Impacts of temperature fluctuations on biohydrogen production and resilience of thermophilic microbial communities

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HIGHLIGHTS

- Transient temperature fluctuation affects H_2 production and microbial stability.
- H_2 production at 35 and 45 °C decreased significantly but rapidly recovered at 55 °C
- H_2 production decreased at 65 °C and stopped completely at 75 °C shift.
- Maximum H_2 yield was not recovered after upward temperature shift especially at 75 °C.
- The major microbial communities were *Clostridium spp*. and *Thermoanaerobacterium spp*.

ABSTRACT

Anaerobic microflora enriched for dark fermentative hydrogen (H_2) production from mixture of glucose and xylose was used in batch cultivations to determine the effects of sudden temperature fluctuations on H_2 yield and microbial community composition. Batch cultures initially cultivated at 55°C (control) were subjected to downward (from 55°C to 35°C or 45°C) or upward (from 55°C to 65°C or 75°C) temperature shifts for 48 hours after which, each culture was transferred to fresh medium and cultivated again at

28 55°C for two consecutive batch cycles. The average H_2 yield obtained during the first
29 cultivation at 55°C was 0.22 ± 0.02 L H_2 g^{-1} $COD_{consumed}$. During the temperature shifts,
30 the obtained H_2 yields were 0.19, 0.17 and 0.2 L g^{-1} $COD_{consumed}$ at 35°C, 45°C and
31 65°C, respectively, while no metabolic activity was observed at 75°C. The sugars were
32 completely utilized during the 48 h temperature shift to 35°C and 45°C but not at 65°C.
33 At the end of the second cycle after the different temperature shifts, the H_2 yield
34 obtained was 96.5, 91.6, 79.9 and 54.1% (second cycle after temperature shift to 35°C,
35 45°C, 65°C and 75°C respectively) when compared to the average H_2 yield produced in
36 the control at 55°C. Characterization of the microbial communities present in the control
37 culture at 55°C showed the predominance of *Thermoanaerobacteriales*, *Clostridiales*
38 and *Bacilliales*. The microbial community composition differed based on the fluctuating
39 temperature with *Thermoanaerobacteriales* being most dominant during upward
40 temperature fluctuation and *Clostridiales* being the most dominant during downward
41 temperatures.

42 **KEYWORDS:** Dark fermentation, Temperature fluctuation, H_2 production, Resilience,
43 Recovery.

44

45 1. INTRODUCTION

46 The increasing global demand for energy and fuels, and the environmental
47 hazards that fossil fuels contribute, strongly demand for alternative energy resources.
48 H_2 has been considered as a possible sustainable alternative (Bartels et al., 2010;
49 Hosseini and Wahid, 2016; Johnston et al., 2005). Although H_2 is very abundant
50 element on Earth, it does not typically exist as H_2 in nature. H_2 is usually found

51 combined with other elements, whilst it can be produced locally from numerous sources.

52 H_2 is produced industrially via electrolysis, coal gasification, and fossil fuel reforming

53 (Dincer, 2012; Holladay et al., 2009). Known BHP methods include direct and indirect

54 biophotolysis by green algae and cyanobacteria (Yu and Takahashi, 2007),

55 photofermentation by phototrophic bacteria (Adessi et al., 2017) and dark fermentation

56 by fermentative bacteria (Chong et al., 2009; Hu et al., 2013). Biological H_2 production

57 (BHP) via dark and photofermentation have drawn increasing interest because of the

58 ability to generate H_2 from various organic resources, such as industrial waste streams

59 and lignocellulosic materials (Akutsu et al., 2009; Li and Fang, 2007; Van Ginkel et al.,

60 2005; Venkata Mohan et al., 2007; Yu et al., 2002; Zhang et al., 2003). Photosythetic H_2

61 production on the other hand is of interest because it needs only light and water and

62 does not produce any CO_2 (Ghirardi, 2006; Melis, 2007).

63 H_2 production by dark fermentation is advantageous over the other BHP

64 processes because of higher H_2 production rates and the possibility to use a wide

65 variety of organic materials as substrates (Lo et al., 2008; Marone et al., 2014). Dark

66 fermentation can be carried out under different temperatures, mesophilic (35-40 ºC),

67 thermophilic (52-60 ºC) and extremely thermophilic (>65 ºC) conditions by different

68 groups of fermentative bacteria (Abreu et al., 2012; Fan et al., 2004; Kongjan et al.,

69 2009; O-Thong et al., 2011; Verhaart et al., 2010). Given the faster rates and higher

70 yields of H_2 , the use of thermophilic dark fermentation is often preferred over

71 mesophilic processes (Pawar and van Niel, 2013). However, thermophilic operations

72 can require higher energy input for heating and are prone to inhibition (Angelidaki and

73 Ahring, 1994) and sudden environmental changes (Biey et al., 2003) which can

74 eventually result in reduced process stability or productivity (Pawar and van Niel, 2013;
75 Wu et al., 2006).

76 Studies on anaerobic biological processes have shown that even small changes
77 in the operating temperature can cause significant changes in microbial community
78 composition and H_2 yields (Dessì et al., 2018b, 2018a; Qiu et al., 2017b). Temporal
79 temperature fluctuations can lead to changes in the enzymatic activities, growth rates
80 and/or loss of microbial diversity, which directly affect the H_2 production (Gadow et al.,
81 2013b). Temperature is therefore a key parameter to be controlled in dark fermentative
82 processes. Previous studies investigating the effects of temperature on fermentative
83 hydrogen production have focused on comparing batch and reactor performances at
84 different fixed operating temperatures (Lee et al., 2006; Dessì et al., 2018; Zhang and
85 Shen, 2006). Several studies focusing on anaerobic digestion have been carried out to
86 establish the relationship between sudden temperature fluctuations and biogas
87 production (Chae et al., 2008; Gao et al., 2012; Kundu et al., 2014). However, only one
88 study has been conducted to evaluate the influence transient of downward temperature
89 fluctuations on the stability of hydrogen production (Gadow et al., 2013).

90 The present study aims to evaluate the effects of sudden downward and upward
91 temperature fluctuations during thermophilic dark fermentative H_2 production. Chemical
92 and molecular methods were used to monitor and compare the metabolic responses to
93 transient temperature conditions (two different upward and two different downward
94 temperature fluctuations), the impact on the resilience of the microbial community profile
95 and evaluate the potential drawbacks thereof.

