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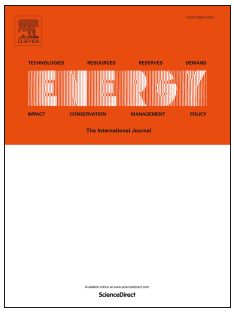

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Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen production from glycerol

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ABSTRACT

Hydrogen is a promising alternative of clean energy carrier which can be biologically produced from glycerol-rich waste an abundant and economic source of substrate. However, continuous hydrogen-producing systems still need to be improved and in particular by manipulating the only few available operating conditions. The aim of this study was to investigate the effect of the two main operational parameters, ie. pH and hydraulic retention time (HRT) on hydrogen yields and microbial community structures. For that, a continuous stirred tank reactor (CSTR) was first inoculated with an enriched mixed microflora and was then fed with glycerol. A strong influence of these two operational parameters was shown on hydrogen yields where the maximum yield (0.58 ± 0.13 $mol_{H2}mol^{-1}_{glycerol}$) was achieved at pH 5.5 - HRT 12 h and was 28.5 times higher than the minimum (0.02 ± 0.02 $mol_{H2}mol^{-1}_{glycerol}$) obtained at pH 5.0 - HRT 14 h. Changes in most dominant microbial populations were mainly influenced by the pH. Interestingly, HRT parameter related to changes in the metabolic patterns and influenced the composition of subdominant

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microorganisms, suggesting they might have a key role in changing the ability of the consortium and/or the activity of dominant microorganisms to produce hydrogen.

Keywords : Oxygen treatment; Mixed culture; Biohydrogen; *Klebsiella*; *Clostridium*; Dark fermentation

1. Introduction

Hydrogen is a compound having a high energy efficiency per unit of mass (142.3 kJ g^{-1}) (Seifert et al., 2009), which is also substantially higher than methane (49.9 kJ g^{-1}) (Xie et al., 2008). Since its combustion only produces water and energy, it can be employed as an energy vector, making hydrogen an efficient and versatile fuel. Currently, hydrogen production is carried out through highly energy-intensive processes such as water gas steam reforming of natural gas and oil, coal gasification of biomass, or water electrolysis (Balat, 2008). In contrast, biological processes for hydrogen production present lower energy requirements and are more environment friendly with regards to global CO_2 reduction (Ghimire et al., 2015). The environmental impact of biohydrogen production is still unknown since bioprocesses have not yet been scaled up at industrial level. However, the use of waste as feedstock from different industries containing highly degradable organic material as economical substrates, has gained importance for escalating the process. Further, there countries that are seriously evaluating the possibility of using hydrogen (H_2) as an alternative fuel in their power systems (Tapia-Venegas et al., 2015a).

Dark fermentation is a biological process where hydrogen is produced with concomitant liquid effluent treatment by reducing its organic load (Dincer, 2012). Glucose has been typically used as model substrate for hydrogen production by dark fermentation (Tapia-Venegas et al., 2013; Wang and Wan, 2008). However, a more economical source of carbon is preferable to avoid the use of

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1st generation carbohydrates that outcompete food usages (Ren et al., 2011). More recently, glycerol as by-product of the biodiesel industry has also been considered as a serious low cost alternative to produce hydrogen by either biological dark fermentation or catalytic reforming (Tapia-Venegas et al., 2015; Selembo et al., 2009). Hydrogen could be efficiently produced from glycerol by pyrolysis and gasification, but contaminants contained in crude glycerol are usually impairing the efficiency of these catalytic processes (Schwengber et al., 2016). Biological processes are more robust to contaminants but H_2 yields are limited by microbial metabolisms. In fact, the effluent of dark fermentation is rich in organic acids that can be further used to produce methane via anaerobic digestion, increasing the total amount of energy produced and making the process economically viable (Tapia-Venegas et al., 2015).

Till now, most of the studies carried out to produce hydrogen from glycerol by dark fermentation have been operated in batch mode with a microbial inoculum previously pre-treated using a thermal shock (Akutsu et al., 2009; Seifert et al., 2009 and literature studies in table 3). Comparatively, the few experiments conducted in continuous stirred tank reactors (CSTR) to produce hydrogen from glycerol-rich effluents have used microbial cultures without pre-treatment, or adapted microbial cultures (which require time for adaptation), or pure cultures at specific operating conditions. These studies did not achieved high H_2 yields, as reported by Temudo et al (2008) (0.05 mol_{H2} $mol_{glycerol}^{-1}$) and Gonzalez-Pajuelo et al., (2005) (less than 0.1 mol_{H2} $mol_{glycerol}^{-1}$). Mostly, these studies focused on generating a suitable hydrogen-producing inoculum from glycerol, punctually achieving yields of 0.4 mol_{H2} $mol_{glycerol}^{-1}$ (Tapia-Venegas et al., 2015b). CSTR reactors are characterized both by the simplicity of their operation and construction as well as by their capacity of continuous generation of a specific product from high volume effluent streams (Ramírez-Morales et al., 2015). An optimal management of the operational parameters may allow improvements of the dark fermentation process regarding hydrogen yields, and among

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70 them, pH and hydraulic retention time (HRT) are two of the most influential factors (Lee et al.,
71 2014; Tapia-Venegas et al., 2013).

72 As performance indicators, the dark fermentation process could be improved to increase its
73 technical efficiency, i.e. increase of the productivity (in terms of product generation per unit of
74 volume and time) and reduce the gap between experimental values and the maximum theoretical
75 yield, ie. 1 mole of H_2 per mole of glycerol for mixed cultures (Ito et al., 2005; Akutsu et al., 2009).
76 Operational parameters such as pH and HRT, together or in combination, are criteria that, along
77 with the origin and pretreatment of the inoculum, select a hydrogen-producing community aving
78 its own very specific structure (Ueno et al., 2006; Chang et al., 2008; Fang and Liu 2002). A better
79 knowledge of the species composition, abundance, and interaction within the global microbial
80 community is key to better understand and control the ability of the consortium to produce
81 hydrogen by dark fermentation (Rafrafi et al., 2013)

82 The aim of this study is to investigate the effect of pH and HRT on hydrogen yields and
83 productivity. Metabolic patterns as well as microbial community structure were characterized
84 during dark fermentation CSTR operation of a reactor fed with glycerol.

85

86

87 ## 2. Materials and methods

88 ### 2.1 Inoculum and culture medium

89 As microbial inoculum, activated sludge was sampled from the wastewater treatment plant
90 located at La Farfana, Santiago, Chile. Prior to use, the sludge was pre-treated by aeration for 24
91 hours in the feeding medium (patent 201402319, INAPI, Chile), which was composed of 10000 mg

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92 l^{-1} of glycerol and the following nutrients (in mg l^{-1}): NH_4Cl 1000; KH_2PO_4 250; $MgSO_4*7H_2O$ 100;
93 NaCl 10; $NaMoO_4*2H_2O$ 10; $CaCl_2*2H_2O$ 10; $MnSO_4*H_2O$ 9.4; $FeCl_2$ 2.78. Then, an anaerobic stage
94 was initiated by applying N_2 for 10 minutes. More medium was added to achieve a concentration
95 of 10 g l^{-1} of glycerol inside of the reactor, and a batch stage of 24 h was performed prior to
96 continuous operation. Duration of the CSTR operation for each test fed with the same medium
97 composition corresponded to at least 20 HRT at steady state. This latter was defined as the period
98 of time when the variation in hydrogen yield was not higher than 30%.

