Science of the Total Environment 1019 (2026) 181556

Contents lists available at ScienceDirect

Science of the Total Environment

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

Acidic-alkaline shocks in vinasse fermentation shape methanogenesis and sulfate reduction dynamics

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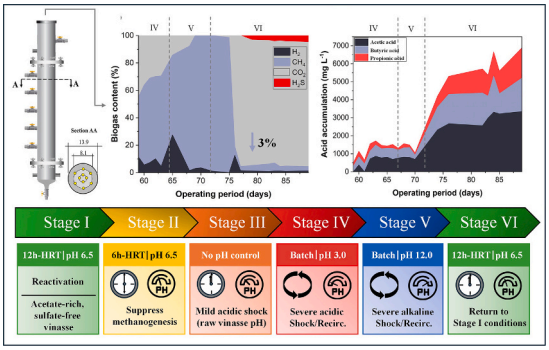
HIGHLIGHTS

- pH shocks hinder methanogenesis, reducing unwanted CH_4 formation.
- SRB communities remain resilient to pH shocks, ensuring system stability.
- Active *Desulfovibrio* sustains sulfate reduction and improves effluent quality.
- Acetate-rich effluent (>3.0 g L^{-1}) is suitable for downstream methanogenesis.
- Fermentative metabolism stays resilient under shocks, enabling waste valorization.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Two-stage anaerobic digestion
Sugarcane biorefinery
In-process operational strategies
Industrial wastewater treatment

ABSTRACT

Efficient two-phase anaerobic digestion (2nd-AD) of sugarcane vinasse hinges on effectively suppressing methanogenesis within the initial sulfate-reducing stage (acidogenesis) to maximize downstream methane production and mitigate safety risks associated with H_2S/CH_4 co-production. This study investigates the strategic in-process application of sequential acidic and alkaline pH shocks to achieve this critical control. An anaerobic structured-bed reactor (AnSTBR), reactivated from prolonged storage (5 months) to mimic off-season conditions and test long-term system resilience, was fed with vinasse for 90 days at 30 °C across six operational stages. The results

Abbreviations: 1st-AD, first stage anaerobic digestion; 2nd AD, two stage anaerobic digestion; AnSTBR, anaerobic structured bed reactor; ATP, adenosine triphosphate; cDNA, converted DNA to RNA; CHt, total carbohydrates; CODc, converted chemical oxygen demand; CODs, soluble COD; CODt, total COD; ER_{SO4}, efficiency removal of sulfate; EC_{HLa}, efficiency conversion of lactic acid; E_{CGly}, efficiency conversion of glycerol; FB, fermentative bacteria; Gly, glycerol; HAc, acetic acid; HBu, butyric acid; HLa, lactic acid; HPr, propionic acid; HRT, hydraulic retention time; LDPE, low density polyethylene; m-AcP, mild acidic pretreatment; MA, methanogenic archaea; OLR, organic loading rate; PA, partial alkalinity; s-AcP, severe acidic pretreatment; s-AP, severe alkaline pretreatment; SLR, sulfate loading rate; SRB, sulfate-reducing bacteria; TDS, total dissolved sulfide; UASB, up flow anaerobic sludge blanket; VFA, volatile fatty acid; VSS, volatile suspended solids.

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https://doi.org/10.1016/j.scitotenv.2026.181556
Received 14 November 2025; Received in revised form 19 January 2026; Accepted 11 February 2026
Available online 16 February 2026

Methanogenesis inhibition
Desulfovibrio

demonstrate that these pH shocks hindered the hydrogenotrophic and, mainly, acetoclastic methanogenesis, reducing methane content to 3% while restoring sulfidogenesis to 82% (Stage VI) even after the system returned to original conditions. This robust sulfate removal in high-rate fermentative systems yielded an effluent rich in acetate (>3.0 g-HAc L^{-1}) with enhanced buffering capacity, ideal for subsequent acetoclastic methanogenesis. Microbial community analysis identified *Desulfovibrio* (28.69–49.38%) as the dominant and most active dissimilatory sulfate reducer, while *Bacteroides* (6.49–3.44%) and *Aminobacterium* (1.73–8.82%) were key acetate producers driving fermentative metabolism. This work establishes a novel operational strategy to efficiently modulate microbial pathways in vinasse biorefineries, advancing biogas production, environmental protection, and sustainable waste management.

1. Introduction

Sugarcane vinasse is the primary wastewater generated during ethanol distillation in sugar-ethanol production facilities. Produced in massive volumes, this effluent represents one of the most critical environmental burdens for the sugar-ethanol industry globally. Owing to its high concentrations of essential nutrients, vinasse is widely utilized for soil fertigation to enhance crop productivity (Hoarau et al., 2018). However, improper or large-scale management of vinasse poses severe environmental risks, including soil salinization, groundwater contamination, and eutrophication of nearby water bodies due to its high organic load and mineral content (Fuess and Garcia, 2014; Christofoletti et al., 2013). Effective, sustainable biorefinery strategies are thus urgently required to manage this residual volume and prevent long-term ecological damage (Carpanez et al., 2022).

Single-phase anaerobic digestion systems (1st-AD) offer a promising strategy for treating sugarcane vinasse and producing renewable biogas (Aquino et al., 2017; Del Nery et al., 2018). However, a major operational and safety challenge arises from the high sulfate content in vinasse (>2.0 g-SO_4^{2-} L^{-1}), which promotes the growth of sulfate-reducing bacteria (SRB). SRB compete with methanogenic archaea (MA) for substrates (acetate and hydrogen), severely reducing methane yields (Fuess et al., 2019, 2023a). Crucially, the resulting H_2S-rich biogas in such mixed systems often contains explosive levels CH_4 and H_2, introducing significant safety and technical challenges for downstream gas desulfurization and utilization (Borges et al., 2025b; Kao et al., 2024). A two-stage anaerobic digestion (2nd-AD) approach, where sulfidogenesis and methanogenesis are kinetically separated, is the proposed solution to address these environmental and safety limitations. In this setup, the first stage facilitates robust sulfate reduction and acidogenesis, yielding an acetate-rich, sulfate-free effluent for optimal methanogenesis in the second stage (Fuess et al., 2023a; Borges et al., 2025a; Rogeri et al., 2023; Piffer et al., 2021).

While sulfate reduction in fermentative systems has been scarcely studied on distinct agro-industrial residues (Reis et al., 1988; Ren et al., 2007; Wang et al., 2008), its application to sugarcane vinasse is a relatively recent development (Fuess et al., 2019). Recent studies have demonstrated successful sulfate reduction (>90%) in thermophilic (55 °C) high-rate fermentative systems processing raw vinasse under controlled conditions (i.e. NaOH and $NaHCO_3$ dosing) (Fuess et al., 2023a; Rogeri et al., 2023; Piffer et al., 2021). These reactors, inoculated with naturally fermented vinasse, showed butyric acid (HBu) as the dominant metabolite. However, feeding butyric-rich effluents into the second stage has been associated with reduced CH_4 production rates (Fuess et al., 2022; Fuess et al., 2020). Additionally, stable thermophilic naturally fermented systems generate not only high concentrations of hydrogen sulfide (H_2S) in the biogas but also low-to-moderate fractions of H_2, which is explosive and can render the desulfurization technology unfeasible due to safety concerns (Borges et al., 2025b; Kao et al., 2024). Moreover, natural fermentation can be unreliable, as seasonal variations in vinasse composition impact microbial community stability (Fuess et al., 2024), likely affecting long-term sulfate removal efficiency.

Conversely, recent research conducted under mesophilic (30 °C) conditions offers potential solutions to these challenges. Sánchez et al. (2021) observed a predilection for acetic-type fermentation during vinasse fermentation in mesophilic temperatures (30–40 °C). Fuess et al. (2023b) observed a shift toward acetate production in mesophilic fermentative systems, albeit with limited (25%) sulfidogenic activity. The acetate-enriched effluent could reduce reliance on thermodynamically unfavorable processes (i.e., acetogenesis) in subsequent methanogenic systems, thereby improving the overall efficiency of the AD process. Building on this, Borges et al. (2025a) evaluated sulfate removal under similar conditions using both naturally fermented vinasse and sludge-enriched inocula. While acetic-type fermentation prevailed in all systems, sludge enrichment resulted in superior sulfate removal (>90%) and improved organic matter conversion (i.e. carbohydrates, lactic acid, and glycerol). However, persistent hydrogenotrophic methanogens were observed even at harsh fermentative conditions (i.e. high organic loads and dissolved sulfides). This raises concerns, as methanogenesis should be confined to the second stage to prevent energy losses. These results highlight the need for refined operational strategies that suppress methanogenesis during mesophilic fermentation, ensuring robust and stable microbial communities for effective long-term operation.

Despite the clear need for microbial control in the first stage of 2nd-AD systems, current research lacks operational strategies capable of maintaining methanogenesis suppression reliably, particularly under the fluctuating conditions of industrial facilities. This study pioneers the strategic, in-process application of sequential acidic and alkaline pH shocks in mesophilic conditions to suppress methanogenesis, specifically targeting the hydrogenotrophic and acetoclastic pathways, thereby enhancing sulfidogenesis and reactor stability. Various operational strategies were evaluated to enhance system performance, including (1) modifications in hydraulic retention time (HRT), and (2) the strategic application of mild and severe chemical shocks (s-AcS, s-AS) during continuous operation, which is an unprecedented approach in vinasse biorefineries. DNA and RNA sequencing were utilized to determine microbial abundance and activity, offering a comprehensive view of functional shifts induced by these shocks and quantifying the resilience of the sulfate-reducing community. Moreover, this study introduces the first metabolic prediction for sulfate reduction in a sludge-enriched mesophilic fermentative system, shedding light on the metabolic pathways active under these conditions. The insights gained are vital for designing resilient, high-rate fermentative systems that minimize environmental risks and maximize resource recovery in the sugar-and-ethanol industry.

2. Material and methods

2.1. Sugarcane vinasse characterization

Vinasse samples were obtained from a full-scale sugar-ethanol plant located in Pradópolis, São Paulo, Brazil. The samples were collected in 20-L plastic containers and stored at −20 °C to ensure preservation. Key physicochemical properties of the vinasse included: Total Chemical Oxygen Demand (CODt) of 28.3 ± 2.8 g L^{-1}, Soluble Chemical Oxygen Demand (CODs) of 23.8 ± 3.3 g L^{-1}, Total Carbohydrates (CHt) of 5.3 ± 0.5 g L^{-1}, Lactic Acid (HLa) of 1.5 ± 0.3 g L^{-1}, Volatile Suspended Solids (VSS) of 1.5 ± 0.2 g L^{-1}, sulfate (SO_4^{2-}) content of 2.4 ± 0.2 g L^{-1},

glycerol (Gly) of 2.9 ± 0.3 g L^{-1}, and a pH of 4.7 ± 0.1.

2.2. Experimental set up, reactor operation, and pretreatment procedures

The reactor evaluated in this study was originally inoculated following the procedures described by Borges et al. (2025a), named as R3, using crushed mesophilic granular sludge (0.04 g TVS g^{-1} sludge) obtained from a full-scale UASB treating poultry wastewater. The inoculum was mixed with raw vinasse, and pH was adjusted using NaOH (50% w v^{-1}) and $NaHCO_3$ to promote the initial establishment of sulfidogenic activity. After the previous experimental cycle, the reactor was stored at 2 °C for three months to simulate the sugarcane off-season. For the present study, R3 was reactivated by resuming recirculation with raw vinasse at 30 °C for 8 days, allowing biomass reactivation and attachment to the structured packing material before continuous feeding. The complete inoculation and start-up procedures are available in Borges et al. (2025a).

Before feeding, frozen vinasse was thawed at room temperature and homogenized. When required, influent pH was adjusted using NaOH (50% w v^{-1}) or H_2SO_4 (6 M), and $NaHCO_3$ was added according to the buffering requirements of each operational stage. For analytical purposes, samples were centrifuged (9000 rpm, 5 min) for CODs measurement, filtered through 0.45 μm membranes for VFA analyses, and immediately stabilized with NaOH (for sulfide) or HCl (for sulfate), preventing volatilization or oxidation artifacts (Fig. 1).

