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Impact of the microbial inoculum source on pre-treatment efficiency for fermentative H₂ production from glycerol

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HIGHLIGHTS

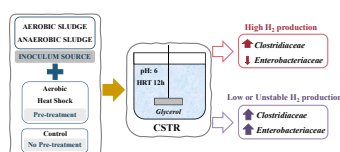
- Microbial community in inocula has a great impact on pre-treatments efficiency.
- In aerobic sludge no pre-treatment is required to increase hydrogen yield.
- Biokinetic control has a strong influence on the *Clostridiaceae* family selection.
- Low/unstable hydrogen production is associated with the *Enterobacteriaceae* family.

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GRAPHICAL ABSTRACT



ABSTRACT

Hydrogen (H₂) production by dark fermentation can be performed from a wide variety of microbial inoculum sources, which are generally pre-treated to eliminate the activity of H₂-consuming species and/or enrich the microbial community with H₂-producing bacteria. This paper aims to study the impact of the microbial inoculum source on pre-treatment behavior, with a special focus on microbial community changes. Two inocula (aerobic and anaerobic sludge) and two pre-treatments (aeration and heat shock) were investigated using glycerol as substrate during a continuous operation. Our results show that the inoculum source significantly affected the pre-treatment efficiency. In aerobic sludge no pre-treatment is necessary, while in anaerobic sludge the heat pre-treatment increased H₂ production but aeration caused unstable H₂ production. In addition, biokinetic control was key in *Clostridium* selection as dominant species in all microbial communities. Lower and unstable H₂ production were associated with a higher relative abundance of *Enterobacteriaceae* family members. Our results allow a better understanding of H₂ production in

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continuous systems and how the microbial community is affected. This provides key information for efficient selection of operating conditions for future applications.

Introduction

The growing environmental pollution of cities has motivated the search for new sources of clean and renewable energy. In this context hydrogen (H_2) appears as a great environment friendly alternative for transportation. Indeed, its combustion produces only water vapor instead of greenhouse gases, with a combustion efficiency 2.75 (122 kJ/g) times higher than traditional fuels and can also be easily converted into electricity in electric vehicle fuel cells [1,2]. Green H_2 is considered a renewable energy since it is produced from renewable resources, such as organic matter by dark fermentation. This latter technology has been widely studied because of a high simplicity and the low operating and maintenance costs when compared to other biological H_2 production systems, such as photofermentation and biophotolysis. In addition, a wide variety of substrates and inocula can be used allowing the production of energy while treating waste [1,3–6]. Different types of waste and organic substrates have already been studied including simple sugars such as glucose and more complex organic matter such as organic industrial wastes [7]. A special interest has been focusing on crude glycerol, the main by-product of the biodiesel industry, as a low-cost feedstock [8–10].

Dark fermentation H_2 production performances from glycerol are mostly dependent to the microbial physiological capacities. As microbial inoculum, strains of known H_2 -producing bacteria could be used in pure cultures, including facultative anaerobes as *Klebsiella* sp. and *Enterobacter* sp. of the *Enterobacteriaceae* family, as well as the strict anaerobes *Clostridium* sp. of the *Clostridiaceae* family [11–14]. Mixed cultures coming from natural and engineered ecosystems such as soil, compost, anaerobic sludge and other anaerobic environments [15–18] have also been used as inocula, with the advantage of providing better adaptation capacity in response to environmental stresses including substrate limitation and abrupt changes in pH and temperature [7,16,19]. The higher robustness of mixed cultures has been attributed to the diversity of the microbial community, enabling positive interspecies interactions such as syntrophy [2,20]. Some mixed community members can also generate adverse effects on the system performance through negative interactions [2]. The origin of the inoculum, its pre-treatment and the operating strategy of the reactors including biokinetic control, i.e. selection pressure on the microbial community imposed by low pH and short HRT, are of crucial importance to ensure H_2 -producer enrichment and achieve high and stable H_2 -production performance [21–27].

Inocula pre-treatments seek to eliminate H_2 -consumers such as hydrogenotrophic methanogenic archaea and enrich the community with H_2 -producers [22,28]. Heat shock pre-

treatment is the most used at lab scale for its efficiency in batch systems. Pre-treatment conditions are generally arbitrary and range from 50 °C to 125 °C and from 20 to 30 min [29–32]. In this case, the microbial community is enriched with spore-forming species such as the H_2 -producer *Clostridium* sp., resisting to high temperature [7,32]. However, other non-spore-forming H_2 -producing species are also depleted such as *Klebsiella* sp., and *Enterobacter* sp. [16,33]. Moreover, heat shock pre-treatment requires additional energy consumption, which is questionable in terms of economic and technical feasibility for a potential industrial application [34,35]. Another less common pre-treatment is aeration, which enriches the inoculum with aerobic and facultative anaerobic H_2 -producers such as *Klebsiella* sp., but also eliminates other oxygen-intolerant H_2 -producing bacteria such as some *Clostridium* sp [36]. Unlike heat shock pre-treatment, aeration could be performed *in-situ* as an industrially viable alternative to the common instability problems of continuous systems during H_2 production [37,38].

This paper aims to study the combined effects of inoculum source and pre-treatment on continuous H_2 production efficiency from glycerol, with special focus on the dynamics of microbial communities. For this, two inocula (aerobic and anaerobic sludge) and two pre-treatments (aeration and heat shock pre-treatment) were compared.

