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

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Onyinye Okonkwo[2], Renaud Escudie[1], Nicolas Bernet[1], Rahul Mangayil[2], Aino-Maija Lakaniemi[2], Eric Trably[1]

[1]LBE, Univ Montpellier, INRA, Narbonne, France

[2]Tampere University, Faculty of Engineering and Natural Sciences, Tampere, Finland.

ABSTRACT

Anaerobic microflora enriched for dark fermentative H_2 production from a mixture of glucose and xylose was used in batch cultivations to determine the effects of sudden short-term 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 a fresh medium and cultivated again at 55°C for two consecutive batch cycles. The average H_2 yield obtained during the first cultivation at 55°C was 2.1 ± 0.14 mol H_2 mol^{-1} hexose equivalent. During the temperature shifts, the obtained H_2 yields were 1.8 ± 0.15, 1.6 ± 0.27 and 1.9 ± 0.00 mol H_2 mol^{-1} hexose equivalent at 35°C, 45°C and 65°C, respectively, while no metabolic activity was observed at 75°C. The sugars were completely utilized during the 48 h temperature shift to 35°C but not at 65°C and 45°C. At the end of the second cycle after the different temperature shifts, the H_2 yield obtained was 96.5, 91.6, 79.9 and 54.1% (second cycle after temperature shift to 35°C, 45°C, 65°C and 75°C, respectively) when compared to the average H_2 yield produced in the control at 55°C. Characterization of the microbial

communities present in the control culture at 55°C showed the predominance of *Thermoanaerobacteriales*, *Clostridiales* and *Bacilliales*. The microbial community composition differed based on the fluctuation temperature with *Thermoanaerobacteriales* being most dominant during the upward temperature fluctuations and *Clostridiales* being the most dominant during the downward temperature fluctuations.

KEYWORDS: Dark fermentation; Temperature fluctuation; biological H_2 production; Resilience; Recovery.

1. INTRODUCTION

The increasing global demand for energy and fuels, and the environmental hazards that fossil fuels contribute, strongly demand for alternative energy resources. H_2 has been considered as a possible sustainable alternative [1,2]. Although H_2 is a very abundant element on earth, it does not typically exist as H_2 in nature. H_2 is usually found combined with other elements, whilst it can be produced locally from numerous sources. H_2 is produced industrially via electrolysis, coal gasification, and fossil fuel reforming [3,4]. Known biological H_2 production (BHP) methods include direct and indirect biophotolysis by green algae and cyanobacteria [5], photofermentation by phototrophic bacteria [6] and dark fermentation by fermentative bacteria [7]. BHP via dark and photofermentation has drawn increasing interest because of the ability to generate H_2 from various organic resources, such as industrial waste streams and lignocellulosic materials [8,9]. Photosynthetic H_2 production on the other hand is of interest because it needs only light and water and does not produce any CO_2 [10,11].

However, H_2 production by dark fermentation is advantageous over the other BHP processes because of higher H_2 production rates and the possibility to use a wide variety of organic materials as substrates [12]. Dark fermentation can be carried out under different temperatures, i.e. mesophilic (35-40 ºC), thermophilic (52-60 ºC) and extremely thermophilic (>65 ºC) conditions by different groups of fermentative bacteria [13,14]. Given the faster rates and higher yields of H_2, the use of thermophilic dark fermentation is often preferred over mesophilic processes [15]. However, thermophilic operations can require higher energy input for heating and are prone to inhibition [16] and sudden environmental changes [17] which can eventually result in reduced process stability or productivity [15,18].

Studies on anaerobic biological processes have shown that even small changes in the operating temperature can cause significant changes in microbial community composition and H_2 yields [14,19,20]. Temporal temperature fluctuations can also lead to changes in the enzymatic activities, growth rates and/or loss of microbial diversity, which directly affect H_2 production [21]. Temperature is therefore a key parameter to be controlled in dark fermentative processes. Previous studies investigating the effects of temperature on fermentative H_2 production have focused on comparing batch incubations and reactor performances at different fixed operating temperatures [20,22]. However, short-term temperature fluctuations can occur e.g., due to technical failures and therefore it is important to study the effects of short-term temperature fluctuations because they might lead to unwanted changes of the microbial growth and activities. Several studies focusing on anaerobic digestion have been carried out to establish the relationship between sudden temperature fluctuations and biogas production [23–25]. However, to

our knowledge only one study has been conducted to evaluate the influence of transient downward temperature fluctuations on the stability of H_2 production [21]. To date, no study has been done to correlate the outcome of temperature fluctuations in dark fermentation to the resilience and stability of the microbial populations involved.

The present study therefore aims to evaluate the effects of sudden short-term downward and upward temperature fluctuations during thermophilic dark fermentative H_2 production and reveal connections between effect of temperature changes and the microbial community structure. Chemical and molecular methods were used to monitor and characterize the microbial responses to transient temperature fluctuations (two different upward and two different downward temperature fluctuations). Understanding the resilience and stability as well as the dynamics of microbial communities to transient temperature fluctuations will help in developing strategies for optimization of dark fermentation processes, especially after possible process disturbances.

