Water Research 128 (2018) 350–361

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

# Biohydrogen production at pH below 3.0: Is it possible?

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ARTICLE INFO

*Article history:*
Received 7 April 2017
Received in revised form
13 September 2017
Accepted 28 October 2017
Available online 30 October 2017

*Keywords:*
Acidogenic reactor
Acid-tolerant bacteria
Biohydrogen
Dark fermentation
Hydrogen
pH

ABSTRACT

Biological hydrogen production was investigated in continuous acidogenic reactors fed with sucrose at 30 °C without pH control. In the first experimental phase, three reactors were compared: a structured fixed-bed (FB), a granular UASB (UG) and a flocculent UASB (UF-1). They were run at 3.3 h HRT and 33 gCOD $L^{-1}d^{-1}$ OLR. Hydrogen production occurred throughout the experimental period with an average effluent pH of only 2.8. The FB, UG and UF-1 reactors presented volumetric hydrogen production rates (VHPR) of 95 ± 69, 45 ± 37 and 54 ± 32 $mLH_2$ $L^{-1}h^{-1}$, respectively; and $H_2$ yields (HY) of 1.5 ± 0.8, 0.8 ± 0.6 and 1.2 ± 0.7 $molH_2$ $mol^{-1}$ $sucrose_{consumed}$, respectively. The UF-1 reactor showed intermediate VHPR and HY, but no declining trend, contrary to what was observed in the FB reactor. Thus, aiming at continuous and long-term $H_2$ production, a flocculent UASB was applied in the second experimental phase. In this phase, the HRT of the acidogenic reactor, which was named UF-2, was raised to 4.6 h, resulting in an OLR of 25 gCOD $L^{-1}d^{-1}$. The VHPR and the HY increased considerably to 175 ± 44 $mLH_2$ $L^{-1}h^{-1}$ and 3.4 ± 0.7 $molH_2$ $mol^{-1}$ $sucrose_{consumed}$, respectively. These improvements were accompanied by greater sucrose removal, higher suspended biomass concentration, less production of lactate and more of acetate, and high ethanol concentration. Contradicting the current published literature data that reports strong inhibition of $H_2$ production by dark fermentation at pH less than 4.0, the UF-2 reactor presented stable, long-term $H_2$ production with satisfactory yields at pH 2.7 on average. 16 S rDNA sequencing revealed that two sequences assigned as *Ethanoligenens* and *Clostridium* accounted for over 70% of the microbiota in all the reactors. The non-necessity of adding alkalizing agents and the successful $H_2$ production under very acid conditions, demonstrated in this study, open a new field of investigation in biological hydrogen production by dark fermentation towards a more sustainable and feasible technology.

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## 1. Introduction

In recent years, more attention has been given to the potential for hydrogen production by dark fermentation (DF). Hydrogen is produced concomitantly with volatile fatty acids (VFA) through acidogenesis during anaerobic treatment, and its recovery is a way of extracting additional energy in wastewater treatment plants. The technology is still evolving and stable, long-term $H_2$ production is challenging due to changes in bacterial metabolic pathways and the concomitant existence of $H_2$-producing and $H_2$-consuming micro-organisms inside the acidogenic reactors. Current efforts are towards optimization of the operating parameters (e.g. reactor designs, environmental conditions, bacterial consortia, substrates) in order to achieve a sustainable $H_2$ net production.

In the DF processes, no more than 4 mol of $H_2$ per mol of hexose is attainable due to the production of products other than gas. The foregoing notwithstanding, usual $H_2$ yields are lower, due to the utilization of the substrate in a variety of pathways that produce less or no $H_2$ and for biomass growth, also due to microbial $H_2$ consumption.

Environmental pH plays a crucial role in hydrogen yields. A neutral pH, besides being onerous to maintain, can favour methanogen growth and be detrimental to the achievement of phase separation. On the other hand, pH values less than 4.5 lead to changes in the metabolic pathways, towards the production of compounds more reduced than the VFA (solvents such as acetone

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https://doi.org/10.1016/j.watres.2017.10.060
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and alcohols, and lactic acid) (Bahl et al., 1982; Lay, 2000; Mizuno et al., 2000; Kim et al., 2004); increased concentrations of undissociated forms of organic acids, which affect microbial growth (Dabrock et al., 1992; Yokoi et al., 1995; Chen et al., 2005; Ruggeri et al., 2015); possible inhibition of hydrogenase activity (Micolucci et al., 2014; Ghimire et al., 2015; Ruggeri et al., 2015; Roy and Das, 2016) as well as ferredoxin's capacity to donate electrons for the protons (Ruggeri et al., 2015). In general, the desirable pH for hydrogen-producing reactors ranges from 4.5 to 6.5. However, even in this pH range, $H_2$-consuming microorganisms such as homoacetogenic and $H_2$-oxidizing methanogens can be found (Lee et al., 2010).

The main drawback to controlling the pH in acidogenic reactors lies in the increased costs. Due to the constant $CO_2$ and acid production, the addition of alkalis to the reactors is usually needed. Ghimire et al. (2015) state that the use of an excessive amount of pH regulators can decrease the economics and sustainability of the process, as well as increase the salt concentration of the DF effluents.

The capacity of acid-tolerant facultative or anaerobic bacteria to produce $H_2$ under extremely acid conditions (pH < 3.5) has not yet been investigated in acidogenic reactors, but only in other environments. In the study by Noguchi et al. (2010), it was found that live cultures of *Escherichia coli* survived at external pH values of 2.5 and 2.0 due to the activity of the [NiFe]-hydrogenase Hyd-3. The reduction of $H^+$ into $H_2$ to control the internal pH in extremely acidic environments such as the stomach is a strategy also reported for *Helicobater pylori* (Bhattacharyya et al., 2000). The capacity to grow in very acid environments has been demonstrated for other $H_2$-producing bacteria, such as Sarcina ventriculi and Clostridium acidisoli. S. ventriculi is a bacterium found in various environments (soil, mud, rabbit and guinea pig stomach contents, elephant dung, human feces and the surface of cereal seeds) and can grow at pH of 2.0–2.5 (Canale-Parola, 1986). However, Goodwin and Zeikus (1987) found that its metabolism shifted from $H_2$-acetate to ethanol production when the pH decreased from 7.0 to 3.0. Kuhner et al. (2000) first isolated *Clostridium akagii* and *Clostridium acidisoli* from acid soils (pH ~3.0) and cultured them at pH 3.7–7.1 and 3.6–6.9, respectively. Their capacity to produce $H_2$ from carbohydrates was demonstrated at pH 5.5 and 6.8, but it was not assayed for other pH values.