Materials and methods

Inocula source

Two mixed cultures were used as inoculum: (i) anaerobic sludge (13.1 gVSS.l⁻¹) from a sludge stabilizing anaerobic reactor and (ii) aerobic sludge (15.8 gVSS.l⁻¹) from an activated sludge reactor. Both were collected from the sewage treatment plant La Farfana located in Santiago, Chile.

Pre-treatments of inocula

Inocula were either used without pre-treatment (in control conditions), or prepared using two different pre-treatments prior to reactors inoculation (Table 1). A heat treatment (HT)

Table 1 – Summary of experimental design.

Assay	Inoculum	Pretreatment	Name of assay
1	Aerobic sludge	Heat treatment	AI-HT
2	Aerobic sludge	Aeration	AI-AT
3	Aerobic sludge	–	AI-C
4	Anaerobic sludge	Heat treatment	AnI-HT
5	Anaerobic sludge	Aeration	AnI-AT
6	Anaerobic sludge	–	AnI-C

was conducted at 105 °C for 2 h. Aeration (AT) was performed by bubbling air for 4 weeks at a rate providing oxygen saturation. Dissolved oxygen was monitored during these treatments, using a probe and a controller. During aeration, glucose was added as carbon source (10 g L⁻¹), as well as other nutrients detailed in [Experimental set-up](#) (patent N° 201402319, INAPI, Chile).

Experimental set-up

Six continuous stirred tank reactors (CSTR) were operated at different conditions, to compare the combined effects of two inocula (aerobic and anaerobic sludge) and two pre-treatments (HT and AT) on continuous H₂-production. In addition, a control (C) without pre-treatment was performed for each inoculum. Tested conditions are summarized in [Table 1](#). Reactors had a useful volume of 2 L, and were operated at 12 h of hydraulic retention time (HRT), pH 5.5 and 37 °C. The reactors were inoculated with 0.4 L of inoculum and then operated in batch mode for 24 h before starting continuous operation. The reactors were operated continuously for at least 16 HRT. The cultivation medium was composed of 7.5 ± 1.1 g L⁻¹ glycerol and others nutrients as follows (mg.L⁻¹) 1000 NH₄Cl, 250 KH₂PO₄, 100 MgSO₄·7H₂O, 10 NaCl, 10 NaMoO₄·2H₂O, 10 g L⁻¹ CaCl₂·2H₂O, 9.4 MnSO₄·H₂O and 2.8 FeCl₂ [27].

Analytical methods

An online MILLIGASCOUNTER® Type MGC-1 was utilized to continuously determine the volume of biogas produced. Biogas composition (H₂, CO₂, and CH₄) was daily measured by gas chromatography (PerkinElmer Clarus 500, Hayesep Q 4 m x 1/8"OD column, thermal conductivity detector). The concentration of ethanol, acetate, propionate and butyrate was daily measured by gas chromatography (PerkinElmer Clarus 500, 60/80 Carboxpack C column, flame ionization detector). The concentration of glycerol, formate and succinate was measured by HPLC with a refractive index detector (Biorad Aminex HPX-87H column, Bio-Rad laboratories, Hercules, CA – US). The biomass concentration was estimated using dry weight in terms of volatile suspended solids (VSS).

Microbial community analysis

For molecular biology analysis, 2 mL biomass samples were collected from original inocula, after pre-treatments and at the end of the continuous operation. Biomass samples were centrifuged, and the pellet was stored in 9% NaCl at -20 °C. Total genomic DNA was extracted with the Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). The V3–V4 region of the bacterial 16S rRNA gene was PCR-amplified according Carmona-Martinez et al. (2015) [39]. The community composition was evaluated by sequencing using the MiSeq v3 chemistry (Illumina) with 2 × 300 bp paired-end reads at the GenoToul platform (<http://www.genotoul.fr>). Sequences were retrieved after demultiplexing, cleaning, clustering (97%) and affiliating sequences using Mothur [40]. A total of 3216 operational taxonomic units (OTU) were found and

then used for statistical analysis. Sequences have been submitted to GenBank with accession No. KX632952-KX636081.

Data analysis

Averages and standard deviations (±SD) of biomass production, H₂ yields and metabolites concentrations were calculated from daily measurements during continuous operation (for at least 16 HRT). H₂ yields was expressed in moles of H₂ produced by moles of glycerol consumed. Chemical oxygen demand (COD) mass balance was performed and the metabolites concentrations were expressed in %COD i.e. COD of metabolites produced by COD of glycerol consumed.

A one-way ANOVA analysis was performed, after checking normal data distribution, to evaluate significant differences in H₂ yields and biomass production between conditions. For H₂ yield, Mann-Whitney as post-hoc test was performed to find out which sample pairs were statistically different. Simpson diversity index was calculated to compare microbial diversity at the beginning and end of each condition. Principal component analysis was performed from (i) initial and final microbial community and, (ii) final microbial community and metabolic patterns. For the PCA were used the microbial community data with a relative abundance >5.0% in at least one sample. All statistical analyses were carried out with PAST 3.24 software (<http://folk.uio.no/ohammer/past/>).

Results & discussion

Microbial communities in original and pre-treated inocula

The initial microbial community was analyzed in the original inocula (AI and AnI) and in the pre-treated inocula (AI-HTi, AnI-HTi, AI-ATi and AnI-ATi). Whereas the Simpson Diversity Index quantifies microbial diversity, where 1 represents infinite diversity and 0 represents no diversity. The original inocula have a high diversity with a Simpson Diversity Index of 0.98 and 0.92 for aerobic and anaerobic sludge, respectively. After any pre-treatments the Simpson Diversity Index showed no great changes compared to the original inocula ([Table 2](#)). When comparing all communities, the highest similarity is observed between both original inocula AI and AnI, as shown on the PCA ([Fig. 1](#)).

