Temperature control as key factor for optimal biohydrogen production from thermomechanical pulping wastewater

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Manuscript submitted to: *Biochemical Engineering Journal*

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Abstract

This study evaluates the use of non-pretreated thermo-mechanical pulping (TMP) wastewater as a potential substrate for hydrogen production by dark fermentation. Batch incubations were conducted in a temperature gradient incubator at temperatures ranging from 37 to 80 °C, using an inoculum from a thermophilic, xylose-fed, hydrogen-producing fluidised bed reactor. The aim was to assess the short-term response of the microbial communities to the different temperatures with respect to both hydrogen yield and composition of the active microbial community. High throughput sequencing (MiSeq) of the reversely transcribed 16S rRNA showed that *Thermoanaerobacterium* sp. dominated the active microbial community at 70 °C, resulting in the highest hydrogen yield of 3.6 (± 0.1) mmol H_2 g^{-1} COD_{tot} supplied. Lower hydrogen yields were obtained at the temperature range from 37 to 65 °C, likely due to consumption of the produced hydrogen by homoacetogenesis. No hydrogen production was detected at temperatures above 70 °C. Thermomechanical pulping wastewaters are released at high temperatures (50 to 80 °C), and thus dark fermentation at 70 °C could be sustained using the heat produced by the pulp and paper plant itself without any requirement for external heating.

Keywords

Dark fermentation, MiSeq, Pulp and paper mill wastewater, *Thermoanaerobacterium*, Thermomechanical pulping, Thermophilic

1. Introduction

Pulp and paper industry is facing an economic challenge due to globalised competition and decreasing paper demand (Machani et al., 2014). The long-term success of the industry is believed to be strictly linked to the ability of companies to innovate and create new value streams, which are predicted to generate 40% of the companies' turnover in 2030 (Toppinen et al., 2017). A biorefinery concept, in which waste from the pulp and paper making process is used as a resource to generate value-added products such as biofuels and biochemicals, is a promising strategy to expand the product platform, reduce waste disposal costs and fulfil the environmental regulations on waste emissions (Kinnunen et al., 2015; Machani et al., 2014; Moncada B. et al., 2016).

Pulping is the major source of polluted wastewaters of the whole papermaking process (Pokhrel and Viraraghavan, 2004). Pulp mill wastewater is typically treated by the traditional activated sludge process, but anaerobic processes have the advantages of coupling wastewater treatment to renewable energy production, produce a lower quantity of waste sludge and require a smaller volume than aerobic processes (Ashrafi et al., 2015). Among pulping processes, thermomechanical pulping (TMP) produces a wastewater more suited for anaerobic biological processes than chemical-based pulping, due to the low concentrations of inhibitory compounds such as sulphate, sulphite, hydrogen peroxide, resin acid and fatty acids (Ekstrand et al., 2013; Rintala and Puhakka, 1994).

Thermomechanical pulping wastewater has been successfully used as a substrate for both mesophilic (Gao et al., 2016) and thermophilic (Rintala and Lepistö, 1992) methane production via anaerobic digestion. However, hydrogen (H_2) is a carbon free fuel expected to play a pivotal role in energy production in the future (Boodhun et al., 2017). Dark fermentative H_2 production has the potential for energy recovery from waste paper hydrolysate (Eker and Sarp, 2017), pulp and paper mill effluent hydrolysates (Lakshmidevi and Muthukumar, 2010) and even from untreated pulps

(Nissilä et al., 2012). Dark fermentative H_2 production has also been reported from carbohydrate-containing wastewaters, such as starch wastewater and palm oil mill effluent (Badiei et al., 2011; Xie et al., 2014). Although TMP wastewaters are characterized by a high content of carbohydrates (25 to 40% of the total COD) (Rintala and Puhakka, 1994), to our knowledge it has not yet been tested as a substrate for H_2 dark fermentation.

Thermophilic dark fermentation of TMP wastewater could be advantageous, as both biological polysaccharide hydrolysis (Elsharnouby et al., 2013) and H_2 yielding reactions (Verhaart et al., 2010) are favoured by high temperature. High temperature also limits the growth of homoacetogenic bacteria and methanogenic archaea (Oh et al., 2003), which may consume the produced H_2 in mixed culture systems. The main drawback of thermophilic processes is the energy required to heat the reactors, but TMP wastewaters are released from the pulping process at a temperature of 50 to 80 °C (Rintala and Lepistö, 1992), and could therefore be treated in thermophilic bioreactors with minimal, or even without external heating.

Temperature is a key factor in dark fermentation, as even a change of a few degrees may result in the development of a different microbial community and thus, affect the H_2 yield (Dessì et al., 2018; Karadag and Puhakka, 2010). Understanding of the composition of the microbial community is also crucial in order to optimize the complex microbial H_2 production process, involving both hydrolytic and fermentative microorganisms (Kumar et al., 2017). Microbial communities from dark fermentation of lignocellulose-based waste and wastewaters have been previously studied at DNA level (Nissilä et al., 2012; Xie et al., 2014), but a RNA-based approach can provide more detailed information on the microorganisms that produce (and consume) H_2. Furthermore, the time response on RNA changes is much faster than on DNA changes (De Vrieze et al., 2016), allowing to detect the response of the microbial community to an environmental change in a relatively short time.

In a previous study, a mixed culture was successfully adapted to thermophilic (70 °C) dark fermentation of xylose in a fluidised bed reactor (FBR) and the H_2 producing *Thermoanaerobacterium* sp. accounted for > 99% of the active microbial community (Dessì et al., 2018). In this study, the same adapted mixed culture was used to test if TMP wastewater is a suitable substrate for dark fermentative H_2 production at various temperatures (from 37 to 80 °C), and to describe how the active microbial community responds to the different temperatures.

2. Materials and methods

2.1 Source of microorganisms

The inoculum used in this study was biofilm-coated activated carbon originating from a thermophilic fluidised bed reactor (FBR) used to study H_2 production from xylose via dark fermentation by gradually increasing the temperature of the reactor from 55 to 70 °C (Dessì et al., 2018). The FBR was initially inoculated with heat-treated (90 °C, 15 min) activated sludge originating from a municipal wastewater treatment plant (Viinikanlahti, Tampere, Finland). The biofilm-coated activated carbon granules were sampled after 185 days of reactor operation, at that point the FBR had been operated at 70 °C for 27 days. No xylose was present in the FBR medium at the sampling time. The granules were stored at 4 °C for one week prior to utilisation. This inoculum was used because the microbial community was dominated by *Thermoanaerobacterium* sp. (Dessì et al., 2018), which previously showed potential for hydrolysis of lignocellulosic substrates and H_2 production from the resulting sugars (Cao et al., 2014).

