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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_2O_2) 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_2 yield (403.6±94.7 mmolH_2/mol$Gly_{consumed}$) was reached at a H_2O_2 concentration of 0.6% (w/w), through the formate, ethanol and butyrate metabolic pathways. The addition of H_2O_2 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_2O_2, Mixed culture, Glycerol.

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1. Introduction

Due to limited energy reserves and an increasing environmental pressure on greenhouse gases released from fossil fuels uses, renewable energy sources have considerably gained in attention over the past decades [1]. In particular, biohydrogen production by dark fermentation has been widely investigated due to a simpler operating configuration than other biological processes [2]. However, the dark fermentation process still needs to be optimized to reach the economic viability [3]. Several solutions have been proposed to reduce the costs of the overall process. Different approaches have been investigated to improve the economic viability of such technology at larger scale, such as (i) the use of low cost substrates (such as waste or industrial by-products), (ii) the operation of bioreactors with mixed cultures instead of pure axenic cultures, and (iii) the improvement of hydrogen yields by changing the operating conditions with a target of a maximum of 4 molH_2/molglucose [4–6]. In particular, glycerol is a by-product generated by the biodiesel industry and represents around 10 % w/w of the total biodiesel end-products. Raw glycerol is a cheap and widely available substrate. Moreover, biodiesel consumption is growing at a rate of 30–50% per year making glycerol resources largely available in the future [7]. The glycerol production is estimated to reach about 40,000 tons per year by 2020 [8].

Up to now, most of the studies dealing with hydrogen production by dark fermentation, and using glycerol as carbon source were carried out with pure microbial cultures [8, 9, 10]. The advantage of using pure culture is the relatively high yields achieved, up to 935 mmolH_2/molGly which is close to the theoretical maximum of 1000 mmolH_2/molGly [11]. In contrast, the use of mixed culture in dark fermentation presents several advantages such as no need of sterile conditions, lower sensitivity to contaminants and thus lower operational costs of the overall process. In mixed cultures, hydrogen-producing bacteria are mainly related to the *Clostridiaceae* (strict anaerobes) [12] or *Enterobacteriaceae* (facultative anaerobes) [13,14] families that are found in many natural environments; e.g. anaerobic digesters, activated sludge-treating bioreactors, compost piles, soil or cow manure [15–17]. The presence of facultative anaerobes in dark fermentation reactors can be beneficial due to their ability to consume oxygen and generate strict anaerobic conditions, optimal to hydrogen production. Such collaborative bacterial growth was already observed by Yokoi et al. [18] who worked with a co-culture of strict and facultative anaerobes (*Clostridium butyricum* and *Enterobacter aerogenes*) in continuous conditions and applied aeration shocks to the medium (20 min). After that shock, the co-culture had the ability to remove O_2 traces by the presence of *Enterobacter aerogens* and recover efficient H_2 production by *Clostridium butyricum*. Moreover, it has

already been reported that aeration shock in dark fermentation can improve the hydrogen production by facultative anaerobes by favouring the oxidative pathway. As an illustration, an aeration shock resulted in the improvement of ethanol and hydrogen production in *Enterobacter aerogens* [19]. Pachapur et al. [19] observed an improvement of hydrogen production performances, i.e. from 21.4 mmolH_2/L to 26.1 mmolH_2/L, when initial aerobic conditions were applied on a co-culture of *Enterobacter aerogenes* and *Clostridium butyricum* if compared to initial strict anaerobic conditions (with nitrogen sparging).

However, due to the high microbial community diversity in mixed cultures, the organic substrates can also be converted into many other metabolic end-products than hydrogen. Mixed cultures may also contain strict anaerobic hydrogen consumers such as methanogenic or homoacetogenic microorganisms, which could affect the overall process performances [17,20]. Thus, one of the methods suitable for selecting strict and facultative anaerobes from mixed cultures is to operate the reactor under micro-oxidative conditions. Li et al. [21] reported an improvement of the hydrogen production using mixed culture and oxidative environment through oxygen addition (0.28 mLO_2/gTS) in batch tests. A 44% increase of the hydrogen yield, from 28.9 mLH_2/gVS to 41.6 mLH_2/gVS, was observed. In order to better control the micro-oxidative environment and improve the diffusion of oxygen, the addition of a chemical reactant such as hydrogen peroxide (H_2O_2) constitutes an alternative to gas injection. However, hydrogen peroxide generates oxygen, H_2O as well as OH• radicals [22]. Owusu-Ansah et al. [23] already showed that excessive reactive oxygen species (ROS) such as OH• radicals could damage microbial cells and negatively impact hydrogen production. Moreover, injection of a strong oxidant can increase the redox potential (ORP) of the medium with potential effect on fermentation pathways. Indeed, the redox potential value should be maintained between -250 and -524 mV to favour the hydrogen producing pathways, i.e. acetate and butyrate [22,23]. Therefore, to improve the hydrogen production using micro-oxidative environment, the concentration of H_2O_2 added in the dark fermenter must remain below the critical concentration of inhibition.