2. MATERIALS AND METHODS

2.1. Enrichment culture: medium composition and inoculum source

The enrichment of H_2 producing microbial community for this study was carried out in a continuous stirred tank reactor (CSTR) after which the enriched culture was used in batch bottle experiments to study the effects of sudden short-term temperature fluctuations on H_2 production and microbial community composition. An anaerobically digested sludge was used as inoculum for the CSTR after heat shock pretreatment at 90°C for 20 min. Two hundred milliliters of the pretreated sludge (10% v/v, final concentration, 40 mg L^{-1} of volatile solids) was inoculated to 1800 mL of the following culture medium (mg/L): K_2HPO_4, 500; NH_4Cl, 100; $MgCl_2 \cdot 6H_2O$, 120; $H_8FeN_2O_8S_2 \cdot$

96 $6H_2O$, 55.3; $ZnCl_2$, 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;
97 H_3BO_3, 0.1; $NiCl_2 \cdot 6H_2O$, 0.1; Na_2O_3Se, 0.01; $CaCl_2 \cdot 2H_2O$, 80; yeast extract, 500 and
98 0.055 mL HCl (37%). The culture was fed with glucose (800 mg L^{-1}) and xylose (1200 mg
99 L^{-1} xylose). Xylose was used as substrate because it is a major component of
100 hemicellulose and therefore commonly present in lignocellulosic biomass, lignocellulose
101 hydrolysates, and pulp and paper wastewaters. Utilizing both glucose and xylose is a
102 practical way to move towards efficient bioconversion of lignocellulosic wastes to H_2. The
103 total working volume of the CSTR was two liters. The reactor was flushed with nitrogen
104 for 5 min and then operated in continuous mode at hydraulic retention time of 6 h and at
105 55 °C for 21 days. The pH was maintained at 6.5.

106 **2.2. H_2 production batch experiments**

107 Prior to exposing the cultures to temporal temperature fluctuations, the H_2-producing
108 enrichment culture (step 1 in Figure 1) was acclimatized to batch growth conditions at
109 55°C using the same cultivation medium as in the CSTR (step 2 in Figure 1). The
110 acclimatized culture was then divided into ten anaerobic cultivation bottles containing
111 fresh medium and subjected to a one-time temperature shock. This was done by placing
112 duplicate bottes to 35 and 45 °C (downward temperature shocks), to 55 °C (control) as
113 well as to 65 and 75 °C (upward temperature shocks) for 48 h (Figure 1, step 3). At the
114 end of the 48 h incubation period, the cultures were centrifuged for 5 minutes, transferred
115 to fresh medium and incubated at the original temperature of 55 °C for 48 h (step 4 in
116 Figure 1). This step was repeated one more time (step 5 in Figure 1).

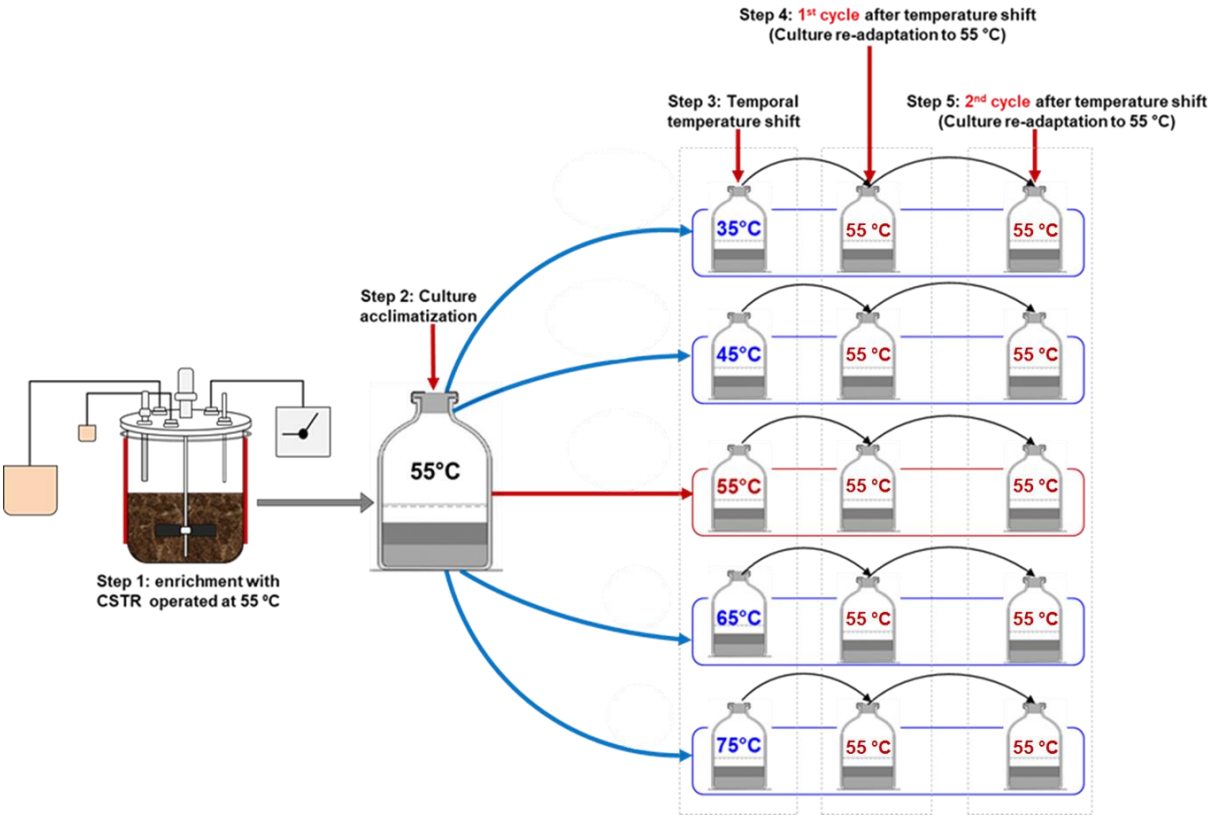

Figure 1. Experimental setup to study the effects of different temperature fluctuations during dark fermentation. First, H_2 producers were enriched in a continuous stirred tank reactor (CSTR) at 55 °C for 21 days (step 1). This was followed by the acclimatization of the enriched mixed culture to batch conditions (step 2) and then, specific temperature shock described as the downward temperature fluctuation (35 °C or 45 °C) and the upward temperature fluctuation (65 °C or 75 °C) were imposed (step 3). Cultures incubated at 55 °C (C) were used as control. After the temperature shocks, the H_2 production was followed for two more batch cycles at 55 °C (step 4 and step 5) to delineate how the culture can recover from the different temperature fluctuations.