Reactor operation consisted of six sequential stages designed to suppress methanogenesis and impose controlled pH shocks under fermentative conditions. Stages differed in hydraulic retention time (HRT), pH control strategy, and application of acidic or alkaline disturbances. A detailed summary of each stage is provided in Table 1. Stages I and II consisted of continuous operation under neutral pH (6.5) with different HRTs (12 and 6 h, respectively) to modulate fermentative activity and inhibit methane formation. The HRT reduction in Stage II intentionally increased organic and sulfate loading, creating conditions that disadvantage slower-growing methanogens while favoring fermentative and sulfate-reducing populations (Fuess et al., 2023a). This strategy provided the operational basis for expecting reduced methane formation at the shorter HRT. In Stage III, no pH correction was applied, allowing the raw vinasse to enter the reactor continuously at its inherent acidity (pH ~4.5). Although no external acid was added, this deliberate omission of alkalinity dosing imposed a reproducible mild acidic shock over a full HRT, enabling assessment of how moderate, operationally relevant pH depression affects methanogenic archaea. Stages IV and V imposed severe acidic (pH 3.0) and severe alkaline (pH 12.0) disturbances under closed-cycle batch recirculation for 24 consecutive cycles, each corresponding to a 6-h turnover (Sun et al., 2020). This configuration ensured full and sustained exposure of the entire biomass to the extreme pH conditions. Finally, Stage VI consisted of recovery under a continuous 12-h HRT and pH 6.5, reinstating conditions comparable to Stage I. This sequence established a controlled transition from methanogenic potential toward a stable acetogenic–sulfidogenic regime. The duration of the recovery phase (Stage VI) was defined using the representativeness index for the operating period (RIOP) in Eq. (1), where Δt corresponds to the phase duration and the mean HRT represents the characteristic cycle time. Following the criterion RIOP ≥30, as proposed by Ruggeri et al. (2015) and applied to vinasse fermentation by Fuess et al. (2023a), Stage VI was maintained for 34 cycles to ensure that the observed metabolic response reflected a stable operating condition rather than transient fluctuations.

$$\mathrm{RIOP} = \frac{\Delta t}{\mathrm{mHRT}} \tag{1}$$

The literature on the application of pH-shock strategies to sugarcane vinasse fermentation is notably scarce. Therefore, the pH values used in this study were selected based on established anaerobic bioprocess studies applying extreme pH disturbances to selectively inhibit specific microbial groups. The severe alkaline shock (pH 12) followed the conditions reported by Jang et al. (2015), who applied pH 12 to eliminate indigenous lactic acid bacteria during food-waste pretreatment. The severe acidic shock (pH 3.0) was selected based on Mota et al. (2018), who demonstrated that hydrogen production can remain stable even under strong acidification, indicating that microbial activity can withstand short-term exposure to pH 3.0. These values were chosen to impose controlled yet intense selective pressures on the microbial community, enabling evaluation of system resilience, mainly SRB, and methanogenic suppression under conditions supported by anaerobic fermentation literature.

Each stage of the study was defined through a problem–solution

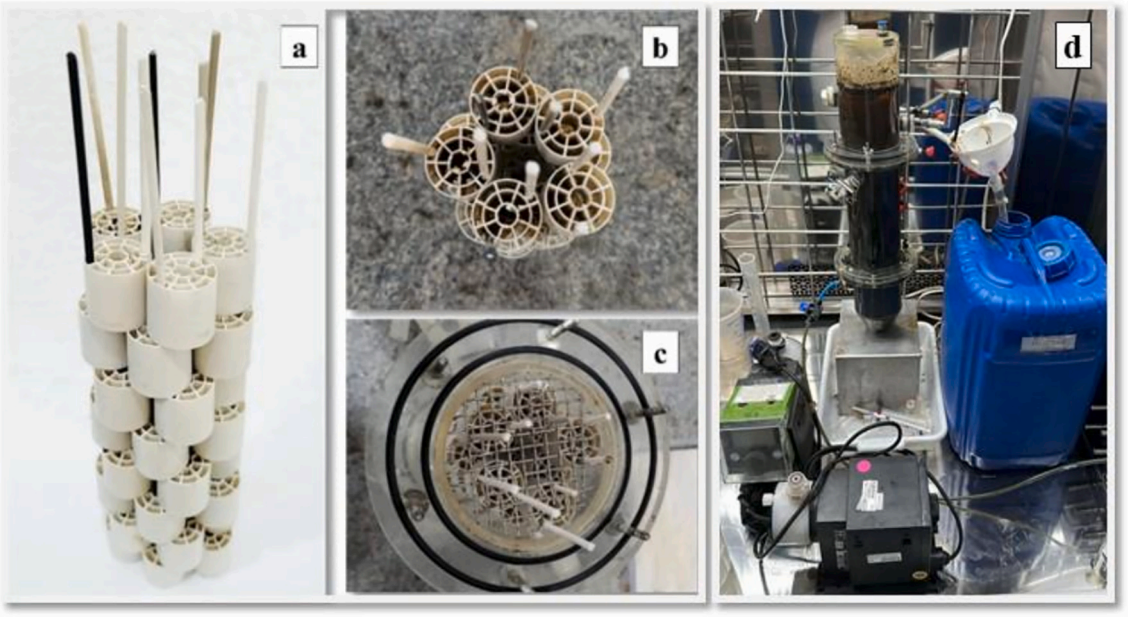

Fig. 1. Assembly of the structured-bed fermentative system: (a) LDPE cylindrical support material; (b) front view of the packed bed; (c) support material integrated into the acrylic frame; (d) AnSTBR system in operation.

Table 1
Operational stages applied to the continuous fermentative system at 30 °C.

Stage	Operating period	HRT	pH correction	$NaHCO_3$ dosage	Description
	(d)	(h)	(NaOH 50% w v^{-1}/6 M H_2SO_4)	(g-$NaHCO_3$ g-COD_t^{-1})	
I	0–27	12.0	6.5 [a]	0.25	System reactivation and establishment of a fermentative–sulfidogenic environment to produce sulfate-free, acetate-enriched fermented vinasse.
II	28–44	6.0	6.5 [a]	0.25	HRT reduction to promote sulfidogenesis and suppress methanogenesis while maintaining stable fermentation conditions.
III	45–58	12.0	–	–	Application of a mild acidic shock (m-AcS) to challenge the microbial community and inhibit methanogenesis.
IV	59–64 [b]	6.0 [c]	3.0	–	Application of a severe acidic shock (s-AcS) to intensify methanogenic suppression.
V	65–72 [b]	6.0 [c]	12.0	–	Application of a severe alkaline shock (s-AS) to test community tolerance to extreme pH and further inhibit methanogenesis.
VI	73–89	12	6.5 [a]	0.25	Recovery phase aimed at re-establishing process stability after the imposed shock treatments.

Abbreviations: CODt – total chemical oxygen demand, HRT – hydraulic retention time.
[a] The pH of raw vinasse (pH 4.3) was adjusted to 6.5 using a 50% w v^{-1} NaOH solution, corresponding to an average dosage of 5 mL L^{-1} (2.5 g NaOH L^{-1}).
[b] Operation carried out in closed-cycle mode.
[c] For each severe pH shock, the system was subjected to 6-h cycles, totaling 24 cycles, as reported by Sun et al. (2020).

sequence rather than a predetermined series of trial (e.g. following reactor reactivation, unexpectedly high methane production persisted despite the strongly fermentative conditions, indicating that methanogens remained highly resilient). Consequently, each new operational adjustment was introduced only after the preceding strategy demonstrated insufficient to suppress methanogenesis without compromising fermentative or sulfidogenic activity. This iterative approach allowed the system's performance to guide the design of subsequent interventions, ensuring that operational changes were evidence-driven and targeted.

2.3. Analytical methods

In the liquid phase, monitoring focused on evaluating COD_t, COD_s (by sample centrifugation at 9000 rpm for 5 min), pH, partial alkalinity (PA) SO_4^{2-}, total dissolved sulfide (TDS), CH_t, Gly, HLa, and volatile fatty acids (VFA). Standard analytical procedures for COD_t, COD_s, pH, SO_4^{2-}, and TDS followed the guidelines specified in the Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012). For TDS and SO_4^{2-} analysis, NaOH (1:1 w v^{-1}) and HCl (6 M), respectively, were added to the samples immediately after collection. CHt, Gly, and HLa concentrations were determined following the methodologies of Dubois et al. (1956), Greenhill (2003), and Taylor (1996), respectively. PA was assessed using Kapp (1984) method. VFA concentrations were measured using gas chromatography (Model GC 2010, Shimadzu Scientific Instruments, Columbia, MD, USA) with a flame ionization detector (FID) and a COMBI-PAL autosampler, following the protocol of Adorno et al. (2014). The carrier gases included H_2 for transport, synthetic air for combustion, and nitrogen for stabilization. Prior to GC analysis, samples were filtered through 0.45 μm syringe filters (Chromafil GF/PET, Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Gas-phase analysis included measuring CO_2, CH_4, and H_2S concentrations using gas chromatography (Model GC-2014, Shimadzu Scientific Instruments®, Columbia, MD, USA) equipped with a thermal conductivity detector (TCD) and an HP-PLOT/Q column (30 m × 0.53 mm × 40 μm) (Lebrero et al., 2016). H_2 was used as the carrier gas. The H_2 concentration was analyzed using a separate gas chromatograph (Model NexisTM GC-2030, Shimadzu Scientific Instruments, Japan) with argon as the carrier gas. Biogas flow rates were measured using an electromechanical pulse system integrated into a U-tube, which was connected to an Arduino setup interfaced with the reactor's headspace, as described by Veiga et al. (1990).

2.4. Microbial characterization and metabolic inference

At the end of Stages I, II, IV, V, and VI, biomass samples were collected from the feeding zone of the AnSTBR. In Stages I and VI, suspended biomass was also sampled. For molecular analyses, samples were preserved in phosphate-buffered saline (PBS) for DNA extraction and in RNALater (Thermo Fisher) for RNA stabilization, following the manufacturer's protocols. The samples were stored at −20 °C until the extraction of nucleic acids (DNA and RNA). DNA and RNA were extracted using the FastDNA SPIN Kit for soil (MP Biomedicals, Irvine, CA, USA) and Trizol, respectively, and stored at −20 °C. RNA samples (1 μ g) were converted to cDNA through the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RNA, DNA and cDNA samples were quantified using a Qubit Fluorometer (Invitrogen) and qualified using a Nanodrop spectrophotometer. All kits were employed following the manufacturer's protocols. Sequencing of the 16S ribosomal RNA region was performed on the Illumina NextSeq 1000 platform (Instituto SENAI de Inovação em Biotecnologia, SP, Brazil) using the following primers targeting both Bacteria and Archaea: V4 - 16S rRNA (~390 bp), 515FB - GTGYCAGCMGCCGCGGTAA, and 806RB - GGACTACNVGGGTWTCTAAT.

The sequencing data were processed using the DADA2 pipeline (v. 1.30.0) in R (v. 4.3.3). Initially, reads were filtered and trimmed with the following parameters: maxN = 0, maxEE = c(2,2), truncQ = 2, and rm. phix = TRUE. Taxonomic assignment was performed against the SILVA database (v. 138.2). All sequence data were submitted to the National Center for Biotechnology Information (NCBI), and the BioProject is available under accession number PRJNA1285913. The metabolic potential of the AnSTBR active microbial community was predicted using the Tax4Fun2 package (v1.1.6) (Wemheuer et al., 2020) in the R environment. Functional inference was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, applying a 97% similarity threshold (Kanehisa et al., 2016). The resulting gene functions were then visualized and mapped through the KEGG Mapper – Reconstruct Pathway tool, with enzyme-specific information retrieved from the BRENDA database.

3. Results and discussion

3.1. Re-establishing sulfidogenesis in vinasse fermentation

At the start of the operation, the fermentative system exhibited stable

effluent pH levels near neutrality (6.7 ± 0.2). Fuess et al. (2023a) identified pH as a critical operational factor for promoting and sustaining sulfidogenic activity in thermophilic (55 °C) fermentative systems treating sugarcane vinasse. Similarly, Borges et al. (2025a) highlighted the strong influence of pH on sulfidogenic pathways in mesophilic (30 °C) systems. In this study, adjusting the influent pH to 6.5 using NaOH (1:1 w v^{-1}) and supplementing the raw vinasse with 0.15 g-$NaHCO_3$ g-COD_t^{-1} provided effective buffering conditions that supported the growth of fermentative and SRB populations. Fig. 2 a depicts the time course of pH, sulfate reduction efficiency ($ERSO_4$, %), PA and TDS concentrations over the initial 58 days of operation (from Stage I to III).

During the initial phase of operation, sulfidogenic activity remained relatively low (20–43%), likely due to the adaptation of SRB communities to the high organic loading rates (OLR) imposed on the system (59.4 ± 1.5 kg-COD_t m^{-3} d^{-1}). Despite this, fermentation activity remained robust, with conversion efficiencies of soluble CHt, HLa, and Gly (% COD converted, COD_c) reaching 86.8 ± 9.4%, comparable to reactor's performance prior to storage in a cold chamber (86.4 ± 2.3%) (Fig. 2 b) (Borges et al., 2025a). These results suggest that the fermentative bacteria (FB) were not adversely affected during the storage period. At similar OLRs (54.8 ± 4.8 kg-COD_t m^{-3} d^{-1}) and effluent pH values (6.7 ± 0.3), Fuess et al. (2023a) reported high fermentative activity in a naturally fermented AnSTBR processing vinasse under mesophilic conditions (30 °C). However, sulfidogenesis establishment remained unsuccessful, with an $ERSO_4$ of 25.4 ± 7.7% after 30 days of operation under a COD/SO_4^{2-} ratio of 18 ± 2. In a continuous naturally fermented AnSTBR treating vinasse at 55 °C, Rogeri et al. (2023) observed COD conversions of 70.0 ± 7.0 at higher OLRs (78.3 ± 6.1 kg-COD_t m^{-3} d^{-1}). Nevertheless, sulfidogenic activity remained limited (47.9 ± 12.3%) due to pH instabilities during the start-up phase. Despite these limitations, the acidified effluent exhibited a COD/SO_4^{2-} ratio exceeding 25, which mitigated potential competition and inhibition effects on methanogenesis (Kiyuna et al., 2017).

