



HAL
open science

Mitigating the variability of hydrogen production in mixed culture through bioaugmentation with exogenous pure strains

Simon Poirier, Jean-Philippe Steyer, Nicolas Bernet, Eric Trably

► **To cite this version:**

Simon Poirier, Jean-Philippe Steyer, Nicolas Bernet, Eric Trably. Mitigating the variability of hydrogen production in mixed culture through bioaugmentation with exogenous pure strains. International Journal of Hydrogen Energy, 2020, 45 (4), pp.2617-2626. 10.1016/j.ijhydene.2019.11.116 . hal-02531498

HAL Id: hal-02531498

<https://hal.inrae.fr/hal-02531498>

Submitted on 21 Jul 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 Mitigating the variability of hydrogen production in mixed culture through bioaugmentation
2 with exogenous pure strains

3 Simon Poirier^{a,\$*}, Jean-Philippe Steyer^a, Nicolas Bernet^a, Eric Trably^a

4 ^a LBE, Univ Montpellier, INRA, 102 Avenue des étangs, 11100, Narbonne, France

5 \$ Present address: Ecole Polytechnique Fédérale de Lausanne (EPFL), ENAC IIE Laboratory
6 for Environmental Biotechnology, 1015, Lausanne, Switzerland

7 simon.poirier@epfl.ch

8 jean-philippe.steyer@inra.fr

9 nicolas.bernet@inra.fr

10 eric.trably@inra.fr

11 * corresponding author: Simon Poirier

12 **Highlights**

13 Mixed culture low reproducibility was related to severe population shifts

14 Bioaugmentation with pure strains reduced the ecosystem variability

15 Despite remaining in minor abundance, *E. coli* and *C. necator* led to reproducible H₂

16 production

17 Best performances correlated with the predominance of *C. acetobutylicum* and *C. butyricum*

18 **Abstract**

19 Process stability is a key operational issue when operating dark fermentation with mixed
20 microbial cultures for hydrogen production. This study aimed at mitigating the instability of
21 hydrogen production by separately adding exogenous pure strains suspected to have key roles
22 in fermentative cultures. Among them, *Clostridium acetobutylicum*, *Clostridium*
23 *pasteurianum* and *Lactobacillus bulgaris* which became predominant within the mixed
24 culture strongly reduced the spectrum of produced metabolites and H₂ production variability.
25 Interestingly, *Escherichia coli* and *Cupriavidus necator*, which remained in minor abundance,
26 maintained a high and stable H₂ production while lowering the metabolic variability. 16S
27 rRNA revealed that this could correlate to a simplification of the microbial diversity and the
28 non-emergence of spore-forming competitors such as *Sporolactobacillus* sp. These results
29 illustrate the potential beneficial role of minor OTUs as keystone species on H₂-producing
30 complex ecosystem and support the possibility of using them to engineer the ecosystem and
31 maintain high and stable performances.

32

33 **Keywords:**

34 - Biohydrogen

35 - Bioaugmentation

36 - Dark fermentation

37 - Ecological engineering

38

39 1 Introduction

40 Due to the current context of global warming as well as the growing scarcity of non-
41 renewable fossil fuels, more research efforts focusing on sustainable energy production are
42 required. Among the possibilities for alternative energy development, hydrogen (H₂) has
43 attracted considerable attention over the past decade due to its high energetic yield (267
44 kJ.mol⁻¹). Nowadays, H₂ is mainly generated through energetically expensive thermochemical
45 processes, such as natural gas reforming and water electrolysis. Therefore, development of
46 clean, sustainable, and cost-competitive H₂ production processes is of major concern
47 (Hosseini and Wahid, 2016).

48 Encouraging alternatives to produce environmentally friendly H₂ are driven by
49 microbial processes that present the lowest environmental impacts (Dincer and Acar, 2015).
50 Among the biological options, dark fermentation appears to be the least restrictive technology
51 compared to biophotolysis and photofermentation since it can be operated as an intensive
52 process and without any external supply of light (Ghimire et al., 2015). Additionally, strict
53 anaerobic or facultative anaerobic bacteria involved in dark fermentation provide higher
54 productivities than photo-fermentative bacteria (Ghimire et al., 2015; Manish and Banerjee,
55 2008). Converting organic biomass in dark fermenters into H₂ is basically a truncated
56 anaerobic digestion process. Indeed, H₂ is a key metabolic intermediate produced mainly
57 during acidogenesis and is then immediately consumed to reduce by-products into mainly
58 methane or acetate. A combination of suitable operational parameters such as moderately low
59 pH and short solids retention time favor H₂ production and suppress the principal consumers:
60 the methanogens. Moreover, the ability of dark fermentation to generate H₂ from a variety of
61 renewable non-sterile and inexpensive feedstock's such as organic waste makes it suitable for

62 the demand of a sustainable development strategy (Hosseini and Wahid, 2016; Lukajtis et al.,
63 2018; Wong et al., 2014).

64 Various pure cultures of fermentative bacteria having the ability to produce H₂ from
65 simple substrates have been identified (Cabrol et al., 2017; Lee et al., 2011). As the most
66 dominant H₂-producing species found in microbial ecosystems, *Clostridium* sp. and
67 *Enterobacter* sp. were the most widely hydrogen-producing bacteria (HPB) used as inoculum
68 (Cabrol et al., 2017; Mishra et al., 2019). Even though pure bacterial strains often exhibit
69 better yields than mixed natural communities, bioprocess operation requires sterile conditions
70 and well controlled conditions that make their development at industrial scale too costly.
71 Moreover, using pure cultures for producing H₂ restricts feedstock's possibilities and does not
72 take advantage of the wide metabolic possibilities inherent to the use of complex ecosystems.
73 Thereby, mixed cultures-based bioprocesses appear as a more suitable solution for H₂
74 production from complex organic wastes. In theory, their robustness allows them to degrade a
75 wide range of substrates, due to their higher resistance and resilience to environmental
76 changes (Cabrol et al., 2017; Chandrasekhar et al., 2015).

77 However, complex microbial ecosystems present numerous interactions between the
78 different communities involved in fermentation. Illustratively, and contrary to the competitive
79 exclusion principle stating that microbial diversity could not be maintained in a chemostat at
80 steady-state, it is regularly observed that low abundant bacterial species are always present.
81 Their roles remain unclear but many recent studies tend to consider them as keystone species
82 (Cabrol et al., 2017; Rafrafi et al., 2013a). Despite their low abundance, they are supposed to
83 significantly participate to ecosystem functioning through various interactions such as trophic
84 competition. Ubiquitous microorganisms such as lactic acid bacteria (LAB) reduce H₂
85 production yields due to substrate competition (replacement of H₂ fermentation by lactic acid

86 fermentation) and to the excretion of bacteriocins (Gomes et al., 2016). Homoacetogenic
87 bacteria which grow on H₂ and CO₂ can also alter the performance of H₂ bioreactors. Besides,
88 a larger range of interactions can occur between microorganisms. Direct exchanges of
89 molecules or electrons via cytoplasmic, pilis or nanotubes connections, with or without
90 conductive materials have also been recently investigated in anaerobic environments (also
91 called DIET for Direct Interspecies Electron Transfer (Baek et al., 2018)). Cellular
92 communication between microorganisms called “quorum sensing” is also attracting more and
93 more attention in fermentation (Nguyen et al., 2019). Whatever the type of interaction,
94 fermentation patterns could be impacted thus redirecting the fermentation pathways towards
95 H₂ production (Benomar et al., 2015). Hence, whether physical or chemical, intra or
96 extracellular, all these interactions are evidences of a complex bacterial communication
97 network in which all species, and even the least abundant, would be able to drive the
98 functioning of the whole ecosystem.

99 Since structure and interactions within microbial communities are crucial for the
100 efficiency of bioprocesses, many studies aimed at better managing complex ecosystems by
101 inoculating exogenous functional microbial consortia or specific strains to mixed culture. This
102 strategy, called bioaugmentation, has been used for several purposes such as withstanding
103 bioprocess fluctuations, accelerating substrate degradation, restoring reactor performance or
104 relieving toxicant inhibition (Bartrolí et al., 2011; Bouchez et al., 2000; Fotidis et al., 2014;
105 Herrero and Stuckey, 2015; Jianlong et al., 2002; Kotay and Das, 2009; Yang and Wang,
106 2018).

107 Bioaugmentation has notably been performed by several authors to enhance the
108 hydrogen production as reviewed by Yang and Wang (2018). Interestingly, Goud *et al.*,
109 (2014) demonstrated that bioaugmentation with three various acidogenic bacteria (*Bacillus*

110 *subtilis*, *Pseudomonas stutzeri* and *Lysinibacillus fusiformis*) can enhance hydrogen
111 productivity from food waste despite overloaded substrate condition (Goud et al., 2014).
112 Moreover, Kumar *et al.*, (2015) showed that bioaugmentation with facultative anaerobes such
113 as *Escherichia coli* XL1-Blue and *Enterobacter cloacae* DSM 16657 can improve hydrogen
114 yield of a sewage sludge inoculum by creating strict anaerobic conditions for *Clostridium*
115 species (Kumar et al., 2015). The potentialities of bioaugmentation with hydrogen producing
116 facultative anaerobes was also pointed by Marone *et al.*, (2012). They reported that the
117 addition of *Rahnella* sp. 10, *Raoultella* sp. 47 and *Butiauxiella* sp. 4 increased hydrogen
118 production rate and yield compared to the indigenous ecosystem during the fermentation of
119 vegetable food waste (Marone et al., 2012). They also suggested synergistic interactions
120 induced by the triple co-culture of these strains. Benomar et al., (2013) demonstrated that
121 physical interactions, including an exchange of molecules such as proteins, between in a co-
122 culture of two anaerobic partners considered as competitors (*Clostridium acetobutylicum* and
123 *Desulfovibrio vulgaris Hildenborough*) allowed a substantial increase in hydrogen production
124 without requiring genetic engineering. Similarly, Laocharoen et al., (2015) also indicated that
125 the addition of a lactic acid-producing strain (*Lactobacillus delbrueckii* ssp. *bulgaricus*
126 TISTR 895) could avoid lactic acid accumulation in the fermentation system and enhance
127 hydrogen production by *Rhodobacter sphaeroides* KKU-PS5 (Laocharoen et al., 2015). The
128 importance of these non-hydrogen producing bacteria, often sub-dominant in bioreactors has
129 also been highlighted by Rafrafi et al., (2013) (Rafrafi et al., 2013b).

