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Performance and composition of bacterial communities in anaerobic fluidized bed reactors for hydrogen production: Effects of organic loading rate and alkalinity

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ABSTRACT

This study evaluated the effects of the organic loading rate (OLR) and pH buffer addition on hydrogen production in two anaerobic fluidized bed reactors (AFBRs) operated simultaneously. The AFBRs were fed with glucose, and expanded clay was used as support material. The reactors were operated at a temperature of 30 °C, without the addition of a buffer (AFBR1) and with the addition of a pH buffer (AFBR2, sodium bicarbonate) for OLRs ranging from 19.0 to 140.6 kg COD m^{-3} d^{-1} (COD: chemical oxygen demand). The maximum hydrogen yields for AFBR1 and AFBR2 were 2.45 and 1.90 mol H_2 mol^{-1} glucose (OLR of 84.3 kg COD m^{-3} d^{-1}), respectively. The highest hydrogen production rates were 0.95 and 0.76 L h^{-1} L^{-1} for AFBR1 and AFBR2 (OLR of 140.6 kg COD m^{-3} d^{-1}), respectively. The operating conditions in AFBR1 favored the presence of such bacteria as *Clostridium*, while the bacteria in AFBR2 included *Clostridium*, *Enterobacter*, *Klebsiella*, *Veillonellaceae*, *Chryseobacterium*, *Sporolactobacillus*, and *Burkholderiaceae*.

1. Introduction

Hydrogen (H_2) is an extremely promising new energy source, as it is clean, recyclable and efficient. The biological production of H_2 can be divided into two processes: photofermentation and dark fermentation. The dark fermentation production of H_2 with anaerobic microorganisms has the advantage of a higher production rate relative to photosynthetic bacteria or algae [1]. The coupling of H_2 production to the utilization of waste materials containing high concentrations of organic compounds, such as solid waste and wastewater, may have economic and environmental benefits [2].

The prevention of the growth of H_2-consuming methanogens is important in the production of H_2 by dark fermentation. A simple heat-shock treatment is often used to remove non-spore-forming bacteria, such as methanogens, from the anaerobic inoculum to enrich the H_2-producing cultures. Another important method is the manipulation of culture

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conditions to block methanogenesis, e.g., operating at low pH and hydraulic retention times (HRTs) [1,3,4].

Several studies have shown that the fermentation component of H_2 production is influenced significantly by factors such as reactor configuration, HRT, organic loading rate (OLR), temperature, substrate concentration, nutritional requirements, and pH [5]. Specifically, pH has the greatest influence on the effluent composition of the acidogenic reactors [5]. Both the metabolic pathway and the hydrogenase activity (hydrogenase is the enzyme that catalyzes H_2 production) may be influenced by pH [6]. In most studies, the optimal pH was observed in the range of 5.2–7.0, with an average pH of 6.0 for H_2 conversion from carbohydrates [7]. However, the literature presents contradictory results regarding the optimum pH value for H_2 production. Khanal et al. [8] reported a value of 4.5, whereas Lee et al. [9] reported a value of 9.0. The possible causes of this lack of consensus are differences in the type of inoculum, substrate, and the pH range under investigation.

Anaerobic fluidized bed reactors (AFBRs) with adhered biofilm have been widely used as biological treatment systems with high efficiency and short HRT for effluents [10]. Numerous studies have explored projected and operational factors of AFBRs, such as the choice of support material, substrate concentration, HRT, and/or OLR [3,11–13], to achieve a high H_2 yield (HY). The pH and variations in the composition of bacterial communities at different OLRs are important in lowering HY [14]. On-line pH control with the addition of acid and base into operating acidogenic reactors is challenging to implement in practice. An alternative approach is to supplement the wastewaters with sufficient buffer to counteract pH drops resulting from the generation of organic acids during anaerobic digestion [15]. However, the use of an agent to increase pH may increase the costs of the process. This approach has not been studied systematically, and the relevant studies have produced contradictory results [16].

This study therefore focused on the performance evaluation of two AFBRs with and without pH buffer addition during H_2 production and analyzed the composition of soluble microbial products in the reactors operated under progressively increasing OLR. The evolution of the microbial community was related to the operational reactor data to better understand the process.

2. Materials and methods

2.1. Anaerobic fluidized bed reactor and support material

Two identical jacketed AFBRs were constructed from transparent acrylic with the following dimensions: 190 cm in height, 5.3 cm in internal diameter, and 4192 cm^3 in total volume. The two reactors employed expanded clay pellets commonly used in gardening. Expanded clay, a cheap material that is resistant to abrasion and that has a high rugosity for biomass immobilization, has been successfully used as a support carrier for H_2 production in anaerobic fluidized bed reactors [3,4,12]. The expanded clay pellets were ground, washed, and sifted to grain sizes between 2.8 mm and 3.35 mm. The real density of the expanded clay was 1.5 g cm^{-3} with a porosity of 23%. Approximately 1200 g of expanded clay was introduced into the reactor, creating an initial height of 94 cm for the static bed support material.

2.2. Heat-treatment of H_2-producing sludge and fermentation medium

The inoculum was obtained from the sludge of an upflow anaerobic sludge blanket reactor treating effluent from swine wastewater. To enrich H_2-producing bacteria, the inoculum was heat-treated at 90 °C for 10 min [17]. The medium used for H_2 fermentation contained glucose (2000 mg L^{-1}) as the sole carbon source with sufficient amounts of inorganic supplements [4].