2.3. Analysis of H_2 production

The biogas volume and composition measurement was carried out at the respective incubation temperatures mentioned in section 2.2 while keeping the culture

bottles in water baths to maintain a constant temperature. Gas production was monitored was periodically measured with a digital manometer to determine the volume of gas produced. H_2 partial pressure can have a significant effect on fermentation as some thermophilic microorganisms may change their metabolism to the production of more reduced substrates such as lactate when the H_2 partial pressure increases. The biogas volume was therefore measured before and after releasing the pressure in each culture bottle. The total volume of produced H_2 was calculated at standard temperature using Equation 1 [26].

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

Where $V_{H_2,t}$ is the cumulative H_2 gas produced at time t, $V_{H_2,t-1}$ is the cumulative H_2 gas produced at t-1, $V_{G,t}$ is the total gas volume at time t, $V_{G,t-1}$ is the total gas volume at time t-1, $C_{H_2,t}$ is the H_2 gas fraction in the headspace at time t, $C_{H_2,t-1}$ is the H_2 gas fraction in the headspace at time t-1 and V_H is the total headspace volume in the culture bottle. H_2 production in moles was calculated on the basis that one mole of an ideal gas will occupy a volume of 22.4 L at standard temperature and pressure according to the ideal gas law. Therefore, the volume of H_2 gas produced was divided by 22.4 L in order to obtain H_2 produced in moles. The gas composition was analyzed using a gas chromatograph (Clarus 580, Perkin Elmer) with a thermal conductivity detector. The columns used were a RtQbond column for H_2, O_2, N_2 and CH_4 quantification and a RtMolsieve column for CO_2 quantification. The carrier gas was argon under a pressure of 3.5 bars. A gas tight Hamilton syringe was used for gas sampling.

2.4. Analysis of the liquid metabolites

Culture suspension samples were collected before and after each experimental step for chemical analysis of the metabolic products. The samples were centrifuged at 13000 rpm for 15 min and the supernatant was filtered with 0.2 μm filter before the analyses. Glucose, xylose, organic acid and alcohol concentrations were measured by high performance liquid chromatography (HPLC) using a refractive index detector (Waters R410) as described previously by [29].

2.5. Microbial community analysis

Genomic DNA was extracted using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The following primers; 515_532U and 909_928U (Wang and Qian, 2009) including their respective linkers, were used to amplify the V4_V5 region of the 16S rRNA gene over 30 amplification cycles at an annealing temperature of 65 °C. The resulting products were purified and loaded onto the Illumina MiSeq cartridge prior to sequencing. Sequencing and library preparation were performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). The sequences analysis was done as described by Venkiteshwaran et al. (2016).

2.6. Calculations

H_2 yield was calculated by dividing the mol H_2 per mole of hexose equivalent using the conversion factor of 5/6 for converting xylose to its hexose equivalent. The theoretical H_2 produced was calculated based on the measured acetate and butyrate concentrations (mM) using the Equation 2 [30] in order to determine the ratio of experimental to theoretical H_2 yield [31]. If the calculated ratio is above 100%, the H_2 is produced also via

other pathways than acetate and butyrate while a ratio below 100% indicates homoacetogenic activity.

$$\text{Theoretical } H_2 \text{ produced} = 2 \times \sum(\text{acetate concentration} + \text{butyrate concentration}) \quad (2)$$

The relative H_2 yield compared to the control was calculated for each culture by comparing the H_2 yields obtained during or after the temperature fluctuation period to the average yield (steps 3 - 5) obtained from the control cultures kept at 55 °C (Equation 3).

$$\text{Relative } H_2 \text{ yield compared to the control } (\%) = \frac{H_2 \text{ yield obtained during/after temperature shift}}{\text{average } H_2 \text{ yield obtained from the control}} \times 100 \quad (3)$$

Total COD of soluble compounds was calculated based on the sum of acids, ethanol and residual sugars by using the following conversion factors: 1 mM glucose = 192 mg COD L^{-1}, 1 mM xylose = 160 mg COD L^{-1}, 1 mM acetate = 64 mg COD L^{-1}, 1 mM propionate = 112 mg COD L^{-1}, 1 mM lactate = 96 mg COD L^{-1}, 1 mM butyrate = 160 mg COD L^{-1} and 1 mM ethanol = 96 mg COD L^{-1} [32,33]. The COD mass balance was calculated using the endpoint concentrations of the residual sugars and soluble metabolites.

3. RESULTS AND DISCUSSION

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

Methane was not detected in any of the incubations performed in this study, which indicates that the initial heat-shock pretreatment was sufficient to totally suppress the activity of methanogens [34,35]. The maximum H_2 yield obtained during the enrichment in the CSTR was 1.9 mol H_2 mol^{-1} hexose equivalent. The H_2 yield obtained in the batch cultivation at 55°C (control) was 2.2 ± 0.07, 2.1 ± 0.06 and 1.9 ± 0.14 mol H_2 mol^{-1} hexose

196 equivalent (in steps 3, 4 and 5 respectively). The H_2 yield was seen to decrease in steps
197 4 and 5.

198 During the cultivation in the different steps (3, 4 and 5) at 55 °C, the concentration
199 of ethanol and acetate increased in step 4 compared to steps 3 and 5 (Table 1). The
200 increase in these metabolites coincided with a decrease in butyrate concentration. The
201 butyrate to acetate ratio (HBu/HAc, mM:mM) has been used in previous studies as an
202 indicator of relative contribution of these two pathways to H_2 production [36,37]. In the
203 cultures incubated at 55 °C, it was 1.2, 0.5 and 1.5 in steps 3, 4 and 5, respectively. The
204 values from step 3 and 5 are in line with previous studies which have reported HBu/HAc
205 ratios ranging between 1.5 and 4.0 [36,37]. The ratio of experimental to theoretical H_2
206 yield was 99, 91 and 83% in steps 3, 4 and 5, respectively. Based on the H_2 yields,
207 HBu/HAc ratios and the ratios of experimental to theoretical H_2 yield, there might have
208 been a slight shift in the metabolic pathway towards acetate production during the
209 thermophilic dark fermentative H_2 production in step 4 and an increasing homoacetogenic
210 activity from step 3 to 5.