2.2 Wastewater characterization

The wastewater was collected from a pulp and paper mill located in Finland. It was the effluent of a TMP process, in which wood was exposed to a high-temperature (120 °C) steam in order to obtain the pulp. The wastewater had a temperature of about 70 °C at the time of the sampling, but was

cooled down and stored at 4 °C to minimise biological activity that might affect its composition. The wastewater had a pH of 5.0 and a composition as given in Table 1.

Table 1 here

2.3 Temperature-gradient batch set–up

The batch cultures were conducted in anaerobic tubes with a total volume of 26 mL (17 mL working volume and 9 mL headspace). The tubes were inoculated by adding 2 mL of biofilm-coated activated carbon granules to 15 mL of TMP wastewater (Table 1). All the tubes were flushed with N_2 for 5 min, and the internal pressure was equilibrated to atmospheric pressure by removing the excess gas using a syringe and a needle before incubation. The initial pH of the batch cultures (wastewater and inoculum) was adjusted to 6.3 (± 0.1) using 1 M NaOH, as higher pH may favour the growth of methanogenic archaea (Jung-Yeol et al., 2012). The tubes were incubated at 200 rpm shaking in a temperature-gradient incubator (Test Tube Oscillator, Terratec Asia Pacific, Australia) at 37, 42, 48, 55, 59, 65, 70, 74 or 80°C (duplicate tubes at each temperature). The experiment was interrupted after 111 hours, when no H_2 production was detected in any of the vials in two consecutive samples, as long inactive periods may affect the RNA-level analysis (De Vrieze et al., 2016).

Gas samples were collected for analysis 1 to 3 times per day. End-point liquid samples were collected and stored at -20 °C before analysis. Non-inoculated control incubations, with fresh activated carbon and TMP wastewater, were prepared at 37, 55 and 70 °C. Control incubations containing 2 mL of fresh activated carbon and a mix of acetate and butyrate in Milli-Q® water (0.86 g COD_{tot} L^{-1} each, 15 mL volume) were also prepared at 42, 65 and 80 °C to assess possible adsorption of VFAs on virgin activated carbon.

153 ***2.4 Microbial community analyses***

154 Biofilm-coated activated carbon granules and liquid medium were collected at the end of the
155 experiment and stored in 5 mL Eppendorf tubes at -80 °C. Microbial community analysis was
156 conducted separately on microbial communities growing attached to the granules and suspended in
157 the liquid medium, as the growth of suspended biomass was clearly visible in the vials after
158 incubation in the temperature range from 42 to 59 °C. Nucleic acids extraction using a modified
159 method from Griffiths et al. (2000), DNA inhibition, complementary DNA (cDNA) synthesis and
160 sequencing (using an Illumina MiSeq platform) were performed as described previously (Dessì et
161 al., 2018). Sequence analysis (1,395,864 sequences in total, 1,238,862 after quality check) was also
162 performed according to Dessì et al. (2018), but using a more recent version of Mothur (v1.39.5) and
163 Silva database (v128). The Illumina sequencing data was deposited to the NCBI Sequence Read
164 Archive under BioProject Number PRJNA428338.

165

166 ***2.5 Analytical methods***

167 Gas production in the tubes was quantified by a volumetric syringe method (Owen et al., 1979), and
168 the gas composition was determined by gas chromatography-thermal conductivity detector (GC-
169 TCD) as reported previously by Dessì et al. (2017). Acetate, butyrate, ethanol, propionate, lactate,
170 and formate concentrations were measured with a high-performance liquid chromatograph (HPLC)
171 equipped with a refractive index detector (RID) (Shimadzu, Japan) and a Rezex RHM-
172 monosaccharide column (Phenomenex, USA) held at 40 °C. The mobile phase was 5 mM H_2SO_4
173 and the flow rate was 0.6 mL min^{-1}. Glucose and xylose concentrations were measured using a
174 HPLC equipped with a RID and a RPM-monosaccharide column (Phenomenex, USA) held at 85 °C
175 with Milli-Q® water at a flow rate of 0.6 mL min^{-1} as the mobile phase. Furfural concentrations
176 were measured by gas chromatography-mass spectrometry (GC-MS) according to Doddapaneni et
177 al. (2018). Samples for HPLC and GC-MS analysis were filtered using 0.2 µm pore size filters.
178 Total chemical oxygen demand (COD_{tot}) and COD of the soluble compounds (COD_s) was measured

using the dichromate method according to the Finnish standard SFS 5504. Initial and final pH of the culture and the pH of the wastewater were determined using a WTW pH 330 meter equipped with a Hamilton® Slimtrode probe (Sigma-Aldrich, USA). Total solids, volatile solids, total nitrogen and PO_4^{3-}-P were determined by the APHA standard procedures (APHA, 1998).

2.6 Calculations

Cumulative H_2 and CO_2 production was calculated according to Logan et al. (2002) and corrected for temperature according to the Arrhenius equation. The theoretical COD_{tot} was estimated from the sum of the compounds detected by HPLC, according to the following equation (Van Haandel and Van der Lubbe, 2012):

$$COD_{tot} = 8\cdot(4x+y-2z)/(12x+y+16z) \text{ g } COD_{tot} \text{ g}^{-1} C_xH_yO_z \quad (1)$$

where x, y and z are the number of C, H and O atoms in the organic molecule, respectively.

2.7 Statistical analysis

One-way analysis of variance (ANOVA) and the Tukey test (Box et al., 1978) at $p = 0.05$ were conducted using the IBM SPSS Statistics package to assess significant differences in H_2 yield after incubation at different temperatures.

3. Results

3.1 Hydrogen production from TMP wastewater at the various temperatures

Batch incubations with TMP wastewater resulted in a different net H_2 yield at different temperatures (Figure 1; Table 2). The highest final H_2 yield of 3.6 (± 0.1) mmol H_2 g^{-1} COD_{tot} was obtained in the batch cultures at 70 °C, in which H_2 production started within 24 h of incubation and remained stable after reaching the maximum (Figure 1). The maximum H_2 yield obtained at 65 °C

was comparable to the one obtained at 70 °C, but the produced H_2 started to be consumed within 36 h resulting in a 51% lower final yield (Figure 1; Table 2). In the batch cultures at temperatures lower than 70 °C, the H_2 produced was always partially (at 37, 42, 59 and 65 °C) or totally (at 48 and 55 °C) consumed. A negligible H_2 production was obtained at both 74 and 80 °C (Figure 1), as well as in the non-inoculated control incubations (Figure S1 in Supplementary Material).