2.3. AFBR setup and operating conditions

The two AFBRs were initially operated in batch mode for 48 h to activate the H_2-producing biomass. During this process, the substrate consumption by microorganisms was recorded periodically. After the activation period, the continuous operation of the reactors began with an HRT of 8 h, decreasing stepwise to 6 h, 4 h, 2 h, and 1 h for 90 days in five experimental phases. The two reactors were fed with synthetic wastewater with an OLR between 19.0 kg m^{-3} day^{-1} and 140.6 kg COD m^{-3} d^{-1}. The total liquid flow rate into the AFBR was maintained at 128 L h^{-1} (expansion = 30%). This flow rate produced a superficial velocity 1.30 times greater than the minimum fluidization velocity. AFBR1 was operated without the addition of a pH buffer, and the reactor AFBR2 was supplemented with alkalinity (1000 mg sodium bicarbonate L^{-1}) and 1 mL L^{-1} of hydrochloric acid (10 M). The reactors were operated at 30 ± 1 °C with an influent pH in the range of 6–7.

The effluent of the AFBR1 and AFBR2 entered a gas–liquid separator in which the gaseous and soluble products were collected separately. A gas meter (Type TG1; Ritter Inc., Germany) was used to measure the amount of H_2 generated. After reaching steady-state operation (based on a constant volumetric H_2 production rate with a variation within 5–10% for 3–5 days), the HRT decreased progressively from 8 h to 1 h.

2.4. Chemical analysis

Volatile organic acids and alcohols were determined using a gas chromatograph (GC-2010, Shimadzu, Tokyo, Japan) equipped with a flame ionization detector (FID). The GC used a COMBI-PAL headspace sample introduction system (AOC 5000 model) and HP-INNOWAX column (30 m long × 0.25 mm internal diameter × 0.25 μm film thickness) [18]. The analyses of solids (total solids, TS; volatile suspended solids, VSS; and total volatile solids, TSS) and chemical oxygen demand (COD) were performed according to Standard Methods [19]. The influent and effluent glucose concentrations were determined using the GOD-PAP enzymatic method [4]. The biogas composition was determined by a gas chromatograph (GC-2010, Shimadzu, Tokyo, Japan) equipped with a thermal conductivity detector (TCD). The carrier gas used was argon with a Carboxen 1010 Plot column (30 m long with an internal diameter of 0.53 mm).

2.5. Molecular biology analysis

The genomic DNA of the samples was obtained following the procedure of Griffiths et al. [20] modified to be a direct method with glass beads and phenol-chloroform extraction. The amplification of the polymerase chain reaction (PCR) was performed with a bacterial domain primer set for the 16S rRNA gene, 27 forward (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1100 reverse (5′-AGGGTTGCGCTCGTTG-3′) [21] as described by Barros et al. [22].

The PCR product purification was performed using a kit (GFX PCR DNA) and Gel Band Purification (GE Healthcare). The clone library was pGEM®-T Easy Vector Systems (Promega), transformed into *Escherichia coli* competent cells according to the manufacturer's instructions. After the extraction of plasmid DNA, the PCR amplification was performed with primers M13F–M13R [22]. The nucleotide sequences were processed and aligned using the SeqMan – DNA-STAR (Lasergene sequence analysis). The phylogenetic affiliations of the obtained sequences were determined using the BLAST search program at the NCBI website compared with the 16S rRNA gene organism sequences represented in Genbank (http://www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (http://rdp.cme.smu.edu). The phylogenetic tree was constructed by the neighbor-joining method [23] using the program MEGA version 4.1 [22,24]. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence level of tree topologies. The *Methanosarcina thermophila* (HB 945419.1) and *Methanosarcina* sp. (AB288262) were used as the outgroups.

3. Results and discussion

3.1. Glucose conversion, biogas contents, pH, and soluble microbial products

Fig. 1 shows the effect of HRT on glucose conversion and biogas content of the reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer). At an HRT in the range of 8–1 h for AFBR1, the glucose conversion was approximately 91%. For AFBR2, in the HRT range of 8–2 h, the glucose conversion was greater than 94%, but the glucose conversion decreased to 79% for the HRT of 1 h. For AFBR1 and AFBR2, the influent glucose concentration ranged from 2065 mg L^{-1} to 2379 mg L^{-1} and 2077 mg L^{-1} to 2370 mg L^{-1}, respectively. Effluent glucose concentrations ranged from 140 mg L^{-1} to 241 mg L^{-1} (AFBR1) and 16 mg L^{-1} to 498 mg L^{-1} (AFBR2).

H_2 and CO_2 were present in the biogas of both reactors, while CH_4 was not detected during any phases of the experiment. The absence of CH_4 in the biogas may be attributed to the heat-treatment of the inoculum and the maintenance of the pH below 5.5 (Fig. 2), factors that inhibit the methanogenic activity responsible for the consumption of H_2 in the system. H_2 content in the biogas increased from 8% to 35% in AFBR1 (without pH buffer) and from 8% to 40% in AFBR2 (with pH buffer) (Fig. 1).

These glucose conversion and biogas content values are in agreement with other studies using AFBRs with glucose concentrations of 2000 mg L^{-1} without pH buffer [3,4], 4000 mg L^{-1} with pH buffer (1000 mg sodium bicarbonate L^{-1}) [12,22], 4000 mg L^{-1} without pH buffer [16], 5000 mg L^{-1} adjusting the buffer concentrations in the feed [25], and 10,000 and 30,000 mg L^{-1} with pH controlled constantly by automatic titration using sodium hydroxide and hydrochloric acid [11], and sucrose concentrations ranging from 5000 to 40,000 mg COD L^{-1} with pH buffer (5240 mg ammonium bicarbonate L^{-1} bicarbonate) [10].

