</sup> at the end of this strategy. It is noteworthy that  
226 pH varied before and after feedings (Fig. 2): pH<sub>r</sub> was 5.8-5.3 whereas pH<sub>adj</sub> 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<sup>-1</sup> 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 H<sub>2</sub> d<sup>-1</sup> at day 47. At this point HY reached its maximum value: 97.8 Nm H<sub>2</sub> g<sub>sucrose</sub><sup>-1</sup>.  
242 Afterwards, production diminished to a minimum of 920 NmL H<sub>2</sub> d<sup>-1</sup> (day 53), following a  
new oscillation. This reduction in hydrogen production was corresponded by a reduction in  
244 the pH<sub>adj</sub>, 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 H<sub>2</sub> d<sup>-1</sup>. The average molar hydrogen yield in this strategy was 0.685 mol H<sub>2</sub>  
mol<sub>hexose</sub><sup>-1</sup>. which was a value much below than the theoretical maximum from concomitant  
248 acetic and butyric fermentation of 3 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup>, or 6 mol H<sub>2</sub> mol<sub>sucrose</sub><sup>-1</sup>, 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<sup>-1</sup>), which was in accordance with lower  
256 hydrogen yields (theoretical HY is 2 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup> in butyric fermentation, against 4 mol  
H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup> in acetic fermentation). Indeed, our HY was in the interval of reported In Table  
258 3 (0.5-1.47 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup>).

In strategy 4h/8c, biogas production ranged from 1006 to 1550 NmL H<sub>2</sub> d<sup>-1</sup>, 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<sub>r</sub> and pH<sub>adj</sub> 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<sup>-1</sup>).  
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<sup>-1</sup>, yield of 100 mL  
268 H<sub>2</sub> (g<sub>sucrose</sub> d)<sup>-1</sup> 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 H<sub>2</sub> (L d)<sup>-1</sup> 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 H<sub>2</sub> production from an acidogenic granule-based reactor,  
finding that at pH 4.2 their HPR<sub>max</sub> and HY<sub>max</sub> were 3.48 L H<sub>2</sub> (L d)<sup>-1</sup> and 1.61 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup>  
274 <sup>1</sup>, 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<sup>-1</sup>) 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)<sup>-1</sup>) 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)<sup>-1</sup>).

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<sup>-1</sup>) its consumption is faster. The initial sugar concentration at  
294 4, 12 and 24 h HRT was 8.3, 19 and 37 g<sub>sucrose</sub> L<sup>-1</sup>. 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 H<sub>2</sub> (L d)<sup>-1</sup>, respectively. The highest HPR from 12h/19c (3.66 NL H<sub>2</sub> (L d)<sup>-1</sup>) was  
310 comparable to those from literature (2.35 – 4 NL H<sub>2</sub> (L d)<sup>-1</sup>) at similar HRT than ours.  
Regarding HY, it varied in the range 0.5 – 1.47 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup> in mesophilic, thermophilic  
312 and hyperthermophilic regimes (35, 38, 55 or 70 °C). Our HY (0.685 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup>)  
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<sup>-1</sup> sucrose  
as substrate. In contrast, the highest HY (0.918 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup>) 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)<sup>-1</sup> 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 H<sub>2</sub> (l<sub>bed</sub> d)<sup>-1</sup>) than mesophilic operation. Other substrate that has shown good results  
was cheese whey at 20 g COD (L d)<sup>-1</sup>, that Carrillo-Reyes et al. [49] used in UASB reactors,  
326 obtaining HPR 1.41 NL H<sub>2</sub> (L d)<sup>-1</sup> and HY of 0.56 mol H<sub>2</sub> mol<sub>hexose</sub><sup>-1</sup> 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<sub>gross</sub>*  
(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<sub>gross</sub>*, except for those reports using psychrophilic or room temperature regimes  
336 despite not having the highest *EPR<sub>gross</sub>*. 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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576 Fig. 2. UASB reactor performance (A) HRT and normalized hydrogen production versus time, (B) pH and VFAs versus time.

