Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70°C

Paolo Dessì [a,*], *Aino–Maija Lakaniemi* [a], *Piet N. L. Lens* [a,b]

[a]*Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, P.O. Box 541, FI-33101 Tampere, Finland*

[b]*UNESCO–IHE, Institute for Water Education, Westvest 7, 2611AX Delft, The Netherlands*

Manuscript submitted to Water Research

*Corresponding author:

Phone: +358 417239696, e-mail: paolo.dessi@tut.fi, mail: Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

Abstract

Two heat–treated inocula, fresh and digested activated sludge from the same municipal wastewater treatment plant, were compared for their H_2 production via dark fermentation at mesophilic (37°C), thermophilic (55°C) and hyperthermophilic (70°C) conditions using xylose as the substrate. At both 37 and 55°C, the fresh activated sludge yielded more H_2 than the digested sludge, whereas at 70°C, neither of the inocula produced H_2 effectively. A maximum yield of 1.85 mol H_2 per mol of xylose consumed was obtained at 55°C. H_2 production was linked to acetate and butyrate production, and there was a linear correlation ($R^2 = 0.96$) between the butyrate and H_2 yield for the fresh activated sludge inoculum at 55°C. Approximately 2.4 mol H_2 per mol of butyrate produced were obtained against a theoretical maximum of 2.0, suggesting that H_2 was produced via the acetate pathway prior to switching to the butyrate pathway due to the increased H_2 partial pressure. *Clostridia* sp. were the prevalent species at both 37 and 55°C, irrespectively of the inoculum type. Although the two inocula originated from the same plant, different thermophilic microorganisms were detected at 55°C. *Thermoanaerobacter* sp., detected only in the fresh activated sludge cultures, may have contributed to the high H_2 yield obtained with such an inoculum.

Keywords

Biohydrogen, inocula, temperature, xylose, butyrate, dark fermentation

1. Introduction

The intensive use of fossil fuels results in their rapid depletion and increased emission of greenhouse gases, in particular CO_2. Therefore, energy production is expected to shift towards renewable and more eco–friendly alternatives in the coming decades. Energy recovery from wastewaters can be a good strategy to pursue the double objective of sustainability and emission reduction. Many industries, such as the pulp and paper industry, produce wastewaters rich in organic compounds, which must be treated prior to discharge, but yet have a high potential for energy recovery (Rajeshwari et al., 2000). Traditional aerobic treatment is expensive, due to the huge amount of oxygen required to oxidize the organic compounds. In contrast, anaerobic processes allow coupling of wastewater treatment and energy production in the form of biogas (Kamali and Khodaparast, 2015).

Methane production from organic compounds is a well–developed technology, but hydrogen (H_2) production is a promising alternative as well because its heating value per gram is the highest among fuels, and because it does not release CO_2 to the atmosphere upon combustion (Dincer and Acar, 2015). Studies on biological H_2 production have focused on bio–photolysis of water, water gas–shift reaction, photo–fermentation and dark fermentation of organic compounds (Bundhoo and Mohee, 2016). The main advantages of dark fermentation over the other technologies are its high H_2 production rate, the simple operation (the reactor configurations are the same of the already well–established anaerobic digestion), and lower energy requirement (Show et al., 2012). Its main drawbacks are the relative low H_2 yield (mol H_2 per mol of substrate) and the formation of by–products, such as CO_2, volatile fatty acids and alcohols (Rittmann and Herwig, 2012).

Dark fermentation is a biological process in which fermentative bacteria produce H_2 to dispose of excessive electrons generated in the oxidation of organic compounds through a hydrogenase enzyme and electron carriers such as nicotinamide adenine dinucleotide (NADH) or reduced ferredoxin (Lee et al., 2011). The maximum H_2 yield by dark fermentation is reached if acetate is the only by–product of the oxidative process. The overall H_2 production is strongly affected by the inoculum and the operating conditions, such as temperature, pH, substrate concentration and H_2 partial pressure (Li and Fang, 2007). Depending on the operating conditions, part of the electrons can be directed to producing compounds more reduced than acetate, such as butyrate or ethanol, resulting in a lower H_2 yield (Li and Fang, 2007).

Temperature is a crucial parameter for most biotechnological processes, because different temperatures can reshape the microbial communities involved in the bioprocess (Karadag and Puhakka, 2010). Furthermore, increasing temperature positively affects both the kinetics and thermodynamics of the process (Verhaart et al., 2010). Thermophilic microorganisms are generally characterized by faster growth and reaction rates than mesophilic species. A direct conversion of sugars to acetate, which yields the maximum amount of H_2, is thermodynamically not favorable at low temperature, but becomes more favorable as the temperature increases, thus making proton reduction to H_2 coupled to NADH oxidation exergonic (Verhaart et al., 2010). Another advantage of high temperature processes is the reduced contamination by pathogens and H_2 consuming bacteria (Van Groenestijn et al., 2002). Industries produce wastewaters at various temperatures, and treating them at their original temperature, without heating or cooling, seems a cost–effective approach. For example, pulp and paper industries typically produce wastewaters with elevated temperatures (50–70°C), which are often cooled down to 30–40°C prior to biological treatment (Suvilampi et al., 2001).

98 Selection of the inoculum is also a key for a successful biohydrogen production process. From the
99 industrial point of view, dark fermentation with mixed cultures is preferable over pure cultures
100 because of easier operation and control, not requiring sterilization, and possibility to use a wide
101 range of feedstocks, as several different microorganisms are often required to degrade completely
102 complex substrates (Wang and Wan, 2009). However, mixed cultures may contain species that
103 degrade organic compounds by other pathways than H_2 production. Hydrogenotrophic
104 methanogens, propionate–producers, homoacetogens, and even sulfate and nitrate reducing bacteria
105 consume H_2 as a part of their metabolism (Bundhoo and Mohee, 2016). Though most H_2 consuming
106 bacteria are non–sporulating and can be removed by pretreating the inoculum, their complete
107 elimination cannot be ensured. For example, the thermophilic homoacetogenic bacterium *Moorella*
108 *glycerini* is a spore–forming microorganism (Slobodkin et al., 1997) and may resist the
109 pretreatment.
110
111 Heat treatment is the most common pretreatment used to select spore–forming, hydrogen–producing
112 microorganisms (Bundhoo et al., 2015). Many heat–treated inocula have been tested in dark
113 fermentation, including sewage sludge (Baghchehsaraee et al., 2008; Hasyim et al., 2011; Lin et al.,
114 2008), aerobic and anaerobic sludge from different plants treating organic waste (Bakonyi et al.,
115 2014; Cavalcante de Amorim et al., 2009), landfill leachate (Wong et al., 2014), hot spring cultures
116 (Koskinen et al., 2008), and compost (Cao et al., 2014). Despite the abundance of data available in
117 the literature, both on H_2 production and the microorganisms involved, the studies often differ in
118 their operating conditions, making it difficult to evaluate and distinguish the effect of the inoculum
119 on the process (Table 1). Although the combined effect of inoculum and temperature on dark
120 fermentation is of both scientific and practical interest, to our knowledge, a direct comparison of the
121 potential of two inocula for H_2 production at mesophilic, thermophilic and hyperthermophilic
122 conditions, keeping the other initial conditions stable, has not yet been performed.

Table 1.

This study aimed to compare two heat–treated inocula, activated sludge and digester sludge from the same municipal wastewater treatment plant, for biohydrogen production under mesophilic (37°C), thermophilic (55°C) and hyperthermophilic (70°C) conditions. Xylose, a pentose sugar commonly present in pulp and paper wastewater, was used as the substrate. The correlations between H_2 and soluble compounds produced via dark fermentation of xylose by the activated sludge inoculum were then determined in order to understand the metabolic pathways at 55°C, the temperature at which the H_2 yield was the highest.

2. Materials and methods

2.1 Source of biomass

The two sludge types used as inoculum were collected in July 2015 from the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland). The first sludge type was fresh activated sludge from the recirculation line between the outdoor aeration tank and the secondary settler. The average outdoor temperature in Tampere usually ranges between -6.7°C in February and +17.4°C in July, although winter temperatures below -20°C are also possible (Finnish Meteorological Institute, see: en.ilmatieteenlaitos.fi/statistics-from-1961-onwards). The second type was digester sludge from a mesophilic (35°C) anaerobic digester treating waste activated sludge. After settling and removing the supernatant, both sludge samples were divided in 10 mL batches to thin 15 mL anaerobic tubes, and heat treated at 90°C for 15 minutes (Maintinguer et al., 2011) by incubation in a pre–heated water bath prior to use as inoculum for the H_2 production experiments.

