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# IMPROVEMENT OF BIOHYDROGEN PRODUCTION FROM GLYCEROL IN MICRO-OXIDATIVE ENVIRONMENT

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## Abstract

Glycerol is a highly available by-product generated in the biodiesel industry. It can be converted into higher value products such as hydrogen using biological processes. The aim of this study was to optimize a continuous dark fermenter producing hydrogen from glycerol, by using micro-aerobic conditions to promote facultative anaerobes. For that, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was continuously added at low but constant flow rate (0.252 mL/min) with three different inlet concentrations (0.2, 0.4, and 0.6% w/w). A mixture of aerobic and anaerobic sludge was used as inoculum. Results showed that micro-oxidative environment significantly enhanced the overall hydrogen production. The maximum H<sub>2</sub> yield (403.6±94.7 mmolH<sub>2</sub>/molGly<sub>consumed</sub>) was reached at a H<sub>2</sub>O<sub>2</sub> concentration of 0.6% (w/w), through the formate, ethanol and butyrate metabolic pathways. The addition of H<sub>2</sub>O<sub>2</sub> promoted the development of facultative anaerobic microorganisms such as *Klebsiella*, *Escherichia-Shigella* and *Enterococcus* sp., likely by consuming oxygen traces in the medium and also producing hydrogen. Despite the micro-oxidative environment, strict anaerobes (*Clostridium* sp.) were still dominant in the microbial community and were probably the main hydrogen producing species. In conclusion, such micro-oxidative environment can improve hydrogen production by selecting specific microbial community structures with efficient metabolic pathways.

Keywords: Biohydrogen, Dark fermentation, Continuous stirred-tank reactor, H<sub>2</sub>O<sub>2</sub>, Mixed culture, Glycerol.

## 38 1. Introduction

39

40 Due to limited energy reserves and an increasing environmental pressure on greenhouse gases released from  
41 fossil fuels uses, renewable energy sources have considerably gained in attention over the past decades [1]. In  
42 particular, biohydrogen production by dark fermentation has been widely investigated due to a simpler operating  
43 configuration than other biological processes [2]. However, the dark fermentation process still needs to be  
44 optimized to reach the economic viability [3]. Several solutions have been proposed to reduce the costs of the  
45 overall process. Different approaches have been investigated to improve the economic viability of such  
46 technology at larger scale, such as (i) the use of low cost substrates (such as waste or industrial by-products), (ii)  
47 the operation of bioreactors with mixed cultures instead of pure axenic cultures, and (iii) the improvement of  
48 hydrogen yields by changing the operating conditions with a target of a maximum of 4 molH<sub>2</sub>/molglucose [4–6]. In  
49 particular, glycerol is a by-product generated by the biodiesel industry and represents around 10 % w/w of the  
50 total biodiesel end-products. Raw glycerol is a cheap and widely available substrate. Moreover, biodiesel  
51 consumption is growing at a rate of 30–50% per year making glycerol resources largely available in the future [7].  
52 The glycerol production is estimated to reach about 40,000 tons per year by 2020 [8].

53 Up to now, most of the studies dealing with hydrogen production by dark fermentation, and using glycerol as  
54 carbon source were carried out with pure microbial cultures [8, 9, 10]. The advantage of using pure culture is the  
55 relatively high yields achieved, up to 935 mmolH<sub>2</sub>/molGly which is close to the theoretical maximum of 1000  
56 mmolH<sub>2</sub>/molGly [11]. In contrast, the use of mixed culture in dark fermentation presents several advantages such  
57 as no need of sterile conditions, lower sensitivity to contaminants and thus lower operational costs of the overall  
58 process. In mixed cultures, hydrogen-producing bacteria are mainly related to the *Clostridiaceae* (strict  
59 anaerobes) [12] or *Enterobacteriaceae* (facultative anaerobes) [13,14] families that are found in many natural  
60 environments; e.g. anaerobic digesters, activated sludge-treating bioreactors, compost piles, soil or cow manure  
61 [15–17]. The presence of facultative anaerobes in dark fermentation reactors can be beneficial due to their ability  
62 to consume oxygen and generate strict anaerobic conditions, optimal to hydrogen production. Such collaborative  
63 bacterial growth was already observed by Yokoi et al. [18] who worked with a co-culture of strict and facultative  
64 anaerobes (*Clostridium butyricum* and *Enterobacter aerogenes*) in continuous conditions and applied aeration  
65 shocks to the medium (20 min). After that shock, the co-culture had the ability to remove O<sub>2</sub> traces by the  
66 presence of *Enterobacter aerogens* and recover efficient H<sub>2</sub> production by *Clostridium butyricum*. Moreover, it has

67 already been reported that aeration shock in dark fermentation can improve the hydrogen production by  
68 facultative anaerobes by favouring the oxidative pathway. As an illustration, an aeration shock resulted in the  
69 improvement of ethanol and hydrogen production in *Enterobacter aerogenes* [19]. Pachapur et al. [19] observed an  
70 improvement of hydrogen production performances, i.e. from 21.4 mmolH<sub>2</sub>/L to 26.1 mmolH<sub>2</sub>/L, when initial  
71 aerobic conditions were applied on a co-culture of *Enterobacter aerogenes* and *Clostridium butyricum* if compared  
72 to initial strict anaerobic conditions (with nitrogen sparging).

73 However, due to the high microbial community diversity in mixed cultures, the organic substrates can also be  
74 converted into many other metabolic end-products than hydrogen. Mixed cultures may also contain strict  
75 anaerobic hydrogen consumers such as methanogenic or homoacetogenic microorganisms, which could affect  
76 the overall process performances [17,20]. Thus, one of the methods suitable for selecting strict and facultative  
77 anaerobes from mixed cultures is to operate the reactor under micro-oxidative conditions. Li et al. [21] reported an  
78 improvement of the hydrogen production using mixed culture and oxidative environment through oxygen addition  
79 (0.28 mL O<sub>2</sub>/gTS) in batch tests. A 44% increase of the hydrogen yield, from 28.9 mLH<sub>2</sub>/gVS to 41.6 mLH<sub>2</sub>/gVS,  
80 was observed. In order to better control the micro-oxidative environment and improve the diffusion of oxygen, the  
81 addition of a chemical reactant such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) constitutes an alternative to gas injection.  
82 However, hydrogen peroxide generates oxygen, H<sub>2</sub>O as well as OH• radicals [22]. Owusu-Ansah et al. [23]  
83 already showed that excessive reactive oxygen species (ROS) such as OH• radicals could damage microbial cells  
84 and negatively impact hydrogen production. Moreover, injection of a strong oxidant can increase the redox  
85 potential (ORP) of the medium with potential effect on fermentation pathways. Indeed, the redox potential value  
86 should be maintained between -250 and -524 mV to favour the hydrogen producing pathways, i.e. acetate and  
87 butyrate [22,23]. Therefore, to improve the hydrogen production using micro-oxidative environment, the  
88 concentration of H<sub>2</sub>O<sub>2</sub> added in the dark fermenter must remain below the critical concentration of inhibition.

