

# Continuous Hydrogen Production from Agricultural Wastewaters at Thermophilic and Hyperthermophilic Temperatures

Lucas Rodrigues Ramos<sup>1</sup> · Edson Luiz Silva<sup>1</sup>

Received: 14 October 2016 / Accepted: 12 December 2016 /  
Published online: 16 December 2016  
© Springer Science+Business Media New York 2016

**Abstract** The objective of this study was to investigate the effects of hydraulic retention time (HRT) (8 to 0.5 h) and temperature (55 to 75 °C) in two anaerobic fluidized bed reactors (AFBR) using cheese whey (AFBR-CW = 10,000 mg sugars L<sup>-1</sup>) and vinasse (AFBR-V = 10,000 mg COD L<sup>-1</sup>) as substrates. Decreasing the HRT to 0.5 h increased the hydrogen production rates in both reactors, with maximum values of 5.36 ± 0.81 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> in AFBR-CW and 0.71 ± 0.16 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> in AFBR-V. The optimal conditions for hydrogen production were the HRT of 4 h and temperature of 65 °C in AFBR-CW, observing maximum hydrogen yield (HY) of 5.51 ± 0.37 mmol H<sub>2</sub> g COD<sup>-1</sup>. Still, the maximum HY in AFBR-V was 1.64 ± 0.22 mmol H<sub>2</sub> g COD<sup>-1</sup> at 4 h and 55 °C. However, increasing the temperature to 75 °C reduced the hydrogen production in both reactors. Methanol and butyric, acetic, and lactic acids were the main metabolites at temperatures of 55 and 65 °C, favoring the butyric and acetic metabolic pathways of hydrogen production. The increased productions of lactate, propionate, and methanol at 75 °C indicate that the hydrogen-producing bacteria in the thermophilic inoculum were inhibited under hyperthermophilic conditions.

**Keywords** Cheese whey · Sugarcane vinasse · Thermophilic fermentation · Hyperthermophilic fermentation · Biohydrogen · Hydraulic retention time · Organic loading rate

## Introduction

The decrease in fossil fuel reserves has made alternative fuel production a major concern in many countries. In this context, fermentative hydrogen production has become a promising

---

✉ Edson Luiz Silva  
edsilva@ufscar.br

<sup>1</sup> Department of Chemical Engineering, Federal University of São Carlos, Rod. Washington Luis, km 235, São Carlos, SP 13565-905, Brazil

alternative because it is a pollution free, renewable, and low-cost alternative to conventional processes of hydrogen production [1].

However, a carbohydrate-rich source must be used to make this process economically feasible because protein or lipid hydrolysis does not generate fermentable components for hydrogen production [2]. The main criteria for selecting adequate substrates for biological hydrogen production are availability, cost, pureness of the substrate, and biodegradability. Potential resources for renewable hydrogen production include the by-products of agroindustrial processes, wood and processing residues, food processing residues, seaweed, and others. Therefore, countries with large agricultural economies have the potential for significant economic development due to this bioenergy [3].

In that context, fermented vinasse and cheese whey (CW) are viable options for biological hydrogen production. Thus, fermentation processes provide alternative uses for these agroindustrial by-products. Vinasse is produced in the distillation step after the fermentation of sugarcane juice. As much as 20 L of vinasse can be generated per liter of ethanol produced, with chemical oxygen demand values (COD) reaching  $100 \text{ g L}^{-1}$  [4]. CW is produced during cheese production. It has a high lactose concentration and COD values greater than  $80 \text{ g L}^{-1}$  [5]. Thus, the hydrogen production from these agroindustrial wastewaters can synergistically provide low-cost, renewable, green energy.

One of the main challenges in continuous hydrogen production is determining the optimal condition that minimizes costs. This condition is determined by evaluating the operational parameters such as the substrate concentration, hydraulic retention time (HRT), organic loading rate (OLR), and temperature [6].

Among these parameters, the temperature increase in a specific range can increase the hydrogen production rate (HPR) by accelerating metabolic functions and cellular growth. According to Pawar and van Niel [7], thermophilic temperatures are more favorable for hydrogen production because they produce high hydrogen yields (HY) and low metabolite concentrations. Higher temperatures, such as hyperthermophilic temperatures ( $>65 \text{ }^\circ\text{C}$ ), can increase the HY and HPR [8–13]. However, few studies have evaluated the effects of temperatures in thermophilic and hyperthermophilic ranges using CW and vinasse as substrates.

Hydrogen production in anaerobic fluidized bed reactors (AFBRs) using vinasse and CW has been studied by different authors [6, 14–17]. Among the high-rate reactors for hydrogen production, AFBRs can efficiently promote mass transfer between the substrate and biofilm [18, 19].

Therefore, the objective of this study is to investigate the best HRT (8 to 0.5 h) for hydrogen production. Then, the temperature effects ( $55$  to  $75 \text{ }^\circ\text{C}$ ) in two AFBRs fermenting CW (AFBR-CW =  $10,000 \text{ mg L}^{-1}$  total carbohydrates) and vinasse (AFBR-V =  $10,000 \text{ mg COD L}^{-1}$ ) are evaluated based on this HRT.

## Materials and Methods

### Inoculum

The inoculum source for the AFBR reactor startup was granular sludge, which was used to treat vinasse in a thermophilic upflow anaerobic sludge bed reactor (UASB) in Usina São Martinho, Pradópolis, SP, Brazil. The pH and total volatile solid concentration in the inoculum

were 7.06 and 41.3 g L<sup>-1</sup>, respectively. Inoculum pretreatment was performed according to the methodology proposed by Kim et al. [20]. The treatment promoted the elimination of methanogenic cells and enriched the acidogenic cells via endospore formation.

## Substrate

This study used the wastewater from sugar and ethanol production, vinasse, and cheese production, CW. The nutrients necessary for cellular growth were added according to Rosa et al. [16].

The CW wastewater was composed of CW powder supplied by Elegê Laticínios S.A., Teotônia, Brazil. The powder is produced by concentrating CW via evaporation and spray drying. The characterization of the CW powder yielded a Kjeldahl nitrogen concentration of  $18 \pm 1$  mg g CW<sup>-1</sup>, which is equivalent to  $116 \pm 10$  mg protein g CW<sup>-1</sup>. The CW powder solutions in 10,000 mg L<sup>-1</sup> of CW included  $7822 \pm 697$  mg L<sup>-1</sup> of total carbohydrates (as lactose),  $11,009 \pm 1030$  mg COD L<sup>-1</sup>, and a pH of  $6.3 \pm 0.1$ .

The vinasse used as the substrate for hydrogen production was obtained from Usina São Martinho (Pradópolis, SP), which produces ethanol and sugar from sugarcane. Because large quantities of vinasse are required to operate the reactors and vinasse is difficult to find in the off-season, the vinasse was stored in a freezer at  $-5$  °C. The pH of raw vinasse was  $4.41 \pm 0.1$ . Table 1 presents the characteristics of vinasse collected during the sugar and ethanol production season.

## Reactor Setup and Operational Conditions

In this study, two similar AFBRs (AFBR-CW and AFBR-V) were built from stainless steel with the following dimensions: heights of 150 cm, internal diameters of 4.1 cm, and total volumes of 1980 cm<sup>3</sup>. The reactors were filled with expanded clay particles with grain sizes between 2.80 and 3.35 mm. The real density and porosity of the expanded clay were 1.5 g cm<sup>-3</sup> and 23 %, respectively. The temperature in the system was controlled at 55, 65, and 75 ± 1 °C by a thermostatic bath that circulated water in the reactor jacket. The reactors were continuously operated for 270 days. After inoculation, the hydrogen production was evaluated by operating the reactor at 55 °C in an HRT of 8 h. When a steady state was observed based on the stabilization of the HPR and HY results, the operational conditions were changed by decreasing the HRT to 6, 4, 2, 1, and 0.5 h. When the best condition for the HY was determined, the temperature was increased to 65 and 75 °C. The AFBR-CW was fed with

**Table 1** Average characterization of vinasse

Parameter	Value	Parameter	Value
pH	4.41 ± 0.1	Citric acid (mg L <sup>-1</sup> )	7482 ± 451
Total carbohydrates (mg L <sup>-1</sup> )	6292 ± 1523	Malic acid (mg L <sup>-1</sup> )	6191 ± 1215
COD (mg L <sup>-1</sup> )	28,636 ± 5204	Succinic acid (mg L <sup>-1</sup> )	2806 ± 660
Sulfate (mg L <sup>-1</sup> )	2256 ± 362	Lactic acid (mg L <sup>-1</sup> )	7516 ± 170
Suspended solids (mg L <sup>-1</sup> )	3184 ± 526	Formic acid (mg L <sup>-1</sup> )	2463 ± 691
Volatile suspended solids (mg L <sup>-1</sup> )	2689 ± 387	Acetic acid (mg L <sup>-1</sup> )	9928 ± 1114
Fixed suspended solids (mg L <sup>-1</sup> )	495 ± 169	Propionic acid (mg L <sup>-1</sup> )	3557 ± 1014
Total Kjeldahl nitrogen (mg L <sup>-1</sup> )	744 ± 77	Isobutyric acid (mg L <sup>-1</sup> )	3755 ± 847
Methanol (mg L <sup>-1</sup> )	2315 ± 707	Caproic acid (mg L <sup>-1</sup> )	809 ± 178

10,000 mg L<sup>-1</sup> of total carbohydrates (lactose), and the AFBR-V was fed with 10,000 mg COD L<sup>-1</sup>.

## Analytical Methods

The COD, pH, suspended solids, total nitrogen, ammoniacal nitrogen, and sulfate were measured according to standard methods [21]. Moreover, the total carbohydrate concentration was estimated as glucose for vinasse and as lactose for CW using the method presented by Dubois et al. [22]. The biogas produced in the reactor was measured based on fluid movement using an acidified saturated saline solution [23]. The biogas composition was analyzed by gas chromatography (Shimadzu GC-2010) using a Supelco Carboxen 1010 Plot column (30 m length and 0.53 mm diameter) and thermal conductivity detector. The concentrations of individual organic acids and alcohols were determined by high-performance liquid chromatography (HPLC, Shimadzu) according to Rosa et al. [17].

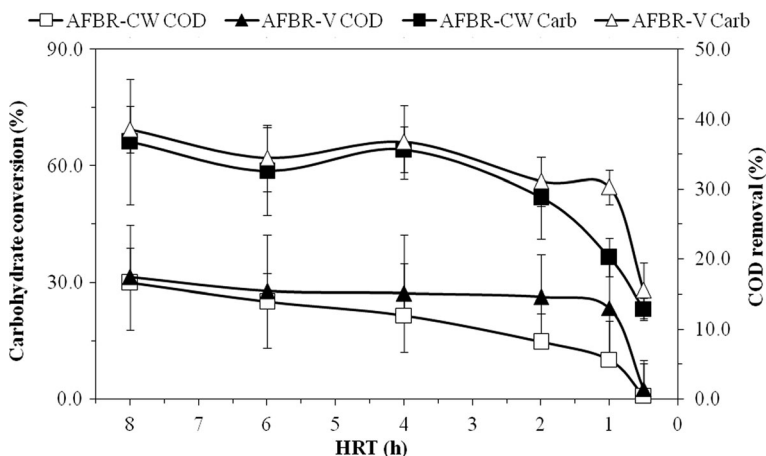
## Results and Discussion

### Fermentative Performance

#### *Effect of HRT*

The pH was controlled daily by adding NaOH to the feed depending on the pH in the effluent. The pH values used to operate the AFBR-CW were kept between the ranges of  $6.48 \pm 0.13$  to  $7.70 \pm 0.48$  in the feed and  $3.96 \pm 0.15$  to  $4.22 \pm 0.13$  in the effluent. The AFBR-V was operated in the ranges of  $4.25 \pm 0.14$  to  $5.23 \pm 0.10$  in the feed and  $4.55 \pm 0.18$  and  $4.95 \pm 0.10$  in the effluent.