At the phylum level, aerobic and anaerobic inocula were dominated by Bacteroidetes and Spirochaetae phyla, representing between 34.8%–40.4% and 20.3%–26.4% of bacterial community respectively. In particular, the most abundant families in aerobic sludge (AI) were *Spirochaetaceae* (12.4%) and *Rikenellaceae* (10.3%), with OTU11 (8.2%) dominating. OTU11 had 91% of 16S rRNA sequence similarity with *Rectinema cohabitans*. In anaerobic sludge (AnI) the most abundant families were *Rikenellaceae* (18.8%) and WCHB1-69 (11.5%), with OTU5 (18.4%) and OTU6 (16.2%) dominating ([Fig. 2](#)). These two OTUs were related to *Mucinivorans hirudinis* (87% 16S rRNA sequence similarity with OTU5) and *Eubacterium minutum* (78% 16S rRNA sequence similarity with OTU6). Although these families have been reported as dominant in other initial microbial communities of H₂-producing reactors [23,41], none of

Table 2 – Simpson diversity index and microbial community composition at the family level, expressed as percentage of total community. DNA samples were collected from original inocula, after pre-treatments and after continuous operation. Only families with a relative abundance $\geq 5.0\%$ in at least one sample are shown. AI, AnI, HT, AT and C represent aerobic inoculum, anaerobic inoculum, heat shock pre-treatment, aeration pre-treatment and control, respectively. The “i” at the end of the sample names refers to samples taken after pre-treatments.

Family	Original inocula		After pre-treatment				After continuous operation					
	AI	AnI	AI-HTi	AnI-HTi	AI-ATi	AnI-ATi	AI-C	AnI-C	AI-HT	AnI-HT	AI-AT	AnI-AT
Simpson diversity index	0.98	0.92	0.99	0.94	0.95	0.96	0.83	0.71	0.67	0.64	0.75	0.79
Bacteroidetes												
<i>Flavobacteriaceae</i>	1.7	3.5	1.6	0.1	4.8	12.1	0.0	0.0	0.0	0.0	0.0	0.1
<i>Porphyromonadaceae</i>	1.8	3.5	0.4	2.5	0.5	1.6	20.8	0.0	0.0	0.0	1.3	0.0
<i>Prevotellaceae</i>	0.2	0.0	0.3	0.3	11.8	6.3	6.4	25.6	35.9	34.1	32.2	24.9
<i>Rikenellaceae</i>	10.3	18.8	8.2	17.3	1.1	1.4	0.0	0.0	0.0	0.0	0.0	0.0
WCHB1-69	5.0	11.5	1.1	1.6	0.5	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	15.8	3.0	11.2	0.5	2.7	1.0	0.2	1.4	1.1	0.2	2.1	2.0
Total	34.8	40.4	22.8	22.4	21.5	22.6	27.5	27.0	37.1	34.3	35.6	27.1
Firmicutes												
<i>Christensenellaceae</i>	0.9	0.1	7.1	0.5	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
<i>Clostridiaceae</i>	0.4	0.3	1.3	0.1	27.1	18.6	39.4	46.2	51.0	56.4	40.1	35.3
<i>Enterococcaceae</i>	0.0	0.0	0.1	0.0	1.6	3.7	27.1	0.3	0.1	0.1	0.6	1.9
<i>Lachnospiraceae</i>	0.0	0.0	0.1	0.1	2.5	4.6	0.2	1.0	0.1	0.1	5.3	2.2
<i>Peptostreptococcaceae</i>	0.5	0.2	4.0	0.2	6.5	0.8	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	6.8	5.5	6.0	8.5	3.8	4.6	4.5	7.5	2.4	1.3	5.1	1.5
Total	8.8	6.3	18.5	9.4	41.7	32.3	71.2	55.0	53.7	57.9	51.1	40.9
Proteobacteria												
<i>Comamonadaceae</i>	4.1	3.3	5.0	4.7	2.7	5.6	0.0	0.0	0.1	0.4	0.0	0.2
<i>Desulfobacteraceae</i>	3.4	0.0	7.7	0.0	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Enterobacteriaceae</i>	0.0	0.0	1.6	0.0	6.7	4.4	0.4	16.7	3.3	1.3	5.4	25.4
<i>Moraxellaceae</i>	0.1	0.1	4.5	0.1	7.5	5.1	0.4	0.0	0.6	0.1	1.5	0.0
<i>Pseudomonadaceae</i>	0.2	0.1	0.1	0.0	8.8	9.6	0.2	0.1	0.8	2.2	6.2	5.8
<i>Sphingomonadaceae</i>	0.6	0.8	1.4	1.0	0.8	9.6	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	11.1	11.7	9.2	23.4	6.1	7.1	0.3	1.1	4.3	3.9	0.3	0.6
Total	19.5	16.0	29.5	29.2	32.9	41.7	1.3	18.0	9.1	7.8	13.3	32.0
Spirochaetae												
<i>Spirochaetaceae</i>	12.4	1.4	6.5	1.2	1.0	0.2	0.0	0.0	0.1	0.0	0.0	0.0
Unknown_Family	0.1	8.8	0.0	1.5	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	7.8	16.3	1.8	14.6	0.9	0.7	0.0	0.0	0.0	0.0	0.0	0.0
Total	20.3	26.4	8.3	17.4	2.0	1.1	0.0	0.0	0.1	0.0	0.0	0.0
Others (<5.0%)	16.7	10.9	20.9	21.6	1.9	2.3	0.0	0.0	0.0	0.0	0.0	0.0

these dominant species in AI or AnI have been reported as H₂-producing.

