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Effect of Substrate Concentration on Dark Fermentation Hydrogen Production Using an Anaerobic Fluidized Bed Reactor

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Abstract The effect of substrate (glucose) concentration on the stability and yield of a continuous fermentative process that produces hydrogen was studied. Four anaerobic fluidized bed reactors (AFBRs) were operated with a hydraulic retention time (HRT) from 1 to 8 h and an influent glucose concentration from 2 to 25 gL^{-1}. The reactors were inoculated with thermally pre-treated anaerobic sludge and operated at a temperature of 30 °C with an influent pH around 5.5 and an effluent pH of about 3.5. The AFBRs with a HRT of 2 h and a feed strength of 2, 4, and 10 gL^{-1} showed satisfactory H_2 production performance, but the reactor fed with 25 gL^{-1} of glucose did not. The highest hydrogen yield value was obtained in the reactor with a glucose concentration of 2 gL^{-1} when it was operated at a HRT of 2 h. The maximum hydrogen production rate value was achieved in the reactor with a HRT of 1 h and a feed strength of 10 gL^{-1}. The AFBRs operated with glucose concentrations of 2 and 4 gL^{-1} produced greater amounts of acetic and butyric acids, while AFBRs with higher glucose concentrations produced a greater amount of solvents.

Keywords Hydrogen production · Anaerobic fluidized bed reactor · Substrate concentration · Hydraulic retention time

Nomenclature

COD	Chemical oxygen demand, mg L^{-1}
HRT	Hydraulic retention time, h
HPR	Hydrogen production rate, L h^{-1} L^{-1}
HY	Hydrogen yield, mol H_2 mol^{-1} glucose
HAc	Acetic acid concentration, mg L^{-1}
HBu	Butyric acid concentration, mg L^{-1}

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HPr Propionic acid concentration, mg L^{-1}
EtOH Ethanol concentration, mg L^{-1}
MetOH Methanol concentration, mg L^{-1}
SMP Soluble microbial products, mg L^{-1}
TVFA Total volatile fatty acids, mg L^{-1}
VFA Volatile fatty acids, mg L^{-1}

Abbreviations
AFBR Anaerobic fluidized bed reactor
FID Flame ionization detector
R2 Reactor fed with glucose concentration of 2 gL^{-1}
R4 Reactor fed with glucose concentration of 4 gL^{-1}
R10 Reactor fed with glucose concentration of 10 gL^{-1}
R25 Reactor fed with glucose concentration of 25 gL^{-1}

Introduction

Today, global attention is moving toward reduction of air pollution and greenhouse gas emissions caused by the combustion of fossil fuels and simultaneous discovery of sustainable future fuel. Hydrogen is considered to be an ideal energy alternative to fossil fuels due to its high conversion efficiency, recyclability, and nonpolluting nature [1–3]. Furthermore, H_2 is a raw material for the synthesis of ammonia, alcohols, and aldehydes, as well as for the hydrogenation of various petroleum and edible oils, coal, and shale oil [4]. Although most H_2 is generated from fossil fuels through thermochemical processes, it may also be produced by biological processes, which are potentially more attractive, especially if wastewater can be used as the raw material [1, 5, 6].

Wastewaters generated from various industrial processes are considered to be ideal substrates because they contain high levels of easily degradable organic material, which results in a net positive energy or economic balance [7]. Anaerobic digestion is widely accepted and used in the biological treatment of wastewater in Brazil due to favorable weather conditions (tropical climate), low implementation and operation costs, low energy consumption, little generation of biologic sludge, and satisfactory tolerance to high organic loads [8]. In addition to contributing to the biological treatment of wastewater, this technology may also help generate hydrogen while degrading the biodegradable fraction of organic residues by interrupting the process at the acidogenic phase (methanogenesis inhibition). H_2 production using wastewater as a fermentative substrate with simultaneous treatment of wastewater is increasing in importance and is an effective way of tapping clean energy from a renewable resource in a sustainable manner [7, 9].

The efficiency of hydrogen and organic acids production is influenced by several operational parameters of a system, each with its own characteristics. Operational parameters, i.e., hydraulic retention time (HRT), carbon source and substrate concentration, pH, temperature, and partial pressure, have been investigated in several reactors including suspended-growth systems and immobilized-growth systems [3, 7]. However, the effect of the substrate concentration on the stability and yield of biological hydrogen production is not well understood despite its technical and economic significance. According to Wang and Wan [3], in an appropriate range, increasing substrate concentration could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but substrate concentrations at much higher levels could decrease this ability [10, 11]. In addition, during biological reaction processes, volatile fatty acids (VFAs) can

accumulate to a high level and thus may be stimulatory, inhibitory, or even toxic to fermentative bacteria, depending on their concentration [12].

According to Lin et al. [13], due to the difficulty in maintaining a sufficient amount of H_2-producing microorganisms in suspended-growth reactors under low HRT, many research efforts have been made to enhance biomass retention using physical or biological immobilization approaches. Furthermore, some studies have shown that cell immobilization techniques including cell entrapment and cell attachment the improve biomass retention and hydrogen production rate (HPR) in immobilized-growth reactors [13–17].

The anaerobic fluidized bed reactor (AFBR) with attached biofilm has been widely used as a biological treatment system for wastewater with high efficiency and low HRT due to its potential advantages, e.g., high concentration of biomass attached to a dense carrier and good mixing characteristics [16, 17]. Therefore, the present study examines the effect of different glucose concentrations on continuous biohydrogen production in AFBRs with mixed-culture biofilms grown on expanded clay support material. The effect of the HRT on the performance of AFBRs and the inhibitory effects of ethanol, acetic acid, and butyric acid were also investigated.