The pH was stable and decreased within the operating range of an acidogenic anaerobic system, i.e., between 3.7 and 4.1 in reactor AFBR1 (without pH buffer) and between 5.1 and 5.5 in reactor AFBR2 (with pH buffer) (Fig. 2). The influent pH was between 6.5 and 7.2 in both reactors.

The distribution of metabolites generated is crucial in assessing the efficiency of H_2-producing cultures. The determination of the composition of soluble microbial products (SMP) implied that the fermentation pathway dominated the metabolic flow [26].

The solventogenic pathway characterized by the formation of reduced end products such as alcohols is unfavorable to H_2 production because the additional free electrons from NADH

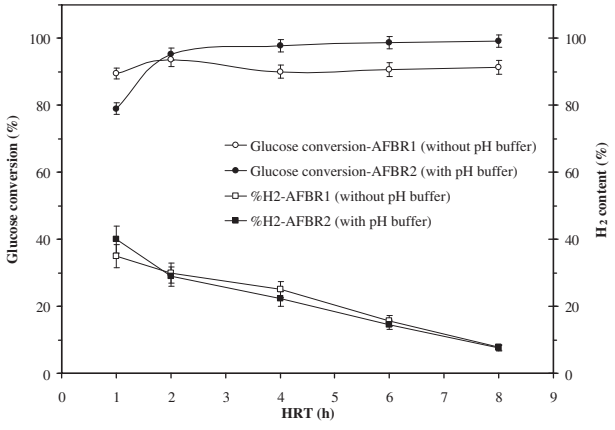

Fig. 1 – Effect of HRT on the glucose conversion and biogas contents in reactors without pH buffer (AFBR1) and with pH buffer (AFBR2).

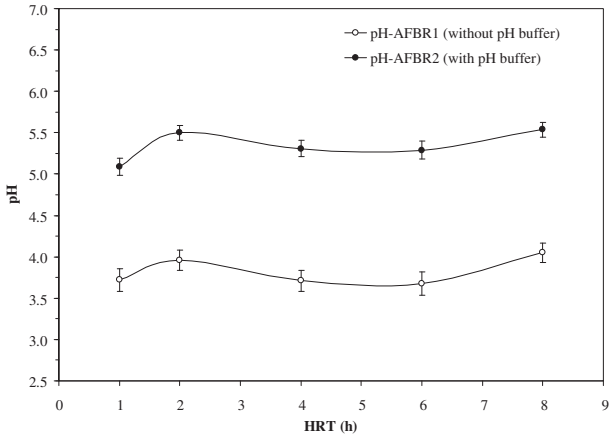

Fig. 2 – Performance of effluent pH in the reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer).

enzyme have been consumed, causing low H_2 yields. On the other hand, high H_2 yields have been associated with an acidogenic pathway that produces a mixture of organic acids, such as acetic acid and butyric acid [27].

Fig. 3 shows that acetic acid (HAc), butyric acid (HBu), and ethanol (EtOH) were major SMPs of reactor AFBR1 (without pH buffer) under different HRTs. Propionic acid (HPr) was not detected in AFBR1 in any experimental phase. The HAc concentration (ranging from 3.76 to 8.87 mM) was greater than the HBu concentration (ranging from 4.66 to 6.60) and the EtOH concentration (ranging from 1.16 to 2.14 mM).

For reactor AFBR2 (with pH buffer), HAc, EtOH, HBu, and HPr were major SMPs under different HRTs. The HAc concentration (4.33–8.67 mM) was greater than the EtOH concentration (2.51–7.61 mM), HBu concentration (1.88–3.13 mM) and HPr concentration (1.22–2.43 mM) (Fig. 4).

According to Koskinen et al. [28], H_2 production from carbohydrates occurs when HAc or HBu is produced, while HPr and EtOH are considered to be unfavorable metabolites for H_2 production, as H_2 is consumed or not produced in the production of HPr and EtOH. Ethanol production thus decreases when H_2 production is optimized (HAc and HBu production) and vice versa. The presence of EtOH is also particularly undesirable due to the added toxicity of EtOH for bacteria. The high EtOH concentration is in agreement with the low H_2 production rates observed during HRT at 8 h because these metabolites represent H_2 that has not been released as gas.

The presence of HAc, HBu, EtOH, and HPr during anaerobic fermentation by *Clostridium* has been widely reported [29]. However, the abundance of EtOH production from the mixed culture used was most likely due to the dominance of *Enterobacter* and/or *Klebsiella*, as EtOH is one of the major products of these facultative anaerobes [13,30].

It is difficult to know whether the EtOH production occurred simultaneously with H_2 generation or if there was a shift in the metabolism at some point during the experiment. Lay et al. [31] have indicated that a shift from H_2/VFA production to solventogenesis occurs at a pH of approximately 5.6, but no significant pH decrease was observed in any of the experiments. Other authors suggest that alcohol production occurs once the bacteria enter the stationary growth phase [32], while still other authors attribute the shift to increasing H_2 partial pressure [33].