Figure 1 here

Table 2 here

3.2 COD_{tot} removal and metabolite production at the various temperatures

Similarly to H_2 production yields, dark fermentation of TMP wastewater at the various temperatures resulted in a different composition of the liquid phase (Figure 2). Acetate was the most abundant metabolite detected in the temperature range from 37 to 70 °C. The final acetate concentration increased with temperature from 0.34 (± 0.04) g COD_{tot} L^{-1} at 37 °C to 0.75 (± 0.18) g COD_{tot} L^{-1} at 55 °C, and then decreased stepwise to 0.07 (± 0.00) and 0.08 (± 0.01) g COD_{tot} L^{-1} at 74 and 80 °C, respectively (Figure 2). Butyrate was found regardless of the incubation temperature, with a final concentration ranging from 0.06 (± 0.00) g COD_{tot} L^{-1} at 70 °C to 0.19 (± 0.00) g COD_{tot} L^{-1} at 59 °C. Ethanol was produced at 37, 42, 59, 65 and 70 °C, with a maximum of 0.14 (± 0.02) g COD_{tot} L^{-1} at 65 °C (Figure 2). Dark fermentation of TMP wastewater caused a pH decrease from the initial value of 6.3: the final pH ranged from 5.7 to 6.1 after incubation at 42, 48, 55, 59, 74 and 80 °C, but was only 5.5 (± 0.1) after incubation at 37 °C, 5.2 (± 0.1) at 65 °C and 5.3 (± 0.0) at 70 °C (Figure 2).

Figure 2 here

In the batch incubations at various temperatures, the COD_{tot} removal efficiency ranged from 69.4% at 74 °C to 79.7% at 42 °C, resulting in a decrease from the initial concentration of 2.86 (± 0.00) g COD_{tot} L^{-1} to a final concentration ranging from 0.58 (± 0.23) g COD_{tot} L^{-1} at 42 °C and 0.88 (± 0.06) g COD_{tot} L^{-1} at 74 °C (Table 3). The COD_{tot} removal efficiency was likely overestimated due to the adsorption of VFAs on the activated carbon: in the adsorption experiment (Figure S2 in Supplementary Material), up to 27% of the acetate and 90% of the butyrate was, in fact, adsorbed on the fresh activated carbon after 111 h of incubation. The COD_{tot} measured was comparable to the COD_{tot} estimated (using Eq. 1) by the sum of sugars and volatile fatty acids in the liquid phase after incubation in the temperature range from 42 to 65 °C (Table 3) . However, the difference between measured and estimated COD_{tot} was about 0.20 g COD_{tot} L^{-1} at 37, 70 and 80 °C, and even higher at 74 °C (0.51 g COD_{tot} L^{-1}).

Table 3 here

3.3 Effect of temperature on the active microbial community

Incubation temperature clearly affected the composition of the active microbial communities growing for 111 h on TMP wastewater (Figure 3, Table 4). At 37 °C, *Clostridium* sp. accounted for 84 and 90% of the attached and suspended active microbial community, respectively. Higher temperature resulted in a gradual decrease of the relative abundance of *Clostridium* sp., being 54% of the attached active microbial community and < 2% of the suspended active microbial community after incubation at 55 °C (Figure 3). *Clostridium* sp. was not detected either in the attached or suspended active community after incubation at temperatures ≥ 59 °C (Figure 3). A bacterium belonging to the order of *Bacillales* closely related to *B. coagulans* (Table 4) was detected in the active attached and suspended microbial communities after incubation at 42 °C, with a relative abundance of 14 and 10%, respectively, and only in suspended form after incubation at 48 °C, with a relative abundance of 50% (Figure 3).

The relative abundance of *Thermoanaerobacterium* sp. (99% similarity to *T. thermosaccharolyticum*) among the attached active microorganisms gradually increased with temperature, being only 2% after incubation at 37 °C and 87% at 59 °C (Figure 3, Table 4). *Thermoanaerobacterium* sp. was also the most common suspended active microorganism after incubation at 55 and 59 °C, with a relative abundance of 96 and 83%, respectively. After incubation at 65 °C, the relative abundance of *Thermoanaerobacterium* sp. in the attached and suspended active microbial community decreased to 57 and 25%, respectively, whereas unclassified *Firmicutes*, with 92% similarity to *Calditerricola* sp. (Table 4) were found with a relative abundance of 30 and 28%, respectively. After incubation at 70 °C, *Thermoanaerobacterium* sp. was again the dominant active microorganism in both attached and suspended form, with a relative abundance of 88 to 89%. After incubation at 59 and 70 °C, *Caldanaerobius* sp. was also found in both attached and suspended form with relative abundance below 10% (Figure 3). After incubation at both 74 and 80 °C, the RNA concentration was not high enough to perform the analysis due to poor microbial growth, and thus microbial communities from 74 and 80 °C could not be analysed.

Figure 3 here

Table 4 here

4. Discussion

4.1 Fermentation of TMP wastewater at different temperatures

Hydrogen production from TMP wastewater inoculated with biofilm-coated activated carbon granules was observed at a wide temperature range from 37 to 70 °C (Figure 1). The highest final H_2 yield of 3.6 (± 0.1) mmol H_2 g^{-1} COD_{tot} supplied, or 4.9 mmol H_2 g^{-1} COD_{tot} consumed, was obtained at 70 °C (Table 2), which could be expected as the inoculum was collected from an FBR

283 operated at 70 °C (Dessì et al., 2018). The H_2 yield obtained in this study is of the same order of
284 magnitude compared to previous studies on thermophilic direct dark fermentation of industrial,
285 sugar-containing wastewaters. For example, Xie et al. (2014) obtained 5.8 mmol H_2 g^{-1} COD_{tot} from
286 starch wastewater at 55°C by a mixed culture dominated by *T. thermosaccharolyticum*, whereas
287 Khongkliang et al. (2017) obtained 11.4 mmol H_2 g^{-1} COD_{tot} from starch wastewater by a pure
288 culture of *T. thermosaccharolyticum*.
289
290 The thermophilic active mixed microbial community previously enriched on xylose in the FBR was
291 dominated by microorganisms closely related to *Thermoanaerobacterium thermosaccharolyticum*
292 (Dessì et al., 2018). Changing of the substrate from xylose to TMP wastewater marginally impacted
293 the active microbial community in the temperature range 59 to 70 °C, as most of the sequences
294 obtained from the RNA samples matched *T. thermosaccharolyticum* (Table 4). A mixed culture
295 dominated by *T. thermosaccharolyticum* has been shown to produce 7 mmol H_2 g^{-1} cellulose at 70
296 °C (Gadow et al., 2013), showing potential for the one-step conversion of lignocellulosic materials
297 to H_2, avoiding a costly hydrolysis step. In fact, the genus *Thermoanaerobacterium* includes strains
298 of cellulolytic microorganisms, such as some strains of *T. thermosaccharolyticum*, able to hydrolyse
299 both cellulose and hemicellulose, and produce H_2 from the resulting monosaccharides (Cao et al.,
300 2014). In this study, however, the microbial community analysis conducted at genus level does not
301 allow to assess possible cellulolytic capabilities of the detected *Thermoanaerobacterium* sp.
302
303 Although the inoculum was enriched for dark fermentation at 70 °C, H_2 production at 70 °C
304 occurred only after 24 h of incubation (Figure 1). This is probably due to the handling of the
305 inoculum, which was stored at 4 °C for one week prior to being used for this experiment. Changes
306 in gene expression and DNA replication were shown to occur in *Thermoanaerobacter*
307 *tengcongensis* as response to a cold shock (Liu et al., 2014), as could be the case for the
308 *Thermoanaerobacterium* sp. dominating the active microbial community of the inoculum used in