Bearing in mind that the application of DF for $H_2$ recovery is only feasible if the environmental balance is beneficial and the economic costs are kept to a minimum, and, that there is a potential for $H_2$ production by acid-tolerant bacteria, the acidogenic reactors were run without addition of pH regulators in the present study. As reactor design and the biomass retention mechanism (biofilm, flocs or granules) affect the biological dynamics, and thus net hydrogen production, different configurations of reactors were evaluated.

## 2. Material and methods

### 2.1. Reactor configurations and inoculum

An up-flow structured fixed-bed reactor, a granular UASB reactor and a flocculent UASB reactor were used. The reactors were made of acrylic, having internal diameters of 6.3 cm, and with total and working volumes of approx. 2.5 and 2.2 L, respectively (Fig. 1). The source of inoculum was granular sludge from a single stage UASB reactor treating poultry slaughterhouse wastewater (Pereiras, São Paulo, Brazil). The granules were completely disrupted with a blender prior to inoculating the structured fixed-bed and flocculent UASB reactors. The structured fixed-bed reactor design (Picanço et al., 2001), as an alternative to the packed-bed reactor, prevents channelling and clogging. Polyethylene cylinders were chosen as

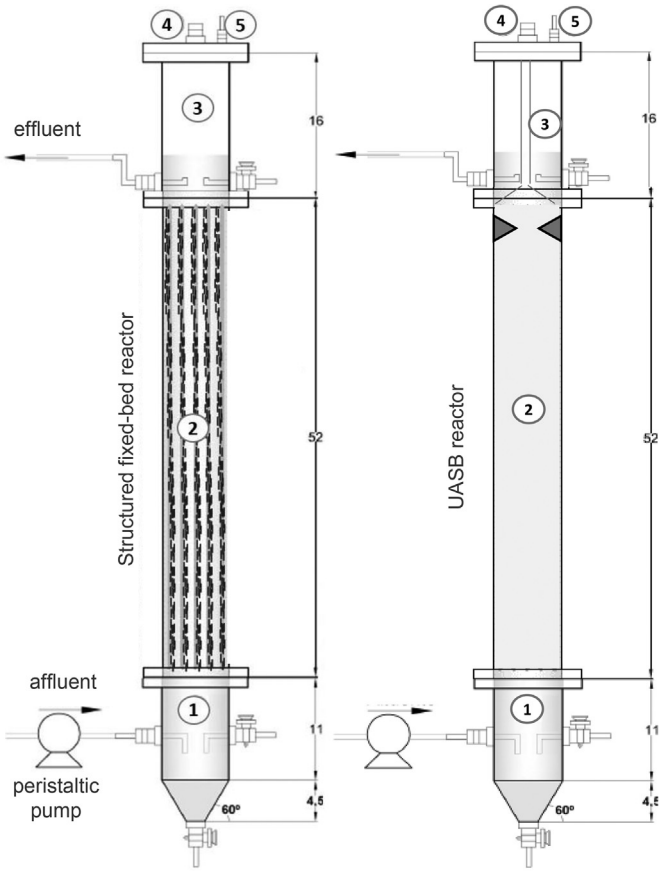


**Fig. 1.** Schematic diagram of the acidogenic reactors. 1: distribution chamber, 2: reactional zone, 3: headspace, 4: biogas sampling, 5: biogas outlet.

the support material in the structured fixed-bed reactor (porosity = 82%), as Ferraz Júnior et al. (2015) found that the reactor filled with polyethylene obtained higher $H_2$ production and yield, also greater abundance of $H_2$-producing bacteria, as compared to the reactors filled with expanded clay, coal and porous ceramics.

The initial concentration of total volatile solids (TVS) was 15 g/l. No sludge pretreatment was used. This allows the survival of non-spore forming $H_2$-producers and makes the inoculation more practical and viable.

### 2.2. Substrate

The reactors were fed with sucrose-based wastewater composed of demerara sugar (Native®) and a nutrient's solution in the following concentrations (mg $L^{-1}$): demerara sugar (4450), $NH_4Cl$ (170), $CaCl_2 \cdot 2H_2O$ (8), $KH_2PO_4$ (37), $MgSO_4 \cdot 4H_2O$ (9), $FeCl_3 \cdot 4H_2O$ (2), $CoCl_2 \cdot 6H_2O$ (2), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.03), $ZnCl_2$ (0.05), $H_3BO_3$ (0.05), $(NH4)_6Mo_7O_{24} \cdot 4H_2O$ (0.09), $Na_2SeO_3 \cdot 5H_2O$ (0.1), $NiCl_2 \cdot 6H_2O$ (0.05), EDTA (1), HCl 36% (1 μL $L^{-1}$).

### 2.3. Operating conditions

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

One configuration was chosen to be applied in the next experimental phase, in order to keep the investigation on continuous

**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 |

hydrogen production. In this phase, a different start-up was applied: after inoculation, the reactor was operated at HRT in the 2.8–6.1 h range for 80 days. It was verified that higher hydrogen production was obtained at HRT between 4 and 5 h (data not shown). Thereafter, the HRT was adjusted to 4.6 h in Phase 2. This corresponded to an OLR of 25.0 gCOD $L^{-1}d^{-1}$.

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

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

### 2.4. Analyses

The biogas flow rate was measured using Milligas counter gas meters (Ritter®). The composition, in terms of $H_2$, $CH_4$ and $CO_2$, was analysed using Shimadzu GC-2010 gas chromatograph with the following specifications: thermal conductivity detector; argon as carrier gas; Carboxen 1010 capillary column; initial detector and injector temperatures of 200 and 230 °C, respectively; oven temperature of 130–135 °C; flow rate of 12 mL $min^{-1}$; and, sample volume of 300 μl.

Sucrose (glucose and fructose) and organic acids (lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric) were determined using Shimadzu System UV/DAD (210 nm) high performance liquid chromatography (HPLC) with Refractive Index (in series) detectors, Aminex HPX-87H column, 0.005 M $H_2SO_4$ solution as eluent, flow of 0.5 mL $min^{-1}$, oven temperature of 43 °C, and 100 μL of sample injection. Ethanol was determined using Shimadzu GC-2010 gas chromatograph with a flame ionization detector (FID), flow of 1.5 mL $min^{-1}$ with ultra-pure hydrogen as the carrier gas, injector and detector temperature of 250 °C and 280 °C, respectively.

Total COD of the affluent, soluble COD of the effluent (filtered in 1.2 μm membrane) and volatile suspended solids (VSS) concentration in the effluent were analysed according to APHA et al. (2005). The pH was measured using a pHmeter (Hach equipment).

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

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

The simplified stoichiometric equations (Equations (1)–(5)) were used to calculate the molar ratio between sucrose consumed and acids produced ([sucrose]/[acid]) and between hydrogen gas and acids produced ([$H_2$]/[acid]). These equations show calculated acidified sucrose (in mmol $L^{-1}$) = S (in mmol sucrose $mmol^{-1}$ acid) x acid concentration (in mmol acid). It is shown that S = 0.25 via lactate, 0.25 via acetate and/or formate, 0.25 via propionate, 0.50 via butyrate, and 0.50 via valerate. The percentage of acidified sucrose for each acid is its respective calculated acidified sucrose divided by the total calculated acidified sucrose.