148 *2.2 Batch experimental set–up*

149 Batch assays were conducted in 120 mL serum bottles with a total working volume of 50 mL. The
150 growth medium was DSMZ 144 (German Collection of Microorganisms and Cell Cultures, 2008)
151 with the following modifications: tryptone was not added, the concentration of yeast extract was
152 reduced to 0.3 g L^{-1} (Nissilä et al., 2011) and xylose (7.50 g L^{-1}, 50 mM) was used as the substrate
153 instead of glucose. The pH of the growth medium was adjusted to 5.5 with 1 M HCl.

154

155 In the first culture, the bottles were inoculated with 11.4 mL activated sludge (8.8 ± 0.1 g VS L^{-1}) or
156 4.2 mL of digester sludge (24.0 ± 0.1 g VS L^{-1}), resulting in an inoculum concentration of about 2 g
157 VS L^{-1}, and medium was added up to 50 mL. The initial xylose concentration of the mixture
158 (medium and inoculum) was 50 mM. The following three batch cultures were inoculated by
159 transferring 5 mL of cultivation from the previous batch culture to 45 mL of fresh medium with
160 55.6 mM of xylose, in order to reach a final xylose concentration of 50 mM. To ensure anaerobic
161 conditions, the serum bottles were flushed with N_2 for 5–10 minutes before and after inoculation.
162 To avoid interference in the gas measurement due to the N_2 flushing, the pressure in the headspace
163 was equilibrated to atmospheric pressure by removing the excessive gas with a syringe before
164 starting the incubation. The bottles were incubated at 37, 55 and 70°C for 6–8 days. All the batch
165 cultures were conducted in triplicate. A control bottle without xylose for all the triplicates was also
166 prepared in all steps.

167

168 *2.3 Microbial community analyses*

169 Samples for microbial community analysis were collected at the end of the last batch culture and
170 stored at -20°C. DNA extraction and polymerase chain reaction–denaturing gradient gel
171 electrophoresis (PCR–DGGE) were performed according to Mäkinen et al. (2012). The forward
172 primer for PCR was GC–BacV3f, while the reverse primer was 907r resulting in a PCR product of

approximately 550 base pairs. All the analyses were done in duplicate. The visible bands were cut using a surgical blade, eluted in sterile water and re–amplified by PCR (primers BacV3f and 907r) as described by Koskinen et al. (2006). The product quality was checked by running the PCR products on a 1% agarose gel before sending the samples to Macrogen (South Korea) for sequencing. The nucleotide sequences obtained were analyzed by Bio-Edit software (version 7.2.5) (Hall, 1999), in order to remove primer sequences, and compared with the sequences in the GenBank nucleotide collection database using BLAST software (Altschul et al., 1990) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Analytical methods

The overpressure of the bottles was measured using a syringe method, which consisted of collecting the produced gas in a graduated syringe until the pressure inside the bottle reached atmospheric pressure and subsequent reading the produced gas volume (Owen et al., 1979). Gas samples from the headspace of the bottles (0.2 mL) were analyzed with a Shimadzu gas chromatograph GC–2014 equipped with a Porapak N column (80/100 mesh) and a thermal conductivity detector (TCD). The temperature of the oven, injector and detector were at 80, 110 and 110°C, respectively. Nitrogen was used as the carrier gas. The gas volume was corrected to standard temperature (0°C). Cumulative H_2 and CO_2 production was calculated with the following equation (Logan et al., 2002):

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

where V_G, V_H and C_H are the current (i) or previous (i–1) measurement of cumulative gas volume, cumulative H_2 volume and fraction of H_2 in the headspace of serum bottles, respectively, and V is the volume of the headspace.

197 Xylose in the liquid phase was determined by using a colorimetric phenol–sulphuric acid method
198 (DuBois et al., 1956) with a Shimadzu Ordior UV–1700 Pharmaspec UV–VIS spectrophotometer at
199 485 nm wavelenght. Acetate, propionate, isobutyrate, butyrate, valerate, ethanol and buthanol were
200 measured by a gas chromatograph equipped with flame ionization detector (GC–FID) according to
201 Kinnunen et al. (2015). Lactate and formate were measured with a Shimadzu high–performance
202 liquid chromatograph (HPLC) equipped with a Rezex RHM–monosaccharide column
203 (Phenomenex, USA) held at 40°C and a refractive index detector (Shimadzu, Japan). The mobile
204 phase was 5 mM H_2SO_4 and flow rate was 0.6 mL min^{-1}.
205
206 **3. Results**
207
208 *3.1 Dark fermentation of xylose by the activated and the digester sludge*
209 At 37 and 55°C, the H_2 yield with the activated sludge inoculum constantly increased during the
210 first three batch cultures (Figure 1a), reaching a maximum of 1.19 (± 0.08) and 1.26 (± 0.11) mol
211 H_2 per mol of xylose (added) at 37 and 55°C, respectively. At 37°C, the H_2 yield was similar at the
212 end of the third and fourth batch culture, but at 55°C, it decreased by approximately 13% at the end
213 of the fourth batch culture compared to the third one. The digester sludge started to produce H_2
214 effectively from the first batch culture at 37°C, reaching a maximum yield of 1.05 (± 0.04) mol H_2
215 per mol of xylose (added) after 84 hours (Figure 1b). In the third batch culture, the yield was similar
216 to the first one, but decreased by 50% and 90% in the second and fourth batch culture, respectively.
217 At 55°C, digester sludge started to produce H_2 effectively after 192 hours, reaching a maximum of
218 0.81 (± 0.15) mol H_2 per mol of xylose (added) at the end of the second batch culture. However, the
219 yield consistently decreased in the following two batch cultures, resulting in a 50% lower yield at
220 the end of the fourth batch culture compared to the second one. Clear consumption of H_2 was
221 observed (H_2 yield dropped) only in the first batch culture at 37°C (Figure 1a and 1b), regardless of

the inoculum. At 70°C, H_2 yield was lower compared to both 37 and 55°C, with a maximum of only 0.22 (± 0.07) mol H_2 per mol of xylose (added) in the first batch culture with digester sludge inoculum (Figure 1a and 1b). Methane in batch cultures was always below the detection limit of the GC–TCD, as well as H_2, CO_2, and methane in the control bottles without substrate.

Figure 1.

At 37°C, xylose was consumed (> 97%) in all four batch cultures with the activated sludge inoculum, while at 55°C, its removal efficiency began to decrease from the third batch culture onwards and was only 67% after the fourth batch culture (Figure 1c). At 70°C, xylose was efficiently consumed (85%) during the first batch culture, but its removal efficiency decreased and was only 15–20% at the end of the third and fourth batch culture (Figure 1c). Batch cultures with the digester sludge inoculum followed the same trend at 55 and 70°C, with a decrease in xylose removal efficiencies from approximately 93% and 71% at the end of the first batch culture to 28% and 12% at the end of the fourth batch culture, respectively (Figure 1d). Unlike the batch cultures with the fresh activated sludge, the xylose removal efficiency decreased drastically also at 37°C in the batch cultures with the digester sludge, being > 97% at the end of the second batch culture and only 20% at the end of the fourth batch culture.

In every batch culture of both inoculum types, the pH started to decrease as soon as the xylose degradation started, and the pH was remarkably below the initial value of 5.5 after 36 h incubation (Figure 1f and 1g). At both 37 and 55°C, during the incubations, the final pH decreased consistently, being below 4.0 at the end of the fourth batch culture. At 70°C, pH was somewhat higher (about 4.0) at the end of the fourth batch culture.

247 *3.2 Carbon distribution and metabolites concentration*

248 H_2 production from xylose at the different temperatures resulted in the production of soluble
249 carbon–based compounds in different proportions (Figure 2). Part of the carbon was removed from
250 the liquid phase mainly as CO_2, while some of it remained in the solution as xylose or was
251 converted to volatile fatty acids (mainly acetate, butyrate and lactate) or alcohols (mainly ethanol).
252 Generally, a higher percentage of xylose was consumed in the batch cultures with the activated
253 sludge inoculum compared to the batch cultures with the digester sludge. Acetate was produced by
254 both inocula at all the temperatures studied (Figure 2). Butyrate was produced by both inocula at 37
255 and 55°C, whereas it was not detected at 70°C but ethanol was produced instead. At 55°C, ethanol
256 production was high (about 37 mM) in the first batch culture with both inocula, but its
257 concentration decreased in the following batch cultures (Figure 2; Table S1 in supplementary
258 material). Lactate was also detected at 70°C with the activated sludge inoculum and at all the
259 studied temperatures in the batch cultures with the digester sludge inoculum (Figure 2). A small
260 concentration of acetate (< 1 mmol of carbon) was detected in the control bottles only in the first
261 batch cultures, regardless of the inoculum and temperature.