Materials and Methods

Anaerobic Fluidized Bed Reactor and Medium Composition

Figure 1 shows a schematic diagram of the four identical jacketed reactors used in this study. The reactors were constructed of transparent acrylic with the following dimensions: a height of 190 cm, an internal diameter of 5.3 cm, and a total volume of 4,192 cm^3. The medium used for H_2 fermentation contained glucose (2, 4, 10, and 25 gL^{-1}) as the main carbon source and was supplemented with nutrients [17], including (in milligrams per liter): CH_4N_2O, 125; $NiSO_4{\cdot}6H_2O$, 1; $FeSO_4{\cdot}7H_2O$, 5; $FeCl_3{\cdot}6H_2O$, 0.5; $CaCl_2{\cdot}6H_2O$, 47; $CoCl_2{\cdot}2H_2O$, 0.08; SeO_2, 0.07; KH_2PO_4, 85; $KHPO_4$, 21.7; and $Na_2HPO_4{\cdot}2H_2O$, 33.4.

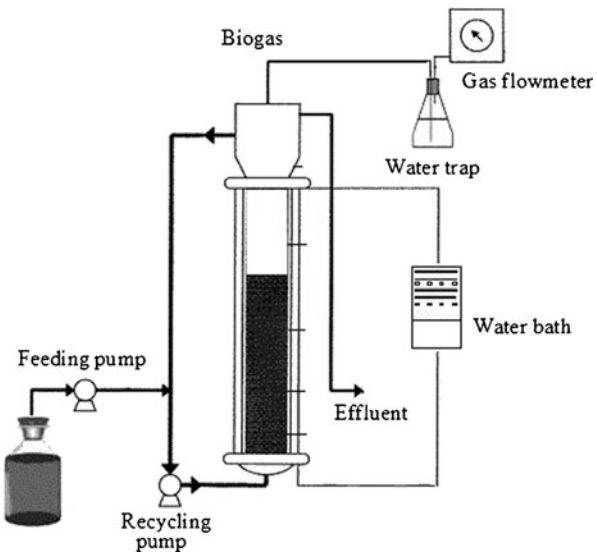

Fig. 1 Schematic description of the anaerobic fluidized bed reactor

Inoculum and Support Material

The inoculum used in this study was obtained from the anaerobic sludge of an upflow anaerobic sludge blanket reactor that treats effluent from swine wastewaters. The H_2 productivity of the sludge was enhanced by heat treatment according to the methodology of Kim et al. [18]. This treatment consisted of preheating the sludge for 10 min at 90 °C to inhibit methanogenic activity [19].

This study employed expanded clay pellets commonly used in gardening. These pellets were ground, washed, and sifted to grain sizes between 2.8 and 3.35 mm. The real density of the expanded clay was 1.5 $g cm^{-3}$. Approximately 1,300 g of expanded clay was introduced into the reactor, which created an initial height of 90 cm for the static bed of support material that was used for the immobilization of the enriched acidogenic biomass.

Setup and Operation Conditions of AFBR for H_2 Production

The four AFBRs with expanded clay as the support material were fed with a medium containing glucose (2, 4, 10, and 25 $g L^{-1}$) and heat-treated sludge (10%, *v*/*v*). Nitrogen gas was used to sparge the fermentation medium to create an anaerobic environment. The temperature in the AFBRs was maintained at 30 °C by recirculating heated water from a thermostatic bath through the column water jackets. For the AFBR system, the total liquid flow rate (Q) was maintained at 128 $L h^{-1}$ (bed expansion=30%). This flow rate produced a superficial velocity that was 1.30-fold greater than the minimum fluidization velocity. The bioreactor was initially operated on batch mode for 48 h to activate the H_2-producing sludge. Afterward, it was switched to a continuous mode with a designated hydraulic retention time (HRT=8 h). To facilitate discussion of results and identify the reactors, each reactor was named according to the influent glucose concentration: reactor operated with 2 g glucose L^{-1} (R2), reactor operated with 4 g glucose L^{-1} (R4), reactor operated with 10 g glucose L^{-1} (R10), and reactor operated with 25 g glucose L^{-1} (R25).

When steady-state was reached (based on a constant H_2 production rate with a variation within 5–10% for 5–10 days), the HRT was decreased progressively from 8 to 1 h. The four reactors were operated for 120 days in five experimental phases without the addition of an alkalinity agent. The compositions of gas products and soluble metabolites produced during H_2 fermentation were monitored as a function of time. The pH and glucose concentration were also recorded. A gas meter (Type TG1; Ritter Inc., Germany) was used to measure the amount of hydrogen generated.

Chemical Analyses

Glucose concentration was measured with an enzymatic GOD-PAP [20]. Chemical oxygen demand (COD), pH, and solids including total solids, volatile suspended solids, and total volatile solids were measured in accordance with Standard Methods [21].

The biogas hydrogen content was determined by gas chromatography (GC-2010, Shimadzu, Japan) using a thermal conductivity detector with argon as the carrier gas, and the column was packed with Supelco Carboxen 1010 Plot (30 m×0.53 mm i.d.) [19]. Concentrations of VFAs and alcohols were also measured by a gas chromatography system (GC-2010, Shimadzu, Japan) that was equipped with a FID and COMBI-PAL headspace injector (AOC 5000 model) and HP-INNOWAX column (30 m× 0.25 mm i.d. × 0.25 μm film thickness) [19].