To determine the HY percentage, the maximum yield or consumption by each route was calculated, as follows: theoretical HY (in mmol $H_2$ $mmol^{-1}$ $sucrose_{consumed}$) = H (in mmol $H_2$ $mmol^{-1}$ acid) x acid yield (in mmol acid $mmol^{-1}$ $sucrose_{consumed}$). It is shown that H = 0 via lactate, 2 via acetate and/or formate, −1 via propionate, 2 via butyrate, and −1 via valerate. The HY percentage is the theoretical HY from each acid divided by the sum of the theoretical HY from acetate and/or formate and butyrate.

Via lactate: $1\ C_{12}H_{22}O_{11} + 1\ H_2O = 4\ CH_3CH(OH)COO^- + 4\ H^+$ (1)

Via acetate and/or formate*: $1\ C_{12}H_{22}O_{11} + 5\ H_2O = 4\ CH_3COO^- + 4\ H^+ + 4\ CO_2 + 8\ H_2$ (2)

*In the mixed-acid fermentation, Equation (2) derives from the sum of the reaction of acetate and formate formation ($1\ C_{12}H_{22}O_{11} + 5\ H_2O = 4\ CHOO^- + 4\ CH_3COO^- + 8\ H^+ + 4\ H_2$) followed by the reaction of formate cleavage ($4\ CHOO^- + 4\ H^+ = 4\ CO_2 + 4\ H_2$). Since [formate] ~0, only [acetate] was included in the calculations.

Via propionate: $1\ C_{12}H_{22}O_{11} + 4\ H_2 = 4\ CH_3CH_2COO^- + 4\ H^+ + 3\ H_2O$ (3)

Via butyrate: $1\ C_{12}H_{22}O_{11} + H_2O = 2\ CH_3CH_2CH_2COO^- + 2\ H^+ + 4\ CO_2 + 4\ H_2$ (4)

Via valerate: $1\ C_{12}H_{22}O_{11} + 2\ H_2 = 2\ CH_3CH_2CH_2CH_2COO^- + 2\ H^+ + 2\ CO_2 + 3\ H_2O$ (5)

### 2.6. Molecular analysis

Biomass were collected from different heights from the FB, UG, UF-1 and UF-2 reactors by the end of operation. Cells were separated by centrifugation (6000 g, 10 min, 4 °C). Genomic DNA was extracted and purified using the protocol of Griffiths et al. (2000). The amount and purity of DNA in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan). The extracted DNA was stored at −20 °C until further use. The 16 S rDNA gene V4-5 region was amplified with the forward primer CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA and the reverse primer GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTRAGT plus the respective linkers over 30 amplification cycles at an annealing temperature of 65 °C. In a second PCR reactor of 12 cycles, an index sequence was added. The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge for sequencing of paired 375–380 bp reads. Sequencing-related work was done at the GeT PlaGe sequencing center of the genotoul life science network in Toulouse, France (get.genotoul.fr). Forward and reverse sequences were retained after assembly and quality checking using a slightly modified version of the Standard Operation Procedure for MiSeq data by Kozich et al. (2013) in

Mothur version 1.35.0 (Schloss et al., 2009). SILVA SSU Ref NR 99, release 128, was used for alignment and as taxonomic outline (Pruesse et al., 2012). The sequences found in this study were submitted to the GenBank (accession numbers MF612196-MF613645). For the construction of a phylogenetic tree, the most abundant sequences found in the reactors were then compared with the available sequences in the GenBank database using the BLAST program (Altschul et al., 1990). Phylogenetic analyses of the sequences were performed using the Molecular Evolutionary Genetic Analysis (MEGA7) software (Kumar et al., 2016). Evolutionary distances were based on the Kimura model (Kimura, 1980) and tree reconstruction on the Neighbor-Joining method with bootstrap values calculated from 500 replicate runs.

## 3. Results and discussion

### 3.1. Volumetric hydrogen production rate and biogas composition

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

Punctual increases in pH, accompanied by reduction of sucrose removal, organic acid and $H_2$ production, were observed. This was more noticeable in the UF-1 reactor, when pH values were above 3.4 on days 30, 80 and 133 (Fig. 2). The lowest organic acid concentrations in effluent were also reported on these days and sucrose removal efficiency was null on days 80 and 133. On days 23, 29, 59, 60, 79 and 130, there were feeding problems in the UF-1 reactor due to clogging of tubes. It was likely that feeding reduction or interruption led to biomass decay, as could be inferred by the lower visible turbidity of the medium and reduction in effluent VSS concentrations after these events. Nevertheless, pH above 3.0 accompanied by a drastically reduced sucrose conversion, was also observed in the UG reactor notably on days 32, 85 and 136, and in the FB reactor on day 85. Consequently, effluent pH was increased due to dilution of medium with non-consumed affluent.

The VHPR were equivalent to: 95 ± 69 $mLH_2\ L^{-1}h^{-1}$ in the FB reactor, 45 ± 37 $mLH_2\ L^{-1}h^{-1}$ in the UG reactor, and 54 ± 32 $mLH_2\ L^{-1}h^{-1}$ in the UF-1 reactor. The non-parametric Kruskall-Wallis ANOVA by Ranks test showed statistically significant differences regarding $H_2$ production (p-value = 0.006). Further analysis of multiple comparisons of mean ranks for all groups showed that $H_2$ production in reactors UG and UF-1 was not significantly different (p-value = 0.575). As shown in Fig. 2, although the FB reactor achieved the highest VHPR at the beginning of the operation, it tended to decrease during the experimental period. This did not occur in the UG and UF-1 reactors. The UF-1 reactor showed superior stability during the entire period of operation, as indicated by its VHPR data. These were the only data that presented normal distribution.

