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Value-added soluble metabolite production from sugarcane vinasse within the carboxylate platform: An application of the anaerobic biorefinery beyond biogas production

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ABSTRACT

Currently, wastewater processing through anaerobic digestion can generate not only biofuels such as biohydrogen and methane, but also value-added soluble-phase intermediates (e.g. carboxylic acids), which have numerous applications. This study investigates the potential of using sugarcane vinasse, the main byproduct from ethanol production, as substrate in dark fermentation to recover soluble metabolites through the biorefinery approach. The impacts of temperature (30–60 °C) and initial pH (5.0–10.0) were initially assessed in batch tests using microbial consortia obtained from the natural fermentation of vinasse. The yield (401 mg-$COD_{organic\ acids}$ $g^{-1}COD t^{initial}$) and productivity (653 mg-$COD_{organic\ acids}$ L^{-1} d^{-1}) of organic acids were maximized at alkaline/mesophilic (pH = 8.8–10.0; 40 °C) conditions. Acetic-type fermentation prevailed at 30–40 °C, whilst butyrate was the primary metabolite at a higher temperature (60 °C). Further chain elongation-based experiments were conducted by adding ethanol and lactate as exogenous carbon sources in vinasse fermentation, also using vinasse-derived microbial consortia as the inoculum. Lactate was added as both chemical reactant and fermented cassava flour wastewater (fCFW). Ethanol addition was irrelevant to the acidogenic activity. Conversely, lactate addition directly increased the production of propionic, butyric, (iso)valeric, and caproic acids, with a predominance of butyrate. Chain elongation was particularly favored when adding fCFW with pH adjusted to 7.0, boosting the generation of caproic acid from lactate and butyrate and (iso)valeric acid from acetate and propionate. These results highlight the potential for producing organic acids from vinasse as an alternative to gaseous fuels, expanding the suitability of dark fermentation targeting bioresource recovery from sugarcane.

1. Introduction

Sugarcane processing aiming at ethanol production generates large volumes of vinasse, the primary byproduct from the distillation step. The Brazilian sugarcane harvest for the biennium 2017/2018 indicated an overall ethanol production of 27.8 billion liters [1], generating over 340 billion liters of vinasse as residual stream. Vinasse consists primarily of potassium and sulfate salts, as well as suspended solids and high concentrations of biodegradable organic compounds [2], such as residual sugars, ethanol and other metabolites resulting from parallel fermentation pathways (e.g. lactic and acetic acids).

Currently, 97% of the vinasse generated in approximately 400 sugarcane mills in Brazil is directed to the fertirrigation of sugarcane fields [3], aiming to recycle potassium and water. However, the lack of a rational soil application may trigger some negative impacts in the environment, including soil salinization, groundwater contamination by the leaching of dissolved organic carbon, metals and sulfates, release of unpleasant odors, insect proliferation and enhanced emission of greenhouse gases [4,5].

The high biodegradable organic content of sugarcane vinasse

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characterizes this wastewater as a suitable substrate for the application of biochemical-based processes. In this context, anaerobic digestion comprises a direct alternative to fertirrigation. Commonly, anaerobic processes target the production of methane-rich biogas streams, which may be further converted into electricity, thermal energy, vehicular fuel (biomethane), among other options [6,7]. However the suppression of methanogenesis directly expands the applications of anaerobic processes, based on the exploitation of acidogenesis, i.e., dark fermentation (DF), as the core processing step to obtain biohydrogen (bioH_2), and a wide variety of organic acids and solvents, such as acetic, propionic and butyric acids, ethanol and butanol [8]. This approach is in agreement with the development of new sources of sustainable energy to replace fossil fuels, and using low- (or zero-) cost feedstocks from residual streams may be considered a strategic advantage of the biodigestion-based recycling of materials.

Studies based on the DF of sugarcane vinasse have widely addressed bioH_2 production in order to systematically define optimal operating conditions in terms of reactor configurations (e.g. packed-, structured- and fluidized-bed), type of support material, temperature conditions (mesophilic *vs.* thermophilic processes), feeding patterns through the organic loading rate and the structure and dynamics of the microbial populations [9–14]. However, the carboxylate platform, which prioritizes the recovery of soluble-phase products [15], is also a valuable platform for adding value to waste streams, as carboxylic acids present numerous industrial applications, including uses as chemical precursors for producing flavor compounds, aviation fuels [16,17], natural antimicrobials with medical uses and additives for animal food and cosmetics [18], microbial lipids [19] and bioplastics [20]. In particular, the bio-based production of longer chain carboxylic acids, such as caproic and caprylic acids, has recently drawn attention due to their high energy content and hydrophobic nature compared to short-chain acids, which facilities the downstream processing in similar applications, such as renewable precursors for aviation fuels [21,22].

Chain elongation of carboxylic acids typically takes place in biological reactors when extra carbon sources and reducing agents are available. For example, mixed cultures can use ethanol or lactic acid as substrates via reversed β-oxidation so that acetate and butyrate are elongated with two units of carbon (acetyl-CoA) derived from a reduced substrate [23–26]. However, chain elongation, similar to the production of conventional volatile fatty acids (VFA) in sugarcane vinasse-fed DF systems, is marginally addressed in the reference literature, which opens up a vast field to study process potentials and limitations.

This study aimed to obtain value-added soluble products from sugarcane vinasse via DF within the context of the carboxylate platform as an approach to diversify anaerobic digestion-related applications. The potential of using mixed microbial consortia from acidogenic systems processing sugarcane vinasse was investigated by providing different temperature and pH conditions in batch reactors. Aspects related to the establishment of favorable conditions to chain elongation using sugarcane vinasse as the primary substrate were also assessed by adding ethanol and lactic acid as external carbon sources, with the particular use of cassava processing wastewater as a potential lactate-rich co-substrate. Overall, this is the first case to systematically assess the potential for recovering soluble metabolites from sugarcane vinasse via DF in order to provide reliable data to guide future studies on continuous acidogenic reactors.

2. Material and methods

2.1. Organic substrates

Vinasse samples collected from a full-scale sugar-, ethanol- and electricity-producing sugarcane biorefinery located in Pradópolis, São Paulo, Brazil, were used as the primary substrate in fermentation tests. Cassava flour wastewater (CFW) samples used in chain elongation experiments were collected from a cassava flour factory located in Santa Maria da Serra, São Paulo, Brazil. Both substrates were stored at −20 °C prior to use. Compositional aspects of the substrates are shown in Table 1.

2.2. Inoculum source and inoculation procedure

Fermentative consortia were obtained through the natural fermentation of sugarcane vinasse, as previously reported elsewhere for vinasse-fed DF systems [9–12,14,27]. A given amount of vinasse was exposed to the local atmosphere for seven days, which primarily stimulated the growth of indigenous microbial populations using vinasse as a culture medium. Fermented vinasse was further recirculated into a bench-scale fixed-film reactor (0.54 L) for five days to enhance cell growth. This procedure was conducted individually at two temperature levels, namely, mesophilic (37 °C) and thermophilic (55 °C) conditions, which provided two sources of vinasse-derived fermentative consortia after draining the reactors. Natural fermentation is an established procedure to obtain strictly acidogenic populations, as pH drops resulting from the progressive accumulation of VFA hinders the growth of methanogens. Desired temperature levels were achieved by using a temperature controlled chamber (model 313/R/E, Adamo Produtos para Laboratórios Ltda, Piracicaba, SP, Brazil). The fermentative consortia were blended at equivalent proportions (1:1 v/v) to obtain a solid-rich inoculum (0.614 g-VS g^{-1}TS; VS = volatile solids and TS = total solids) prior to running the experiments (see Sections 2.3 and 2.4), in order to favor metabolic flexibility of the biomass towards the different incubation conditions assessed.

2.3. Batch tests for vinasse fermentation: influence of temperature and initial pH

Batch fermentation of sugarcane vinasse was carried out in 500 mL Duran® flasks filled with 250 mL of culture media (inoculum + substrate). Culture media was prepared using diluted vinasse to an initial total chemical oxygen demand (CODt) of 10 g L^{-1} blended with 15 mL of inoculum. No sterilization procedure was applied in vinasse preparation, because the experimental arrangements aimed at exploiting indigenous microbial populations from both raw (non-fermented) and naturally fermented vinasses. The bottles were flushed with nitrogen (100%) for 10 min to guarantee an anaerobic micro-environment and were then sealed with rubber stoppers and plastic caps. The bottles were incubated

Table 1
Sugarcane vinasse, cassava flour wastewater (CFW) and fermented cassava flour wastewater (fCFW; used as lactate source) compositional aspects.