Results and Discussion

Effect of HRT and Glucose Concentration on H_2 Production

Figures 2, 3, and 4 show the variation in hydrogen yield (HY), HPR, and H_2 content, respectively, as a function of HRT for the four AFBRs used in this study. The HY values improved for all AFBRs when the HRT was decreased from 8 to 2 h, achieving maximum values of 2.49, 1.78, 1.26, and 0.60 mol H_2 mol^{-1} glucose for influent glucose concentrations of 2, 4, 10, and 25 g L^{-1}, respectively. These results suggest that the HY values increased when the HRT and the influent glucose concentration decreased. Furthermore, for reactors R2, R4, and R10, the HY values increased significantly, while for reactor R25, the values remained virtually constant. However, for the HRT of 1 h, the HY values decreased to 2.41, 1.25, 0.78, and 0.56 mol H_2 mol^{-1} glucose for reactors R2, R4, R10, and R25, respectively (Fig. 2).

The HPR values for reactors R2, R4, and R10 increased from 0.11 to 0.97, 0.14 to 1.06, and 0.13 to 1.46 L h^{-1} L^{-1}, respectively, when the HRT was decreased from 8 to 1 h. For reactor R25, the HPR increased from 0.08 to 0.71 L h^{-1} L^{-1} when the HRT was reduced from 8 to 2 h, but it decreased to 0.61 L h^{-1} L^{-1} when the HRT was reduced to 1 h (Fig. 3).

The biogas was composed of hydrogen and carbon dioxide for all AFBRs in the experimental phases of this study. No methane was found in the biogas. H_2 content increased significantly from 8% to 35% (R2), 47% to 59% (R4), 27% to 51% (R10), and 15% to 48% (R25) as the HRT decreased from 8 to 1 h, reaching the highest level (59%) when the reactor was operated with an influent glucose concentration of 4 g L^{-1} and HRT of 1 h. The results obtained from this work confirm that heat-treated anaerobic sludge associated with the preservation of acidogenic conditions could inhibit the methanogenic activity in AFBRs [20].

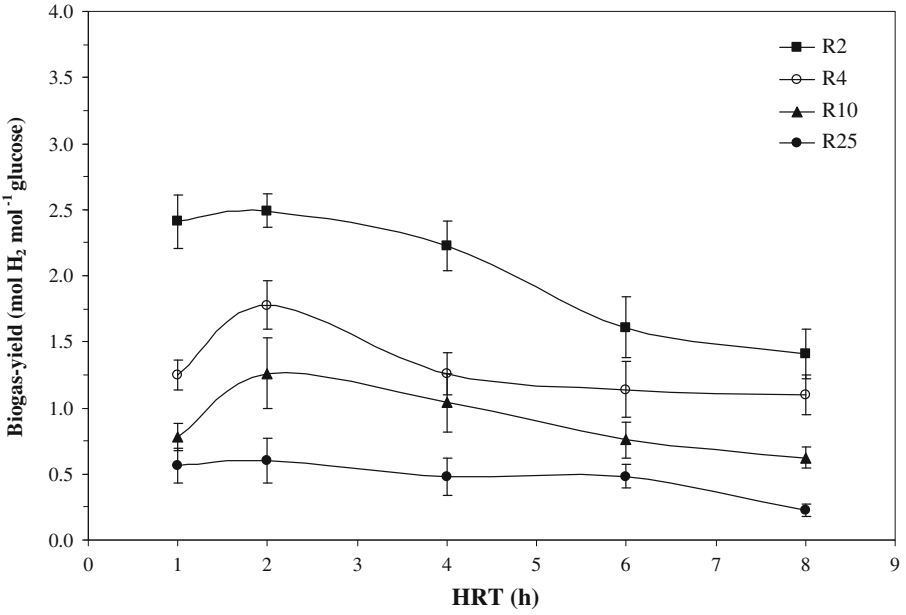

Fig. 2 Effect of HRT on biogas yield in AFBRs with feed strengths of 2, 4, 10, and 25 g L^{-1}

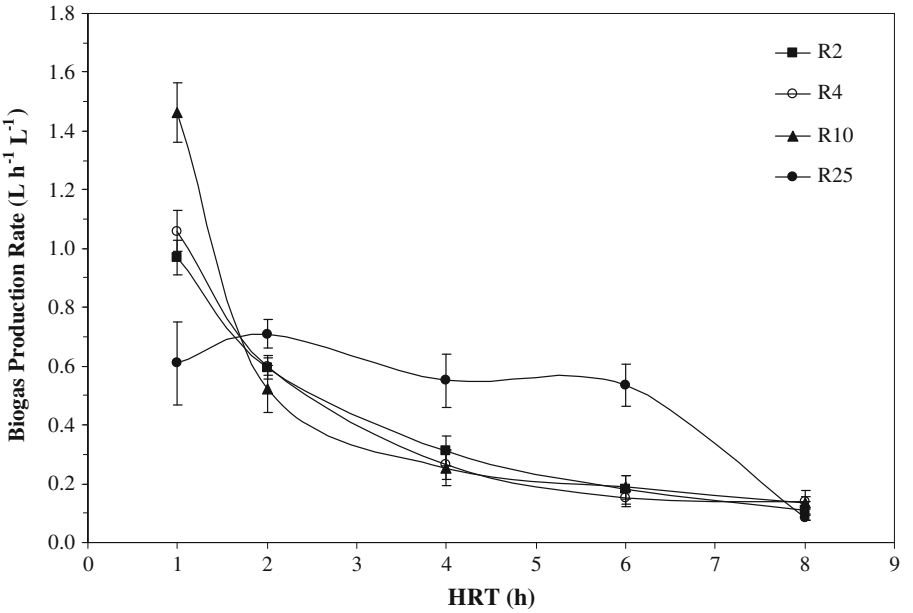

Fig. 3 Effect of HRT on biogas production rate in AFBRs with feed strengths of 2, 4, 10, and 25 gL^{-1}

