



Improved methane production from sugarcane vinasse with filter cake in thermophilic UASB reactors, with predominance of *Methanothermobacter* and *Methanosarcina* archaea and Thermotogae bacteria

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ABSTRACT

Biogas production from sugarcane vinasse has enormous economic, energy, and environmental management potential. However, methane production stability and biodigested vinasse quality remain key issues, requiring better nutrient and alkalinity availability, operational strategies, and knowledge of reactor microbiota. This study demonstrates increased methane production from vinasse through the use of sugarcane filter cake and improved effluent recirculation, with elevated organic loading rates (OLR) and good reactor stability. We used UASB reactors in a two-stage configuration, with OLRs up to 45 g COD L⁻¹ d⁻¹, and obtained methane production as high as 3 L L⁻¹ d⁻¹. Quantitative PCR indicated balanced amounts of bacteria and archaea in the sludge (10⁹–10¹⁰ copies g⁻¹ VS), and of the predominant archaea orders, Methanobacteriales and Methanosarcinales (10⁶–10⁸ copies g⁻¹ VS). 16S rDNA sequencing also indicated the thermophilic Thermotogae as the most abundant class of bacteria in the sludge.

1. Introduction

Bioethanol production from sugar cane in Brazil reached 30.2 billion liters in 2015, representing 30.6% of world bioethanol production (Seyboth et al., 2016; UNICA, 2016). Each liter of sugarcane bioethanol distilled generates 10–14 L of vinasse (Ortegón et al., 2016), a by-product that can be used as a fertilizer, although incorrect use or disposal represents a serious threat to the environment. This is mainly due to its high organic material content (Moraes et al., 2014), which, however, has a high energy potential if it can be converted into a readily usable form, such as biogas, mostly made up of methane.

Methane production relies on anaerobic digestion which, however, is hindered by the presence of inhibitory compounds and other characteristics of the vinasse, such as volatile acids (Mota et al., 2013; de Barros et al., 2016). The vinasse is constituted 94–97% water, macronutrients (mainly K, Ca, N, Mg and S), melanoidins, and residual

amounts of sugar, alcohol, and volatile components such as chloroform, pentachlorophenol, phenol, and methylene chloride, and the amount of these substances depends on the feedstock and the process of ethanol production (de Barros et al., 2016; Janke et al., 2016a; Prado et al., 2013).

Nutrient availability, different configurations of anaerobic reactors, operational strategies to obtain stability in methane production, and quality of biodigested vinasse are other issues that must be solved before widespread production of biogas from vinasse. Many of these issues could be solved with the use of filter cake in the vinasse. Filter cake is readily available in abundant amounts as it is one of the main solid residues of sugar production from sugarcane, and is rich in macro and micronutrients, mainly, Ca, N, P, Mg (Janke et al., 2016b; Prado et al., 2013), Fe, Mn and Zn (Janke et al., 2015), which can improved the nutritional conditions to obtain stable anaerobic digestion.

However, currently there is no established technology for the use of

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filter cake in vinasse anaerobic digestion reactors. Thus, this study assessed optimal parameters for vinasse anaerobic digestion in a two-reactor system with filter cake addition, including reactor configuration, influent parameters, and other operational parameters. Additionally, the microbiota present in the reactors was characterized, allowing possible improvement of its composition for optimal process efficiency.

The study used a two-stage process with reactors in series to prevent organic and acidic overloads, attenuate the inhibitory effect of toxic compounds (Camarillo and Rincón, 2012), and promote the stability and control of the process (Demirel and Yenigün, 2006), allowing application of high organic loading rates (OLR) and higher energy production (Zhong et al., 2015), besides favoring the enrichment of specific microorganisms in separate reactors. A thermophilic anaerobic treatment for vinasse was employed, as this effluent exits the distillation process at high temperatures, which may influence biochemical reactions, possibly allowing us to use shorter hydraulic retention times (HRT), reducing capital costs and increasing the degradation of organic solids and the production of biogas (Sentürk et al., 2010).

The purpose of this study was to evaluate the thermophilic anaerobic conversion of vinasse and the diversity and quantity of bacteria and archaea in upflow anaerobic sludge blanket (UASB) reactors, in two stages, with high OLR to obtain high methane production. For this, the OLR was increased from 5 to 55 g COD L⁻¹ d⁻¹, with supplementation of alkalinity and nutrients through the filter cake and effluent recirculation.

2. Material and methods

2.1. Experimental setup

The experimental setup consisted of two UASB reactors installed in series (R1 and R2), with volumes of 12.1 L (R1) and 5.6 L (R2), in a climate chamber with storage tanks for influent and effluent, diaphragm pump, and fiberglass gasometers (Fig. 1). The reactors were constructed with PVC tubes, with phase separators in a Y shape, at a 45° angle to the vertical (Cavalcanti et al., 1999 adapted by Bruno and Oliveira (2013); Villa-Montoya et al., 2016). The influent and UASB

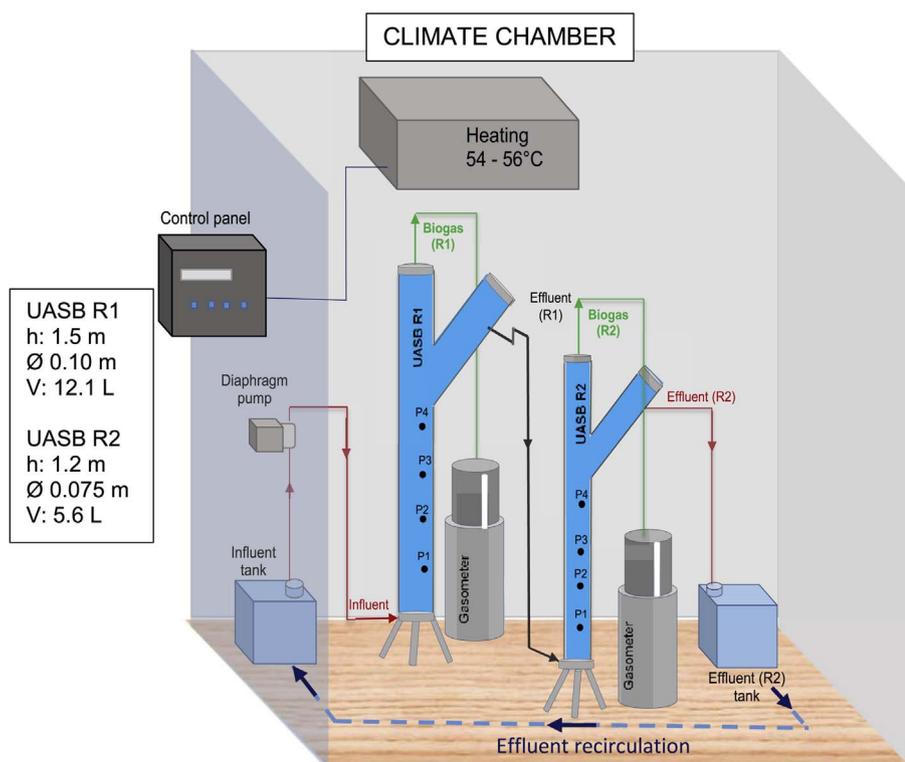


Fig. 1. Schematic representation of the treatment system composed of two-stage UASB reactors (R1 and R2) under thermophilic conditions.

Table 1
Characteristics of vinasse and filter cake.