130 In this framework, the aim of this study was to investigate the impact of the addition of
131 seven exogenous strains, reported as keystone species, on H₂ production performances and on
132 the microbial ecosystem balance of a complex community. Based on the literature and on
133 previous work, seven (*Clostridium acetobutylicum*, *Clostridium pasteurianum*, *Escherichia*

134 *coli*, *Lactobacillus bulgaris*, *Enterococcus casseliflavus*, *Desulfovibrio vulgaris* and
135 *Cupriavidus necator*) were selected for their potential interaction with H₂ producers.
136 Microbial dynamics were monitored with 16S rRNA gene sequencing. Inoculum originating
137 from an industrial methanogenic reactor was tested.

138 2 Materials and methods

139 2.1 Experimental set-up

140 Experiments were carried out in 600 mL batch flasks with a working volume of
141 200 mL. Growth medium composition is detailed in Rafrafi et al. (2013). Main components
142 were 2g/L of yeast extract, 10 g/L of glucose, 7.4 g/L MES Buffer, oligo-element solution 40
143 mL/L, H₂O *q.s.p* 1 L. pH was initially adjusted to 6.0 using NaOH (5N). Bioreactors were
144 sealed with a screw cap and a rubber septum and headspaces were flushed with N₂ (purity >
145 99.99%, Linde gas SA) during 10 min in order to ensure strict anaerobic conditions. They
146 were then autoclaved (121°C, 20 min) and stored at 4°C prior to inoculation.

147 An anaerobic inoculum was sampled from the outlet of a lab-scale methanogenic reactor
148 fed with wine distillery wastewater (Ornaisons, France). To avoid hydrogen consumption by
149 methanogenic populations, a heat-shock treatment (90°C, 10 min) was performed on this
150 anaerobic sludge. It was then incubated at 35°C in a 1000 mL flask. A set of 24 anaerobic
151 batch flasks were initially seeded with 10 mL of this anaerobic inoculum with a syringe
152 through the rubber septum. One pure strain was added to each bioreactor. A set of seven pure
153 strains were used for bioaugmentation assays on the basis of previous works, ie. *Clostridium*
154 *acetobutylicum*, *Clostridium pasteurianum*, *Escherichia coli*, *Lactobacillus bulgaris*,
155 *Enterococcus casseliflavus*, *Desulfovibrio vulgaris* and *Cupriavidus necator* (Benomar et al.,
156 2015; Rafrafi et al., 2013). A total of 10 mL of each pure strain culture, corresponding to 10⁷
157 CFU and 0.1% of the total biomass of the mixed culture, was sampled during their respective
158 exponential growth phase and added to the bioreactor using a syringe through the rubber
159 septum. Control flasks were supplemented with sterile water. Three replicates were carried
160 out for each condition.

161 Time zero (T_0) samples were taken and all reactors were incubated at 37°C. Agitation
162 was manually performed before and after each sampling campaign. Liquid samples (2 mL)
163 were periodically taken through the septum and centrifuged at 10,000 g for 10 min. The
164 pellets and supernatant obtained were stored separately at -20°C for analysis of biomass and
165 chemical indicators respectively. The incubations were run up to 120 hours at 35 °C until all
166 daily biogas productions decreased below 1 mL/h.

167 2.2 Analytical methods

168 Biogas production and composition (H_2 , CO_2 , O_2 , CH_4 and N_2 content) were analyzed
169 using a gas chromatograph R3000 (SRA Instruments) as detailed in Chatellard *et al.*, (2016).
170 The volume of produced biogas was normalized according to the ambient temperature and
171 pressure.

172 To determine H_2 production parameters, the cumulative H_2 production ($H(t)$) data was
173 fitted to a modified Gompertz equation (Eq. (1)):

$$174 \quad H(t) = P \times \exp \left\{ -\exp \left[\frac{R_{\max} \times e}{P} \times (\lambda - t) + 1 \right] \right\} \quad (\text{Eq.1})$$

175 where P is the maximum cumulative H_2 production (mL H_2 /L), R_{\max} is the maximum H_2
176 production rate (mL H_2 /L/day), λ is the lag-phase time (day), t is the incubation time (day) and
177 e is $\exp(1)$. The cumulative H_2 production was expressed in mL per L of culture taking into
178 account the variations in volume due to gas and liquid sampling. The values of P , R_{\max} and λ
179 were estimated using a non-linear regression algorithm developed with Matlab software
180 (version 6.5, MathWorks). H_2 production yield was calculated by dividing the maximum H_2
181 production (P) by the molar amount of glucose consumed.

182 Fermentation end products (lactate, ethanol) were quantified using high performance
183 liquid chromatography (HPLC) coupled to a refractometer (Waters R410). The
184 chromatograph was equipped with an HPX 87 column (Biorad) and the eluent corresponded
185 to a H₂SO₄ solution (0.222 ml/L) under isocratic elution at 0.4 mL/min. The operating
186 conditions were: temperature of column, 35°C; temperature of refractometer, 40°C.

187 2.3 Molecular analyses of microbial communities

188 2.3.1 DNA extraction and PCR amplification

189 Total DNA was extracted from the pellet using the Wizard Genomic DNA Purification
190 kit (Promega) according to the manufacturer's instructions. The purity and amount of DNA in
191 the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan). DNA
192 were stored at -20°C prior sequencing. The procedure for 16S rRNA gene sequencing and raw
193 data processing was described elsewhere by Carmona-Martinez (2015). The V3-V4 region of
194 the 16S rRNA gene was amplified with the forward primer
195 CTTTCCCTACACGACGCTCTTCCGATCTTACGGRAGGCAGCAG and the reverse
196 primer GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT plus
197 the respective linkers over 30 amplification cycles at an annealing temperature of 65.0°C. In a
198 second PCR reactor of 12 cycles, an index sequence was added using the primers
199 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC and
200 CAAGCAGAAGACGGCATAACGAGAT-index-GTGACTGGAGTTCAGACGTGT. The
201 PCR products were then sent for sequencing on a Illumina MySeq cartridge in GeT PlaGe
202 sequencing center of the genotoul life science network in Toulouse, France (get.genotoul.fr)
203 according to the procedure described in (Carmona-Martínez et al., 2015).

204 2.3.2 Data processing and statistical analysis

205 A slightly modified version of the Standard Operation Procedure was used to process
206 the raw data for alignment and assignation of the sequences in MOTHUR version 1.33.0.
207 SILVA release 102. This version is more detailed by Carmona-Martinez et al. (2015). R
208 CRAN software (version 3.3.3) was used to examine 16S abundance file. Considering the
209 dispersion in the total number of reads identified in each sample, bacterial OTUs abundances
210 were normalized. Only OTUs that exceeded 0.5% in at least one sample have been taken into
211 account for the histogram representation.

212 3 Results and discussion

213 3.1 H₂ production dynamics

214 To accurately assess the H₂ production performances, cumulative H₂ production curves
215 were fitted to a modified Gompertz equation for each batch experiment. Gompertz model
216 showed a good fit with the experimental H₂ production data, with determination coefficients
217 r^2 over 0.99 for all batch tests. H₂ production curves obtained with the mixed culture were
218 decomposed to two distinct fitting curves, each corresponding to a different H₂ production
219 phase. Gompertz parameters determined for all incubations are presented in Table. 1.

220 3.1.1 Mixed culture (controls)

221 Cumulated H₂ production dynamics obtained from the different replicates of the mixed
222 culture are detailed in Fig. 1A. Kinetics evidenced a low reproducibility between the three
223 replicates. H₂ production started in all bioreactors after a lag time of 6 hours and consisted of
224 2 phases. The duration of this first production phase was variable between the three replicates.
225 It lasted respectively from 15 hours in replicates 2 and 3 ending with a plateau phase, up to 45
226 hours in replicate 1. The plateau phase duration ranged from 25 hours to 50 hours in replicates
227 3 and 2, respectively. Cumulated H₂ production volumes at the end of this first phase varied
228 between 180 mL and 280 mL. For all the replicates, a second H₂ production phase followed
229 the plateau whose duration was also variable from one replicate to another. Total H₂
230 production was also not consistent, ranging from 70 mL to 280 mL during this phase.
231 Consequently, ultimate H₂ production yield (H_{\max}) varied from 271 mL up to 562 mL. The
232 variability observed in the analysis of H₂ production kinetics could be explained by an
233 instability of the bacterial communities during both growth phases that directly affected the

234 H₂ production of the consortium as already described by a recent study (Chatellard et al.,
235 2016).

236 3.1.2 Pure strains behaviours

237 H₂ production was highly reproducible among each triplicate of hydrogen producing
238 strains (*Clostridium acetobutylicum*, *Clostridium pasteurianum* and *Escherichia coli*). Data
239 are respectively presented in presented in Supplementary Fig. S1-S3. H₂ production dynamics
240 revealed that *Clostridium pasteurianum* reached higher maximum production yields
241 (514.4±32.5 mL, 2.02±0.04 molH₂/mol glucose) than *Clostridium acetobutylicum*
242 (463.1±26.6 mL, 1.94±0.14 molH₂/mol glucose) and *Escherichia coli* (54.0±7.7 mL,
243 0.90±0.12 molH₂/mol glucose). These yields are similar to those recovered in many studies
244 (Cabrol et al., 2017).

245 Both *Clostridia* reached comparable H₂ yield as those obtained with replicates 2 and 3
246 of the mixed culture, ie. 471 mL and 562 mL of H₂, respectively. Pure bacterial strains such as
247 *Clostridiales* are known to give better yields when producing H₂ than mixed natural
248 communities suggesting that, despite a low reproducibility, two out of the three replicates of
249 the mixed culture used for this study were able to reach good performances.

