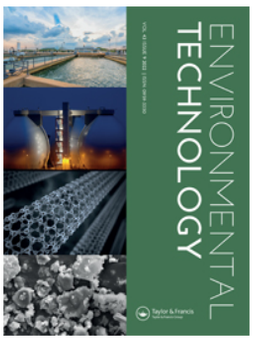
Microbial and functional characterization of granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment

Franciele Pereira Camargo, Isabel Kimiko Sakamoto, Tiago Palladino Delforno, Cédric Midoux, Iolanda Cristina Silveira Duarte, Edson Luiz Silva, Ariane Bize, Maria Bernadete Amâncio Varesche



Environmental Technology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tent20

Microbial and functional characterization of granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment

Franciele Pereira Camargo, Isabel Kimiko Sakamoto, Tiago Palladino Delforno, Cédric Midoux, Iolanda Cristina Silveira Duarte, Edson Luiz Silva, Ariane Bize & Maria Bernadete Amâncio Varesche

To cite this article: Franciele Pereira Camargo, Isabel Kimiko Sakamoto, Tiago Palladino Delforno, Cédric Midoux, Iolanda Cristina Silveira Duarte, Edson Luiz Silva, Ariane Bize & Maria Bernadete Amâncio Varesche (2022): Microbial and functional characterization of granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment, Environmental Technology, DOI: 10.1080/09593330.2022.2052361

To link to this article: https://doi.org/10.1080/09593330.2022.2052361

Published online: 07 Apr 2022.

ENVIRONMENTAL TECHNOLOGY
https://doi.org/10.1080/09593330.2022.2052361

Taylor & Francis
Taylor & Francis Group

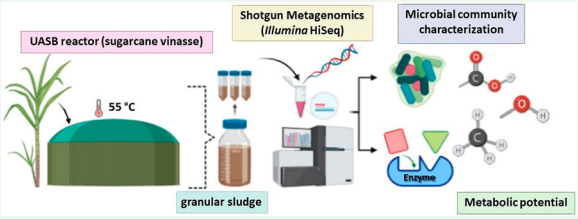
Microbial and functional characterization of granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment

Franciele Pereira Camargo [a], Isabel Kimiko Sakamoto [a], Tiago Palladino Delforno [b], Cédric Midoux [c], Iolanda Cristina Silveira Duarte [d], Edson Luiz Silva [e], Ariane Bize [c] and Maria Bernadete Amâncio Varesche [a]

[a]Department of Hydraulics and Sanitation, School of Engineering of São Carlos, University of São Paulo (USP), São Carlos, Brazil; [b]Innovation Institute for Biotechnology (SENAI-Biotech), Bom Retiro, SP, Brazil; [c]Université Paris-Saclay, INRAE, PRocédés biOtechnologiques au Service de l'Environnement (PROSE), Antony, France; [d]Department of Biology, Federal University of São Carlos (UFSCar), Sorocaba, Brazil; [e]Department of Chemical Engineering, Federal University of São Carlos (UFSCar) São Carlos, Brazil

ABSTRACT

Considering the scarcity of data in the literature regarding phylogenetic and metabolic composition of different inocula, especially those from thermophilic conditions, this research aimed at characterizing the microbial community and preferable metabolic pathways of an UASB reactor sludge applied to the thermophilic treatment (55°C) of sugarcane vinasse, by means of shotgun metagenomics. After its metabolic potential was depicted, it was possible to observe several genes encoding enzymes that are of great importance to anaerobic digestion processes with different wastes as substrate, especially regarding the biodegradation of carbohydrates and ligninolytic compounds, glycerolypids, volatile fatty acids and alcohols metabolism and biogas (H_2 and CH_4) production. The genera identified in higher relative abundances for Bacteria domain were *Sulfirimonas* (37.52 ± 1.8%), possibly related to the sludge endogenic activity due to its strong relation with a peptidoglycan lyase enzymes family, followed by *Fluviicola* (5.01 ± 1.0%), *Defluviitoga* (4.36 ± 0.2%), *Coprothermobacter* (4.32 ± 0.5%), *Fervidobacterium* (2.93 ± 0.3%), *Marinospirillum* (2.75 ± 0.2%), *Pseudomonas* (2.14 ± 0.2%) and *Flavobacterium* (1.78 ± 0.1%), mostly related with carbohydrates fermentations and/or H_2 production. For Archaea domain, *Methanosarcina* (0.61 ± 0.1%), *Methanothermobacter* (0.38 ± 0.0%), *Methanoculleus* (0.30 ± 0.1%), *Thermococcus* (0.03 ± 0.0%), *Methanolobus* (0.02 ± 1.8%), *Methanobacterium* (0.013 ± 0.0%), *Aciduliprofundum* and *Pyrococcus* (0.01 ± 0.0%) were the most dominant ones, being *Methanosarcina* the most related with methanogenesis. It was concluded that the robust inoculum description performed in this study may subside future biotechnological researches by using similar inocula (UASB sludges), focusing on the obtainment of value-added by-products by means of anaerobic digestion, such as volatile fatty acids, alcohols and biogas (H_2 and CH_4), by using several types of waste as substrate.



ARTICLE HISTORY
Received 5 December 2021
Accepted 3 March 2022

KEYWORDS
Shotgun metagenomics; metabolic potential; phenol degradation; biohydrogen; biomethane

1. Introduction

Anaerobic Digestion (AD) is a widely known process by which it is possible to obtain several value-added compounds, due to the metabolic activity of several microorganisms, each of them highly specialized in hydrolysis, acidogenesis and acetogenesis or methanogenesis steps [1,2]. One of the main AD advantages, is the wide range of possible substrates, such as solid wastes and wastewater, which makes this process a two-way street, where it is possible to reuse these wastes to obtain energy in a environmental-friendly way [3,4].

Among the several parameters affecting the AD processes, such as reactor configuration, nutritional and physicochemical characteristics, type of substrate and hydraulic detention time, it is worth highlighting the

CONTACT Franciele Pereira Camargo francielep.camargo@hotmail.com Department of Hydraulics and Sanitation, School of Engineering of São Carlos, University of São Paulo (USP), Av. Trabalhador São Carlense, 400, zipcode 13566-590, São Carlos, SP, Brazil
Supplemental data for this article can be accessed at https://doi.org/10.1080/09593330.2022.2052361

importance of the microbial source used as inoculum [5–7]. According to Sierocinski et al. [8], due to coevolution between the species in a microbial community, generally one species can prevail as the most dominant one, being predicted to be the one using the resources available in the system in a most efficiently way, even when grown in isolation. Since the dominance of some microbial communities can drive to metabolic shifts, leading to the formation of not desired metabolites, the selection of the inoculum should be carefully considered before the reactor start-up, in order to avoid these pathways and favour the ones in which the desired compound is more efficiently produced [6,9].

Despite the remarkable importance of the inoculum source to ensure process efficiency, most of the current AD research is focused only on the physicochemical parameters, analyzing the microbial community involved in it only after the reactor operation, or even not analyzing it at all. When the microbial community is analyzed, it is usually carried out by using a target DNA sequence, such as the 16S rRNA metabarcoding, which regardless of being a great method to phylogenetically describe the communities, does not enable the inference of the gene encoding enzymes that are involved in the metabolic pathways [10,11]. The previous characterization of the inoculum by metagenomic analyzes, such as shotgun method, may enable its enrichment [12,13], in order to favour specific organisms, as well as facilitate the selection of the most appropriate microorganisms sources and reactor parameters to favour the desired process [14]. It is also worth to mention that thermophilic bio-reactors applied to biogas production are less explored and characterized then mesophilic ones, especially regarding its microbial composition [15].

Zhu et al. [13] reported that the enrichment of sewage sludge in carboxymethylcellulose to be used as inoculum in anaerobic digestion using cellulose as substrate increased about 69.81% the CH_4 production, where *Ruminiclostridium* was the most abundant genus. Candry et al. [12] evaluated several inocula (3 anaerobic digesters, 2 animal faeces and 1 caproic acid producing environment) in order to select the most efficient chain elongating communities, using ethanol and acetic acid as substrates (pH 7.0 and 5.5, respectively). These authors concluded that, since the inoculum origin determines the pH along the process, it consequently affects caproic acid production (caproic acid = 6.4 g L^{-1} at pH 7.0 and 2.3 at pH 5.5), being *Clostridium kluyveri* the most related to the chain elongation. These results emphasizes the need for careful inoculum selection during bioprocess development, even when different by-products are desired, such as H_2, CH_4 and/or organic compounds.

The sludge from the same reactor used in this research (UASB reactor applied to vinasse thermophilic treatment) has been widely applied over the years as microbial source in continuous and batch reactors with different substrates. Among these, it was worth to mention sugarcane vinasse [16–21], sugarcane juice [22], sugarcane molasses [7], cheese whey [23], glucose [24,25], glycerol [26,27], cellulosic hydrolyzate [28] and sucrose-based synthetic wastewater [29].

Considering the scarcity of data in the scientific literature regarding the phylogenetic composition and functional potential of this type of inoculum, the present research aimed at characterizing its microbial community and preferable metabolic pathways, focusing on its use in anaerobic digestion processes. By means of metagenomic shotgun approach, the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment was studied regarding the pathways related to carbohydrates, lignolytic compounds, glycerolypids, volatile fatty acids and alcohols, resistance and degradation of potential inhibitors (limonene, furfural, phenols) and biogas (hydrogen and methane) production. In this sense, this study is a robust description of the UASB sludge microbiome, which may guide future biotechnological researches in this field.

2. Material and methods

2.1. Full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment

The UASB reactor was operated at São Martinho sugarcane mill (http://www.saomartinho.ind.br/), the largest Brazilian organization of sugarcane power, located at Pradópolis, São Paulo, Brazil, constructed in 1990s. The main objective of this plant is to produce biogas for subsequent use in the generation of thermal energy for drying yeast from vinasse, one of the major residues generated in the alcohol industry [30]. It is the only vinasse treatment plant mentioned in the literature [31], being this large amount of vinasse (about 1.5×10^{11} L/year) produced by the ethanol industry. Sludge pH, total solids (TS) and total volatile solids (TVS) concentrations were about 7.06, 16.2 and 41.3 g L^{-1}, respectively [16,32].

2.2. Granulated sludge collection and storage

The sludge was collected during sugarcane harvest, directly from a full-scale UASB reactor (75 m^3) at Usina São Martinho (Pradópolis, São Paulo, Brazil) [31], operated at 55°C applied to the treatment of sugarcane

vinasse. The material was collected at a single point in a valve at the base of the UASB's tank. After collection, the sludge was immediately stored in plastic containers (30 L) at 4°C, for up to 12 months. Before its use, the sludge underwent trituration using a household blender for 0.5 min for granule disruption. The sludge had a pH of 7.1, and comprised 36.7 gTS L^{-1} and 20.4 gTVS L^{-1}, 65 mg L^{-1} of acetic acid, 39 mg L^{-1} of lactic acid [14].

2.3. Microbial community and functional characterization

2.3.1. Sampling and DNA extraction

Before DNA extraction, three samples of approximately 50 mL of the sludge previously collected and stored were washed in 10 mL of PBS buffer (NaCl 8%, KCl 0.2%, Na_2HPO_4 1.44%, KH_2PO_4 0.24%) [33] and stored at 4°C for approximately 12 h. The extraction kit FastDNA SPIN Kit for Soil (MP Biomedicals™) was used to perform the DNA extractions of biological replicates, according to the manufacturer's guide. A Nanodrop 2000 Spectrophotometers (ThermoFisher Scientific) was used to analyze the genetic material extracted regarding its concentration (ng/µL) and purity (260 nm/280 nm ratio).

2.3.2. Sequencing and data processing

Library preparation and metagenomic sequencing were performed in triplicates on a *Illumina* HiSeq 4000 platform PE150 (2 × 150 bp), using the NEBNext Ultra II DNA Library Prep kit for NEB #E7645, by GenOne Biotechnologies (Rio de Janeiro, Brazil). Raw sequences were submitted to the National Center for Biotechnology Information (NCBI) SRA database under accession numbers SRX7704207 to SRX7704209 (BioProject PRJNA605706).

Quality control steps were performed using FastQC and MultiQC tools. Reads and bases with poor quality, with length ≤50 and ontaining PolyG and PolyX tails at the 3′ ends were discarded with the preprocessing tool Fastp (v. 0.20.0) [34]. A co-assembly approach was applied using metaSPAdes (v.3.14.0) [35] on the trimmed reads, selecting contigs ≥1000 bp. Finally, the Quality Assessment Tool for Genome Assemblies (QUAST) was applied for the assembly quality control, computing metrics on contig size and GC percent, according to Gurevich [36].

2.3.3. Microbial community diversity, structure and composition

Gene prediction was done with Prodigal tool (v. 2.6.3) [37], while Bowtie2 was applied for mapping the reads back to the contigs (https://doi.org/10.1038/nmeth.1923). Taxonomic annotation of the detected genes was performed with the tool Kaiju [38] against sequences available in NCBI nr database, being the annotation was performed with a 1E-5 cut-off for E-value and a minimum identity of 60%. Phyloseq package using R basic functions (https://www.R-project.org/) was used for composition visualization.