89

90 The present study aims to investigate the effect of hydrogen-peroxide (H<sub>2</sub>O<sub>2</sub>) addition to maintain micro-oxidative  
91 conditions in a mixed culture-based reactor operated in continuous mode with glycerol as substrate. The impact of  
92 different concentrations of the oxidizing agent (0.2, 0.4, 0.6% (w/w)) on hydrogen production performances,  
93 microbial communities and metabolic pathways were evaluated to understand the improvement of hydrogen  
94 performances under micro-oxidative conditions.

95

## 96 **2. Materials and Methods**

### 97 **2.1. Continuous H<sub>2</sub> production experiments**

98

99 The experiments were carried out in a continuous stirred tank reactor (CSTR) with a working volume of 2 L. Fig. 1  
100 shows the scheme of the reactors and the overall equipment design. The reactor was operated at a temperature  
101 of 37 °C, hydraulic retention time (HRT) of 12 h and mixing rate of 350 rpm [26]. Pure glycerol (15 g/L) from  
102 SIGMA ALDRICH (CAS: 56-81-5) was used as sole carbon source. The pH was initially adjusted at 6.5 and  
103 controlled during the whole experiment by adding NaOH (1.5M). A mixture of anaerobic and aerobic sludge  
104 collected from a municipal wastewater treatment plant (1:1 based on Volatile Solids (VS)) was used as inoculum.  
105 A substrate/inoculum (S/X) ratio of 10 (on VS basis) was used in all the experiments. The culture medium was  
106 prepared according to Varrone et al. [30], and was composed of the following elements: K<sub>2</sub>HPO<sub>4</sub> (0.125 g/L),  
107 NH<sub>4</sub>Cl (0.5 g/L), MgSO<sub>4</sub>\*7H<sub>2</sub>O (0.05 g/L), CaCl<sub>2</sub>\*2H<sub>2</sub>O (0.005 g/L), FeSO<sub>4</sub>\*7H<sub>2</sub>O (0.005 g/L), yeast extract (0.5  
108 g/L). Micro-oxidative environment was provided by continuous injection of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as oxidizing  
109 agent, under a flow rate of 0.252 mL/min. One control (no H<sub>2</sub>O<sub>2</sub>) and three different concentrations of H<sub>2</sub>O<sub>2</sub> were  
110 tested: 0.0, 0.2, 0.4 and 0.6 (w/w). These concentrations were chosen to avoid excessive stress conditions on the  
111 microbial community.

112 To ensure continuous operation of the reactor, the fermentation medium was continuously fed with a peristaltic  
113 pump and the working volume was maintained by a level sensor connected to an effluent discharge pump. The  
114 volume of biogas was measured by a water displacement method and was daily analysed for its composition.  
115 Temperature, pH and ORP parameters were monitored online. Each experiment was carried out for at least 10  
116 days, equivalent to 20 HRT. Liquid samples were periodically collected for metabolites and microbial community  
117 analysis.

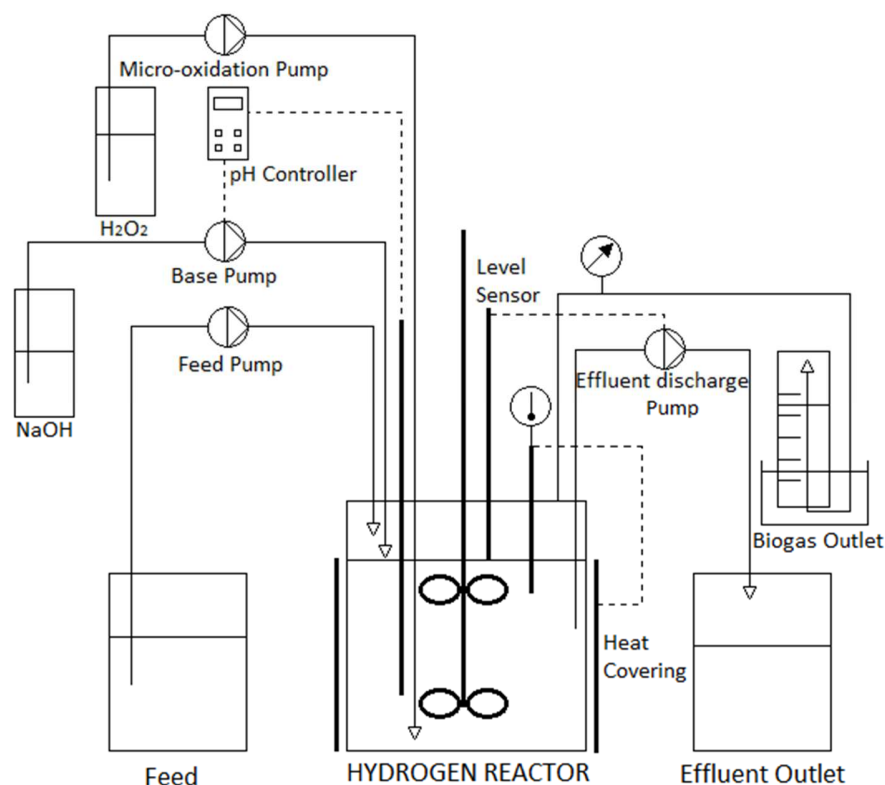


Figure 1 : Schematic diagram of the continuous stirred tank reactor (CSTR) and the experimental equipment

## 2.2. Analytical methods

Gas composition was measured using a gas chromatograph Perkin Clarus 580 composed of an injector heated at 250°C and two capillary columns heated at 60°C each. The first column was an RtUbond for the detection and quantification of CO<sub>2</sub>. The second column was an RtMolsieve used for detection and quantification of O<sub>2</sub>, H<sub>2</sub>, N<sub>2</sub> and CH<sub>4</sub>. The carrier gas was argon at a pressure of 350 kPa at 31.8 mL/min. The detection was ensured by a thermal conductivity detector at 150°C.

Volatile fatty acids (VFAs) were quantified with an Elite-FFAP crossbond@carbowax® 15 m column connected to a flame ionization detector at 280°C and N<sub>2</sub> at 6 mL/min as carrier gas, in a gas chromatograph Perkin Clarus 580, as described elsewhere [31]. Glycerol, formate, ethanol and 1,3-propanediol were quantified using high performance liquid chromatograph (HPLC). The chromatograph was composed of an automatic sampler (Water 717), a pre-column to filter residues (Micro guard cation H refill cartridges, Bio-rad) and an Aminex HPX-87H column (300 mm on 7.8 mm, Bio-rad) at 35°C. The carrier liquid used was sulfuric acid at 4 mM at 0.4 mL/min

134 [31]. For the control, metabolite analysis were performed on the days 0.5, 3, 6.5 and 10, for 0.2% analysis were  
135 performed on the day 1, 4, 5, 6, 7, 8, 9, for 0.4% on the day 0, 1, 2, 3, 6, 7, 8, 9 and 10 and for 0.6% metabolites  
136 from day 2.5, 4.5, 6.5, 7.5, 10 were analysed.

### 137 **2.3 Microbial community analysis**

138 For each reactor, molecular analysis of the bacterial community was periodically performed. Microbial cells were  
139 collected after centrifugation at 13,000 g for 15 min of 2 mL of fermentation broth. DNA was extracted, as  
140 previously described [32]. Total extracted DNA was purified using a QiAmp DNA microkit (Qiagen, Hilden,  
141 Germany). The amount and purity of DNA in the extracts were measured by spectrophotometry (Infinite  
142 NanoQuant M200, Tecan).