The present study aims to investigate the effect of hydrogen-peroxide (H_2O_2) addition to maintain micro-oxidative conditions in a mixed culture-based reactor operated in continuous mode with glycerol as substrate. The impact of different concentrations of the oxidizing agent (0.2, 0.4, 0.6% (w/w)) on hydrogen production performances, microbial communities and metabolic pathways were evaluated to understand the improvement of hydrogen performances under micro-oxidative conditions.

2. Materials and Methods

2.1. Continuous H_2 production experiments

The experiments were carried out in a continuous stirred tank reactor (CSTR) with a working volume of 2 L. Fig. 1 shows the scheme of the reactors and the overall equipment design. The reactor was operated at a temperature of 37 °C, hydraulic retention time (HRT) of 12 h and mixing rate of 350 rpm [26]. Pure glycerol (15 g/L) from SIGMA ALDRICH (CAS: 56-81-5) was used as sole carbon source. The pH was initially adjusted at 6.5 and controlled during the whole experiment by adding NaOH (1.5M). A mixture of anaerobic and aerobic sludge collected from a municipal wastewater treatment plant (1:1 based on Volatile Solids (VS)) was used as inoculum. A substrate/inoculum (S/X) ratio of 10 (on VS basis) was used in all the experiments. The culture medium was prepared according to Varrone et al. [30], and was composed of the following elements: K_2HPO_4 (0.125 g/L), NH_4Cl (0.5 g/L), $MgSO_4*7H_2O$ (0.05 g/L), $CaCl_2*2H_2O$ (0.005 g/L), $FeSO_4*7H_2O$ (0.005 g/L), yeast extract (0.5 g/L). Micro-oxidative environment was provided by continuous injection of hydrogen peroxide (H_2O_2) as oxidizing agent, under a flow rate of 0.252 mL/min. One control (no H_2O_2) and three different concentrations of H_2O_2 were tested: 0.0, 0.2, 0.4 and 0.6 (w/w). These concentrations were chosen to avoid excessive stress conditions on the microbial community.

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

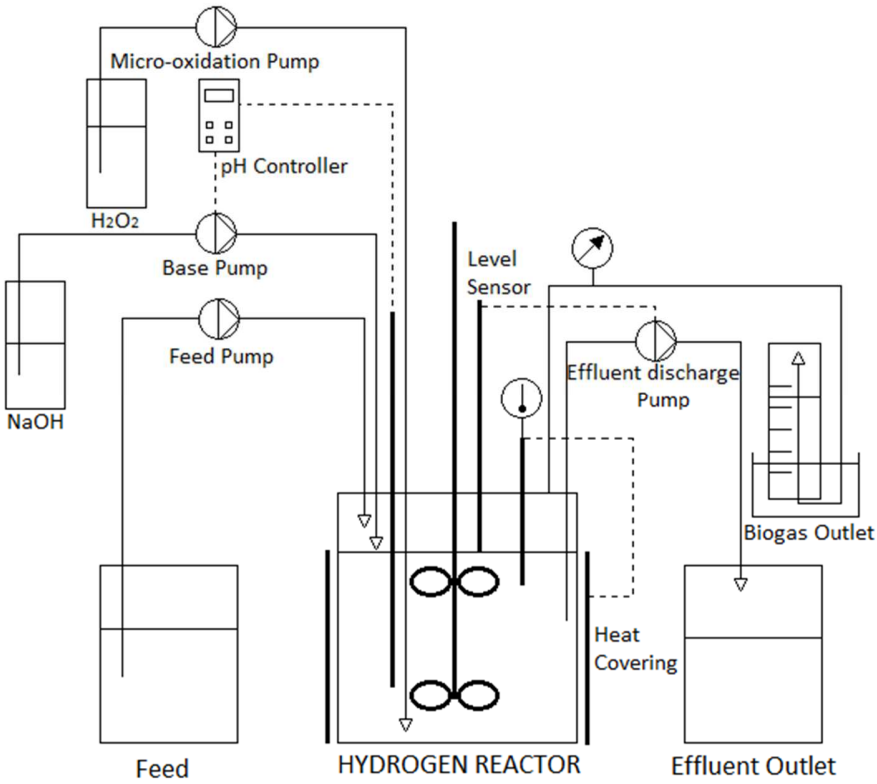

Figure 1 : Schematic diagram of the continuous stirred tank reactor (CSTR) and the experimental equipment