Heat shock pre-treatment has a rather limited impact on total microbial community, as shown on the PCA in Fig. 1. After heat shock, the total abundance of the initially dominant Bacteroidetes and Spirochaetae phyla decreased in both inocula. By contrast, the relative abundance of Firmicutes and Proteobacteria increased, representing between 9.4 – 18.5% and 29.2–29.5% of the bacterial community respectively (Table 2). Proteobacteria became dominant in both inocula. However, at the family level, the same Rikenellaceae family as before the heat shock was maintained dominant in the anaerobic inoculum (AnI-HTi) (17.3%) and became dominant in the aerobic inoculum (AI-HTi) (8.2%), even if its relative abundance slightly decreased with respect to the original inocula. In anaerobic inoculum the same OTU5 (17.1%) and OTU6 (14.5%) remained dominant, while in aerobic inoculum OTU25 (5.3%) became dominant. The OTU25 had 95% of 16S rRNA sequence similarity with *Desulfonatrobacter acetoxydans*. As expected, the aerobic inoculum community was more evenly distributed than the

anaerobic one, even after heat-treatment. Besides, heat treatment not only favored families with known spore-forming species such as *Peptostreptococcaceae*, but also families with non-spore forming species such as *Desulfobacteraceae* and *Christensenellaceae* [42,43]. However, this finding is not unusual since other studies have reported that non-spore forming species can survive drastic treatments such as heat shock [2]. Surprisingly, the heat treatment resulted in a very limited enrichment of the community with members of well-known H₂-producing families such as *Clostridiaceae* or *Enterobacteriaceae*.

The aeration pre-treatment resulted in more drastic composition changes than the heat shock, and more divergent communities depending on the inoculum source, as shown on PCA in Fig. 1. Especially, aeration decreased the abundance of the initially dominant Bacteroidetes and Spirochaetae phyla, strongly increasing the relative abundance of Firmicutes and Proteobacteria representing between 32.2 – 41.7% and 32.9–41.7% of the bacterial community respectively (Table 2). After aeration, *Clostridiaceae* became the most abundant family in both aerobic (AI-ATi) and anaerobic

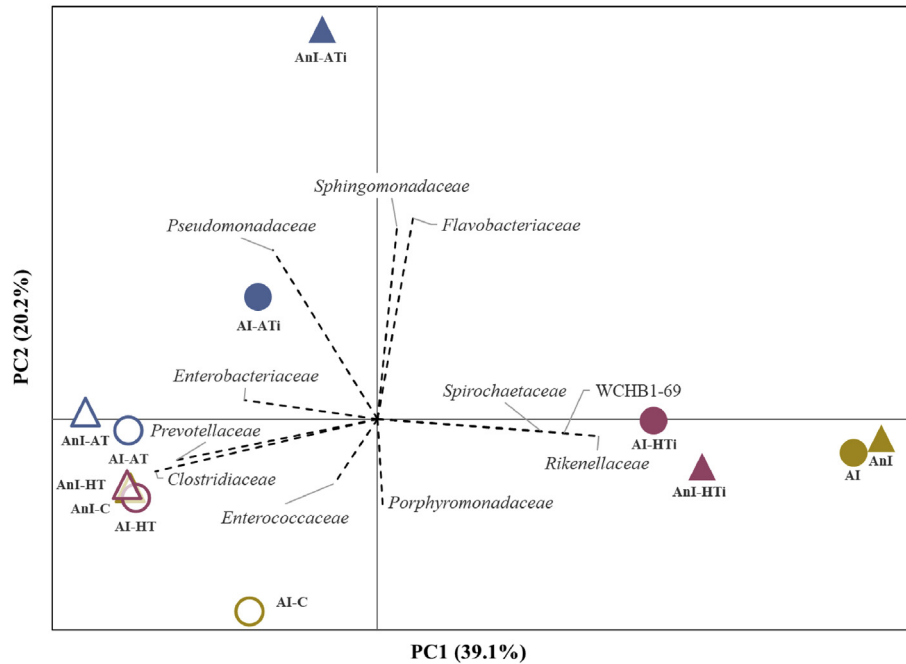


Fig. 1 – Principal component analysis (PCA) based on initial and final microbial population distribution. PCA was performed from correlation matrix. Triangle and circle shapes represent the anaerobic (AnI) and aerobic (AI) sludge, respectively. Filled and empty symbols represent the initial and final samples, respectively. Purple, blue and yellow symbols represent the samples with heat shock pre-treatment (HT), aeration pre-treatment (AT) and control (C), respectively. The “i” at the end of the sample names refers to samples taken prior to reactor inoculation i.e. after pre-treatments. Dotted lines represent Euclidean distances between PCA axes and taxonomic families. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(AnI-ATi) inocula, representing 27.1% and 18.6% of microbial community, respectively. The second most abundant family was *Prevotellaceae* (11.8%) and *Flavobacteriaceae* (12.1%) for aerobic and anaerobic inocula, respectively. *Clostridiaceae*

and *Prevotellaceae* families have strict anaerobic species commonly found in H_2 producing systems [2]. The selection of strict anaerobic species after aerobic treatment and/or from aerobic inocula is not unusual and has already been reported in the literature [44,45]. *Flavobacteriaceae* is mainly composed of aerobic species not commonly found in H_2 -producing reactors [2,46]. Besides, in aerobic inoculum OTU3 (11.4%) and OTU2 (8.9%) were dominant, while OTU240 (8.9%) and OTU1 (8.8%) in anaerobic inoculum. These four OTUs were related to *Clostridium butyricum* (100% 16S rRNA sequence similarity with OTU3), *Prevotella paludivivens* (90% 16S rRNA sequence similarity with OTU2), *Sphingobium yanoikuyae* (100% 16S rRNA sequence similarity with OTU240) and *Clostridium pasteurianum* (98% 16S rRNA sequence similarity with OTU1).

