Bioresource Technology 286 (2019) 121379

Contents lists available at ScienceDirect

Bioresource Technology

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

Novel insights on the versatility of biohydrogen production from sugarcane vinasse via thermophilic dark fermentation: Impacts of pH-driven operating strategies on acidogenesis metabolite profiles

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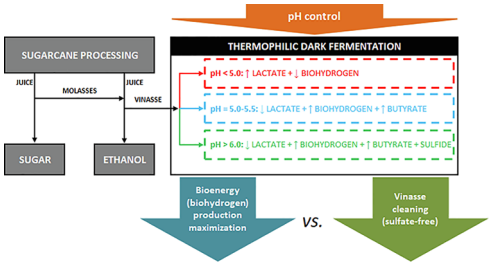
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Sugarcane biorefinery
Vinasse management
Biohydrogen production
Lactate fermentation
Sulfate reduction
pH-dependence

ABSTRACT

An innovative application of the anaerobic structured-bed reactor (AnSTBR) in thermophilic dark fermentation of sugarcane vinasse targeting biohydrogen ($bioH_2$) production was assessed. A detailed metabolite monitoring program identified the major substrates and primary metabolic pathways within the system. Increasing the applied organic loading rate positively affected $bioH_2$ production, reaching 2074 N mL-H_2 L^{-1} d^{-1} and indicating an optimal load of approximately 70 kg-COD m^{-3} d^{-1}. Controlling the fermentation pH (5.0–5.5) was the primary strategy to maintain $bioH_2$-producing conditions, offsetting negative impacts associated with the compositional variability of vinasse. Metabolic correlations pointed out lactate as the primary substrate for $bioH_2$ production, indicating its accumulation as evidence of impaired reactors. The versatility of the acidogenic system was confirmed by identifying three major metabolic pathways according to the pH, i.e., lactate-producing (pH < 5.0), $bioH_2$-/butyrate-producing (pH = 5.0–5.5) and $bioH_2$-producing/sulfate-reducing (pH > 6.0) systems, which enables managing the operation of the reactors for diversified purposes in practical aspects.

1. Introduction

Using sugarcane vinasse as substrate in fermentative processes has drawn much attention in recent years, considering the potential conversion of residual carbon from ethanol fermentation in bioenergy and/or liquid phase bioproducts via biohydrogen ($bioH_2$) and soluble metabolites (e.g. short-chain organic acids and solvents) production. This approach concerns the application of the biorefinery concept in the management of residual streams, which aims to improve the global energy conversion efficiency in processing chains. Considering

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https://doi.org/10.1016/j.biortech.2019.121379
Received 25 March 2019; Received in revised form 22 April 2019; Accepted 24 April 2019
Available online 25 April 2019
0960-8524/

specifically the case of vinasse, studies focus on investigating the bioH_2 production potential (Albanez et al., 2016; Ferraz et al., 2014, 2015a,b; Fuess et al., 2016, 2018a; Santos et al., 2014a,b,c), often directing the fermented vinasse to sequential methanogenic systems (Ferraz et al., 2016; Fuess et al., 2017).

In this context, several technological approaches have been proposed in an effort to optimize bioH_2 production from sugarcane vinasse, which includes: [i] studying different reactor configurations, i.e., conventional packed-beds (Ferraz et al., 2014, 2015a,b; Fuess et al., 2016, 2018a), fluidized-beds (Santos et al., 2014a,b,c) and sequencing batch (Albanez et al., 2016) systems; [ii] defining adequate support materials in fixed-film systems (Ferraz et al., 2015b); [iii] defining optimized operating conditions, primarily regarding the applied organic loading rate (OLR), temperature (Ferraz et al., 2014; Santos et al., 2014c) and diversified operating strategies (Fuess et al., 2016) and [iv] co-digesting vinasse with different organic substrates (Albanez et al., 2016; Santos et al., 2014a). Despite the remarkable scientific progress achieved, which also includes a better understanding of the microbial communities involved in vinasse conversion (Fuess et al., 2018a), there are still limitations to a better energetic exploitation of vinasse in acidogenic systems.

In particular, two aspects require complementary investigations to improve the bioH_2 production from sugarcane vinasse, considering the definition of adequate bioreactor configurations and a better understanding of the metabolic pathways associated with the conversion of the organic substrates found in vinasse. Regarding reactor configurations, fixed-film systems are, theoretically, the best options to achieve high hydrogenogenic activities due to their efficient cell retention capacity (Fernandes et al., 2013). However, studies indicate negative effects from the excessive biomass accumulation in bioH_2-targeted acidogenic systems (Dinamarca and Bakke, 2009), as the food-to-microorganism (F/M) ratio is unbalanced and non-bioH_2-producing and/or bioH_2-consuming pathways are favored (Anzola-Rojas et al., 2015). Thus, adequate acidogenic systems should simultaneously provide surface for biomass attachment and a relatively high void index, which directly reflects more balanced biomass retention levels within the system. Both characteristics may be provided by structured-bed reactors (Anzola-Rojas and Zaiat, 2016; Fuess et al., 2017), characterizing an alternative reactor configuration to conventional packed-bed systems.

Regarding compositional aspects, the complexity of vinasse makes it difficult to identify the metabolic pathways primarily associated with bioH_2 production, which limits both the establishment of monitoring routines to carefully analyze the performance of the reactors (e.g. calculating the yield in conversion processes) and the definition of operating strategies to minimize/overcome performance losses. Assessing the metabolic pathways in sugarcane vinasse dark fermentation still requires a less “black box” approach, as previous studies (Albanez et al., 2016; Ferraz et al., 2014, 2015a,b; Fuess et al., 2016, 2018a; Santos et al., 2014a,b,c) only considered the conversion of carbohydrates as a response to understand substrate uptake in those systems, always neglecting the role of relevant substrates, such as lactate and glycerol. Moreover, the role of sulfate in the fermentation process also demands a clear understanding, as a series of implications may be associated with the establishment of sulfidogenic conditions, namely, the consumption of hydrogen, the provision of acetate for subsequent methanogenic processes, and inherent aspects of inhibition by sulfide. In particular, driving sulfate-reducing conditions in the acidogenic phase may be an effective “vinasse cleaning” approach, focusing on the bioenergy recovery via methane production in two-phase biodigestion systems. Despite the relevance to the overall bioenergy production, managing sulfate in the biodigestion of sugarcane vinasse is still marginally considered in correlated studies, although the negative impacts of sulfidogenesis on methane production have been clearly demonstrated (Kiyuna et al., 2017).