Figure 1 presents the average values of total carbohydrate conversion to lactose in the AFBR-CW and glucose in the AFBR-V, as well as the COD removals of both reactors as a function of HRT.



**Fig. 1** Effect of HRT on total carbohydrate conversion and COD removal in the AFBR-CW and AFBR-V

The carbohydrate conversion and COD removal values observed during HRTs of 8, 6, 4, and 2 h in AFBR-CW were similar and ranged between  $51.8 \pm 10.5$  and  $66.1 \pm 16.1$  % for carbohydrates and  $8.2 \pm 4.1$  and  $16.6 \pm 5.0$  % for COD removal. The maximum values were observed in an HRT of 8 h. When the HRT was decreased to 1 and 0.5 h, the carbohydrate conversion and COD removal values decreased. They reached minimum values of  $23.2 \pm 2.9$  and  $0.5 \pm 5.1$  % in an HRT of 0.5 h.

Reductions in carbohydrate conversion and COD removal with decreasing HRT have also been observed in other studies of CW [16, 24]. Azbar et al. [24] used a continuous stirred tank reactor (CSTR) for hydrogen production from cheese production wastewater in thermophilic conditions (55 °C) to evaluate the effects of HRTs of 3.5, 2, and 1 days for a COD of  $47 \text{ g L}^{-1}$ . The maximum COD removal of 90 % was obtained in an HRT of 3.5 days; however, the HRT of 1 day had a removal of 47 %. Rosa et al. [16] used an AFBR in mesophilic conditions (30 °C) to evaluate different HRTs (4, 2, and 1 h) in hydrogen production from CW with a COD of  $5000 \text{ mg L}^{-1}$ . They observed a reduction in lactose conversion from 95 to 85 % when the HRT decreased from 4 to 1 h.

In the AFBR-V, the carbohydrate conversion and COD removal values observed as the HRT decreased from 8 to 1 h were similar and ranged from  $54.5 \pm 4.4$  to  $69.3 \pm 6.0$  % for carbohydrate conversion and  $13.0 \pm 7.2$  to  $17.4 \pm 7.5$  % for COD removal. Maximum conversion values were observed in an HRT of 8 h. When the HRT decreased to 0.5 h, the carbohydrate conversion and COD removal values decreased to minimums of  $27.9 \pm 7.2$  and  $1.4 \pm 3.6$  %, respectively.

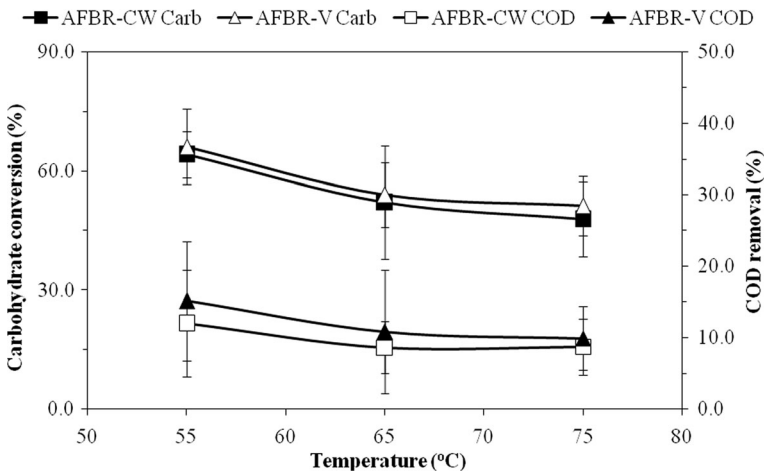
Decreases in carbohydrate conversion and COD removal with decreasing HRT in reactors fed with vinasse were also observed by Santos et al. [14], who evaluated the effect of HRT (8 to 1 h) on thermophilic (55 °C) hydrogen production from vinasse and glucose ( $5000 \text{ mg COD L}^{-1}$ ). The maximum carbohydrate conversion was observed in an HRT of 2 h. When the HRT was decreased to 1 h, the carbohydrate conversion decreased to 41 %. Similarly, Intanoo et al. [25] used an anaerobic sequencing batch reactor (ASBR) to evaluate the effect of HRT (32, 25.6, 21.3, and 18.3 h) on hydrogen production from alcohol wastewater and observed that the maximum value of COD removal was 32 % when the HRT decreased from 32 to 21.3 h. However, decreasing the HRT to 18.3 h caused COD removal to decrease due to the increase in the volatile fatty acid (VFA) concentration, which caused microorganism toxicity.

### *Effect of Temperature*

The effects of temperature on carbohydrate conversion and COD removal in the AFBR-CW and AFBR-V are illustrated in Fig. 2. When evaluating the effect of temperature in the AFBR-CW in an HRT of 4 h and temperatures of 55, 65, and 75 °C, the results suggest that increasing the temperature decreased carbohydrate conversion, which reached a minimum value of  $47.8 \pm 9.5$  % at 75 °C. However, the values of COD removal observed at different temperatures were similar, ranging between  $8.6 \pm 3.7$  and  $12.0 \pm 7.4$  %.

In AFBR-V, as the temperature increased from 55 to 75 °C in an HRT of 4 h, the carbohydrate conversion decreased from  $66.1 \pm 9.5$  % (55 °C) to  $51.2 \pm 7.5$  % (75 °C). The COD removal values were similar at different temperatures, ranging between  $9.9 \pm 4.5$  and  $15.1 \pm 8.4$  %.

The temperature increase negatively affected total carbohydrate conversion by the fermentative microorganisms in AFBR-CW and AFBR-V. Similar results have been observed in other studies [26, 27]. Gadow et al. [26] evaluated the effects of mesophilic (37 °C), thermophilic (55 °C), and hyperthermophilic (80 °C) temperatures on hydrogen production in a CSTR using cellulose ( $10 \text{ g L}^{-1}$ ). A maximum cellulose degradation of 71.3 % was observed under



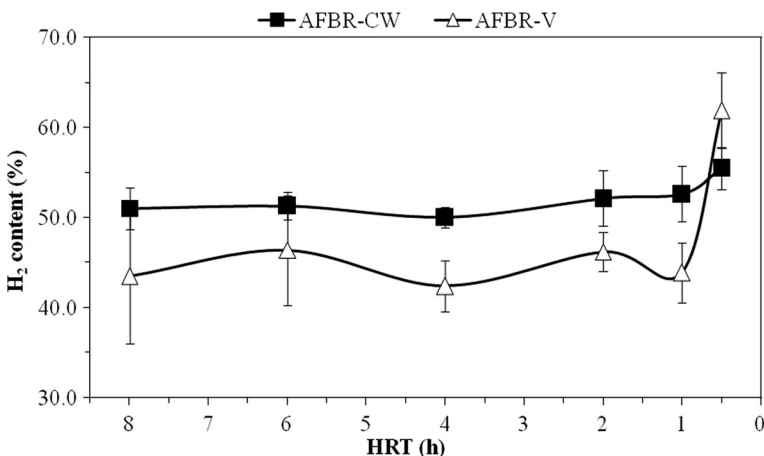
**Fig. 2** Effect of temperature on total carbohydrate conversion and COD removal in AFBR-CW and AFBR-V

mesophilic conditions, followed by 60.3 % under thermophilic conditions and 53 % under hyperthermophilic conditions. Zhong et al. [27] used an induced bed reactor (IBR) for hydrogen production from dairy processing waste (98.7 g COD L<sup>-1</sup>) to evaluate the effects of different HRTs (5, 3, and 1 day) and mesophilic (40 °C) and thermophilic (60 °C) temperatures. The authors observed that the average COD removal values were 8 % higher when the temperature was increased to 60 °C.

### Hydrogen Content

#### *Effect of HRT*

During the operation of AFBR-CW and AFBR-V, the composition of the produced biogas was analyzed to evaluate the hydrogen production and methane presence. The variations in H<sub>2</sub> content (H<sub>2</sub>%) as a function of HRT are illustrated in Fig. 3.



**Fig. 3** Variations in H<sub>2</sub> content as a function of HRT in AFBR-CW and AFBR-V

In both reactors, only  $H_2$  and  $CO_2$  were observed in the biogas, and the respective values of  $H_2\%$  in the produced biogas were stable. Methane was not formed during the operation of the systems, reflecting the thermal treatment efficiency of the sludge used. In AFBR-CW, the  $H_2\%$  remained stable between  $47.62 \pm 2.1$  and  $55.5 \pm 2.5 \%$  when the HRT was decreased from 8 to 0.5 h. The maximum value of  $55.5 \pm 2.5 \%$  was observed in an HRT of 0.5 h. These results are similar to those observed by Azbar et al. [24], who observed an  $H_2\%$  between 41 and 44 % using a CSTR for hydrogen production from cheese processing wastewater in thermophilic conditions ( $55 \text{ }^\circ\text{C}$ ) while varying HRT from 3.5 to 1 day. Davila-Vazquez et al. [28] observed  $H_2\%$  values between 49 and 58 % using a CSTR for hydrogen production from CW in mesophilic conditions ( $37 \text{ }^\circ\text{C}$ ).

In AFBR-V, the values of  $H_2\%$  were stable when the HRT was varied between 8 and 1 h. The  $H_2\%$  values ranged from  $43.5 \pm 7.5$  to  $46.4 \pm 6.1 \%$ . Decreasing the HRT to 0.5 h increased the  $H_2\%$  to a maximum value of  $61.9 \pm 6.1 \%$ . A similar  $H_2\%$  value of 40 % was observed by Intanoo et al. [29] in an HRT of 24 h when evaluating the effect of adding fermentation residue to hydrogen production in a thermophilic ASBR ( $55 \text{ }^\circ\text{C}$ ) using alcohol wastewater as the substrate. In the study conducted by Santos et al. [15] using two thermophilic ( $55 \text{ }^\circ\text{C}$ ) AFBRs with vinasse as the substrate,  $H_2\%$  values between 43.3 and 48.9 % were observed without the presence of methane when the HRT was decreased from 6 to 1 h, with COD concentrations of 15 and 20 g COD  $L^{-1}$ .

### *Effect of Temperature*

The effects of temperature on  $H_2\%$  in AFBR-CW and AFBR-V are illustrated in Fig. 4. In AFBR-CW, the increase in temperature did not change the  $H_2\%$ , with a maximum value of  $52.0 \pm 2.6 \%$  at  $65 \text{ }^\circ\text{C}$  and minimum value of  $47.6 \pm 2.1 \%$  at  $75 \text{ }^\circ\text{C}$ . Conversely, when the temperature changed from 55 to  $75 \text{ }^\circ\text{C}$  in AFBR-V, the values of  $H_2\%$  at 55 and  $65 \text{ }^\circ\text{C}$  were similar at 46 %. However, increasing the temperature to  $75 \text{ }^\circ\text{C}$  caused the  $H_2\%$  to decrease to  $23.3 \pm 5.9 \%$ .

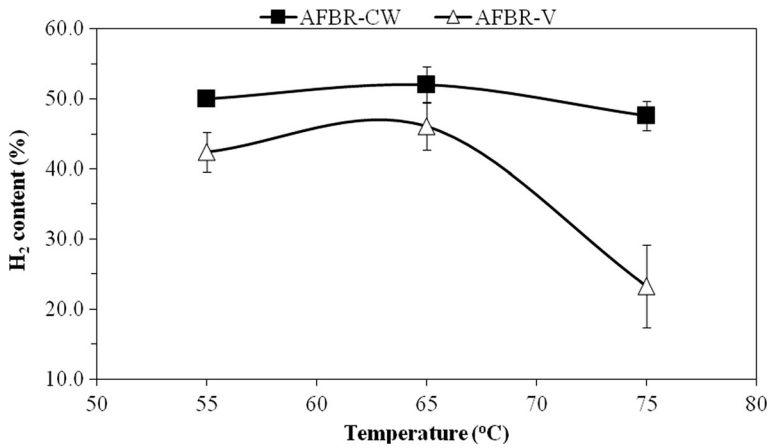
A decrease in  $H_2\%$  when temperature conditions varied from thermophilic to hyperthermophilic was also observed in other studies [13, 30]. Luo et al. [30] evaluated the effect of temperature (37, 60, and  $70 \text{ }^\circ\text{C}$ ) on fermentative hydrogen production from cassava stillage in batch tests and observed a maximum  $H_2\%$  of 50 % at a thermophilic temperature ( $60 \text{ }^\circ\text{C}$ ), with lower values under mesophilic ( $37 \text{ }^\circ\text{C}$ ) and extreme thermophilic ( $70 \text{ }^\circ\text{C}$ ) conditions. Algapani et al. [13] did not observe significant hydrogen production at a hyperthermophilic temperature ( $70 \text{ }^\circ\text{C}$ ) when comparing hydrolysis and hydrogen production in two CSTR reactors using food waste; however, they observed a maximum  $H_2\%$  of 58.6 % in an HRT of 5 h under thermophilic conditions ( $55 \text{ }^\circ\text{C}$ ).