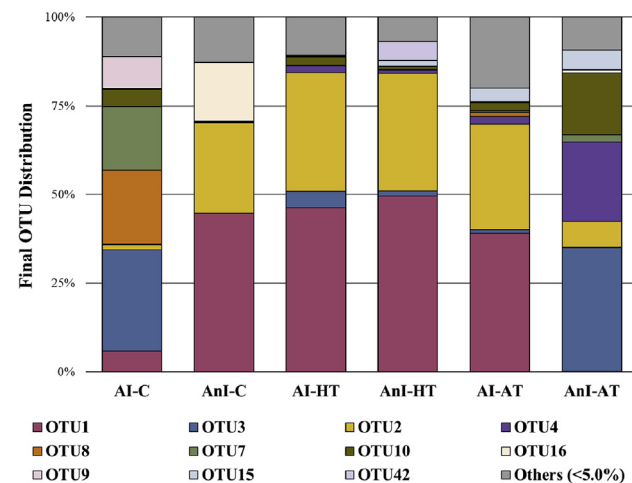


Fig. 2 – Microbial community distribution based on OTU at the end of continuous operation. AI, AnI, HT, AT and C represent aerobic inoculum, anaerobic inoculum, heat pre-treatment, aeration pre-treatment and control, respectively. OTUs with a relative abundance <5.0% are grouped as “Others”.

Our results show that aerobic pre-treatment allows the selection of species with aerobic and facultative anaerobic metabolisms belonging to families such as *Pseudomonadaceae* and *Moraxellaceae* (Fig. 2). Especially, the increase of the *Enterobacteriaceae* family known for its facultative anaerobic H_2 producing members was observed [47]. Surprisingly, the important presence of the *Clostridiaceae* family was also observed, whose members managed to remain and multiply despite theoretically lethal aeration conditions. This could show positive interactions, where non oxygen tolerant microorganisms could be protected by others through oxygen consumption during stressful conditions such as aeration.

Performance indicators during continuous H₂ production

Biomass production for all experiments did not differ significantly (See ANOVA in Supplementary Material), with average growth yields reaching $6.0 \pm 2.1 \text{ g}_{\text{VSS}} \cdot \text{mol}_{\text{gly}}^{-1} \cdot \text{consumed}$ (Table 3). All experiments produced H₂ with yields ranging from 0.29 ± 0.10 to $0.55 \pm 0.08 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{gly}}^{-1} \cdot \text{consumed}$, except for the anaerobic sludge after aeration treatment (AnI-AT), which produced H₂ unsteadily (Table 3). In general, the H₂ yields obtained in this study are within the ranges reported in literature ($0.05\text{--}0.58 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{gly}}^{-1} \cdot \text{consumed}$) for dark fermentation from glycerol using mixed cultures in continuous systems [15,27,48–50]. Soluble metabolites produced concomitantly with H₂ are detailed in Table 3. Butyrate was the main metabolite in all experiments, reaching between $22.0 \pm 8.8\% \text{COD consumed}$ and $39.7 \pm 21.5\% \text{COD consumed}$. Succinate production represented between $12.0 \pm 4.5\% \text{COD consumed}$ and $16.1 \pm 5.6\% \text{COD consumed}$ in experiments that used heat-treated sludge (AI-HT and AnI-HT) and aeration-treated aerobic sludge (AI-AT), but was in less amount $2.5 \pm 1.2\% \text{COD consumed}$ in the control experiments (AI-C and AnI-C) and in the experiment with unstable H₂ production (AnI-AT). Ethanol production represented less than $12.4 \pm 5.4\% \text{COD consumed}$ in all experiments except in AI-AT reaching $27.3 \pm 10.1\% \text{COD consumed}$. Acetate and propionate were also detected in all experiments but at low concentrations ($<5.7 \pm 2.0\% \text{COD consumed}$) except in AnI-AT where acetate accumulated $13.2 \pm 9.4\% \text{COD consumed}$. Formate was also produced at very low concentrations ($<2.9 \pm 4.4\% \text{COD consumed}$), only in aerobic sludge experiments (AI-C, AI-HT and AI-AT). Overall, glycerol removal was between $74 \pm 22\% \text{COD consumed}$ and $90 \pm 15\% \text{COD consumed}$.

Comparing the two-original sludge (i.e., not pre-treated) in the control experiences (AI-C and AnI-C), a 72% higher H₂ yield was obtained along with 31.2% more butyrate and 47.2% less ethanol using aerobic sludge than using anaerobic sludge. This is consistent with literature where higher H₂ production is often associated with higher butyrate production [2,51,52]. This demonstrates a better adaptability for H₂ production of untreated aerobic sludge compared to anaerobic sludge in a continuous system using glycerol as substrate. As already reported, untreated anaerobic sludge may require more time to adapt to glycerol [53]. Besides,

although no methane production was observed in any reactor, a part of H₂ could have been consumed by other H₂-consuming microorganisms present in the untreated anaerobic sludge such as homoacetogens or hydrogenotrophic methanogenic archaea.