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_2. The second column was an RtMolsieve used for detection and quantification of O_2, H_2, N_2 and CH_4. 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_2 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 cartbridges, 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_2 recovered (in mmolH_2) per mole of glycerol consumed per period of 12 h (1
154 HRT). The hydrogen yield was calculated as follows (Eq. 1):

155 $$Y_{max} = \frac{n\,H_2}{n\,glycerol_{consumed}} \quad (mmolH_2/molGly_{consumed}) \qquad (1)$$

156 Where n H_2 is the cumulative amount of moles of H_2 produced during 12 h (1 HRT) and n $glycerol_{consumed}$ the total
157 consumed moles of glycerol over the same period of time.

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

3. Results and discussion

3.1. Effect of micro-oxidative conditions on continuous hydrogen production from glycerol

During all the experiments, no methane was observed, showing that hydrogen consumption was not caused by a methanogenic activity. As shown in Table 1, the average redox value in the medium remained in the same range of -495.3 mV and -543.9 mV in the control and for all concentrations of H_2O_2. All these redox potentials were within an optimal range for hydrogen production, i.e. from -250 to -524 mV, as previously reported by Lin et al. [24]. According to this observation, injection of H_2O_2 at the tested concentrations (0.2, 0.4 and 06% (w/w)) did not macroscopically affect the redox potential of the medium in comparison to the control (i.e. -511.54 mV) and enabled the microbial community to remain in a favourable environment for hydrogen production. Such redox potential stability could be explained by the fact that the injected H_2O_2 was rapidly consumed by the microbial community since no oxygen was observed in the gas phase. As mentioned in Table 1, the hydrogen content in the biogas ranged from 33% to 49% for the experiments performed with H_2O_2 addition at 0.4 and 0.6 %, respectively. Consistently, hydrogen content in dark fermenters using glycerol as substrate is usually ranging from 33 to 54% [35,10].

Nonetheless, the micro-oxidative conditions impacted the overall hydrogen performances since the maximal hydrogen yields were significantly different at each H_2O_2 concentration. At low H_2O_2 concentration (0.2%), the maximum yield was found at 44.1±1.3 mmolH_2/mol$Gly_{consumed}$ which was lower than the maximum yield found in the control (90.6±2.8 mmolH_2/mol$Gly_{consumed}$). The increase of H_2O_2 concentration to 0.4% (w/w) led to a

187 significant increase of the maximum hydrogen yield to 290.7±136.2 mmolH_2/mol$Gly_{consumed}$. The highest yield was

188 found at 0.6% (w/w) H_2O_2 with 403.6±94.7 mmolH_2/mol$Gly_{consumed}$.

189

190 Table 1 : Maximum hydrogen production in CSTR operated at different inlet H_2O_2 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)

$[H_2O_2]_{added}$ (w/w)	Total H_2O_2 added (mol)	Steady state $Yield_{max}$ (mmolH_2/mol$Gly_{consumed}$)	H_2 cumulated (mmolH_2)	Average redox (mV)	H_2 content (%)
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_2O_2 (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_2/L/h was found at day 6.5 and a stable but low production
198 rate around 13.8±0.5 mLH_2/L/h was then observed from day 7.5 to 10. At the lowest concentration of H_2O_2 (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±0.2
200 mLH_2/L/h from day 6 to 7 (during 4 HRT). Injection of H_2O_2 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±4.3 mLH_2/L/h. An improvement of hydrogen production rate
204 was then observed from day 7 to 8 reaching 45.5±19.1 mLH_2/L/h with a maximum of 66.8 mLH_2/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_2/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±4.1
209 mLH_2/L/h. Then, after a sharp increase, a stationary phase was reached with a constant hydrogen production rate

of 49.4±9.0 mLH_2/L/h and was maintained during 4 days (i.e. 8 HRT). After that period, the hydrogen production rate rapidly decreased down to 5.7 mLH_2/L/h at day 10 suggesting an instability of the process.