Metagenome-Assembled Genome (MAG) were reconstructed with Maxbin and MetaBAT2 tool [39], to obtain individual genomes from complex microbial communities, enabling us to accurately predict some of the species involved in the consortium. The CheckM software [40] was used to estimate the completeness, contamination and strain heterogeneity of each MAG, and genomes recovered with contamination <10% and completeness >50% were considered as medium-quality genomes, whereas genomes with contamination <5% and completeness >90% were considered as high-quality genomes [41], being only the medium and high-quality genomes considered in this study.

2.4. Functional analysis and metabolic mapping

The platforms GhostKOALA against the Kyoto Encyclopedia of Genes and Genomes (KEGG), KEGG Orthology (KO) database [42] and dbCAN [43] against the Carbohydrate-Active Enzymes (CAZymes) (http://www.cazy.org/) defined according to protein homology (HMMER server) were used for functional and metabolic annotation. graphical visualizations were obtained with ggplot2 [44] package, and R basic functions. The main steps followed for the microbial and functional characterization of the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment are summarized in the pipeline presented in Figure A.1.

3. Results and discussion

3.1. Sequencing statistics

After sequencing and read trimming, the total number of 150 pb read-pairs obtained for the three samples of UASB sludge (Sample 1–3) were 17,290,172, 12,5267,54 and 19,814,318, respectively. About 70% of the total reads could be mapped, using the bowtie tool, highlighting an assembly of sufficient quality. After coassembly 78,123 contigs were obtained, being 478 contigs greater than 50,000 bp, and the largest contig was 932,907 bp (Table A.1).

3.2. Microbial community of UASB reactor microbiome

In Table A.2, it is possible to visualize the diversity indexes obtained for the UASB sludge biomass samples calculated at the species level. 10,202 taxa were observed for these sequences. The Shannon-Wiener diversity index (H′) indicated was 6.0 for these samples, while the Simpson index calculated, commonly used to indicate dominance, was 1.0. Through the analysis of rarefaction curves (Figure A.2), it was observed that the curves reached a plateau, that is, the sequencing depth was sufficient to detect the high majority of the richness of the microbial community in these samples.

After analyzing the microbial community associated with the dataset obtained from the UASB sludge biomass, it was observed 3 different domains (Archaea, Bacteria, Eukaryota) and Viruses, 163 phyla, 466 orders, 423 classes, 848 families and 2581 genera (Figure 1). Also, it could be noticed that there was low variation between the biological replicates (Sample 1–3), showing that the data has great reproducibility. The phyla observed in higher relative abundance to the Bacteria Domain were Proteobacteria (40.51 ± 2.3%), Bacteroidetes (21.44 ± 3.3), Firmicutes, (9.76 ± 1.1), Thermotogae (8.04 ± 0.6), Synergistetes (1.42 ± 0.1%), Tenericutes (1.38 ± 0.1%) and Nitrospirae (1.27 ± 0.1%).

Among the most abundant Bacteria genera, it was observed greater relative abundance of *Sulfirimonas* (37.52 ± 1.8%), *Fluviicola* (5.01 ± 1.0%), *Defluviitoga* (4.36 ± 0.2%), *Coprothermobacter* (4.32 ± 0.5%), *Fervidobacterium* (2.93 ± 0.3%), *Marinospirillum* (2.75 ± 0.2%), *Pseudomonas* (2.14 ± 0.2%) and *Flavobacterium* (1.78 ± 0.1%). Euryarchaeota (1.59 ± 0.3%) was the most dominant Archaea phylum observed, being the others (Crenarchaeota, Candidatus Woesearchaeota, Ca. Thorarchaeota,

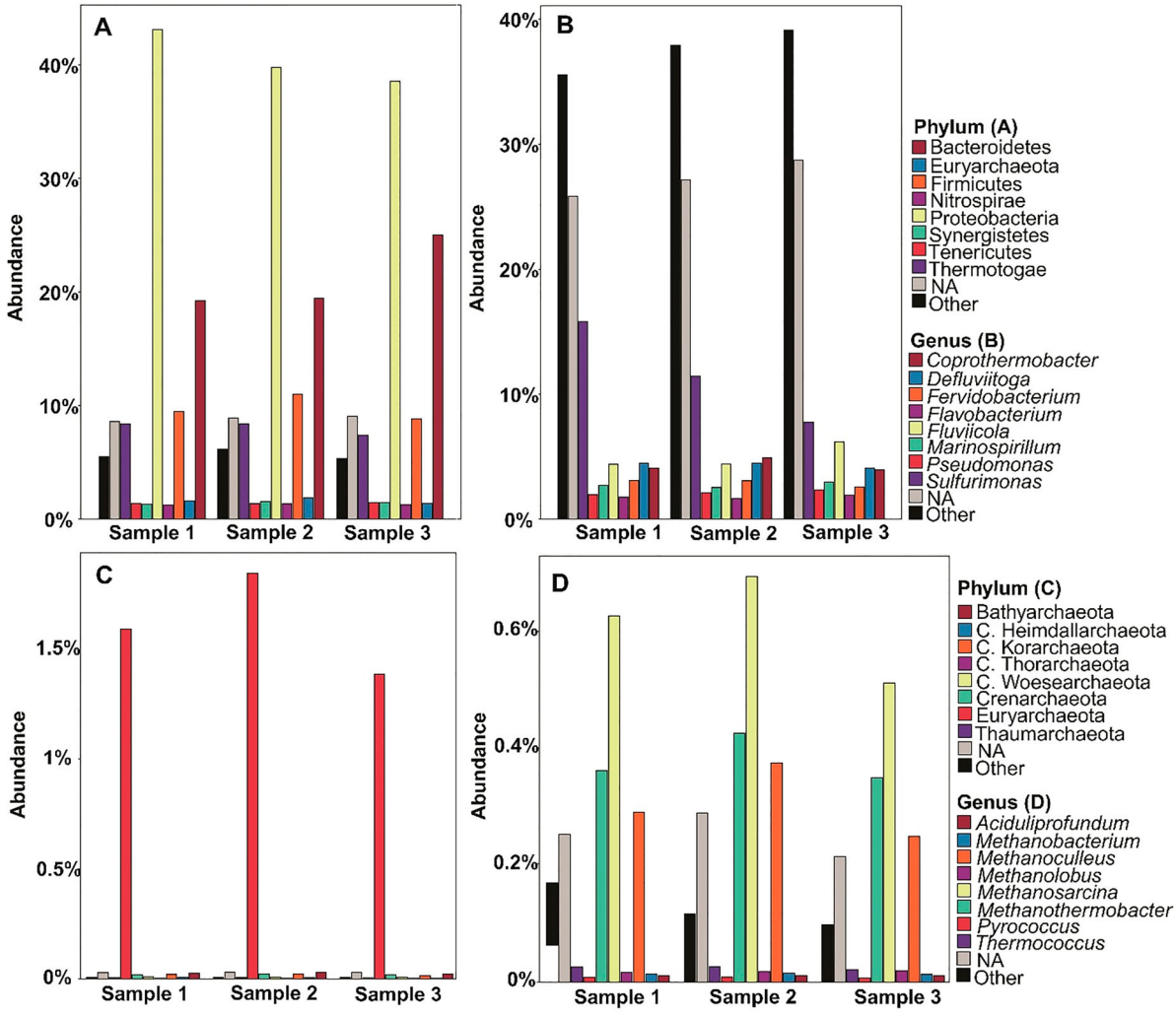


Figure 1. Relative abundance of the 10 most abundant Bacteria phyla (A) and genera (B) and Archaea phyla (C) and genera (D) in the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment (biological replicates, Samples 1–3). NA = not annotated. The relative abundances were calculated considering the total number of preprocessed reads in the samples.

Ca. Korarchaeota, Ca. Heimdallarchaeota, Ca. Bathyarchaeota and Thaumarchaeota) observed in relative abundances ≤0.05%. The genera observed in higher relative abundances were *Methanosarcina* (0.61 ± 0.1%), *Methanothermobacter* (0.38 ± 0.0%), *Methanoculleus* (0.30 ± 0.1%), *Thermococcus* (0.03 ± 0.0%), *Methanolobus* (0.02 ± 1.8%), *Methanobacterium* (0.013 ± 0.0%), *Aciduliprofundum* and *Pyrococcus* (0.01 ± 0.0%).

After the reconstruction of single with medium or high-quality genomes from complex microbial communities, it was possible to accurately predict 21 high-quality bins, from the total of 59 bins (Table A.3). Some of the most abundant species predicted and involved in this consortium were the Bacteria *Defluviitoga tunisiensis*, *Desulfuromonas acetexigens*, *Lysobacter concretionis*, *Thermodesulfovibrio thiophilus* and the Archaea *Methanoculleus thermophiles*.

Defluviitoga tunisiensis, a commonly isolated strain in anaerobic reactors, is a chemo-organotrophic, non-sporulating, rod-shaped bacteria, besides being a thermophilic (37–65°C – optimum 55°C), anaerobic and slightly halophilic (optimum NaCl 0.5%) species, reducing thiosulphate and elemental sulphur to H_2S. This species is able to use a wide range of electron donnors, such as arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, microcrystalline cellulose, xylan and yeast extract, being acetate, H_2 and CO_2 as the main by-products after glucose fermentation [45]. Its intrinsic characteristics show the great potential of this species in several biorefinary processes, especially by using wastes with high salt concentrations. By means of genome reconstruction, it was possible to confirm the presence of several KO related to this species in the sludge analyzed, such as the ones involved in Glycolysis, Pentose phosphate pathway, Citrate cycle, Fructose and mannose metabolism, among others.

In the same way, the bioaugmentation of *Desulfuromonas acetexigens*, an anaerobic Gram-negative sulphur-reducing bacterium, which exclusively uses acetate as energy and carbon source, was considered in recent researches as very promising to maximize resource recovery from wastes, favouring maximum recovery of energy as H_2 [46]. After genome reconstruction, it was possible to confirm the presence of K00640 (cysE) and K00641 (metX) genes in relation with this species, being both related to Sulphur metabolism, with serine O-acetyltransferase and homoserine O-acetyltransferase/O-succinyltransferase activity, respectivelly.

Lysobacter concretionis strains were first isolated from UASB granules treating wastewater from brewery [47], however, besides its metabolic potential by degrading several types of substrate (acetate, glycogen, hydroxybutirate and proline), there are still few studies regarding its characterization and potential use in biorefinery processes. By means of genome reconstruction, it was possible to confirm that this species is related to several genes encoding enzymes of biotechnological interest, such as the ones involved in fructose and mannose metabolism (K00754; K00846; K01623 and K03841) and fatty acids metabolism (K00128; K00626; K01782; K05297 and K06445).

Thermodesulfovibrio thiophilus is also an anaerobic, thermophilic (55–60°C), sulphate-reducing bacteria, which can be isolated from thermophilic methanogenic sludges, using mostly H_2, formate, pyruvate and lactate as electron donors, being excellent syntrophic organisms in methanogenic systems, since they can regulate the H_2 pressure, or alternatively, use lactate instead when co-inoculated with hydrogenotrophic methanogens, such as *Methanothermobacter* [48].

The species *Ruminiclostridium* [*Clostridium*] *cellulosi*, is widely studied in order to degrade several types of lignocellulolytic substrates, especially reducing cellulose crystallinity, thanks to its multi-protein cellulolytic complex, the cellulosome [49]. As reported by Tsavkelova et al. [50], the absence of *Ruminiclostridium cellulosi* in a consortium applied to waste paper degradation under thermophilic conditions (55°C) dramatically reduced their total cellulolytic activity, since it is a known active hydrolytic of lignocellulose at elevated temperatures. Its major end products are H_2, CO_2, ethanol and acetic acid. *Paraclostridium bifermentans*, as other bacteria from *Paraclostridium* genus, are one of the main contributors to H_2 production in anaerobic reactors [51]. Rabelo et al. [52] isolated a *Paraclostridium* strain (CR4) from sugarcane bagasses, monitoring its cellulase gene expression, obtaining 162.4 mL of H_2 from glucose via acetic acid pathway (2.9 g L^{-1}), besides 78.4 mL of H_2 from cellulose, mainly via butyric acid pathway (2.9 g L^{-1}). Other cellulolytic species that could be inferred, *Bacteroidales bacterium*, is usually found in rumen, as reported by Zhang et al. [53], where the inoculation of straw with cow rumen consortium dominated by this genus reached 81% of biodegradation at pH 6.5, concomitantly producing 178.16 mL L^{-1} of H_2. It is worth mentioning that the cellulolytic genera mentioned (*Clostridium*, *Ruminiclostridium*, *Paraclostridium* and *Bacteroidales*) were observed in low relative abundances (≤1.0%) in the present study; however, could be favoured depending on the operational conditions applied, as reported by Camargo et al. [14].

