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Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added short-chain organic acids and alcohol production

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The role of sugarcane vinasse as a nutrient source and the impacts of different inoculum pretreatment methods (acid-thermal and thermal treatment) were assessed in acidogenic systems aiming to produce value-added short-chain organic acids (SCOA) and alcohols. In-depth microbiome characterization was also conducted by high-throughput sequencing of the 16S rRNA gene using the Miseq Illumina platform. SCOA production was 47.3 % higher in vinasse-fed reactors, with isobutyric (up to 10.3 g L^{-1}) and butyric (up to 10.6 g L^{-1}) acids as the primary metabolites most likely resulting from lactate conversion. Ethanol comprised the main product from solventogenic pathways in all conditions, with values ranging between 2.7 and 5.2 g L^{-1}, whereas no butanol was detected. Microbial analyses revealed high relative abundance values for the *Clostridium*, *Lactobacillus*, *Bacillus* and *Ruminococcus* genera, with the predominance of the *Clostridium* genus (17%) in acid-thermal treatment reactors and the *Lactobacillus* genus (37%) in thermal treatment reactors. Overall, vinasse proved to be a suitable substrate for value-added SCOA production, which characterizes a potential management approach to this wastewater stream. In this sense, the biochemical production of butyrate from vinasse could diversify the product portfolio of sugarcane biorefineries, also minimizing bioenergy losses by converting residual carbon fractions.

[**Key words:** Sugarcane biorefinery; Vinasse management; Dark fermentation; Value-added soluble products; Inoculum pretreatment; Molecular analyses]

Concern about greenhouse gas emissions and the scarcity of fossil fuels has been stimulating research that seeks the best use of waste before its disposal in the environment (1). Considering this, the anaerobic digestion (AD) process stands out as an alternative for waste treatment allied to energy recovery and/or to the generation of by-products potentially used as raw materials or renewable energy sources for other processes/products. Among such by-products, the production of organic acids and alcohols has attracted attention, although conclusive research on them is still insufficient (2).

Butyric acid, $CH_3(CH_2)_2COOH$, is an intermediate compound of AD systems with a wide range of applications such as chemical, textile, plastic, food, beverages, dairy and pharmaceutical industries (3). Chemical synthesis is the most common process for butyric acid production using crude oil as a raw material due to lower production costs and large-scale supply (4). However, the growing demand for natural products, the rising concerns about the environment and the decreasing availability of crude oil has intensified efforts to develop microbial fermentation technologies for butyric acid production from renewable biomass (5). On the other hand, large-scale production of butyric acid from anaerobic processes is a challenge due to the high complexity and difficulty to control the process. AD comprises a wide microbial community acting in synergy and in a self-regulatory way. The metabolic pathway of the butyric acid producers should be favored in order to direct the fermentation to the formation of this product by evaluating various factors of the process, e.g., substrate concentration, pH, hydrogen partial pressure, distribution and inhibitory effects of final products. Considering that specific conditions and process parameters can favor different microorganisms, the use of microbial consortia can minimize inhibition problems associated to intermediary and/or final products (6). Additionally, obtaining a mixed native culture from microbial consortia may be a potential way to reduce costs of a process when compared to the use of pure cultures, as the costly methodologies for isolating and purifying microorganisms can be sometimes prohibitive. The use of an undefined microbial culture processing step is also advantageous to handle the complexity of organic waste streams when addressing to a commercial process to convert waste to a bio-product (7).

Apart from the common uses of butyric acid, this soluble metabolite can also be a source of a biofuel production as it is a

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precursor of butanol (8). Biobutanol is a promising alternative vehicular fuel to replace gasoline in the future. It also offers some advantages over ethanol, especially regarding higher energy combustion, and is able to substitute gasoline in internal combustion engines without any mechanical modifications (9). The biological process for butanol production occurs by acetone–butanol–ethanol (ABE) fermentation conducted by ABE-producing clostridia microorganisms. The ABE fermentation has two phases in an energy acquiring pathway, one acidogenic and the other solventogenic. During acidogenesis, cells grow exponentially producing acetic and butyric acid. The accumulation of organic acids results in a decrease of pH (below 5.0), favoring the metabolic route shift to solventogenic phase. Then, *Clostridium* sp. produces endospores and the acids are reutilized to produce ABE solvents in stationary growth. Studies have shown that the concentration of butyric acid is essential for the synthesis of butanol. Tashiro et al. (10) achieved an increase in the butanol specific yield (g-butanol g^{-1} cells) and productivity (g-butanol g^{-1} glucose) of 54% and 72%, respectively, by adding butyric acid (5 g L^{-1}) or adjusting the butyrate/glucose ratio to 1:4. Al-Shorgani et al. (11) also reported the enhancement of butanol production by adding butyric acid to the ratio 2:15 (w/w) relatively to glucose. The final butanol concentration, productivity and yield increased by 82.3%, 50% and 15%, respectively.

Sugarcane vinasse, a voluminous wastewater from ethanol production, may have the potential for butyric acid generation as the AD of this stream is applicable (12). Most studies have focused on biogas production from vinasse for energy and biomethane generation (13,14), showing gaps in the literature regarding short-chain organic acids (SCOA) and alcohol production, except when there is imbalance of an AD process leading to the collapse of the system. It is believed that the application and control of specific fermentation parameters and conditions favoring butyric acid producers could lead to the controlled generation of this intermediate. Although biological butyric acid production and separation is costly and biotechnologically more immature (15) when compared to AD focusing on biogas, the added-value of the former product might be higher than this latter, allowing a more positive economic balance. However, the carboxylate platform where is inserted the butyric acid bio-production is a new trend (7), deserving attention the fundamental research about it.