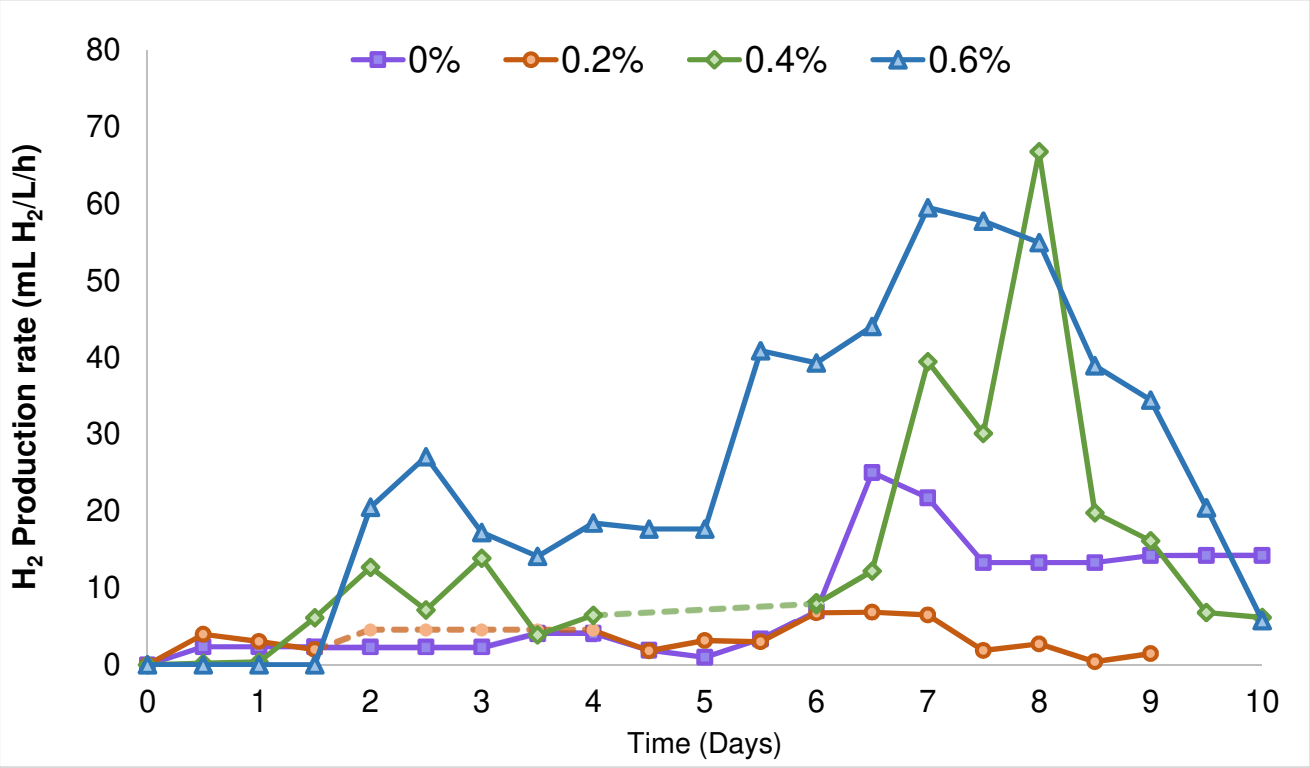

Figure 2 : Hydrogen production rate from fermentation of glycerol in micro-oxidative environment adding oxidant (H_2O_2) at different concentrations (0.0, 0.2, 0.4 and 0.6%), dotted line: average productivity based on hydrogen cumulated.

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

227 Then, the second phase of hydrogen production was significantly improved by injecting H_2O_2 at 0.4 and 0.6%
228 (w/w) when compared to the control, showing another beneficial impact of H_2O_2. 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 $mLH_2/L/h$ 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_2O_2 addition on the process
232 stability.
233 With the objective to evaluate the impact of high concentration of H_2O_2, 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 $mLH_2/L/h$ 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 $mmolH_2/molGly$, which is
242 close to the theoretical maximum of 1000 $mmolH_2/molGly$ [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 $mmolH_2/molGly$ [35–38]
248 which are very comparable to the best performances observed in our study (403.6±94.7 $mmolH_2/molGly_{consumed}$).
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_2O_2 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

had a positive impact on the hydrogen production rate. The productivity was even higher than the value reported in the study of Tapia-Venegas et al. [38] under similar conditions (CSTR, pH: 5.5, 35°C, HRT of 12 h and mixed culture). Nevertheless, an instability of hydrogen production was observed in long term operation, i.e. after 9 days (18 HRT) at 0.4 and 0.6% H_2O_2. This suggests that such micro-oxidation conditions can also cause a stressful environment after a period of high hydrogen production probably by accumulation of OH• radicals [22]. Owusu-Ansah et al. [23] already showed that excessive reactive oxygen species (ROS) such as OH• radicals could damage microbial cells and negatively impact the hydrogen production. To avoid an excess of OH• radicals in the continuous dark fermenter, injection of H_2O_2 by pulse (e.g. 6 h of injection followed by 6 h without injection) combined with a long hydraulic retention time of 12h could be used.