96 ## 2. MATERIALS AND METHODS

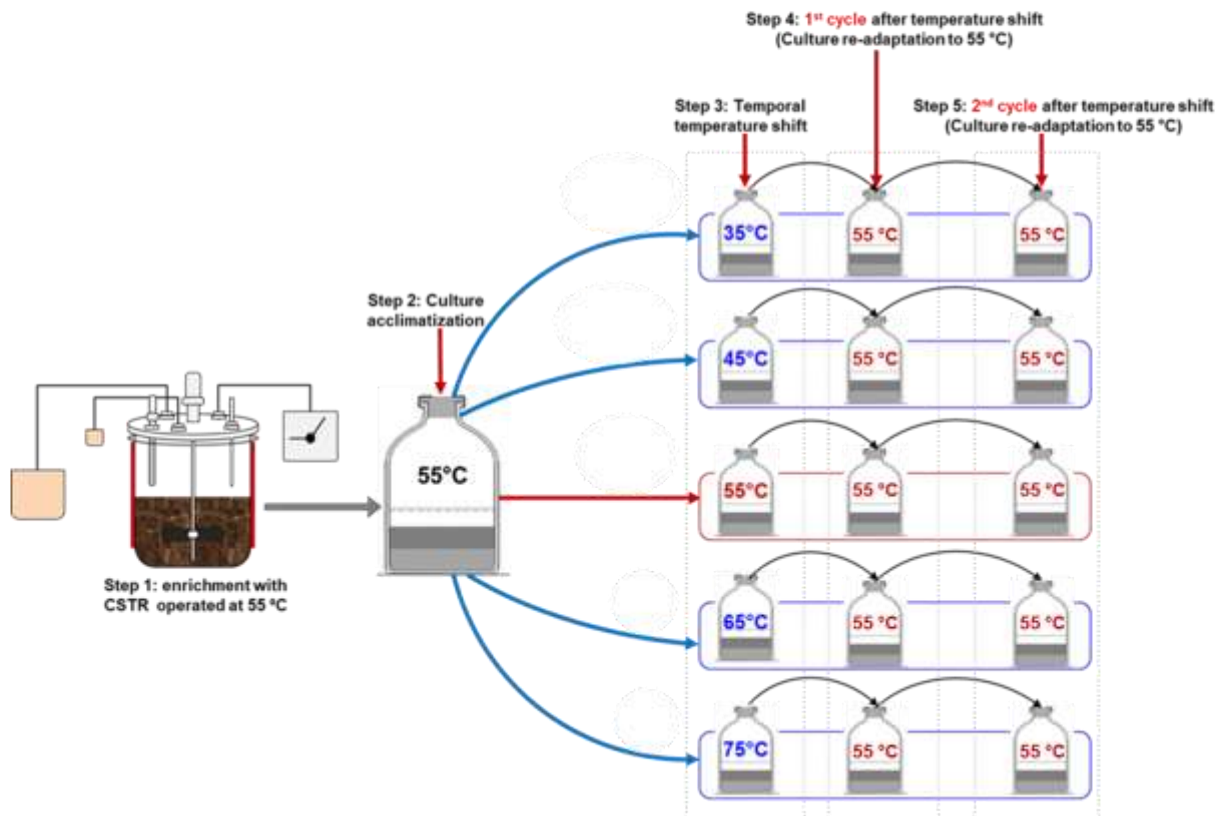
97 ### 2.1. Enrichment culture: medium composition and inoculum source

98 The enrichment of H_2 producing microbial community for this study was carried
99 out in a continuous stirred tank reactor (CSTR) after which the enriched culture was
100 used in batch bottle experiments to study the effects of sudden temperature fluctuations
101 on H2 production and microbial community composition. An anaerobically digested
102 sludge was used as inoculum for the CSTR after heat shock pretreatment at 90°C for 20
103 min. Two hundred milliliters of the pretreated inoculum (10% v/v, final concentration, 40
104 mg L^{-1} of volatile solids) was inoculated to 1800 mL of the following culture medium
105 (mg/L): K_2HPO_4, 500; NH_4Cl, 100; $MgCl_2 \cdot 6H_2O$, 120; $H_8FeN_2O_8S_2 \cdot 6H_2O$, 55.3; $ZnCl_2$,
106 1.0; $MnCl_2 \cdot 4H_2O$, 2.0; $CuSO_4$, 000.4; $(NH_4)_6Mo_7O_{24}$, 1.2; C_OSO_4, 1.3; H_3BO_3, 0.1; $NiCl_2$
107 $\cdot$ $6H_2O$, 0.1; Na_2O_3Se, 0.01; $CaCl_2 \cdot 2H_2O$, 80; yeast extract, 500 and 0.055 mL HCl
108 (37%). The cultures were fed with glucose and xylose mixture (total sugar
109 concentration of 2000 mg L^{-1}) in the ratio of 2:3 (800 mg L^{-1} glucose and 1200 mg L^{-1}
110 xylose). The total working volume of the CSTR was two liters. The reactor was flushed
111 with nitrogen for 5 min and then operated in continuous mode at hydraulic retention time
112 of 6 h and at 55 °C for 21 days. The pH was maintained at 6.5.

113 ### 2.2. H_2 production batch experiments

114 Prior to exposing the cultures to temporal temperature fluctuations, the H_2-producing
115 enriched culture (step 1 in Figure 1) was acclimatized to batch growth conditions at
116 55°C (step 2 in Figure 1). The acclimatized culture was then divided into ten anaerobic
117 cultivation bottles containing fresh medium (same medium as in the CSTR) and
118 subjected to a one-time temperature shock. This was done by placing duplicate bottes
119 to 35 °C and 45°C (downward temperature shocks of 10 and 20 °C), to 55 °C (control)

120 as well as to 65 and 75 °C (upward temperature shocks) and incubated for 48 h (Figure
121 1, step 3). At the end of the 48 h incubation period, the cultures were centrifuged for 5
122 minutes, transferred to fresh medium and incubated at the original temperature of 55 °C
123 for 48 h (step 4 in Figure 1). This step was repeated one more time (step 5 in Figure 1).



124

125 Figure 1. Experimental setup to study the effects of different temperature fluctuations
126 during dark fermentation. First, H_2 producers were enriched in a continuous stirred tank
127 reactor (CSTR) at 55 °C for 21 days (step 1). This was followed by the acclimatization of
128 the enriched mixed culture to batch conditions (step 2) and then, specific temperature
129 shock described as the downward temperature shock (35 °C or 45 °C) and upward
130 temperature shock (65 °C or 75 °C) were imposed (step 3). Cultures incubated at 55 °C
131 (C) were used as control. After the temperature shocks, the H_2 production was followed

132 for two more batch cycles at 55 °C (step 4 and step 5) to delineate how the culture can
133 recover from the different temperature fluctuations.

134 **2.3. Analysis of H_2 production**

135 Gas production was monitored through the increase in gas pressure, which was
136 periodically measured with a digital manometer while the gas composition was analyzed
137 using a gas chromatograph (Clarus 580, Perkin Elmer) with a thermal conductivity
138 detector. The columns used were a RtQbond column for H_2, O_2, N_2 and CH_4
139 quantification and a RtMolsieve column for CO_2 quantification. The carrier gas was
140 argon under a pressure of 3.5 bars. A gas tight Hamilton syringe was used for gas
141 sampling. The biogas volume and composition measurement was carried out at the
142 respective incubation temperatures mentioned in section 2.2 by continuously keeping
143 the culture bottles in water baths.

