BioEnergy Research (2019) 12:733–742
https://doi.org/10.1007/s12155-019-09994-5

The Source of Inoculum and the Method of Methanogenesis Inhibition Can Affect Biological Hydrogen Production from Crude Glycerol

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Published online: 15 June 2019

Abstract
Crude glycerol has become an attractive feedstock for biohydrogen production via dark fermentation. However, it is necessary to determine the inoculation conditions that provide the highest biohydrogen yield. The aim of this work was to evaluate in batch assays the effect of the inoculum and methanogenesis inhibition method on biohydrogen production from crude glycerol. Four inocula were tested: anaerobic sludge treating municipal wastewater, anaerobic sludge treating brewery wastewater, goat ruminal liquid, and a mixture of the first three inocula. Each inoculum was subjected to three different treatments to inhibit methanogenesis: addition of chloroform, acid shock, and heat shock. Denaturing gradient gel electrophoresis (DGGE) of the polymerase chain reaction (PCR) products was used to assess microbial communities present in the pretreated inocula. The results indicate that the ruminal liquid treated with chloroform produced the highest biohydrogen yield (0.208 mol H_2 mol^{-1} glycerol). The microbial community present after all treatments tested preserved good functionality and stability in terms of species composition, and could endure changing environmental conditions.

Keywords Dark fermentation · Glycerine · Hydrogen-producing inocula · Sludge pretreatment

Introduction

It is estimated that 4,000,000 tons of glycerol will be generated by 2027 as a by-product of transesterification of vegetable oils and animal fat for biodiesel production [1]. Although it is used in the chemical industry, crude glycerol derived from biodiesel production is approximately 20% impure, making it a low value-added product. Currently, crude glycerol produced in biodiesel plants has been sold at low cost to be purified through vacuum distillation, ion exchange adsorption, adsorption using activated carbon, and membrane separation [2]. To expand its use, this type of crude glycerol was tested as a feedstock for physico-chemical and biological synthesis of value-added products as such the following: formate, butanol, dihydroxyacetone, propanediols, ethanol, poly(hydroxyalkanoates), organic acids, methane, and hydrogen [3–6].

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H_2 is an advantageous energy source because its combustion practically does not generate greenhouse gases, besides possessing an energetic potential greater than other hydrocarbon-based fuels (1 kg of H_2 can replace 3.55 L of conventional diesel, for example) [5]. Crude glycerol is a potential feedstock to synthetize H_2 biologically because it is highly biodegradable (can reach up to 100% of anaerobic biodegradability [7]), and it contains a high organic matter fraction in its constitutions, with about 1260 g of chemical oxygen demand (COD) per liter [8]. The main limitations related to biohydrogen production from crude glycerol are its high salinity (> 46 g L^{-1} of chloride) [9] and the low biohydrogen yield (maximum of 1.0 mol H_2 mol^{-1} glycerol) [10] compared with other carbon sources (the maximum biohydrogen yield to glucose is between 2.0 and 4.0 mol H_2 mol^{-1} glucose [11]). On the other hand, glycerol has a high degree of carbon reduction (4.67) compared with glucose (4.0) [12].

Biohydrogen can be produced via photo or dark fermentation, the latter being the most viable from an economic point of view [5]. The mixed cultures used promote the degradation of organic matter, producing acids, alcohols, and H_2 as the main by-products [6]. Although the dark fermentation may occur without inoculum addition [13], such strategy reduces the start-up period of H_2-producing bioreactors [14]. Mixed cultures contain H_2-consuming microorganisms (mainly methanogenic archaea and homoacetogenic bacteria), which can considerably decrease biohydrogen yield. Thus, it is necessary to inoculate the H_2-producing bioreactors with a sludge free of these undesirable microorganisms in order to achieve a good biohydrogen yield and make the start-up period shorter [15, 16]. When a mixed culture is exposed to an environmental high stress level, usually only spore-forming bacteria (especially *Clostridium* and *Bacillus*) can survive, resulting in a selection of this microbiota [17].

Several methods may suppress methanogenic and homoacetogenic activities in mixed cultures such as heat, acid and alkaline pretreatments, freezing, thawing [16], sterilization, microwave [14], and addition of inhibiting chemicals such as chloroform [18]. In addition, Wang et al. [19] studied the biohydrogen production from activated sludge as a feedstock and observed that the effect of calcium peroxide (at 0.25 g g^{-1} VSS) was much more efficient to inactivate the enzymes related to hydrogen consumption than that to those related to hydrogen production. Similar experiments were carried out by Wang et al. [20], but adding different concentrations of ammonium instead of calcium peroxide. These researchers observed that the maximum biohydrogen yield was obtained at an ammonium concentration of 266 mg L^{-1}. Chen et al. [21] used a nitrite-rich wastewater to maximize the biohydrogen production from waste activated sludge. They observed that free nitrous acid formed at acid pH (5.5) accelerated sludge disruption and promoted the biodegradability of organics; this phenomenon led to a biohydrogen yield increase from 8.5 to 15.0 mL H_2 g^{-1} VSS when the initial nitrite concentration increased from 0 to 250 mg L^{-1}. There is no consensus on which method is the most suitable for hydrogen-producing bacteria (HPB) selection [7], which is a result of the specific microbial diversity and resistance of each inoculum when exposed to a given substrate and/or imposed condition [22].

The aim of this work was to evaluate in batch assays the effect of the inoculum and methanogenesis inhibition method on biohydrogen production from crude glycerol. Four inocula were tested: anaerobic sludge treating municipal wastewater, anaerobic sludge treating brewery wastewater, goat ruminal liquid, and a mixture of the first three inocula. Each inoculum was subjected to three different treatments to inhibit methanogenesis: addition of chloroform, acid shock, and heat shock. Denaturing gradient gel electrophoresis (DGGE) of the polymerase chain reaction (PCR) products was used to assess microbial communities present in the pretreated inocula.

Materials and Methods

Crude Glycerol

Residual glycerol was used as a feedstock and provided by Petrobras (Quixadá Biodiesel Plant, State of Ceará, Brazil) generated from transesterification of soybean oil (56%) and beef tallow (44%) with the following characteristics: 78.4% glycerol, 4.8% sodium chloride, 1.2% non-glycerol organic matter (NGOM), 3.0% methanol, 4.8% ashes, and COD of 1374 g L^{-1}.

