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ORIGINAL ARTICLE

Microbial consortia composition on the production of methane from sugarcane vinasse

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Abstract

Vinasse is a by-product from the ethanol industry and can be used for methane production through anaerobic digestion process driven by microbial consortia. The microorganisms involved must be known to obtain an ideal methane production. The present work evaluated the production of methane and byproducts from different ratios substrate/biomass (S_0/X_0), using sludge from an effluent treatment of the vegetable oil industry as inoculum in media containing vinasse. Also, the microbial community of the best methane production bioassay was characterized by high-throughput DNA sequencing. The following chemical parameters were evaluated: methanogenic activity, chemical oxygen demand, carbohydrate consumption, and production of volatile fatty acids. The highest methane production occurred at S_0/X_0 ratio of 1.5, which produced 59.78 mmol CH_4 L^{-1}. A great variety of microorganism genera was identified by high-throughput DNA sequencing, showing differences in the microbial consortia of the initial and final sampling times. At the final sampling point, the classes Bacteroidia (Porphyromonadaceae—OTU genera unknown 42.26% and Bacteroides genus 10.58%) and the class Betaproteobacteria (Proteobacteria-Comamonadaceae OTU) were identified as the dominant bacteria. The most abundant archaeal genera in the bioassay were *Methanosaeta*, *Methanomassiliicoccaceae* OTU *vadinCA11*, and *Methanobacterium.* The identification of the microorganisms of consortia involved in anaerobic digestion can collaborate on technologies to increase methane production through microbial isolation, bioaugmentation, and co-cultures.

Keywords Biogas · High-throughput sequencing · 16S rRNA · Bacteria · Archaea · Anaerobic digestion

1 Introduction

Ethanol represents one of the major alternatives for reducing dependence on fossil fuels. Brazil is the world's largest producer of ethanol from sugarcane, producing about 28 billion ton per year [1]. The bioethanol production is expected to keep growing for the next decade to reach 134 billion liters in 2024 with biggest producers expected to be USA, Brazil, European Union, and China [2]. However, for each liter of ethanol produced, 12–15 l of vinasse is generated. Vinasse is a liquid by-product of high organic load, dark brown color, low pH, high salt content, and highly polluting [3, 4]. It contains a chemical oxygen demand (COD) of 20 to 100 g $COD.L^{-1}$, some organic solids in suspension as minerals, residual sugar, and some volatile compounds. Currently, vinasse is discharged directly onto farmland by fertigation, but its prolonged use as fertilizer leads to the salinization of the soil and desertification [5, 6]. Also, sprinkling vinasse on sugarcane crops delays their maturation and leads to a decreased sugar content [7]. The high production of vinasse demands a search for better environmental management of this by-product.

Methane is a sustainable alternative for energy production, being produced from agricultural byproducts as a substrate [8, 9]. The production of methane through vinasse biodigestion

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helps to reduce the polluting potential of this residue, producing value-added byproducts as well as a high-quality biofertilizer for agriculture [3].

The process of vinasse bioconversion can be performed using inocula of natural microbial communities, such as sludge from effluent treatment plants [8, 10]. The microorganism consortia involved in this process shows a series of complex metabolic reactions involving several species of bacteria and archaea [11]. They act symbiotically at intermediary stages, such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis [12–14]. The knowledge of the microbial communities is essential for understanding and developing the process of methanogenesis [15–17]. In this context, the objective of this work was to evaluate the production of methane and by-product of vinasse in bioassays with different S_0/X_0 ratios and to use high-throughput sequencing to identify the microbial community associated with the best methane bioassay.

2 Materials and methods

2.1 Operational conditions

Sugarcane vinasse provided by the company Guarani SP, Brazil, was used as a culture medium for the bioassays (Table 1). It was centrifuged at 10,000 rpm for 5 min to remove coarse solids. The biomass used was granular sludge from UASB reactor, from vegetable oil treatment station (S 29° 51′ 41.04″; W 51° 10′ 45.12″). The inoculum that was lightly macerated to reduce granulometry and was directly inoculated in the culture medium with vinasse.

The experiments were performed in triplicate, using four different substrate/biomass ratios ($S_0/X_0 = 0.5$, 1.0, 1.5, and 1.7) (Table 2). Bioreactors with a total volume of 610 mL (366 mL of medium and 244 mL of headspace) run for 14 days under mesophilic conditions. The pH was adjusted periodically to 6.8–7.2 using KOH 3M to maintain optimum conditions for archaea development. The experiments were kept under stirring in an orbital shaker at 140 rpm. Nitrogen sprinkled for 10 min to maintain in anaerobiosis of the medium. During the fermentation, initial (0 h), intermediary (168 h), and final (336 h) samples were collected, and sent to chemical and biological analyses. Gaseous samples were collected from the headspace every 24 h.