250 3.1.3 Confrontation between mixed culture and pure strains

251 The mixed culture confrontation with pure strains induced the establishment of new H₂
252 production kinetics. Three tendencies were observed:

253 Interestingly, adding strains such as *Clostridium acetobutylicum* (Fig. 1B), *Clostridium*
254 *pasteurianum* (Fig. 1C) and *Lactobacillus bulgaris* (Fig. 1D) generated the disappearance of
255 the first plateau phase. In presence of exogenous *Clostridies*, the entire substrate was
256 consumed after 60 hours of incubation which implied that the addition of these two pure

257 strains increased substrate consumption rate. However, lower H₂ production yields were
258 obtained. In presence of *Clostridium acetobutylicum*, a yield of only 271.1±42.4 mL was
259 reached, which was still comparable to the H_{max} obtained with one of the mixed cultures
260 replicate. *Clostridium pasteurianum* led to higher H_{max} values ranging between 330 mL and
261 370 mL of H₂. Addition of *Lactobacillus bulgaris* within the mixed culture generated a strong
262 reduction in H₂ production. Only 15 hours after seeding this strain, H₂ productivity reached
263 zero. As a result, the global H₂ yield reached only 169.5±18.1 mL. This suggested that
264 *Lactobacillus bulgaris* addition caused trophic competition between this lactic acid bacteria
265 (LAB) and the native ecosystem, likely favored by a release of bacteriocins in the medium as
266 already reported by several authors (Cabrol et al., 2017; Gomes et al., 2016; Noike et al.,
267 2002; Palomo-Briones et al., 2018). Considering that H₂ generation completely stopped after
268 only 15 hours after this strain addition, this observation well supports the fact that
269 *Lactobacillus bulgaris* outcompeted efficiently hydrogen-producing bacteria.

270 Overall, all pure strain bioaugmentations led to a decrease in H₂ yields. However, these
271 seeding generated an increase of the reproducibility of the H₂ production dynamics when
272 compared to the mixed culture control. It can be assumed that adding these pure strains led to
273 new interactions in the global ecosystem. With regard to the hydrogen yields, it was presumed
274 that trophic interactions could have taken place in the mixed culture leading to an increased
275 competition for the substrate.

276 In addition, bioaugmentation of *Escherichia coli* (Fig. 1E) and *Cupriavidus necator* (Fig. 1F)
277 did not prevent H₂ production kinetics from being decomposed into two phases. However, it
278 greatly reduced the variability of H₂ production during the second phase. While the average
279 production of this phase remained stable within these three replicates, the standard deviation
280 dropped from 159.3 mL for the mixed culture alone down to 79.9 mL in presence of *C.*

281 *necator* and even to 49.5 mL in presence of *E. coli*. The addition of these two strains may
282 have led to new interactions taking place during the second phase of production and
283 suggesting that they managed to remain active in the global ecosystem.

284 Finally, *E. casseliflavus* (Fig. 1G) and *D. vulgaris* (Fig. 1H) did not have any beneficial
285 influence on the variability within the mixed culture during the second phase of H₂
286 production. It could either indicate that both strains did not grow throughout the incubation or
287 that their growth did not modify the interactions between the key hydrogen producers and
288 consumers.

289 3.2 Metabolites accumulation

290 Fig. 2 presents the distribution of the soluble metabolites produced concomitantly with
291 H₂.

292 3.2.1 Mixed culture (controls)

293 Metabolite production kinetics by the mixed culture is presented in Supplementary Fig.
294 S4. It was divided into two bar charts corresponding to two H₂ production phases. High
295 variability observed within the triplicate of bioreactors required to distinguish the results for
296 each replicate. Contrary to what could be expected, a great reproducibility was revealed in the
297 metabolic pathways used by the mixed culture during the first H₂ production phase. All
298 replicates produced four types of metabolite (acetate, butyrate, formate and lactate) in
299 relatively unchanged proportions. Acetate and butyrate production were the major
300 fermentation products (3.22 ± 0.05 mmol and 3.58 ± 0.11 mmol, respectively) suggesting that
301 *Clostridium spp* could be predominant within the mixed culture during the first phase.
302 Replicate 1 revealed a high lactate accumulation of 8.66 mmol during the second phase that
303 consequently led to lower H₂ production. In replicate 2, caproate and ethanol that were not

304 detected in the first phase, accumulated at a level of 2.32 mmol and 1.20 mmol respectively.
305 This result suggests that additional metabolic pathways were used for substrate conversion
306 during the second phase. Interestingly, replicate 3 did not reveal any metabolic shift during
307 the second phase. An increase in acetate (4.6 mmol) and butyrate (8.5 mmol) production was
308 observed confirming the shape of the metabolite distribution present in the first phase. The
309 difference observed in the three replicates indicated that the ecosystem was governed by a
310 high variability in metabolic patterns with subsequent changes in metabolite distribution.
311 Compared to the other replicates, the metabolites distribution of replicate 3 was consistent
312 between both phases and led to the highest H₂ production. Instability found in replicates 1 and
313 2 could be the consequence of a trophic competition between the communities leading to
314 different shifts in bacterial distribution of the ecosystem.

315 3.2.2 Pure strains

316 Final metabolite production of all pure strains is presented in supplementary Fig. S5.
317 Both *Clostridies* used similar metabolic pathways and produced four types of metabolites
318 (acetate, butyrate, propionate and formate), in relatively unchanged proportions. *C.*
319 *acetobutylicum* produced 3.37±0.50 mmol of acetate, 5.03±0.21 mmol of butyrate, 1.20±0.17
320 mmol of propionate and 2.17±0.06 mmol of formate while *C. pasteurianum* produced
321 3.90±0.20 mmol of acetate, 6.17±0.11 mmol of butyrate, 0.30±0.01 mmol of propionate and
322 1.76±0.06 mmol of formate. It could be noted that *C. acetobutylicum* produced more
323 propionate than *C. pasteurianum*. Propionic acid pathway is a hydrogen consuming pathway
324 that leads to limited hydrogen production. Here, it seems that in these conditions *C.*
325 *acetobutylicum* metabolic pathways were less efficient for hydrogen production than *C.*
326 *pasteurianum* which is underlined by a lower butyrate level and consequently to lower H₂
327 yield.

328 *E. coli* synthesized 1.64 ± 0.15 mmol of acetate, 1.65 ± 0.05 mmol of propionate and
329 0.73 ± 0.06 mmol of lactate while no butyrate was detected during these incubations. Hosseini
330 and Wahid (2016) demonstrated that *Escherichia coli* was able to use acetate and formate
331 pathways to synthesize H_2 . However, formate is hardly detectable. The presence of lactate
332 suggested that *E. coli* partly shunted theoretical hydrogen potential towards solventogenesis.
333 This could be induced by acetate inhibition which could orientate *E. coli* metabolic pathways
334 towards non-acetate producing pathways.

335 *L. bulgaris* and *E. casseliflavus* produced respectively between 8.7 ± 0.1 mmol and
336 6.5 ± 0.5 mmol of lactate from glucose while *D. vulgaris* and *C. necator* did not produce any
337 metabolite confirming their incapacity to produce H_2 .

338 3.2.3 Confrontation of mixed culture with pure strains

339 Results presented in Figure 2 showed that *C. acetobutylicum*, *C. pasteurianum*, *E. coli*;
340 and *C. necator* additions strongly directed ecosystem metabolic pathways towards acetate and
341 butyrate, although small amounts of other metabolites (lactate, formate and ethanol) were still
342 produced. Interestingly, metabolites synthesized by these four triplicates as well as by the
343 triplicate consisting of the mixed culture confronted with *L. bulgaris* were more reproducible
344 within each other than for the mixed culture alone. *Lactobacillus bulgaris*, promoted the
345 synthesis of lactate, repressed acetate and butyrate pathways thus inducing a decrease in H_2
346 production. This strain addition could moreover limit the growth of bacterial population
347 degrading lactate to butyrate, which apparently occurred in replicates 2 and 3 of the mixed
348 culture. *Clostridium pasteurianum* promoted acetate production. However, the presence of
349 ethanol and lactate in some replicates may indicate that some metabolic instability persisted in
350 the ecosystem. Similarly, *E. coli* and *C. necator* induced an increase in butyrate production
351 leading to higher and more stable H_2 production levels. Contrastingly, *E. casseliflavus* and *D.*

352 *vulgaris*, which do not produce H₂ led to highly diverse metabolites production suggesting
353 that they do not influence H₂ production pathways.

354 The repeatability of the results obtained when adding these five strains contrasted with
355 the variability of metabolites in the mixed culture replicates. This finding tended to imply that
356 these strains were metabolically active in the ecosystem and that their inclusion would have a
357 stabilizing effect on the overall consortium metabolism. Except when adding *Lactobacillus*,
358 which seemed to activate lactate pathway while preventing the acetate and butyrate pathways,
359 no trend appeared to emerge about the influence of *Clostridium* sp. on the metabolite patterns
360 of the ecosystem. Indeed, the excessive variability of microbial communities observed during
361 the mixed culture growth induced significant fluctuations in the production of metabolites
362 which prevent us to conclude about a possible impact of adding *Clostridium* sp. on the choice
363 of the ecosystem metabolic pathways.

364 3.3 Microbial population dynamics

365 Microbial community dynamics were investigated with 16S rRNA gene high-
366 throughput sequencing. The objective was to identify key microorganisms involved in
367 structural variations of the microbiota. A total of 15 samples were selected. One sample was
368 used to characterize the initial composition of the mixed culture (inoculum). A second sample
369 was collected after 15 hours of incubation of the mixed culture, during the first exponential H₂
370 production phase. Due to the high variability observed within H₂ production kinetics of the
371 mixed culture, two samples per replicate were subsequently collected at the end of each
372 plateau phase. For the incubation confronting the seven exogenous strains tested, one sample
373 per triplicate, collected at the end of the H₂ production was sequenced.

374 Several thousands of sequences were identified for each sample. They were assembled
375 into 493 bacterial OTUs. OTUs belonging to the same bacterial order were represented with
376 the same color palette. Bacterial community composition dynamics were represented in
377 histograms at the OTU level in Fig. 3.

378 3.3.1 Mixed culture instability

379 Inoculum bacterial composition was highly diverse with a total of 486 different OTUs.
380 For sake of clarity, OTUs for which relative abundance did not exceed 1% were aggregated
381 and considered as minor OTUs.

382 Mixed culture was mainly composed of OTUs assigned to phyla *Firmicutes* (18%),
383 *Bacteroidetes* (16%) and *Proteobacteria* (12%). Two H₂ producing OTUs were notably
384 predominant: *Clostridium acetobutylicum* (8%) and *Clostridium butyricum* (3%).