143 The V3 region of 16S rRNA genes were amplified using universal primers for bacteria (W49 and W104) according  
144 to Milferstedt et al. [32]. DNA samples were sequenced by Illumina MiSeq (get.genotoul.fr) as described  
145 elsewhere [33]. Sequences were analysed using the bioinformatics pipeline described in Moscoviz et al. [34] and  
146 were grouped into operational taxonomic units (OTUs) with 97% similarity. Sequences were submitted to the  
147 GenBank database, under the accession numbers N° KY048455 - KY049838. Pearson correlation matrix was  
148 calculated using bacterial OTUs relative abundance and fermentation production concentrations as variables, as  
149 reported in [34].

### 150 **2.3 Data analysis**

151 Total substrate degradation and metabolites productions were assessed with a theoretical Chemical Oxygen  
152 Demand (COD) mass balance. The hydrogen yield corresponded to the best performances observed during at  
153 least 3 HRT (1.5 day) in terms of H<sub>2</sub> recovered (in mmolH<sub>2</sub>) per mole of glycerol consumed per period of 12 h (1  
154 HRT). The hydrogen yield was calculated as follows (Eq. 1):

$$155 Y_{\max} = \frac{n H_2}{n \text{ glycerol}_{\text{consumed}}} \quad (\text{mmolH}_2/\text{molGly}_{\text{consumed}}) \quad (1)$$

156 Where n H<sub>2</sub> is the cumulative amount of moles of H<sub>2</sub> produced during 12 h (1 HRT) and n glycerol<sub>consumed</sub> the total  
157 consumed moles of glycerol over the same period of time.

158 Pearson correlations and significance calculations were made with the R 3.1.3 software (R Development Core  
159 Team 2010). For correlation coefficient calculations, the function “rcorr” of the package Hmisc was used.  
160 Significance levels were assessed using 9999 random permutations with the function “sample” of the package  
161 combinat (p-values  $\pm 0.0001$ ). Only OTUs with a relative abundance of at least 3% in one of the samples were  
162 considered. Clusters were assessed from the Pearson correlation network topology using the R 3.1.3 software  
163 with the “cluster\_edge\_betweenness” function of the package “igraph”. The weight considered for this function  
164 was the absolute value of the correlations.

### 165 **3. Results and discussion**

166

#### 167 **3.1. Effect of micro-oxidative conditions on continuous hydrogen** 168 **production from glycerol**

169

170 During all the experiments, no methane was observed, showing that hydrogen consumption was not caused by a  
171 methanogenic activity. As shown in Table 1, the average redox value in the medium remained in the same range  
172 of -495.3 mV and -543.9 mV in the control and for all concentrations of H<sub>2</sub>O<sub>2</sub>. All these redox potentials were  
173 within an optimal range for hydrogen production, i.e. from -250 to -524 mV, as previously reported by Lin et al.  
174 [24]. According to this observation, injection of H<sub>2</sub>O<sub>2</sub> at the tested concentrations (0.2, 0.4 and 0.6% (w/w)) did not  
175 macroscopically affect the redox potential of the medium in comparison to the control (i.e. -511.54 mV) and  
176 enabled the microbial community to remain in a favourable environment for hydrogen production. Such redox  
177 potential stability could be explained by the fact that the injected H<sub>2</sub>O<sub>2</sub> was rapidly consumed by the microbial  
178 community since no oxygen was observed in the gas phase. As mentioned in Table 1, the hydrogen content in  
179 the biogas ranged from 33% to 49% for the experiments performed with H<sub>2</sub>O<sub>2</sub> addition at 0.4 and 0.6 %,  
180 respectively. Consistently, hydrogen content in dark fermenters using glycerol as substrate is usually ranging from  
181 33 to 54% [35,10].

182

183 Nonetheless, the micro-oxidative conditions impacted the overall hydrogen performances since the maximal  
184 hydrogen yields were significantly different at each H<sub>2</sub>O<sub>2</sub> concentration. At low H<sub>2</sub>O<sub>2</sub> concentration (0.2%), the  
185 maximum yield was found at  $44.1 \pm 1.3$  mmolH<sub>2</sub>/molGly<sub>consumed</sub> which was lower than the maximum yield found in  
186 the control ( $90.6 \pm 2.8$  mmolH<sub>2</sub>/molGly<sub>consumed</sub>). The increase of H<sub>2</sub>O<sub>2</sub> concentration to 0.4% (w/w) led to a



187 significant increase of the maximum hydrogen yield to  $290.7 \pm 136.2$  mmolH<sub>2</sub>/molGly<sub>consumed</sub>. The highest yield was  
 188 found at 0.6% (w/w) H<sub>2</sub>O<sub>2</sub> with  $403.6 \pm 94.7$  mmolH<sub>2</sub>/molGly<sub>consumed</sub>.

189

190 Table 1 : Maximum hydrogen production in CSTR operated at different inlet H<sub>2</sub>O<sub>2</sub> concentrations (0.0, 0.2, 0.4  
 191 and 0.6 %) over a period of 10 days. (operating conditions: Temperature, 37°C; pH, 6.5; glycerol concentration,  
 192 15 g/l; HRT, 12h)

| [H <sub>2</sub> O <sub>2</sub> ] <sub>added</sub><br>(w/w) | Total H <sub>2</sub> O <sub>2</sub><br>added<br>(mol) | Steady state Yield <sub>max</sub><br>(mmolH <sub>2</sub> /molGly <sub>consumed</sub> ) | H <sub>2</sub><br>cumulated<br>(mmolH <sub>2</sub> ) | Average<br>redox<br>(mV) | H <sub>2</sub> content<br>(%) |
|--|---|--|--|--------------------------|-------------------------------|
| 0%   | 0   | 90.6±2.8   | 270.3  | -511.5                   | 45                            |
| 0.2%   | 0.309   | 44.1±1.3   | 98.1   | -495.3                   | 41                            |
| 0.4%   | 0.619   | 290.7±136.2  | 501.5  | -543.9                   | 33                            |
| 0.6%   | 0.927   | 403.6±94.7   | 790.1  | -506.2                   | 49                            |