Inocula

Four different inocula were used as sources of hydrogen-producing bacteria: (1) flocculent sludge collected in a full-scale upflow anaerobic sludge blanket (UASB) treating municipal wastewater (MW); (2) granular sludge collected from an UASB reactor treating brewery wastewater (BW); (3) goat ruminal liquid obtained by stomach tubing (RL); and (4) mixture of the three inocula (1:1:1 $v{\cdot}v^{-1}$) (MX). The total volatile solids (TVS) of the mixed cultures used were 55 g L^{-1} (MW), 26 g L^{-1} (BW), and 34 g L^{-1} (RL).

All the inocula were subjected to three different treatments to inhibit methanogenesis: addition of chloroform (CF), acid shock (AS), and heat shock (HS). There was no pre-cultivation of the inocula before being used in the biohydrogen production tests. For CF, 0.05% ($v{\cdot}v^{-1}$) chloroform was added to the batch assays [23]. For AS, 2 M HCl was added to the assays to reach pH 3.0; after which, an incubation period of 24 h at ambient temperature was used and a pH adjustment to 8.12 was done with 2 M NaOH [16].

For HS, a temperature of 90 °C and an incubation time of 10 min were used as treatment strategy [24]. Control experiments were conducted without subjecting the inocula to any pretreatment.

Experimental Design

The experimental set-up, including macro- and micronutrient composition, was based on the procedures described in Davila-Vasquez et al. [25]. Crude glycerol (16 g L^{-1} of COD) was used as the feedstock, and the four different inocula (MW, BW, RL, and MX) were used at 4.5 g L^{-1} of TVS. The initial pH was adjusted to 8.12 with 2 M NaOH. Batch experiments were performed in 250-mL Durham flasks using 200 mL working volume and 50 mL as headspace. Before being sealed, flasks were flushed with N_2 gas for 1 min to promote anaerobic atmosphere. Each test was performed in triplicate in an orbital shaker at 120 rpm and 37 °C for approximately 70 h (final of fermentation time). The biogas volume was measured by using the liquid displacement method [26], and H_2 concentration was determined by gas chromatography (GC); these measurements were conducted on a daily basis. Hydrogen yield was calculated according to Eq. 1, adapted from Chookaew et al. [27].

$$HY = \frac{V_{\mathrm{H2prod}} / V_{\mathrm{molarH2}}}{n_S} \quad (1)$$

where HY is the yield of biohydrogen (mol H_2 mol^{-1} glycerol); V_{H2prod} is the volume of biohydrogen produced (in L); $V_{\mathrm{molar\ H2}}$ is the volume occupied by a mol of H_2 under standard temperature and pressure (STP) (L mol^{-1}); and n_s is the number of mols of substrate added to each flask (mol).

Statistically significant differences among the treatments applied were evaluated by Tukey's paired comparison using the software package Sisvar (version 5.1) at 95% of confidence level ($p \leq 0.05$).

Analytical Methods

Gas composition (CH_4, H_2, and CO_2) was analyzed by gas chromatography (C2V-200 micro GC, Thermo Fisher Scientific, The Netherlands), with a limit of detection (LOD) of 2 ppm. Two milliliter aliquots of the supernatant was collected from each flask at the beginning and end of the experiment. These samples were kept frozen at − 20 °C until being analyzed by a high-performance liquid chromatography (HPLC) (Shimadzu, Japan) in order to assess the carboxylic acid and alcohol production, whose limits of detection were 0.100 and 0.250 g L^{-1}, respectively. The conditions and columns used for GC and HPLC analyses were described in Dams et al. [28]. Standard methods [29] were used to determinate the TVS concentrations and final pH.

Microbial Community Evaluation

DNA extraction and amplification were performed as previously described [28]. Briefly, aliquots were taken at the beginning and end (day 14) of batch experiments, collected from all batch assays, and nucleic acids were extracted from 0.5 g of centrifuged mixed culture samples using a PowerSoil DNA Isolation Kit (MO Bio, Carlsbad, CA). For *Bacteria* domain, similar primers were used as previously [28] containing GC clamps for further analysis by DGGE. All primers were synthesized commercially by IDT Integrated DNA Technologies (IA, USA). PCR fragments were amplified using the following protocols: 95 °C for 2 min, 31 cycles at 95 °C for 30 s, 58 °C for 45 s, 72 °C for 60 s, and 72 °C for 6 min. PCR products were stained with SYBR Safe DNA gel (Invitrogen, Cergy Pontoise, France), analyzed using agarose gel electrophoresis (1.8%) with HyperLadder II (Bioline, USA Inc.), and subsequently separated by denaturing gradient gel electrophoresis (DGGE) using a denaturing gradient ranging from 57 to 72%. DGGE was used to determine the diversity of microbial communities in pretreated sludges and ruminal liquid. The aim was to investigate the effect of the pretreatments applied without further characterization of individual species.

DGGE and analysis of the fingerprints were conducted using a Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) as previously described [28] using 8% ($w{\cdot}v^{-1}$) polyacrylamide DGGE gels with a denaturing gradient ranging from 57 to 72%. Electrophoresis was conducted for 16 h at 60 °C and 65 V. Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to obtain a matrix of relative band intensity values according to band positions. DGGE dendrograms were constructed, and similarity coefficient was calculated using the Jaccard correlation [30]. Ranged-weighted richness (Rr) and functional organization (Fo) [31] were calculated based on Pareto–Lorenz curves [32]. Lorenz distribution curves were organized based on DGGE profiles, where for each DGGE lane, the respective bands are ranked from high to low based on their intensities [32].

