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

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11

12 **ABSTRACT**

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

24 unstable H₂ production were associated with a higher relative abundance of
25 *Enterobacteriaceae* family members. Our results allow a better understanding of H₂
26 production in continuous systems and how the microbial community is affected. This
27 provides key information for efficient selection of operating conditions for future
28 applications.

29 **Keywords:** *Aeration Treatment, Biohydrogen, Biokinetic control, Dark fermentation,*
30 *Microbial Community.*

31

32 **1. INTRODUCTION**

33 The growing environmental pollution of cities has motivated the search for new sources of
34 clean and renewable energy. In this context hydrogen (H₂) appears as a great environment
35 friendly alternative for transportation. Indeed, its combustion produces only water vapor
36 instead of greenhouse gases, with a combustion efficiency 2.75 (122 kJ/g) times higher than
37 traditional fuels and can also be easily converted into electricity in electric vehicle fuel cells
38 [1], [2]. Green H₂ is considered a renewable energy since it is produced from renewable
39 resources, such as organic matter by dark fermentation. This latter technology has been
40 widely studied because of a high simplicity and the low operating and maintenance costs
41 when compared to other biological H₂ production systems, such as photofermentation and
42 biophotolysis. In addition, a wide variety of substrates and inocula can be used allowing the
43 production of energy while treating waste [1], [3]–[6]. Different types of waste and organic
44 substrates have already been studied including simple sugars such as glucose and more
45 complex organic matter such as organic industrial wastes [7]. A special interest has been

46 focusing on crude glycerol, the main by-product of the biodiesel industry, as a low-cost
47 feedstock [8]–[10].

48 Dark fermentation H₂ production performances from glycerol are mostly dependent to the
49 microbial physiological capacities. As microbial inoculum, strains of known H₂-producing
50 bacteria could be used in pure cultures, including facultative anaerobes as *Klebsiella sp.*
51 and *Enterobacter sp.* of the *Enterobacteriaceae* family, as well as the strict anaerobes
52 *Clostridium sp.* of the *Clostridiaceae* family [11]–[14]. Mixed cultures coming from natural
53 and engineered ecosystems such as soil, compost, anaerobic sludge and other anaerobic
54 environments [15]–[18] have also been used as inocula, with the advantage of providing
55 better adaptation capacity in response to environmental stresses including substrate
56 limitation and abrupt changes in pH and temperature [7], [16], [19]. The higher robustness
57 of mixed cultures has been attributed to the diversity of the microbial community, enabling
58 positive interspecies interactions such as syntrophy [2], [20]. Some mixed community
59 members can also generate adverse effects on the system performance through negative
60 interactions [2]. The origin of the inoculum, its pre-treatment, the operating strategy of the
61 reactors and the biokinetic control in continuous systems are of crucial importance to
62 ensure H₂-producer enrichment and achieve high and stable H₂-production performance
63 [21].

64 Inocula pre-treatments seek to eliminate H₂-consumers such as hydrogenotrophic
65 methanogenic archaea and enrich the community with H₂-producers [22], [23]. Heat shock
66 pre-treatment is the most used at lab scale for its efficiency in batch systems. Pre-treatment
67 conditions are generally arbitrary and range from 50°C to 125°C and from 20 to 30 min

68 [24]–[27]. In this case, the microbial community is enriched with spore-forming species
69 such as the H₂-producer *Clostridium sp.*, resisting to high temperature [7], [27]. However,
70 other non-spore-forming H₂-producing species are also depleted such as *Klebsiella sp.*, and
71 *Enterobacter sp.* [16], [28]. Moreover, heat shock pre-treatment requires additional energy
72 consumption, which is questionable in terms of economic and technical feasibility for a
73 potential industrial application [29], [30]. Another less common pre-treatment is aeration,
74 which enriches the inoculum with aerobic and facultative anaerobic H₂-producers such as
75 *Klebsiella sp.*, but also eliminates other oxygen-intolerant H₂-producing bacteria such as
76 some *Clostridium sp.* [31]. Unlike heat shock pre-treatment, aeration could be performed
77 *in-situ* as an industrially viable alternative to the common instability problems of
78 continuous systems during H₂ production [32], [33].

79 This paper aims to study the combined effects of inoculum source and pre-treatment on
80 continuous H₂ production efficiency from glycerol, with special focus on the dynamics of
81 microbial communities. For this, two inocula (aerobic and anaerobic sludge) and two pre-
82 treatments (aeration and heat shock pre-treatment) were compared.

83 **2. MATERIALS AND METHODS**

84 **2.1 Inocula source**

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

89 **2.2 Pre-treatments of inocula**

90 Inocula were either used without pre-treatment (in control conditions), or prepared using
91 two different pre-treatments prior to reactors inoculation (Table 1). A heat treatment (HT)
92 was conducted at 105 °C for 2 h. Aeration (AT) was performed by bubbling air for 4 weeks
93 at a rate providing oxygen saturation. Dissolved oxygen was monitored during these
94 treatments, using a probe and a controller. During aeration, glucose was added as carbon
95 source (10 g.L⁻¹), as well as other nutrients detailed in Section 2.3 (patent N° 201402319,
96 INAPI, Chile).

97 **2.3 Experimental set-up**

98 Six continuous stirred tank reactors (CSTR) were operated at different conditions, to
99 compare the combined effects of two inocula (aerobic and anaerobic sludge) and two pre-
100 treatments (HT and AT) on continuous H₂-production. In addition, a control (C) without
101 pre-treatment was performed for each inoculum. Tested conditions are summarized in
102 Table 1. Reactors had a useful volume of 2 L, and were operated at 12 hours of hydraulic
103 retention time (HRT), pH 5.5 and 37 °C. The reactors were inoculated with 0.4 L of
104 inoculum and then operated in batch mode for 24 hours before starting continuous
105 operation. The reactors were operated continuously for at least 16 HRT. The cultivation
106 medium was composed of 7.5±1.1 g.L⁻¹ glycerol and others nutrients as follows (mg.l⁻¹) 1
107 000 NH₄Cl, 250 KH₂PO₄, 100 MgSO₄·7H₂O, 10 NaCl, 10 NaMoO₄·2H₂O, 10 g.L⁻¹
108 CaCl₂·2H₂O, 9.4 MnSO₄·H₂O and 2.8 FeCl₂ [34].