There is only a few information about the species *Acholeplasma laidlawii*, *Weissella cibaria*, *Marinospirillum minutulum* and *Flavobacterium ummariense* regarding

their possible contributions to anaerobic digestions processes. According to Xiong et al. [54], *Acholeplasma laidlawii* is able to ferment several carbohydrates to acetate, lactate and alcohols under alkalyne pH (~8.5), when fermenting food waste in a Leach Bed Reactor.

3.3. Functional characterization of UASB reactor microbiome

Most of the annotated sequences are related to Metabolism KEGG category (12.15%), being about 78.90% not annotated in any KEGG category. The difficulty of increasing the amount of genes annotated is known as one of the limitations of the method used (shotgun metagenomics), since the higher percentages of non-annotated genes may lead to underestimations of the microbial community as well as its functional potential.

The main pathways (Figure 2) observed in Metabolism category were the metabolism of carbohydrates (5.64%), nucleotides (0.66%), amino acids (1.87%), glycan biosynthesis (1.29%), vitamins and cofactors (1.84%), terpenoids and polyketides (0.40%), secondary metabolites (0.39%), among others (0.012%) (Figure 3). The Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems and Human Diseases categories were not considered in this study. These results are in accordance with the results described by Delforno et al. [55], where the authors reported about 35% of the same KEGG category in UASB reactors applied to laundry wastewater containing LAS as standard compound, which can be indicative of high metabolic potential.

Among the main carbohydrate metabolism pathways, the most abundant one, glycolysis and gluconeogenesis (0.50%), citric acid cycle (0.55%), pentose phosphate (0.52%), galactose (0.32%), starch and sucrose (0.42%), amino sugar and nucleotide sugar (0.45%), pyruvate (0.83%), glyoxylate and dicarboxylate (0.70%), butanoate (0.45%), among others (0.91%), stand out. The most abundant genera were *Sulfirimonas* (11.04%), *Defluviitoga* (8.02%), *Coprothermobacter* (6.77%), *Fervidobacterium* (4.09%), *Fluviicola* (3.72%), *Pseudomonas* (2.93%), *Marinospirillum* (2.67%) and *Acetomicrobium* (1.99%). Also, *Defluviitoga* was the most abundant genus in the galactose, starch and sucrose and pentose phosphate pathways.

Despite the variety of substrates studied with this inoculum, it can be seen in Table 1 that there are few researches where the microbial community involved in the process was evaluated. Among the studies where this characterization was carried out via metabarcoding (RNAr 16S), [76] observed the genera *Thermoanaerobacterium* and *Thermohydrogenium* in greater relative abundance in batch reactors operated with glucose as substrate, under thermophilic conditions (55°C) and pH 7.0. In the present study, it was observed relative abundances ≤0.01% for the mentioned genera. It is worth mentioning that the aforementioned authors submitted the inoculum to a thermal pre-treatment, in order to eliminate the potentially H_2-consuming organisms and inhibit the CH_4 production, achieving 13.3 mmol H_2 L^{-1} in the process.

The H_2 concentrations obtained by [76] were similar to the ones obtained by Camargo et al. [56], where the authors achieved 13.31 mmol H_2 L^{-1} in batch reactors operated under mesophilic conditions and using citrus peel waste as substrate. However, despite the use of similar inoculum, the genera observed by [76] were not observed by Camargo et al. [56] in relative abundance ≥0.01%, probably due to the mesophilic conditions used by these authors (37°C). Similarly, Santos et al. [21] observed a greater relative abundance of *Thermoanaerobacterium* and *Clostridium* in an anaerobic fluidized bed reactor (AFBR) operated at 55°C, fed with sugarcane vinasse, obtaining 30.7 mmol H_2 L^{-1}, being these genera observed in relative abundances ≤0.01% in the present study.

Rodrigues et al. [26] applied heat pre-treatment in order to inhibit methanogenic organisms and other H_2-consumers in the inoculum, and observed greater relative abundance of the *Enterobacter* genus (≤0.01% in the present study) in batch reactors operated at 37°C, pH 5.5 and fed with glycerol as the sole carbon source. As observed through the inoculum functional characterization, there are several genes involved in the glycerolipids degradation, produced by the microorganisms present in the granular sludge from the UASB reactor applied in the treatment of sugarcane vinasse, the most abundant being the oxidoreductase K00128 (0.049%), aldehyde dehydrogenase (NAD^+). Vilela et al. [7] observed dominance of the genera *Thermoanaerobacterium*, *Clostridium*, *Lactobacillus* and *Pseudomonas* in a thermophilic UASB reactor (55°C) fed with sugarcane molasses, obtaining 45.7 mmol H_2 L^{-1}. Regarding methane production, Barros et al. [16] obtained 67.2 mmol CH_4 L^{-1} in UASB thermophilic reactors (54–56°C) fed with sugarcane vinasse, where the main organisms observed were Methanosarcinales, Thermotogae and Methanobacteriales. The previous adaptation of this inoculum to the potential inhibitors present in sugarcane vinasse, and the wide variety of specific genes for degradation of carbohydrates present in the inoculum, makes it ideal for this type of substrate, which explains the greater amount of research using vinasse as the main substrate.

Both the CAZymes and the KO related with carbohydrates and/or lignocellulosis degradation were

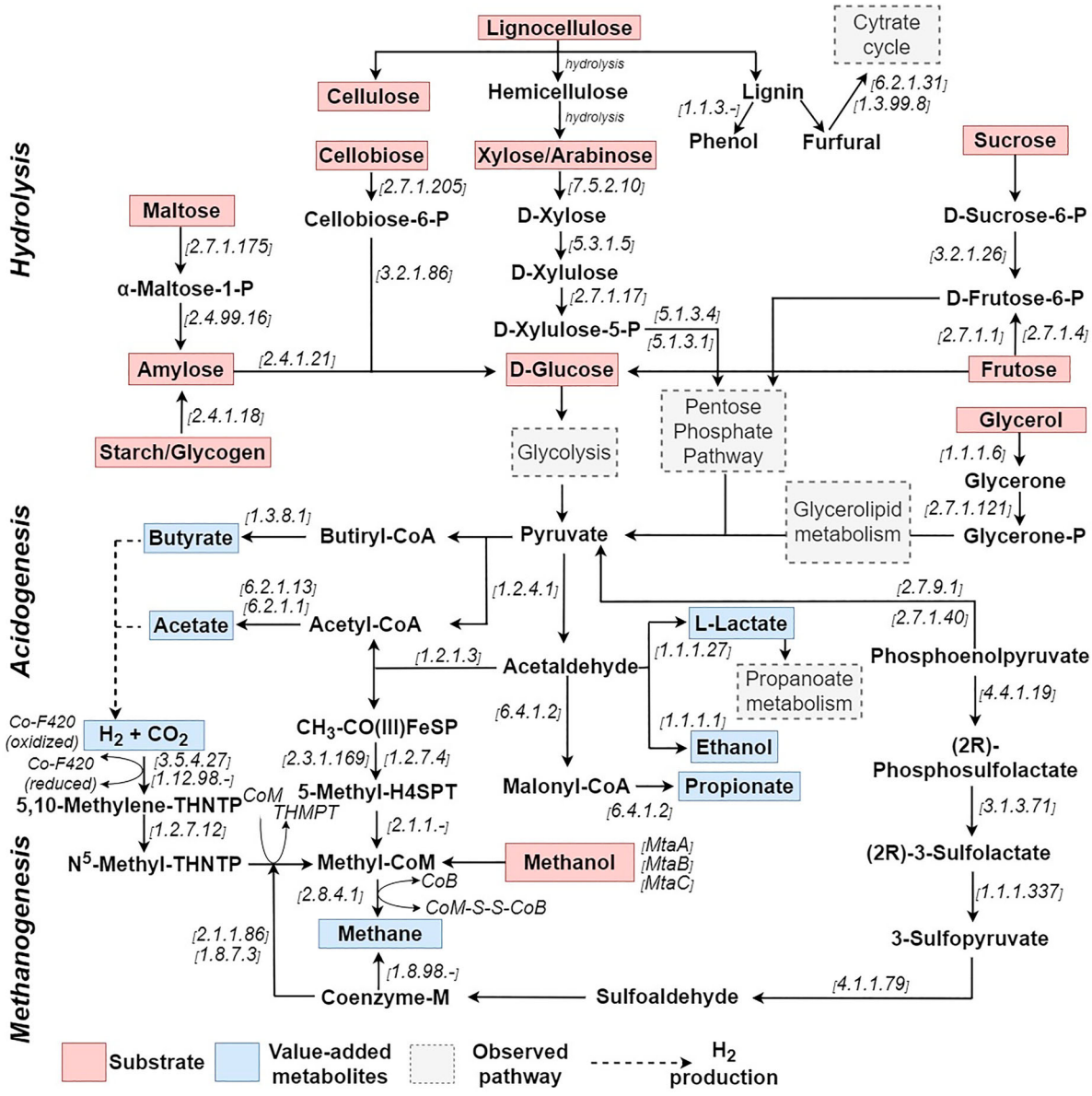


Figure 2. Metabolic potential of the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment, focusing on the potential substrates (red) and value-added metabolites (blue). The genes encoding enzymes involved in each reaction are showed in square brackets as EC numbers.

evaluated (Figure 4). Several Carbohydrate-Active Enzymes from the classes Auxiliary Activities (AA) or Glycoside Hydrolysis (GH) could be identified, being the families AA3 (0.042%) and GH23 (0.103%) the most abundant ones. Since CAZymes are defined by protein homology [43], it is worth to emphasize that distinct enzymes with distinct activities could be represented by the same CAZy.

The glucose-methanol-choline (GMC) oxidoreductase family AA3 is related with several activities, such as cellobiose dehydrogenase, glucose 1-oxidase, aryl alcohol oxidase, alcohol oxidase and pyranose oxidase. This family was mainly related with the bacterial genera *Aureimonas* (30.09%), *Pseudomonas* (28.94%) and *Burkholderia* (28.75%).

It is important to state that even if the Auxiliary Activities (AA) group may not be directly related to carbohydrates degradation, it is considered a ligninolytic group, since it acts in a synergistic manner, comprising redox enzymes which catalyses reactions together with them [57]. For instance, the lignocellulosic biomass is composed of cellulose fibrils embedded by a lignin matrix [58].

Li et al. [59] stated that the lignin depolimerization is related with several AA enzymes, such as ligninolytic peroxidases and laccases, however, this mecanism

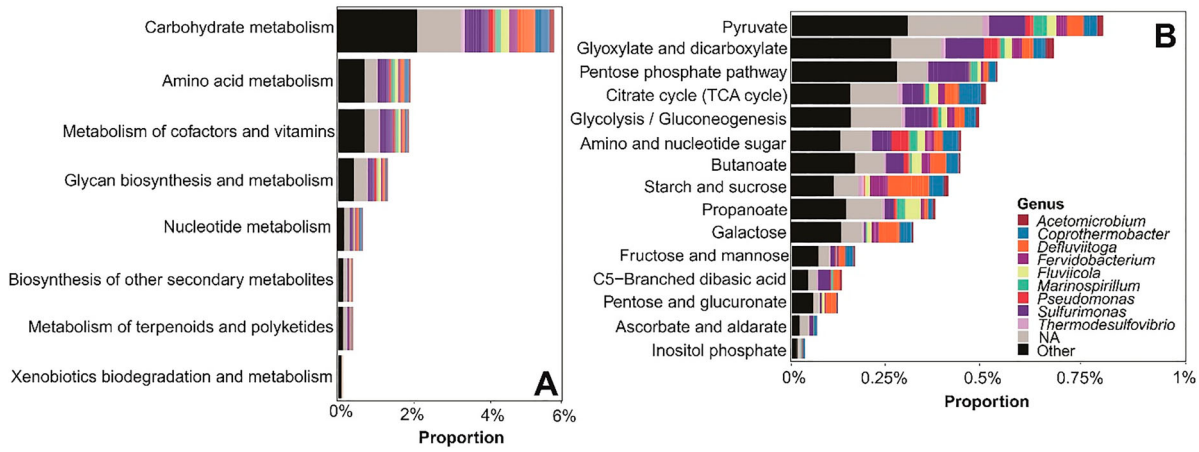


Figure 3. Proportions of the most abundant pathways (A) in the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment and for carbohydrate metabolism category (B), with the contributions of the 10 most abundant genera. The proportions were calculated considering the means of the total number of preprocessed reads in the biological triplicates. NA = not annotated.

is more clarified in aerobic organisms (e.g. fungi), being still unclear in anaerobic bacteria. In this study, a bacterial genus described by Akita et al. [60] as lignolytic, *Burkholderia*, could be related with AA3 (cellobiose dehydrogenase) family. Moreover, Melo-Nascimento et al. [57] linked this genus to the production of extracellular cellobiose dehydrogenase enzymes.