Parameters	Vinasse (mg L ⁻¹)	Filter cake (mg g ⁻¹ TS)
pH	4.5	8.2
COD	44,500	20
KN	430	6.19
P _{total}	26	9.04
K	149	0.050
Cu	5	0.125
Ca	16	0.002
Zn	2	0.042
Mg	14	0.125
Na	16	0.900
Mn	75	0.312
Fe	7	0.018

COD: chemical oxygen demand; KN: Kjeldahl nitrogen; P_{total}: total phosphorus; TS: total solids.

reactors temperatures were maintained in thermophilic range (54–56 °C) with a heating and temperature control system (equipped with thermocouple) installed in climate chamber.

2.2. Influent and inoculum sludge

The influent used to feed the UASB reactors was sugarcane vinasse from the distillation of hydrous ethanol, collected monthly at the exit of the distillation column. The filter cake, a byproduct from the processing of sugarcane, was collected monthly (Table 1).

Fresh vinasse and filter cake were obtained from a sugarcane plant located in Ribeirão Preto region, state of São Paulo, southeast Brazil.

The inoculum sludge was collected in a full scale thermophilic UASB reactor treating vinasse in a sugarcane plant (Usina São Martinho) located in Pradópolis city, state of São Paulo, southeast Brazil. The values of total solids (TS) and volatile solids (VS) were 16.2 and 8.7 g L⁻¹, respectively.

The UASB reactors used in this work were already in operation for 160 days treating vinasse and the characteristics of the sludge

Table 2
Operating conditions of two-stage UASB reactors (R1 and R2), thermophilic, treating sugarcane vinasse.

Days in operation	1–110	110–170	171–230	231–310	311–380	381–460
OLR – R1	8	18	28	32	45	37
OLR – R2	13	26	30	38	49	55
Proportion (%) vol.) vinasse: effluent R2	8:92	12:88	23:77	36:64	38:62	23:77
HRT – R1					17	
HRT – R2					8	
Source of N and P	Filter cake					
Alkalinity source	Recirculation of the effluent R2					

OLR: organic loading rate ($\text{g COD L}^{-1} \text{d}^{-1}$), HRT: hydraulic retention time (h).

contained in the reactors were: 49.7 g L^{-1} TS and 36.1 g L^{-1} VS (R1); and 42.4 g L^{-1} TS and 29.7 g L^{-1} VS (R2).

2.3. Operating conditions of reactors

OLR was gradually increased during 460 days. The main operational conditions (ORL, HRT, and sources of N, P, and alkalinity) for UASB reactors are described in Table 2.

After 310 days of operation, HRT was changed from 24 h to 17 h in R1 and from 11 h to 8 h in R2 (Table 2) with the purpose of increasing the OLR to $45 \text{ g COD L}^{-1} \text{d}^{-1}$ in R1 and maintaining the effluent recirculation rate.

With gradual increasing of OLR in reactors, and R2 effluent recirculation, the pH values of the influent ranged from 6.59 to 7.70 (Table 3). These values were kept within the range recommended for activity of methanogenic microorganisms (Uçkun Kiran et al., 2016).

For use of the filter cake as a source of nutrients, a solution was prepared with 40 g of filter cake per 1 L of crude vinasse and kept for 24 h. After this period it was sieved in a 2 mm mesh and filtered in cotton cloth to separate the fibers present in the filter cake and to avoid clogging of the pump and pipes. The amount of filter cake used per liter of vinasse was based on the COD:N:P ratio of 350:5:1 recommended for anaerobic digestion of carbohydrates with biomass of high yield coefficient (Chernicharo, 2007).

Table 3
Average values of critical parameters during the operation of the two-stage UASB reactors (R1 and R2).

Parameters		OLR											
		8		18		28		32		45		37	
		13		26		30		38		49		55	
		v.c		v.c		v.c		v.c		v.c		v.c	
pH	Influent	7.03	5	6.59	5	7.61	2	7.42	2	7.70	3	7.60	3
	R1	7.97	4	7.98	2	7.76	4	8.09	3	8.19	3	8.44	1
	R2	8.49	2	8.19	3	8.43	3	8.55	1	8.76	1	8.64	2
PA	Influent	1077	32	1436	35	2974	10	2499	9	3181	7	2999	21
	R1	1627	36	2467	14	3127	9	2855	10	3330	2	3066	29
	R2	1582	33	2623	12	3499	7	3330	6	3921	13	3280	24
N-am	Influent	78	37	141	18	93	16	72	27	64	14	81	33
	R1	99	42	205	14	135	17	101	29	73	27	87	33
	R2	93	47	203	12	140	18	113	35	82	31	65	44
TVA/TA	R1	0.55	34	0.57	15	0.56	31	0.68	14	0.70	18	0.78	20
	R2	0.55	34	0.51	14	0.38	34	0.54	10	0.58	13	0.70	16
IA/PA	R1	0.84	53	1.02	29	0.89	39	0.56	13	0.86	23	1.28	50
	R2	0.81	44	0.96	21	0.54	27	0.43	22	0.63	17	1.11	48

OLR: organic loading rate; PA: partial alkalinity; N-am: ammoniacal nitrogen; TVA: total volatile acids; TA: total alkalinity; IA: intermediate alkalinity; v.c.: variation coefficient. Units: PA, N-am, TVA, TA, IA: mg L^{-1} ; OLR: $\text{g COD L}^{-1} \text{d}^{-1}$; v.c.: %.

2.4. Tests and determinations in the influent, effluent, sludge, and production of biogas

Composite samples of the influent and effluent of the UASB reactors (R1 and R2) were collected twice a week for the determination of pH, total alkalinity (AT), and chemical oxygen demand (COD), according to the methodologies described by APHA (2005); the partial alkalinity (PA) and intermediate (IA), as described by Ripley et al. (1986); and total volatile acids (TVA), according to DiLallo and Albertson (1961).

Kjeldahl nitrogen (KN) and ammoniacal nitrogen (N-am) determinations were performed weekly by steam distillation as described by APHA (2005). The concentrations of total phosphorus (P_{total}), K, Ca, Mg, Cu, Fe, Mn, and Zn were determined weekly after acid (nitric-perchloric) digestion of the sample and reading in an atomic absorption spectrophotometer, as described by APHA (2005). The sludge samples were collected monthly and determination of the total (TS) and volatile solids (VS) was performed according to methodologies described by APHA (2005). The biogas production was measured daily using gasometers (Bruno and Oliveira, 2013; Villa-Montoya et al., 2016) and its composition determined biweekly by gas chromatograph (APHA, 2005). The results of the methane production were reported at standard temperature and pressure (STP, 101.325 kPa, 273.15 K).

At day 336 of the experiment, when the OLR in R1 and methane production were higher, quantification of the acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids of the influent and effluent from UASB reactors was performed, by gas chromatography according to the methodology described by Van Cleef et al. (2015).

2.5. Sample information and genomic DNA from microorganisms

The genomic DNA samples sequenced in the Ion Torrent were from the sludge collected at the collection points of the reactors P1 to P4 in Fig. 1, located in the UASB reactors, R1 and R2, at 340 days of operation, in phase V, when OLR in R1 and methane production were higher. The sludge samples from these collection points were mixed to form a composite sample from R1, as well as a composite sample from R2. The sludge volume from each point was collected in order to obtain the same amount of volatile solids (VS) from each point. The total (TS) and volatile solids (VS) of the composite sample sludge were 38.1 and 23.9 g L^{-1} (R1) and 36.1 and 21.7 g L^{-1} (R2).