309 this study. Although *Thermoanaerobacterium* sp. was the most abundant microorganism (relative
310 abundance close to 90%) in both the attached and suspended microbial community at both 59 and
311 70 °C, its relative abundance was lower at 65 °C (Figure 3). The same phenomenon was observed in
312 the FBR from where the inoculum originated (Dessì et al., 2018), and was attributed to either the
313 decreased activity of *Thermoanaerobacterium* sp. or to the increased activity of competing
314 microorganisms at 65 °C.
315
316 Despite the inoculum was enriched for thermophilic dark fermentation, H_2 was already produced
317 after 12 h of incubation at 37 °C, reaching a maximum yield of 3.2 (± 0.1) mmol H_2 g^{-1} COD_{tot}
318 supplied within 24 h (Figure 1). A maximum yield of only 0.9 mmol H_2 g^{-1} COD_{tot} supplied was
319 previously obtained at 37 °C from a paper mill wastewater (production process type and wastewater
320 fraction used not specified) using heat treated digested sludge as inoculum (Marone et al., 2017).
321 The H_2 yields obtained in this study are also higher than those reported by Lucas et al. (2015) by
322 mesophilic (37 °C) dark fermentation of cassava, dairy and citrus wastewaters, which produced 1.4,
323 1.7 and 1.3 mmol H_2 g^{-1} COD_{tot} supplied, respectively. This confirms the potential of TMP
324 wastewater for dark fermentation.
325
326 *Clostridium* sp. proliferated at 37 °C accounting for more than 80% of both the attached and
327 suspended active microbial community at the end of the batch incubation (Figure 3). It is plausible
328 that *Clostridium* sp. were present in the parent activated sludge but inactive in the FBR operated at
329 70 °C (Dessì et al., 2018). In fact, *Clostridium* spp. produce spores to survive under harsh
330 conditions, and are able to restore their metabolic activity after desporulation as soon as the
331 environmental conditions become more favourable (Li and Fang, 2007). *Clostridium* sp. cells might
332 also have been present in the TMP wastewater, which was not sterilised. However, the absence of
333 H_2 and CO_2 in the non-inoculated control incubation at 37 °C (Figure S1 in Supplementary
334 Material) suggests that *Clostridium* sp. did not proliferate in the absence of the inoculum.

335

336 In this study, no H_2 was produced at 74 or 80 °C (Figure 1) and the RNA concentration was too low
337 to allow sequencing analysis, suggesting a lack of active species. This was attributed to the source
338 of inoculum used, as species within the *Thermoanaerobacterium* genus, such as *T.*
339 *thermosaccharolyticum*, may be inhibited by temperatures higher than 70 °C (Ren et al., 2008).
340 Gadow et al. (2013) obtained H_2 production from cellulose by a mixed microflora from a sewage
341 sludge digester even at 75 and 80 °C. However, H_2 production at such high temperatures was
342 attributed to *Thermoanaerobacter tengcongensins* (Gadow et al., 2013), which was not part of the
343 active microbial community in this study. Some degradation products of hemicellulose such as
344 furfural or hydromethylfurfural may inhibit fermentative microorganisms (Jönsson et al., 2013),
345 including *Thermoanaerobacterium*, at a concentration over 1 g L^{-1} (Cao et al., 2010). However, the
346 TMP process is conducted at temperatures below 120 °C, which is too low to produce such high
347 concentrations of these inhibitory compounds (Baêta et al., 2017). In fact, the concentration of
348 furfural in the TMP wastewater used in this study was below the detection limit of the GC-MS
349 (Table 1).
350
351 A decrease in the cumulative H_2 production occurred in all the incubations at temperatures lower
352 than 70 °C (Figure 1), probably due to the activity of homoacetogenic bacteria. Homoacetogenesis,
353 in which 4 moles of H_2 and 2 mol of CO_2 are consumed per mol of acetate produced, often occurs
354 in batch H_2 production experiments within the first 80 h of incubation, especially under mesophilic
355 conditions (for a review, see Saady, 2013). However, in this study, H_2 seems to be consumed faster
356 under thermophilic (from 48 to 65 °C) as compared to mesophilic (37 °C) conditions (Figure 1),
357 suggesting that homoacetogenic microorganisms were mainly thermophiles or moderate
358 thermophiles. The CO_2 concentration in the batch incubations did not decrease as expected in case
359 of homoacetogenesis (Figure S3 in Supplementary Material). However, this could be explained
360 considering that CO_2 production may occur also through non-hydrogenic pathways, mainly the

361 ethanol production pathway (Figure 2). In the non-inoculated control incubations, CO_2 was also
362 detected, together with acetate, at both 55 and 70 °C, where H_2 production was not observed (Figure
363 S1 in Supplementary Material). This suggests that non-hydrogenic, CO_2 producing pathways other
364 than ethanol production could have occurred as well.
365
366 Homoacetogens are among the most phylogenetically diverse functional groups of bacteria (Drake
367 et al., 2006). Among the thermophiles, *Moorella thermoacetica*, which accounted for 5% of the
368 suspended active community at 55 °C and 6% of the attached active community at 65 °C (Figure 3),
369 is a known homoacetogenic bacterium with an optimum growth temperature ranging from 55 to 60
370 °C (Drake et al., 2006). *Clostridium* spp. have also been previously found in thermophilic
371 fermentative reactors and associated with homoacetogenesis (Ryan et al., 2008). It is plausible that
372 the shift to autotrophic metabolism (e.g. homoacetogenesis) occurred after substrate depletion, as
373 suggested by Oh et al. (2003).
374
375 ***4.2 COD_{tot} balance and metabolite production***
376 The COD_{tot} measured in the beginning of the incubations (Table 3) was 15% lower than the value
377 obtained while characterizing the TMP wastewater (Table 1). Apparently, some biological or non-
378 biological reaction occurred while storing the TMP wastewater at 4 °C before the experiment,
379 resulting in a slight COD_{tot} concentration decrease. The COD_{tot} removal efficiency during the
380 incubations was 69 to 80% regardless the incubation temperature (Table 3). It is in line with the
381 COD_{tot} removal from anaerobic digestion of pulp and paper wastewater reported in the literature
382 (Meyer and Edwards, 2014), but higher than expected for dark fermentation which usually removes
383 only 30 to 40% of the COD_{tot} (Sharma and Li, 2010). This was likely due to the adsorption of VFAs
384 on the activated carbon (Figure S2 in Supplementary Material), which caused an overestimation of
385 the COD_{tot} removal. However, it should be noted that the adsorption experiment (Figure S2 in
386 Supplementary Material) was performed with fresh activated carbon, whereas the main experiment