This study addresses characteristics of both limiting aspects previously highlighted in order to propose technological approaches to better understand the steps, i.e., the main metabolic pathways of the thermophilic (55 °C) dark fermentation of sugarcane vinasse, as well as to improve the recovery of bioenergy through bioH_2 production. Thus, an anaerobic structured-bed reactor (AnSTBR) was continuously monitored under increasing OLR conditions, using sugarcane vinasse samples with different compositional characteristics. BioH_2 production using the AnSTBR has already been addressed; however, lab-made wastewaters were used as substrate in all cases (Anzola-Rojas and Zaiat, 2016; Anzola-Rojas et al., 2016; Blanco et al., 2017), which characterizes this case as the first application of the AnSTBR in the acidogenic processing of high-strength wastewaters. Optimized operating conditions were defined for the assessed reactor configuration, as well as a detailed monitoring program of the soluble phase was applied to identify the primary fermentative pathways established within the system to obtain either bioH_2 or other types of metabolites. This study is the first case to critically characterize the versatility of sugarcane vinasse dark fermentation, also discussing aspects of the establishment of sulfidogenic pathways. Overall, understanding the potential achievable substrate conversion pathways provides subsides to manage the operation of the reactor according to specific targets, which encompasses maximizing the bioenergy production via bioH_2 within the biorefinery context.

2. Materials and methods

2.1. Sugarcane vinasse characterization

Sugarcane vinasse samples were regularly collected from a full-scale sugar and ethanol plant located in Pradópolis, São Paulo, Brazil. The sampling was carried out between May and November 2017 following the progress of the sugarcane harvesting period, leading to five collections (C1–C5). Compositional aspects of the sugarcane vinasse throughout the harvest are detailed in Table 1. The compositional variations observed in vinasse resulted from shifts in the production profile of the sugarcane biorefinery, i.e., from an annexed (sugar and ethanol production) to an autonomous (primarily ethanol production) character throughout the harvest. The co-production of sugar and ethanol implies using blends of molasses and juice in ethanol fermentation, leading to more concentrated vinasses.

2.2. Experimental approach and operating conditions

BioH_2 production from sugarcane vinasse was assessed in a bench-scale (1.9 L) AnSTBR continuously monitored for 150 days. The fixed-bed was structured through orderly placing low-density polyethylene (LDPE) rings, performing a total porosity of 0.92. Details of the experimental apparatus are depicted in Fig. 1. The AnSTBR has been previously used in the recovery of bioenergy from sugarcane vinasse (Aquino et al., 2017; Fuess et al., 2017); however, in both cases methanogenic systems were assessed, as well as polyurethane foam was used as the support material. The pH of vinasse was adjusted between 6.5 and 7.5 prior to feeding the reactor through dosing concentrated NaOH (50% m/V) or concentrated NaOH (50% m/V) in association with $NaHCO_3$ (0.1–0.02 g-$NaHCO_3$ g^{-1} CODt), based on the pH measured in fermented vinasse. Despite the known pH-dependence of vinasse fermentation (Fuess et al., 2016), no specific target pH values were initially defined, so that the behavior of the reactor toward the different operating conditions was used to direct pH control. In this context, dosing $NaHCO_3$ aimed to provide buffer capacity to the reactor to overcome pH-related limitations in fermentation.

The inoculation of the AnSTBR was carried out using the natural fermentation of sugarcane vinasse under thermophilic temperature conditions (55 °C), following the protocol described elsewhere (Ferraz et al., 2014; Fuess et al., 2016). Subsequently, the reactor was maintained under controlled thermophilic conditions (55 °C) and subjected to decreasing hydraulic retention time (HRT) values, i.e., 24, 16, 12, 6

Table 1
Compositional characterization of sugarcane vinasse throughout the 2017 harvesting period.

Parameter	Sampling period				
	May/2017	June/2017	September/2017	October/2017	November/2017
Collection	C1 (inoculation)[a]	C2 (1–54)[a]	C3 (55–80)[a]	C4 (81–124)[a]	C5 (125–150)[a]
CODt[b]	40,425	42,162	49,000	24,760	19,512
CODs[b]	37,725	34,962	43,420	20,975	15,287
BOD[b]	19,450	16,650	23,182	11,562	8792
BOD/CODt	0.48	0.39	0.47	0.47	0.45
CH[b]	7275	5275	5020	5475	3875
GLY[b]	2617	3914	3278	3537	2517
HAc[b]	1722	1457	1117	429	153
HPr[b]	37	127	64	0	0
HLa[b]	2550	1620	1735	1690	1050
HBu[b]	0	40	468	0	0
EtOH[b]	570	713	5490	462	154
MeOH[b]	67	180	395	0	0
SO_4^{2-}[b]	2450	2500	1800	1042	1183
CODt/SO_4^{2-}	16.50	16.86	27.22	23.76	16.49
CODs/SO_4^{2-}	15.40	13.99	24.12	20.13	12.92
VSS[b]	2053	5553	5507	3312	2700
pH	4.54	4.67	4.63	4.29	4.25

Parameters: CODt – total chemical oxygen demand (unfiltered samples), CODs – soluble chemical oxygen demand (0.45 μm-filtered samples), BOD – biochemical oxygen demand, CH – total carbohydrates, GLY – glycerol, HAc – acetic acid, HPr – propionic acid, HLa – lactic acid, HBu – butyric acid, EtOH – ethanol, MeOH – methanol, SO_4^{2-} – sulfate, VSS – volatile suspended solids.
Notes: [a]Period in which vinasse samples from a given collection were used in the feeding of the reactor, [b]Values in mg L^{-1}.

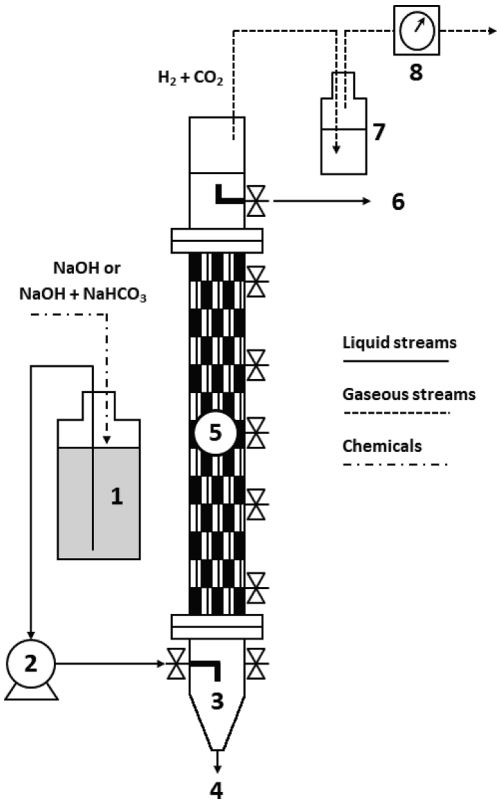

Fig. 1. Schematic representation of the vinasse-fed acidogenic system. Legend: 1- feeding tank, 2- peristaltic pump, 3- AnSTBR (basal portion), 4- bottom biomass discharge port, 5- AnSTBR (structured-bed), 6- effluent port, 7- water seal and 8- gas meter.

and 4 h in order to perform increasing OLR values (40–120 kg-COD m^{-3} d^{-1}). Details of the operating conditions are presented in Table 2. Increasing the OLR aimed to define optimal operating conditions for bioH_2 production from vinasse in the AnSTBR. Bottom biomass discharges (BBD) were carried out as complementary operating strategies for maintaining the hydrogenogenic activity, as proposed by Fuess et al. (2016). No periodicity was initially defined to perform the BBD.