99 **2.2 Operating conditions and experimental design**

100 The experiments were carried out in a 2-L (working volume) continuous stirred tank reactor (CSTR)
101 operated under anaerobic conditions. Temperature was controlled and maintained at 37°C, and
102 agitation was maintained at 400 rpm. pH was monitored and regulated in a 5 - 6.5 range, by a
103 sensor/controller connected to a pump with a NaOH 0.8M solution. pH values were recorded
104 using ODIN® (INRIA) software. HRT was investigated in the range between 8 and 14 hrs by varying
105 the feed pump flow. A total of 8 cultures were performed in continuous mode, as indicated in
106 Table 1. To maintain the anaerobic environment inside of the reactor, the feed tank was daily
107 bubbled with nitrogen for 5 minutes.

108

109 **2.3 Analytical methodology**

110 Concentration of glycerol and metabolites such as volatile fatty acids (VFA), ethanol, and 1,3-
111 propanediol were quantified using HPLC (Biorad HPX-87H column, Bio-Rad Laboratories, Hercules,
112 CA, US). Biogas flow rate was measured with a Ritter MilliGascounter®. Biogas composition
113 (hydrogen, nitrogen, methane, and carbon dioxide) was determined by gas chromatography using

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Perkin Elmer Clarus 500, equipped with the Hayesep Q 4 m x 1/8''OD column (VICI, Bandera, TX, US). Volatile solids were determined according to Standard Methods #2540 D (2012).

2.4 Bacterial community characterization

Microbial communities of each culture were characterized by sampling the initial inoculum (prior to enrichment) and reactor outlets at steady state. Samples were first centrifuged at 10000 rpm for 10 min. DNA was then extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) and was stored at -20°C until use. The community was analyzed using phylogenetic analysis of the V3-V4 bacterial region of the *16S*-rRNA gene. Extracted DNA was amplified via PCR for Capillary Electrophoresis Single Stranded Conformational Polymorphism (CE-SSCP) analysis, using the primers w49 (5'-ACGGTCCAGACTCCTACGGG-3') and W104 (5'-6FAM-TTACCGCGGCTGCTGGCAC-3') through *Pfu* Turbo DNA polymerase (Stratagene). A capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) method was used for PCR product diversity characterization. Samples were heat-denatured at 95°C for 5 min and re-cooled directly in ice for 5 min. CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) in 50 cm capillary tubes filled with 10% glycerol, conformation analysis polymer, and corresponding buffer (Applied Biosystems). Samples were eluted at 12 kV and 32°C for 30 min, as described elsewhere (Wéry et al., 2008). CE-SSCP profiles were aligned with an internal standard (ROX) to consider the inter-sample electrophoretic variability. CE-SSCP profiles were normalized using the Stat- Fingerprints library (Michelland et al., 2009) in R software version 2.9.2 (R. Development Core Team 2010) (Rafrafi et al., 2013). Relative abundance was assessed from the individual area of OTU peaks in the CE-SSCP profile on the total area of the peaks on each profile.

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Community composition was evaluated using the MiSeq v3 chemistry (Illumina) with 2 × 300 bp paired-end reads at the GenoToul platform (www.genotoul.fr). Sequences were retrieved after demultiplexing, cleaning, and affiliating sequences using mothur (Schloss et al., 2009). Sequences were submitted to GenBank and deposited in the NCBI Sequence Read Archive under the accession number SUB1737472.

2.5 Statistical and numerical methodology

The average value of each steady state regarding hydrogen yield, hydrogen volumetric productivity, VFA concentration, 1,3-propanediol, and ethanol concentration corresponded to the mean value of daily measurements at steady state for each test (for at least 20 HRT). The hydrogen yield was expressed in moles of H_2 produced divided by the moles of glycerol consumed. VFAs, 1,3-propanediol, and ethanol concentration were expressed in %COD (Chemical Oxygen Demand) of the effluent. An ANOVA analysis was performed to determine the significant difference between hydrogen yields of each condition with Minitab® software17.

Principal component analysis (PCA), Pearson correlation, and UPGMA clustering graphs were used to compare the variability in microbial community structure dynamics and were performed using XLSTAT software. For better clarity, only microorganisms with abundances higher than 1% were considered since they accounted for more than 90% of the total community (3054 OTUs).

3. Results

3.1 Effect of pH and HRT on hydrogen yield and productivity

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Table 2 shows hydrogen productivities and yields obtained in the different CSTR cultures according to pH and HRT. According ANOVA analysis, hydrogen yields for each condition were statistically different (with a significance of 95%). Values for productivities and yields ranged between 0.6-2.1 $l_{H2}l^{-1}$ d^{-1} (2.5-88 $mmol_{H2}l^{-1}$ d^{-1}) and 0.02-0.58 $mol_{H2}mol^{-1}_{glycerol}$, respectively. The highest hydrogen yield (0.58 $mol_{H2}mol^{-1}_{glycerol}$) was reached by operating the CSTR at pH 5.5 and HRT 12 h. The process was therefore substantially influenced by both pH and HRT.

The specific effect of pH and HRT on hydrogen yields can be seen in Figure 1. Overall, the system was very sensitive to changes of pH and HRT in the range of the studied conditions. A pH increase from 5.5 to 6.0 reduced the H_2 yield by 40 to 50%, and a HRT change from 12 h to 8 h showed a similar effect. The optimal hydrogen yield was observed for HRT ranging between 10 h and 14 h in the vicinity of the condition pH 5.5 and HRT 12 h. According to the Pearson correlation matrix of the hydrogen productivities yields, metabolite production profiles, pH and HRT from steady states (see Fig. 2), the longer HRT significantly and positively correlated to hydrogen yields and productivities.

3.2 Effect of pH and HRT on metabolite and microbial biomass production

In Table 2, the average microbial biomass (gr VSS l^{-1}) and metabolites accumulated during CSTR operation at steady state are represented and expressed in COD percentage (%COD).

Despite the variability in metabolite production observed at the different steady states, the metabolites found at the highest concentration were 1,3-propanediol (1,3 PDO), succinate, and butyrate, that represented between 29 and 100% of the total COD of the effluent. According to Fig. 2, butyrate was directly related to higher hydrogen production, and the latter was in turn

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181 related to longer HRT. In addition, higher pH correlated with succinate concentration and was
182 inversely related to butyrate or 1,3-propanediol accumulation.

183 Furthermore, the metabolites that were produced at lesser extent (formate, acetate, propionate,
184 and ethanol) accounted only for 21 to 4% of the total effluent COD. Amongst them, only an
185 increase in pH negatively correlated with ethanol accumulation.

186 As shown in Table 2, glycerol removal was above 90% in all tests, and in terms of microbial
187 biomass concentration at steady state for the different cultures, this varied between 0.42 and
188 0.64$g_{VSS\ l}^{-1}$. As a consequence, high variation in the specific hydrogen productivity, ranging from 6
189 to 163 $mmol_{H2}\ g^{-1}_{VSS}\ d^{-1}$, was observed.

