Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: biohydrogen production and active microbial community

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Abstract

Dark fermentative biohydrogen production in a thermophilic, xylose-fed (50 mM) fluidised bed reactor (FBR) was evaluated in the temperature range 55–70 °C with 5-degree increments and compared with a mesophilic FBR operated constantly at 37 °C. A significantly higher ($p = 0.05$) H_2 yield was obtained in the thermophilic FBR, which stabilised at about 1.2 mol H_2 mol^{-1} xylose (36% of the theoretical maximum) at 55 and 70 °C, and at 0.8 mol H_2 mol^{-1} xylose at 60 and 65 °C, compared to the mesophilic FBR (0.5 mol H_2 mol^{-1} xylose). High-throughput sequencing of the reverse-transcribed 16S rRNA, done for the first time on biohydrogen producing reactors, indicated that *Thermoanaerobacterium* was the prevalent active microorganism in the thermophilic FBR, regardless of the operating temperature. The active microbial community in the mesophilic FBR was mainly composed of *Clostridium* and *Ruminiclostridium* at 37 °C. Thermophilic dark fermentation was shown to be suitable for treatment of high temperature, xylose-containing wastewaters, as it resulted in a higher energy output compared to the mesophilic counterpart.

Keywords: Active community, Biohydrogen, FBR, MiSeq, *Thermoanaerobacterium*, Thermophilic

1. Introduction

H_2 is a carbon free fuel considered as a promising candidate to replace fossil fuels in the near future [1]. Although hydrocarbons are currently the main feedstock for H_2 production, biomass is a renewable and environmentally friendly alternative feedstock [2]. Dark fermentation is the most studied among the biological H_2 production technologies because the variety of usable organic substrates, and the high achievable conversion rates, may promote the scale-up of the process [3]. However, due to the thermodynamics of the reactions involved, which are more favourable at high temperature and low H_2 partial pressure, operation and optimisation of full-scale dark fermentation is more challenging than traditional anaerobic digestion [4].

Many pathways are possible for dark fermentation, depending on the microbial species, operating parameters, and the substrate used. Glycolysis is the most common route for degradation of hexoses by *Clostridium* [5] and most thermophiles, including *Thermoanaerobacter* [6]. Glucose is oxidised to pyruvate, resulting in the generation of reduced nicotinamide adenine dinucleotide (NADH) and energy in the form of adenosine triphosphate [7]. Pyruvate may be further oxidised to acetylcoenzyme A through reduction of ferredoxin and then to volatile fatty acids (VFAs), or alcohols [7]. Metalloenzymes called hydrogenases use NADH or reduced ferredoxin as electron donor for proton oxidation [8], resulting in the formation of molecular H_2. The oxidation of glucose to H_2 and CO_2 yields 12 mol H_2 mol^{-1} glucose. However, the dehydrogenation of acetate to CO_2 is endergonic, and the spontaneous oxidative process will thus end with acetate production, yielding only 4 mol H_2 mol^{-1} glucose [9]. This thermodynamic limitation is also valid for pentose sugars such as xylose, which will then yield a maximum of 3.33 mol H_2 mol^{-1} xylose.

In practice, the H_2 yield by mixed cultures varies from 14% to 70% of the theoretical limit [10]. For high H_2 partial pressures (> 60 Pa), proton reduction by NADH is not thermodynamically favourable

and the reaction switches, e.g. to the butyrate pathway [11], resulting in a lower H_2 yield. Temperature and pH also strongly affect the microbial community and thus, the substrate degradation pathway [12]. Butyrate accumulation can trigger solventogenesis [13], which does not yield H_2. Some microorganisms, including various *Clostridium* sp., are facultative autotrophic and can reduce CO_2 with H_2 forming acetate [10]. Other known H_2-consuming microorganisms include hydrogenotrophic methanogens, propionate producers, and sulfate or nitrate reducing bacteria [14].

H_2 production at high temperatures can be advantageous in terms of H_2 yield and production rates [15,16]. High temperature positively affects the kinetics of the oxidative reactions and the growth of microorganisms [17]. Furthermore, the direct conversion of sugars to acetate becomes thermodynamically more favourable as the temperature increases, thus resulting in a high H_2 yield [18]. Thermophilic anaerobic microorganisms such as *Thermotoga* and *Thermoanaerobacter* are excellent H_2 producers, as they use most of the reductants produced during glycolysis to form H_2, allowing yields between 3 and 4 mol H_2 mol^{-1} hexose [18]. Although H_2 yields from pure cultures are typically higher [5], mixed cultures are preferable for industrial application, as they offer more stability and versatility, and sterilisation is not required [12]. Most studies on H_2 production at high temperature by mixed cultures have been conducted at 55, 60, or 70 °C, generally obtaining higher H_2 yields than mesophilic trials with a similar inoculum and substrate [16,19,20].

Handling and processing of organic substrates and the low H_2 yield are two of the main deterrents for the establishment of dark fermentation at commercial scale [21]. Despite the high H_2 yield obtained at high temperatures, the net energy gain (the difference between the energy input needed to heat the reactor and output) seems to be indirectly proportional to the operation temperature [22]. However, some industrial wastewaters, such as thermomechanical pulping wastewaters, are produced at high (50–70 °C) temperatures [23] and could be treated in situ avoiding cooling and minimising energy

100 loss. Such wastewaters contain readily fermentable sugars, both hexoses (mainly glucose) and
101 pentoses (mainly xylose), suitable for H_2 production by dark fermentation. Continuous dark
102 fermentation of glucose has been widely studied at various temperatures [24–28], while much less
103 attention was given to dark fermentation of xylose, especially at high temperature. In a previous study,
104 H_2 production from xylose was compared in batch cultures at 37, 55 and 70 °C, using heat treated (90
105 °C, 15 min) activated sludge from a wastewater treatment plant as the inoculum [16]. That study
106 showed effective H_2 production at 55 °C, but not at 70 °C. However, the effect of temperature in the
107 55–70 °C temperature range is worth investigating, as a difference of a few degrees can affect the
108 microbial community inside the reactor and thus, the H_2 production efficiency [28].
109
110 Recently, the establishment of next-generation sequencing techniques has improved the knowledge
111 on H_2-producing microbial communities. Etchebehere et al. [29] performed 454 pyrosequencing on
112 microbiological samples from 20 H_2 producing lab-scale bioreactors operated within a temperature
113 range of 25–37 °C. Although the microbial communities were diverse due to the different operating
114 conditions of the bioreactors, the authors observed a predominance of *Firmicutes* and distinguished
115 high-yield H_2 producers (*Clostridium*, *Kosmotoga*, and *Enterobacter*), low-yield H_2 producers
116 (*Veillonellaceae*) and competitors (*Lactobacillus*). Nitipan et al. [30] reported
117 *Thermoanaerobacterium* as the dominant genus in a thermophilic (60 °C) sequencing batch reactor
118 producing H_2 from palm oil mill effluent. Zhang et al. [31] showed that *Firmicutes* such as
119 *Caldanaerobius*, *Caldicellulosiruptor*, and *Thermoanaerobacter* become dominant in a
120 hyperthermophilic (70 °C) H_2 producing, glucose-fed chemostat. To date, analysis of dark
121 fermentative microbial communities by next-generation sequencing has been mainly based on the
122 presence of 16S rRNA genes, which provide information on the structure of the microbial community.
123 However, an analysis based on the expression of 16S rRNA genes describes more accurately the
124 composition of the microbial community actively involved in dark fermentation [32].

