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Composition and ecology of bacterial and archaeal communities in anaerobic reactor fed with residual glycerol

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ABSTRACT

Glycerol, the main residue of biodiesel production, can be used to produce organic acids and energy through anaerobic digestion. This study aimed to assess microbial structure, diversity, productivity, and stability and the influence of these parameters on the performance of an anaerobic reactor. The experimental setup consisted of an upflow anaerobic sludge blanket (UASB) reactor fed residual glycerol and nutrients. The organic loading rate (OLR) was gradually increased through five stages, and sludge samples were collected at each, followed by DNA extraction and PCR denaturing gradient gel electrophoresis (PCR-DGGE). The resulting bands were excised, amplified, and purified. The results showed increased bacterial diversity and richness from the inoculum (Rr 38.72 and H 2.32) and along stages I and II, reaching the highest populational parameters (Rr 194.06 and H 3.32). The following stages promote decreases in richness and diversity, achieving the lowest populational parameters on this study (Rr 11.53 and H 2.04). Biogas production increased along with functional organization due to the specialization of the bacterial community and a decrease in the methanogenic population, both promoted by the increase in OLR.

1. Introduction

Anaerobic digestion is one of the most widely used bioconversion technologies, since it can be used to convert residues into added value products through low cost processes, low energy consumption and low footprint equipment [1,2]. Glycerol is a byproduct of the transesterification of oils used for biodiesel production [3]. During this process, for every 10 kg of biodiesel [4], 1 kg glycerol is generated. Glycerol can be used as a substrate for anaerobic digestion in bioreactors, resulting in various products, such as 1,3-propanediol [5], ethanol [6], propionic [7], butyric, acetic [8], caproic [9], and succinic acid [10], butanol [11], dihydroxyacetone [12], hydrogen [13], and methane [14].

Glycerol is a highly biodegradable substrate [15]. Stoichiometrically, it is possible to produce an average of 1 mol H_2 per mol glycerol [16] and some authors have obtained even more [17,18]. In a simulation, Viana [19] observed that a two-stage system (hydrogenogenic followed by a methanogenic reactor) can produce up to 233 MJ per m^3 of reactor per day. Therefore, besides by-products, glycerol fermentation could provide a significant amount of energy.

During anaerobic digestion of biodegradable material, hydrogen and methane can be produced by microorganisms of the domains Bacteria and Archaea in competing pathways [20]. Therefore, to select the optimal operational parameters and improve H_2 or CH_4 yield in anaerobic reactors, it is necessary to understand the microbial ecology of the biological processes involved [21]. Significant changes in one or more operational parameters affects the anaerobic process and can produce a disturbance in community structure and dominance between established species [22]. During this transient period, microorganisms will readapt, some inoculated species may disappear and some previously undetected species can

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outgrow others [23,24].

Mixed bacterial populations have complex ecological interactions, such as cooperation or competition, as well as temporal effects during the different stages of a reactor. Therefore, to favour the selection of a productive and stable community, it is necessary to know its ecological functions, population characteristics and reactor efficiency of both Bacteria and Archaea domains [25].

Anaerobic bioreactors act as controlled ecosystems, allowing accurate evaluation of the community structure and diversity and the succession processes that modify these communities over time, since ecological interactions can directly affect stability and/or function, as seen by Koskinen et al. [26]. These authors monitored bacterial community inside a dark fermentation fluidized-bed bioreactor with PCR-DGGE in order to verify the cause of the instability in hydrogen production. The authors concluded that the instability occurred due to rapid enrichment, which changed the microbial community structure and its metabolism from acetate–butyrate to acetate–propionate, thus decreasing hydrogen production. The use of molecular biological techniques is essential for assessing the microbial diversity, ecology, and dynamics at different levels in bioreactors.

Therefore, this study aimed to assess: (i) ecological relationships (Shannon diversity index, range-weighted richness, and species specialization and/or dominance); (ii) the community structure through populational shifts during the operation, and (iii) the relationship between the community structure, the ecological processes in the production yield, and the volatile fatty acids (VFAs) produced during the operation of the reactors. This information could enable the selection of a microbial consortium and operational conditions with greater efficiency, improving the performance of anaerobic reactors.

2. Materials and methods

2.1. Substrate and inoculum

The reactor was fed with residual glycerol from the transesterification process during biodiesel production. Bovine tallow (44%) and soy (56%) were the feedstock for biodiesel production. The characteristics of the glycerol were: 1% non-glycerol organic matter, pH 5.5, 81.5% purity, 4.8% ash, 12.71% moisture, 0.03% methanol, 5.3% NaCl, and density 1255.9 kg m^{-3}. The glycerol presented 1374 mg chemical oxygen demand (COD) per liter of organic matter concentration and was diluted to reach the desired COD for each stage of operation. The dilution of the glycerol in the influent was gradually reduced, until it reached the lowest amount of water possible for the operation to be feasible. In every operational stage, a nutrient solution was added, adapted from Lin & Lay (2005) [27], in the following concentrations (mg L^{-1}): 40.0 $MgCl_2 \cdot 6H_2O$, 5.0 $CoCl_2 \cdot 6H_2O$, 0.1 $CaCl_2 \cdot 2H_2O$, 2.5 $NiCl_2 \cdot 6H_2O$, 10.6 $MnCl_2 \cdot 4H_2O$, 1.1 KCl, 107.5 $NH_4H_2PO_4$, 0.1 $ZnCl_2$, 5.0 $FeSO_4 \cdot 7H_2O$, 0.3 $MnSO_4 \cdot H_2O$, 5.0 $CuSO_4 \cdot 5H_2O$, and 51.1 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