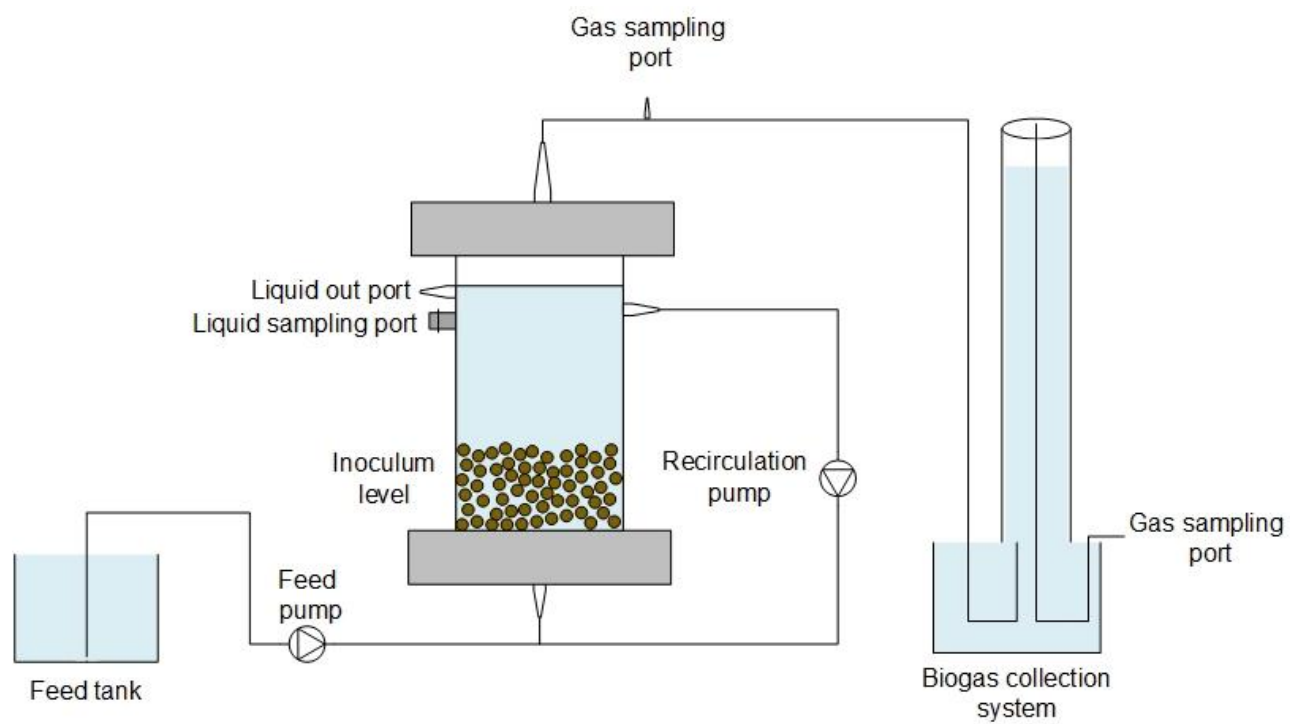
578 Fig. 3. Influence of HRT on total sugar concentration in function on time.