Operating a single-phase system exclusively with vinasse during the sugarcane off-season presents significant challenges. Extended feed interruptions (e.g., at least 4 months) can disrupt the reactor's microbial community, prolonging start-up times needed to reestablish efficient operation under high OLRs when the system restarts (Fuess et al., 2023b; Santana Jr. et al., 2019; Jáuregui-Jáuregui et al., 2014). Consequently, this underutilization of vinasse's energy potential at the start of the harvest season often coincides with the accumulation of large wastewater volumes. To address these challenges, studies on co-digestion with various substrates, such as molasses (Fuess et al., 2023b; Barbosa et al., 2022), glycerol (Menezes et al., 2023; Takeda et al., 2022; Borges et al., 2021, 2022), and filter cake (Volpi et al., 2021) have been conducted to enable year-round biogas production in sugar-ethanol facilities. While co-digestion can enhance methane production, several disadvantages have been reported in the literature, including: (i) imbalance in nutrient composition (Xie et al., 2018); (ii) substrate competition (Wan et al., 2011); (iii) increased risk of methanogens inhibition (Farhat et al., 2018); (iv) logistical challenges (including storage, transportation, and handling costs) (Jasińska et al., 2023); and (v) variable biogas quality (Xu et al., 2018). In this study, the temporary shutdown of the

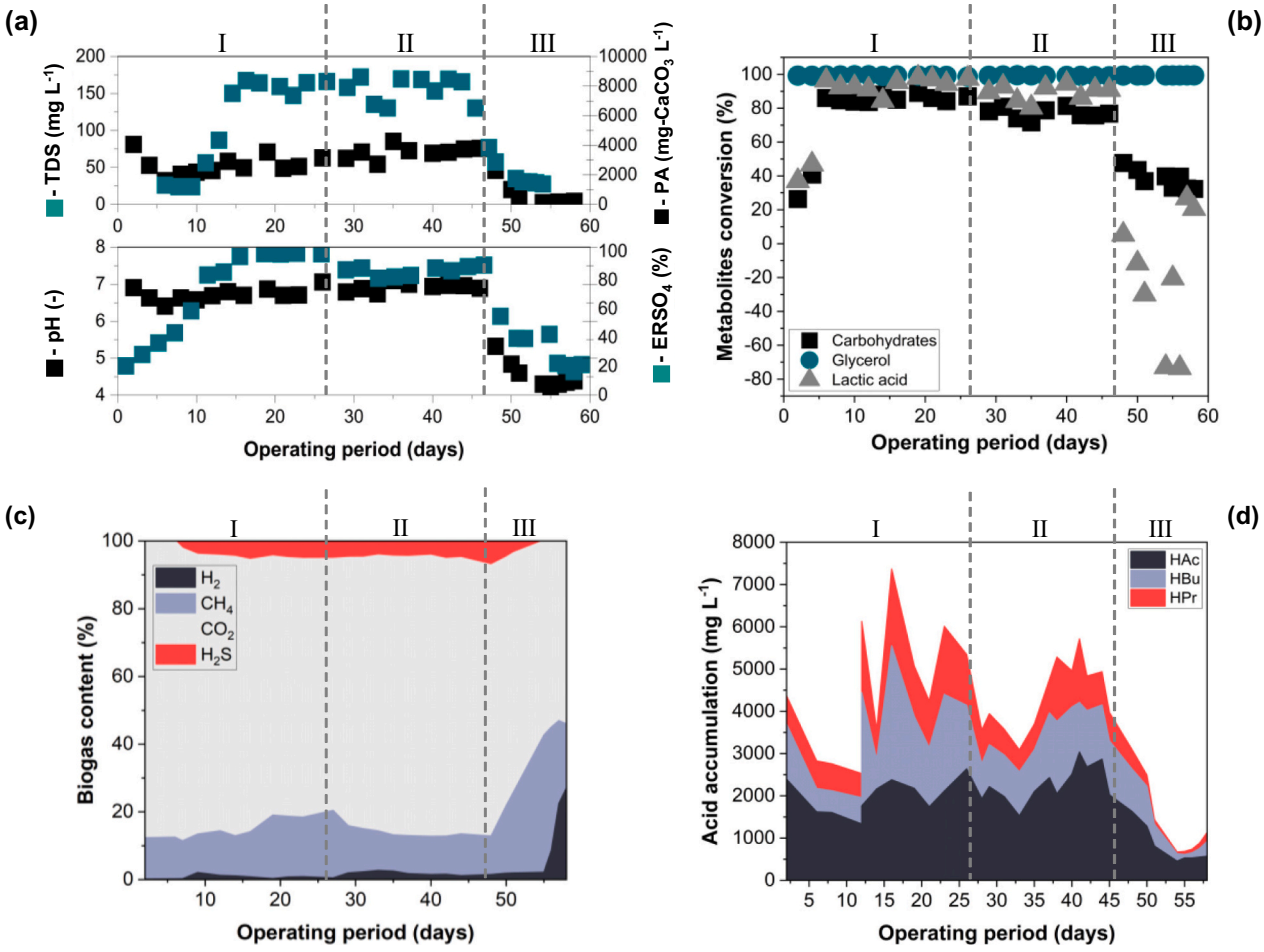

Fig. 2. Time course of: (a) total dissolved sulfide (TDS), partial alkalinity (PA), pH and sulfate removal efficiency (ER_{SO4}), (b) soluble metabolites conversion (i.e., CHt, HLa and Gly), (c) biogas content, and (d) volatile fatty acid (VFA) accumulation. Negative values indicate the accumulation (rather than consumption) of the metabolite in the bulk liquid.

mesophilic, sludge-enriched system, simulating the sugarcane off-season, did not adversely impact performance upon restarting. This observation highlights the system's resilience and the microbial community's ability to rapidly restore functionality following prolonged storage. Furthermore, rapid acidification in the first AD stage is likely to accelerate methanogenesis in the subsequent stage, enhancing overall system efficiency and reducing downtime.

From the 12th day onwards, an $ERSO_4$ of 85% was observed, reaching 99% in subsequent days, with effluent pH levels maintained above 6.5. PA values increased throughout Stage I, ranging from 1612 to 4263 mg-$CaCO_3$ L^{-1} (average of 3063 ± 724 mg-$CaCO_3$ L^{-1}) (Fig. 2 a). These findings indicate a suitable and conducive buffering capacity for fermenters and sulfate reducers, despite the higher conversion of organic compounds (CODc>80%) into volatile acids. Fuess et al. (2023a) have shown that a thermophilic, naturally fermented system supplemented with 0.1 g-$NaHCO_3$ g-COD_t^{-1} exhibit lower effluent PA values (1085 mg-$CaCO_3$ L^{-1}) compared to a mesophilic, sludge-enriched fermentative system (2365 mg-$CaCO_3$ L^{-1}) supplemented with similar bicarbonate dosing (0.15 g-$NaHCO_3$ g-COD_t^{-1}) to raw vinasse (Fuess et al., 2023a; Borges et al., 2025a). Improvements in effluent PA levels from mesophilic fermentation can potentially reduce the high chemical consumption needed to stabilize methanogenic systems, particularly by exploring alternative alkalinization methods (i.e., effluent recirculation); however, further studies are required to validate this approach. Borges et al. (2025a) further revealed that lower bicarbonate consumption can be achieved by the granular sludge system, despite neutralizing a greater number of protons in the solution. This observation was not only attributed to the inoculum source but also to the higher sulfidogenic activity, as bicarbonate ions can be generated during the metabolism of organic compounds or hydrogen in conjunction with sulfate reduction. As a result of high sulfidogenic activity in this study, soluble TDS concentrations increased, ranging from 8 to 183 mg-TDS L^{-1} (Fig. 2 a). No apparent adverse effects on sulfidogenic communities were observed, consistent with findings previously reported by Borges et al. (2025a). Although reports on sulfide concentrations in fermentative systems are scarce, Gil-Garcia et al. (2018) identified inhibitory levels for SBR populations at concentrations exceeding 250 mg-TDS L^{-1}, supporting the conclusion that the concentrations observed in this study were non-critical.

Stage I exhibited high conversion efficiencies for CHt (EC_{CHt} = 86.0 ± 1.8%), HLa (EC_{HLa} = 94.1 ± 4.3%), and Gly (EC_{Gly} = 99.2 ± 0.3%), driven by robust fermentative and sulfidogenic activities (Fig. 2 b). Among the carbon sources, Gly (−339.6 kJ $mol^{-1}SO_4^{2-}$) and HLa (−160.1 kJ $mol^{-1}SO_4^{2-}$) were likely preferentially utilized by SRB communities due to their simpler structure and more favorable thermodynamic properties compared to complex organic substrates (Bertolino et al., 2014). During Stage I, CH_4 concentrations in the biogas increased significantly, reaching up to 20%, despite the elevated OLR applied. H_2 levels remained consistently below 1%, while H_2S concentrations reached 5.5% (55,000 ppm_v). Fig. 2 c presents the temporal trends in biogas composition.

3.2. Decreasing HRT to control methanogenesis

The increase in CH_4 content prompted a reduction in HRT from 12 to 6 h in Stage II to inhibit methanogenesis. While pH (6.9 ± 0.1) and PA (2720–4263 mg-$CaCO_3$ L^{-1}) remained stable and comparable to Stage I, doubling the OLR resulted in a 13% decline in $ERSO_4$, with CH_4 content ranging from 10% to 14%. The reduced HRT also influenced fermentative activity, as seen in lower average conversion rates of CHt (77.1 ± 3.4%) and HLa (88.9 ± 4.8%) (Fig. 2 b). VFA accumulation started at lower levels and gradually increased during Stage I, with HAc showing the highest average concentrations (1994 mg-HAc L^{-1}) in comparison to HBu (1495 mg-HBu L^{-1}) and HPr (1076 mg-HPr L^{-1}) (Fig. 2 d). Acetic production remained dominant regardless of HRT variations, reaching its highest peak of 3037 mg-HAc L^{-1} on day 41. HAc-rich fermentative systems due to the activity of incomplete SRB oxidizers under mesophilic conditions has also been reported in other studies (Borges et al., 2025a; Sánchez et al., 2021).

The main disadvantage of having methane in the early stages of a fermentation system, where sulfate reduction is the primary objective, lies in the reduced efficiency of energy recovery. Methane production, primarily via hydrogenotrophic pathways, diverts H_2 from SRB to MA, thereby competing for substrates and reducing the overall efficiency of sulfate reduction (Weijma et al., 2002). Since acetoclastic methanogens are highly sensitive to harsh fermentation conditions, such as elevated organic loads, carbon sources are not converted to methane under these conditions. This leads to a reduction in carbon recovery, with methane being released into the atmosphere rather than being utilized for energy recovery, ultimately compromising the intended performance of the bioprocess. The presence of CH_4 and H_2 in H_2S-rich off-gas streams, the latter particularly in thermophilic naturally fermented systems (up to 25%), also elevates safety concerns in aerobic desulfurization systems due to their flammability and explosive potential (Borges et al., 2025b; Kao et al., 2024). Methane has a flammability range of 5–16% in air, while hydrogen's range is even broader (4–75%) and is highly prone to accidental ignition due to its low ignition energy (Jeon and Kim, 2020; Cui et al., 2016). Combined with the toxicity of H_2S and the potential for reactive compound formation, such as pyrophoric iron sulfides, these risks require stringent safety protocols. Desulfurization systems must be equipped with explosion-proof equipment, continuous sulfur monitoring, controlled oxygen supply, and emergency ventilation to mitigate these hazards, which add to operational and maintenance costs (Li et al., 2022).

3.3. Mild to severe chemical shocks to suppress methanogens

During Stage III, characterized as a mild acidic shock (m-AcS), sulfidogenic activity was markedly inhibited ($ERSO_4$ = 55–21%) as pH levels decreased from 7.0 to 4.5 ± 0.3 (Fig. 2 a). This underscores the critical dependence of sulfidogenesis on maintaining pH levels above 6.5, corroborating previous findings (Fuess et al., 2019, 2023a; Borges et al., 2025a). This shift also coincided with a reduction in TDS (138–9 mg-TDS L^{-1}) and the complete depletion of PA concentrations, indicating that the inhibition of SRB populations was primarily driven by unfavorable pH conditions rather than elevated sulfide concentrations. Fermentative activity also declined, with the average EC_{CHt} dropping to 38.2 ± 5.5% (Fig. 2 b). However, Gly fermentation remained robust (EC_{Gly} > 99%) across Stages II and III, consistent with glycerol's status as a readily available and highly thermodynamically favorable carbon source for both active SRB and fermenters (Zhou et al., 2022). Fuess et al. (2019) reported nearly complete glycerol conversion (> 95%) in a high-rate naturally fermented system processing vinasse at 55 °C. This high efficiency was observed under both favorable (pH > 5.0) and unfavorable (pH < 5.0) conditions for H_2 production. A marked drop in VFA accumulation was observed during this stage (Fig. 2 d), especially for HAc (2870–571 mg-HAc L^{-1}), due to environmental stress, affecting overall microbial metabolism. The reduced effluent pH, approaching that of raw vinasse (4.5–4.7), likely shifted to HLa-type fermentation, as shown in Fig. 2 b. Lactate accumulation at pH levels below 5.0 has been documented in previous studies (Fuess et al., 2019; Piffer et al., 2022). Notably, CH_4 concentrations in the biogas sharply increased, peaking at 40% on day 55, while H_2 reached a maximum of 27%. According to Ferraz Junior et al. (2014), optimal conditions for H_2 production (1212 mL-H_2 d^{-1}) and content (36%) in the biogas during thermophilic (55 °C) fermentation of sugarcane vinasse were observed at comparable parameters to this study, including a pH of around 4.9, an HRT of 12 h, and OLR of 72.4 kg-COD_t m^{-3} d^{-1}.