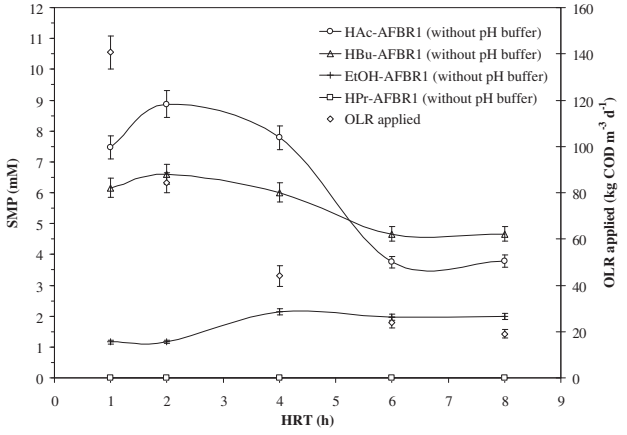

Fig. 3 – Effect of HRT on the SMP for the reactor AFBR1 (without pH buffer).

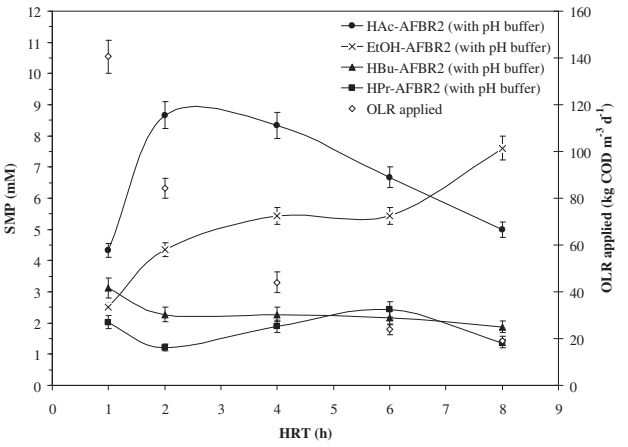

Fig. 4 – Effect of HRT on the SMP for the reactor AFBR2 (with pH buffer).

Fig. 5 shows the amount of total volatile fatty acids (TVFA) (TVFA = HAc + HBu + HPr) and the HAc/HBu ratio for reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer). The TVFA and the HAc/HBu ratio for both systems exhibit a similar trend; these factors increase with decreasing HRT and reach a maximum at the optimum HRT of 2 h (OLR of 84.3 kg COD m^{-3} d^{-1}). Beyond this optimum, TVFA and the HAc/HBu ratio decreased with decreasing HTR. The HAc/HBu ratio can therefore be used to indicate the optimum HRT (or OLR) for H_2 production [34].

Some authors also found that lower HAc/HBu ratios resulted in greater HY. This inconsistency might be attributed to the different types of fermentation pathways used by the microorganisms [13]. According to Wu et al. [35], the HAc/HBu ratio appears to be insufficient for predicting HY and/or H_2 content. Other factors should therefore be considered simultaneously. For instance, the amounts of the metabolites that

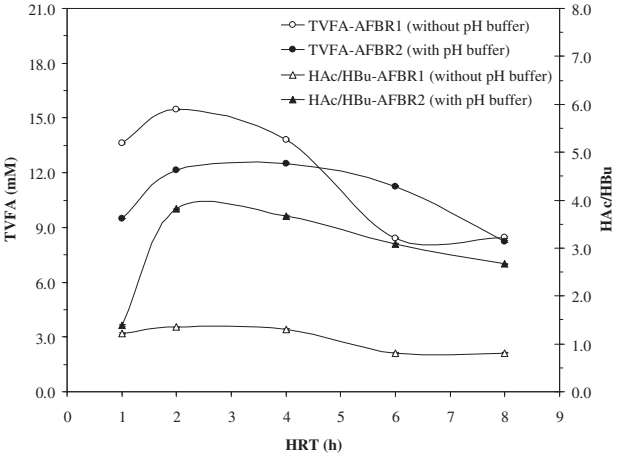

Fig. 5 – Effect of HRT on the TVFA and HAc/HBu ratio for the reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer).

are favorable (e.g., HAc and HBu) or unfavorable (e.g., EtOH, HPr and lactic acid) to H_2 production may also play critical roles in the HY. Moreover, Wu et al. [35] show that there might be an optimal HAc/HBu ratio for H_2 production but that ratio may be highly dependent on the anaerobic culture or the carbon substrate used.

The greater production of HAc and HBu can explain why reactor AFBR1 showed higher HY and H_2 content in biogas than reactor AFBR2. The metabolic pathway used by reactor AFBR1 can be considered more favorable for obtaining satisfactory H_2 production than the metabolic pathway used by reactor AFBR2. To maximize HY, the substrate metabolism should be steered away from alcohols and TVFA production (solventogenesis).

3.2. *Effect of OLR in the H_2 production*

Fig. 6 shows the effect of OLR on the hydrogen production rate (HPR) and HY values of the reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer).

The HPR values for AFBR1 and AFBR2 increased linearly from 0.10 to 0.95 and from 0.12 to 0.76 L h^{-1} L^{-1}, respectively, when OLR increased from 19.0 to 140.6 kg COD m^{-3} d^{-1}. For AFBR1, linear regression results show that the correlation between HPR (y_1) and OLR (x_1) can be expressed as $y_1 = 0.0069x_1 - 0.0153$ ($r^2 = 0.9989$). For AFBR2, linear regression results show that the correlation between HPR (y_2) and OLR (x_2) can be expressed as $y_2 = 0.0057x_2 + 0.0107$ ($r^2 = 0.9383$) (Fig. 6).