2.2 Chemical analyses

The analysis of methane production was performed using gas chromatography (DaniMaster AS), with CarboxenTM 1006 PLOT capillary column (30 m × 0.53 mm), with thermal conductivity detector (TCD), ultrapure nitrogen gas as the carrier gas. The amount of methane produced was analyzed every 24 h for 14 days. A calibration curve was performed using standard methane gas. Analyses were also made from liquid samples of volatile fatty acids (acetic, propionic, butyric, isobutyric, valeric, and isovaleric), where samples were centrifuged at 1000 rpm for 10 min, filtered at 0.2 membrane (Millipore), and diluted in 5 × methanol. They were analyzed using gas chromatography (GC/MS, Shimadzu-QP 2010 Ultra) equipped with a DN-FFAP (30 mx 0.32 mm × 0.25 μm) column with flame ionization detector (FID), with helium as carrier gas and synthetic, and air and nitrogen as auxiliary gases. The column temperature was 100 °C for 5 min, increasing 7 °C per min to 200 °C. The temperatures of the injector and detector were 200 °C and 250 °C, respectively. The volatile acids were identified by the respective retention times, compared with those obtained in the standard curves. For the development of the standard curves, synthetic solutions of the studied compounds were used. The analyses of the composition of the vinasse and the COD were determined following the standard methods [18]. The total carbohydrate concentration of the samples was determined by a colorimetric method using sucrose as standard [19].

2.3 Experimental data fitting

The experimental data in triplicate were adjusted using the Statistica software (version 10). The modified Gompertz model [20] was used to estimate the kinetic parameters (R_m, H_{max} and λ) for the methane production in the different substrate/biomass ratios (Eq. 1).

$$M = P.\exp\left\{-\exp\left[\frac{R.e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where M is the cumulative methane production (mL); λ is the lag phase time (h); P is the ultimate methane production (mL); R_m is methane production rate ($mL.h^{-1}$); and e is the constant (2.72).

2.4 High throughput DNA sequencing

To identify which microorganisms are parts of the methane production, the initial sample (IS, 0 h) and final sample (FS, 336 h) from the bioassay, $S_0/X_0 = 1.5$ ratio, were sequenced. The genomic DNA from the microbial consortia showing the best methane production was extracted from 250 μL of the sample with the DNeasy PowerSoil Kit (Qiagen), according to the manufacturer's protocol. The extracted DNA was amplified by PCR with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) for the 16S rRNA gene. The amplification conditions followed 35 cycles (95 °C, 30 s; 50 °C, 1 min; 72 °C, 1 min) after an initial denaturation at 95 °C for 3 min [21] in an Applied Biosystems Model 2720 thermal cycler. The samples were analyzed on 1% agarose

Table 1 Composition of vinasse used in bioassay So/Xo 1.5

Parameters	IS	FS	Methodology
Calcium (mg Ca L^{-1})	266.30	240.00	3030-E/3111-B
COD* (mg L^{-1})	11,918	3468	*APHA 5220C
Total phosphorus (mg L^{-1})	35.10	58.00	4500-P B and E
Total magnesium (mg MgL^{-1})	193.00	165.30	3030-E/3111-B
Total manganese (mg L^{-1})	1.35	2.65	3030-E/3111-B
Ammoniacal nitrogen	77.70	68.01	4500-NH_3 B-C
Nitrogen kjeldahl (mg NH_3-N L^{-1})	313.57	243.89	4500-Norg-B
Potassium (mg K L^{-1})	2030.90	253.96	3500-K-B
Iron (mg FeL^{-1})	7.94	3.20	3030-E/3111-B
Sodium (mg Na L^{-1})	36.99	54.25	3500-Na-B
Sulfates (mg L^{-1})	731	347	4500-SO_4-E

According Standard Methods s for Examination of Water and Wastewater (2017), 23ND Edition

Tests recognized by the Metrology Network/RS, according to ABNT NBR ISO/IEC 17025: 2017; Certificate of Recognition No. 3415A/3415B and 3415 C valid until July/2019