The genus Clostridium is a major H_2 producer found in the microbial community of bioreactors, and especially prevalent in sewage sludge and wastewater treatment reactors sludge [28]. Therefore, the inoculum consisted of a mixed sludge obtained from a full-scale upflow anaerobic sludge blanket (UASB) reactor used for treatment of municipal wastewater. The inoculum did not undergo any form of pre-treatment; therefore, the operational conditions were the only factors to induce or prevent shifts in microbial diversity.

2.2. Bioreactor setup

The bioreactor was a lab-scale UASB reactor, with a working volume of 14.65 L. Eight taps were installed vertically along the reactor to allow sludge sampling. The pH was controlled automatically using a dosing pump with a 0.5% (v/v) NaOH solution. The values of COD and total volatile solids were determined using titration and gravimetry, respectively [29]. The biogas flow rate was monitored by a drum-type gas meter, and the gas composition was determined by gas chromatography (C2V-200 micro GC; Thermo Fisher Scientific, Waltham, MA). The concentrations of VFAs were determined by HPLC, under the following conditions: Agilent Zorbax C18 column (150 × 4.6 mm) maintained at 25 °C; ultraviolet/visible detector Varian UV 325 Polaris 215 nm, with acetonitrile/water (3:7) with 0.01% sulfuric acid as the mobile phase, at flow of 0.4 mL min^{-1}. The injected sample volume was 20 μL. The samples were previously filtered on the cellulose acetate membrane ME25 with 0.45 μm pores.

2.3. Operation strategy

The reactor was operated in five stages, with OLRs being increased when the reactor was considered to be adapted to the substrate (from 14.4 to 54.5 kg COD m^{-3} d^{-1}), i.e., when the theoretical OLR for each stage was achieved and the pH, biogas volume, and COD were within the planned range. To assess the acidogenic conditions and verify the decrease in methane production, the CH_4 and H_2 levels were measured.

2.4. DNA extraction and PCR amplification of 16S rRNA

DNA was extracted using the Fast extraction® DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) per the manufacturer's protocol, with the following modifications: centrifugation at 13500×*g* (13900 rpm) for 20 min, 30 s of cell disruption (Mini-BeadBeater; BioSpec, Bartlesville, OK), incubation for 1 min on ice, 20 s of disruption, and incubation of samples for 1 h to allow DNA adsorption to the silica matrix. The extracted DNA was identified and stored at −18 °C. The extracted DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). After quantification, the samples were diluted to appropriate concentrations for PCR.

PCR amplification of DNA was performed for both bacteria and archaea, using primers containing 40-bp GC-clamps for further analysis by DGGE. The 16S rRNA gene hypervariable regions V2–V3 for archaea were amplified using the primers 0515R-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC-3′) and 0109F-T (5′-ACT GCT CAG TAA CAC GT-3′) (Sigma Aldrich, St. Louis, MO) [30,31]. For bacteria, the regions V6–V8 were amplified using the universal primers 1401R (5′-CGG TGT GTA CAA GAC CC-3′) and 0968F-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) (Sigma Aldrich) [32].

The PCR mix contained 10 μL reaction buffer (5 ×), 5 μL $MgCl_2$ (25 mM), 0.25 μL Taq polymerase (5 U/μL) (Promega, USA), 1 μL deoxynucleotide triphosphates (10 mM), 1 μL extracted DNA, 1 μL primers (10 μM), and nuclease-free water (Promega, Madison, WI) up to a final volume of 50 μL. The PCR was carried out in a T100 Thermal Cycler iCycler (Bio-Rad Laboratories, Hercules, CA). For bacteria, the following conditions were applied: initial denaturation at 95 °C for 2 min; 31 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 1 min; and final elongation at 72 °C for 6 min. For archaea, the following conditions were applied: initial denaturation at 95 °C for 2 min, 31 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s, and elongation at 72 °C for 90 s; and final elongation at 72 °C for 6 min. The size of the PCR products was estimated using the 1000 bp Hyperladder II DNA marker (Bioline, London, UK) in 1.8% agarose

gel electrophoresis and using SYBR Green I staining (Sigma-Aldrich). The PCR products were verified in 1.7% (w/v) agarose gel electrophoresis, using a 1 kb DNA Ladder (Promega) as the molecular weight marker. The gels were stained with SYBR Green I stain for 40 min, and the result was analyzed in a Hood II universal transilluminator (Bio-Rad Laboratories).

