

# Adaptation of acidogenic sludge to increasing glycerol concentrations for biohydrogen production

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**Abstract** Hydrogen is a promising alternative as an energetic carrier and its production by dark fermentation from wastewater has been recently proposed, with special attention to crude glycerol as potential substrate. In this study, two different feeding strategies were evaluated for replacing the glucose substrate by glycerol substrate: a one-step strategy (glucose was replaced abruptly by glycerol) and a step-by-step strategy (progressive decrease of glucose concentration and increase of glycerol concentration from 0 to 5 g L<sup>-1</sup>), in a continuous stirred tank reactor (12 h of hydraulic retention time (HRT), pH 5.5, 35 °C). While the one-step strategy led to biomass washout and unsuccessful H<sub>2</sub> production, the step-by-step strategy was efficient for biomass adaptation, reaching acceptable hydrogen yields (0.4±0.1 mol<sub>H<sub>2</sub></sub> mol<sup>-1</sup><sub>glycerol consumed</sub>) around 33 % of the theoretical yield independently of the glycerol concentration. Microbial community structure was investigated by single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) fingerprinting techniques, targeting either the total community (16S ribosomal RNA (rRNA) gene) or the functional

*Clostridium* population involved in H<sub>2</sub> production (hydA gene), as well as by 454 pyrosequencing of the total community. Multivariate analysis of fingerprinting and pyrosequencing results revealed the influence of the feeding strategy on the bacterial community structure and suggested the progressive structural adaptation of the community to increasing glycerol concentrations, through the emergence and selection of specific species, highly correlated to environmental parameters. Particularly, this work highlighted an interesting shift of dominant community members (putatively responsible of hydrogen production in the continuous stirred tank reactor (CSTR)) according to the gradient of glycerol proportion in the feed, from the family Veillonellaceae to the genera *Prevotella* and *Clostridium* sp., putatively responsible of hydrogen production in the CSTR.

**Keywords** Acidogenic sludge adaptation · Biohydrogen · Glycerol · Dark fermentation · Community structure · Pyrosequencing

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## Introduction

Hydrogen is an energetically promising alternative, mainly because it is an energetic vector that allows electricity generation with high efficiency, has high calorific value per weight unit, and its combustion in the presence of oxygen emits water vapor only (Lattin et al., 2007; Niu et al. 2010). There is a high variety of hydrogen-producing technologies that require the use of external non-renewable energy, representing a strong economical and environmental disadvantage (Elam et al. 2003). Hydrogen can also be produced by biological fermentation process, through the use of microorganisms. Dark fermentation is one of the most attractive biological processes compared to photofermentation because a variety of carbon

sources can be used as substrates and hydrogen is produced continuously without depending on solar energy (Das et al. 2008; Guo et al. 2010). Hydrogen production through dark fermentation from wastewaters is environmentally sustainable and economically attractive since it allows both energy recovery and waste mitigation (Khanal et al. 2004).

During the last decade, glycerol generation as a by-product of biodiesel production has significantly increased. The annual growth of biodiesel market is estimated to reach 42 % by 2016 (Yang et al., 2013; Siles et al. 2010). Therefore, crude glycerol production exceeds the current commercial demand of purified glycerol since the last decade and has been recently proposed as a carbon source for bioenergy production (Singhabhandhu and Tezuka 2010). It is a potential promising feedstock for hydrogen production, since it is a simple substrate that is easily biodegradable during glycolysis by an oxidative pathway (Siles et al. 2010; Varrone et al. 2013).

Several microbial glycerol degradation routes producing hydrogen as a co-product have been suggested, including ethanol, acetate, butyrate, and formate pathways, but they have still not been clearly elucidated (Da Silva et al., 2009; Trchounian and Trchounian 2009). Most previous studies used pure microbial cultures such as *Enterobacter aerogenes*, obtaining hydrogen yields from 0.69 to 0.89 mol<sub>H<sub>2</sub></sub> mol<sup>-1</sup><sub>glycerol</sub> (Ito et al. 2005; Sakai et al., 2007; Akutsu et al. 2009; Markov et al., 2011; Wu et al. 2011). However, to make the process economically profitable, operation costs must be lowered, for instance, by working under non-sterile conditions and using mixed microbial cultures. A limited diversity of adapted microorganisms can be selected by specific operating conditions, such as the type of substrate (Ito et al. 2005; Seifert et al. 2009) or the hydraulic retention time (HRT). When mixed cultures coming from an anaerobic digester were used in continuous systems with low HRT, low hydrogen yields were reported (0.05 mol<sub>H<sub>2</sub></sub> mol<sup>-1</sup><sub>glycerol</sub>), but higher yields were observed after inoculum pretreatment through thermal shock (0.11–0.41 mol<sub>H<sub>2</sub></sub> mol<sup>-1</sup><sub>glycerol</sub>) (Temudo et al. 2008). However, this type of pretreatment is unsuitable at industrial scale because it implies an extra energetic cost and a lack of robustness of the biomass to face operational failures (Ren et al. 2008; Akutsu et al. 2009; Seifert et al. 2009; Varrone et al. 2013). Therefore, it is interesting to study a glycerol adaptation strategy of the mixed culture without heat-pretreatment of the inoculum.

The success of biohydrogen production strongly depends on species composition and interactions within the bacterial community (Rafrafi et al. 2013). Investigation of the dynamics of the microbial community diversity in mixed cultures provides new insights to understand the community-level adaptations, that could help controlling the process stability (Tolvanen et al., 2011; Cabrol and Malhautier 2011; Pu et al., 2014). Despite some well-known methodological biases (e.g., preferential amplification, co-migration of different

species, multiple copies of the targeted gene with different sequences) (Muyzer and Smalla; 1998), fingerprinting techniques such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-single-strand conformation polymorphism (SSCP) are useful to rapidly evidence changes in community structure of dominant species associated with operating and/or functional changes in hydrogen-producing reactors (Tolvanen et al., 2011; Rafrafi et al. 2013). In addition, next-generation high-throughput sequencing tools provide deeper characterization of the community including minor species (Goud et al. 2012; Laothanachareon et al. 2014), which are known to play a key functional role in hydrogen production (Rafrafi et al. 2013).

The analysis of key functional genes involved in H<sub>2</sub> production is a smart process monitoring tool, enabling to adjust operating parameters during the operation so as to enhance the growth of targeted microorganisms responsible for hydrogen production (Chen et al. 2006; Tolvanen et al., 2011). Given that the most abundant hydrogen producers have been mainly affiliated to the *Clostridium* genus, special attention has been paid to monitor the abundance and diversity of clostridial *hydA* gene coding for the Fe–Fe hydrogenase enzyme, through the development of specific primers (Quéménéur et al. 2011). Several authors reported the strong correlation between the hydrogen production performance and the abundance and expression level of clostridial hydrogenase gene and transcript (Chen et al. 2006; Quéménéur et al. 2011; Winkler et al., 2013)

With the purpose of broadening the range of potential substrates for hydrogen production, we evaluated the conversion of glycerol to hydrogen, using anaerobic sludge (previously adapted to glucose) in chemostat operated at low HRT without thermal pretreatment. The aim of this work is to study the adaptation of the biomass to glycerol substrate, according to different strategies, either a one-step strategy (abrupt substrate change from glucose to glycerol) or a step-by-step strategy (progressive increase of glycerol concentration). The adaptation process was evaluated at both functional and microbial levels through 16S ribosomal RNA (rRNA) and *hydA* genes fingerprinting and pyrosequencing, in order to assess the relationships between operating conditions, functional performance, and community structure.