When inoculum pre-treatments were performed, different effects were observed depending on the inoculum sources. Within aerobic sludge experiments (AI-C, AI-HT, and AI-AT), the compared pre-treatments did not have any significant effect on H₂ yield (See ANOVA and test of Mann-Whitney pairwise in Supplementary Material). On the contrary, within anaerobic sludge experiments, heat treatment (AnI-HT) increased H₂-yields by 45% compared to control (AnI-C), as already reported by other authors [44,54]. In addition, the heat pre-treatment resulted in two similar H₂ production systems (AnI-HT and AI-HT) with slightly different metabolite production but statistically equal H₂ yields (See ANOVA and test of Mann-Whitney pairwise in Supplementary Material), despite the inocula came from different sources. This shows the reproducibility and effectiveness of heat treatments, leaving evidence why has been widely reported in the literature to prepare different inocula for the H₂ production by dark fermentation [33,52,55–58].

Unlike heat treatment, aerobic treatment on anaerobic sludge (AnI-AT) generated a negative effect respect to the control (AnI-C), causing unstable H₂ production during all operation days. Consequently, when comparing the behavior of both sludge when exposed to aerobic treatment, again aerobic sludge showed a better adaptability to H₂ production compared to anaerobic sludge.

In conclusion, and depending on the inoculum source, three effects of pre-treatment on H₂ production can be observed respect to the control: Positive effect (i.e. heat pre-treatment on anaerobic sludge), negative effect (i.e. aerobic pre-treatment on anaerobic sludge) and neutral effect (i.e. heat pre-treatment and aerobic pre-treatment on aerobic sludge). Consistently, the inoculum source importance on the efficiency of the pre-treatment was already evidenced but in a study performed in batch mode operation using glucose, and comparing two pre-treatments: heat treatment and acidification [59].

Table 3 – Performance indicators during continuous operation of H₂ producing reactors, including biomass yield, H₂ yield, and soluble metabolites production. Average values and standard deviations (\pm SD) were calculated from daily measurements during continuous operation.

Parameter	Unit	AI-C	AnI-C	AI-HT	AnI-HT	AI-AT	AnI-AT
Biomass yield	$\text{g}_{\text{VSS}} \cdot \text{mol}_{\text{gly}}^{-1} \cdot \text{consumed}$	5.8 (\pm 2.1)	6.3 (\pm 1.5)	7.0 (\pm 3.1)	5.8 (\pm 2.4)	5.3 (\pm 2.0)	6.7 (\pm 2.7)
H ₂ yield	$\text{mol}_{\text{H}_2} \cdot \text{mol}_{\text{gly}}^{-1} \cdot \text{consumed}$	0.50 (\pm 0.19)	0.29 (\pm 0.10)	0.47 (\pm 0.17)	0.42 (\pm 0.08)	0.55 (\pm 0.08)	^a
Ethanol	%COD	6.5 (\pm 2.6)	12.3 (\pm 5.1)	12.4 (\pm 5.4)	3.0 (\pm 1.3)	27.3 (\pm 10.1)	9.5 (\pm 6.4)
Acetate	%COD	3.7 (\pm 1.9)	5.7 (\pm 2.0)	4.8 (\pm 1.5)	4.1 (\pm 1.6)	3.4 (\pm 1.2)	13.2 (\pm 9.4)
Propionate	%COD	1.4 (\pm 0.7)	3.2 (\pm 1.5)	2.7 (\pm 1.0)	1.4 (\pm 0.6)	1.6 (\pm 0.4)	5.2 (\pm 3.5)
Butyrate	%COD	32.8 (\pm 11.1)	25.0 (\pm 8.7)	22.0 (\pm 8.8)	30.7 (\pm 13.8)	23.4 (\pm 8.2)	39.7 (\pm 21.5)
Succinate	%COD	1.9 (\pm 0.8)	2.5 (\pm 1.2)	12.0 (\pm 4.5)	16.1 (\pm 5.6)	15.5 (\pm 5.9)	1.8 (\pm 3.9)
Formate	%COD	1.1 (\pm 0.5)	–	1.8 (\pm 0.7)	–	1.6 (\pm 0.4)	2.9 (\pm 4.4)
Glycerol removal efficiency	%	90 (\pm 15)	79 (\pm 19)	78 (\pm 20)	80 (\pm 31)	87 (\pm 16)	74 (\pm 22)

Metabolite distribution based on COD mass balance. %COD were calculated based on total glycerol consumed.

^a During AnI-AT the H₂ production was unstable.

Link between final microbial community and metabolic patterns during continuous H₂ production