211 In a dark fermentation, the maximum theoretical H_2 that can be obtained from
212 glucose under standard temperature and pressure is 4 mol H_2 per mol glucose with
213 acetate as the only metabolite while 2 mol H_2 per mol of glucose is produced with when
214 butyrate is the only metabolite [38]. However, during dark fermentation, H_2 is produced
215 along with other metabolites such as alcohols, lactate and propionate, the production of
216 which are either consume substrate without production of any H_2 or even consume H_2
217 and therefore lead to low H_2 yields [39,40]. The metabolites produced by a bacterium or
218 mixed cultures depend on the environmental conditions.

Table 1: Concentration of soluble metabolites and their contribution to end-point COD in the control cultures incubated at 55 °C including results from the incubations steps 3, 4 and 5.

Parameters	Concentration (mM)			Percentage of end-pointCOD		
	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

n.d: Not detected

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

During the downward temperature shifts, the H_2 yield slightly decreased compared to the control (55 °C), being 1.8 ± 0.15 and 1.6 ± 0.27 mol H_2 mol^{-1} hexose equivalent at 35 and 45 °C, respectively. However, H_2 production recovered rapidly when the cultures were transferred to a fresh medium and incubated at the original temperature of 55 °C. H_2 yield of 2.2 ± 0.07 and 2.0 ± 0.01 mol H_2 mol^{-1} hexose equivalent (steps 4 and 5, respectively) were obtained for cultures exposed to 35°C shift, and 2.0 ± 0.08 and 1.9 ± 0.00 mol H_2 mol^{-1} hexose equivalent (steps 4 and 5, respectively) for cultures exposed to 45°C shift. The substrates were fully consumed during and after the temperature shift to 35°C. Meanwhile during the temperature shift to 45°C, 6.4 ± 0.5% of the substrate was not consumed (Table 2) but all substrates given were completely depleted after the cultures were returned to 55 °C. During the downward temperature

fluctuation, similar metabolic patterns were observed at 35 and 45 °C shifts except that the concentration of lactate and butyrate was higher at the 35 °C than 45 °C shift (Figure 2, Table 2). Meanwhile, the concentration of acetate was higher at 45 °C than 35 °C. When cultures after both fluctuating temperatures where returned to 55 °C, they both showed similar patterns in their metabolite distribution with only slight variations in their frequencies (Figure 2, Table 2).

The proportion of experimental to theoretical H_2 yield (calculated based on acetate and butyrate concentrations) during and after the temperature fluctuation to 35 °C was 79, 99 and 90% in steps 3, 4 and 5, respectively. For cultures exposed to 45 °C, the ratio was 71, 88 and 89% in steps 3, 4 and 5, respectively. Lactate and propionate, have been reported to be involved in H_2 consuming pathways, which leads to H_2 yields which are significantly lower than the theoretical values calculated using only the concentrations of acetate and butyrate [39,40]. However, it is also possible that the decrease in H_2 production during the downward temperature fluctuation was influenced by homoacetogenic activity and to minor extent lactate production. This lead to a reduction in the H_2 yield compared to the controls incubated at 55 °C.

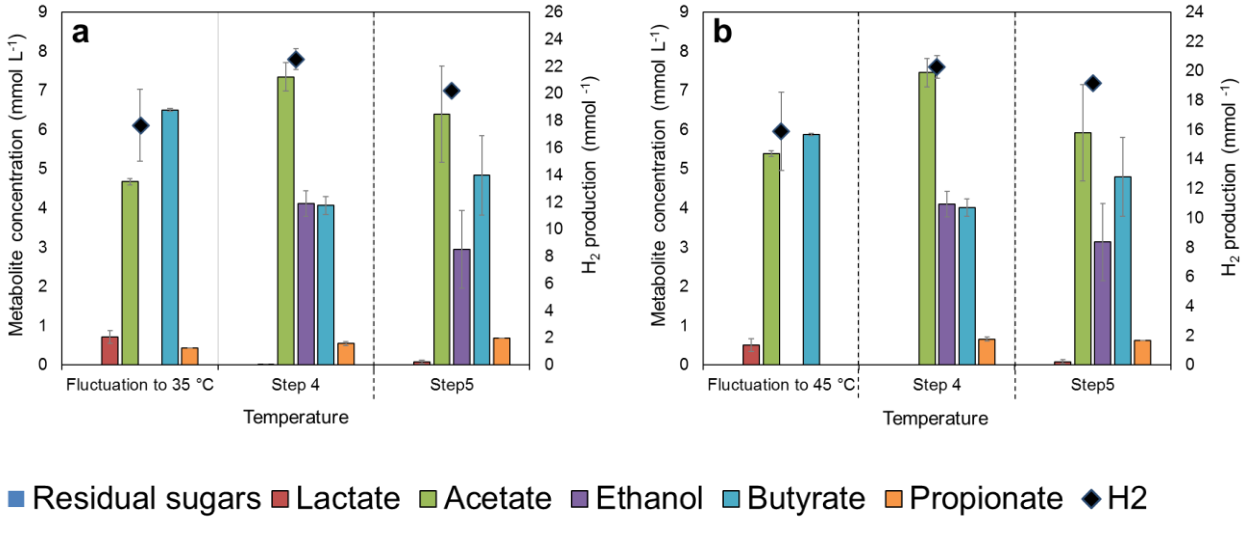

Figure 2. Metabolites and H_2 produced during the downward temperature fluctuations. H_2 and soluble metabolite production during temperature fluctuation at A) 35 °C (step 3) and B) 45 °C and after returning the cultures back to 55 °C (steps 4 and 5).