Parameter	Unit	Wastewater		
		Sugarcane vinasse	CFW	fCFW
pH	(–)	4.70 ± 0.02	4.03 ± 0.02	3.98 ± 0.01
EC	mS cm^{-1}	11.78 ± 0.13	6.95 ± 0.12	6.09 ± 0.12
CODt	g L^{-1}	48.2 ± 3.0	78.3 ± 0.1	–
CODs	g L^{-1}	44.9 ± 2.2	53.7 ± 0.1	50.8 ± 2.3
CODs/CODt	(–)	0.93	0.68	–
BOD	g L^{-1}	23.2 ± 2.4	35.6 ± 0.0	–
BOD/COD	(–)	0.48 ± 0.03	0.45 ± 0.02	–
CHt	g L^{-1}	5.2 ± 0.2	17.6 ± 1.0	11.7 ± 1.5
HLaO	g L^{-1}	1.70 ± 0.12	5.15 ± 0.21	17.20 ± 0.10
EtOH	g L^{-1}	0 (zero)	0.42 ± 0.02	1.70 ± 0.02
HAcO	g L^{-1}	0.33 ± 0.02	2.03 ± 0.02	3.01 ± 0.02
GlyOH	g L^{-1}	2.81 ± 0.04	0 (zero)	0 (zero)
TKN	g L^{-1}	0.59 ± 0.08	1.14 ± 0.02	0.99 ± 0.02
P-PO_4^{3-}	g L^{-1}	0.04 ± 0.01	0.28 ± 0.01	0.22 ± 0.01
SO_4^{2-}	g L^{-1}	2.05 ± 0.10	0 (zero)	0 (zero)

Parameters: EC - electrical conductivity, CODt - total chemical oxygen demand, CODs - soluble chemical oxygen demand (0.45 μm-filtered samples), BOD - biochemical oxygen demand, CHt - total carbohydrates, HLaO - lactic acid, EtOH - ethanol, HAcO - acetic acid, GlyOH - glycerol, TKN - total Kjeldahl nitrogen, P-PO_4^{3-} - phosphate-phosphorus, SO_4^{2-} - sulfate.

in an orbital shaker (HS 501 Digital Shaker, IKA® -Werke GmbH & Co. KG, Staufen, Germany) at 130 rpm and monitored for 7 to 12 days.

The influence of the temperature was initially investigated at four levels, i.e., 30, 40, 50 and 60 °C, associating three initial pH levels (i.e., 6.0, 7.0 and 8.0) to each temperature condition. Triplicates were monitored for each combination of variables. A second batch fermentation test was further carried out assessing six initial pH levels (uncontrolled, 5.0, 6.0, 7.0, 8.8 and 10.0) at a fixed temperature of 40 °C. pH adjustments to desired levels were conducted using 6 M HCl and/or NaOH 50% solution (m/V) only after blending diluted vinasse with the inoculum, i.e., no additional pH adjustments were carried out during the incubation periods. Fermentation performance was assessed by periodically collecting 2 mL-aliquots of the fermentation broth. Minimally invasive sampling efforts were carried out through the rubber stoppers using needles and syringes, i.e., the headspace of the systems was not directly exposed to local atmosphere during the entire incubation periods aiming to preserve anaerobic conditions. The samples were centrifuged (9000 rpm for 10 min) and filtered through 0.45 μm nylon membrane filters for further chemical analysis, namely, pH, total carbohydrates (CHt), soluble COD (CODs) and fermentation metabolites, i.e., organic acids and solvents.

2.4. Chain elongation tests: ethanol vs. lactate as exogenous carbon sources

The setup of the reactors and incubation procedure used in chain elongation tests were equivalent to those described in Section 2.3, except for specificities regarding the pulse-like addition of external carbon sources, namely ethanol and lactic acid. The effect of adding ethanol and lactic acid on the chain elongation of carboxylic acids was studied specifically at initial pH and temperature conditions of 8.8 and 40 °C, respectively. Ethanol was added at 2 g L^{-1} following an initial ethanol-to-acetate ratio of 2:1 in terms of molar concentration, as reported elsewhere [26]. Ethanol addition was carried out on days 5 and 9 of ongoing fermentation experiments, so that defining proper amounts was based on the previous quantification of both acetate and ethanol levels in the fermented substrate in days 4 and 8.

In turn, lactic acid was added as both the chemical reactant (L-lactic acid for analysis ACS with purity grade of 85.0–90.0%; Labsynth Produtos para Laboratórios Ltda, Diadema, SP, Brazil) and biologically produced lactate from the fermentation of CFW, i.e., fCFW. Biological lactic acid production was previously obtained from the natural fermentation of CFW in batch tests. In this case, fermentation tests were also carried out in 500 mL Duran® flasks filled with 400 mL of culture media (CFW with initial pH of 4.5 without adding external source of inoculum) under anaerobic conditions (after nitrogen flushing) and incubated at 30 °C for 10 days. This approach aimed to stimulate populations of lactate-producing bacteria (LAB) naturally found in cassava processing wastewaters [28]. Regarding the chain elongation experiments, lactic acid was added twice or three times (depending on the concentration of lactic acid in fermented vinasse) to ongoing experiments at concentrations of 1.35 g L^{-1} [29]. Dosing lactic acid was promptly carried out after the full consumption of lactic acid by the acidogenic consortia, as monitored by the temporal decay profiles. The addition of non-sterilized fCFW was assessed without pH adjustment and with pH adjusted to 7.0 in different assays. Control reactors, i.e., without adding either ethanol or lactic acid, were also monitored for comparison purposes.

In this case, reactor sampling was based on the periodic collection of 2 mL of the fermentation broth throughout the incubation period, in order to obtain up to 14 samples. The samples were centrifuged at 9000 rpm for 10 min, filtered through 0.45 μm nylon membrane filters and further analyzed for pH, CHt, CODs and fermentation metabolites.

2.5. Analytical methods

Liquid phase measurements regarding pH (pH meter model DM-22, Digimed Instrumentação Analítica, São Paulo, SP, Brazil), electrical conductivity (EC meter model DM-31, Digimed Instrumentação Analítica, São Paulo, SP, Brazil), CODt, CODs, biochemical oxygen demand (BOD), total organic carbon (TOC), total Kjeldahl nitrogen (TKN), phosphorus and sulfate were based on protocols described in the Standard Methods for the Examination of Water and Wastewater [30]. Lactic acid was analyzed by colorimetry according to Taylor [31]. Total carbohydrates were also analyzed by colorimetry using the phenol-sulfuric method proposed by Dubois et al. [32]. VFA (C2–C6) and solvents (acetone, methanol, ethanol and n-butanol) were analyzed using a gas chromatography (GC) set (model GC-2010, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a COMBI-PAL auto sampler, as described by Adorno et al. [33]. Hydrogen, synthetic air and nitrogen were used as the carrier, flame and make-up gases, respectively. A second GC set (model GC-2010, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a thermal conductivity detector (TCD) was used to monitor biogas composition during the natural fermentation of sugarcane vinasse (Section 2.2). In this case, argon was used as the carrier gas [34].

2.6. Data interpretation and statistical analyses

The extent of hydrolysis (HydExt; %) associated to sugarcane vinasse conversion was calculated according to Garcia-Aguirre et al. [35], as shown in Eq. (1). The acidification extent (AcdExt; %) was calculated through Eq. (2), which relates the total amount of organic acids obtained at the end of the fermentation process and the total COD of the fermentation broth. The net organic acid production was considered to be the final organic acid production minus the concentration of organic acids initially measured in raw vinasse (Table 1). In Eqs. (1) and (2), the terms $CODs^{final}$, $CODs^{initial}$, $CODt^{initial}$ and $COD_{organic\ acids}$ are, respectively, the CODs of vinasse at the end of the incubation period (g L^{-1}), the CODs of vinasse prior to fermentation (g L^{-1}), the CODt of vinasse prior to fermentation (g L^{-1}) and the concentration of organic acids in COD equivalents at the end of the incubation period (g L^{-1}). The following coefficients were used to calculate COD equivalents for organic acids: 1.07 g-COD g^{-1}HAcO (HAcO = acetic acid), 1.07 g-COD g^{-1}HLaO (HLaO = lactic acid), 1.51 g-COD g^{-1}HPrO (HPrO = propionic acid), 1.82 g-COD g^{-1}HBuO (HBuO = butyric acid), 2.04 g-COD g^{-1}HVaO (HVaO = valeric acid), 2.04 g-COD g^{-1}iHVaO (iHVaO = isovaleric acid) and 2.20 g-COD g^{-1}HCaO (HCaO = caproic acid).

$$\mathrm{HydExt} = \left(\frac{CODs^{final} - CODs^{initial}}{CODt^{initial} - CODs^{initial}}\right) \cdot 100 \quad (1)$$

$$\mathrm{AcdExt} = \left(\frac{COD_{organicacids}}{CODs^{final}}\right) \cdot 100 \quad (2)$$

Fermentation performance was also assessed by calculating both the yield ($Y_{organic\ acids}$; mg-$COD_{organic\ acids}$ $g^{-1}CODt^{initial}$) and productivity ($P_{organic\ acids}$; mg-$COD_{organic\ acids}$ L^{-1} d^{-1}) of organic acids, as indicated in Eqs. (3) and (4), respectively. In these cases, the terms $COD_{organic\ acids}$, $CODt^{initial}$ and Δt are the concentration of organic acids in COD equivalents at the end of the incubation period (mg L^{-1}), the CODt of vinasse prior to fermentation (g L^{-1}) and the incubation period (d).

$$Y_{organic\ acids} = \frac{COD_{organic\ acids}}{CODt^{initial}} \quad (3)$$

$$P_{organic\ acids} = \frac{COD_{organic\ acids}}{\Delta t} \quad (4)$$

All fermentations tests and measurements were conducted in triplicate and duplicate, respectively, and the average and standard deviation values were reported. Analysis of variance (ANOVA) and *t*-test were

performed with Statgraphics Centurion version 15 (Statgraphics Technologies, Inc., The Plains, Virginia, USA) with a threshold p-value of 0.05.