In this work, the production of SCOA and alcohols from dark fermentation of sugarcane vinasse was assessed by using a pretreated mixed culture from anaerobic sludge. Different pretreatments were applied aiming to favor the acidogenic and solventogenic microbial populations. The sequential enrichment of the pretreated cultures was evaluated in batch tests using controlled organic substrate (sucrose) with and without vinasse supplementation as nutrient and complementary carbon source. Molecular biology techniques were further applied to identify the main microbial populations associated with the predominant fermentative pathways. This is the first study assessing the potential of sugarcane vinasse to produce value-added SCOA and alcohols in acidogenic systems, which frequently aim to recover only gaseous products (e.g., biohydrogen).

MATERIALS AND METHODS

Sugarcane vinasse Sugarcane vinasse was collected from a full-scale ethanol and sugar mill located in Pradópolis, São Paulo State, Brazil. A single sampling was carried out specifically at the beginning of the 2014/2015 harvesting period (May). Vinasse samples were kept at −15°C to prevent previous degradation. Aliquots of vinasse were centrifuged at 6000 rpm for 5 min due to high total solids concentration (26.63 g L^{-1} on average) and only the liquid phase was further used. The composition of vinasse was analyzed in terms of organic acids, carbohydrates, ethanol and micronutrients after solids removal, as follows (g L^{-1}): malic acid (10.46 ± 0.61), succinic acid (3.72 ± 0.45), lactic acid (10.13 ± 0.27), acetic acid (0.22 ± 0.03), iso-butyric acid (3.41 ± 0.19), butyric acid (0.61 ± 0.25), glucose (0.41 ± 0.08), sucrose (1.28 ± 0.10), ethanol (0.25 ± 0.05). Additionally, total nitrogen (TN), nitrate (NO_3^-) and sulfate (SO_4^{2-}) concentrations of 840, 460, and 900 mg L^{-1}, respectively, were measured in vinasse, whereas concentrations of specific metals, i.e., Fe^{2+}, Ca^{2+}, Mg^{2+} and K^+, reached 9.6, 510, 260 and 2650 mg L^{-1}, respectively. Trace levels of Pb, Cd, Cu, Cr and Ni were also detected.

Inoculum and pretreatment methods The inoculum was obtained from a full-scale 3-chamber anaerobic baffled reactor used to treat cattle free-stalls and dairy barn wastewater. Equal aliquots of sludge were collected in each chamber. Sludge samples were mixed and stored at 4°C prior to the assays.

Two methods of pretreatment, i.e., thermal (TT) and acid-thermal treatment (AT), were applied to the inoculum to enhance the prevalence of spore-forming bacteria, based on previous reports (15,16). Coarse solids were removed from the sludge samples by sieving them prior to the pretreatment.

For the TT method, a sludge aliquot of 300 mL was heated up to boiling for 30 min, then cooled in an ice bath and stored at 4°C for 24 h. The AT method was initially conducted by heating 300 mL of sludge for 30 min at 80°C. The sample was then cooled to room temperature and the pH was adjusted to 3.0 by adding HCl 0.1 N. Finally, the pretreated inoculum was stored at 4°C for 24 h. Sequentially, 20 mL (10% v/v) of each pretreated inoculum (I_0) was added separately to Duran flasks containing 180 mL of autoclaved (120°C, 30 min at 14.7 psi) Reinforced Clostridium Medium (RCM) (17) with pH adjusted to 6.8. The flasks were fluxed with nitrogen (N_2) for 5 min and then incubated for 24 h at 35°C. Then 20 mL of each inoculum was transferred to 180 mL of fresh RCM, following the same steps described above. This procedure was repeated three times, resulting in three generations of enriched inoculum (I_1, I_2 and I_3) for each pretreatment type.

Batch fermentation tests Batch fermentation tests were performed using 500 mL Duran flasks in triplicates for each condition. The reactors were fed with synthetic culture medium (SCM; Table 1) modified from Monot et al. (18) containing sucrose as the primary organic source. The working volume (300 mL) of the reactors was composed of: micronutrients (30 mL), macronutrients (30 mL), vitamins (30 mL), buffer (30 mL), reducing agent (0.75 mL) and inoculum (15 mL), with the remaining volume filled with water. The effect of vinasse as a nutrient source for VFA and alcohol production was assessed by replacing the macro and micronutrients of SCM by increasing vinasse proportion (v/v), i.e., 1:3 (B1), 1:1 (B2) and 2:1 (B3). Initial carbohydrate concentration in both sucrose and vinasse tests was set as 20 g L^{-1} and the initial pH was adjusted to 6.9 by adding 1N HCl. Enriched inocula AT and TT from generation I_3 were used in the sucrose and vinasse assays, so that triplicates were monitored for each condition: ATV, inoculum from acid-thermal treatment in SCM supplemented with vinasse; ATS, inoculum from acid-thermal treatment in SCM without vinasse; TTV, inoculum from thermal treatment in SCM supplemented with vinasse; and TTS, inoculum from thermal treatment in SCM without vinasse. Serial subculturing from the enriched inocula (I_3) was performed in B1, B2 and B3 aiming at adapting microorganisms to the increasing vinasse doses. In this case, the microbial consortia resulting from vinasse fermentation in B1 flasks was used as the inoculum (15 mL) in B2 flasks. Similarly, the acidogenic biomass from B2 flasks was used as inoculum (15 mL) in B3 flasks.