Table 2: Contribution of each metabolite and the residual sugars to end-point COD during and after the downward temperature fluctuations.

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

n.d: Not detected

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

During the upward temperature shift at 65 °C, the H_2 yield was 1.9 ± 0.0 mol H_2 mol^{-1} hexose equivalent which corresponded 10.5% decrease compared to the average H_2 yield in the controls. The H_2 yield in the subsequent cultivation steps at 55°C was 12.1% (1.9 ± 0.05 mol H_2 mol^{-1} hexose equivalent) and 21.3% (1.7 ± 0.27 mol H_2 mol^{-1} hexose equivalent) lower in steps 4 and 5, respectively, compared to the average H_2 yield in the control. Temperature shift to 75 °C resulted in a complete stop of the dark fermentative microbial activity. Hence, no substrate consumption was observed. H_2 production recovered as soon as the cultures were transferred to a fresh medium and incubated again at 55°C. However, the H_2 yield was only 0.7 ± 0.26 mol H_2 mol^{-1} hexose

equivalent (67.3% decrease) and 1.13 ± 0.17 mol H_2 mol^{-1} hexose equivalent (44.9% H_2 decrease) in steps 4 and 5, respectively.

During the temporal temperature shift to 65°C, 25.5 ± 2.4% of the substrates was not consumed at the end of the 48 h period (Table 3). However, all the substrates added were consumed when the cultures were returned to 55°C. Upon the shift to 65°C, the share of acetate and ethanol increased while that of butyrate and lactate reduced (Figure 3). This was different compared to the metabolite formation obtained in the control at 55 °C where butyrate was the most abundant metabolite, followed by acetate. The ratio of experimental to theoretical H_2 yield calculated from the sum of acetate and butyrate was higher than expected (145%) for the acetate–butyrate pathway. When the temperature was returned to 55 °C, butyrate and acetate once again became the major liquid metabolites (Table 3). The ratio of experimental to theoretical H_2 yield calculated from the sum of acetate and butyrate were 82 and 74% in steps 4 and 5, respectively. Due to the higher percentage of acetate and ethanol than butyrate, it is possible that the temperature fluctuation to 65 °C induced a metabolic shift towards ethanol–acetate pathway. The ethanol–acetate pathway have a theoretical maximum of 2 mol of H_2 mol^{-1} glucose [41,42]

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

Similar findings where butyrate and acetate were the major liquid metabolites at 35–60°C while at 65°C the main by-product was ethanol have been reported by Qiu et al. [19]. Some members of the order *Clostridiales* such as *Ruminococcus albus* are capable of H_2 production via the ethanol-acetate pathway. Additionally, *Thermoanaerobacterium spp.* has been reported to produce ethanol and acetate as the major by-products at 65 °C from xylose [19].

As mentioned earlier, H_2 production came to a complete stop during the temperature shift to 75°C with no substrate consumption. However, only 95% of the total substrate given was detected in the end of the incubation, indicating that the existing microbial population used 5% of the substrates for their survival mechanisms or other metabolic pathways. When the cultures were returned to 55°C after the high rise in temperature to 75°C, the substrate removal was again complete at the end of the incubation period in steps 4 and 5. After the fluctuation period at 75 °C, H_2 producing activity commenced with varying concentrations of the metabolites (Figure 3, Table 3). The ratios of experimental to theoretical H_2 yield calculated from the sum of acetate and butyrate were 40 and 51% in steps 4 and 5, respectively.

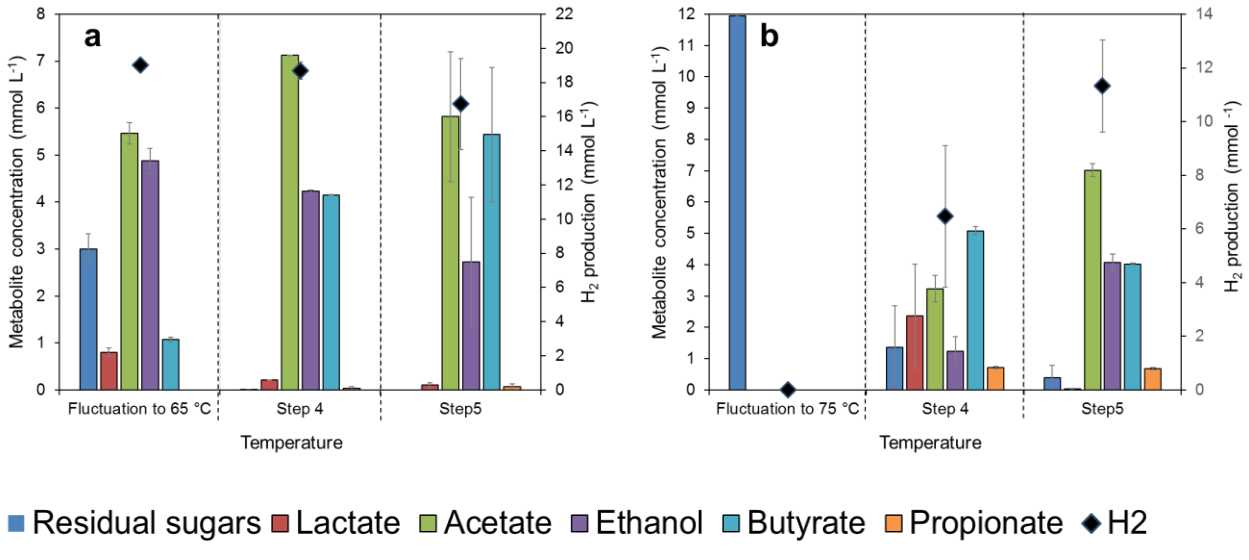

Figure 3. Metabolites and H_2 produced during the upward temperature fluctuations at A) 65 °C (step 3) and B) 75 °C and after returning the cultures back to 55 °C (steps 4 and 5).