## **Hydrogen Production Rate**

### *Effect of HRT*

Figure 5 illustrates the HPR behaviors in AFBR-CW and AFBR-V at different HRTs.

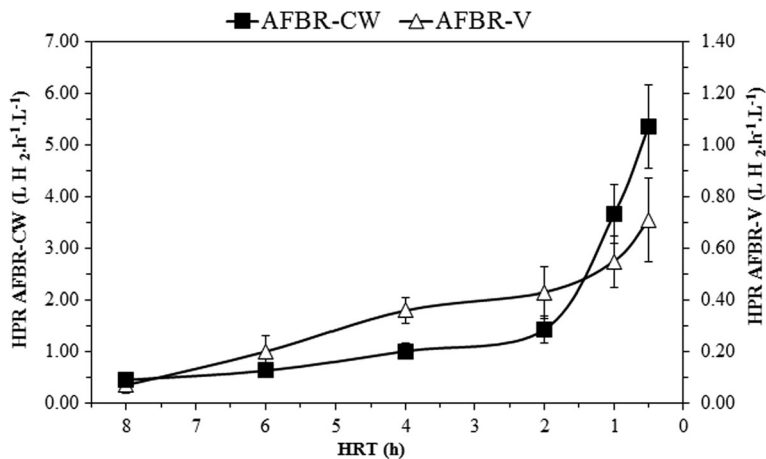
The HPR values in AFBR-CW increased with decreasing HRT, reaching a maximum of  $5.36 \pm 0.81 \text{ L } H_2 \text{ h}^{-1} L^{-1}$  in an HRT of 0.5 h. A similar trend was identified by Davila-Vazquez et al. [28] using CW as substrate in a mesophilic ( $37 \text{ }^\circ\text{C}$ ) CSTR. They observed that the HPR increased from 496 to 583  $\text{mL } H_2 \text{ h}^{-1} L^{-1}$  when the HRT decreased from 10 to 6 h. Similarly,



**Fig. 4** Variations in H<sub>2</sub>% as a function of temperature in AFBR-CW and AFBR-V

Carrilo-Reyes et al. [31] observed an increase in the HPR from 3.45 to 4.30 L H<sub>2</sub> L<sup>-1</sup> day<sup>-1</sup> when the HRT decreased from 13 to 10 h in a mesophilic (37 °C) UASB using CW as the substrate. Perna et al. [32] evaluated mesophilic (30 °C) hydrogen production from CW at OLRs of 22, 33, and 37 g COD L<sup>-1</sup> day<sup>-1</sup> and an HRT of 24 h. They obtained a maximum HPR value of 1.0 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> at an OLR of 37 g COD L<sup>-1</sup> day<sup>-1</sup>.

In AFBR-V, decreasing the HRT from 8 to 0.5 h increased the HPR from 0.07 ± 0.03 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>, reaching a maximum value of 0.71 ± 0.16 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>. The same trend was observed by Santos et al. [15] using two thermophilic (55 °C) AFBRs. When the HRT was decreased from 6 to 1 h, the HPR increased in both reactors, reaching maximum values of 1.49 L H<sub>2</sub> L<sup>-1</sup> h<sup>-1</sup> (15 g COD L<sup>-1</sup>) and 1.2 L H<sub>2</sub> L<sup>-1</sup> h<sup>-1</sup> (20 g COD L<sup>-1</sup>) in an HRT of 1 h. In a thermophilic (55 °C) AFBR with vinasse and a feed COD of 5000 mg COD L<sup>-1</sup>, Santos et al. [14] observed that the HPR increased with decreasing HRT, with a maximum value of 0.78 L H<sub>2</sub> L<sup>-1</sup> h<sup>-1</sup> in an HRT of 1 h. Similarly, Ferraz Júnior et al. [33] observed a maximum HPR of 2284 mL H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> in an HRT of 10.2 h high OLR (84.2 kg COD m<sup>-3</sup> day<sup>-1</sup>) when evaluating



**Fig. 5** Variation in the HPR as a function of HRT in AFBR-CW and AFBR-V



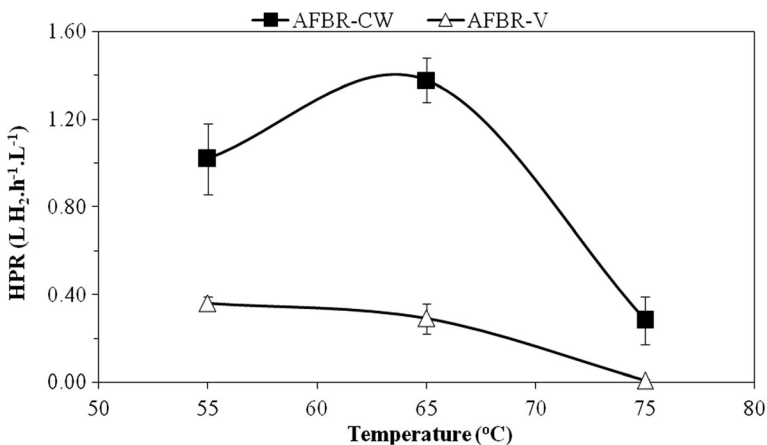
thermophilic (55 °C) hydrogen production in an APBR using vinasse as the substrate (35.2 g COD L<sup>-1</sup>).

### Effect of Temperature

The effects of temperature on the HPRs in AFBR-CW and AFBR-V are illustrated in Fig. 6. In AFBR-CW, the temperatures of 55 and 65 °C yielded similar HPR values of  $1.01 \pm 0.16$  and  $1.38 \pm 0.10$  L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>. However, the HPR decreased to  $0.28 \pm 0.11$  L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> when the temperature increased to 75 °C. In AFBR-V, temperatures of 55 and 65 °C yielded similar HPR values of  $0.36 \pm 0.5$  and  $0.29 \pm 0.10$  L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>. However, the HPR decreased to  $0.008 \pm 0.005$  L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> when the temperature increased to 75 °C.

To our knowledge, few studies have compared the effects of thermophilic and hyperthermophilic conditions on the HPR in continuous reactors. Gadow et al. [9] evaluated the effect of temperature in three CSTRs under mesophilic (37 °C), thermophilic (55 °C), and hyperthermophilic (80 °C) conditions in an HRT of 10 days based on a concentration of 5 g cellulose L<sup>-1</sup>. They observed maximum HPR values of 0.286 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> (80 °C), 0.24 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> (55 °C), and 0.008 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> (37 °C). Additionally, Gadow et al. [26] evaluated the effect of temperature in three CSTRs under mesophilic (37 °C), thermophilic (55 °C), and hyperthermophilic (80 °C) conditions in an HRT of 10 days based on a concentration of 10 g cellulose L<sup>-1</sup>. They observed a maximum HPR of 0.255 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> in the thermophilic reactor. Similarly, Zhong et al. [27] observed a maximum HPR of 110 mL H<sub>2</sub> L<sup>-1</sup> h<sup>-1</sup> in an IBR using dairy processing waste (98.7 g COD L<sup>-1</sup>) for hydrogen production when the temperature was increased from 40 to 60 °C at different HRTs (5, 3, and 1 days).

Although the HPR decreased as the temperature increased to 75 °C, the AFBR-CW and AFBR-V results under thermophilic and hyperthermophilic conditions are comparable to those of other studies [10, 11, 34]. Qiu et al. [34] observed a maximum HPR of 209.5 mL H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> in an extreme thermophilic (70 °C) CSTR using distillery wastewater in an HRT of 4 days. Gadow et al. [10] operated a hyperthermophilic (70 °C) CSTR with 10 g cellulose L<sup>-1</sup> and an HRT of 10 days. They observed a maximum HPR of 0.158 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup>. Additionally, Jiang et al. [11] observed a maximum HPR of 0.242 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> in a thermophilic (55 °C) CSTR with 10 g cellulose L<sup>-1</sup> and an HRT of 10 days.



**Fig. 6** Variation in the HPR as a function of temperature in AFBR-CW and AFBR-V

## Hydrogen Yield

### Effect of HRT

The HY variation during the operation of AFBR-CW and AFBR-V is illustrated in Fig. 7 as a function of HRT. As the HRT decreased in AFBR-CW at a temperature of 55 °C, the HY reached a maximum value of  $4.10 \pm 0.68$  mmol H<sub>2</sub> g COD<sup>-1</sup> in an HRT of 4 h. Low HRTs in AFBR-CW decreased HY values, with a minimum value of  $2.58 \pm 0.39$  mmol H<sub>2</sub> g COD<sup>-1</sup> in an HRT of 0.5 h.

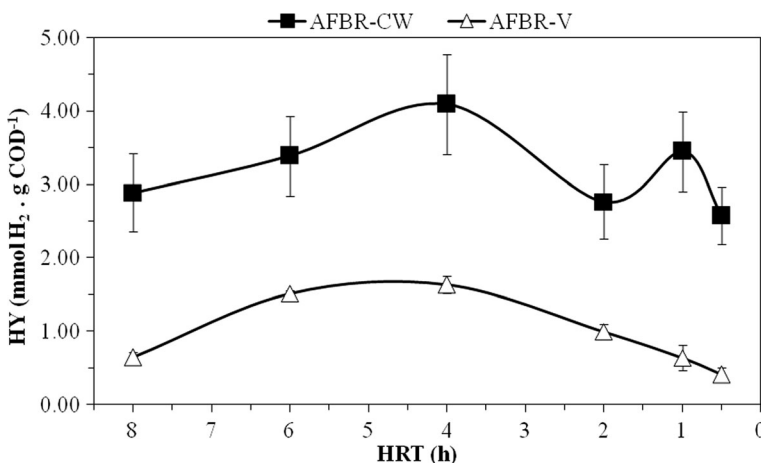
In AFBR-V, the HY reached a maximum value of  $1.64 \pm 0.22$  mmol H<sub>2</sub> g COD<sup>-1</sup> in an HRT of 4 h. Low HRTs in AFBR-V decreased the HY, with a minimum value of  $0.41 \pm 0.09$  mmol H<sub>2</sub> g COD<sup>-1</sup> in an HRT of 0.5 h. Thus, when the HRT decreased below 4 h, with a consequent increase in the OLR, the HY decreased.

Table 2 shows the average values of H<sub>2</sub>%, HPR, and HY in AFBR-CW as functions of different operational conditions, such as the HRT, temperature, and OLR. These results suggest that the increase in the OLR associated with decreasing HRT increased the HPR and H<sub>2</sub>%. The optimal condition for HY was an OLR of 78.1 kg COD m<sup>-3</sup> day<sup>-1</sup>.

Table 3 presents the HY values observed in this study and other studies for comparison and evaluation of the effects of the HRT and OLR.

Lower values of HRT in AFBR-CW decreased the HY by increasing the organic matter available for microbial consumption, which increased higher the accumulation of inhibitory VFA. Similar effects were observed in studies that evaluated the effect of the OLR, in which high OLRs inhibited hydrogen production and decreased the HY [16, 24, 27, 32, 37, 38]. Azbar et al. [24] observed that increasing the OLR from 21 to 47 kg COD m<sup>-3</sup> day<sup>-1</sup> by decreasing the HRT from 3.5 to 1 day decreased the HY from 22 to 5 mmol H<sub>2</sub> g COD<sup>-1</sup>. Additionally, decreasing the OLR from 47 to 35 kg COD m<sup>-3</sup> day<sup>-1</sup> by decreasing the COD concentrations from 47 to 35 g COD L<sup>-1</sup> increased the HY from 3.5 to 9 mmol H<sub>2</sub> g COD<sup>-1</sup>.