The HY values for AFBR1 and AFBR2 increased linearly from 1.38 to 2.18 mol H_2 mol^{-1} glucose and from 0.96 to 1.78 mol H_2 mol^{-1} glucose, respectively, when OLR increased from 19.0 to 44.0 kg COD m^{-3} d^{-1}. For an OLR of 84.3 kg COD m^{-3} d^{-1} (HRT of 2 h), the maximum HY values of 2.45 and 1.90 mol H_2 mol^{-1} glucose were achieved for AFBR1 and AFBR2. However, HY decreased to 2.37 and 1.24 mol H_2 mol^{-1} glucose for AFBR1 and AFBR2, respectively, when OLR increased to 140.6 kg COD m^{-3} d^{-1} (Fig. 3). Lin et al. [10] operated an AFBR with a draft tube using silicone gel for trapping anaerobic sludge, and a maximum HY of 4.98 mol H_2 mol^{-1} sucrose (which corresponds to 62.3% yield considering that the maximum theoretical HY for sucrose is 8 mol H_2 mol^{-1} sucrose) was obtained at an OLR of 107.9 kg COD m^{-3} d^{-1}. For an AFBR operated with activated carbon as a support material, Zhang et al. [11] obtained a maximum HY of 1.19 mol H_2 mol^{-1} glucose (which corresponds to 29.8% yield considering that the maximum theoretical HY for sucrose is 4 mol H_2 mol^{-1} glucose) at an OLR of 240 kg COD m^{-3} d^{-1}.

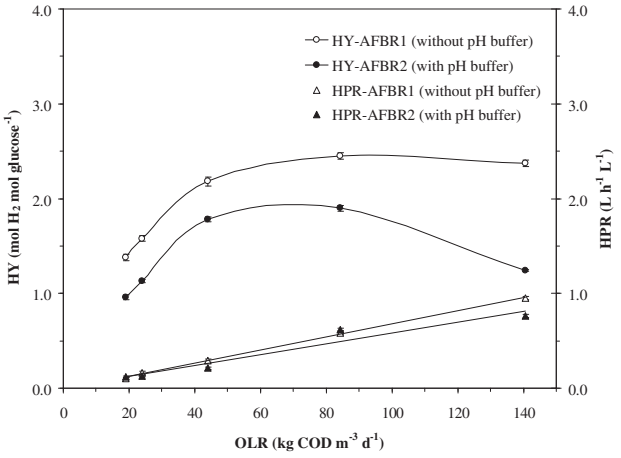

Fig. 6 – Effect of HRT on the HY and HPR for the reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer).

According to the literature review of Kraemer and Bagley [36], there is disagreement as to whether higher HY can be achieved with lower or higher OLR, and the mechanisms causing the HY diversity at different OLRs are unclear. TVFA inhibition at higher OLR has been the best supported explanation.

The HY observed in the current study was maximized at an OLR of 84.3 kg COD m^{-3} d^{-1} in both reactors, decreasing as the OLR increased further. However, TVFA also decreased from 15.5 to 13.6 mM for AFBR1 and from 12.16 to 9.48 mM for AFBR2, when OLR increased from 84.3 to 140.6 kg COD m^{-3} d^{-1} (Fig. 3). The results of this work are somewhat similar to those of Shen et al. [37], suggesting that an optimum OLR that maximizes HY may be near the OLR that causes overload with respect to substrate conversion.

The main products in AFBR1 (without pH buffer, pH range 3.7–4.1) were HAc and HBu, while in AFBR2 (with pH buffer, pH range 5.1–5.5), the main products were HAc and EtOH, and HPr was detected in all HRTs. The results from this work suggest that the absence of methanogenic activity can be a consequence of heat-treatment of the inoculum [3], a pH range of 3.7–5.5 [11], a lower HRT [11], and a high recycle flow rate applied in both AFBRs (ranging from 243 to 30 when HRT decreased from 8 to 1 h) [4]. The results also indicate the competition between the microorganisms of mixed culture for the glucose substrate, and the changes in the fermentation pathway at pH below 5.5 were dependent on the OLR (or HRT) and alkalinity supplementation.

3.3. *Composition of bacterial communities*

Analyses of composition of bacterial communities obtained from a sample of biomass adhering to the support material in reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer) were conducted for HRT of 2 h (OLR of 84.3 kg COD m^{-3} d^{-1}). Through the cloning and sequencing of fragments of 16S rRNA, a total of 63 and 101 clones were obtained from AFBR1 and AFBR2. The identified clones are shown in Table 1.

Figs. 7 and 8 show the consensus phylogenetic tree obtained with primers for the bacteria domain from the cloning and sequencing of the microbial consortium used in reactors AFBR1 and AFBR2. The coefficients of similarity observed between the clones and the NCBI database ranged from 96% to 99% and indicated the presence of phylogenetically related bacteria, based on the evaluation of partial sequences of the 16S rRNA gene.

For reactor AFBR1, for OLR increasing from 19.0 to 44.0 kg COD m^{-3} d^{-1} (HRT decreased from 8 to 4 h), the HAc and HBu concentrations increased from 3.76 to 7.78 mM and from 4.66 to 6.01 mM, respectively, while the EtOH concentration remained near 2 mM. When the OLR increased to

Table 1 – Microorganisms identified in reactors AFBR1 (without pH buffer) and AFBR2 (with pH buffer).