Table 1. Researches carried out over the years using the anaerobic sludge from the same UASB reactor applied to vinasse thermophilic treatment as inoculum, applied to different substrates, in batch and continuous reactors, besides the main organisms identified in each of them. (–) data not shown.

Substrate	By-product	Sludge pre-treatment	t (°C)	pH	Reactor	Main organism	Reference
sugarcane stillage	5.57 L CH_4.g COD^{-1}	–	55		AFBR	–	Ramos and Silva [19]
cellulosic hydrolysate	115.7 mL H_2 $h.L^{-1}$	heat	55		AFBR	–	Lopes et al. [28]
glycerol	15.14 moL H_2 mol^{-1} glycerol	heat	37	5.5	Batch	*Enterobacter*	Rodrigues et al. [26]
sugarcane juice	501 mL H_2 h^{-1} L^{-1}	heat	55	3.9-4.6	AFBR	–	Ferreira et al. [22]
citrus peel waste	13.31 $mmol.L^{-1}$	–	37	7.0	Batch	*Clostridium*, *Ruminiclostridium*	Camargo et al. [56]
sugar cane molasse	2041mL H_2 d^{-1} L^{-1}	–	55	–	USBR	*Thermoanaerobacterium*, *Clostridium*, *Lactobacillus*, *Pseudomonas*	Vilela et al. [7]
glucose	1.73 mol-EtOH/mol-glucose	–	55	7.0	Batch	*Caloramator*, *Fervidobacterium*, *Thermoanaerobacterium*, *Ethanoligenens*	Silva et al. [25]
sugarcane vinasse and cheese whey	1.01 mmol H_2.g COD^{-1}	heat	55	4.26-4.95	AFBR	–	Ramos and Silva [23]
sucrose-based synthetic wastewater	194.9 mL $H_2.h^{-1}$ L^{-1}	heat	55	–	AFBR	–	Ferreira et al. [29]
sugarcane vinasse	4505 $mLCH_4$ L^{-1} d^{-1}	–	55	7.0	ASTBR	–	Fuess et al. [18]
sugarcane vinasse	3 L CH_4 L^{-1} d^{-1}	–	55	6.59-7.70	UASB	Methanosarcinales, Thermotogae Methanobacteriales,	Barros et al. [16]
acidified sugarcane vinasse	306 $NmLCH_4.gCOD_{removed}$	–	55	6.8-7.2	UASB	–	Ferraz-Júnior et al. [17]
glycerol	28.49 mmol H_2 L^{-1}	heat	37	5.5	Batch	–	Rodrigues et al. [27]
glucose	13.3 $mmolH_2.L^{-1}$	heat	55	7.0	Batch	*Thermoanaerobacterium*, *Thermohydrogenium*	[76]
sugarcane stillage and glucose	30.7 mmol H_2 h^{-1} L^{-1}	–	55	–	AFBR	*Thermoanaerobacterium*, *Clostridium*	Santos et al. [21]
sugarcane vinasse	0.80 L H_2 h^{-1} L^{-1}	heat	55	–	AFBR	–	Santos et al. [20]

Note: Anaerobic Fluidized-Bed Reactor (AFBR); Anaerobic Structured-Bed Reactor (ASTBR); Anaerobic Upflow Structured Bed Reactor (USBR); Upflow Anaerobic Sludge Blanket (UASB).

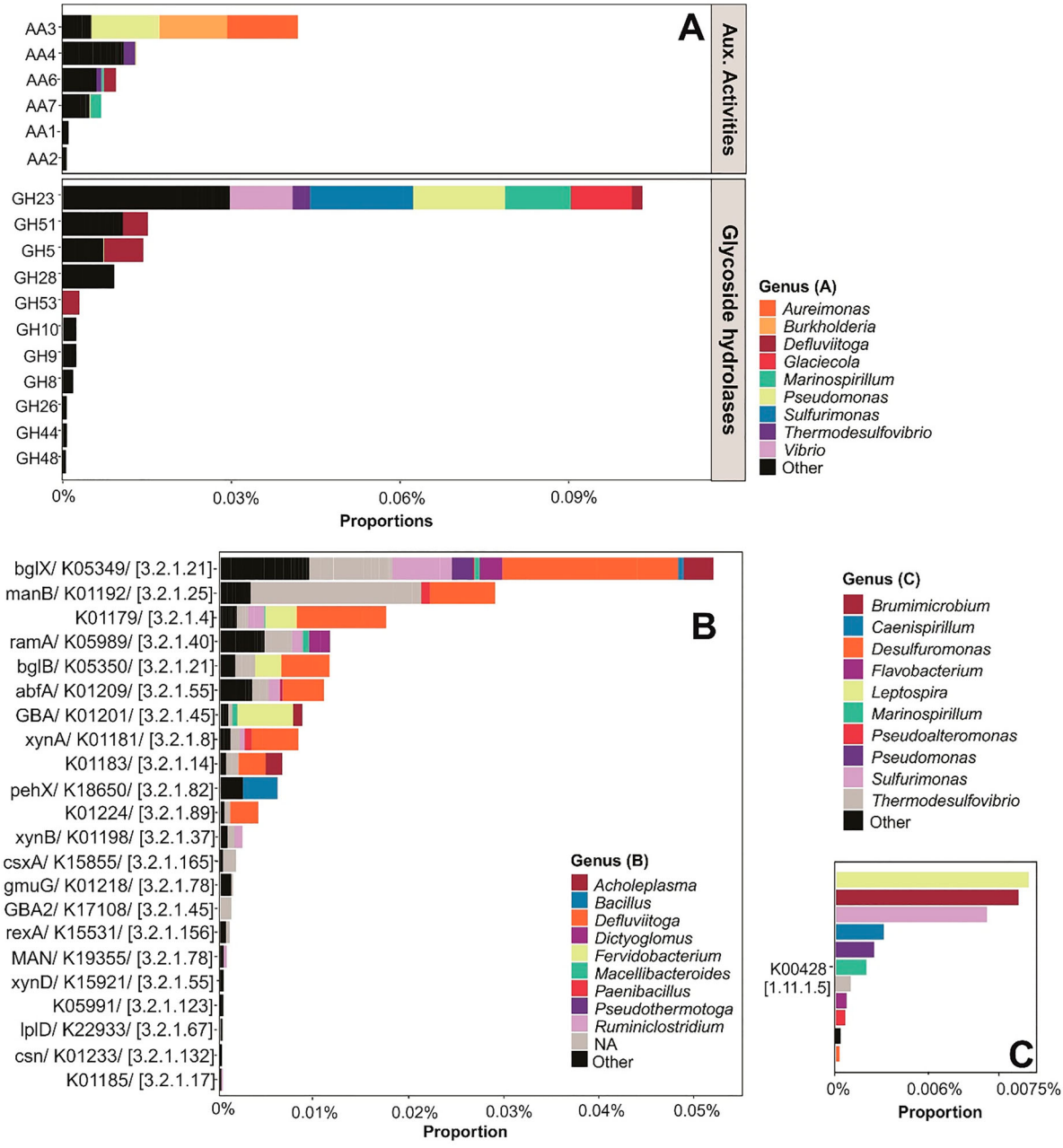


Figure 4. Proportions of the Carbohydrate-Active Enzymes (CAZymes) from the classes Auxiliary Activities (AA) or Glycoside Hydrolysis (GH) (A), the proportions of the most abundant KO related with cellulose and/or hemicellulosis (B) and lignin degradation (C), with the 10 most abundant genera associated to them in the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment. The proportions were calculated considering the total number of processed reads in the biological triplicates. NA = not annotated. The genes encoding enzymes involved in each reaction are showed in square brackets as EC numbers.

Among the other AA families identified in lower proportions, it is possible to highlight the AA4 (0.013%), AA6 (0.009%) and AA7 (0.007%), mainly related with the genera *Thermodesulfovibrio* (15.66%), *Defluviitoga* (22.73%) and *Marinospirillum* (28.0%), respectively. AA4 is a vanillyl-alcohol oxidase, which is involved in the conversion of several phenolic compounds, while the AA6 family is related with the 1,4-benzoquinone reductase activity, an intracellular enzyme also related with the biodegradation of aromatic compounds. The family AA7 is a glucooligosaccharide chitooligosaccharide oxidase.

Besides the CAZymes, a KO related with lignin-like compounds degradation could be observed, the K00428 (0.043%), a cytochrome c peroxidase. This KO was mainly related with the *Leptospira* (20.56%), *Sulfurimonas* (40.12%) and *Brumimicrobium* (19.52%) genera. However, even though peroxidases may be associated

with lignolytic activity, their activity may be related to other metabolic pathways, such as in the protection against oxygen molecules in strictly aerobic organisms [61], being not possible to state that such mentioned genera are lignolytic only due to the presence of this enzyme. Being that, it is worth mentioning that *Brumimicrobium* [62] and *Leptospira* [63] were already described in the literature as potentially related to lignin degradation.

Regarding the enzymes involved in carbohydrate degradation, the GH23 family was by far the most abundant one (0.103%), being associated with the activities of peptidoglycan lytic transglycosylase and chitinase, mostly in bacterial cell wall degradation. Probably its higher proportions occurred due to the sludge origins, since it was collected from a well-established industrial UASB reactor, where the endogenic activity in the biomass may be high. This family could be associated with several bacterial genera, being the main ones *Sulfurimonas* (17.82%), *Pseumodomas* (15.83%) and *Marinospirillum* (11.41%). Other CAZymes related to the GH family could be observed in lower proportions, such as GH51 (0.015%), GH5 (0.014%) and GH53 (0.003%), related especially with the presence of the bacteria *Defluviitoga* (28.75, 47.83 and 100%, respectively). It is worth mentioning that the K01179, the endoglucanase referring to these GH, was confirmed in association with the *Defluviitoga tunisiensis* species after genome reconstruction.

The GH51 family has several activities, such as endoglucanase, endo-β-1,4-xylanase, β-xylosidase, α-L-arabinofuranosidase and lichenase/endo-β-1,3-1,4-glucanase. On the other hand, the family GH53 is related only with the endo-β-1,4-galactanase activity. The GH5 is a family of exocellulases with a wide range of activities, being worth mentioning the endo-β-1,4-glucanase/cellulase, endo-β-1,4-xylanase, β-glucosidase, β-mannosidase and cellulose β-1,4-cellobiosidase activities. Besides that, this family includes the KO related with carbohydrates degradation observed in the highest proportions, the K05349 (0.053%), a β-glucosidase active in the Starch and sucrose metabolism and cellulose degradation pathway, converting the cellobiose to D-glucose and cellodextrin, the first intermediate of cellulose biodegradation, to cellobiose. This KO was mostly related to *Defluviitoga* (35.28%) genus, especially with *D. tunisiensis* species, as could be confirmed by genome reconstruction.

The second KO observed in higher proportions was the K01192 (0.029%), a β-mannosidase also belonging to the GH5 family and related especially to the *Defluviitoga* (23.79%) genus. However, the Clostridiales order is still the most related with this activity in the literature, since these organisms are capable to break the crystalline cellulose structure by expressing an extracellular enzymatic complex (cellulosomes) or non-cellulosomal free CAZymes, degrading [64]. Villa and Veiga-Crespo [65] reported the genus *Sulfurimonas* as β-1,3 – endoglucanase producer, being this genus also observed in the present study (40.12%) as one of the most abundant Bacteria genera.

The K01179 was observed in proportions of 0.017%. This enzyme is an endoglucanase (GH5 family) active in the Starch and sucrose metabolism and cellulose degradation pathway, converting the cellulose to its first intermediate, the cellodextrin, and also the cellodextrin to cellobiose. Other KO observed in lower proportions and mostly related with *Defluviitoga* genus were the β-glucosidase K05350 (0.012%), the endo-1,4-β-xylanase K01181 (0.008%) active in xylose degradation pathway, the chitinase K01183 (0.007%) and the α-L-arabinofuranosidase K01209 (0.011%), being this last one in association with the species *D. tunisiensis*.

About 11 KO related with the glycerolipids degradation pathway could be observed in the UASB sludge biomass (Figure 5). The most abundant one was the oxidoreductase K00128 (0.049%), aldehyde dehydrogenase (NAD+). This enzyme has a wide specificity, and can catalyze the reduction of the NAD^+ to NADH using an aldehyde and a molecule of H_2O, concomitantly generating a carboxylate and H^+ in this reaction. In this case, the D-glyceraldehyde is then converted to D-glycerate, in one of the steps of the glycerol degradation before glycolysis. In this way, besides the glycerolipids metabolism, this enzyme is also related to the Glycolysis/Gluconeogenesis, Fatty Acids Degradation, Limonene and pinene degradation and Pyruvate metabolisms, Aminoacids metabolism, among others, which can justify its high proportions in this biomass. In this study, this KO could be mostly related to the bacterial genera *Oxobacter* (20.4%), *Lutibacter* (18.4%), *Polaribacter* (16.3%) and *Spirosoma* (4.1%).