262

263 **Figure 2.**

264

265 *3.3 Microbial community analysis*

266 The microbial community composition shown by DGGE (number and location of the bands) after
267 four successive batch cultures was different with the different inocula and incubation temperatures
268 (Figure 3). At 37°C, the enriched microbial communities were dominated by bacteria having 91-
269 100% similarity to *Clostridia* sp., based on the partial 16S rRNA sequencing. More specifically,
270 sequencing of the selected bands indicated the presence of microorganisms having 98–100%
271 similarity to *Clostridium butyricum* and *Clostridium acetobutylicum* in the batch cultures with both

inocula (Table 2). At 37°C, genes possibly related to *Sporolactobacillus* sp. (92% similarity to *Sporolactobacillus putidus*) were detected only with the digester sludge inoculum. At 55°C, *Thermoanaerobacter thermosaccharoliticum* (98% similarity) and *Caloramator australicus* (97–99% similarity) were present in the batch cultures with the fresh activated and digester sludge inoculum, respectively. At 70°C, *Caloramator australicus* (97–99% similarity) was detected in the batch cultures with both inocula, while genes related to *Thermoanaerobacter* sp. (100% similarity) and *Caldanaerobius* sp. (99% similarity) were found in the batch cultures with the fresh activated and the digester sludge, respectively (Table 2).

Figure 3.

Table 2.

3.4 H_2 production pathways by the activated sludge inoculum at 37 and 55°C

Although a similar H_2 production was obtained at both 37 and 55°C in the batch cultures with activated sludge (Figure 1a), approximately 97% of the xylose was consumed at 37°C, whereas only 67% in the fourth batch culture at 55°C (Figure 1c), indicating a higher H_2 yield per mol of xylose consumed at 55°C (Figure 4a). Therefore, the microbial community at 55°C has the potential to yield more H_2 compared to the community at 37°C, and this is probably related to a different biodegradation pathway. At 37°C, the H_2 yield stabilized to 1.20 (± 0.10) mol H_2 per mol of xylose consumed, while at 55°C, it constantly increased reaching a maximum of 1.85 (± 0.51) mol H_2 per mol of xylose consumed after the first 84 h of the fourth batch culture, before decreasing to 1.64 (± 0.19) mol H_2 per mol of xylose consumed at the end of the experiment (Figure 4a). At 55°C, both acetate and butyrate followed the same trend as the H_2 production (Figure 4b). The acetate and butyrate yields constantly increased during the consecutive batch cultures reaching a maximum of

297 approximately 0.7 and 0.8 mol per mol of xylose consumed for acetate and butyrate, respectively,
298 84 h after initiating the fourth batch culture. Then, the yields decreased to 0.5 and 0.7 mol per mol
299 of xylose consumed, respectively, at the end of the experiment. Ethanol production was high in the
300 first batch culture (0.7 mol ethanol per mol of xylose consumed) and consistently decreased in the
301 following cultures, becoming negligible in the fourth culture (Figure 4b).
302
303 A linear correlation ($R^2 = 0.96$) was found between the H_2 and butyrate yield at 55°C (Figure 4c).
304 Based on the linear regression, approximately 2.4 mol H_2 per mol of butyrate were produced.
305 Conversely, the H_2 yield and ethanol yield seem to be inversely proportional (Figure 4b).
306
307 **Figure 4.**
308
309 **4. Discussion**
310
311 *4.1 Dark fermentation of xylose by the activated sludge and the digester sludge*
312 At both 37 and 55°C, the activated sludge inoculum yielded more H_2 than the digester sludge.
313 Although both inocula originated from the same wastewater treatment plant, different microbial
314 communities developed after four batch cultures at all three incubation temperatures. Except for the
315 first culture at 37°C, the H_2 produced was never consumed (Figure 1), which confirmed that the
316 heat treatment effectively eliminated most H_2 consuming microorganisms. In the first culture at
317 37°C, H_2 consumption was likely attributed to homoacetogenesis, as methane was not detected.
318 Few species of spore forming homoacetogenic bacteria may resist heat treatment (Slobodkin et al.,
319 1997), but their growth is hindered in the pH range (3.5–5.5) of this experiment (Figure 1e and 1f).
320 However, *Clostridium acetobutylicum*, present in the batch cultures at 37°C with both inocula
321 (Table 2) can switch its metabolism from acidogenesis (and H_2 production) to solventogenesis (and

322 H_2 consumption) in case of low pH (< 4.5) and high H_2 partial pressure (Kim and Zeikus, 1992).
323 Simultaneous production and consumption of H_2 can thus not be excluded, and the presented results
324 are the net H_2 production (difference between H_2 produced and consumed). Furthermore, only the
325 dominant microorganisms can be detected by PCR–DGGE and thus, the contribution of some
326 species which might had a role in either H_2 production or consumption could be missing.
327
328 For both inocula, and all the temperatures investigated, the pH profile (Figure 1e and 1f) does not
329 correlate well with the xylose concentration profiles (Figure 1c and 1d). This is especially evident in
330 the last two batch cultures of the digestate inoculum, in which the pH dropped to < 4 even when
331 xylose consumption was lower than in the previous batch cultures. One possible explanation is that,
332 from the first batch culture, bacteria accumulated undissociated volatile fatty acids, which then
333 dissociated inside the cell due to the neutral cytosolic pH, causing an intracellular overload of
334 protons which were subsequently forced out from the cytoplasm (Jönsson et al., 2013), causing the
335 pH drop observed in the last two batch cultures. This might also explain the decreased xylose
336 degradation rate in the last two batch cultures of both inocula. Excretion of the protons outside the
337 cells costs energy, e.g. in the form of adenosine triphosphate (ATP), thus limiting the energy
338 available for microbial growth (Bundhoo and Mohee, 2016). Also the carbon balances support this
339 hypothesis: in the first two batch cultures of both inocula, and for all temperatures investigated, up
340 to 30% of the carbon introduced as xylose was not detected as CO_2 or soluble metabolites (Figure
341 2). It is plausible that part of the carbon was retained inside the cells in the form of volatile fatty
342 acids, alcohols or storage products. Conversely, in the third and fourth batch culture, the sum of
343 carbon detected as CO_2 and soluble metabolites sometimes exceeded (by 10% at the most) the
344 amount of carbon provided as xylose. Accordingly, the accumulated volatile fatty acids inhibited
345 the H_2 producing bacteria (Van Ginkel and Logan, 2005), possibly inducing their death and cell
346 lysis, thus releasing the cell content and causing an overestimation of carbon detected in the
347 medium. Also acids in the dissociated form, which cannot penetrate the cell membrane, can cause

348 cell lysis by increasing the ionic strength of the medium (Van Niel et al., 2003). It should be noted
349 that the contribution of growth of microorganisms, dissolved CO_2, and yeast extract has not been
350 considered in the carbon balance, and further investigation is required to confirm their role in the
351 carbon balance.
352
353 *4.2 Comparative H_2 production by the activated sludge and the digester sludge at 37°C*
354 At 37°C, the microbial community was dominated by *Clostridia* species (Table 2). Due to the high
355 percentages of acetate and butyrate in the liquid phase, *Clostridium butyricum* and *Clostridium*
356 *acetobutylicum*, detected at 37°C with both inocula, were likely associated with H_2 production.
357 *Clostridium butyricum* produces H_2 by dark fermentation via the acetate and butyrate pathway, and
358 it is active at a pH as low as 4.4 (Seppälä et al., 2011). *Clostridium acetobutylicum* produces H_2,
359 acetate and butyrate via acidogenesis at a pH as low as 4.7, before switching the metabolic pathway
360 to solventogenesis (Grupe and Gottschalk, 1992). However, as evidenced by the low (< 2.2 mM)
361 ethanol concentration in the liquid phase of batch cultures at 37°C (Table S1 in supplementary
362 material), solventogenesis did not occur even at the lowest pH values achieved in the batch cultures.
363 This is likely due to the insufficient butyrate concentration in the medium, as a butyrate
364 concentration of 2 g L^{-1} is required to trigger solventogenesis (Cheng et al, 2012). The highest
365 butyrate concentration detected in this study was about 30 mM (2.6 g L^{-1}) at the end of the first and
366 fourth batch culture with the activated sludge inoculum at 37°C (Table S1 in supplementary
367 material), but most of the xylose was already consumed at that point (Figure 1c).
368
369 The low pH likely gave good conditions for the growth of *Sporolactobacillus* sp., a lactic acid–
370 producing mesophilic bacterium growing in the pH range 3.5–5.5, with an optimum of pH 4.5
371 (Fujita et al., 2010), which was found only in the batch cultures at 37°C with the digester sludge
372 inoculum. At 37°C, lactate (about 2 mM) was found only in the fourth batch culture of the digester