109 **2.4 Analytical methods**

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

119 **2.5 Microbial community analysis**

120 For molecular biology analysis, 2 mL biomass samples were collected from original
121 inocula, after pre-treatments and at the end of the continuous operation. Biomass samples
122 were centrifuged, and the pellet was stored in 9 % NaCl at –20 °C. Total genomic DNA
123 was extracted with the Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA,
124 USA). The V3-V4 region of the bacterial 16s rRNA gene was PCR-amplified according
125 Carmona-Martinez *et al.* (2015) [35]. The community composition was evaluated by
126 sequencing using the MiSeq v3 chemistry (Illumina) with 2x300 bp paired-end reads at the
127 GenoToul platform (<http://www.genotoul.fr>). Sequences were retrieved after
128 demultiplexing, cleaning, clustering (97%) and affiliating sequences using Mothur [36]. A
129 total of 3216 operational taxonomic units (OTU) were found and then used for statistical

130 analysis. Sequences have been submitted to GenBank with accession No. KX632952-
131 KX636081.

132 **2.6 Data analysis**

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

139 An ANOVA analysis was performed to evaluate significant differences in H₂ yields and
140 biomass production between the conditions. Simpson diversity index was calculated to
141 compare microbial diversity at the beginning and end of each condition. Principal
142 component analysis was performed from (i) initial and final microbial community and, (ii)
143 final microbial community and metabolic patterns. For the PCA were used the microbial
144 community data with a relative abundance >5.0% in at least one sample. All statistical
145 analyses were carried out with PAST 3.24 software (<http://folk.uio.no/ohammer/past/>).

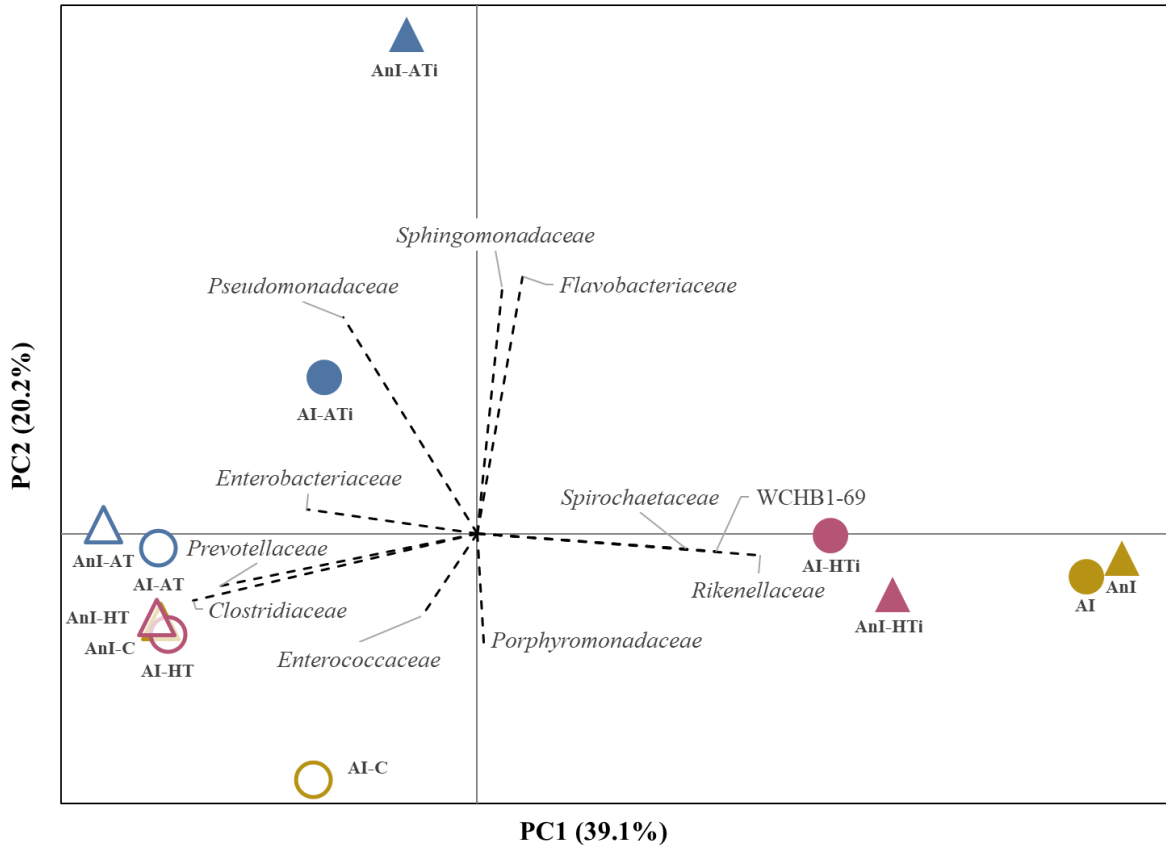
146 **3. RESULTS & DISCUSSION**

147 **3.1 Microbial communities in original and pre-treated inocula**

148 The initial microbial community was analyzed in the original inocula (AI and AnI) and in
149 the pre-treated inocula (AI-HT_i, AnI-HT_i, AI-AT_i and AnI-AT_i). Whereas the Simpson
150 Diversity Index quantifies microbial diversity, where 1 represents infinite diversity and 0
151 represents no diversity. The original inocula have a high diversity with a Simpson Diversity