3.2. Effect on micro-oxidative conditions on metabolic pathways

To evaluate the impact of H_2O_2 on fermentative metabolic pathways, the other by-products were considered. Fig. 3 shows the COD mass balances under the different concentrations of H_2O_2, based on 5 samples for the control 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 representative periods for each conditions. Regarding the outlet composition, the COD mass balance ranged between 82 and 89% with a reasonable variability error of 10%. Since the electron percentage towards biomass growth can be around 12%, this suggests that no major metabolite was missing in this study [39]. In the control reactor (i.e. no H_2O_2 injection) the metabolic pattern was stable during the whole experiment. The main metabolites were ethanol, butyrate, acetate, propionate, succinate, 1,3-propanediol and valerate. Among these, 1,3-propanediol, succinate, propionate and valerate were produced by metabolic pathways that do not generate directly or indirectly hydrogen (Table 3). These pathways represented 62.1±1.5% of the total COD converted. Butyrate, representing 10.8±0.9% of the total COD, is a product resulting from a metabolic pathway that cogenerates hydrogen. Butyrate pathway is widely found in strict anaerobes such as *Clostridium butyricum* [40]. As shown in Table 3, formate and acetate pathways could also have directly generated hydrogen. However, formate and acetate were produced at a low proportion of 0.3±0.2%$_{COD}$ and 3.4±0.2%$_{COD}$, respectively. Only 14.5±1.1%$_{COD}$ of the metabolites in the control were issued from hydrogen-producing pathways. During this experiment, ethanol was also observed at low proportion (3.1±0.4%$_{COD}$).

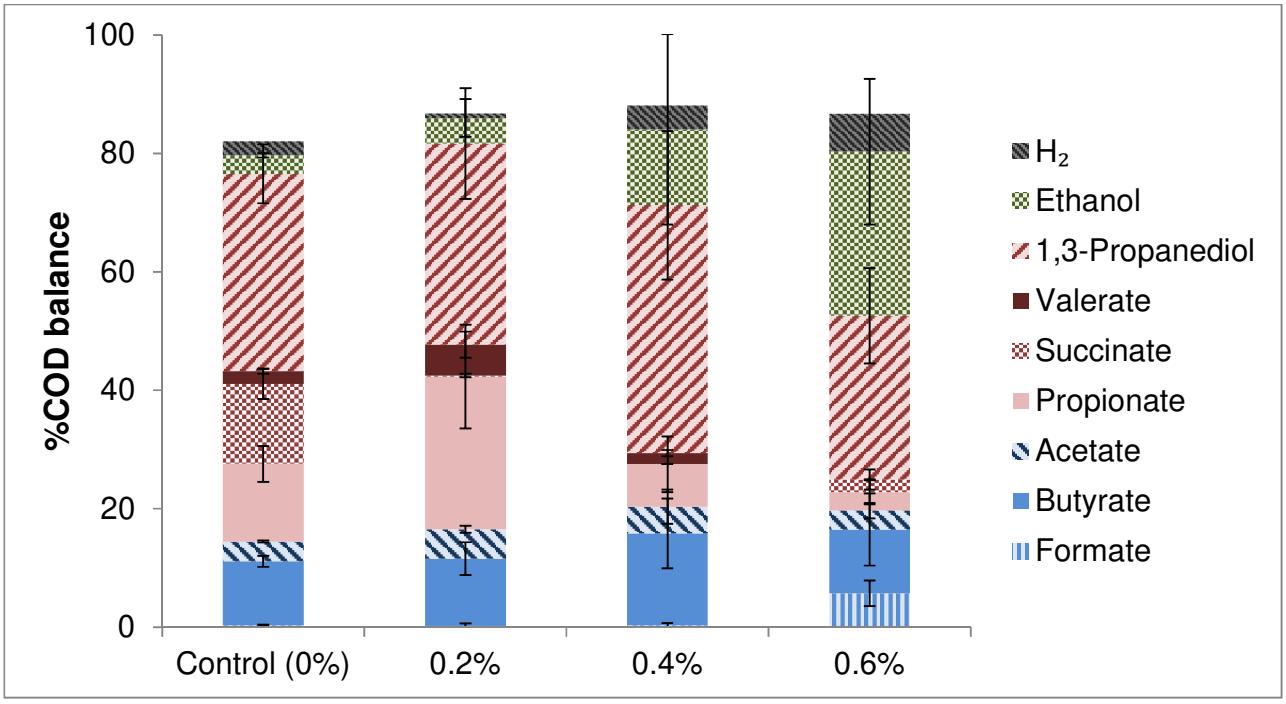

Figure 3 : Metabolite distribution based on COD mass balance in dark fermentation bioreactor with addition of oxidant (H2O2) at different concentration (control (0.0), 0.2, 0.4, 0.6 %) using pure glycerol as carbon source.