Similarly, Venetsaneas et al. [37] evaluated mesophilic (37 °C) hydrogen production from CW in a CSTR and observed maximum values of HY (1.56 mol H<sub>2</sub> mol lactose<sup>-1</sup>) and HPR (120 mL H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>) at an OLR of 30 kg COD m<sup>-3</sup> day<sup>-1</sup>. Under similar operational



**Fig. 7** Variation in the HY as a function of HRT in AFBR-CW and AFBR-V

**Table 2** Average values of the main results obtained in AFBR-CW

T (°C)	HRT (h)	OLR (kg COD m <sup>-3</sup> day <sup>-1</sup> )	H <sub>2</sub> (%)	HPR (L H <sub>2</sub> h <sup>-1</sup> L <sup>-1</sup> )	HY (mmol H <sub>2</sub> g COD <sup>-1</sup> )	HY (mol H <sub>2</sub> mol lactose <sup>-1</sup> )
55	8	39	51.1 ± 2.3	0.45 ± 0.13	2.89 ± 0.53	3.06 ± 0.34
55	6	52.1	51.3 ± 1.5	0.63 ± 0.09	3.39 ± 0.44	3.51 ± 0.44
55	4	78.1	50.0 ± 1.1	1.01 ± 0.16	4.10 ± 0.68	3.77 ± 0.48
55	2	156.2	52.1 ± 3.1	1.43 ± 0.26	2.77 ± 0.51	3.55 ± 0.32
55	1	312.4	52.6 ± 3.1	3.66 ± 0.57	3.45 ± 0.55	3.50 ± 0.36
55	0.5	624.8	55.5 ± 2.3	5.36 ± 0.81	2.58 ± 0.39	3.46 ± 0.29
65	4	78.1	52.0 ± 2.6	1.38 ± 0.10	5.51 ± 0.37	4.67 ± 0.37
75	4	78.1	47.6 ± 2.1	0.28 ± 0.11	1.20 ± 0.39	1.04 ± 0.12

conditions, Perna et al. [32] observed a maximum HY when the OLR was increased to 37 kg COD m<sup>-3</sup> day<sup>-1</sup> by increasing the feed concentration from 22 to 37 g COD L<sup>-1</sup> in a mesophilic (37 °C) APBR with an HRT of 24 h.

Rosa et al. [16] used CW as the substrate in an AFBR under mesophilic (30 °C) conditions and found that the HY decreased from 1.33 to 1.06 mol H<sub>2</sub> mol lactose<sup>-1</sup> as the OLR increased from 30 kg COD m<sup>-3</sup> day<sup>-1</sup> (HRT = 4 h) to 120 kg COD m<sup>-3</sup> day<sup>-1</sup> (HRT = 1 h).

Similarly, Zhong et al. [27] used an IBR for hydrogen production from dairy processing waste (98.7 g COD L<sup>-1</sup>) and observed a maximum HY of 5.58 mmol H<sub>2</sub> g COD<sup>-1</sup> when the HRT decreased from 5 to 3 days and the OLR increased from 19.74 to 32.9 kg COD m<sup>-3</sup> day<sup>-1</sup>. However, when operating the reactor at an OLR of 98.7 kg COD m<sup>-3</sup> day<sup>-1</sup>, which was equivalent to an HRT of 1 day, the HY was 3.87 mmol H<sub>2</sub> g COD<sup>-1</sup>.

Lima et al. [38] observed maximum HPR (45 mL H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>) and HY (1.12 mol H<sub>2</sub> mol lactose<sup>-1</sup>) in an ASBR in the OLR of 18.5 kg COD m<sup>-3</sup> day<sup>-1</sup>. When increasing the feed concentration from 5400 to 6350 mg COD L<sup>-1</sup>, which was equivalent to 5671 mg carbohydrate L<sup>-1</sup>, the authors observed a decrease in HY to 0.37 mol H<sub>2</sub> mol lactose<sup>-1</sup>.

However, Dareioti and Kornaros [39] observed a maximum HY of 0.54 mol H<sub>2</sub> mol carbohydrate<sup>-1</sup> in a CSTR by decreasing the HRT from 5 to 0.75 days, thus increasing the OLR to 126.67 kg COD m<sup>-3</sup> day<sup>-1</sup> in the mesophilic co-digestion of olive-mill wastewater, CW, and liquid cow manure. The same positive effect in HY by increasing OLR to 171.6 kg COD m<sup>-3</sup> day<sup>-1</sup> was observed by Dareioti and Kornaros [40] in the mesophilic co-digestion of ensiled sorghum, CW, and liquid cow manure. The maximum HY of 0.7 mol H<sub>2</sub> mol carbohydrate<sup>-1</sup> was observed by decreasing the HRT from 5 to 0.5 day.

In these studies where the increase in OLR favored the HY, a higher HRT range was used. While in AFBR-CW, the HRT was decreased from 8 to 0.5 h, obtaining maximum HY of

**Table 3** Maximum values of HY observed in CW studies

Reference	Reactor (temperature)	HRT (h)	OLR (kg COD m <sup>-3</sup> day <sup>-1</sup> )	HY (mmol H <sub>2</sub> g COD <sup>-1</sup> )	HY (mol H <sub>2</sub> mol lac <sup>-1</sup> )
Azbar et al. [24]	CSTR (55 °C)	84	21	22	2.4
Kargı et al. [35]	Batch (55 °C)	–	–	–	2.06
Romão et al. [36]	Batch (25 °C)	–	–	–	4.13
Rosa et al. [16]	AFBR (30 °C)	4	30	–	1.33
Zhong et al. [27]	IBR (60 °C)	72	32.9	5.58	–
This study	AFBR-CW (65 °C)	4	78.1	5.51	4.67

4.10 ± 0.68 mmol H<sub>2</sub> g COD<sup>-1</sup> in the HRT of 4 h. Dareioti and Kornaros [39] and Dareioti and Kornaros [40] obtained lower HY of 0.54 mol H<sub>2</sub> mol carbohydrate<sup>-1</sup> (HRT = 0.75 days) and 0.7 mol H<sub>2</sub> mol carbohydrate<sup>-1</sup> (HRT = 0.5 day). Thus, decreasing HRT to values lower than 4 h decreases the HY.

The HY of 4.67 mol H<sub>2</sub> mol lactose<sup>-1</sup> obtained in AFBR-CW is equivalent to 2.33 mol H<sub>2</sub> mol glucose<sup>-1</sup>, representing 58 % of the theoretical HY of 4 mol H<sub>2</sub> mol glucose<sup>-1</sup>. Kargi et al. [35] performed batch tests of hydrogen production from CW that yielded high HY values similar to those observed in AFBR-CW. The highest HY of 2.06 mol H<sub>2</sub> mol lactose<sup>-1</sup>, representing 26 % of the theoretical value, was obtained at a concentration of 9.5 g total carbohydrates L<sup>-1</sup>. Increasing the carbohydrate concentration increased the acid concentrations, mainly acetate (HAc), butyrate (HBu), and lactate (HLA), which inhibited production. The HYs observed in the AFBR-CW are also higher than those observed by Romão et al. [36] (4.13 mol H<sub>2</sub> mol lactose<sup>-1</sup>), who evaluated mesophilic (25 °C) hydrogen production from cheese whey permeate in a batch test with concentration of 20 g lactose L<sup>-1</sup>. Indeed, the HY of 58 % of the theoretical value observed in AFBR-CW is higher than any value published in the literature.

Table 4 shows the average values of H<sub>2</sub>%, HPR, and HY in AFBR-V as functions of the different operational conditions, such as HRT and temperature. In Table 5, the HY values observed in AFBR-V and those from other studies are presented for comparison to evaluate the effect of the HRT.

Table 4 shows that the results obtained in this study agree well with those in the literature, and the decrease in HY is associated with the increase in the OLR applied to the reactor. Increasing the OLR increases the amount of organic matter available for microbial conversion, and a maximum conversion of carbohydrates to hydrogen, as indicated by HY, occurred at an OLR of 60 kg COD m<sup>-3</sup> day<sup>-1</sup>. However, negative OLR effects are observed as the HRT decreases, suggesting that high OLRs inhibit recalcitrant and toxic compounds present in the vinasse feed, resulting in the accumulation of VFA. These results indicate that hydrogen production at HRTs lower than 4 h occurred in organic overload conditions.

The same inhibition from the recalcitrant substances was also observed by Buitrón et al. [41] studying the feasibility of continuous hydrogen production from tequila vinasse in a mesophilic APBR. According to the authors, the presence of inhibitory substances and low concentration of simple sugars limits the hydrogen production from vinasse. The maximum HY observed was 1.38 mmol H<sub>2</sub> g COD<sup>-1</sup> in the HRT of 4 h (OLR = 51.6 kg COD m<sup>-3</sup> day<sup>-1</sup>), similarly to the maximum HY of AFBR-V (1.64 ± 0.22 mmol H<sub>2</sub> g COD<sup>-1</sup>) in the OLR of 60 kg COD m<sup>-3</sup> day<sup>-1</sup>.

**Table 4** Average values of the main results obtained in AFBR-V

T (°C)	HRT (h)	OLR (kg COD m <sup>-3</sup> day <sup>-1</sup> )	H <sub>2</sub> (%)	HPR (L H <sub>2</sub> h <sup>-1</sup> L <sup>-1</sup> )	HY (mmol H <sub>2</sub> g COD <sup>-1</sup> )
55	8	30	43.5 ± 7.5	0.07 ± 0.03	0.65 ± 0.32
55	6	40	46.4 ± 6.1	0.20 ± 0.06	1.52 ± 0.32
55	4	60	42.4 ± 2.8	0.36 ± 0.05	1.64 ± 0.22
55	2	120	46.2 ± 2.2	0.43 ± 0.1	1.00 ± 0.24
55	1	240	43.9 ± 3.3	0.55 ± 0.19	0.64 ± 0.22
55	0.5	480	61.9 ± 4.2	0.71 ± 0.16	0.41 ± 0.09
65	4	60	46.1 ± 3.4	0.29 ± 0.09	1.29 ± 0.39
75	4	60	23.3 ± 5.9	0.008 ± 0.005	0.04 ± 0.02

**Table 5** Maximum values of HY observed in vinasse studies

Reference	Reactor (temperature)	HRT (h)	OLR (kg COD m <sup>-3</sup> day <sup>-1</sup> )	HY (mmol H <sub>2</sub> g COD <sup>-1</sup> )
Santos et al. [14]	AFBR (55 °C)	4	52.7	5.73
Santos et al. [15]	AFBR (55 °C)	6	60	2.23
Santos et al. [6]	AFBR (55 °C)	6	40	2.86
Ferraz Júnior et al. [42]	APBR (55 °C)	12	72.4	0.77
This study	AFBR-V (55 °C)	4	60	1.64

Negative HY and HPR effects were also observed by Ferraz Júnior et al. [42] as the OLR increased when evaluating the effect of the OLR on thermophilic (55 °C) hydrogen production in an APBR at HRTs of 24 and 8 h, corresponding to OLR values ranging from 36.2 to 108.8 kg COD m<sup>-3</sup> day<sup>-1</sup>. The maximum HPR and HY values of 1023 mL H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> and 2.4 mol H<sub>2</sub> mol carbohydrate<sup>-1</sup> (0.7 mmol H<sub>2</sub> g COD<sup>-1</sup>) were observed in an HRT of 12 h, corresponding to an OLR of 72.4 kg COD m<sup>-3</sup> day<sup>-1</sup>. When the OLR was increased to 108.6 kg OLR m<sup>-3</sup> day<sup>-1</sup>, the HY and HPR decreased to 63.9 and 42.8 %, respectively.