## Material and methods

### Operating conditions and experimental design

Experiments were carried out in a 2-L (working volume) continuous stirred tank reactor (CSTR), under anaerobic conditions. pH and temperature were controlled and maintained at 5.5 and 37 °C, respectively, and the agitation rate was maintained at 240 rpm. The reactor was inoculated at 4 g<sub>VSS</sub>L<sup>-1</sup>

with sludge from a full-scale anaerobic digester (UASB) treating wastewater from a tobacco plant, with a methanogenic activity of  $0.17 \text{ g}_{\text{COD}}\text{CH}_4 \cdot \text{g}^{-1}_{\text{VSS}}\text{day}^{-1}$ . Before inoculation, sludge was washed and sieved (1-mm pore) to disrupt the granules. The hydraulic retention time (HRT) was set at 12 h as previously reported by Tapia-Venegas et al. (2013).

After inoculation, the system was kept in a batch configuration with glucose feeding for 24 h before the continuous culture began. Two different feeding strategies were evaluated for replacing glucose by glycerol substrate, as shown in Table 1. In the one-step strategy, glucose was replaced abruptly by continuous glycerol feeding at  $5.2 \pm 0.1 \text{ g L}^{-1}$  from day 0. In the step-by-step strategy, after an initial continuous glucose-feeding phase at  $5.1 \pm 0.3 \text{ g L}^{-1}$  (stage 0), the reactor was fed with a mixture of glucose (at  $2.4 \pm 0.1 \text{ g L}^{-1}$ ) and glycerol at increasing concentrations (from  $2.0 \pm 0.1$  to  $5.0 \pm 0.1 \text{ g L}^{-1}$ ) during stages 1 to 4 (Table 1). Finally, the reactor was fed only with glycerol at  $5.0 \pm 0.1 \text{ g L}^{-1}$  (stage 5). The synthetic feeding medium was supplemented with salts and minerals according to Tapia-Venegas et al. 2013. Every stage was maintained for approximately 20 HRTs after reaching a steady state in terms of hydrogen yield (i.e., once the relative standard deviation of  $\text{H}_2$  yield was kept around 21 % on average).

### Chemical analysis

Chemical oxygen demand (COD) and volatile suspended solids (VSS) were measured according to Standard Method 5220D. Glucose was measured by dinitrosalicylic acid method and glycerol by HPLC (Bio-Rad HPX-87-H column, Bio-Rad Laboratories, Hercules, CA, USA). Volatile fatty acids (VFA) and ethanol were quantified by gas chromatography, using respectively a GC-8A (Shimadzu, Kyoto, Japan) chromatograph equipped with an ID GP 60/80 Carbowax C/0.3 % Carbowax 20 M/0.1 %  $\text{H}_3\text{PO}_4$  packaged column (Sigma Aldrich, St Louis, MO, USA) and a Clarus 500 chromatograph

(PerkinElmer, Waltham, MA, USA) equipped with a wide bore, semi-capillary, equity 1 column (Sigma-Aldrich, St. Louis, MO, USA). The biogas flow was measured by water displacement and the biogas composition was determined by gas chromatography in a PerkinElmer Clarus 500 chromatograph equipped with a Hayesep Q 4  $\text{m} \times 1/8$ "OD column (VICI, Bandera, TX, USA).

### Bacterial community characterization

Biomass samples from each stage were collected each period and centrifuged at 10,000 rpm for 10 min. Total genomic DNA was extracted from the pellet using Power Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) and stored at  $-20^\circ\text{C}$  before use. Community characterization was carried out at both phylogenetic (16 rRNA gene) and functional (*hydA* gene) levels, through different methodological approaches (DGGE, SSCP, and pyrosequencing), in order to compare different molecular techniques and strengthen the conclusions drawn from each technique.

**16S rRNA gene analysis by DGGE** The V3 region from the ribosomal 16S RNA (around 200 bp) was amplified by PCR for DGGE analysis, using Immolase DNA-Polymerase (Bioline, London, UK), with w49F and w104R bacterial primers. A 40-bp GC clamp was added at the 5' end of the forward primer to perform the DGGE analysis. The PCR products (500 ng) were separated through denaturing gradient gel electrophoresis (DGGE) on a 10 % polyacrylamide gel with a linear gradient ranging from 20 to 70 %, using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc, Hercules, CA, USA) and the gels were stained with SYBR®Green (Invitrogen, Life Technology, Carlsbad, CA, USA) and photographed for further analysis.

**16S rRNA gene analysis by SSCP** The same V3 region from the 16S rRNA gene was amplified by PCR for SSCP analysis,

**Table 1** Experimental design of two types of feeding strategy: one-step strategy and step-by-step strategy

Experiment	Stage	Number of HRTs	Period (days)	Glycerol percentage (% COD)	Glucose percentage (% COD)	Total COD ( $\text{g}_{\text{COD}}\text{L}^{-1}$ )
One-step strategy	–	20	0–10	100	0	$6.3 \pm 0.1$
Step-by-step strategy	S0	22	0–11	0	100	$5.4 \pm 0.3$
	S1	30	12–27	52	48	$5.0 \pm 0.3$
	S2	32	28–44	60	40	$6.0 \pm 0.4$
	S3	28	45–59	65	35	$7.5 \pm 0.4$
	S4	32	60–76	71	29	$8.6 \pm 0.3$
	S5	34	77–94	100	0	$6.1 \pm 0.1$

using *Pfu* Turbo DNA polymerase (Stratagene) and a 5'-fluorescein phosphoramidite labeled reverse primer, with w49F and w104R bacterial primers. The PCR products were 5- to 2000-fold diluted and 1  $\mu$ L was mixed with 18.8  $\mu$ L of formamide and 0.2  $\mu$ L of internal standard Gene Scan ROX (Applied Biosystems, Foster City, CA, USA). Samples were heat-denatured at 95 °C for 5 min and immediately cooled in ice. SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) with 50-cm-long capillary tubes filled with a non-denaturing 5.6 % conformation analysis polymer (Applied Biosystems). Samples were eluted at 12 kV and 32 °C for 30 min.

**Hydrogenase gene analysis by DGGE** A fragment of the *hydA* gene (around 250 bp) coding for clostridial Fe–Fe hydrogenase was amplified by PCR for DGGE analysis using Platinum Taq-DNA-Polymerase (Invitrogen, Life Technology, Carlsbad, CA, US) with *hydA*-F and *hydA*-R primers (Quéméneur et al. 2011). Three PCR reactions were carried out in parallel and the PCR products were mixed and concentrated through the use of the gel/PCR purification mini kit (Favorgen Biotech Corp, Ping-Tung, Taiwan). The PCR products were separated by DGGE using the same protocol as previously described for *16S rRNA* gene amplicons, excepted that a 8 % polyacrylamide gel was used.