3. Results and discussion

3.1. Effect of temperature and initial pH on vinasse fermentation

The impact of temperature on sugarcane vinasse fermentation was studied at 30, 40, 50 and 60 °C at different initial pH levels (i.e., 6.0, 7.0 and 8.0), aiming to identify possible interactions between both parameters. Table 2 depicts the overall performance of batch tests regarding substrate hydrolysis, acidification and metabolite production. Increasing the temperature enhanced substrate hydrolysis, despite the relatively low overall HydExt values (<35%; Table 2). This pattern may be explained by the high fraction of soluble organic matter naturally observed in raw sugarcane vinasse, as indicated by the high CODs/CODt ratio prior to fermentation (0.93; Table 1). High temperatures positively impacted the production of organic acids, except at 50 °C, a condition in which the performance of both the hydrolytic activity and fermentation reached the lowest levels, irrespective of the initial pH (Table 2). This discrepant pattern may have resulted from experimental errors, such as limitations in system inoculation (e.g. by providing less amounts of active cells) and sampling procedures. Nevertheless, the amount of organic acids in fermented vinasse exceeded that of non-fermented vinasse by at least 3.5-fold, with peak values observed at 40 °C (7.1-fold; initial pH = 8.0) and 60 °C (7.5-fold; initial pH = 8.0) (Table 2). Previous studies indicate that higher temperatures tend to increase accessibility to substrates (by facilitating the solubilization of complex materials), in addition to enhancing enzymatic activity [36,37], which supports the results observed herein.

Table 2
Overall performance regarding hydrolysis (HydExt) and acidification (AcdExt), maximum yield ($Y_{organic\ acids}$) and productivity ($P_{organic\ acids}$) of organic acids in sugarcane vinasse fermentation (7 days of incubation) at varying temperature conditions and initial pH values.

Experimental condition		Response-variable			
Temperature	pH	HydExt	AcdExt	$Y_{organic\ acids}$	$P_{organic\ acids}$
(°C)	(–)	(%)	(%)	(mg-$COD_{organic\ acids}$ $g^{-1}CODt^{initial}$)	(mg-$COD_{organic\ acids}$ L^{-1} d^{-1})
30.0	6.0	23 ± 7	37 ± 1	253 ± 8 *(5.6-fold)*[a]	473 ± 15
	7.0	19 ± 2	29 ± 2	203 ± 12 *(4.5-fold)*[a]	379 ± 23
	8.0	10 ± 2	23 ± 3	168 ± 19 *(3.7-fold)*[a]	314 ± 35
40.0	6.0	35 ± 2	31 ± 3	156 ± 21 *(3.5-fold)*[a]	366 ± 39
	7.0	29 ± 4	37 ± 3	248 ± 19 *(5.5-fold)*[a]	464 ± 35
	8.0	26 ± 4	47 ± 4	319 ± 29 *(7.1-fold)*[a]	595 ± 55
50.0	6.0	8 ± 0	20 ± 2	158 ± 13 *(3.5-fold)*[a]	296 ± 24
	7.0	10 ± 2	25 ± 8	195 ± 65 *(4.3-fold)*[a]	365 ± 122
	8.0	7 ± 2	25 ± 10	188 ± 74 *(4.2-fold)*[a]	352 ± 138
60.0	6.0	24 ± 3	46 ± 2	315 ± 14 *(7.0-fold)*[a]	588 ± 33
	7.0	29 ± 4	44 ± 4	295 ± 32 *(6.5-fold)*[a]	552 ± 60
	8.0	34 ± 4	52 ± 6	337 ± 41 *(7.5-fold)*[a]	631 ± 76

Response-variables: HydExt - hydrolysis extent, AcdExt - acidification extent, $Y_{organic\ acids}$ - yield of organic acids, $P_{organic\ acids}$ - productivity of organic acids. Note: [a]Refers to the increase in the amount of organic acids relative to non-fermented vinasse (45.1 mg-$COD_{organic\ acids}$ g^{-1}CODt).

Maximum fermentation performance was usually observed when initially providing slight basic conditions (pH = 8.0) for both mesophilic (40 °C; AcdExt = 47%, $Y_{organic\ acids}$ = 319 $COD_{organic\ acids}$ $g^{-1}CODt^{initial}$ and $P_{organic\ acids}$ = 595 mg-$COD_{organic\ acids}$ L^{-1} d^{-1}) and thermophilic (60 °C; AcdExt = 52%, $Y_{organic\ acids}$ = 337 $COD_{organic\ acids}$ $g^{-1}CODt^{initial}$ and $P_{organic\ acids}$ = 631 mg-$COD_{organic\ acids}$ L^{-1} d^{-1}) temperature ranges (Table 2), regardless of the similar pH decay on the course of fermentation for all assessed conditions. This finding suggests the versatility of the microbial consortia used in the inoculation of the reactors, leading to efficient substrate conversion at a relatively wide temperature range, regardless of the discrepant pattern observed at 50 °C. Lee et al. [38] observed a similar pattern in the production of organic acids from kitchen garbage, in order to associate maximum VFA production levels to high temperatures (60–70 °C) at relatively high pH conditions (7.0). An opposite pattern was reported by He et al. [39], in which higher production levels of organic acids were observed in mesophilic conditions (35°) compared to the thermophilic range (55 and 70 °C). Process specificities, such as the source of inoculum and substrate complexity, will certainly impact fermentation performance to different extents, so that selecting well-balanced microbial consortia tends to minimize temperature- and pH-related limitations. In particular, using the natural fermentation approach proved to be an effective strategy for obtaining strictly acidogenic consortia (no methane has been detected) adapted to both substrate and operating conditions.

Experimental conditions defined herein to obtain an enhanced production of organic acids from sugarcane vinasse are considerably different from those previously reported in the optimization of $bioH_2$ production in vinasse-fed acidogenic systems, particularly regarding pH values. Overall, maximum hydrogenogenic activity levels have been reported for pH values in a slightly acidic narrow range (5.0–5.5) in thermophilic (55 °C) and hyperthermophilic (70 °C) temperature conditions [9,10,12,14,27], with much lower $bioH_2$ production rates associated to the mesophilic range (25 °C [11]). In practical aspects, the versatility of acidogenesis may be exploited more effectively when the production of organic acids is targeted, as a wider range of operating conditions may be used, depending on the source of the inoculum. In this context, selecting specific operating conditions will depend basically on the type of organic acid desired, which is strongly related with the temperature.