193

194

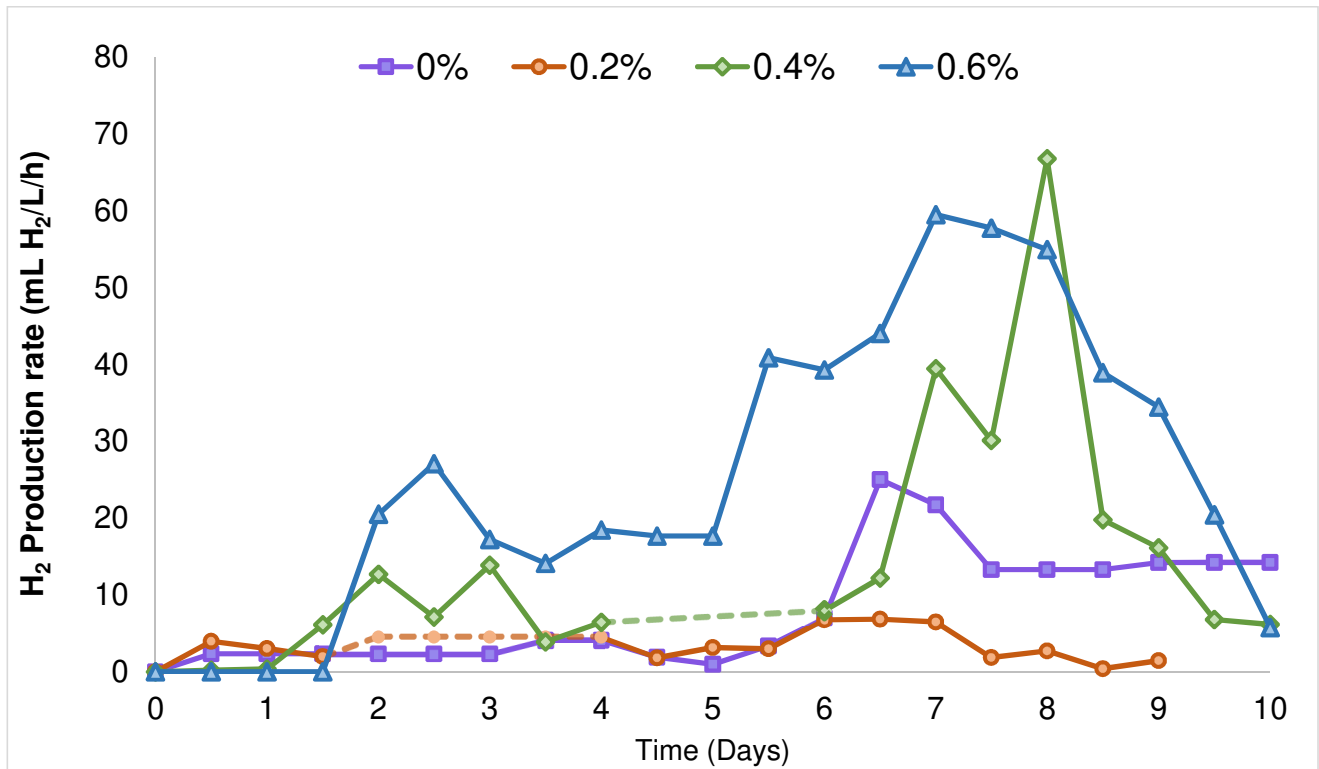
195 Fig. 2 shows the time course profiles of the hydrogen production rate at the four different inlet concentrations of  
 196 H<sub>2</sub>O<sub>2</sub> (0.0, 0.2, 0.4 and 0.6%). In the control, hydrogen production started after 6 days which can be assimilated to  
 197 the lag phase. The maximum productivity of 25.1 mLH<sub>2</sub>/L/h was found at day 6.5 and a stable but low production  
 198 rate around  $13.8 \pm 0.5$  mLH<sub>2</sub>/L/h was then observed from day 7.5 to 10. At the lowest concentration of H<sub>2</sub>O<sub>2</sub> (0.2%  
 199 w/w), the hydrogen lag phase was similar to the control (6 days) with a stable but lower production rate of  $6.7 \pm 0.2$   
 200 mLH<sub>2</sub>/L/h from day 6 to 7 (during 4 HRT). Injection of H<sub>2</sub>O<sub>2</sub> at 0.2% had a detrimental effect on the hydrogen  
 201 production rate when compared to the control.

202 Interestingly, at 0.4%, the lag phase decreased to 1.5 days with a first phase of hydrogen production from day 1.5  
 203 to day 6.5, with a low hydrogen production rate of  $6.0 \pm 4.3$  mLH<sub>2</sub>/L/h. An improvement of hydrogen production rate  
 204 was then observed from day 7 to 8 reaching  $45.5 \pm 19.1$  mLH<sub>2</sub>/L/h with a maximum of 66.8 mLH<sub>2</sub>/L/h at day 8.  
 205 However, a severe drop of the hydrogen production rate was observed after 8 days and decreased to 6.2  
 206 mLH<sub>2</sub>/L/h at day 10.

207 For the last condition (0.6%), as already found with the 0.4% experiments, the lag phase was shorter compared to  
 208 the control with a first phase of hydrogen production from day 1.5 to 5 with an average value of  $19.0 \pm 4.1$   
 209 mLH<sub>2</sub>/L/h. Then, after a sharp increase, a stationary phase was reached with a constant hydrogen production rate

210 of  $49.4 \pm 9.0$  mL $H_2$ /L/h and was maintained during 4 days (i.e. 8 HRT). After that period, the hydrogen production  
211 rate rapidly decreased down to 5.7 mL $H_2$ /L/h at day 10 suggesting an instability of the process.

212  
213



214

215 Figure 2 : Hydrogen production rate from fermentation of glycerol in micro-oxidative environment adding oxidant  
216 ( $H_2O_2$ ) at different concentrations (0.0, 0.2, 0.4 and 0.6%), dotted line: average productivity based on hydrogen  
217 cumulated.

218

219 Overall, a significant variation on the hydrogen performances related to the inlet concentration of the oxidizing  
220 agent was clearly shown. The micro-oxidative environment under continuous injection of  $H_2O_2$  at 0.4 and 0.6%  
221 significantly reduced the lag phase of hydrogen production when compared to the control. Since this first period of  
222 lag phase was longer in the control and in the experiment operated at 0.2%, this observation suggests that a  
223 minimum of oxidant addition is initially required. A primary development of  $H_2$ -producing facultative anaerobes at  
224 0.4 and 0.6%  $H_2O_2$  can be one of the hypotheses that can explain the rapid production of  $H_2$ . Consistently, Yokoi  
225 et al. [18] reported similar two phases of hydrogen production in batch conditions, using a co-culture of strict and  
226 facultative anaerobes (*Clostridium Butyricum* and *Enterobacter aerogenes*).

227 Then, the second phase of hydrogen production was significantly improved by injecting H<sub>2</sub>O<sub>2</sub> at 0.4 and 0.6%  
228 (w/w) when compared to the control, showing another beneficial impact of H<sub>2</sub>O<sub>2</sub>. Although the highest production  
229 rate was similar (not significantly different using ANOVA test,  $p > 0.5$ ) at 0.4 and 0.6% (45.5±19.1 and 49.4±9.0  
230 mLH<sub>2</sub>/L/h respectively), the stability of the process was improved at 0.6% (w/w). In both cases, the production rate  
231 started to decrease after 8.5 days showing a detrimental effect of continuous H<sub>2</sub>O<sub>2</sub> addition on the process  
232 stability.

233 With the objective to evaluate the impact of high concentration of H<sub>2</sub>O<sub>2</sub>, a concentration of 0.8% (w/w) was also  
234 evaluated following the experiment at 0.6% after day 10 (data not shown). During the injection at 0.8%, a low  
235 hydrogen productivity was observed with a maximum of 15.7±5.6 mLH<sub>2</sub>/L/h showing that the concentration of  
236 0.6% (w/w) was the optimal condition for improving hydrogen performances and higher concentration was  
237 detrimental to the hydrogen production.