gel. PCR products from the initial samples and final fermentation sample after the highest methane production were submitted to high throughput DNA sequencing on the Ion PGM System (Thermo Fisher) following the manufacturer's instructions (Thermo Fisher). The libraries construction was performed with the Ion Plus Fragment Library kit for short amplicons (≤ 350 pb), from 100 ng of the amplification product. A barcode adapter of the IonXpress Barcode adapters was added to each sample. All procedures were performed according to the manufacturer's protocol. For quantification and equalization of the libraries, the Ion Library Equalizer kit was used, according to the manufacturer's instructions. For emulsion PCR and enrichment steps, the Ion PGM Template OT2 200 kit was used with the Ion One Touch 2 system. For the sequencing, an Ion 316 chip was used with the Ion PGM Sequencing 200 v2 kit, according to the manufacturer's instructions. Fragments of the 16S rRNA gene generated by the sequencing were submitted to quality control using the PRINSEQ program [22]. The replicate sequences were identified, sorted, and filtered to exclude unique sequences using the USEARCH v7.0.1090 program [23]. The clusters were assembled using a 99% minimum identity and chimeras were removed using the RDP reference database [24]. The taxonomic attribution was obtained using QIIME v1.7 based on 97% sequence similarities with the GreenGenes 13.8 database [25, 26]. Sequences were deposited in the National Center for Biotechnology Information (NCBI) under BioProject PRJNA471743.

3 Results and discussion

3.1 Methane production

All bioassays produced methane independent of the S_0/X_0 ratio used (Table 3). The best final production of methane $P = 613.7$ mL was found in the bioassay with a ratio of $S_0/X_0 = 1.5$, with a maximum production rate of 1.9 mL CH_4 h^{-1} and lag phase (λ) at 59 h.

Comparing these results with other studies, it can be seen that Kiyuna et al. [27] found their best methane production of 541.4 ml CH_4 in a vinasse study, and Shin et al. [28] in a work using another substrate (swine manure and food debris) presented 601 mL of CH_4. The best methane yield (59.78 mmol CH_4 L^{-1}) was observed in 264 h (Fig. 1).

3.2 Carbohydrate consumption and removal COD

In the bioassays, the highest percentages of carbohydrate intake were observed in the $S_0/X_0 = 1.7$ (89.3%), 1.5 (78.6%), and 1.0 (72.7%) ratios. The removal of COD was less

Table 2 Parameters of the substrate/biomass ratio S_0/X_0 of vinasse in different bioassays

So/Xo	SVT (mg $L^{-1)}$	mSVT (mg)	mCOD	COD (mg O_2/L)	SVT (mL)	V vinasse (mL)	Δv	COV (kgDQO/m3)
0.5	10,000	3660	1830	20,840	54.19	87.81	224.00	5
1	10,000	3660	3660	20,840	54.19	175.62	136.19	10
1.5	10,000	3660	5490	20,840	54.19	263.43	48.37	15
1.7	10,000	3660	6222	20,840	54.19	298.56	13.25	17

Table 3 Estimated methane production, maximum methane production, lag phase time, carbohydrate consumption, and COD removal from experiments in the conversion of vinasse to methane by microbial consortia in different S_0/X_0 ratios

S_0/X_0 ratio	Ultimate methane production (mL CH_4) *	Methane production rate (mL CH_4 h^{-1}) *	Lag phase time (h)*	Carbohydrate consumption (%)	COD removal (%)	Conversion (%)	mL/$gCOD_0$	mL/$gCOD_{removed}$
0.5	286.4 ± 7.4	18.5 ± 0.6	97.0 ± 0	68.9	89.4	14.7	52.2	58.3
1	363.7 ± 9.3	2.1 ± 0.1	22.4 ± 2.4	72.7	85.2	14.8	50.2	58.8
1.5	613.7 ± 3.0	1.9 ± 0.1	59.3 ± 3.8	78.6	70.9	9.3	26.3	37.2
1.7	308.5 ± 50.0	2.7 ± 1.5	23.1 ± 2.6	89.3	53.7	8.43	18.0	33.5

*The modified Gompertz model [20] was used to estimate the kinetic parameters (*R*m, Hmax, and λ) for the methane production in the different substrate/biomass ratios

effective in the $S_0/X_0 = 1.7$ (53.7%) ratio and the higher removal efficiency occurred in $S_0/X_0 = 0.5$ with 89.4% (Table 3). In general, it is observed that the higher the availability of organic load in the medium, the higher the difficulty in the removal of COD. The ability of microbial consortia to reduce the organic load of vinasse is already well reported in the literature, reducing the impact in the environment [29, 30].

When analyzing the methane volume per gram of COD removed, it is observed that the S_0/X_0 value 0.5 to 1 is more advantageous than the higher ratios. In the same way, the conversion of substrate to methane is higher in S_0/X_0 0.5 and 1 (14.7%) and lower in S_0/X_0 (8.4 to 9.3%). The ratio $S_0/X_0 = 1.5$ was the dilution chosen because it presented the best-accumulated methane production, although COD removal and the lag phase time were not more efficient. These results can be explained by the influence of the fermentation parameters in the microbial communities. As can be observed, the higher substrate concentration ($S_0/X_0 = 1.7$) obtained higher intakes of carbohydrates, indicating a possible change in the metabolic pathways or predominance of microorganisms. These results, however, require further investigation. On the other hand, it is a fact that the microbial communities strongly alter the substrate composition, as can be observed in the fermentation of $S_0/X_0 = 1.5$ (Table 1). After 336 h of bioassay,