2.3. Reactor monitoring, analytical methods and performance assessment

Monitoring the AnSTBR was based on measuring the following parameters: pH, total (CODt) and soluble (CODs) chemical oxygen demand, total carbohydrates (CH), glycerol (GLY), lactic acid (HLa), volatile fatty acids (VFA) and solvents, volatile suspended solids (VSS) and sulfate (SO_4^{2-}) for the liquid phase; biogas flow rate and composition for the gaseous phase. pH, CODt, CODs, VSS and SO_4^{2-} analyses were based on protocols described in the Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012). CH, GLY and HLa determinations were based on Dubois et al. (1956), Greenhill (2003) and Taylor (1996), respectively. VFA and solvent concentrations were obtained through gas chromatography (GC/FID) according to Adorno et al. (2014). Vinasse samples were filtered in 0.45 μm filters prior to CODs, CH, GLY, HLa, VFA/solvents and SO_4^{2-} analyses. Regarding the gaseous phase, the biogas flow rate was monitored through coupling a gas meter (model MGC-1 V30; Dr.-Ing. Ritter Apparatebau GMBH & CO. KG, Bochum, Germany) directly in the headspace of the reactor (Fig. 1). Biogas composition (H_2, CO_2 and CH_4) was measured via gas chromatography (GC/TCD), as detailed in Perna et al. (2013).

The performance of the reactor was assessed based on the following response-variables: CODt removal (ER_{CODt}; %), CODs removal (ER_{CODs}; %), CH conversion (EC_{CH}; %), GLY conversion (EC_{GLY}; %), HLa conversion (EC_{HLa}; %), SO_4^{2-} reduction efficiency (ER_{SO42-}; %), biogas flow rate (BFR; mL d^{-1}), volumetric hydrogen production rate (VHPR; NmL-H_2 L^{-1} d^{-1}) and hydrogen yield (HY; mmol-H_2 g^{-1} CH and mmol-H_2 g^{-1} $COD_{applied}$). Particularly, a new approach for calculating the HY from sugarcane vinasse was considered in this study to obtain values in terms of mmol-H_2 g^{-1} $COD_{converted}$, in which the term "$COD_{converted}$" includes the conversion of CH, GLY and HLa. The biomass growth per consumed substrate factor ($Y_{X/S}$; g-VSS g^{-1} COD), biomass retention within the reactor and specific organic loading rate (SOLR; g-COD g^{-1} VSS d^{-1}) were calculated at the end of the operating period, as proposed by Anzola-Rojas et al. (2015).

Table 2
Operating conditions applied in the AnSTBR.

Operating phase	HRT[a] (h)	Period (d)	Vinasse (collection)	OLR[a] (kg-COD m^{-3} d^{-1})	$NaHCO_3$ dosing (g-$NaHCO_3$ g^{-1} CODt)
24	24	1–35	C2	40	0.0
16-I[b]	16	36–47	C2	60	0.0
16-II[c]	16	48–54	C2	60	0.0
		55–70	C3	60	0.0
12-I[d]	12	71–80	C3	80	0.0
12-II[e]	12	81–84	C4	50	0.0
6-I[f]	6	85–91	C4	100	0.0
6-II	6	92–110	C4	100	0.1–0.05
6-III	6	111–114	C4	100	0.05–0.02
6-IV	6	115–124	C4	100	0.05
		125–138	C5	100	0.05
4	4	139–150	C5	120	0.05

Operating parameters: HRT – hydraulic retention time, OLR – organic loading rate. Notes: [a]Theoretical values, [b]Period prior to BBD #1, [c]Period after BBD #1, [d]Prior to feeding the reactor with vinasse from C4 collection, [e]After feeding the reactor with vinasse from C4 collection, [f]Prior to $NaHCO_3$ dosing.

3. Results and discussion

3.1. Overall performance: bioH_2 production and substrate conversion

Overall, increasing the applied OLR (through decreasing the HRT) positively affected the bioH_2 production from sugarcane vinasse, with a maximum VHPR (2074 ± 258 N mL-H_2 L^{-1} d^{-1}; Table 3) observed for an OLR of approximately 90 kg-COD m^{-3} d^{-1} (HRT = 12 h; phase 12-I). Fig. 2a depicts the temporal profile of the VHPR according to the different operating conditions. Comparatively, the maximum value observed was at least 30% higher than the ones reported for conventional vinasse-fed packed-bed acidogenic systems, also considering thermophilic conditions (55 °C): 527 N mL-H_2 L^{-1} d^{-1} (OLR = 72.4 kg-COD m^{-3} d^{-1}) and 391 N mL-H_2 L^{-1} d^{-1} (108.6 kg-COD m^{-3} d^{-1}) (Ferraz et al., 2014), 762 N mL-H_2 L^{-1} d^{-1} (OLR = 84.2 kg-COD m^{-3} d^{-1}) (Ferraz et al., 2015a) and 1604 N mL-H_2 L^{-1} d^{-1} (OLR = 84.2 kg-COD m^{-3} d^{-1}) (Fuess et al., 2016). However, only increasing the OLR was not sufficient to maintain a continuous long-term hydrogenogenic activity in the AnSTBR, considering impacts of the compositional variation of vinasse throughout the sugarcane harvest. The accumulation of biomass in the basal portion of the reactor also had a negative impact on the bioH_2 production to a lesser extent.