238

239 A literature review of hydrogen production from glycerol by fermentation is reported in Table 2. Up to date, most of  
240 the studies were performed in batch mode and only few were carried out in continuous systems [14,27,30,35].  
241 The best hydrogen yield was reported in batch tests by Murarka et al. [14] with 935 mmolH<sub>2</sub>/molGly, which is  
242 close to the theoretical maximum of 1000 mmolH<sub>2</sub>/molGly [9]. In comparison, hydrogen yields are significantly  
243 lower in continuous reactors (Table 2). To the best of our knowledge, only four studies have been carried out in  
244 continuous mode using glycerol as substrate : three of them used operating conditions similar to the present study  
245 with a HRT of 12 h [35,36], while Kumar et al. [37] used a longer HRT of 2 days. Using a longer HRT did not  
246 improve hydrogen performances since all the hydrogen yields were very close (Table 2). Overall, continuous  
247 bioreactors operated with pure glycerol showed maximum yields ranging from 380 to 500 mmolH<sub>2</sub>/molGly [35–38]  
248 which are very comparable to the best performances observed in our study (403.6±94.7 mmolH<sub>2</sub>/molGly<sub>consumed</sub>).  
249 However, these studies used pure cultures of *Clostridium pasteurianum*, *Clostridium butyricum* or *Bacillus*  
250 *thuringiensis* to select specific metabolic pathway towards hydrogen production. In our study, the use of a micro-  
251 oxidative environment by H<sub>2</sub>O<sub>2</sub> injection, especially at 0.6% and 0.4% (w/w), and mixed microbial culture allowed  
252 to reach hydrogen yields very similar to pure culture conditions. In term of production rates, lower performances  
253 were achieved in our control operated in continuous mode, when compared to the batch reactor, showing the  
254 difficulty to obtain a high rate of hydrogen production in such configuration. However, micro-oxidative conditions

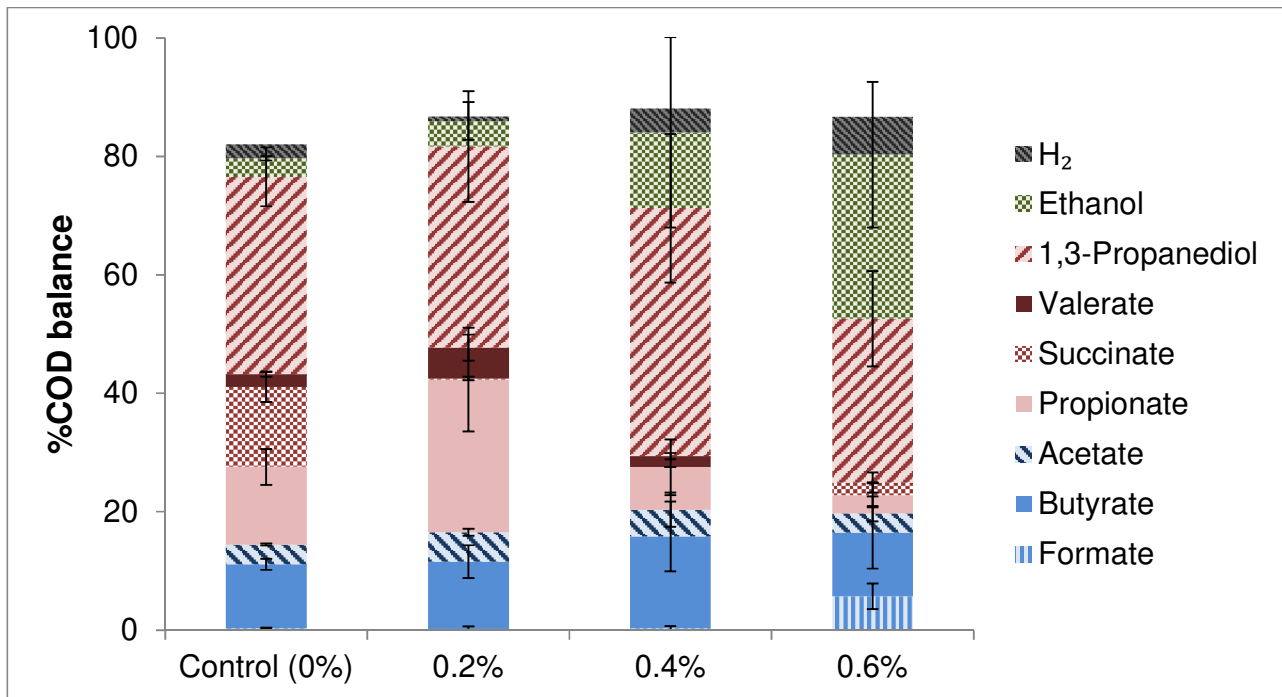
255 had a positive impact on the hydrogen production rate. The productivity was even higher than the value reported  
256 in the study of Tapia-Venegas et al. [38] under similar conditions (CSTR, pH: 5.5, 35°C, HRT of 12 h and mixed  
257 culture). Nevertheless, an instability of hydrogen production was observed in long term operation, i.e. after 9 days  
258 (18 HRT) at 0.4 and 0.6% H<sub>2</sub>O<sub>2</sub>. This suggests that such micro-oxidation conditions can also cause a stressful  
259 environment after a period of high hydrogen production probably by accumulation of OH• radicals [22]. Owusu-  
260 Ansah et al. [23] already showed that excessive reactive oxygen species (ROS) such as OH• radicals could  
261 damage microbial cells and negatively impact the hydrogen production. To avoid an excess of OH• radicals in the  
262 continuous dark fermenter, injection of H<sub>2</sub>O<sub>2</sub> by pulse (e.g. 6 h of injection followed by 6 h without injection)  
263 combined with a long hydraulic retention time of 12h could be used.

264

### 265 **3.2. Effect on micro-oxidative conditions on metabolic pathways**

266

267 To evaluate the impact of H<sub>2</sub>O<sub>2</sub> on fermentative metabolic pathways, the other by-products were considered. Fig.  
268 3 shows the COD mass balances under the different concentrations of H<sub>2</sub>O<sub>2</sub>, based on 5 samples for the control  
269 and 0.6%, 7 samples for 0.2% and 9 samples for 0.4% (as describe in section 2.2) which correspond to the most  
270 representative periods for each conditions. Regarding the outlet composition, the COD mass balance ranged  
271 between 82 and 89% with a reasonable variability error of 10%. Since the electron percentage towards biomass  
272 growth can be around 12%, this suggests that no major metabolite was missing in this study [39]. In the control  
273 reactor (i.e. no H<sub>2</sub>O<sub>2</sub> injection) the metabolic pattern was stable during the whole experiment. The main  
274 metabolites were ethanol, butyrate, acetate, propionate, succinate, 1,3-propanediol and valerate. Among these,  
275 1,3-propanediol, succinate, propionate and valerate were produced by metabolic pathways that do not generate  
276 directly or indirectly hydrogen (Table 3). These pathways represented 62.1±1.5% of the total COD converted.  
277 Butyrate, representing 10.8±0.9% of the total COD, is a product resulting from a metabolic pathway that  
278 cogenerates hydrogen. Butyrate pathway is widely found in strict anaerobes such as *Clostridium butyricum* [40].  
279 As shown in Table 3, formate and acetate pathways could also have directly generated hydrogen. However,  
280 formate and acetate were produced at a low proportion of 0.3±0.2%<sub>COD</sub> and 3.4±0.2%<sub>COD</sub>, respectively. Only  
281 14.5±1.1%<sub>COD</sub> of the metabolites in the control were issued from hydrogen-producing pathways. During this  
282 experiment, ethanol was also observed at low proportion (3.1±0.4%<sub>COD</sub>).