**Hydrogenase gene analysis by SSCP** The same clostridial *hydA* gene fragment was amplified by PCR for SSCP analysis using *Pfu* Turbo DNA polymerase (Stratagene) and a 5'-fluorescein phosphoramidite labeled reverse primer, with *hydA*-F and *hydA*-R primers (Quéméneur et al. 2011). The PCR products were separated by SSCP using the same protocol as previously described for *16S rRNA* gene amplicons, the only difference being that samples were eluted at 12 kV and 32 °C for 64 min.

**Pyrosequencing analysis** Genomic DNA samples were sent to Molecular Research Laboratory (Shallowater, TX, USA) for determination of the community composition using 454-pyrosequencing of the V4–V5 region of the *16S rRNA* gene which captures most of the bacterial and archaeal diversity (Wang and Qian 2009). Sequences were deposited in the National Center for Biotechnology Information (NCBI) database under the accession number SRP058142.

### Numerical and statistical analysis

Each metabolite concentration was converted to COD and expressed in percentage of total measured metabolites. The hydrogen yield was calculated in  $\text{mmol}_{\text{H}_2}\text{g COD}^{-1}_{\text{consumed}}$ . Hydrogen yield was also expressed as a percentage of the theoretical yield when comparing the different substrates. The theoretical yield for each intermediary stage was

calculated as a combination of the theoretical  $\text{H}_2$  yields of pure glucose ( $4 \text{ mol}_{\text{H}_2} \text{mol}^{-1}_{\text{glucose consumed}}$ ) and glycerol ( $1 \text{ mol}_{\text{H}_2} \text{mol}^{-1}_{\text{glycerol consumed}}$ ), weighted by their respective proportions in the feeding mixture (Ito et al. 2005; Akutsu et al. 2009).

DGGE profiles were aligned and analyzed with GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium), to obtain the matrix of relative band intensity according to band position. The DGGE band intensities were normalized by profile before further analysis.

Raw SSCP data were aligned with the internal standard ROX to correct any change in the electrophoretic motility between run and SSCP peak areas were normalized before statistical analysis with the *Stat Fingerprints* library from R (R Development Core Team 2009).

All band- or peak-intensity matrices from (respectively) DGGE and SSCP analyses were further computed using R software. The pair-wise similarity between community profiles was calculated by the Bray-Curtis index and the samples were clustered by unweighted pair group method with arithmetic mean (UPGMA) dendrogram using the *cluster* library. Principal component analysis (PCA) was computed with the *vegan* library. Correlations between the bacterial community structure and various (normalized) environmental variables (EV) were investigated by fitting the EV onto the ordination (*envfit* function from *vegan*). The significance was tested by a permutation test. The most discriminant EV ( $p$  value < 0.05) were represented on the PCA plot as arrows whose direction and length indicate, respectively, (i) the direction of the increasing EV gradient and (ii) the magnitude of the correlation between the EV and the ordination. Moreover, a bubble representation was superimposed on the PCA plot, by allocating to each sample a symbol size proportional to the percentage of glycerol in the feed at the corresponding sampling date.

Raw 454-pyrosequencing data were processed through the Mothur pipeline. Sequence data were first trimmed to remove the sequences containing wrong barcodes or primers, as well as the sequences shorter than 200 bp and those containing long homopolymers. Putative chimeras were removed with the *uchime* function of Mothur. Finally, 38,812 high-quality sequences were conserved from the initial data set. The number of sequences ranged from 2820 to 9432 per sample. Sequences were randomly subsampled to the minimal number of sequences per sample (i.e., 2820). Sequences were aligned against the Silva v119 database. Sequences were grouped in operational taxonomic units (OTUs) at 97 % similarity level, resulting in 436 different OTUs. The OTU abundances were standardized for each sample before further analysis. Taxonomic affiliation up to the genus level was realized using Mothur by comparison with the Silva v119 database. To assign the sequences at the species level, a BLAST analysis was carried out. PCA was computed on standardized OTU abundance table using the same procedure as for fingerprinting



patterns. The discriminant OTUs displaying most of the variance in community ordination were identified by the *envfit* function from *vegan*. The fitting relationship significance was tested by a permutation test. The 20 most abundant OTUs were represented on the PCA plot as arrows and the most discriminant ones ( $p$  value < 0.05) were highlighted in bold.

## Results

### Glycerol degradation efficiency and biomass yield

With the one-step strategy, the microorganisms coming from the anaerobic sludge inoculum were rapidly washed out of the reactor. The biomass yield dropped from 1 to 0.04 g<sub>VSS</sub>L<sup>-1</sup> in 10 days. The biomass was not able to degrade glycerol and to produce biogas under these conditions.

With the step-by-step strategy, the glycerol degradation efficiency increased from 52.8±3.5 % (for glycerol concentrations between 2 and 3 g L<sup>-1</sup>), to 68.5±2.7 % (for glycerol concentrations between 4 and 5 g L<sup>-1</sup>) (Table 2). In all stages where glucose was present in the feed, glucose degradation efficiency remained similar and high (87±3.9 %). The biomass yield in the step-by-step strategy was reduced by 53.9 % between extreme feeding conditions with glucose only (stage 0) and glycerol only (stage 5) substrates (Table 2).

### Hydrogen production performance

Hydrogen productivity in the step-by-step strategy was variable according to the operation stage. The maximum productivity (61.9±12.2 mL<sub>H<sub>2</sub></sub>L<sup>-1</sup> h<sup>-1</sup>) was obtained with glucose as sole substrate at a concentration of 5.4 g<sub>COD</sub>L<sup>-1</sup>. It decreased to 31.4±3.8 % after the first substrate shift, corresponding to the addition of glycerol at 2 g L<sup>-1</sup> in the mixed feed. Afterwards, hydrogen productivity increased up to 46.7±10.3 % with the increase of glycerol availability in the feeding mixture from 3 to 5 g L<sup>-1</sup>, suggesting a progressive adaptation of the microbial community to the changing substrate mixture. However, the lowest hydrogen productivity (24.7±9.1 mL<sub>H<sub>2</sub></sub>L<sup>-1</sup> h<sup>-1</sup>) was obtained during the last stage, once glucose had been completely removed from the feed and glycerol was the sole substrate.