The flasks were fluxed with N_2 for 5 min to provide anaerobic conditions and then incubated for 165 h in a shaker at 37°C and 60 rpm. The fermentation products (SCOA and solvents) were analyzed in the raw (time = 0) and fermented (time = 6 d) substrate, whereas pH was monitored every two days.

Analytical methods and performance assessment The pH measurements were carried out according to the standard methods (19), whereas carbohydrates (glucose, fructose and sucrose), SCOA and alcohols were analyzed with a high-performance liquid chromatography system (HPLC, Shimadzu Scientific Instruments, Columbia, MD, USA), as reported elsewhere (20). The HPLC was equipped with a pump (LC-10ADVP), autosampler (SIL-20A HT), column oven

TABLE 1. Composition of the synthetic culture medium used in batch tests.

Solution	Compound	Concentration (g L^{-1})
Micronutrient	$MgSO_4 \cdot 7H_2O$	2.0
	$FeSO_4 \cdot 7H_2O$	0.5
	$MnSO_4$	0.2
Macronutrient	KH_2PO_4	10.0
	K_2HPO_4	10.0
	$NH_4CH_3CO_2$	11.0
	$(NH_4)_2SO_4$	25.0
Vitamin	Yeast extract	30.0
	$C_6H_5NO_2$	0.01
	$C_8H_{11}NO_3$	0.02
	$C_{17}H_{20}N_4O_6$	0.01
	$C_{12}H_{17}N_4OS$	0.01
	$C_7H_7NO_2$	0.001
Reducing Agent	$C_3H_7NO_2S$	40.0
Buffer	$NaHCO_3$	30.0

(CTO-20A), UV-diode array detector (SDP-M10 AVP), refraction index detector (RID-10A), system controller (SCL-10AVP) and an Aminex HPX-87H column (300 mm, 7.8 mm, BioRad). Sulfuric acid (0.005 M) at a rate of 0.5 mL min^{-1} was used as the mobile phase, whereas the oven temperature was set at 43°C. Excessive levels of suspended solids were removed from the vinasse prior to determining SCOA and alcohols (21). An aliquot of 4 mL of vinasse was mixed with 0.5 mL of a saturated solution of barium hydroxide ($BaOH_2$) and 0.5 mL of a 5% (m/v) solution of zinc sulfate ($ZnSO_4$), and then centrifuged for 10 min at 3200 rpm. The supernatant was stored at −20°C prior to the analyses.

The performance of the fermentation tests was assessed by calculating the yield (Y; mg g^{-1}) and productivity (P; mg L^{-1} h^{-1}) of selected metabolites, as detailed in Eqs. 1 and 2. The terms [SCOA], [CH], and Δt are the final concentration of a given organic acid (mg L^{-1}), the initial concentration of carbohydrates (g L^{-1}) and the incubation period (h), respectively.

$$Y = \frac{[\text{SCOA}]}{[\text{CH}]} \tag{1}$$

$$P = \frac{[\text{SCOA}]}{\Delta t} \tag{2}$$

Statistical analysis Statistical analysis was performed by using the RStudio Desktop 0.99.441 software. Data regarding SCOA and alcohol average concentrations were compared through by analysis of variance with an interaction of three factors (three-way ANOVA), i.e., culture medium, pretreatment and batch test, to evaluate whether there were differences between and within groups at a 95% confidence interval. Variance homogeneity from the interactions was first verified by the Levene test. Group differences were identified by Tukey's honest significant difference test.

Microbial diversity analysis by 16S rRNA gene sequencing For the in-depth microbiome characterization, the microbial community was analyzed by massive amplicon sequencing using a Miseq−Illumina, 2 × 250 (Illumina Inc., San Diego, CA, USA). Two compose samples obtained from the three replicates for ATV- and TTV-flasks after B3 tests were analyzed. The samples (15 mL) were washed with phosphate buffer and centrifuged at 4000 rpm for 15 min. This procedure was repeated to obtain pellets of 10 mL, which were kept at −20°C prior to DNA extraction. Total DNA extraction was performed using a modified phenol−chloroform protocol described elsewhere (22). The DNA quality was assessed by a standard of 260/280 nm >1.8, as measured by an ND-2000 spectrophotometer (Nanodrop Inc., Wilmington, DE, USA) and agarose gel electrophoresis. A forward primer 16S rRNA (S-D-Bact-0341-b-S-17) (CCTACGGGNGGCWGCAG) and a reverse primer S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) were used to flank the V3 and V4 16S rRNA gene hypervariable region (23).

The CASAVA (1.8.2) software was used for pre-processing the samples. Adaptors, barcodes, primers and sequences containing ambiguous 'N', Phred quality <24 and sequences shorter than 65 bp were removed using the Software Seqyclean (https://bitbucket.org/izhbannikov/seqyclean). The UNIVEC software was used for vector contamination removal. The sequences were aligned using the Pynast (24) and the operational taxonomic units (OTU) were determined using 97% sequence similarity. The taxonomic classification was performed using the Ribosomal Database Project (25). For this purpose, the adopted confidence threshold was 80% for genus and 50% for other taxonomic levels (Phylum-Family). Alfa (Chao1 and Shannon) diversity was quantified using Past software (26). Data were normalized before the diversity analysis and comparison of the relative abundances using a QIIME script. Moreover, the Statistical Analyses of Metagenomic Profiles (STAMP) software v 2.0.9 was used for statistical assessment in order to detect biologically relevant differences in the relative proportion of sequences between the samples. As recommended by STAMP developers, the significance of the differences between samples was assessed by the Two-sided Fisher's Exact test, and Storey's FDR method was used for multiple test correction (27). The most important taxonomies were selected by filtering using the q-value ($p > 0.05$).

The sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk) under the accession number ERS1396357 (AT) and ERS1396356 (TT). The project accession number is PRJEB15712.

RESULTS AND DISCUSSION

Fermentation performance: products from acidogenesis and solventogenesis The results obtained for SCM containing only sucrose and supplemented with vinasse were compared to evaluate the influence of vinasse on the production, yield and productivity of the main SCOA and alcohols in dark fermentation process. The results were also compared relatively to the two pretreatments as well as the microbial diversity and vinasse dosage. The assessed conditions promoted the dominance of acidogenesis process, with high SCOA production levels, whilst solventogenesis only marginally occurred.

The assessed pretreatment methods of inoculum (acid-thermal and thermal treatments) did not differ relatively to the SCOA production. Conversely, dosing vinasse to the reactors enhanced SCOA production compared to systems fed only with sucrose: isobutyric and butyric acids were the main SCOA produced with the first substrate, while lactic acid was the main acid produced in the latter. Table 2 shows SCOA and ethanol concentrations detected in the fermented substrate of each batch test. SCOA production was 47.3% higher in SCM supplemented with vinasse compared to the use of sucrose as the only carbon source, with no meaningful variations ($p > 0.05$) between biomass from ATV and TTV pretreatments (22.3 ± 5.1 g L^{-1} and 21.3 ± 1.4 g L^{-1}). The main metabolites were isobutyric and butyric acid for ATV and TTV, respectively (Table 2). Butyric acid production increased with the increasing dilution of vinasse, while isobutyric acid concentrations remained similar for dilutions 1:1 (B2) and 2:1 (B3). For SCM with sucrose, there was no difference ($p > 0.05$) in isobutyric and butyric acid production between the ATS and TTS treatments.

Lactic acid concentrations in the SCM with sucrose and vinasse in all treatments were similar, showing a decreasing behavior, i.e., lactate was consumed as the acidogenic biomass was subcultured (from B1 to B3), regardless of the vinasse concentration. The results suggest an adaptation of the inoculum, in order to promote the use of lactate as substrate for butyric and iso-butyric acid production. Differences in pH variation most likely did not impact the observed metabolite profiles, with values closely ranging between 6.14 and 6.87 (Table 2), regardless of dosing vinasse and type of inoculum treatment. In particular, using vinasse as substrate in acidogenic systems tends to favor butyrate production, as reported elsewhere

TABLE 2. Metabolite concentrations in the acidified substrates according to the fermentation test and pretreatment method.

Fermentation test	Pretreatment	Concentration (g L^{-1})				Ethanol	pH
		Lactic acid	Acetic acid	Iso-butyric acid	Butyric acid		
B1	ATS	8.80 ± 1.2	1.07 ± 0.14	1.62 ± 1.04	3.50 ± 0.33	2.72 ± 0.19	6.87
	TTS	8.10 ± 0.0	2.03 ± 0.07	1.15 ± 0.15	5.09 ± 0.95	3.63 ± 0.58	6.20
	ATV	6.59 ± 1.60	0.45 ± 0.02	3.76 ± 0.22	6.55 ± 0.93	3.42 ± 0.38	6.87
	TTV	8.79 ± 2.33	1.23 ± 0.60	3.73 ± 0.15	6.40 ± 1.23	3.04 ± 0.41	6.15
B2	ATS	5.94 ± 1.48	0.46 ± 0.35	2.55 ± 1.04	5.15 ± 0.49	3.37 ± 0.37	6.55
	TTS	6.39 ± 0.31	0.81 ± 0.27	4.98 ± 0.28	4.83 ± 0.64	3.00 ± 0.34	6.39
	ATV	5.81 ± 0.95	0.07 ± 0.02	10.34 ± 0.43	10.60 ± 0.62	3.85 ± 0.16	6.37
	TTV	6.15 ± 0.89	0.87 ± 0.07	6.84 ± 0.56	8.41 ± 0.62	2.94 ± 0.27	6.39
B3	ATS	−0.10 ± 0.04	0.51 ± 0.03	4.11 ± 0.82	7.50 ± 0.72	4.66 ± 0.23	6.41
	TTS	−0.22 ± 0.03	0.60 ± 0.28	3.12 ± 0.30	8.29 ± 0.05	4.75 ± 0.59	6.29
	ATV	−2.84 ± 0.46	2.19 ± 1.01	8.63 ± 1.31	14.13 ± 0.77	5.16 ± 0.57	6.66
	TTV	−4.17 ± 0.37	4.13 ± 0.06	7.61 ± 0.89	13.21 ± 0.60	5.10 ± 0.38	6.14

Vinasse proportion relative to the total batch volume (300 mL): B1, 1:3; B2, 1:1 and B3, 2:1. Negative values indicate consumption of the compound.

TABLE 3. Yield and productivity for selected metabolites (acetic acid, butyric acid and ethanol) according to the fermentation test and pretreatment method.