At the lowest concentration of H_2O_2 (0.2% w/w), a variation in the propionate and succinate composition was observed with regards to the control. A metabolic shift was found with an absence of succinate and a statistically significant increase of the propionate proportion to 25.8±8.7%$_{COD}$ ($p < 0.005$). A similar proportion of butyrate was observed which correspond to 11.4±2.7%$_{COD}$ of the metabolic distribution. The slight increase of acetate proportion under this condition (5.0±0.6%$_{COD}$) was correlated with the reduction of hydrogen yield, that was probably due to the homoacetogenic reaction (Eq. 2). Indeed, as previously reported in the literature, 33 to 57% of the acetate could result from hydrogen consumption pathway in continuous stirred tank reactor [41].

$$4H_2 + CO_2 \rightarrow \text{Acetate} + 2H_2O \qquad (2)$$

At 0.4% of H_2O_2 (w/w), the proportion of ethanol and butyrate significantly increased when compared to the control ($p < 0.05$). In this experiment, an average of 20.3±4.2%$_{COD}$ of the metabolites was due to metabolic pathways related to hydrogen production. At the maximal hydrogen production time in this condition (day 8), the metabolite proportion related to hydrogen-production pathway was similar to the average observed in Fig. 3 (i.e. 17.1%$_{COD}$). In the experiment carried out at higher concentration of H_2O_2 (0.6% w/w), a significant metabolic shift

with an improvement of formate and ethanol production ($p < 0.005$), representing 33.4±14.4%$_{COD}$ of the total COD, and a decrease of the 1,3-propanediol proportion (27.7±8.0%$_{COD}$) was shown. Ethanol and formate are known as metabolic end-products of facultative anaerobes which supports the development of facultative anaerobic bacteria under such condition [8,38]. In total, 19.7±3.5%$_{COD}$ of the metabolites were generated from hydrogen-producing pathways (i.e. formate, acetate, butyrate) which is similar to the proportion observed in the experiment at 0.4% (20.3±4.2%$_{COD}$). Alone, metabolic pathways that directly produce hydrogen could not explain the increase of hydrogen performances observed during the injection of H_2O_2 at 0.6%. One of the hypotheses is that the metabolic pathway for ethanol production can indirectly produce hydrogen by generating formate, which can further be converted into H_2 (Table 3). This hypothesis is consistent with the study of Varrone et al. [43] where a metabolic shift from 1,3-propanediol to ethanol led to an improvement of hydrogen production from glycerol. Ito et al. [9] also observed hydrogen production (63 mmolH_2/L/h) from glycerol with an important accumulation of ethanol (850 mmol/molGly) using pure culture of facultative anaerobes (*Enterobacter aerogenes*). Moreover, our results confirmed the hypothesis that during dark fermentation, the oxidant added in the form of H_2O_2 was used as electron acceptor and subsequently favoured the oxidative pathway in facultative anaerobes through an improvement of ethanol and hydrogen production [19]. Choi et al. [44] showed that the presence of oxygen as electron acceptor at limited concentration led to the consumption of excess of reducing equivalent (redox balance) generated during biomass growth. Consequently, the addition of oxygen could improve cellular growth and favour the microbial activity, explaining the improvement of hydrogen and ethanol production as observed in our experiments when H_2O_2 is added at 0.4% and 0.6%.

Although a low concentration of H_2O_2 (0.2%) did not substantially impact the fermentation patterns in term of hydrogen producing pathways compared to a classic dark fermenter reactor (without micro-oxidative condition). Increasing the H_2O_2 concentration to 0.4 and 0.6% was favourable for the hydrogen production through the ethanol, butyrate and formate pathways.