373 sludge (Table S1 in supplementary material), when the low pH of 3.5 could have reduced the
374 substrate competition among the H_2 producing microorganisms. In the batch cultures with the
375 activated sludge inoculum, the absence of lactate may indicate a low concentration of
376 *Sporolactobacillus* sp. in the microbial community. This bacterium is likely one of the causes for
377 the low H_2 yield obtained in the fourth batch culture of the digester sludge at 37°C (Figure 1b), as
378 part of the electrons were directed to reduce pyruvate to lactate via NADH oxidation instead of
379 reducing protons to molecular H_2. Furthermore, lactic acid bacteria can excrete bacteriocins, which
380 are toxic to other bacteria, including *Clostridium* (Noike et al., 2002). However, a protein and
381 enzyme–level study is required to assess the inhibitory effect of bacteriocins on H_2 producing
382 bacteria, which is out of the scope of this paper.
383
384 At 37°C, in batch cultures with activated sludge, the H_2 yield per mol of xylose consumed was
385 lower than the one obtained at 55°C (Figure 4a). H_2 yields by mesophilic mixed cultures are
386 generally lower than by thermophilic cultures (Table 1), but yields of 2.25 and 2.64 mol H_2 per mol
387 of xylose have been obtained by Lin and Cheng (2006) and Chaganti et al. (2012) at 35 and 37°C,
388 respectively, using a similar inoculum to the ones used in this study. However, Lin and Cheng
389 (2006) worked at an initial pH of 6.5 and substrate concentration of 124.9 mM, whereas Chaganti et
390 al. (2012) used a statistical approach to optimize several chemical and physical parameters, such as
391 pH, oleic acid concentration and biomass concentration.
392
393 *4.3 Comparative H_2 production by the activated sludge and the digester sludge at 55°C*
394 *Clostridia* species were also detected at 55°C with both inocula (Table 2) and associated with H_2
395 production via the acetate and butyrate pathway. *Clostridium thermopalmarium*, found in batch
396 cultures with the digester inoculum at 55°C, mainly ferments sugars to butyrate, producing H_2, CO_2
397 and small amounts of acetate, lactate and ethanol (Lawson Anani Soh et al., 1991). At 55°C, the

398 different activity of *Clostridium* sp. with the activated and the digester sludge can be attributed to
399 the different pH. During the third and fourth batch culture of the digester sludge, as happened at
400 37°C, the pH dropped to as low as 3.5 (Figure 1f), resulting in low xylose degradation. Xylose
401 degradation was low also in the fourth batch culture of the activated sludge, in which the pH
402 dropped below 4.0 (Figure 1e). *Thermoanaerobacter thermosaccharoliticum*, found at 55°C with
403 the activated sludge inoculum, has been used to ferment a variety of monomeric sugars, including
404 33.3 mM xylose (Cao et al., 2014), resulting in the total degradation of the substrate and the
405 production of 1.7 mol H_2 per mol of xylose with acetate and butyrate as the main soluble end
406 products (Cao et al., 2014). However, the initial pH of their experiment was set to 7.0, whereas in
407 this study the initial pH was 5.5. *T. thermosaccharoliticum* effectively produces H_2 from xylose in a
408 pH range 5–7, whereas its H_2 yield dramatically decreases at lower pH values (Ren et al., 2008).
409
410 The highest H_2 yield of 1.85 mol H_2 per mol of xylose consumed was obtained in this study during
411 the fourth batch culture of activated sludge at 55°C (Figure 4a). This is in line with the results
412 obtained by Calli et al. (2008) who reported a maximum yield of 1.7 mol H_2 per mol xylose at 55°C
413 (Table 1). Interestingly, even if the compost used as inoculum by Calli et al. (2008) was not
414 pretreated, methane was not detected, confirming that thermophilic conditions reduce the risk of
415 contamination by methanogens. A similar H_2 yield (1.65 mol H_2 per mol xylose) was obtained at
416 65°C with a geothermal spring inoculum (Zeidan and Van Niel, 2009). A slightly higher H_2 yield of
417 2.07–2.19 mol H_2 per mol of xylose has been reported in thermophilic (60°C) batch incubations
418 (Table 1) by using a pure culture of *T. thermosaccharolyticum* (Khamtib and Reungsang, 2012; Ren
419 et al., 2008; Zhang et al., 2011). This bacterium may have a significant contribution to the H_2 yield
420 by activated sludge at 55°C.
421
422 *4.4 Comparative H_2 production by the activated sludge and the digester sludge at 70°C*

423 At 70°C, hyperthermophilic bacteria were found present even in the activated sludge, despite the
424 temperature in Finland seldom exceeds 25°C in summer. In the wastewater treatment plant where
425 the sludge was collected, the aeration basins are exposed to ambient temperatures. All the
426 hyperthermophilic species detected after four batch cultures with the activated and digester sludge,
427 including *Caldanaerobius* sp., *Caloramator australicus* and *Thermoanaerobacter* sp., generate H_2
428 from carbohydrates producing acetate and ethanol as the end product at a pH optimum of 7 or even
429 slightly higher (Lee et al., 2008; Ogg and Patel, 2009; Vipotnik et al., 2016). The low xylose
430 degradation and H_2 yield (Figure 1), the presence of ethanol and acetate in the medium and the
431 absence of butyrate (Figure 2) indicate that the bacteria were barely active at the beginning of the
432 batch cultures at 70°C, when the pH was > 5, before being completely inhibited after a further pH
433 decrease.
434
435 H_2 production at 70°C was achieved by Kongjan et al. (2009) and Zhao et al. (2010) with a
436 maximum yield of 1.62 and 1.84 mol H_2 per mol of xylose, respectively, but the experiments were
437 conducted at a higher initial pH and lower substrate concentration compared to this study (Table 1).
438 Furthermore, in both cases, the inoculum was previously enriched for H_2 production at 70°C.
439
440 *4.5 H_2 production pathways in the fresh activated sludge inoculum at 55°C*
441
442 The linear regression between the H_2 and butyrate yield at 55°C with the fresh activated sludge
443 inoculum (Figure 4c) shows a production of approximately 2.4 mol H_2 per mol of butyrate.
444 However, only 2.0 mol H_2 per mol of butyrate is theoretically obtainable (2), suggesting that H_2
445 was produced also through the acetate pathway (3).
446
447 $C_5H_{10}O_5 \rightarrow 0.83\ CH_3CH_2CH_2COOH + 1.67\ H_2 + 1.67\ CO_2$ (2)

448 $C_5H_{10}O_5 + 1.67\ H_2O \rightarrow 1.67\ CH_3COOH + 3.33\ H_2 + 1.67\ CO_2$ (3)

449

450 A direct conversion of xylose to acetate, despite being thermodynamically more favorable under
451 thermophilic than mesophilic conditions, is strongly affected by the H_2 partial pressure. At 55°C, H_2
452 production through the acetate pathway is thermodynamically feasible only at H_2 partial pressures
453 of far less than 1 kPa, and then the pathway shifts to butyrate production (Verhaart et al., 2010).
454 Based on our calculations done using the ideal gas law (Figure S1 in supplementary material), 1 kPa
455 was reached during the first 36 h in batch cultures of activated sludge at 55°C (despite overpressure
456 removal during each sampling). It is, therefore, plausible that H_2 first evolved through the acetate
457 pathway, and then the metabolic pathway shifted to butyrate production due to the accumulation of
458 H_2 in the headspace. This would explain the higher total H_2 yield than the theoretical production
459 through the butyrate pathway. Furthermore, according to Valdez-Vazquez et al. (2006), a H_2 partial
460 pressure of 0.75 atm (74 kPa) or even lower is sufficient to inhibit thermophilic H_2 producing
461 microorganisms. In this study, the highest H_2 partial pressures reached are in the range of 60–85
462 kPa (Figure S1 in supplementary material), suggesting that the H_2 partial pressure, as well as low
463 pH, could have negatively affected the process at 55°C.