DNA samples were collected at the end of the continuous operation to assess changes in the microbial community. As shown in Table 2, the *Clostridiaceae* family was most abundant in all conditions, with a relative abundance between 35.3% and 56.4% and was mainly represented by OTU1 and OTU3 (Fig. 2). OTU1 was dominant in AnI-C (44.7%), AI-HT (46.2%), AnI-HT (49.5%) and AI-AT (39.2%), while OTU3 in AI-C (28.5%) and AnI-AT (34.9%). The *Prevotellaceae* family was the second most abundant in AnI-C (25.6%), AI-HT (35.9%), AnI-HT (34.1%) and AI-AT (32.2%) reactors and was represented by OTU2 (Fig. 2). The second and third most abundant family in AI-C were *Enterococcaceae* (27.1%) and *Porphyromonadaceae* (20.8%) and were mainly represented by OTU7 (18.0%) and OTU8 (20.8%), respectively (Fig. 2). These two OTUs were related to *Enterococcus gallinarum* (99% 16S rRNA sequence similarity with OTU7) and *Dysgonomonas mossii* (100% 16S rRNA sequence similarity with OTU8). In AnI-AT, the second and third most abundant family were *Enterobacteriaceae* (25.4%) and *Prevotellaceae* (24.9%) and were mainly represented by OTU4 (22.4%) and OTU10 (17.5%), respectively. These two OTUs were related to *Klebsiella aerogenes* (99% 16S rRNA sequence similarity with OTU4) and *Prevotella dentalis* (90% 16S rRNA sequence similarity with OTU10). In AnI-C the *Enterobacteriaceae* (16.7%) family was the third most abundant and was mainly represented by OTU16 (16.4%). The OTU16 had 100% of 16S rRNA sequence similarity with *Raoultella ornithinolytica*.

Illustratively Fig. 3 shows a principal component analysis (PCA) performed from final microbial community at family level and metabolic patterns to observe the relations between them according to each experiment. The PCA shows that the control experiences (AI-C and AnI-C) are negatively related, probably due to the great impact of the inoculum origin on both the final microbial communities and reactor behavior. Particularly, AI-C is related to butyrate production and with *Enterococcaceae* and *Porphyromonadaceae* families. While, AnI-C is slightly related to acetate and ethanol production. The heat-treated reactors, independently of the inoculum (AI-HT and AnI-HT), were characterized by higher abundance of the *Clostridiaceae* and *Prevotellaceae* families along with the production of ethanol, acetate and butyrate, as is observed in Fig. 3. This is consistent with the literature, since some species of the *Clostridiaceae* family could present an acidogenic or solventogenic metabolism, associated to a higher H₂ production along with acetate-butyrate pathway and a lower H₂ production along with the production of alcohols such as ethanol, respectively [60]. Unlike heat pre-treatment, aerobic pre-treatment generated two slightly different microbial communities. Fig. 3 shows how AnI-AT is related to the *Enterobacteriaceae* family, while AI-AT is related to the succinate production.

In addition, Fig. 3 shows that microbial diversity is positively related to AnI-AT and negatively related to AI-HT and AnI-HT. The literature is not clear on how microbial diversity could affect H₂ production. Contradictorily, it has been reported that greater diversity may increase the possibilities of

selecting H₂-producing bacteria, but it may also increase competition among members of the microbial community, leading to a decrease in the H₂ production [35,61–63]. Our results show that the microbial community was considerably simplified, and that the Simpson diversity index decreased by 15.8–33.0% compared to the initial inocula. In particular, heat shock pre-treatment reduced microbial diversity by $32.7 \pm 0.5\%$, while aeration decreased by $19.5 \pm 1.0\%$ (Table 2). However, greater microbial diversity could be linked to lower H₂ production efficiency.

Combined effect of inoculum source and pre-treatments on microbial community

Fig. 1 shows a PCA performed from samples taken before inoculating the reactors and at the end of continuous operation. Three main groups are observed, in which the change of the microbial community from the original sludge, after pre-treatment and after continuous operation is clearly evidenced. In the first group (Fig. 1, on the right) the original sludge (AI and AnI) is associated with the sludge after heat pre-treatment (AI-HTi and AnI-HTi). In turn, this group is associated with the most important families of their microbial community, i.e., *Rikenellaceae*, *Spirochaetaceae* and WCHB1-69. The second group (Fig. 1, top) includes sludge after aeration pre-treatment (AI-ATi and AnI-ATi) and are related to families that increased their relative abundance in at least one of these samples such as *Sphingomonadaceae*, *Flavobacteriaceae* and *Pseudomonadaceae*. While the third group (Fig. 1, left down) is composed of all the samples taken at the end of the continuous operation and are related to the families that dominated the final microbial communities in each case, i.e. *Porphyromonadaceae*, *Enterococcaceae*, *Clostridiaceae*, *Prevotellaceae* and *Enterobacteriaceae*. In all cases, there is more similarity between reactor communities inoculated with different sludge exposed to same pre-treatment, suggesting that the pre-treatment has more impact than the inoculum source on the total community structure. Despite the pre-treatments performed and the inoculum origin, the selection pressure imposed by biokinetic control appears to be crucial in determining the dominant families of the H₂-producing microbial community, particularly in the selection of *Clostridiaceae* family members. This is consistent with the literature, as members of this family are often selected during continuous H₂ production operated at low pH (values between 5.0 and 6.0) and short HRT (<12 h) [4,27,64,65].

When considering the experiments that used untreated inoculum, it is observed that despite the impact of biokinetic control (as discussed above) on selection of *Clostridiaceae* family members, AI-C had a 72% higher H₂ yield than in AnI-C (Table 3). Among the dominant species of the AI-C microbial community is *Dysgonomonas mossii* (Fig. 2), a fermentative but not H₂-producing bacteria [66–68]. AI-C reach the maximum H₂ yield of this study, suggesting a positive interaction of *Dysgonomonas mossii* with the microbial community and especially with the known H₂-producing bacteria. Unlike AI-C, all dominant families in the AnI-C microbial community (i.e., *Clostridiaceae*, *Prevotellaceae*, and *Enterobacteriaceae*) have known H₂-producing members, but the low H₂ yield obtained