144 **2.4. Analysis of the liquid metabolites**

145 Culture suspension samples were collected before and after each experimental
146 step for chemical analysis of the metabolic products. The samples were centrifuged at
147 13000 rpm for 15 min and the supernatant was filtered with 0.2 µm filter before the
148 analyses. Glucose, xylose, organic acids and alcohol concentrations were measured by
149 high performance liquid chromatography (HPLC) using a refractive index detector
150 (Waters R410) as described previously by (Monlau et al., 2013).

151 **2.5. Microbial community analysis**

152 Genomic DNA was extracted using the PowerSoil™ DNA Isolation Sample Kit
153 (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's
154 instructions. The following primers; 515_532U and 909_928U (Wang and Qian, 2009)

155 including their respective linkers, were used to amplify the V4_V5 region of the 16S
156 rRNA gene over 30 amplification cycles at an annealing temperature of 65 °C. The
157 resulting products were purified and loaded onto the Illumina MiSeq cartridge prior to
158 sequencing. Sequencing and library preparation were performed at the Genotoul
159 Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France
160 (get.genotoul.fr). The sequences analysis was done as described by Venkiteshwaran et
161 al. (2016).

162 **2.6. Calculations**

163 The total volume of produced H_2 was calculated using Equation 1 (Logan et al.,
164 2002):

165 $$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (1)$$

166 where $V_{H,i}$ is the cumulative H_2 gas produced at the current time interval, $V_{H,i-1}$ is the
167 cumulative H_2 gas produced at previous time interval. $V_{G,i}$ and $V_{G,i-1}$ are the total gas
168 volume at current and previous time interval respectively. $C_{H,i}$ is the H_2 gas fraction in
169 the headspace at the current time interval, $C_{H,i-1}$ is the H_2 gas fraction in the headspace
170 at previous time interval and V_H is the total headspace volume in the culture bottle.
171 H_2 yield was calculated by dividing the total volume of H_2 (mL) by the amount of
172 substrate consumed (g-COD). The theoretical H_2 produced was calculated based on the
173 equation 2 (Luo et al., 2010) in order to determine the ratio of experimental to
174 theoretical H_2 yield (Akinbomi and Taherzadeh, 2015) as shown in equation 3.
175 Additionally, the maximum H_2 yield recovered was calculated for cultures during
176 temperature fluctuation and after the fluctuation period (equation 4).

177

178 $$\text{Theoretical H}_2\text{ produced} = 2 \times \sum(\text{acetate yield} + \text{butyrate yield}) \quad (2)$$

179 $$\text{Relative Yield} = \frac{\text{Experimental yield}}{\text{Theoretical yield}} \times 100 \quad (3)$$

180 $$\text{Maximum } H_2 \text{ recovered } (\%) = \frac{H_2 \ \textit{yield obtained during/after temperature shift}}{\text{average } H_2 \text{ yield obtained from the control}} \times 100$$

181 (4)

182 Total COD of soluble compounds was calculated based on the sum of acids, ethanol
183 and residual sugars by using the following conversion factors: 1 mM glucose = 192 mg
184 COD L^{-1}, 1 mM xylose = 160 mg COD L^{-1}, 1 mM acetate = 64 mg COD L^{-1}, 1 mM
185 propionate = 112 mg COD L^{-1}, 1 mM lactate = 96 mg COD L^{-1}, 1 mM butyrate = 160 mg
186 COD L^{-1} and 1 mM ethanol = 96 mg COD L^{-1}. COD mass balance of thermophilic dark
187 fermentation was carried out to in order to estimate the metabolic end-products that
188 converted to ethanol, volatile fatty acids (VFAs) and H_2. The COD mass balance was
189 calculated from the composition of the endpoint cultivation in the gas and soluble
190 components using the COD coefficient of each product according to (Gonzales and
191 Kim, 2017; Sivagurunathan and Lin, 2016).

192

3. RESULTS AND DISCUSSION

193
194

3.1. Thermophilic H_2 production at constant temperature in CSTR and first batch test (55°C)

195 Methane was not detected in any of the incubations performed in this study,
196 which indicates that the initial heat-shock pretreatment was sufficient to totally suppress
197 the activity of methanogens (Cai et al., 2004; Venkata Mohan et al., 2008). The
198 maximum H_2 yield obtained during the enrichment in the CSTR was 0.2 ± 0.001 L H_2 g
199 $COD^{-1}_{consumed}$. The H_2 yield obtained in the batch cultivation at 55°C (control) was 0.23 ±
200 0.01, 0.22 ± 0.01 and 0.2 ± 0.02 L H_2 g^{-1} $COD_{consumed}$ (in steps 3, 4 and 5 respectively).

201 The COD mass balance calculated based on the added concentration of substrate and
202 measured metabolic products ranged between 72 and 77%, while the missing COD
203 fraction of the total COD was probably used for growth and biomass production. In a
204 dark fermentation in vivo system, the maximum theoretical H_2 that could be obtained
205 from glucose under standard temperature and pressure is 4 mol H_2 per mol glucose
206 with acetate as the only metabolite (Vardar-Schara et al., 2008). However, during dark
207 fermentation, H_2 is produced along with other metabolites such as alcohols, lactate and
208 propionate, which are involved in the H_2 consuming pathways that leads to low H_2
209 yields. (Buyukkamaci and Filibeli, 2004; Hawkes et al., 2007). Practically, high H_2 yields
210 are linked to acetate and butyrate accumulation. However, the end-products produced
211 by a bacterium or mixed cultures depend on the environmental conditions. Reduced
212 fermentation end-products like ethanol and lactate, contain H_2 that has not been
213 released as gas. In this study, different concentrations of acetate, butyrate, lactate,
214 ethanol and propionate were produced as shown in table 1. During the cultivation in the
215 different steps (3, 4 and 5) at 55 °C, the concentration of ethanol and acetate increased
216 in step 4 compared to steps 3 and 4 (table 1). The increase in these metabolites led to a
217 decrease in butyrate concentration. The butyrate to acetate ratio (B/A, mM:mM) which
218 has been used in previous studies as an indicator of the amount of bioH_2 produced (Lin
219 et al., 2006; Sangyoka et al., 2016) was used to evaluate the efficiency of H_2 production
220 between step 3, 4 and 5. In this present study, the (HBu/HAcration (mM:mM) was 1.2,
221 0.5 and 1.5 (in steps 3, 4 and 5 respectively). The result was in correlation with previous
222 studies which obtained HBu/HAc ratios ranging between 1.5 and 4.0 (Lin et al., 2006;
223 Sangyoka et al., 2016). However, step 4 had a much lower HBu/HAc ratio compared to

224 step 3 and 5, suggesting a metabolic shift or homoacetogenic activity or both. HBu/HAc
225 ratios might not always provide a positive correlation to high H_2 production due to
226 homoacetogenic activity (Guo et al., 2014) which has been reported to influence the
227 concentration of end-point metabolites due to the formation of acetate from H_2 and CO_2.
228 The ratio of experimental to theoretical H_2 yield was 99, 91 and 83% in steps 3, 4 and 5,
229 respectively. These values clearly indicate homoacetogenesis. The effect was more
230 intense in step 5. Combining the results obtained thus far, the data obtained in step 4
231 also highlights the fact that there might have been a slight shift in the metabolic pathway
232 toward acetate production.