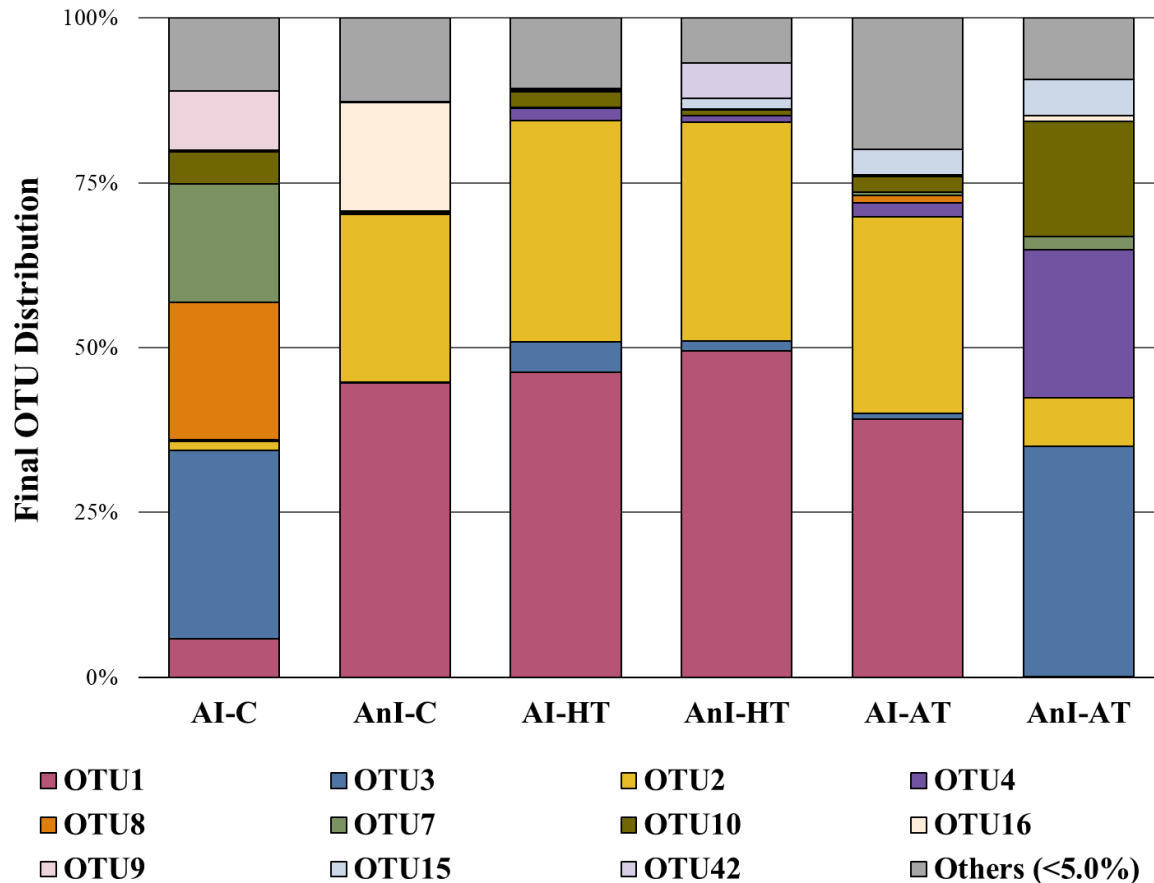
152 Index of 0.98 and 0.92 for aerobic and anaerobic sludge, respectively. After any pre-
153 treatments the Simpson Diversity Index showed no great changes compared to the original
154 inocula (Table 2). When comparing all communities, the highest similarity is observed
155 between both original inocula AI and AnI, as shown on the PCA (Fig. 1).

156 At the phylum level, aerobic and anaerobic inocula were dominated by Bacteroidetes and
157 Spirochaetae phyla, representing between 34.8% – 40.4% and 20.3% – 26.4% of bacterial
158 community respectively. In particular, the most abundant families in aerobic sludge (AI)
159 were *Spirochaetaceae* (12.4%) and *Rikenellaceae* (10.3%), with OTU11 (8.2%)
160 dominating. OTU11 had 91% of 16S rRNA sequence similarity with *Rectinema*
161 *cohabitans*. In anaerobic sludge (AnI) the most abundant families were *Rikenellaceae*
162 (18.8%) and WCHB1-69 (11.5%), with OTU5 (18.4%) and OTU6 (16.2%) dominating
163 (Fig. 2). These two OTUs were related to *Mucinivorans hirudinis* (87% 16S rRNA
164 sequence similarity with OTU5) and *Eubacterium minutum* (78% 16S rRNA sequence
165 similarity with OTU6). Although these families have been reported as dominant in other
166 initial microbial communities of H₂-producing reactors [37], [38], none of these dominant
167 species in AI or AnI have been reported as H₂-producing.