Reactor	Clones	Microorganism	Access number (GenBank)	Similarity (%)	Reference
AFBR1	1, 2, 3, 5, 6, 13, 44, 45, 47, 48	*Clostridium* sp.	EU331374	99	Li et al. (2007) – not published
	7, 8, 11, 12, 17, 46, 53, 54, 57	Uncultured bacterium	EF393081	98	D'Angelo et al. (2007) – not published
	4, 9, 10, 15, 16, 22, 38, 60, 61, 62	Clostridiaceae	AB081585	96	Sato et al. (2007) – not published
	14, 18, 19, 26, 28, 35, 39, 41, 42, 43, 50, 52, 63	*Clostridium* sp.	AY862515	98	Zhang et al. (2004) – not published
	20, 21, 29, 30, 32, 33, 34, 51, 55, 56, 59	Clostridia	AY607121	96	[38]
	23, 24, 25, 31, 36, 37, 40, 49, 58	*Clostridium* sp.	EF040827	99	Kim et al. (2006) – not published
	4, 9, 10, 14, 16, 20, 26, 44, 48, 87, 101	Uncultured	GQ203648.1	98	Li (2009) – not published
		Enterobacter sp.	FJ189785.1		Math et al. (2008) – not published [39]
		Enterobacter sp.	AB461711.1		
	11, 22, 27, 29, 34, 50, 79, 82, 105, 116, 117, 118	*Clostridium* sp.	GU129927.1 FJ938128.1	99	Kuang et al. (2009) – not published [40]
	25, 61, 109	Uncultured Burkholderiaceae bacterium	AM420125.1 FJ375495.1	98	Bolivar et al. (2006) – not published [41]
	23, 64, 73, 89, 110, 113	Uncultured *Klebsiella* sp.	GQ416853.1	99	Boucher et al. (2009) – not published [42]
AFBR2	7, 8, 18, 21, 30, 31, 38, 43, 46, 54, 63, 66, 69, 72, 76, 83, 86, 95, 103, 119	*Sporolactobacillus laevolacticus*	AB362643.1 AB362649.1 D16274.1	99	Tanaka et al. (2007) – not published [43] [44]
	5, 12, 15, 24, 28, 33, 53, 55, 65, 68, 70, 78, 84, 88, 90, 92, 96, 102, 104, 114, 115	*Chryseobacterium* sp.	EU724053.1 DQ673675.1	98	Berg et al. (2008) – not published [45]
	6, 13, 17, 19, 32, 36, 37, 39, 47, 49, 56, 59, 67, 71, 74, 80, 85, 93, 94, 99, 100, 106, 107, 108, 111, 112, 120	Uncultured *Veillonellaceae* bacterium	FJ393139.1 FJ393127.1	96	[46]

84.3 kg COD m^{-3} d^{-1} (HRT decreased to 2 h), the HAc and HBu concentrations increased to 8.87 and 6.60 (maximum concentrations), respectively, while the EtOH concentration decreased to 1.17 mM. The maximum HY value for reactor R1 was observed. For OLR of 140.4 kg COD m^{-3} d^{-1}, the HAc and HBu concentrations decreased to 7.48 and 6.16 mM, and the EtOH concentration remained at 1.16 mM.

The operating conditions in the reactor AFBR1 (without pH buffer) mainly favored the presence of such bacteria as *Clostridium*. Clostridia are straight, gram-positive, endospore-forming bacilli that thrive at pH values of approximately 4.0. Most species are obligately anaerobic, although tolerance to oxygen varies widely; some species will grow but not sporulate in the presence of air at atmospheric pressure [47]. The

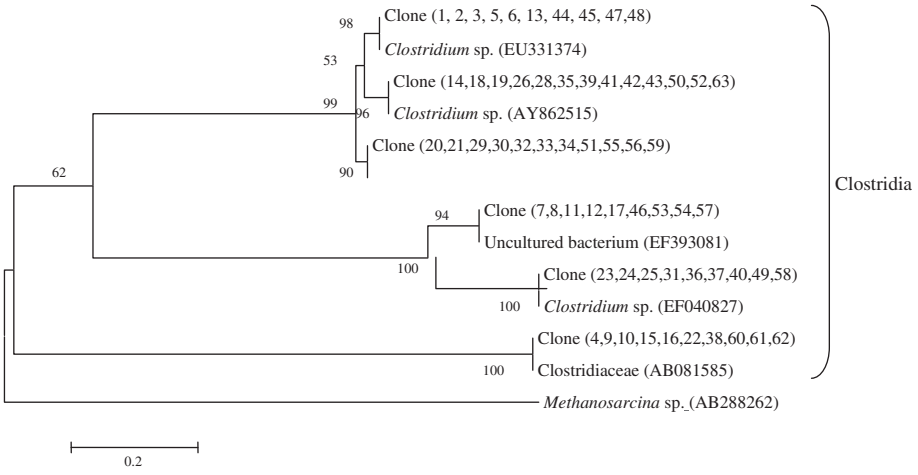

Fig. 7 – Phylogenetic relationships of representative bacterial 16S rRNA gene sequences determined by the neighbor-joining method. Sample obtained from the biomass adhering to support material in reactor AFBR1 (without pH buffer). Bootstraps obtained with 500 resamplings are shown at the nodes. The scale bar indicates 0.2 nucleotide substitution per site. *Methanosarcina* sp. (outgroup).