For real-time quantitative PCR (qPCR), after 340 days of operation,

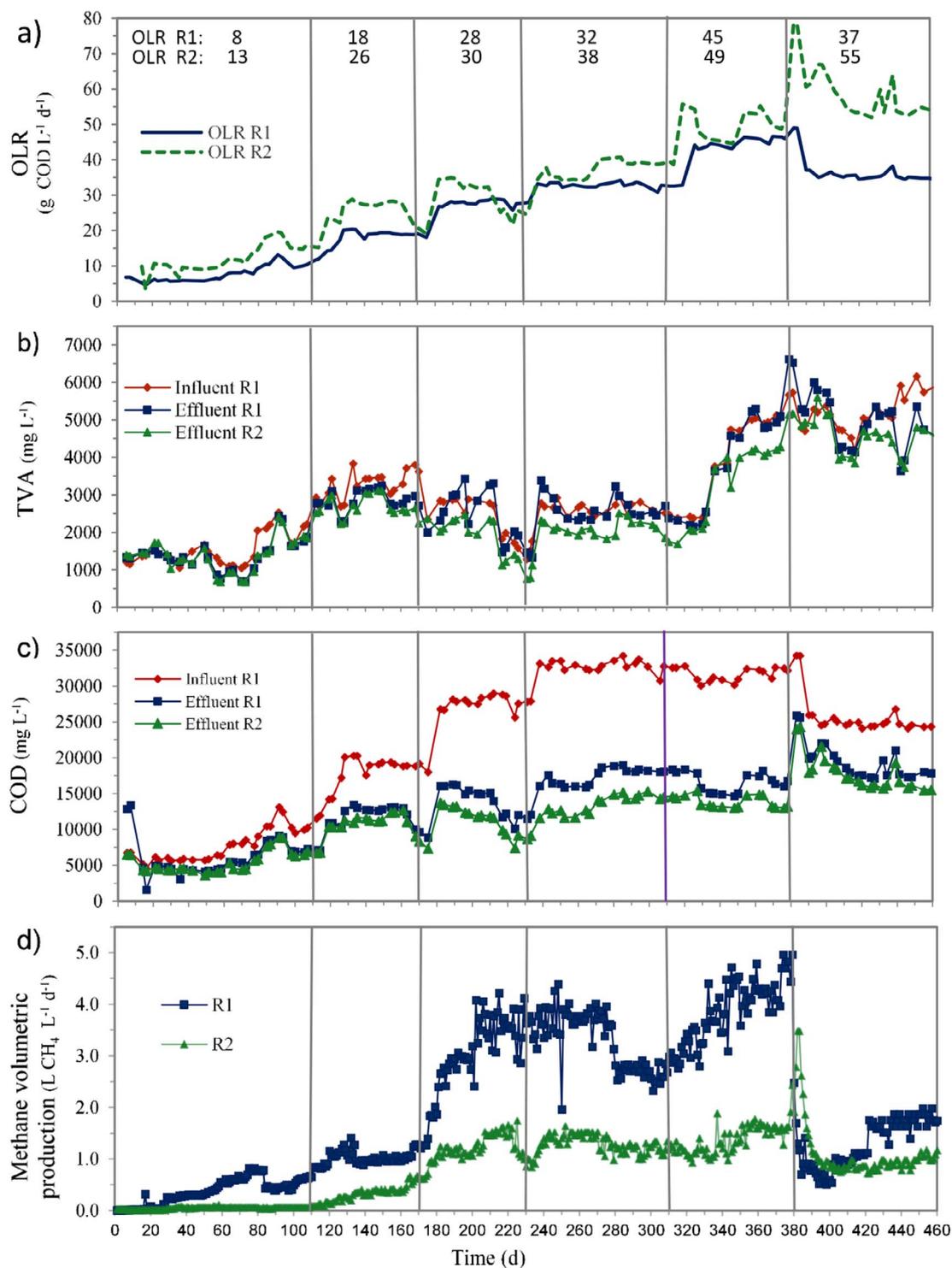


Fig. 2. Values of organic loading rate (OLR) (a), total volatile acids (TVA) (b), chemical oxygen demand (COD) (c), and methane volumetric production (d) in two-stage UASB reactors (R1 and R2).

the genomic DNA from the sludge was separated into three samples of R1 and three samples of R2, collected at the collection points (P1, P2, P3, and P4 in Fig. 1) along each reactor (sample 1 (P1), sample 2 (P2) and sample 3 (P3 and P4)). This procedure was performed in order to verify whether there are significant differences in the quantification of the microorganisms along the height of the digestion chamber of the UASB reactors. The TS and VS of the sludge from the collection points in R1 were 113.0 and 73.3 g L⁻¹ (P1), 29.2 and 17.2 g L⁻¹ (P2), 30.5 and 18.9 g L⁻¹ (P3), and 34.8 and 19.9 g L⁻¹ (P4), respectively. In R2, the

TS and VS were 107.2 and 75.1 g L⁻¹ (P1), 36.5 and 17.0 g L⁻¹ (P2), 31.2 and 20.3 g L⁻¹ (P3), and 29.1 and 15.0 g L⁻¹ (P4), respectively.

The extractions of genomic DNA of both sludge samples described above were performed from 0.37 mg of sludge by means of the PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc.), stored at -20 °C. The quality of the genomic DNA was analyzed by both Nanodrop ND-1000 (Thermo Fisher Scientific Inc.) and 1% agarose gel with 1 × TAE buffer, and the quantity of the genomic DNA extracted was assessed using the Qubit® dsDNA HS Assay Kit 100 assays,

0.2–100 ng, according to the manufacturer's instructions.

2.6. Amplification of the 16S rRNA gene with the ion tag and sequencing

The 16S ribosomal RNA gene was partially amplified (V4–V5 regions) from the samples using universal primers 515F and 926R, which were designed with adapters and barcodes (22 unique sequences for each sample). The PCR reaction consisted of 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 90 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min.

The PCR reactions were performed using 8 µl of KAPA HiFi HotStart ReadyMix (2 ×) PCR kit (Kapa Biosystems, Boston, USA), 1 µl of each primer (10 µM) and 50 ng of DNA template. The amplicons were purified on 2% agarose gel and recovered with Zymoclean™ Gel DNA Recovery Kit w/Zymo-Spin™ IC Columns (Zymo Research, Irvine, CA) according to manufacturer's instructions. The quantification of the purified amplicons was performed in Qubit Fluorometric Quantitation (Thermo Fisher Scientific, USA) and, after equimolar mixing, single-read (1 × 400) sequencing was performed on the Ion Torrent PGM platform (Thermo Fisher Scientific, USA) using the 318 v2 chip, following the manufacturer's instructions.

2.7. Processing of raw data

The raw data were trimmed from barcode/primer adapters using the programs Scythe 0.991 (<https://github.com/vsbuffalo/scythe>), to the 3' position of the sequences, and Cutadapt 1.7.1 (Martin, 2011) to the 5' position of the sequences. Then, the data were filtered for sequences that presented quality PHRED < 20 and sequences smaller than 300 bp using the program Prinseq (Schmieder and Edwards, 2011). The determination of the Operational Taxonomic Units (OTUs) and alpha- and beta-diversity analyses for archaea and bacteria were performed using QIIME v. 1.9.0 (Caporaso et al., 2011). The chimeric sequences were removed using the protocol UPARSE and the USEARCH v. 1.1.1 program. The taxonomic classification of the OTUs was performed with the Ribosomal Database Project (RDP II) trainset 14 using mothur v. 1.25.0 (Schloss et al., 2009). The alignment of the UTOs was performed against the database SILVA v. 119. The quality of the raw and trimmed data was evaluated using the program FastQC v. 0.11.3 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). The amplicon 16S rRNA project has been deposited at Sequence Read Archive (SRA) under the Biosample SAMN07415631, BioProject PRJNA395859, and under the accession number SRR5870508.