The breakdown of the metabolite profiles obtained for the different incubation conditions indicated HAcO and HBuO as the primary products from sugarcane vinasse fermentation (Fig. 1). Mesophilic temperature conditions, i.e., 30 and 40 °C, directly favored the acetic-type

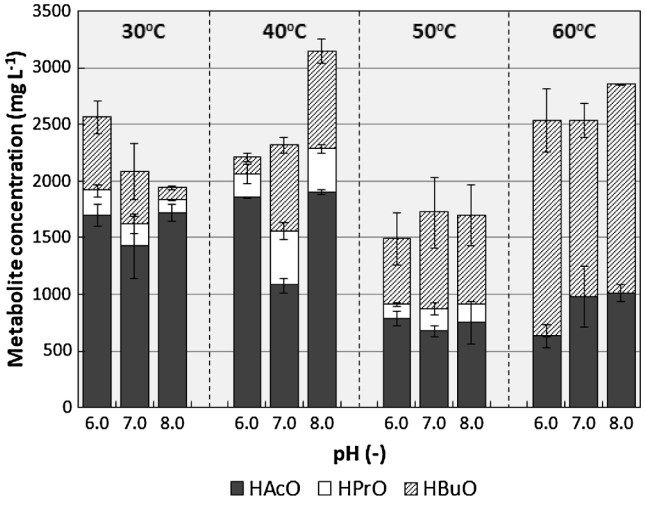

Fig. 1. Organic acid distribution profiles in sugarcane vinasse fermentation (7 days of incubation) at varying temperature and initial pH values. Legend: HAcO = acetic acid, HPrO = propionic acid and HBuO = butyric acid.

fermentation regardless of the pH, with maximum HAcO concentrations of approximately 1900 mg L^{-1} observed at 40 °C (initial pH 6.0 and 8.0) (Fig. 1). Conversely, butyric type fermentation prevailed under thermophilic conditions (60 °C), also reaching maximum HBuO concentrations of 1900 mg L^{-1} at initial pH values of 6.0 and 8.0 (Fig. 1). HPrO was also detected as a minor fermentation intermediate (100–480 mg L^{-1}; Fig. 1) in all assessed conditions except at 60 °C. Previous studies [9,10,27,40] also reported the predominance of HBuO in thermophilic (55 °C) and hyperthermophilic (70 °C) bioH_2-producing sugarcane vinasse-fed acidogenic systems, which corroborates the pattern observed herein at 60 °C. Overall, the highest production of organic acids (in terms of total concentration) was observed at 40 °C and initial pH of 8.0 (Fig. 1).

Similar patterns regarding metabolite profiles in acidogenic systems have also been reported for different substrates. Jiang et al. [36] observed HAcO and HPrO as major fermentation intermediates in the mesophilic temperature range (35–45 °C), whilst HBuO was the primary metabolite (81%) at 55 °C when fermenting food waste. Enhanced butyric-type fermentation has been also observed in thermophilic (55 °C) xylose fermentation using mixed microbial consortia by Qiu et al, [41]. In this case, the authors identified an enrichment in HBuO-producing microorganisms as a direct consequence of temperature increase (37 to 55 °C), which typically decreases the diversity of bacterial populations. Yang et al. [42] also reported an increase in HBuO concentrations from the co-fermentation of hemicellulose and starch at 55 °C; however, a further increase in the temperature to 70 °C led to a sharp decline in butyric-type fermentation. A similar pattern was observed by He et al. [39] during the fermentation of food waste, in which HBuO and iso-butyric acid (iHBuO) concentrations reached peak values at 55 °C and the further temperature increase to 70 °C favored the acetic-type fermentation. These authors also reported relevant concentrations of EtOH at lower temperatures (35 °C).

3.2. Effect of initial pH on sugarcane vinasse fermentation

Considering the determining role of pH on microbial growth and metabolism, further fermentation tests were carried out at a wider initial pH range (i.e., 5.0–10.0; Section 2.3) and a fixed temperature of 40 °C. pH does not only influence the hydrolysis of organic materials, but primarily substrate fermentation. In particular, emphasis has been given to the alkaline pH range in this step, based on the positive response of vinasse fermentation at an initial pH of 8.0, irrespective of incubation temperature (Section 3.1). Monitoring the pH throughout the fermentation process indicated sharp drops within the first two days of incubation (Fig. 2), leading to values varying between 5.0 and 6.0. However, variations were minimal up to 12 days of incubation, with values concentrated in a narrow range (5.2–5.5; Fig. 2), regardless of the initial pH. The initial pH decrease (0–2 d) was directly associated with enhanced substrate fermentation, as further observed in metabolite concentration profiles (Fig. 3). A discrepant pattern was identified only in reactors fed with vinasse with initial pH lower than 5.0, in which a slight increase to 4.9–5.1 was observed. These results indicate that effects of varying the initial pH of sugarcane vinasse resulted directly from the different fermentation conditions in the first 2 days of incubation, most likely due to differences in the availability of fermentable substrates.

Overall performance data regarding the fermentation of vinasse at different initial pH values are summarized in Table 3. The results indicated the favoring of both substrate hydrolysis and acidification at higher initial pH values (>7.0; Table 3), leading to maximum HydExt and AcdExt values of 80% and 58% at a pH of 8.8. Comparatively, the performance of hydrolysis was increased over 5-fold times in relation to pH values usually required for enhanced bioH_2 production, i.e., close to 5.0 (14 *vs.* 80%; Table 3). The increase in acidification also reached relevant patterns (2.2-fold higher) when comparing fermentation performance at initial pH values of 5.0 (30%) and 8.8 (58%). The enhanced

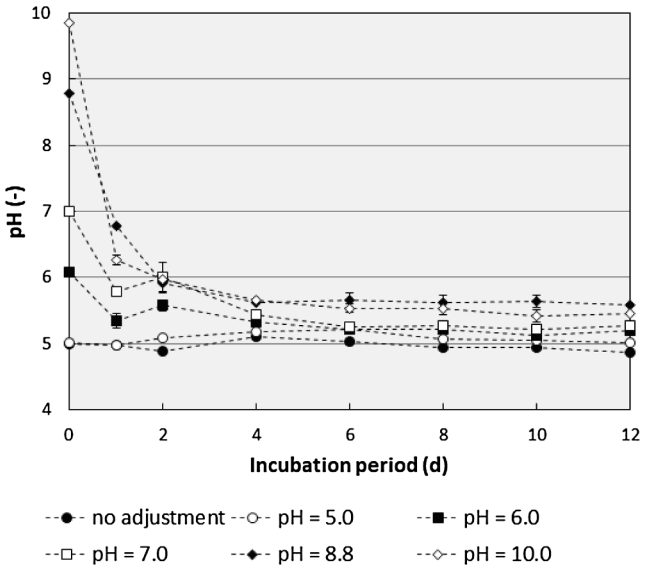

Fig. 2. Temporal variation profiles for pH in sugarcane vinasse fermentation (12 d of incubation) at 40 °C and different initial pH values (no adjustment, 5.0, 6.0, 7.0, 8.8 and 10.0).

hydrolysis at alkaline conditions most likely resulted from NaOH dosing, as previously suggested elsewhere [43]. The alkaline shock may have acted as a pre-treatment step by enhancing hydrolysis rates, releasing primarily soluble carbohydrates [44,45]. Despite the higher HydExt, the impacts of high NaOH-dosing levels on acidification reached lower magnitudes, considering similar AcdExt values for initial pH values in the range of 6.0–10.0 (43–47%, except of the pH of 8.8; Table 3). The observed pattern most likely resulted from detrimental effects of strong alkaline conditions (pH > 8.8) on microbial metabolism. In fact, extremely alkaline conditions (pH > 12.0) have been previously referred to as inhibitory for unspecialized, i.e., non-alkaliphilic, acidogenic populations [7].

Increasing the initial pH of sugarcane vinasse also led to higher $Y_{organic\ acids}$ values (Table 3). The maximum $Y_{organic\ acids}$ was slightly higher at pH of 10.0 (401 mg-$COD_{organic\ acids}$ $g^{-1}COD_t^{initial}$) compared to reactors incubated with initial pH of 6.0, 7.0 and 8.8 (311–315 mg-$COD_{organic\ acids}$ $g^{-1}COD_t^{initial}$. Consequently, the increase in the amount of organic acids in fermented vinasse relative to non-fermented samples varied negligibly within the pH range of 6.0–8.8 (6.9–7.0-fold; in particular, no significant differences have been observed for these incubation conditions, $p < 0.05$), whilst a 8.9-fold increase was observed at pH of 10.0 (Table 3). $P_{organic\ acid}$ values were also similar (653–679 mg-$COD_{organic\ acids}$ L^{-1} d^{-1}; Table 3) within the initial pH range of 7.0–10.0. Both the $Y_{organic\ acids}$ and $P_{organic\ acids}$ were roughly 1.5-fold higher at neutral-to-basic conditions (initial pH > 6.0) compared to acidic conditions (initial pH < 5.0). The favoring of organic acid production under alkaline conditions is in line with previous results obtained in the acidogenesis of food waste, activated sludge and urban/agro-industrial waste streams [35,46–48]. Lower pH values may slow down the hydrolysis rate of organic waste and subsequently hinder the fermentation step [47,49], as the availability of fermentable substrates (e.g. sugars) is limited. Moreover, lower pH values increase the concentration of undissociated acids in the bulk liquid, which may easily permeate cell membranes and inhibit normal cell functions by negatively affecting the pH of the cytoplasm [50].