Similarly, Santos et al. [15] decreased the HRT from 6 to 1 h in two thermophilic (55 °C) AFBRs, with consequent increases in the OLR from 60 to 360 kg COD m<sup>-3</sup> day<sup>-1</sup> (15 g COD L<sup>-1</sup>) and from 80 to 480 kg COD m<sup>-3</sup> day<sup>-1</sup> (20 g COD L<sup>-1</sup>). This increase in OLR decreased the HY, with maximum values of 2.23 mmol H<sub>2</sub> g COD<sub>ap</sub><sup>-1</sup> (15 g COD L<sup>-1</sup>) and 1.85 mmol H<sub>2</sub> g COD<sub>ap</sub><sup>-1</sup> (20 g COD L<sup>-1</sup>) in an HRT of 6 h. These results indicate that hydrogen production occurred under organic overload conditions in the reactor. Santos et al. [6] also observed organic overload in a thermophilic (55 °C) AFBR with continuous hydrogen production using vinasse concentrations of 10,000 mg COD L<sup>-1</sup> and H<sub>2</sub> g COD<sub>ap</sub><sup>-1</sup> in an HRT of 6 h. The concentration of 30,000 mg COD L<sup>-1</sup> yielded a maximum HY of 0.79 mmol H<sub>2</sub> g COD<sub>ap</sub><sup>-1</sup> in an HRT of 6 h.

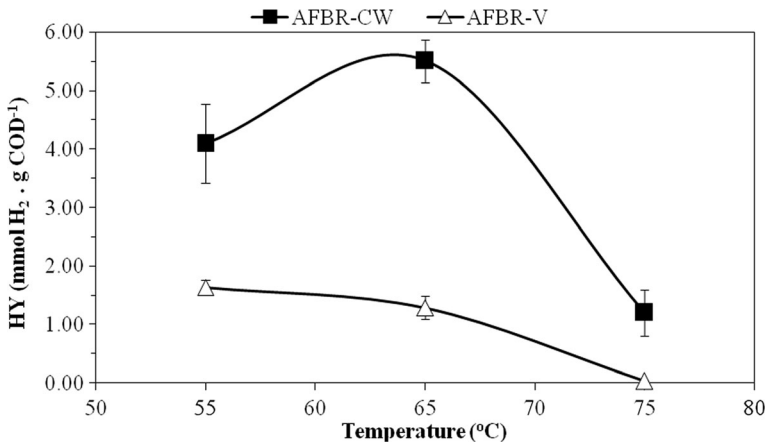
Santos et al. [14] optimized the OLR to maximize hydrogen production in a reactor using vinasse as organic substrate by decreasing the HRT from 8 to 1 h, with a subsequent increase in the OLR from 26.6 to 225.3 kg COD m<sup>-3</sup> day<sup>-1</sup> under thermophilic conditions. An OLR of 52.7 kg COD m<sup>-3</sup> day<sup>-1</sup> yielded a maximum HY of 5.73 mmol H<sub>2</sub> g COD<sub>ap</sub><sup>-1</sup> in an HRT of 4 h. Fuess et al. [43] observed a maximum HPR of 1604 mL H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> and maximum HY of 3.4 mol H<sub>2</sub> mol sucrose<sup>-1</sup>, which is equivalent to 0.87 mmol H<sub>2</sub> g COD<sup>-1</sup>, at an H<sub>2</sub>% of 37 %, HRT of 7.5 h, and OLR of 84.2 kg COD m<sup>-3</sup> day<sup>-1</sup> in a thermophilic (55 °C) APBR using vinasse as the substrate.

### Effect of Temperature

Figure 8 illustrates the effect of temperature on HY in an HRT of 4 h in AFBR-CW and AFBR-V.

The HY values observed in AFBR-CW were similar at temperatures of 55 and 65 °C, with a maximum value of  $5.51 \pm 0.37$  mmol H<sub>2</sub> g COD<sup>-1</sup>, the highest value observed in this study, observed at 65 °C. However, the increase in temperature to 75 °C decreased the HY to  $1.20 \pm 0.39$  mmol H<sub>2</sub> g COD<sup>-1</sup>.

In AFBR-V, the HY of  $1.29 \pm 0.39$  mmol H<sub>2</sub> g COD<sup>-1</sup> at 65 °C was similar to the value observed at 55 °C. However, increasing the temperature to 75 °C decreased the HY to  $0.04 \pm 0.02$  mmol H<sub>2</sub> g COD<sup>-1</sup>. Thus, increasing the temperature was detrimental to the HY in both reactors.



**Fig. 8** Variation in HY as a function of temperature in AFBR-CW and AFBR-V

Note that the HY values observed in AFBR-CW at thermophilic temperatures of 55 °C ( $4.10 \pm 0.68$  mmol H<sub>2</sub> g COD<sup>-1</sup>) and 65 °C ( $5.51 \pm 0.37$  mmol H<sub>2</sub> g COD<sup>-1</sup>) are equivalent to  $3.77 \pm 0.48$  and  $4.67 \pm 0.37$  mol H<sub>2</sub> mol lactose<sup>-1</sup>, respectively. These values are higher than the value observed by Rosa et al. [16] and Romão et al. [36]. Rosa et al. [16], who used CW for hydrogen production in a mesophilic (30 °C) AFBR, observed a maximum HY of 1.33 mol H<sub>2</sub> mol lactose<sup>-1</sup> in an HRT of 4 h and observed methane production in an HRT of 1 h. Similarly, Romão et al. [36] observed a maximum HY of 4.13 mol H<sub>2</sub> mol lactose<sup>-1</sup> during mesophilic (25 °C) hydrogen production from whey permeate in batch tests with a concentration of 20 g lactose L<sup>-1</sup>.

In AFBR-V, the maximum HY of  $1.64 \pm 0.22$  mmol H<sub>2</sub> g COD<sup>-1</sup> and maximum HPR of 0.71 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> were observed at 55 °C. Comparatively, Reis et al. [44] observed a maximum HPR of 0.51 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> in an HRT of 1 h and for a concentration of 5 g COD L<sup>-1</sup> when evaluating the hydrogen production from vinasse in two mesophilic (30 °C) AFBRs with concentrations of 5 and 10 g COD L<sup>-1</sup>. Additionally, methane was produced concomitantly with hydrogen, with a maximum composition of 40 % in an HRT of 2 h.

Therefore, we conclude that thermophilic hydrogen production yields higher HPRs and HYs and better represses methanogenic microorganisms, which repress hydrogen consumption pathways, compared to using mesophilic production. These observations agree with those of Kargi et al. [45], who conducted batch tests using CW (10.77 g total carbohydrates L<sup>-1</sup>) for hydrogen production under mesophilic (35 °C) and thermophilic (55 °C) conditions. The HY of 0.81 mol H<sub>2</sub> mol glucose<sup>-1</sup> obtained at the thermophilic temperature was higher than that generated at the mesophilic temperature (0.47 mol H<sub>2</sub> mol glucose<sup>-1</sup>) due to the elimination of hydrogen-consuming bacteria and the selective enrichment of more efficient hydrogen producers.

The advantages of thermophilic production over mesophilic fermentative hydrogen production were also noted by Luo et al. [46]. The authors found that HY increased from 14 to 69.6 mL H<sub>2</sub> g VS<sup>-1</sup> as the temperature increased from 37 to 60 °C when evaluating mesophilic (37 °C) and thermophilic (60 °C) hydrogen production from cassava stillage in a CSTR. Gupta et al. [47] also observed higher HYs under thermophilic conditions, with a maximum value of 1.13 mol H<sub>2</sub> mol hexose<sup>-1</sup>, when evaluating the performance of mesophilic anaerobic digester sludge under mesophilic (37 °C) and thermophilic (60 °C) conditions for hydrogen production from starch and cellulose.

Table 6 provides important parameters that have been used in various studies of hydrogen production, including the temperature, inoculum source, and complexity of the substrate.

Based on the different studies presented in Table 6, the effect of temperature varies with multiple factors, including substrate complexity, inoculum source, and microbial community diversity. Continuous hydrogen production can yield optimal results in the mesophilic, thermophilic, or hyperthermophilic range depending on each of these factors. The increase in temperature from 55 to 65 °C increased the HY and HPR in AFBR-CW. In AFBR-V, the HY and HPR values were similar under both conditions. These HY and HPR increases in AFBR-CW at 65 °C are associated with the selection of a thermophilic microbial community that more efficiently produces hydrogen from complex substrates and improves the mass transfer conditions.

The advantages of increasing the temperature of hydrogen production have also been observed in other studies. Luo et al. [30] used cassava stillage in batch tests and found that, although a mesophilic inoculum was used, the mixed culture had different microbial communities that could be selectively enriched in different temperature conditions. Thus, the maximum HY of 67.8 mL H<sub>2</sub> g VS<sup>-1</sup> was observed at a temperature of 60 °C, followed by the HYs under mesophilic (35 °C) conditions and extreme thermophilic conditions (70 °C).

When the temperature was increased from 40 to 60 °C in an IBR, Zhong et al. [27] observed a maximum HY of 5.58 mmol H<sub>2</sub> g COD<sup>-1</sup> at 60 °C using a high concentration of dairy wastewater (98.7 g COD L<sup>-1</sup>). This result is similar to that produced by the temperature condition of 65 °C in AFBR-CW, which yielded a maximum HY of 5.58 mmol H<sub>2</sub> g COD<sup>-1</sup>.

However, the HYs and HPRs reached minimum values in AFBR-CW and AFBR-V when the temperature was increased to 75 °C. At this temperature, an efficient hydrogen-producing microbial community could not be created due to the selection of microorganisms suited for hyperthermophilic temperatures. Additionally, temperatures over 70 °C can decrease hydrogen production via deactivation of enzymatic machinery [48].

Other studies have also evaluated microbial community selection when increasing the temperature. The microbial community is reorganized when temperatures increase from mesophilic to thermophilic conditions, and higher microbial diversity is associated with mesophilic conditions rather than thermophilic conditions [50, 51]. Gupta et al. [47] found that applying a temperature shock to a mesophilic inoculum by increasing the temperature to thermophilic conditions could increase the HY; however, the increased HY was still lower than HYs generated by inoculums that were thermophilic and enriched in thermophiles.

Zhang et al. [49] found that microbial community diversity decreases with increasing temperature, and this process was characterized by the selective enrichment of some species.

**Table 6** Comparison of HY values in AFBR-CW and AFBR-V with those used in other studies to evaluate the effect of temperature

Reference	Reactor	Substrate	T (°C)	HY (mol H <sub>2</sub> mol hexose <sup>-1</sup> )	HY (mmol H <sub>2</sub> g COD <sup>-1</sup> )
Gadow et al. [10]	CSTR	Cellulose (10 g L <sup>-1</sup> )	55	2.21	–
Roy et al. [48]	Batch	Glucose (10 g L <sup>-1</sup> )	60	2.71	–
Zhong et al. [27]	IBR	Dairy wastewater (98.7 g COD L <sup>-1</sup> )	60	–	5.58
Zhang et al. [49]	Batch	Corn stover hydrolysate (6.2 g sugar L <sup>-1</sup> )	55	1.4	–
This study	AFBR-CW	Cheese whey (10 g lac L <sup>-1</sup> )	65	2.33	5.51
This study	AFBR-V	Vinasse (10 g COD L <sup>-1</sup> )	55	2.45	1.64



When the effect of temperature on hydrogen production from corn stover hydrolysate was evaluated at temperatures of 30, 37, 55, and 70 °C, the efficiency of fermentation based on temperature exhibited the following order: 55 > 70 > 37 ~ 30 °C. The superior production at 55 °C can be attributed to the selective enrichment of thermophilic hydrogen producers that are capable of producing more hydrogen using complex substrates. The poor hydrogen production at 70 °C is a result of not forming an extreme thermophilic community due to the low efficiency in consuming complex substrates and the negative effects of the associated by-products.