This study aims to evaluate, for the first time, how dark fermentative H_2 production and the composition of the active microbial community are affected by a stepwise (5 °C) temperature increase in the 55–70 °C temperature range in a xylose-fed fluidised bed reactor (FBR) inoculated with heat treated activated sludge. A second FBR was operated in parallel with the same inoculum, but at a lower temperature (37 °C) in order to compare its performance to the thermophilic counterpart, prior to increasing the temperature to 55 °C to observe the response of this mesophilic microbial community to the temperature shift.

2. Materials and methods

2.1 Source of biomass

Activated sludge was collected from the recirculation line between the aeration tank and the secondary settler in the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland). It was settled, and, after removing the supernatant, heat-treated at 90 °C for 15 minutes as described by Dessì et al. [16] before being used as inoculum for the FBRs.

2.2 Composition of synthetic wastewater

Both FBRs were fed with a synthetic wastewater based on the DSMZ 144 medium, with xylose (50 mM) as electron donor instead of glucose. However, tryptone, resazurin and Na_2S were omitted and the concentration of KH_2PO_4, K_2HPO_4, and yeast extract was reduced 10 times (to 0.55 mM, 0.86 mM, and 0.3 g L^{-1}, respectively).

2.3 FBR configuration

Two FBRs (Figure 1), 1 L volume each, were operated with 300 mL activated carbon as carrier material for biomass adhesion. A recirculation flow rate of about 1900 mL min^{-1} was applied by using

150 a peristaltic pump (Masterflex, USA) to expand the activated carbon bed by 30% (the flow rate of
151 minimum fluidization was 1400 mL min^{-1}). To maintain a constant expansion, the recirculation flow
152 was increased up to a maximum of 20% due to the adhesion of the biomass, which made the bed
153 heavier. To achieve a stable temperature, the FBRs were operated inside incubators (Labilo, Finland).
154 A water jacket (Julabo, Germany) was also installed, as the heat provided by the incubator was not
155 enough to reach 70 °C inside the FBR.
156
157 To avoid microbial growth, the xylose solution was prepared in a different tank than the medium
158 containing the nutrients and trace elements. Both solutions were flushed with N_2 into the feeding
159 tanks, and their pH was adjusted to 5.0–5.5 with HCl prior to being fed to the FBRs through peristaltic
160 pumps (Masterflex, USA). To minimise the growth of methanogenic archaea [33], the pH inside the
161 reactor was kept at 5 (± 0.1) by automatic titration (Metrohm, Switzerland). The FBRs were sealed
162 on the top, and both liquid and gas were directed to a gas-liquid separator. The liquid was directed to
163 an effluent tank, while the gas was directed to a gas meter (Ritter, Germany) before being released
164 into a fume hood (Figure 1).
165
166

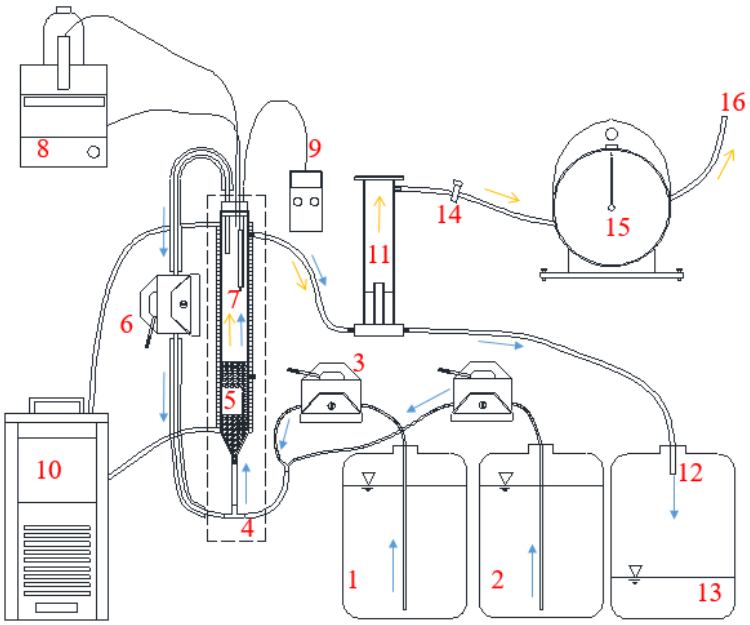

Figure 1. Overview of the experimental set-up used in this study. Medium influent tank (1), xylose influent tank (2), peristaltic pumps for influent feeding (3), influent sampling point (4), activated carbon bed with the active biomass (5), peristaltic pump for recirculation (6), pH probe (7), automatic titrator (8), temperature control (9), water jacket (10), gas-liquid separator (11), effluent sampling point (12), effluent tank (13), gas sampling point (14), gas meter (15), and gas outlet (16). Liquid path (↗), gas path (↗). The dashed rectangle represents the part of the set-up located inside the incubator.

2.4 FBR operation and sampling

Both FBRs were inoculated by 50 mL of heat-treated activated sludge (8.8 g VS L^{-1}). After two days of start-up in batch mode, the FBRs were switched to continuous mode (day 0) and operated for 7 days at a hydraulic retention time (HRT) of 12 hours. On day 7, the HRT was decreased to 6 hours. The thermophilic FBR was operated in continuous mode for a total of 185 days. It was initially operated at 55 (± 1) °C. Temperature was then increased to 60 (± 1) °C on day 77, to 65 (± 1) °C on day 119, and to 70 (± 1) °C on day 158 until day 185. The mesophilic FBR was operated at 37 (± 1) °C for 185 days, and then at 55 (± 1) °C until day 228.

Influent and effluent samples, and gas samples, were collected from the sampling points specified in Figure 1. Biofilm-containing activated carbon was collected from the thermophilic FBR before every change in operating conditions (plus a mid-term sample collected on day 53), and stored at -20 °C for DNA analysis. Samples from the mesophilic FBR were also collected on the same days. An additional sample of carrier material was collected from the thermophilic FBR on day 119 (60 °C), 158 (65 °C), and 185 (70 °C) and, from the mesophilic reactor on day 185 (37 °C), and 228 (55 °C), and stored at -80 °C for RNA-level analysis. The collection system consisted of a syringe connected to a tube, which was used to suck 4–6 mL biomass out of the FBR bed. While collecting the biomass samples, the FBRs were open from the top but flushed with N_2 to avoid exposure to air.

2.5 Chemical analysis

Influent and effluent composition was determined either with GC-FID according to Kinnunen et al. [34], or with HPLC as reported by Dessì et al. [16]. Gas samples were analysed for H_2, CH_4, and CO_2 with a Shimadzu gas chromatograph GC-2014 equipped with a Porapak N column (80/100 mesh) and a thermal conductivity detector. Oven, injector and detector temperatures were at 80, 110 and 110 °C, respectively.