Figure 5 shows the effect of glucose concentration on the performance of the AFBRs with feed strengths of 2, 4, 10, and 25 gL^{-1}. The H_2 content increased significantly from 35% to 59% when the glucose concentration was increased from 2 to 4 gL^{-1}. After increasing the

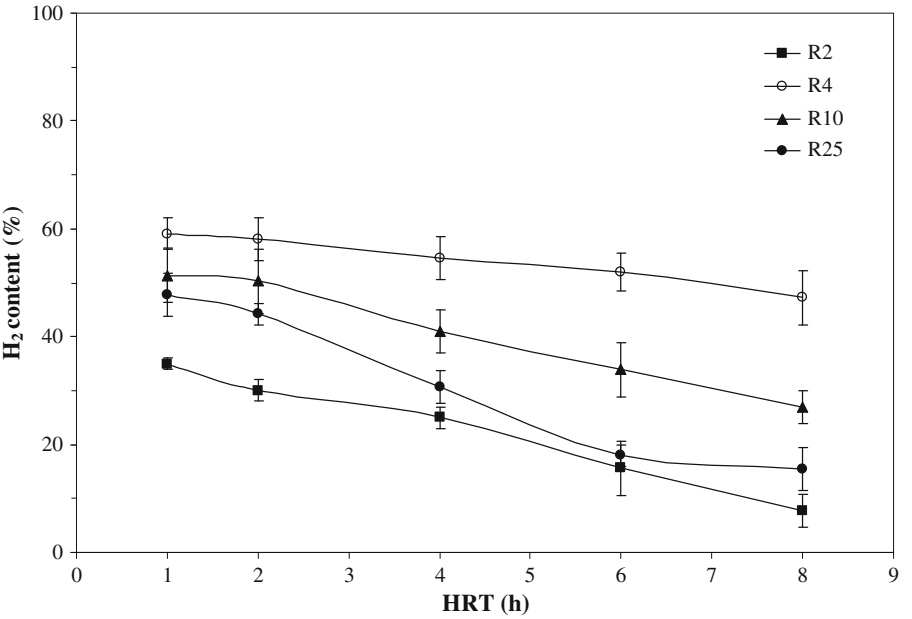

Fig. 4 Effect of HRT on H_2 content in AFBRs with feed strengths of 2, 4, 10, and 25 gL^{-1}

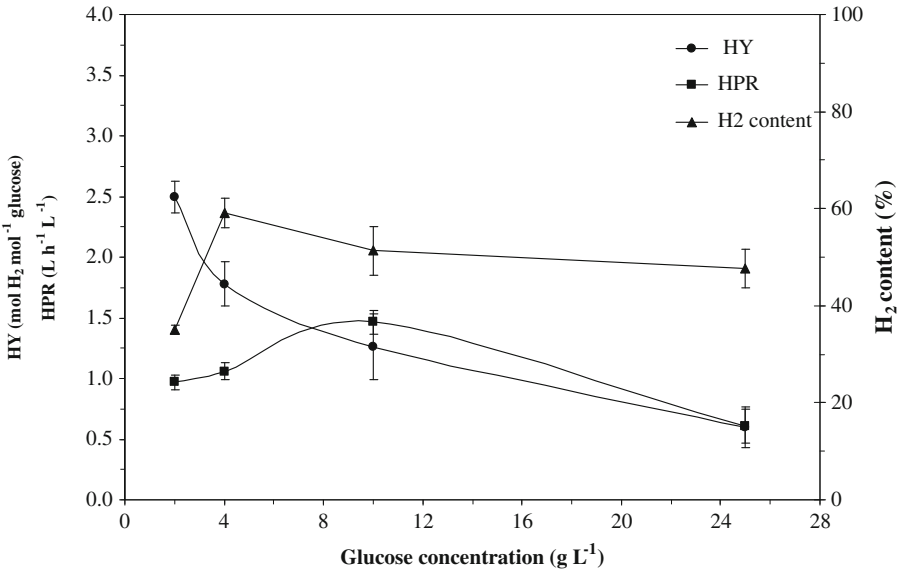

Fig. 5 Effect of glucose concentration on HY, HPR, and H_2 content in AFBRs

glucose concentration from 10 to 25 gL^{-1}, the content remained virtually constant, reaching values close to 50%.

As shown in Figs. 2, 3, and 4, the highest HY values for reactors R2, R4, R10, and R25 were obtained when the reactors were operated at a HRT of 2 h. However, the highest HPR and H_2 content values were obtained when the reactors were operated at a HRT of 1 h, except for reactor R25. Thus, the approach of Zhang et al. [16] was adopted to evaluate the effect of glucose concentration on the performance of AFBRs to choose between maximum HY values at a HRT of 2 h (Fig. 2) and maximum HPR and H_2 content values at a HRT of 1 h (Figs. 3 and 4, respectively).

Thus, Fig. 5 shows that the HY values decreased from 2.49 to 0.60 mol H_2 mol $glucose^{-1}$ upon an increase in the glucose concentration from 2 to 25 gL^{-1}. However, the HPR values increased from 0.97 to 1.46 Lh^{-1} L^{-1} when the glucose concentration increased from 2 to 10 gL^{-1}, and when the glucose concentration was increased to 25 gL^{-1}, the HPR value dropped to 0.61 Lh^{-1} L^{-1}. The operational condition with the highest HPR value (1.46 Lh^{-1} L^{-1}) was HRT 1 h, with 10 gL^{-1} of glucose. However, the HPR is deemed as a less important parameter for reactor performance analysis during hydrogen production. Therefore, the optimum condition for hydrogen production was 2 gL^{-1} at a HRT of 2 h because under this condition, the HY (2.49 mol H_2 mol $glucose^{-1}$) was higher than under the other conditions.