190

191 **3.3 Effect of pH and HRT on microbial community structures**

192

193 CE-SSCP analysis was performed to provide a first overview of the microbial community
194 dynamics and make selection prior to sequencing analysis. Fig. 3 presents the CE-SSCP profiles
195 for the established microbial communities at steady state for the different tests together
196 with UPDMA clustering. The average community of all inocula is presented in
197 supplementary material (Fig. S1). Low diversity was observed in all CE-SSCP profiles of the
198 communities found at steady state (PICs 1 to 7) in comparison to the methanogenic
199 inoculum (Fig. 3 and Fig. S1).

200

201 According to the UPGMA clustering based on Pearson correlation made on the data issued from
202 SSCP analysis (Fig. 3), two major groups were statistically distinguished: a first group was formed

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by the communities found at pH 5 and pH 5.5, and a second corresponded to the communities related to pH 6 and 6.5. Surprisingly, the condition of pH 6.5 and HRT 8 h was more linked to the group of pH 5 and pH 5.5 and the community was closer to another community found at HRT 8h (pH 5.5 and HRT 8 h).

For the first group, PIC 1 was dominant, with a relative abundance ranging from 58 to 99 % of the total community. In contrast, in the second group, PIC 1 represented a very low abundance percentage ranging from 18 to 4% only, with a dominance of PIC 4 and 5, within a range of14 to 44% and 28 to 65% of the total abundance, respectively. An intermediate group was also identified for the communities found at HRT 8 h whatever the pH (5.5 or 6.5). In this group, PIC 1 represented 24 to 58% with a co-dominance of PICs 4 and 5, with 14 to 44% and 28 to 32%, respectively.

According to the statistical grouping of the microbial communities found at steady state, it was concluded that the pH substantially influenced the microbial community structure, except at HRT of 8h where the communities were consistently similar regardless the pH (6.5 or 5.5).

3.4 Bacterial population identification

Since two groups were clearly distinguished, four of the nine tests were selected for further sequencing analysis. As shown in figure 4, a similar amount of dominant OTUs was observed throughout the studied conditions except at pH 6 and a HRT of 14 h. In all cases, the phylum Firmicutes was dominant (above 50%), and the phyla Bacteroidetes and Proteobacteria showed percentages between 14 to 26% and 6 to 23%, respectively.

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For three conditions (pH 5.5 and HRT 12 h, pH 5 and HRT 10 h, and pH 5 and HRT 14 h), the most dominant microorganism was related to a *Clostridium sp* (OTU 1; more than 50%), followed by *Prevotella* sp. (OTU 5) with between 13-17.4%. Interestingly, *Klebsiella* sp. (OTU 8) was only found at pH 5.5 and HRT 12 h where it represented 11.3%. At pH 6 and 14 h HRT, a wider distribution of the abundance within the community was observed with microorganisms related to several genera such as *Clostridium sp* (OTU 1), *Enterococcus sp.* (OTU 3), *other Clostridia* (OTU 4), *Prevotella sp.* (OTU 5), and *Snodgrassella sp.* (OTU 13) with 8.4%, 20.6%, 18.3%, 22.2%, and 13.9% of the total abundance, respectively.

In order to clarify the prevalence of the main dominant bacterial species, *Clostridum sp* was mainly dominant at all steady states at pH 5 and 5.5 (pH 5.5 and HRT 12 h; pH 5 and HRT 10 h; pH 5.5 and HRT 8 h). This OTU was likely represented by Peak n°1 on the CE-SSCP analysis, since it corresponded to the highest relative abundance (>80%; Fig. 3) in the same conditions. According to relative abundance (figure 4), *Enterococcus* (OTU3) *and Prevotella* (OTU4) could be represented by the peaks n°4 and n° 5, but the relationship is not as clear when correlations with hydrogen productivities yields, metabolite production profiles, pH and HRT from steady states are examined (Fig. 4 and Fig. S3).

PCA analysis performed with the microbial community obtained from sequencing analysis of the four steady states confirms that the samples could be differentiated by their pH, as shown in UPDMA clustering based on the Pearson correlation coefficient from the SSCP analysis data (Fig. 3 and Fig. S2). The communities found at pH 5.0 were mostly related to OTUs 1, 2, 12, and 15, reported as *Clostridium, Veillonellaceae, Pseudomonas, and Sutterella sp.* respectively. The condition at pH 6 was in favour of a higher diversity in OTUs, with especially OTU 4, 3, 13, and 16 as other *Clostridia, Enterococcus, Snodgrassella, and Desulfovibrio,* respectively. OTUs 10, 15, and

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247 31 (*Klebsiella, other Alphaproteobacteria, and Kluyvera*) were only related to the condition at pH
248 5.5 and HRT 12 h.

249

250 **3.5 Relationship between community structure and pH and HRT**

251 In order to highlight correlations between the composition of the microbial communities
252 (sequencing analysis), metabolite profiles, hydrogen yield, hydrogen productivity and
253 independedent variables as pH and HRT, a Pearson correlation matrix was calculated (Fig. 5). This
254 Pearson correlation matrix also highlighted two groups of bacteria. Moreover, one group was
255 linked to hydrogen and butyrate production (OTU 7, 8 and 14) while another group was linked to
256 acetate, propinate and formate production (OTUs 3, 4, 6, 13, and 16). OTUs 7, 8, and 14
257 corresponding to *Enterobacter, Klebsiella,* and *other alphaproteobacteria*, positively correlated
258 with high H_2 yields. Interestingly, *Klebsiella* and *other alphaproteobacteria* were also associated to
259 an increase in butyrate and ethanol concentration, at high HRT and low pH, and also negatively
260 correlated with high succinate concentration. In addition, OTUs 3, 4, 6, 13, and 16 (*Enterococcus,*
261 *Other Clostridia, Bacteroides, Snodgrassella, and Desulfovibrio,* respectively) were related to
262 higher acetate concentrations. OTUs 3, 4, 6, 13, and 16, along with OTU 2 (*Prevotella*) were linked
263 to formate production. Among them, only OTUs 3, 4, 13, and 16 were related to an increase in
264 propionate. The presence of OTU 1 (*Clostridium sp*) correlated with a decrease in acetate, formate,
265 and propionate concentration, and reaffirming this, also there a similar trend relating to the study
266 of microbial diversity by 16S-CE-SSCP with the most dominant OTU (PIC. 1, see Fig. S.3). Finally, 1,3
267 propanediol was not found to be correlated to a specific group of bacteria suggesting that the
268 overall community was able to produce 1,3-PDO whatever the conditions.

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4. Discussion

4.1 Effect of pH and HRT on functional performances

According to the Pearson correlation matrix built on hydrogen productivities, yields, metabolite production profiles, pH and HRT from steady states (Fig 2), the HRT showed a great impact on H_2 yields, H_2 productivities, and butyrate concentrations, especially within the range of 10-14 h (see Fig. 1). In counterpart, pH influenced more the production of succinate. The maximum of H_2 yield and productivity were observed for pH 5.5 and 12 h HRT. This condition showed a performance more than 28.5 times higher than the minimum yield (pH 5.0 and HRT 14 h). Similar trends were previously observed with other substrates when different HRT conditions were studied, and reporting that the most affected variables were H_2 yield and productivity (see Table 3). According to literature, HRT might be used as a tool to select microbial populations (based on growth rates) suitable for the hydrodynamic dilution created in continuous operation (Tapia et al., 2014). It is also described that pH plays an important role on cell membrane polarity and the equilibrium between different chemical forms of compounds, resulting in more or less availability and/or toxicity of the substrates, and thus influencing the enzymatic activity, metabolites profiles and hydrogen producers composition (see Table 3, Arooj et al., 2008; Tapia-Venegas et al., 2013; Fang and Liu 2002; Moscoviz et al., 2016; Cai et al., 2010). Changes in microbial composition influenced by HRT as pH will be discussed later.