A possible explanation for the higher initial VHPR in the FB reactor could be the lower biomass wash-out, owing to the presence of the support material. Biomass was observed to be "trapped" in the polyethylene cylinders, although it did not form a thick biofilm. The flocs formed in the sludge bed at the bottom of the FB reactor were visually larger than those from the UF-1 reactor. This was probably due to the shear stress and physical selection caused by the support material, which retained larger particles, while the smaller ones passed easily through the pores. Low interspecies distances are a key point of efficient interspecies hydrogen transfer

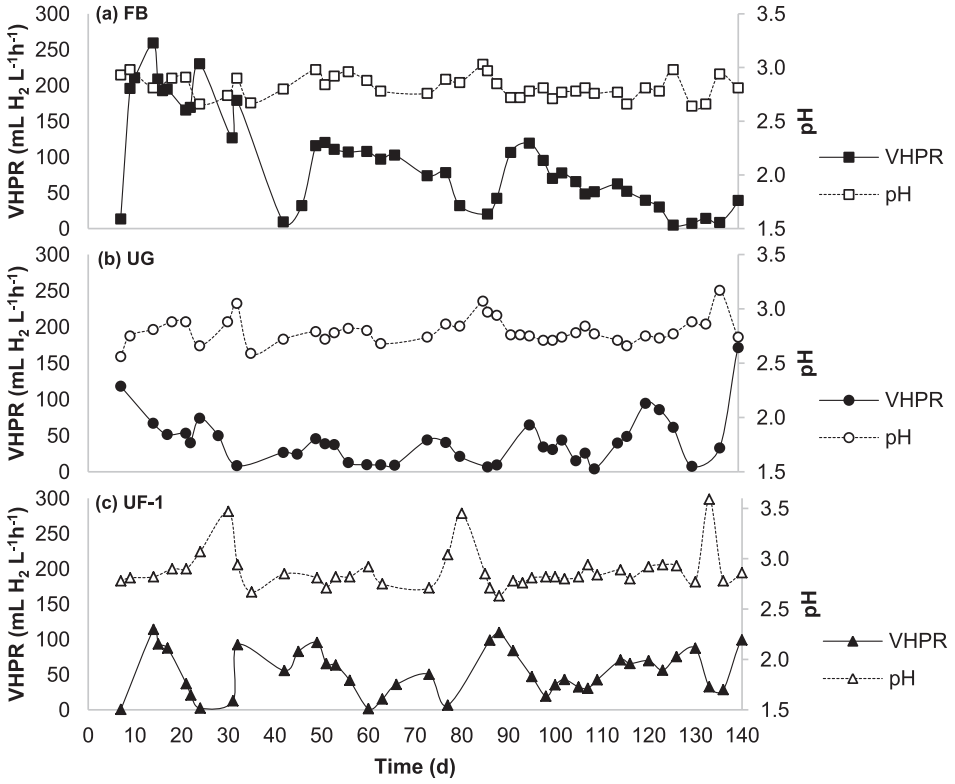


**Fig. 2.** Volumetric hydrogen production rate and pH in the first experimental phase: (a) FB reactor, (b) UG reactor, (c) UF-1 reactor.

between acetogenic bacteria and hydrogenotrophic methanogenic archaea in anaerobic aggregates, biofilms and granules (MacLeod et al., 1990; Davey and O'toole, 2000; Hulshoff Pol et al., 2004; Felchner-Zwirello et al., 2013). As stated by Dinamarca et al. (2011), this mechanism also can play a relevant role in non-methanogenic mixed cultures, through hydrogen transfer between hydrogen producers and consumers, limiting sustainable hydrogen production due to homoacetogenesis. Therefore, in the present study, the biomass agglutination in larger flocs, granules and biofilm could have had an adverse effect on long-term $H_2$. Acetate formation was observed to increase from day 77 in the FB reactor. However, this was not followed by an increase in hydrogen production, which can be an indicator of homoacetogenic activity. Penteado et al. (2013) studied seven structured fixed-bed reactors having different sources of inoculum, fed with sucrose. VHPR decreased over time in all reactors, and it was observed that HY decreased as the percentage of acetic acid produced by homoacetogenesis increased. In addition, the filling in the FB and UG reactors with support material and granules, respectively, may have hindered the escape of the produced biogas, increasing the $H_2$ partial pressure in the medium, which inhibits its own production (Sikora et al., 2013).

The granules inside the UG reactor were originally dark colored with an average diameter of 2.1 mm. They became whitened and smaller, with an average diameter of 1.5 mm by the end of operation. Floc formation and suspended biomass growth were also observed. On the 135$^{th}$ day of operation, the UG reactor lost most of its biomass due to a remarkable wash-out of the granules. The granule flotation likely occurred due to the adherence of gas bubbles to their surfaces, and reduction of their inner densities. This assumption is based on the fact that the environmental conditions were not favourable for the maintenance of the methanogenic microorganisms, leading to biomass decay in the inner layers of the granules. Then, in the outer layers, the granules became most colonized by acidogenic bacteria that survived in the acid environment. The increasing substitution of mixed-consortia granules by specific acidogenic bacteria inside the UG reactor probably had a positive effect on $H_2$ production, as indicated by the increased VHPR at the end of operation (Fig. 2).

In the UF-1 reactor, it is probable that faster biomass decay and washing-out occurred at the beginning of the operation due to the larger contact surface of the biomass with the medium and the initial absence of a biomass retention mechanism. This may be the reason that $H_2$ production started later in this reactor and with less intensity. Reyes et al. (2012) also observed a delay in $H_2$ production in the reactors inoculated with disintegrated granules. This production started after about 40–70 h of continuous operation, compared to the reactors inoculated with intact granules, in which $H_2$ production started within the first 12 h. On the other hand, the selection of bacteria resistant to the adverse conditions (low pH and high organic acids concentration) as well as the increasing biomass concentration due to the self-flocculation phenomenon provided a superior stability to the UF-1 reactor. The higher selectivity of the desired bacteria from the disaggregated granules was verified by Reyes et al. (2012), who found that this form of inoculation resulted in greater specific hydrogenogenic activity compared to that from intact granules.

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, UG and UF-1 reactors, respectively. In each reactor, the effluent VSS concentration correlated positively with the VHPR according to the Spearman non-parametric test ($\alpha$ = 5%). The values of the R correlation coefficients were 0.45 ($p = 0.0063$), 0.56 ($p = 0.0014$) and 0.40 ($p = 0.0198$) for the FB, UG and UF-1 reactors, respectively.

For the application in the second experimental phase, the UG reactor was considered less advantageous as it had the lowest VHPR and HY. The FB reactor, however, showed the highest VHPR and HY mean values, although with a tendency toward performance decrease over time. The UF-1 reactor showed intermediate VHPR and HY values, but no declining trend was observed. From the 80$^{th}$ day of operation, the $H_2$ yield in the UF-1 reactor also showed progressive improvement, contrasted to the FB reactor. Thus, aiming at continuous and long-term $H_2$ production, this configuration seemed to be the most adequate among those studied. It is also pertinent that the flocculated UASB reactor design has the greatest potential to use the entire reactor volume to be filled with active biomass, thus maximizing the reactor space utility and virtually increasing cell density in the reactor, without formation of close microbial associations such as biofilms and granules. For these reasons, this configuration was chosen for the second experimental phase.