2.5. Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis was performed in 8% polyacrylamide gel with a urea/formamide denaturing gradient of 42–67% and 25–50% for bacteria and archaea, respectively [33]. The denaturing gradient for bacteria followed the standard range observed in the literature. The denaturing gradient for archaea was adapted from the standard range to better express the bands in the gel. A D-Code Universal Mutation Detection System (Bio-Rad Laboratories), with 0.5 × TAE buffer at 60 °C, at 85 V, for 16 h, and at 60 °C, at 65 V, for 18 h, was used for bacterial and archaeal PCR products, respectively. After the DGGE, the gels were stained with SYBR Green I (1:10.000 dilution) for 1 h and subsequently examined using a UV transilluminator (Bio-Rad Laboratories). Individual bands were excised from the gels with a sterile blade, resuspended in 50 μL ultrapure water, and kept at 60 °C for 1 h to allow the extraction of the PCR products from the gel. Excised PCR products were purified with the GenElute DNA Purification Kit (Sigma-Aldrich). The excised bands were chosen based on presence, intensity, and frequency in the gel.

2.6. Statistical analysis

The DGGE patterns obtained were normalized and analyzed with Bionumerics software v. 6.1 (Applied Maths, Sint-Martens-Latem, Belgium) to score the band pattern. Bands with more than 1% intensity after background analysis were considered. The Jaccard coefficient was used to determine profile similarities. Cluster analyses were constructed using the UPGMA algorithm (Bionumerics software). Three ecological parameters were calculated, as described by Marzorati et al. [34]: the range-weighted richness (Rr) was calculated as the total number of bands, the Shannon diversity index (H) was calculated by the distribution of bands, and the functional organization (Fo) was calculated by measuring the normalized area between the Pareto–Lorenz curve and the perfect evenness line. Rr reflects the carrying capacity of a system; H reflects the distribution of species in a given environment; Fo represents the relations in the functional distribution (structure vs. functionality) of the microbial community [34,35].

2.7. Sequencing and phylogenetic analysis

Sequencing was performed by the Sanger method [36] at the Molecular Biology Laboratory, Institute of Marine Science. The taxonomic relationships of the sequences were determined using the RDP classification tool [37], with 90% confidence level. The obtained sequences (accession numbers upon acceptance) were compared with GenBank through the BLAST search tool (National Centre for Biotechnology Information, NCBI) [38].

3. Results and discussion

3.1. Hydrogen production and yield

For higher acidogenesis, methanogenic organisms must be inhibited. In our study, the inhibition occurred due to the increase in OLR, which also increased biogas and hydrogen production and yield, as well as acetic and valeric acid concentration. Furthermore, methane production gradually decreased during the operation. The biogas detected in the reactors contained hydrogen, methane, and carbon dioxide. The operational stages, time, OLR, pH, hydrogen partial pressure, biogas production, H_2 and CH_4 percentages in biogas, and H_2 production and yield of the reactor from stages I to V are presented in Table 1 and Fig. 1.

Originally, the operation was set to reach the higher OLR possible. However, when the OLR increase above 60 kg COD $m^{-3}.d^{-1}$, there were events of sludge loss with the effluent, indicating operational instability. Therefore, the last stable stage was Stage V, when the OLR reached 54.5 kg COD m^{-3} d^{-1}. At this stage, the bioreactor reached its highest average yield (0.076–0.096 mol H_2 mol^{-1} glycerol). The highest biogas and hydrogen production also occurred at this stage (20.00 l biogas d^{-1} and 13.30 L H_2 d^{-1}), indicating that this stage had the most specialized microbiota. The yield was 27% higher than that observed by Vlassis et al. [39], since these authors produced only 0.070 mol H_2 mol^{-1} glycerol, with the same type of inoculum, despite the fact that this was considered to be below average [18,40,41]. Furthermore, at stage V (at an OLR of 54.5 kg COD m^{-3} d^{-1}), the reactor presented its highest instability period, including sludge flotation.

Although the H_2 yield was higher at stage V, it corresponded to only 8% of the maximum theoretical yield of 1 mol H_2 mol^{-1} glycerol [42]. Some authors obtained yields higher than the maximum theoretical yield mentioned above, since it is stoichiometrically possible to produce up to 3 mol H_2 mol^{-1} glycerol [4,18]. However, if glycerol is the only carbon source in the reactor, the maximum theoretical yield of H_2 is reduced to 1 mol H_2 mol^{-1} glycerol [41–43].