Regarding the accumulation of solids, discharging approximately 9.5 g-VSS (BBD #1; Fig. 2c) between operating phases 16-I and 16-II (Table 2) enabled a 260% increase in the VHPR (from 521 ± 132 to 1355 ± 242 N mL-H_2 L^{-1} d^{-1}; Table 3), showing evidence of negative impacts of the biomass accumulation over the bioH_2 production. However, BBD #1 led to a negligible variation in the SOLR (1.77–1.98 g-COD g^{-1} VSS d^{-1}; Fig. 2d), which suggests that the positive effects resulted from eliminating bioH_2-consuming microorganisms and/or from facilitating the liquid–gas mass transfer, considering the removal of excess solid levels. Four additional BBD (BBD #2–BBD #5; Fig. 2c) were carried out throughout the operation of the AnSTBR; however, the observed results indicated negligible effects of solid removal over bioH_2 production (Fig. 2a) in such cases. Overall, using the AnSTBR enabled maintaining SOLR values (2.0–7.0 g-COD g^{-1} VSS d^{-1}; Fig. 2c) relatively close to the optimal range reported elsewhere (4.0–6.0 g-COD g^{-1} VSS d^{-1}; Anzola-Rojas et al., 2015; Fuess et al., 2016; Hafez et al., 2010), especially from the application of OLR values higher than 80 kg-COD m^{-3} d^{-1} (Fig. 2d). It is worth mentioning that SOLR values within the range 1.5–2.0 g-COD g^{-1} VSS d^{-1} were observed by the end of the operating period (Fig. 2d), even for retained biomass concentrations exceeding 25 g-VSS L^{-1} (Fig. 2c), which characterizes values at least two-fold higher than the ones reported in the long-term operation of conventional packed-bed systems (Fuess et al., 2016).

Controlling the operating pH was the determining factor to maintain the hydrogenogenic activity within the AnSTBR (Table 3) in order to drive the predominance of different metabolic pathways in the system. Using less concentrated vinasses (CODt = 24.7–19.5 g L^{-1}; collections C4–C5; Table 2), which resulted from directing higher total reducing sugar proportions to ethanol production in the sampled distillery, led to a considerable drop in the effluent pH (< 4.5; Table 3 and Fig. 2a – phase 12-II), having a direct impact on bioH_2 production and favoring the accumulation of HLa within the AnSTBR (Fig. 2b). Initially, the HRT was decreased from 12 h to 6 h (phase 6-I; Table 2) as a strategy to increase the bioH_2 production based on the behavior previously described by Palomo-Briones et al. (2017), i.e., lower HRT are less favorable to lactic fermentation. However, the applied strategy did not enhance the hydrogenogenic activity, as low pH values (< 5.0) were still observed in the fermented vinasse (Table 3), with the subsequent accumulation of HLa (Fig. 2b).

Dosing $NaHCO_3$ to the acidogenic system (initially at a dose of 0.1 g-$NaHCO_3$ g^{-1} CODt, which was further decreased to 0.05 g-$NaHCO_3$ g^{-1} CODt; phase 6-II) led to the recovery of the bioH_2 production from vinasse, i.e., VHPR = 62 ± 56 N mL-H_2 L^{-1} d^{-1} (phase 6-I) *vs.* 1463 ± 387 N mL-H_2 L^{-1} d^{-1} (phase 6-II) (Fig. 2a), which was strictly associated with the increase in the effluent pH (Fig. 2a). Increasing the pH (> 6.0) also stimulated the activity of sulfate-reducing bacteria (SRB) within the AnSTBR in this operating phase, considering the complete removal of the influent SO_4^{2-} concentrations (Fig. 3c) associated with the detection of hydrogen sulfide (H_2S) at proportions of approximately 2% (20000 ppm_v) in the biogas. The establishment of acidogenic-sulfidogenic conditions in the AnSTBR most likely limited identifying the real bioH_2 production capacity in the applied operating condition (HRT = 6 h, OLR = 100 kg-COD m^{-3} d^{-1}; Table 2), considering the consumption of fractions of the bioH_2 by the hydrogenotrophic sulfidogenic pathway (Zhou and Xing, 2015). Average-to-low sulfidogenic activity levels (ER_{SO42-} 14.3–51.3%) were also identified during the first five days of operation (phase 24) prior to the establishment of pH values below 6.0.

Decreasing the $NaHCO_3$ dose (0.02 g-$NaHCO_3$ g^{-1} CODt; phase 6-III) successfully led to suppressing SRB activity in the AnSTBR. However, a sharp drop was observed again in the effluent pH (< 5.0; Fig. 2a), leading to the complete cessation of the hydrogenogenic activity followed by the accumulation of HLa (Fig. 2a). The observed pattern is in accordance with the behavior of SRB, as low-to-negligible growth rates are associated with acidic environments, i.e., pH = 4.0–6.0 (Fortin et al., 1996; Reis et al., 1988, 1991). Previous studies assessing acidogenic systems fed with various types of organic sources usually reported the inhibition of SRB at pH values below 5.5–5.8 (Hwang et al., 2009a, 2009b; Lin and Chen, 2006), even when applying SO_4^{2-} concentrations as high as 20 g L^{-1}. Regarding specifically sugarcane vinasse, previous studies on bioH_2 production (Ferraz et al., 2014, 2015a,b; Fuess et al., 2016, 2018a; Santos et al., 2014a,b,c) always reported fermentation pH values below 6.0, and therefore the

Table 3
Performance data for the conversion of diversified substrates and biogas/bioH_2 production in the AnSTBR.