Results and Discussion

Biohydrogen Yield

The biohydrogen yields for each inocula and technique of methanogenesis inhibition are presented in Table 1, in which it can be verified that almost all techniques used for methanogenesis inhibition increased biohydrogen production. One exception was observed in the case of MX inoculum, in

Table 1 Biohydrogen yield according to the inocula and methanogenesis inhibition method (MIM). Values given in mol mol^{-1} of glycerol

MIM	Inocula				
	MW	BW	LR	MX	Average
Ctrl	$^{C}0.036^{b,c}$	$^{H}0.019^{c}$	$^{E}0.087^{a}$	$^{J}0.072^{a,b}$	$^{M}0.053$
CF	$^{A}0.186^{d}$	$^{F}0.117^{e}$	$^{D}0.208^{d}$	$^{I}0.172^{d}$	$^{L}0.171$
AS	$^{B,C}0.072^{f}$	$^{G}0.068^{f}$	$^{E}0.109^{f}$	$^{K}0.018^{g}$	$^{M}0.067$
HS	$^{B}0.100^{h}$	$^{G,H}0.042^{j}$	$^{E}0.098^{h,i}$	$^{J}0.080^{i}$	$^{M}0.080$
Average	0.098^{l}	0.062^{m}	0.126^{k}	$0.085^{l,m}$	

Tukey's test = values followed by different letters differ statistically. The comparative analysis between the methanogenesis inhibition methods must be done vertically, having the capital letters as reference. The comparative analysis between the inocula should be made horizontally, with the lower case letters as a reference

which the biohydrogen yield of the control flask (0.072 mol H_2 mol^{-1} glycerol) was greater than the yield achieved when the AS treatment was applied (0.018 mol H_2 mol^{-1} glycerol). Among the treatments tested, addition of chloroform (CF) exerted the most significant positive effect on biohydrogen production ($p \leq 0.05$), achieving an average yield of 0.171 mol H_2 mol^{-1} glycerol. Other studies have already demonstrated the effectiveness of chloroform in inhibiting methanogenic activity [23, 33]. This is because chloroform (used at 0.05%) is a selective agent and can only inhibit methanogenic archaea, which are H_2 consumers. This selective inhibition with CF was confirmed in the biogas analysis, where no biomethane was detected regardless of the inocula (Fig. 1). The inhibitory activity of chloroform is due to the suppression of methyl-coenzyme M reductase, which is only present in methanogenic archaea [33]. In addition, chloroform can bind to free corrinoids (an analogous and active form of vitamin B_{12}) in the microbial cell, therefore affecting corrinoid-containing enzymes [19]. Thus, inhibiting the methanogenesis via suppression of enzyme activity may be more efficient than the mechanisms involved in HS or AS [34, 35].

The cumulative biohydrogen production for each inoculum and technique for methanogenesis inhibition as function of experimental time is shown in Fig. 2. It can be observed that the biohydrogen accumulated in the beginning of the experiment and was consumed after the second day of incubation, except for CF, showing that some H_2-consuming microorganisms could survive even under the stress conditions applied. This was confirmed by biogas analysis, which detected methane in MW inoculum treated with AS, indicating that biohydrogen was probably consumed by hydrogenotrophic methanogenic archaea. Bacteria can also directly use H_2 as a donor of protons and electrons in chain elongation processes, which proceed via ß-reverse oxidation to form caproic acid (C6) from short-chain carboxylic acids such as acetic and butyric acids [35, 36]. In such cases, the consumption of H_2 occurs mainly when there is a shortage of ethanol in the medium, as this latter compound is a preferential electron donor [37]. The concentrations of C6 in LR+AS and LR+HS were 1.2 and 0.9 g L^{-1}, respectively (Table 2). With respect to the average yield for each inocula, BW treated with CF showed the lowest average of biohydrogen yield (0.117 mol H_2 mol^{-1} glycerol), probably because of the presence of a lower number of acidogenic bacteria in this microbial community.

In RL, both AS and HS treatments had no influence on the biohydrogen production, as the H_2 yields obtained were statistically equal to the controls (Table 1). Additionally, these

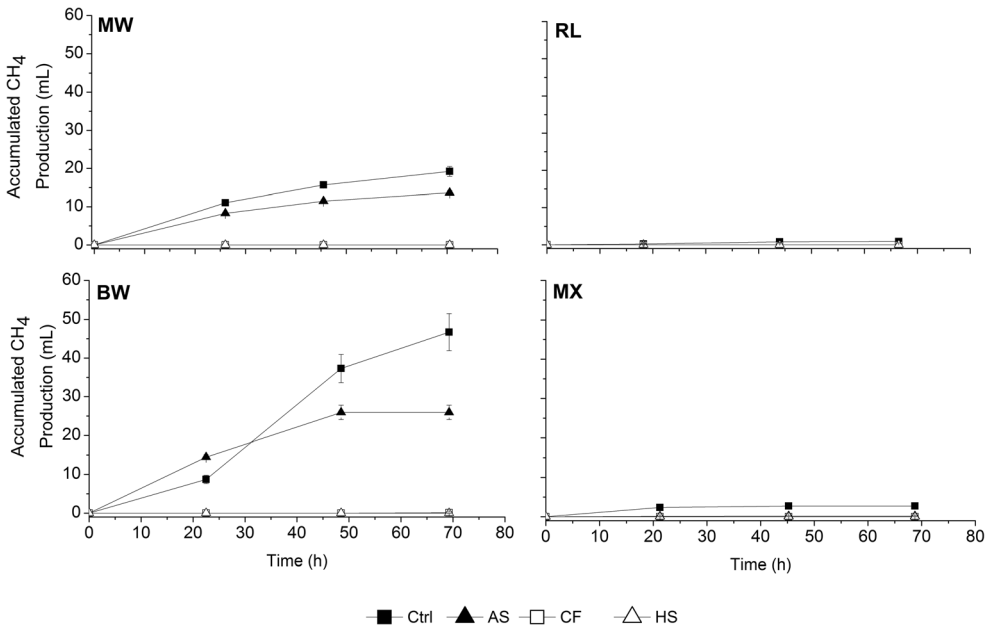

Fig. 1 Cumulative biomethane production for each inocula and technique for methanogenesis inhibition presented as function of experimental time. MW = flocculent sludge originating from a full-scale UASB reactor used to treat municipal wastewater; BW = granular sludge withdrawn from an UASB reactor used to treat brewery wastewater; RL = goat ruminal liquid obtained by stomach tubing; and MX = mixture of the three inocula (1:1:1 $v{\cdot}v^{-1}$); Ctrl = control (no pretreatment); CF = addition of chloroform; AS = acid pH shock; HS = heat shock