The evolution of organic acid production at different initial pH values is depicted in Fig. 3. Metabolite profile in raw (non-fermented) sugarcane vinasse (t = 0) indicated the presence of HAcO, HBuO and HLaO at relatively low concentrations (<400 mg L^{-1} each; Fig. 3), which naturally result from the bacterial contamination of broth in ethanol-targeted fermentation vessels [51,52]. Overall, HAcO and HBuO were

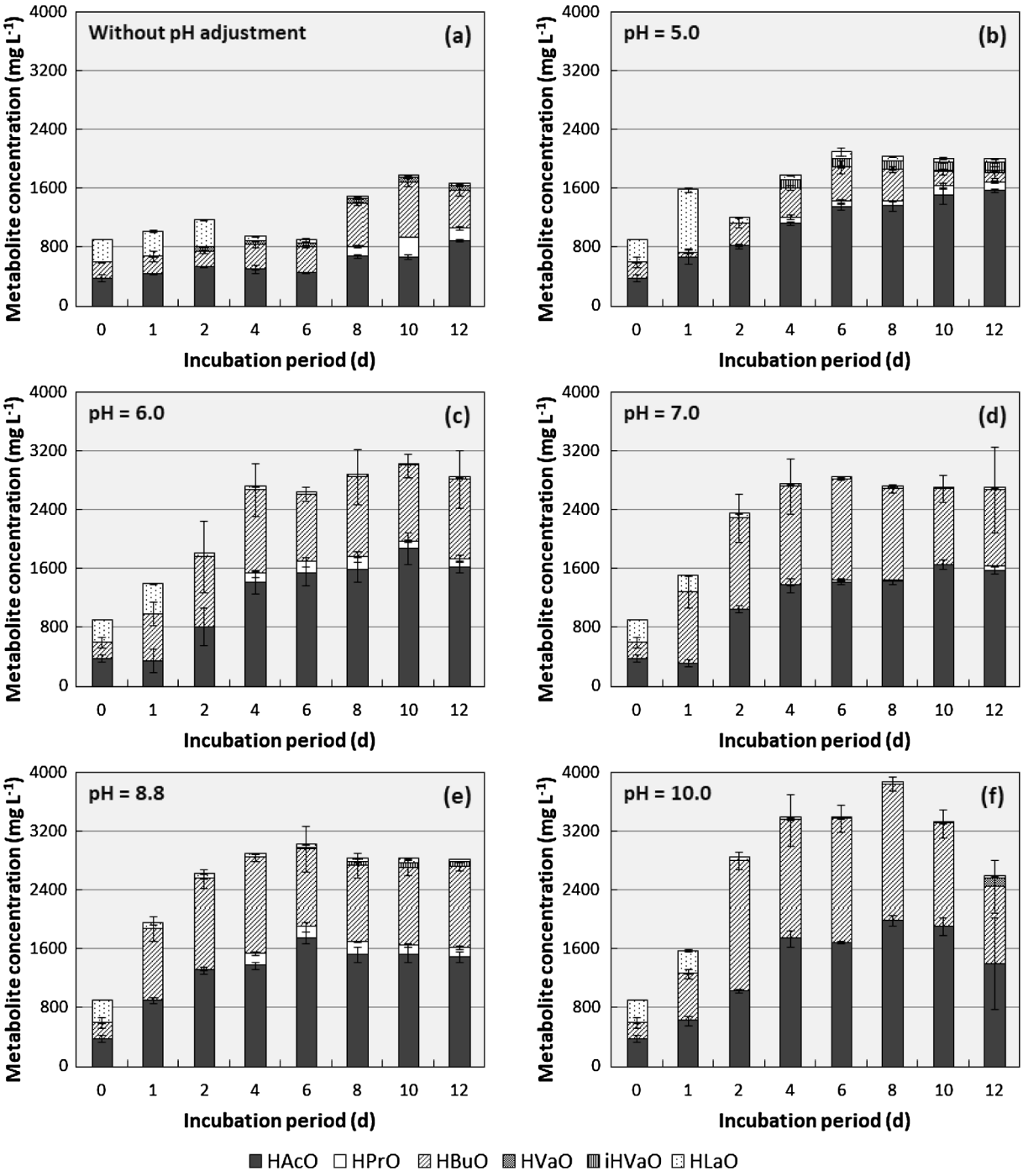

Fig. 3. Organic acid distribution profiles in sugarcane vinasse fermentation (12 d of incubation) at 40 °C and different initial pH values: (a) vinasse without pH adjustment, (b) pH = 5.0, (c) pH = 6.0, (d) pH = 7.0, (e) pH = 8.8 and (f) pH = 10.0. Legend: HAcO = acetic acid, HPrO = propionic acid, HBuO = butyric acid, HVaO = valeric acid, iHVaO = iso-valeric acid and HLaO = lactic acid.

the major fermentation intermediates in all assessed conditions, similar to the patterns observed in the first round of experiments (Section 3.1). Final HAcO concentrations (after 12 d of incubation) varied narrowly (1400–1600 mg L^{-1}) within the initial pH range 5.0–10.0 (Fig. 3b-f), indicating that acetic-type fermentation was most likely not affected. These concentrations are in accordance with the ones previously measured in the first round of experiments under similar initial pH conditions (1800–1900 mg L^{-1} – 40 °C and pH = 8.0; Fig. 1). However, increasing the initial pH (>6.0) directly favored butyric-type fermentation pathways, with concentrations higher than 1000 mg L^{-1} (Fig. 3c-f). Comparatively, HBuO concentrations below 500 mg L^{-1} were obtained in reactors with an initial pH lower than 5.0 (Fig. 3a-b).

Enhanced butyric-type fermentation may have resulted from HLaO consumption via reverse ß-oxidation [17,24]. HLaO was fully consumed within 1–2 d of incubation in all conditions assessed (Fig. 3), which coincides with the increase in HBuO concentrations and strongly suggests the establishment of HLaO and HAcO co-fermentation to produce HBuO (Reaction (1) [53]). This particular metabolic pathway has been previously observed in acidogenic systems fed with vinasses from different feedstocks, namely, sugarcane [13,14] and agave [54,55], in order to strictly depend on fermentation pH. Experimental results indicate that HLaO consumption to produce HBuO is strongly inhibited

Table 3
Overall performance regarding hydrolysis (HydExt) and acidification (AcdExt), maximum yield ($Y_{organic\ acids}$) and productivity ($P_{organic\ acids}$) of organic acids in sugarcane vinasse fermentation (12 days of incubation) at 40 °C and different initial pH values.

pH (–)	Response-variable			
	HydExt	AcdExt	$Y_{organic\ acids}$	$P_{organic\ acids}$
	(%)	(%)	(mg-$COD_{organic\ acids}$ $g^{-1}CODt^{initial}$)	(mg-$COD_{organic\ acids}$ L^{-1} d^{-1})
Withou adjustment	17 ± 7	25 ± 2	204 ± 11 *(4.5-fold)*[a]	265 ± 11
5.0	14 ± 3	30 ± 4	213 ± 28 *(4.7-fold)*[a]	464 ± 61
6.0	35 ± 5	47 ± 9	311 ± 60 *(6.9-fold)*[a]	405 ± 78
7.0	35 ± 9	43 ± 19	312 ± 122 *(6.9-fold)*[a]	679 ± 133
8.8	80 ± 5	58 ± 8	315 ± 42 *(7.0-fold)*[a]	683 ± 91
10.0	55 ± 10	47 ± 24	401 ± 144 *(8.9-fold)*[a]	653 ± 157

Response-variables: HydExt - hydrolysis extent, AcdExt - acidification extent, $Y_{organic\ acids}$ - yield of organic acids, $P_{organic\ acids}$ - productivity of organic acids. Note: [a]Refers to the increase in the amount of organic acida relative to non-fermented vinasse (45.1 mg-$COD_{organic\ acids}$ g^{-1}CODt).

at pH values below 5.0 [14]. In fact, metabolite profiles obtained for initial pH values higher than 6.0 (Fig. 3c-f) indicate enhanced HBuO production patterns within 1–2 d of incubation (>1000 mg L^{-1}), which coincides with fermentation pH values within 5.5–6.0 (Fig. 2), i.e., favorable conditions to HLaO-derived butyric fermentation. In line with this hypothesis, much lower HBuO concentrations were observed when the initial pH was lower than 5.0 (<300 mg L^{-1}; Fig. 3a-b).

$$HAcO + 2HLaO \rightarrow H_2 + 3/2HBuO + 2CO_2 + H_2O \quad \text{(Reaction 1)}$$