was conducted with biofilm-covered activated carbon. The latter could have been partially saturated with VFAs at the moment of inoculation, as VFAs were also produced in the FBR from where the inoculum originated (Dessì et al., 2018).

In the temperature range from 42 to 65 °C, more than 85% of the residual COD_{tot} was detected as acetate, butyrate or ethanol by HPLC analysis (Table 3). However, 30 to 37% of the residual COD_{tot} was not detected as compounds identified by HPLC analysis after incubation at 37, 70 and 80 °C, and even 58% of the residual COD_{tot} was not identified after incubation at 74 °C. At 74 and 80 °C, most of the undetected COD_{tot} was likely constituted by polysaccharides such as cellulose, which were not degraded due to the lack of bacterial activity at such high temperatures. At 74 and 80 °C, CO_2 was also not produced (Figure S3 in Supplementary Material), supporting this conclusion. Lignin, which accounts for 16-49% of the COD_{tot} in TMP wastewater (Rintala and Puhakka, 1994) can release VFAs at temperatures around 80 °C (Veluchamy and Kalamdhad, 2017), suggesting that the acetate and butyrate detected at 74 and 80 °C (Figure 2) were produced physically rather than biologically.

The simultaneous production of acetate and butyrate suggests that H_2 was produced via both the acetate and butyrate pathway in the temperature range from 37 to 70 °C. Acetate was the main metabolite found in the liquid phase at all temperatures tested, excluding 74 and 80 °C (Figure 2), and was associated either to H_2 production through the acetate dark fermentative pathway or H_2 consumption by homoacetogenesis. Interestingly, acetate production increased with temperature in the range from 37 to 55 °C, and then decreased stepwise at temperatures above 55 °C (Figure 2). In particular, the high (> 0.7 g COD_{tot} L^{-1}) acetate (Figure 2) and concomitant low (< 0.5 mmol g^{-1} COD_{tot}) cumulative H_2 yield (Figure 1) suggest that the optimum growth temperature for homoacetogenic bacteria was about 55 °C in this study. At 70 °C, however, the H_2 produced was

not consumed during the incubation (Figure 1), suggesting inhibition of homoacetogenic microorganisms.

Solventogenesis occurred both in mesophilic (37 and 42 °C) and thermophilic (59, 65, and 70 °C) batch cultures, resulting in ethanol production (Figure 2). *Clostridium* sp., which dominated the active microbial communities under mesophilic conditions (Figure 3), may shift its metabolism from acidogenesis to solventogenesis as response to a change of pH or volatile fatty acids concentration, but the mechanism which triggers solventogenesis is not well understood (Kumar et al., 2013). A pure culture of *T. thermosaccharolyticum* has been reported to produce ethanol together with acetate and butyrate by dark fermentation of cellulose and complex lignocellulosic substrates such as corn cob, corn straw and wheat straw (Cao et al., 2014). Similarly, in this study, acetate, butyrate and ethanol were the main metabolites (Figure 2) of the dark fermentation of TMP wastewater at 65 and 70 °C by a mixed culture dominated by *T. thermosaccharolyticum* (Figure 3; Table 4).

4.3 Practical implications

Hydraulic retention times lower than 24 hours are typically used for dark fermentation of wastewater in bioreactors operated in continuous mode (Lin et al., 2012). Therefore, based on the results obtained in this batch experiment (Figure 1), dark fermentation of TMP wastewater at 37 and 65 °C appears favourable if suspended biomass bioreactors are used, as homoacetogenic bacteria would be flushed out (Figure 1). However, bioreactors retaining high active biomass content, such as FBRs or upflow anaerobic sludge bioreactors (UASBs), would enable higher organic loadings and conversion rates than suspended biomass bioreactors (Koskinen et al., 2006). Therefore, attached biomass bioreactors operated in continuous mode at 70 °C are recommended for H_2 production via dark fermentation of TMP wastewater. A proper insulation and temperature control are nevertheless necessary to keep the temperature inside the bioreactor accurately at 70 °C, as a

438 decrease of 5 °C may already result in a decreased efficiency due to H_2 consumption by
439 homoacetogenic bacteria. However, H_2 production at 70 °C can be quickly restored in case of
440 failure of the temperature control. In fact, H_2 production was detected at 70 °C within only 24 h
441 (Figure 1) with a thermophilic inoculum previously stored at 4 °C for one week.
442
443 Despite the surprisingly high COD_{tot} removal efficiency of 69 to 80 % obtained in this study (Table
444 3), dark fermentation of TMP wastewater resulted in the generation of an effluent containing 0.5 to
445 1.0 g COD_{tot} L^{-1} (Table 3), mainly in the form of VFAs, thus requiring further treatment prior
446 discharge to the environment. Such effluent can be either treated by a traditional activated sludge
447 plant, or further valorised by producing energy or high value chemicals. Promising strategies for the
448 valorisation of dark fermentation effluents include further H_2 production by photofermentation or
449 microbial electrolysis cells, methane production by anaerobic digestion, and bioplastics or
450 electricity production using microbial fuel cells (for reviews, see Ghimire et al., 2015 and Bundhoo,
451 2017).
452
453 **5. Conclusions**
454 Hydrogen was produced by dark fermentation from TMP wastewater at a wide range of
455 temperatures (37 to 70 °C) using a mixed microbial community enriched on xylose at thermophilic
456 conditions. An operation temperature of 70 °C was the most favourable for dark fermentative H_2
457 production and effectively repressed the activity of homoacetogenic bacteria. Therefore,
458 considering that TMP wastewater is produced at elevated temperature, dark fermentation at 70 °C
459 may be a cost-effective approach for the treatment and valorisation of this wastewater. However,
460 temperature must be efficiently controlled, as a shift of only a few degrees may decrease the H_2
461 yield.
462
463 **Acknowledgements**

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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Figures

Figure 1 – Hydrogen yield from batch incubation of thermomechanical pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.