The hydrogen yield decreased from 6.1±1.2 mol<sub>H<sub>2</sub></sub> g<sup>-1</sup><sub>CODconsumed</sub> to 4.6±0.9 mol<sub>H<sub>2</sub></sub> g<sup>-1</sup><sub>CODconsumed</sub> with glucose or a mixture of glucose and glycerol as substrate, respectively (Table 2). The hydrogen yield seemed to be negatively correlated to the proportion of glycerol in the feed mixture. The lowest hydrogen yield (3.2±1.1 mol<sub>H<sub>2</sub></sub> g<sup>-1</sup><sub>CODconsumed</sub>) was obtained with glycerol as sole substrate, corresponding to a 50 % loss compared to the glucose-feeding conditions. However, the comparison of hydrogen yields obtained with different substrates and substrate mixtures is complex, since

**Table 2** Performance indicators of hydrogen production during the different stages of the step-by-step strategy, including glycerol and glucose degradation efficiency, hydrogen yield and productivity, biomass

yield, and by-products production. Average values and standard deviations (in parenthesis) were calculated during steady state of the continuous operation during 20 HTRs

Parameter	Unit	Stages						
		0	1	2	3	4	5	
Glycerol RE	%	–	55.3 (5.9)	50.3 (4.4)	66.6 (4.8)	70.4 (6.1)	69.8 (1.3)	
Glucose RE	%	95.6 (1.4)	86.6 (2.2)	86.6 (1.7)	87.5 (2.0)	87.2 (1.2)	–	
H <sub>2</sub> yield	mmol <sub>H<sub>2</sub></sub> g <sub>COD</sub> <sup>-1</sup>	6.1 (1.2) <sup>a</sup>	4.5 (0.6)	5.4 (1.2)	4.6 (0.9)	3.7 (1.1)	3.2 (1.1) <sup>b</sup>	
H <sub>2</sub> yield	% <sup>c</sup>	29 (6)	31 (5)	40 (9)	35 (6)	28 (8)	36 (12)	
H <sub>2</sub> productivity	mL <sub>H<sub>2</sub></sub> L <sup>-1</sup> h <sup>-1</sup>	61.9 (12.2)	31.4 (3.8)	41.7 (6.2)	50.1 (10.3)	46.7 (10.3)	24.7 (9.1)	
Biomass yield	mg <sub>VSS</sub> g <sub>COD</sub> <sup>-1</sup>	223.5 (26)	210.6 (17)	238.5 (22)	171.7 (23)	129.3 (13)	145.3 (28)	
Acetate	%COD <sub>m</sub>	40.8 (2.1)	39 (1.7)	41.5 (2.2)	36.9 (0.4)	36.3 (0.8)	29.3 (3.4)	
Ethanol	%COD <sub>m</sub>	35.6 (1.2)	35.3 (6.9)	25.7 (1.2)	26.3 (0.1)	23.5 (0.6)	29.8 (1.1)	
Butyrate	%COD <sub>m</sub>	20 (2.3)	9 (4.8)	16.2 (1.5)	19.7 (0.9)	23.1 (0.4)	26.7 (4.2)	
Propionate	%COD <sub>m</sub>	3.6 (0.7)	11.5 (2.5)	8.5 (1.3)	6.2 (1.3)	9.3 (0.3)	5.6 (2.4)	
Valerate	%COD <sub>m</sub>	0	5.2 (0.5)	8.1 (0.6)	10.8 (0.2)	7.9 (0.7)	8.6 (2.5)	
Total COD	g <sub>COD</sub> L <sup>-1</sup>	2.3 (0.2)	3.7 (0.5)	4.8 (0.3)	5.1 (0.1)	5.7 (0.1)	1.5 (0.0)	
A/B	g <sub>COD</sub> g <sub>COD</sub> <sup>-1</sup>	4.13	1.88	1.57	2.05	2.54	1.09	

<sup>a</sup> For pure glucose feeding, this yield corresponds to 1.2±0.2 mol<sub>H<sub>2</sub></sub>mol<sup>-1</sup><sub>glucose consumed</sub>

<sup>b</sup> For pure glycerol feeding, this yield corresponds to 0.4±0.1 mol<sub>H<sub>2</sub></sub>mol<sup>-1</sup><sub>glycerol consumed</sub>

<sup>c</sup> The hydrogen yield is expressed as the percentage of the maximal theoretical yield, depending on the substrate considered (see “Material and Methods”)

Total COD total metabolite concentration expressed in COD; %COD<sub>m</sub> metabolite concentration expressed in percentage of total metabolite concentrations (converted to COD), in the outlet; A/B ratio of acetate to butyrate concentrations, expressed in COD

glucose and glycerol have different theoretical maximal yields. The actual yield was therefore assessed as a percentage of the theoretical maximum yield (as explained in the “Material and Methods” section) and it appeared that the potential for producing hydrogen was maintained within a relatively close range, independently of the substrate ratio, at around  $33\pm 5\%$  of the theoretical yield for all stages.

The hydrogen production performances reached with the step-by-step strategy with glycerol as sole substrate were better than the ones obtained after a direct shift from glucose to glycerol without progressive transition (one-step strategy), which led to unsuccessful  $H_2$  production and a rapid biomass washout. Therefore, the stepwise increase of glycerol ratio in the feed and the progressive decrease of glucose concentration was a successful strategy for the adaptation of biomass to hydrogen production from glycerol.

### By-products of hydrogen production

The main volatile fatty acids and alcohols detected as by-products of hydrogen production during the different operation stages in the step-by-step strategy are presented in Table 2. According to the COD mass balance, the detected by-products represented about 85 % of the COD in the outlet of the reactor for each operation stage. Therefore, the metabolites that were not targeted by our analysis, e.g., 1,3-propanediol, represented less than 15 % of the total COD as previously reported in dark fermentation systems (Gonzales-Pajuelo et al. 2005 and Wu et al. 2011).

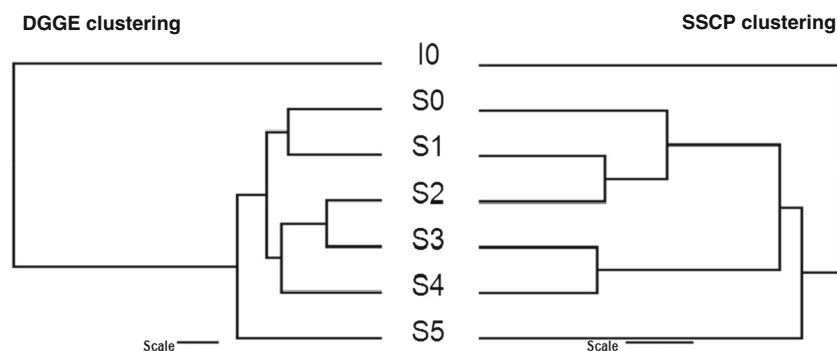
The intermediate product distribution was very similar whatever the inlet substrate composition and concentration. The main intermediate products in all operation stages were acetate and ethanol, representing about  $66\pm 8\%$  of the measured metabolites. Propionate and butyrate were also detected in all cases, although in lower proportions, i.e., less than 27 % of the measured metabolites. Valerate was only detected when glycerol was present in the feed (stages 1 to 5), at very low concentrations (less than 10 %).

### Effect of increasing glycerol concentration on total community structure

According to the UPGMA clustering based on Bray Curtis similarity from 16S rRNA gene DGGE profiles, the anaerobic sludge inoculum I0 (producing methane as end-product) exhibited the most different community structure, and fell clearly apart from all other stages of hydrogen-producing acidogenic community (S0 to S5) (Fig. 1). The strongest community shift occurred between inoculum and  $H_2$ -producing communities, whereas the  $H_2$ -producing communities had a more stable structure, sharing several bands in common (Fig. S1). Their structure was mostly driven by the specific conditions common to stages S0 to S5 and different from the inoculum I0 (i.e., shorter HRT and lower pH).