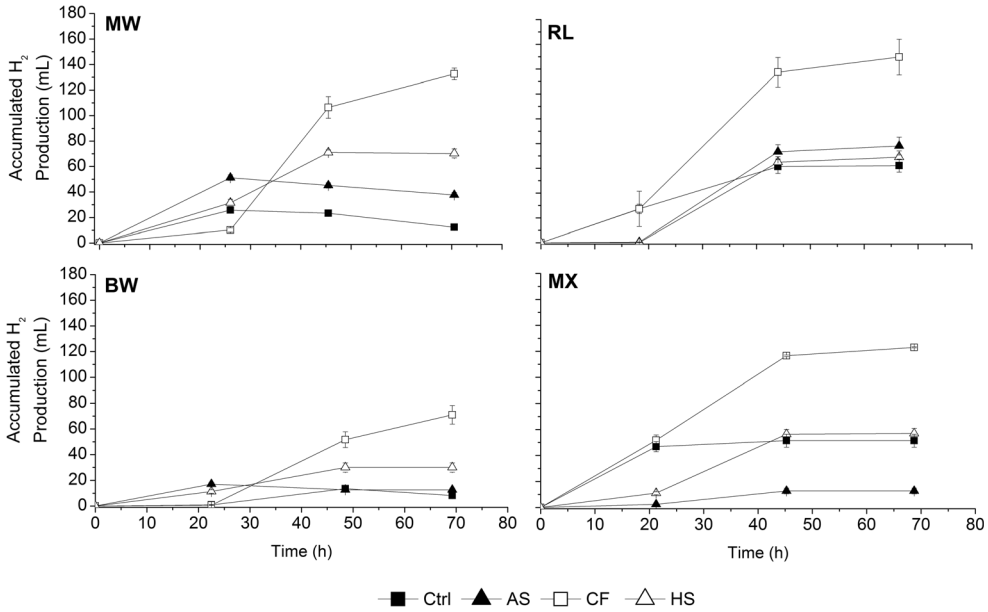

Fig. 2 Cumulative biohydrogen production for each inocula and technique for methanogenesis inhibition as function of experimental time. MW = flocculent sludge originating from a full-scale UASB reactor used to treat municipal wastewater; BW = granular sludge withdrawn from an UASB reactor used to treat brewery wastewater; RL = goat ruminal liquid obtained by stomach tubing; and MX = mixture of the three inocula (1:1:1 $v{\cdot}v^{-1}$); Ctrl = control (no pretreatment); CF = addition of chloroform; AS = acid pH shock; HS = heat shock

treatments inhibited both H_2-consuming and H_2-producing microorganisms in MW and BW (Figs. 1 and 2), and the methanogenic archaea present in BW showed to be quite resistant to acid treatment. This higher resistance of BW to AS occurred likely because of its granular structure, known for higher microbial population density (which includes methanogenic archaea) and size compared to flocculent sludge [38], which serves as protection for methanogenic archaea, located predominantly in the core of the granule [39]. Therefore, maybe a higher concentration of acid and/or longer exposure time could be required to completely inhibit methanogenic activity present in BW sludge. AS was not effective in increasing biohydrogen production, which is in agreement with the results reported in other studies [16, 33, 34]. Regarding to HS treatment, other procedures could be used to avoid the decay of spore-forming HPB such as lower temperatures and prolonged contact time [40].

The findings of the present investigation also showed that, as predicted, the inocula source can strongly influence biohydrogen yield (Table 1). Taking into account all techniques of methanogenesis inhibition investigated, the highest yield average value (0.126 mol H_2 mol^{-1} glycerol) was achieved for RL, followed by those obtained using MW, MX, and BW. However, CF addition showed to be the best methanogenesis inhibition strategy investigated, resulting the highest biohydrogen yields, i.e., 0.208, 0.186, and 0.172 mol H_2 mol^{-1} glycerol, for RL, MW, and MX, respectively.

The predominance of spore-forming *Clostridium* species in the microbial community of the municipal wastewater treatment sludge [33] and ruminal fluid [41] may be related to the higher yields obtained when compared with the other inocula tested. In addition, the results indicated that the increased H_2 yield in RL was attributed to a low concentration of methanogens in the inoculum, as almost no methane was detected even in the control flasks (Fig. 1). However, the microbial diversity of the RL can vary greatly according to the type and diet of the animal [42].

In theory, the mixture of the three inocula (MX) could result in an increase of the biohydrogen yield because a more diversified microbial population would expand syntrophic relationships between species, as demonstrated by Chang et al. [43]. The yield, however, showed intermediate values (mean 0.085 mol H_2 mol^{-1} glycerol) compared with those obtained using other inocula. It seems that RL strongly influenced the inoculum mixture, as the production of gases and metabolites was very similar to that achieved in RL ($p > 0.05$). Therefore, the syntrophic relationships were likely not well established among the species present in the mixture, and a longer adaptation time would be required.

Soluble Metabolites

The results obtained in the present investigation indicated that almost all the glycerol added as carbon source was converted to soluble and gaseous metabolites. The exceptions were MW + Ctrl, MW + CF, and MW + AS, whose conversion rates were 85, 83, and 83%, respectively. The concentration of soluble metabolites formed at the end of glycerol fermentation for each inoculum and technique of methanogenesis inhibition is shown in Table 2.

The difference between the metabolic routes used and respective by-products formed is related to the diversity of the microbial population of each inoculum before and after methanogenesis inhibition [44]. In the present research, the predominating metabolic routes did not favor biohydrogen production. In terms of concentration, the main products formed were propionic acid, 1,3-PPD, ethanol, and caproic acid. 1,3-PPD was also the main soluble metabolite achieved

Table 2 Concentration of soluble metabolites formed at the end of fermentation of glycerol for each inoculum and methanogenesis inhibition method (MIM), including the control assay. Values followed by "±" represent the standard deviation

