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Enhancing thermophilic dark fermentative hydrogen production at high glucose concentrations via bioaugmentation with *Thermotoga neapolitana*

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ABSTRACT

The aim of the present study was to investigate the effect of gradually increasing glucose concentrations (from 5.6 to 111 mmol L^{-1}) on the fermentative H_2 production with and without bioaugmentation. A stirred tank reactor was operated at 70 °C and inoculated with a hyperthermophilic mixed culture or a hyperthermophilic mixed culture bioaugmented with *Thermotoga neapolitana*. With both the unaugmented (control) and augmented cultures, the H_2 production rate was improved when the initial glucose concentration was increased. In contrast, the highest H_2 yield (1.68 mol H_2 mol^{-1} glucose consumed) was obtained with the augmented culture at the lowest glucose concentration of 5.6 mmol L^{-1} and was 37.5% higher than that obtained with the unaugmented culture at the same feed glucose concentration. Overall, H_2 production rates and yields were higher in the bioaugmented cultures than in the unaugmented cultures whatever the glucose concentration. Quantitative polymerase chain reaction targeting *T. neapolitana hydA* gene and MiSeq sequencing proved that *Thermotoga*

32 was not only present in the augmented cultures but also the most abundant at the
33 highest glucose concentrations.

34

35 Keywords: Biohydrogen, Dark fermentation, Metabolic pathways, Microbial dynamics.

36

37 INTRODUCTION

38 Recent developments in dark fermentation by anaerobic bacteria have consistently
39 shown this process to be promising for the transformation of carbohydrate-rich
40 substrates to hydrogen. The theoretical stoichiometric yield for fermentative hydrogen
41 production is 4 mol H_2 mol^{-1} glucose consumed. However, this is exclusively possible
42 when acetate is the sole volatile fermentative product [1,2]. Invariably, the hydrogen
43 yield is lowered by the production of more reduced molecules. Fermentative hydrogen
44 (H_2) production is possible with both pure and mixed microbial cultures originated from
45 natural or engineered environments [3–5]. Typically, the use of mixed cultures is the
46 only option for H_2 generation from non-sterile organic waste and biomass residues
47 unless selective conditions, such as extremely high temperatures, are used during the
48 bioprocess [6–11]. In addition to the microbial culture, the H_2 production performance
49 depends on the operating conditions such as temperature, pH, substrate type and
50 concentration, as well as hydraulic retention time [3,12–15].

51 Substrate concentration can have a significant effect on the rate, yield and stability
52 of H_2 production. Increasing substrate concentrations have been shown to result in
53 higher production rates but lower H_2 yields with, for example, a mesophilic or
54 thermophilic H_2-producing mixed cultures [16,17]. Increasing the substrate
55 concentration and, thus, the organic load of the system allows to save the energy
56 required for heating the H_2-producing bioreactors, as high substrate concentrations

57 lead to increased microbial activity and heat generation by microbial metabolism [18].
58 However, the use of extremely high substrate concentrations can cause substrate
59 and/or product inhibition and result in sub-optimal pH for the H_2-producers due to
60 volatile fatty acid accumulation [19,20]. In addition, the low H_2 yields observed at
61 increasing organic loads can be due to a shift in metabolic flux towards
62 solventogenesis (e.g. formation of butanol, acetone and ethanol) and other reduced
63 end-products, the generation of which is not accompanied by H_2 production [21].

64 Bioaugmentation has been proposed in several studies as a potential strategy for
65 enhancing dark fermentation under stress conditions [16,22,23]. Bioaugmentation can
66 be defined as the addition of pre-grown highly specialized microorganisms or
67 populations of several microorganisms to improve the capacity of a treatment or
68 production system [24–26]. Bioaugmentation is an emerging strategy for industrial
69 wastewater treatment [27] and has been used to shorten the lag phase and improve
70 the chemical oxygen demand (COD) removal during dark fermentation of the organic
71 fraction of municipal solid waste [28]. It has also been used to enhance thermophilic
72 H_2 production from corn stover hydrolysate [29] and beverage wastewater [30].
73 Okonkwo et al. [29] applied bioaugmentation with a synthetic co-culture to enhance
74 the H_2 production during or after temporal temperature fluctuation. Given the success
75 of bioaugmentation strategy in several previous studies, bioaugmentation might be a
76 useful tool for enhancing H_2 production also at high substrate concentrations [31].
77 However, one of the most difficult issues in bioaugmentation is to ensure the survival
78 of the microorganisms introduced in the established mixed culture as the number of
79 exogenous microorganisms has been reported to shortly decrease after inoculation
80 either as a result of abiotic or biotic influence [32]. Some studies used strategies such
81 as repeated bioaugmentation to promote the persistence of the added bacterium in

82 the system [33,34]. This strategy might be effective for a transient system recovery but
83 might not ensure long-term process enhancement, if the added bacterium or bacteria
84 are not able to compete with the existing microbial consortium. Furthermore, sudden
85 process disturbances such as increased operation temperature can lead to reduced
86 microbial diversity in the mixed culture and lead to a lower process efficiency, requiring
87 bioaugmentation with bacteria that can stably coexist with the existing microbial
88 consortium.