464

465 Although acetate production followed a similar trend to butyrate (Figure 4b), no correlation with H_2
466 yield was found, suggesting that acetate was produced also through other pathways with no H_2
467 production. The correlation between butyrate and H_2 yield was not at all found at 37°C (data not
468 shown), probably due to a more diverse microbial community and thus, a wider variety of metabolic
469 pathways.

470

Ethanol was the main metabolite produced during the first batch culture at 55°C (Figure 4b). In the subsequent cultures, its yield decreased while the butyrate and H_2 yield increased. This suggests that butyrate (2) and ethanol (4) production were competitive pathways.

$$C_5H_{10}O_5 \rightarrow 1.67\ CH_3CH_2OH + 1.67\ CO_2 \qquad (4)$$

The shift from ethanol to butyrate fermentation can be attributed to either a change in microbial community or a shift in the metabolic pathway of the active microbial species during the four successive batch cultures. The metabolic shift is confirmed by the fact that, in the first batch culture with the activated sludge at 55°C, gas composition was approximately 65% CO_2 and only 35% H_2 (Figure S2c in supplementary material), but the share of H_2 constantly increased in the subsequent batch cultures being about 57% of the total gas at the end of third and fourth batch culture.

This study demonstrated that activated sludge can be used as inoculum for thermophilic H_2 production from xylose containing wastewaters. However, a further study with a continuously fed bioreactor is required to evaluate the potential and stability of this process for full–scale applications.

Conclusions

- Using heat treated activated sludge as the inoculum, xylose containing wastewaters can be treated at 55°C obtaining higher H_2 yields than at 37°C
- The highest H_2 yield of 1.85 mol H_2 per mol of xylose consumed was obtained with activated sludge during the fourth batch culture at 55°C. At the beginning of every culture,

H_2 production was likely associated with the acetate pathway and then shifted towards the butyrate pathway due to the increased H_2 partial pressure

- At 55°C, ethanol was produced in the first batch culture. In the following cultures, ethanol production steadily decreased while butyrate and H_2 production steadily increased, indicating a clear shift in the xylose degradation pathway towards dark fermentation. This suggests that for non–adapted inocula, a start–up period may be required prior to obtaining high H_2 yields.
- H_2 production at 70°C was negligible, possibly because the pH was below the optimum for the detected hyperthermophiles present in the inoculum.

Acknowledgements

The authors gratefully thank Mira Sulonen (Tampere University of Technology, Finland) for the support with the microbial community analyses, Timo Lepistö (Tampere University of Technology, Finland) for improving the grammar of the manuscript and the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland) for providing the activated and digester sludge.

Funding

This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under grant agreement no. 643071.

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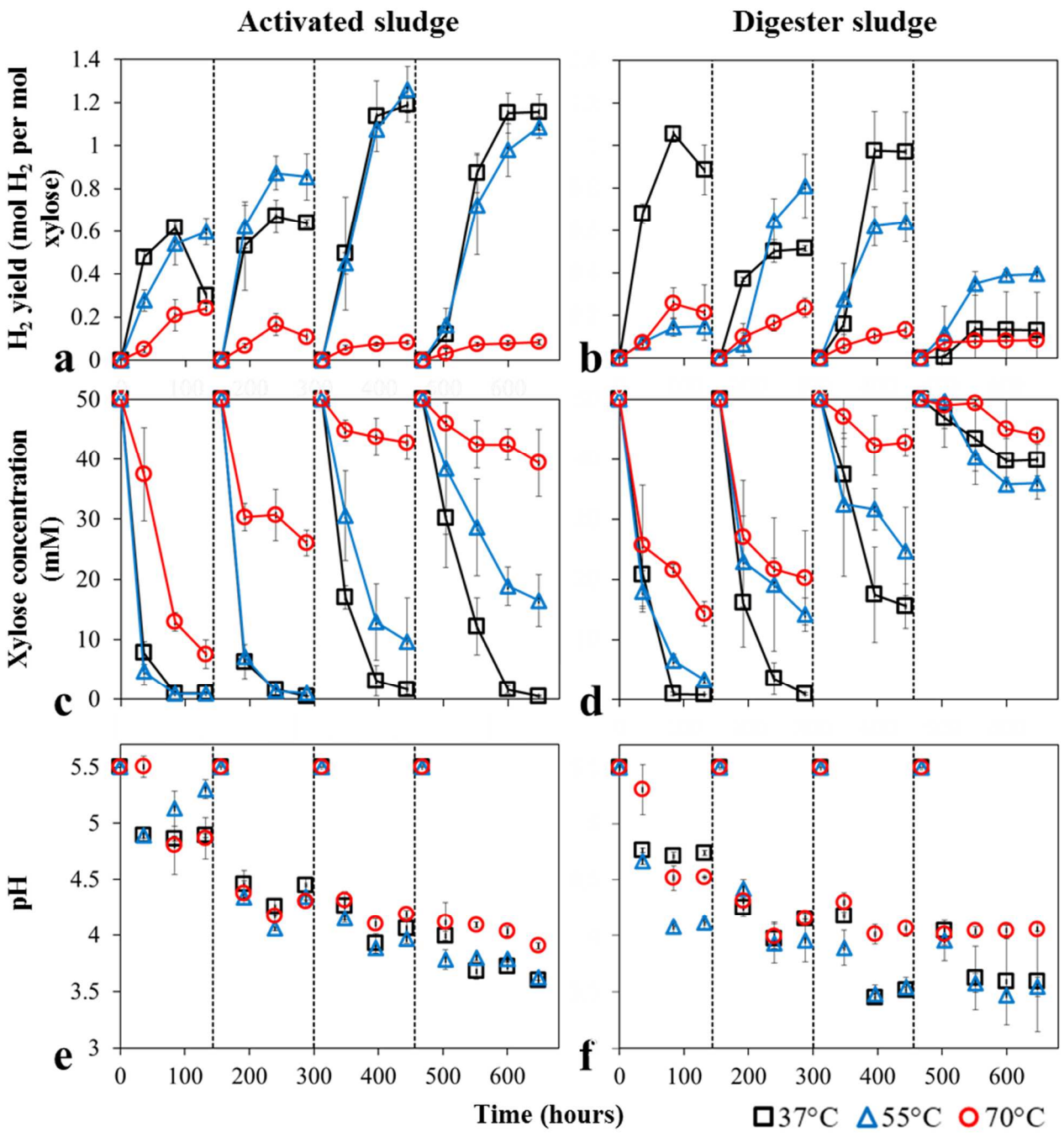

Figure 1. H_2 yield (mol H_2 per mol of xylose added), residual xylose and pH trend with the activated and the digester sludge at 37, 55 and 70°C. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates. The dotted lines refer to the end of every batch culture and start of a new one.