The reduction observed in stage V could be caused by a deviation in the metabolic path due to operational conditions, like the one that produces 1,3-propanediol. According to Seifert et al. [44], this can be caused by glycerol concentration exceeding 10 g glycerol L^{-1}. Furthermore, this concurrent route not only reduces the H_2 yield but also consumes 1 mol of H_2 [41]. In order to produce H_2, the metabolic route must be via pyruvate-formate, which produces acetic acid, butyric acid, and ethanol [11]. If glycerol is used as a substrate to produce H_2 through butyric fermentation, the main fermentation products are 1,3-propanediol, butyric acid, lactic acid, and ethanol [44].

During stages IV and V, the high partial pressure of H_2 (pH_2 of 0.3 atm) caused the inhibition of hydrogenogenic bacteria. This increase in partial pressure changed NADH and ferredoxin mechanisms, causing a decrease in H_2 production [45]. Due to these changes, i.e., due to the NADH/NAD^+ ratio, the microbiota was induced to change its metabolic path from butyric acid to propionic acid, since the propionic acid route produces more NAD^+ than the butyric route [46].

Fermentation products, like propionic acid, which is one of the main products and the most abundantly produced acid (32–46%) in this experiment, can have a negative impact on H_2 yield. A higher concentration of propionic acid can inhibit acidogenic and H_2-producing bacteria, further reducing the yield. The fermentation products produced during the operation are presented in Fig. 2.

3.2. Microbial community characterization

Ecological interactions between microorganisms inside a reactor can directly affect the stability and/or function of the system. The diversity inside a reactor can constantly shift due to population changes caused by competition for resources. Furthermore, allochthonous and indigenous microorganisms could compete with the acidogenic population for available resources, directly affecting production and yield [47–49]. The increase in OLR produced different effects on the microbial community during the

Table 1
Operational parameters of the UASB reactor fed with residual glycerol at each stage.

Stage	Time[a]	OLR[b]	pH	pH_2[c]	Biogas[d]	CH_4 (%)[e]	H_2 (%)[e]	H_2 Production[f]	H_2 yield[g]
I	24	14.4 ± 3.9	5.7 ± 0.5	0.04	2.43 ± 1.6	12.49	17.62	0.30 ± 0.4	0.005 ± 0.01
II	45	26.5 ± 5.3	5.3 ± 0.2	0.11	6.81 ± 2.1	25.03	20.13	1.39 ± 0.4	0.017 ± 0.01
III	59	39.2 ± 5.5	5.4 ± 0.2	0.19	8.82 ± 1.5	29.74	19.24	1.70 ± 0.3	0.015 ± 0.00
IV	73	48.6 ± 1.6	5.2 ± 0.1	0.63	13.12 ± 1.9	13.45	67.92	6.84 ± 2.4	0.049 ± 0.01
V	84	54.5 ± 14.7	5.2 ± 0.1	0.73	17.11 ± 2.1	0.0	85.17	13.30 ± 2.6	0.076 ± 0.02

[a] Accumulated operating time (days).
[b] Organic loading rate (KgCOD.m^{-3}. d^{-1}).
[c] H_2 partial pressure (atm).
[d] Biogas production (L.d^{-1}).
[e] Gas concentration in biogas.
[f] H_2 Production (L.d^{-1}).
[g] H_2 yield (mol H_2. mol^{-1}glycerol). Values after ± stand for standard deviation.

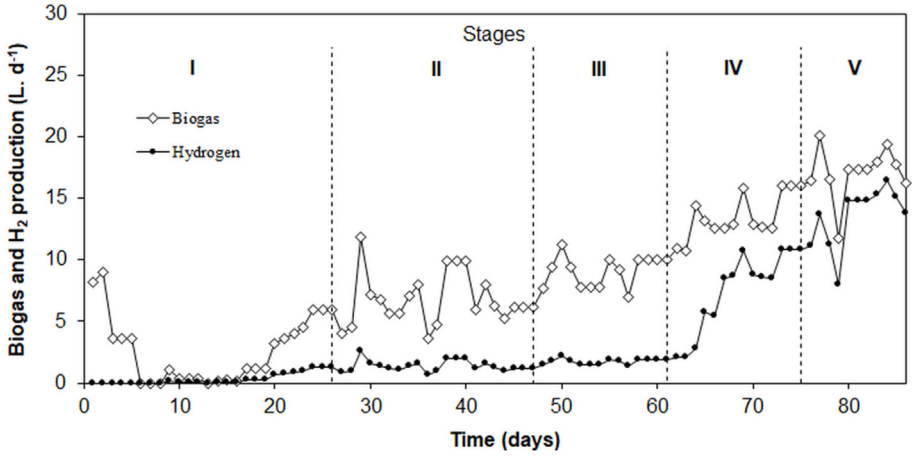


Fig. 1. Daily production of biogas and hydrogen.