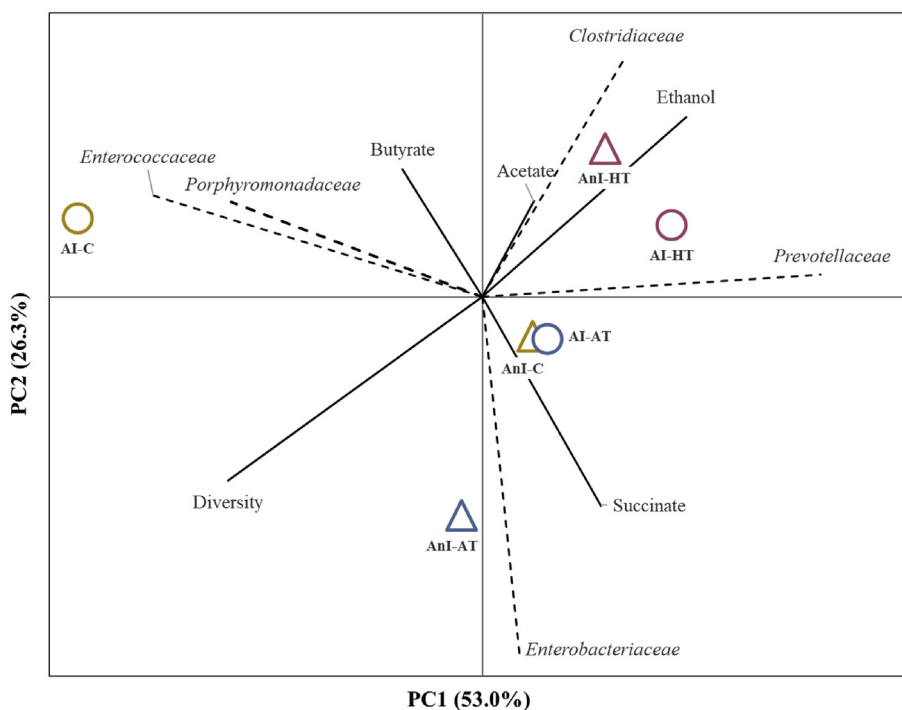


Fig. 3 – Principal component analysis (PCA) based on metabolic patterns and final microbial population distribution. PCA was performed from variance-covariance matrix. Triangle and circle shapes represent the anaerobic (AnI) and aerobic (AI) inoculum, respectively. Purple, blue and yellow symbols represent the samples with heat treatment (HT), aeration (AT) and control (C), respectively. Plain lines and dotted lines represent Euclidean distances between PCA axes and taxonomic families and metabolic yields, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in this experiment suggests the predominance of negative interactions in the microbial community. Therefore, the inoculum source plays a key role in determining the final microbial community when no pre-treatment is performed.

Comparing the metabolic patterns when a heat-treated inoculum (AI-HT and AnI-HT) was used, and especially the H_2 yields, no statistically significant differences are observed, although the inocula come from different sources. Surprisingly, the final microbial community of both is very similar, with *Clostridium* and *Prevotella* as dominant genus. The relative abundance of *Clostridium* at the end of these experiments was more than 50%, which was expected since the heat treatment objective is to enrich the microbial community with spore-forming species such as *Clostridium*. Contrary to our results, Baghchehsaraee et al. (2008) obtained lower H_2 yield when using heat-treated aerobic sludge, attributed to a decrease in microbial diversity due to pre-treatment [35]. However, they worked with glucose as substrate in batch mode operation and heat pre-treatment conditions were 65°, 80° or 95° for 30 min. Consequently, they are all important parameters affecting the microbial community composition.

Aeration as pre-treatment generated important differences in microbial communities and H_2 yields depending on the inoculum source (AI-AT and AnI-AT). The main difference was the relative abundance of *Klebsiella aerogenes* in AnI-AT, a known H_2 producing bacteria. However our results show that it is negatively related to H_2 production suggesting a negative

interaction with other known H_2 producers in the community, which are in the ratio 1.1:1.6:1.0 for *Prevotella*:*Clostridium*:*Klebsiella*, respectively [2]. In contrast to our results Silva-Illanes et al. (2017) evidenced the existence of positive interactions between all H_2 -producing bacteria present in the microbial community, which are in the ratio 1.2:5.4:1.0 for *Prevotella*:*Clostridium*:*Klebsiella*, respectively [27]. Consequently, the difference in the results is the relative abundance of H_2 producers and their ratio, while in our results the genera are in a ratio around 1.0, in Silva-Illanes et al. (2017) *Clostridium* is the most important being 5.4 times more abundant.

In conclusion it was shown that the inoculum source played a key role for H_2 production in continuous reactors. The inoculum source determines not only the metabolic patterns when using untreated sludge, but also affects the efficiency of the pre-treatments performed. A combined effect between pre-treatments and inoculum sources was evidenced by probably affecting the microbial interactions and final selection of the microbial community.

Conclusion

Inoculum source has a strong impact on the reactor behaviors when non-pretreated sludge is used, but also on pre-treatment efficiency. Heat pre-treatment of anaerobic sludge increased H_2 yield, while aeration resulted in unstable H_2

production. Whereas when aerobic sludge is used no pre-treatment is necessary, as there are no statistically significant differences in H₂ yields when comparing all experiments, including control. In addition, biokinetic control was key in the *Clostridium* sp. selection as dominant in the microbial community of all assays. While, lower or intermittent H₂ production were associated with higher relative abundance of *Enterobacteriaceae* family members. Our results allow a better understanding of H₂ production in continuous systems, providing key information for an efficient selection of operating conditions for future industrial applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijhydene.2019.11.113>.

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