It is worth noticing that the hydrogen yields reported in this study represented one of the highest values reported to date for continuous cultures operated with mixed microflora and glycerol (pure and/or crude) as substrate. Such high value was probably due to the optimized range of the operating parameters (pH and HRT), and the use of an adequately treated inoculum as already

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shown elsewhere (Tapia-Venegas et al, 2015b; Temudo et al, 2008; Dounavis et al 2015). However, continuous stirred tank reactors may limit the establishment of a high cell density, being a limiting factor for high hydrogen production rates due to a reduced contact-time between the biomass and the substrate (Han et al., 2012). Therefore, it would be interesting to study this inoculum developed in an immobilized system.

In addition, main metabolites, such as 1,3 propanediol, succinate, and butyrate were similar for all studied steady states, this results are consistent with Moscoviz et al (2016), who showed a consistent production of 1,3 propanodiol whatever the pH in batch tests. A direct correlation was observed between butyrate concentration and hydrogen yield, suggesting that butyrate was the main metabolic pathway to produce hydrogen. Consistently, this relationship was previously statistically demonstrated in other studies operated with mixed cultures (Rafrafi et al., 2013). The inverse correlation of H_2 accumulation with succinate accumulation could be easily explained since the succinate pathway of glycerol degradation, uses NADH potential, that thus lead to a decrease in the potential for hydrogen production (Rafrafi et al. 2013; Temudo et al., 2008). This metabolite has not been reported as fundamental in hydrogen production via fermentation (see table 3). However, it remains a basic chemical product with applications in agriculture as well as in the food and pharmaceutical industries and may be of wider interest. For this reason, further purification from the effluent might be of secondary interest (Mienda et al., 2016). As another compound of industrial interest, the 1,3 propanediol is produced through direct glycerol reduction in opposition with the oxidative pathway where hydrogen is produced. 1,3-PDO leads therefore to lowering the hydrogen yields (see table 3; González-Pajuelo et al 2005; Temudo et al 2008; Dounavis et al 2015). Other studies reported higher yields with major metabolites as acetate and ethanol, in a continuous system (Tapia-Venegas et al., 2015; 0.4 mol_{H2} mol^{-1} $_{glycerol}$). Thus, it is important to pursue future strategies that avoid the production of this metabolite.

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317 In the present study, glycerol degradation efficiency was above 90% in all tests suggesting that the
318 carbon source was the limiting factor of the CSTR, the growth rate of microorganisms responsible
319 of glycerol degradation was always higher than their wash-out rate. For this reason, HRT did not
320 have a great effect on substrate degradation efficiency as previously concluded elsewhere
321 (Romero Aguilar et al., 2013). According to table 2, intermediate hydrogen yield values are
322 obtained at higher organic loading rate (OLR) values and lowest hydrogen yields at lowest and
323 intermediate OLR values, depending on pH (pH higher or lower than 5.5). Other studies have also
324 seen an influence of OLR on hydrogen production, however this influence is unclear (Etchebehere
325 et al., 2016). According to the COD mass balance, the detected by-products represented between
326 55-85% of the COD in the outlet of the reactor for each condition, so it is probably that in some
327 cases, a major metabolite was missing during the analytical procedure.

328 **4.2 Effect of pH and HRT on fermentative microbial communities**

329 The microbial community distribution found at steady state were mainly influenced by pH and not
330 by HRT in the range tested, which is confirmed by the UPGMA clustering analysis (Fig. 1) based on
331 the Pearson coefficient and principal component analysis (Fig. S2). A similar trend was also
332 reported in other studies (see table 3; Ueno et al., 2006). In contrast, Chang et al., (2008) observed
333 a change in the dominant *Clostridium* sp. to *Acidaminococcus* sp. by reducing HRT, with a
334 concomitant effect on community metabolism (Ueno et al., 2006). In the present study, changes in
335 HRT impacted the composition of subdominant members in the mixed culture as observed at pH
336 5.0 (*Veillonellaceae*, *Enterococcus, Kluyvera*, *Pseudomonas Sutteralla*). These subdominant
337 members may be in competition for the substrate and determine the consortium's ability to
338 produce hydrogen by generating positive or negative interactions with hydrogen producers, as
339 suggested elsewhere (Rafrafi et al., 2013). Indeed, HRT may also have an effect on the ability to

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produce hydrogen of the dominant microorganisms in the mixed culture though changes in low abundant keystone species. The importance of subdominant microorganisms has already been reported in literature (Rafrafi et al., 2013).

At lower pHs (5 and 5.5), a primary dominant OTU was obtained that represented near to or above 50% of the population total, while at higher pHs (6), the dominant OTUs had a more equitable distribution (figure 4). Other studies have also reported the effect of pH on microbial diversity, by increasing or decreasing the number of OTUs (Fang and Liu 2002; Zhang et al., 2016).

Two scenarios are proposed as a result of microbial community changes according to the pH (Table 2 and Fig. 3): 1) different microbial structures selected by different pHs had similar yields (pH 5.5 & HRT 8h/pH 6.0 &HRT 10h; pH 6.5 & HRT 8h/pH 5.0 & HRT 10h and pH 6.5&HRT 8h/pH 5.0-HRT 14h), an observation that is consistent with the literature (Cabrol et al., 2016) and 2) similar microbial structures determined by simalar pHs had different hydrogen yields (pH 5.0 & HRT 10h/pH 5.0 & HRT 14h; pH 5.5 &HRT 12h/pH 5.0 & HRT 10h and pH 6.0 & HRT 10h; pH 6.5-HRT 12h). In this case, the ability of the consortium to produce hydrogen is not only determined by the environmental conditions.

In the conditions at pH 5.0 and 5.5, *Clostridium sp,* which is an obligate anaerobic bacteria with the capacity to produce hydrogen, was mostly dominant (Kamalaskar et al., 2016; Tapia-Venegas et al., 2015; Moreno-Andrade et al., 2015). Other microorganisms found in all sequenced samples were related to *Prevotella*, *Sutterella*, *Pseudomonas*, and *Acinetobacter*. *Prevotella*, representing over 13%, could produce H2 at low concentrations (Marques dos Reis et al., 2015). Besides, Pseudomonas groups are a large amount of bacterial species and some of them can produce hydrogen (Aguayo-Villarreal et al., 2016). *Sutterella*, subdominant microorganisms in the samples (0.1-2.6%), have also been found in hydrogen-producing systems, but its function is still unknown

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363 and in some cases, it has been suggested that it could be hydrogen consumers representing a
364 decrease in the production (Chang et al., 2008; Nasr et al., 2015). Similarly, *Acinetobacter* was also
365 found as subdominant in all samples, so its exact function is still unknown within the community,
366 although it consume oxygen traces in the medium (Sun et al., 2016).