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

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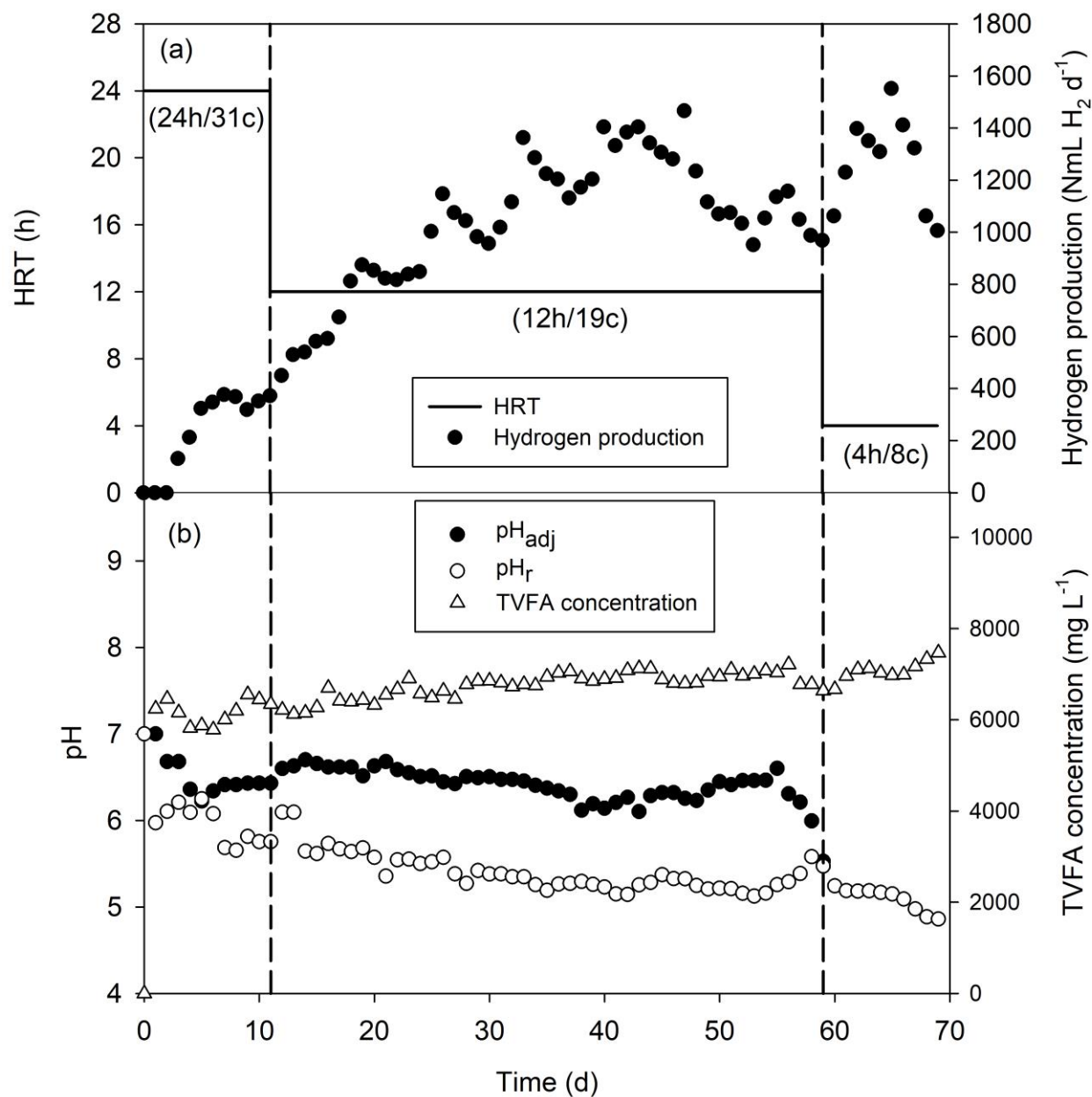