2.6 Microbiological analysis

DNA extraction and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of the partial 16S rRNA gene were performed according to Mäkinen et al. [35]. The forward and reverse primers for PCR were GC-BacV3f and 907r, respectively. The visible bands in the polyacrylamide gel were cut by using a surgical blade, eluted in sterile water and re-amplified by PCR (primers BacV3f and 907r) before sending them to Macrogen (South Korea) for sequencing as described by Koskinen et al. [36]. The nucleotide sequences obtained were analysed using Bio-Edit

209 [37] software version 7.2.5, and compared with the sequences in the GenBank nucleotide collection
210 database using BLAST software [38].
211
212 Nucleic acids for MiSeq analysis were co-extracted from the biofilm-containing activated carbon
213 samples according to Griffiths et al. [39], but 3M sodium acetate (1/10 of sample volume) and cold
214 (-20 °C) 100% isopropanol (1 sample volume) were added for precipitation instead of polyethelene
215 glycol. Furthermore, nucleic acids were re-suspended in sterile water instead of tris-EDTA buffer.
216 DNA and RNA were quantified by a Nanodrop spectrophotometer (NanoDrop Technologies, USA).
217 Their quality was assessed by measuring the absorbance ratio at 260/280 nm, and 260/230 nm
218 wavelength. Nucleic acid samples were diluted to a final concentration of 25 ng mL^{-1}. DNA was
219 removed by adding 1 µL turbo DNase and 2.5 µL turbo DNase buffer (Invitrogen, Thermo Fisher,
220 USA), followed by incubation at 37 °C for 30 minutes. DNase was then inactivated by addition of
221 2.5 µL DNase inactivator (Invitrogen). After centrifugation (10000xg, 1.5 minutes), the RNA-
222 containing supernatant was transferred to a fresh RNase free tube. The absence of DNA was
223 confirmed by 16S rRNA gene PCR (primers 338f and 805r) followed by electrophoresis in 1%
224 agarose gel (no bands obtained). Complementary DNA (cDNA) was obtained from RNA using M-
225 MuLV Reverse Transcriptase (New England, BioLabs, USA), following the instructions provided by
226 the supplier. The success of the reverse transcription was confirmed by 16S rRNA gene PCR (primers
227 338f and 805r) and electrophoresis in 1% agarose gels (bands appeared). Samples of cDNA were sent
228 to FISABIO (Valencia, Spain) for high-throughput sequencing of partial 16S rRNA genes on an
229 Illumina MiSeq platform. Forward and reverse primers for PCR were 515f and 806r, respectively
230 [40]. A total of 427,163 raw sequences was obtained from 5 samples. Sequence screening, alignment
231 to Silva (v123) database, clustering, chimeras removal and taxonomic classification (cut-off = 97%)
232 were performed using Mothur v1.39.3 [41], following the procedure described by Kozich et al. [42].
233

234 *2.7 Net energy gain calculation*

235 The net energy gain (kJ L^{-1}) (Equation 2) was estimated by the sum of the energy recovered by
236 combustion of the H_2 produced per litre of wastewater treated (Equation 3) and the energy required
237 to heat the FBR to the desired temperature (Equation 4) [22]:

238

239 Net energy gain: $NE_G = E_G + E_L$ (2)

240 Energy gain: $E_G = Y_H \cdot MV_H \cdot C_X \cdot \rho_H \cdot LHV_H$ (3)

241 Energy loss: $E_L = C_W \cdot (T_F - T_I) \cdot \rho_W$ (4)

242

243 where Y_H is the H_2 yield (mol H_2 mol^{-1} xylose), MV_H is the molar volume of H_2 (22.414 L mol^{-1}), C_X
244 is the influent xylose concentration (50 mol L^{-1}), ρ_H is the density of gaseous H_2 (8.9×10^{-5} kg L^{-1}),
245 LHV_H is the lower heating value of H_2 (120×10^3 kJ kg^{-1}), C_W is the specific heat of water (4.2 kJ
246 kg^{-1} K^{-1}), T_F and T_I (K) is the temperature of the wastewater after and before heating, respectively,
247 and ρ_W is the density of water (1 kg L^{-1}).

248

249 *2.8 Statistical analysis*

250 To assess significant differences in H_2 yield at the various temperatures investigated, analysis of
251 variance (ANOVA) and the Tukey test [43] at $p = 0.05$ were conducted using the IBM SPSS Statistics
252 package. The statistical analysis was conducted on the last 4 sampling points collected at the various
253 temperatures investigated in both the mesophilic and thermophilic FBR (8-10 operation days). The
254 output of the statistical analysis is provided in the supporting material (File S1).

255

256 **3. Results**

257 *3.1 H_2 production in the thermophilic FBR*

For all the temperatures investigated, the thermophilic FBR yielded more H_2 than the mesophilic FBR (Figure 2a versus 2b). At 55 °C (days 1-77), the H_2 yield increased steadily in the first days of operation, reaching 1.2 mol H_2 mol^{-1} xylose on day 18, and remaining relatively stable on days 18–25 (Figure 2a). In the following days, due to a problem with the liquid-gas separator (foam produced by the FBR partially clogged the gas line and part of the gas was likely lost with the effluent), the H_2 yield decreased reaching a minimum of 0.7 mol H_2 mol^{-1} xylose on day 35. On day 52, after solving the issue by washing the liquid-gas separator, the H_2 yield increased sharply reaching a maximum of about 1.3 mol H_2 mol^{-1} xylose on day 56 and then stabilising to 1.2 mol H_2 mol^{-1} xylose on days 65–77 (Figure 2a). On days 7–77, xylose was detected in the effluent at a concentration < 2.5 mM (> 95% removal). Acetate and butyrate were the main metabolites produced. Their concentration in the effluent increased on days 1–21, reaching a concentration of about 30 and 35 mM for acetate and butyrate, respectively (Figure 2c). Ethanol was produced in the first days of operation, reaching a maximum concentration of about 10 mM on day 7 before decreasing to < 2 mM on day 27. On days 65–77, the acetate concentration ranged between 20 and 24 mM, while the butyrate concentration ranged between 19 and 25 mM (Figure 2c).

After increasing the temperature to 60 °C on day 77, the H_2 yield remained stable at 1.2 mol H_2 mol^{-1} xylose for one day and then decreased to a minimum of 0.6 mol H_2 mol^{-1} xylose on day 81 (Figure 2a). H_2 remained low on days 81–91 and started to increase again, reaching a maximum of about 1.0 mol H_2 mol^{-1} xylose on day 95. From day 98, the H_2 yield slightly decreased again and stabilised to 0.8 mol H_2 mol^{-1} xylose on days 112-119 (Figure 2a). The increase in temperature caused an increase in the VFAs concentration in the effluent (Figure 2c). The acetate concentration almost doubled, ranging between 29 and 42 mM on days 88–119. The butyrate concentration increased as well, ranging between 25 and 35 mM on days 88–119 (Figure 2c).