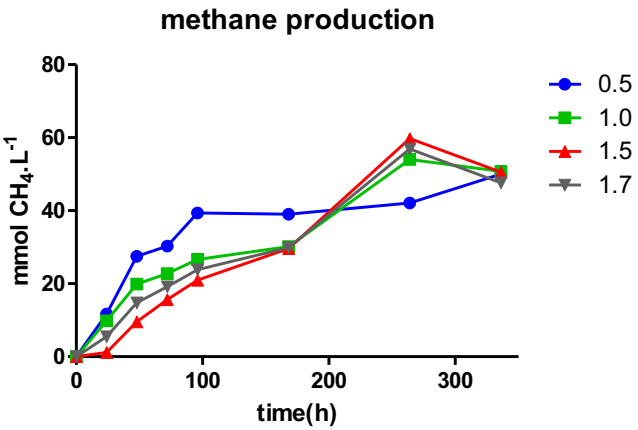

Fig. 1 Production of methane in mmol CH_4 L^{-1} from vinasse of archaeal consortia from different S_0/X_0 ratios

it was noted the reduction of the concentration of potassium, iron, calcium, and the increase of phosphorus and total manganese.

3.3 Volatile fatty acids present in the fermentation

The acids analyzed in the fermentation process production of methane from sugarcane vinasse were acetic, propionic, butyric, isobutyric, valeric, and isovaleric (Fig. 2).

Chromatographic data show that in the initial vinasse samples volatile fatty acids were detected and that these acids were consumed during the anaerobic decomposition which can be seen in Fig. 2b–d, with exception of the sample $S_0/X_0 = 0.5$.

The volatile fatty acids have been reported as the primary precursors of methane production. *Syntrophobacter* groups, as sulfate reducers, are capable of degrading propionate in syntrophic association with methanogens, are the main propionate oxidizers [31, 32]. The *Syntrophobacter* group was found to be the most abundant among propionate degraders present in anaerobic digesters and the species described in the literature are *S. fumaroxidans*, *S. wolinii*, *S. pfennigii*, *S. sulfatireducens*, and HP1.1 strain of the *Syntrophobacter* genus [33–37]. On the other hand, a massive accumulation of the acids can also act as inhibitors of methanogenic microorganisms due to a decrease in the pH of the medium, to thermodynamic reasons, and the acids toxicity, destabilizing the cellular biochemical reactions of the consortia [38]. Acid production has become a useful process for the chemistry of renewable energies. These acid derivatives can be purified and used in the food, textile, pharmaceutical, leather, and plastic industries. The values obtained in the market are higher for the acids than for the methane, not to mention the ease of storage, transport, and greater safety [39, 40].

3.4 Microbial community identification

Samples from the $S_0/X_0 = 1.5$ ratio assay were submitted to high throughput DNA sequencing. A total of 78,892 and

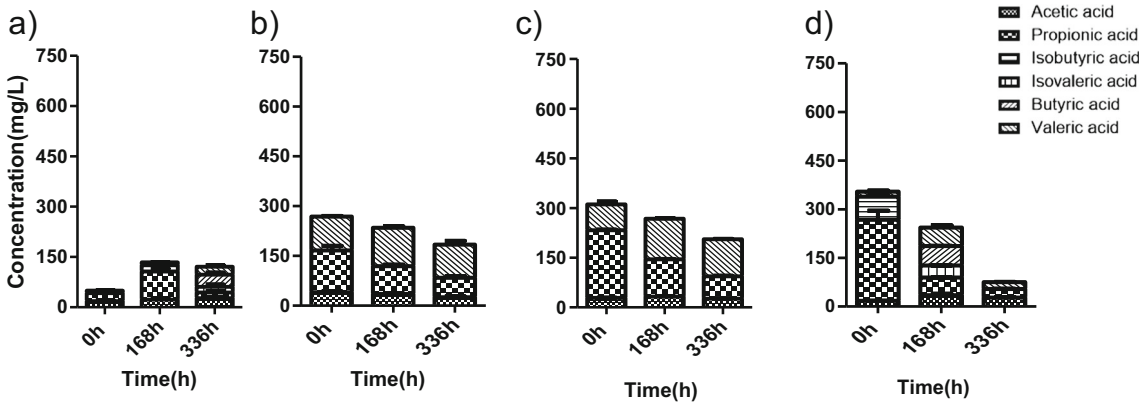

Fig. 2 Volatile fatty acids from vinasse in the bioassay of methane production by microbial consortia at different S_0/X_0 ratios: **a** 0.5, **b** 1.0, **c** 1.5, and **d** 1.7

86,051 DNA sequences were obtained from samples IS and FS, respectively.