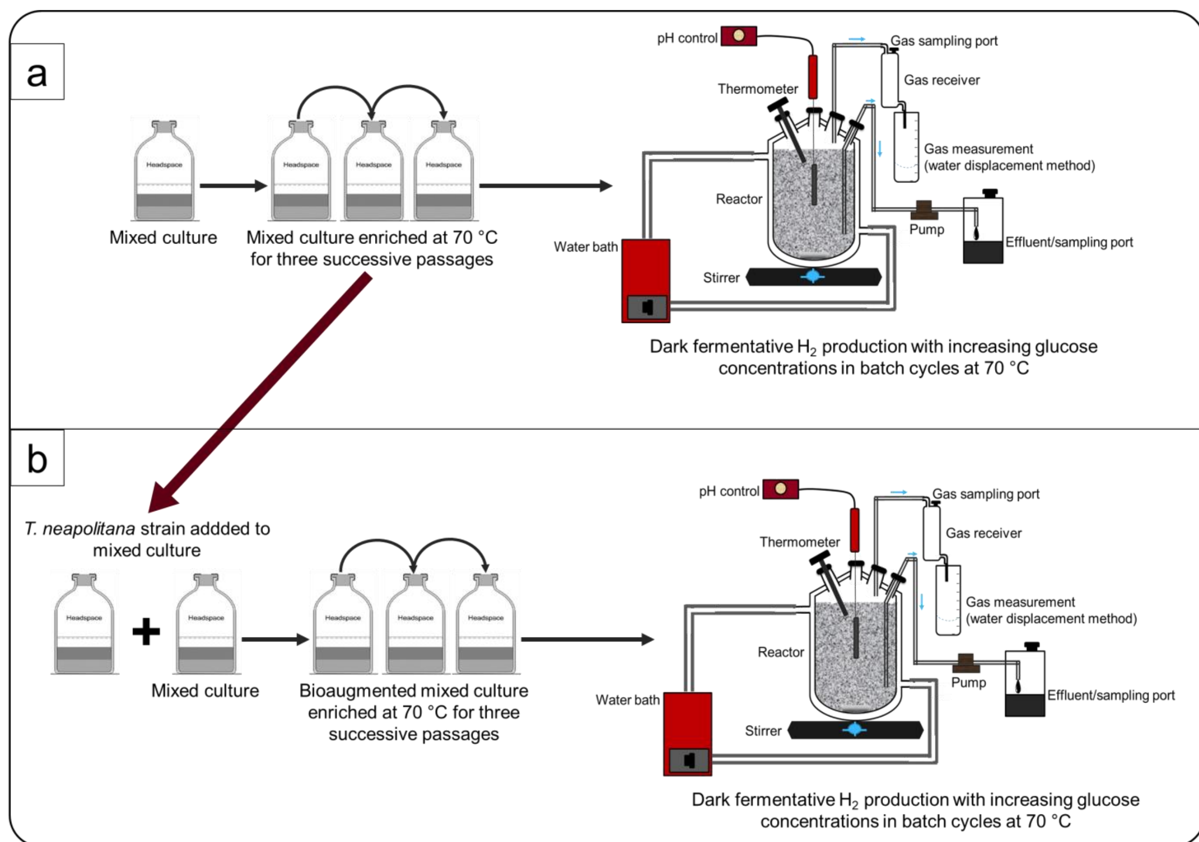
89 The aim of this study was to study the dynamics of *Thermotoga neapolitana* in a
90 mixed microbial consortium after a period of pre-adaptation as a strategy to make *T.*
91 *neapolitana* a stable member of the native microbial community. This study further
92 examined the effects of different feed glucose concentrations on H_2 production in a
93 thermophilic mixed culture with and without *T. neapolitana*, which is a
94 hyperthermophilic bacterium capable of utilizing a wide range of organic substrates as
95 carbon source and able to produce high hydrogen yields [35,36]. Previous reports
96 showed that *Thermotoga neapolitana* is capable of producing up to 3.8 mol H_2 mol^{-1}
97 glucose, which is close to the theoretical limit of 4 mol H_2 mol^{-1} glucose, and producing
98 acetate, lactate and CO_2 as other major metabolic end products [35,37]. This makes
99 *T. neapolitana* ideal for bioaugmentation purposes. To the best of our knowledge, this
100 is the first study to use pre-adaptation as a strategy for allowing *T. neapolitana* to be
101 a stable member of a native H_2-producing microbial community and for enhancing H_2
102 production.