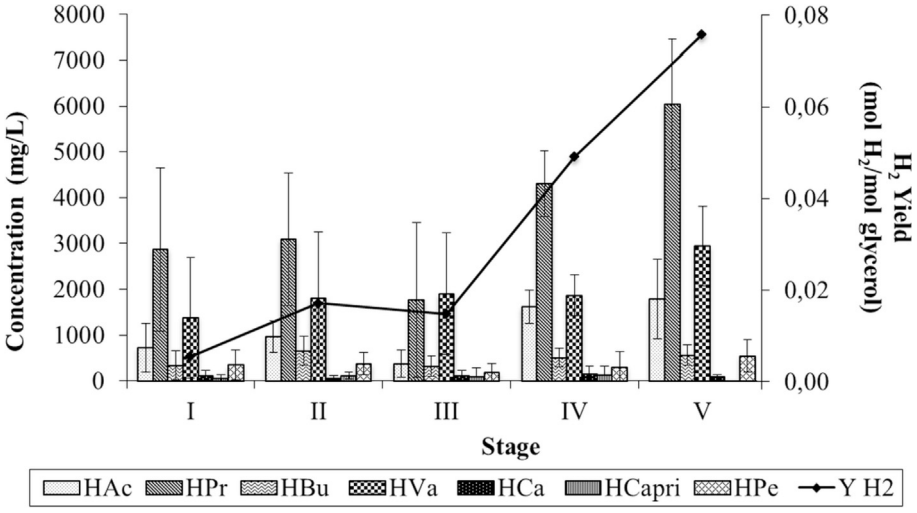
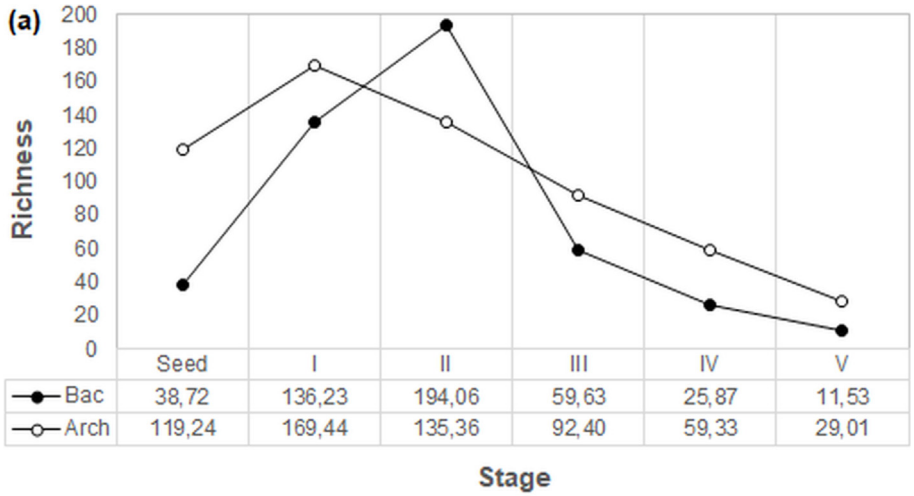
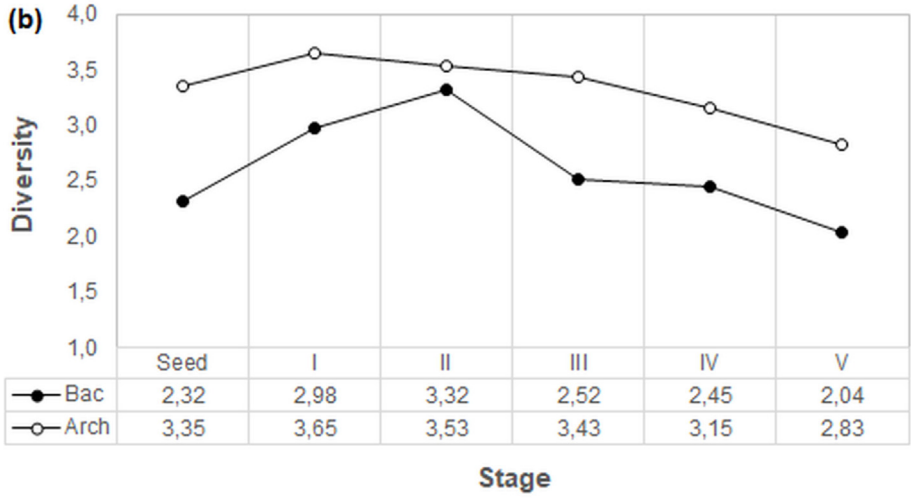


Fig. 2. Average concentration of the main acids produced during each stage of operation of the UASB reactor fed with residual glycerol.

operation, according to the ecological parameters presented in Fig. 3.