In response to unsuccessful efforts to suppress methanogenesis, the system underwent a severe acidic shock (s-AcS) (Stage IV), maintained over six consecutive days in a closed cycle to ensure at least 24 HRTs for consistent evaluation of fermentative performance (in terms of

metabolic patterns), as evaluated by Sun et al. (2020). By sustaining pH levels at 3.0 ± 0.2, the fermentative system demonstrated a 27% reduction in the initial SO_4^{2-} concentration (5.5–4.0 g-SO_4^{2-} L^{-1}) on days 59 and 60, with minimal accumulation of TDS (8.5 ± 1.0 mg-TDS L^{-1}) (Fig. 3 a). The reduction in sulfate at the beginning of the s-AcS might be related to residual active SRB, although no H_2S was detected in the gas phase. From day 61 onwards, no sulfidogenic activity was observed. The increase in average sulfate loading rates (SLR) from 5.1 ± 0.4 (Stages I to III) to 9.0 ± 1.0 kg-SO_4^{2-} m^{-3} d^{-1} due to sulfuric acid addition for pH adjustment did not favor sulfidogenesis. Instead, the extreme acidic conditions imposed (pH 3.0 ± 0.2) exerted a far stronger inhibitory effect on these neutrophilic microorganisms than variations in sulfate supply, leading to rapid suppression of sulfidogenic metabolism after the initial two days. This stage also exhibited a sharp decline in H_2 content in the biogas (27–4%), while methane levels surged to 66% (Fig. 3 b).

Acidic pretreatments/shocks suppress key microbial groups (i.e. SRB, MA) by lowering pH and improved substrate availability by breaking down complex molecules (Qiu et al., 2023; Tran et al., 2021; Sun et al., 2020). Bench-scale studies employing dark fermentation of sugarcane vinasse, either as a sole substrate or in co-digestion with other feedstocks, have consistently applied inoculum pretreatments aimed at suppressing methanogenic activity and enriching hydrogen-producing microbial communities. These pretreatments, performed prior to inoculation, include thermal (typically 80–121 °C for short durations) (Ribeiro et al., 2024; Rego et al., 2022; Bernal et al., 2021; Magrini et al., 2021; Ramos et al., 2021; Ramos and Silva, 2017, 2018), acid (pH ≈ 3 for 24 h) (Volpi et al., 2024; Lamaison et al., 2015), or combined acid-thermal methods (Moraes et al., 2019; Fuess et al., 2020b). Such strategies have been employed across both batch and continuous reactor configurations, primarily targeting biohydrogen production or the generation of volatile fatty acids and other soluble metabolites. Despite this, there is no evidence in the literature of pretreatment applications being integrated into the continuous operation phase of these systems. Furthermore, no studies to date have reported the use of such pretreatments for methanogen inhibition in high-rate fermentative-sulfidogenic systems processing vinasse, highlighting a gap in current research for systems operating under active sulfidogenic conditions.

In this study, the acidic conditions during Stage III likely stressed the FB communities, reducing their metabolic activity and resulting in lower concentrations of HAc (2023–571 mg-HAc L^{-1}), HBu (1274–350 mg-HBu L^{-1}), and HPr (673–216 mg-HPr L^{-1}), as shown in Fig. 2 d. Operating the system in a closed-cycle mode at pH 3.0 likely intensified the inhibitory effects on fermentative microorganisms, particularly HAc producers, due to the accumulation of toxic compounds, such as undissociated VFAs, while restricting nutrient replenishment. Consequently, HAc accumulation declined to undetectable levels for 12 HRTs, reaching 863 mg-HAc thereafter, while HBu and HPr concentrations remained relatively stable (Fig. 3 c).

Unlike most FB species, certain neutrophilic SRB, such as the non-spore-forming genus *Desulfovibrio*, commonly identified in mesophilic fermentative systems treating vinasse, can survive under acidic conditions through adaptive strategies. These include intracellular pH homeostasis via proton pumps (Padan et al., 2005), stress-induced protein regulation (Azcarate-Peril et al., 2005), adjustments in metabolic pathways to minimize acidic by-products formation (Yu et al., 2021), and the exploitation of microniches with localized favorable conditions (Tran et al., 2021). Furthermore, sulfate reduction generates in situ bicarbonate alkalinity, which can partially counteract the effects of low pH (Rogeri et al., 2024). These mechanisms likely account for the limited but persistent SRB activity observed during Stage III and the initial period of Stage IV.

Methanogens, particularly acetoclastic species, are highly sensitive to acidic environments, with optimal activity occurring at pH 6.5–8.0 (Demirel and Scherer, 2008). Acidic conditions inhibit their metabolism by disrupting intracellular pH homeostasis and enzymatic functions, a process further aggravated by the presence of harmful membrane-permeant free acid molecules (Qiu et al., 2023). Hydrogenotrophic methanogens display slightly greater resilience due to superior pH regulation mechanisms but remain suppressed under prolonged acidic conditions (pH < 5.0) (Wang et al., 2020; Li. et al., 2018; Shin et al., 2015). Nevertheless, methanogenic consortia in anaerobic bioreactors can adapt to acidic environments, particularly under high organic loads, as proposed by this study. In this case, methanogens in granular matrix cores are shielded from direct acid exposure by outer-layer bacteria, facilitating survival and activity (Han et al., 2019).

After unsuccessful attempts to suppress methanogenesis, a severe alkaline shock (s-AS) was applied during Stage V in a closed-cycle mode. As expected, the CO_2 content in the biogas decreased from 14% to 0%, with CO_2 primarily existing as HCO_3^- and carbonate (CO_3^{2-}) in the bulk liquid. Meanwhile, CH_4 became the dominant gas, accounting for 91–99% of the biogas composition. At pH 12, a 13% increase in $ERSO_4$ (4088–3580 g-SO_4^{2-} L^{-1}) was observed associated with TDS concentrations ranging from 8 to 31 mg-TDS L^{-1}, suggesting marginal sulfidogenesis. Notably, no H_2S in the biogas was detected during Stage V. No significant changes in metabolite accumulation were observed between Stages IV and V (Fig. 3 c).

Several studies have demonstrated that alkaline stress can adversely impact bacterial population by increasing intracellular pH, disrupting membrane potential, and damaging proteins and the cell envelope (Saito and Kobayashi, 2003). To withstand such conditions, SRB have been reported to adopt several adaptive strategies, including maintaining pH homeostasis, modifying cell membrane structures for enhanced resilience, increasing metabolic activity to counteract stress, and altering metabolic pathways to minimize the production of toxic by-products (Slonczewski et al., 2009; Stolyar et al., 2007). These mechanisms likely enabled SRB to maintain functionality and growth under the

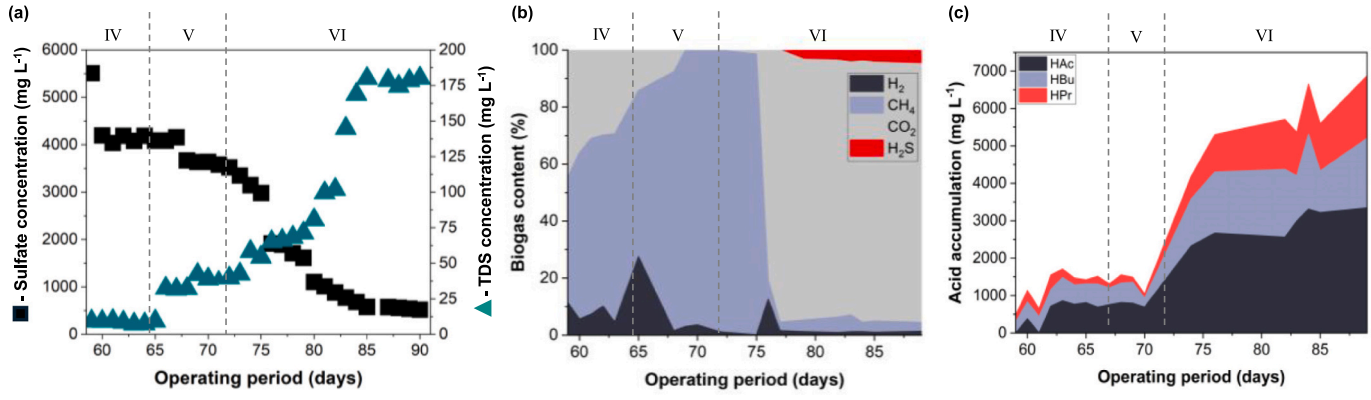

Fig. 3. Time course of: (a) sulfate and total dissolved sulfide (TDS) concentrations, (b) biogas content, and (c) metabolites accumulation from Stages IV to VI.

highly alkaline conditions observed during Stage V. As for methanogens, an in-depth investigation by Qiu et al. (2023) demonstrated that extreme pH conditions inhibited critical metabolic pathways and intracellular processes, reducing the abundance of acetoclastic methanogens while enriching hydrogenotrophic species in granular sludge (16.9%–19.5-fold). Key enzymes involved in acetoclastic methanogenesis, such as acetate kinase and formylmethanofuran dehydrogenase, exhibited reduced gene expression and activity (up to 93.1%). Additionally, disrupted electron transport, evidenced by decreased coenzyme F420 content (46.3%–70.4%) and lower abundance of CO dehydrogenase and NADH:ubiquinone reductase, further suppressed methanogenic activity. Energy metabolism was also severely affected, with ATP synthesis declining by up to 95.3%.

Following the demonstration of SRB and MA resilience under harsh fermentative conditions, a new operational stage (Stage VI) was implemented to restore sulfidogenic activity by reverting to the initial conditions of Stage I. After 48 h of continuous operation, $ERSO_4$ reached 54%, increasing to 82% by the end of the stage. This was accompanied by a rise in TDS concentrations, from 58 to 180 mg-TDS L^{-1}. A sharp increase in HAc accumulation (693–3347 mg-HAc L^{-1}) was also observed, suggesting full recovery or adaptation of the microbial community to extreme pH conditions. The return to the initial operational conditions led to a significant decrease in CH_4 content, which dropped to a minimum of 3%. These findings suggest that the sequential induced-acidic alkaline shock effectively inhibited the growth of specific methanogenic populations, contributing to the observed shifts in gas composition and sulfidogenic activity. The demonstrated resilience of SRB and MA under harsh fermentative conditions highlights potential for operational flexibility in biorefineries, although cost implications of pH regulation warrant further assessment.

3.4. Microbial characterization and metabolic inference

The 16S rRNA gene sequencing of samples from Stages I, I.s (suspended biomass), III, IV, V, VI, and VI.s (suspended biomass) yielded good coverage, as indicated by the plateau observed in the rarefaction curves (Fig. S1). The highest relative abundance of organisms from the domain Archaea ranged from 3.9% (Stage I) to 6.9% (Stage VI), while the domain Bacteria ranged from 96.1% (Stage VI) to 93.1% (Stage I). The most abundant family (Table S1) in Stages I, I.s, III, IV, and V was Bacteroidaceae (14.3–15.8%), typically known for its fermentative metabolism (Krieg et al., 2010). In contrast, in Stages VI and VI.s, the most abundant family was Desulfovibrionaceae (18.3–19.6%), which includes SRB (Brenner et al., 2005). However, the same trend was not observed for the active microorganisms (cDNA) and Desulfovibrionaceae (Table S2) was the most active family (21.6–60.9%), except in Stage IV, where it represented only 10.7% of active families, with relative abundance of Bacteroidaceae activity ranging from 6.5 to 13.4%. The lower activity of Methanomicrobiaceae in Stage VI (2.3–2.6%), compared to Stages III, IV and V (2.6–4.2%), was likely due to the strategies employed to suppress the methanogenic community, including reduced HRT, mild acidic shock (m-AcS), severe acidic shock (s-AcS), and severe alkaline shock (s-AS). These treatments promoted the selection of a microbial community dominated by SRB. While this selection led to a reduction in methane production, it did not eliminate the methanogenic community from the AnSTBR, indicating the resilience of these organisms.