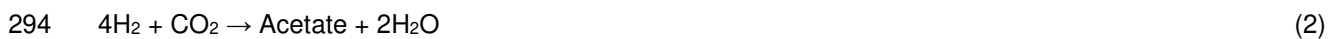


283

284 Figure 3 : Metabolite distribution based on COD mass balance in dark fermentation bioreactor with addition of  
 285 oxidant (H<sub>2</sub>O<sub>2</sub>) at different concentration (control (0.0), 0.2, 0.4, 0.6 %) using pure glycerol as carbon source.

286

287 At the lowest concentration of H<sub>2</sub>O<sub>2</sub> (0.2% w/w), a variation in the propionate and succinate composition was  
 288 observed with regards to the control. A metabolic shift was found with an absence of succinate and a statistically  
 289 significant increase of the propionate proportion to 25.8±8.7%<sub>COD</sub> (p < 0.005). A similar proportion of butyrate was  
 290 observed which correspond to 11.4±2.7%<sub>COD</sub> of the metabolic distribution. The slight increase of acetate  
 291 proportion under this condition (5.0±0.6%<sub>COD</sub>) was correlated with the reduction of hydrogen yield, that was  
 292 probably due to the homoacetogenic reaction (Eq. 2). Indeed, as previously reported in the literature, 33 to 57% of  
 293 the acetate could result from hydrogen consumption pathway in continuous stirred tank reactor [41].



295

296 At 0.4% of H<sub>2</sub>O<sub>2</sub> (w/w), the proportion of ethanol and butyrate significantly increased when compared to the  
 297 control (p < 0.05). In this experiment, an average of 20.3±4.2%<sub>COD</sub> of the metabolites was due to metabolic  
 298 pathways related to hydrogen production. At the maximal hydrogen production time in this condition (day 8), the  
 299 metabolite proportion related to hydrogen-production pathway was similar to the average observed in Fig. 3 (i.e.  
 300 17.1%<sub>COD</sub>). In the experiment carried out at higher concentration of H<sub>2</sub>O<sub>2</sub> (0.6% w/w), a significant metabolic shift

301 with an improvement of formate and ethanol production ( $p < 0.005$ ), representing  $33.4 \pm 14.4\%_{\text{COD}}$  of the total  
302 COD, and a decrease of the 1,3-propanediol proportion ( $27.7 \pm 8.0\%_{\text{COD}}$ ) was shown. Ethanol and formate are  
303 known as metabolic end-products of facultative anaerobes which supports the development of facultative  
304 anaerobic bacteria under such condition [8,38]. In total,  $19.7 \pm 3.5\%_{\text{COD}}$  of the metabolites were generated from  
305 hydrogen-producing pathways (i.e. formate, acetate, butyrate) which is similar to the proportion observed in the  
306 experiment at 0.4% ( $20.3 \pm 4.2\%_{\text{COD}}$ ). Alone, metabolic pathways that directly produce hydrogen could not explain  
307 the increase of hydrogen performances observed during the injection of  $\text{H}_2\text{O}_2$  at 0.6%. One of the hypotheses is  
308 that the metabolic pathway for ethanol production can indirectly produce hydrogen by generating formate, which  
309 can further be converted into  $\text{H}_2$  (Table 3). This hypothesis is consistent with the study of Varrone et al. [43] where  
310 a metabolic shift from 1,3-propanediol to ethanol led to an improvement of hydrogen production from glycerol. Ito  
311 et al. [9] also observed hydrogen production ( $63 \text{ mmolH}_2/\text{L/h}$ ) from glycerol with an important accumulation of  
312 ethanol ( $850 \text{ mmol/molGly}$ ) using pure culture of facultative anaerobes (*Enterobacter aerogenes*). Moreover, our  
313 results confirmed the hypothesis that during dark fermentation, the oxidant added in the form of  $\text{H}_2\text{O}_2$  was used as  
314 electron acceptor and subsequently favoured the oxidative pathway in facultative anaerobes through an  
315 improvement of ethanol and hydrogen production [19]. Choi et al. [44] showed that the presence of oxygen as  
316 electron acceptor at limited concentration led to the consumption of excess of reducing equivalent (redox balance)  
317 generated during biomass growth. Consequently, the addition of oxygen could improve cellular growth and favour  
318 the microbial activity, explaining the improvement of hydrogen and ethanol production as observed in our  
319 experiments when  $\text{H}_2\text{O}_2$  is added at 0.4% and 0.6%.

320 Although a low concentration of  $\text{H}_2\text{O}_2$  (0.2%) did not substantially impact the fermentation patterns in term of  
321 hydrogen producing pathways compared to a classic dark fermenter reactor (without micro-oxidative condition).  
322 Increasing the  $\text{H}_2\text{O}_2$  concentration to 0.4 and 0.6% was favourable for the hydrogen production through the  
323 ethanol, butyrate and formate pathways.

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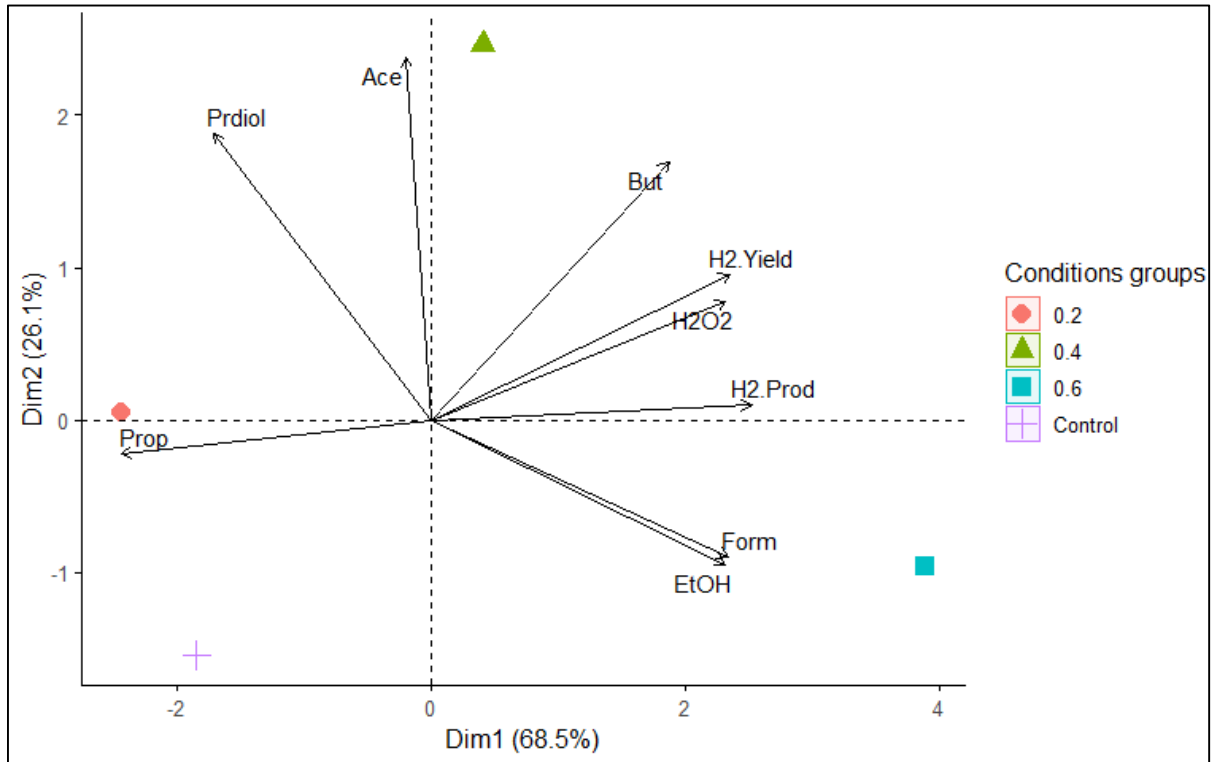
328 Table 3: Main metabolic pathways of glycerol fermentation observed in mixed culture. [19,34,45,46]