103 MATERIALS AND METHODS

104 Experimental Design

105 The medium used for the cultivation consisted of the following components (g L^{-1}):
106 NH_4Cl, 1.0; K_2HPO_4, 0.3; KH_2PO_4, 0.3; $MgCl_2 \times 6 H_2O$, 0.2; $CaCl_2 \times 2H_2O$, 0.1; NaCl,

107 5.0; KCl, 0.1; cysteine-HCl, 1.0; yeast extract, 2.0; 10.0 ml L^{-1} of vitamin and trace
108 element solution (DSMZ 141, Germany). Nitrogen gas was used to sparge the
109 fermentation medium and create an anaerobic environment. Dark fermentation
110 experiments were carried out in batch mode in a double jacketed glass stirred tank
111 reactor (STR) with a working volume of 2 L (Figure 1). The reactor temperature was
112 kept constant at 70 °C using a heated water bath. The reactor was equipped with a
113 pH electrode and temperature probe connected to a programmable controller (Bluelab
114 pH Controller, New Zealand) to maintain the pH of the cultures at 6.5 by automatic
115 dosing of potassium hydroxide (2 molar). The fermentation broth inside the reactor
116 was mixed by a magnetic stirrer (Argolab, Italy) at 150 rpm.



117

118 Figure 1. Experimental design to study the effects of bioaugmentation at various feed
119 glucose concentrations during dark fermentation with a thermophilic mixed culture
120 without augmentation (a) and augmented with *Thermotoga neapolitana* (b).

121

122 The seed source used in this study was a mixed culture obtained from a laboratory
123 scale continuously stirred tank bioreactor producing H_2 from glucose and xylose at 55
124 °C [22]. Instead, *T. neapolitana* was purchased from DSMZ, Braunschweig, Germany.
125 The cultivation of the mixed culture was initiated at 70 °C in 250 mL batch bottles
126 with a working volume of 200 mL at an initial pH of 6.5 with 27.8 mmol L^{-1} glucose as
127 substrate. Twenty milliliters of the inoculum (10% v/v) was transferred to 180 mL of
128 the culture medium (mg L^{-1}). The cultivation was carried out in batch for three transfers
129 prior to the start of the experiment to acclimatize the culture to the higher incubation
130 temperature (Figure 1a).
131 To determine the influence of bioaugmentation at increasing substrate
132 concentrations, *T. neapolitana* DSM 4359 (DSMZ, Germany) was added to the mixed
133 culture in a 1:1 ratio (based on optical density measurements, OD_{600}). The
134 bioaugmented culture was then cultivated with glucose in batch mode in 250 mL
135 anaerobic serum bottles with a working volume of 200 mL for three successive
136 transfers at 70 °C (Figure 1b) to adapt *T. neapolitana* to growing alongside the native
137 microbial community. For each successive transfer, 20 mL of the inoculum (10% v/v)
138 was transferred to 180 mL of the culture medium (mg L^{-1}) to a final volume of 200 mL.
139 H_2 production with the unaugmented and the bioaugmented mixed culture was
140 separately investigated in batch mode in the STR described in section 2.1 and each
141 experiment lasted for a period of 48 h. The initial glucose concentration was stepwise
142 increased from 5.6 to 27.8, 55.5 and 111.0 mmol L^{-1} in order to determine the impact
143 of increasing substrate concentration on H_2 production, biomass concentration and
144 metabolic patterns.

145 **Analytical methods and calculation procedures**

146 The gas produced in the STR was quantified using a water displacement method
147 with 500 mL glass containers. The H_2 containing gas produced was sampled from the
148 gas sampling port using a gas-tight syringe (Hamilton, USA) and the H_2 concentration
149 of the biogas was measured using a 3400 gas chromatograph (GC) (Varian, USA)
150 equipped with a thermal conductivity detector (TCD) and a Restek packed column
151 using argon as the carrier gas. The total volume of the produced H_2 at each time point
152 was calculated using Equation 1 [38]:

153 $$V_{H_2,t} = V_{H_2,t-1} + C_{H_2,t}(V_{G,t} - V_{G,t-1}) + V_H(C_{H_2,t} - C_{H_2,t-1}) \quad (1)$$

154 where $V_{H_2,t}$ is the cumulative H_2 produced at time t, $V_{H_2,t-1}$ is the cumulative H_2
155 produced at time t-1, $V_{G,t}$ is the total gas volume at time t, $V_{G,t-1}$ is the total gas volume
156 at time t-1, $C_{H_2,t}$ is the H_2 fraction in the headspace at time t, $C_{H_2,t-1}$ is the H_2 fraction
157 in the headspace at time t-1 and V_H is the total headspace volume in the bioreactor.