HLaO consumption at more acidic conditions could be associated with the production of HPrO, as observed in Reaction (2) [56–58]. Metabolite profiles indicate regular HPrO concentrations of approximately 100 mg L^{-1} in reactors with initial pH lower than 6.0 (Fig. 3a-c), so that no specific pattern was observed at higher pH values. This particular pathway has also been suggested to occur in sugarcane vinasse acidogenesis as an alternative to produce metabolic energy under low carbohydrate availability [13]. Enhanced propionic-type fermentation has also been commonly identified at relatively high pH values in DF systems [12,59,60], as HPrO-producing bacteria grow faster at neutral pH values [37]. Conventional HPrO production (Reaction (3) [61]) takes place when high H_2 partial pressures are established within acidogenic systems. However, given the low carbohydrate availability after enhanced substrate acidification, i.e., after 2 d of incubation, unfavorable conditions may have limited this particular pathway, despite the most likely enhanced bioH_2 production resulting from HLaO conversion into HBuO (Reaction (1)). The minor-to-null HPrO concentrations at pH values higher than 6.0 corroborate this hypothesis.

$$3HLaO \rightarrow 2HPrO + HAcO + CO_2 + H_2O \quad \text{(Reaction 2)}$$

$$Glucose + H_2 \rightarrow 2HPrO + 2H_2O \quad \text{(Reaction 3)}$$

Minimal HVaO and iHVaO concentrations (<100 mg L^{-1} each) were also determined at both acidic (pH < 5.0) and alkaline (pH > 8.8) initial incubation conditions, so that clear patterns regarding favorable or unfavorable producing conditions were not identified. Ferraz Jr. et al. [11] reported relative higher HVaO concentrations (280–350 mg L^{-1}) in sugarcane vinasse mesophilic (25 °C) DF at pH values within the range of 5.4–5.5, whilst Jiang et al. [36] also observed HVaO production from food waste fermentation at acidic conditions (pH < 5.0). Although HVaO has been indicated as the primary metabolite from HLaO fermentation by specific bacterial strains, namely, *Megasphaera elsdenii* [62], the incubation conditions used herein most likely did not favor the valeric-type fermentation, based on the much higher predominance of HAcO and HBuO (Fig. 3).

3.3. Chain elongation tests: Effect of ethanol and lactate addition

EtOH and HLaO are characterized as carbon sources and reducing equivalents capable of promoting the chain elongation of carboxylic acids using both isolated strains and mixed cultures [23–26,63–65]. Examples of using both EtOH and HLaO in chain elongation processes are presented through Reactions (4) to (8) [26,66], in addition to HLaO and HAcO co-fermentation to produce HBuO (Reaction (1)). In this context, both carbon sources were used separately as external electron donors in sugarcane vinasse acidification at pH of 8.8 and 40 °C, which were previously defined as adequate conditions to the enhanced production of organic acids (primarily HAcO). The effect of EtOH addition on days 5 and 9 on vinasse fermentation is depicted in Fig. 4. EtOH concentration was decreased by half after the first addition (day 5; Fig. 4a). A similar pattern was observed after the second addition of ethanol to the fermentation broth, so that a residual concentration of about 2000 mg L^{-1} was measured by the end of the incubation period (Fig. 4a). HAcO concentration remained unchanged during the entire incubation period in both EtOH-supplemented and non-supplemented reactors (Fig. 4b). HPrO accumulation was observed only in control reactors (Fig. 4c) most likely as a result from HLaO consumption (Reaction (2)), as previously described in Section 3.2. Curiously, EtOH addition negatively impacted HPrO production via HLaO uptake, so the provision of EtOH did not even stimulate the production of both HPrO and HAcO (Reaction 9), as previously observed in sugarcane vinasse fermentation [13]. HBuO, HVaO and iHVaO concentrations remained unchanged, irrespective of EtOH addition (data not shown), indicating that no chain elongation-associated pathways were at least favored in such fermentation conditions. CHt conversion reached approximately 80% in both conditions (data not shown either), also indicating no particular effects of EtOH addition on carbohydrate metabolization.

$$EtOH + HAcO \rightarrow n-HBuO + H_2O \quad \text{(Reaction 4)}$$

$$2EtOH + HAcO \rightarrow n-HCaO + 2H_2O \quad \text{(Reaction 5)}$$

$$EtOH + n-HBuO \rightarrow n-HCaO + H_2O \quad \text{(Reaction 6)}$$

$$EtOH + HPr \rightarrow n-HVaO + H_2O \quad \text{(Reaction 7)}$$

$$HLaO + n-HBuO\ H^+ \rightarrow n-HCaO + H_2O + CO_2 \quad \text{(Reaction 8)}$$

$$3EtOH + CO_2 \rightarrow 2HPrO + HAcO + H_2O \quad \text{(Reaction 9)}$$

Despite using EtOH-to-HAcO molar concentration ratios of 2:1 (day 5) and 4:1 (day 9), which should favor HCaO formation (Reaction 5 [26]), no chain elongation was observed, so that EtOH was most likely used as a simple carbon source. In fact, some studies have pointed out that the use of EtOH and HAcO as sole substrates in higher molar ratios, i.e., from 3:1 to 10:1, could lead to enhanced HCaO production [19,67]. Limiting conditions to the use of EtOH in chain elongation may have been associated with fermentation pH (<6.0; Fig. 4d), as this metabolic pathway is favored at neutral conditions (pH = 7.0 [26]). Decreasing the pH from 7.0 to 5.0 has led to approximately 40% lower growth rates of *Clostridium kluyveri* strain 3231B, which is known as a HCaO-producing bacterial strain [68]. Conversely, Zhu et al. [65] reported similar HCaO production rates from *Ruminococcaceae* bacteria strain CPB6 within the initial pH range of 5.0–6.5, in order to observe much lower production levels at neutral-to-alkaline conditions (pH = 7.0–8.0). These contradictory results reinforce the need to address further studies on the use of EtOH to promote chain elongation in vinasse-fed systems, primarily when using mixed consortia.

Previous results on the impacts of initial pH on vinasse fermentation (Section 3.2) suggested the relevance of HLaO in HBuO and HPrO

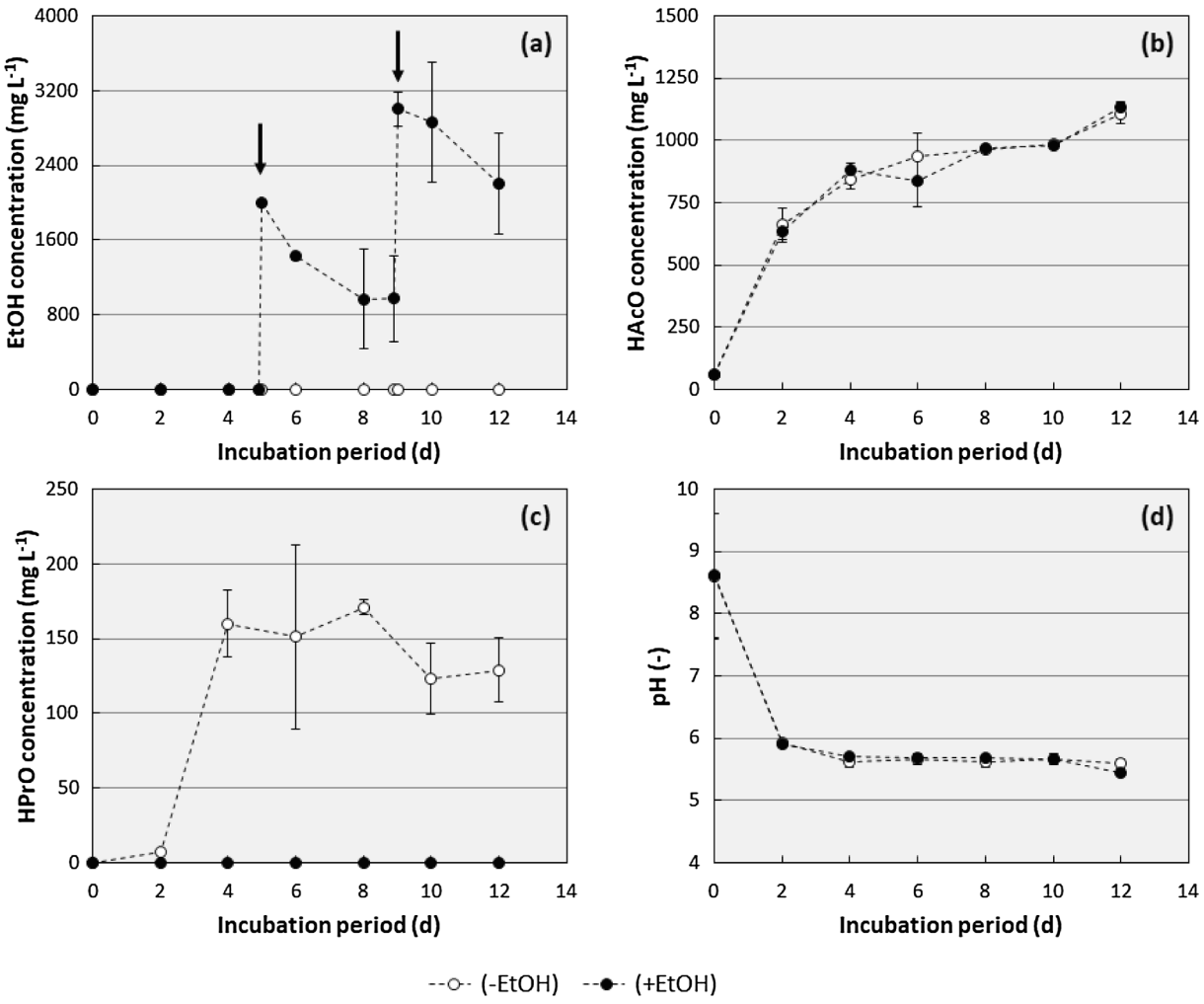

Fig. 4. Chain elongation tests in sugarcane vinasse fermentation using ethanol as the additional carbon source – temporal profiles for: (a) ethanol (EtOH) concentration, (b) acetic acid (HAcO) concentration, (c) propionic acid (HPrO) concentration and (d) fermentation pH. Notes: (−EtOH) = control reactors (without EtOH addition), (+EtOH) = EtOH-supplemented reactors. Arrows denote EtOH addition on days 5 and 9.