233 Table 1: Concentration of accumulated products in the control cultures incubated at 55
234 °C during the incubations steps 3, 4 and 5.

Parameters	Concentration (mM)			Distribution (Percentage COD)		
	Step 3	Step 4	Step 5	Step 3	Step 4	Step 5
Residual sugars	n.d	n.d	n.d	n.d	n.d	n.d
Lactate	n.d	n.d	0.11 ± 0.0	n.d	n.d	0.5 ± 0.2
Acetate	5.01 ± 0.4	7.63 ± 0.0	4.54 ± 0.4	15.3 ± 1.1	22.89 ± 0.1	13.6 ± 1.3
Ethanol	1.22 ± 0.3	3.85 ± 0.1	1.54 ± 0.2	5.5 ± 1.4	17.34 ± 0.3	6.9 ± 0.9
Butyrate	5.96 ± 0.4	4.00 ± 0.1	6.61 ± 0.2	44.7 ± 2.7	30.01 ± 0.4	49.5 ± 1.4
Propionate	0.73 ± 0.0	0.63 ± 0.0	0.73 ± 0.0	3.8 ± 0.1	3.3 ± 0.0	3.3 ± 0.1

235 n.d: Not detected

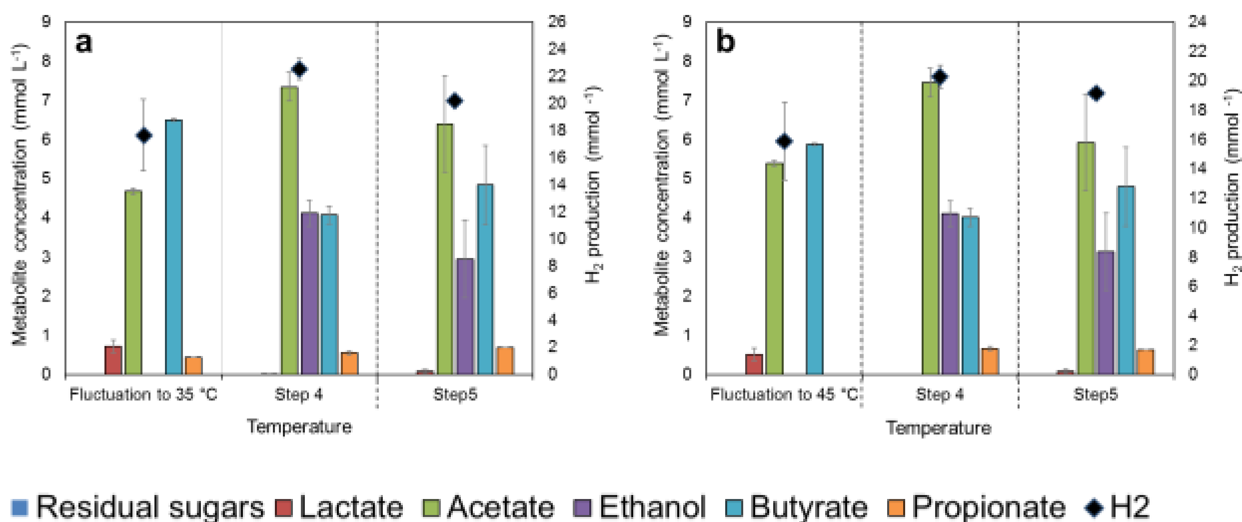
236 **3.2. Sudden transient downward temperature shift and its effects on H_2**
237 **metabolism**

238 During the downward temperature shifts, the H_2 yield slightly decreased
239 compared to the control (55 °C), being 0.19 and 0.17 L g^{-1} $COD_{consumed}$ at 35 and 45 °C,
240 respectively. However, H_2 production recovered rapidly when the cultures were

241 transferred to a fresh medium and incubated at the original temperature of 55 °C. H_2
242 yield of 0.21 and 0.20 L g^{-1} $COD_{consumed}$ (steps 4 and 5, respectively) were obtained for
243 cultures exposed to 35°C shift, and 0.24 and 0.21 L g^{-1} $COD_{consumed}$ (steps 4 and 5,
244 respectively) for cultures exposed to 45°C shift. All the substrates were consumed
245 during and after the temperature shift to 35°C. Meanwhile during the temperature shift
246 to 45°C, 6.4 ± 0.5% of the substrate was not consumed (table 2). The different
247 concentrations of acetate, butyrate, lactate, ethanol and propionate were produced as
248 shown in figure 2. During the downward temperature fluctuation, similar metabolic
249 patterns were observed at 35 and 45 °C shifts except that the concentration lactate and
250 butyrate was higher at the 35 °C than 45 °C shift (table 2). Meanwhile, the concentration
251 of acetate was higher at 45 °C than 35 °C. When both fluctuating temperatures where
252 returned to 55 °C, they both showed similar metabolite distribution patterns with slight
253 variations in their frequencies.

254 The ratio of experimental to theoretical H_2 yield during and after the temperature
255 fluctuation to 35 °C was 79, 99 and 90% in steps 3, 4 and 5 respectively. For cultures
256 exposed to 45 °C, the ratio was 71, 88 and 89% in steps 3, 4 and 5 respectively.
257 Alcohols, lactate and propionate, have been reported to be involved in the H_2
258 consuming pathway which leads to H_2 yields which are significantly lower than the
259 theoretical values (Buyukkamaci and Filibeli, 2004; Hawkes et al., 2007). It is therefore
260 suggested that the decrease in H_2 production during the downward temperature
261 fluctuation was significantly influenced by homoacetogenic acitivity as well as lactate
262 (though the influence of lactate seemed insignificant due to the low concentrations).
263 Meanwhile, the increase in the concentration of ethanol after the temperatures were

264 returned to 55 °C suggested that ethanol production coupled with homoacetogenic
265 activity might have been the reason why 100% of H2 yield obtained in the control was
266 not achieved. The results obtained during and after the downward temperature
267 fluctuations, therefore suggest that homoacetogenic activity occurred during cultivation
268 and was more severe during the temperature fluctuation, leading to a reduction in the
269 H_2 yield.

270 

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272 Figure 2. Metabolites and H_2 produced during the downward temperature fluctuations.
273 H_2 and soluble metabolite production during temperature fluctuation at A) 35 °C (step 3)
274 and B) 45 °C and after returning the cultures back to 55 °C (steps 4 and 5).

275

276 Table 2: COD mass balance obtained from the sum of frequencies of each metabolite
277 and the residual sugars during and after downward temperature fluctuation.