158 H_2 production was converted into moles on the basis that one mole of an ideal gas
159 occupies a volume of 22.4 L at standard temperature and pressure according to the
160 ideal gas law. Therefore, the volume of H_2 gas produced was divided by 22.4 L in order
161 to obtain H_2 produced in moles. The H_2 yield and productivity were calculated using
162 Equations 2 and 3, respectively.

163 $$H_2\ yield = \frac{mol\ H_2}{mol\ glucose\ consumed} \quad (2)$$

164 $$H_2\ productivity = \frac{mmol\ H_2}{reaction\ volume \times fermentation\ time\ (hour)} \quad (3)$$

165

166 **Microbial analyses**

167 Genomic DNA was extracted using the PowerSoil™ DNA Isolation Kit (MoBio
168 Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

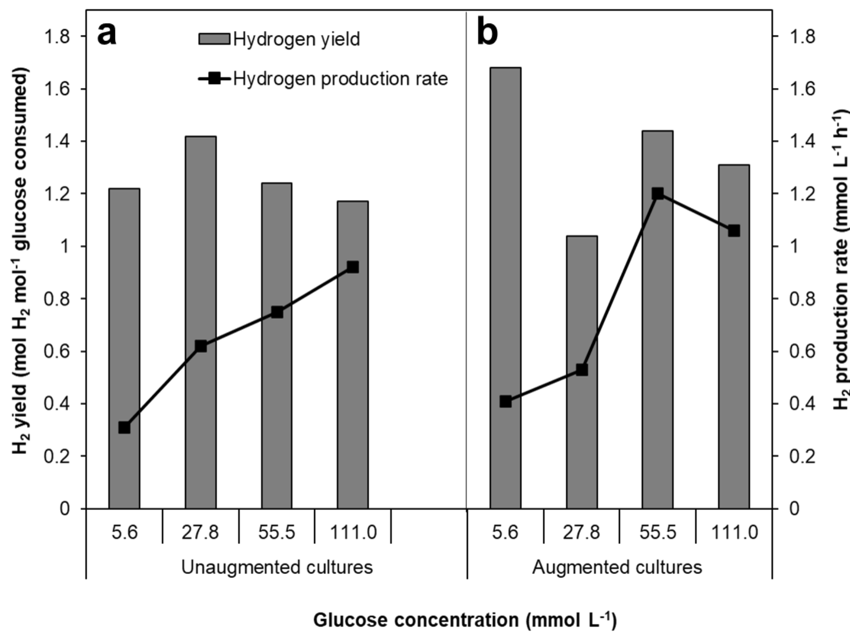
169 Primers 515_532U and 909_928U [39] including their respective linkers were used to
170 amplify the V4_V5 region of the 16S rRNA gene. The resulting products were purified
171 and loaded onto Illumina MiSeq cartridge for sequencing. Sequencing and library
172 preparation were performed at the Genotoul Lifescience Network Genome and
173 Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). The sequence
174 analysis was done as described by [40]. Real-time quantitative polymerase chain
175 reaction (qPCR) monitoring of *T. neapolitana* was carried out using *HydA* primers
176 following the method described by [41]. The 16S rRNA sequences used to support the
177 findings of this study have been deposited in the NCBI Sequence Read Archive under
178 project file SUB6057042: MN203737 - MN203763.

179 RESULTS AND DISCUSSION

180 H_2 production rates and yields at increasing glucose concentrations

181 In the unaugmented cultures, the highest H_2 yield was 1.42 mol H_2 mol^{-1} of glucose
182 consumed at an initial concentration of 27.8 mmol L^{-1} of glucose. The H_2 yield dropped
183 to 1.17 mol H_2 mol^{-1} of glucose consumed at 111 mmol L^{-1} of feed glucose
184 concentration (Figure 2a). The H_2 yield obtained in the augmented cultures was higher
185 than that obtained in the unaugmented cultures. Nonetheless, similar to the
186 unaugmented cultures, the H_2 yield decreased by increasing the substrate
187 concentration. With bioaugmentation, H_2 yield increased by 37, 16 and 12% at 5.6,
188 55.5 and 111 mmol L^{-1} of feed glucose, respectively, compared to the unaugmented
189 cultures. The highest H_2 yield (1.68 mol H_2 per mol of consumed glucose) was
190 obtained at the feed glucose concentration of 5.6 mmol L^{-1}. Qiu et al. [42] studied the
191 effect of xylose concentrations (ranging from 16.7 to 100.0 mmol L^{-1}) on dark
192 fermentative H_2 production by an extreme thermophilic culture, and reported that the
193 fermentation reached the highest H_2 yield of 1.29 mol H_2 mol^{-1} xylose consumed at