production, which motivated adding HLaO as an exogenous carbon source to promote chain elongation in vinasse acidogenesis. Both chemically- and biologically-originated HLaO sources were used in this step (in the second case fCFW was used as the HLaO source without pH adjustment and with pH corrected to 7.0). Fig. 5 depicts temporal concentration profiles for diversified metabolites considering four experimental conditions, namely, control systems (without the addition of HLaO), chemically-derived HLaO-supplied reactors and biologically-derived HLaO-supplied reactors (using fCFW without previous pH adjustment and with pH corrected to 7.0). HLaO addition was carried out on days 3, 6 and 9 (Fig. 5a), after marked concentration drops were observed. HLaO availability ceased after approximately 2 days in control reactors, similar to previous experiments (Section 3.2). In HLaO-supplied reactors, HLaO was quickly consumed (Fig. 5a), which suggests its central role as substrate for the microbial consortia used herein. A different pattern was observed only after the second addition of fCFW without pH adjustment, as the HLaO consumption rate sharply decreased (Fig. 5a). A plausible hypothesis to explain such a performance could be associated with inhibitory effects of bacteriocins, which are commonly detected in CFW [28]. However, the inhibitory activity of bacteriocins, such as nisin, should be enhanced only at very acidic conditions (pH $\ll$ 5.0 [69]), which were not observed herein (pH $>$ 5.0; Fig. 5j). CHt conversion reached values of approximately 80% and 60% in fCFW-non-supplemented and fCFW-supplemented reactors (data not shown), indicating carbohydrate metabolization as an independent step in relation to HLaO addition. The lower conversion patterns observed when dosing fCFW resulted from the presence of residual CHt concentrations in fCFW (Table 1).

HAcO concentration increased to similar levels (1000–1400 mg L^{-1}) in all assessed conditions up to day 2 (Fig. 5c), and a slight increase (1600 mg L^{-1}) was observed only in fCFW-supplied reactors most likely due to endogenous concentrations of HAcO found in fCFW (Table 1). HAcO concentrations in HLaO-supplied reactors were always lower than in control reactors from day 6 onwards, suggesting the favoring of HAcO consumption. At this point, marked increases were observed in HPrO (Fig. 5d), HBuO (Fig. 5e) and HCaO (Fig. 5i) concentrations in these reactors, reaching levels significantly different ($p < 0.05$) to those observed in control systems. These findings suggest an initial favoring of the butyric-type fermentation from the co-fermentation of HAcO and HLaO (Reaction (1)) in all cases, so that final HBuO concentrations were at least 2.5-fold higher compared to control reactors. iHBuO production was observed only when fCFW without previous pH adjustment was added to the fermentation broth (Fig. 5f). HBuO isomerization to iHBuO, previously characterized as a pH-independent process [70], may be a potential pathway to explain the observed pattern. However, no clear mechanisms were identified, based on the variation patterns associated to the other metabolites.

HCaO was promptly favored in fCFW-supplied systems (Fig. 5i) after the first supplementation, which may have been stimulated by increasing HBuO concentrations (Reaction 7). The findings reported by

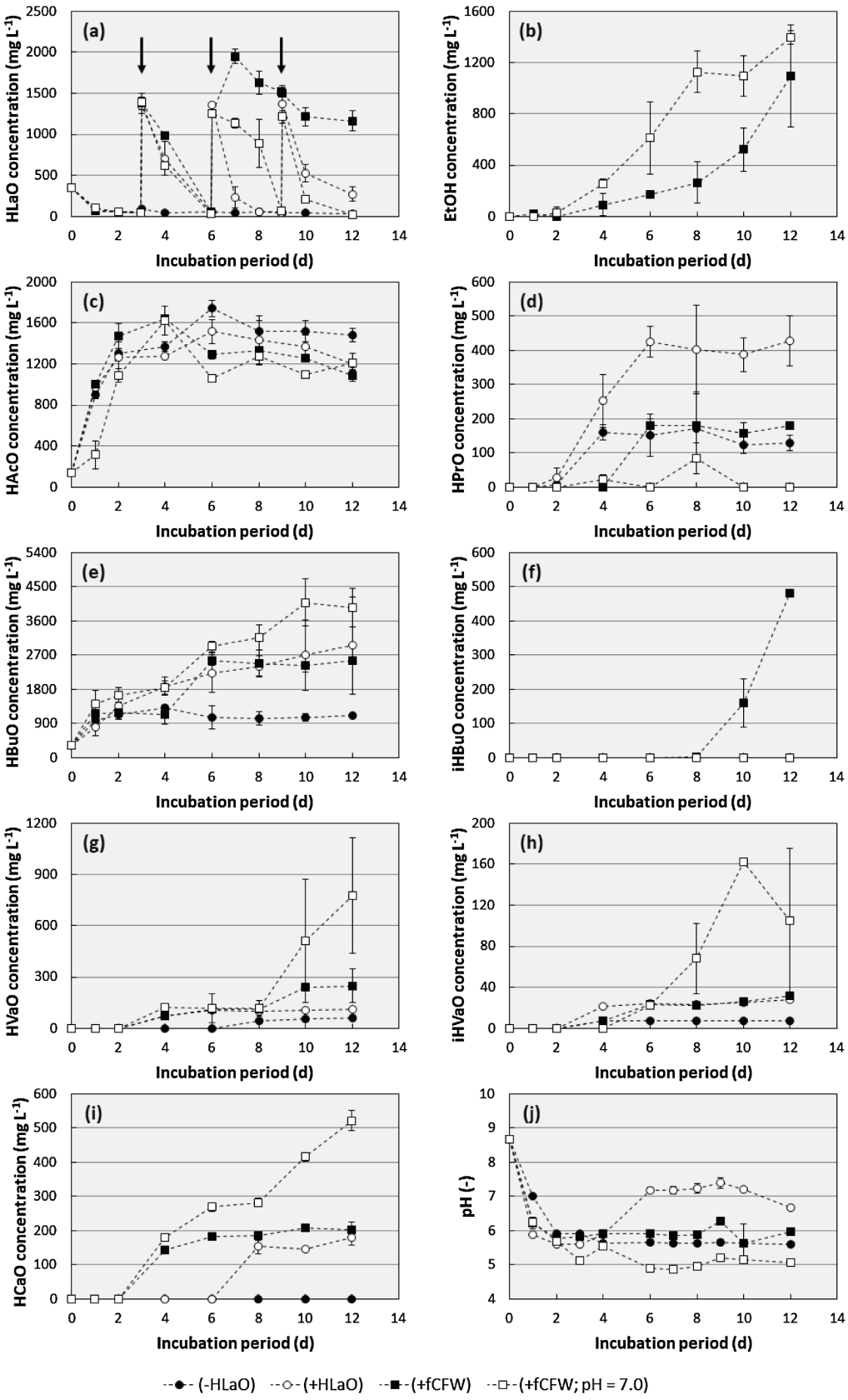

Fig. 5. Chain elongation tests in sugarcane vinasse fermentation using lactic acid as the additional carbon source – temporal profiles for: (a) lactic acid (HLaO) concentration, (b) ethanol (EtOH) concentration, (c) acetic acid (HAcO) concentration, (d) propionic acid (HPrO) concentration, (e) butyric acid (HBuO) concentration, (f) iso-butyric acid (iHBuO) concentration, (g) valeric acid (HVaO) concentration, (h) iso-valeric acid (iHVaO) concentration, (i) caproic acid (HCaO) concentration and (j) fermentation pH. Notes: (−HLaO) = control reactors (without HLaO addition), (+HLaO) = HLaO-supplemented reactors (HLaO as chemical reactant), (+fCFW) = fCFW-supplemented reactors (fCFW without pH adjustment) and (+fCFW; pH = 7.0) = fCFW-supplemented reactors (fCFW with pH adjusted to 7.0). Arrows denote HLaO addition (as either chemical reactant or fCFW) on days 3, 6 and 9.