The experimental parameters on the reactor operation could set up different niche and microbial community structure, thus it is expected that when hydrogen producing bacteria became dominant populations, the hydrogen production efficiency would be high. The production and yields were related to influent COD and microbial interactions. The rapid increase in the Rr and H from the inoculum (Rr 38.72 and H 2.32) to stages I (Rr 136.23 and H 2.98) and II (Rr 194.06 and H 3.32) indicates that these loading rates induce the diversification and stabilization of the community, since OLRs between 20 and 30 kgCOD m^{-3}. d^{-1} promote a more diverse community [36–38]. Despite these stages not corresponding to higher productivity (Fo 54.73 and H_2 production of 1.70), they did correspond to highest diversity and richness. Elevated richness and diversity promote increased stability in an ecosystem [50]; thus,





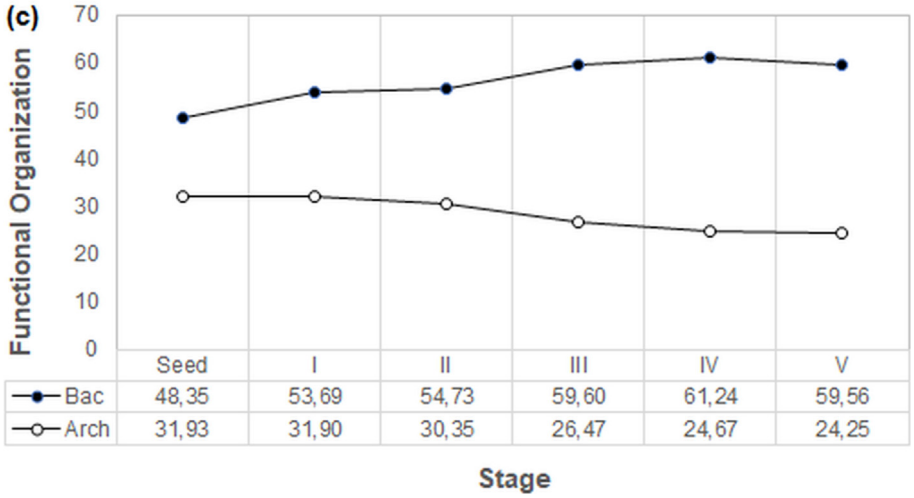


Fig. 3. Ecological parameters of the reactor along the OLRs applied in the operation. Bacterial (Bac - ◆) and Archaeal (Arch - ■) richness (Rr), diversity (H) and functional organization (Fo).

these stages are the most stable stages of the operation.

Increases in OLR cause an increase the amount of organic matter in the environment, favouring acidogenesis and, therefore, a drop in pH, with consequent selective pressures [37] for H_2-producing bacteria and methanogen archaea, thus promoting changes in microbial community structure and ecological niches. However, if the organic matter increases even further, it could cause re-sporulation of H_2-producing bacteria, due to substrate excess, thus decreasing H_2 production [41].

Mariakakis et al. [47] similarly found that the diversity decreased when the OLR increased above the range of 20–30 kg COD m^{-3} d^{-1}. The stages IV and V presented a decrease in richness (25.87 and 11.53, respectively) and diversity (2.45 and 2.04, respectively), which indicates that these species were probably sensitive to substrate concentration; thus, an increased OLR resulted in their inhibition. Furthermore, some authors found that a significant increase in bacterial richness could adversely influence reactor performance [26,51,52], probably due to competition. Our results are similar to these, since the stages with the lowest richness presented the highest levels of productivity (Fig. 3).

The Fo gradually increased from the inoculum (48.35) to stages I (53.69), II (54.73), III (59.36), and IV (61.24), slightly decreasing in stage V (59.56). Stage V presented the highest values of H_2 production (13.30), yield (0.076), and concentration (85.17%), as well as the second highest Fo for bacteria. According to Marzorati et al. [34], the higher the Fo, the more specialized a community becomes, wherein a small number of species becomes dominant. This is considered a highly functionally organized community, which, however, lacks resistance to external changes because the low diversity and equitability promote lower functional redundancy and the recovery of less available species after the disturbance [34].

The decrease in Rr and H and the increase in Fo indicates that the community is becoming more specialized towards the use of the substrate, which can be confirmed by the increased production of biogas, H_2, acetic, and valeric acid. Furthermore, archaeal species were progressively inhibited, until no CH_4 was detected at stage V; thus, methanogenic activity could only be completely inhibited at the final stage of operation.

The Rr and H of the archaeal community increased significantly from the inoculum (Rr 119.24 and H 3.35) to stage I (Rr 169.44 and H 3.65) and decreased afterwards. The Fo gradually decreased from the inoculum (31.93) up to stage V (24.25). Methane production increased at stages II and III, when it peaked (29.74), despite the fact that the richness and diversity of archaeal species were both significantly reduced at this stage (Rr 92.40 and H 3.43). This occurred because of the average functional organization (26.47), which implies that these species are probably resistant and stable enough to endure the constantly increasing OLR.

These findings corresponds to what has been reported about *Methanosarcina* sp.: it has high growth rates and is resistant to changes caused by overloading when compared to other methanogens [53–55], being able to grow at low retention times (i.e. as low as 4 d) [53] and high OLR [56]. Despite the general information that methanogens can proliferate in acidic environments, the genus Methanosarcina is known for having a high resistance to low pH, capable of having methanogenic activity in pH 5.5 or even lower [22,57].