Fermentation test	Pretreatment	Acetic acid		Butyric acid		Ethanol	
		Yield (mg g^{-1})	Productivity (mg L^{-1} h^{-1})	Yield (mg g^{-1})	Productivity (mg L^{-1} h^{-1})	Yield (mg g^{-1})	Productivity (mg L^{-1} h^{-1})
B1	ATS	50 ± 10	6.5 ± 0.9	160 ± 10	21.2 ± 2.0	130 ± 10	16.5 ± 1.2
	TTS	70 ± 60	12.3 ± 0.4	250 ± 50	30.9 ± 5.8	180 ± 30	22.0 ± 3.5
	ATV	10 ± 10	2.7 ± 0.1	320 ± 30	39.7 ± 5.6	110 ± 90	20.7 ± 2.3
	TTV	60 ± 30	7.5 ± 3.6	320 ± 60	38.8 ± 7.5	100 ± 90	18.5 ± 2.5
B2	ATS	10 ± 20	2.8 ± 2.1	250 ± 30	31.6 ± 3.0	170 ± 20	20.7 ± 2.2
	TTS	40 ± 10	4.9 ± 1.7	250 ± 30	29.7 ± 3.9	150 ± 20	18.4 ± 2.1
	ATV	0	0.4 ± 0.1	540 ± 40	65.0 ± 3.8	200 ± 10	23.6 ± 1.0
	TTV	30 ± 30	5.3 ± 0.4	440 ± 50	51.6 ± 3.8	150 ± 20	18.0 ± 1.7
B3	ATS	20 ± 10	3.0 ± 0.2	370 ± 40	43.8 ± 4.2	230 ± 10	27.3 ± 1.3
	TTS	20 ± 20	3.5 ± 1.6	290 ± 250	48.5 ± 0.3	250 ± 30	27.8 ± 3.4
	ATV	110 ± 50	12.8 ± 5.9	690 ± 40	82.7 ± 4.5	250 ± 30	30.2 ± 3.3
	TTV	140 ± 120	24.2 ± 0.4	680 ± 40	77.3 ± 3.3	260 ± 20	29.9 ± 2.2

Vinasse proportion relative to the total batch volume (300 mL): B1, 1:3; B2, 1:1 and B3, 2:1.

(28,29). Lappa et al. (30) found similar results when comparing the use of sucrose and simulated sugar beet vinasse for SCOA and ethanol production, observing the decrease of lactic acid followed by the increase of butyric acid productivity in the tests with vinasse. Fuess et al. (31) also highlighted the key-role lactate to produce butyric acid and hydrogen in a sugarcane vinasse-fed acidogenic system. In turn, Bourriaud et al. (32) evaluated lactate fermentation using labelled compounds, in order to observe that lactate is characterized as a precursor for butyrate synthesis by human intestinal microflora.

Concentrations of *n*-butanol were below the detection limit of the chromatographic method employed (<0.2 g L^{-1}) in all assessed conditions, indicating the establishment of unfavorable conditions for butanol-producing bacteria, despite the predominance of the main butanol precursor, i.e., butyric acid, in vinasse-supplemented reactors. Increasing concentrations of butyric acid may have inhibited the growth of butanol-producing clostridia species (33). Tashiro et al. (34) reported very low butanol production levels (0.2 g L^{-1}) from 10 g L^{-1} butyric acid as the only carbon source using non-growing cells, revealing that the combination of this acid with glucose is the main factor for the determination of butanol production from butyric acid. The absence of a C6 source balancing the butyric acid produced in the current experiment may have hampered the potential for butanol production from vinasse.

Table 3 shows the yield and productivity for the main SCOA and ethanol produced during the fermentation process. The yields were calculated based on the initial total carbohydrate concentration measured as glucose, fructose and sucrose. The butyric acid yield rose by increasing the vinasse dosage for both pretreated inoculum, achieving 690 mg g^{-1} in B3, i.e., approximately two-fold higher than the yield from sucrose medium without vinasse (Table 3). The type of inoculum treatment did not influence butyric acid and