Parameters (% COD)	Fluctuation to 35 °C			Fluctuation to 45 °C		
	Step 3	Step 4	Step 5	Step 3	Step 4	Step 5
Residual sugars	n.d	0.2 ± 0.2	n.d	6.4 ± 0.5	0.3 ± 0.3	n.d
Lactate	3.2 ± 1.7	0.1 ± 0.1	0.4 ± 0.3	2.3 ± 0.7	n.d	0.4 ± 0.2
Acetate	14.0 ± 0.2	22.1 ± 0.0	19.2 ± 2.4	16.1 ± 0.2	22.4 ± 1.1	17.8± 3.7
Ethanol	n.d	18.5 ± 0.4	13.3 ± 4.6	n.d	18.5 ± 1.5	14.1 ± 4.4
Butyrate	48.8 ± 1.0	30.6 ± 0.5	36.3 ± 6.7	44.1 ± 0.3	30.1 ± 1.7	36.0 ± 7.6

Propionate	n.d	2.9 ± 0.0	3.6 ± 0.1	n.d	3.5 ± 0.3	3.3 ± 0.0
Sum	66.0	74.4	72.8	68.9	74.8	71.6

278 n.d: Not detected

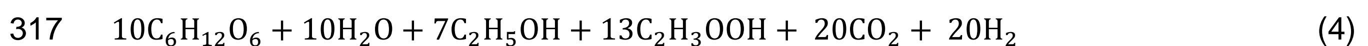
279 **3.3. Sudden transient upward temperature shift and its effects on H_2**
280 **metabolism**

281 During the upward temperature shift at 65 °C, the H_2 yield reached 0.2 ± 0.0 L H_2
282 g^{-1} $COD_{consumed}$ which corresponded to 10.5% decrease when compared to the average
283 H_2 yield in the controls. The subsequent cultivation steps at 55°C after the temperature
284 shift back to 55°C to 12.1% (0.19 ± 0.01 L H_2 g^{-1} $COD_{consumed}$) and 21.3% (0.18 ± 0.03
285 L H_2 g^{-1} $COD_{consumed}$) decrease in H_2 yield in steps 4 and 5 respectively when compared
286 to the average H_2 yield in the control. On the other hand, temperature shift to 75 °C
287 resulted in a complete stop of the dark fermentative microbial activity. Hence, no
288 substrate consumption was observed. H_2 production recovered as soon as the cultures
289 were transferred to a fresh medium and incubated again at 55°C. However, the H_2 yield
290 was only 0.07 ± 0.03 L H_2 g^{-1} $COD_{consumed}$ (67.3% H_2 decrease) and 0.12 ± 0.02 L H_2 g^{-1}
291 $COD_{consumed}$ (44.9% H_2 decrease) in steps 4 and 5, respectively, which is much lower
292 compared to the H_2 yields obtained in the corresponding control cultures incubated at
293 55 °C.

294 During the temporal temperature shift to 65°C, 25.5 ± 2.4% of the substrate was
295 not consumed at the end of the 48 h period (table 3). However, all the substrates added
296 were consumed when the temperature returned to 55°C.

297 There was distinct effect of temperature seen with respect to the different upward
298 temperature fluctuations experienced. Upon the shift to 65°C, acetate and ethanol
299 became the major metabolites with a low amount of butyrate as well as lactate (figure
300 3). This was a huge contrast to the metabolic distribution obtained in the control as

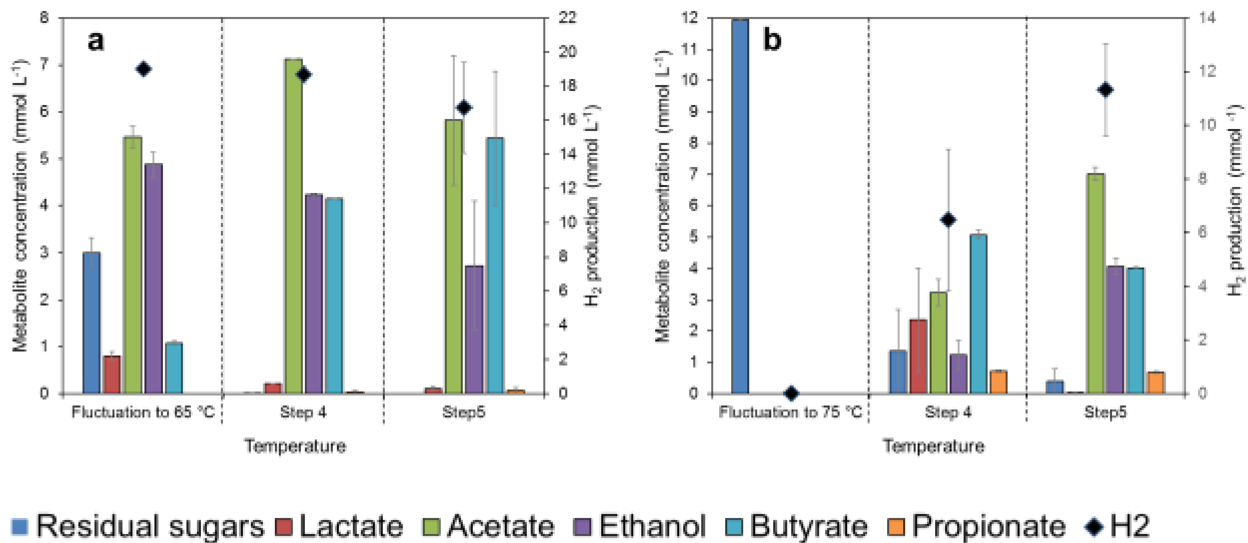
301 ethanol was seen to have the highest percentage of COD compared to the control.
302 When the temperature returned to 55 °C, butyrate and acetate became the major liquid
303 metabolites. The ratio of experimental to theoretical H_2 yield calculated from the sum of
304 acetate and butyrate were 145, 82 and 74% in steps 3, 4 and 5 respectively. The ratio
305 of experimental to theoretical H_2 yield obtained during the temperature fluctuation to
306 65°C was greater >100% which is rather unlikely. Based on the results, it is suggested
307 that the shift in temperature to 65 °C led to a shift, away from the acetate–butyrate
308 pathway. (Qiu et al., 2017b) reported similar findings where butyrate and acetate were
309 the major liquid metabolites at 35–60°C while at 65°C the main by-product was ethanol.
310 The catabolic redox processes of many anaerobic bacteria is branched, leading to
311 variability in ATP as well as the thermodynamic efficiency of ATP synthesis. Fluxes in
312 the different branches are adjusted so that the ATP gain and the thermodynamic
313 efficiency are optimal for the respective growth conditions (Thauer et al., 1977). Thus,
314 glucose fermentation to ethanol, acetate, CO2, and H_2 might have been the outcome of
315 such catabolic redox processes. An example of such regulatory adjustment is
316 *Ruminococcus albus* belonging to the order, *Clostridiales*.