385 After 15 hours of incubation, *Clostridium butyricum* became highly predominant
386 reaching a relative abundance of 81%. At the end of the first plateau phase, this OTU was
387 equally represented within each replicate, with a relative abundance varying between 96% and
388 97%. This great microbial stability confirmed the good reproducibility observed within H₂
389 production kinetics.

390 Interestingly, at the end of the second plateau phase, the microbial composition of the
391 three replicates greatly varied. In all cases, *Clostridium butyricum* proportion dropped while
392 different hydrogen producers took advantage on substrate consumption.

393 In the first replicate (R1), *Clostridium acetobutylicum* became predominant,
394 representing 51% of the total OTUs, while *Clostridium algifaecis* and *Clostridium sporogenes*
395 reached comparable proportions of 12-13%. *Clostridium butyricum* relative abundance
396 dropped down to 19%. Moreover, two OTUs belonging to *Sporolactobacillus* genus became

397 detectable in the ecosystem with a total proportion of 4%. Their appearance can thus be
398 directly correlated to the presence of lactate within the final metabolic pattern and to the
399 inhibition of the second H₂ production phase. Although, these lactic acid bacteria were not
400 detectable in the inoculum, their ability to form spore could explain their resistance to the
401 initial heat treatment.

402 In the second replicate, *Clostridium butyricum* proportion decreased down to 51% while
403 two other hydrogen producers, *Clostridium pasteurianum* and *Clostridium acetobutylicum*
404 emerged, reaching relative abundance of 29% and 19%, respectively. Meanwhile, a minor
405 OTU assigned to *Clostridium autoethanogenum* also emerged. This genus has been reported
406 to perform medium-chain fatty acids synthesis via chain elongation, utilizing acetate and
407 ethanol as main substrates. Its presence within the ecosystem could explain the production of
408 caproate and ethanol in the final metabolites of this bioreactor.

409 Contrastingly, the third bioreactor was almost exclusively dominated by two OTUs
410 assigned to *Clostridium acetobutylicum* (50%) and to *Clostridium butyricum* (45%). A small
411 amount of lactate was produced and could be linked to the presence of *Sporolactobacillus*
412 *vinae* (1%). However, contrary to the first replicate, this lactic acid bacterium did not seem to
413 reach sufficient proportion to impact the H₂ production.

414 The 16S rRNA sequences confirmed trends observed for H₂ production and metabolites
415 accumulation. It also evidenced the instability in microbial community composition and
416 related metabolic patterns occurring during the second H₂ production phase. This highlights
417 the microbial rearrangements occurring between H₂ producers and the emergence of
418 competitors such as lactic acid bacteria. Considering their ability to form spores of
419 *Sporolactobacillus* and *Clostridiaceae*, this instability could be explained by the fact that after

420 a certain lag phase, these bacteria managed to emerge and to create new competitions within
421 the ecosystem.

422 3.3.2 Confrontation with exogenous pure strains

423 Three strains (*C. acetobutylicum*, *C. pasteurianum*, and *L. bulgaris*) completely
424 annihilated the second H₂ production phase, thus reducing the global performance variability
425 of the microbiota. The 16S rRNA sequences revealed that after its addition in the bioreactor,
426 *C. acetobutylicum* became ultra predominant in the final ecosystem, reaching a final
427 proportion of 99%. Furthermore, the addition of *C. pasteurianum* induced equilibrium
428 between this strain (59%) and the major H₂ producer during the first production phase of the
429 mixed culture, *C. butyricum* (39%). This result suggested that *C. acetobutylicum* outcompeted
430 for the substrate against *C. butyricum* in higher extends than *C. pasteurianum*. However, in
431 terms of performance, higher production yields were observed in presence of *C. pasteurianum*
432 which tended to indicate that this co-culture consisted of two *Clostridies* was more efficient in
433 producing as the single strain. *L. bulgaris* also became predominant within the ecosystem,
434 which completely simplified the final composition of the microbiota. It was exclusively
435 dominated by this lactic acid bacterium (45%) and *C. butyricum* (54%). Nevertheless, besides
436 the metabolic stability of this triplicated reactors, *L. bulgaris* growth partly rerouted glucose
437 fermentation towards lactate production, which greatly reduced the H₂ production yields.
438 Thus, the annihilation of the second H₂ production phase seemed to be explained by the
439 extreme simplification of the ecosystem induced by the addition of good competitors for the
440 substrate consumption.

441 Moreover, adding the two strains (*E. coli* and *C. necator*) significantly stabilized the
442 performances during the second H₂ production phase. Sequencing results evidenced that *E.*
443 *coli* slightly emerged (2%). Nevertheless, its presence seemed to induce beneficial effects

444 leading to a simplified microbiota quasi exclusively composed of *C. acetobutylicum* (67%)
445 and *C. butyricum* (27%). Similarly, while *C. necator* was not even detected in the final
446 ecosystem, its addition in the mixed culture led to a great simplification of its composition
447 tending towards a co-culture consisting of *C. acetobutylicum* (40%) and *C. butyricum* (54%).
448 These results suggested that the initial addition of these strains drove the microbiota towards a
449 more stable composition similar to the one observed in the third replicate of the mixed culture
450 alone. This type of ecosystem only consisted of two *Clostridium* sp. strains showed the best
451 performance indicating that both strains had positive effects in terms of stability and final H₂
452 production volumes.

453 In presence of *E. casseliflavus* or *D. vulgaris*, performance instability was not mitigated.
454 Samples were selected in bioreactors for which the second H₂ production phase did not occur
455 to evidence potential OTUs that could be responsible for the inhibition of this phase.
456 Sequencing results indicated that although *E. casseliflavus* reached a final proportion of 3%,
457 its presence was also associated to the emergence of *Sporolactobacillus* which became
458 predominant (28%). This lactic acid bacterium was also recovered in the first replicate of the
459 mixed culture for which no second H₂ production phase was observed. As a result, H₂
460 producers such as *C. acetobutylicum* and *C. butyricum* were less represented in the ecosystem
461 (40% and 22 %, respectively). Interestingly, *C. algifaecis* was significantly present with a
462 relative abundance of 8%. *D. vulgaris* was not detected in the final ecosystem. But similarly
463 to the bioreactors within which no second H₂ production phase occurred, *Sporolactobacillus*
464 and *C. algifaecis* gained importance (34% and 8%, respectively) at the expense of *C.*
465 *acetobutylicum* (28%) and *C. butyricum* (30%). Thus, it can be hypothesized that either these
466 strains were not metabolically active or that they did not influence the interactions within the
467 mixed culture.

468 Nevertheless, it confirms that the mixed culture instability could lead to situations
469 where strong spore forming competitors such as lactic acid bacteria could emerge and greatly
470 affect H₂ production. Interestingly, this instability always appeared after a first H₂ production
471 phase during which rearrangements occurred within the ecosystem, probably because of a
472 shift in substrate composition from glucose to metabolites such as VFA. It can be
473 hypothesized that acetate production could create inhibitory conditions that were less
474 favorable to *C. butyricum*. During this shift, new interactions were implemented and spore-
475 forming bacteria could grow. Results prove that this instability could be avoided in batch
476 reactors by two strategies: either by bioaugmenting with specific H₂ producers which were
477 able to resist to acetate production and pH drop, or by using other strains (eg., *E. coli* or *C.*
478 *necator*) which seemed to be able to prevent the growth of competitors such as lactic acid
479 bacteria.

480 4 Conclusions

481 The distinct addition of *E. coli* and *C. necator* was proved to reduce metabolic and H₂
482 production instability of a mixed culture. Bioaugmentations with *C. acetobutylicum*, *C.*
483 *pasteurianum* and *L. bulgaris* also mitigated this variability but induced stronger trophic
484 competition that greatly limited final H₂ production. Divergence in performances were
485 associated to populations shifts in the mixed culture but also to the appearance of spore
486 forming, lactate producing genera such as *Sporolactobacillus*. This outcome highlighted
487 potentialities to use pure strains to enhance process stability and limit the stochastic
488 emergence of substrate competitors within mixed cultures.

489

490 E-supplementary data of this work can be found in online version of the paper.

491 **Acknowledgments**

492 We gratefully acknowledge the financial support of the French National Research Agency
493 (ANR) through the InGEcoH Project entitled “Ecological engineering of microbial
494 ecosystems producing biohydrogen by dark fermentation” (contract number 2008- BIOE-005-
495 01). We also thank Yan Rafrafi and Anaïs Bonnafous for having participated to this work.