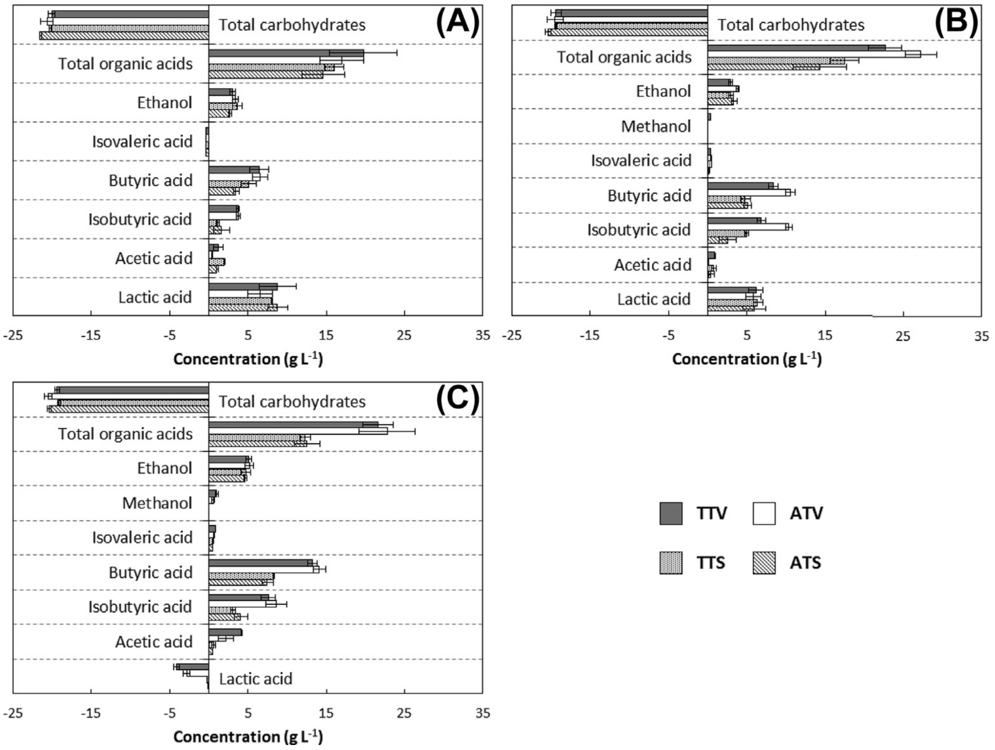


FIG. 1. Primary metabolites consumed/produced in dark fermentation tests according to subcultures (A) B1, (B) B2 and (C) B3. Vinasse proportion relative to the total batch volume (300 mL): B1, 1:3; B2, 1:1 and B3, 2:1.

ethanol production. Acetic acid production was lower than the butyric acid production, with the highest yield in the B3 assay with vinasse supplementation. The highest ethanol productivity occurred in the B3 assay in the ATV condition (30.17 ± 3.34 mg L h^{-1}; Table 3). However, the differences between the fermentation conditions (TTV and ATV) were not significant ($p > 0.05$). Compared to the SCOA data, ethanol productivity was considerably lower, indicating a low solventogenic activity.

According to Lappa et al. (30), the ethanol yield factor tends to decrease when the continuous acidogenesis proceeds. Total acid (dissociated + undissociated, acetic + butyric acid) concentration of 240–250 mmol L^{-1} in the medium is reported as an inhibitor of solvetogenesis (35), thus hampering the ethanol production relative to SCOA generation, as observed in the present work. The average production of SCOA and alcohols detected in the fermentation tests is presented in Fig. 1, where negative values indicate consumption of the metabolite. The sum of acetic, isobutyric and butyric acid concentrations were 110 ± 26 mmol L^{-1}, 162 ± 64 mmol L^{-1} and 220 ± 94 mmol L^{-1} in B1, B2 and B3, respectively, with a significant difference ($p < 0.05$) between B1 and B3. Thus, vinasse dosing had a significant impact on the SCOA production. On the other hand, there was no significant difference among the results concerning inoculum pretreatment (ATS, TTS, ATV and TTV).

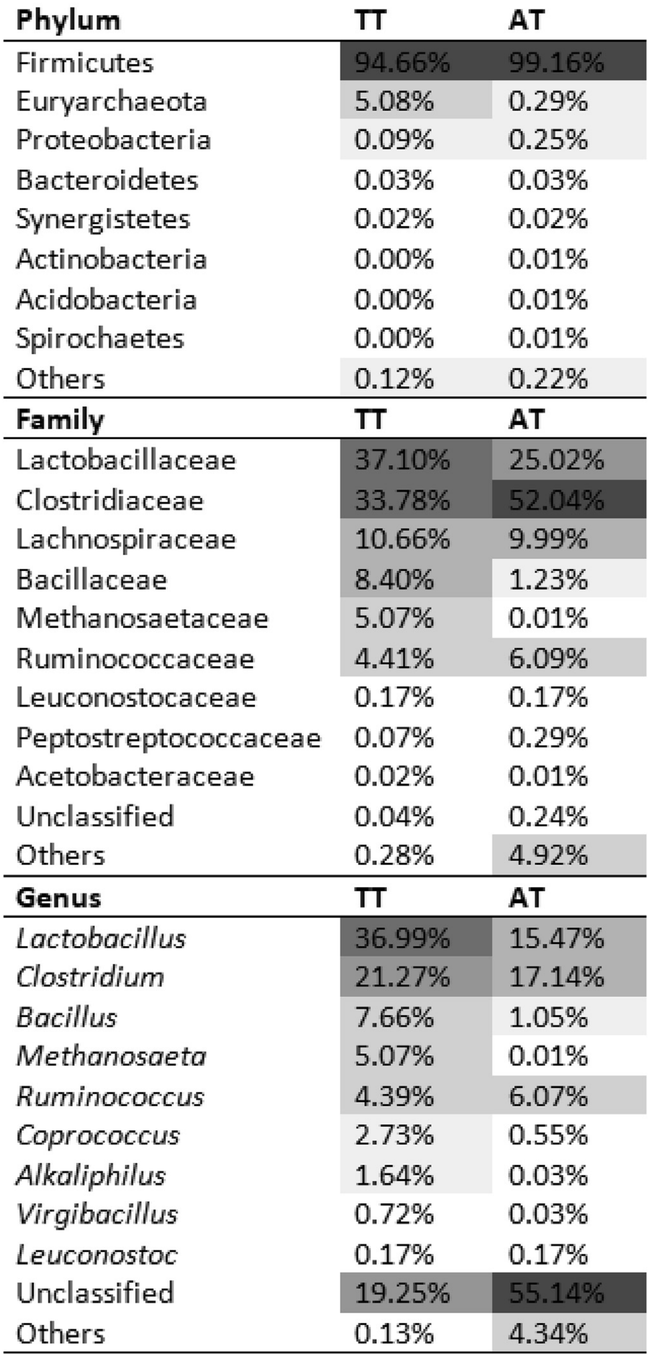
The current results are comparable to experiments aiming the maximization of butyric acid production from waste applying a mixed microbial fermentation (36). Specifically from vinasse, some works were reported by using pre-treated anaerobic inoculum or from natural fermentation, but focusing on bio-hydrogen production (28,37,38). In this case, SCOA production occurred marginally, being the maximum butyric acid concentrations (reported in the rage of 0.1–4.8 g L^{-1}) achieved when the hydrogen productivity was unsatisfactory due to the generation of more reduced byproducts as propionate and ethanol (39,40). On the other hand, higher butyric acid can be reached when the bioprospecting of SCOA producing consortia is performed: Sydney et al. (41) achieved 10 g L^{-1} of butyric acid from sugarcane vinasse conversion when using an enriched consortium obtained from soil used for sugarcane cultivation.