The transferase K11529 (0.012%) glycerate 2-kinase was the second most abundant KO related with glycerolipids degradation pathway, being *Defluviitoga* (27.5%), *Fervidobacterium* (22.5%) and *Acetomicrobium* (6.7%) the main genera related with it. Besides the glycerolipids metabolism, this KO is also related, among others, with Pentose phosphate pathway, Carbon and Methane metabolism. This enzyme catalyzes the convertion of D-glycerate to 2-Phospho-D-Glycerate, the final step of glycerol degradation before glycolysis, and needs an ATP molecule for this purpose.

In similar proportions (0.011%) the K13979 alcohol dehydrogenase ($NADP^+$) was observed, with the genus *Pedobacter* (72.7%) being the most related one. This enzyme is one of the enzymes that can convert glycerol to D-glyceraldehyde, since it reduces a $NADP^+$ molecule to NADPH by

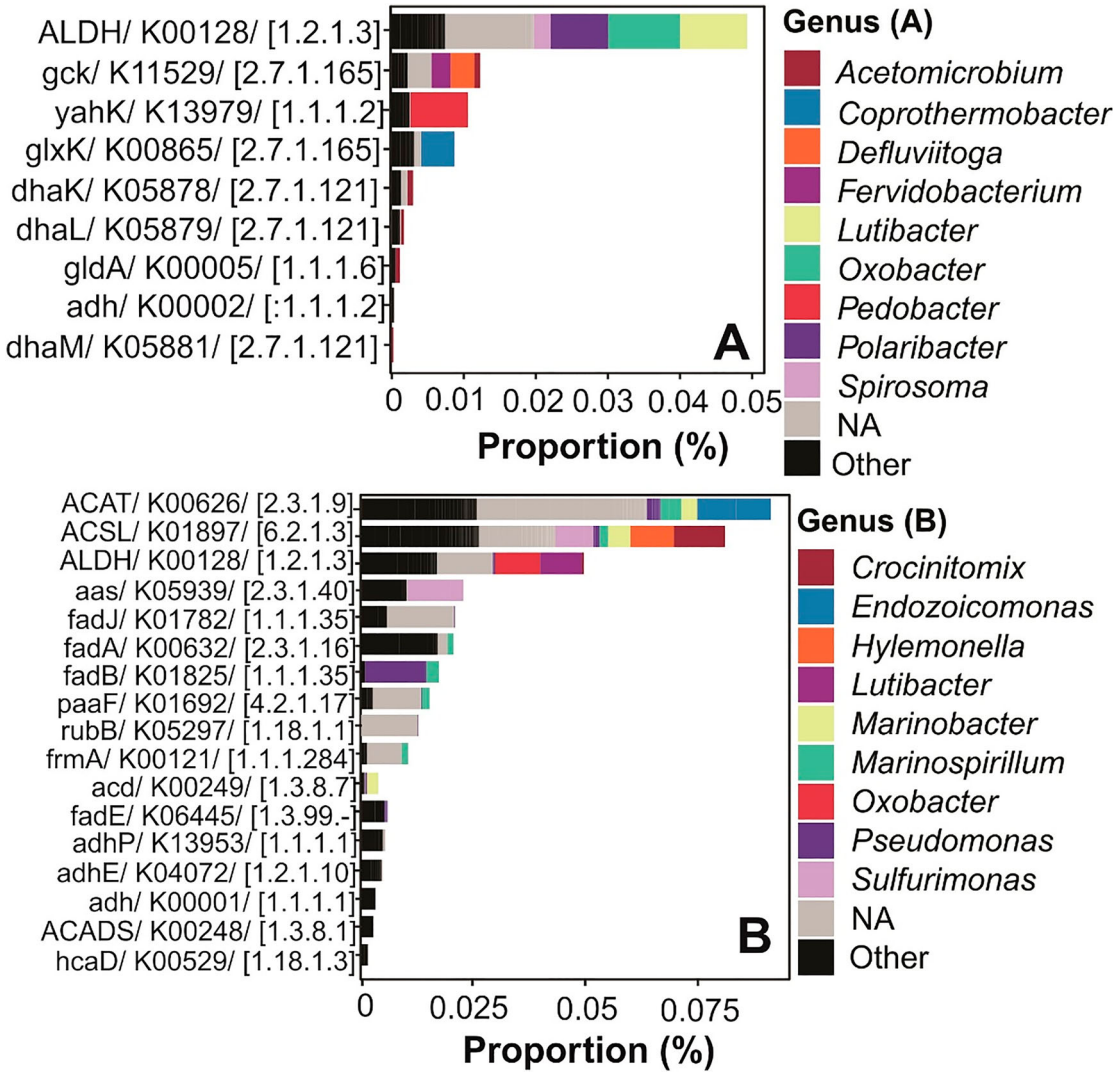


Figure 5. Proportions of the KO related with glycerolypids degradation (A) and organic compounds (VFA and/or alcohol) production (B) in the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment, with the 10 most abundant associated genera. The proportions were calculated considering the total number of processed reads in biological triplicates. NA = not annotated. The genes encoding enzymes involved in each reaction are showed in square brackets as EC numbers.

oxidyzing a primary alcohol, generating an aldehyde and H^+ in this process. Being that it is possible to infer that the glycerol degradation before glycolysis can occur using the studied UASB sludge as inoculum, especially thanks to the K13979, K00128 and K11529, respectively, as described in the simplified Equations (1)–(3).

$$Glycerol + NADP^+ \rightarrow D-glyceraldehyde + NADPH + H^+ \quad (1)$$

$$D-glyceraldehyde + NAD^+ + H_2O \rightarrow D-glycerate + NADH + H^+ \quad (2)$$

$$D-glycerate + ATP \rightarrow 2-phospho-D-glycerate + ADP \quad (3)$$

Other KOs observed in lower proportions in this pathway were K00865 (0.009%), K05878 (0.003%) and K05879 (0.002%), being the first one also related to the reaction described in Equation (3), and the other ones both related with the activity phosphoenolpyruvate-glycerone phosphotransferase subunit DhaK and DhaL, respectively. In this alternative path, glycerol can be converted to glycerone and glyceronephosphate before being degradated in the glycolysis or glycerophospholipid metabolism, also generating a pyruvate molecule in this process by using a molecule of phosphoenolpyruvate. It is worth highlighting the high relation of *Coprothermobacter* with the K00865 (55.6%), and of *Acetomicrobium* with both of the other ones (25.3 and 21.0%, respectively).

The KOs related with enzymes that have an important role in organic acids or alcohols production were filtered and analyzed based on the KEGG pathways. Most of these KO are present in the Glycolysis/Gluconeogenesis, Fatty acid degradation, Benzoate, Propanoate and Butanoate metabolism and degradation, Carbon metabolism, Pyruvate metabolism, starch and sucrose metabolism, among others. The one observed in higher proportions was the K00626 (0.091%), an acetyl-CoA C-acetyltransferase, mainly related with the genus *Endozoicomonas* (18.0%) and *Marinospirillum* (5.1%). The higher proportions of this enzyme can be justified by its wide function, converting two molecules of Acetyl-CoA to a free CoA and Acetoacetyl-CoA, being found in eukaryotes, prokaryotes and viruses. In anaerobic digestion processes, it is worth mentioning its importance in the last step of both Hexanoyl-CoA, Glutaryl-CoA and Butyryl-CoA conversion into Acetyl-CoA in the Fatty acids degradation and Benzoyl-CoA in de Benzoate degradation pathway. The Acetyl-CoA is a key intermediate in the production of Acetic, Butyric, Propionic, Lactic, among other acids and organic compounds produced during the acidogenesis and acetogenesis in the anaerobic digestion and dark fermentation.

The K01897 (0.081%), a long-chain acyl-CoA synthetase, could be related to several genera, such as *Crocinitomix* (14.1%), *Hylemonella* (12.0%), *Sulfurimonas* (10.5%), *Marinobacter* (6.1%), *Marinospirillum* (2.4%) and *Pseudomonas* (1.6%). It acts in the fatty acids biosynthesis from Acetyl-CoA in a wide range of long-chain saturated and unsaturated fatty acids, binding them to a CoA molecule, generating a diphosphate and a long-chain fatty acyl-CoA, requiring an ATP molecule in the process. In the same way, it can also acts in the Hexadecanoate degradation in the Fatty acids degradation pathway. The K06445 (0.0057%), acts in the same pathways mentioned, as an acyl-CoA dehydrogenase.

The K00128 was already discussed in relation to the Glycerolypids degradation pathway, being involved in the conversion of an aldehyde to a carboxylate and H^+ molecules (Equation (2)). This KO was observed in proportions of 0.049% and was mostly related to *Oxobacter* (20.4%) and *Lutibacter* (18.4%) genera.

Regarding other KOs observed in lower proportions and related with organic compounds and/or alcohols production in anaerobic reactors, it is worth mentioning the K13954, K13953, K04072 (alcohol dehydrogenase), involved in the interconversion of acetaldehyde to ethanol, by means of the NAD^+ reduction to NADH, with concomitant production of H^+ (Equation (4)) and/or acetaldehyde to Acetyl-CoA (Equation (5)).

$$Ethanol + NAD^+ \leftrightarrow acetaldehyde + NADH + H^+ \quad (4)$$

$$Acetaldehyde + CoA + NAD^+ \rightarrow AcetylCoA + NADH + H^+ \quad (5)$$

It is known that both H_2 and CH_4 productions are strictly related with the organic intermediates produced during the process. The H_2 production occurs in previous steps than methanogenesis, during anaerobic digestion, during the acidogenesis or acetogenesis, concomitantly with the production of some organic acids, being worth to highlight the acetic acid and butyric acid, being produced 4 molecules of H_2 in the first case and 2 molecules of H_2 in the second case (Equations (6) and (7)). HBu cannot be used directly as a substrate for methanogenesis; therefore, it should be converted to HAc (Equation (8)) [66,67].

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2CH_3COOH + 4H_2 \quad (6)$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + CH_3CH_2CH_2COOH + 2H_2 \quad (7)$$

$$CH_3COOH + 2CH_3CH(OH)COOH \rightarrow H_2 + \frac{3}{2}CH_3(CH_2)_2 + COOH + 2CO_2 + H_2O \quad (8)$$

The CH_4 production occurs at the last step thanks to methanogenic archaea, through hydrogenotrophic (Equation (9)), acetoclastic (Equation (10)) or methylotrophic methanogenesis, after methanol conversion to methyl-CoM (Equation (11)), being directly or indirectly dependent of the byproducts generated in the previous steps [68].

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O_2 \quad (9)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (10)$$

$$[methylCo(III)methanol\ specific\ corrinoid\ protein] + CoM \rightarrow methylCoM + methyl[Co(I)methanol\ specific\ corrinoid\ protein] \quad (11)$$

Based on the Figure 6-A, it is possible to notice that several KO related to H_2 production could be observed in the studied samples, being K00123, K00169, K00170 and K03737 the most abundant ones, in proportions of 0.031, 0.026, 0.022 and 0.021%, respectively. The K00123 is a formate dehydrogenase (major subunit) which can be found in the glyoxylate and dicarboxylate, methane and carbon metabolisms, and was mainly related to the bacterial genera *Sulfurimonas* (23%), *Acetomicrobium* (18.7%) and *Alkalilimnicola* (15.2%). Together with hydrogen dehydrogenase, this enzyme forms a system previously known