In Stages I, I.s, III, and IV, the total (DNA, Fig. 4) and active (cDNA, Fig. 5 a) microbial community in the AnSTBR was primarily composed of fermentative genera, particularly *Bacteroides* (14.25–15.07%-DNA; 9.61%–13.44%-cDNA) followed by *Lacticaseibacillus* (5.79–8.13%-DNA; 5.81%–14.18%-cDNA), *Aminobacterium* (5.41–8.48%-DNA; 4.47%–8.03%-cDNA), and *Propionispira* (4.77–5.78%-DNA; 3.56%–5.98%-cDNA). *Bacteroides* species are anaerobic, Gram-negative, chemo-organotrophic, and saccharolytic bacteria whose major fermentation products are succinate and acetate (Krieg et al., 2010). They play an important role in the hydrolysis and acidogenesis stages, while *Aminobacterium* have species involved in acetogenesis, converting aminoacids into acetate (Baena et al., 2000). *Propionispira* species are anaerobic bacteria that ferment carbohydrates and produce acetate and propionate as major fermentation products (Ueki et al., 2014). These microorganisms have been part of the FB community in anaerobic reactors, including those fed with vinasse (Iltchenco et al., 2021; Bueno et al., 2024; Ao et al., 2024; An et al., 2020) and are consistent with the VFA profile observed, in which HAc showed the highest mean concentration (1994 mg-HAc L^{-1}), followed by HBu (1495 mg-HBu L^{-1}) and HPr (1076 mg-HPr L^{-1}). In phase III under the raw vinasse pH of 4.5–4.7, HLa-type fermentation predominated (Fig. 2 b), carried out mainly by lactic acid bacteria of the genus *Lacticaseibacillus*. In fact, as pointed out in Section 3.3, lactate accumulation at pH levels below 5.0 has been often documented in sugarcane vinasse fermentation (Fuess et al., 2019, 2023a; Piffer et al., 2022).

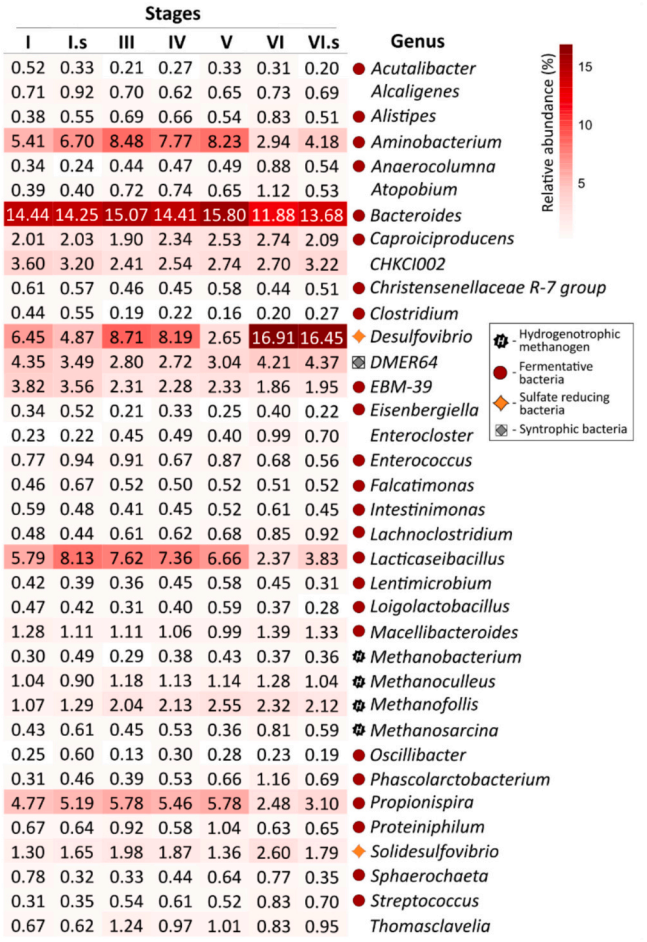

Fig. 4. Taxonomic characterization of the observed genus (DNA samples) of the AnSTBR across Stages I, I.s, III, IV, V, VI, and VI.s.

In Stages IV and V, where the microbial community was exposed to pH values of 3.0 and 12.0, respectively, the abundance of total (DNA, Fig. 4) and active (cDNA, Fig. 5 a) fermentative bacteria did not differ markedly. These results demonstrate the resilience of fermentative microorganisms to abrupt pH variations, specially the acetogenic bacteria *Bacteroides* and the lactic acid bacteria *Lacticaseibacillus,* which showed the highest cDNA levels in Stages IV and V (Figs. 5 a). This is most likely a result of increased transcription of stress-response genes (e.g., chaperones, acid or alkaline resistance proteins), along with the upregulation of carbohydrate metabolism pathways, such as fructose, mannose and galactose metabolism, to generate more ATP, as well as amino sugar and nucletotide sugar metabolism (Fig. 5 c), which are involved in cell

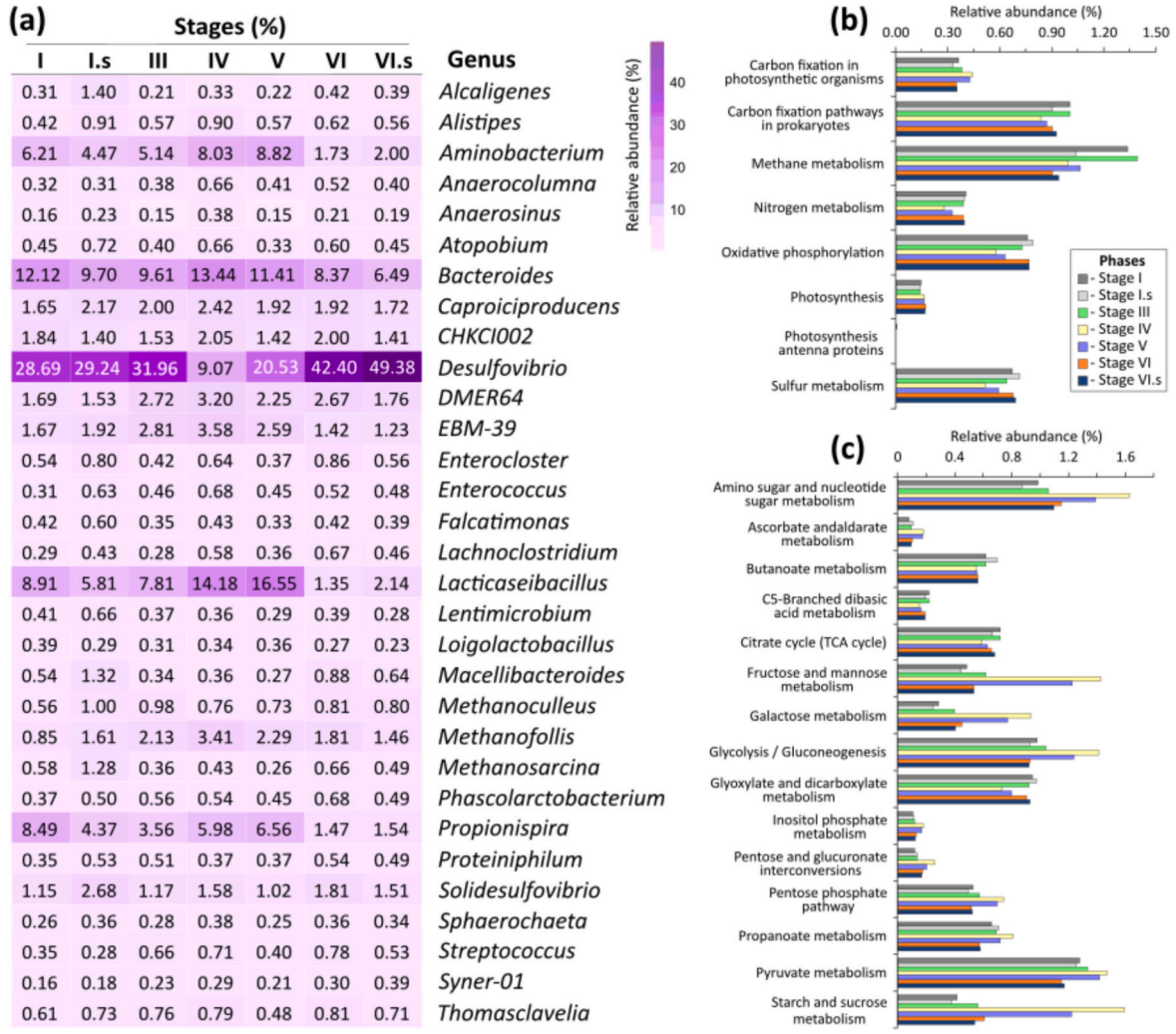

Fig. 5. Microbial and metabolic activity (RNA conversion to cDNA) of the AnSTBR across Stages I, I.s, III, IV, V, VI and VI.s. (a) Relative abundance of top 25 active organisms for each stage; (b) Relative abundance of energy metabolism pathways and (c) Carbohydrate metabolism pathway relative abundance based on KEGG functional categories.

repair, biofilm formation and osmoprotection. These adaptations allow cell survival under pH stress conditions (Padan et al., 2005; Azcarate-Peril et al., 2005; Schumacher et al., 2023), as indicated by the reduction in total organic acids production from Stage III (3971–673 mg L^{-1}) to Stages IV (1550–488 mg L^{-1}) and V (1556–1044 mg L^{-1}) (Fig. 3 c).

The severe acid condition (Stage IV) significantly inhibited sulfidogenesis, as confirmed by the decreased relative abundance of *Desulfovibrio* in the cDNA (Fig. 5 a), the downregulation of sulfur metabolism pathways (Fig. 5 b), and the operational monitoring, in which no H_2S was detected in the biogas (Fig. 3 b). These findings corroborate previous results indicating that sulfidogenesis is dependent on pH levels above 6.0 (Fuess et al., 2019, 2023a; Borges et al., 2025a). Although *Desulfovibrio* can survive under acidic conditions through adaptive strategies, as discussed in Section 3.3, its sulfate-reducing activity is suppressed under such conditions.

In Stage VI, the AnSTBR conditions returned to the initial operational setup, with the pH adjusted to 6.5 using $NaHCO_3$ and the HRT set to 12 h. Despite the reduction in *Bacteroides* relative abundance of DNA and cDNA (Fig. 4 and Fig. 5 a) with values of 11.9% in the biofilm sample (VI) and 13.7% in the suspended biomass (VI.s) for DNA and of 8.4% (VI) and 6.5% (VI.s) for cDNA, the HAc accumulation increased, indicating pH dependence. The fermentative community remained diverse, with notable acetate producers, such as *Aminobacterium* (4.2%-DNA; 2.0%-cDNA in VI.s) and *Caproiciproducens* (2.7%-DNA; 1.9%-cDNA in VI) (Fig. 4 and Fig. 5 a) as well was *Lacticaseibacillus* (3.8%-DNA; 2.1%-cDNA in VI.s). *Desulfovibrio* reached its highest abundance (16.4–16.9%, Fig. 4) and activity (42.4–49.4%, Fig. 5 a) and sulfur metabolism recovered once the reactor returned to its initial condition (Fig. 5 b).

The strategies used to inhibit the activity of the archaeal group demonstrated effective in suppressing methanogenesis. However, the persistent relative abundance of hydrogenotrophic methanogens across all stages highlights the resilience of this group, which may quickly reestablish itself once the reactor is exposed to more favorable conditions for methanogenesis. The most abundant and active genera were *Methanofollis* (1.1–2.1%-DNA; 0.8–3.4%-cDNA), *Methanoculleus* (1.0–1.3%-DNA; 0.6–1.0%-cDNA), *Methanosarcina* (0.4–0.8%-DNA; 0.3–1.3%-cDNA), and *Methanobacterium* (0.3–0.5%-DNA). Genera such as *Methanofollis*, *Methanoculleus*, and *Methanobacterium* exhibit robust physiological traits that confer tolerance to environmental disturbances, including pH fluctuations and substrate limitations (Garcia et al., 2000). Their simple metabolism, relying on H_2 and CO_2 as substrates, requires minimal enzymatic complexity and is energetically favorable under anaerobic conditions, especially when other methanogenic pathways are inhibited (Thauer et al., 2008; Zheng et al., 2020). These archaea may also form multicellular aggregates or biofilms, which protect them from environmental shocks and enable rapid recovery when conditions improve (Whitman et al., 2006). Together, these traits explain their persistence and emphasize their ecological importance in anaerobic

digestion processes. In fact, the most abundant energy metabolism pathway (based on KEGG Orthology categories) was methane metabolism (Fig. 5 b), especially in the initial stages, with a notable decrease observed after the s-AcS and s-AS.

The relatively high methane fractions (up to 20%) observed in the biogas during the early operational stages are consistent with this microbial and metabolic profile. In Stage I, the inoculum contained active hydrogenotrophic methanogens (e.g., *Methanofollis, Methanoculleus, Methanosarcina, Methanobacterium*), which rapidly exploited the high H_2 availability generated during intense saccharolytic fermentation. The initial conditions (pH 6.5, HRT 12 h, no inhibitory shocks applied yet) favored hydrogenotrophic methanogenesis, allowing these archaea to remain metabolically active and convert H_2 and CO_2 into CH_4, despite the 3-month refrigerated storage (Section 2.2). Only after the application of the acidic and alkaline shocks, designed to suppress methanogenesis, did methane production decline sharply, confirming that the early methane content was a direct consequence of the physiological traits of the inoculated methanogens and the favorable early-stage conditions.