194 initial pH 7.0 and 50.0 mmol L^{-1} of feed xylose. However, based on other literature
195 reports, it seems that the optimal initial substrate concentration depends on the
196 inoculum, substrate type, reactor configuration, temperature and pH range [43,44].
197 In this study, the H_2 production rate increased with increased feed glucose
198 concentration and reached the highest value of 0.92 mmol-$L^{-1}h^{-1}$ at 111 mmol L^{-1} of
199 feed glucose in the unaugmented culture. In the augmented culture, the H_2 production
200 rate increased from 0.41 mmol L^{-1} h^{-1} at feed glucose concentration of 5.6 mmol L^{-1} up
201 to 1.44 mmol L^{-1} h^{-1} at 55.5 mmol L^{-1} and then decreased to 1.13 mmol L^{-1} h^{-1} at 111
202 mmol L^{-1} of feed glucose (Figure 2b). Higher H_2 production rates than observed in this
203 study have been observed with mixed cultures under different operating conditions
204 [45,46]. The obtained H_2 production rate and yield was generally higher in the culture
205 augmented with *T. neapolitana* than in the unaugmented culture at the various glucose
206 concentrations studied. This indicates that *T. neapolitana* was able to survive
207 alongside the native microbial communities.
208
209
210



211

212 Figure 2. H_2 yield and production rate obtained with the unaugmented (a) and
213 augmented (b) cultures at different initial glucose concentrations.

214

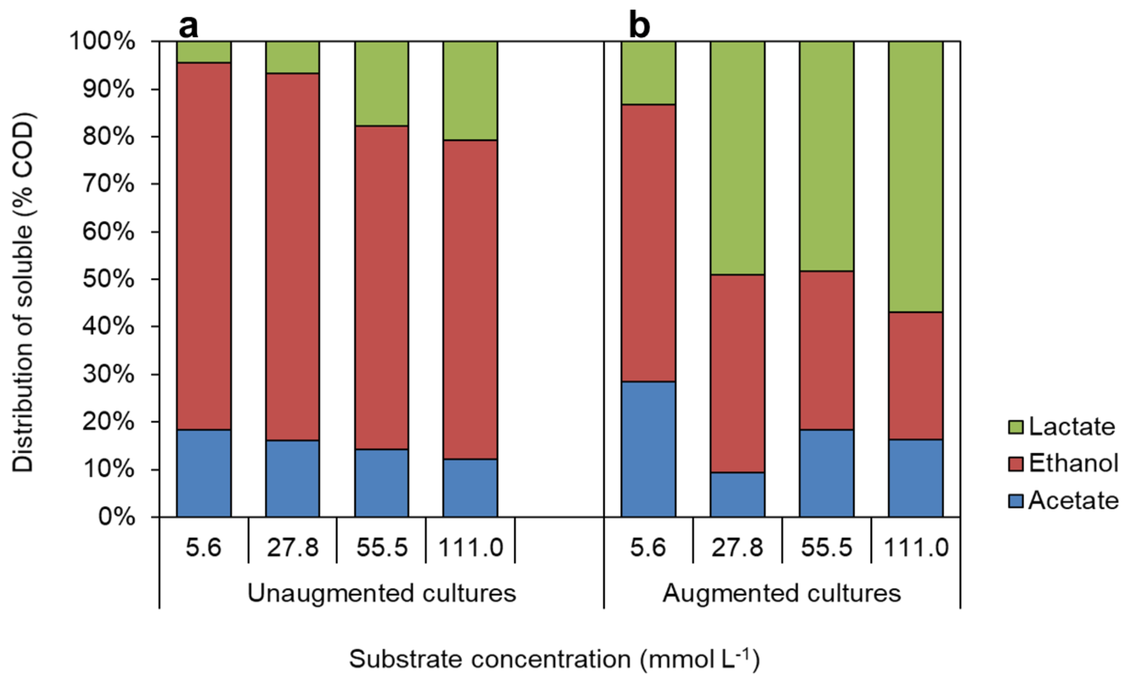
215 **Effect of glucose concentration on the composition of soluble metabolites**

216 The main soluble microbial products associated with glucose degradation were
217 ethanol, acetate and lactate (Figure 3). In the unaugmented cultures, ethanol was the
218 main soluble metabolite produced and corresponded to 67–77% of the total soluble
219 metabolites produced as COD equivalents. The share of acetate decreased with
220 increasing glucose concentration (from 18 to 12%), while that of lactate increased from
221 4.5 to 18% (Figure 3a). In the augmented cultures, the share of ethanol decreased
222 with increasing glucose concentration (from 58% at 5.6 mmol L^{-1} glucose to 27% at
223 111 mmol L^{-1} glucose). Thus, the share of ethanol was lower in the augmented culture
224 compared to the unaugmented culture at all studied glucose concentrations. The
225 percentage of acetate decreased in the augmented culture from 29% at 5.6 mmol L^{-1}
226 of feed glucose to 16% at 111 mmol L^{-1} of feed glucose, while the share of lactate

227 significantly increased from 13% at initial concentration of 5.6 mmol L^{-1} glucose to 57%

228 at 111 mmol L^{-1} of feed glucose (Figure 3b).