Within the  $H_2$ -producing communities, the samples clustered according to the substrate type and concentration. A microbial succession was observed along with the progressive substrate change. Samples fed with null or low glycerol concentration (S0 and S1) clustered together, separated from samples fed with higher glycerol concentration (S2 to S4), indicating the influence of the feeding strategy on the community structure and strongly suggesting a progressive adaptation of the community to increasing glycerol concentrations. Interestingly, the sample corresponding to pure glycerol feeding as sole substrate (S5) exhibited the most different community structure, clearly discriminated from the samples fed with a mixture of glucose and glycerol (Fig. 1). Therefore, the metabolic changes associated to the shift from mixed to pure substrate could be explained by the changes in the community structure. However, the community changes associated with the increase of glycerol concentration within the  $H_2$ -producing communities revealed by DGGE were mostly linked to slight modifications of band intensities, rather than appearance/disappearance of bands.

The effect of increasing glycerol concentration on total community structure revealed by DGGE was strongly confirmed by the SSCP patterns (Fig. 1 and Fig. S1). Sample



**Fig. 1** UPGMA clustering based on Bray Curtis similarity from 16S rRNA gene-DGGE (left) and 16S rRNA gene-SSCP (right) profiles. I0 is the methanogenic inoculum. S0 to S5 correspond to steady states of the

progressive increase of glycerol concentration in the step-by-step strategy, as stated in Table 1. The scale bar indicates 5 % of dissimilarity

clustering was extremely similar, despite some slight differences concerning the intermediate-concentration sample (S2). This consistency between SSCP and DGGE profiles, while the discriminating power of these techniques do not rely on the same characteristics of DNA molecules, confirms the robustness of our methodological approach. It also validates the common assumption made for the analysis of dominant species in low-diversity communities, i.e., that one DGGE band corresponds to one single ribotype and that relative band intensity is proportional to ribotype concentration, even though this assumption is subjected to conceptual limits and potential methodological biases (Loisel et al. 2006). The effect described here on the community structure is stronger than the variation that may be explained solely by the choice of the molecular technique used.

### Effect of increasing glycerol concentrations on hydrogenase-containing clostridial population

While the 16S rRNA gene approach is not specifically restricted to hydrogen-producing microorganisms in the mixed cultures, the functional *hydA* approach should give a better estimate of clostridial-type hydrogen producers (Québécois et al. 2011). The presence of bands in all *hydA*-PCR-DGGE profiles reveals the presence of hydrogen producers from clostridial populations in all samples. Even in the methanogenic inoculum sample where H<sub>2</sub> production may be low, a small fraction of 16S rRNA gene sequences were affiliated to clostridia from pyrosequencing data (Tables 3 and 4).

The clustering obtained from *hydA*-DGGE and 16S rRNA gene-DGGE profiles were highly similar (Fig. 2). Despite some minor clustering differences for intermediate-concentration samples, both methods reveal the same microbial succession and adaptation in response to increasing glycerol concentration. Therefore, the *hydA*-containing clostridial population and the total bacterial community shifted in a similar way as glycerol concentration increased. However, the structural effect of the highest glycerol concentrations seems to be more important on the *hydA*-containing fraction of the community than on the total community, since samples corresponding to stages S4 and S5 appeared to be more discriminated in *hydA*-DGGE clustering than in 16S rRNA gene-DGGE clustering.

The correspondence of sample clustering between 16S rRNA- and *hydA*-targeting fingerprint profiles has also been verified with the SSCP methodology (Fig. S2). *Clostridium* population dynamics therefore reflect the total community dynamics in response to glycerol concentration changes, but this does not imply that *Clostridium* populations would be dominant in the community.

### Link between community structure and environmental variables

Once verified the consistency between DGGE and SSCP methods as well as 16S rRNA and *hydA* genes analysis, the relationship between community structure and environmental variables was only presented for the 16S rRNA gene-DGGE approach. The PCA representation (explaining more than 90 % of the sample variance), revealed the following: (i) the strong differentiation of the inoculum sample (according to PC1 axis), (ii) the community dynamism clearly differentiating the samples corresponding to low (S0, S1) and high (S2 to S4) glycerol concentrations (according to PC2 axis), and (iii) the clear divergence of the sample corresponding to pure glycerol feeding as sole substrate (S5), all confirming that the influence of glycerol concentration on the community structure was level dependent (Fig. 3a).

Statistically significant correlations were evidenced between the PCA ordination and several environmental variables ( $p$  values < 0.05). Butyrate and valerate production, as well as hydrogen yield when expressed as a percentage of the theoretical yield, were positively correlated to the community structure at high glycerol concentration. Ethanol and acetate production, as well as biomass yield, were positively correlated to intermediate stage community structures, corresponding to medium glycerol concentrations. In contrast, the acetate-to-butyrate ratio (A/B) was associated to low glycerol concentration communities. The correlation between propionate production and community structure was not significant.

### Identification of bacterial populations by 454-pyrosequencing

The microbial populations for each stage of the step-by-step increase strategy were identified by 454 pyrosequencing (Table 4). The samples were represented on PCA ordination according to their microbial composition (Fig. 3b). The correlation between the PCA ordination and some discriminant OTUs was evidenced statistically, as discussed below.

After subsampling to 2820 sequences per sample, the highest diversity and evenness level were found in the methanogenic inoculum sample (240 OTUs). No strongly dominant genus or family was found in the inoculum, where the most abundant community members belonging to the *Cloacomonas* genus, Rikenellaceae family and Xanthomonadaceae family represented 14.5, 10.5, and 15.4 % of the total number of sequences, respectively. These community members were not conserved under the hydrogen-producing conditions. It is interesting to point out that, even though the inoculum came from an anaerobic digester with methanogenic activity, only two OTUs were identified as methanogenic Archaea (belonging to *Methanosaeta* and *Methanobacterium* genera),

**Table 3** Hydrogen yields from glycerol fermentation reported in the literature. The main operating conditions and the most abundant metabolites are also indicated

	Inoculum	Inoculum treatment	Operating conditions	Glycerol concentration (g L <sup>-1</sup> )	H <sub>2</sub> yield (mol <sub>H<sub>2</sub></sub> mol <sup>-1</sup> glycerol)	H <sub>2</sub> yield (mmol <sub>H<sub>2</sub></sub> g <sup>-1</sup> COD)	Main metabolites	Reference
Mixed cultures	Anaerobic sludge and acidogenic sludge	Low HRT	Chemostat HRT 10 h pH 8.0 T 30 °C	4	0.05	0.4	Ethanol and 1,3 propanediol	Temudo et al. (2008)
	Kitchen Waste	Thermal shock	Batch pH 6.5 T 35 °C	4	0.11	1.0	Aceticacid and 1,3 propanediol	Akutsu et al. (2009)
	Soil	Thermal shock	Batch pH 6.5 T 35 °C	3	0.28	2.5	Acetate and ethanol	Selembo et al.(2009)
	Anaerobic sludge	Thermal shock	Batch pH 6 T 37 °C	10	0.41	3.7	1,3 propanediol and lactate	Seifert et al.(2009)
	Activity sludge from wastewater treatment plant	Enrichment	Batch PH initial 8 T 37 °C	15	0.9	8.0	NR	Varrone et al.(2013)
Pure Cultures	Anaerobic sludge	Low HRT	Chemosta HRT 12 h pH 5.5 T 37 °C	5	0.4	3.2	Ethanol and acetate	This study
	<i>Klebsiella</i> Ssp.	NT	Batch pH 6.0 T 35 °C	50	0.35	3.1	1,3 propanediol and ethanol	Wu et al.(2011)
	<i>Enterobacter aerogenes</i>	NT	Batch pH 6.8 T 37 °C	10	0.89	7.9	Ethanol	Ito et al.(2005)
	<i>Enterobacter aerogenes</i> HU-101	NT	Batch pH 6.3 T 37 °C	10	0.62	5.5	Ethanol	Nakashimada et al.(2002)
	<i>Clostridium acetobutylicum</i> ATCC 824	NT	Chemostat (HRT NR) pH 6.5 T 35 °C	10	<0.1	0	1,3 propanediol and butyrate	González-Pajuelo et al. (2005)
	<i>Klebsiella pneumoniae</i>	NT	Batch pH T NR	20	0.53	4.7	Ethanol	Liu and Fang (2007)