283 At 65 °C (days 120–158), the average H_2 yield was comparable to the yield obtained at 60 °C, but the
284 production was more unstable compared to both 55 °C and 60 °C, ranging between 0.7 and 1.3 mol
285 H_2 mol^{-1} xylose (Figure 2a). Increasing the temperature to 65 °C had minimal impact on the VFA
286 concentration when compared to the 60 °C condition (Figure 2c).
287
288 After increasing the temperature further to 70 °C, H_2 production ceased on day 161, but increased
289 again from day 163, stabilising to values comparable to those obtained at 55 °C (1.2 mol H_2 mol^{-1}
290 xylose) on days 172–185 (Figure 2a). A high concentration of xylose was detected in the effluent on
291 days 163–174, with a peak of about 50 mM (0% removal) on day 163. On days 163–174, the acetate
292 and butyrate concentration decreased and slowly increased again, reaching values comparable to the
293 ones obtained at 60 and 65 °C (21–32 mM acetate and 22–32 mM butyrate) on days 175–185 (Figure
294 2c).
295
296 *3.2 H_2 production in the mesophilic FBR*
297 The mesophilic FBR had a fluctuating H_2 production, especially during days 1–90. The H_2 yield was
298 above 1.0 mol H_2 mol^{-1} xylose only on days 53–65. On day 7, 74 and 81, the H_2 yield ceased and
299 increased again within 1–3 days. On days 90–185, the H_2 production fluctuated between 0.3 and 1.0
300 mol H_2 mol^{-1} xylose. Between days 0–185, the xylose concentration in the effluent was < 5 mM (>
301 90% removal).
302
303 Similar to the H_2 yield, acetate and butyrate production was unstable during days 1–90 (Figure 2d).
304 The acetate concentration was often close to 40 mM and was even higher on day 18 and 76.
305 Interestingly, the peaks of acetate (Figure 2d) seem to mirror the drops in H_2 yield (Figure 2b). On
306 days 90–185, the butyrate concentration ranged between 10 and 19 mM, while the acetate
307 concentration was more unstable, ranging between 20 and 36 mM (Figure 2d).

After increasing the temperature to 55 °C (day 186), H_2 production ceased on day 206 (Figure 2b), and then increased sharply to values comparable to those obtained in the thermophilic FBR at 55 °C on days 219-228 (1.0 mol H_2 mol^{-1} xylose). After raising the temperature from 37 to 55 °C, xylose was still consumed (> 97%). Ethanol was detected in the effluent on days 195–213, with a maximum of 15 mM on day 208 (Figure 2d). However, the liquid phase composition was similar to the one obtained in the thermophilic FBR at 55 °C on days 217–228 (about 18 and 22 mM acetate and butyrate, respectively).

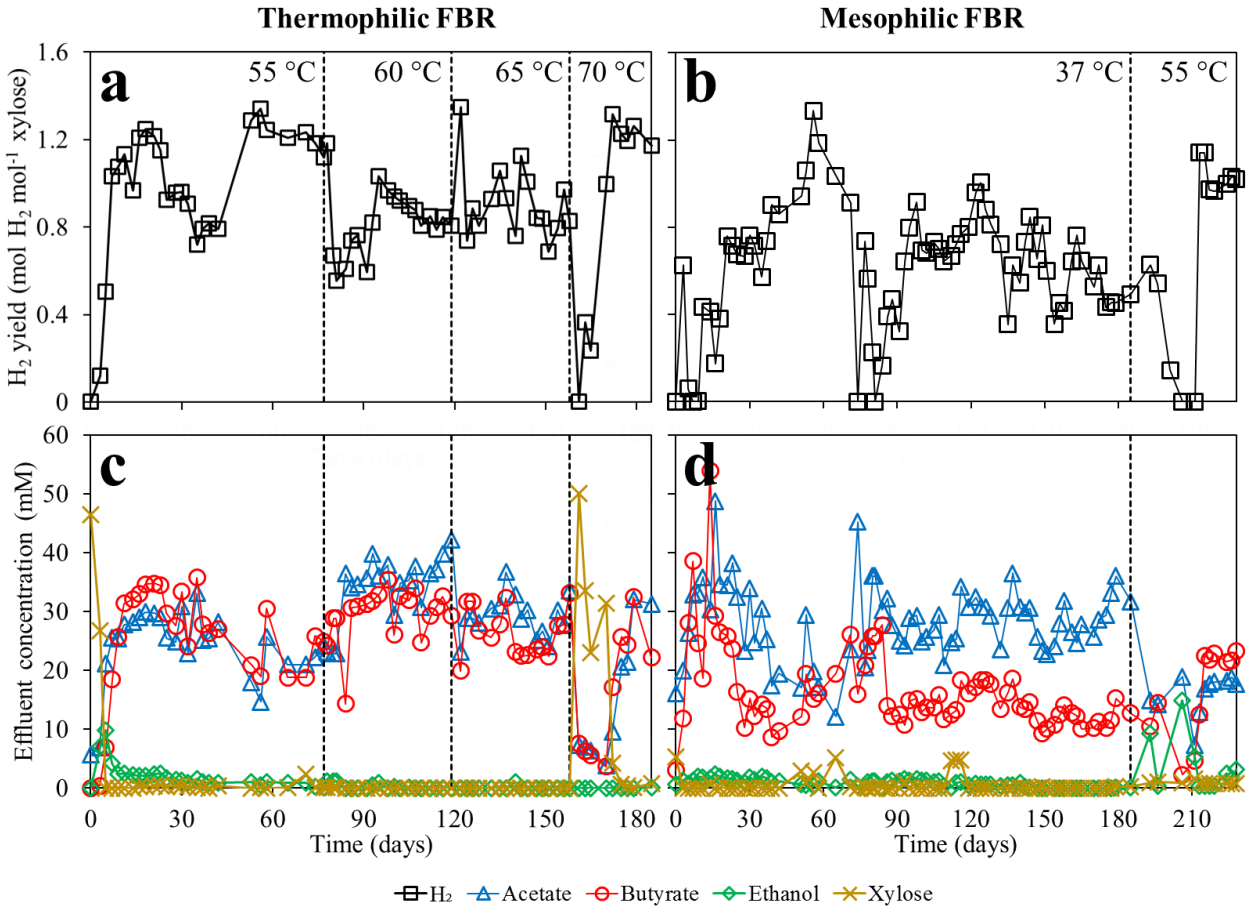

Figure 2. H_2 yield (mol H_2 mol^{-1} xylose added) (a, b) and acetate, butyrate, ethanol and xylose concentration (mM) in the effluent (c, d) of the thermophilic (a, c) and mesophilic (b, d) FBR. The vertical dotted lines indicate a change of temperature.

321

322 *3.3 Microbial community analysis*

323 Different eubacterial community profiles from the thermophilic and mesophilic FBR were obtained
324 by PCR-DGGE analysis from the biofilm-containing activated carbon (Figure 3; Table 1). In the
325 thermophilic FBR, *Clostridium acetobutylicum* (100% similarity) was found at 55 °C on day 0 (after
326 two days of start-up in batch mode), but was not detected in subsequent samples (Figure 3). On days
327 7–185, regardless the temperature, *Thermoanaerobacter thermosaccharolitycum* (98–99% similarity)
328 was the dominant microorganism in the thermophilic FBR (Figure 3). *Alicyclobacillus* sp. (96–97%
329 similarity) was found on day 7, after which its concentration was below detection limit at 55 °C on
330 day 77 and at 60 °C on day 119, but it was detected again when temperature was further increased to
331 65 and 70 °C (Figure 3).

332

333 A wider eubacterial community was found in the mesophilic FBR compared to the thermophilic FBR.
334 On day 0 and day 7, nucleotide sequences with 99−100% similarity to *Clostridium pasteurianum*
335 were detected. Species with 99−100% similarity to *Clostridium acetobutylicum* were detected on all
336 sampling days (days 7−185). On day 77, nucleotide sequences close to *Pseudomonas* and *Delftia* sp.
337 were also found. On days 119, 158, and 185, the DGGE profiles of the mesophilic community were
338 similar to each other and dominated by *Clostridium* sp. (Figure 3).