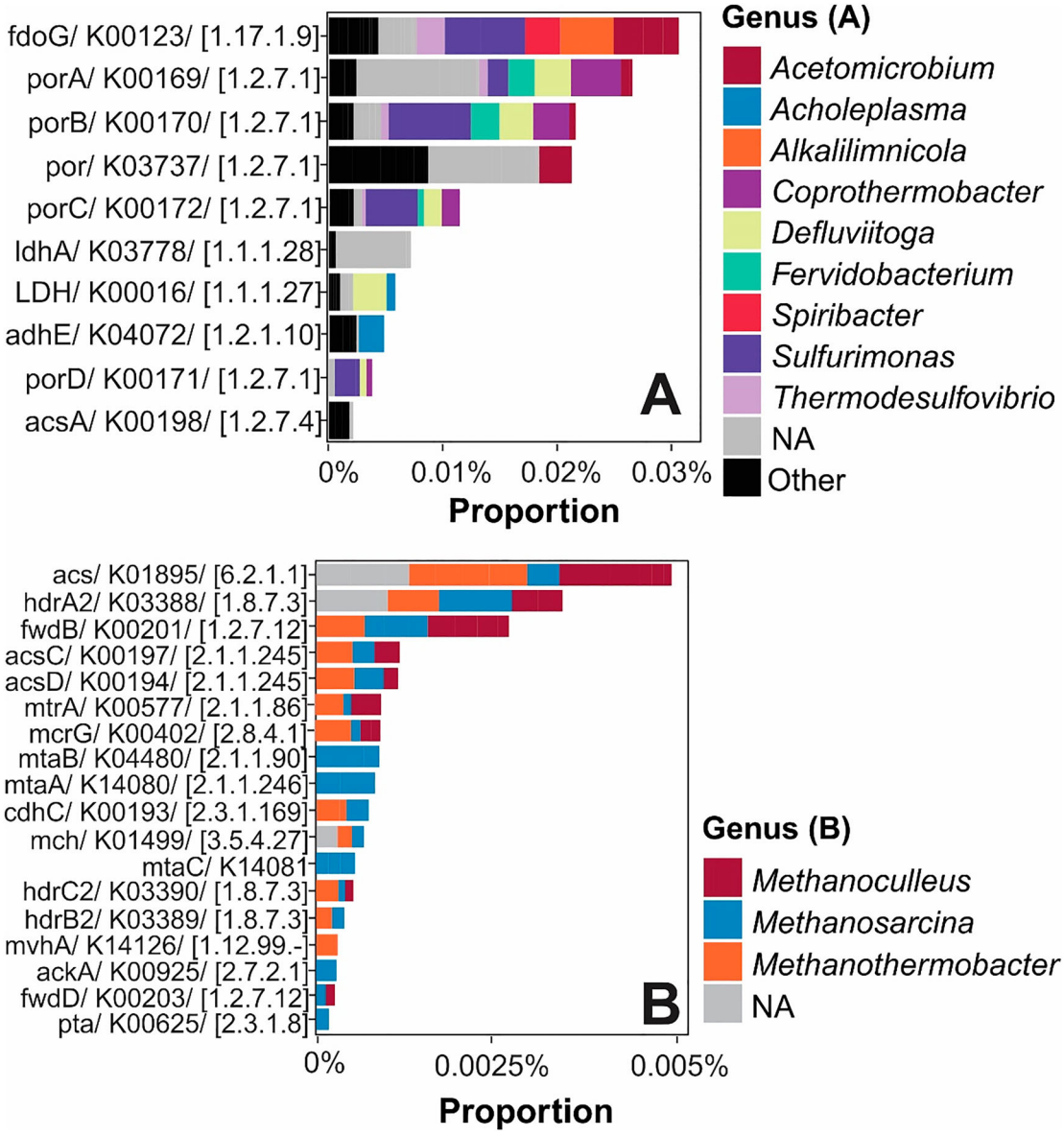


Figure 6. Proportions of the KO related with H_2 (A) and CH_4 (B) production in the granulated sludge from full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment, with the 10 most abundant associated genera. The proportions were calculated considering the total number of processed reads in biological triplicates. NA = not annotated. The genes encoding enzymes involved in each reaction are showed in square brackets as EC numbers.

as formate hydrogenlyase, which catalyzes the reduction of NAD^+ with formate to NADH, CO_2 and H^+.

The second and third most abundant KO were K00169 and K00170, the pyruvate synthase/ pyruvate ferredoxin oxidoreductase (alpha subunit and beta subunit, respectively), being mostly related to *Sulfurimonas* (33.3%), *Corprothermobacter* (14.7) and *Defluviitoga* (13.6%). These enzymes are important to several pathways, such as Glycolysis/Gluconeogenesis, Pyruvate, Propanoate, Butanoate and Methane metabolisms, among others, which can explain their higher proportions in comparison to other KO observed. It is an important enzyme both to methanogenesis and H_2 production, since the reaction catalyzed by this enzyme generates a key molecule to these pathways, the Acetyl-CoA from pyruvate, generating H^+ along the reaction (Equation (12)). In the same way, the pyruvate-ferredoxin/flavodoxin oxidoreductase K03737, related mainly with *Acetomicrobium* (13.3%), can be related with the same reaction, and this enzyme can be with the activity of pyruvate-Acetyl-CoA interconversion [69].

$$\begin{aligned} &Pyruvate + CoA + ferredoxin(oxidized) \\ &\leftrightarrow AcetylCoA + CO_2 + ferredoxin(reduced) \\ &+ 2H^+ \end{aligned} \tag{12}$$

The enzymes related with H_2 production and observed in lower proportions were K00172 (0.011%), K03778 (0.007%), K00016 (0.006%), K04072 (0.005%), K00171 (0.004%) and K00198 (0.002%). Among the other KO, the K00172 (0.011%) and K00171 (0.004%) are also related with the same functions described above regarding pyruvate synthase (Equation (12)). Also, it is worth to mention the K03778 (0.007%) and K00016 (0.006%), both related to lactate dehydrogenase (D and L-lactate, respectively), being the K00016 was mainly related to *Defluviitoga* genus (50.7%). In this study, after genome reconstruction, it was possible to confirm that the species *Defluviitoga tunisiensis* was related with K00169 (porA), K001700 (porB), K00171 (porD), K00172 (porG), besides K00016 (LDH).

In the Pyruvate metabolism, the mentioned enzymes can generate a pyruvate and an H^+ molecule by reducing NAD^+ to NADH with lactate, as described in the Equation (13). These enzymes are active in glycolysis/gluconeogenesis pathways and usual in all living being, and besides being active in lactate and alcohols synthesis, it can also perform the reverse route, which enables the hydrogen production by the conversion of lactic acid into pyruvic acid after reduction of NAD^+, that can be converted into H_2, CO_2 and butyric acid [9].

$$Lactate + NAD^+ \leftrightarrow Pyruvate + NADH + H^+ \quad (13)$$

The K04072 is an acetaldehyde dehydrogenase/alcohol dehydrogenase involved in several pathways, such as Glycolysis/Gluconeogenesis, Fatty acid degradation, Pyruvate metabolism, Butanoate metabolism, Degradation of aromatic compounds, among others. In this study, the K04072 could be related to the genus *Acholeplasma* (45.9%). This enzyme can be involved in the meta-cleavage pathway for the degradation of phenols, methylphenols and catechols, and catalyzed the reaction described in Equations (14) and/or (15) and (16).

$$Primaryalcohol + NAD^+ \leftrightarrow an\ Aldehyde + NADH + H^+ \quad (14)$$

$$Secondaryalcohol + NAD^+ \leftrightarrow a\ Ketone + NADH + H^+ \quad (15)$$

$$Acetaldehyde + CoA + NAD^+ \leftrightarrow AcetylCoA + NADH + H^+ \quad (16)$$

Finally, the K00198 is an anaerobic carbon-monoxide dehydrogenase catalytic subunit directly related to methanogenesis and carbon metabolism. This enzyme catalyses the reversible reduction of CO_2 to CO in prokaryotes, according to Equation (17), being the oxidation of CO to CO_2 catalyzed by methanogenic archaea or purple sulphur bacteria, and the reduction of CO_2 to CO catalyzed by acetogenic and sulphate-reducing microbes, which can be incorporated into Acetyl-CoA by other enzymes activity.

$$CO + H_2O + 2ferredoxin(oxidized) \leftrightarrow CO_2 + 2ferredoxin(reduced) + 2H^+ \quad (17)$$

Figure 6-B summarized the KO related with Methane metabolism, in the methanogenesis pathway, which were filtered according to Macedo et al. [70]. The K01895 is an acetyl-CoA synthetase and was the most abundant KO observed in the UASB biomass samples related with this pathway (0.0049%). The main Archaea genera related with this KO were *Methanothermobacter* (33.2%), *Methanoculleus* (31.6%) and *Methanosarcina* (9.0%). The species *Methanoculleus thermophiles* could be identified after genome reconstruction, where the presence of the gene K01895 (acs) was confirmed. Besides the Methane metabolism, this enzyme can be related with others, such as Glycolysis/Gluconeogenesis, Pyruvate metabolism, Glyoxylate and dicarboxylate metabolism, Propanoate metabolism, among others, which can explain its higher proportions. It is involved in the interconversion of Acetyl-CoA and Acetate (Equation (18)), which can be directly used as substrate in the acetoclastic methanogenesis (Equation (10)).

$$ATP + Acetate + CoA \leftrightarrow AMP + Diphosphate + AcetylCoA \quad (18)$$

The K03388 was the second most abundant KO related with methanogenesis (0.0034%). It is a heterodisulfide reductase (subunit A2), which can be found in most methanogenic organisms and catalyzes the regeneration of the Coenzyme B and Coenzyme M heterodisulfide (Equation (19)), using two molecules of ferredoxin (oxidized, [4Fe-4S] cluster). In *Methanosarcina*, it can also catalyze the reactions involving the coenzyme F420:CoB-CoM heterodisulfide and ferredoxin reductase (reduced, [4Fe-4S] cluster) (Equation (20)). Also, this enzyme can be found in formate-oxidizing CO_2-reducing methanogenic archaea, such as *Methanococcus*, reducing both ferredoxin and CoB-CoM heterodisulfide to formate (Equation (21)) [15]. Being that, it is possible to state that this enzyme is involved in the three types of methanogenesis: acetoclastic, hydrogenotrophic and methylotrophic. In this study, the genera *Methanosarcina* (29.6%), *Methanothermobacter* (20.9%) and *Methanoculleus* (20.7%) were related to

this KO.

$$2Fd(oxidized) + CoB + CoM \leftrightarrow 2Fd(reduced) + CoMSSCoB + 2H^{+} \quad (19)$$

$$2CoF420(ox.) + 2Fd(red.) + CoB + CoM + 2H^{+} \leftrightarrow 2CoF420 + 2Fd(red.) + CoMSSCoB \quad (20)$$

$$2CO_2 + 2Fd(reduced) + CoB + CoM + 2H^{+} \leftrightarrow 2formate + Fd(oxidized) + CoMSSCoB \quad (21)$$

The K00201 was observed in proportions of 0.0027% and was related with the genera *Methanoculleus* (42.2%), *Methanothermobacter* (25%) and *Methanosarcina* (22.9%), being the two last ones already widely described in association with this enzyme [71,72]. It is a formylmethanofuran dehydrogenase (subunit B), involved strictly in the Methane metabolism since it is involved in methanogenesis from CO_2 as well as the oxidation of coenzyme M to CO_2 (Equation (22)).

$$Formylmethanophuran + H_2O + 2Fd(ox.) \leftrightarrow 2CO_2 + methanofuran + 2Fd(red.) + 2H^{+} \quad (22)$$

Regarding the other KO observed in lower proportions (≤0.001%), most of them were related with the genera *Methanothermobacter* and *Methanosarcina*. It is worth to mention that K14081, K14080 and K04480 were exclusively related to the genus *Methanosarcina* in the present study. The two first enzymes are part of the complex [methyl-Co (III) methanol-specific corrinoid protein]: coenzyme M methyltransferase, and it is involved in methanogenesis from methanol. In the same way, the K04480 is also involved only in methanogenesis from methanol, being this KO a methanol-5-hydroxybenzimidazolylcobamide Co-methyltransferase, which catalyses the transfer of methyl groups from methanol to a methanol-specific corrinoid protein.

The K14128 and K14126 were both related only with the genus *Methanothermobacter*, since the enzyme F420-non-reducing hydrogenase small and large subunits are related only with hydrogenotrophyc methanogenesis (Equation (19)). Besides that, the methyl-coenzyme M reductase gamma subunit (K00402) is a transferase which catalysis the last methanogenesis step, where the biological methane production occurs. Among the genera commonly related with the K00402, were *Methanosarcina* and *Methanothermobacter* [73–75]. The K00193 can be associated with the synthesis of methyl-Co (III) Fe-S corrinoid protein (CH_3-CO (III)FeSP), using the Co(I) corrinoid Fe-S protein and acetyl-CoA. After this conversion, the enzymes acetyl-CoA decarbonylase/synthase (K00194 and K00197) act in the conversion of the CH_3-CO(III)FeSP to Co(I) corrinoid Fe-S protein and 5-methyl tetrahydro sarcina pterin (5-Methyl-H4SPT).

Finally, the K00577, related to the tetrahydromethanopterin S-methyltransferase subunit A (mtrA) and part of the multienzymatic complex archaeon *Methanobacterium thermoautotrophicum*, is also involved in the CH_4 production from CO_2 and H_2 (Equation (9)), by means of Methyl-CoM biosynthesis. However, the genus *Methanobacterium* was observed only in very low relative abundances in the studied samples (0.013 ± 0.0%).

The degradation pathways of several AD inhibitors were also considered. Only two KO related with the Limonene and pinene degradation pathway could be observed (Figure A.3), being the K00128 (0.049%) and K14731 (0.001%), the first one related only to (−)-S-limonene and the second one being related, in addition, to (+)-(R)-limonene degradation. The K00128, an aldehyde dehydrogenase (NAD^+), is an enzyme with wide specificity, being related to several metabolic pathways, such as Glycolysis/Gluconeogenesis, Fatty acid degradation, Glycerolipid, Pyruvate and Aminoacids metabolisms, among others, catalyzing the conversion of an aldehyde to a carboxylate, as described before in the Equation (2). In the Limonene degradation pathway, it converts the perillyl aldehyde to perillyc acid.