Table 3: Contribution of each metabolite and the residual sugars to the end-point COD during and after upward temperature fluctuations.

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

n.d.: Not detected

3.4. Microbial community composition during the altered temperature conditions

The microbial community in the thermophilic control cultures incubated at 55°C was dominated by three major orders: *Thermoanaerobacterales* (94, 98 and 77% in steps 3, 4 and 5, respectively) > *Clostridiales* (3, 1 and <1%) > *Bacillales* (2, <1 and 22%). The sudden increase in *Bacillales* in step 5 was concomitant with the decreased H_2 yield. The dominant member of this order was *Tumebacillus* spp., which corresponded up to 20% of relative abundance of all microorganisms detected in step 5. *Tumebacillus* spp. are gram positive, aerobic, rod shaped, and spore forming bacteria, which are able to degrade carbohydrates and have been detected from anaerobic processes [43]. However, their role in the consortium is not known and it is not certain whether its presence was the reason of the lower yield observed in step 5, during incubation at 55°C.

3.4.1. Downward temperature shifts

Decreasing the temperature to 35°C or to 45°C for 48 hours considerably influenced the microbial community composition. *Clostridiales* became the dominant order in the community (relative abundance of 84% and 74% at 35 °C and 45 °C, respectively) as seen in Figure 4. During both downward shifts, *Thermoanaerobacteriales* was present in lower abundance (10% and 25% at 35 °C and 45 °C, respectively)

compared to the control cultures. *Bacillales* (6% and <1% at 35 °C and 45 °C, respectively) was also present at low relative abundance at this point. The members of the order of Clostridiales identified belonged to *Clostridium* spp. The increase in temperature back to 55 °C lowered the relative abundance of *Clostridiales,* and *Thermoanaerobacteriales* became again the dominant order (Figure 4). The share of other members of the consortium, some of which were known homoacetogens, was below 1%. Though in very low abundance, the metabolic capacities of this group of bacteria might have had noticeable influence on the dark fermentative metabolism [44]. When compared to the control cultures (incubated constantly at 55 °C), *Thermoanaerobacteriales* quickly became the most dominant species in steps 4 and 5 after downward temperature fluctuations which means that short-term downward temperature fluctuation leads to the suppression of thermophilic microorganisms but due to their resilient nature, are able quickly re-adapt when the temperature becomes again favorable for their growth.

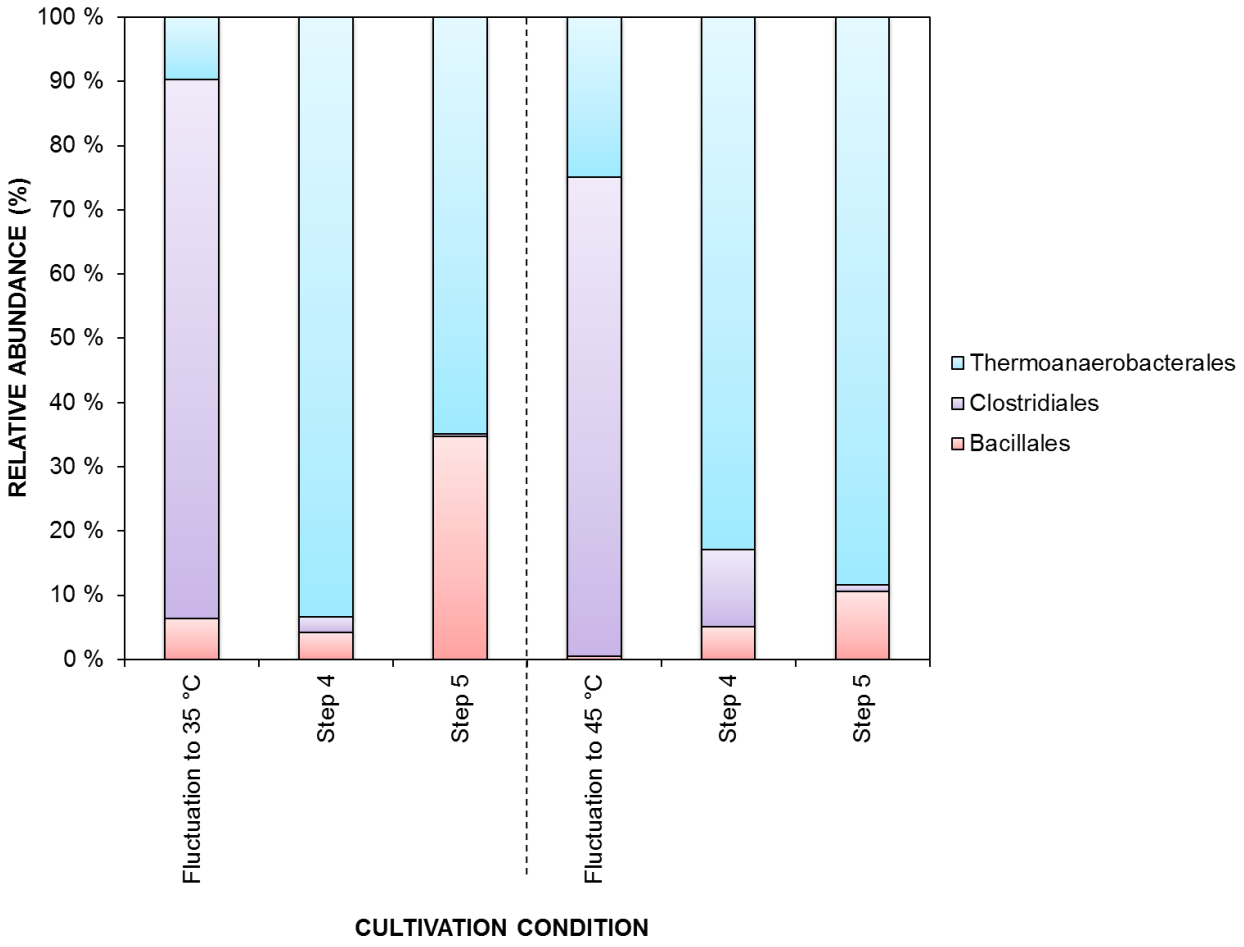

Figure 4. Microbial community composition as relative abundance of different microbial orders (>1%) during (step 3) and after (steps 4 and 5) the downward temperature shifts.