However, Obazu et al. [52] observed a superior operational condition at 70 °C, with maximum values of HPR (231.3 mmol H<sub>2</sub> L<sup>-1</sup> h<sup>-1</sup>) and HY (3.55 mol H<sub>2</sub> mol glucose<sup>-1</sup>). Overall, the results of previous studies that contradict our results were conducted with pure substrates such as sucrose [52] and cellulose [9]. Sucrose is a simple substrate that is easily biodegradable by the microbial community. Thus, the microbial community can easily adapt to this substrate at any temperature. Conversely, at high concentrations or OLRs [11, 26], thermophilic conditions yielded the best results, followed by those produced under hyperthermophilic conditions. Therefore, the selection and adaptation of a hyperthermophilic microbial community are more difficult in complex substrates or at higher OLRs.

Based on previous studies and the results obtained using AFBR-CW and AFBR-V in this study, it can be difficult to select a microbial community capable of producing H<sub>2</sub> under hyperthermophilic conditions (75 °C) from an originally thermophilic inoculum (55 °C) due to the complexity of CW and vinasse [11, 26, 30, 41, 49]. Higher values of substrate complexity and OLR decreased the optimal temperature of hydrogen production due to the decrease in microbial diversity, which reduces the synergetic capacities of substrate degradation and hydrogen production at these higher temperatures.

The HY decrease in AFBR-CW from 5.51 ± 0.37 mmol H<sub>2</sub> g COD<sup>-1</sup> at 65 °C to 1.20 ± 0.39 mmol H<sub>2</sub> g COD<sup>-1</sup> at 75 °C was similar to that in AFBR-V from 1.64 ± 0.22 mmol H<sub>2</sub> g COD<sup>-1</sup> at 55 °C to 0.04 ± 0.02 mmol H<sub>2</sub> g COD<sup>-1</sup> at 75 °C. Both reductions were associated with the denaturation of hydrogen-producing microorganisms that were dominant in the thermophilic inoculum used in this study. Due to the high complexity of vinasse containing inhibitory compounds such as phenol and melanoidins in high concentrations [6, 14, 15], the HY and optimal temperature values were lower than those associated with CW.

Notably, although the values of HY observed in the hyperthermophilic range were lower, they are in agreement with other results published in the literature. AFBR-CW yielded a HY similar to that noted by Frascari et al. [53], who evaluated hyperthermophilic (77 °C) hydrogen production from CW with maximum HY of 5.8 mmol H<sub>2</sub> g lactose<sup>-1</sup> (1.04 mol H<sub>2</sub> mol hexose<sup>-1</sup>). Algapani et al. [13] observed no significant variation in hydrogen production when the temperature was increased from 55 to 70 °C, which was similar to the trend observed in AFBR-V.

## Soluble Metabolic Products (SMPs)

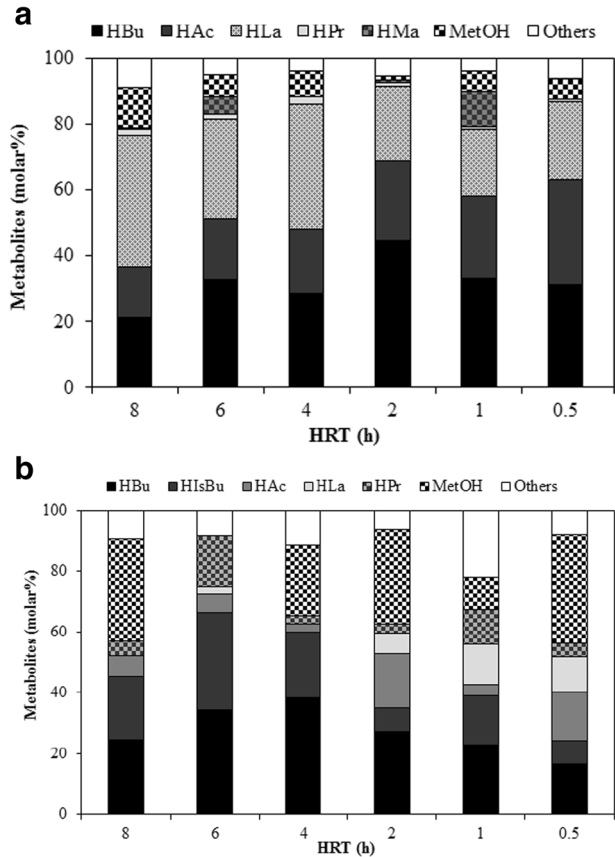
### *Effect of HRT*

The distributions of the liquid metabolites produced in AFBR-CW and AFBR-V are illustrated in Fig. 9.

The main metabolites found in the effluent of AFBR-CW under all conditions were butyric (HBu), lactic (HLA), and acetic (HAc) acids, with percentages ranging from 21.6 to 43.0, 4.7 to 37.1, and 15.8 to 36.9 %, respectively. Propionic (HPr), isobutyric (HIsBu), and malic (HMa) acids were also observed, with percentages ranging from 0.7 to 6.6, 0.8 to 2.6, and 0.4 to 8.1 %, respectively.



**Fig. 9** Distribution of the main metabolites produced in **a** AFBR-CW and **b** AFBR-V



In AFBR-V, the main metabolites were HBU, HisBu, and methanol (MetOH), with percentages ranging from 12.2 to 37.0, 7.6 to 32.0, and 10.8 to 35.7 %, respectively. Other metabolites such as HPr, HAc, and HLa were also observed, with percentages between 3.1 and 16.9, 3.8 and 17.8, and 3.8 to 13.2 %, respectively.

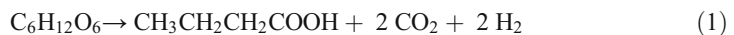
Acidogenesis was more common than solvetogenesis, producing only MetOH. The presence of MetOH was observed for all operational conditions in AFBR-CW and AFBR-V, with the exception of the HRTs of 2 h (AFBR-CW) and 6 h (AFBR-V). The molar fraction of MetOH varied between 6.5 % in AFBR-CW in an HRT of 0.5 h and 35.7 % in AFBR-V in an HRT of 0.5 h. Under such conditions, HPRs of  $5.36 \pm 0.81$  and  $0.71 \pm 0.16$  L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> were observed in AFBR-CW and AFBR-V, respectively. Additionally, in an HRT of 4 h at 55 °C, in which the maximum HYs of  $4.10 \pm 0.68$  68 mmol H<sub>2</sub> g COD<sup>-1</sup> (AFBR-CW) and  $1.64 \pm 0.22$  mmol H<sub>2</sub> g COD<sup>-1</sup> (AFBR-V) were observed, the MetOH molar fractions in AFBR-CW and AFBR-V were 13.9 and 27.1 %, respectively. Therefore, the performances of both reactors were not affected by the different compositions of this alcohol based on the metabolites produced.

Schink and Zeikus [54] suggest that hydrogen-producing *Clostridium* sp. bacteria can produce MetOH as their main metabolite. Similar results were observed by Rosa et al. [16] based on the operation of a mesophilic AFBR for hydrogen production from CW. The

maximum MetOH yield of 1.44 mol mol lactose<sup>-1</sup> was observed in an HRT of 4 h; the same conditions yielded a maximum HY of 1.33 mol mol lactose<sup>-1</sup>.

As the HRT decreased from 8 to 4 h, the molar fraction of HBU in AFBR-CW remained constant between 37 and 32 %. In AFBR-V, the molar fraction of HBU increased from 25 to 37 %, with a maximum value in an HRT of 4 h. HIsBu was identified only in AFBR-V. The HIsBu molar fraction varied between 16 and 32 %, with a maximum value in an HRT of 6 h. HBU and HIsBu molar fractions were associated with an HRT of 4 h, in which the HY values were highest in both reactors at 4.10 ± 0.68 mmol H<sub>2</sub> g COD<sup>-1</sup> (AFBR-CW) and 1.64 ± 0.22 mmol H<sub>2</sub> g COD<sup>-1</sup> (AFBR-V). However, when the HRT decreased from 4 to 0.5 h, the molar fractions of HBU in AFBR-CW and AFBR-V decreased to 24 and 17 %, respectively. In this case, the HY decreased, but the maximum HPR values of 5.36 ± 0.81 and 0.71 ± 0.16 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> were observed in AFBR-CW and AFBR-V, respectively.

The butyric metabolic pathway, which produces 2.0 mol H<sub>2</sub> mol glucose<sup>-1</sup>, is shown in Eq. 1 [1]. The production of HBU and HIsBu and the maximum HY values reflect the predominance of the butyric pathway for hydrogen production. Similarly, Santos et al. [15] observed a maximum HPR of 0.45 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> at an OLR of 60 kg COD m<sup>-3</sup> day<sup>-1</sup> without decreasing the HY in two thermophilic AFBRs with COD concentrations of 15,000 and 20,000 mg COD L<sup>-1</sup>, which produced an HBU molar fraction of 16 %.

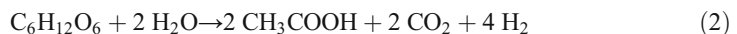


This pathway was also indicated by Perna et al. [32] in an APBR using CW as the substrate. They observed an increase in the HY when HBU production increased and HLA production decreased.

The main metabolites observed by Santos et al. [14] were succinic acid, HLA, and HBU. The use of raw vinasse favored the production of HBU, HAc, and HIsBu and the reduction of the succinic acid concentration, representing a metabolic pathway change that favored hydrogen production. The microbial community was dominated by *Thermoanaerobacterium* sp. and *Clostridium* sp.

Similarly, Santos et al. [15] observed HLA, HBU, HIsBu, and HPr as the major metabolites when HRT decreased, favoring the butyric pathway for hydrogen production. Hydrogen producers (*Megasphaera* sp.) and other organisms (*Lactobacillus* sp.) were observed in the microbial community.

The production of HAc was favored due to the HRT decrease from 8 to 0.5 h in both reactors, increasing the molar fraction from 16 to 33 % in AFBR-CW and from 7 to 16 % in AFBR-V. Under these conditions, the maximum HPR values were 5.36 ± 0.81 and 0.71 ± 0.16 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> in AFBR-CW and AFBR-V, respectively. Das and Veziroğlu [1] noted that the production of HAc makes it possible to generate 4 mol H<sub>2</sub> mol glucose<sup>-1</sup>, as shown in Eq. 2. Thus, the decrease in the HRT increased the hydrogen production efficiency and the HPR by favoring the acetate pathway.



This same pathway was noted by Azbar et al. [24] using a CSTR for thermophilic (55 °C) hydrogen production from CW. The authors observed a maximum HPR of 6 L H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> and an increase in the HAc concentration by decreasing the HRT from 3.5 to 1 day.

Similarly, Ferraz Júnior et al. [42] observed HAc, HBU, and HPr as the major metabolites, with the butyric pathway favored by high OLRs and the acetic pathway favored by low OLRs. Under the conditions that yielded a maximum HY, both HAc and HBU were produced in high concentrations.

The simultaneous production of HAc and HBU was also indicated by Fues et al. [43], who observed HAc (1355 mg L<sup>-1</sup>), HBU (1378 mg L<sup>-1</sup>), and HPr (721 mg L<sup>-1</sup>) as the main metabolites in a thermophilic (55 °C) APBR using vinasse as the substrate. Additionally, they observed a maximum HPR of 1604 mL H<sub>2</sub> day<sup>-1</sup> L<sup>-1</sup> in an HRT of 7.5 h and maximum OLR of 84.2 kg COD m<sup>-3</sup> day<sup>-1</sup>.

The production of HPr is responsible for the consumption of hydrogen. As the HRT varied, the molar fraction of HPr ranged from approximately 0.7 to 2 % in AFBR-CW and from 3.1 to 17 % in AFBR-V. A significant amount of this acid was produced in AFBR-V, and the maximum H<sub>2</sub>% of 17 % (6 h) was associated with a decrease in the HRT from 8 to 6 h, resulting in an increase in the HY from 0.65 to 1.52 mmol g COD<sup>-1</sup>.

This behavior was also observed by Davila-Vasquez et al. [28] using CW in a CSTR. They observed prevalent HPr at an OLR of 184.4 kg lactose m<sup>-3</sup> day<sup>-1</sup> without detecting hydrogen production due to substrate overload.