On the other hand the K14731, monoterpene epsilon-lactone hydrolase, is more specific to the aromatic compounds degradations, being found only in Limonene and pinene degradation, Degradation of aromatic compounds and Caprolactam degradation pathways, catalyzing two different reactions in the first one, both related to the ring opening of epsilon-lactones which are formed during degradation of dihydrocarveol. Since the enzymes found to be related to limonene degradation were observed in very low proportions, are not specific to this pathway and specially since other needed enzymes that should be involved in this pathway were not observed in the UASB sludge biomass, it can be inferred that this sludge may not be a very adequate inoculum to be used in the process with high concentrations of this compound.

In Figure A.3, it is possible to notice that several KOs related to benzoate and phenols degradation, in the Xenobiotics/Benzoate degradation and metabolism pathway, however, it is possible to highlight four of them with proportions higher than 0.002% in the studies samples. The K00632 (0.021%), an acetyl-CoA acyltransferase, was the main KO observed in this pathway, being also related with other metabolic pathways, such as Fatty acid degradation and metabolism, Ethylbenzene degradation, among others. In the conversion path of benzoate/phenol to succinyl-CoA, the K00632 is involved in one of the last steps, the conversion

of 3-Oxoadipyl-CoA to succinyl-CoA. *Bruminicrobium* (41.0%), *Halomonas* (30.0%), *Marinospirillum* (6.7%), *Lysobacter* (3.3%) and *Flavobacterium* (2.4%) were related to this KO. It is worth highlighting that the K00632 was not observed in association with *Lysobacter concretionis* after the genome reconstruction, enabling to infer that possibly other species of this genus could be associated with this gene in the present study.

The lyase K01666 (0.008%), a 4-hydroxy 2-oxovalerate aldolase, was mostly related to the genus *Burkholderia* (87.5%), and it is involved in the last steps of benzoate/phenol degradation, in the convertion of (S)-4-hydroxy-2-oxopentanoate to acetaldehyde and pyruvate, requiring Mn_2^+ for its maximal activity. *Burkholderia xenovorans* is a known species that produces this enzyme. Besides their low proportions, the other KO related to enzymes needed in benzoate and phenols degradation were observed in the UASB sludge biomass, being worth mentioning the K05784, K05549, defined as activities benzoate/toluate 1,2-dioxygenase reductase component and benzoate/toluate 1,2-dioxygenase subunit alpha, respectively, being the enzymes responsible for the convertion of benzoate to the first intermediate of its complete convertion to its subproducts, the (1R,6S)-1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate and NAD^+, requiring for that a molecule of NADH, H^+ and O_2. That means, these enzymes require aerobic environments to work properly, and are related especially with the genus *Pseudomonas*, a facultative anaerobic genus, which can explain its presence in the studied samples.

Finally, three KOs related with furfural degradation were observed in the UASB sludge biomass (Figure A.3), K16877 (0.0006%), K16876 (0.0004%) and K16880 (HmfE) (0.0003%), being the two first ones mostly related with the *Herbaspirillum* (100%) genus and the last one related with *Bradyrhizobium* (100%). These enzymes catalyze the conversion of the 2-Furoate, the first intermediate of the furfural degradation, to 2-Oxoglutarate. The conversion of furfural to 2-Furoate is firstly catalyzed by the enzyme 5-(hydroxymethyl) furfural/furfural oxidase, being then converted to 2-Furoyl-CoA by the enzyme 2-furoate-CoA ligase (K16876). The 2-Furoyl-CoA is then converted to 5-Hydroxy-2-furoyl-CoA by the enzyme 2-furoyl-CoA dehydrogenase (K16879), which is finally converted to 2-Oxoglutaryl-CoA and then to 2-Oxoglutarate by the enzyme 2-oxoglutaroyl-CoA hydrolase (K16880).

4. Conclusion

The metabolic potential of a granulated sludge from a full-scale UASB thermophilic reactor applied to sugarcane vinasse treatment was depicted. It was possible to observe several genes encoding enzymes that are of great importance to AD processes using different types of wastes as substrate, especially regarding carbohydrates and ligninolytic compounds biodegradation, glycerolypids pathway, VFA and alcohols metabolism, resistance and degradation of potential inhibitors (limonene, furfural, phenols) and biogas (H_2 and CH_4) production.

Among the most abundant genera, it is worth highlighting that *Sulfurimonas* great relative abundance is probably related to the GH23 family, mostly associated with bacterial cell wall degradation, being possible to assume that it occurred due to the sludge endogenic activity, since it was collected from a well-established industrial UASB, where this activity in the biomass may be high. In addition, *Methanosacina* was the most abundant archaea, enabling to infer that it had great contribution to the methanogenesis, both acetoclastic and hydrogenotrophic, since this genus was related to the presence of genes encoding acetyl-CoA synthetase and several dehydrogenases, such as formylmethanofuran dehydrogenase, since it is involved in methanogenesis from CO_2 as well as the oxidation of coenzyme M to CO_2.

However, when analyzing different research studies using the same inoculum as microbial source, it was observed that the relative abundances of the dominant genera varied according to the physicochemical parameters and/or substrate used, as well as the reactor efficiency. Besides that, the most abundant genera observed by means of 16S rRNA sequencing are not always related to the dominant metabolism in the reactor. In this way, it is worth highlighting the importance of the KO and genera observed in smaller proportions and involved in the target pathway, in order to better comprehend the reactor dynamics. The results reported here may expectedly subside future research work in anaerobic digestion worldwide, using both the same or similar inocula, since the robust description of the UASB sludge microbiome could guide the experimental set-up, in order to favour specific organisms and metabolic pathways, improving the value-added metabolites obtainment.

Acknowledgements

This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP processes 2018/26182-1, 2017/01722-0 and 2015/06246-7), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Financial code 001 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are grateful to the INRAE MIGALE bioinformatics facility (MIGALE, INRAE, 2020). Migale bioinformatics Facility, doi:10.15454/1.5572390655343293E12) for providing help, computing and storage resources.

Data availability statement

The data that support the findings of this study are openly available in National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA605706, reference number PRJNA605706, and accession numbers SRX7704207 to SRX7704209.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by CAPES; CNPQ; São Paulo Research Foundation (FAPESP) [grant number 2015/06246-7,2017/01722-0,2018/26182-1].

ORCID

Franciele Pereira Camargo http://orcid.org/0000-0002-0246-0399
Isabel Kimiko Sakamoto http://orcid.org/0000-0003-4475-1116
Tiago Palladino Delforno http://orcid.org/0000-0002-1705-0763
Cédric Midoux http://orcid.org/0000-0002-7964-0929
Iolanda Cristina Silveira Duarte http://orcid.org/0000-0002-9141-1010
Edson Luiz Silva http://orcid.org/0000-0003-3194-4912
Ariane Bize http://orcid.org/0000-0003-4023-8665
Maria Bernadete Amâncio Varesche http://orcid.org/0000-0003-3124-7471

References

[1] Jiang C, Peces M, Andersen MH, et al. Characterizing the growing microorganisms at species level in 46 anaerobic digesters at Danish wastewater treatment plants: a six-year survey on microbial community structure and key drivers. Water Res. 2021;193:116871, doi:10.1016/j.watres.2021.116871.
[2] Venkiteshwaran, K, Bocher, B, Maki, J, Zitomer, D. Relating anaerobic digestion microbial community and process function : supplementary issue: water microbiology. Microbiol Insights. 2015;8s2, MBI.S33593. doi:10.4137/MBI.S33593
[3] Cecilia, JA, García-Sancho, C, Maireles-Torres, PJ, et al Industrial food waste valorization: a general overview. In: Bastidas-Oyanedel, JR., Schmidt, J., editors. Biorefinery. Springer: New York; 2019. p. 25.
[4] Pachiega R, Rodrigues MF, Rodrigues CV, et al. Hydrogen bioproduction with anaerobic bacteria consortium from brewery wastewater. Int J Hydrogen Energy. 2019;4:155–163. doi:10.1016/j.ijhydene.2018.02.107.
[5] Kumar V, Nabaterega R, Khoei S, et al. Insight into interactions between syntrophic bacteria and archaea in anaerobic digestion amended with conductive materials. Renew Sustain Energy Rev. 2021;144:110965, doi:10.1016/j.rser.2021.110965.
[6] Soares LA, Silva Rabelo CAB, Sakamoto IK, et al. Screening and bioprospecting of anaerobic consortia for biofuel production enhancement from sugarcane bagasse. Appl. Biochem. Biotechnol. 2020;190:232–251. doi:10.1007/s12010-019-03074-2.
[7] Vilela R, Saia FT, Gregoracci GB, et al. Hydrogen production in reactors: the influence of organic loading rate, inoculum and support material. Int J Hydrogen Energy. 2019;44:27259–27271. doi:10.1016/j.ijhydene.2019.08.180.
[8] Sierocinski P, Milferstedt K, Bayer F, et al. A single community dominates structure and function of a mixture of multiple methanogenic communities. Curr Biol. 2017;27:3390–3395. e4. doi:10.1016/j.cub.2017.09.056.
[9] Mazareli RCdS, Villa-Montoya AC, Delforno TP, et al. Metagenomic analysis of autochthonous microbial biomass from banana waste: screening design of factors that affect hydrogen production. Biomass Bioenergy. 2020;138. doi:10.1016/j.biombioe.2020.105573
[10] Cabezas A, de Araujo JC, Callejas C, et al. How to use molecular biology tools for the study of the anaerobic digestion process? Rev Environ Sci Bio/Technol. 2015;14:555–593. doi:10.1007/s11157-015-9380-8.
[11] Delforno TP, Lacerda Júnior GV, Noronha MF, et al. Microbial diversity of a full-scale UASB reactor applied to poultry slaughterhouse wastewater treatment: integration of 16S rRNA gene amplicon and shotgun metagenomic sequencing. Microbiologyopen. 2017;6:e00443, doi:10.1002/mbo3.443.
[12] Candry P, Huang S, Carvajal-Arroyo JM, et al. Enrichment and characterisation of ethanol chain elongating communities from natural and engineered environments. Sci Rep. 2020;10:3682, doi:10.1038/s41598-020-60052-z.
[13] Zhu Q, Dai L, Wang Y, et al. Enrichment of waste sewage sludge for enhancing methane production from cellulose. Bioresour Technol. 2021;321:124497, doi:10.1016/j.biortech.2020.124497.
[14] Camargo FP, Sakamoto IK, Delforno TP, et al. Microbial and functional characterization of an allochthonous consortium applied to hydrogen production from Citrus Peel Waste in batch reactor in optimized conditions. J Environ Manage. 2021;291:112631), doi:10.1016/j.jenvman.2021.112631.
[15] Straub CT, Counts JA, Nguyen DMN, et al. Biotechnology of extremely thermophilic archaea. FEMS Microbiol Rev. 2018;42:543–578. doi:10.1093/femsre/fuy012.
[16] de Barros VG, Duda RM, Vantini J, et al. Improved methane production from sugarcane vinasse with filter cake in thermophilic UASB reactors, with predominance of Methanothermobacter and Methanosarcina archaea and Thermotogae bacteria. Bioresour Technol. 2017;244:371–381. doi:10.1016/j.biortech.2017.07.106.
[17] Ferraz-Júnior ADN, Koyama MH, de Araújo Júnior MM, et al. Thermophilic anaerobic digestion of raw sugarcane vinasse. Renew Energy. 2016;89:245–252. doi:10.1016/j.renene.2015.11.064.
[18] Fuess LT, Kiyuna LSM, Ferraz ADN, et al. Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. Appl Energy. 2017;189:480–491. doi:10.1016/j.apenergy.2016.12.071.

[19] Ramos LR, Silva EL. Thermophilic hydrogen and methane production from sugarcane stillage in two-stage anaerobic fluidized bed reactors. Int J Hydrogen Energy. 2020;45:5239–5251. doi:10.1016/j.ijhydene.2019.05.025.

[20] Santos SC, Rosa PRF, Sakamoto IK, et al. Hydrogen production from diluted and raw sugarcane vinasse under thermophilic anaerobic conditions. Int J Hydrogen Energy. 2014b;39:9599–9610. doi:10.1016/j.ijhydene.2014.04.104.

[21] Santos SC, Ferreira Rosa PR, Sakamoto IK, et al. Continuous thermophilic hydrogen production and microbial community analysis from anaerobic digestion of diluted sugar cane stillage. Int J Hydrogen Energy. 2014a;39:9000–9011. doi:10.1016/j.ijhydene.2014.03.241.

[22] Ferreira TB, Rego GC, Ramos LR, et al. HRT control as a strategy to enhance continuous hydrogen production from sugarcane juice under mesophilic and thermophilic conditions in AFBRs. Int J Hydrogen Energy. 2019;44:19719–19729. doi:10.1016/j.ijhydene.2019.06.050.

[23] Ramos LR, Silva EL. Continuous hydrogen production from cofermentation of sugarcane vinasse and cheese whey in a thermophilic anaerobic fluidized bed reactor. Int J Hydrogen Energy. 2018;43:13081–13089. doi:10.1016/j.ijhydene.2018.05.070.