NR: not reported; NT: no treatment



**Table 4** Identification of the bacterial populations by pyrosequencing of 16S rRNA gene. Percentage in communities are displayed. Only the groups of sequences with a relative abundance higher than 0.1 % in at

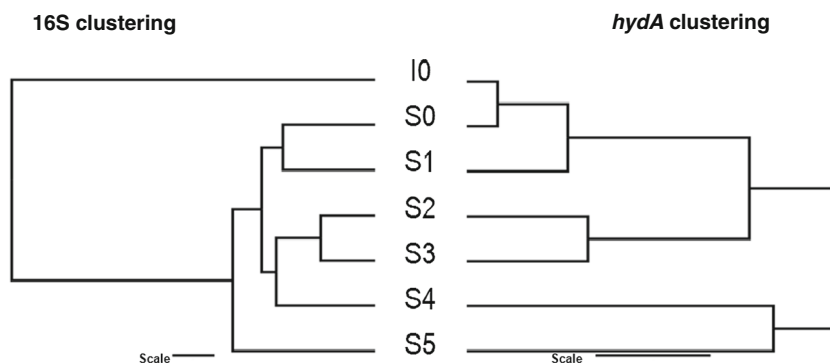
least one sample are presented. A gray scale proportional to sequence abundances is used for a better readability

		Samples	I0	S0	S1	S2	S3	S4	S5	Total		
		rarefied # sequences	2820	2820	2820	2820	2820	2820	2820	19740		
		Number of OTUs	240	53	78	68	69	59	39	436		
Higher taxonomic rank	Genus	Number of OTUs	Sequence relative abundance, per sample (% all sequences)							Most representative OTU		
Phylum Bacteroidetes	<i>Prevotellaceae</i>	<i>Prevotella</i>	9	-	35.2	42.0	45.1	47.6	64.0	32.9	OTU 1	
	<i>Rikenellaceae</i>		1	10.5	-	-	-	-	-	-	OTU 6	
	<i>Bacteroidaceae</i>	<i>Bacteroides</i>	2	-	-	1.2	1.7	5.2	0.5	0.1	OTU 14	
	<i>Porphyromonadaceae</i>	<i>Proteiniphilum</i>	9	3.0	-	-	-	-	-	-	OTU 19	
		Other Bacteroidales	9	1.3	0.8	0.1	0.2	1.5	0.0	-		
		<i>Cryomorphaceae</i>	<i>Fluviicola</i>	2	5.7	-	-	-	-	-	-	OTU 13
		Other Flavobacteriales	10	1.2	0.1	0.5	0.7	1.3	0.4	-		
		<i>Chitinophagaceae</i>		2	2.4	-	-	-	-	-	-	OTU 16
		Other <i>Spingobacteriales</i>	4	0.5	-	-	-	0.1	-	-		
		Other Bacteroidetes	18	2.7	-	0.1	0.3	1.8	0.2	0.3		
Phylum Firmicutes	<i>Veillonellaceae</i>		28	-	32.0	9.0	12.5	25.1	17.0	15.1	OTU 2	
	<i>Bacilli (Bacillales and Lactobacillales)</i>		16	1.6	1.0	0.5	1.3	0.5	0.9	0.4		
	<i>Ruminococcaceae</i>		13	0.0	0.0	0.1	0.4	1.5	0.2	0.0		
	<i>Clostridiaceae</i>	<i>Clostridium sp.</i>	7	0.1	0.4	8.6	5.4	2.1	8.0	40.9	OTU 3	
	<i>Clostridiaceae</i>	<i>Clostridium beijerinckii</i>	13	-	0.3	17.5	11.8	0.1	0.5	0.9	OTU 4	
	<i>Clostridiaceae</i>	<i>Clostridium sp.</i>	7	-	7.0	6.5	4.6	-	0.0	0.1	OTU 9	
		Other <i>Clostridia unclassified</i>	55	0.5	2.1	7.7	7.0	0.6	1.6	1.6	OTU 11	
Phylum Spirochaetae	<i>Spirochaetes</i>	<i>Cloacamonas</i>	2	14.5	-	-	-	-	-	-	OTU 5	
		Other <i>Spirochaetes</i>	4	0.4	-	-	-	-	-	-		
Phylum Proteobacteria	<i>Rhodobiaceae</i>	<i>Parvibaculum</i>	1	2.8	-	-	-	-	-	-	OTU 17	
	<i>Acetobacteraceae</i>	<i>Acetobacter</i>	2	0.0	16.8	0.0	0.3	0.1	0.1	3.8	OTU 12	
		Other <i>Alphaproteobacteria</i>	27	4.6	0.0	-	0.1	0.0	-	0.0		
	<i>Comamonadaceae</i>		10	5.7	0.3	0.0	0.3	0.3	-	-	OTU 15	
	<i>Hydrogenophilaceae</i>	<i>Thiobacillus</i>	1	2.0	-	-	-	-	-	-		
		Other <i>Betaproteobacteria</i>	25	4.3	0.0	0.0	0.1	0.4	0.0	-	OTU 18	
		<i>Deltaproteobacteria</i>	11	1.8	-	-	-	-	-	-		
		<i>Enterobacteriaceae</i>		16	-	1.7	1.3	2.0	3.3	4.9	3.4	OTU 7
		<i>Moraxellaceae</i>	<i>Perlucidibaca</i>	7	2.8	0.9	0.5	0.4	0.8	0.3	0.1	OTU 20
		<i>Xanthomonadaceae</i>		18	15.4	0.2	-	-	0.2	-	-	OTU 10
	Other <i>Gammaproteobacteria</i>		15	1.6	0.7	0.0	0.2	0.4	0.1	0.0		
Phylum Actinobacteria	<i>Corynebacteriaceae</i>	<i>Corynebacterium</i>	3	-	0.2	4.1	5.2	6.8	0.9	0.1	OTU 8	
		Other <i>Actinobacteria</i>	15	1.1	0.1	0.1	0.2	0.2	0.1	0.1		
Other Bacteria	<i>Thermotogaceae</i>		1	1.6	-	-	-	-	-	-		
	<i>Planctomycetes</i>		8	0.7	-	-	-	-	-	-		
	Unclassified		64	10.2	0.0	-	0.0	0.1	0.1	0.1		
Archaea	<i>Euryarchaeota</i>	<i>Methanosaeta</i>	1	0.0	-	-	-	-	-	-		
	<i>Euryarchaeota</i>	<i>Methanobacterium</i>	1	1.0	-	-	-	-	-	-		

representing less than 1 % of the inoculum community. As expected, these methanogenic Archaea were not found in hydrogen-producing samples.