Microbial analyses: taxonomic profiles Overall, Illumina sequencing yielded approximately 260,000 sequences for both samples. After the quality control and normalization process, a total of approximately 123,000 sequences were retained for each sample with an average sequence length of 200 bp. The total number of OTUs ranged from 1087 to 1415 with a Good's coverage estimate of over 99%. These results were corroborated by richness estimators (Chao 1) and diversity measured (Rarefaction) data. The Chao 1 values ranged from 1866 to 2342 for the TT and AT, respectively. A slight difference of the Shannon Index values was obtained comparing TT (H' = 3.03) and AT (H' = 3.29). In general, values greater than 5.0 indicate a biomass environment with a very high diversity as in soil (42) and anaerobic reactors (43,44).

After ranking the representative sequences of each OTU by the RDP-Classifier, over 99% of the sequences were classified for the family and phylum level, a great amplitude was observed for the genus level (81% and 45% for TT and AT, respectively). Since the samples were submitted to the same sequence analysis workflow, the observed differences in the percentage of unclassified reads are related to the intrinsic characteristics of each treatment. In this way, the most plausible hypothesis is that the association of two types of treatment (acid and thermal, AT sample) may have selected microorganisms with a low match in the database used.

Despite, AT and TT samples showed similar concentrations of SCOA production, STAMP analysis identified a significant statistical difference of some taxonomies. For the phylum level, only Euryarchaeota, Firmicutes and Proteobacteria showed statistical differences (Figs. 2 and 3). On the other hand, at the genus level, 15 distinct genera showed significant statistical differences. The main ones were *Lactobacillus*, *Methanosaeta* and *Bacillus*. Different microorganisms with metabolic potential for same pathways are common in microbial consortium. This strategy is defined as functional redundancy and it is considered as insurance to maintain ecosystem functions under changing environmental conditions. Additionally, this strategy can make the anaerobic digestion a process robust and plastic (45).

The results on the highest percentage of the Archaea domain and *Methanosaeta* genus in the TT sample suggest that applying TT method was not effective in completely eliminating methanogens.



Phylum	**TT**	**AT**
Firmicutes	94.66%	99.16%
Euryarchaeota	5.08%	0.29%
Proteobacteria	0.09%	0.25%
Bacteroidetes	0.03%	0.03%
Synergistetes	0.02%	0.02%
Actinobacteria	0.00%	0.01%
Acidobacteria	0.00%	0.01%
Spirochaetes	0.00%	0.01%
Others	0.12%	0.22%
Family	**TT**	**AT**
Lactobacillaceae	37.10%	25.02%
Clostridiaceae	33.78%	52.04%
Lachnospiraceae	10.66%	9.99%
Bacillaceae	8.40%	1.23%
Methanosaetaceae	5.07%	0.01%
Ruminococcaceae	4.41%	6.09%
Leuconostocaceae	0.17%	0.17%
Peptostreptococcaceae	0.07%	0.29%
Acetobacteraceae	0.02%	0.01%
Unclassified	0.04%	0.24%
Others	0.28%	4.92%
Genus	**TT**	**AT**
Lactobacillus	36.99%	15.47%
Clostridium	21.27%	17.14%
Bacillus	7.66%	1.05%
Methanosaeta	5.07%	0.01%
Ruminococcus	4.39%	6.07%
Coprococcus	2.73%	0.55%
Alkaliphilus	1.64%	0.03%
Virgibacillus	0.72%	0.03%
Leuconostoc	0.17%	0.17%
Unclassified	19.25%	55.14%
Others	0.13%	4.34%

FIG. 2. Heat map analysis of the most abundant phyla, families and genera detected in TT and AT samples after B3 tests.