According to Prakasham et al. [22], the substrate concentration is one of the most important fermentation parameters for effective biohydrogen production as noticed with other microbial fermentations. However, higher concentrations can also negatively impact biohydrogen production [10, 11]. Therefore, the authors hypothesized that the progressive increase in H_2 production with increased glucose concentration might be due to the limitation of substrate concentration at lower substrate conditions for effective metabolism and further biohydrogen production process. However, the observed reduction in H_2 yield above the optimum glucose concentration could be due to limited glucose utilization either at the transport level or the metabolism level; it could also be due to substrate concentration.

Van Ginkel et al. [10], Chen et al. [23], and Skonieczny and Yargeau [24] also hypothesized that high substrate concentrations become inhibitory to the microorganisms as a result of a pH drop and/or hydrogen pressure increase. Conversely, at low substrate concentrations, bacteria are thought to utilize the carbon source mainly for biomass growth and not biogas production.

Soluble Microbial Products

Table 1 shows the distribution of soluble microbial products (SMP) associated with an increase in glucose concentration and HRT reduction in the AFBRs. The SMP for reactor R2 were acetate (HAc) (38.11–55.12%), butyrate (HBu) (39.62–47.35%), and ethanol (EtOH) (3.89–14.61%). For reactor R4, the SMP were HBu (42.87–64.62%), EtOH (5.25–35.00%), HAc (22.13–28.14%), and methanol (MetOH) (0.00–10.36%). For reactor R10, the SMP were EtOH (17.10–47.26%), MetOH (0.00–42.33%), HAc (21.77–33.20%), and HBu (7.84–31.30%). For reactor R25, the SMP were EtOH (48.54–62.65%), HAc (15.00–40.53%), HBu (2.58–24.03%), MetOH (0.00–19.77%), and propionate (HPr) (0.00–0.90%). Generally, the SMP produced in this study were similar to those in several other studies [13, 14, 16]; however, the metabolite distribution did not agree that of the previous studies.

Table 1 Effect of glucose concentration on the SMP distribution in AFBRs

Reactor	HRT (h)	HAc/SMP (%)	HBu/SMP (%)	HPr/SMP (%)	EtOH/ SMP (%)	MetOH/ SMP (%)	TVFA (mM)	SMP (mM)	HAc/HBu
R2	8	38.28	47.11	0.00	14.61	0.00	8.45	9.89	0.81
	6	38.11	47.35	0.00	14.54	0.00	8.42	9.85	0.80
	4	51.30	39.62	0.00	9.08	0.00	13.80	15.17	1.29
	2	55.12	40.99	0.00	3.89	0.00	15.47	16.10	1.34
	1	52.47	43.19	0.00	4.34	0.00	13.63	14.25	1.22
R4	8	27.32	64.62	0.00	5.25	2.81	16.38	17.81	0.42
	6	23.36	56.29	0.00	9.99	10.36	16.82	21.12	0.42
	4	28.14	60.02	0.00	11.85	0.00	16.66	18.90	0.47
	2	25.20	45.69	0.00	29.10	0.00	15.94	22.48	0.55
	1	22.13	42.87	0.00	35.00	0.00	11.06	17.02	0.52
R10	8	23.01	7.84	0.00	31.12	38.03	15.88	51.47	2.94
	6	24.69	15.88	0.00	17.10	42.33	11.26	27.77	1.55
	4	31.25	23.67	0.00	45.08	0.00	8.00	14.56	1.32
	2	33.20	31.30	0.00	35.50	0.00	11.05	17.13	1.06
	1	21.77	20.71	0.00	47.26	10.26	8.96	21.10	1.05
R25	8	40.53	3.63	0.90	48.54	6.41	21.03	46.68	11.17
	6	15.00	2.58	0.00	62.65	19.77	14.08	80.11	5.82
	4	21.37	8.04	0.00	53.27	17.31	9.72	33.04	2.66
	2	22.47	15.32	0.00	54.51	7.70	9.81	25.96	1.47
	1	21.51	24.03	0.00	54.46	0.00	8.90	19.53	0.90

HAc acetate, *HBu* butyrate, *HPr* propionate, *EtOH* ethanol, *MetOH* methanol, *TVFA* total volatile fatty acids, *TVFA* HAc + HBu + HPr, *SMP* TVFA + EtOH + MetOH, *HAc/SMP* molar acetate-to-SMP ratio, *HBu/SMP* molar butyrate-to-SMP ratio, *HPr/SMP* molar propionate-to-SMP ratio, *EtOH/SMP* molar ethanol-to-SMP ratio, *MetOH/SMP* molar methanol-to-SMP ratio, *HAc/HBu* molar acetate-to-butyrate ratio

As indicated in Table 1, HAc and HBu contributed to the majority of SMP for reactor R2 (85.48–96.09%) and reactor R4 (64.90–70.90%), while EtOH was also produced in a considerable amount in R2 (3.89–14.61%) and R4 (5.25–35.00%). These values are in agreement with those of other studies using AFBR with expanded clay and glucose (2 gL^{-1} [20]; 4 gL^{-1} [25]), celite and glucose (5 gL^{-1} [26]), and activated carbon and glucose (10 and 30 gL^{-1} [16]).