229



230

231 Figure 3. The distribution of soluble metabolites as chemical oxygen demand (COD)

232 equivalents at the endpoint of fermentation at the different initial glucose

233 concentrations with the unaugmented (a) and augmented (b) cultures.

234

235 In many previous studies, dark fermentation of glucose has resulted in the

236 production of mainly butyrate and acetate as soluble metabolites under mesophilic,

237 thermophilic and hyperthermophilic conditions [47–51]. However, it seems that

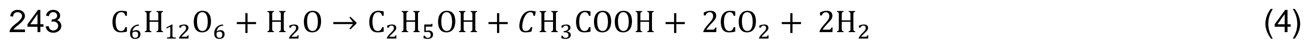
238 ethanol-based fermentation was the major pathway leading to H_2 production in this

239 study due to the high ethanol yields obtained especially with the unaugmented

240 cultures. The ethanol-type fermentation (Equation 4) has a theoretical maximum of 2

241 mol of H_2 per mol of glucose and has been reported to occur under mesophilic

242 conditions [52,53] but not for mixed cultures at temperatures as high as 70 °C.

243 

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2 \quad (4)$$

244 Previous studies have reported yields of 1.8 mol ethanol mol^{-1} glucose with pure
245 culture of *T. ethanolicus* [54] and 1.5 mol ethanol mol^{-1} glucose from *T. hydrosulfuricus*
246 [55] at 72 and 69 °C respectively. The highest ethanol yield obtained with the
247 unaugmented culture in this study was 1.4 mol H_2 mol^{-1} of glucose. Meanwhile, the
248 highest ethanol yield in the augmented culture was 1.2 mol ethanol mol^{-1} of glucose
249 and was obtained at initial glucose concentration of 27.8 mmol L^{-1}.

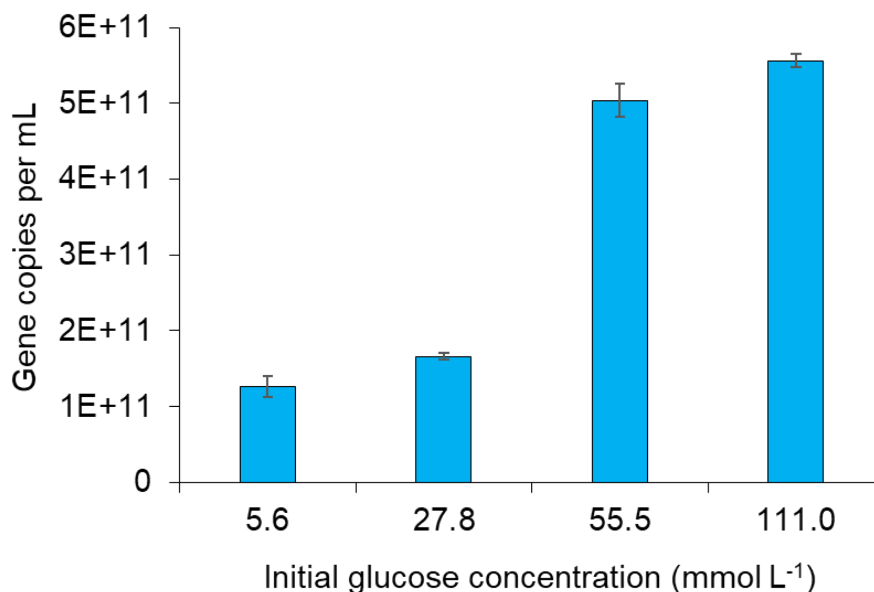
250 In addition to the increased H_2 yield obtained by bioaugmentation, a shift in the
251 metabolic networks was observed with the *T. neapolitana*-augmented culture when
252 compared to the unaugmented culture. Indeed, the *T. neapolitana*-augmented culture
253 produced a lower share of ethanol and a higher share of acetate and lactate to the
254 fermentation broth (Figure 3). Thus, the bioaugmentation with *T. neapolitana* directed
255 the metabolic pathway towards acetate and lactate production. Previous reports on
256 pure cultures of *T. neapolitana* have shown that acetate, lactate and alanine are the
257 major soluble metabolites produced by *T. neapolitana* [41,56,57]. The direction of the
258 metabolic pathway towards acetate production allows producing more H_2 and seemed
259 to be the case with the bioaugmented culture in this study. Nonetheless, as *T.*
260 *neapolitana* is also capable of producing high concentrations of lactate at increased
261 substrate concentrations [17], the increase in the share of lactate observed in the
262 augmented culture was at least partly attributed to presence of *T. neapolitana*. Lactate
263 as an electron sink takes a large amount of reducing power away from H_2 production
264 thereby reducing the H_2 yield [58,59].