The flocculated UASB applied in Phase 2 was identified as the UF-2 reactor and was operated at a higher HRT (4.6 h) and lower OLR (25.0 gCOD $L^{-1}d^{-1}$). It obtained constant and stable $H_2$ production, and achieved significant improvement over the previous experimental phase (Fig. 3). The VHPR was very satisfactory, corresponding to 175 ± 44 $mLH_2\ L^{-1}h^{-1}$. Continuous acid and $CO_2$ production in the reactor led to strong acidification of the effluent, and pH was self-adjusted to values consistently less than 3.0, with an average value of 2.7 (Fig. 3). The $H_2$ production in the UF-2 reactor presented normal distribution and was statistically higher, according to Kolmogorov-Smirnov test (p-value < 0.001), compared to the other reactors.

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

The biogas of the FB, UG, UF-1 and UF-2 reactors presented a $H_2$ content equal to (%): 59.6 ± 11.0, 62.1 ± 10.8, 62.2 ± 7.1 and 59.8 ± 5.9, respectively. The percentage of hydrogen in the biogas was not significantly different, according to the Kruskal-Wallis ANOVA (FB, UG, UF-1 reactors) and the Kolmogorov-Smirnov tests (UF-2 reactor vs. FB, UG, UF-1 reactors). Methane was not detected in the biogas in any of the reactors. This leads to the assumption that the environmental conditions established by the pH self-adjustment and low HRT were sufficient to completely inhibit methanogenesis. Operating with extreme pH values seems to be an efficient strategy for avoiding methanogenic activity, as verified by Wang et al. (2015), studying hydrogen production in waste activated sludge at pH 10.

### 3.2. Hydrogen yield and sucrose removal

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

The mean sucrose removal in the UF-2 reactor was 81% while, in the FB, UG and UF-1 reactors, it was 64, 67 and 56%, respectively (Fig. 4). However, as the OLR applied in the UF-2 reactor was less than in the other reactors, the mean volumetric sucrose removal rate was in the same range as the other reactors: 2.22, 2.38, 1.91 and 2.16 mmol $sucrose_{consumed}\ L^{-1}h^{-1}$ in the FB, UG, UF-1 and UF-2 reactors, respectively. Thus, the substantial increase in VHPR obtained in the UF-2 reactor was mainly due to the improvement in the $H_2$ yield. The mean HY of 1.50, 0.76 and 1.19 mol $H_2$ $mol^{-1}$ $sucrose_{consumed}$ obtained in the FB, UG and UF-1 reactors, respectively, was surpassed by a level of 3.35 mol $H_2$ $mol^{-1}$ $sucrose_{consumed}$ obtained in the UF-2 reactor (Fig. 5). Statistical analyses

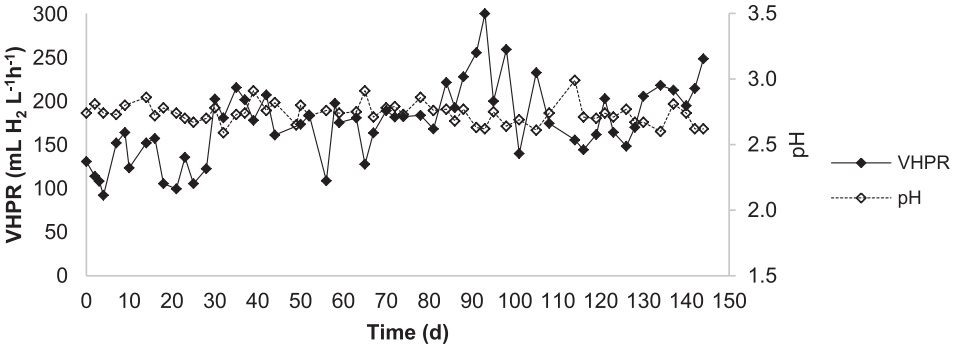


**Fig. 3.** Volumetric hydrogen production rate and pH in the second experimental phase: UF-2 reactor.

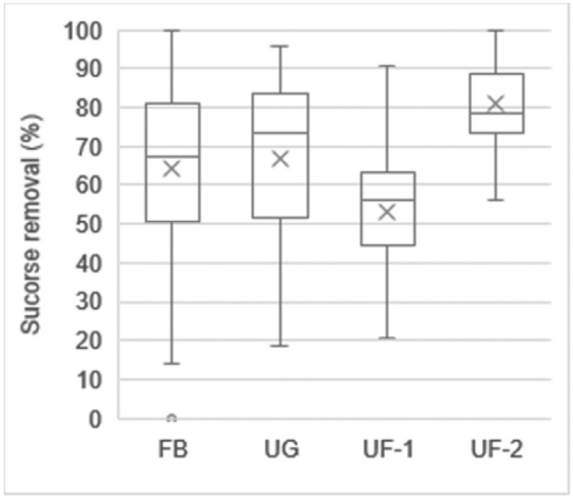


**Fig. 4.** Sucrose removal.

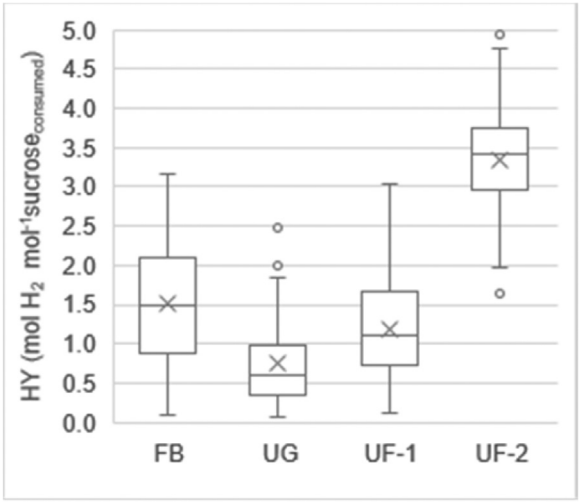


**Fig. 5.** $H_2$ yield.

revealed that $H_2$ yield in Phase 1 differed significantly among reactors (Kruskall-Wallis ANOVA, p-value = 0.002); however, the difference between the FB and UF-1 reactors was not significant (multiple comparisons of mean ranks, p-value = 0.413). Nevertheless, $H_2$ yield obtained in the UF-2 reactor was statistically higher than that obtained in the FB, UG and UF-1 reactors (Kolmogorov-Smirnov test, p-value<0.001).

Table 2 shows the organic acid concentrations, and the percentages of respective acidified sucrose and $H_2$ yield. Comparing the effluent organic acid composition of the UF-2 reactor to the other reactors, it was concluded that there was a shift from less lactate to more acetate production, thus accounting for UF-2 reactor superior performance. Lactate production involves the consumption of NADH and pyruvate, reducing the potential production of $H_2$ by both the NADH-pathway and, mainly, substrate competition due to pyruvate consumption. On the other hand, the acetate production represented by the reactions in Equation (2) is desired for both Clostridial- and Enterobacterial-type fermentation (mixed acid fermentation), as the acetate route provides the highest $H_2$ yield in Clostridial-type fermentation and acetate is produced along with formate in Enterobacterial-type fermentation. Since $H_2$ can be produced from formate cleavage, acetate indicates that the formate route took place in the mixed acid fermentation. Also, the concentrations of propionate and valerate, which are produced at the expense of $H_2$ consumption, were lower in the UF-2 reactor.