367 Regarding others OTUs found of the condition pH 5.5 and HRT 12 h, *Klebsiella* sp., a facultative
368 anaerobic bacterium, was dominant and was already reported as a glycerol consumer and
369 hydrogen producer that can reach a theoretical yield of 1 $mol_{H2}mol^{-1}{}_{glycerol}$ (Chookaew et al., 2012;
370 Niu et al., 2010). In addition, *Alphaproteobacteria* is a very diverse group comprising phototrophic
371 microorganisms such as *Methylobacterium spp* that are generally used for biological gas treatment
372 since they oxidize H_2S (Solcia et al., 2014). *Rhizobium spp* is characterized by its ability to fix
373 biologically N_2 (Yadav et al., 2014) while *Kluyvera* sp are microorganisms found in hydrogen-
374 producing systems, that can produce ethanol from glycerol, but not hydrogen (Zhan et al., 2016).
375 Therefore, hydrogen-producing microorganisms at pH 5.5 and HRT 12 h were represented by
376 *Clostridium sp, Prevotella and Klebsiella sp*. H_2 yield and productivity were maximal at pH 5.5 and
377 HRT 12h, that can be attributed to the presence of *Klebsiella* in this condition, where a positive
378 synergy with other microorganisms that are responsible for hydrogen production such as
379 *Clostridium sp* could also be possible (Seppälä et al., 2011; Tapia-Venegas et al., 2015).

380 For the conditions at pH 6, hydrogen-producing microorganisms were represented by other
381 *Clostridia* and *Clostridium sp.* In the case of *Enterococcus*, some species have previously been
382 described as hydrogen-producing microorganisms, but mainly they are classified as lactic acid
383 bacteria that outcompete H_2-producing bacteria for the substrate (Valdez-Vazquez et al., 2015).
384 *Snodgrassella*, a species that has recently been found in the guts of bees, is a fermentative
385 bacteria. However, it is still unclear whether it can produce hydrogen (Moran et al., 2015;
386 Godálová et al., 2016). *Desulfovibrio* is a sulfate-reducing bacteria that utilizes hydrogen as an

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387 energy source and CO_2 and acetate as a carbon source, but in the absence of sulfate can be a
388 hydrogen producer or interact with *Clostridium* species resulting in H_2 production enhancement
389 (Kpebe et al., 2016).

390 5. Conclusions

391 HRT and pH showed a joint effect on hydrogen production, the production of certain metabolites
392 (such as butyrate and succinate), and microbial structure. However, hydrogen yield and
393 productivity were mainly influenced by HRT, with an observed optimum at pH 5.5 and HRT 12 h.
394 The yield for this condition was 0.58 ± 0.13 $mol_{H2}mol^{-1}{}_{glycerol}$, a value that is much higher than the
395 ones that have been reported in the literature for continuous cultures of mixed inoculum fed with
396 glycerol. However, in order to improve hydrogen productivity, it is still necessary to try other
397 strategies to improve process productivities through the immobilization of specific microbial
398 consortium. The effect of pH was mainly related to changes in the dominant microbial structure of
399 the consortium while the HRT was related to hydrogen yield and hydrogen productivity and
400 subdominant microorganisms, changing the ability of the consortium and/or dominant
401 microorganisms to produce hydrogen. In conclusion, all the strategies investigated in the present
402 study have to be investigated at larger scale for better evaluating the economic and technical
403 feasibilities of the dark fermentation processes to produce cost-effective H_2 prior to
404 commercialization.

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409 away.

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564

565

566 **Fig. 1.** H2 Yield (mole H2/mole gly) obtained in steady state at different pH and HRT represented
567 as response surface (A) and contour plot (B).

568 **Fig.2.** Pearson correlation between hydrogen productivities and yields, metabolite production
569 profiles from steady states (SS) at different pH and HRT studied. Statistically significant
570 correlations are marked according to p-value ≥ 90% (*) y ≥ 95% (*), degree of gray is
571 representative of the correlation, white correspond to negative correlations and black to positive
572 correlations. H2 Yield: hydrogen yield H2 Prod: hydrogen productivity, 1,3 P.D: 1,3 Propanediol,
573 HRT: hydraulic retention time.

574

575 **Fig. 3.** Dynamics of microbial community structures represented by 16S DNA gene CE-SSCP profiles
576 of the all cultures studied in steady state (SS) at different pH and HRT (left) and UPGMA clustering
577 base on Pearson coefficient similarity from 16S rRNA gene-SSCP profiles (right). These CE-SSCP
578 profiles were aligned on the basis of the common ROX internal standard, and areas normalized.
579 The X- and Y-axes represent the relative peak migration distance and the relative peak intensity,
580 respectively. The numbers in SSCP profiles represent the names of pics.

581 **Fig. 4.** Characterization of the four bacterial communities by Illumina analysis of 16S rRNA gene.
582 Percentage in communities are displayed and only the groups of sequences with a relative

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583 abundance higher than 1% in the sample are presented. The shown community represents over
584 90% of the total community (3054 OTUs). A gray scale proportional to sequence abundances is
585 used to better readability.

586 **Fig. 5.** Pearson correlation between hydrogen productivities and yields, metabolite production
587 profiles and sequencing from steady states (SS) for different pH and HRT. Statistically significant
588 correlations are marked according to p-value ≥ 90% (*) and ≥ 95% (**) degree of gray is
589 representative of the correlation, white correspond to negative correlations and black to positive
590 correlations. H2 Yield: hydrogen yield, H2 Prod: Hydrogen productivity, 1,3 P.D: 1,3 Propanediol,
591 HRT: Hydraulic retention time.

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1 **Table 1.** Summary the experimental plan (pH and HRT ranges) and related number of the assays

Assay	pH	HRT
1	6.5	8 h
2	6.5	12 h
3	5.5	8 h
4	5.5	12 h
5	6	10 h
6	6	14 h
7	5	10 h
8	5	14 h

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Table 2. Reactor performances (H_2 yield, H_2 productivity, metabolite composition) according to pH and Hydraulic Retention Times (HRT)

.