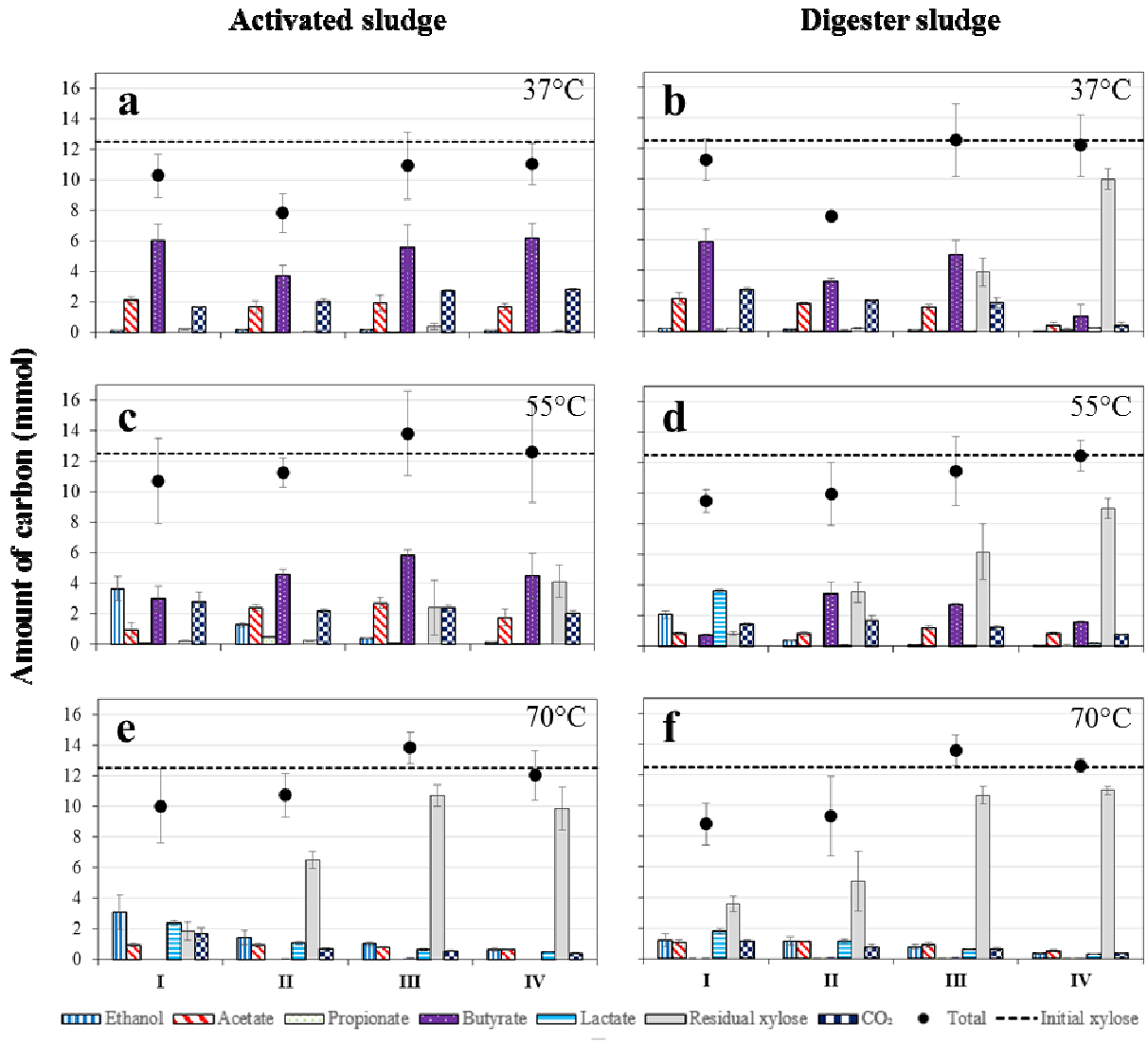

Figure 2. Carbon distribution at the end of each batch culture. The columns refer to the mmol of carbon found in the different metabolites at the end of every batch cultures and the black dots represent their sum. The dotted line refer to the 12.5 mmol of carbon introduced as xylose at the beginning of each incubation. Every column or point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates.

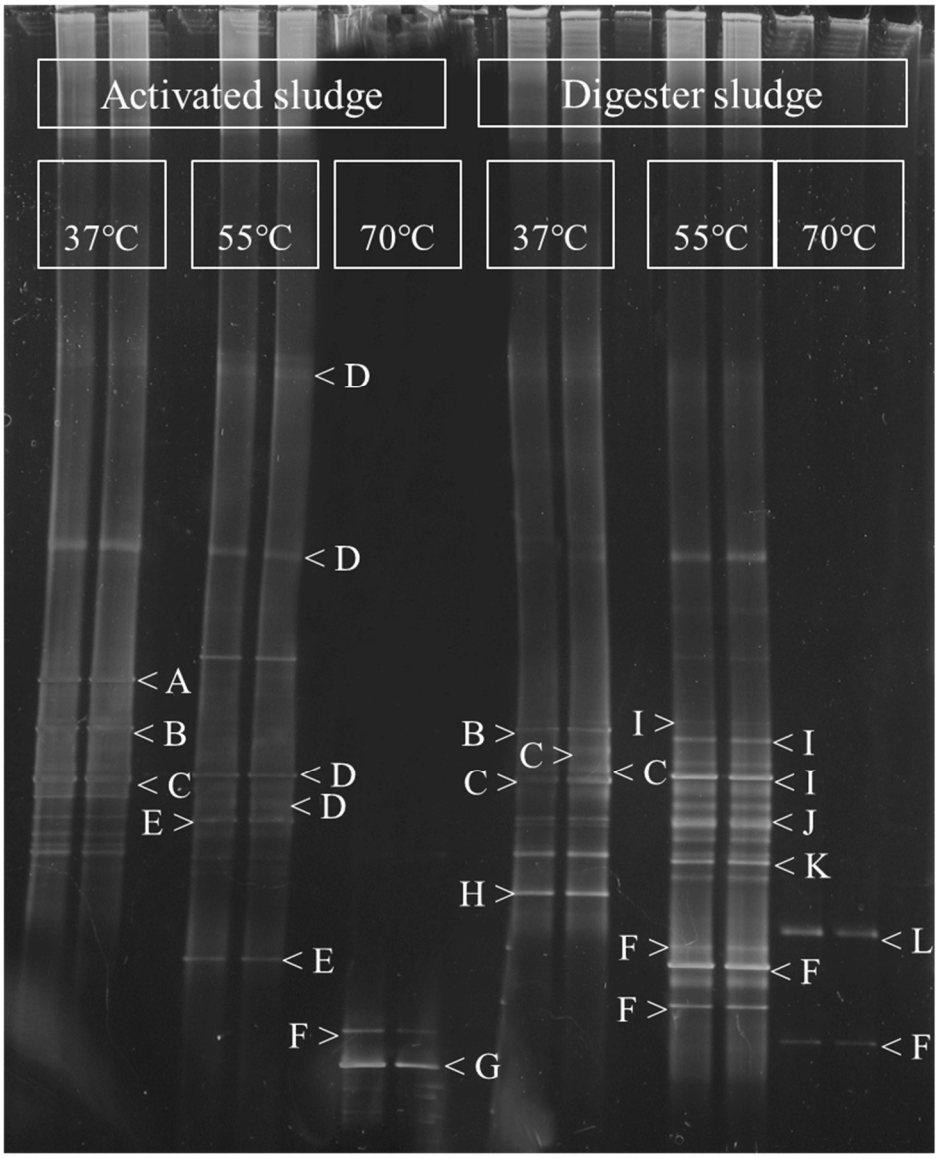

Figure 3. Bacterial community composition analyzed by PCR–DGGE from the batch cultures with the fresh activated and digester sludge inocula after the four batch cultures at 37, 55 and 70°C. The band labels refer to Table 3.