Thirty-six phyla were identified in the two samples, and a larger number of taxa were observed in the SI than in the FS. A higher number of phyla in the SI compared with FS was also observed in other studies of anaerobic digestion of residues [11, 29, 41]. The environment to which the microorganisms are submitted in the fermentation tests to produce methane leads to a selection of microorganisms that act in the process and the number of taxa tends to decrease. Species in syntrophy can occur by the combined metabolic activity of microorganisms, allowing the microbial community to survive with minimal energy resources [8, 42]. Preliminary studies on biogas plants and anaerobic reactors presented similar results about the identified microorganisms [43–45].

Proteobacteria (25.6%), Bacteroidetes (18.7%), and Euryarchaeota (17.6%) were the most representative phyla in IS. In the FS, the most prevalent phyla were Bacteroidetes (58.5%), Firmicutes (14.1%), and Proteobacteria (13.1%) (Fig. 3). These phyla were also the most prevalent microorganisms in anaerobic fermentation of vinasse in the study of methanogenic analysis of desulphurization system used to treat biogas from vinasse methanization [29]. Bacteroidetes, the most abundant phylum in our study, is composed of strict anaerobes capable of degrading protein-rich substrates involved in the hydrolysis and acidogenesis of anaerobic digestion [46, 47].

Phylum Firmicutes contain individuals capable of fermenting various organic substrates and producing spores. The genus *Clostridia*, for example, can catabolize proteins, lipids, and complex carbohydrates [17, 43, 44, 48]. Proteobacteria, Deltaproteobacteria, and Gammaproteobacteria consist of microorganisms involved in acidogenesis, nitrification, and other metabolic processes [49, 50].

An OTU belonging to the order Bacteroidales was the most prevalent microorganism in the IS, corresponding to 10.96% of the total sequences (Table 4). Bacteria of the genera *Syntrophomonas*, *Syntrophus*, *Flavobacterium*, Candidatus *Cloacomonas*, *Anaerolinaceae*, *Sulfuricurvum*, *Sulfurimonas*, *Thermomonas*, and *Kosmotoga* also occurred

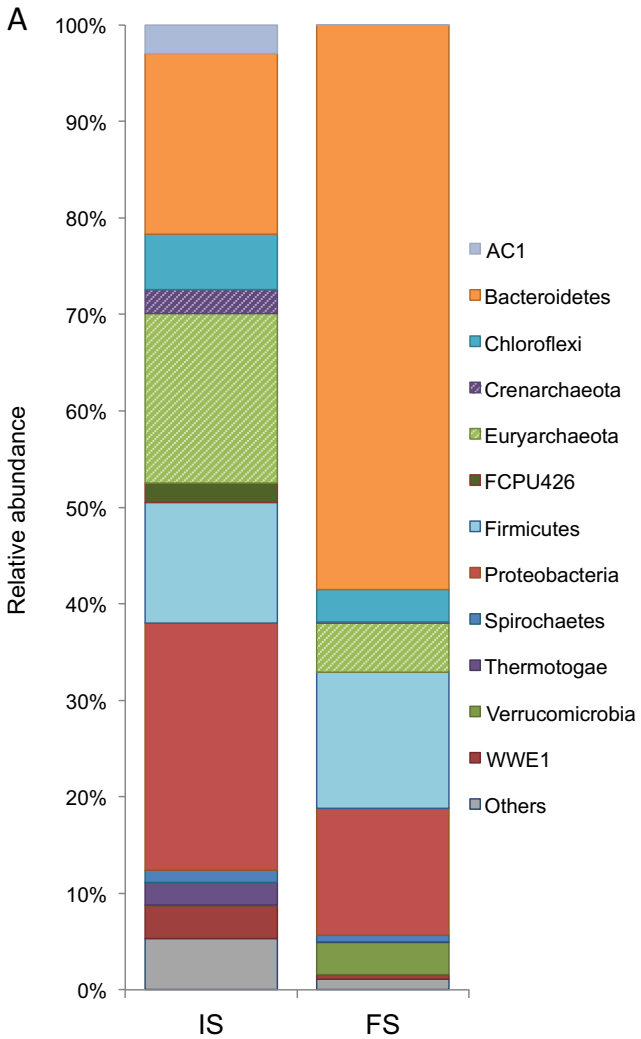

Fig. 3 Relative abundance of phyla (or candidate divisions) in microbial consortia composition on the production of methane from sugarcane vinasse (IS) and final sample (FS) (S_0/X_0 = 1.5 vinasse ratios)