Inocula	MIM	Concentration (g L^{-1})						
		C2	C3	C4	C6	C8	EtOH	1,3-PPD
MW	Ctrl	N.D	0.6 ± 0.002	0.2 ± 0.001	1.7 ± 0.100	N.D	N.D	N.D
	CF	0.2 ± 0.001	0.5 ± 0.001	0.2 ± 0.001	N.D	N.D	N.D	N.D
	AS	N.D	0.6 ± 0.180	0.2 ± 0.001	0.6 ± 0.140	N.D	N.D	N.D
	HS	0.4 ± 0.001	0.9 ± 0.040	0.2 ± 0.001	0.2 ± 0.001	0.6 ± 0.100	N.D	N.D
BW	Ctrl	0.4 ± 0.050	1.4 ± 0.200	0.1 ± 0.001	0.5 ± 0.020	N.D	N.D	0.7 ± 0.050
	CF	N.D	0.1 ± 0.001	0.5 ± 0.004	1.2 ± 0.050	N.D	N.D	0.6 ± 0.050
	AS	0.3 ± 0.070	1.3 ± 0.200	0.1 ± 0.001	0.7 ± 0.020	N.D	N.D	0.7 ± 0.050
	HS	0.3 ± 0.060	0.7 ± 0.070	0.1 ± 0.001	1.2 ± 0.050	N.D	N.D	0.7 ± 0.040
RL	Ctrl	N.D	0.5 ± 0.001	N.D	0.7 ± 0.180	N.D	0.1 ± 0.001	1.2 ± 0.100
	CF	N.D	0.4 ± 0.002	0.1 ± 0.001	1.1 ± 0.200	N.D	1.2 ± 0.100	0.4 ± 0.010
	AS	0.2 ± 0.001	0.5 ± 0.001	N.D	1.2 ± 0.070	N.D	0.5 ± 0.100	0.7 ± 0.200
	HS	0.4 ± 0.004	0.5 ± 0.002	0.1 ± 0.001	0.9 ± 0.040	N.D	0.3 ± 0.080	1.0 ± 0.120
MX	Ctrl	N.D	0.6 ± 0.060	0.4 ± 0.020	1.0 ± 0.100	N.D	N.D	1.4 ± 0.200
	CF	0.4 ± 0.060	0.3 ± 0.010	0.3 ± 0.010	0.8 ± 0.100	N.D	0.6 ± 0.000	0.4 ± 0.020
	AS	N.D	0.8 ± 0.020	0.6 ± 0.020	1.3 ± 0.200	N.D	0.5 ± 0.000	1.4 ± 0.200
	HS	0.1 ± 0.020	N.D	0.1 ± 0.001	1.0 ± 0.200	N.D	0.2 ± 0.000	1.5 ± 0.100

Values followed by "±" represent the standard deviation. C*n*, where "*n*" is the number of carbons equivalent to the organic acid produced; *EtOH*, ethanol; *1,3-PPD*, 1,3-propanediol; *N.D*, not detected

by many other studies for biohydrogen production from crude glycerol [15, 45]. The production of biohydrogen occurs via pyruvate-formate pathway [46], and the main by-products formed upon substrate (including glycerol) degradation are acetic and butyric acids, and ethanol [47]. 1,3-PPD can be obtained biologically from glycerol fermentation but using a metabolic pathway parallel to the route of biohydrogen production (reductive route). Based on the stoichiometry, it is necessary to consume 1 mol of H_2 to form 1 mol of 1,3-PPD [15], reducing the biohydrogen yield.

Synthesis of propionic acid occurs when phosphoenolpyruvate is converted to succinate, and is subsequently converted to propionate. In order to produce H_2, phosphoenolpyruvate needs to be converted to pyruvate [3]. However, in addition to the culture itself, other conditions may have favored the accumulation of propionic acid, such as (i) concentration of glycerol (15 g L^{-1}) [48]; (ii) pH, which was in optimal range for acidogenesis (between 5.0 and 6.0) and outside the optimal range for acid consumption (between 7.2 and 7.5) [49]; and (iii) partial pressure of H_2 (p_{H2}). Under conditions of high p_{H2}, the yield of NADH increases, and to maintain an appropriate $NADH/NAD^+$ ratio within the cell, microorganisms are induced to form propionic acid because this is the route that produces more NAD^+ [50].

High concentrations of caproic acid (C6) were found and the highest production of C6 (1.7 g L^{-1}) was achieved using MW + Ctrl. The caproic acid may have been formed through the chain elongation reaction from short-chain carboxylic acids (ß-reverse oxidation), as previously explained, or via ß oxidation, where the long-chain fatty acids (LCFA) contained in the crude glycerol are converted to medium-chain fatty acids, such as caproic (C6) and caprylic acids (C8). However, the ß oxidation might not have had much influence on the C6 and C8 production, because the high dilution (about 86 times) would cause a decrease in the LCFA concentration in the flasks from 12,000 to 140 mg L^{-1} (considering that all the NGOM represent the LCFAs). Caproic acid is widely used as a precursor for the production of biofuels [51], but is also used as flavoring [52] and supplement in pig and poultry feed to control enteric diseases [53].

Microbial Analysis

Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) are techniques used to evaluate changes in the microbial communities in hydrogen-producing biological reactors [54]. Ecological parameters such as ranged-weighted richness (Rr) and functional organization (Fo) can be estimated to indicate the degree of diversity in a bacterial community [32]. For each DGGE lane, the respective bands are ranked from high to low, based on their intensities: 25% Pareto–Lorenz (PL) curves represent a low functional organization, while 80% PL curves indicate a specialized and highly organized microbial community [31].

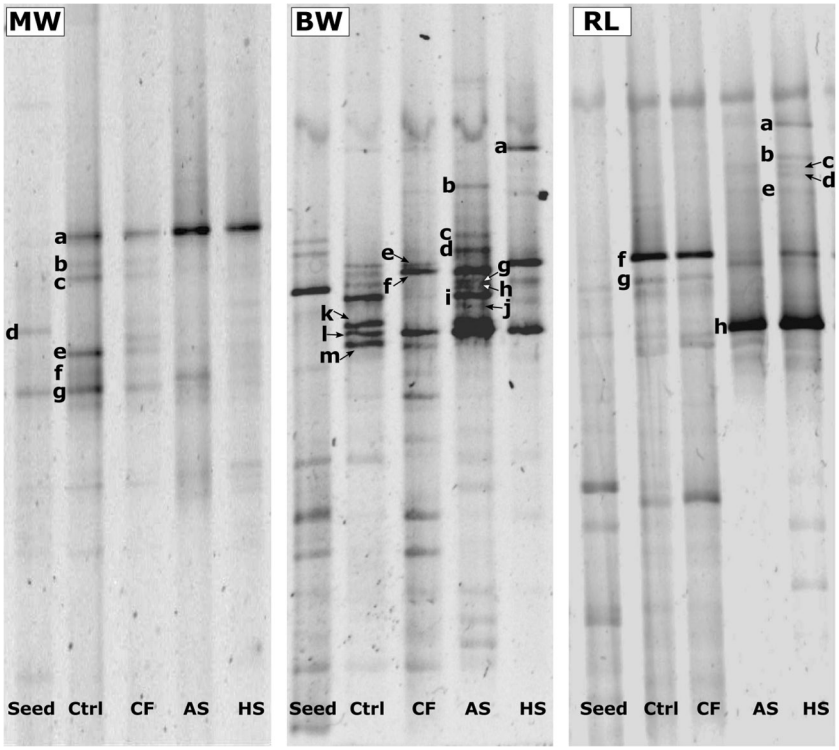

Fig. 3 Bacterial profiles of the inocula tested in this study, examined using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE)