Experiment Measurements	pH 5.5 HRT 8h	pH 5.5 HRT 12h	pH 6.0 HRT 10h	pH 6.5 HRT 8h	pH 6.5 HRT 12h	pH 5.0 HRT 10h	pH 5.0 HRT 14h	pH 6.0 HRT 14h
H_2 Yield ($mol_{H2}mol^{-1}_{glycerol}$)	0.22 ± 0.05	0.58 ± 0.13	0.24 ± 0.02	0.17 ± 0.08	0.05 ± 0.03	0.17 ± 0.04	0.02 ± 0.02	0.26±0.04
H_2 Productivity ($mmol_{H2}l^{-1}\ d^{-1}$)	43.4 ± 6.7	88.0 ± 20.2	53.7 ± 9.9	58.7 ± 16.9	7.7 ± 3.9	38.8 ± 7.0	2.5 ± 1.3	51.0±10.1
H_2 % in biogas	56±15	61±7	68±3	64±12	37±14	61±10	21±8	57±2
Glycerol degradation (%)	98.4	97.5	99.7	96.8	97.1	99.6	92.2	100.0
Succinate %COD	55.1 ± 1.0	7.4 ± 6.0	30.8 ± 1.0	15.2 ± 18.1	33.2 ± 4.5	32.4 ± 1.7	38.8 ± 7.3	28.9 ± 2.2
Formate %COD	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	2.5 ± 1.6	1.0 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.5 ± 0.4
Acetate %COD	5.5 ± 0.5	6.1 ± 2.9	8.7 ± 1.3	7.2 ± 1.3	10.8 ± 1.6	4.6 ± 1.9	4.2 ± 1.6	7.3 ± 2.7
1,3-Propanediol %COD	24.5 ± 2.8	48.0 ± 19.0	42.4 ± 2.6	53.2 ± 17.7	30.8 ± 7.4	42.1 ± 2.9	30.8 ± 6.8	32.6 ± 1.8
Propionate %COD	0.7 ± 0.5	4.5 ± 10.0	5.0 ± 0.9	4.2 ± 1.5	7.2 ± 3.1	5.5 ± 0.5	8.5 ± 0.7	15.8 ± 1.5
Ethanol %COD	0.7 ± 0.5	3.5 ± 2.9	0.8 ± 0.1	5.7 ± 2.1	7.5 ± 8.7	2.0 ± 0.6	2.0 ± 1.0	1.0 ± 1.9
Butyrate %COD	13.4 ± 0.5	30.5 ± 14.1	12.2 ± 2.0	12.0 ± 1.8	9.5 ± 3.1	14.3 ± 3.2	15.8 ± 3.2	14.0 ±4.1
Biomass g VSS/l	0.42 ± 0.08	0.54 ± 0.17	0.45 ± 0.09	0.64 ± 0.07	0.46 ± 0.06	0.56 ± 0.03	0.42 ± 0.21	0.5 ± 0.06
Effluent total COD mg/l (x%)	4623 (60.1)	4635 (55.1)	8173 (77.9)	8111 (72.2)	6005 (58.9)	8836 (85.4)	6694 (70.6)	8562 (84.0)
Specific Productivity (mmole H_2/g VSS/d)	103.3	163.0	119.3	91.7	16.7	69.3	6.0	102.0
Organic loading speed (OLR) ($Kg_{COD}m^{-3}\ d^{-1}$)	37	24	29	37	24	29	21	21

%COD metabolite concentration expressed in percentage of total metabolite concentrations (converted to COD).

(x%) Percentage measured of total COD output.

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Table 3. Hydrogen production assays from fermentation reported in literature.

Inocula/ treatment	Operating conditions	H2 yield ($_{molH2}$ mol^{-1} $_{susbrate}$)	Microbial Observations	Environmental Observations	Reference
Anaerobic sludge/ heat treated at 90 °C for 10min	Starch CSTR pH 5.3 T 35°C HRT 18 to 4h	0.92	-	Optimal yield at HRT 9 h. Yield is the most affected variable Butyrate and acetate were main metabolites	Arooj et al., 2008
Acidogenic sludge	Glucose CSTR pH 4 to 7 T 36°C HRT 6 h	2.1	The number of OTUs increases as pH increases	Optimum at pH 5.5 for hydrogen yield and specific hydrogen production rate Propionate concentration is one of the most affected variables	Fang and Liu 2002
Anaerobic sludge/Low HRT	Glucose CSTR pH 4 to 7 T: 35°C HRT 6 to 14h	1.54	-	Optimal yield at pH 5.5 and HRT 12 h. Hydrogen yield are productivity are the most affected variables	Tapia-Venegas et al., 2013
Anaerobic sludge/ Low HRT and progressive adaptation	Glycerol CSTR pH 5.5 HRT 12 h T: 37°C	0.4	Shift in dominant community members according to the gradient of glycerol proportion in the feed.	Ethanol and acetate as main metabolites	Tapia-Venegas et al., 2015
Activated sludge/ Aerobic enrichment	Glycerol CSTR pH 5 to 6.5 T: 35°C HRT 8 to 14h	0.02-0.58	The effect of pH is related to changes in the dominant microbial structure of the consortium	Optimal yield at pH 5.5 and HRT 12 h. Hydrogen yield and productivity are the variables that are most affected by HRT Succinate concentarion is the variable most affected by pH. Succinate, 1.3 propanediol, and butyrate were the main metabolites	This study
Anaerobic sludge/ heat treated 100 °C for 20 min	Crude glycerol up-flow, packed bed column bioreactor T: 35°C $pH_{initial}$ 6 to 7 HRT 36 to 24 h	0.15-0.53	-	1,3 propanediol and butyrate were the main metabolites. Higher hydrogen production at HRT 24 h	Dounavis et al 2015

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Clostridium acetobutylicum ATCC 824	Glycerol CSTR pH 6.5 35°C HRT NR	<0.1	-	1.3 propanediol and butyrate were the main metabolites	Gonzalez-Pajuelo et al., 2005
	Glucosa pH 4.5-7 T: 70°C		Increase in community richness at lower pHs Higher yield attributed to the enrichment of the consortium with a specific microorganism	Optimal yield at pH 5.5	Zhang et al., 2016
Activated sludge/heat treated 100 °C for 45min	Molasses (hexose) CSTR T 35 °C HRT 12-3h pH 5.5	2.1	Change in the dominance of *Clostridium sp.* to *A Acidaminococcus* sp at lower HRT	Higher yield at lower HRT. Acetate and butyrate were the main metabolites	Chang et al., 2008
Compost/ Enrichment	Solid waste CSTR HRT 141 to 12h pH 5 to 8 T 60°C	645 mLH2 / gCOD	*Thermoanaerobacterium thermosaccharolyticum* was the dominant hydrogen-producing micro-organism and unidentified organisms became dominant after HRT 96 h and pH 7 or 8 . Hydrogen is not observed at these conditions.	Acetate and butyrate were the main metabolites Higher H2 yields at lower HRT and lower pH. The effect of HRT on community metabolism.	Ueno et al., 2006
Co-culture *E. coli* CECT432 and *Enterobacter* spH1	Crude glycerol Batch T 37°C pH initial 6.8	1.53	Co-culture 1:1	Ethanolm was the main metabolite	Maru et al., 2016