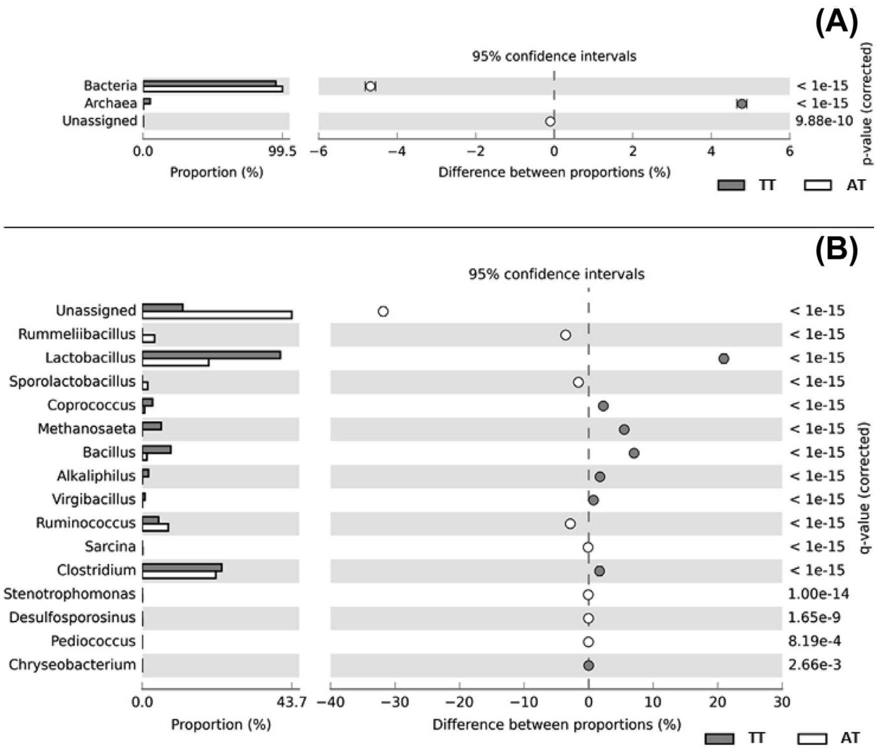


FIG. 3. Statistical comparison of the inoculum pretreatment methods (acid-thermal, AT; thermal treatment, TT) for (A) phyla and (B) genera. The statistical significance of the differences between samples was assessed using the Stamp software.

The sum of relative abundance of methanogens genus were 0.3% and 0.002% in the samples TT and AT, respectively. The low percentage of AT samples indicates a better efficiency of the combined pretreatment methods to eliminate methanogens microorganisms that are undesirable to SCOA production.

Furthermore, *Clostridium*, *Lactobacillus*, *Bacillus* and *Ruminococcus* genera showed significant statistical difference between the samples. All genera were closely related with the production of metabolites in the monitored reactors. *Lactobacillus* (37%) and *Clostridium* (21%) genera were predominant in the TT sample, whereas, *Clostridium* (17%) was the most representative in AT (followed by *Lactobacillus*, i.e., 15%). In addition, *Bacillus*, *Ruminococcus* and *Coprococcus* genera found in both samples showed relative abundance above 3%. Considering phylogenetical and biochemical aspects, both *Coprococcus* and *Ruminococcus* genera are very closely related, i.e., both belong to the Lachnospiraceae family and are obligate chemoorganotrophic anaerobes capable of fermenting cellobiose. The main fermentation products include butyric acid and acetic acid, followed by formic acid and propionic acid. For example, the *Coprococcus* eutactus species is recognized by the production of butyric acid, formic acid and lactic acid from glucose (46).

Similar to *Coprococcus* and *Ruminococcus*, the *Lactobacillus* genus comprises non-spore-forming lactate-producing bacteria which use glucose as substrate (47). On the other hand, both *Clostridium* and *Bacillus* genera are spore-forming bacteria. Both genera were found with higher relative abundance in TT compared to AT. The *Bacillus* genus includes rod-shaped, gram-positive, aerobic or (under some conditions) anaerobic bacteria widely found in soil and water. Species such as *Bacillus coagulans* and *Bacillus* sp. *SGP1* are able to produce lactic acid (4,46). The *Clostridium* genus includes spore-forming, gram-positive anaerobes, although some species grow under microaerophilic conditions (46). Several species have shown the ability to produce acid lactic and butyric such as *Clostridium butyricum*, *Clostridium tyrobutyricum* and *C. thermobutyricum* (5,48,49). The dominant genera among bacteria that produce hydrogen by butyrate metabolic pathway include *Clostridium* and *Bacillus*, whereas the propionate pathway is associated with *Bacteroides*, *Peptostreptococcus*, *Ruminococcus* genera (50). On the other hand, there is no consensus concerning the dominant genera for ethanol production between the hydrogen producing bacteria; some authors cite the occurrence of mainly *Ethanoligenens*, *Acetanaerobacterium*, *Clostridium* and *Rhodopseudomona* genera. In relation to *Coprococcus* genus, Pryde et al. (51) found specific genes for butyryl-CoA and acetyl-CoA, both associated with the butyrate synthesis.

In summary, the physicochemical results indicated that vinasse proved to be a suitable substrate for value-added SCOA production and that the microbiological results indicated different taxonomic profiles depending on the applied treatment (AT and TT), but both associated with the microbial community producing SCOA. Different taxonomic profiles, however, both with metabolic potential for SCOA production demonstrate the functional redundancy of the microbial consortium used, evidencing the microbiological robustness of the bioprocess. In addition, for the production of SCOA, the AT treatment was more effective in removing undesirable microorganisms as the methanogens.

Concluding remarks Dark batch-fermentation from sucrose and vinasse by pre-treated anaerobic inoculum under specific controlled conditions promoted the dominance of acidogenesis process, with high SCOA production levels. Solventogenesis only marginally occurred at the assessed conditions. The assessed pretreatment methods of inoculum (acid-thermal and thermal treatments) did not differ relatively to the SCOA production. On the other hand, the fed composition of batch fermentation supplemented with vinasse showed higher SCOA production when compared with sucrose: isobutyric and butyric acids were the main SCOA produced with the first substrate, while lactic acid was the main acid produced in the latter. The high-throughput sequencing evaluated the microbial community in depth. Overall, the *Clostridium* genus was predominant from AT sample. On the other hand, the *Lactobacillus* was predominant from the TT samples. Although the mixed native culture achieved by the pretreatments proved to be a potential producer consortium of short chain organic acids when supplemented with vinasse, the development of superior separation technologies should be pursued as the liquid extraction in a variable and mixed product spectrum is still a drawback within the waste to bio-product conversion platform.

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