496 **References**

- 497 Baek, G., Kim, Jaai, Kim, Jinsu, Lee, C., 2018. Role and Potential of Direct Interspecies
498 Electron Transfer in Anaerobic Digestion. *Energies* 11, 107.
499 <https://doi.org/10.3390/en11010107>
- 500 Bartrolí, A., Carrera, J., Pérez, J., 2011. Bioaugmentation as a tool for improving the start-up
501 and stability of a pilot-scale partial nitrification biofilm airlift reactor. *Bioresour.*
502 *Technol.* 102, 4370–4375. <https://doi.org/10.1016/j.biortech.2010.12.084>
- 503 Benomar, S., Ranava, D., Cárdenas, M.L., Trably, E., Rafrafi, Y., Ducret, A., Hamelin, J.,
504 Lojou, E., Steyer, J.-P., Giudici-Ortoni, M.-T., 2015. Nutritional stress induces
505 exchange of cell material and energetic coupling between bacterial species. *Nat*
506 *Commun* 6, 6283. <https://doi.org/10.1038/ncomms7283>
- 507 Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Doré, J., Delgenès, P., Moletta, R.,
508 Wagner, M., 2000. Ecological study of a bioaugmentation failure. *Environ. Microbiol.*
509 2, 179–190.
- 510 Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J.-P., Ruiz-Filippi, G., Trably, E., 2017.
511 Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights
512 for driving the ecosystem function. *FEMS Microbiol. Rev.* 41, 158–181.
513 <https://doi.org/10.1093/femsre/fuw043>
- 514 Carmona-Martínez, A.A., Trably, E., Milferstedt, K., Lacroix, R., Etcheverry, L., Bernet, N.,
515 2015. Long-term continuous production of H₂ in a microbial electrolysis cell (MEC)
516 treating saline wastewater. *Water Research* 81, 149–156.
517 <https://doi.org/10.1016/j.watres.2015.05.041>
- 518 Chandrasekhar, K., Lee, Y.-J., Lee, D.-W., 2015. Biohydrogen Production: Strategies to
519 Improve Process Efficiency through Microbial Routes. *Int J Mol Sci* 16, 8266–8293.
520 <https://doi.org/10.3390/ijms16048266>
- 521 Chatellard, L., Trably, E., Carrère, H., 2016. The type of carbohydrates specifically selects
522 microbial community structures and fermentation patterns. *Bioresource Technology*
523 221, 541–549. <https://doi.org/10.1016/j.biortech.2016.09.084>
- 524 Dincer, I., Acar, C., 2015. Review and evaluation of hydrogen production methods for better
525 sustainability. *International Journal of Hydrogen Energy* 40, 11094–11111.
526 <https://doi.org/10.1016/j.ijhydene.2014.12.035>
- 527 Fotidis, I.A., Wang, H., Fiedel, N.R., Luo, G., Karakashev, D.B., Angelidaki, I., 2014.
528 Bioaugmentation as a Solution To Increase Methane Production from an Ammonia-
529 Rich Substrate. *Environ. Sci. Technol.* 48, 7669–7676.
530 <https://doi.org/10.1021/es5017075>
- 531 Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015.
532 A review on dark fermentative biohydrogen production from organic biomass: Process
533 parameters and use of by-products. *Applied Energy* 144, 73–95.
534 <https://doi.org/10.1016/j.apenergy.2015.01.045>
- 535 Gomes, S.D., Fuess, L.T., Mañunga, T., Feitosa de Lima Gomes, P.C., Zaiat, M., 2016.
536 Bacteriocins of lactic acid bacteria as a hindering factor for biohydrogen production
537 from cassava flour wastewater in a continuous multiple tube reactor. *International*

- 538 Journal of Hydrogen Energy, Special Issue on Progress in Hydrogen Production and
539 Applications (ICH2P-2015), 3-6 May 2015, Oshawa, Ontario, Canada 41, 8120–8131.
540 <https://doi.org/10.1016/j.ijhydene.2015.11.186>
- 541 Goud, R.K., Sarkar, O., Chiranjeevi, P., Venkata Mohan, S., 2014. Bioaugmentation of potent
542 acidogenic isolates: A strategy for enhancing biohydrogen production at elevated
543 organic load. *Bioresource Technology*, Special Issue: CESE 2013 & Special Issue:
544 ICABB 2013 165, 223–232. <https://doi.org/10.1016/j.biortech.2014.03.049>
- 545 Herrero, M., Stuckey, D.C., 2015. Bioaugmentation and its application in wastewater
546 treatment: A review. *Chemosphere, Wastewater-Energy Nexus: Towards Sustainable
547 Wastewater Reclamation* 140, 119–128.
548 <https://doi.org/10.1016/j.chemosphere.2014.10.033>
- 549 Hosseini, S.E., Wahid, M.A., 2016. Hydrogen production from renewable and sustainable
550 energy resources: Promising green energy carrier for clean development. *Renewable
551 and Sustainable Energy Reviews* 57, 850–866.
552 <https://doi.org/10.1016/j.rser.2015.12.112>
- 553 Jianlong, W., Xiangchun, Q., Libo, W., Yi, Q., Hegemann, W., 2002. Bioaugmentation as a
554 tool to enhance the removal of refractory compound in coke plant wastewater. *Process
555 Biochemistry* 38, 777–781. [https://doi.org/10.1016/S0032-9592\(02\)00227-3](https://doi.org/10.1016/S0032-9592(02)00227-3)
- 556 Kotay, S.M., Das, D., 2009. Novel dark fermentation involving bioaugmentation with
557 constructed bacterial consortium for enhanced biohydrogen production from
558 pretreated sewage sludge. *International Journal of Hydrogen Energy, IWBT 2008* 34,
559 7489–7496. <https://doi.org/10.1016/j.ijhydene.2009.05.109>
- 560 Kumar, G., Bakonyi, P., Sivagurunathan, P., Kim, S.-H., Nemestóthy, N., Bélafi-Bakó, K.,
561 Lin, C.-Y., 2015. Enhanced biohydrogen production from beverage industrial
562 wastewater using external nitrogen sources and bioaugmentation with facultative
563 anaerobic strains. *Journal of Bioscience and Bioengineering* 120, 155–160.
564 <https://doi.org/10.1016/j.jbiosc.2014.12.011>
- 565 Laocharoen, S., Reungsang, A., Plangklang, P., 2015. Bioaugmentation of *Lactobacillus*
566 *delbrueckii* ssp. *bulgaricus* TISTR 895 to enhance bio-hydrogen production of
567 *Rhodobacter sphaeroides* KCU-PS5. *Biotechnology for Biofuels* 8, 190.
568 <https://doi.org/10.1186/s13068-015-0375-z>
- 569 Lee, D.-J., Show, K.-Y., Su, A., 2011. Dark fermentation on biohydrogen production: Pure
570 culture. *Bioresource Technology*, Special Issue: Biofuels-III: Biohydrogen 102,
571 8393–8402. <https://doi.org/10.1016/j.biortech.2011.03.041>
- 572 Łukajtis, R., Hołowacz, I., Kucharska, K., Glinka, M., Rybarczyk, P., Przyjazny, A.,
573 Kamiński, M., 2018. Hydrogen production from biomass using dark fermentation.
574 *Renewable and Sustainable Energy Reviews* 91, 665–694.
575 <https://doi.org/10.1016/j.rser.2018.04.043>
- 576 Manish, S., Banerjee, R., 2008. Comparison of biohydrogen production processes.
577 *International Journal of Hydrogen Energy, IWHE 2006* 33, 279–286.
578 <https://doi.org/10.1016/j.ijhydene.2007.07.026>
- 579 Marone, A., Massini, G., Patriarca, C., Signorini, A., Varrone, C., Izzo, G., 2012. Hydrogen
580 production from vegetable waste by bioaugmentation of indigenous fermentative
581 communities. *International Journal of Hydrogen Energy, XII International Symposium*

- 582 on Polymer Electrolytes: New Materials for Application in Proton Exchange
583 Membrane Fuel Cells 37, 5612–5622. <https://doi.org/10.1016/j.ijhydene.2011.12.159>
- 584 Mishra, P., Krishnan, S., Rana, S., Singh, L., Sakinah, M., Ab Wahid, Z., 2019. Outlook of
585 fermentative hydrogen production techniques: An overview of dark, photo and
586 integrated dark-photo fermentative approach to biomass. *Energy Strategy Reviews* 24,
587 27–37. <https://doi.org/10.1016/j.esr.2019.01.001>
- 588 Nguyen, P.D.T., Mustapha, N.A., Kadokami, K., Garcia-Contreras, R., Wood, T.K., Maeda,
589 T., 2019. Quorum sensing between Gram-negative bacteria responsible for methane
590 production in a complex waste sewage sludge consortium. *Appl. Microbiol.*
591 *Biotechnol.* 103, 1485–1495. <https://doi.org/10.1007/s00253-018-9553-9>
- 592 Noike, T., Takabatake, H., Mizuno, O., Ohba, M., 2002. Inhibition of hydrogen fermentation
593 of organic wastes by lactic acid bacteria. *International Journal of Hydrogen Energy*,
594 *BIOHYDROGEN* 2002 27, 1367–1371. <https://doi.org/10.1016/S0360-3199>
595 (02)00120-9
- 596 Palomo-Briones, R., Trably, E., López-Lozano, N.E., Celis, L.B., Méndez-Acosta, H.O.,
597 Bernet, N., Razo-Flores, E., 2018. Hydrogen metabolic patterns driven by
598 *Clostridium-Streptococcus* community shifts in a continuous stirred tank reactor. *Appl*
599 *Microbiol Biotechnol* 102, 2465–2475. <https://doi.org/10.1007/s00253-018-8737-7>
- 600 Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., Giudici-
601 Orticoni, M.-T., Steyer, J.-P., 2013a. Sub-dominant bacteria as keystone species in
602 microbial communities producing bio-hydrogen. *International Journal of Hydrogen*
603 *Energy* 38, 4975–4985. <https://doi.org/10.1016/j.ijhydene.2013.02.008>
- 604 Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., Giudici-
605 Orticoni, M.-T., Steyer, J.-P., 2013b. Sub-dominant bacteria as keystone species in
606 microbial communities producing bio-hydrogen. *International Journal of Hydrogen*
607 *Energy* 38, 4975–4985. <https://doi.org/10.1016/j.ijhydene.2013.02.008>
- 608 Wong, Y.M., Wu, T.Y., Juan, J.C., 2014. A review of sustainable hydrogen production using
609 seed sludge via dark fermentation. *Renewable and Sustainable Energy Reviews* 34,
610 471–482. <https://doi.org/10.1016/j.rser.2014.03.008>
- 611 Yang, G., Wang, J., 2018. Various additives for improving dark fermentative hydrogen
612 production: A review. *Renewable and Sustainable Energy Reviews* 95, 130–146.
613 <https://doi.org/10.1016/j.rser.2018.07.029>
614

615 **Figure caption**

616 Figure 1: Cumulated H₂ production (mL) over time (number of hours) of the mixed culture
617 (A), of the mixed culture bioaugmented with *Clostridium acetobutylicum* (B), with
618 *Clostridium pasteurianum* (C), with *Lactobacillus bulgaris* (D), with *Escherichia coli* (E),
619 with *Cupriavidus necator* (F) with *Enterococcus casseliflavus* (G) and with *Desulfovibrio*
620 *vulgaris* (H).

621 Replicates 1 are represented in blue while replicates 2 are represented in red and replicates 3
622 in green.

623 Figure 2: Ultimate metabolites concentration (mmol) of the different reactors inoculated with
624 the mixed alone or bioaugmented with the different pure strains after the total conversion of
625 the substrate.

626 Figure 3: Relative abundances of the bacterial genera generated by 16S rRNA gene
627 sequencing of the different reactors inoculated with the mixed alone or bioaugmented with the
628 different pure strains.