3.4.2. Upward temperature shifts

In contrast to the downward temperature shifts, temporal upward temperature shifts did not cause a significant alteration in the microbial community. *Thermoanarobacteriales* remained the dominant order during and after the temperature shifts. *Thermoanaerobacteriales* (97%) and *Clostridiales* (3%) were the main orders during the temperature shift at 65°C. Interestingly, during this step, it was observed that H_2 might have also been produced via the acetate–ethanol pathway due to the high

amount of ethanol produced and on the fact that the ratio of experimental to theoretical H_2 produced was greater than 100%. Members of the group *Thermoanaerobacterium*, which were the most dominant group of the order *Thermoanaerobacteriales*, are known to produce H_2 via the acetate and butyrate pathway. However, the ratio of experimental to theoretical H_2 yield (calculated based on acetate and butyrate) reported in the previous section, indicated that H_2 production could not have been achieved only via the acetate and butyrate pathways. A fraction of the H_2 produced might have come from some members of the order *Clostridiales* via the acetate–ethanol pathway [42], despite their low abundance. It has been shown that sub-dominant bacteria can influence the global microbial metabolic network in mixed cultures [44,45]. It would be important to identify bacteria, which are able to utilize this pathway for H_2 production in order to optimize of H_2 production efficiency even under unstable conditions.

After the cultures were taken back from 65°C to 55°C, the relative abundance of *Thermoanaerobacteriales* was 97%, *Clostridiales* was 1% and *Baciallales* was 2% (step 4). In step 5, proportion of *Thermoanaerobacteriales* decreased to 79%, *Clostridiales* seemed to disappear from the microbial consortium, and *Bacilliales* increased to 21% (Figure 5).

No activity was observed during the temperature shift to 75°C suggesting that the bacteria present, did not have enough time to initiate H_2 production activity and perhaps, needed more time to adapt to such a high temperature. However, H_2 production started as soon as the culture was returned to 55 °C. In the first cycle after the fluctuation (step 4), the microbial community consisted of *Thermoanerobacteriales* (91%) and *Bacillales* (9%). In the second cycle after the temperature shift to 75°C (step 5),

Thermoanaerobacteriales dominated the microbial community with 99.9% abundance. While *Clostridiales* are able to withstand temperatures up to 55° C [46,47], a further increase in the temperature to 65° C led to the decrease in their relative abundance and even to complete disappearance after the 75°C fluctuation. The decrease in the H_2 yield after the 65°C and 75°C shift can be linked to the disappearance of *Clostridiales* from the consortium. Although most of the studies on dark fermentative H_2 production have focused on key-stone species as having the most significant impact on biological processes, it has been shown that sub-dominant bacteria can also have a significant effect despite their low abundance [44]. Therefore, it is suggested that the presence of *Clostridiales* in the consortium had a significant role in H_2 productivity, hence the low yield obtained following its disappearance from the microbial consortium. When compared to the control cultures (incubated constantly at 55 °C), Thermoanaerobacteriales remained the most abundant species in steps 4 and 5 after upward temperature fluctuations. However, the relative abundance was higher, compared to the control cultures (especially after the 75 °C fluctuation) while *Clostridiales* disappeared from the consortium.