In this study, a narrower denaturing gradient range (57–72%) was used to mitigate the possible limitations of DGGE, because under the adverse environment of chloroform, acid, or thermal treatment, few microorganisms would survive and be part of the microbial community. Other studies have utilized a smaller gradient with environmental samples, such as those obtained to examine hydrogen production by anaerobic sludge [23] and for functional studies of ammonia-oxidizing bacterial communities in sequential batch reactors [55].

The results showed that the community composition of the sludge and RL shifted, and differences in the number and pattern between communities were observed. After about 70 h of incubation, the bacterial community in the untreated MW was enriched and at least six bands were visualized. As treatments were applied, bands *d*, *e*, *f*, and *g* disappeared or their respective intensities were reduced, while band *a* persisted and its intensity increased (Fig. 3), indicating the degree of abundance of each microbial group [56].

RL is composed of a rich microbial community, as shown in Fig. 3. The bacterial profile of RL suggests a predominance of bacteria resistant to high temperatures and acidic conditions as the intensity of band *h* in the fourth and fifth lanes increased. Most of the bands disappeared or weakened after treatments were applied. Under conditions of AS and HS, the cellulolytic and methanogenic organisms were likely eliminated, while endospore-producers, such as members of the *Clostridia* genus, were favored and remained in the system. According to Kim et al. [13], microbial analysis by next-generation sequencing, used to assess food waste during biohydrogen production, revealed that at highly acidic conditions (pH 3, 2, and 1), clostridial sequences gradually increased as pH decreased. This suggests that these organisms can survive by forming endospores under acidic conditions, as suggested by Kim et al. [57].

In the BW sludge, some bands (*i*, *k*, *l*, *m*) clearly increased its intensity, indicating a selection of a few members of the community. Enrichment of the microbial community was observed after treatment with CF and AS (third and fourth lanes). Treatment with AS showed that certain members of the microbial community can be selected by controlling the level of acidity (bands *f*, *g*, *h*) and treatment with HS (fifth lane) spore formers was possibly selected. Indeed, Nissilä et al. [58] indicated that HS, used to treat activated sludge from domestic sewage, enriched for spore formers and reduced species diversity. These authors reported that in addition to *Thermoanaerobacterium thermosaccharolyticum*, the community was enriched for thermophilic and cellulolytic microorganisms related to *Clostridium caenicola*, which can produce biohydrogen, bioethanol, and acetate as fermentation products, along with numerous uncultured Clostridia species that may have contributed to biohydrogen production.

The Rr values, obtained after treatments with AS and HS, were ≤ 10, especially for MW; this was attributed to the

particularly adverse environments induced by these treatments [31]. However, for BW treated with CF, the Rr value was > 30, indicating a habitable environment [31]; this result also indicates that the halogen chloroform exerted a considerable effect on the microbial community of BW. Indeed, Ning et al. [23] showed enrichment of a microbial community exposed to different concentrations of chloroform, and suggested that a microbial population can be selected by maintaining the concentration of chloroform at a reasonable level. Conversely, when the ruminal microbial community (RL) was exposed to acidic conditions (AS), it showed a medium richness (Rr = 14) and higher diversity than the seed inoculum (raw ruminal liquid); this shows that the population adapted to the adverse environment.

The microbial community present in MW, BW, and RL showed Fo > 30 and good stability when exposed to all the pretreatments tested. According to Marzorati et al. [31], this indicates a balanced community with medium Fo. Furthermore, the community was able to endure environmental stress and preserve its functionality under perturbed conditions. However, studies have shown that exposure to certain environmental conditions affects the functionality of a community as demonstrated by Wittebolle et al. [56], which observed that nitrification remained high throughout the experiment. The authors pointed out that certain microbial characteristics, such biodiversity and dynamics, are more important in indicating microbial functionality than the presence of certain specific species.

Conclusions

In this study, crude glycerol was used as a feedstock for biohydrogen production to evaluate the effect of different inocula and techniques for methanogenesis inhibition. The results indicated that ruminal liquid (RL) had the highest potential for biohydrogen production among the tested inocula. The addition of 0.05% ($v{\cdot}v^{-1}$) chloroform (CF) was the most appropriate strategy for selecting HPB. For biohydrogen production, the best combinations of inocula and technique for methanogenesis inhibition were LR + CF, MW + CF, and MX + CF. The microbial community present after all treatments tested preserved good functionality and stability in terms of species composition, and could endure changing environmental conditions.

Funding Information This work was financially supported by the Brazilian Agricultural Research Corporation (Embrapa) (Call 11/2012-Macroprograma 3); the Brazilian National Council for Research and Technology (CNPq) (Grant 473352/2011-7); and the Ceará State Foundation for the Support of Scientific and Technological Development (FUNCAP) (Grant No. 350365/2013-0 and DCR 002400477.02.00/12).