Although the rate of methane production decreased to undetectable, archaea were still present. This is possibly due to the inoculum being rich in *Methanosarcina* sp., which could have started growing faster than other archaea and dominated the methanogenic community, thus promoting enhanced stability [53,58]. Some authors suggest that certain substances present in crude glycerol, such as palmitic acid (between 1.5 and 3.0 g L^{-1}), from which significantly more CH_4 may be produced than from pure glycerol, may increase methane production [43,59].

Although the increases in OLR select hydrogenogenic bacteria over methanogenic archaea, the latter were present in high loads, which indicates that the increase in OLR by itself is sufficient to inhibit methanogenic activity, but not to be completely free of the remaining resistant archaeal species, especially from the genus Methanosarcina.

3.3. Microbial community identification

Sequencing was performed to identify the main species in the reactor. The taxonomic relationships of the sequences were determined with 80% confidence level. The sequences were also compared with GenBank through BLAST [38]. Table 2 presents the main species, which had over 90% similarity. Fig. 4 presents the DGGE patterns of both archaea and bacteria. The excised bands were enumerated based on their sequence in Table 2, from 1 to 4 for archaea and 1 to 8 for bacteria. Fig. 5 presents the dendrograms of both domains.

The bacterial sequences were mainly related to *Firmicutes* and *Proteobacteria*, while the archaeal domain were mainly related to Euryarchaeota, especially *Methanosarcina*, *Methanosarcinales*, and *Methanobrevibacter*. *Methanosarcina* and *Methanosarcinales* include methane-producing prokaryotes that utilize carbon dioxide [60]. Another study found these archaea, along with *Methanosaeta*, in granular sludge of UASB reactors used to treat brewery wastes [61].

Three main bacterial phyla, namely *Actinobacteria*, *Firmicutes*, and Proteobacteria, and two archaeal phyla, namely *Euryarchaeota* and *Methanosarcinales*, were detected using sequencing.

Firmicutes comprise widely studied and dominant bacteria that are capable of tolerating harsh conditions due to their spore-forming ability and are able to colonize many types of environments [62]. One of the identified species belonging to the phylum *Firmicutes* is *Clostridium sticklandii*, which produces organic acids such as acetate, butyrate, and propionate via the Stickland reaction [63]. Kim et al. [18]suggest that this species can be related to protein digestion, especially from the substrate or cellular remains.

The dendrograms and patterns indicate that diversity and richness were more similar between stages I to III than between the

Table 2
Main species found in the UASB reactor fed with residual glycerol. Over 90% similarity.

Bacteria					Archaea				
Band	Closest Relative	% G + C	% identity	Phylum	Band	Closest Relative	% G + C	% identity	Phylum
B1	*Desulfovibrio sp.*	66.5	100%	Proteobacteria	A1	Uncultured *Methanosarcina sp.*	42.7	99%	Methanosarcinales
B2	*Klebsiella pneumoniae*	57.5	99%'	Proteobacteria	A2	Uncultured *Methanobrevibacter sp.*	31.3	99%	Euryarchaeota
B3	Uncultured *Firmicutes* bacterium	55	99%	Firmicutes	A3	Uncultured *euryarchaeote*	–	92%	Euryarchaeota
B4	*Acidaminococcus sp.*	50.2	99%	Firmicutes	A4	Uncultured *Methanosarcinales*	–	91%	Methanosarcinales
B5	Uncultured *Ruminococcus sp.*	41.3	98%	Firmicutes					
B6	Uncultured *Atopobium sp.*	35	99%	Actinobacteria					
B7	*Clostridium sticklandii*	33	98%	Firmicutes					
B8	*Pelomonas saccharophila*	–	100%	Proteobacteria					