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

The overloading in Phase 1 seemed to be the main factor accounting for reduced hydrogen yields. According to Cohen et al. (1984), lactate pathway is energetically less favourable and its formation in acid digestion could be associated with an imbalance between electron donating and electron accepting reactions, in conditions of high accessibility of the substrate, such as low HRT and shock loading. Apart from the influence of organic loading on metabolic routes, the *Lactobacillus* genus was found in greater relative abundance in the reactors of Phase 1 (Section 3.3).

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

The results presented in Table 2 are only for comparison, based on the equations shown in Section 2.5. Many other pathways could have taken place in the reactors. The calculated acidified sucrose corresponded to 55%, 66%, 64% and 39% of the consumed sucrose in the FB, UG, UF-1 and UF-2 reactors, respectively. Naturally, part of the sucrose could have been used for cellular growth. Moreover, it is likely that other pathways leading to hydrogen formation were also

**Table 2**
Organic acid concentrations and the respective percentages of acidified sucrose and $H_2$ 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) |

present. The mean calculated HY in the FB, UG, UF-1 and UF-2 reactors was equivalent to 1.00, 0.78, 0.85 and 1.62 mmol$H_2$ mmol$^{-1}$sucrose$_{consumed}$, respectively, which corresponded to 67%, 103%, 71% and 48% of the measured HY, respectively.

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

The high concentrations of ethanol in the UF-2 reactor is contrary to what was first expected, because ethanol is a more reduced compound than organic acids and its formation is usually associated with HY reduction. Nevertheless, some pathways are proposed for ethanol formation along with hydrogen (Xu et al., 2008; Lee et al., 2009). Equation (6) shows the reaction proposed by Hwang et al. (2004) for bacterial conversion of glucose into ethanol, acetate and hydrogen. Although the ethanol-acetate pathway yields less hydrogen than the acetate-pathway (Equation (2)), the hydrogen yield could be 4.0 mol of $H_2$ per mol of sucrose consumed (which is in the range achieved in the UF-2 reactor), considering sucrose as substrate. Since the hydrogen yield per mol of acetate produced is the same of as shown in Equation (2) (H = 2 mmol $H_2$ mmol$^{-1}$ acetate), the assumption of this reaction would not change the HY percentage values depicted in Table 2, whereas the acidified sucrose percentage would be higher from acetate (S = 0.50 mmol sucrose mmol$^{-1}$ acetate). However, as ethanol was not analysed in all effluents, however, it was not possible to account for it in the estimations presented in Table 2. Ethanol formation is in agreement with the findings of sequencing analyses (Section 3.3), that revealed an abundance of microorganisms affiliated with *Ethanoligenens harbinense*.

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2OH + CH_3COO^- + H^+ + 2H_2 + 2CO_2 \quad (6)$$

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

There were 123838 partial 16 S ribosomal DNA gene sequences obtained from the microbial sequencing, of which 94–99% were assigned to the phylum Firmicutes in the reactors versus 17% in the inoculum. Sequences assigned to the domain Archaea were 9.6% of the inoculum and less than 0.1% of the reactors, indicating that the conditions applied in this study dispensed with an inoculum pretreatment. Based on the operational taxonomic units (OTU), the Shannon-diversity index was 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, respectively, by the end of operation. The self-established harsh environment likely played a key role in the reduction of biomass diversity. An annotated abundance relative description is given in Table 3. Representative sequences (abundance of more or equal to 1.0%) were selected from the acidogenic reactors to infer a phylogenetic tree (Fig. 6).

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

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

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

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

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

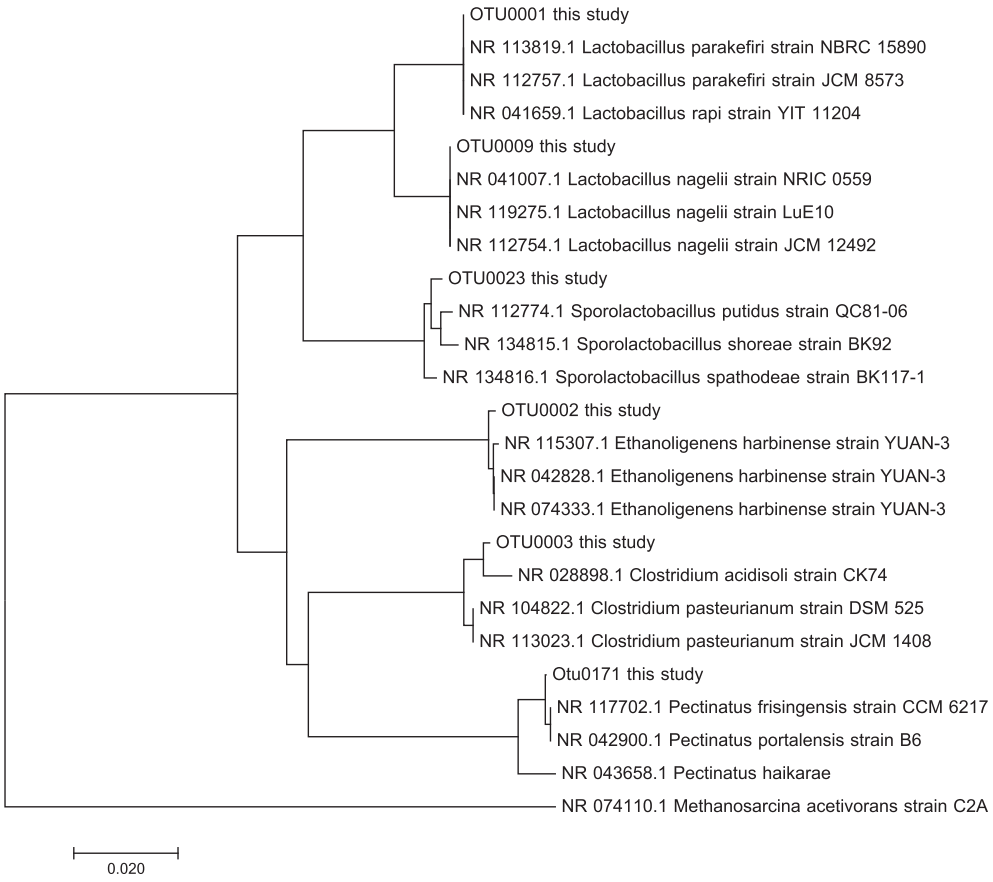


**Fig. 6.** Consensus phylogenetic tree based on 16 S rDNA for bacteria domain obtained from the highly abundant OTUs found in the reactors. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There was a total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

increased production and yield of hydrogen (Section 3.2).