629 Figure S1: Cumulated H₂ production (mL) over time (number of hours) of a pure culture of
630 *Clostridium acetobutylicum*.

631 Replicate 1 is represented in blue while replicate 2 is represented in red and replicate 3 in
632 green.

633 Figure S2: Cumulated H₂ production (mL) over time (number of hours) of a pure culture of
634 *Clostridium pasteurianum*.

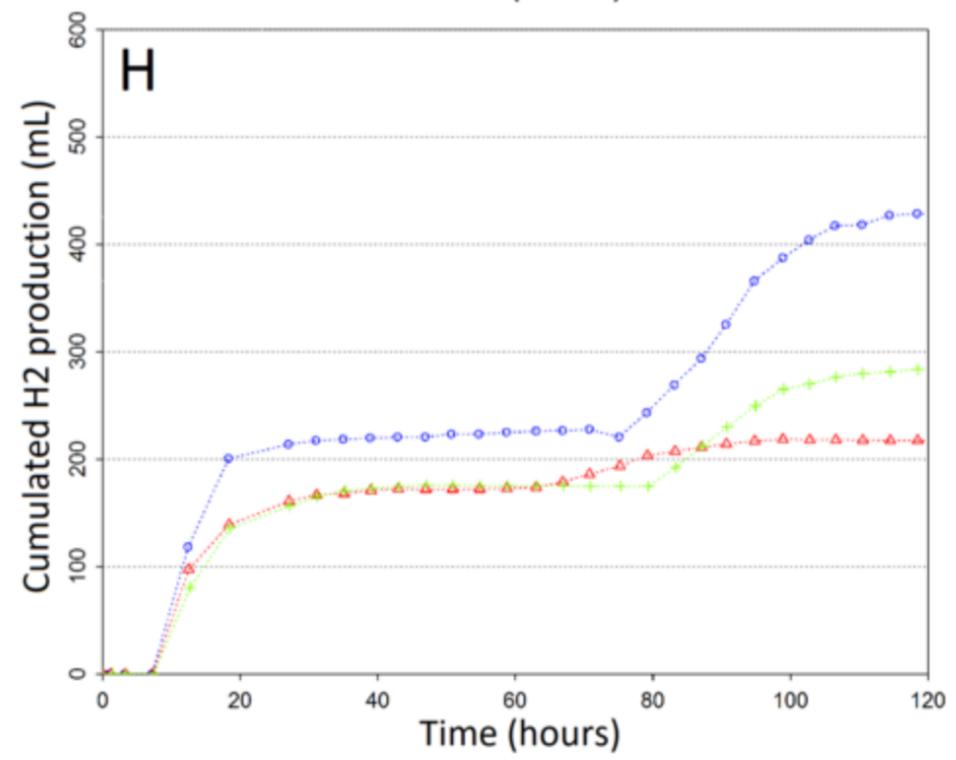
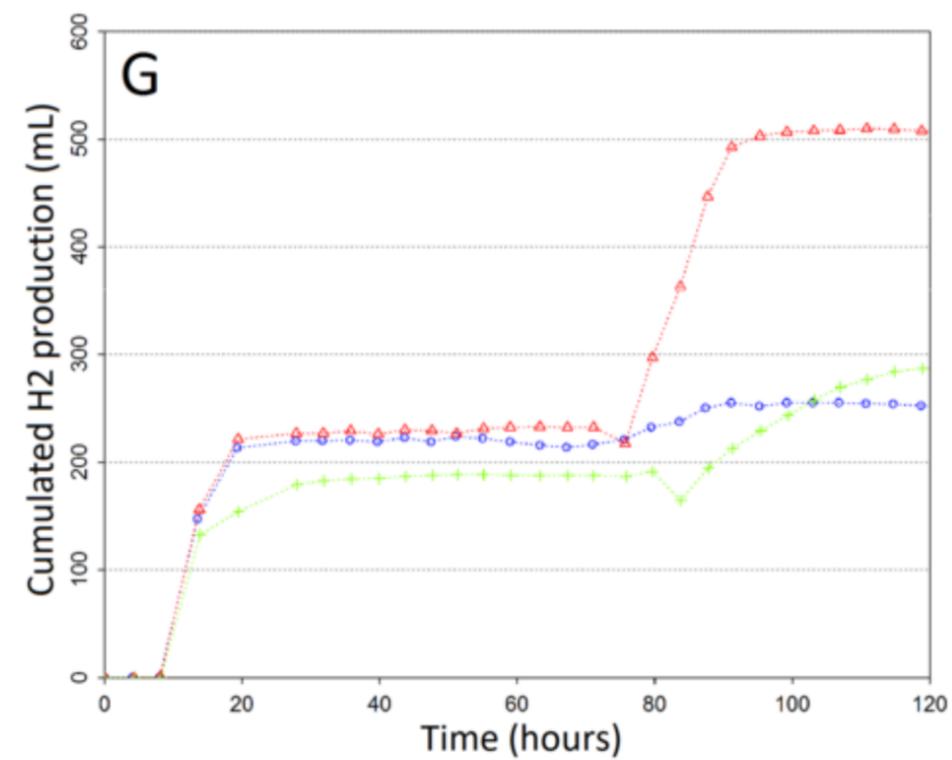
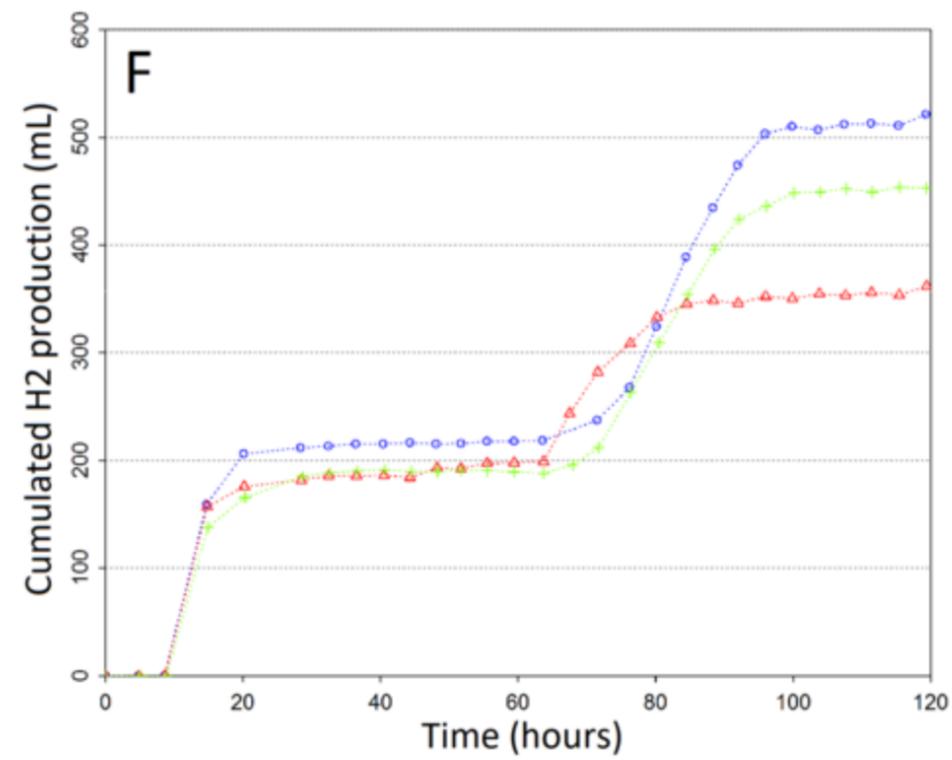
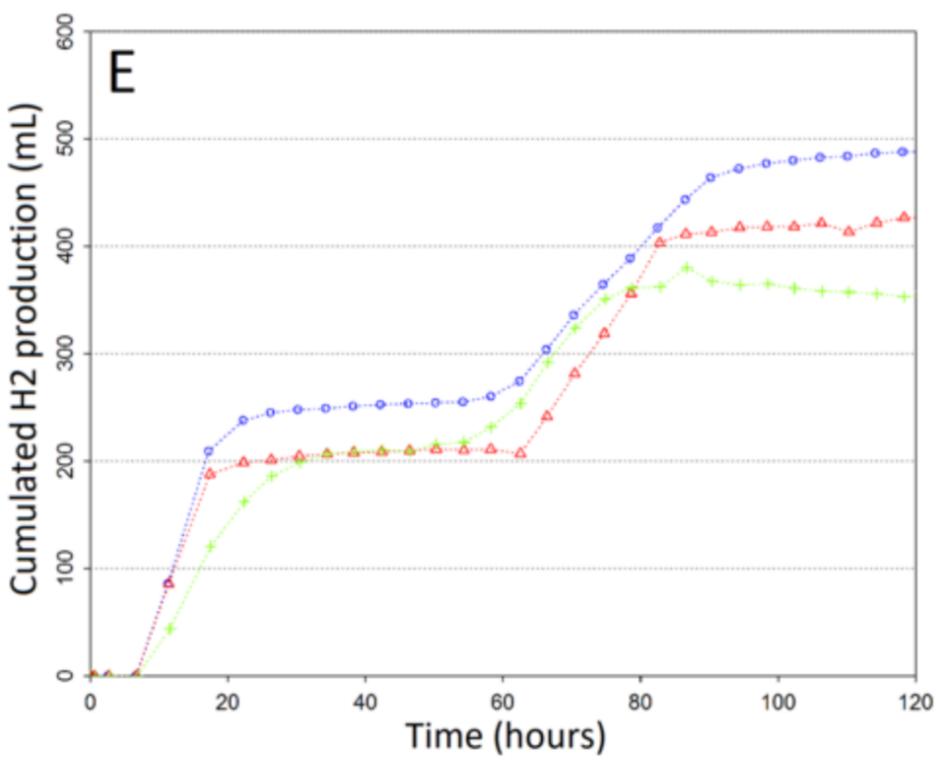
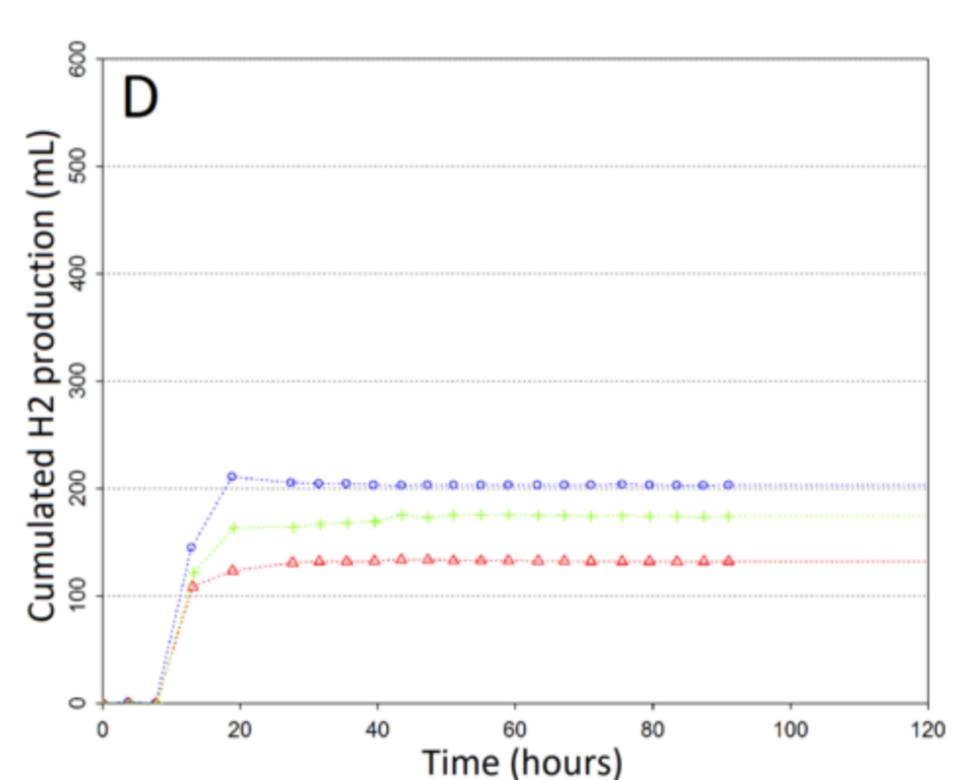
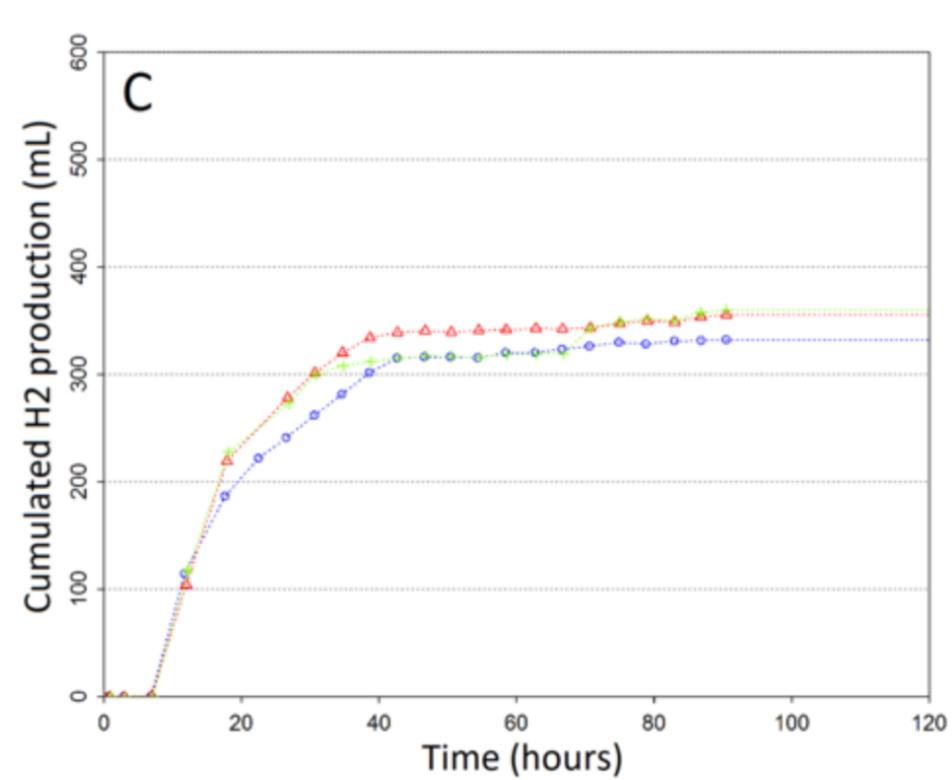
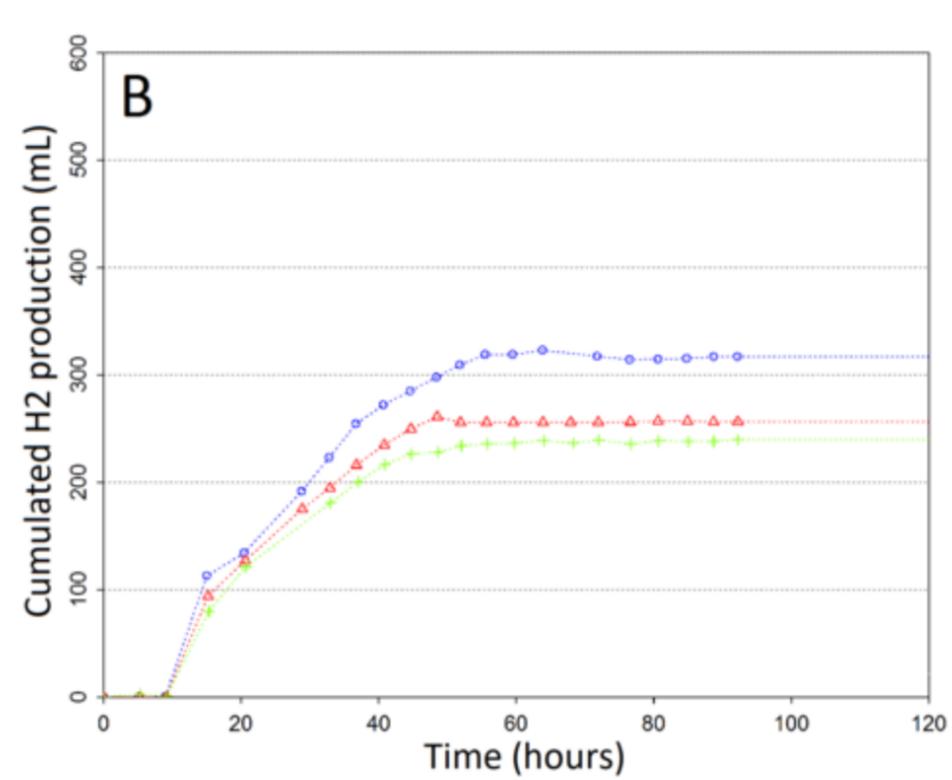
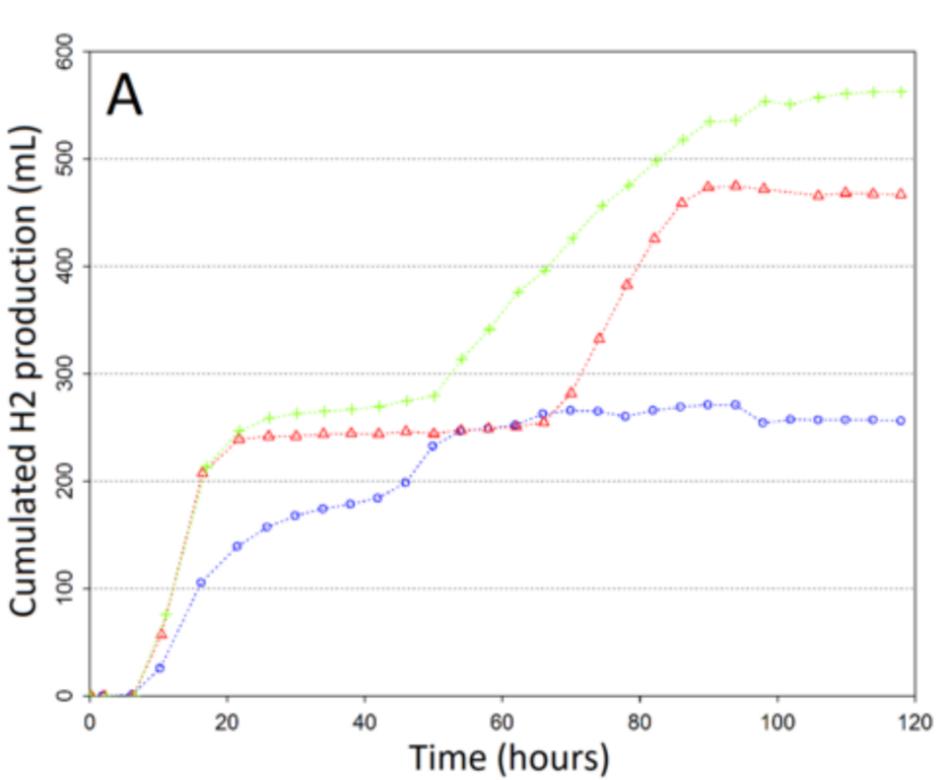
635 Replicate 1 is represented in blue while replicate 2 is represented in red and replicate 3 in
636 green.