339

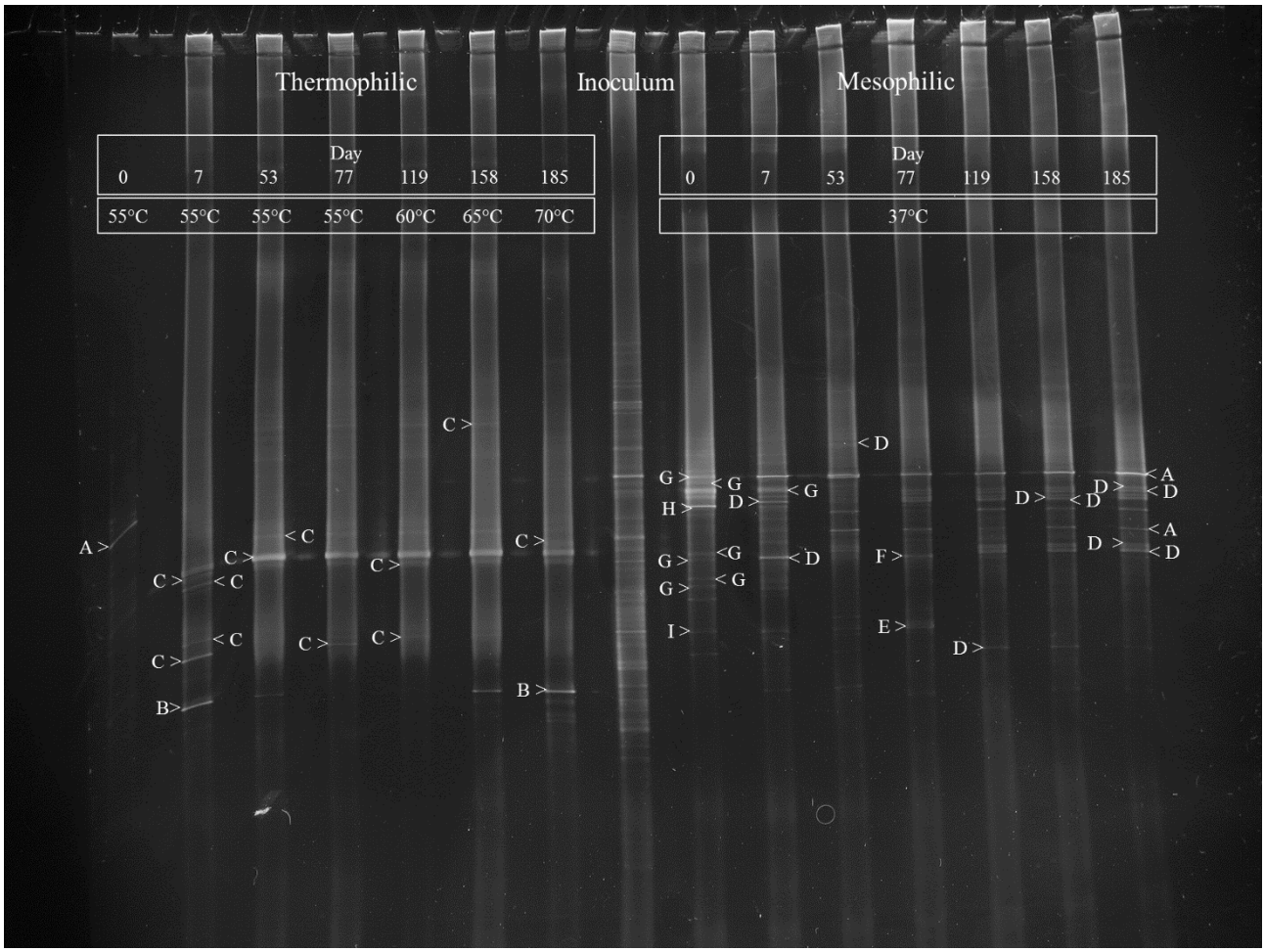

Figure 3. Microbial community profiles obtained by PCR−DGGE from biofilm-containing activated carbon samples collected from the thermophilic and the mesophilic H_2 producing FBR. The band labels refer to Table 1.

353 **Table 1.** Association of 16S rRNA gene sequences of DGGE bands to those collected in the GenBank.

BL[a]	Affiliation[b]	Accession number	Matching sequence lenght[c]	Similarity (%)[d]
A	*Clostridium acetobutylicum*	KP410577 KP410579	446−472	99−100
B	*Alicyclobacillus* sp.	JX505129	485−524	96−97
C	*Thermoanaerobacter thermosaccharolyticum*	JX984968 KJ831072 AF247003	405−499	98−99
D	*Clostridium* sp.	EU887966 EU887970 KU886097 LC020495	367−491	94−99
E	*Pseudomonas* sp.	GQ903481	442	97
F	*Delftia* sp.	KT865666	465	99
G	*Clostridium pasteurianum*	KX378861	440−506	99−100
H	*Ralstonia* sp.	KT183537	496	99
I	*Lysobacter* sp.	JQ791571	429	93

354 [a] Band label in Figure 3a

355 [b] Closest cultured species in GenBank

356 [c] Number of nucleotide pairs used in the sequence comparison

357 [d] Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest species in GenBank

358

359 Based on the high-throughput sequencing of the reverse-transcribed 16S rRNA (Figure 4), species
360 belonging to the *Thermoanaerobacterium* genus dominated the active microbial community (> 99%
361 of the relative abundance) of the thermophilic FBR at both 60 and 70 °C (days 119 and 185). At 65
362 °C (day 158), about 22% of the relative abundance matched the genus *Clostridium* (Figure 4). In the
363 mesophilic FBR, microorganisms of the genus *Clostridium* and *Ruminiclostridium* dominated the

active microbial community at 37 °C (Figure 4). Increasing the FBR temperature to 55 °C resulted in a shift of the active microbial community towards the *Thermoanaerobacterium* genus (>90% of the relative abundance).