At the different stages of the operation, the number of OTUs ranged from 39 to 78, suggesting that the specific operating conditions imposed in acidogenic reactors (i.e., lower

**Fig. 2** UPGMA clustering based on Bray Curtis similarity from *16S rRNA* (a) and *hydA* (b) DGGE profiles. I0 is the methanogenic inoculum. S0 to S5 correspond to steady states of the progressive increase of glycerol concentration in the step-by-step strategy, as stated in Table 1. The scale bar indicates 5 % of dissimilarity

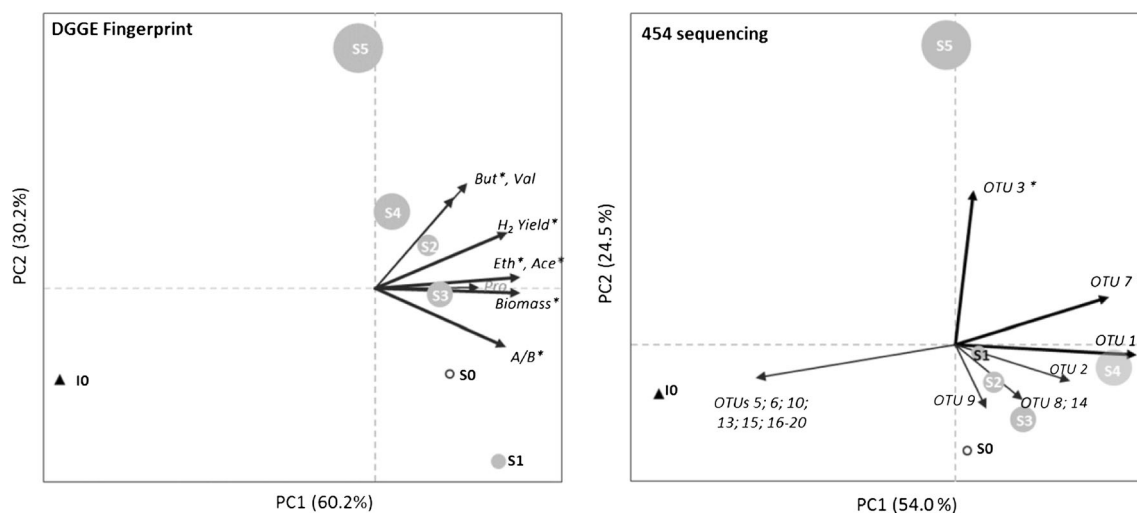


pH and lower HRT) led to species selection and community simplification, in comparison with the inoculum community.

Interestingly, all the abundant community members in hydrogen-producing communities were very rare in the inoculum and only emerged during hydrogen production. The most abundant genus under all feeding strategies was *Prevotella* (OTU 1), which was particularly dominant (64 % of the community) in stage 4, i.e., at the highest glycerol and glucose concentrations in the feed. Members of the Veillonellaceae family (OTU 2) were also important members of the community when glycerol was present in the feed (representing from 9.0 to 25.1 % of the community), but they were even more abundant with no glycerol (32.0 % in stage 0). Similarly, *Acetobacter* (OTU 12) seems to be specific of hydrogen production from pure glucose substrate and represented 16.8 % of the total community. Meanwhile, *Acetobacter* sp. abundance remained below 3.5 % of the community when glycerol was present in the feed. Therefore, sample S0—fed

with glucose only—differentiates from other samples through a highest percentage of sequences affiliated to the *Veillonellaceae* family and the *Acetobacter* genus. On the ordination from sequencing data (Fig. 3, right panel), a large number of OTUs were associated with low or intermediate glycerol concentration communities.

*Clostridium* spp., including OTU 9, were present as minor community members, i.e., between 4.6 and 7.0 % of the community, either with glucose substrate only, or with a mixture of glucose and low glycerol concentrations. *Clostridium beijerinckii* (OTU 4) and most of the Clostridiaceae members unidentified at the genus level were more abundant when glycerol was present in the feed, at low concentration (stages S1 and S2). The abundance of these *Clostridium* species decreased with increasing glycerol concentrations. Some *Clostridium* sp., including OTU 3, remained at low abundance (<8.6 % of the community) during all the H<sub>2</sub>-producing stages, while



**Fig. 3** Principal component analyses (PCA) of microbial community patterns generated by *16S rRNA*-DGGE analysis (left) and 454 pyrosequencing (right). In the left panel, the arrows represent the significant (\*) correlations with environmental variables (*But* butyrate, *Val* valerate, *Eth* ethanol, *Ace* acetate; *H<sub>2</sub> Yield* hydrogen yield expressed in percentage of the theoretical yield, *Biomass* biomass yield, *A/B* acetate to butyrate ratio), calculated as stated in Table 2. In the right panel, the arrows

represent the most discriminant OTUs from 454 sequencing. In both representations, the size of the circles is proportional to glycerol percentage in the feed, I0 is the methanogenic inoculum, and S0 to S5 correspond to steady states of the progressive increase of glycerol concentration in the step-by-step strategy, as stated in Table 1. The percentages of variance expressed in the first and second axes of PCA ordinations are displayed

they were highly dominant (40.9 % of the community) when glycerol was the only substrate at high concentration (S5). During stage S5, these sequences were more abundant than the previously dominant *Prevotella*. This specific *Clostridium* OTU 3 drove the community structure segregation during stage 5 and might be responsible for the high H<sub>2</sub> yield performance during stage S5.

Members of the Enterobacteriaceae family (OTU 7) were statistically significant drivers of the community differentiation during stages 4 and 5 (i.e., when glycerol concentration was the highest), even though they represented less than 4.9 % of the community. Therefore, these members of the Enterobacteriaceae family can be considered as putative hydrogen producers under high glycerol concentrations.

Some community members belonging to *Bacteroides* and *Corynebacterium* genus (OTU 8 and OTU 14) appeared transitory under intermediate glycerol concentrations, but their abundance profiles did not follow the glycerol concentration gradient. Contrary to other studies (Goud et al. 2012), Bacillales and Lactobacillales were very minor species, representing always less than 1 %.

## Discussion

### Functional performances

In the present study, the one-step strategy led to biomass washout and unsuccessful H<sub>2</sub> production, but the step-by-step approach was an efficient strategy for biomass adaptation. Step-by-step strategy showed a reduction of biomass yield with glycerol substrate compared to the glucose-feeding conditions (53.9 % loss factor). This reduction in biomass yield is in agreement with Temudo et al. (2008), who compared glucose versus glycerol fermentation for hydrogen production and observed a 50 % decrease of the biomass yield with glycerol. These differences can be explained by the higher amount of ATP synthesized with glucose than glycerol, which negatively affects the microbial growth, since the growth rate is proportional to the ATP quantity that can be obtained from the mineralization of the energy source in the growth medium.