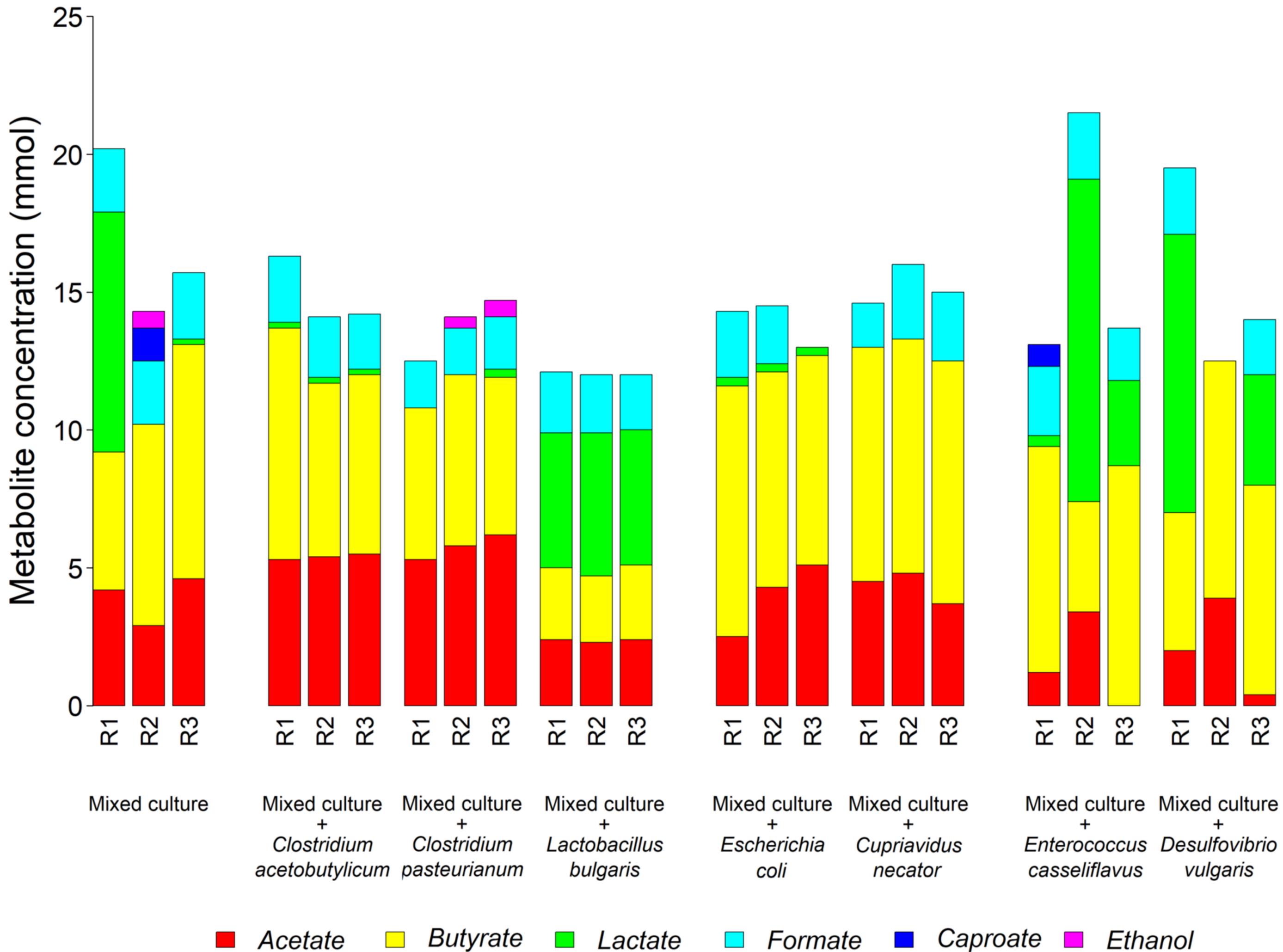
637 Figure S3: Cumulated H₂ production (mL) over time (number of hours) of a pure culture of
638 *Escherichia coli*.