*C. acidisoli* was isolated from acidic peat-bog soil and was grown at pH 3.6–6.9, with no distinct optimum between pH 3.6–6.6, in a temperature range of 5–37 °C, with an optimum of 25–30 °C (Kuhner et al., 2000). At pH 4.0, 5.5 and 6.5, glucose fermentation yielded lactate, acetate, butyrate, $H_2$ and $CO_2$ as end-products. At pH 5.5, the molar ratio of $H_2$ to lactate, acetate and butyrate produced was 6.4, 4.1 and 3.6, respectively, and the HY was 1.8 mol$H_2$ mmol$^{-1}$ glucose$_{consumed}$. Lee et al. (2009) found great abundance of a species affiliated with *Clostridium* sp. HPB-16, which is phylogenetically close to *C. acidisoli*, during batch fermentation with hydrogen production at final pH of 3.5. Acetate and butyrate were the dominant organic products. These authors assumed that hydrogen was formed by the pyruvate decarboxylation-ferredoxin-hydrogenase pathway, which is the common mechanism for $H_2$ formation by the *Clostridium* and *Ethanoligenens* species.

Xing et al. (2006) isolated *E. harbinense* YUAN-3 from anaerobic activated sludge of molasses wastewater. They found that it grows in the pH range 3.5–9.0 at 20–44 °C, and the optima for growing were pH 4.5–5.0 and 35 °C. Acetate, ethanol, hydrogen and carbon dioxide were formed as end products of glucose fermentation. At 35 °C a hydrogen yield up to 2.8 mol$H_2$ mol$^{-1}$ glucose was achieved, along with production of 1.1 mol ethanol and 0.7 mol acetate per mol of glucose. In the UF-2 reactor, the mean production was

1.1 mol ethanol per mol of sucrose (= 0.6 per mol of hexose) and 0.8 mol acetate per mol of sucrose (= 0.4 mol per mol of hexose). The differences in the yields of ethanol and acetate were expected because the fermentation in the UF-2 reactor was carried out by a microbial consortium, which means that many more pathways were possible, and relatively high amounts of lactate were formed (Section 3.2). Nevertheless, the molar proportion of ethanol and acetate is similar between the UF-2 reactor (= 1.4 ethanol: acetate) and that reported by Xing et al. (2006) (= 1.6 ethanol: acetate). Since the maximum achieved hydrogen yield with the pure culture of *E. harbinense* (Xing et al., 2006) was higher than the theoretical yield depicted in Equation (6) (Section 3.2), it is probable that this bacterium is able to produce hydrogen and ethanol by pathways other than ethanol-acetate fermentation. Xu et al. (2008) also found an HY higher than 2 mol$H_2$ mol$^{-1}$glucose with the *Ethanoligenens harbinense* B49 strain. They suggested oxidative decarboxylation of pyruvate as the possible route for the hydrogen production observed, in accordance with Lee et al. (2009). However, the ethanol-type hydrogen production mechanism by *E. harbinense* is still unclear (Zhao et al., 2017).

*Lactobacillus* sp. ranged from 20% to 27% in the reactors of Phase 1 and was less than 1% in the UF-2 reactor. The most representative sequence of *Lactobacillus* was affiliated with *L. nagelii* (Fig. 6), which is characterized as producing lactic acid from glucose without gas formation (Edwards et al., 2000). Then, it is probable that the presence of *Lactobacillus* in the FB, UG and UF-1 reactors contributed to higher lactic acid formation and less hydrogen yield, due to the reduction of pyruvate availability for the $H_2$-producing pathways (Section 3.2). The excretion of extracellular polymeric substances (EPS) by lactic acid bacteria protects them against hostile environments and favors the formation of flocs and biofilm (Rafrafi et al., 2013), which may have implied competitive advantages at higher OLR.

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

### 3.4. Interaction among performance evaluation parameters

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

The VHPR and HY improvements in the UF-2 reactor are noteworthy, with respect to the others. These improvements were attributed mainly to the HRT increasing from 3.3 to 4.6 h and, therefore, the OLR decreasing from 33.1 to 25.0 gCOD L$^{-1}$d$^{-1}$, since these were the only operational parameters changed intentionally. Several operating indicators accompanied the UF-2 improvement in $H_2$ production. These indicators include: increased VSS concentration; higher sucrose removal; less production of lactate and more of acetate, and high production of ethanol; pH always below 3.0; and, longer chains of rods. Fig. 7 presents a proposed model of the relationship among these parameters that led to higher $H_2$ production in the UF-2 reactor.

Based on the proposed model, we suggest that the increased HRT led to greater removal of sucrose, due to the longer contact time between the substrate and the biomass, and to a higher VSS concentration (biomass) that resulted from the lower wash-out and longer time for bacterial growth. The higher HRT allowed the formation of long chains of bacteria, increasing their adaptability to the harsh environmental conditions (low pH) and contributing to the increased biomass in the reactor. The growth of acid tolerant bacteria such as *Ethanoligenens* was favoured and the competitive advantage of *Lactobacillus* was reduced. The higher VSS and HRT resulted in a lower specific organic loading rate, which enhanced the sucrose removal efficiency. The increased sucrose removal resulted in higher concentrations of fermentation products, such as acids, $CO_2$ and $H_2$. This latter directly reflected in higher VHPR. The high levels of acids and $CO_2$/carbonic acid caused a reduction in the pH of the medium. The maintenance of a very acid environment and less relative abundance of *Lactobacillus* resulted in reduced lactate formation. The increased pyruvate availability to other $H_2$-producing pathways, such as acetate and ethanol formation, thus increased hydrogen yield and production.

On the other hand, increasing HRT over the suitable values is not recommended as it leads to OLR reduction. In addition to increasing reactor volume requirements, this can reduce volumetric substrate removal rates, reducing the attainable VHPR. Very low OLR can also lead to cellular decay, reducing the biomass concentration. In addition, reduced HRT values can increase the pH (through the consumption and release of $CO_2$, $H^+$ and acids) and the $H_2$ in the medium, due to the mass transfer reduction caused the less turbulence. Increasing the pH and $H_2$ in the liquid medium then favours the growth of $H_2$-consuming bacteria; and, it can reduce the competitive advantage of the $H_2$-producing bacteria tolerant to very acid conditions.