168

169 **Fig. 1: Principal component analysis (PCA) based on initial and final microbial**
 170 **population distribution. PCA was performed from correlation matrix. Triangle and**
 171 **circle shapes represent the anaerobic (AnI) and aerobic (AI) sludge, respectively.**
 172 **Filled and empty symbols represent the initial and final samples, respectively. Purple,**
 173 **blue and yellow symbols represent the samples with heat shock pre-treatment (HT),**
 174 **aeration pre-treatment (AT) and control (C), respectively. The "i" at the end of the**
 175 **sample names refers to samples taken prior to reactor inoculation *i.e.* after pre-**
 176 **treatments. Dotted lines represent Euclidean distances between PCA axes and**
 177 **taxonomic families.**



178

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

183 Heat shock pre-treatment has a rather limited impact on total microbial community, as
 184 shown on the PCA in Fig. 1. After heat shock, the total abundance of the initially dominant
 185 Bacteroidetes and Spirochaetae phyla decreased in both inocula. By contrast, the relative
 186 abundance of Firmicutes and Proteobacteria increased, representing between 9.4 – 18.5%
 187 and 29.2 – 29.5% of the bacterial community respectively (Table 2). Proteobacteria became

188 dominant in both inocula. However, at the family level, the same *Rikenellaceae* family as
189 before the heat shock was maintained dominant in the anaerobic inoculum (AnI-HTi)
190 (17.3%) and became dominant in the aerobic inoculum (AI-HTi) (8.2%), even if its relative
191 abundance slightly decreased with respect to the original inocula. In anaerobic inoculum
192 the same OTU5 (17.1%) and OTU6 (14.5%) remained dominant, while in aerobic inoculum
193 OTU25 (5.3%) became dominant. The OTU25 had 95% of 16S rRNA sequence similarity
194 with *Desulfonatronobacter acetoxydans*. As expected, the aerobic inoculum community
195 was more evenly distributed than the anaerobic one, even after heat-treatment. Besides,
196 heat treatment not only favored families with known spore-forming species such as
197 *Peptostreptococcaceae*, but also families with non-spore forming species such as
198 *Desulfobacteraceae* and *Christensenellaceae* [39], [40]. However, this finding is not
199 unusual since other studies have reported that non-spore forming species can survive drastic
200 treatments such as heat shock [2]. Surprisingly, the heat treatment resulted in a very limited
201 enrichment of the community with members of well-known H₂-producing families such as
202 *Clostridiaceae* or *Enterobacteriaceae*.

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

210 27.1% and 18.6% of microbial community, respectively. The second most abundant family
211 was *Prevotellaceae* (11.8%) and *Flavobacteriaceae* (12.1%) for aerobic and anaerobic
212 inocula, respectively. *Clostridiaceae* and *Prevotellaceae* families have strict anaerobic
213 species commonly found in H₂ producing systems, while *Flavobacteriaceae* is mainly
214 composed of aerobic species not commonly found in H₂-producing reactors [2], [41].
215 Besides, in aerobic inoculum OTU3 (11.4%) and OTU2 (8.9%) were dominant, while
216 OTU240 (8.9%) and OTU1 (8.8%) in anaerobic inoculum. These four OTUs were related
217 to *Clostridium butyricum* (100% 16S rRNA sequence similarity with OTU3), *Prevotella*
218 *paludivivens* (90% 16S rRNA sequence similarity with OTU2), *Sphingobium yanoikuyae*
219 (100% 16S rRNA sequence similarity with OTU240) and *Clostridium pasteurianum* (98%
220 16S rRNA sequence similarity with OTU1).

221 Our results show that aerobic pre-treatment allows the selection of species with aerobic and
222 facultative anaerobic metabolisms belonging to families such as *Pseudomonadaceae* and
223 *Moraxellaceae* (Fig. 2). Especially, the increase of the *Enterobacteriaceae* family known
224 for its facultative anaerobic H₂ producing members was observed [42]. Surprisingly, the
225 important presence of the *Clostridiaceae* family was also observed, whose members
226 managed to remain and multiply despite theoretically lethal aeration conditions. This could
227 show positive interactions, where non oxygen tolerant microorganisms could be protected
228 by others through oxygen consumption during stressful conditions such as aeration.

229 **3.2 Performance indicators during continuous H₂ production**

230 Biomass production for all experiments did not differ significantly (ANOVA, *p*-value
231 ≤ 0.05), with average growth yields reaching $6.0 \pm 2.1 \text{ g}_{\text{VSS}} \cdot \text{mol}_{\text{gly-consumed}}^{-1}$ (Table 3). All

232 experiments produced H₂ with yields ranging from 0.29±0.10 to 0.55±0.08 mol_{H₂}.mol_{gly-}
233 consumed⁻¹, except for the anaerobic sludge after aeration treatment (AnI-AT), which
234 produced H₂ unsteadily (Table 3). In general, the H₂ yields obtained in this study are within
235 the ranges reported in literature (0.05 to 0.58 mol_{H₂} mol_{gly-consumed}⁻¹) for dark fermentation
236 from glycerol using mixed cultures in continuous systems [15], [34], [43]–[45]. Soluble
237 metabolites produced concomitantly with H₂ are detailed in Table 3. Butyrate was the main
238 metabolite in all experiments, reaching between 22.0±8.8%_{COD consumed} and 39.7±21.5%_{COD}
239 consumed. Succinate production represented between 12.0±4.5%_{COD consumed} and 16.1±5.6%_{COD}
240 consumed in experiments that used heat-treated sludge (AI-HT and AnI-HT) and aeration-
241 treated aerobic sludge (AI-AT), but was in less amount 2.5±1.2%_{COD consumed} in the control
242 experiments (AI-C and AnI-C) and in the experiment with unstable H₂ production (AnI-
243 AT). Ethanol production represented less than 12.4±5.4%_{COD consumed} in all experiments
244 except in AI-AT reaching 27.3±10.1%_{COD consumed}. Acetate and propionate were also
245 detected in all experiments but at low concentrations (<5.7±2.0%_{COD consumed}) except in AnI-
246 AT where acetate accumulated 13.2±9.4%_{COD consumed}. Formate was also produced at very
247 low concentrations (<2.9±4.4%_{COD consumed}), only in aerobic sludge experiments (AI-C, AI-
248 HT and AI-AT). Overall, glycerol removal was between 74±22%_{COD consumed} and
249 90±15%_{COD consumed}.