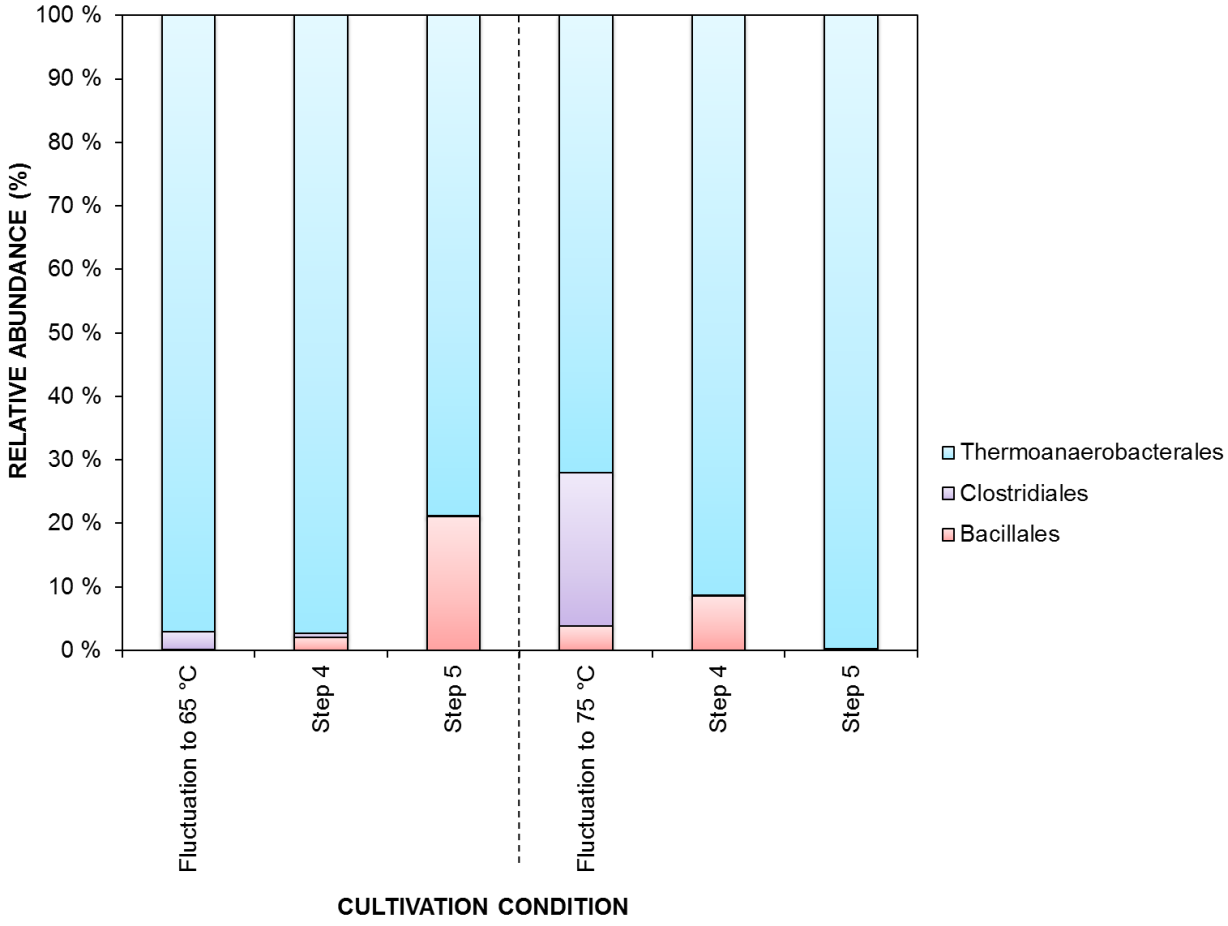

Figure 4. Microbial community composition as relative abundance of different microbial orders (>1%) during (step 3) and after (steps 4 and 5) the upward temperature shifts.

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

H_2 production recovered faster after the downward temperature fluctuations than after of the upward temperature fluctuations (Table 2). This is in line with results of Huang et al. (2004) [48] who suggested that for thermophilic systems, a longer adaptation time is often required for new temperature conditions.

Table 4. Percentage maximum relative H_2 yield (compared to H_2 yield obtained in the controls) obtained during the temperature fluctuations and after the fluctuation periods.

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

The resilience of a microbial consortium is the capacity of the microorganisms in the consortium to recover quickly from process disturbances. Resilience and functional redundancy are the basic mechanisms via which microbial communities are able to maintain community function when disturbance is introduced into a biological system [49,50]. The current result suggests that the performance during and after the temperature fluctuations was a consequence of a microbial community with higher resilience to the downward temperature shifts than to the upward temperature shifts. Change in the relative abundance of *Clostridiales* and *Thermoanaerobacteriales* due to the transient changes in the incubation temperature illustrates the robustness and adaptability of the mixed microbial community to the new incubation conditions. The presence of members of these orders helped to maintain continuous H_2 production process during the fluctuating conditions, despite the lower H_2 yield observed. The species belonging to *Thermoanaerobacteriales* are known for their abilities to survive in environments of extremely high temperature [51,52]. *Thermoanaerobacterium* spp. belonging to the *Thermoanaerobacterales* was the most abundant genus in the consortium and demonstrated the ability to function at all the studied temperature. *Clostridiales* was the most abundant order at 35 °C. Its level of relative abundance

decreased with increasing temperatures up to 65 °C and disappearance at 75 °C. *Clostridiales* differ in their optimal growth conditions compared to *Thermoanaerobacteriales* [53,54]. However, both groups are metabolically similar which allows for flexibility in H_2 production performance when process disturbances occur. Other bacteria present in the consortium enabled higher microbial diversity and increase of the system robustness. Nonetheless, the presence of homoacetogens, though in very low abundance, created a negative impact on the H_2 production. The results obtained in this study showed that H_2 production due to temperature disturbances is a result of changes occurring in the metabolic networks and the microbial community composition. Temperature therefore plays an important role in microbial community stability and resilience.

Additionally, as observed in this study, the effects of short-term temperature fluctuations on the microbial communities depend on the extent of the temperature fluctuation. For example, after the downward temperature fluctuations, the thermophilic microorganisms were able to re-adapt to their original conditions without any identifiable loss in microbial diversity in the community. On the contrary, after the upward temperature fluctuations, the microbial community was different from that observed in the control incubated constantly at 55 °C. In the control, *Clostridiales* were present, although in low abundance and were considered to play a role in H_2 production, as their disappearance after the temperature fluctuations led to reduced H_2 production in steps 4 and 5. This might lead to reduced H_2 production also in the long term due to loss of microbial diversity. Therefore, recovery strategies such as bioaugmentation to optimize the H_2 production

could be required after unexpected upward temperature fluctuations. However, this subject requires further studies.

3. CONCLUSIONS

Sudden, even temporal upward and downward temperature fluctuations had a direct impact on microbial community structure, the soluble metabolites produced and the H_2 production. A mixed microbial culture enriched for H_2 production at 55 °C recovered more rapidly enabling similar H_2 yield (92-108%) after returning to original temperature of 55 °C as the control culture kept at constant temperature (55 °C). Upward temperature shifts from 55 to 65 or 75 °C had more significant negative effect on dark fermentative H_2 production than downward temperature shifts and H_2 yield remained lower than the control (55 °C) after the temperature was returned to 55 °C (31-89%). The likely reason for this was that upward temperature shifts resulted in more significant loss of microbial diversity. Based on the observations made in this study, attention should be paid towards optimizing operational parameters during bioreactor operations, especially with regards to factors that may lead to unexpected increase in temperature such as high organic loading rate. Thus, cooling systems are recommended. Alternatively, adding known H_2 producers (i.e. bioaugmentation) characterized by wide temperature ranges might help to improve the robustness of the system by making up for the loss in microbial diversity enhancing the stability and resilience of the microbial consortium to adverse environmental changes and consequently improve the performance of the H_2 production process.

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