2.8. qPCR: absolute quantification

Real-time quantitative PCR (qPCR) was conducted with DNA samples isolated from the sludge collected from each reactor after 340 days of continuous operation, as described in item 2.5. The main microorganisms identified with Ion Torrent sequencing were quantified. The Archaea and Bacteria domains, the orders Methanobacteriales and Methanomicrobiales, and two families, Methanosarcinaceae and Methanosaetaceae, belonging to the order Methanosarcinales, had regions of 16S rDNA amplified using specific primers (Song et al., 2010). The qPCR was realized according to Duda et al. (2015).

3. Results and discussion

3.1. Buffering capacity with high OLRs

The system displayed operational stability and satisfactory buffering capacity during the experimental period, as demonstrated by the increase in both the values of PA and of TVA in the R2 effluent with high OLR, 3921 mg L⁻¹ (Table 3) and 5000 mg L⁻¹ (Fig. 2b), respectively. Moreover, TVA concentration was lower in the R2 effluent compared to influent (Fig. 2b), consistent with consumption of TVA methanogenic

archaea. A common strategy used for maintaining the stability of anaerobic reactors treating vinasse, is to apply alkalinizing substances (Janke et al., 2016a; de Barros et al., 2016). According to the results obtained in this work, using effluent recirculation, with the purpose of maintaining the bicarbonate produced from methanogenic metabolism, allows an adequate balance between pH, alkalinity, and TVA, without the need for additional chemicals.

Additionally, TVA/TA ratios did not rise above 0.78 (R1) and 0.70 (R2) when OLR increased above 37 and 55 g COD L⁻¹ d⁻¹, respectively (Table 3). According to Zhao and Viraraghavan (2004), TVA/TA ratios higher than 0.8 can indicate inhibition of methanogenic archaea. This was not observed in this study, however, probably due to the addition of filter cake, which can provide nutrients essential for adequate performance of anaerobic digestion, as well as to the use of a higher effluent recirculation rate, as needed (Table 2). IA/PA ratio is another important indicator of process stability, with values of IA/PA below 0.3 considered optimal for anaerobic digestion in domestic wastewater treatment (Ripley et al., 1986). However, the results obtained in this study suggest that, using vinasse as substrate, IA/PA ratios higher than 0.6 allow an adequate process stability, as indicated by the pH, PA, and TVA/TA values with OLR above 45 and 55 COD L⁻¹ d⁻¹, for R1 and R2, respectively (Table 3).

Even with the application of high OLRs, concentrations of ammoniacal nitrogen (N-am) in R1 and R2 effluents remained stable and lower than 205 mg L⁻¹ (Table 3). Ammoniacal nitrogen are important for stability because is a essential nutrient for microbial digestion, and additionally, is an important factor for improve alkalinity (Ariunbaatar et al., 2015). N-am concentrations reported in this study were close to those observed by España-Gamboa et al. (2012), and are considered as beneficial for anaerobic digestion.

Relatively high proportions of acetic acid were observed in both reactors (69% in R1 and 72% in R2 at OLR of 45 and 49 g COD L⁻¹ d⁻¹ respectively). The quantification of volatile acids, such as acetic, propionic, butyric, isobutyric, and isovaleric, provides important information about the metabolic pathways involved in the production of methane. These acids are formed from the anaerobic digestion of carbohydrates, proteins, and fats (Sentürk et al., 2010), and a high proportion of acetic acid is desirable, as this acid is one of the main precursors of methanogenesis (Sentürk et al., 2010). The ratio between propionate/acetate can be used as indication of process imbalance, if the ratio exceeds 1.4 (Ahring et al., 1995). In this work we obtained propionate/acetate ratios of 0.28 (R1) and 0.25 (R2) with highest OLR evaluated indicating acceptable balance of acids in the UASB reactors. Consequently, addition of filter cake, effluent recirculation, and two-stage operation allows the generation of adequate environmental conditions for propionate consumption and acetic acid generation even if high OLRs are applied. Thus, the conditions established in this study allow for high OLR input and optimal conditions for methanogenesis.

The oxidation of propionic acid is thermodynamically unfavorable, and the degradation of these acids occurs due to many enzymatic reactions. Degradation of propionic acid in thermophilic systems is slower than is the case for the other organic acids found in anaerobic processes. Fuess et al. (2017), treating vinasse in a UASB reactor in thermophilic conditions and OLR of 20–25 g COD L⁻¹ d⁻¹, reported that from 60 to 80% of the acids present in the effluent were composed of propionic acid. In this work the values of propionic acid in the effluent of R1 and R2 represent approximately 19% of the organic acids determined, which is below the values described by Fuess et al. (2017), probably due to the addition of filter cake, which contributed the nutrients needed to maintain low propionic acid values.

The addition of iron, cobalt, nickel and/or yeast extract were required to enhance the propionate utilization rate to about two times that of the control (Boonyakitsombut et al., 2002). In propionate degradation, propionate must first be oxidized by propionate-oxidizing bacteria to acetate and hydrogen as intermediate products which are then converted to methane by acetotrophic and hydrogenotrophic

methanogenic archaea, respectively. The role of nutrient requirement to stimulate the activity of each bacterial and archaeal groups in the consortia is important in order to enhance propionate degradation. The supplementation of the vinasse with filter cake increased the concentrations of macro and micronutrients, mainly N, P, Fe and Zn.

Speece (2008) cited that addition of Fe^{2+} to a mesophilic anaerobic reactor having 1000 mg L^{-1} propionate, which was otherwise impossible to lower, reduced concentrations to about 100 mg L^{-1} . Yamada et al. (2015) demonstrated that the supplementation of conductive iron oxide particles (magnetite and ferrihydrite) accelerated methanogenic degradation from acetate and propionate by thermophilic microbial communities via electric syntrophy. The genera *Methanobacterium* and *Methanosarcina* were the methanogenic archaea predominant during propionate degradation by flooded rice field soil samples (Lueders et al., 2004). In this work, orders Methanobacteriales and Methanosarcinales dominated the methanogenic archaeal community and the genus *Methanothermobacter*, *Methanosarcina* and *Methanoculleus* showed higher relative abundance (81.3, 7.4 and 7.1%, respectively) when propionate/acetate ratios were low (0.28 – R1 and 0.25 – R2). These results suggested that nutrients, mainly Fe, increased the specific microbial activity or the biomass concentration of at least one group of the bacteria or methanogenic archaea involved in propionate degradation and allowed stable operation and more efficiency of thermophilic anaerobic digestion of vinasse with acceleration of degradation of acetate and propionate for methane production.

3.2. Removal of COD and biogas production

The highest COD removal efficiency for the system (60%, R1 + R2) was attained at the highest COD tested ($31,545 \text{ mg L}^{-1}$ in the influent) (Fig. 2c). Values obtained for COD removal were similar to these obtained by Fuess et al. (2017), who evaluated the performance of a two-stage system conformed UASB and anaerobic structured-bed reactor. In this work, however, using filter cake and effluent recirculation in thermophilic conditions, we were able to reach the highest OLRs even with a substrate composition that was highly variable during the experimental period.