| <b>Metabolic pathways for hydrogen production</b>         | <b>Metabolic pathways in competition with hydrogen production</b> |
|---|---|
| $glycerol + H_2O \rightarrow acetate + formate + H_2$     | $glycerol \rightarrow propionate + H_2O$                          |
| $2glycerol \rightarrow butyrate + 4H_2 + 2CO_2$           | $glycerol + CO_2 \rightarrow succinate + 2H_2O$                   |
| $formate \rightarrow H_2 + CO_2$                          | $glycerol \rightarrow lactate + H_2O$                             |
| <b>Indirect metabolic pathway for hydrogen production</b> | $glycerol \rightarrow 1,3 - propanediol + H_2O$                   |
| $glycerol \rightarrow ethanol + formate$                  |   |
| <b>Metabolic pathways for hydrogen consumption</b>        |   |
| $4H_2 + 2CO_2 \rightarrow acetate + 2H_2O$                |   |
| $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$                    |   |

329

330 In order to compare hydrogen performances and metabolic patterns, a statistical principal component analysis  
 331 (PCA) was performed (Fig. 4). The two first dimensions explained 68.5 and 26.1% of the total variance,  
 332 respectively. Consistently, maximum hydrogen yields and hydrogen production rates positively correlated with the  
 333  $H_2O_2$  concentration and the accumulation of butyrate and formate. These results also supports the fact that  
 334 ethanol-based metabolic pathway might indirectly produce hydrogen through the partial degradation of formate  
 335 (Table 3). As expected, the inlet concentration of  $H_2O_2$  negatively correlated with the production of propionate and  
 336 1,3-propanediol. Consistently, and in accordance with Table 3, the production of propionate and 1,3-propanediol  
 337 are two metabolic pathways in competition with the hydrogen production while formate, ethanol and butyrate are  
 338 metabolic pathways producing hydrogen and carried by a mixture of facultative anaerobes (formate and ethanol)  
 339 and strict anaerobes (butyrate) [37-38]. Interestingly, acetate did not correlate with hydrogen production, that can  
 340 be easily explained by the fact that acetate is generated by two pathways: one producing  $H_2$  from glycerol and  
 341 another one consuming  $H_2$  by homoacetogenesis (Eq. 2) [20].

342



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Figure 4 : PCA performed on the global performances of each conditions of H<sub>2</sub>O<sub>2</sub> injected, Prop : propionate, Prdiol : 1,3-propanediol, Ace : acetate, But : butyrate, Form : formate, EtOH : ethanol, H<sub>2</sub>O<sub>2</sub> : Quantity of H<sub>2</sub>O<sub>2</sub> added (mol), H<sub>2</sub> yield : Maximum hydrogen yield reach (mLH<sub>2</sub>/molGlyc<sub>consumed</sub>, H<sub>2</sub> Prod : Average hydrogen productivity (mLH<sub>2</sub>/L/h).

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### 3.3. Fermentative microbial communities developed in micro-oxidant condition

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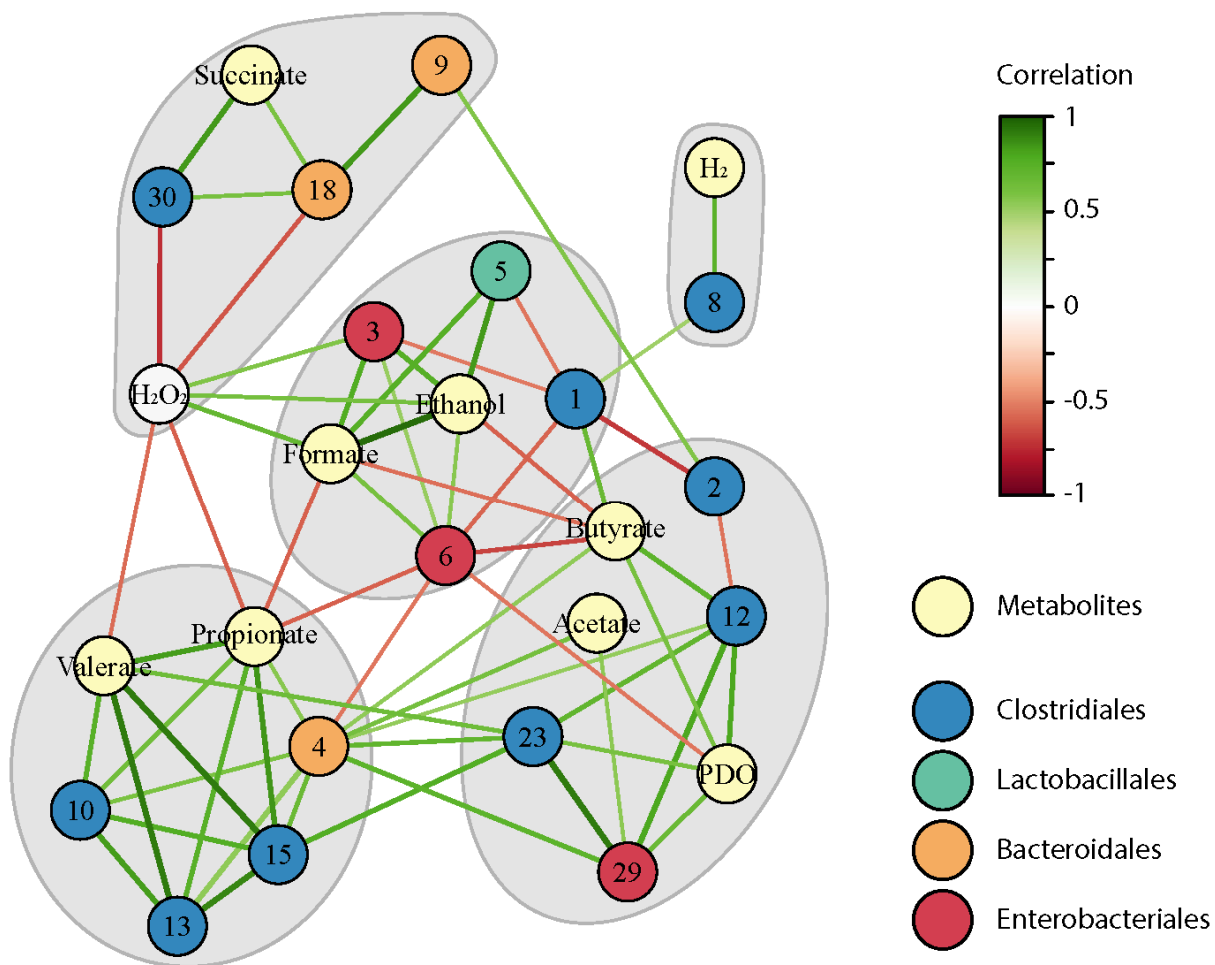
358