Operating phase		24	16-I	16-II	12-I	12-II	6-I	6-II	6-III	6-IV	4
Operating parameters	HRT[a]	25.4 ± 1.5	16.6 ± 0.6	16.3 ± 0.5	13.2 ± 1.2	12.5 ± 0.8	6.0 ± 0.7	6.4 ± 0.7	6.0 ± 0.3	6.2 ± 0.5	4.3 ± 0.3
	OLR[b]	41.9 ± 5.1	59.2 ± 4.2	60.3 ± 8.9	87.5 ± 13.5	48.2 ± 3.7	98.5 ± 12.3	99.1 ± 14.2	97.6 ± 5.4	88.2 ± 11.9	114.7 ± 10.0
Response-variables	pH	5.45 ± 0.29	5.11 ± 0.14	5.32 ± 0.14	5.21 ± 0.09	4.45 ± 0.05	4.44 ± 0.06	6.51 ± 0.64	4.78 ± 0.41	5.24 ± 0.16	5.07 ± 0.09
	ER_{CODt}[c]	13.4 ± 9.2	10.9 ± 6.3[i]		13.7 ± 6.72[j]		13.2 ± 9.3[k]				8.7 ± 5.5
	ER_{CODs}[c]	7.9 ± 5.3	6.3 ± 5.4[i]		5.4 ± 4.2[j]		10.1 ± 6.8[k]				8.8 ± 2.1
	EC_{CH}[c]	56.4 ± 10.2	57.4 ± 9.2	54.2 ± 5.5	56.6 ± 1.9	60.2 ± 7.9	50.8 ± 6.7	74.1 ± 7.1	76.0 ± 10.4	74.0 ± 4.1	74.6 ± 3.4
	EC_{GLY}[c]	97.5 ± 2.5	96.6 ± 4.0	98.3 ± 0.4	98.5 ± 0.2	87.5 ± 2.6	92.7 ± 5.1	98.5 ± 0.1	98.0 ± 1.2	97.9 ± 0.9	98.1 ± 0.0
	EC_{HLa}[c]	80.0 ± 18.4	85.3 ± 2.4	63.6 ± 22.4	78.2 ± 10.6	0	0	73.6 ± 32.0	0	34.4 ± 39.2	18.9 ± 23.3
	ER_{SO42-}[c]	4.8 ± 13.6	0	0	0	0	0	72.3 ± 36.3	0	0	0
	BFR[d]	6204 ± 1191	4833 ± 1146	9717 ± 1714	15212 ± 644	1422 ± 1814	616 ± 499	19345 ± 3435	9625 ± 9382	13729 ± 2567	17096 ± 644
	VHPR[e]	755 ± 231	521 ± 132	1355 ± 242	2074 ± 258	234 ± 29	62 ± 56	1463 ± 387	1222 ± 1189	1510 ± 236	1830 ± 127
	HY[f]	10.3 ± 4.5	4.6 ± 2.1	14.4 ± 2.6	15.6 ± 2.8	1.4 ± 1.7	0.2 ± 0.2	3.8 ± 1.5	3.0 ± 2.8	4.4 ± 0.8	3.7 ± 0.5
	HY[g]	3.1 ± 1.3	1.0 ± 0.4	0.9 ± 0.2	1.0 ± 0.2	0.2 ± 0.3	0	0.6 ± 0.2	0.5 ± 0.5	0.7 ± 0.2	0.6 ± 0.1
	HY[h]	3.9 ± 1.4	1.7 ± 0.4	4.9 ± 1.2	5.2 ± 1.0	0.6 ± 0.8	0.1 ± 0.1	1.6 ± 0.6	1.3 ± 1.2	2.0 ± 0.3	1.8 ± 0.2

Notes: [a]h; [b]kg-COD m^{-3} d^{-1}; [c]%; [d]mL d^{-1}; [e]NmL-H_2 L^{-1} d^{-1}; [f]mmol-H_2 g^{-1} CH; [g]mmol-H_2 g^{-1} $COD_{applied}$ (Calculation based on the CODt applied in the AnSTBR); [h]mmol-H_2 g^{-1} $COD_{converted}$ (Calculation based on the conversion of total carbohydrates, glycerol and lactic acid); [i]Values include data regarding phases 16-I and 16-II; [j]Values include data regading phases 12-I and 12-II; [k]Values include data regarding phases 6-I, 6-II, 6-III and 6-IV.

Fig. 2. Overall performance of the AnSTBR: temporal profiles of the (a) volumetric hydrogen production rate (VHPR, ●) and effluent pH (○), (b) lactic acid (HLa) concentrations in raw (●) and fermented (○) vinasse, (c) biomass retention within the reactor (○) and (d) specific organic loading rate (SOLR, ○). Note: Arrows represent bottom biomass discharges (BBD) – c.

impacts of sulfate reduction have never been considered relevant in vinasse fermentation before. Despite the negative effects over bioH_2 production, establishing sulfidogenic conditions in acidogenic systems prior to methanogenesis may positively impact the global energy recovery in two-phase anaerobic biodigestion systems (Gil-Garcia et al., 2018), considering the elimination of both the competition by substrate and inhibitory effects of H_2S in the methanogenic phase.

BioH_2 production was stimulated only by increasing the $NaHCO_3$ dose to 0.05 g-$NaHCO_3$ g^{-1} CODt (phase 6-IV), reaching values equivalent to the ones observed in phase 6-II (VHPR = 1510 ± 236 N mL-H_2 L^{-1} d^{-1}; Table 3). However, the hydrogenogenic activity gradually increased (Fig. 2a) in this case, as well as the drop in HLa concentrations (Fig. 2b), so that pH values below 5.0 were observed up to the complete recovery of the buffer capacity of the system (Fig. 2a). Finally, high bioH_2 production levels were also observed when decreasing the HRT to 4 h (phase 4), still considering a $NaHCO_3$ dosing (0.05 g-$NaHCO_3$ g^{-1} CODt). The increase observed in the VHPR (1830 ± 127 N mL-H_2 L^{-1} d^{-1}; Table 3) compared to phase 6-IV, was most likely related to the complete inhibition of the