References

1. OECD/FAO (2018) Biofuels. Agric. outlook, OECD Publishing, pp. 191–206
2. Ardi MS, Aroua MK, Hashim NA (2015) Progress, prospect and challenges in glycerol purification process: a review. Renew Sust Energ Rev 42:1164–1173. https://doi.org/10.1016/j.rser.2014.10.091
3. da Silva GP, Mack M, Contiero J (2009) Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol Adv 27:30–39. https://doi.org/10.1016/j.biotechadv.2008.07.006
4. Boga DA, Liu F, Bruijnincx PCA, Weckhuysen BM (2016) Aqueous-phase reforming of crude glycerol: effect of impurities on hydrogen production. Catal Sci Technol 6:134–143. https://doi.org/10.1039/c4cy01711k
5. Sarma SJ, Brar SK, Le Bihan Y, Buelna G (2012) Bio-hydrogen production by biodiesel-derived crude glycerol bioconversion: a techno-economic evaluation. Bioprocess Biosyst Eng. https://doi.org/10.1007/s00449-012-0755-8
6. Viana QM, Viana MB, Vasconcelos E a F, Santaella ST, Leitão RC (2014) Fermentative H2 production from residual glycerol: a review. Biotechnol Lett 36:1381–1390. https://doi.org/10.1007/s10529-014-1507-4
7. Siles López JA, Martín Santos MDLA, Chica Pérez AF, Martín Martín A (2009) Anaerobic digestion of glycerol derived from biodiesel manufacturing. Bioresour Technol 100:5609–5615. https://doi.org/10.1016/j.biortech.2009.06.017
8. Viana MB, Freitas AV, Leitão RC, Pinto GAS, Santaella ST (2012) Anaerobic digestion of crude glycerol: a review. Environ Technol Rev 1:81–92
9. Viana MB, Freitas AV, Leitão RC, Santaella ST (2012) Biodegradability and methane production potential of glycerol generated by biodiesel industry. Water Sci Technol 66:2217–2222. https://doi.org/10.2166/wst.2012.455
10. Markov SA, Averitt J, Waldron B (2011) Bioreactor for glycerol conversion into H2 by bacterium Enterobacter aerogenes. Int J Hydrog Energy 36:262–266. https://doi.org/10.1016/j.ijhydene.2010.09.090
11. Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domíguez-Espinosa R (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 22:477–485. https://doi.org/10.1016/j.tibtech.2004.07.001
12. Nielsen J, Villadsen J, Gunnar L (2014) Principles, bioreaction engineering, modeling of reactions kinetics. https://doi.org/10.1007/s11883-005-0040-2
13. Kim DH, Jang S, Yun YM, Lee MK, Moon C, Kang WS et al (2014) Effect of acid-pretreatment on hydrogen fermentation of food waste: microbial community analysis by next generation sequencing. Int J Hydrog Energy 39:16302–16309. https://doi.org/10.1016/j.ijhydene.2014.08.004
14. Guo XM, Trably E, Latrille E, Carrère H, Steyer J-P (2010) Hydrogen production from agricultural waste by dark fermentation: a review. Int J Hydrog Energy 35:10660–10673. https://doi.org/10.1016/j.ijhydene.2010.03.008
15. Selembo PA, Perez JM, Lloyd WA, Logan BE (2009) Enhanced hydrogen and 1,3-propanediol production from glycerol by fermentation using mixed cultures. Biotechnol Bioeng 104:1098–1106. https://doi.org/10.1002/bit.22487
16. Rossi DM, Costa JB, Souza EA, Peralba MCR, Samios D, Ayub MAZ (2011) Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. Int J Hydrogen Energy 36:4814–4819. https://doi.org/10.1016/j.ijhydene.2011.01.005
17. Madigan MT, Martinko JM (2000) Brock - Biology of microorganisms, 12th edn. Prentice hall international, London