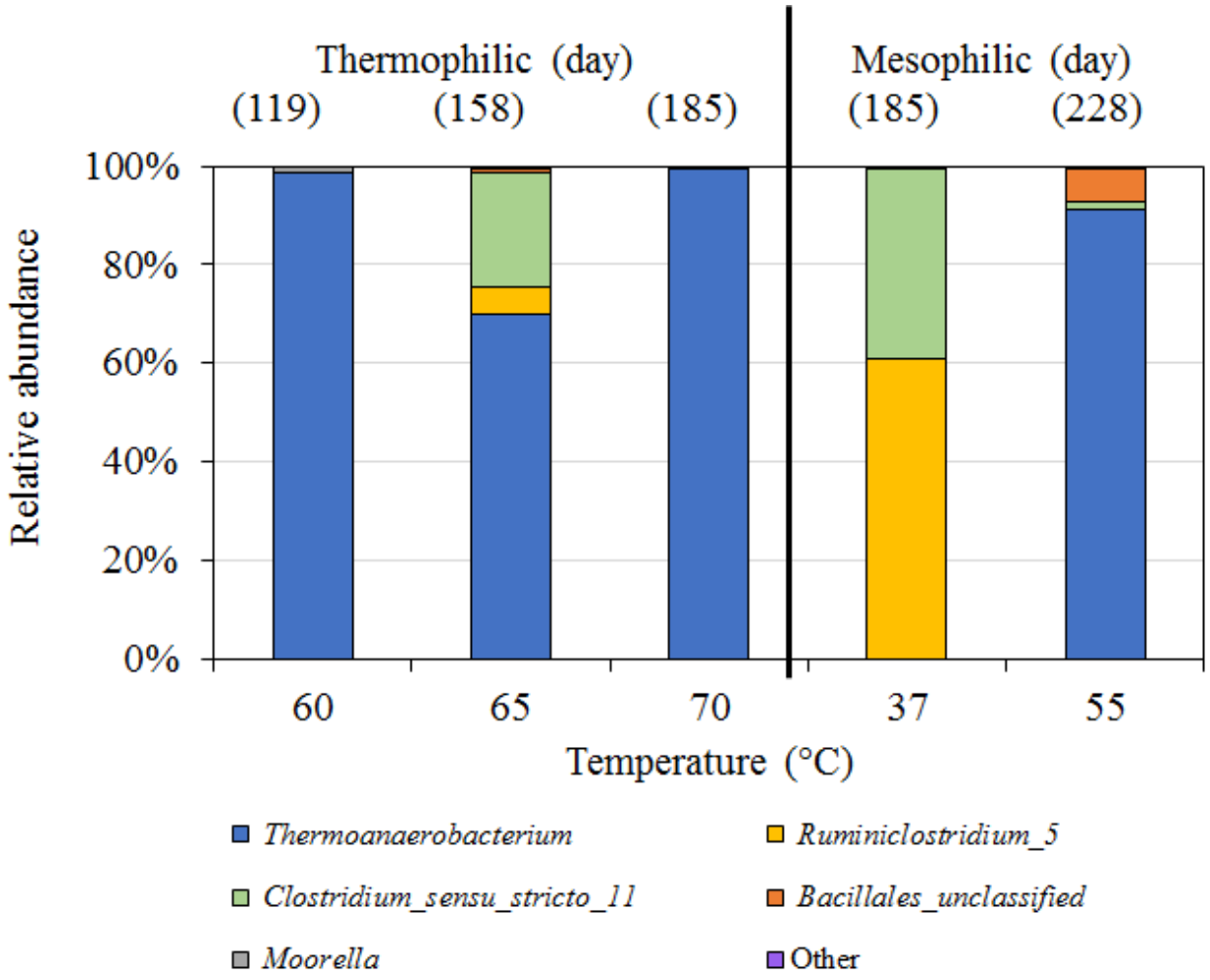

Figure 4. Relative abundance of the active microbial community, classified at genus level, obtained by MiSeq sequencing analysis of the 16S rRNA from biofilm-containing activated carbon samples, reverse transcribed to 16S cDNA. "Other" refers to the sum of genus with a relative abundance < 1%.

4. Discussion

4.1 Thermophilic H_2 production and microbial community dynamics

375 The significantly higher and more stable H_2 yield obtained when performing dark fermentation of
376 xylose under thermophilic than under mesophilic conditions (Figure 2a versus 2b) was mainly due to
377 the composition of the microbial community (Figure 3; Table 1), particularly the active microbial
378 community (Figure 4). Generally, thermophilic microorganisms yield more H_2 than mesophilic
379 species [5]. A thermophilic community dominated by *Thermoanaerobacterium*, the prevalent active
380 genus in the thermophilic FBR regardless of the temperature applied (Figure 4), has already been
381 reported to yield more H_2 than a mesophilic community dominated by *Clostridium* [33]. Furthermore,
382 thermophilic conditions are not favourable for most H_2 consuming microorganisms [44].
383
384 For all the temperatures investigated (55–70 °C), the thermophilic microbial community was
385 dominated by microorganisms closely related to *Thermoanaerobacter thermosaccharolyticum*
386 (Figure 3; Table 1). Accordingly, the genus *Thermoanaerobacterium* was prevailing in the active
387 thermophilic microbial community at 60, 65 and 70 °C (Figure 4). In the thermophilic FBR, although
388 the sample at 55 °C is missing in the active microbial community analysis (Figure 4), the DGGE
389 profile (Figure 3) and the reactor performance (Figure 2a), suggest that *Thermoanaerobacterium* was
390 also the prevalent active genus at 55 °C. In fact, even in the mesophilic FBR, *Thermoanaerobacterium*
391 became dominant upon increasing the temperature from 37 to 55 °C (Figure 4). *T.*
392 *thermosaccharolyticum* has been utilised in batch reactors to produce H_2 from xylose at various
393 temperatures, obtaining a maximum yield of about 2.2 mol H_2 mol^{-1} xylose at 60 °C [45,46].
394 However, in this study, the highest H_2 yield was only about 1.2 mol H_2 mol^{-1} xylose at 55 °C and
395 even 30% lower at 60 °C (Figure 2a), although DGGE profiles appear similar (Figure 3). One
396 explanation is that, in this study, the pH was set to 5.0, whereas the optimum pH for *T.*
397 *thermosaccharolyticum* is 6.5 [46]. Furthermore, Koskinen et al. [36] showed that in a mesophilic
398 H_2-producing FBR, the attached microbial community is slightly different than the suspended one,

399 which was not analysed in this study. It is thus plausible that the contribution of some H_2 producing
400 or H_2 consuming microorganism on the net H_2 yield was not considered.
401
402 In the thermophilic FBR, sequences belonging to the *Clostridium* genus were detected only at 65 °C,
403 accounting for 22% of the active microbial community (Figure 4). However, at 65 °C, *Clostridium*
404 was not detected by PCR-DGGE. A possible explanation is that the concentration of *Clostridium* was
405 under the detection limit of PCR-DGGE, but these microorganisms were particularly active at 65 °C
406 and thus detected through the RNA-level sequencing analysis [32]. The activity of *Clostridium* is
407 likely linked to the unstable H_2 yield obtained at 65 °C, as some *Clostridium* sp., such as the
408 thermophilic *C. thermoaceticum*, may oxidize sugars through competitive pathways to H_2 or can even
409 carry out homoacetogenesis [47]. The role of *Clostridium* in the xylose degradation at 65 °C could
410 be further detailed by a proteomics analysis. At 65 °C, a bacterium closely related to *Alicyclobacillus*
411 sp. appeared in the DGGE profile (Figure 3). Genera with high similarity (99%) to *Alyciclobacillus*
412 sp. have been found in a thermophilic FBR used for dark fermentation of cheese whey [48], but further
413 studies are required to understand the role of this microorganism in the anaerobic processes.
414
415 The drop and subsequent increase in H_2 yield that occurred during the first days of operation at 70
416 °C, as well as the decreased xylose removal efficiency during these days (Figure 2a and 2c) can be
417 attributed to the acclimation of the microbial community to the higher temperature. Meng et al. [49]
418 performed a proteomic study on *Thermoanaerobacter tengcongensis*, cultured at 55, 75 and 80 °C,
419 and showed the existence of temperature-dependent protein complexes, which may affect the H_2
420 yield. The stable H_2 yield obtained at 70 °C after the acclimation (Figure 2a) is due to the homogeneity
421 of the active microbial community growing on the carrier material at that temperature: > 99% of the
422 total cDNA sequences at 70 °C matched the genus *Thermoanaerobacterium* (Figure 4).
423

The highest yield of 1.2 mol H_2 mol^{-1} xylose was obtained at both 55 and 70 °C, whereas the H_2 yield was significantly lower (0.8 mol H_2 mol^{-1} xylose) at 60 and 65 °C. Similarly, Yokoyama et al. [50] studied, in batch, dark fermentation of cow waste in the temperature range 37–85 °C, obtaining the highest H_2 production at 60 °C and 75 °C, and a lower H_2 production at 67 °C. However, their cultures were dominated by *Clostridium* sp. and *Caldanaerobacter* sp. at 60 and 75 °C, respectively, in contrast to this study with *Thermoanaerobacter* sp. as the dominant microorganism at both 55 and 70 °C.