### 3.5. Comparative studies

Hydrogen production in extremely acidic environments, average pH of 2.8 in the FB, UG, and UF-1 reactors, and of 2.7 in the UF-2 reactor, was unexpected. Extensive data in the literature indicate

**Table 4**
Performance evaluation parameters of all reactors.

| Parameter | Reactor | Minimum | Maximum | Mean | SD | CV |
|---|---|---|---|---|---|---|
| VHPR - mL$H_2$ L$^{-1}$h$^{-1}$ | 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% |
| $H_2$ 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 - mol$H_2$ 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% |

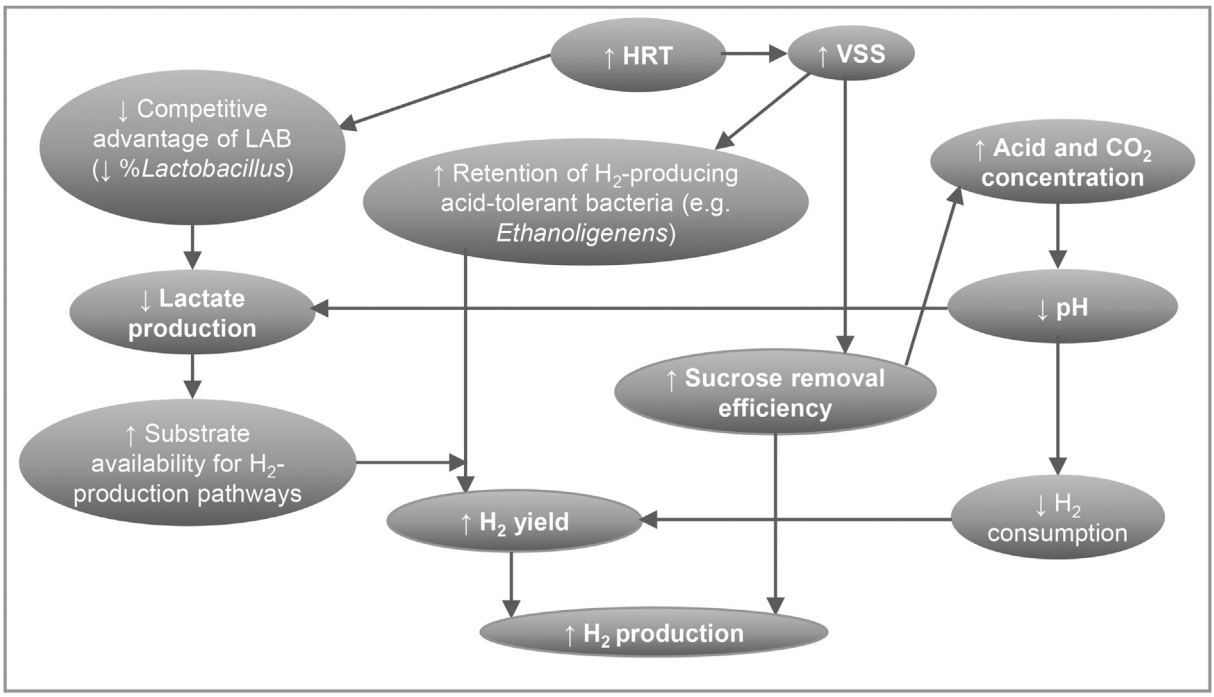


**Fig. 7.** Proposed model to explain changes in the UF-2 reactor that led to increased $H_2$ production.

**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[a] - 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 et al. (2002) |
| stirred tank | 80 | 5.25 | 35 | 55 | 506 | 2.3 | Kyazze et al. (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 et al. (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 et al. (2013) |
| granular UASB | 21.6 | 4.0 | 36 | 40 (approx.) | 92 (approx.) | 1.6 (approx.) | Ning et al. (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 |

[a] The reference conditions adopted were 25 °C and 1 atm.

drastic reduction or cessation of hydrogen production by dark fermentation at pH values below 4.5–4.0 (Yokoi et al., 1995; Lay, 2000; Mizuno et al., 2000; Lee et al., 2002; Kim et al., 2004; Liu and Shen, 2004; Hwang et al., 2004; Chen et al., 2005; Liu et al., 2006; Chojnacka et al., 2011; Ruggeri et al., 2015). It is only in specific cases, in continuous acidogenic reactors, that $H_2$ production at pH values below 4.0 is reported (Xing et al., 2008; Tähti et al., 2013; Carosia et al., 2017). The capacity of Ethanoligenens harbinense strain YUAN-3 to produce $H_2$ was evaluated by Xing et al. (2008) in a continuous stirred reactor at 35 °C for 21 days. The pH value was kept above 3.5 by a pH controller and they observed that $H_2$ production was not severely affected when the pH reached the minimum values (i.e., around 3.6), obtaining HY of approx. 1.5 $molH_2$ $mol^{-1}$glucose. Carosia et al. (2017) found bacteria similar to *Ethanoligenens harbinense* to be dominant bacteria in $H_2$-producing anaerobic fluidized bed reactors, inoculated with heat-treated sludge. Although buffers (hydrochloric acid and sodium bicarbonate) were added, effluent pH was approximately 3.7, and the optimum HY obtained was 0.76 $molH_2$ $mol^{-1}$glucose. Tähti et al. (2013) used an extreme thermophilic (70 °C) UASB reactor for $H_2$ production from glucose by mixed culture. However, a low HY was obtained, equivalent to 0.73 mol $mol^{-1}glucose_{added}$, which was accompanied by a decrease in pH to around 3.7. In the present study, despite the lowest pH values already being reported, the HY and VHPR obtained are in the highest-range. For comparison purposes, Table 5 shows the results obtained in the UF-2 reactor with results from other studies applying continuous hydrogen-producing reactors fed with sucrose-based wastewater, in the mesophilic range.

These results indicate that the formation of a very acidic environment allowed the growth of acid-tolerant bacteria that were able to produce $H_2$ under very acid conditions, especially *Clostridium* sp. and *Ethanoligenens* sp.

## 4. Conclusions

This study stands out as the first to demonstrate the real possibility for continuous, long-term, stable $H_2$ production at pH below 3.0, with a mean yield of 3.4 mol of $H_2$ per mol of sucrose consumed. Proper HRT and OLR were crucial for enhancing hydrogen production. This was associated with increased sucrose consumption, reduced lactate formation, high acetate and ethanol concentrations, reduction of relative abundance of *Lactobacillus* sp. and increase of *Ethanoligenens* sp.

The operating requirements were keep at minimum and the non-pH control, along with the production of $H_2$ in extremely acid environments, presents several operating and economic advantages, including: the non-addition of alkalizing agents, which contributes to reduction of the costs; elimination of the demand for sludge pretreatment, due to the naturally acid environment; and, the non-necessity of constant sludge removal, since higher biomass concentration leads to enhanced $H_2$ production. These results open a new field of investigation in biological hydrogen production by dark fermentation towards a more sustainable and feasible technology.

## Acknowledgements

The authors are grateful for the financial support provided by the São Paulo Research Foundation (FAPESP) under grant agreement numbers #2014/22475-3, #2015/06246-7, #2017/05819-9, #2013/15665-8 and #2015/21650.

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