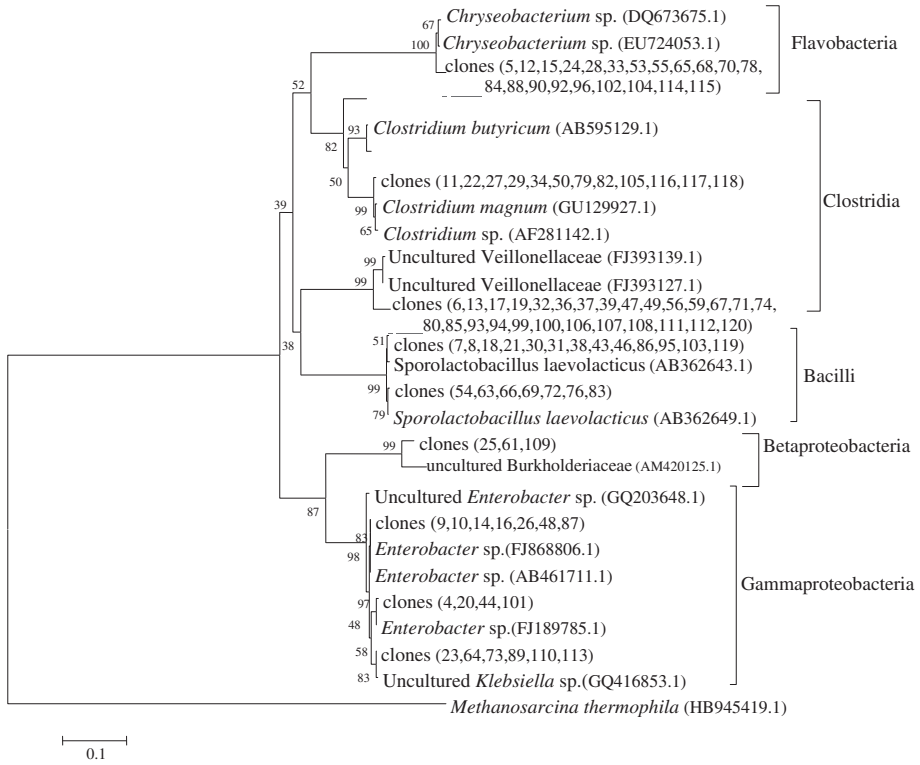

Fig. 8 – Phylogenetic relationships of representative bacterial 16S rRNA gene sequences determined by the neighbor-joining method. Sample obtained from the biomass adhering to support material in reactor AFBR2 (with pH buffer). Bootstraps obtained with 1000 resamplings are shown at the nodes. The scale bar indicates 0.1 nucleotide substitution per site. *Methanosarcina thermophile* (outgroup).

members of genus *Clostridium* are among the most extensively studied H_2 producers, fermenting a wide variety of carbohydrates, including polysaccharides. The main fermentation products from glucose are not only H_2, CO_2, butyrate and acetate but also ethanol, lactate, formate, acetone and butanol [25]. The high efficiency of H_2 production in this bioreactor should be achieved in the bacterial composition presented as Clostridia dominant.

The effect of pH buffering on AFBR2 subject to increasing of OLR from 19.0 to 44.0 kg COD m^{-3} d^{-1} (HRT decreased from 8 to 4 h) caused the HAc and HBu concentrations to increase from 5.00 to 8.83 mM and 1.88 to 2.27 mM, respectively, while the EtOH concentration decreased from 7.61 to 5.43 mM. The HPr concentration ranged from 1.35 to 1.89 mM, reaching a maximum value of 2.43 mM for an OLR of 23.9 kg COD m^{-3} d^{-1} (HRT of 6 h). When the OLR increased to 84.3 kg COD m^{-3} d^{-1} (HRT decreased to 2 h), the HAc concentration increased to 8.67 mM and the HBu concentration remained at 2.27 mM, while the EtOH concentration decreased to 4.35 mM. The maximum HY value for AFBR2 was observed under these conditions. For an OLR of 140.4 kg COD m^{-3} d^{-1}, the HAc concentration decreased to 4.33 mM, while the HBu concentration increased to 3.13 mM and the EtOH concentration decreased to 2.51 mM.

A wider diversity of bacteria, including Clostridium, Enterobacter, Klebsiella, Sporolactobacillus, Chrysebacterium, Burkholderiaceaea and Veillonellaceae, was found in reactor AFBR2 (with pH buffer).

A literature review indicates that in reactors with H_2-producing mixed cultures, *Clostridia* species are commonly accompanied by *Enterobacter* [48] or *Klebsiella* species [35,49–51]. Facultative anaerobes (such as *Enterobacter* and *Klebsiella*) are efficient in producing H_2 compared to strict anaerobes (such as *Clostridium*). H_2 production at partially anaerobic conditions is technically feasible for facultative anaerobes [50,51].

In AFBR2, 11 clones were similar to gram-stain-negative *Enterobacter* sp.. *Enterobacter* strains are facultatively anaerobic and chemoorganotrophic, having both a respiratory and a fermentative metabolism. D-glucose and other carbohydrates are catabolized with the production of acid and, in many species, gas [52]. Yokoi et al. [53] studied the performance of *Enterobacter aerogenes* HO 39 and reported HY values of 1.0 mol H_2 mol^{-1} glucose at an optimum temperature of 38 °C and pH 4 for H_2 production. According to Song et al. [54], *Enterobacter* strains are considered suitable for industrial scale H_2 production due to their rapid growth rates, ability to utilize a wide range of carbon sources, and low sensitivity to dissolved oxygen, H_2 pressure and pH.

The higher levels of EtOH and HPr in AFBR2 may have been caused by the control of the pH between 5.1 and 5.5 by alkalinity supplementation (1000 mg sodium bicarbonate L^{-1}), which could have favored the prevalence of solvent-producing microorganisms such as *Klebsiella* sp. As reported by Wu et al. [49], formation of alcohols is known to consume free electrons from NADH and is therefore unfavorable for H_2

production. The production of electron-consuming solvents (such as EtOH) therefore decreased H_2 production. According to Rossi et al. [55], facultative anaerobes such as *Enterobacter* and *Klebsiella* have shown a very restricted optimal pH range (between 5.0 and 6.0) for H_2 production. In AFBR2, six clones similar to *Klebisiella* sp. were identified. These clones can utilize various types of substrates and produce alcohols such as 2,3-butanediol, isopropanol and ethanol as well as hydrogen and carbon dioxide as soluble and gaseous metabolites [56].