The methane production in the anaerobic digestion of vinasse is extremely important to make feasible the technology application. The methane concentrations in biogas varied between 49 and 65% in R1, and 49–70% in R2. According to Mazareli et al. (2016), in acidic conditions high TVA occurs, carbonic acid predominates, and more CO_2 can be released in the biogas, which may have contributed to the lower methane concentrations in R1. The highest concentrations of methane in the biogas occurred in R2, suggesting high hydrolytic and acidogenic activity in R1, with higher TVA concentrations (Fig. 2b). High volumetric methane production was obtained in this work, reaching levels up to $4.0 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ in R1 and $1.4 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ in R2 (Table 4). In recent studies on anaerobic treatment of sugarcane vinasse with UASB reactors operated under mesophilic conditions, de Barros et al. (2016) reported volumetric methane production of up to $0.9 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ with an OLR of $6.0 \text{ g COD L}^{-1} \text{ d}^{-1}$, and Janke et al. (2016b) obtained values up to $1.4 \text{ L CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ with OLR of $5.9 \text{ g COD L}^{-1} \text{ d}^{-1}$. In this study, the higher volumetric methane production was probably obtained by means of the thermophilic conditions.

The temperature may influence biochemical reactions through increased reaction rates, which would allow the use of shorter hydraulic retention times (HRT), increasing the degradation of organic solids and the production of biogas (Sentürk et al., 2010), as observed in this study.

After 375 days of system operation, methane volumetric production markedly decreased (Fig. 2d), and TVA/TA mean values increased (Table 3). This may have occurred due to the low amount of Fe observed after 320 days of operation. Although the performance of the system (R1 + R2) declined abruptly, the system did not collapse

Table 4

Biogas characteristics in function of the OLR applied in two-stage UASB reactors (R1 and R2).

Parameters	OLR					
	8	18	28	32	45	37
R1	8	18	28	32	45	37
R2	13	26	30	38	49	55
<i>Percentage of methane in biogas (a)</i>						
R1	50	49	65	62	59	61
v.c	29	11	7	16	9	12
R2	49	62	79	69	70	76
v.c	26	6	6	7	8	6
<i>Methane volumetric production (b)</i>						
R1	0.41	1.14	3.32	3.20	4.00	1.50
v.c	65	22	14	17	14	56
R2	0.06	0.41	1.23	1.30	1.40	1.12
v.c	43	46	18	12	19	48
R1 + R2	0.32	1.00	2.72	2.59	3.12	1.28
v.c	62	38	12	15	17	27
<i>Specific methane production (c)</i>						
R1	0.17	0.18	0.24	0.21	0.19	0.15
v.c	54	31	12	16	13	32
R2	0.09	0.16	0.20	0.15	0.17	0.25
v.c	100	49	29	19	30	47
R1 + R2	0.19	0.17	0.23	0.20	0.19	0.16
v.c	77	31	13	12	12	19

OLR: organic loading rate ($\text{g COD L}^{-1} \text{ d}^{-1}$); v.c: variation coefficient (%). Units: (a) %, (b) $\text{L CH}_4 \text{ L}^{-1} \text{ reactor d}^{-1}$, (c) $\text{L CH}_4 \text{ g}^{-1} \text{ COD removed}$.

because the volumetric methane production was maintained at lower values and the effluent presented buffering capacity, maintaining the pH values of the influent in the range considered optimal for the methanogenic microorganisms through effluent recirculation.

The average values of specific methane production ranged from 0.17 to $0.24 \text{ L CH}_4 \text{ g}^{-1} \text{ COD removed}$ in R1 and from 0.09 to $0.25 \text{ L CH}_4 \text{ g}^{-1} \text{ COD removed}$ in R2 (Table 4).

España-Gamboa et al. (2012), evaluating methane production by vinasse treatment using a modified UASB reactor, observed an optimum OLR of $17 \text{ g COD L}^{-1} \text{ d}^{-1}$, achieving a specific methane production of $0.263 \text{ L CH}_4 \text{ g}^{-1} \text{ COD added}$, and the system collapsed at an OLR of $22 \text{ g COD L}^{-1} \text{ d}^{-1}$. In another study carried out on two UASB reactors treating vinasse under thermophilic conditions, Van Haandel et al. (2014) observed an abrupt drop in the reactor performance, which could not be recovered. Therefore, the results obtained in our study highlight the importance of filter cake application, since its use allows operational stability, even with application of high OLRs.

3.3. Effect of the filter cake on nutrient availability and removal efficiencies

We observed adequate nutrient availability for anaerobic digestion of vinasse in the system influent. This condition was favored by the incorporation of the filter cake into the substrate, in amounts sufficient to guarantee a COD:N:P ratio of 350:5:1 (Table 5). Consequently, with OLR increase, KN and P_{total} concentrations increased from 174 to 488 mg L^{-1} and 41 to 194 mg L^{-1} respectively.

The KN and P_{total} removal efficiency values in the series reactors (R1 + R2) varied from 14 to 30% and from 53 to 73%, respectively. According to Oliveira et al. (1997), one of the possible mechanisms for higher nitrogen and phosphorus removal is the formation of struvite ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$) and vivianite ($\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$), which precipitate and are immobilized in the sludge blanket. No removals were observed for potassium (K) (Table 5).

In recent studies, Janke et al. (2016a), using phosphate in the form of KH_2PO_4 , induced TVA accumulation possibly due to inhibition of potassium in methanogenesis. However, in this work, the use of filter cake for supplementation mainly of nitrogen and phosphorus, reduced the effects of possible shock loads and prevented the flotation of

Table 5
Relation COD:N:P, concentration and removal efficiency of macro nutrients, KN, P_{total}, and K, in the influent and effluent of the two-stage UASB reactors (R1 and R2).

Parameters	OLR					
	8	18	28	32	45	37
R1	8	18	28	32	45	37
R2	13	26	30	38	49	55
<i>Influent – COD:N:P (350:5:1)^a</i>						
COD	350	350	350	350	350	350
N	7.6	7.8	5.8	5.0	5.0	5.0
P	1.7	2.1	1.9	1.9	2.2	2.6
<i>Kjeldahl nitrogen (KN)</i>						
Influent	174	411	471	483	445	488
v.c.	26	12	5	11	8	10
R1	162	337	370	402	352	376
v.c.	52	23	5	8	17	11
R2	149	288	355	385	337	365
v.c.	53	14	14	12	12	7
Removal efficiency	14	30	25	20	24	25
<i>Total phosphorus (P_{total})</i>						
Influent	41	111	150	180	193	194
v.c.	52	10	4	9	4	18
R1	25	50	75	71	79	85
v.c.	31	34	7	8	12	22
R2	11	46	70	66	77	80
v.c.	65	34	9	15	8	18
Removal efficiency	73	59	53	63	60	59
<i>Potassium (K)</i>						
Influent	27	77	75	49	115	114
v.c.	88	7	9	30	21	12
R1	28	61	82	51	110	121
v.c.	81	36	5	34	15	21
R2	21	85	95	46	115	117
v.c.	104	17	13	47	16	2

OLR: organic loading rate (COD L⁻¹ d⁻¹), v.c: variation coefficient (%), ^a recommended by [Chernicharo \(2007\)](#). Units for KN, P_{total} and K: mg L⁻¹; Removal efficiency: %.

granules in UASB reactors. This condition allows TVA consumption and high methane production (Fig. 2d).

The removal efficiencies of KN, K (Table 6), and Ca, Mg, Fe, Mn, Zn, and Cu were low, and these elements remained in the effluent. This characteristic is interesting, because effluent can be used in fertigation sugar cane. Thus, nutrients available in the effluent and retained in the sludge can be used by replacing part of the mineral fertilizer and reducing production costs.

The concentrations of Fe, Zn, and Mn increased, but the increase in Fe concentration was more pronounced. According to [Singh et al. \(1999\)](#), Fe, Mn, and Zn are among the trace elements necessary for the synthesis of several anaerobic microorganisms, since these elements are enzymatic cofactors for several enzymes.