HLa was produced in all HRTs in AFBR-CW. Decreasing the HRT from 8 to 0.5 h decreased the HLa production from 36 to 24 %. In AFBR-V, the HLa molar fractions were observed between 4 and 18 %. Collet et al. [55] suggested that HLa production uses the substrate necessary for hydrogen production, decreasing the HY. However, even at high HLa concentrations, the reactors exhibited maximum HPRs of 5.36 ± 0.81 and 0.71 ± 0.16 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> in an HRT of 0.5 h. Santos et al. [6] decreased the HRT from 8 to 1 h and observed the production of HAc (6.3–22.8 %) and HLa (25.4–32.7 %) under all operational conditions, with a maximum HPR of 1.96 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> in an HRT of 1 h. Santos [14] also observed the presence of HLa (21.8 %) and an increased HPR (1.20 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>) at low HRTs when evaluating the feasibility of hydrogen production in a thermophilic (55 °C) AFBR using vinasse.

### *Effect of Temperature on the Microbial Metabolism*

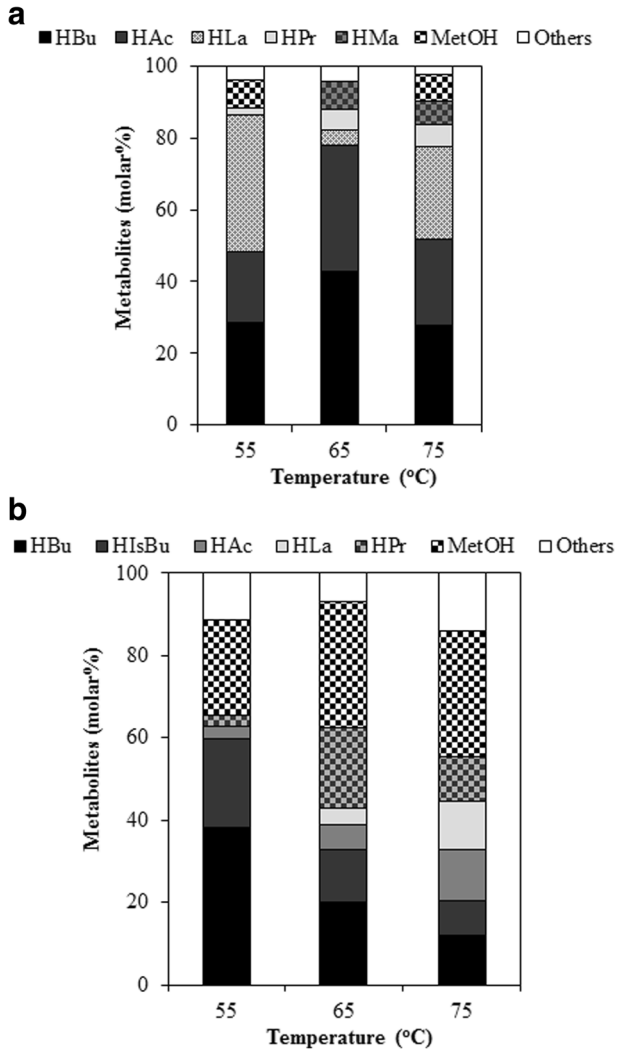
The effect of temperature on the production of metabolites in AFBR-CW and AFBR-V is illustrated in Fig. 10.

In AFBR-CW, acidogenesis prevailed over solvetogenesis, producing only MetOH as a solvent at temperatures of 55 °C (13.9 %) and 75 °C (8.1 %), with no MetOH produced at 65 °C. Additionally, the molar fractions of MetOH in AFBR-V were 27.1 % (55 °C), 30.8 % (65 °C), and 28.4 % (75 °C).

The increase in temperature from 55 to 65 °C in AFBR-CW resulted in the increase in the molar fractions of HBU and HAc from 35 to 40 % and 19 to 37 %, respectively. Moreover, the production of HLa decreased from 32 to 4.7 %. By inhibiting the HLa-producing bacteria, more substrate was available for hydrogen production via the butyric and acetic pathways. This result is in agreement with the maximum HY of 5.51 ± 0.37 mmol H<sub>2</sub> g COD<sup>-1</sup> observed at the temperature of 65 °C and HRT of 4 h.

Nokie et al. [56] investigated the influence of the coexistence of hydrogen-producing (*Clostridium acetobutylicum* or *Clostridium butylicum*) and HLa-producing (*Lactobacillus paracasei* and *Enterococcus durans*) bacteria in the fermentation of tofu processing wastewater. They observed that the metabolic pathway of HLa is preferred over hydrogen production if HLa bacteria are present in the community. The authors found that applying thermal treatments between temperatures of 50 and 90 °C could inhibit HLa bacteria, resulting in the production of more hydrogen.

**Fig. 10** Distribution of produced metabolites as a function of temperature in **a** AFBR-CW and **b** AFBR-V



However, when the temperature was increased to 75 °C, the molar fractions of HBu and HAc observed in AFBR-CW decreased to 25 and 25 %, respectively. Moreover, HLa was produced at a molar fraction of 26 %, and HPr had a maximum value of 6.6 %.

In AFBR-V, gradually increasing the temperature from 55 to 75 °C decreased the molar fractions of HBu and HIsBu from 37 to 12 % and from 16 to 8.9 %, respectively. The molar fraction of HAc increased from 4 to 13 % by increasing the temperature from 55 to 75 °C.

However, the increase in temperature favored the production of metabolites not associated with hydrogen production. HLa and HPr were favored when the temperature increased to 75 °C. HLa, which was not detected at 55 °C, had a molar fraction of 12 % at 75 °C. The maximum values of HPr were 11 % at 65 °C and 8 % at 75 °C. Moreover, the total concentration of metabolites decreased from 6027 to 2968 mg L<sup>-1</sup>.

In a study by Luo et al. [30], the main metabolites were HBU and ethanol at 60 °C (total = 7000 mg L<sup>-1</sup>); HAc, HPr, and HBU at 37 °C (total = 7000 mg L<sup>-1</sup>); and only HAc and ethanol at 70 °C (but smaller total concentration = 2500 mg L<sup>-1</sup>). Thus, HBU production was inhibited and the HY decreased at 70 °C. Thermophilic hydrogen production was associated with the highest concentration of HBU and inhibition of HPr.

The same result was verified by Gadow et al. [26], who evaluated the effects of mesophilic (37 °C), thermophilic (55 °C), and hyperthermophilic (80 °C) temperatures on hydrogen production in a CSTR using cellulose (10 g L<sup>-1</sup>) in an HRT of 10 h. Under thermophilic conditions and a maximum HPR of 0.255 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>, the maximum concentrations of HAc and HBU were 2.36 and 1.61 g L<sup>-1</sup>, respectively. Under hyperthermophilic conditions, the HPR decreased to 0.219 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup>, decreasing the concentrations of HAc and HBU to 1.90 and 1.32 g L<sup>-1</sup>, respectively.

Similarly, Zhang et al. [49] evaluated the effects of temperatures of 30, 37, 55, and 70 °C using corn stover hydrolysate for hydrogen production from activated sludge and granular anaerobic sludge. They observed more efficient fermentation and higher concentrations of HAc and HBU at 55 °C compared to those at 70 °C. The analysis of the microbial community suggested that the increase in temperature decreased the microbial community diversity, which promoted the growth of specific species.

Thus, the increase in temperature from 55 to 75 °C decreased the values of HY in AFBR-CW and AFBR-V from 5.51 ± 0.37 to 1.20 ± 0.39 mmol H<sub>2</sub> g COD<sup>-1</sup> and 1.64 ± 0.22 to 0.04 ± 0.02 mmol H<sub>2</sub> g COD<sup>-1</sup>, respectively. This behavior was a result of the shift in the metabolic pathway, which repressed the hydrogen production of HBU in favor of the production of HPr and HLa. The repression of the butyric pathway is associated with the selection of a hyperthermophilic microbial community at a temperature of 75 °C. Because vinasse and CW are complex, it was not possible to form an effective hyperthermophilic community from the thermophilic inoculum.

## Conclusions

The temperature and HRT are factors capable of altering thermophilic and hyperthermophilic hydrogen production in AFBRs using CW or vinasse as substrates. In AFBR-CW, the decrease in HRT and the increase in temperature benefited hydrogen production until the optimal conditions, an HRT of 4 h and a temperature of 65 °C, were reached, yielding a maximum HY of 5.51 ± 0.37 mmol H<sub>2</sub> g COD<sup>-1</sup>. In this case, hydrogen production was favored via the butyric and acetic pathways, and the production of HLa was repressed by increasing the temperature. The maximum values of HPR and H<sub>2</sub>% were 5.36 ± 0.81 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> and 55.5 ± 2.3 % in an HRT of 0.5 h. In AFBR-V, the decrease in HRT favored hydrogen production via the butyric pathway, with a maximum HY of 1.64 ± 0.22 mmol H<sub>2</sub> g COD<sup>-1</sup> in an HRT of 4 h and at a temperature of 55 °C. The maximum HPR values of 0.71 ± 0.16 L H<sub>2</sub> h<sup>-1</sup> L<sup>-1</sup> and H<sub>2</sub>% of 61.9 ± 4.2 % were observed in an HRT of 0.5 h. However, increasing the temperature to hyperthermophilic (75 °C) conditions decreased the hydrogen production in both reactors because the microbial community that was originally selected in thermophilic (55 °C) conditions had difficulty adapting.

**Acknowledgements** The authors gratefully acknowledge the financial support from the CNPq—National Council for Scientific and Technological Development, CAPES—Coordination for the Improvement of Higher Education Personnel, and FAPESP—São Paulo Research Foundation.