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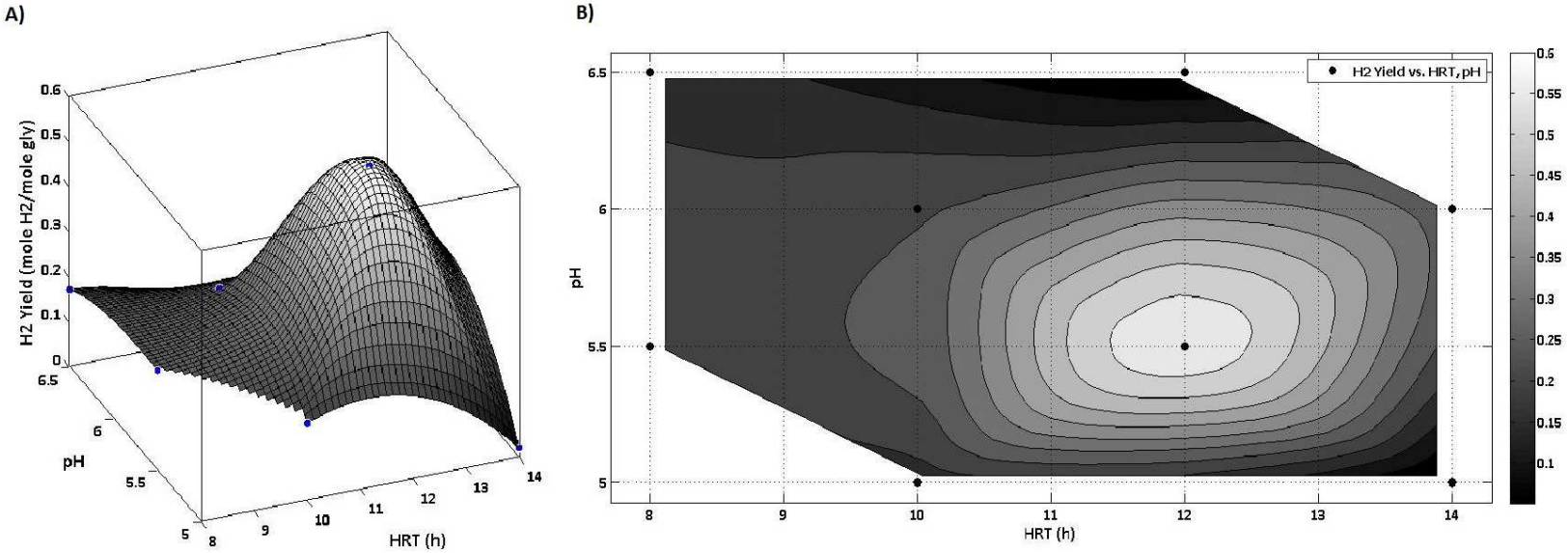

Fig. 1. H_2 Yield (mole H_2/mole gly) obtained in steady state at different pH and HRT represented as response surface (A) and contour plot (B).

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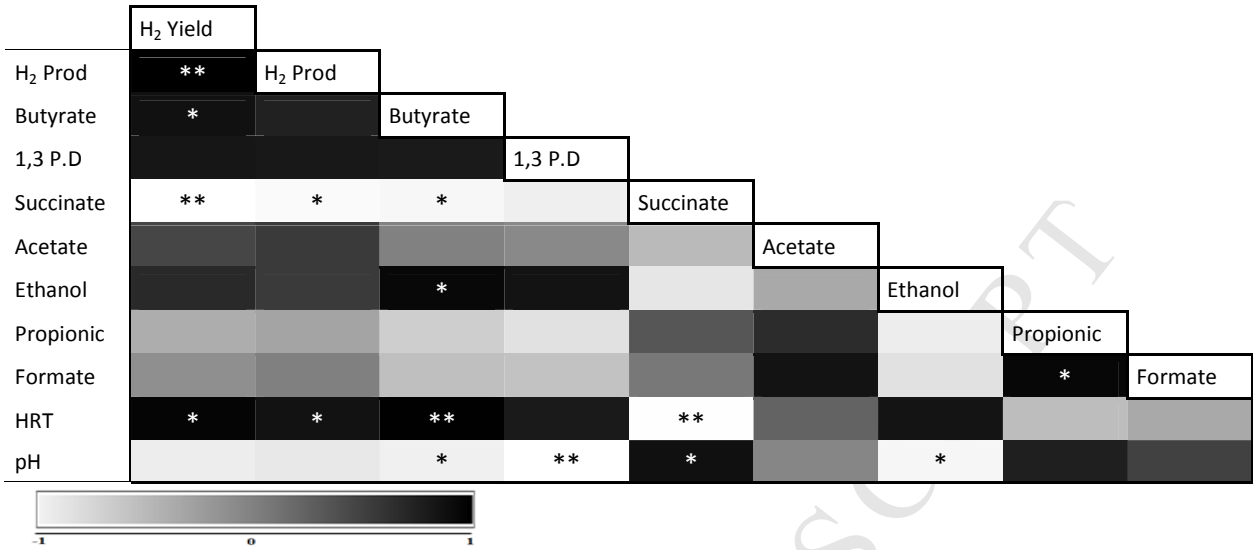

Fig.2. Pearson correlation between hydrogen productivities yields, metabolite production profiles, pH and HRT from steady states (SS) at different pH and HRT studied. Statistically significant correlations are marked according to p-value ≥ 90% (*) y ≥ 95% (*), degree of gray is representative of the correlation, white correspond to negative correlations and black to positive correlations. H_2 Yield: hydrogen yield H_2 Prod: hydrogen productivity, 1,3 P.D: 1,3 Propanediol, HRT: hydraulic retention time.

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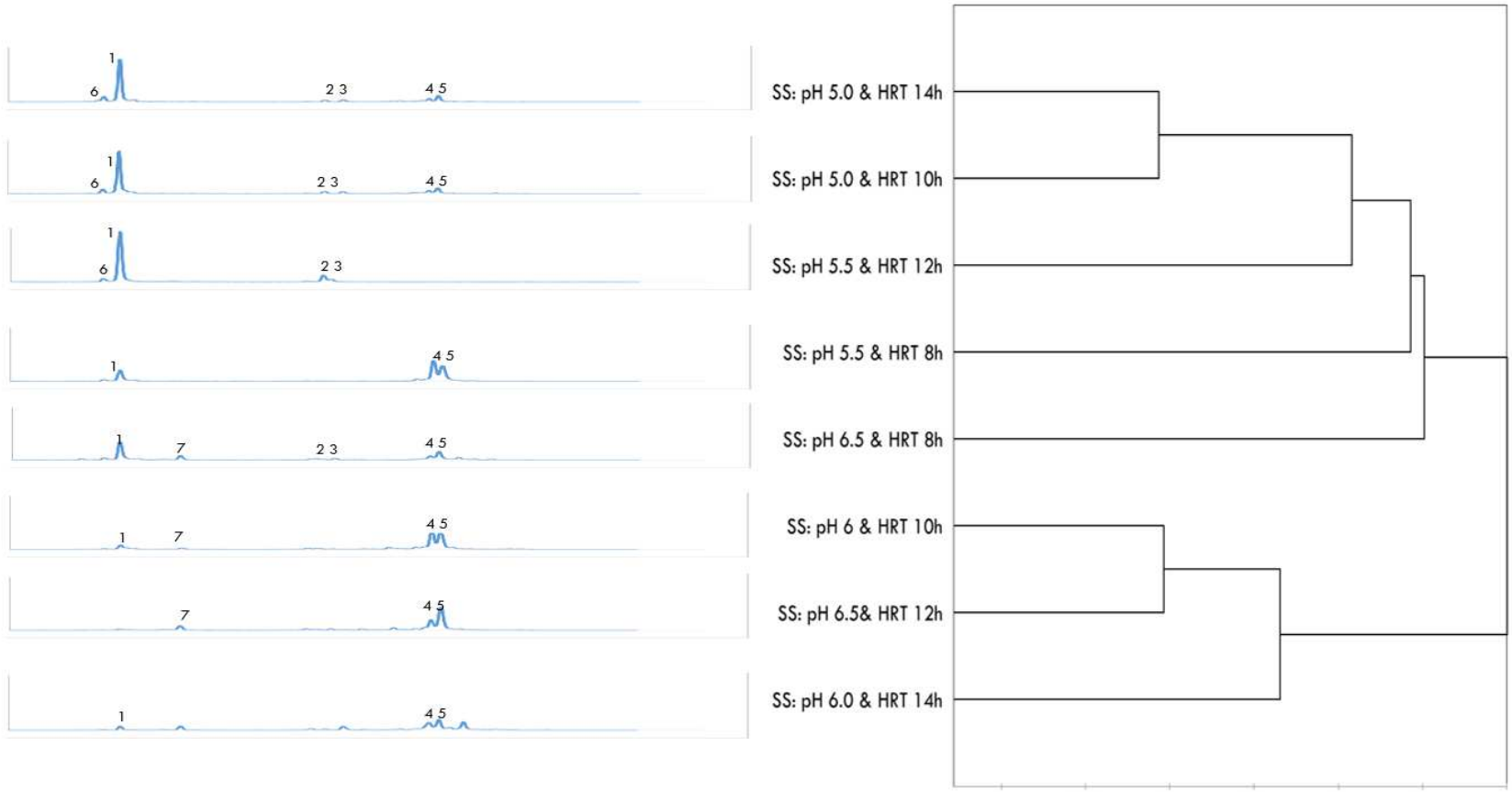