Table 4 Relative abundance of bacteria (or respective OTU) in microbial consortia composition on the production of methane from sugarcane vinasse (IS) and final sample (FS) ($S_0/X_0 = 1.5$ vinasse ratios)

Domain	Phylum	Class	Order	Family	Genus	Relative abundance (%)	
						IS	FS
Bacteria	AC1	SHA-114 OTU				2.93	0.02
	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales OTU		10.96	3.02
				BA008 OTU		1.32	0.02
				Bacteroidaceae	*Bacteroides*	0.42	10.58
				p-2534-18B5 OTU		0.03	2.01
				Porphyromonadaceae OTU		2.79	42.26
		Flavobacteriia	Flavobacteriales	*Flavobacteriaceae*	*Flavobacterium*	1.88	0.02
	Chloroflexi	Anaerolineae	Anaerolineales	*Anaerolinaceae*	*Anaerolinaceae* OTU	2.20	0.38
					T78	1.75	2.52
	FCPU426 OTU					1.99	0.02
	Firmicutes	Bacilli	Lactobacillales OTU			1.23	0.00
		Clostridia	Clostridiales	*Clostridiaceae*	*Clostridium*	1.14	1.98
				Ruminococcaceae	*Ruminococcaceae* OTU	0.13	1.13
					Ruminococcus	0.00	1.77
				Syntrophomonadaceae	*Syntrophomonas*	7.33	0.79
				Veillonellaceae	BSV43 OTU	0.01	2.76
					vadinHB04 OTU	0.03	3.02
	Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Comamonadaceae* OTU	0.25	7.94
		Deltaproteobacteria	Desulfobacterales	*Desulfobulbaceae*	*Desulfobulbaceae* OTU	0.00	1.44
			Desulfovibrionales	*Desulfovibrionaceae*	*Desulfovibrio*	0.11	1.81
			Syntrophobacterales	*Syntrophaceae*	*Syntrophaceae* OTU	2.04	0.03
					Syntrophus	4.43	0.04
				Syntrophorhabdaceae	*Syntrophorhabdaceae* OTU	3.20	0.06
		Epsilonproteobacteria	Campylobacterales	*Helicobacteraceae*	*Sulfuricurvum*	1.41	0.04
					Sulfurimonas	6.70	0.01
		Gammaproteobacteria	Xanthomonadales	*Xanthomonadaceae*	*Thermomonas*	1.15	0.06
	Thermotogae	Thermotogae	Thermotogales	*Thermotogaceae*	*Kosmotoga*	2.36	0.11
	Verrucomicrobia	Pedosphaerae	Pedosphaerales	R4-41B OTU		0.02	3.29
	WWE1	Cloacamonae	Cloacamonales	*Cloacamonaceae*	*Candidatus* Cloacamonas	3.30	0.06

in abundance higher in IS than in FS, and all were observed in abundance higher than 1% of the total sequences.

This result is in accordance with Krause et al. [51] that performed a study on a total community DNA sample from an agricultural biogas reactor continuously fed with maize silage, green rye, and small proportions of chicken manure. Microorganisms from the class Bacteroidia were identified in other studies using a methanogenic reactor to treat livestock waste, and they might be related to the fermentation of glucose, producing acetate and propionate [52–54]. Firmicutes is an acetate-oxidizing bacteria, in syntrophic cooperation with hydrogenotrophic methanogens [55].

Syntrophomonas is a bacterial genus capable of fermenting fatty acids in syntrophy with microorganisms that use hydrogen and formate [56]. Since vinasse is rich in organic acids—previous studies have reported concentrations of volatile fatty acids in the order of 19 g L^{-1} in sugarcane molasses [49].

When analyzing the most prevalent microorganisms in FS, we can see the unknown genus of the family *Porphyromonadaceae* with the highest number of copies of RNA16S genes with 42.26% followed by Bacteroides with 10.58% and OTU *Comamonadaceae* with 7.94% of abundance (Table 4). These microorganisms have been related to some stage of anaerobic digestion, such as hydrolysis, acidogenesis, or acetogenesis [49, 50, 57].