According to [Schmidt et al. \(2014\)](#), trace element deficiency causes decreased performance and increased TVA concentrations, and the authors observed that Fe depletion has an influence on methanogenic microorganisms. In this study, the lowest Fe concentrations occurred after 320 days of operation. Coinciding in this period, the highest concentrations of TVA and lower process performance were observed, indicating that the accumulation in the concentration of TVA may have occurred due to the low amount of Fe. The chemical composition of filter cake is a function of the variety and maturation of the sugar cane, type of soil, procedure of juice clarification, and various other factors ([Prado et al., 2013](#)). Thence, filter cake characteristics vary throughout harvest of sugar cane. Thus, to achieve low TVA concentrations, stable operation and higher methane production in anaerobic reactors treating vinasse with filter cake supplementation its chemical characteristics should be periodically monitored to keep suitable Fe concentrations in the influent.

3.4. Characterization of the generated sludge

The values of volatile solids (VS) in the sludge of the UASB R1 and R2 reactors increased with the gradual increase of OLR (Fig. 3), indicating that there was growth in the sludge blanket of the reactors.

The VS/TS ratios were below 0.7 at the collection points for P2, P3, and P4 sludge, in R1 and R2, except at P1 of R1 and R2, which presented values above 0.7 (Fig. 3). According to Brazilian legislation, the sewage sludge or derived product is considered stable for agriculture use if VS/TS < 0.70 ([CONAMA, 2006](#)), which avoids high organic matter concentrations in the soil and microbial activity ([Villa-Montoya et al., 2016](#)). Based on this resolution, stabilized sludge was observed, mainly at the upper collection points, P2, P3, and P4. Thus, when necessary, sludge disposal should be performed from P2 upwards, which has stable sludge (VS/TS < 0.7), maintaining in the reactors the sludge with VS/TS ratios higher than 0.7, which indicates a greater amount of organic matter and consequently greater activity.

3.5. Microbial diversity in the anaerobic reactors

Analysis of archaea and bacteria diversity in the reactor identified 11 different phyla. Over 96.00% (R1), 99.70% (R2) and 58.00% (R1), 23.40% (R2) belonged to the phyla Euryarchaeota and Thermotogae, respectively. Among the nine bacterial phyla found, the most abundant were the Thermotogae, Firmicutes, and Bacteroidetes (Fig. 4a and b).

The phylum Thermotogae, found in both the reactors, although especially in R1, is of great interest from the biotechnological point of view, due to its stability at high temperatures and its ability to use several complex carbohydrates, such as cellulose and xylans, for hydrogen production ([Gupta and Bhandari, 2011](#)). The highest abundances of the Thermotogae phylum in R1 may have occurred due to characteristics of the vinasse, which may contain phenolic compounds such as tannic and humic acids, carotenoids, chlorophyll, anthocyanins, betalains, riboflavins, quinones, and caramels ([Kaster et al., 2011](#)).

The Firmicutes phylum was the opposite of Thermotogae, with the highest percentage in R2 (Fig. 4a). These microorganisms are facultative, and are known to produce cellulases, lipases, proteases, and other extracellular enzymes ([Yin et al., 2016](#)) as well as various organic acids (C3, C4 acids) such as propionic, butyric and its derivatives ([Venkata Mohan et al., 2011](#)). The phylum Firmicutes includes many bacteria in the class Clostridia that break down complex organic material ([Wirth et al., 2012](#)), which are probably present in the reactors R1 and R2 due to the characteristics of the vinasse.

The phylum Bacteroidetes, the third most abundant, is composed of bacteria that are involved in the hydrolysis and acidogenesis steps of anaerobic digestion ([Dias et al., 2016](#)), and is important in the degradation of complex polymers ([Jabari et al., 2016](#)).

Thermotogae, Firmicutes, and Bacteroidetes are mainly responsible for the degradation of complex organic compounds, so the presence of these phyla in the R1 and R2 reactors indicates that microorganisms capable of degrading these compounds were present in the anaerobic digestion of vinasse, contributing to the stability of the system with high methane production.

The Nitrospirae phylum, with a single class, order, and family, represents several metabolisms, and many genera are aerobic, including nitrifying and sulfate reducers ([Garrity et al., 2001](#)). In the R1 and R2, respectively, 14.30% and 26.50% of this phylum were denoted as unidentified. The use of unclassified microorganisms is an artifact used to group sequences that have bacterial characteristics but do not fit into a known phylum. It shows that many bacteria present in thermophilic conditions are still unknown and could be explored as genetic and metabolic potential.

In the Nitrospirae phylum we find the sulfate-reducing *Thermodesulfovibrio* genus. These bacteria are thermophiles, acidophiles, and obligate anaerobe that oxidize the organic material into acetate and uses sulfate and thiosulfate as acceptor electrons to growth,

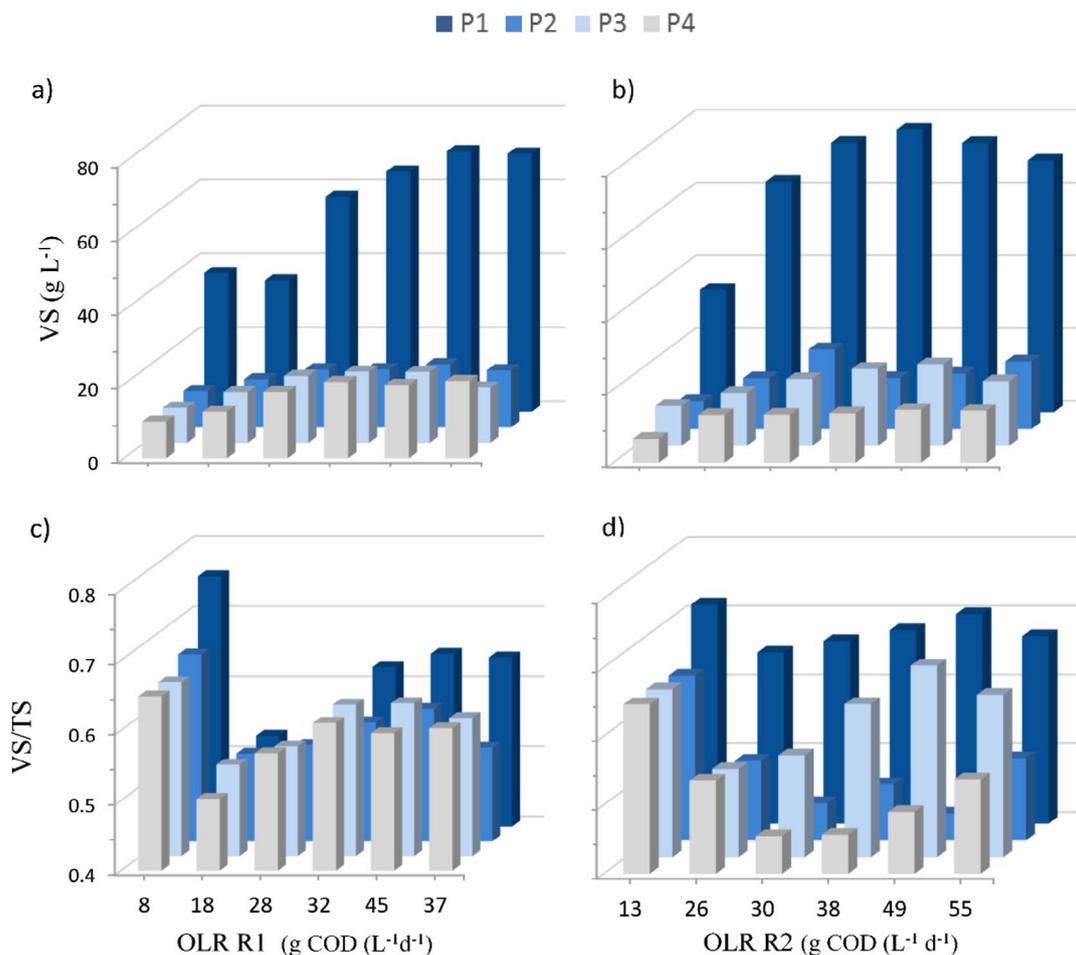


Fig. 3. Sludge bed characteristics of the two-stage UASB reactors (R1 and R2). (a) and (c) R1, (b) and (d) R2. VS/TS: volatile and total solids ratio, OLR: organic loading rate.

but can also utilize sulfite and nitrite (Garrity et al., 2001).