359

Analysis of the microbial communities was performed on samples periodically collected from the outlet of the continuous reactors (at least 3 samples per reactor, for a total of 15 samples). Considering all the samples, a total of 1448 operating taxonomic units (OTUs) with 97% similarity were detected. Although high dynamics of the microbial communities were observed over time, the most dominant bacteria (representing at least > 62 % of the total abundance) was always affiliated to one of the four most dominant taxonomic orders : The *Clostridiales* order from the *Clostridia* class, with abundances ranging from 28 to 84% of the total sequences, was generally the most abundant order, followed by *Enterobacteriales* (2-52 %), *Bacteroidales* (0-31 %) and *Lactobacillales* (0-43 %) orders.



360 In order to estimate the correlations existing between microbial community compositions and fermentation  
361 patterns, a Pearson correlation matrix was calculated using OTU relative abundances and fermentation product  
362 concentrations as variables [34]. The graphical representation of the resulting correlation network is shown in Fig.  
363 5. OUT 1 related to *Clostridium intestinale* was positively correlated to butyrate ( $r = 0.68$ ,  $p < 0.01$ ,  $n = 15$ ). As  
364 mentioned in Table 3, butyrate pathway leads to hydrogen production through the hydrogenase enzyme [49]. This  
365 pathway was probably the main metabolic route responsible of hydrogen production. As shown in Fig. 5, only  
366 OTU 8 was found to positively correlate with hydrogen ( $r = 0.75$ ,  $p < 0.05$ ). This OTU is related to *Robinsoniella*  
367 *peoriensis* (100 % 16S rRNA sequence similarity), a Gram-positive, non-motile, spore-forming bacterium,  
368 generally isolated from swine-manure and human faeces [50]. The major end products of its fermentative  
369 metabolism included acetate and succinate but not butyrate or hydrogen [50]. This OTU was generally in low  
370 abundance (if present) and reaches 6% only in the condition of 0.4% at the day 8 which correspond to the outlier  
371 of the maximum hydrogen productivity. Thus, a reason of this observation is probably that hydrogen is not linked  
372 to a single species.



373

374 Figure 5: Correlation network obtained from Pearson correlation matrix calculated using bacterial OTUs relative  
 375 abundance and fermentation production concentrations as variables. Circles represent the variables, while the  
 376 lines represent the found correlations. The size and color of each correlation is proportional to its value. Bacterial  
 377 genera are represented with different colors, while the numbers into the circles represent the respective number  
 378 of OTU. Grey areas represent clusters as provided by the edge betweenness method.  
 379

380 In addition, Fig. 5 shows that certain metabolic products could be associated with bacteria belonging to  
 381 phylogenetically highly distant taxonomic groups. In particular, the metabolic pathways leading to ethanol and  
 382 formate could be associated to the presence of OTUs 3 and 5, respectively affiliated to *Klebsiella pneumoniae*  
 383 (100% 16S rRNA sequence similarity) and *Enterococcus cecorum* (100% 16S rRNA sequence similarity). Both  
 384 positively correlated ( $p < 0.01$ ) with ethanol ( $r = 0.76$  for *Klebsiella pneumoniae* and  $r = 0.84$  for *Enterococcus*  
 385 *cecorum*) and formate ( $r = 0.78$  for *Klebsiella pneumoniae* and  $r = 0.76$  for *Enterococcus cecorum*). Interestingly,  
 386 *Klebsiella pneumoniae* abundance also positively correlated with the  $H_2O_2$  inlet concentration ( $r = 0.58$ ,  $p < 0.05$ ).

387 At a lower extent ( $p < 0.05$ ), OTU 6 positively correlated to formate and ethanol pathways. This OTU is related to  
388 the genus *Escherichia-Shigella* (100% sequence similarity), belonging, as the genus *Klebsiella*, to the  
389 Enterobacteriaceae family. The *Klebsiella*, *Escherichia-Shigella* and *Enterococcus* genera, although belonging to  
390 different phylogenetic groups (*Proteobacteria* for *Klebsiella* and *Escherichia-Shigella*, and *Firmicutes* for  
391 *Enterococcus*), are all facultative anaerobic fermentative microorganisms with similar metabolic pathways that  
392 generates acetate, ethanol and formate. They are able to produce hydrogen from the conversion of formate into  
393 hydrogen and CO<sub>2</sub> through the FHL pathway (Formate Hydrogen Lyase) [51]. Consistently, in the study of  
394 Varrone et al. [43] the production of hydrogen from glycerol was associated with a high production of ethanol  
395 (*Klebsiella*, *Escherichia/Shigella* and *Cupriavidus*). High abundances of the OTUs 3 and 6 were observed in the  
396 reactor operated with 0.6% of H<sub>2</sub>O<sub>2</sub>, during the phases in which high ethanol production was observed (see Fig.  
397 5). This observation is consistent with an indirect production of hydrogen from the ethanol pathway by the  
398 conversion of formate and explains the improvement of hydrogen performances at 0.6% of H<sub>2</sub>O<sub>2</sub>. Our  
399 observations suggest that facultative anaerobes had the ability to produce hydrogen by the formate and ethanol  
400 pathways and could participate to the improvement of the overall hydrogen performances. Since they are also  
401 able to consume oxygen, the anaerobic conditions were optimal for the growth of strict anaerobes such as  
402 bacteria from the *Clostridiales* order, which are well-known efficient hydrogen producers.

403

#### 404 **4. Conclusion**

405

406 This study aimed to investigate the hydrogen production using micro-oxidative condition by injecting H<sub>2</sub>O<sub>2</sub> at  
407 different concentrations (0.2, 0.4 and 0.6%). It was shown that constant injection of H<sub>2</sub>O<sub>2</sub> at a concentration of  
408 0.6% (w/w) improved the hydrogen performances. A hydrogen yield reached 403.6±94.7 mmolH<sub>2</sub>/molGly which  
409 was 345% higher than the yield without H<sub>2</sub>O<sub>2</sub> addition (90.6±2.8 mmolH<sub>2</sub>/molGly). Interestingly, micro-oxidative  
410 conditions favoured the development of facultative anaerobes, such as bacteria from *Klebsiella*, *Escherichia-*  
411 *Shigella* and *Enterococcus* genera, which very likely produced hydrogen through ethanol and formate pathways.  
412 Strict anaerobes (*Clostridium intestinale*) dominated the microbial community and were likely responsible of the  
413 hydrogen produced during the experiments, suggesting that optimal conditions for strict anaerobic bacteria were

414 preserved. In conclusion, the dark fermentation process can be improved by driving the metabolic pathway  
415 through a micro-oxidative environment.

416

## 417 **Acknowledgements**

418

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422

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424

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