The reactors with higher glucose concentrations produced greater amount of solvents (35.5–82.4%). For these glucose concentrations (10 and 25 gL^{-1}), the higher ethanol concentrations observed in this study are similar to the results of Wu et al. [27], but they are significantly different from the results of our recent studies using the same medium composition, inoculum, and support material [17, 20, 25]. In those studies, HBu and HAc contributed to the majority of SMP (often over 80%) when H_2 production was optimized. For reactors R10 and R25, the abundance of EtOH production from the mixed culture used was probably due to the dominance of *Enterobacter* and/or *Klebsiella*, as EtOH is one of the major products of these facultative anaerobes.

Propionate was not detected during the operation of the reactors, except in reactor R25 (HRT 8 h); however, even in reactor 25, the amount was insignificant (0.90%). The absence of propionate at all HRT suggests that the activity of bacteria that form this acid can be inhibited under low pH conditions. This activity may also be sensitive to low HRT [16] and high organic loads. Moreover, the absence of propionate during reactor operation is associated with improved hydrogen yield because propionate production consumes 2 mol of hydrogen for every 2 mol of propionate produced (Eq. 1).

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (1)$$

The production of acetic and butyric acids indicates that a greater quantity of SMP favor hydrogen production because hydrogen production occurs when these products are generated (Eqs. 2 and 3).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (3)$$

Ethanol was produced in large proportions in most reactors, except in reactor R2 (containing 2 gL^{-1} glucose). In this reactor, the percentage of ethanol was lower than 14.61% at the beginning of the operation and then dropped and stabilized at values close to 3.89%. Ethanol is known to be detrimental to hydrogen production, as no hydrogen is consumed or produced (Eq. 4).

$$C_6H_{12}O_6 \rightarrow CH_3CH_2OH + 2CO_2 \quad (4)$$

Similar to the findings of Skonieczny and Yargeau [24], the concentration of ethanol is higher in this study than those reported elsewhere [28, 29]. Acetic and butyric acids are key intermediate products for the production of hydrogen and volatile fatty acids, as hydrogen production is explained by Eqs. 2 and 3. The presence of ethanol is particularly detrimental because it is toxic to bacteria [24].

The SMP distribution in reactors R2 and R4 strongly suggests that the predominant fermentation was of the HBu type. Furthermore, H_2 production depends on the ratio of acetic acid and butyric acid (HAc/HBu), as shown in Table 1. Also, larger HAc/HBu ratios resulted in greater measured yields of hydrogen. This is consistent with previous research; many researchers have observed butyrate-type fermentation in continuous reactors and stated that

the HAc/HBu ratio can be used as an indicator of H_2 production in acidogenic systems [30, 31]. In general, the largest HAc/HBu ratio corresponds to the highest H_2 yield, according to the theoretical stoichiometric equations (Eqs. 2 and 3). The results of this study are evidence of this because HAc was the main soluble metabolite in reactors R2 and R4.

However, some authors also found lower HAc/HBu ratios resulted in greater H_2 yield. This inconsistency might be attributed to the different types of fermentation pathways used by the microorganisms. As discussed by Wu et al. [32], the HAc/HBu ratio appears to be insufficient to predict H_2 yield and/or content. Therefore, other factors should be considered simultaneously.

The pH remained stable throughout the system operation within the operating range of acidogenic anaerobic systems, i.e., between 3.7 and 4.1 in R2, 3.5 and 3.7 in R4, 3.4 and 3.6 in R10, and 3.3 and 3.5 in R25. The influent pH remained between 5.2 and 6.0 in R2, 5.2 and 5.9 in R4, 4.8 and 5.6 in R10, and 5.5 and 5.9 in R25 (Table 2).

To estimate glucose consumption during fermentation, glucose levels were measured in the fermentation medium (Table 2). The data indicate that glucose consumption steadily increased at all concentrations, indicating a divergence between H_2 production and glucose utilization above optimal levels. This may further confirm that glucose metabolism can be influenced by initial external concentration and that H_2 production can be inhibited by soluble metabolites derived from glucose fermentation. The data reveal that glucose consumption increased with decreasing glucose concentration, suggesting a similarity between H_2 production and glucose utilization above optimal glucose levels.

The substrate conversion achieved in reactors R2 and R4 is consistent with that of other studies using AFBRs for hydrogen production with sucrose (20 g COD L^{-1} [14]; 5–40 COD L^{-1}

Table 2 pH, glucose concentrations, and conversion values at different steady states of AFBRs

Reactor	HRT (h)	Influent pH	Effluent pH	Influent glucose (g L^{-1})	Effluent glucose (g L^{-1})	Glucose conversion (%)
R2	8	5.2±0.6	4.1±0.1	2.379±0.051	0.206±0.097	91±5
	6	5.7±0.7	3.7±0.1	2.377±0.069	0.222±0.094	91±4
	4	5.3±0.7	3.7±0.1	2.408±0.332	0.241±0.038	90±10
	2	5.7±0.7	4.0±0.1	2.173±0.097	0.140±0.039	94±3
	1	6.0±0.7	3.7±0.1	2.065±0.133	0.217±0.012	89±7
R4	8	5.9±0.5	3.7±0.1	4.203±0.053	0.887±0.025	79±3
	6	5.8±0.1	3.5±0.1	4.003±0.064	0.446±0.037	89±5
	4	5.3±0.7	3.5±0.1	4.055±0.101	0.845±0.065	79±2
	2	5.2±0.7	3.5±0.1	3.985±0.097	0.625±0.094	84±4
	1	5.6±0.5	3.5±0.1	4.043±0.056	1.308±0.057	68±3
R10	8	5.6±0.7	3.4±0.1	9.925±0.102	4.235±0.097	57±4
	6	5.6±0.7	3.4±0.1	9.519±0.110	5.448±0.085	43±3
	4	5.6±0.6	3.4±0.1	9.789±0.123	6.506±0.093	34±2
	2	5.3±0.9	3.4±0.3	9.780±0.099	6.247±0.101	36±2
	1	4.8±0.6	3.6±0.2	10.528±0.126	6.228±0.114	41±3
R25	8	5.8±0.6	3.4±0.1	25.187±0.201	16.113±0.198	36±4
	6	5.7±0.5	3.3±0.1	23.434±0.210	15.563±10.87	34±3
	4	5.9±0.2	3.3±0.1	25.610±0.223	18.249±0.139	29±2
	2	5.6±0.2	3.3±0.1	25.618±0.204	19.814±0.201	23±2
	1	5.5±0.2	3.5±0.2	26.809±0.234	21.572±0.242	20±1