## References

1. Das, D., & Veziroğlu, T. N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*, *26*, 13–28.
2. Hallenbeck, P. C. (2009). Fermentative hydrogen production: principles, progress and prognosis. *International Journal of Hydrogen Energy*, *34*, 7379–7389.
3. Show, K. Y., Lee, D. J., Tay, J. H., Lin, C. Y., & Chang, J. S. (2012). Biohydrogen production: current perspectives and the way forward. *International Journal of Hydrogen Energy*, *37*, 15616–15631.
4. Wilkie, A. C., Riedesel, K. J., & Owens, J. M. (2000). Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass and Bioenergy*, *19*, 63–102.
5. Karadag, D., Kôroğlu, O. E., Ozkaya, B., Cakmakci, M., Heaven, S., & Banks, C. (2014). A review on fermentative hydrogen production from dairy industry wastewater. *Journal of Chemical Technology and Biotechnology*, *89*, 1627–1636.
6. Santos, S. C., Rosa, P. R. F., Sakamoto, I. K., Varesche, M. B. A., & Silva, E. L. (2014). Hydrogen production from diluted and raw sugarcane vinasse under thermophilic anaerobic conditions. *International Journal of Hydrogen Energy*, *39*, 9599–9610.
7. Pawar, S. S., & van Niel, E. W. J. (2013). Thermophilic biohydrogen production: how far are we? *Applied Microbiology and Biotechnology*, *97*, 7999–8009.
8. Lee, D. J., Show, K. Y., & Su, A. (2011). Dark fermentation on biohydrogen production: pure culture. *Bioresource Technology*, *102*, 8393–8402.
9. Gadow, S. I., Li, Y. Y., & Liu, Y. (2012). Effect of temperature on continuous hydrogen production of cellulose. *International Journal of Hydrogen Energy*, *37*, 15465–15472.
10. Gadow, S. I., Jiang, H., Hojo, T., & Li, Y. Y. (2013). Cellulosic hydrogen production and microbial community characterization in hyper-thermophilic continuous bioreactor. *International Journal of Hydrogen Energy*, *38*, 7259–7267.
11. Jiang, H., Gadow, S. I., Tanaka, Y., Cheng, J., & Li, Y. Y. (2015). Improved cellulose conversion to biohydrogen with thermophilic bacteria and characterization of microbial community in continuous bioreactor. *Biomass and Bioenergy*, *75*, 57–64.
12. Gadow, S. I., Jiang, H., & Li, Y. Y. (2016). Characterization and potential of three temperature ranges for hydrogen fermentation of cellulose by means of activity test and 16S rRNA sequence analysis. *Bioresource Technology*, *209*, 80–89.
13. Algapani, D. E., Qiao, W., Su, M., Pumpo, F., Wandera, S. M., Adani, F., & Dong, R. (2016). Biohydrolysis and bio-hydrogen production from food waste by thermophilic and hyperthermophilic anaerobic process. *Bioresource Technology*, *216*, 768–777.
14. Santos, S. C., Rosa, P. R. F., Sakamoto, I. K., Varesche, M. B. A., & Silva, E. L. (2014). Continuous thermophilic hydrogen production and microbial community analysis from anaerobic digestion of diluted sugar cane stillage. *International Journal of Hydrogen Energy*, *39*, 9000–9011.
15. Santos, S. C., Rosa, P. R. F., Sakamoto, I. K., Varesche, M. B. A., & Silva, E. L. (2014). Organic loading rate impact on biohydrogen production and microbial communities at anaerobic fluidized thermophilic bed reactors treating sugar cane stillage. *Bioresource Technology*, *159*, 55–63.
16. Rosa, P. R. F., Santos, S. C., Sakamoto, I. K., Varesche, M. B. A., & Silva, E. L. (2014). Hydrogen production from cheese whey with ethanol-type fermentation: effect of hydraulic retention time on the microbial community composition. *Bioresource Technology*, *161*, 10–19.
17. Rosa, P. R. F., Santos, S. C., & Silva, E. L. (2014). Different ratios of carbon sources in the fermentation of cheese whey and glucose as substrates for hydrogen and ethanol production in continuous reactors. *International Journal of Hydrogen Energy*, *39*, 1288–1296.
18. Shida, G. M., Barros, A. R., Reis, C. M., Amorim, E. L. C., Damianovic, M. H. R. Z., & Silva, E. L. (2009). Long-term stability of hydrogen and organic acids production in an anaerobic fluidized-bed reactor using heat treated anaerobic sludge inoculum. *International Journal of Hydrogen Energy*, *34*, 3679–3688.
19. Reis, C. M., & Silva, E. L. (2011). Effect of upflow velocity and hydraulic retention time in anaerobic fluidized-bed reactors used for hydrogen production. *Chemical Engineering Journal*, *172*, 28–36.
20. Kim, D. H., Han, S. K., Kim, S. H., & Shin, H. (2006). Effect of gas sparging on continuous fermentative hydrogen production. *International Journal of Hydrogen Energy*, *31*, 2158–2169.
21. Standard methods for the examination for water and wastewater (2012). American Public Health Association, American Water Works Association, Water Environmental Federation. 22nd ed. Washington DC, USA.
22. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.



23. Walker, M., Zhang, Y., Heaven, S., & Banks, C. (2009). Potential errors in the quantitative evaluation of biogas production in anaerobic digestion processes. *Bioresource Technology*, *100*, 6339–6346.
24. Azbar, N., Dokgoz, F. T., Keskin, T., Korkmaz, K. S., & Syed, H. M. (2009). Continuous fermentative hydrogen production from cheese whey wastewater under thermophilic anaerobic conditions. *International Journal of Hydrogen Energy*, *34*, 7441–7447.
25. Intanoo, P., Rangsunvigit, P., Namprohm, W., Thamprajamchit, B., Chavadej, J., & Chavadej, S. (2012). Hydrogen production from alcohol wastewater by an anaerobic sequencing batch reactor under thermophilic operation: nitrogen and phosphorous uptakes and transformation. *International Journal of Hydrogen Energy*, *37*, 11104–11112.
26. Gadow, S. I., Jiang, H., Hojo, T., & Li, Y. Y. (2013). Effect of temperature and temperature shock on the stability of continuous cellulosic-hydrogen fermentation. *Bioresource Technology*, *142*, 304–311.
27. Zhong, J., Stevens, D. K., & Hansen, C. L. (2015). Optimization of anaerobic hydrogen and methane production from dairy processing waste using a two-stage digestion in induced bed reactors (IBR). *International Journal of Hydrogen Energy*, *40*, 15470–15476.
28. Davila-Vazquez, G., Cota-Navarro, C. B., Rosales-Colunga, L. M., de León-Rodríguez, A., & Razo-Flores, E. (2009). Continuous biohydrogen production using cheese whey: improving the hydrogen production rate. *International Journal of Hydrogen Energy*, *34*, 4296–4304.
29. Intanoo, P., Suttikul, T., Leethochawalit, M., Gulari, E., & Chavadej, S. (2014). Hydrogen production from alcohol wastewater with added fermentation residue by an anaerobic sequencing batch reactor (ASBR) under thermophilic operation. *International Journal of Hydrogen Energy*, *39*, 9611–9620.
30. Luo, G., Xie, L., Zou, Z., Zhou, Q., & Wang, J. Y. (2010). Fermentative hydrogen production from cassava stillage by mixed anaerobic microflora: effects of temperature and pH. *Applied Energy*, *87*, 3710–3717.
31. Carrillo-Reyes, J., Celis, L. B., Alariste-Mondragón, F., & Razo-Flores, E. (2014). Decreasing methane production in hydrogenogenic UASB reactors fed with cheese whey. *Biomass and Bioenergy*, *63*, 101–108.
32. Perna, V., Castelló, E., Wenzel, J., Zampol, C., Fontes Lima, D. M., Borzacconi, L., Varesche, M. B., Zaiat, M., & Etchebehere, C. (2013). Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. *International Journal of Hydrogen Energy*, *38*, 54–62.
33. Ferraz Júnior, A. D. N., Etchebehere, C., & Zaiat, M. (2015). High organic loading rate on thermophilic hydrogen production and metagenomic study at an anaerobic packed-bed reactor treating a residual liquid stream of a Brazilian biorefinery. *Bioresource Technology*, *186*, 81–88.
34. Qiu, C., Wen, J., & Jia, X. (2011). Extreme-thermophilic biohydrogen production from lignocellulosic bioethanol distillery wastewater with community analysis of hydrogen-producing microflora. *International Journal of Hydrogen Energy*, *36*, 8243–8251.
35. Kargi, F., Eren, N. S., & Ozmihiç, S. (2012). Hydrogen gas production from cheese whey powder (CWP) solution by thermophilic dark fermentation. *International Journal of Hydrogen Energy*, *37*, 2260–2266.
36. Romão, B. B., Batista, F. R. X., Ferreira, J. S., Costa, H. C. B., Resende, M. M., & Cardoso, V. L. (2014). Biohydrogen production through dark fermentation by a microbial consortium using whey permeate as substrate. *Applied Biochemistry and Biotechnology*, *172*, 3670–3685.
37. Venetsaneas, N., Antonopoulou, G., Stamatelatu, K., Kornaros, M., & Lyberatos, G. (2009). Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. *Bioresource Technology*, *100*, 3713–3717.
38. Lima, D. M. F., Lazaro, C. Z., Rodrigues, J. A. D., Ratusznei, S. M., & Zaiat, M. (2016). Optimization performance of an AnSBBR applied to biohydrogen production treating whey. *Journal of Environmental Management*, *169*, 191–201.
39. Dareioti, M. A., & Kornaros, M. (2014). Effect of hydraulic retention time (HRT) on the anaerobic co-digestion of agro-industrial wastes in a two-stage CSTR system. *Bioresource Technology*, *167*, 407–415.
40. Dareioti, M. A., & Kornaros, M. (2015). Anaerobic mesophilic co-digestion of ensiled sorghum, cheese whey and liquid cow manure in a two-stage CSTR system: effect of hydraulic retention time. *Bioresource Technology*, *175*, 553–562.
41. Buitrón, G., Prato-García, D., & Zhang, A. (2014). Biohydrogen production from tequila vinasses using a fixed bed reactor. *Water Science & Technology*, *70*, 1919–1925.
42. Ferraz Júnior, A. D. N., Wenzel, J., Etchebehere, C., & Zaiat, M. (2014). Effect of organic loading rate on hydrogen production from sugarcane vinasse in thermophilic acidogenic packed bed reactors. *International Journal of Hydrogen Energy*, *39*, 16852–16862.
43. Fuess, L. T., Kiyuna, L. S. M., Garcia, M. L., & Zaiat, M. (2016). Operational strategies for long-term biohydrogen production from sugarcane stillage in a continuous acidogenic packed-bed reactor. *International Journal of Hydrogen Energy*, *41*, 8132–8145.

44. Reis, C. M., Carosia, M. F., Sakamoto, I. K., Varesche, M. B. A., & Silva, E. L. (2015). Evaluation of hydrogen and methane production from sugarcane vinasse in an anaerobic fluidized bed reactor. *International Journal of Hydrogen Energy*, *40*, 8498–8509.
45. Kargi, F., Eren, N. S., & Ozmihci, S. (2012). Bio-hydrogen production from cheese whey powder (CWP) solution: comparison of thermophilic and mesophilic dark fermentations. *International Journal of Hydrogen Energy*, *37*, 8338–8342.
46. Luo, G., Xie, L., Zou, Z., Wang, W., Zhou, Q., & Shim, H. (2010). Anaerobic treatment of cassava stillage for hydrogen and methane production in continuously stirred tank reactor (CSTR) under high organic loading rate (OLR). *International Journal of Hydrogen Energy*, *35*, 11733–11737.
47. Gupta, M., Gomez-Flores, M., Nasr, N., Elbeshbishy, E., Hafez, H., Naggar, M. H. E., & Nakhla, G. (2015). Performance of mesophilic biohydrogen-producing cultures at thermophilic conditions. *Bioresource Technology*, *192*, 741–747.
48. Roy, S., Vishnuvardhan, M., & Das, D. (2014). Continuous thermophilic biohydrogen production in packed bed reactor. *Applied Energy*, *136*, 51–58.
49. Zhang, K., Ren, N. Q., & Wang, A. J. (2015). Fermentative hydrogen production from corn stover hydrolysate by two typical seed sludges: effect of temperature. *International Journal of Hydrogen Energy*, *40*, 3838–3848.
50. Pap, B., Györkei, Á., Boboescu, I. Z., Nagy, I. K., Bíró, T., Kondorosi, É., & Maróti, G. (2015). Temperature-dependent transformation of biogas-producing microbial communities points to the increased importance of hydrogenotrophic methanogenesis under thermophilic operation. *Bioresource Technology*, *177*, 375–380.
51. Cisneros-Pérez, C., Carrillo-Reyes, J., Celis, L. B., Alatríste-Mondragón, F., Etchebehere, C., & Razo-Flores, E. (2015). Inoculum pretreatment promotes differences in hydrogen production performance in EGSB reactors. *International Journal of Hydrogen Energy*, *40*, 6329–6339.
52. Obazu, F. O., Ngoma, L., & Gray, V. M. (2012). Interrelationships between bioreactor volume, effluent recycle rate, temperature, pH, %H<sub>2</sub>, hydrogen productivity and hydrogen yield with undefined bacterial cultures. *International Journal of Hydrogen Energy*, *37*, 5579–5590.
53. Frascari, D., Cappelletti, M., Mendes, J. D. S., Alverini, A., Scimonelli, F., Manfreda, C., Longanesi, L., Zannoni, D., Pinelli, D., & Fedi, S. (2013). A kinetic study of biohydrogen production from glucose, molasses and cheese whey by suspended and attached cells of *Thermotoga neapolitana*. *Bioresource Technology*, *147*, 553–561.
54. Schink, B., & Zeikus, J. G. (1980). Microbial methanol fermentation: a major end product of pectin metabolism. *Current Microbiology*, *4*, 387–389.
55. Collet, C., Adler, N., Schwitzguébel, J. P., & Péringer, P. (2004). Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose. *International Journal of Hydrogen Energy*, *29*, 1479–1485.
56. Noike, T., Takabatake, H., Mizuno, O., & Ohba, M. (2002). Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. *International Journal of Hydrogen Energy*, *27*, 1367–1371.