Fig. 3. Dynamics of microbial community structures represented by *16S DNA* gene CE-SSCP profiles of the all cultures studied in steady state (SS) at different pH and HRT (left) and UPGMA clustering base on Pearson coefficient similarity from 16S rRNA gene-SSCP profiles (right). These CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and areas normalized. The X- and Y-axes represent the relative peak migration distance and the relative peak intensity, respectively. The numbers in SSCP profiles represent the names of pics.

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Samples			SS: pH 5.5 & HRT 12	SS: pH 6.0 & HRT 14	SS: pH 5.0 & HRT 10	SS: pH 5.0 & HRT 14	
identified OTUs			3054	3054	3054	3054	
	Family	Genus	Number of OTUS	Sequence relative abundance per sample (all 5 sequences)			OTU
Phylum Firmicutes	***Clostridiaceae***	***Clostridium sp***	6	58.9%	8.4%	61.9%	64.2%	*1*
	Veillonellaceae		2	1.8%	2.5%	-	5.8%	*2*
	Enterococcaceae	***Enterococcus***	1	-	20.6%	0.5%	-	*3*
	Other Clostridia	***unclassified***	5	-	18.3%	-	-	*4*
Phylum Bacteroidetes	***Prevotellaceae***	***Prevotella***	4	13.0%	22.2%	17.4%	16.0%	*5*
	Bacteroidaceae	***Bacteroides***	2	1.5%	4.1%	0.2%	-	*6*
Phylum Proteobacteria	***Enterobacteriaceae***	***Enterobacter***	1	0.1%	0.1%	-		*7*
	Enterobacteriaceae	***Klebsiella***	2	11.3%	-	0.1%	0.1%	*8*
	Enterobacteriaceae	***Kluyvera***	1	1.1%	-	2.4%	0.1%	*9*
	Moraxellaceae	***Acinetobacter***	3	0.5%	5.1%	4.7%	1.0%	*10*
	other Gammaproteobacteria		4	-	-	-	-	*11*
	Pseudomonadaceae	***Pseudomonas***	1	0.1%	0.4%	0.4%	2.2%	*12*
	Neisseriaceae	***Snodgrassella***	1	0.3%	13.9%	1.0%	0.1%	*13*
	Other Alphaproteobacteria		2	0.1%	-	-	-	*14*
	Alcaligenaceae	***Sutterella***	2	1.5%	0.1%	1.8%	2.6%	*15*
	Desulfovibrionaceae	***Desulfovibrio***	1	-	3.4%	-	-	*16*

Fig. 4. Characterization of the four bacterial communities by *Illumina* analysis of *16S* rRNA gene. Percentage in communities are displayed and only the groups of sequences with a relative abundance higher than 1% in the sample are presented. The shown community represents over 90% of the total community (3054 OTUs). A gray scale proportional to sequence abundances is used to better readability.

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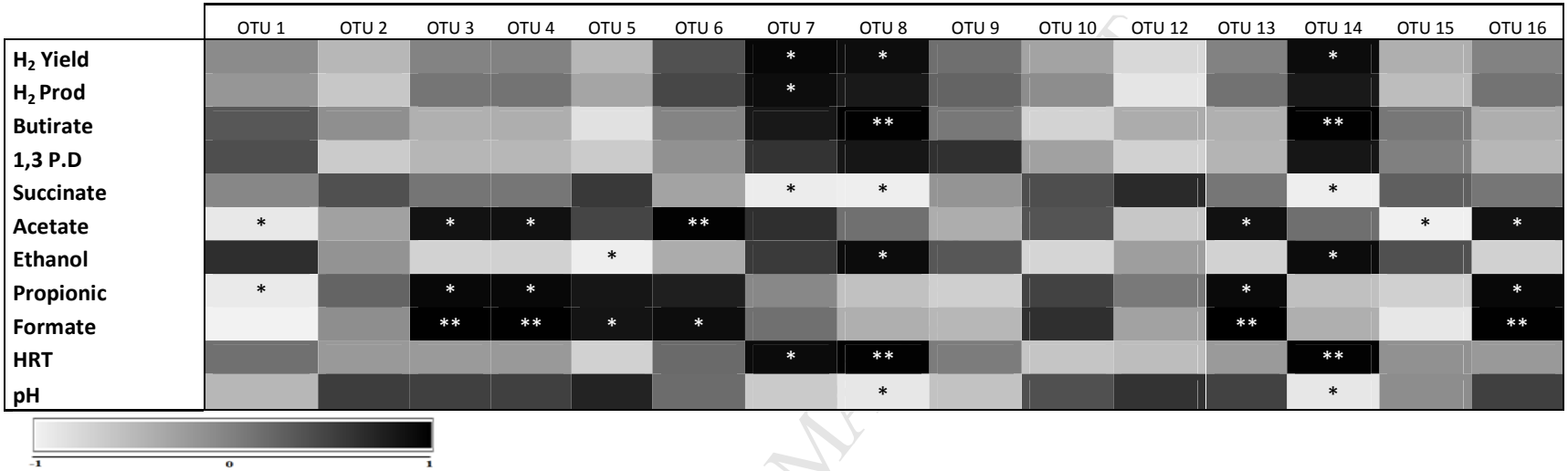

Fig. 5. Pearson correlation between environmental variables and sequencing at steady state (SS)for different pH and HRT. Statistically significant correlations are marked according to p-value ≥ 90% (*) and ≥ 95% (**) degree of gray is representative of the correlation, white correspond to negative correlations and black to positive correlations. H_2 Yield: hydrogen yield, H_2 Prod: Hydrogen productivity, 1,3 P.D: 1,3 Propanediol, HRT: Hydraulic retention time.

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Highlights

- The optimum for H_2 yield was found in the vicinity of pH 5.5 and HRT 12 h
- Hydrogen yield and productivity were mainly influenced by HRT
- Changes in dominant microbial community structures were mainly influenced by pH
- In all cases, the *Firmicutes* phylum was dominant (above 50%)
- Some bacteria from *Proteobacteria* phylum are correlated to high H_2 yields

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