250 Comparing the two-original sludge (*i.e.*, not pre-treated) in the control experiences (AI-C
251 and AnI-C), a 72% higher H₂ yield was obtained along with 31.2% more butyrate and
252 47.2% less ethanol using aerobic sludge than using anaerobic sludge. This is consistent
253 with literature where higher H₂ production is often associated with higher butyrate

254 production [2], [46], [47]. This demonstrates a better adaptability for H₂ production of
255 untreated aerobic sludge compared to anaerobic sludge in a continuous system using
256 glycerol as substrate. As already reported, untreated anaerobic sludge may require more
257 time to adapt to glycerol [48]. Besides, although no methane production was observed in
258 any reactor, a part of H₂ could have been consumed by other H₂-consuming
259 microorganisms present in the untreated anaerobic sludge such as homoacetogens or
260 hydrogenotrophic methanogenic archaea.

261 When inoculum pre-treatments were performed, different effects were observed depending
262 on the inoculum sources. Within aerobic sludge experiments (AI-C, AI-HT, and AI-AT),
263 the compared pre-treatments did not have any significant effect on H₂ yield (ANOVA, *p*-
264 value ≤0.05). On the contrary, within anaerobic sludge experiments, heat treatment (AnI-
265 HT) increased H₂-yields by 45% compared to control (AnI-C). In addition, the heat pre-
266 treatment resulted in two similar H₂ production systems (AnI-HT and AI-HT) with slightly
267 different metabolite production but statistically equal H₂ yields (ANOVA, *p*-value ≤0.05),
268 despite the inocula came from different sources. This shows the reproducibility and
269 effectiveness of heat treatments, leaving evidence why has been widely reported in the
270 literature to prepare different inocula for the H₂ production by dark fermentation [28], [47],
271 [49]–[51].

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

275 to aerobic treatment, again aerobic sludge showed a better adaptability to H₂ production
276 compared to anaerobic sludge.

277 In conclusion, and depending on the inoculum source, three effects of pre-treatment on H₂
278 production can be observed respect to the control: Positive effect (*i.e.* heat pre-treatment on
279 anaerobic sludge), negative effect (*i.e.* aerobic pre-treatment on anaerobic sludge) and
280 neutral effect (*i.e.* heat pre-treatment and aerobic pre-treatment on aerobic sludge).
281 Consistently, the inoculum source importance on the efficiency of the pre-treatment was
282 already evidenced but in a study performed in batch mode operation using glucose, and
283 comparing two pre-treatments: heat treatment and acidification [52].

284 **3.3 Link between final microbial community and metabolic patterns during** 285 **continuous H₂ production**

286 DNA samples were collected at the end of the continuous operation to assess changes in the
287 microbial community. As shown in Table 2, the *Clostridiaceae* family was most abundant
288 in all conditions, with a relative abundance between 35.3% and 56.4% and was mainly
289 represented by OTU1 and OTU3 (Fig. 2). OTU1 was dominant in AnI-C (44.7%), AI-HT
290 (46.2%), AnI-HT (49.5%) and AI-AT (39.2%), while OTU3 in AI-C (28.5%) and AnI-AT
291 (34.9%). The *Prevotellaceae* family was the second most abundant in AnI-C (25.6%), AI-
292 HT (35.9%), AnI-HT (34.1%) and AI-AT (32.2%) reactors and was represented by OTU2
293 (Fig. 2). The second and third most abundant family in AI-C were *Enterococaceae* (27.1%)
294 and *Porphyromonadaceae* (20.8%) and were mainly represented by OTU7 (18.0%) and
295 OTU8 (20.8%), respectively (Fig. 2). These two OTUs were related to *Enterococcus*
296 *gallinarum* (99% 16S rRNA sequence similarity with OTU7) and *Dysgonomonas mossii*

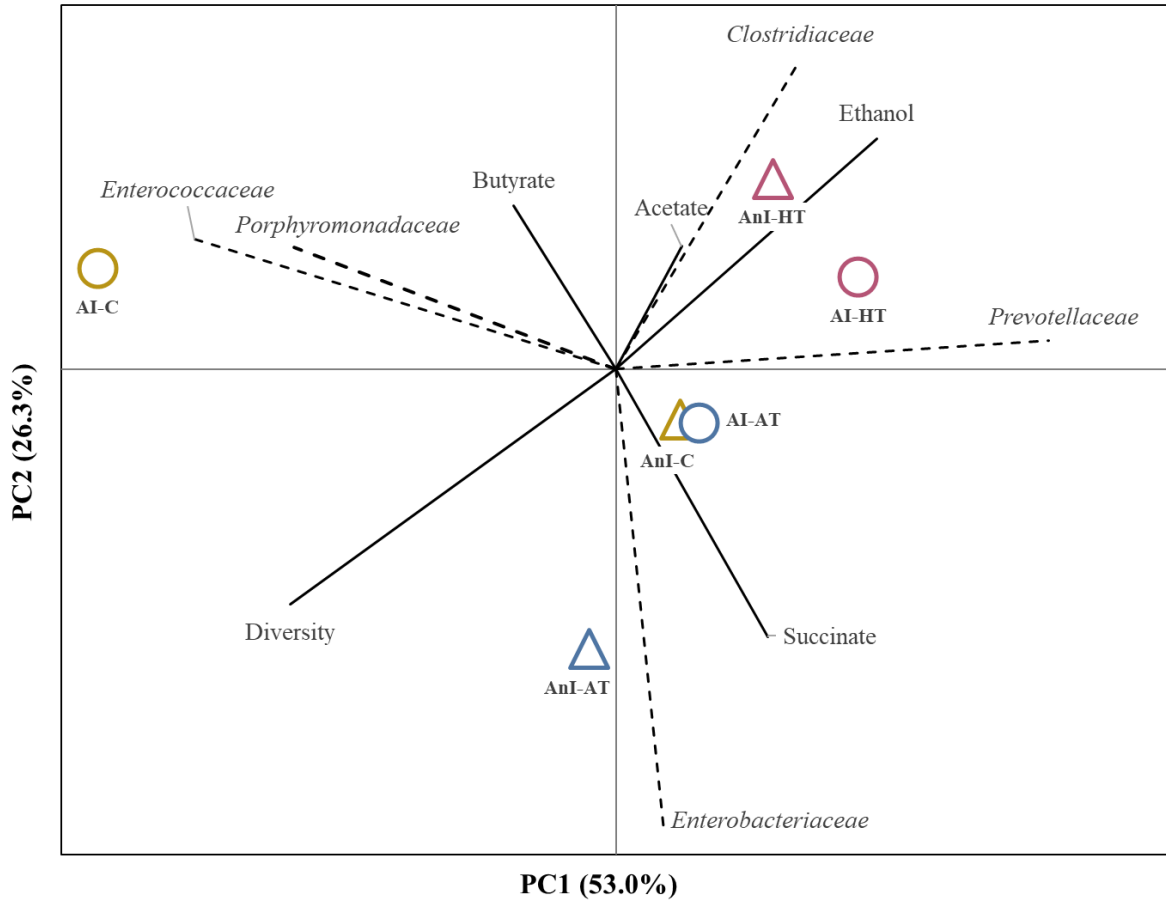
297 (100% 16S rRNA sequence similarity with OTU8). In AnI-AT, the second and third most
298 abundant family were *Enterobacteriaceae* (25.4%) and *Prevotellaceae* (24.9%) and were
299 mainly represented by OTU4 (22.4%) and OTU10 (17.5%), respectively. These two OTUs
300 were related to *Klebsiella aerogenes* (99% 16S rRNA sequence similarity with OTU4) and
301 *Prevotella dentalis* (90% 16S rRNA sequence similarity with OTU10). In AnI-C the
302 *Enterobacteriaceae* (16.7%) family was the third most abundant and was mainly
303 represented by OTU16 (16.4%). The OTU16 had 100% of 16S rRNA sequence similarity
304 with *Raoultella ornithinolytica*.