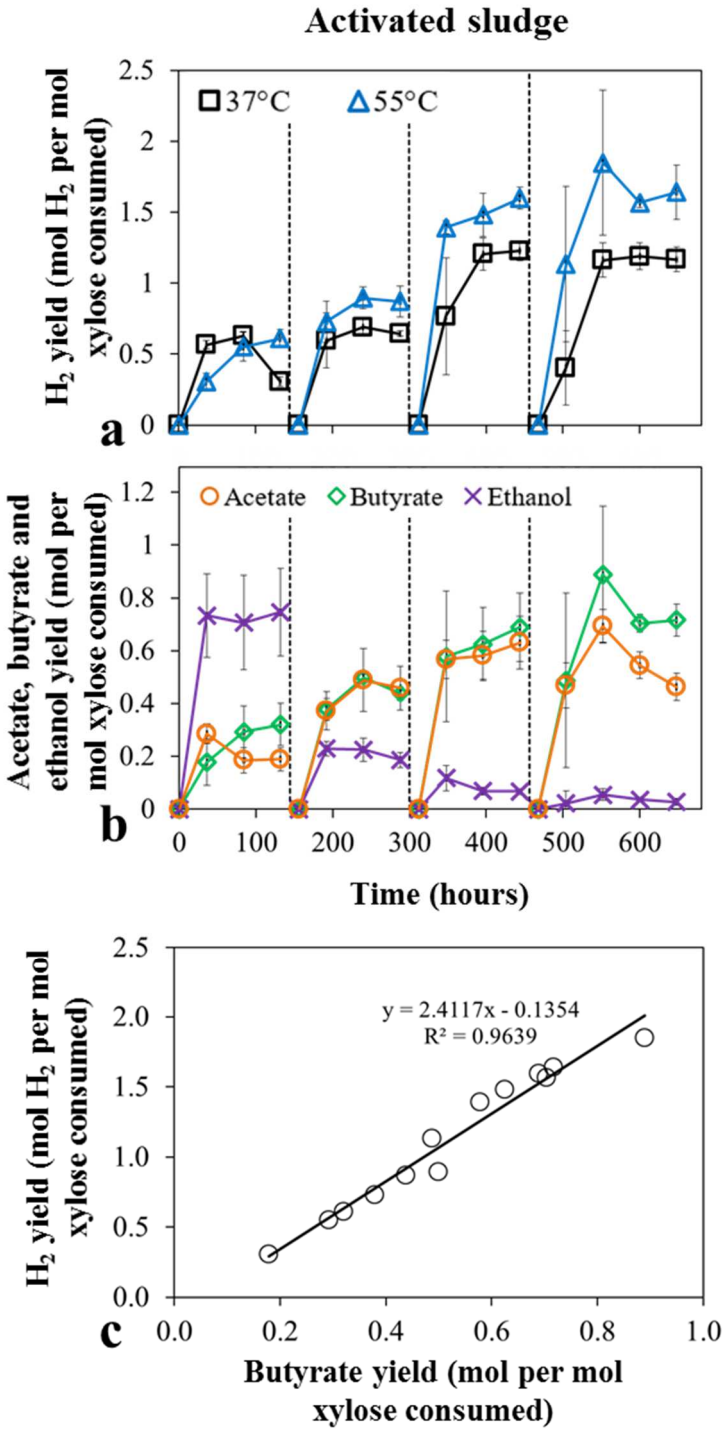

Figure 4. H_2 yield (mol H_2 per mol of xylose consumed) obtained with the activated sludge at 37 and 55°C (a) and the acetate, butyrate and ethanol yields obtained with the activated sludge inoculum at 55°C (b) with respect to time. H_2 yield was shown to be directly proportional to butyrate (c) when activated sludge was used as inoculum at 55°C. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars show the standard deviation of the triplicates.

Table 1. H_2 yields obtained in various batch studies conducted at different temperatures and using different initial pH and xylose concentrations. The reported H_2 yield refer to the highest one obtained in the cited studies.

Inoculum	**Pre-treatment**	**T (°C)**	**Initial pH**	**Initial xylose (mM)**	**H_2 yield[a] (mol per mol xylose)**	**Reference**
Activated sludge	Heat treatment	35	6.5	124.9	1.30	Lin et al. (2006)
Activated sludge	Heat treatment	35	5.5	66.6	1.88	De Sá et al. (2013)
Activated sludge	Heat treatment	35	6.5	124.9	2.25	Lin and Cheng (2006)
Clostridium butyricum	-[b]	37	7.5	124.9	0.73	Lo et al. (2008)
Granulated sludge	Heat treatment	37	5.5	23.9	0.80	Maintinguer et al. (2011)
Digested activated sludge	-	37	6.7	33.3	2.64	Chaganti et al. (2012)
Clostridium beijerinckii	-	40	8	66.6	2.31	An et al. (2014)
Activated sludge	Heat treatment	40	7.1	124.9	1.30	Lin et al. (2008)
Mixed culture compost	-	55	5	13.3	1.70	Calli et al. (2008)
Thermoanaerobacter thermosaccharolyticum	-	60	6.7	33.3	2.07	Zhang et al. (2011)
Thermoanaerobacter thermosaccharolyticum	-	60	6.5	66.6	2.09	Khamtib and Reungsang (2012)
Thermoanaerobacter thermosaccharolyticum	-	60	6.5	66.6	2.19	Ren et al. (2008)
Thermoanaerobacter thermosaccharolyticum	-	60	7.0	33.3	1.72	Cao et al. (2014)
Geothermal spring	-	60	7.9	66.6	1.65	Zeidan and Van Niel (2009)
Biomass from H_2 producing reactor	-	70	7.0–8.0	3.3	1.62	Kongjan et al. (2009)
Biomass from H_2 producing reactor	-	70	7.0	13.3	1.84	Zhao et al. (2010)

[a] Highest H_2 yield obtained in the experiment

[b] Not applied

Table 2. Identification of the DGGE bands obtained after four successive batch cultures at 37, 55 and 70°C based on the comparison of their 16S rRNA gene sequences to those collected in the GenBank and their presence (+) or absence (-) in the different batch cultures.

BM[a]	Microorganism[b]	Access number	Matching sequence lenght[c]	Similarity (%)[d]	Activated sludge			Digester sludge		
					37	55	70	37	55	70
A	*Clostridium* sp.	FJ361757	477	99	+	-	-	-	-	-
B	*Clostridium acetobutilycum*	KP410577 KP410579	457-515	99	+	-	-	+	-	-
C	*Clostridium butyricum*	CP013352 KT072767	418-492	98-100	+	-	-	+	-	-
D	*Clostridium* sp.	KR052807	381-490	92-100	-	+	-	-	-	-
E	*Thermoanaerobacter thermosaccharoliticum*	KT274717	426	98	-	+	-	-	-	-
F	*Caloramator australicus*	HM228391	385-449	97-99	-	-	+	-	+	+
G	*Thermoanaerobacter* sp.	KR007668	452	100	-	-	+	-	-	-
H	*Sporolactobacillus putidus*	NR_112774	486	92	-	-	-	+	-	-
I	*Clostridium* sp.	AB504378 AB537983	433-451	91-98	-	-	-	-	+	-
J	*Clostridium thermopalmarium*	KM036191	428	98	-	-	-	-	+	-
K	*Clostridium isatidis*	NR_026347	425	93	-	-	-	-	+	-
L	*Caldanaerobius* sp.	JX984966	429	99	-	-	-	-	-	+

[a] Band mark in Figure 3

[b] Closest species in GenBank

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

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

Table 3. Identification of the DGGE bands obtained from batch cultures at 37, 55 and 70°C after four successive batch cultures based on their 16S rRNA gene sequences to those collected in the GenBank and their presence (+) or absence (-) in the different batch cultures.

BM[a]	Microorganism[b]	Access number	Sequence lenght[c]	Similitude (%)[d]	Activated sludge			Digester sludge		
					37	55	70	37	55	70
A	*Clostridium* sp.	FJ361757	477	99	+	-	-	-	-	-
B	*Clostridium acetobutilycum*	KP410577 KP410579	457-515	99	+	-	-	+	-	-
C	*Clostridium butyricum*	CP013352 KT072767	418-492	98-100	+	-	-	+	-	-
D	*Clostridium* sp.	KR052807	381-490	92-100	-	+	-	-	-	-
E	*Thermoanaerobacter thermosaccharoliticum*	KT274717	426	98	-	+	-	-	-	-
F	*Caloramator australicus*	HM228391	385-449	97-99	-	-	+	-	+	+
G	*Thermoanaerobacter* sp.	KR007668	452	100	-	-	+	-	-	-
H	*Sporolactobacillus putidus*	NR_112774	486	92	-	-	-	+	-	-
I	*Clostridium* sp.	AB504378 AB537983	433-451	91-98	-	-	-	-	+	-
J	*Clostridium thermopalmarium*	KM036191	428	98	-	-	-	-	+	-
K	*Clostridium isatidis*	NR_026347	425	93	-	-	-	-	+	-
L	*Caldanaerobius* sp.	JX984966	429	99	-	-	-	-	-	+