The apparent increase in methane fraction during the extreme pH shocks (Stages IV and V) does not indicate sustained methanogenic activity. Instead, it results from selective depletion of CO_2 from the gas phase combined with the extremely low biogas production observed during the shocks. Under strongly acidic conditions, the Bjerrum equilibrium collapses fully toward $CO_{2(aq)}$, which is rapidly stripped from the recirculating liquid and continuously lost through the open headspace. During the alkaline shock, CO_2 is again depleted, this time through chemical conversion into bicarbonate and carbonate species, further suppressing its gas-phase partial pressure. Because CH_4 does not participate in acid–base equilibria and is considerably less soluble, its partial pressure remains comparatively stable. With the biogas flowrate dropping to marginal levels during the shocks, these abiotic processes artificially elevated the measured CH_4 fraction even though methanogenesis was most likely negligible.

From a biological perspective, the dominant neutrophilic methanogens (*Methanoculleus, Methanofollis, Methanosarcina*) were subjected to extreme energetic stress. Exposure to severe pH gradients forces methanogens to reallocate ATP away from methanogenesis toward survival mechanisms such as proton-pumping, cytoplasmic buffering, and membrane stabilization, ultimately generating a cumulative "metabolic debt." The functional collapse of methanogenesis only became evident in Stage VI, once pH returned to neutrality and CO_2 partial pressure stabilized, revealing an archaeal community that lacked the energetic reserves required to resume metabolism.

Evidence from the literature supports this interpretation of delayed inhibition rather than instantaneous inactivation. Qiu et al. (2023) reported that exposure to low pH (4.0) accelerates methanogenic decay by nearly an order of magnitude compared to neutral conditions, indicating progressive physiological deterioration. Similarly, Sun et al. (2020) demonstrated that low-pH shocks increase decay rates of methanogenic archaea by up to one order of magnitude while acidogenic bacteria remain largely unaffected. Importantly, methane production in their study did not cease immediately but declined progressively even after pH normalization, consistent with cumulative membrane damage, impaired proton-pumping, and enzyme inactivation. Together, these findings reinforce that pH shocks act as pre-conditioning stressors that induce profound but delayed methanogenic inhibition, aligning with the behavior observed in our system.

Regarding carbohydrate metabolism, pyruvate metabolism (Fig. 5 c) was the dominant pathway, as the most abundant substrate was the sugar present in sugarcane vinasse. Based on the taxonomic profile and metabolic prediction, the predominant metabolic processes in the AnSTBR are displayed in Fig. 6. Initially, the sucrose from sugarcane vinasse was hydrolyzed into D-glucose and D-fructose by the enzymes alpha-glucosidase [EC3.2.1.20] and beta-fructofuranosidase [EC3.2.1.26], respectively, primarily by *Bacteroides*, *Aminobacterium*, and *Macellibacteroides*, which utilize sucrose for acetate production. Glycerol was oxidized by glycerol dehydrogenase [EC1.1.1.6], likely by *Enterococcus*, due to its known affinity for this substrate (Gilmore et al., 2014). Glycolysis then directed energy flow toward pyruvate metabolism as a central route. Hydrogen production occurred via the conversion of pyruvate to acetyl-CoA by acetaldehyde dehydrogenase

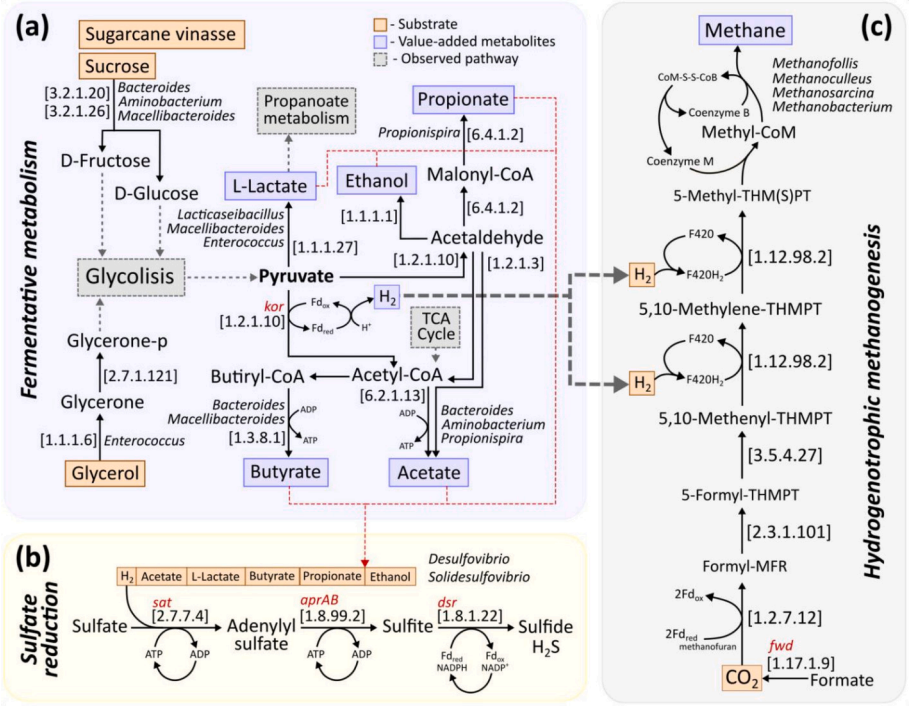

Fig. 6. Metabolic inference of predominant pathways in the AnSTBR: (a) Fermentative metabolism; (b) Dissimilatory sulfate reduction; and (c) Hydrogenotrophic methanogenesis.

[EC1.2.1.10], leading to the formation of butyrate, lactate, and acetate, the main VFAs observed in the reactor.

The H_2 and VFA produced through fermentative metabolism were used as electron donors for dissimilatory sulfate reduction (Fig. 6 b), especially by *Desulfovibrio* and *Solidesulfovibrio*. Initially, sulfate adenylyltransferase [EC2.7.7.4], encoded by the *sat* gene, transferred electrons from organic substrates to sulfate, forming adenylyl sulfate, which was then reduced to sulfite by adenylyl-sulfate reductase [EC1.8.99.2], and finally to sulfide via the dissimilatory sulfite reductase system [EC1.8.1.22].

Hydrogenotrophic methanogenesis was the dominant methane-producing pathway in the AnSTBR (Fig. 6 c), especially during the initial stages (until Stage III, Fig. 5 b). The carbon source for methane synthesis was CO_2, which was first reduced to formyl-methanofuran by formylmethanofuran dehydrogenase [EC1.2.7.12]. H_2 consumption occurred during the regeneration of coenzyme F_{420}, catalyzed by methenyltetrahydromethanopterin hydrogenase [EC1.12.98.2]. The s-AcS and s-AS strategies hindered this pathway while promoting the dominance of fermentative metabolism (Fig. 5 a) and sulfate reduction (Fig. 5 b).

To deepen insight into how pH shocks modulate metabolic pathways and electron-transfer networks, future research should combine metagenomic and metatranscriptomic analyses with targeted assays of enzymes mediating interspecies electron transfer (e.g., hydrogenases, formate dehydrogenases, DsrAB). Such integrative approaches will enable a direct link between community structure, functional capacity, and real-time metabolic responses to pH perturbations. Moreover, although the sequential pH shocks applied during operation helped elucidate community resilience, future studies should examine the application of pH shocks directly to the inoculum prior to the continuous operation of the fermentative-sulfidogenic system in a longer-term monitoring to fully assess the resilience of the microbial community and the potential re-emergence of methanogens. From a techno-economic perspective, the use of extreme pH shocks also imposes practical constraints, as scaling the strategy to continuous mode would substantially increase reagent demand and associated environmental impacts. Therefore, future work should quantify chemical consumption, evaluate operational costs, and explore alternative low-input approaches for methanogen suppression.

4. Conclusions

The mesophilic AnSTBR inoculated with granular sludge exhibited strong sulfidogenic activity (86–99%) at near-neutral pH. Methane formation persisted under high organic and sulfate loads, driven by resilient hydrogenotrophic methanogens such as *Methanofollis*, *Methanoculleus*, and *Methanosarcina*. Acidic and alkaline pre-treatments temporarily increased methane in biogas (66–99%), highlighting the tolerance of methanogens to harsh fermentative conditions. However, restoring effluent pH to 7.0 under continuous flow reestablished sulfidogenesis while reducing methane to ~3%, confirming the competitiveness and reversibility of sulfate-reducing populations. Fermentative metabolism was supported by acetate producers (*Bacteroides* and *Aminobacterium*), leading to ~3.0 g-HAc L^{-1} acetic acid accumulation. Carbohydrate catabolism proceeded mainly through the pyruvate pathway, while *Desulfovibrio* and *Solidesulfovibrio* mediated dissimilatory sulfate reduction. These results suggest that selecting a methanogen-free or methanogen-depleted inoculum may be advantageous for systems aiming to maximize sulfidogenic performance and minimize methanogenic interference.

CRediT authorship contribution statement

André do Vale Borges: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Conceptualization. **Lucas Tadeu Fuess:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Henrique Dornelles:** Writing – review & editing, Software, Methodology. **Paula Yumi Takeda:** Writing – review & editing, Methodology, Data curation. **Flávia Talarico Saia:** Writing – review & editing, Software, Methodology. **Renan Coghi Rogeri:** Writing – review & editing, Methodology. **Kaio Gustavo Gomes:** Writing – review & editing. **Márcia Helena Rissato Zamariolli Damianovic:** Resources, Project administration, Funding acquisition, Conceptualization.

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.

Acknowledgment

This work was financially supported by the São Paulo Research Foundation (FAPESP, grant numbers 2022/10615-1, 2021/15245-5 and 2015/06246-7). This study was financed in part by the Coordination for the Improvement of Higher Education Personnel – Brasil (CAPES) – Finance Code 001.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2026.181556.

Data availability

Data will be made available on request.

References

Adorno, M., Hirasawa, J., Varesche, M., 2014. Development and validation of two methods to quantify volatile acids. Am. J. Anal. Chem. 5, 406–414. https://doi.org/10.4236/ajac.2014.57049.

An, Q., Cheng, J.R., Wang, Y.T., Zhu, M.J., 2020. Performance and energy recovery of single and two stage biogas production from paper sludge: clostridium thermocellum augmentation and microbial community analysis. Renew. Energy 148, 214–222. https://doi.org/10.1016/j.renene.2019.11.142.

Ao, T.J., Wu, J., Li, K., Chandra, R., Zhao, X.Q., Tang, Y.Q., Bai, F.W., 2024. Cellulosic ethanol stillage for methane production by integrating single-chamber anaerobic digestion and microbial electrolysis cell system. Sci. Total Environ. 951, 175814. https://doi.org/10.1016/j.scitotenv.2024.175814.

APHA, AWWA, WEF (Eds.), 2012. Standard Methods for Examination of Water and Wastewater, 22nd ed. APHA, Washington, D.C.

Aquino, S.De., Fuess, L.T., Pires, E.C., 2017. Media arrangement impacts cell growth in anaerobic fixed-bed reactors treating sugarcane vinasse: structured vs. randomic biomass immobilization. Bioresour. Technol., [s. l.] 235, 219–228. https://doi.org/10.1016/j.biortech.2017.03.120.

Azcarate-Peril, M.A., McAuliffe, O., Altermann, E., Lick, S., Russell, W.M., Klaenhammer, T.R., 2005. Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in Lactobacillus acidophilus. Appl. Environ. Microbiol. 71, 5794–5804. https://doi.org/10.1128/AEM.71.10.5794-5804.2005.

Baena, S., Fardeau, M.L., Labat, M., Ollivier, B., Garcia, J.L., Patel, B.K., 2000. Aminobacterium mobile sp. nov., a new anaerobic amino-acid-degrading bacterium. Int. J. Syst. Evol. Microbiol. 50 (1), 259–264. https://doi.org/10.1099/00207713-50-1-259.

Barbosa, M.Y.U., Alves, I., Del Nery, V., Sakamoto, I.K., Pozzi, E., Damianovic, M.H.R.Z., 2022. Methane production in a UASB reactor from sugarcane vinasse: shutdown or exchanging substrate for molasses during the off-season? J. Water Process Eng. 47, 102664. https://doi.org/10.1016/j.jwpe.2022.102664.

Bernal, A.P., de Menezes, C.A., Silva, E.L., 2021. A new side-looking at the dark fermentation of sugarcane vinasse: improving the carboxylates production in mesophilic EGSB by selection of the hydraulic retention time and substrate concentration. Int. J. Hydrog. Energy 46 (24), 12758–12770. https://doi.org/10.1016/j.ijhydene.2021.01.161.

Bertolino, S.M., Melgaço, L.A., Sá, R.G., Leão, V.A., 2014. Comparing lactate and glycerol as a single-electron donor for sulfate reduction in fluidized bed reactors. Biodegradation 25 (5), 719–733. https://doi.org/10.1007/s10532-014-9694-1.

Borges, A.V., Fuess, L.T., Alves, I., Takeda, P.Y., Damianovic, M.H.R.Z., 2021. Co-digesting sugarcane vinasse and distilled glycerol to enhance bioenergy generation in biofuel-producing plants. Energy Convers. Manag. 250, 114897. https://doi.org/10.1016/j.enconman.2021.114897.