305 Illustratively Fig. 3 shows a principal component analysis (PCA) performed from final
306 microbial community at family level and metabolic patterns to observe the relations
307 between them according to each experiment. The PCA shows that the control experiences
308 (AI-C and AnI-C) are negatively related, probably due to the great impact of the inoculum
309 origin on both the final microbial communities and reactor behavior. Particularly, AI-C is
310 related to butyrate production and with *Enterococaceae* and *Porphyromonadaceae* families.
311 While, AnI-C is slightly related to acetate and ethanol production. The heat-treated
312 reactors, independently of the inoculum (AI-HT and AnI-HT), were characterized by higher
313 abundance of the *Clostridiaceae* and *Prevotellaceae* families along with the production of
314 ethanol, acetate and butyrate, as is observed in Fig. 3. This is consistent with the literature,
315 since some species of the *Clostridiaceae* family could present an acidogenic or
316 solventogenic metabolism, associated to a higher H₂ production along with acetate-butyrate
317 pathway and a lower H₂ production along with the production of alcohols such as ethanol,
318 respectively [53]. Unlike heat pre-treatment, aerobic pre-treatment generated two slightly

319 different microbial communities. Fig. 3 shows how AnI-AT is related to the
320 *Enterobacteriaceae* family, while AI-AT is related to the succinate production.

321 In addition, Fig. 3 shows that microbial diversity is positively related to AnI-AT and
322 negatively related to AI-HT and AnI-HT. The literature is not clear on how microbial
323 diversity could affect H₂ production. Contradictorily, it has been reported that greater
324 diversity may increase the possibilities of selecting H₂-producing bacteria, but it may also
325 increase competition among members of the microbial community, leading to a decrease in
326 the H₂ production [30], [54], [55]. Our results show that the microbial community was
327 considerably simplified and that the Simpson diversity index decreased by 15.8 to 33.0%
328 compared to the initial inocula. In particular, heat shock pre-treatment reduced microbial
329 diversity by 32.7±0.5%, while aeration decreased by 19.5±1.0% (Table 2). However,
330 greater microbial diversity could be linked to lower H₂ production efficiency.

331



332

333 **Fig. 3: Principal component analysis (PCA) based on metabolic patterns and final**
 334 **microbial population distribution. PCA was performed from variance-covariance**
 335 **matrix. Triangle and circle shapes represent the anaerobic (AnI) and aerobic (AI)**
 336 **inoculum, respectively. Purple, blue and yellow symbols represent the samples with**
 337 **heat treatment (HT), aeration (AT) and control (C), respectively. Plain lines and**
 338 **dotted lines represent Euclidean distances between PCA axes and taxonomic families**
 339 **and metabolic yields, respectively.**

340

341 3.4 Combined effect of inoculum source and pre-treatments on microbial 342 community

343 Fig. 1 shows a PCA performed from samples taken before inoculating the reactors and at
344 the end of continuous operation. Three main groups are observed, in which the change of
345 the microbial community from the original sludge, after pre-treatment and after continuous
346 operation is clearly evidenced. In the first group (Fig. 1, on the right) the original sludge
347 (AI and AnI) is associated with the sludge after heat pre-treatment (AI-HTi and AnI-HTi).
348 In turn, this group is associated with the most important families of their microbial
349 community, *i.e.*, *Rikenellaceae*, *Spirochaetaceae* and WCHB1-69. The second group (Fig.
350 1, top) includes sludge after aeration pre-treatment (AI-ATi and AnI-ATi) and are related to
351 families that increased their relative abundance in at least one of these samples such as
352 *Sphingomonadaceae*, *Flavobacteriaceae* and *Pseudomonadaceae*. While the third group
353 (Fig. 1, left down) is composed of all the samples taken at the end of the continuous
354 operation and are related to the families that dominated the final microbial communities in
355 each case, *i.e.* *Porphyromonadaceae*, *Enterococcaceae*, *Clostridiaceae*, *Prevotellaceae* and
356 *Enterobacteriaceae*. In all cases, there is more similarity between reactor communities
357 inoculated with different sludge exposed to same pre-treatment, suggesting that the pre-
358 treatment has more impact than the inoculum source on the total community structure.
359 Despite the pre-treatments performed and the inoculum origin, the selection pressure
360 imposed by biokinetic control appears to be crucial in determining the dominant families of
361 the H₂-producing microbial community, particularly in the selection of *Clostridiaceae*
362 family members. This is consistent with the literature, as members of this family are often