317 

$$10C_6H_{12}O_6 + 10H_2O + 7C_2H_5OH + 13C_2H_3OOH + 20CO_2 + 20H_2 \quad (4)$$

318 When the temperature was returned to 55 °C after the temporal temperature shift to 65
319 °C, the metabolic distribution switched back to acetate butyrate production as seen from
320 the frequency of the metabolic products (table 3). As mentioned earlier, H_2 production
321 came to a complete stop during the temperature shift to 75°C with no substrate
322 consumption. However, only 95% of the total substrate given was recovered of which
323 the existing microbial population for survival might have used 5%. When the

324 temperature returned to 55°C after the high rise in temperature to 75°C, the substrate
325 given was completely depleted at the end of the incubation period for both steps 4 and
326 5. After the fluctuation period at 75 °C, H_2 producing activity commenced with varying
327 frequencies of the metabolites. Ten percent of the residual sugar was observed in step
328 3. Meanwhile the proportions of the metabolites were acetate (9.7 ± 1.3%), ethanol (5.5
329 ± 2.1%), butyrate (38.1 ± 1.0%) and propionate (3.8 ± 0.2%). Except for butyrate,
330 acetate and ethanol were significantly low compared to the control. In step 5, the
331 proportion of butyrate decreased compare to step 4 while acetate and ethanol
332 increased. The ratio of experimental to theoretical H_2 yield calculated from the sum of
333 acetate and butyrate were 40 and 51% in steps 4 and 5 respectively, showing the level
334 of impact of higher fluctuation temperature to 75 °C.



335
336
337 Figure 3. Metabolites and H_2 produced during the downward temperature fluctuations.
338 H_2 and soluble metabolite production during temperature fluctuation at A) 65 °C (step 3)
339 and B) 75 °C and after returning the cultures back to 55 °C (steps 4 and 5).
340

341 Table 3: COD mass balance obtained from the sum of frequencies of each metabolite

342 and the residual sugars during and after upward temperature fluctuation.

Parameters (% COD)	Fluctuation to 65 °C			Fluctuation to 75 °C		
	Step 3	Step 4	Step 5	Step 3	Step 4	Step 5
Residual sugars	22.5 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	95.85 ± 0.2	10.1 ± 10.1	3.0 ± 2.8
Lactate	3.6 ± 0.4	1.0 ± 0.0	0.5 ± 0.3	n.d	10.6 ± 7.5	0.2 ± 0.1
Acetate	16.4 ± 0.7	21.4 ± 0.0	17.5 ± 4.1	n.d	9.7 ± 1.3	21.1 ± 0.6
Ethanol	21.9 ± 1.2	19.1 ± 0.1	12.3 ± 6.2	n.d	5.5 ± 2.1	18.3 ± 1.2
Butyrate	8.0 ± 0.4	31.2 ± 0.1	40.8 ± 10.7	n.d	38.1 ± 1.0	30.2 ± 0.2
Propionate	2.8 ± 0.0	2.9 ± 0.2	3.7 ± 0.4	n.d	3.8 ± 0.2	3.6 ± 0.2
Sum	75.2	75.6	74.8	95.85	77.8	76.4

343 n.d: Not detected

344 **3.4. Microbial community composition during the altered temperature**
345 **conditions**

346 The microbial community in the thermophilic control cultures incubated at 55°C

347 was dominated by three major orders: *Thermoanaerobacterales* (94, 98 and 77% in

348 steps 3, 4 and 5, respectively) > *Clostridiales* (3, 1 and <1%) > *Bacillales* (2, <1 and

349 22%). The sudden increase in *Bacillales* in the fifth step was concomitant with

350 decreased H_2 yield. The dominant member of this order was *Tumebacillus spp.*, which

351 corresponded up to 20% of relative abundance of all microorganisms detected.

352 *Tumebacillus* spp. are gram positive, aerobic, rod shaped, and spore forming bacteria,

353 which are able to degrade carbohydrates and have been detected from anaerobic

354 processes (Gagliano et al., 2015). However, their role in the consortium is not known

355 and it is not certain whether its presence was the reason of the lower yield observed in

356 the step 5, during incubation at 55°C.

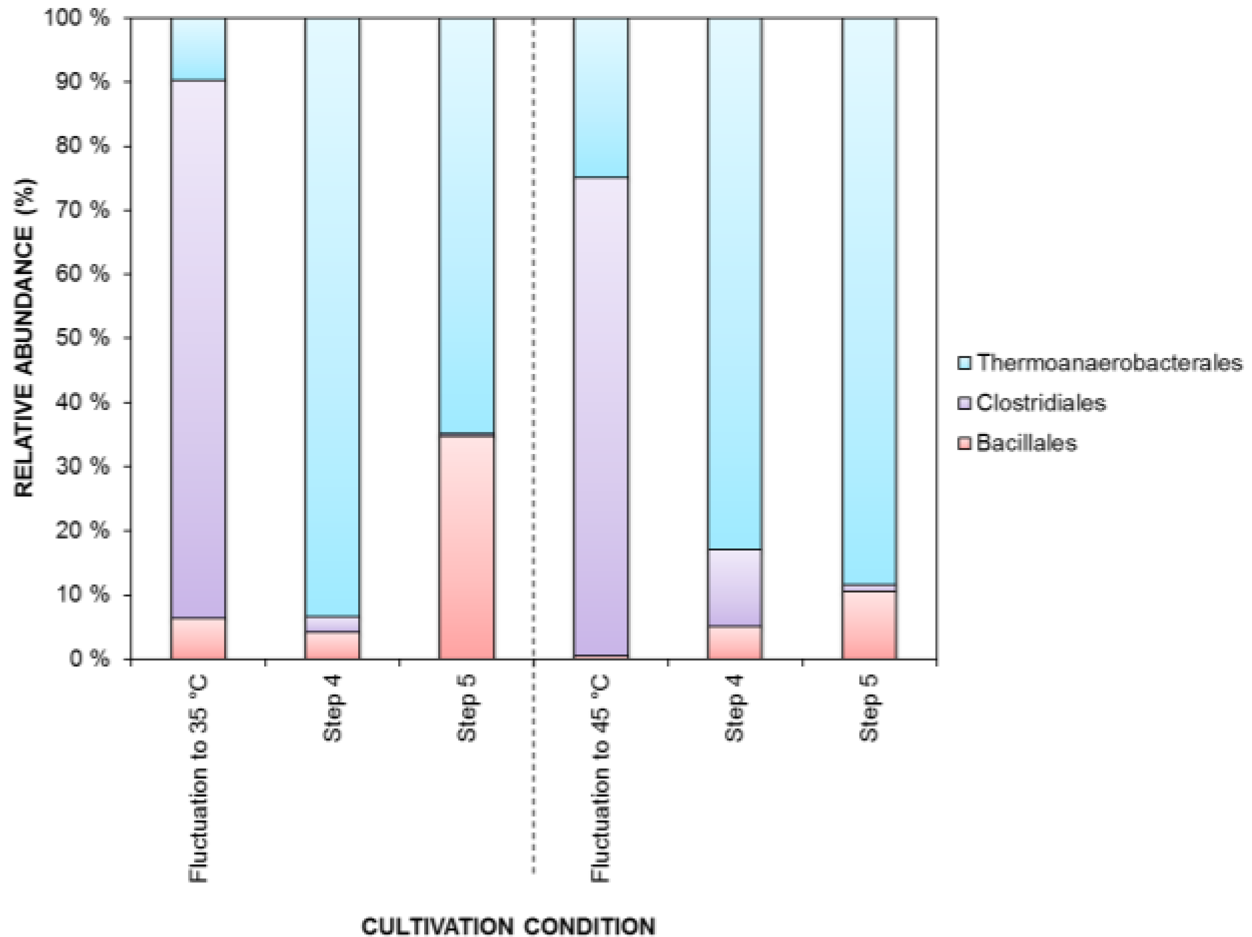
357 **3.4.1. Downward temperature shifts**