18. Venkata Mohan S, Lalit Babu V, Sarma PN (2008) Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresour Technol 99:59–67. https://doi.org/10.1016/j.biortech.2006.12.004
19. Wang D, Zhang D, Xu Q, Liu Y, Wang Q, Ni BJ et al (2019) Calcium peroxide promotes hydrogen production from dark fermentation of waste activated sludge. Chem Eng J 355:22–32. https://doi.org/10.1016/j.cej.2018.07.192
20. Wang D, Duan Y, Yang Q, Liu Y, Ni B-J, Wang Q et al (2018) Free ammonia enhances dark fermentative hydrogen production from waste activated sludge. Water Res 133:272–281. https://doi.org/10.1016/j.watres.2018.01.051
21. Chen F, Liu Y, Ni B-J, Wang Q, Yuan Z, Li X et al (2018) Free nitrous acid promotes hydrogen production from dark fermentation of waste activated sludge. Water Res 145:113–124. https://doi.org/10.1016/j.watres.2018.08.011
22. Chae KJ, Choi MJ, Lee JW, Kim KY, Kim IS (2009) Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. Bioresour Technol 100:3518–3525. https://doi.org/10.1016/j.biortech.2009.02.065
23. Ning Y-Y, Jin D-W, Sheng G-P, Harada H, Shi X-Y (2012) Evaluation of the stability of hydrogen production and microbial diversity by anaerobic sludge with chloroform treatment. Renew Energy 38:253–257. https://doi.org/10.1016/j.renene.2011.07.038
24. Kim D, Han S, Kim S, Shin H (2006) Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrog Energy 31:2158–2169. https://doi.org/10.1016/j.ijhydene.2006.02.012
25. Davila-Vazquez G, Alatriste-Mondragón F, de León-Rodríguez A, Razo-Flores E (2008) Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: influence of initial substrate concentration and pH. Int J Hydrog Energy 33:4989–4997. https://doi.org/10.1016/j.ijhydene.2008.06.065
26. Soto M, Mendez R, Lema JM (1993) Methanogenic and non- methanogenic activity tests: theoretical basis and experimental setup. Water Res 27:1361–1376
27. Chookaew T, O-Thong S, Prasertsan P (2012) Fermentative production of hydrogen and soluble metabolites from crude glycerol of biodiesel plant by the newly isolated thermotolerant Klebsiella pneumoniae TR17. Int J Hydrog Energy 37:13314–13322. https://doi.org/10.1016/j.ijhydene.2012.06.022
28. Dams RI, Viana MB, Guilherme AA, Silva CM, dos Santos AB, Angenent LT et al (2018) Production of medium-chain carboxylic acids by anaerobic fermentation of glycerol using a bioaugmented open culture. Biomass Bioenergy 118:1–7. https://doi.org/10.1016/j.biombioe.2018.07.023
29. American Public Health Association (2005) Standard methods for the examination of water and wastewaters, 21st edn
30. Lazaro CZ, Perna V, Etchebehere C, Varesche MBA (2014) Sugarcane vinasse as substrate for fermentative hydrogen production: the effects of temperature and substrate concentration. Int J Hydrog Energy 39:6407–6418. https://doi.org/10.1016/j.ijhydene.2014.02.058
31. Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol 10:1571–1581. https://doi.org/10.1111/j.1462-2920.2008.01572.x
32. Wittebolle L, Vervaeren H, Verstraete W, Boon N (2008) Quantifying community dynamics of nitrifiers in functionally stable reactors. Appl Environ Microbiol 74:286–293. https://doi.org/10.1128/AEM.01006-07
33. Hu B, Chen S (2007) Pretreatment of methanogenic granules for immobilized hydrogen fermentation. Int J Hydrog Energy 32:3266–3273. https://doi.org/10.1016/j.ijhydene.2007.03.005
34. Zhu H, Beland M (2006) Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. Int J Hydrog Energy 31:1980–1988. https://doi.org/10.1016/j.ijhydene.2006.01.019
35. Ding HB, Tan GYA, Wang JY (2010) Caproate formation in mixed-culture fermentative hydrogen production. Bioresour Technol 101:9550–9559. https://doi.org/10.1016/j.biortech.2010.07.056
36. Steinbusch KJJ, Hamelers HVM, Plugge CM, Buisman CJN (2011) Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. Energy Environ Sci 4:216. https://doi.org/10.1039/c0ee00282h
37. Kenealy WR, Waselefsky DM (1985) Studies on the substrate range of Clostridium kluyveri; the use of propanol and succinate. Arch Microbiol 141:187–194
38. Hulshoff Pol LW, de Castro Lopes SI, Lettinga G, Lens PNL (2004) Anaerobic sludge granulation. Water Res 38:1376–1389. https://doi.org/10.1016/j.watres.2003.12.002
39. Guiot SR, Pauss a, Costerton JW (1992) A structured model of the anaerobic granule consortium. Water Sci Technol 25:1–10
40. Lay J, Fan K, Hwang J, Chang J, Hsu P (2005) Factors affecting hydrogen production from food wastes by Clostridium-rich composts. J Environ Eng 131:595–602. https://doi.org/10.1061/(ASCE)0733-9372(2005)131:4(595)
41. Ratti RP, Botta LS, Sakamoto IK, Silva EL, Varesche MBA (2013) Production of H2 from cellulose by rumen microorganisms: effects of inocula pre-treatment and enzymatic hydrolysis. Biotechnol Lett. https://doi.org/10.1007/s10529-013-1395-z
42. Tajima K, Aminov RI, Nagamine T, Ogata K, Nakamura M, Matsui H et al (1999) Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol Ecol 29:159–169. https://doi.org/10.1016/S0168-6496(99)00008-2
43. Chang JJ, Chou CH, Ho CY, Chen WE, Lay JJ, Huang CC (2008) Syntrophic co-culture of aerobic Bacillus and anaerobic Clostridium for bio-fuels and bio-hydrogen production. Int J Hydrog Energy 33:5137–5146. https://doi.org/10.1016/j.ijhydene.2008.05.021
44. Mu Y, Yu HQ, Wang G (2007) Evaluation of three methods for enriching H2-producing cultures from anaerobic sludge. Enzym Microb Technol 40:947–953. https://doi.org/10.1016/j.enzmictec.2006.07.033
45. Wu KJ, Lin YH, Lo YC, Chen CY, Chen WM, Chang JS (2011) Converting glycerol into hydrogen, ethanol, and diols with a Klebsiella sp. HE1 strain via anaerobic fermentation. J Taiwan Inst Chem Eng 42:20–25. https://doi.org/10.1016/j.jtice.2010.04.005
46. Biebl H, Menzel K, Zeng a P, Deckwer WD (1999) Microbial production of 1,3-propanediol. Appl Microbiol Biotechnol 52:289–297
47. Mangayil R, Karp M, Santala V (2012) Bioconversion of crude glycerol from biodiesel production to hydrogen. Int J Hydrog Energy 37:12198–12204. https://doi.org/10.1016/j.ijhydene.2012.06.010
48. Barbirato F, Chedaille D, Bories A (1997) Propionic acid fermentation from glycerol: comparison with conventional substrates. Appl Microbiol Biotechnol 47:441–446
49. Chernicharo CA de L (2007) Anaerobic Reactors, vol 04. IWA Publishing, London
50. Lettinga G, Hulshoff Pol LW, Zeeman G (1999) Biological wastewater treatment. Part I: anaerobic wastewater treatment. Lectures notes. Wageningen University, Wageningen
51. Verser DW (2012) Method for the indirect production of butanol and hexanol. US8252567
52. Kenealy WR, Cao Y, Weimer PJ (1995) Production of caproic acid by cocultures of ruminal cellulolytic bacteria and Clostridium kluyveri grown on cellulose and ethanol. Appl Microbiol Biotechnol 44:507–513. https://doi.org/10.1007/BF00407210
53. Boyen F, Haesebrouck F, Vanparys A, Volf J, Mahu M, Van Immerseel F et al (2008) Coated fatty acids alter virulence

properties of Salmonella typhimurium and decrease intestinal colonization of pigs. Vet Microbiol 132:319–327. https://doi.org/10.1016/j.vetmic.2008.05.008

54. Kumar G, Cho SK, Sivagurunathan P, Anburajan P, Mahapatra DM, Park JH et al (2018) Insights into evolutionary trends in molecular biology tools in microbial screening for biohydrogen production through dark fermentation. Int J Hydrog Energy 43:19885–19901. https://doi.org/10.1016/j.ijhydene.2018.09.040
55. Wittebolle L, Verstraete W, Boon N (2009) The inoculum effect on the ammonia-oxidizing bacterial communities in parallel sequential batch reactors. Water Res 43:4149–4158. https://doi.org/10.1016/j.watres.2009.06.034
56. Zhang T, Fang HHP (2000) Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. Biotechnol Lett 22:399–405. https://doi.org/10.1023/a:1005680803442
57. Kim J, Shin SG, Han G, O'Flaherty V, Lee C, Hwang S (2011) Common key acidogen populations in anaerobic reactors treating different wastewaters: molecular identification and quantitative monitoring. Water Res 45:2539–2549. https://doi.org/10.1016/j.watres.2011.02.004
58. Nissilä ME, Tähti HP, Rintala JA, Puhakka JA (2011) Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: effects of different heat treatments. Int J Hydrog Energy 36:1482–1490. https://doi.org/10.1016/j.ijhydene.2010.11.010