The genus *Fervidobacterium* can oxidize the hemicelluloses present in the influent (Pandit et al., 2016), and relative abundances of 12.10 and 3.00% were found in R1 and R2, respectively. The highest abundances in R1 may have occurred due to the higher concentrations of hemicelluloses, probably coming from filter cake. The observed large abundance of the genus *Fervidobacterium* in R1 has a high correlation with the volumetric production of methane, KN, P_{total} , and Fe. Another abundant genus was *Petrimonas*, microorganisms which can be found under alkaline conditions with pH 8–9. They are fermentative bacteria, mainly producing acetic acid, hydrogen, and carbon dioxide, precursors of methanogenic (Maspolim et al., 2015). The greater abundance of this microorganism in R2 can be related to the more alkaline pH 8.62 (Table 3), and also has correlates with the volumetric production of methane, KN, P_{total} , and Fe.

In relation to the archaea, two phyla, Euryarchaeota (dominant in both reactors) and Crenarchaeota were found (Fig. 4c). In the Euryarchaeota phylum, the most dominant class was Methanobacteria (Fig. 4d).

The genus *Methanothermobacter* (~80.5%/R1, ~90%/R2) showed dominance in both reactors. In general, *Methanothermobacter* have an optimum growth temperature near 65 °C and the ability to grow on H_2 and CO_2 as carbon and energy source, NH_3 as nitrogen source, and H_2S or sulfite but not sulfate as sulfur sources. The *Methanothermobacter* require Na^+ , K^+ , Fe^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} , MoO_4^{2-} , and/or WO_4^{2-} , and possibly Ca^{2+} for growth (Gupta and Bhandari, 2011). These microorganisms have a genomic architecture and coding potential responsible for the CO_2 reduction to methane (methanogenesis from H_2 and CO_2). In addition, they are can easily be mass cultured (Kaster

et al., 2011). We suggest that the filter cake used in this study created the best conditions for the dominance of *Methanothermobacter*. Thus, we suggest these microorganisms as candidates in the consortium of methanogenesis in the treatment of vinasse associated with filter cake. The large abundance of the genus *Methanothermobacter* has high correlation with the volumetric production of methane, KN, P_{total} , and Fe in R1 and R2.

However, the hydrogenotrophic methanogens *Methanoculleus* and the acetoclastic and hydrogenotrophic methanogens *Methanosarcina* which presented high relative abundance in R1, are considered as robust and effective methane producers occurring in high performance anaerobic digestion processes. These microorganisms are best adapted to environments with pH closer to 7 (Table 3) and with the presence of trace elements. In a study carried out by Wintsche et al. (2016), the trace elements relative abundance of *mcrA* transcripts from *Methanosarcina* sp. was positively correlated with the concentrations of cobalt, manganese, molybdenum, nickel, and tungsten trace elements. Thus, we can affirm that the physicochemical conditions of R1 promoted the development of these microorganisms. The distribution of the archaea between hydrogenotrophic and acetoclastic in the sludge of the UASB reactors R1 and R2, as observed in this work, allowed the high production of methane using the produced acetic acid, H_2 and CO_2 .

3.6. Absolute quantification of methanogenic

Among the several microbial communities identified, we selected some microorganisms to determine the 16S rDNA gene concentration at each point of the two-stage UASB reactors (R1 and R2). The Bacteria and Archaea domains, the Methanobacteriales, Methanomicrobiales,

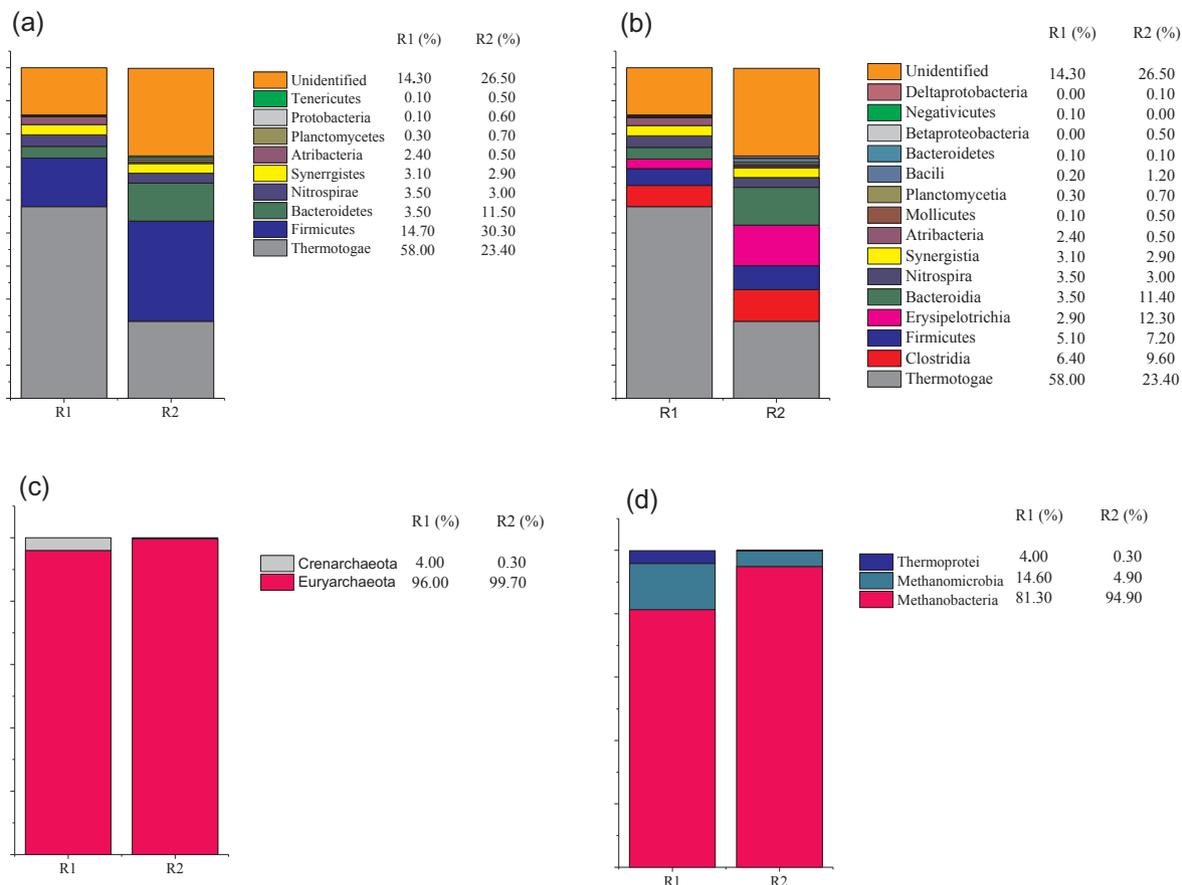


Fig. 4. Taxonomic affiliations and relative abundance of phyla (a) and classes (b) of bacterial and taxonomic affiliations and relative abundance of the phyla (c) and classes (d) of the archaea found in the sludge samples of the two-stage UASB reactors (R1 and R2). Normalized by the size of the smallest sample (37,074 sequences).

and Methanosarcinales orders, as well as the families Methanosarcinaceae and Methanosaetacea were quantified (Fig. 5).