358 Decreasing the temperature to 35°C or to 45°C for 48 hours considerably

359 influenced the microbial community composition. *Clostridiales* became the dominant

360 order in the community (84% and 74% at 35 °C and 45 °C, respectively) as seen in
361 Figure 4. During both downward shifts, *Thermoanaerobacteriales* was present at lower
362 abundance (10% and 25% at 35 °C and 45 °C, respectively) compared to the control
363 cultures. *Bacillales* (6 and <1% at 35 °C and 45 °C, respectively) was also present at
364 low relative abundance at this point. The members of the order of Clostridiales identified
365 belonged to Clostridium spp, The increase in temperature back to 55 °C lowered the
366 relative abundance of *Clostridiales,* and *Thermoanaerobacteriales* became again the
367 dominant order in the cultures (Figure 4). The share of other members of the consortium
368 was below 1%, some of which were known homoacetogens. Though in very low
369 abundance, the metabolic capacities of this group of bacteria might have noticeable
370 influence on the dark fermentative metabolism (Rafrafi et al., 2013).

371



372 Figure 4. Microbial community composition as relative abundance of different
373 microbial orders (>1%) during (step 3) and after (steps 4 and 5) the downward
374 temperature shifts.

375 **3.4.2. Upward temperature shifts**

376 In contrast to the downward temperature shifts, temporal upward shifts did not
377 cause a significant alteration in the microbial populations. *Thermoanarobacteriales*
378 remained the dominant order during and after the temperature shifts.
379 *Thermoanaerobacteriales* (97%) and *Clostridiales* (3%) were the main orders during the
380 temperature shift at 65°C. Interestingly, during this step, it was suggested that H_2 was
381 also produced via the acetate–ethanol pathway. Members of the group

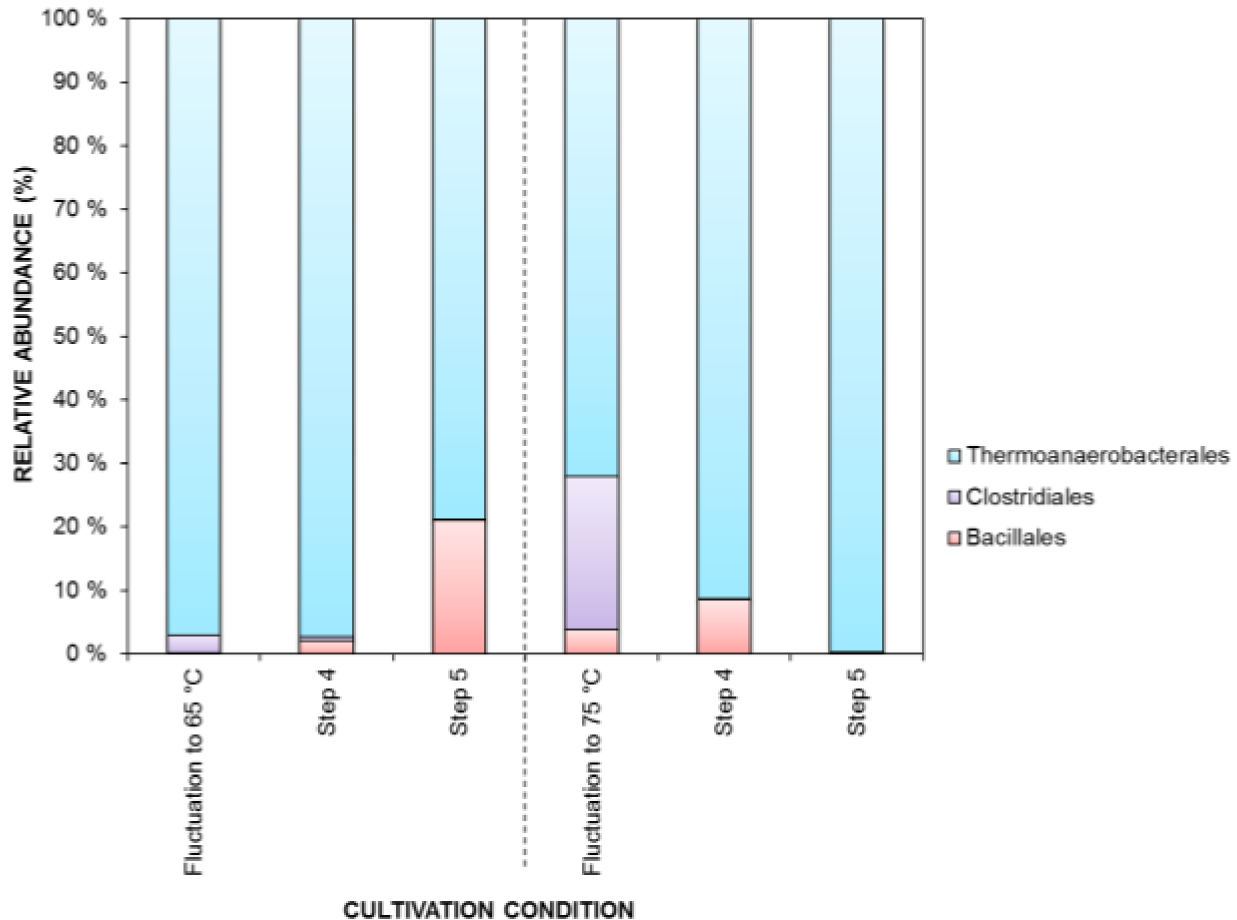
382 *Thermoanaerobacterium*, which were the most dominant group of the order
383 Thermoanaerobacteriales, are known to produce H_2 via the acetate and butyrate
384 pathway. However, the ratio of experimental to theoretical H_2 yield obtained in the
385 previous section, showed that H_2 production could not have been achieved only via the
386 acetate and butyrate pathway. A fraction of the H_2 produced might have come from
387 *Clostridium* via the acetate and ethanol pathway, despite the low abundance. However,
388 the dominant genera are unknown (Ren et al., 2007). Studied by (Rafrafi et al., 2013)
389 have shown that Sub-dominant bacteria can influence the global microbial metabolic
390 network in mixed cultures. Therefore, the acetate–ethanol pathway observed was
391 influenced by *Clostridium spp.* Since acetate-ethanol pathway is more stable way for H_2
392 production (Oztekin et al., 2008), it would be interesting to study this pathway in detail
393 and the bacteria responsible for the process in order to optimize of H_2 production even
394 under unstable conditions.