4.2 Mesophilic H_2 production and microbial community dynamics

The lower H_2 yield obtained under mesophilic conditions (Figure 2b) was attributed to the microbial community. The DGGE profiles obtained on days 119, 158, and 185 were similar to each other and mainly composed of *Clostridium* (Figure 3; Table 1), which was also shown to be the dominant active genus together with the closely related *Ruminiclostridium* genus (Figure 4). Similarly, Si et al. [51] studied the microbial diversity of their mesophilic H_2 producing reactors by MiSeq sequencing, reporting *Clostridiaceae* as the most abundant family. Chatellard et al. [52] showed that, in particular, *Clostridium* dominated mesophilic microbial communities fermenting pentose-based substrates, as was also the case in this study.

Mesophilic H_2 production was unstable (Figure 2b), likely due to the accumulation of VFAs (Figure 2d). At high concentrations, undissociated VFAs penetrate the cell membrane, lowering the internal pH and inhibiting H_2 production [14]. In fact, the H_2 yield (as well as the acetate and butyrate concentration) cyclically increased and decreased in the mesophilic FBR between days 91–185, and the H_2 yield was higher when the concentration of VFAs was lower (Figure 2b and 2d). Wang et al. [53] reported a decrease in H_2 yield at acetate concentrations higher than 50 mM. In this study, a drop in H_2 yield occurred at acetate concentrations of 40 mM.

449

450 The significantly higher H_2 yield obtained after increasing the temperature of the mesophilic FBR
451 from 37 to 55 °C (Figure 2b) is clearly due to the shift in the active microbial community from the
452 *Clostridium* and *Ruminiclostridium* genus to the *Thermoanaerobacterium* genus (Figure 4).
453 Interestingly, the H_2 yield at 55 °C remained comparable to the one obtained at 37 °C for 13 days,
454 suggesting that the change in the dominant active community did not occur immediately upon the
455 temperature change. The production of ethanol upon exposing the microbial community to 55 °C,
456 and the subsequent shift to acetate, butyrate and H_2 production, was observed also in the first days of
457 operation in the thermophilic FBR (days 1-20, Figure 2c) and in a previous batch study with the same
458 inoculum [16]. This can be attributed to either a change in the microbial community or a gradual shift
459 from ethanol to butyrate fermentation as a response to the temperature shift [16].
460
461 *4.3 The role of homoacetogenesis on mesophilic and thermophilic H_2 yield*
462 A decrease in H_2 yield to below 0.2 mol H_2 mol^{-1} xylose occurred in the mesophilic FBR on days 9,
463 16, 74, and 89 (Figure 2b). On the same days, the CO_2 concentration also decreased (File S2 in the
464 supporting material) and a peak of 35–50 mM acetate was detected within 1–2 days from the H_2
465 decrease (Figure 2d), suggesting the occurrence of homoacetogenesis. H_2 and CO_2 were likely
466 consumed to produce acetate autotrophically. Several *Clostridium* sp., which dominated the active
467 mesophilic microbial community, are known homoacetogens [54]. The causes that trigger
468 microorganisms from heterotrophic to the less energy yielding autotrophic metabolism are
469 controversial. A H_2 partial pressure > 500 Pa can favour homocetogenesis and H_2 can be
470 simultaneously produced and consumed, but radioactive label tracking techniques would be required
471 to distinguish the two processes [10]. Oh et al. [55] argued that in mixed culture fermentation, the
472 switch to autotrophic metabolism occurs only after substrate depletion. This hypothesis seems less
473 probable in the studied FBR, as xylose was fed continuously. However, it is plausible that H_2

474 producing microorganisms quickly consume the xylose, inducing the facultative bacteria to shift to
475 autotrophic metabolism.
476
477 Koskinen et al. [36] studied the microbial community dynamics over time in a mesophilic (35 °C)
478 FBR reactor inoculated with digested activated sludge and concluded that the adhesion of H_2
479 consuming microorganisms, including homoacetogens, to the carrier material may cause an unstable
480 H_2 production. Similarly to this study, Dinamarca and Bakke [56] reported a decrease from 1.5 to
481 below 0.25 mol H_2 mol^{-1} glucose after 57 days of reactor operation at 35 °C. The authors concluded
482 that homoacetogenesis is directly correlated with the HRT and dependent on biomass density and
483 sludge age [56]. Also Luo et al. [33] argued that, even if the inoculum is pretreated, methanogenic
484 and homoacetogenic microorganisms could develop again during long-term operation. Methanogens
485 are typically inhibited by a pH below 6, while homoacetogenic bacteria are also inhibited by a pH
486 below 6, but only under thermophilic conditions [33].
487
488 In the thermophilic FBR, excluding day 161 on which a decrease in H_2 yield was attributed to the
489 increased temperature, sudden drops in H_2 yield were not observed (Figure 2a) and the acetate
490 concentration was more stable (Figure 2c). This indicates a minor role of homoacetogenesis at
491 thermophilic conditions. This is in agreement with Luo et al. [33], who reported no homoacetogenesis
492 in thermophilic (55 °C) batch incubations at pH 5.5. Their thermophilic microbial community was
493 dominated by *Thermoanaerobacter* sp., as was the case in this study.
494
495 *4.4 Comparison of H_2 production with previous studies*
496 At 55 °C, despite the HRT of only 6 hours, the H_2 yield per mol xylose added is consistent with a
497 previous batch study with same inoculum and substrate [16]. At 70 °C, however, H_2 was effectively
498 produced during FBR operation, but not in the batch incubation [16], likely due to the longer time for

499 acclimation of the biomass to the high temperature. The maximum H_2 production rate (HPR) of 282.1
500 mL H_2 h^{-1} L^{-1} obtained at 70 °C is among the highest reported in continuous studies on thermophilic
501 dark fermentation of sugars by mixed cultures (Table 2). This is likely due to the composition of the
502 active microbial community, dominated by an effective H_2 producer such as *Thermoanaerobacterium*
503 (Figure 4). A 12-times higher HPR (3470 mL H_2 h^{-1} L^{-1}) has been obtained with a pure culture of *T.*
504 *thermosaccharolyticum* immobilized on heat-treated methanogenic granules [57], but the sugar
505 concentration (in terms of chemical oxygen demand) was almost 3 times higher than the concentration
506 used in this study. Furthermore, O-Thong et al. [57] obtained the maximum HPR at an HRT of only
507 1 hour, whereas in this study the HRT was set to 6 hours.
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527 **Table 2.** Highest stable H_2 production rate (HPR) obtained by dark fermentation of simple sugars in various continuous

528 studies conducted at high temperature (T ≥ 55°C) and using different inoculum, reactor type, pH and HRT.