In addition to *Clostridium*, *Enterobacter*, and *Klebsiella*, *Chryseobacterium* sp., *Veillonellaceae*, *Sporolactobacillus laevolacticus*, and *Burkholderiaceae* were also detected in reactor AFBR2 (with pH buffer). However, the potential functions of some microorganisms present in AFBR2 remain unclear.

In AFBR2, 27 clones were similar to the uncultured *Veillonellaceae* bacterium (96%). The *Veillonellaceae* are a family of the Fimicutes and Clostridia class. Members of this family are all obligate anaerobes and occur in habitats such as rivers, lakes, and the intestines of vertebrates. The members of this family range from spherical forms, such as *Megasphaera* and *Veillonella*, to curved rods, as typified by the Selenomonads. *Selenomonas* has a characteristic crescent shape, with flagella inserted on the concave side, while *Sporomusa* is similar but non-motile. The optimum temperatures are between 30 and 37 °C with optimum pH between 6.5 and 8.0. Pyruvate, lactate, malate, fumarate and oxaloacetate are fermented. The major metabolic end products in trypticase-glucose-yeast extract broth are acetic and propionic acids. CO_2 and H_2 are produced from lactate [57]. The physiological diversity of these bacteria favored by the maintenance of effluent pH in the range of 5.09–5.54 likely explains the HPr production in AFBR2.

In AFBR2, 21 clones were similar to *Chryseobacterium* (similarity 98%) with strains occurring in soil, fresh water, and marine environments, while others are found in dairy products; yet others are opportunistic pathogens in humans and animals. *Chryseobacterium* cells are gram-negative, non-motile, non-spore-forming rods with parallel sides and rounded ends. Most *Chryseobacterium* strains are chemo-organotrophs with a strictly respiratory type of metabolism except for *Chryseobacterium scophthalmum*, which displays both respiratory and fermentative metabolisms. Moreover, some strains exhibited anaerobic respiration with nitrate or fumarate as the terminal electron acceptor and were able to produce acids from arabinose, cellobiose, ethanol, fructose, glucose, glycerol, lactose, maltose, sucrose, and xylose [58]. The maintenance of effluent pH between 5.1 and 5.5 most likely favored the growth of these bacteria, which can utilize glucose and produce organic acids, including HPr.

In AFBR2, 21 clones were identified as *S. laevolacticus* (99% similarity). *S. laevolacticus* cells are Gram-positive, with endospores resistant to heating at 80 °C for 10 min. *S. laevolacticus* cells are facultatively anaerobic or microaerophilic; good growth occurs on media containing glucose, and D- or DL-lactic acid is produced homofermentatively. Acid is produced from glucose, fructose, galactose, mannose, maltose, sucrose and trehalose. *S. laevolacticus* is responsible for lactic acid production and employed to ferment fructose and glucose at pH values below 4.0 [59], which might be responsible for the lower HY values obtained.

In AFBR2, three clones were similar to the uncultured *Burkholderiaceae* bacterium (98% similarity). According to Maintinguer et al. [30], most of the bacteria belonging to the *Burkholderia* genus are commonly found in soil, water and plant roots and are associated with the fungi mycelium. These bacteria, which are Gram-negative rods, are known to degrade sugars such as sucrose [60], but there are no reports associating these bacteria with H_2 production.

The heat-treatment of the inoculum and establishing a high recycle flow rate for expanded clay fluidization on AFBR1 favored the maintenance of pH near 4.0. For AFBR2, in addition to the conditions mentioned for AFBR1, the addition of an agent to raise pH favored the maintenance of pH near 5.0. These operating conditions of AFBRs defined the initial composition of the microbial communities present in the reactors, until they were altered by increasing OLR.

4. Conclusions

In both AFBRs, the HY values increased with reduction of HRT from 8 to 2 h, and the HY values decreased when HRT was reduced to 1 h. The HPR values increased with decreasing HRT from 8 to 1 h. AFBR1 (without pH buffer) showed higher HY and HPR values in all HRTs evaluated, and the maximum values reached were 2.45 mol H_2 mol^{-1} glucose and 0.95 L h^{-1} L^{-1}, respectively. The H_2 content in the biogas was approximately the same in both reactors (maximum near 40% for HRT of 1 h). The main products were HAc and HBu for AFBR1 (pH between 3.7 and 4.1, without pH buffer), and, for AFBR2, the main products were HAc and EtOH (pH between 5.1 and 5.5, with pH buffer) for OLRs ranging from 19.0 to 140.6 kg COD m^{-3} d^{-1} (HRT decreasing from 8 to 1 h). From these results, pH control and applied OLR appeared to cause variations in the composition of the microbial communities, and pH control and applied OLR play an important role in determining the type of anaerobic fermentation pathway.

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Notation

Symbols

COD	Chemical oxygen demand
EtOH	Ethanol concentration
HAc	Acetic acid concentration
HBu	Butyric acid concentration
HPr	Propionic acid concentration
HPR	Hydrogen production rate
HRT	Hydraulic retention time
HY	Hydrogen yield
OLR	Organic loading rate
SMP	Soluble microbial products
TVFA	Total volatile fatty acids

VFA Volatile fatty acids
VSS Volatile suspended solids

Abbreviations:
AFBR Anaerobic fluidized bed reactor
FID Flame ionization detector
TCD Thermal conductivity detector

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