Borges, A.V., Fuess, L.T., Takeda, P.Y., Alves, I., Dias, M.E.S., Damianovic, M.H.R.Z., 2022. Co-digestion of biofuel by-products: enhanced biofilm formation maintains high organic matter removal when methanogenesis fails. J. Environ. Manag. 310, 114768. https://doi.org/10.1016/j.jenvman.2022.114768.

Borges, A.V., Fuess, L.T., Takeda, P.Y., Rogeri, R.C., Saia, F.T., Gregoracci, G.B., Damianovic, M.H.R.Z., 2025a. Unleashing the full potential of vinasse fermentation in sugarcane biorefineries. Renew. Sust. Energ. Rev. 208, 115096. https://doi.org/10.1016/j.rser.2024.115096.

Borges, A.D.V., Damianovic, M.H.R.Z., Torre, R.M., 2025b. Assessment of aerobic-anoxic biotrickling filtration for the desulfurization of high-strength H_2S streams from sugarcane vinasse fermentation. J. Hazard. Mater. 489, 137696. https://doi.org/10.1016/j.jhazmat.2025.137696.

Brenner, D.J., Krieg, N.R., Staley, J.T., 2005. Bergeys's Manual of Systematic Bacteriology - The Proteobacteria Part C. Springer. https://doi.org/10.1177/001872086000200109.

Bueno, B.E., Garcia Rea, V.S., Saia, F.T., Gregoracci, G.B., Dacanal, G., van Lier, J.B., Zaiat, M., 2024. Treatment of sugarcane vinasse in AnMBR and UASB: process performance and microbial community comparison. Front. Bioeng. Biotechnol. 12, 1489807. https://doi.org/10.3389/fbioe.2024.1489807.

Carpanez, T.G., Moreira, V.R., Assis, I.R., Amaral, M.C.S., 2022. Sugarcane vinasse as organo-mineral fertilizers feedstock: opportunities and environmental risks. Sci. Total Environ. 832, 154998. https://doi.org/10.1016/j.scitotenv.2022.154998.

Christofoletti, C.A., Escher, J.P., Correia, J.E., Marinho, J.F.U., Fontanetti, C.S., 2013. Sugarcane vinasse: environmental implications of its use. Waste Manag. 33 (12), 2752–2761. https://doi.org/10.1016/j.wasman.2013.09.005.

Cui, G., Yang, C., Li, Z., Zhou, Z., Li, J., 2016. Experimental study and theoretical calculation of flammability limits of methane/air mixture at elevated temperatures and pressures. J. Loss Prev. Process Ind. 41, 252–258. https://doi.org/10.1016/j.jlp.2016.02.016.

Del Nery, V., Alves, I., Damianovic, M.H.R.Z., Pires, E.C., 2018. Hydraulic and organic rates applied to pilot scale UASB reactor for sugar cane vinasse degradation and biogas generation. Biomass Bioenergy 119, 411–417. https://doi.org/10.1016/j.biombioe.2018.10.002.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Rev. Environ. Sci. Bio Technol. 7, 173–190. https://doi.org/10.1007/s11157-008-9131-1.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356. https://doi.org/10.1021/ac60111a017.

Farhat, A., Miladi, B., Hamdi, M., Hassib, B., 2018. Fermentative hydrogen and methane co-production from anaerobic co-digestion of organic wastes at high loading rate coupling continuously and sequencing batch digesters. Environ. Sci. Pollut. Res. 25, 27945–27958. https://doi.org/10.1007/s11356-018-2796-2.

Ferraz Junior, A.D.N., Wenzel, J., Etchebehere, C., Zaiat, M., 2014. Effect of organic loading rate on hydrogen production from sugarcane vinasse in thermophilic acidogenic packed bed reactors. Int. J. Hydrog. Energy 39, 16852–16862. https://doi.org/10.1016/j.ijhydene.2014.08.017.

Fuess, L.T., Garcia, M.L., 2014. Implications of stillage land disposal: A critical review on the impacts of fertigation. J. Environ. Manag. 145, 210–229. https://doi.org/10.1016/j.jenvman.2014.07.003.

Fuess, L.T., Zaiat, M., Nascimento, C.A.O., 2019. Novel insights on the versatility of biohydrogen production from sugarcane vinasse via thermophilic dark fermentation: impacts of pH-driven operating strategies on acidogenesis metabolite profiles. Bioresour. Technol. 286, 121379. https://doi.org/10.1016/j.biortech.2019.121379.

Fuess, L.T., Zaiat, M., Nascimento, C.A.O., 2020. Molasses vs. juice: maximizing biohydrogen production in sugarcane biorefineries to diversify renewable energy generation. J. Water Process Eng. 37, 101534. https://doi.org/10.1016/j.jwpe.2020.101534.

Fuess, L.T., dos Santos, G.M., Delforno, T.P., de Souza Moraes, B., da Silva, A.J., 2020b. Biochemical butyrate production via dark fermentation as an energetically efficient alternative management approach for vinasse in sugarcane biorefineries. Renew. Energy 158, 3–12. https://doi.org/10.1016/j.renene.2020.05.063.

Fuess, L.T., Piffer, M.A., Zaiat, M., Nascimento, C.A.O., 2022. Phase separation enhances bioenergy recovery in sugarcane vinasse biodigestion: absolute or relative truth? Bioresour. Technol. Rep. 18, 101026. https://doi.org/10.1016/j.biteb.2022.101026.

Fuess, L.T., Braga, A.F.M., Eng, F., Gregoracci, G.B., Saia, F.T., Zaiat, M., Lens, P.N.L., 2023a. Solving the bottlenecks of sugarcane vinasse biodigestion: impacts of temperature and substrate exchange on sulfate removal during dark fermentation. Chem. Eng. J. 455 (Part 2). https://doi.org/10.1016/j.cej.2022.140965.

Fuess, L.T., Braga, A.F.M., Zaiat, M., Lens, P.N.L., 2023b. Solving the seasonality issue in sugarcane biorefineries: high-rate year-round methane production from fermented sulfate-free vinasse and molasses. Chem. Eng. J. 478, 147432. https://doi.org/10.1016/j.cej.2023.147432.

Fuess, L.T., Rogeri, R.C., Eng, F., do Vale Borges, A., Bovio-Winkler, P., Etchebehere, C., Zaiat, M., 2024. Thermophilic fermentation of sugarcane vinasse: process flexibility explained through characterizing microbial community and predicting metabolic functions. Int. J. Hydrog. Energy 77, 1339–1351. https://doi.org/10.1016/j.ijhydene.2024.06.200.

Garcia, J.-L., Patel, B.K.C., Ollivier, B., 2000. Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. Anaerobe 6, 205–226. https://doi.org/10.1006/anae.2000.0345.

Gil-Garcia, C., De Godoi, L.A.G., Fuess, L.T., Damianovic, M.H.R.Z., 2018. Performance improvement of a thermophilic sulfate-reducing bioreactor under acidogenic conditions: effects of diversified operating strategies. J. Environ. Manag. 207, 303–312. https://doi.org/10.1016/j.jenvman.2017.11.043.

Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N., 2014. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Massachusetts Eye and Ear Infirmary.

Greenhill, S., 2003. Method for Determination of Free and Combined Glycerin in Biodiesel. U.S. Patent Application 10/744,272, 22 dez.

Han, W., He, P., Lin, Y., Shao, L., Lü, F., 2019. A methanogenic consortium was active and exhibited long-term survival in an extremely acidified thermophilic bioreactor. Front. Microbiol. 10, 2757. https://doi.org/10.3389/fmicb.2019.02757.

Hoarau, J., Caro, Y., Grondin, I., Petit, T., 2018. Sugarcane vinasse processing: toward a status shift from waste to valuable resource. A review. J. Water Process Eng. 24, 11–25. https://doi.org/10.1016/j.jwpe.2018.05.003.

Iltchenco, J., Peruzzo, V., Eva Magrini, F., Marconatto, L., Paula Torres, A., Luiz Beal, L., Paesi, S., 2021. Microbiota profile in mesophilic biodigestion of sugarcane vinasse in batch reactors. Water Sci. Technol. 84 (8), 2028–2039. https://doi.org/10.2166/wst.2021.375.

Jang, S., Kim, D.H., Yun, Y.M., Lee, M.K., Moon, C., Kang, W.S., Kim, M.S., 2015. Hydrogen fermentation of food waste by alkali-shock pretreatment: microbial community analysis and limitation of continuous operation. Bioresour. Technol. 186, 215–222. https://doi.org/10.1016/j.biortech.2015.03.031.

Jasińska, A., Grosser, A., Meers, E., 2023. Possibilities and limitations of anaerobic co-digestion of animal manure—a critical review. Energies 16 (9), 3885. https://doi.org/10.3390/en16093885.

Jáuregui-Jáuregui, J.A., Méndez-Acosta, H.O., González-Álvarez, V., Snell-Castro, R., Alcaraz-González, V., Godon, J.J., 2014. Anaerobic treatment of tequila vinasses under seasonal operating conditions: start-up, normal operation and restart-up after a long stop and starvation period. Bioresour. Technol. 168, 33–40. https://doi.org/10.1016/j.biortech.2014.04.006.

Jeon, J., Kim, S.J., 2020. Recent progress in hydrogen flammability prediction for the safe energy systems. Energies 13 (23), 6263. https://doi.org/10.3390/en13236263.

Kanehisa, M., Sato, Y., Morishima, K., 2016. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. J. Mol. Biol. 428, 726–731. https://doi.org/10.1016/j.jmb.2015.11.006.

Kao, T.-C., Lin, Y.-C., Yang, H.-N., Tsai, H.-Y., Chen, J.-R., 2024. Incident investigation of hydrogen explosion and fire in a residue desulfurization process. J. Loss Prev. Process Ind. 92, 105458. https://doi.org/10.1016/j.jlp.2024.105458.

Kapp, H., 1984. Schlammfaulung mit hohem Feststoffgehalt. In: Stuttgarter Berichte zur Siedlungswasserwirtschaft (Sludge with a High Solids Content, Stuttgart reports for Urban Water Management), Band 86. Oldenbourg Verlag, München, p. 300.

Kiyuna, L.S.M., Fuess, L.T., Zaiat, M., 2017. Unraveling the influence of the COD/sulfate ratio on organic matter removal and methane production from the biodigestion of sugarcane vinasse. Bioresour. Technol. 232, 103–112. https://doi.org/10.1016/j.biortech.2017.02.028.

Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., Paster, B.J., L. Naomi, W., Ludwig, W., Whitman, W.B., 2010. Bergey's Manual of Systematic Bacteriology, 4. Springer-Verlag New York Inc. https://doi.org/10.1007/978-0-387-68572-4.

Lamaison, do C.F., de Andrade, P.A.M., Bigaton, A.D., Andreote, F.D., Antônio, R.V., Reginatto, V., 2015. Long-term effect of acid and heat pretreatment of sludge from a sugarcane vinasse treatment plant on the microbial community and on thermophilic biohydrogen production. Int. J. Hydrog. Energy 40 (41), 14124–14133. https://doi.org/10.1016/j.ijhydene.2015.08.096.

Lebrero, R., Toledo-Cervantes, A., Muñoz, R., Del Nery, V., Foresti, E., 2016. Biogas upgrading from vinasse digesters: a comparison between an anoxic biotrickling filter and an algal-bacterial photobioreactor. J. Chem. Technol. Biotechnol. 91 (9), 2488–2495. https://doi.org/10.1002/jctb.4843.

Li, P., Li, M., Liu., Z., Zhao, Y., Qian, X., Huang, P., 2022. Effect of high temperature and sulfur vapor on the flammability limit of hydrogen sulfide. J. Clean. Prod. 337 (20), 130579. https://doi.org/10.1016/j.jclepro.2022.130579.

Li., Y., Sun, Y., Li, L., Yuan, Z., 2018. Acclimation of acid-tolerant methanogenic propionate-utilizing culture and microbial community dissecting. Bioresour. Technol. 250, 117–123. https://doi.org/10.1016/j.biortech.2017.11.034.

Magrini, F.E., de Almeida, G.M., da Maia Soares, D., et al., 2021. Effect of different heat treatments of inoculum on the production of hydrogen and volatile fatty acids by dark fermentation of sugarcane vinasse. Biomass Convers. Biorefinery 11, 2443–2456. https://doi.org/10.1007/s13399-020-00687-0.

Menezes, C.A., Almeida, P.S., Camargo, F.P., Delforno, T.P., Oliveira, V.M., Sakamoto, I. K., Varesche, M.B.A., Silva, E.L., 2023. One versus two-stage codigestion of sugarcane vinasse and glycerol: assessing combinations at mesophilic and (hyper) thermophilic conditions. Sci. Total Environ. 904, 166294. https://doi.org/10.1016/j.scitotenv.2023.166294.

Moraes, de S.B., Dos Santos, G.M., Delforno, T.P., Fuess, L.T., da Silva, A.J., 2019. Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added short-chain organic acids and alcohol production. J. Biosci. Bioeng. 127 (5), 594–601. https://doi.org/10.1016/j.jbiosc.2018.10.008.