Quantification of microorganisms indicated a balance between domains Archaea and Bacteria, with 1.20×10^9 to 3.96×10^9 copies/g VS in R1 and 1.19×10^9 to 4.17×10^9 copies/g VS in R2, and 7.39×10^9 to 1.99×10^{10} copies/g VS in R1 and 1.04×10^{10} to 3.74×10^{10} copies/g VS in R2, respectively. In an experiment conducted by Duda et al. (2015), a balance between bacteria and archaea was also observed, but in high-rate horizontal anaerobic reactors in series for the treatment of swine wastewater. In order for stability to occur in the anaerobic reactors, the archaea concentration must be close to that of the bacteria (Fig. 5), so that they can efficiently use the substrates produced by the bacteria, since the archaea microorganisms have a low growth and substrate utilization rates than bacteria. Imbalances in the proportion of bacteria to archaea can affect the entire system, leading to a reduction in the amount of methane produced (Akuzawa et al., 2011). This effect did not occur with UASB reactors treating vinasse with suitable filter cake supplementation, which maintained low TVA concentrations (Fig. 2b) and high levels of methane production (Fig. 2d) in face of similar numbers of bacteria and archaea.

In relation to Methanobacteriales, Methanomicrobiales, and Methanosarcinales orders, the concentrations of Methanobacteriales and Methanosarcinales were higher, and all of them kept in equilibrium at the points of R1, but increased from P1 to P3 in R2. Thus, we suggest that the use of the generated hydrogen occurs in all parts of the reactor R1 and mainly in the upper parts of the reactor R2 (Fig. 5). The higher hydrogenotrophic archaea concentrations in the sludge of the reactors (R1 and R2) favor the efficient transfer of hydrogen to the methane production, consequently maintaining low hydrogen pressure, allowing

the equilibrium of the reactions.

The Methanosarcinaceae showed a slightly higher concentration in R1, when compared to R2. However, Methanosarcinaceae and Methanosaetacea presented a similar concentration pattern in R2 (Fig. 5). In Duda et al. (2015), the absolute quantities of both families were similar in the four reactors. But in this work, the increase in concentration of Methanosarcinaceae in R1 may have occurred due to the higher TVA concentrations and consumption of other compounds besides acetate.

4. Conclusion

The highest methane production reached was $4.0 \text{ L CH}_4 (\text{L d})^{-1}$ with OLR of $45 \text{ g COD } (\text{L d})^{-1}$ under conditions of stability. The thermophilic digestion in two-stage UASB reactors with filter cake supplementation and effluent recirculation contributed to the increase of conversion from vinasse to methane and the process stability. In the sludge of the reactors, the three most abundant bacterial phyla were Thermotogae, Firmicutes, and Bacteroidetes; and the largest methanogenic archaea quantities were of the orders Methanobacteriales and Methanosarcinales. The equilibrium in the concentration between the microbial communities for the domains Bacteria and Archaea favored the good functionality of the reactors.

Conflicts of interest

The authors declare no conflict of interest.

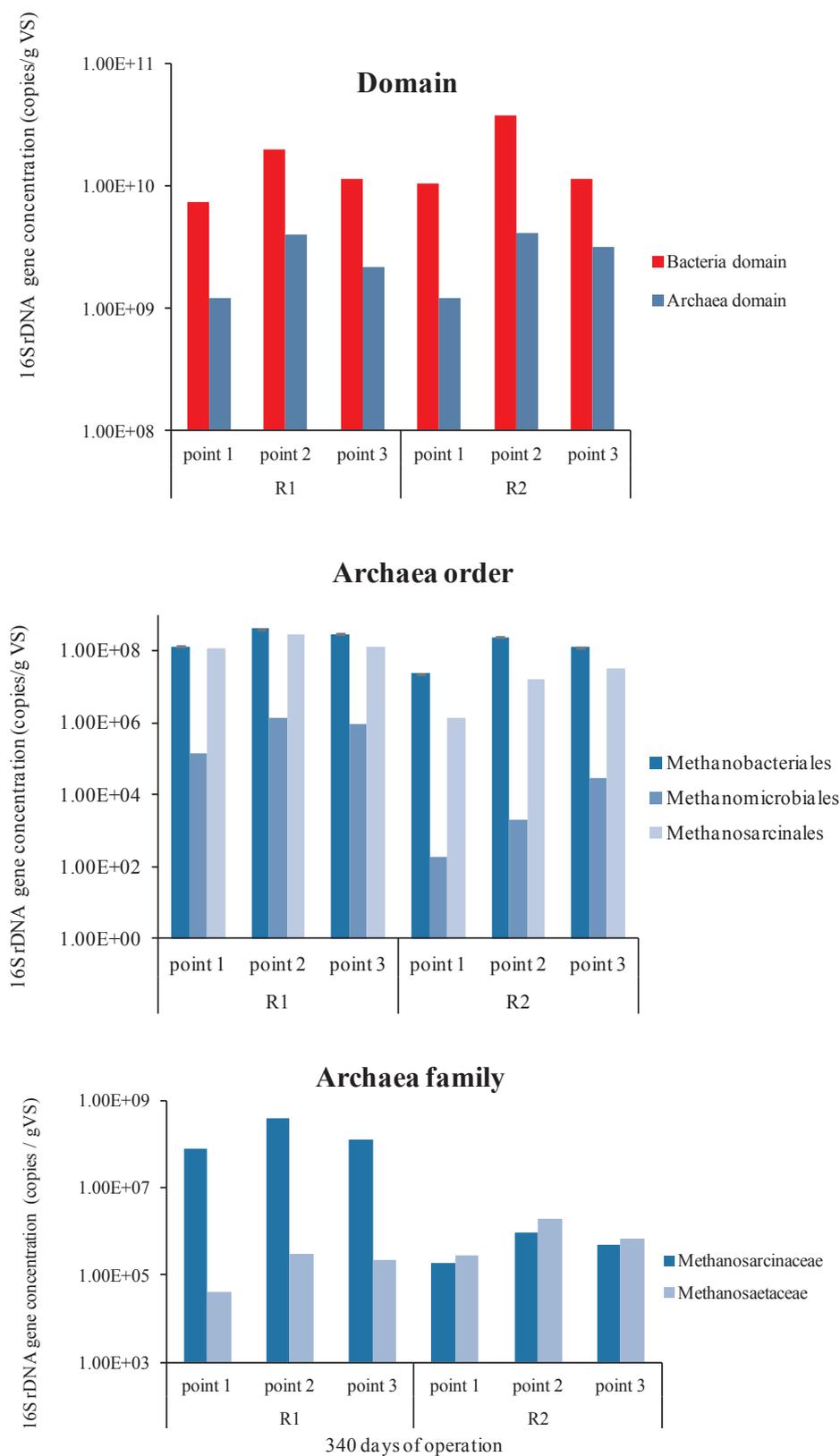


Fig. 5. Absolute quantification of the microorganisms with qPCR of the Bacteria and Archaea domains, three orders (Methanobacteriales, Methanomicrobiales, and Methanosarcinales) and two families (Methanosarcinaceae and Methanosactaceae) of the archaeal domain present in the sludge of the two-stage UASB reactors (R1 and R2). VS: volatile solids; points 1, 2 and 3 (Fig. 1).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.07.106>.

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