The hydrogen yields obtained in the present study with glycerol substrate and step-by-step strategy are comparable with previous results (Table 3). With glycerol substrate, the use of mixed cultures enabled to reach hydrogen yields between 0.4 and 8 mmolH<sub>2</sub>g<sup>-1</sup>COD consumed (4–89 % of theoretical yield), while the H<sub>2</sub> yields ranged from 3.1 to 7.9 mmol H<sub>2</sub>g<sup>-1</sup>COD<sub>consumed</sub> with pure cultures of *Enterobacter* and *Klebsiella* genus (34–88 % of theoretical yield) (Table 3). The experimental hydrogen yields reached with pure cultures and batch systems are generally higher than

the ones reported for mixed cultures. Therefore, it is difficult to compare productivities obtained in this study with literature because few studies have been carried out in a continuous system with glycerol as substrate and also had low yields of hydrogen (Table 3).

The hydrogen production performances reached with the step-by-step strategy with glycerol as sole substrate were better than the ones obtained after a direct shift from glucose to glycerol without progressive transition (one-step strategy). Therefore, the stepwise increase of glycerol ratio in the feed and the progressive decrease of glucose concentration was a successful strategy for the adaptation of biomass to hydrogen production from glycerol. Varrone et al. (2013) observed comparable results with an adaptation strategy called “Enrichment of activity” in successive batch systems with glycerol as sole carbon source. An aerobic sludge was inoculated and the most effective microbial populations were successively selected on the basis of their higher hydrogen production and fresh medium was supplemented after substrate exhaustion, for several months. The authors reported yields of 7.2 mmolH<sub>2</sub>g<sup>-1</sup>COD<sub>consumed</sub> with an initial glycerol concentration of 20 g L<sup>-1</sup>. To date, no strategy of adaptation to increasing substrate concentrations has been reported in the context of hydrogen production from glycerol in continuous systems.

In the present study, the main metabolites obtained during the step-by-step strategy were ethanol, acetate, and butyrate. The proportions of the fermentation products were similar in all stages, and comparable to those reported for mixed cultures from glucose and glycerol (Temudo et al. 2008). In the literature, the main metabolites obtained during hydrogen production from glycerol are usually acetate, ethanol, and 1,3-propanediol (Table 3), with different proportions probably coming from different operating conditions and different groups of selected microorganisms. Butyrate was positively associated with the hydrogen yield obtained in this study, as widely reported (Rafrafi et al. 2013).

### Microbial succession

Our results highlighted the shift of microbial community between inoculum and hydrogen-production stages. The divergence between the bacterial communities during the transition from methane to hydrogen production has been previously reported in other acidogenic reactors, through T-RFLP fingerprint analysis (Castelló et al. 2009).

In the step-by-step strategy, the most abundant genus under all feeding strategies was *Prevotella* and the maximal metabolite concentration observed during stages 3 and 4 might be related to its predominance of during these stages. *Prevotella* (non-spore forming and obligate anaerobe) is considered as a late microorganism in sucrose fermentation, which consumes by-products such as acetate, succinate, or lactate. Recently, Mariakakis et al. (2011) found that *Prevotella* species

produced hydrogen in small amounts. Moreover, this microorganism has been detected by DGGE band sequencing and cloning/sequencing methods in ASBR and CSTR hydrogen producing systems operated under conditions similar to those of the present study (temperature between 35 and 37 °C, pH 5.3–5.5), fed with sucrose, glucose, or starch, inoculated with anaerobic sludge with and without thermal shock pretreatment (Mariakakis et al. 2011; Arooj et al. 2007). *Prevotella* presence was correlated in one case to the highest hydrogen production period (Mariakakis et al. 2011).

Members of the Veillonellaceae family were also important members of the community in this study and were also detected in hydrogen production systems fed with glucose or sugar refinery wastewater, inoculated with anaerobic sludge with and without thermal shock, but their function remains unclear. In some cases, they were considered as potential hydrogen consumers, while in other cases, they were considered as hydrogen producers or lactate consumers (Hung et al. 2011; Momoe et al. 2012; Won et al. 2013).

*Clostridium beijerinckii* is a hydrogen and butanol producer generally isolated from anaerobic sludge (Zhao et al. 2011). Previous studies of hydrogen production systems from glucose substrate comparing different *Clostridium* species for pure culture inoculation, reported ones of the highest hydrogen yields with *Clostridium beijerinckii* pure cultures, depending on the operation conditions (Zhao et al. 2011; Hu et al. 2014). With glycerol substrate, *Clostridium* genus have been frequently reported at high abundance within mixed cultures treated with thermal shock (Table 3). However, there are no reports of hydrogen production in pure cultures with glycerol substrate, preferring the reductive glycerol degradation pathway (1,3 propanediol production). The case of the sequences affiliated to the Clostridiaceae family is interesting since they exhibited different abundance profiles according to their taxonomic affiliation. *Clostridium beijerinckii* and most of the *Clostridiaceae* members unidentified at the genus level were more abundant at low glycerol concentration in the feed. However, some *Clostridium* sp., including OTU 3 were highly dominant when glycerol was the only substrate, more abundant than the previously dominant *Prevotella*. Therefore, it can be concluded that all *Clostridium* species did not participated in the same way in hydrogen production from increasing glycerol concentration and evidenced an interesting shift within the *Clostridium* genus in relation with the glycerol concentration.

Members of the Enterobacteriaceae family were statistically significant drivers of the community differentiation when glycerol concentration was the highest. Members of the Enterobacteriaceae family have been isolated from various sources such as agricultural soils, wastewater sludge, and cow dung, and have been used in hydrogen production studies, exhibiting very different H<sub>2</sub> production capacities (Seol et al. 2008). In mixed cultures fed with glucose and inoculated

from active sludge without pretreatment, Enterobacteriaceae OTUs have been detected in much lower abundance than *Clostridium* species (Song et al. 2011). In packed bed reactor fed with molasses, Enterobacteriaceae developed in a granular form and have been considered as the dominant hydrogen producers, probably because they were not exposed to the competition with heterolactic fermentation bacteria, contrary to what occurred to the Clostridiaceae dominant in the attached biofilm (Chojnacka et al. 2011).

In conclusion, step-by-step strategy was an efficient strategy for community structure adaptation, maintaining around 33 % of the theoretical yield independently of the glycerol proportion in the feed. The fingerprinting and pyrosequencing results revealed the influence of the feeding strategy on the bacterial community structure and suggested the progressive adaptation of the community. The emergence and selection of specific species were highly correlated to environmental parameters. A microbial succession was observed according to the gradient of glycerol in the feed, with successive dominance of Veillonellaceae and *Prevotella* sp. and *Clostridium* sp. identified as major community members putatively involved in efficient hydrogen production from glycerol.

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**Conflict of interest** The authors declare that they have no competing interests.

**Ethical approval** This manuscript is an original work of authors and does not contain any studies with human participants or animals performed by any of the authors.

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