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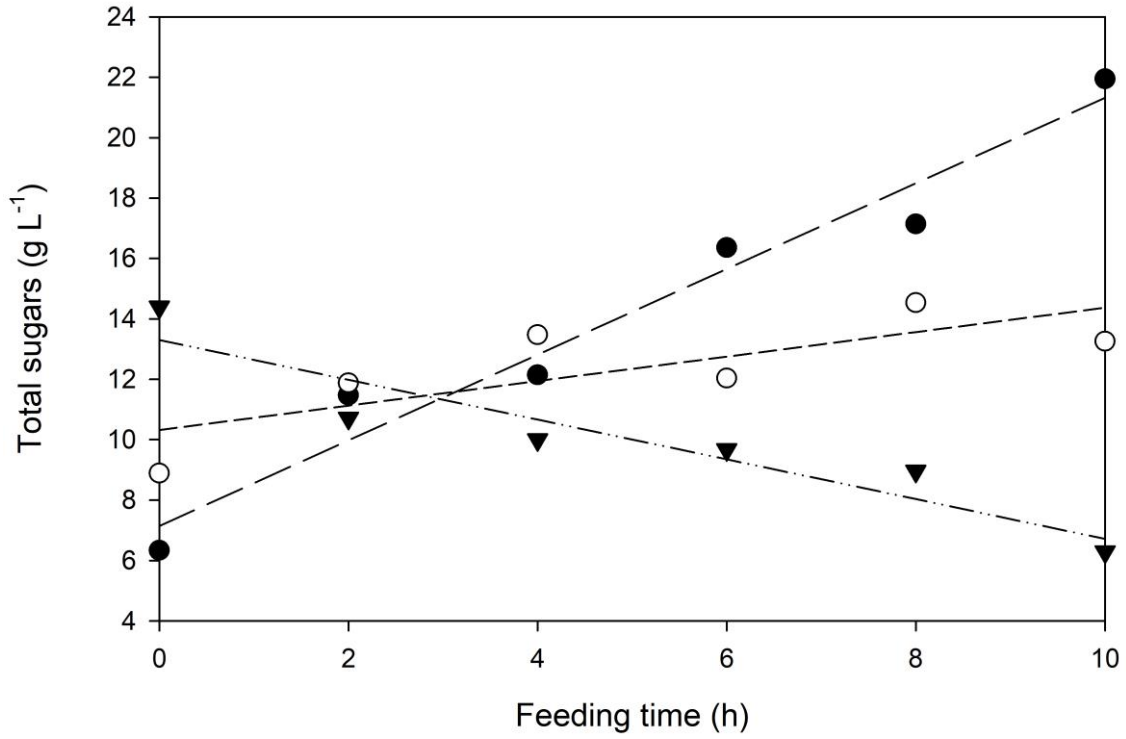
Fig. 1. Schematic diagram of the experimental apparatus.