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

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

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

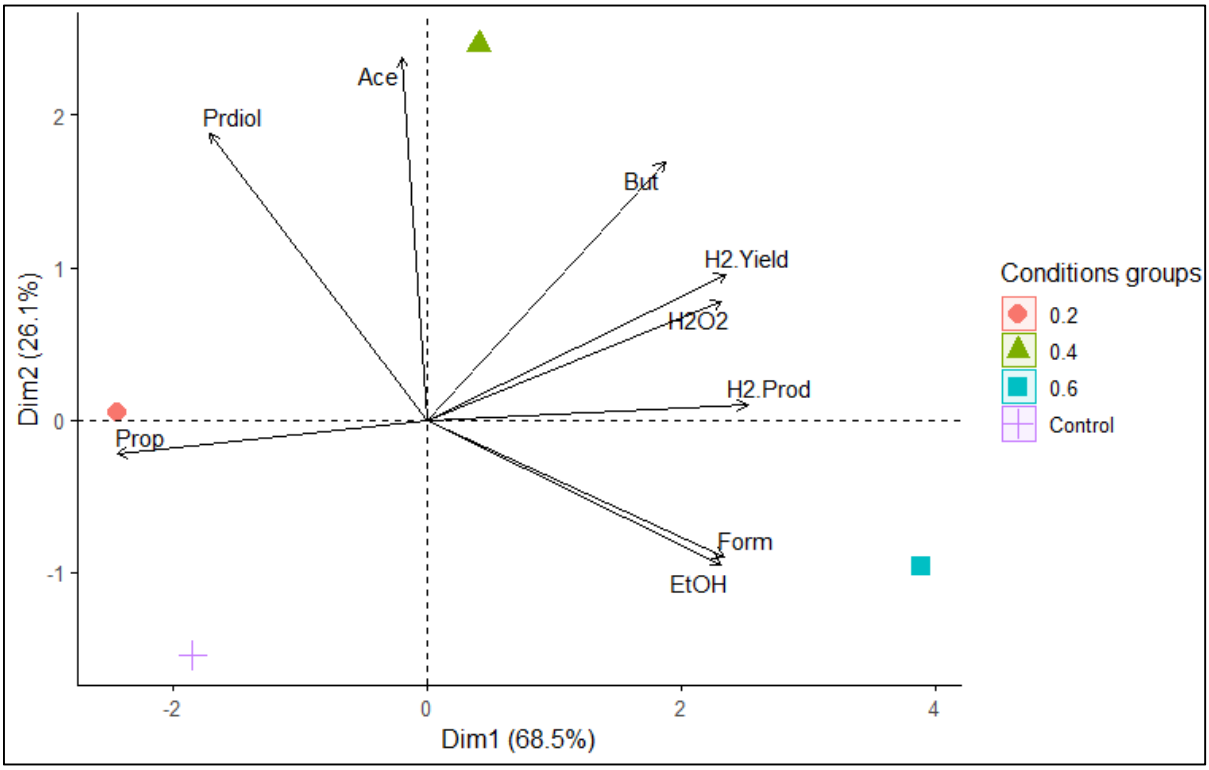

Figure 4 : PCA performed on the global performances of each conditions of H_2O_2 injected, Prop : propionate, Prdiol : 1,3-propanediol, Ace : acetate, But : butyrate, Form : formate, EtOH : ethanol, H_2O_2 : Quantity of H_2O_2 added (mol), H_2 yield : Maximum hydrogen yield reach ($mLH_2/molGlyc_{consumed}$, H_2 Prod : Average hydrogen productivity ($mLH_2/L/h$).

3.3. Fermentative microbial communities developed in micro-oxidant condition

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.