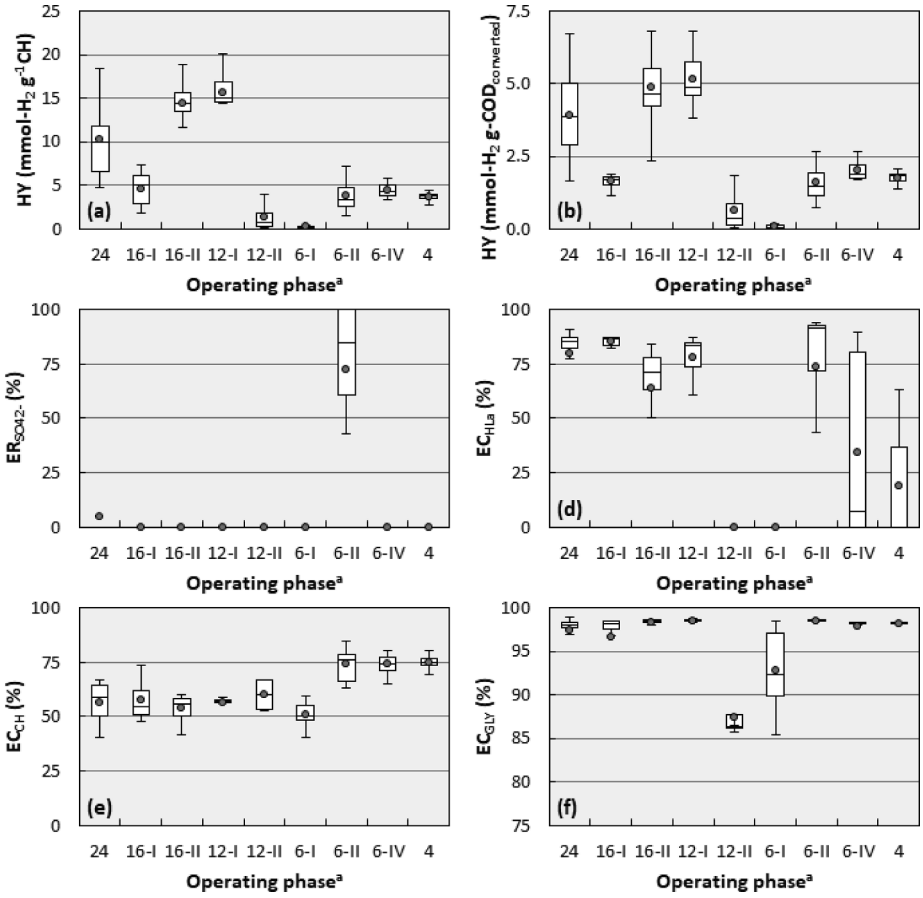

Fig. 3. Performance of the AnSTBR according to the different operating phases: (a) hydrogen yield (HY, mmol-H_2 g^{-1} CH), (b) hydrogen yield (mmol-H_2 g^{-1} $COD_{converted}$), (c) sulfate reduction efficiency (ER_{SO42-}, %), (d) lactic acid conversion (EC_{HLa}, %), (e) total carbohydrates conversion (EC_{CH}, %) and (f) glycerol conversion (EC_{GLY}). Notes: [a]Numbers describing the operating phases represent the theoretical HRT in each phase. Data regarding phase 6-III were not included in the figure.

sulfidogenic activity within the AnSTBR.

Additional details regarding the bioH_2 production (considering specifically the HY) and organic matter conversion are depicted in Fig. 3. The similar pattern observed for the different calculations used to report the HY, i.e., in terms of mmol-H_2 g^{-1} CH (Fig. 3a) and mmol-H_2 g^{-1} $COD_{converted}$ (Fig. 3b), indicates that considering only the conversion of total carbohydrates as a reference may provide a satisfactory understanding of the performance of vinasse-fed acidogenic systems, as reported in previous studies (Ferraz et al., 2014, 2015a,b; Fuess et al., 2016, 2018a). However, diversifying the considered substrates by including the conversion of HLa and glycerol in the calculation provides a more precise performance assessment. The conversion of the monitored organic substrates presented discrepant patterns, so that the different operating conditions applied impacted more effectively the dynamics of HLa production/consumption (Figs. 2b and 3d). In turn, carbohydrate conversion presented relative stability throughout the entire operation of the system, characterizing only two specific periods, i.e., EC_{CH} = 50.8–60.2% (phases 24 to 6-I) and EC_{CH} = 74.0–76.0% (phases 6-II to 4) (Table 3 and Fig. 3e). The increase observed in EC_{CH} coincided with the dosing of $NaHCO_3$ (phase 6-II onwards; Fig. 3e), suggesting the provision of more favorable conditions for carbohydrate assimilation by the acidogenic biomass, regardless of the establishment of sulfidogenic conditions.

Similarly to carbohydrate uptake, glycerol conversion achieved stable and high levels (usually > 95%) throughout the entire operating period (Table 3 and Fig. 3f) under both favorable (pH > 5.0) and unfavorable (pH < 5.0) conditions for bioH_2 production. These results indicate that glycerol fermentation might not be a determining aspect for the direct bioH_2 production from sugarcane vinasse in the studied conditions, most likely due to the pH maintained in the AnSTBR. Mangayil et al. (2012) reported an optimal pH of 6.5 for the production of bioH_2 from glycerol, associating minimal hydrogenogenic activities to lower pH values, i.e., 5.0 and 6.0. In this case, pH values measured during 90% of the operating period, i.e., < 6.0 (Fig. 2a, excluding phase 6-II), suggest the establishment of unfavorable conditions for bioH_2 production from glycerol in the AnSTBR. The high operating temperature may also have limited this particular process, as performance losses were also associated with temperature conditions higher than 40 °C (Mangayil et al., 2012). Nevertheless, glycerol fermentation may have provided substrates for bioH_2 production in sequential metabolic pathways, especially lactate (Haron et al., 2018; Moscoviz et al., 2016).

3.2. Fermentation intermediates, observed correlations and mass balance

The hydrogenogenic activity in the AnSTBR was directly associated with the production of butyric acid (HBu) (Fig. 4a), with concentrations usually above 2 g L^{-1} and peak values of 5 g L^{-1} in phases 12-I and 6-II. Conversely, HLa was the primary metabolite identified in the periods of low bioH_2 production (phases 12-II and 6-I), reaching concentrations in the range of 3.5–3.6 g L^{-1} (Fig. 4a). The production/consumption of the additional quantified metabolites, i.e., methanol (MeOH), ethanol (EtOH), acetic acid (HAc) and propionic acid (HPr), was most likely not directly correlated with the production of bioH_2. The sharp increase in EtOH concentrations between the 55th and 79th days of operation (Fig. 4a) coincided with the application of vinasses from collection C3 (Table 1). In this case, the high EtOH concentrations resulted from a leaking EtOH pipe in the distillery during the sampling of vinasse, i.e., the accumulation of EtOH in the AnSTBR was not associated with any specific metabolic shift in the acidogenic biomass. Nevertheless, it is worth mentioning the capacity of the microbial communities to withstand ethanol concentrations as high as 6.0 g L^{-1}. HAc concentrations presented a decreasing pattern throughout the operating period, i.e., from 1.8 to 2.3 g L^{-1} (phases 24 and 16-I) to 0.6–0.8 g L^{-1} (phases 6-IV