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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<sub>sucrose</sub> L<sup>-1</sup>; 12h/19c  
 596 operated at 12 h HRT fed with 19 g<sub>sucrose</sub> L<sup>-1</sup>; 4h/8c operated at 4 h HRT fed with 8.3 g<sub>sucrose</sub>  
 L<sup>-1</sup>.



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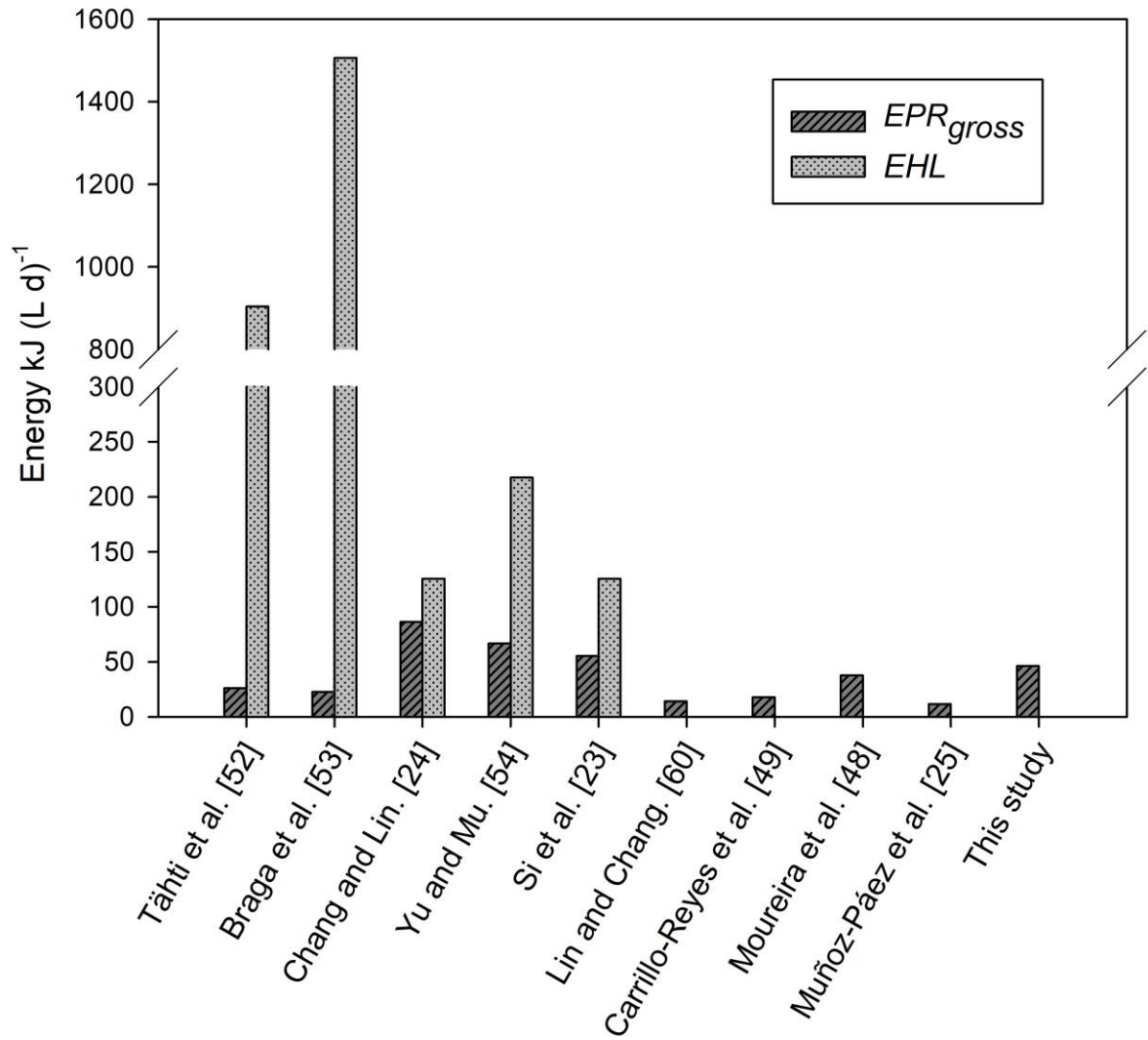
602 Fig. 3. Influence of HRT on total sugar concentration in function on time. Linear regression  
 604 curves were represented by dashed lines. (●) 24h/31c,  $Y = 7.14 + 1.41X$ ,  $R^2 = 0.96$ ; (○)  
 604 12h/19c,  $Y = 10.32 + 0.40X$ ,  $R^2 = 0.60$ ; (▼) 4h/8c,  $Y = 13.29 - 0.66X$ ,  $R^2 = 0.87$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

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

628



630

Table 1. Characteristics of inoculum.

<b>Parameter</b>	
pH	6.66 ± 0.10
Humidity (% <sub>wb</sub> )	93.80 ± 0.07
Total solids (% <sub>wb</sub> )	6.20 ± 0.07
Volatile solids (% <sub>db</sub> )	34.82 ± 3.59
Ash (% <sub>db</sub> )	65.18 ± 3.59

632

Notes: wb, wet basis; db, dry basis

634

636

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

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

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

646 Notes: <sup>a</sup>solid retention time (HRT equivalent 28.8 h); <sup>b</sup> calculated as hexose equivalent; PBR, packed bed reactor; AFBR,  
648 anaerobic fluidized bed reactors; [H<sub>2</sub>]<sub>avg</sub>, 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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