[13]) and glucose (10 and 30 gL^{-1} [16]; 2 gL^{-1} [20]; 4 gL^{-1} [25]). These findings may be attributed to the high solid retention times because of the system of attached growth that promotes greater biomass accumulation in the system [15].

However, the accumulation of ethanol, acetic, and butyric acids may have had an inhibitory effect on the microorganisms, especially in R10 and R25, with regard to the parameters of substrate degradation, hydrogen production, and hydrogen yield during fermentative hydrogen production. Higher the concentrations of these metabolites lead to greater inhibitory effects. Other researchers have come to similar conclusions when studying the inhibitory effects of sodium butyrate [33] and ethanol, acetic acid, propionic, and butyric acids [34].

Carbon Balance and COD Removal

As proposed by Gavala et al. [15], Eq. 5 can be used to calculate the carbon balance in the reactors. Measured versus calculated COD concentrations for each steady state are also presented in this study, and the COD calculations were performed as follows: The products ($COD_{products}$) and the glucose ($COD_{glucose}$) COD concentration were calculated according to Eqs. 5 and 6, respectively. COD_{others} was the remaining COD after subtraction of the sum of the $COD_{products}$ and $COD_{glucose}$ from the $COD_{measured}$ (Eq. 7). The COD_{others} corresponds to the non-identified metabolic products during glucose fermentation.

$$\begin{aligned} COD_{products} = {} & a\left(\frac{mmol\ HAc}{1}\right)\cdot 64\frac{mgCOD}{mmol\ HAc} \\ & + b\left(\frac{mmol\ HBu}{1}\right)\cdot 160\frac{mg\ COD}{mmol\ HBu} \\ & + c\left(\frac{mmol\ H\,\mathrm{Pr}}{1}\right)\cdot 112\frac{mg\ COD}{mmol\ H\,\mathrm{Pr}} \\ & + d\left(\frac{mmol\ MetOH}{1}\right)\cdot 48\frac{mg\ COD}{mmol\ MetOH} \\ & + e\left(\frac{mmol\ EtOH}{1}\right)\cdot 96\frac{mg\ COD}{mmol\ EtOH} \end{aligned} \tag{5}$$

where a, b, c, d, and e are the measured concentrations of the acetic acid, butyric acid, propionic acid, methanol, and ethanol, respectively.

$$COD_{glucose} = f\left(\frac{mg\ Glucose}{1}\right)\frac{192\ mg\ COD}{180\ mg} \tag{6}$$

where f is the measured concentration of glucose.

The difference between $COD_{measured}$ and COD based on SMP may be attributed to the presence of other soluble metabolites not detectable by the chromatographic method used. Equation 7 shows how this difference was calculated:

$$COD_{others} = COD_{mensured} - (COD_{products} + COD_{glucose}) \tag{7}$$

Table 3 presents influent and effluent COD values and standard deviations as well as efficiencies for all reactors. Influent COD represents glucose added to the wastewater and carbonaceous matter present in urea. Effluent COD corresponds to the carbonaceous matter

Table 3 Influent COD, effluent COD, and COD removal in AFBRs with feed strengths of 2, 4, 10, and 25 gL^{-1}

Reactor	HRT (h)	Influent COD (mg L^{-1})	Effluent COD (mg L^{-1})	COD removal (%)
R2	8	2,395±156	1,504±208	37±10
	6	2,443±124	1,527±135	37±10
	4	2,628±170	1,997±178	24±7
	2	2,698±202	1,996±265	26±6
	1	2,395±95	1,916±100	20±2
R4	8	4,216±210	3,788±153	10±6
	6	4,140±206	3,349±146	19±9
	4	4,139±270	3,718±165	10±4
	2	4,487±220	3,805±191	15±2
	1	4,312±226	3,680±136	15±4
R10	8	11,298±954	8,617±457	24±5
	6	10,439±843	9,056±419	13±6
	4	10,693±977	8,639±433	19±3
	2	10,175±799	8,589±447	16±2
	1	10,969±901	8,705±512	21±2
R25	8	26,126±1,024	20,202±978	23±3
	6	26,447±1,201	22,352±883	15±2
	4	27,285±1,392	22,207±791	19±2
	2	26,116±1,273	23,502±943	10±1
	1	28,216±1,321	25,242±967	11±2

in the effluent that was oxidized. Carbonaceous matter present in the effluent consists of non-consumed glucose, soluble metabolites, e.g., organic acids, solvents, and other intermediary compounds, and biomass detached from the support medium.

To estimate the carbon balance, the theoretical effluent COD was calculated based on stoichiometric relationships for oxidation of glucose, acetic acid, butyric acid, propionic acid, biomass, ethanol, and methanol. Table 4 presents theoretical COD values for the remaining glucose, soluble metabolites, and biomass as well as the difference between the theoretical total COD and the COD measured for all reactors.