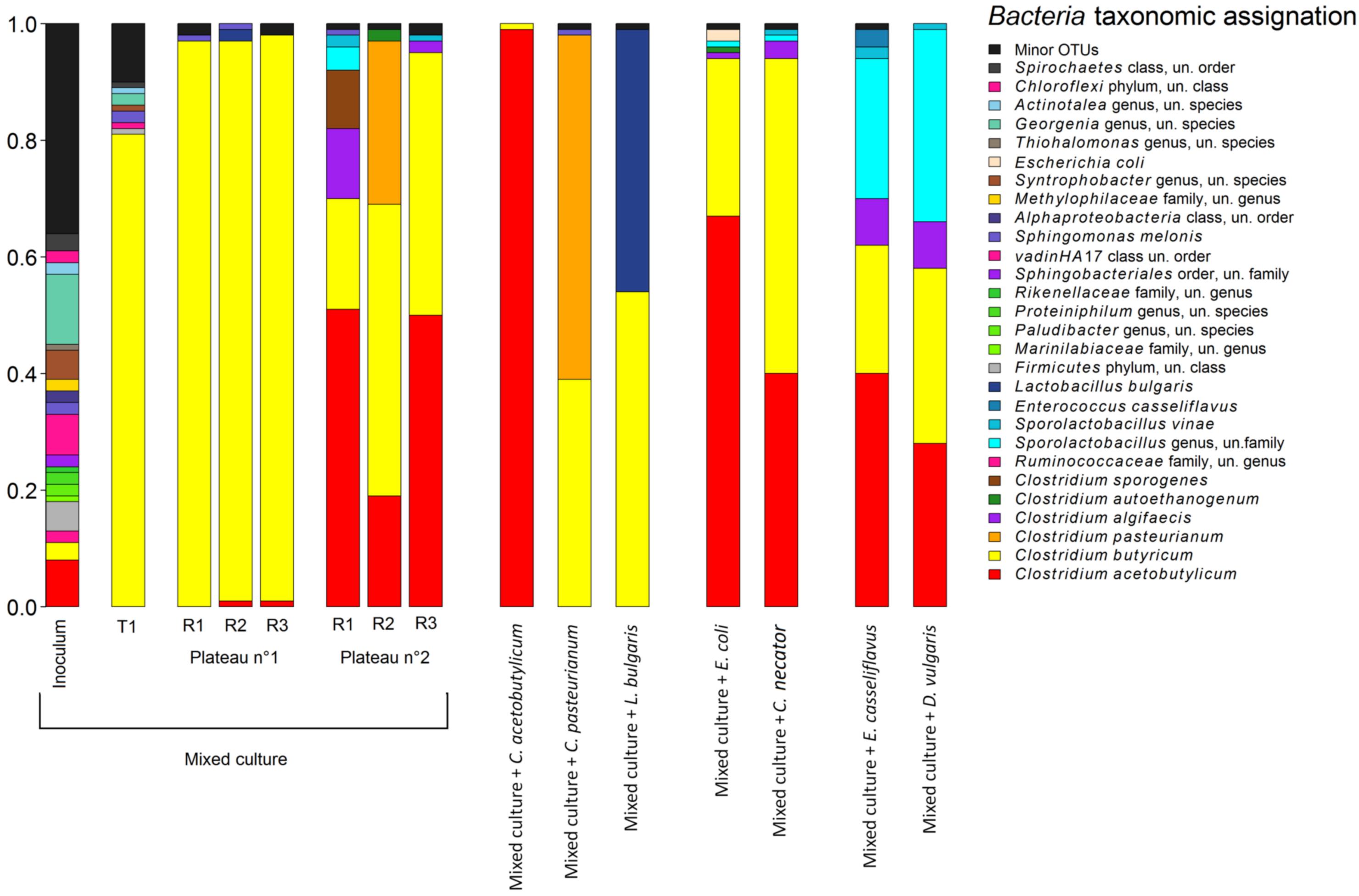
639 Replicate 1 is represented in blue while replicate 2 is represented in red and replicate 3 in
640 green.

641 Figure S4: Metabolites concentration (mmol) of the different reactors inoculated with the
642 mixed culture after the first and the second plateau phase of H₂ production.

643 Figure S5: Ultimate metabolites concentration (mmol) of the different reactors inoculated
644 with the different pure strains after the total conversion of the substrate.







		V_{\max} (mL H ₂ ·L ⁻¹ ·h ⁻¹)	H_{\max} (mL H ₂)	λ (hour)	Carbon conversion yield (mol H ₂ ·mol glucose ⁻¹)
Mixed culture	<i>Production phase n°1</i>	126.8 ± 52.6	224.2 ± 49.2	8.4 ± 0.3	1.95 ± 0.10
	<i>Production phase n°2</i>	49.5 ± 23.8	176.3 ± 151.5	-	1.50 ± 1.28
	Total	-	431.9 ± 159.3	-	1.68 ± 0.62
<i>Clostridium acetobutylicum</i>		303.0 ± 40.4	463.1 ± 26.6	19.4 ± 0.3	1.65 ± 0.10
<i>Clostridium pasteurianum</i>		77.5 ± 7.0	514.4 ± 32.5	14.2 ± 0.5	1.79 ± 0.12
<i>Escherichia coli</i>		18.3 ± 0.7	54.0 ± 7.7	19.2 ± 0.6	0.18 ± 0.03
Mixed culture + <i>Clostridium acetobutylicum</i>		50.1 ± 3.5	274.8 ± 42.4	9.7 ± 0.2	1.17 ± 0.21
Mixed culture + <i>Clostridium pasteurianum</i>		93.9 ± 23.1	349.1 ± 14.8	8.3 ± 0.1	1.45 ± 0.06
Mixed culture + <i>Lactobacillus bulgaris</i>		142.7 ± 8.3	169.5 ± 36.1	8.7 ± 0.4	1.21 ± 0.23
Mixed culture + <i>Escherichia coli</i>	<i>Production phase n°1</i>	112.0 ± 40.1	224.5 ± 23.9	7.9 ± 0.1	1.61 ± 0.27
	<i>Production phase n°2</i>		207.2 ± 33.6	-	2.04 ± 0.09
	Total	-	436.0 ± 49.5	-	1.77 ± 0.28
Mixed culture + <i>Cupriavidus necator</i>	<i>Production phase n°1</i>	164.0 ± 26.4	197.7 ± 15.4	9.7 ± 0.1	1.47 ± 0.16
	<i>Production phase n°2</i>		247.3 ± 68.3	-	2.08 ± 0.55
	Total	-	445.6 ± 79.9	-	1.75 ± 0.31
Mixed culture + <i>Enterococcus casseliflavus</i>	<i>Production phase n°1</i>	106.3 ± 36.5	189.6 ± 26.7	8.3 ± 0.2	1.46 ± 0.20
	<i>Production phase n°2</i>		120.9 ± 81.7	-	1.00 ± 0.65
	Total	-	310.5 ± 107.4	-	1.24 ± 0.42
Mixed culture + <i>Desulfovibrio vulgaris</i>	<i>Production phase n°1</i>	169.5 ± 36.4	212.2 ± 21.3	9.4 ± 0.1	1.47 ± 0.18
	<i>Production phase n°2</i>		138.4 ± 126.5	-	1.21 ± 1.05
	Total	-	350.6 ± 138.5	-	1.35 ± 0.51