395 After the temperature was taken back from 65°C to 55°C, the relative abundance
396 of *Thermoanaerobacteriales* was 97% in step 4 while it reduced to 79% in step 5.
397 *Thermoanaerobacteriales* belong to the class *Clostridia*, which consist of,
398 *Thermoanaerobacteraceae* (having abundance below 0.1%), *Thermodesulfobiaceae*,
399 and *Thermoanaerobacterales* families, and various undetermined genera. The species
400 in this order are known for their abilities to survive in environments of extremely
401 elevated temperature (Gadow et al., 2013a; Koskinen et al., 2008, 2008; O-Thong et al.,
402 2011; Qiu et al., 2017a). *Thermoanaerobacterium* spp. belonging to the
403 *Thermoanaerobacterales* family was the most abundant genus in the consortium. On
404 the other hand, *Clostridiales* was 1% in the first cycle after the temperature shift (step 4)

405 and disappeared in step 5. *Baciallales* on the other hand had a relative abundance of
406 2% in step 4 and further increased to 21% in step 5 (Figure 5).

407 Though no metabolic activity was observed during the temperature shift to 75 °C,
408 H_2 production started as soon as the culture was returned to 55 °C. No activity was
409 observed during the temperature shift to 75°C suggesting that the bacteria present, did
410 not have enough time to initiate H_2 production activity and perhaps, needing more time
411 to adapt to such a high temperature. In the first cycle after the fluctuation (step 4), the
412 microbial community consisted of *Thermoanerobacteriales sp.* (91%) and *Bacillales* (9
413 %). In the second step after the temperature shift to 75°C (step 5),
414 *Thermoanaerobacteriales* dominated the microbial community with 99.9% abundance.
415 While *Clostridiales* are able to withstand temperatures up to 55° (Chen et al., 2012; Liu
416 et al., 2008)C. However, a further increase in the temperature up to 48 hours led to the
417 decrease in their relative abundance and complete disappearance at after 75°C
418 fluctuation. The decrease in the H_2 yield after the 65°C and 75°C shift can be linked to
419 the disappearance of *Clostridiales* in the consortium. Although most of the studies on
420 dark fermentative H_2 production have focused on dominant species, Rafrafi et al. (2013)
421 showed that sub-dominant bacteria can also have a significant effect despite their low
422 abundance. Therefore, it is suggested that the presence of *Clostridiales* in the
423 consortium had a significant role in H_2 productivity, hence the low yield obtained
424 following its disappearance from the microbial consortium.

425



426 Figure 4. Microbial community composition as relative abundance of different
427 microbial orders (>1%) during (step 3) and after (steps 4 and 5) the upward temperature
428 shifts.

429 **3.4.1. Comparison between cultures exposed to temporal downward and**
430 **upward temperatures**

431 H_2 production after periods of downward temperature fluctuation was recovered
432 faster than after periods of upward fluctuation (Table 2). This is in line with results of
433 (Huang et al., 2004) who suggested that for thermophilic systems, a longer adaptation
434 time is often required for new temperature conditions.

435

436 Table 4. Maximum H_2 yield recovered during temperature fluctuation and after the
437 fluctuation period.

Culture conditions	Temperature Fluctuation	Step 4 (55 °C)	Step 5 (55 °C)
Shift to 35 °C	84.3	107.6	96.5
Shift to 45 °C	75.9	96.8	91.6
Shift to 65 °C	90.9	89.3	79.9
Shift to 75 °C	0	30.9	54.1

438

439 Resilience of a microbial consortium is the capacity of the microorganisms in the
440 consortium to recover quickly from process disturbances. Resilience and functional
441 redundancy are the basic mechanisms via which microbial communities are able to
442 maintain community function when disturbance is introduced into a biological system
443 (Konopka et al., 2015; Werner et al., 2011). The current result suggests that the
444 performance during and after the temperature fluctuation was a consequence of a
445 microbial community with comparatively high resilience to the downward temperature
446 shifts. Change in the relative abundance of *Clostridiales* and *Thermoanaerobacteriales*
447 due to the transient changes in the incubation temperature illustrates the robustness
448 and adaptability of the mixed microbial community to new incubation conditions. This
449 helped to maintain continuous H_2 production process during the fluctuating conditions,
450 although with a lowered H_2 yield. *Clostridiales* differ in their optimal growth conditions
451 compared to *Thermoanaerobacterium*, which are strictly thermophilic (Bader and
452 Simon, 1980; Mtimet et al., 2016; O-Thong et al., 2008). However, they are
453 metabolically similar which allows for flexibility in H_2 production performance when
454 processes occur. Other bacteria present in the consortium, allowed for microbial
455 diversity and increase of the system robustness. Nonetheless, the presence of

456 homoacetogens though in very low abundance created a negative impact on the H_2
457 yield obtained. In general the results obtained show that temperature disturbances not
458 only affect the H_2 production performance but also the microbial community composition
459 which is closely linked to the metabolic networks (Wang and Wan, 2008).

460 **3. CONCLUSIONS**

461 Sudden or even temporal upward and downward temperature fluctuation had a
462 direct impact H_2 production, the frequencies of the metabolite distribution and the
463 microbial community structure. In this study, downward temperature fluctuation was
464 seen to recover more rapidly to a maximum H2 yield relative to the control (55 °C). On
465 the other hand, upward temperature shifts from 55 to 65 and 75 °C had more significant
466 negative effect on by dark fermentative H_2 production than downward temperature shifts
467 and did not reach maximum H_2 yield relative to the control (55 °C).The likely reason for
468 this was that upward temperature shifts resulted in more significant loss of microbial
469 diversity. A change in microbial community structure due to temperature fluctuation is
470 strictly determined by the direction of the fluctuation, either upward or downward.
471 Different microbial populations become dominant at different conditions, thereby
472 influencing the metabolic routes and ultimately, H_2 yield. However, as seen during the
473 upward temperature fluctuation at 65 °C, H_2 production from acetate and ethanol
474 pathway reiterated the fact that sub-dominant species (in this case, *Clostridium*) might
475 also have a significant contribution to dark fermentative H_2 production. The impact of
476 sudden or temporal temperature fluctuation investigated in this study has already been
477 seen to be more significant impact on cultures exposed to upward temperature
478 fluctuation period. Therefore, attention should be paid towards operational parameters

479 during bioreactor operations, especially with regards to factors that may lead to changes
480 in temperature fluctuation such as organic loading or self-heating. In consequence,
481 thermophilic H_2 producing bioreactors should be designed especially to prevent sudden
482 increases in temperature. Thus, cooling systems is recommended.Alternatively,
483 optimization of bioreactors with known H_2 producers characterized by wide temperature
484 ranges might help to improve the robustness of the system by making up for the loss in
485 microbial diversity enhancing the stability and resilience of the microbial consortium to
486 adverse environmental changes and consequently improve the performance of the H_2
487 production process. Additionally, with the ecological dynamics (due to temperature
488 changes) observed between *Clostridiales* and *Thermoanaerobacteriales*, certain
489 members of these groups can serve as useful tools in developing molecular methods for
490 complementary monitoring of the stability of the dark fementative H_2 production.
491

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499

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