Lin and Hu [70] support this hypothesis, indicating HCaO production from HBuO at relatively similar pH values (5.7). In turn, HPrO production was initially increased when dosing HLaO as chemical reactant (Fig. 5d). In this case, HLaO conversion into HAcO and HPrO may have been favored (Reaction (2)), similar to that observed in the control system. This pattern may be explained by the increase in pH values (>7.0; Fig. 5b) from day 6 onwards in this condition, which most likely favored the growth of HPrO-producing bacteria. This particular microbial group is strongly inhibited by undissociated acids, especially at pH values below 6.0 [37], which also explains the lower HPrO concentrations in the other conditions (pH always < 6.0; Fig. 5b).

HVaO and iHVaO concentrations also presented marked increasing patterns, but only in fCFW-supplied reactors, specifically with pH adjusted-fCFW (Fig. 5g-h), after 8 d of incubation. Concomitantly, HPrO concentrations presented a slight increase followed by a marked drop from this point onwards (Fig. 5d), suggesting its uptake in HVaO production. Previous studies reported on high chain elongation-related HVaO production levels from HPrO and HAcO from *Megasphaera* sp. MH [71] and *M. hexanoica* [72], corroborating the hypothesis of HPrO consumption herein. HVaO production in fCFW-supplied systems may also have been favored by the "side-effect-like" addition of EtOH, following Reaction (7). However, EtOH concentration profiles (Fig. 5b) always presented increasing patterns, mainly after adding fCFW on days 3, 6 and 9, which suggests marginal-to-zero consumption rates. Unfavorable pH conditions may have limited the use of EtOH in chain elongation-related pathways, similarly when directly dosing EtOH in the reactors (Fig. 4).

3.4. Outlook: perspectives for sugarcane vinasse fermentation

The microbial consortia used in this study were capable of converting HBuO and HPrO into longer chain organic acids, primarily HCaO and HVaO. In particular, HLaO supplying played an essential role in chain elongation by acting distinctly, depending on its source, specifically when adding fCFW with pH adjusted to 7.0 in vinasse fermentation. On the one hand, HLaO provision favored enhanced HBuO production (from the co-fermentation with HAcO; Reaction (1)), which further stimulated HCaO production (Reaction 8), both via reverse ß-oxidation [17]. HLaO is converted into acetyl-CoA, generating metabolic energy to promote chain elongation [65]. On the other hand, the addition of HLaO also favored HPrO and HAcO production (Reaction (2)), which was further converted into HVaO (and iHVaO at lower proportions). Both HCaO and HVaO present similar market prices, i.e., respectively 2000–2500 USD/ton [73] and 2500–3000 USD/ton [72], which can theoretically obtain similar revenues regardless of the targeted metabolite.

In particular, the natural predisposition for producing HBuO from HLaO and HAcO in sugarcane vinasse-fed acidogenic systems, as seen elsewhere [13,14,27] and in this study (Section 3.2), should be carefully considered. Targeting HBuO as an end fermentation product should require lower retention times in continuous systems (i.e., lower operating costs), as HBuO concentrations obtained after 6 d (~3000 mg L^{-1}; Fig. 5e) were 3.5-to-7.5-fold higher than HVaO (~800 mg L^{-1}; Fig. 5g) and HCaO (~550 mg L^{-1}; Fig. 5i) concentrations obtained after 12 d of incubation, in addition to demanding lower inputs of HLaO. Costs associated with HLaO supplying may be directly minimized by using low-cost sources, such as in the use of fCFW proposed herein, and/or by stimulating lactate-type fermentation prior to chain elongation within the same fermenter [74]. Nevertheless, the market price of HBuO is similar (2000–2200 USD/ton [72]) to the ones of HCaO and HVaO, so that the small difference in prices may be offset by the much higher production levels of HBuO relative to longer chain organic acids.

However, limiting aspects still require proper considerations prior to an effective exploitation of vinasse in fermentative processes. Despite the proven potential associated to vinasse fermentation presented herein, yield and productivity values for selected metabolites are still low, as a result of both the complex compositional character of vinasse and the use of microbial consortia as the source of biocatalysts. First, some fundamental aspects require further understanding, particularly knowing the mechanisms associated to using HLaO in chain elongation processes. This step requires studies dealing with systematic transcriptomic analysis, isotope-labeled flux analysis or genetic engineering manipulation, in order to regulate the production of targeted metabolites by microbial consortia.

In practical aspects, process performance may be improved through direct engineering interventions, such as the use of cell immobilization to favor the growth of selected microbial groups; the application of extraction methods to remove fermentation products online, therefore stimulating specific metabolic pathways (e.g. continuous HBuO removal to maintain HLaO consumption and limit further HBuO conversion); and the conventional process control through specific parameters, i.e., pH, the pair hydraulic retention time-organic loading rate, operation mode of bioreactors, among other aspects. Extraction- and purification-based processes must be invariably developed and/or improved in parallel to the optimization of acidogenesis, aiming to provide cost-competitive processing chains relative to the conventional purpose of anaerobic digestion, i.e., the production of methane-rich biogas streams aiming at direct energetic applications.

Applying concepts of bioenergy recovery within the carboxylate platform is indeed required to both measure process efficiency on an energy basis and provide subsides to compare different applications of anaerobic consortia, namely, direct (hydrogen- and/or methane-rich biogas) and indirect (soluble metabolites) bioenergy production. The inhibition of methane-producing pathways conserves higher energy densities within the organic compounds, as energy losses are little in the transportation of electrons during substrate fermentation [16,75]. Thus, exploiting soluble phase metabolites from acidogenesis will provide highly energetic products, regardless of their application, i.e., direct usable energy production or as precursors in chemical platforms. Previous research [76] indicated that the energy recovery potential associated with HBuO production (30.9–33.4 MW) from sugarcane vinasse could outperform that of highly efficient methane-producing systems (25.5 MW) by 21.2–31.0%. Despite considering only the energetic content of the products, i.e., processing-related energetic costs were not included, this estimate suggests that the exploitation of soluble metabolites has potential to minimize energy losses (substrate) in vinasse conversion, which improves the reach of the biorefinery concept by favoring the cascading principle. This principle aims at maximizing product yields with minimum energy losses [77], which again highlights the importance of developing competitive extraction/purification processes to achieve favorable energy return on invested ratios. Combining the recovery of both liquid- and gas-phase products may also characterize a strategy to maximize the energetic efficiency of the vinasse-based anaerobic biorefinery, considering the biomethanation of the residual carbon (non-recovered metabolites and non-converted substrates) in the fermentation broth [76,78]. A second principle of the biorefinery concept could be successfully satisfied by this approach, as the versatility of anaerobic consortia provides flexibility to substrate conversion, supplying different market demands as resource recovery is maximized.

4. Conclusions

Sugarcane vinasse fermentation has been successfully achieved at varied operating conditions using microbial consortia in this study. Overall, the production of organic acids was not a highly temperature-dependent process, as total concentrations varied narrowly within a relatively high temperature range (30–60 °C). Temperature-related effects were directly associated with metabolite profiles, as acetic (up to 1897 mg L^{-1}) and butyric (up to 1906 mg L^{-1}) fermentation pathways were favored at mesophilic and thermophilic conditions, respectively. In turn, pH dependence was more prominently, with enhanced acidification patterns observed at neutral-to-alkaline conditions (pH > 6.0),

which also favored butyric-type fermentation. Both the yield and productivity were maximized at pH and temperature conditions of 8.8 and 40 °C, respectively. Further chain elongation-based studies have proven the determining role of lactic acid as a precursor of caproic (up to 521 mg L^{-1}) and valeric (up to 778 mg L^{-1}) acid production from vinasse, respectively by promoting reverse ß-oxidation and by producing propionic acid, characterized as a precursor of valeric acid. Ethanol supplementation has not stimulated chain elongation pathways in the studied conditions. Overall, sugarcane vinasse may be considered a potential substrate for the carboxylate platform within the residue biorefinery context, and special attention should be directed to the production of butyric acid, based on its relatively high market price and the natural predominance of butyric-type fermentation in vinasse-fed acidogenic systems.

CRediT authorship contribution statement

Felipe Eng Sánchez: Investigation, Formal analysis, Conceptualization, Writing - original draft. **Lucas Tadeu Fuess:** Conceptualization, Writing - review & editing, Visualization. **Guilherme Soares Cavalcante:** Investigation. **Maria Ângela Talarico Adorno:** Methodology. **Marcelo Zaiat:** Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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