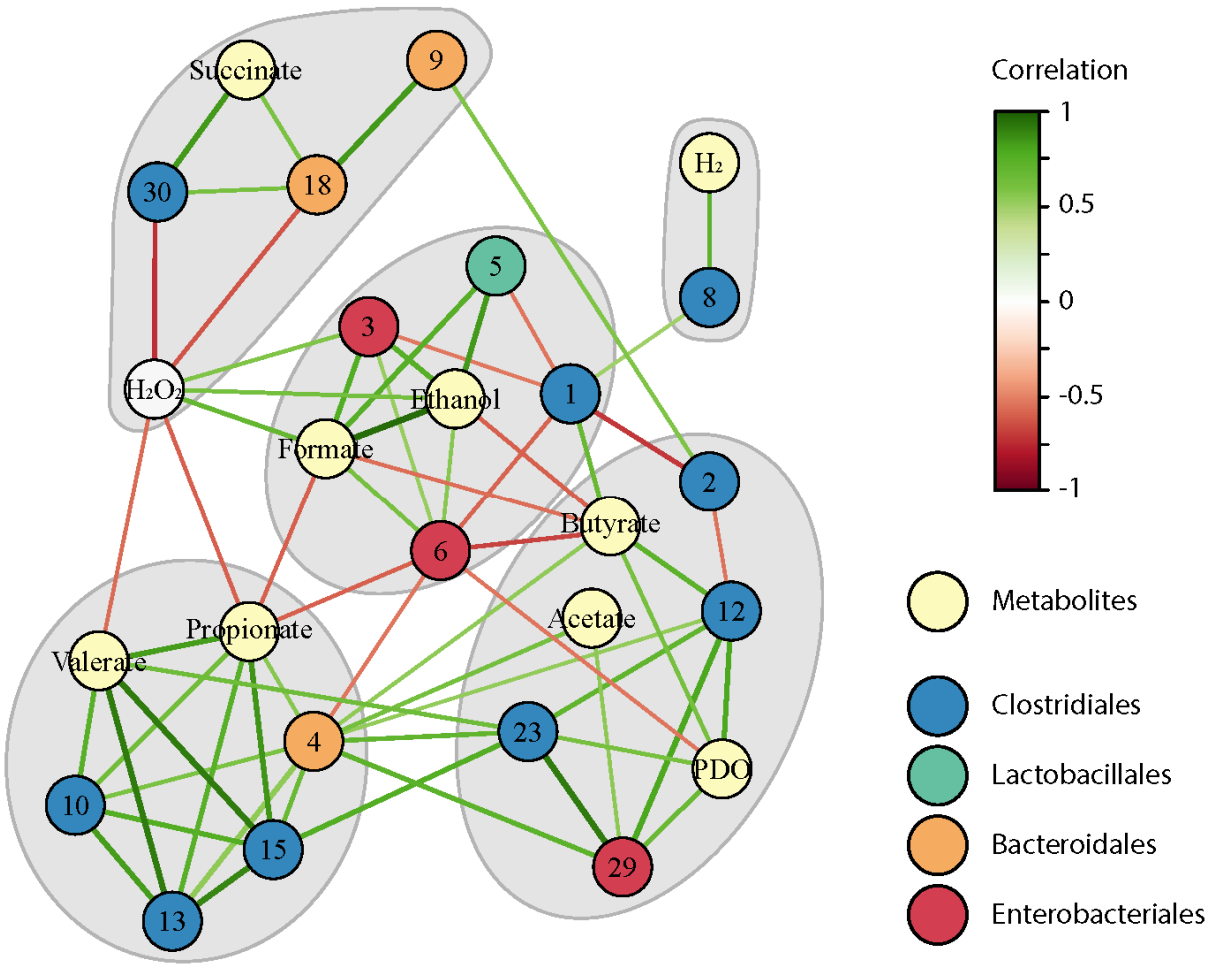

Figure 5: Correlation network obtained from Pearson correlation matrix calculated using bacterial OTUs relative abundance and fermentation production concentrations as variables. Circles represent the variables, while the lines represent the found correlations. The size and color of each correlation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

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

At a lower extent ($p < 0.05$), OTU 6 positively correlated to formate and ethanol pathways. This OTU is related to the genus *Escherichia-Shigella* (100% sequence similarity), belonging, as the genus *Klebsiella*, to the Enterobacteriaceae family. The *Klebsiella*, *Escherichia-Shigella* and *Enterococcus* genera, although belonging to different phylogenetic groups (*Proteobacteria* for *Klebsiella* and *Escherichia-Shigella*, and *Firmicutes* for *Enterococcus*), are all facultative anaerobic fermentative microorganisms with similar metabolic pathways that generates acetate, ethanol and formate. They are able to produce hydrogen from the conversion of formate into hydrogen and CO_2 through the FHL pathway (Formate Hydrogen Lyase) [51]. Consistently, in the study of Varrone et al. [43] the production of hydrogen from glycerol was associated with a high production of ethanol (*Klebsiella, Escherichia/Shigella* and *Cupriavidus*). High abundances of the OTUs 3 and 6 were observed in the reactor operated with 0.6% of H_2O_2, during the phases in which high ethanol production was observed (see Fig. 5). This observation is consistent with an indirect production of hydrogen from the ethanol pathway by the conversion of formate and explains the improvement of hydrogen performances at 0.6% of H_2O_2. Our observations suggest that facultative anaerobes had the ability to produce hydrogen by the formate and ethanol pathways and could participate to the improvement of the overall hydrogen performances. Since they are also able to consume oxygen, the anaerobic conditions were optimal for the growth of strict anaerobes such as bacteria from the *Clostridiales* order, which are well-known efficient hydrogen producers.

4. Conclusion

This study aimed to investigate the hydrogen production using micro-oxidative condition by injecting H_2O_2 at different concentrations (0.2, 0.4 and 0.6%). It was shown that constant injection of H_2O_2 at a concentration of 0.6% (w/w) improved the hydrogen performances. A hydrogen yield reached 403.6±94.7 mmolH_2/molGly which was 345% higher than the yield without H_2O_2 addition (90.6±2.8 mmolH_2/molGly). Interestingly, micro-oxidative conditions favoured the development of facultative anaerobes, such as bacteria from *Klebsiella, Escherichia-Shigella* and *Enterococcus* genera, which very likely produced hydrogen through ethanol and formate pathways. Strict anaerobes (*Clostridium intestinale*) dominated the microbial community and were likely responsible of the hydrogen produced during the experiments, suggesting that optimal conditions for strict anaerobic bacteria were

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

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