For the reactor operated with 2 gL^{-1} of glucose, the difference between $COD_{measured}$ and COD based on SMP ranged from 23 to 57 mg L^{-1} and corresponded to a variation of 1.02% and 2.86%. In the reactor operated with 4 gL^{-1} of glucose, this difference varied between 12 and 350 mg L^{-1}, which corresponded to a variation of 0.34% and 9.19%. The reactor operated at 10 gL^{-1} of glucose showed a difference ranging from 91 to 301 mg L^{-1} (variation of 1.05% and 3.28%), whereas in the reactor operated with 25 gL^{-1} of glucose, the difference varied between 17 and 1,026 mg L^{-1} (variation of 0.07% and 4.62%). The observed differences may be attributed to the presence of other metabolites that were not detected, e.g., lactic acid and formic acid, probably due to the chromatographic method adopted (headspace extraction), as this method can only detect volatile acids and alcohols.

Based on the carbon balance, the largest variation between COD measured in the effluent and the theoretical COD (corresponding to glucose, soluble metabolites, and biomass in the effluent) was 9.19%. However, it is important to note that the methods of determination of COD and metabolites produce errors of close to 10%, according to Standard Methods [21]. Thus, this variation may be attributed to the margin of error of the determination methods used.

Table 4 Theoretical COD values of soluble metabolites, biomass COD, and effluent COD measured in AFBRs with feed strengths of 2, 4, 10, and 25 gL^{-1}

Reactor	HRT (h)	$COD_{t,glucose}$ ($mg\ L^{-1}$)	$COD_{t,acetate}$ ($mg\ L^{-1}$)	$COD_{t,butyrate}$ ($mg\ L^{-1}$)	$COD_{t,propionate}$ ($mg\ L^{-1}$)	$COD_{t,biomass}$ ($mg\ L^{-1}$)	$COD_{t,ethanol}$ ($mg\ L^{-1}$)	$COD_{t,methanol}$ ($mg\ L^{-1}$)	$COD_{t,total}$ ($mg\ L^{-1}$)	$COD_{measured}$ ($mg\ L^{-1}$)	COD_{others} ($mg\ L^{-1}$)
R2	8	220	242	746	0	134	139	0	1,481	1,504	23
	6	237	240	747	0	129	138	0	1,490	1,527	37
	4	257	498	962	0	128	132	0	1,977	1,997	20
	2	149	567	1,055	0	107	60	0	1,939	1,996	57
	1	231	478	985	0	134	59	0	1,888	1,916	27
R4	8	946	245	1,382	0	192	90	24	3,405	3,788	39
	6	475	192	1,000	0	157	203	105	3,157	3,349	32
	4	901	320	1,563	0	161	215	0	3,432	3,719	12
	2	666	320	1,763	0	155	629	0	3,455	3,805	350
	1	1,394	235	964	0	181	573	0	3,556	3,680	124
R10	8	4,514	757	645	0	148	1,540	940	8,545	8,617	159
	6	5,807	438	705	0	157	457	564	8,129	9,056	104
	4	6,935	291	551	0	140	631	0	8,548	8,639	91
	2	6,659	364	858	0	134	585	0	8,600	8,589	254
	1	6,639	294	699	0	168	959	104	8,862	8,705	301
R25	8	17,177	1,210	271	47	148	2,178	144	21,174	20,202	1,026
	6	16,590	769	330	0	145	4,825	760	23,419	22,352	486
	4	19,454	452	425	0	141	1,692	275	22,439	22,207	107
	2	21,122	373	636	0	134	1,360	96	23,722	23,502	17
	1	22,996	269	751	0	168	1,023	0	25,206	25,242	35

Conclusions

Based on the experimental results, we concluded that for all AFBRs, the HY values increased when the HRT decreased from 8 to 2 h, and the influent glucose concentration increased from 2 to 25 gL^{-1}. For a HRT of 1 h, the HY values decreased considerably. The AFBRs with a HRT of 2 h and feed strengths of 2, 4, and 10 gL^{-1} showed satisfactory H_2 production performance (2.49, 1.78, 1.26 mol $H_2 mol^{-1}$ glucose, respectively), but the reactor fed with 25 gL^{-1} of glucose (0.60 mol $H_2 mol^{-1}$ glucose) did not. The highest HY value was obtained for reactor R2 (2 gL^{-1}) when it was operated at a HRT of 2 h.

For AFBRs with a feed strength of 2, 4, and 10 gL^{-1}, the HPR values increased when the HRT was reduced. This was not true for the reactor operated with a glucose concentration of 25 gL^{-1}. The maximum HPR value was achieved for the reactor with a HRT of 1 h and a feed strength of 10 gL^{-1}.

All AFBRs that increased glucose concentration had an impact on HY, HPR, and SMP distribution. Furthermore, the reactors operated with higher glucose concentrations (10 and 25 gL^{-1}) produced a greater amount of solvents.

Also, HRT and glucose concentration were found to influence the SMP distribution in the reactors. Propionic acid was not detected in either reactor. Thus, low pH values and high glucose concentrations can inhibit the production of propionic acid. In addition, the accumulation of ethanol and acetic and butyric acids can inhibit hydrogen production.

The hydrogen content in biogas increased when the HRT was reduced in all reactors, and its highest value (59%) was obtained in the reactor operated at a HRT of 1 h and fed with a glucose concentration of 4 gL^{-1}. The inoculum enrichment method was shown to be efficient, as methane was not detected in the biogas of the reactors.

The reactors operated with glucose concentrations of 2 and 4 gL^{-1} displayed the most auspicious distribution of soluble metabolites for hydrogen production. The predominant metabolites were acetic and butyric acids.

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