265

266 **Quantification of *Thermotoga neapolitana* within the mixed microbial**
267 **communities**

268 The qPCR method applied in this study to quantify and confirm the presence of *T.*
269 *neapolitana* in the bioaugmented mixed cultures has previously been successfully
270 used in quantitation of *T. neapolitana* from pure and mixed cultures [41]. The
271 quantitative analysis of *T. neapolitana hydA* gene from the bioaugmented cultures
272 showed an increase of the *hydA* gene copies per mL of culture as the initial glucose
273 concentration was increased (Figure 4). Thus, the qPCR results indicated that after
274 bioaugmentation, *T. neapolitana* became an active member of the microbial
275 consortium and likely responsible for the shift in the soluble metabolites and
276 enhancement of H_2 production compared to the unaugmented culture. The qPCR
277 carried out on the unaugmented culture confirmed that *T. neapolitana* was not present
278 in the culture.

279



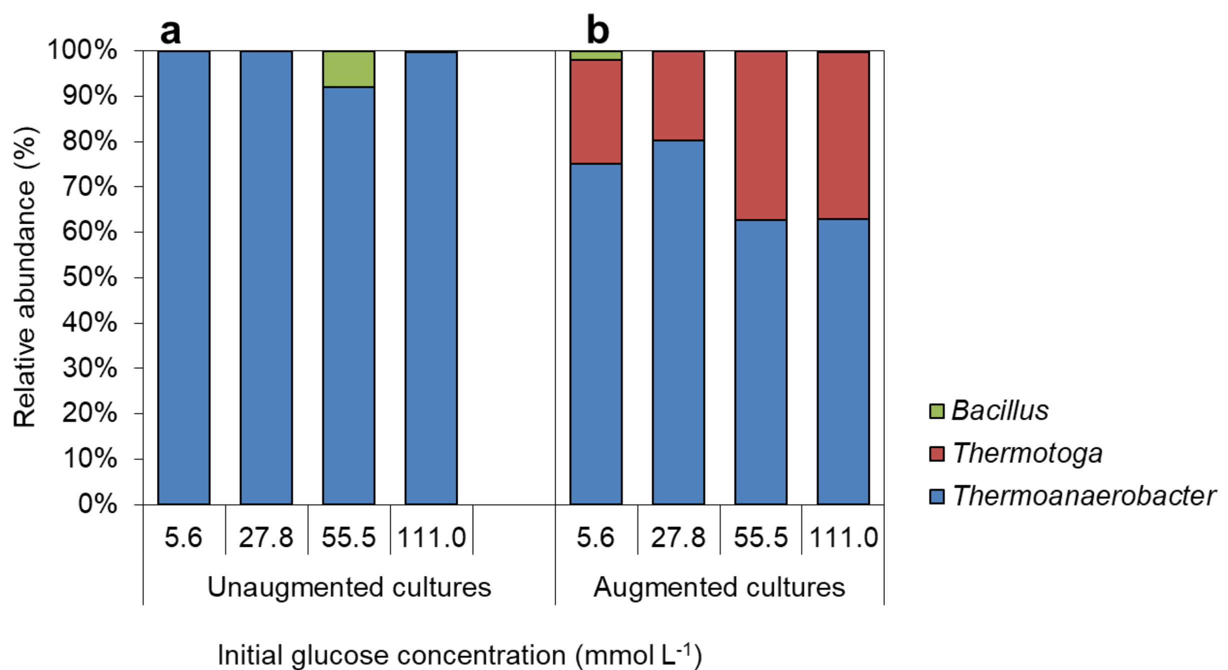
280

281 Figure 4. Real-time qPCR monitoring of hydA gene copy numbers of *T. neapolitana* in
282 the augmented culture at different initial glucose concentrations.

283

284 **Microbial community profiles at different glucose concentrations**

285 The MiSeq data obtained from the cultures assessed in this study covered over 33000
286 effective sequences with the lowest number of sequences being 24967. The number
287 of operational taxonomic units (OTUs) was relatively low, indicating that the microbial
288 communities in both the unaugmented and the augmented culture were rather simple
289 because high temperature environments are extremely selective [60]. The
290 unaugmented culture was dominated by *Thermoanaerobacter* spp. at all initial glucose
291 concentrations. The share of *Thermoanaerobacter* in the microbial community was
292 99.9% at all other glucose concentrations than 55.5 mol L^{-1}, when *Bacillus* was
293 detected at an abundance of 8%.