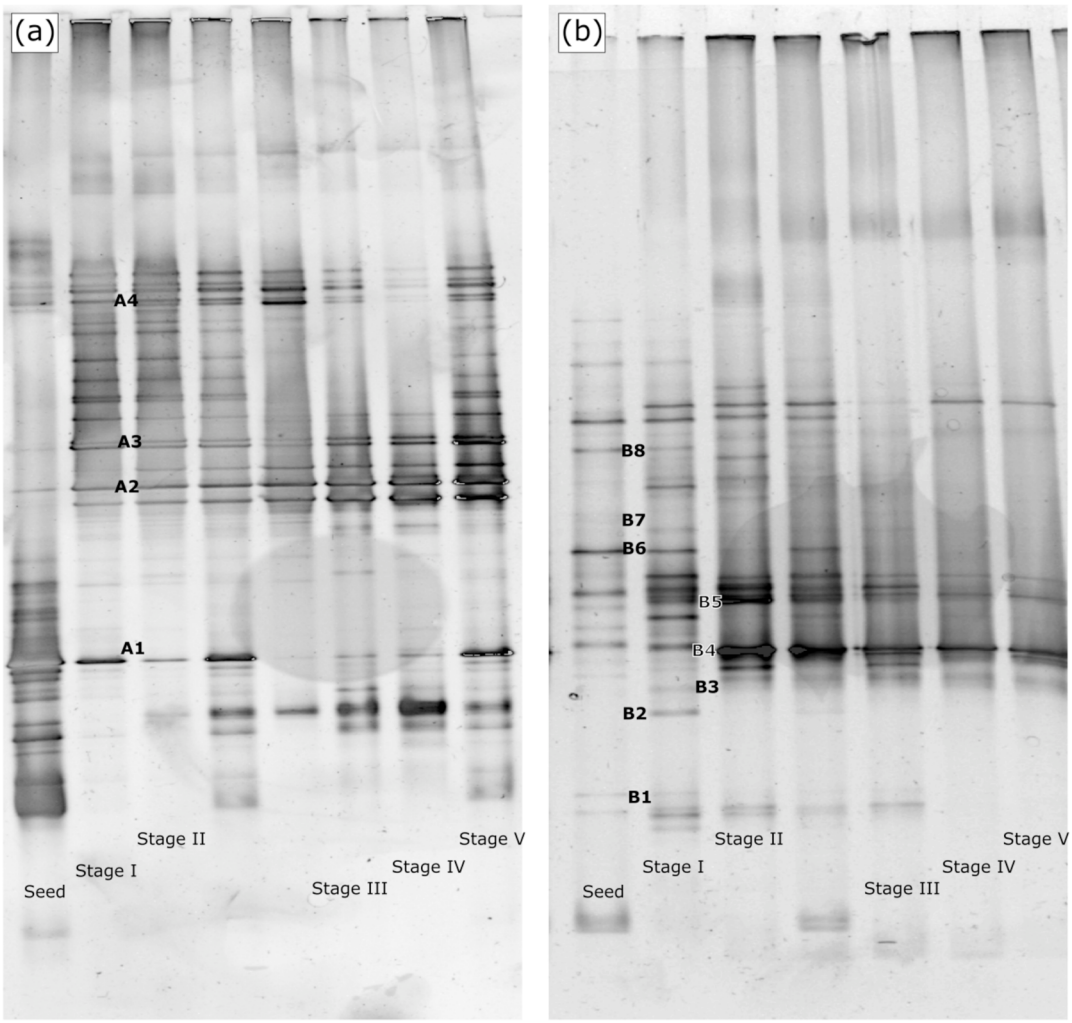


Fig. 4. Denaturing gradient gel electrophoresis (DGGE) patterns of anaerobic communities from both archaea (A) and bacteria (B).

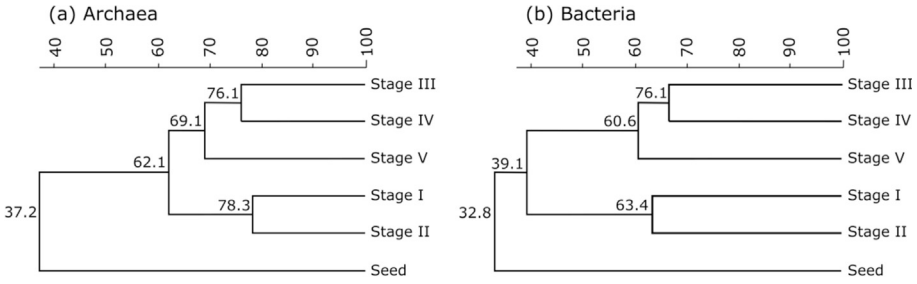


Fig. 5. Archaeal (a) and bacterial (b) dendrograms.

final stages IV and V. The reasons were different for each domain: for bacteria, the similarity in the initial stages was probably due to generalist species, since residual glycerol at low OLRs favours a diverse and rich community with low productivity [34,35,43]. The similarity in bacteria decreased, due to increases in OLR and consequent initial dominance of most adapted and productive species, which is corroborated by the 10% increase in Fo and the decrease in Rr and H observed along the operation. The selection in final stages reduced similarity even further.

The impurities in residual glycerol and low OLRs also explain the maintenance of general community diversification similarity in archaea [34,43,47,48], thus the high similarity between stages I and II. However, the similarity decreases along the operation, especially after stage II, since most of methanogens are not resistant to increasing OLR concentrations; thus, only a few restricted species were capable of surviving [60,61]. This indicates the selection of resistant organisms until the final stages.

4. Conclusions

The bacterial Rr and H at stages I, II, and III indicate the favoring of generalist species due to the increased carrying capacity and

niche diversification. Thus, higher Rr and H promote higher stability, while higher Fo promotes lower stability and/or resilience due to a decrease in the genetic pool.

Increases in Fo and decreases in Rr and H indicate community specialization; therefore, substrate degradation becomes more efficient. Archaeal parameters at stages III and IV decreased due to the inhibition of archaea. Methane was not detected at stage V, indicating that the generalist species were inhibited.

H_2 yield was limited by pH_2, Fo, and propionic acid concentration. OLR increases negatively impacted methanogens; however, even high OLRs could not eliminate the remaining archaea.

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