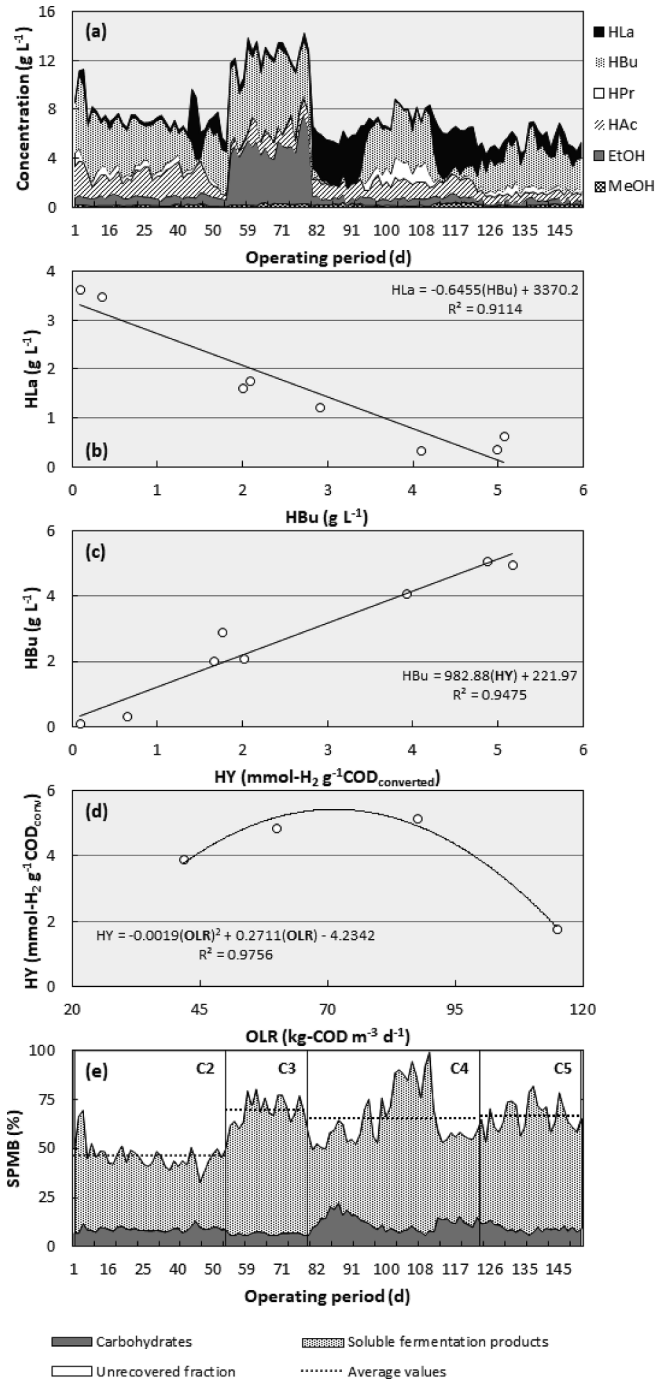

Fig. 4. Soluble metabolites and observed correlations: (a) concentration of fermentation intermediates, (b) correlation between lactic acid (HLa) and butyric acid (HBu) concentrations, (c) correlation between butyric acid concentrations (HBu) and the hydrogen yield (HY), (d) correlation between the hydrogen yield (HY) and the applied OLR and (e) soluble phase mass balance (SPMB) for the fermented vinasse. Legend: experimental values (○) and fitted models (−).

and 4) (Fig. 4a), also following the pattern observed in raw vinasse (Table 1). Finally, the accumulation of HPr during phase 6-II was most likely associated with the establishment of favorable conditions to propionic fermentation at high pH values (> 6.0), similarly to the findings reported by Dareioti et al. (2014).

Overall, the correlation of selected operating data, i.e., HBu and HLa concentrations and the HY values related to the different applied HRT/OLR, corroborated the patterns observed from the temporal profiles. HLa and HBu concentrations were inversely correlated (R^2 = 0.9114; Fig. 4b), whereas HBu concentrations and the HY presented a direct correlation (R^2 = 0.9475; Fig. 4c). These results strongly suggest that the primary bioH_2-producing metabolic pathway from sugarcane vinasse resulted from the fermentation of both HLa and HAc, which produces H_2, HBu and CO_2 (Matsumoto and Nishimura, 2007). In fact, the slope of the equation relating both HLa and HBu concentrations (−0.6455; Fig. 4b) corresponds to almost 90% of the stoichiometric ratio between HBu and HLa in the lactate-acetate pathway (0.75; Matsumoto and Nishimura, 2007), which supports the proposed hypothesis. Similarly, Fuess et al. (2018a) proposed the occurrence of the referred metabolic pathway in the thermophilic dark fermentation of sugarcane vinasse. However, in that case the authors considered bioH_2 production from HLa only as an alternative pathway to carbohydrate shortage conditions. Monitoring data obtained in this study highlighted the role of HLa as a primary substrate for bioH_2 production from sugarcane vinasse. Thus, periods associated with low bioH_2 production levels and the subsequent accumulation of HLa in the AnSTBR most likely resulted from the inhibition of lactate-consuming bacteria at low pH values, favoring the predominance of products from the metabolism of lactic acid bacteria (LAB), which are frequently found at high proportions in vinasse-fed acidogenic systems (Ferraz et al., 2015a; Fuess et al., 2018a). Vinasse itself may be considered a source of LAB, which was recently identified among the primary microbial groups in vinasse samples resulting from sugarcane processing (Cassman et al., 2018).

BioH_2 and HBu production from HLa and HAc has been associated with several *Clostridium* species, such as *C. acetobutylicum*, *C. diolis JPCC H3*, *C. butyricum*, *C. beijerinckii* and *C. tyrobutyricum* JM1 (Diez-Gonzalez et al., 1995; Jo et al., 2008; Matsumoto and Nishimura, 2007). Studies on the dark fermentation of sugarcane vinasse frequently report on the presence of the *Clostridium* genus in acidogenic systems (Ferraz et al., 2015a,b; Fuess et al., 2018a; Santos et al., 2014a), regardless of the temperature. Despite the high heterogeneity and the intrinsic metabolic diversity of this microbial group (Ferraz et al., 2015a), the *Clostridium* genus has been particular associated with HBu production and the *Lactobacillus* genus (i.e., HLa availability) through a principal component analysis in Fuess et al. (2018a), which suggests the determining role of this microbial group in lactate fermentation in vinasse-fed reactors. Results reported by García-Depraect et al. (2019a,b) on the fermentation of tequila vinasses are in accordance with the findings presented herein, indicating the core role of Clostridial populations within the lactate-acetate pathway for bioH_2 production. Using HLa as substrate prevents the inhibition of the hydrogenogenic activity by LAB, which corroborates the versatility of vinasse-derived acidogenic populations.

Regarding operating aspects, the scanning of different feeding conditions imposed on the fermentative system led to defining an approximately optimal OLR (Fig. 4d) of 71.3 kg-COD m^{-3} d^{-1}, using the HY as reference to quantify the production of bioH_2. In this case, data regarding the application of a HRT of 6 h (phases 6-II and 6-IV, specifically) were not included, considering the interference of bioH_2-consuming metabolic pathways, i.e., sulfate reduction and propionic fermentation, due to the increase in fermentation pH (> 6.0). The obtained correlation also considered the OLR values effectively applied in the AnSTBR, i.e., 41.9, 59.9, 87.5 and 114.7 kg-COD m^{-3} d^{-1} in phases 24, 16-II, 12-I and 4, respectively (Table 3). The optimal OLR estimated for the thermophilic bioH_2 production from sugarcane vinasse in the AnSTBR presented a similar magnitude to the value previously defined for packed-bed systems operated under equivalent conditions (84.2 kg-COD m^{-3} d^{-1}; Ferraz et al., 2014, 2015a; Fuess et al., 2016). This particular result suggests that optimizing sugarcane vinasse dark fermentation is irrespective of the reactor configuration, specifically of the arrangement of the support material in fixed-film systems. However, using structured-bed systems tends to considerably improve bioH_2 production from vinasse, as seen by the experimental data obtained for different systems (Section 3.1).