294

295 Figure 5. Microbial community composition and relative abundance of genera
296 identified at different feed glucose concentrations in the unaugmented (a) and
297 augmented (b) culture.

298

299 At the lowest initial glucose concentration of 5.6 mmol L^{-1}, the augmented cultures
300 had 75, 23 and 2% abundance of *Thermoanaerobacter, Thermotoga* and *Bacillus*

301 spp., respectively. However, at the higher initial glucose concentrations, *Bacillus* spp.
302 were not detected anymore from the microbial community and the shares of
303 *Thermoanaerobacter* and *Thermotoga* spp. were 62-80% and 20-37%, respectively
304 (Figure 5). The abundance of *Thermotoga* in the community was higher at the two
305 highest initial glucose concentrations, which is accordance with the qPCR results
306 (Figure 4).

307 The bioaugmentation of a *Thermoanaerobacter*-dominated mixed culture with *T.*
308 *neapolitana* improved both the H_2 production yield and rate. *Thermoanaerobacter*
309 species are well known thermophilic bacteria capable of producing H_2, ethanol and
310 acetate [61–63]. Thus, their presence explains also the high ethanol production
311 observed. Bacteria within this genus have also been reported to use the Embden–
312 Meyerhof–Parnas pathway for sugar degradation and produce ethanol, acetate and
313 lactate as major volatile end products [64], which is in accordance with the metabolite
314 profiles observed in this study. In the bioaugmented culture, the presence of *T.*
315 *neapolitana* resulted in lower ethanol production, while the shares of acetate and
316 lactate increased compared to the unugmented cultures. Thus, the differences
317 observed in the abundance of different soluble metabolites in the unaugmented and
318 augmented cultures can be explained with the observed differences in the microbial
319 community composition.

320 The pre-adaptation as a strategy to make *T. neapolitana* a stable member of the
321 native microbial community was successful based on the molecular monitoring
322 methods used this study, as both the *T. neapolitana* hydA gene copy numbers and
323 relative abundance of *Thermotoga* were shown to increase towards the end of the
324 study. The pre-adaptation of a bacteria to a mixed culture prior to its application to a
325 large scale process could thus be beneficial for enhancing microbial activity levels,

326 treating complex waste materials and driving the metabolic pathway towards the
327 desired products. Bioaugmentation also has the potential to improve the microbial
328 community structure and enhance resistance and resilience in case of unforeseen
329 disturbances [65]. However, pre-adaptation may not be feasible in the case of sudden
330 transient disturbances due to the fact that it is time consuming.

331 Based on the results obtained from chemical analysis and molecular data, it is
332 evident that *T. neapolitana* contributed to the H_2 production in the mixed culture.
333 Reports from this and previous studies have shown that *T. neapolitana* is able to
334 produce H_2, CO_2, acetate and lactate from mono and polysaccharides as the major
335 products of metabolism. However, its primary role in nature is to reduce sulfur to
336 hydrogen sulfide through the oxidation of organic molecules [66]. Nonetheless, no
337 extensive research exists on the interactions of this organism with other organisms up
338 to now. Except for the switch in the metabolic pathways and an enhanced H_2
339 production, it is not known what kind of interactions occurred between *T. neapolitana*
340 and the native microbial community. It would be useful to further investigate the
341 characteristics of *T. neapolitana* in the augmented culture at a functional level by
342 studying the protein expression to identify the mechanisms responsible for its
343 adaptation and survival within the native microbial community [67–69], as this could
344 enable a further process optimization.

345 CONCLUSIONS

346 The bioaugmentation of a *Thermoanaerobacter*-dominated mixed culture with
347 *Thermotoga neapolitana* improved both the H_2 production yield and rate. Thus, the
348 results of this study indicate that the addition of a single strain with required
349 characteristics can be enough for improving the performance of a biological process.
350 The H_2 production rate of the augmented cultures increased when the initial glucose

351 concentration was increased from 5.6 to 55.5 mmol L^{-1}, while the highest H_2 production
352 yield, 1.68 mol H_2 per mol of consumed glucose, was obtained at the lowest initial
353 glucose concentration of 5.6 mmol L^{-1}. The pre-adaptation of *T. neapolitana* to the
354 mixed culture during three successive batch incubations prior to the reactor
355 experiments was demonstrated to be a successful strategy to ensure that *T.*
356 *neapolitana* was able to co-exist within the mixed microbial consortium. However,
357 further experiments utilizing continuously-fed bioreactor systems are recommended to
358 evaluate the long-term effects of the selected bioaugmentation strategy.

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