A broad community of microorganisms is involved, including methanogens. These *Archaea* are the biologic key to the process because they accomplish the methane-forming reaction [58]. In this study, eight archaeal genera (or respective OTU) were observed in IS and FS in abundance higher than 1% of the

Table 5 Relative abundance of archaea (or candidate divisions) in microbial consortia composition on the production of methane from sugarcane vinasse (IS) and final sample (FS) ($S_0/X_0 = 1.5$ vinasse ratios)

Phylum	Class	Order	Family	Genus	IS (%)	FS(%)
Euryarchaeota	Methanomicrobia	Methanosarcinales	*Methanosaetaceae*	*Methanosaeta*	7.33	1.97
Euryarchaeota	Thermoplasmata	E2	*[Methanomassiliicoccaceae]*	vadinCA11	0.43	1.15
Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2		2.05	0.13
Euryarchaeota	Methanobacteria	Methanobacteriales	*Methanobacteriaceae*	*Methanobacterium*	4.15	0.7
Euryarchaeota	Methanomicrobia	Methanosarcinales	*Methanosarcinaceae*	*Methanosarcina*	0	0.43
Euryarchaeota	Thermoplasmata	E2	*[Methanomassiliicoccaceae]*	–	0.84	0.35
Euryarchaeota	Methanomicrobia	Methanomicrobiales	*Methanoregulaceae*	*Methanolinea*	0.22	0.05
Euryarchaeota	Thermoplasmata	E2	*[Methanomassiliicoccaceae]*	–	1.05	0.42
Euryarchaeota	Thermoplasmata	E2	*[Methanomassiliicoccaceae]*	*Methanomassiliicoccus*	1.98	0.16
Crenarchaeota	MCG	pGrfC26	–	–	2.39	0.15
Euryarchaeota	Methanomicrobia	Methanomicrobiales	–	–	0.35	0.05
Euryarchaeota	Methanomicrobia	Methanomicrobiales	*Methanospirillaceae*	*Methanospirillum*	0.02	0.01
Euryarchaeota	Methanomicrobia	Methanosarcinales	*Methanosarcinaceae*	*Methanomethylovorans*	0.02	0
Crenarchaeota	MCG	–	–	–	0.03	0
Crenarchaeota	Thermoprotei	–	–	–	0.02	0

total sequences. The most abundant microorganism of the phylum Crenarchaeota was an OTU belonging to the class MCG (pGrfC26) (2.39% in IS and 0.1% in FS). The most prevalent genera or respective OTU from the phylum Euryarchaeota in FS were *Methanosaeta* 1.97%, VadinCA11 (OTUs belonging to the family *Methanomassiliicoccaceae*) (1.15%), and *Methanobacterium* 0.7% (Table 5). These genera were probably the methane producers observed in the biogas [15].

The genus *Methanosaeta* is composed by acetoclastic species, capable of forming long and thin filaments that provide granules and syntrophy. *Methanobacterium* also produce filaments, but short, and are the most closely related hydrogenotrophic species to the final anaerobic digestion phase [59]. The genus *Methanosarcina* is well known to be together with Methanosaeta, the largest producer of methane, but it appears only in 0.43% in the FS sample. This reduced participation of *Methanosarcina* can be explained because this microorganism is sensible to propionic acid [60] and this acid was found in SF from 206.2 to 69.8 mg L^{-1} in this study.

The genus *Methanosaeta* was related to anaerobic digestion in the analysis of the microbial community of 22 anaerobic mesophilic digesters treating urban sewage [61]. In a study with anaerobic reactors for large-scale methane production, using 16S rRNA gene sequencing, [62, 63] found archaeal OTUs affiliated to the orders Methanosarcinales, Methanomicrobiales, phylum Crenarchaeota, and the candidate group Arc I. In summary, these groups of prokaryotes found in our anaerobic digestion ensure the production of methane, including hydrolysis, acidogenesis, acetogenesis, and vinasse methanogenesis. Also at low abundance (lower than 0.02% of the total sequences), the genus *Methanospirillum* was reported by Tsushima et al. [64], a hydrogenotrophic archaea at low temperatures.

With regard to the abundance of each taxon, there was an increase of some microorganisms in IS when compared with FS. The genera *Chryseobacterium*, *Flavobacterium*, *Sphingobacterium*, *Pseudomonas*, Syntrophus, *Sulfurimonas*, and the OTUs SHA-114, FCPU426, Hyd24-12, and ABY1 were at least 100 times more abundant in IS than in FS. The genus *Ruminococcus* and the OTUs BSV43 and R4-41B were at least 100 times more abundant in FS than in IS (Fig. 4).

4 Conclusions

The evaluation of the methanogenic activities showed that the bioassay with higher efficiency in producing methane after 236 h was in the $S_0/X_0 = 1.5$ ratio, with a value of 59.78 mmol CH_4 L^{-1}. In the microbial consortia, not all the microorganisms present could be identified at the genus level. Although not all the microorganisms present in the consortia could be identified at the genus level, a great variety was identified by high-throughput sequencing. This technique was able to distinguish the communities from the initial and final sampling times.