Mota, V.T., Júnior, A.F., Trably, E., Zaiat, M., 2018. Biohydrogen production at pH below 3.0: is it possible? Water Res. 128, 350–361. https://doi.org/10.1016/j.watres.2017.10.060.

Padan, E., Bibi, E., Ito, M., Krulwich, T.A., 2005. Alkaline pH homeostasis in bacteria: new insights. Biochim. Biophys. Acta Biomembr. 1717, 67–88. https://doi.org/10.1016/j.bbamem.2005.09.010.

Piffer, M.A., Zaiat, M., Nascimento, C.A.O. Do, Fuess, L.T., 2021. Dynamics of sulfate reduction in the thermophilic dark fermentation of sugarcane vinasse: a biohydrogen-independent approach targeting enhanced bioenergy production. J. Environ. Chem. Eng. 9 (5), 105956. https://doi.org/10.1016/j.jece.2021.105956.

Piffer, M.A., Oliveira, C.A., Bovio-Winkler, P., Eng, F., Etchebehere, C., Zaiat, M., Nascimento, C.A.O., Fuess, L.T., 2022. Sulfate- and pH-driven metabolic flexibility in sugarcane vinasse dark fermentation stimulates biohydrogen evolution,

sulfidogenesis or homoacetogenesis. Int. J. Hydrog. Energy 47 (73), 31202–31222. https://doi.org/10.1016/j.ijhydene.2022.07.039.
Qiu, S., Zhang, X., Xia, W., Li, Z., Wang, L., Chen, Z., Ge, S., 2023. Effect of extreme pH conditions on methanogenesis: methanogen metabolism and community structure. Sci. Total Environ. 877, 162702. https://doi.org/10.1016/j.scitotenv.2023.162702.
Ramos, L.R., Silva, E.L., 2017. Continuous hydrogen production from agricultural wastewaters at thermophilic and hyperthermophilic temperatures. Appl. Biochem. Biotechnol. 182 (2), 846–869. https://doi.org/10.1007/s12010-016-2366-3.
Ramos, L.R., Silva, E.L., 2018. Continuous hydrogen production from cofermentation of sugarcane vinasse and cheese whey in a thermophilic anaerobic fluidized bed reactor. Int. J. Hydrog. Energy 43 (29), 13081–13089. https://doi.org/10.1016/j.ijhydene.2018.05.070.
Ramos, L.R., Lovato, G., Rodrigues, J.A.D., Silva, E.L., 2021. Anaerobic digestion of vinasse in fluidized bed reactors: process robustness between two-stage thermophilic-thermophilic and thermophilic-mesophilic systems. J. Clean. Prod. 314, 128066. https://doi.org/10.1016/j.jclepro.2021.128066.
Rego, G.C., Ferreira, T.B., Ramos, L.R., et al., 2022. Bioconversion of pretreated sugarcane vinasse into hydrogen: new perspectives to solve one of the greatest issues of the sugarcane biorefinery. Biomass Convers. Biorefinery 12, 5527–5541. https://doi.org/10.1007/s13399-020-00984-8.
Reis, M.A.M., Goncalves, L.M.D., Carrondo, M.J.T., 1988. Sulphate removal in acidogenic phase anaerobic digestion. Environ. Technol. Lett. 9 (8), 775–784. https://doi.org/10.1080/09593338809384632.
Ren, N.Q., Chua, H., Chan, S.Y., TSang, Y.F., Sin, N., 2007. Effects of COD/SO_4^{2-} ratios on an acidogenic sulfate-reducing reactor. Ind. Eng. Chem. Res. 46 (6), 1661–1666. https://doi.org/10.1021/ie060589w.
Ribeiro, A.R., Devens, K.U., Camargo, F.P., Sakamoto, I.K., Varesche, M.B.A., Silva, E.L., 2024. Evaluation of the effect of increasing the organic load in the thermophilic co-fermentation of sugarcane industry by-products on hydrogen, ethanol and lactic acid generation. Int. J. Hydrog. Energy 57, 549–561. https://doi.org/10.1016/j.ijhydene.2024.01.028.
Rogeri, R.C., Fuess, L.T., Eng, F., Borges, A.V., Araujo, M.N., Damianovic, M.H.R.Z., Silva, A.J., 2023. Strategies to control pH in the dark fermentation of sugarcane vinasse: impacts on sulfate reduction, biohydrogen production and metabolite distribution. J. Environ. Manag. 325 (Part B). https://doi.org/10.1016/j.jenvman.2022.116495.
Rogeri, R.C., Fuess, L.T., de Araujo, M.N., Eng, F., do Vale Borges, A., Damianovic, M.H.R.Z., da Silva, A.J., 2024. Methane production from sugarcane vinasse: the alkalinizing potential of fermentative-sulfidogenic processes in two-stage anaerobic digestion. Energy Nexus 14, 100303. https://doi.org/10.1016/j.nexus.2024.100303.
Ruggeri, B., Tommasi, T., Sanfilippo, S., 2015. BioH2 & BioCH4 Through Anaerobic Digestion: From Research to Full-Scale Applications. Springer. https://doi.org/10.1007/978-1-4471-6431-9.
Saito, H., Kobayashi, H., 2003. Bacterial responses to alkaline stress. Sci. Prog. 86 (4), 271–282. https://doi.org/10.3184/003685003783238635.
Sánchez, F.E., Fuess, L.T., Cavalcante, G.S., Adorno, M.A.A., Zaiat, M., 2021. Value-added soluble metabolite production from sugarcane vinasse within the carboxylate platform: an application of anaerobic biorefinery beyond biogas production. Fuel 286, 119378. https://doi.org/10.1016/j.fuel.2020.119378.
Santana Jr., A.E.S., Duda, R.M., Oliveira, R.A., 2019. Improving the energy balance of ethanol industry with methane production from vinasse and molasses in two-stage anaerobic reactors. J. Clean. Prod. 238, 117577. https://doi.org/10.1016/j.jclepro.2019.07.052.
Schumacher, K., Brameyer, S., Jung, K., 2023. Bacterial acid stress response: from cellular changes to antibiotic tolerance and phenotypic heterogeneity. Curr. Opin. Microbiol. 75, 102367. https://doi.org/10.1016/j.mib.2023.102367.
Shin, H.C., Ju, D.-H., Jeon, B.S., Choi, O., Kim, H.W., Um, Y., et al., 2015. Analysis of the microbial community in an acidic hollow-fiber membrane biofilm reactor (Hf-MBfR) used for the biological conversion of carbon dioxide to methane. PLoS One 10 (12), e0144999. https://doi.org/10.1371/journal.pone.0144999.
Slonczewski, J.L., Fujisawa, M., Dopson, M., Krulwich, T.A., 2009. Cytoplasmic pH measurement and homeostasis in bacteria and archaea. Adv. Microb. Physiol. 55, 1–317. https://doi.org/10.1016/S0065-2911(09)05501-5.
Stolyar, S., He, Q., Joachimiak, M.P., He, Z., Yang, Z.K., Borglin, S.E., Stahl, D.A., 2007. Response of *Desulfovibrio vulgaris* to alkaline stress. J. Bacteriol. 189 (24), 8944–8952. https://doi.org/10.1128/jb.00284-07.
Sun, M., Liu, B., Yanagawa, K., Ha, N.T., Goel, R., Terashima, M., Yasui, H., 2020. Effects of low pH conditions on decay of methanogenic biomass. Water Res. 179, 115883. https://doi.org/10.1016/j.watres.2020.115883.
Takeda, P.Y., Oliveira, C.A., Dias, M.E.S., Paula, C.T., Borges, A.V., Damianovic, M.H.R.Z., 2022. Enhancing the energetic potential of sugarcane biorefinery exchanging vinasse and glycerol in sugarcane off-season in an anaerobic reactor. Renew. Energy 195, 1218–1229. https://doi.org/10.1016/j.renene.2022.06.128.
Taylor, K.A.C.C., 1996. Simple colorimetric assay for muramic acid and lactic acid. Appl. Biochem. Biotechnol. 56 (1), 49–58. https://doi.org/10.1007/BF02787869.
Thauer, R.K., Kaster, A., Seedorf, H., Buckel, W., Hedderich, R., 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6, 579–591. https://doi.org/10.1038/nrmicro1931.
Tran, T.T.T., Kannoorpatti, K., Padovan, A., Thennadil, S., 2021. Sulphate-reducing bacteria's response to extreme pH environments and the effect of their activities on microbial corrosion. Appl. Sci. 11. https://doi.org/10.3390/app11052201.
Ueki, A., Watanabe, M., Ohtaki, Y., Kaku, N., Ueki, K., 2014. Description of Propionispira arcuata sp. nov., isolated from a methanogenic reactor of cattle waste, reclassification of Zymophilus raffinosivorans and Zymophilus paucivorans as Propionispira raffinosivorans comb. nov. and Propionispira paucivorans comb. nov. and emended description of the genus Propionispira. Int. J. Syst. Evol. Microbiol. 64 (Pt_10), 3571–3577. https://doi.org/10.1099/ijs.0.063875-0.
Veiga, M.C., Soto, M., Méndez, R., Lema, J.M., 1990. A new device for measurement and control of gas production by bench scale Anaerobic digesters. Water Res. 24 (12), 1551–1554. https://doi.org/10.1016/0043-1354(90)90090-S.
Volpi, M.P.C., Junior, A.D.N.F., Franco, T.T., Moraes, B.S., 2021. Operational and biochemical aspects of co-digestion (co-AD) from sugarcane vinasse, filter cake, and deacetylation liquor. Appl. Microbiol. Biotechnol. 105 (23), 8969–8987. https://doi.org/10.1007/s00253-021-11635-x.
Volpi, M.P.C., Adarme, O.F.H., Araújo, M.F., Bella, T.R., Procópio, P.F., de Abreu, L.G.F., Mockaitis, G., 2024. Biohydrogen and methane production via silage-based dark co-fermentation using vinasse and filter cake. Bioresour. Technol. Rep. 27, 101927. https://doi.org/10.1016/j.biteb.2024.101927.
Wan, C., Zhou, Q., Fu, G., Li, Y., 2011. Semi-continuous anaerobic co-digestion of thickened waste activated sludge and fat, oil and grease. Waste Manag. 2011 (31), 1752–1758. https://doi.org/10.1016/j.wasman.2011.03.025.
Wang, A., Ren, N., Wang, X., Lee, D., 2008. Enhanced sulfate reduction with acidogenic sulfate-reducing bacteria. J. Hazard. Mater. 154 (1–3), 1060–1065. https://doi.org/10.1016/j.jhazmat.2007.11.022.
Wang, C., Li, Y., Sun, Y., 2020. Acclimation of acid-tolerant methanogenic culture for bioaugmentation: strategy comparison and microbiome succession. ACS Omega 5 (11), 6062–6068. https://doi.org/10.1021/acsomega.9b03783.
Weijma, J., Gubbels, F., Hulshoff Pol, L.W., Stams, A.J.M., Lens, P., Lettinga, G., 2002. Competition for H_2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. Water Sci. Technol. 45 (10), 75–80. https://doi.org/10.2166/wst.2002.0294.
Wemheuer, F., Taylor, J.A., Daniel, R., Johnston, E., Meinicke, P., Thomas, T., Wemheuer, B., 2020. Tax4Fun2: prediction of habitat-specific functional profiles and functional redundancy based on 16S rRNA gene sequences. Environ. Microb. 15, 11. https://doi.org/10.1186/s40793-020-00358-7.
Whitman, W.B., Bowen, T.L., Boone, D.R., 2006. The methanogenic bacteria. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (Eds.), The Prokaryotes, third ed., The Prokaryotes. Springer, New York. https://doi.org/10.1007/0-387-30743-5_9.
Xie, S., Higgins, M.J., Bustamante, H., Galwayc, B., Nghiem, L.D., 2018. Current status and perspectives on anaerobic codigestion and associated downstream processes. Environ. Sci.: Water Res. Technol. 4, 1759. https://doi.org/10.1039/c8ew00356d.
Xu, R., Zhang, K., Liu, P., Khan, A., Xiong, J., Tian, F., Li, X., 2018. A critical review on the interaction of substrate nutrient balance and microbial community structure and function in anaerobic co-digestion. Bioresour. Technol. 247, 1119–1127. https://doi.org/10.1016/j.biortech.2017.09.095.
Yu, H., Jiang, Z., Lu, Y., Yao, X., Han, C., Ouyang, Y., Wang, H., Guo, C., Ling, F., Dang, Z., 2021. Transcriptome analysis of the acid stress response of *Desulfovibrio vulgaris* ATCC 7757. Curr. Microbiol. 77, 2702–2712. https://doi.org/10.1007/s00284-020-02051-x.
Zheng, S., Liu, F., Wang, B., Zhang, Y., Lovley, D.R., 2020. Methanobacterium capable of direct interspecies electron transfer. Environ. Sci. Technol. 54, 15347–15354. https://doi.org/10.1021/acs.est.0c05525.
Zhou, X., Fernández-Palacios, E., Dorado, A.D., Gamisans, X., Gabriel, D., 2022. Assessing main process mechanism and rates of sulfate reduction by granular biomass fed with glycerol under sulfidogenic conditions. Chemosphere 286 (1), 131649. https://doi.org/10.1016/j.chemosphere.2021.131649.