363 selected during continuous H₂ production operated at low pH (values between 5.0 – 6.0)
364 and short HRT (<12 h) [4], [34], [56], [57].

365 When considering the experiments that used untreated inoculum, it is observed that despite
366 the impact of biokinetic control (as discussed above) on selection of *Clostridiaceae* family
367 members, AI-C had a 72% higher H₂ yield than in AnI-C (Table 3). Among the dominant
368 species of the AI-C microbial community is *Dysgonomonas mossii* (Fig. 2), a fermentative
369 but not H₂-producing bacteria [58]–[60]. AI-C reach the maximum H₂ yield of this study,
370 suggesting a positive interaction of *Dysgonomonas mossii* with the microbial community
371 and especially with the known H₂-producing bacteria. Unlike AI-C, all dominant families in
372 the AnI-C microbial community (*i.e.*, *Clostridiaceae*, *Prevotellaceae*, and
373 *Enterobacteriaceae*) have known H₂-producing members, but the low H₂ yield obtained in
374 this experiment suggests the predominance of negative interactions in the microbial
375 community. Therefore, the inoculum source plays a key role in determining the final
376 microbial community when no pre-treatment is performed.

377 Comparing the metabolic patterns when a heat-treated inoculum (AI-HT and AnI-HT) was
378 used, and especially the H₂ yields, no statistically significant differences are observed,
379 although the inocula come from different sources. Surprisingly, the final microbial
380 community of both is very similar, with *Clostridium* and *Prevotella* as dominant genus. The
381 relative abundance of *Clostridium* at the end of these experiments was more than 50%,
382 which was expected since the heat treatment objective is to enrich the microbial community
383 with spore-forming species such as *Clostridium*. Contrary to our results, Baghchehsaraee *et*
384 *al.* (2008) obtained lower H₂ yield when using heat-treated aerobic sludge, attributed to a

385 decrease in microbial diversity due to pre-treatment [30]. However, they worked with
386 glucose as substrate in batch mode operation and heat pre-treatment conditions were 65°,
387 80° or 95° for 30 min. Consequently, they are all important parameters affecting the
388 microbial community composition.

389 Aeration as pre-treatment generated important differences in microbial communities and H₂
390 yields depending on the inoculum source (AI-AT and AnI-AT). The main difference was
391 the relative abundance of *Klebsiella aerogenes* in AnI-AT, a known H₂ producing bacteria.
392 However our results show that it is negatively related to H₂ production suggesting a
393 negative interaction with other known H₂ producers in the community, which are in the
394 ratio 1.1:1.6:1.0 for *Prevotella:Clostridium:Klebsiella*, respectively [2]. In contrast to our
395 results Silva-Illanes *et al.*, (2017) evidenced the existence of positive interactions between
396 all H₂-producing bacteria present in the microbial community, which are in the ratio
397 1.2:5.4:1.0 for *Prevotella:Clostridium:Klebsiella*, respectively [34]. Consequently, the
398 difference in the results is the relative abundance of H₂ producers and their ratio, while in
399 our results the genera are in a ratio around 1.0, in Silva-Illanes *et al.*, (2017) *Clostridium* is
400 the most important being 5.4 times more abundant.

401 In conclusion it was shown that the inoculum source played a key role for H₂ production in
402 continuous reactors. The inoculum source determines not only the metabolic patterns when
403 using untreated sludge, but also affects the efficiency of the pre-treatments performed. A
404 combined effect between pre-treatments and inoculum sources was evidenced by probably
405 affecting the microbial interactions and final selection of the microbial community.

406 **4. CONCLUSION**

407 Inoculum source has a strong impact on the reactor behaviors when non-pretreated sludge
408 is used, but also on pre-treatment efficiency. Heat pre-treatment of anaerobic sludge
409 increased H₂ yield, while aeration resulted in unstable H₂ production. Whereas when
410 aerobic sludge is used no pre-treatment is necessary, as there are no statistically significant
411 differences in H₂ yields when comparing all experiments, including control. In addition,
412 biokinetic control was key in the *Clostridium sp.* selection as dominant in the microbial
413 community of all assays. While, lower or intermittent H₂ production were associated with
414 higher relative abundance of *Enterobacteriaceae* family members. Our results allow a
415 better understanding of H₂ production in continuous systems, providing key information for
416 an efficient selection of operating conditions for future industrial applications.