At the final sampling point from the highest methane production, the most prevalent phyla were Bacteroidetes, Firmicutes, and Proteobacteria. It can be observed the consumption of the acids during the production of methane was observed, especially the propionic and the presence of

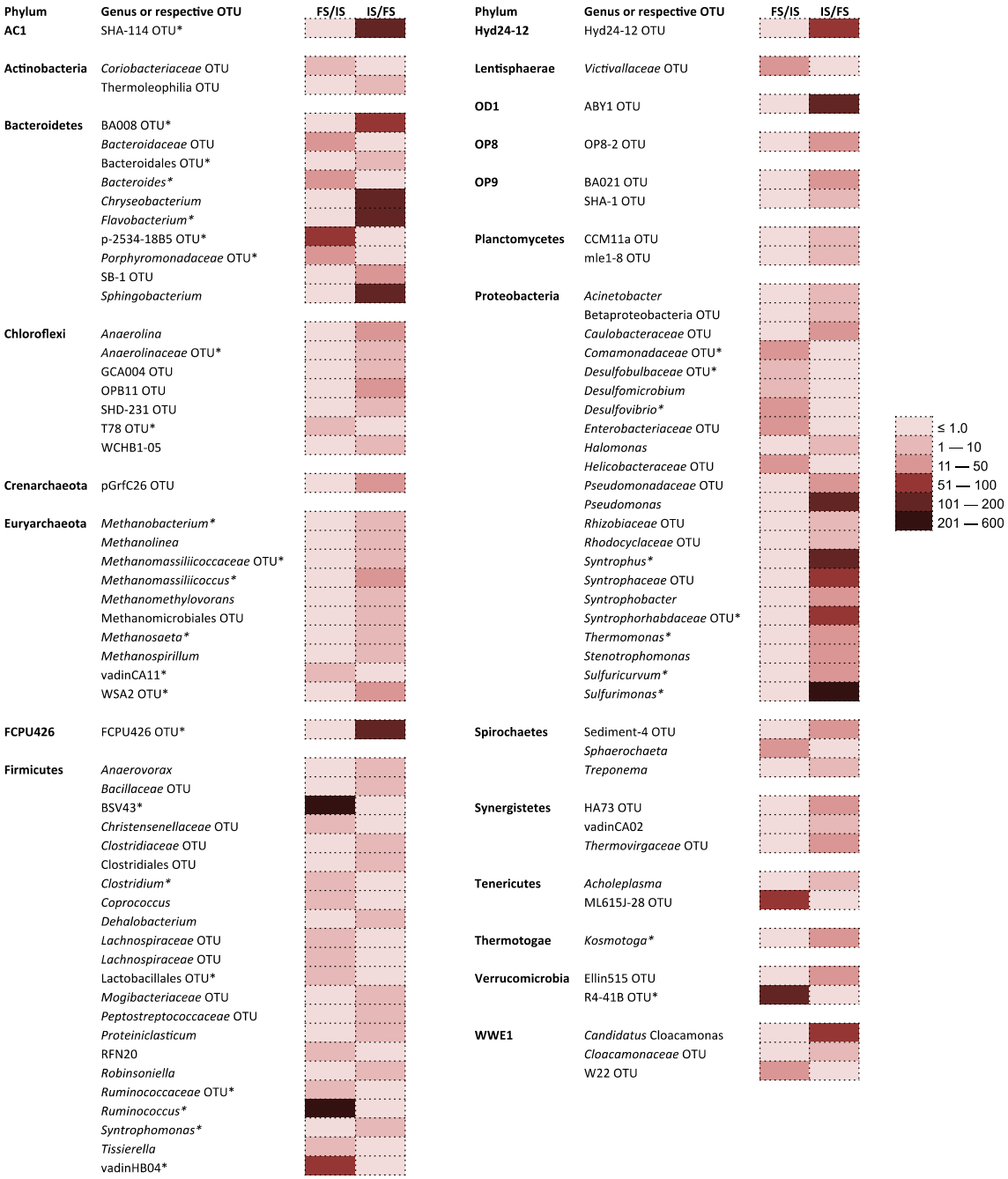

Fig. 4 Ratio of microbial genera (or respectively OTUs) in the bioassay of methane production in initial (IS) and final (FS) sample ($S_0/X_0 = 1.5$ vinasse ratios)

syntrophic groups like Syntrophobacter. A genus unknown from the order Bacteroidales was the most prevalent in the highest production of methane, followed by *Bacteroides* and an OTU belonging to the family *Comamonadaceae*. As regards Archaea, the most prevalent were the genera *Methanosaeta*, *Methanobacterium, Methanosarcina* (Euryarchaeota), and OTU VadinCA1.

The identification of the most prevalent genera and the relation of compounds generated in the anaerobic digestion serve to understand the participation of the microorganisms in the process and develop strategies in the bioprospection of products of biotechnological interest. The identification of the microorganisms of consortia involved in anaerobic digestion can collaborate on technologies to increase methane production through microbial isolation, bioaugmentation, and co-cultures.

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