Inoculum	**Pretreatment**	**Reactor type[a]**	**Substrate (g COD L^{-1})**	**T (°C)**	**pH[b]**	**HRT (h)**	**HPR (mL H_2 h^{-1} L^{-1})**	**Reference**
Biomass from H_2-producing CSTR	-	UASB	Sucrose (11.2)	55	4.5-5.0 (nc)	3	112.5	[58]
Sludge from anaerobic digester	Heat treatment (105°C, 5 min)	UASB	Sucrose (11.2)	55	5.0-5.5 (nc)	1.5	124.2	[59]
Anaerobic sludge from previously operated reactor	Heat treatment (80°C, 60min)	FBR	Sucrose (5.0)	60	5.5 (i)	12	60.5	[60]
Sludge from H_2 producing CSTR	-	TBR	Glucose (7.3)	60	5.5 (c)	2	980.6	[24]
Thermoanaerobacter thermosaccharolyticum immobilised on methanogenic granules	Heat treatment (121°C, 30min)	UASB	Sucrose (22.5)	60	5.0-5.5 (nc)	1	3470	[57]
Biomass from H_2-producing CSTR	-	CSTR	Xylose (1.1)	70	6.7 (nc)	72	2.6	[61]
Sludge from H_2 producing CSTR	-	UASB	Glucose (2.1)	70	7.0 (i)	24	12.7	[25]
Methanogenic biomass from CSTR	Chemical treatment (BES)[c]	UASB	Glucose (4.8)	70	7.2 (i)	27	47.3	[62]
Anaerobic sludge from thermophilic CSTR	-	CSTR	Glucose (4.6)	70	5.5 (c)	21.6	91.7	[26]
Activated sludge	**Heat treatment (90°C, 15 min)**	**FBR**	**Xylose (8.0)**	**70**	**5.0 (c)**	**6**	**282.1**	**This study**
Caldicellulosiruptor saccharolyticus	-	TBR	Sucrose (11.5)	73	6.5 (c)	3-5	493.1[d]	[63]

529 [a] Continuous stirred tank reactor (CSTR), expanded granular sludge blanket (EGSB), fluidised bed reactor (FBR),
530 sequential batch reactor (SBR), trickling bed reactor (TRB), upflow anaerobic sludge blanket (UASB).
531 [b] The reported pH refers either to the initial pH (i) or the operation pH, which can be either controlled to a stable value (c)
532 or not controlled (nc).
533 [c] Added to the reactor feeding.
534 [d] Per L of filtering bed.
535

4.5 Practical implications

Organic carbon-rich wastewaters are produced by industries at various temperatures. The energy gain from combustion of the H_2 produced and the energy required to heat the FBR to the desired temperature are two important aspects to take into consideration for a proper economic analysis. For a rough estimation of the effect of operation and feed wastewater temperature on energy gain of a H_2 producing system, a correlation between the wastewater temperature, fermentation temperature and net energy gain is presented in Figure 5.

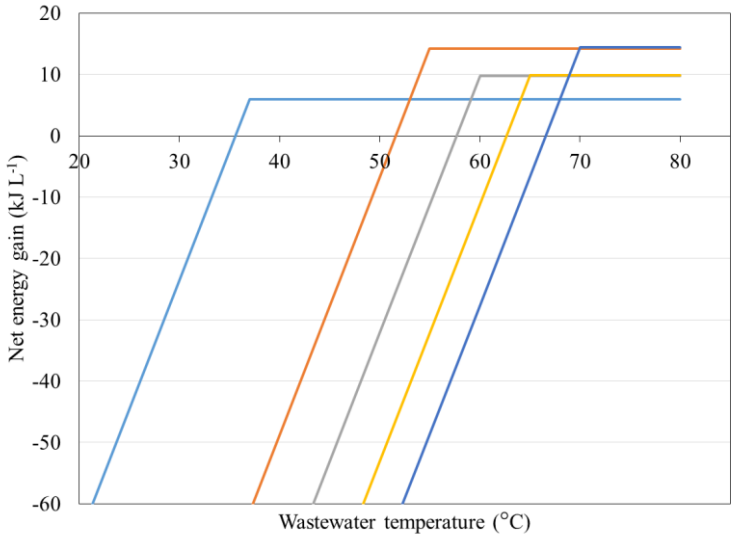

Figure 5. Effect of fermentation temperature on net energy gain from a xylose containing wastewater. X-axis shows the hypothetical temperature at which the wastewater is released. The coloured lines represent the net energy gain obtainable per litre of xylose (50 mM) containing wastewater treated under mesophilic (37 °C) or thermophilic (55, 60, 65, 70 °C) conditions. The net energy gain was calculated according to Perera et al. [22] from the average H_2 yields of the last 4 sampling points collected at the various temperatures investigated in both the mesophilic and thermophilic FBR (8-10 operation days).

553 Despite the comparatively low H_2 yield obtained (Figure 2b), dark fermentation at 37 °C is still the
554 best option to treat wastewaters produced at temperatures up to about 50 °C based on the net energy
555 gain. Thermophilic treatment at 55 °C is to be preferred for wastewaters produced at above 50 °C,
556 and the net energy gain obtained at 70 °C is comparable to the one obtained at 55 °C for wastewaters
557 produced at temperatures exceeding 70 °C (Figure 5). However, many other aspects can influence the
558 net energy gain both in a positive (heat recovery from hot wastewater, energy content of the effluent
559 and removal of pathogens) or negative (heat losses, H_2 to energy conversion efficiency and
560 maintenance and operation costs of the FBRs) way. Thermophilic processes could be advantageous
561 to treat wastewaters produced at high temperature. The data presented in this study, both on FBR
562 performance and composition of the active microbial community, helps understanding the process
563 and selecting the operation temperature in H_2 producing bioreactors depending on the temperature of
564 the waste stream to be treated. However, more research is required to further increase the net energy
565 gain. Post-treatment of the dark fermentation effluent, for recovery of value-added products such as
566 VFAs and alcohols or for further energy harvesting (e.g., H_2 production through photo-fermentation
567 or microbial electrolysis cells, methane production through anaerobic digestion or bioelectricity
568 production using microbial fuel cells) is a key factor towards process scale-up [7,64].
569
570 **5. Conclusions**
571 *Thermoanaerobacterium* dominated the thermophilic active microbial community, resulting in a
572 higher and more stable H_2 yield in the thermophilic FBR compared to the mesophilic FBR dominated
573 by *Clostridium*. Treating high temperature, xylose-containing wastewaters by thermophilic dark
574 fermentation can thus lead to a higher energy output compared to the mesophilic counterpart.
575 Temperatures of 55 and 70 °C resulted in the net maximum H_2 yield of 1.2 mol H_2 mol^{-1} xylose,
576 whereas the competition by *Clostridium* caused unstable H_2 production at 65 °C. This study
577 contributes to the understanding of dark fermentation of xylose in FBRs, and the microorganisms

578 actively involved in the mesophilic or thermophilic process, which supports the development of high
579 rate H_2 producing bioreactors.
580
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585
586 **Conflict of interest**
587 The authors declare no conflict of interest.
588
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593
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