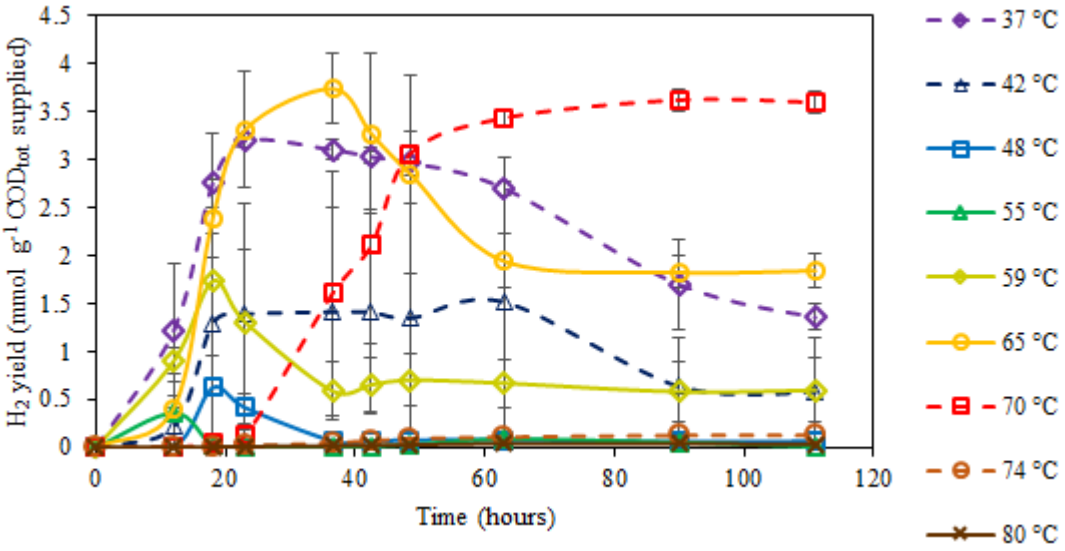


Figure 2 – Concentration of detected sugars, volatile fatty acids and alcohols (on primary y-axis) and pH (on secondary y-axis) after 111 h of incubation of thermomechanical pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.

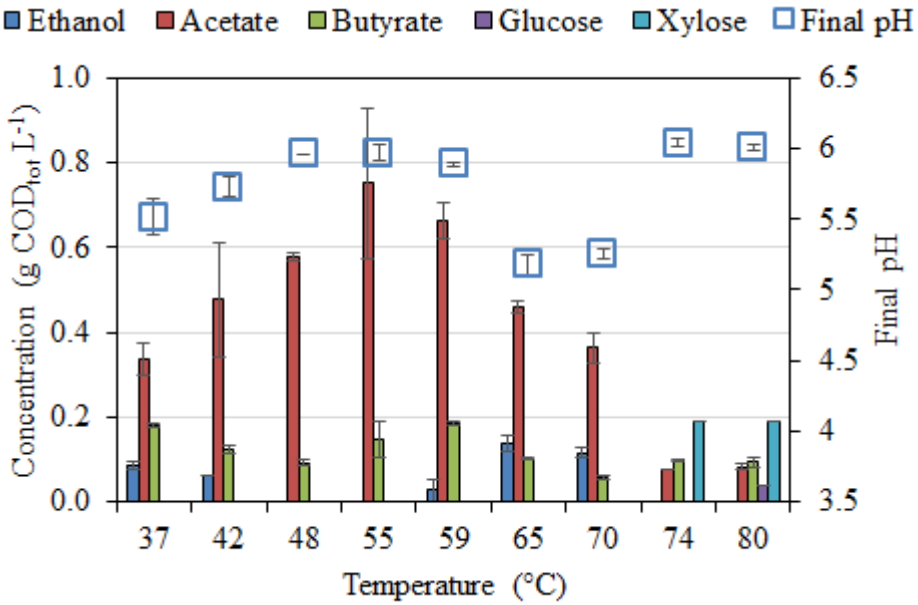
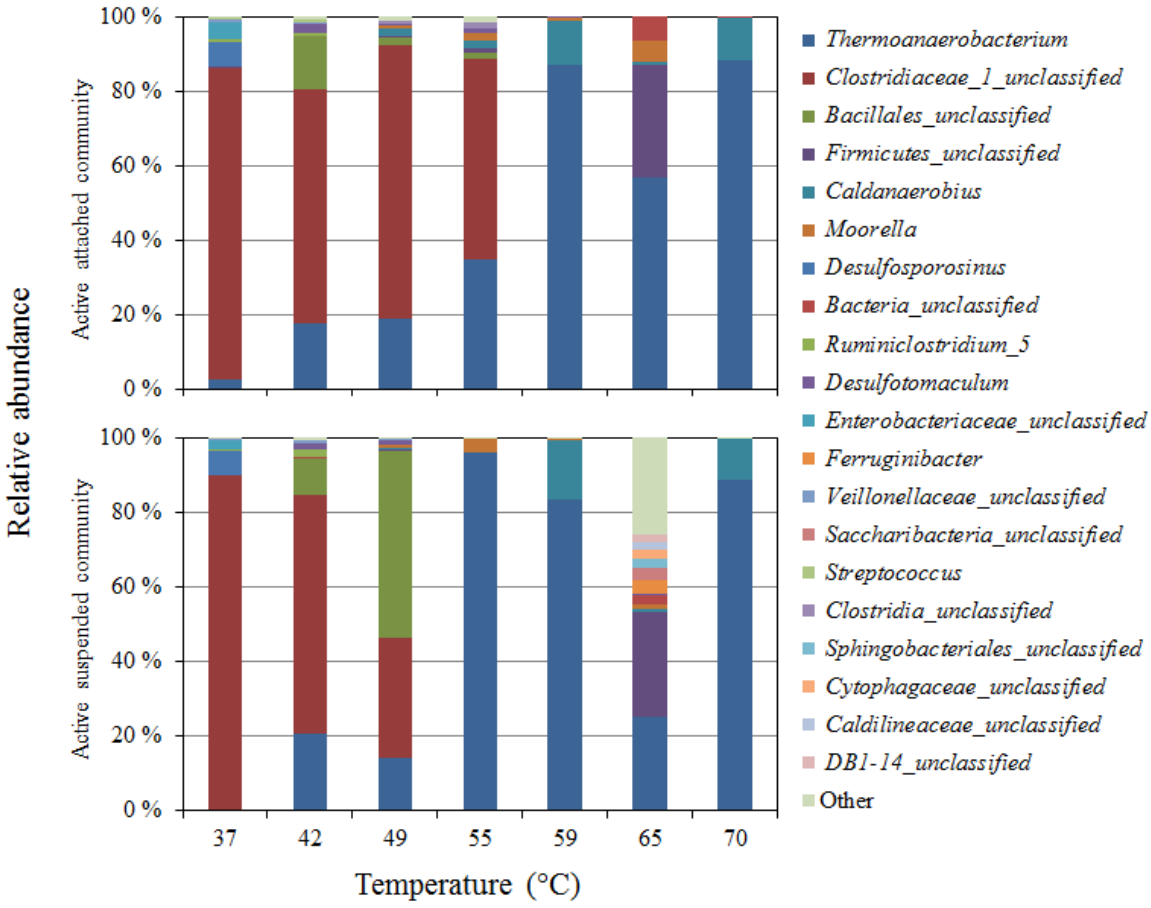