[24] Ratti RP, Delforno TP, Sakamoto IK, et al. Thermophilic hydrogen production from sugarcane bagasse pretreated by steam explosion and alkaline delignification. Int J Hydrogen Energy. 2015;40:6296–6306. doi:10.1016/j.ijhydene.2015.03.067.

[25] Silva V, Ratti RP, Sakamoto IK, et al. Biotechnological products in batch reactors obtained from cellulose,: glucose and xylose using thermophilic anaerobic consortium. Renew Energy. 2018;125:537–545. doi:10.1016/j.renene.2018.02.124.

[26] Rodrigues CV, Nespeca MG, Sakamoto IK, et al. Bioconversion of crude glycerol from waste cooking oils into hydrogen by sub-tropical mixed and pure cultures. Int J Hydrogen Energy. 2019;4:144–154. doi:10.1016/j.ijhydene.2018.02.174.

[27] Rodrigues CV, Santana KO, Nespeca MG, et al. Crude glycerol by transesterification process from used cooking oils: characterization and potentialities on hydrogen bioproduction. Int J Hydrogen Energy. 2016;41:14641–14651. doi:10.1016/j.ijhydene.2016.06.209.

[28] Lopes HJS, Ramos LR, de Menezes CA, et al. Simultaneous hydrogen and ethanol production in a thermophilic AFBR: a comparative approach between cellulosic hydrolysate single fermentation and the fermentation of glucose and xylose as co-substrates. Cellulose. 2020;27:2599–2612. doi:10.1007/s10570-020-03000-4.

[29] Ferreira TB, Rego GC, Ramos LR, et al. Selection of metabolic pathways for continuous hydrogen production under thermophilic and mesophilic temperature conditions in anaerobic fluidized bed reactors. Int J Hydrogen Energy. 2018;43:18908–18917. doi:10.1016/j.ijhydene.2018.08.177.

[30] Klein BC, de Mesquita Sampaio IL, Mantelatto PE, et al. Beyond ethanol, sugar, and electricity: a critical review of product diversification in Brazilian sugarcane mills. Biofuels Bioprod Bioref. 2019;13:809–821. doi:10.1002/bbb.1969.

[31] Souza ME, Fuzaro G, Polegato AR. Thermophilic anaerobic digestion of vinasse in pilot plant UASB reactor. Water Sci Technol. 1992;25:213–222. doi:10.2166/wst.1992.0153.

[32] Ramos LR, Silva EL. Continuous hydrogen production from agricultural wastewaters at thermophilic and hyperthermophilic temperatures. Appl. Biochem. Biotechnol. 2017;182:846–869. doi:10.1007/s12010-016-2366-3.

[33] Green MR, Sambrook J. Molecular cloning: a laboratory manual. 4th ed. New York: Cold Spring Harbor Laboratory Press; 2012. doi:10.3724/SP.J.1141.2012.01075

[34] Chen S, Zhou Y, Chen Y, et al. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–i890. doi:10.1093/bioinformatics/bty560.

[35] Nurk S, Meleshko D, Korobeynikov A, et al. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 2017;27:824–834. doi:10.1101/gr.213959.116.

[36] Gurevich A, Saveliev V, Vyahhi N, et al. QUAST: quality assessment tool for genome assemblies. Bioinformatics. 2013;29:1072–1075. doi:10.1093/bioinformatics/btt086.

[37] Hyatt D, Chen GL, LoCascio PF, et al. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010;11; doi:10.1186/1471-2105-11-119.

[38] Menzel P, Ng KL, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun. 2016;7:1–9. doi:10.1038/ncomms11257.

[39] Kang DD, Froula J, Egan R, et al. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. Peer J. 2015;3:e1165, doi:10.7717/peerj.1165.

[40] Parks DH, Imelfort M, Skennerton CT, et al. Checkm: assessing the quality of microbial genomes recovered from isolates: single cells, and metagenomes. Genome Res. 2015;25:1043–1055. doi:10.1101/gr.186072.114.

[41] Bowers RM, Kyrpides NC, Stepanauskas R, et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nat Biotechnol. 2017;35:725–731. doi:10.1038/nbt.3893.

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

[43] Yin Y, Mao X, Yang J, et al. DbCAN: a web resource for automated carbohydrate-active enzyme annotation. Nucleic Acids Res. 2012;40:445–451. doi:10.1093/nar/gks479.

[44] Wickham H. ggplot2: elegant graphics for data analysis. 1st ed. New York (NY): Springer. doi:10.1007/978-0-387-98141-3

[45] Ben Hania W, Godbane R, Postec A, et al. Defluviitoga tunisiensis gen. nov.: sp. nov., a thermophilic bacterium isolated from a mesothermic and anaerobic whey digester. Int J Syst Evol Microbiol 2012;62:1377–1382. doi:10.1099/ijs.0.033720-0.

[46] Katuri KP, Kamireddy S, Kavanagh P, et al. Electroactive biofilms on surface functionalized anodes: the anode respiring behavior of a novel electroactive bacterium: desulfuromonas acetexigens. Water Res. 2020;185:116284, doi:10.1016/j.watres.2020.116284.

[47] Bae H-S, Im W-T, Lee S-T. Lysobacter concretionis sp. nov.: isolated from anaerobic granules in an upflow anaerobic

sludge blanket reactor. Int J Syst Evol Microbiol. 2005;55:1155–1161. doi:10.1099/ijs.0.63399-0.
[48] Sekiguchi Y, Muramatsu M, Imachi H, et al. Thermodesulfovibrio aggregans sp. nov. and Thermodesulfovibrio thiophilus sp. nov., anaerobic: thermophilic, sulfate-reducing bacteria isolated from thermophilic methanogenic sludge, and emended description of the genus Thermodesulfovibrio. Int J Syst Evol Microbiol. 2008;58:2541–2548. doi:10.1099/ijs.0.2008/000893-0.
[49] Yoav S, Barak Y, Shamshoum M, et al. How does cellulosome composition influence deconstruction of lignocellulosic substrates in Clostridium (Ruminiclostridium) thermocellum DSM 1313? Biotechnol Biofuels. 2017;10:222, doi:10.1186/s13068-017-0909-7.
[50] Tsavkelova E, Prokudina L, Egorova M, et al. The structure of the anaerobic thermophilic microbial community for the bioconversion of the cellulose-containing substrates into biogas. Process Biochem. 2018;66:183–196. doi:10.1016/j.procbio.2017.12.006.
[51] Yang G, Yin Y, Wang J. Microbial community diversity during fermentative hydrogen production inoculating various pretreated cultures. Int J Hydrogen Energy. 2019;44:13147–13156. doi:10.1016/j.ijhydene.2019.03.216.
[52] Silva Rabelo CAB, Okino CH, Sakamoto IK, et al. Isolation of Paraclostridium CR4 from sugarcane bagasse and its evaluation in the bioconversion of lignocellulosic feedstock into hydrogen by monitoring cellulase gene expression. Sci Total Environ. 2020;715:136868, doi:10.1016/j.scitotenv.2020.136868.
[53] Zhang L, Chung J, Jiang Q, et al. Characteristics of rumen microorganisms involved in anaerobic degradation of cellulose at various pH values. RSC Adv. 2017;7:40303–40310. doi:10.1039/C7RA06588D.
[54] Xiong Z, Hussain A, Lee J, et al. Food waste fermentation in a leach bed reactor: reactor performance, and microbial ecology and dynamics. Bioresour Technol. 2019;274:153–161. doi:10.1016/j.biortech.2018.11.066.
[55] Delforno TP, Macedo TZ, Midoux C, et al. Comparative metatranscriptomic analysis of anaerobic digesters treating anionic surfactant contaminated wastewater. Sci Total Environ. 2019;649:482–494. doi:10.1016/j.scitotenv.2018.08.328.
[56] Camargo FP, Sakamoto IK, Duarte ICS, et al. Influence of alkaline peroxide assisted and hydrothermal pretreatment on biodegradability and bio-hydrogen formation from citrus peel waste. Int J Hydrogen Energy. 2019;44:22888–22903. doi:10.1016/j.ijhydene.2019.07.011.
[57] Melo-Nascimento A, Treumann C, Neves C, et al. Functional characterization of ligninolytic Klebsiella spp. strains associated with soil and freshwater. Arch Microbiol. 2018;200:1267–1278. doi:10.1007/s00203-018-1532-0.
[58] Janusz G, Pawlik A, Sulej J, et al. Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol Rev. 2017;41:941–962. doi:10.1093/femsre/fux049.
[59] Li C, Chen C, Wu X, et al. Recent advancement in lignin biorefinery: with special focus on enzymatic degradation and valorization. Bioresour Technol. 2019;291:121898, doi:10.1016/j.biortech.2019.121898.
[60] Akita H, Kimura Z, Yusoff MZM, et al. Identification and characterization of Burkholderia multivorans CCA53. BMC Res Notes. 2017;10:249, doi:10.1186/s13104-017-2565-1.
[61] Lu Z, Imlay JA. When anaerobes encounter oxygen: mechanisms of oxygen toxicity: tolerance and defence. Nat Rev Microbiol. 2021. doi:10.1038/s41579-021-00583-y
[62] Duan Y, Awasthi SK, Liu T, et al. Positive impact of biochar alone and combined with bacterial consortium amendment on improvement of bacterial community during cow manure composting. Bioresour Technol. 2019;280:79–87. doi:10.1016/j.biortech.2019.02.026.
[63] Tian J, Pourcher A, Bouchez T, et al. Occurrence of lignin degradation genotypes and phenotypes among prokaryotes. Appl Microbiol Biotechnol. 2014: 1–18. doi:10.1007/s00253-014-6142-4.
[64] Xia Y, Wang Y, Fang HHP, et al. Thermophilic microbial cellulose decomposition and methanogenesis pathways recharacterized by metatranscriptomic and metagenomic analysis. Sci Rep. 2014;4:1–9. doi:10.1038/srep06708.
[65] Villa TG, Veiga-Crespo P. Enzybiotics: antibiotic enzymes as drugs and therapeutics. Enzybiot: Antibiot Enzym Drugs Therapeut. 2009. doi:10.1002/9780470570548
[66] Mawson AJ, Earle RL, Larsen VF. Degradation of acetic and propionic acids in the methane fermentation. Water Res. 1991;25:1549–1554. doi:10.1016/0043-1354(91)90187-U.
[67] Mazareli RCdS, Sakamoto IK, Silva EL, et al. Bacillus sp. isolated from banana waste and analysis of metabolic pathways in acidogenic systems in hydrogen production. J Environ Manage. 2019;247:178–186. doi:10.1016/j.jenvman.2019.06.040.
[68] Neshat SA, Mohammadi M, Najafpour GD, et al. Anaerobic co-digestion of animal manures and lignocellulosic residues as a potent approach for sustainable biogas production. Renew Sustain Energy Rev. 2017;79:308–322. doi:10.1016/j.rser.2017.05.137.
[69] Nakayama T, Yonekura SI, Yonei S, et al. Escherichia coli pyruvate: flavodoxin oxidoreductase: YdbK – regulation of expression and biological roles in protection against oxidative stress. Genes Genet. Syst. 2013;88:175–188. doi:10.1266/ggs.88.175.
[70] Macedo TZ, de Souza Dornelles H, do Valle Marques AL, et al. The influence of upflow velocity and hydraulic retention time changes on taxonomic and functional characterization in fluidized bed reactor treating commercial laundry wastewater in co-digestion with domestic sewage. Biodegradation. 2020;31:73–89. doi:10.1007/s10532-020-09895-x.
[71] Kaster A-K, Moll J, Parey K, et al. Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. Proc Natl Acad Sci. 2011;108:2981–2986. doi:10.1073/pnas.1016761108.
[72] Matschiavelli N, Rother M. Role of a putative tungsten-dependent formylmethanofuran dehydrogenase in Methanosarcina acetivorans. Arch Microbiol. 2015;197:379–388. doi:10.1007/s00203-014-1070-3.
[73] Gräwert T, Hohmann H-P, Kindermann M, et al. Inhibition of Methyl-CoM reductase from methanobrevibacter ruminantium by 2-Bromoethanesulfonate. J Agric Food Chem. 2014;62:12487–12490. doi:10.1021/jf505056g.
[74] Wagner T, Kahnt J, Ermler U, et al. Didehydroaspartate modification in methyl-coenzyme M reductase catalyzing methane formation. Angew Chemie Int Ed. 2016;55:10630–10633. doi:10.1002/anie.201603882.

[75] Wrede C, Walbaum U, Ducki A, et al. Localization of methyl-coenzyme M reductase as metabolic marker for diverse methanogenic archaea. Archaea. 2013;2013:1–7. doi:10.1155/2013/920241.

[76] Ratti RP, Delforno TP, Okada DY, et al. Bacterial communities in thermophilic H2-producing reactors investigated using 16S rRNA 454 pyrosequencing. Microbiol Res. 2015;173:10–17. doi:10.1016/j.micres.2015.01.010.