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Table 1: Summary of experimental design

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

610

611

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

Family	Original inocula		After pre-treatment				After continuous operation					
	AI	AnI	AI-HTi	AnI-HTi	AI-ATi	AnI-ATi	AI-C	AnI-C	AI-HT	AnI-HT	AI-AT	AnI-AT
Simpson diversity index	0.98	0.92	0.99	0.94	0.95	0.96	0.83	0.71	0.67	0.64	0.75	0.79
Bacteroidetes												
<i>Flavobacteriaceae</i>	1.7	3.5	1.6	0.1	4.8	12.1	0.0	0.0	0.0	0.0	0.0	0.1
<i>Porphyromonadaceae</i>	1.8	3.5	0.4	2.5	0.5	1.6	20.8	0.0	0.0	0.0	1.3	0.0
<i>Prevotellaceae</i>	0.2	0.0	0.3	0.3	11.8	6.3	6.4	25.6	35.9	34.1	32.2	24.9
<i>Rikenellaceae</i>	10.3	18.8	8.2	17.3	1.1	1.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>WCHB1-69</i>	5.0	11.5	1.1	1.6	0.5	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	15.8	3.0	11.2	0.5	2.7	1.0	0.2	1.4	1.1	0.2	2.1	2.0
Total	34.8	40.4	22.8	22.4	21.5	22.6	27.5	27.0	37.1	34.3	35.6	27.1
Firmicutes												
<i>Christensenellaceae</i>	0.9	0.1	7.1	0.5	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
<i>Clostridiaceae</i>	0.4	0.3	1.3	0.1	27.1	18.6	39.4	46.2	51.0	56.4	40.1	35.3
<i>Enterococcaceae</i>	0.0	0.0	0.1	0.0	1.6	3.7	27.1	0.3	0.1	0.1	0.6	1.9
<i>Lachnospiraceae</i>	0.0	0.0	0.1	0.1	2.5	4.6	0.2	1.0	0.1	0.1	5.3	2.2
<i>Peptostreptococcaceae</i>	0.5	0.2	4.0	0.2	6.5	0.8	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	6.8	5.5	6.0	8.5	3.8	4.6	4.5	7.5	2.4	1.3	5.1	1.5
Total	8.8	6.3	18.5	9.4	41.7	32.3	71.2	55.0	53.7	57.9	51.1	40.9
Proteobacteria												
<i>Comamonadaceae</i>	4.1	3.3	5.0	4.7	2.7	5.6	0.0	0.0	0.1	0.4	0.0	0.2

<i>Desulfobacteraceae</i>	3.4	0.0	7.7	0.0	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0
<i>Enterobacteriaceae</i>	0.0	0.0	1.6	0.0	6.7	4.4	0.4	16.7	3.3	1.3	5.4	25.4
<i>Moraxellaceae</i>	0.1	0.1	4.5	0.1	7.5	5.1	0.4	0.0	0.6	0.1	1.5	0.0
<i>Pseudomonadaceae</i>	0.2	0.1	0.1	0.0	8.8	9.6	0.2	0.1	0.8	2.2	6.2	5.8
<i>Sphingomonadaceae</i>	0.6	0.8	1.4	1.0	0.8	9.6	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	11.1	11.7	9.2	23.4	6.1	7.1	0.3	1.1	4.3	3.9	0.3	0.6
Total	19.5	16.0	29.5	29.2	32.9	41.7	1.3	18.0	9.1	7.8	13.3	32.0
Spirochaetae												
<i>Spirochaetaceae</i>	12.4	1.4	6.5	1.2	1.0	0.2	0.0	0.0	0.1	0.0	0.0	0.0
Unknown_Family	0.1	8.8	0.0	1.5	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Others (<5.0%)	7.8	16.3	1.8	14.6	0.9	0.7	0.0	0.0	0.0	0.0	0.0	0.0
Total	20.3	26.4	8.3	17.4	2.0	1.1	0.0	0.0	0.1	0.0	0.0	0.0
Others (<5.0%)	16.7	10.9	20.9	21.6	1.9	2.3	0.0	0.0	0.0	0.0	0.0	0.0

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

Parameter	Unit	AI-C	AnI-C	AI-HT	AnI-HT	AI-AT	AnI-AT
Biomass yield	g _{VSS} mol _{gly-consumed} ⁻¹	5.8 (\pm 2.1)	6.3 (\pm 1.5)	7.0 (\pm 3.1)	5.8 (\pm 2.4)	5.3 (\pm 2.0)	6.7 (\pm 2.7)
H ₂ yield	mol _{H₂} mol _{gly-consumed} ⁻¹	0.50 (\pm 0.19)	0.29 (\pm 0.10)	0.47 (\pm 0.17)	0.42 (\pm 0.08)	0.55 (\pm 0.08)	*
Ethanol	%COD	6.5 (\pm 2.6)	12.3 (\pm 5.1)	12.4 (\pm 5.4)	3.0 (\pm 1.3)	27.3 (\pm 10.1)	9.5 (\pm 6.4)
Acetate	%COD	3.7 (\pm 1.9)	5.7 (\pm 2.0)	4.8 (\pm 1.5)	4.1 (\pm 1.6)	3.4 (\pm 1.2)	13.2 (\pm 9.4)
Propionate	%COD	1.4 (\pm 0.7)	3.2 (\pm 1.5)	2.7 (\pm 1.0)	1.4 (\pm 0.6)	1.6 (\pm 0.4)	5.2 (\pm 3.5)
Butyrate	%COD	32.8 (\pm 11.1)	25.0 (\pm 8.7)	22.0 (\pm 8.8)	30.7 (\pm 13.8)	23.4 (\pm 8.2)	39.7 (\pm 21.5)
Succinate	%COD	1.9 (\pm 0.8)	2.5 (\pm 1.2)	12.0 (\pm 4.5)	16.1 (\pm 5.6)	15.5 (\pm 5.9)	1.8 (\pm 3.9)
Formate	%COD	1.1 (\pm 0.5)	-	1.8 (\pm 0.7)	-	1.6 (\pm 0.4)	2.9 (\pm 4.4)
Glycerol removal efficiency	%	90 (\pm 15)	79 (\pm 19)	78 (\pm 20)	80 (\pm 31)	87 (\pm 16)	74 (\pm 22)

622 Metabolite distribution based on COD mass balance. %COD were calculated based on total glycerol consumed
 623 *During AnI-AT the H₂ production was unstable.
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