Figure 3 – Relative abundance of the active genera resulting from MiSeq sequencing of the partial 16S rRNA (transcribed to 16S cDNA) on microbiological samples obtained from the biofilm-containing activated carbon (attached) and from the liquid medium (suspended) after batch incubation with thermomechanical pulping wastewater at various temperatures (from 37 to 70 °C). The microbial genera are listed in order of relative abundance. Samples at 74 and 80 °C could not be analysed due to the low RNA concentration present in the samples.



657 **Table 1 -** Composition of the thermomechanical pulping wastewater used in this study

Parameter	**Concentration (mg L^{-1})**
Total solids	3771 ± 10
Volatile solids	2452 ± 8
Total COD	3352 ± 82
Soluble COD	3289 ± 54
Total nitrogen	< 10
Total PO_4^{3-}-P	2.8
Acetate	< 30
Furfural	< 10
Glucose	43 (± 2)
Xylose	38 (± 0)

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667 **Table 2 -** Maximum and final hydrogen yield obtained from batch incubation of thermomechanical
668 pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-
669 containing activated carbon as inoculum

Temperature (°C)	**H_2 yield (mmol H_2 g^{-1} COD_{tot} supplied)**		**H_2 yield (mmol H_2 g^{-1} COD_{tot} consumed)**	**Time required for maximum H_2 yield (h)**
	Maximum	Final	Final	
37	3.2 (± 0.1)	1.4 (± 0.1)	1.9 (± 0.2)	23
42[a]	1.5	0.6	1.3	63
48	0.6 (± 0.1)	0.1 (± 0.0)	0.1 (± 0.0)	18
55	0.4 (± 0.1)	0.0 (± 0.0)	0.0 (± 0.0)	12
59	1.7 (± 0.8)	0.6 (± 0.3)	0.9 (± 0.5)	18
65	3.7 (± 0.4)	1.8 (± 0.2)	2.6 (± 0.3)	36
70	3.6 (± 0.1)	3.6 (± 0.1)	4.9 (± 0.4)	90
74	0.1 (± 0.0)	0.1 (± 0.0)	0.2 (± 0.0)	n.a.[b]
80	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	n.a.

670 [a] Hydrogen was produced only in one of the duplicate tubes;

671 [b] Not applicable.

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679 **Table 3 -** COD_{tot} balances after incubation of thermomechanical pulping wastewater at various
680 temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as
681 inoculum

Temperature (°C)	**Final COD_{tot} measured[a] (g L^{-1})**	**Final COD_{tot} estimated[b] (g L^{-1})**	**Difference (measured – estimated)**	**COD_{tot} removal (%)[c]**
37	0.79 (± 0.00)	0.60 (± 0.04)	0.19 (± 0.04)	72.5
42	0.58 (± 0.23)	0.66 (± 0.12)	-0.08 (± 0.11)	79.7
48	0.70 (± 0.01)	0.67 (± 0.00)	0.03 (± 0.02)	75.7
55	0.82 (± 0.14)	0.90 (± 0.22)	-0.07 (± 0.08)	71.2
59	0.84 (± 0.03)	0.88 (± 0.01)	-0.04 (± 0.04)	70.7
65	0.80 (± 0.04)	0.70 (± 0.03)	0.10 (± 0.00)	72.0
70	0.73 (± 0.10)	0.54 (± 0.03)	0.20 (± 0.07)	74.3
74	0.88 (± 0.06)	0.37 (± 0.00)	0.51 (± 0.07)	69.4
80	0.62 (± 0.06)	0.41 (± 0.02)	0.21 (± 0.05)	78.4

682 [a] Data obtained by measurement according to the standard procedure; the initial COD_{tot} was 2.86 g L^{-1};
683 [b] Data obtained by the sum of the COD_{tot} equivalents (Eq. 1) of organic compounds measured in the liquid
684 phase;
685 [c] Calculated from COD_{tot} measured.
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691 **Table 4 -** Association of the six most abundant 16S rRNA gene sequences to species collected in
692 the GenBank

Family	**Genus and species**[a]	**Accession number**	**Matching sequence**[b]	**Similarity (%)**[c]
Thermoanaerobacteraceae	*Thermoanaerobacterium thermosaccharolyticum*	JX984971	474-765	99
Clostridiaceae	*Clostridium* sp.	AY548785	450-741	99
Bacillaceae	*Bacillus coagulans*	MF373392	512-803	100
Bacillaceae	*Calditerricola yamamurae*	NR_112684	529-820	92
Thermoanaerobacteraceae	*Caldanaerobius* sp.	LC127102	482-773	99
Thermoanaerobacteraceae	*Moorella thermoacetica*	CP017237	145404-145695	100