[a] Band mark in Figure 3

[b] Closest species in GenBank

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

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

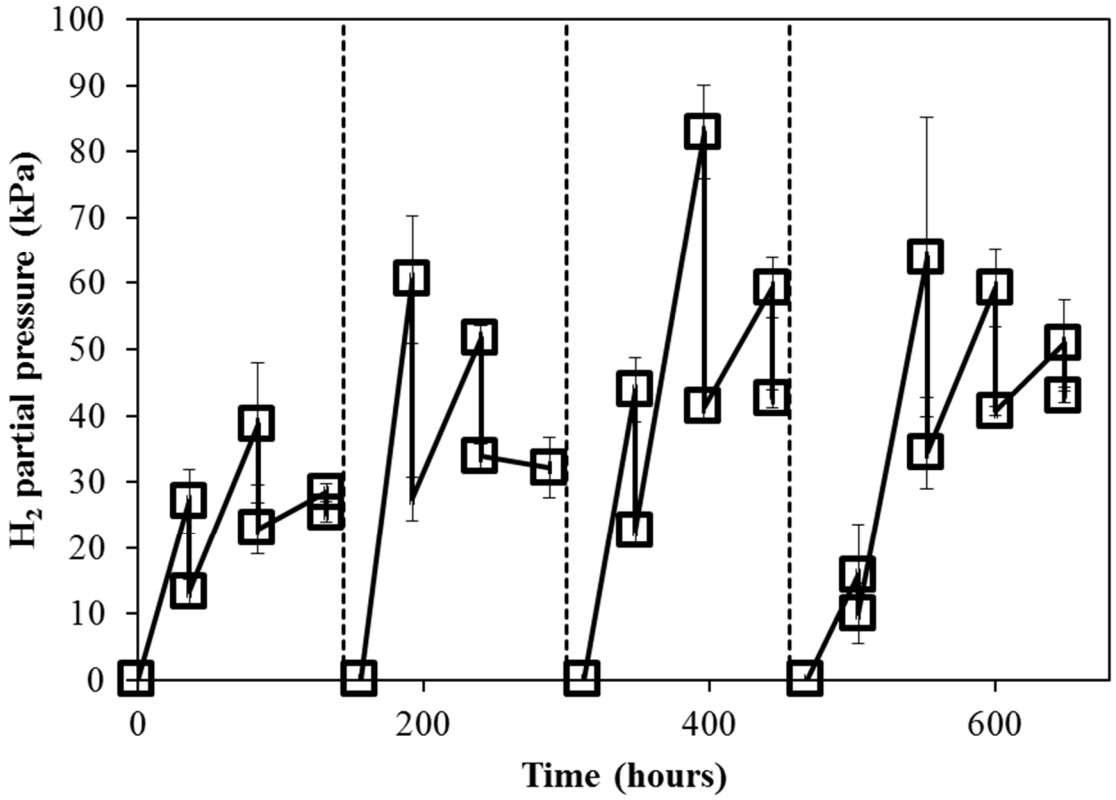

Figure S1. Evolution of H_2 partial pressure with time, assuming equilibrium conditions between the liquid and gas phase. The two points represented for every sampling time refer to the H_2 partial pressure before (the highest) and after (the lower) removing the overpressure. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars show the standard deviation of the triplicates. The dotted lines refer to the end of a batch culture and start of a new batch culture.

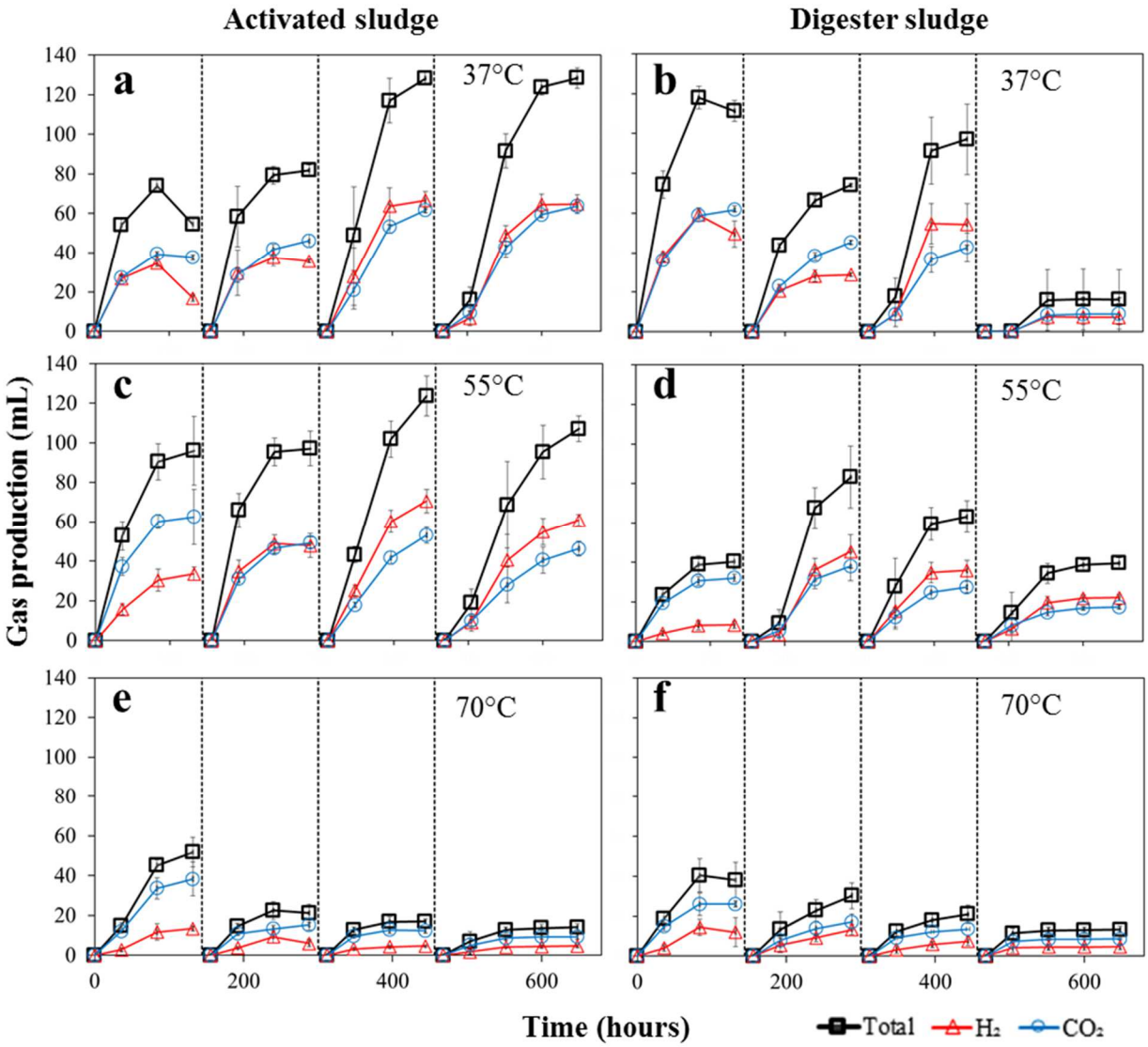

Figure S2. Cumulative gas production obtained with the fresh activated and the digester sludge inoculum. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates. The dotted lines refer to the end of every batch culture and start of a new culture.

Table S1. Concentration of most abundant metabolites found at the end of the four consecutive batch cultures.

Inoculum	T (°C)	Batch culture	Concentration (mM)			
			Acetate	Butyrate	Ethanol	Lactate
Activated sludge	37	I	21.4 (± 2.2)	30.2 (± 5.2)	1.6 (± 0.3)	-
		II	16.8 (± 4.1)	18.6 (± 3.4)	2.0 (± 0.0)	-
		III	19.5 (± 5.0)	28.0 (± 7.2)	2.1 (± 0.1)	-
		IV	16.8 (± 2.1)	31.0 (± 4.9)	1.6 (± 0.0)	-
	55	I	9.3 (± 4.7)	15.0 (± 4.0)	36.6 (± 8.1)	-
		II	24.2 (± 1.8)	23.0 (± 1.6)	12.8 (± 1.4)	-
		III	27.0 (± 3.4)	29.2 (± 1.9)	3.9 (± 0.3)	-
		IV	17.6 (± 5.6)	22.5 (± 7.3)	1.3 (± 0.3)	-
	70	I	9.7 (± 0.9)	-	30.8 (± 11.3)	15.8 (± 1.2)
		II	9.5 (± 1.4)	-	14.5 (± 4.7)	7.3 (± 0.8)
		III	8.2 (± 0.4)	0.5 (± 0.2)	10.2 (± 1.3)	4.3 (± 0.4)
		IV	6.3 (± 0.1)	-	6.3 (± 1.2)	3.2 (± 0.0)
Digester sludge	37	I	21.6 (± 4.0)	29.5 (± 4.0)	2.2 (± 0.3)	-
		II	18.3 (± 0.8)	16.4 (± 0.9)	1.6 (± 0.1)	-
		III	15.8 (± 1.8)	25.3 (± 4.5)	0.9 (± 0.2)	-
		IV	4.2 (± 2.0)	5.1 (± 3.9)	-	1.7 (± 0.0)
	55	I	8.2 (± 0.8)	3.6 (± 0.3)	20.7 (± 2.5)	24.2 (± 0.7)
		II	8.2 (± 1.5)	17.1 (± 3.9)	3.9 (± 0.4)	-
		III	12.1 (± 1.7)	13.6 (± 0.6)	1.1 (± 0.1)	-
		IV	8.4 (± 0.6)	7.9 (± 0.6)	0.6 (± 0.1)	1.2 (± 0.7)
	70	I	10.7 (± 1.9)	-	12.0 (± 4.1)	11.9 (± 1.3)
		II	11.5 (± 0.6)	-	11.6 (± 2.6)	7.6 (± 0.9)
		III	9.1 (± 1.8)	-	7.8 (± 1.3)	4.1 (± 0.0)
		IV	5.3 (± 0.7)	-	3.4 (± 0.4)	2.1 (± 0.3)