Monitoring data obtained during the operation of the AnSTBR were also used to calculate the COD-based mass balance in the acidogenic phase specifically for soluble compounds – soluble phase mass balance (SPMB; Fig. 4e). Overall, the percentage recovery was directly associated with the type of vinasse, i.e., more concentrated vinasses (collections C2 and C3) were usually associated with lower recovery percentages, mainly for vinasses from sampling C2 (days 1–54; average SPMB of 46.3%). In turn, using less concentrated vinasses (collections C4 and C5) led to higher recovery percentages, with values above 65% (Fig. 4e). The observed pattern most likely resulted from the type of substrate used in ethanol fermentation, specifically considering the use of molasses as substrate. Coupling sugar and ethanol production from sugarcane implies blending juice and molasses prior to fermentation, which increases the concentration of colored components in the broth, primarily melanoidins (Fuess et al., 2018c). Melanoidins are highly recalcitrant compounds resulting from a Maillard reaction between sugars and proteins at high temperatures (Pant and Adholeya, 2007). In particular, lower recovery percentages of the SPMB were observed for high-colored vinasses (collections C2 and C3; Supplementary data), suggesting a high contribution of melanoidins for the unrecovered organic fraction of the mass balance (Fig. 4e). Conversely, the higher recovery percentages observed were also associated with less-colored vinasses, which corroborate the hypothesis. Finally, including the continuous monitoring of glycerol and HLa led to higher recovery percentages for the SPMB compared to previous studies on bioH_2 production from vinasse in fixed-film systems, which usually reported values below 45–50% (Ferraz et al., 2014, 2015a,b; Fuess et al., 2016).

3.3. Outlook: targeting bioH_2 production or sulfate reduction?

Using the phase separation, i.e., operating sequential acidogenic and methanogenic reactors, in vinasse-fed anaerobic systems has primarily aimed to increase the recovery of bioenergy from two perspectives: [i] enhancing the methanogenic activity in the second-phase reactors by preventing the accumulation of organic acids and [ii] exploiting the energy potential of bioH_2 (Ferraz et al., 2016; Fuess et al., 2017, 2018b). In particular, studies indicate a low contribution of bioH_2 in energy balances for bioenergy production from sugarcane vinasse, reporting values below 5% relative to the overall energy production capacity from biogas streams (Ferraz et al., 2016; Fuess et al., 2017). Therefore, enhancing the methanogenic activity may be considered the key-factor to improve the energy extraction from vinasse, which highlights the benefits of operating acidogenic-sulfidogenic systems, i.e., targeting sulfate reduction, instead of only acidogenic systems.

Kiyuna et al. (2017) reported that decreasing the CODs/SO_4^{2-} ratio in sugarcane vinasse from 12.0 to 7.5 decreased the methane production by 35% in thermophilic anaerobic reactors, with a diversion of 13.6% of the electron flow to sulfidogenesis and the accumulation of acetate due to sulfide inhibition. These authors also reported that the establishment of sulfate-reducing conditions could be considered negligible only at CODs/SO_4^{2-} ratio values higher than 25 in the assessed systems. CODs/SO_4^{2-} ratio values (usually within the range 12.92–24.12) observed for the raw vinasse samples used in this study (Table 1) suggest a relatively comfortable situation considering potential impacts of sulfidogenesis in methanogenic reactors throughout the harvest. However, considering an average CODs removal efficiency of 8.3% (based on the obtained experimental data) and no SO_4^{2-} reduction in the acidogenic phase, CODs/SO_4^{2-} ratio values would reach levels below 14.0 during almost the entire operating period, most likely hindering the bioenergy recovery via methane production from vinasse, regardless of the pre-fermentation of the complex organic matter.

Overall, defining the best approach, i.e., targeting bioH_2 production from vinasse or sulfate reduction in the acidogenic phase still requires a more critical understanding, considering both technical (experimental) aspects and a technological assessment, which should take into consideration the use/final destination of a H_2- and H_2S-rich biogas stream, as observed in phase 6-II in this study. Recovering elemental sulfur through H_2S partial oxidation may be a potential biotechnological approach for managing the biogas streams from acidogenic-sulfidogenic systems, as proposed for sulfide managing in methanogenic reactors (Muñoz et al., 2015). In this case, a H_2S-free and H_2-rich biogas stream could be obtained and further used in diversified applications within the biodigestion-power plant, as proposed by Fuess et al. (2018b). However, blending oxygen and hydrogen may lead to safety problems in industrial plants, which hinders a direct exploitation of H_2- and H_2S-rich biogas streams and favors the application of conventional H_2S removal methods, such as in-situ precipitation.

Regardless of the limitations in managing the gaseous phase of acidogenic-sulfidogenic reactors, preliminary assumptions strongly suggest that the establishment of sulfate-reducing conditions in sugarcane vinasse dark fermentation may be the most attractive approach on an energy basis. Moreover, the results observed herein indicate the capacity of SRB to maintain high activities under relatively unfavorable operating conditions, namely, high OLR/low HRT (100 kg-COD m^{-3} d^{-1}/ 6 h) and relatively high COD/SO_4^{2-} (> 10.0, using the theoretical value of 0.67 for the complete sulfate reduction as the reference; Lens et al., 1998).

4. Conclusions

The results obtained herein indicated that operating strategies are imperative to direct the metabolic pathways in the thermophilic dark fermentation of vinasse, defining three major pathways according to the pH: lactate-producing (pH < 5.0), bioH_2-/butyrate-producing (pH = 5.0–5.5) and bioH_2-producing/sulfate-reducing (pH > 6.0) systems. Controlling the fermentation pH offsets negative impacts associated with the compositional variability of sugarcane vinasse, and metabolic correlations suggested that lactate is the primary substrate used in bioH_2 production in vinasse-fed reactors. In practical aspects, the versatility of acidogenic systems may be used according to specific targets, such as bioenergy recovery (bioH_2 production) or “vinasse cleaning” (sulfate removal) prior to methanogenesis.

Acknowledgements

The authors are grateful for the financial support provided by the São Paulo Research Foundation (FAPESP) [grant numbers 2017/00080-5, 2015/50684-9 and 2015/06246-7] and the Coordination for the Improvement of Higher Education Personnel via the Academic Excellence Program (PROEX-CAPES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.121379.

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