693 [a] Closest cultured species in GenBank;
694 [b] Section of the 16S rRNA gene (in bp) matching the sequence obtained by MiSeq analysis;
695 [c] Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest cultured
696 species in GenBank.
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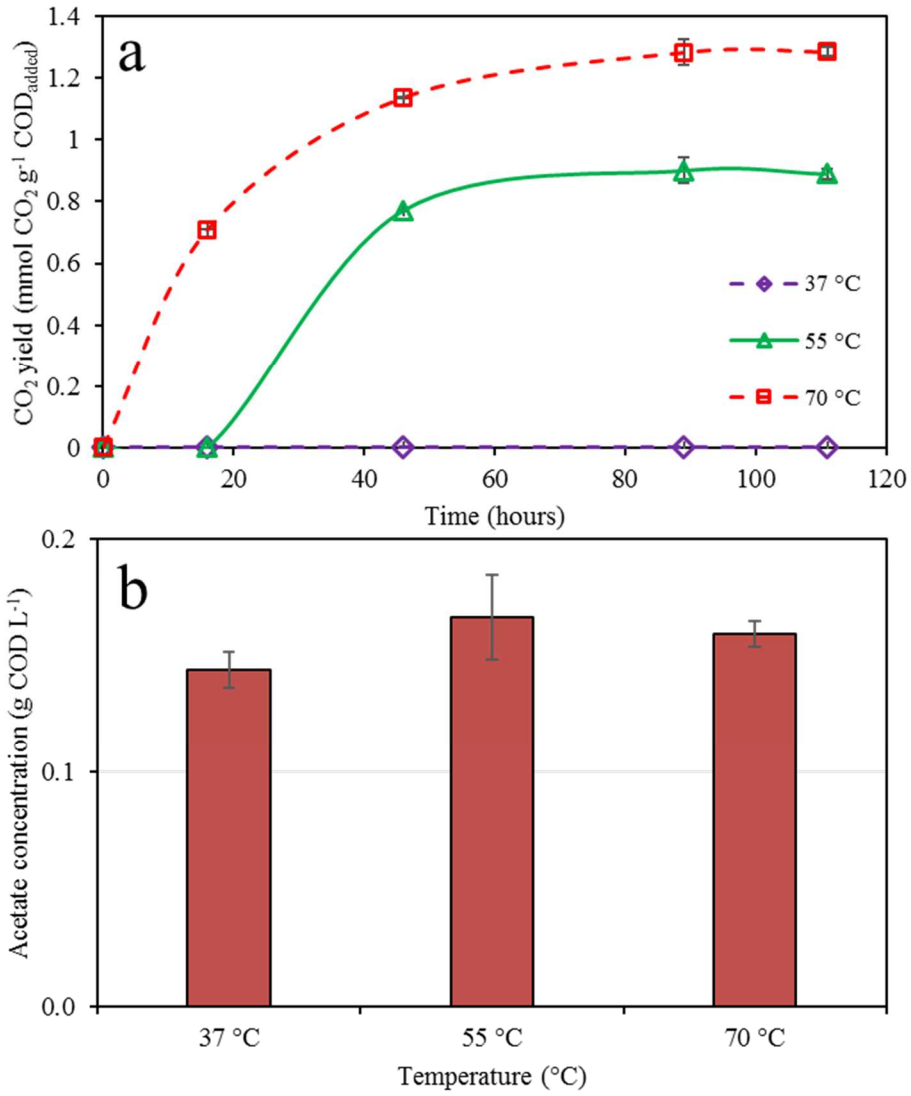
Supporting material

Figure S1 – Carbon dioxide yield profiles (a) and acetate yield after 111 h of incubation (b) obtained in the non-inoculated incubation of thermomechanical pulping wastewater at 37, 55 and 70 °C. Hydrogen was not detected at any of the temperatures tested. Error bars refer to the standard deviations of the duplicates.

Figure S2 – Adsorption of VFAs on activated carbon. Acetate and butyrate concentration before and after 111 h of incubation with fresh activated carbon at 42, 65 and 80 °C. The initial concentration of VFAs was chosen hypothesizing that only 40% of the 2.86 g COD_{tot} L^{-1} was removed through dark fermentation, and equally distributing the remaining 1.71 g COD_{tot} L^{-1} between acetate and butyrate. Error bars refer to the standard deviations of the duplicates.

Figure S3 – Carbon dioxide yield from batch incubation of thermomechanical pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.

Figure S1 – Carbon dioxide yield profiles (a) and acetate yield after 111 h of incubation (b) obtained in the non-inoculated incubation of thermomechanical pulping wastewater at 37, 55 and 70 °C. Hydrogen was not detected at any of the temperatures tested. Error bars refer to the standard deviations of the duplicates.



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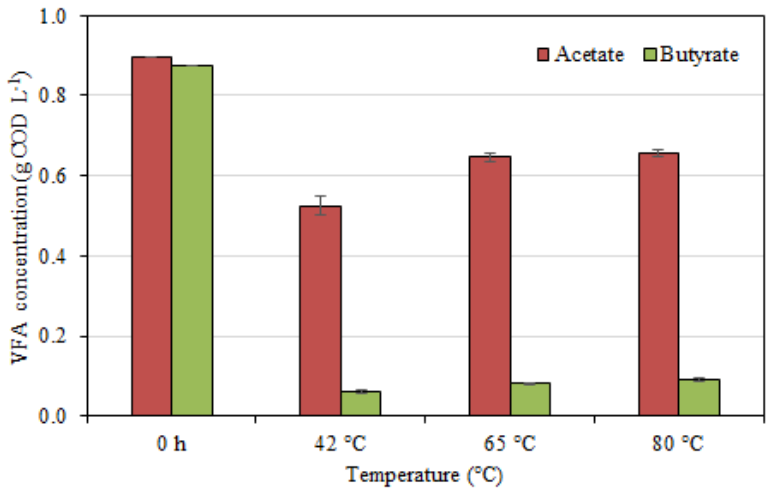
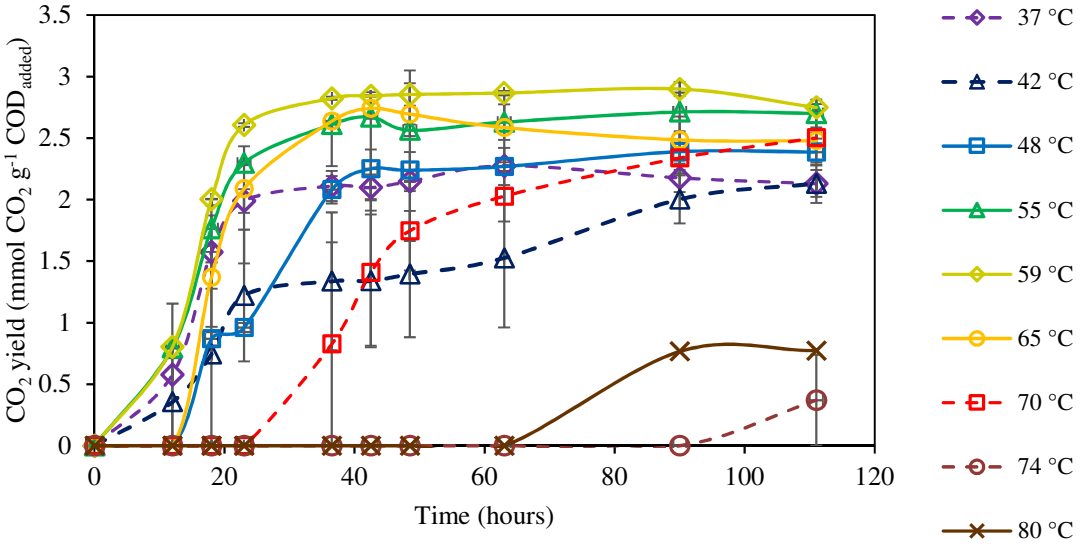
Figure S3 – Carbon dioxide yield from batch incubation of thermomechanical pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.