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

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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 H_2 yield of 3.6 (± 0.1) mmol H_2 mol^{-1} COD_{tot} supplied. Lower hydrogen yields were obtained at the temperature range 37-65 °C, likely due to consumption of the produced hydrogen by homoacetogenesis. No hydrogen production was detected at temperatures above 70 °C. TMP wastewaters are released at high temperatures (50-80 °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

Hydrogen, lignocellulose, MiSeq, pulp and paper mill, *Thermoanaerobacterium*, 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 or biochemicals, is a promising strategy to expand the product platform, reduce waste disposal costs and fulfil the environmental policies 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, resulting in high energy consumption, emission of CO_2 to the atmosphere and in the production of large volumes of waste sludge, which require further treatment prior to disposal (Kinnunen et al., 2015). Instead, anaerobic processes have the advantages of coupling wastewater treatment to renewable energy production, produce a lower amount of waste sludge and require a smaller volume than aerobic processes (Ashrafi et al., 2015).

In thermomechanical pulping (TMP), the wood fibres are treated by hot steam under pressure (Pokhrel and Viraraghavan, 2004). TMP 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 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) and pulp and paper mill effluent hydrolysates (Hay et al., 2015; Lakshmidevi and Muthukumar, 2010) and even

from untreated pulps (Nissilä et al., 2012). H_2 production has also been reported from carbohydrates-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 (25-40% of the total COD) (Rintala and Puhakka, 1994), to our knowledge it has not yet been tested as a substrate for dark fermentation. The typically low concentration or even absence of possible inhibitory compounds such as sulphate, sulphite, hydrogen peroxide, resin acid and fatty acids, makes TMP wastewater a more potential substrate for dark fermentation than wastewaters from chemical-based pulping (Ekstrand et al., 2013; Rintala and Puhakka, 1994).

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 50-80 °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 (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 (De Vrieze et al., 2016). 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* 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 (37-80 °C), and 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 originated 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 (FBR 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 FBR granules were stored at 4 °C for one week prior utilisation. This inoculum was used because the microbial community was dominated by *Thermoanaerobacterium* (Dessì et al., 2018), which previously showed potential for hydrolysis of lignocellulosic substrates and H_2 production from the resulting sugars (Abreu et al., 2012; 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 minimize biological activity that might affect its composition. The wastewater had a pH of 5.0 and its composition as given in Table 1.

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 FBR 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 before incubation using a syringe and a needle. 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, Germany) 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 inactivity times may affect the RNA-level analysis (De Vrieze et al., 2016).

Gas samples were collected for analysis 1-3 times per day. End-point liquid samples were collected and stored at -20 °C before analysis. Abiotic negative controls, 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 MQ water (0.86 g COD 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.

2.4 Microbial community analyses

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

2.5 Analytical methods

Gas production in the tubes was quantified by a volumetric syringe method (Owen et al., 1979), and the gas composition was determined by gas chromatography – thermal conductivity detector (GC-TCD) as reported previously (Dessì et al., 2017). Acetate, butyrate, ethanol, propionate, lactate, and formate concentrations were measured with a high–performance liquid chromatograph (HPLC) equipped with a refractive index detector (RID) (Shimadzu, Japan) and a Rezex RHM–monosaccharide column (Phenomenex, USA) held at 40 °C. The mobile phase and flow rate were 5 mM H_2SO_4 and 0.6 mL min^{-1}, respectively. Glucose and xylose concentrations were measured using a HPLC equipped with a RID and a RPM-monosaccharide column (Phenomenex, USA) held at 85 °C with MQ water at a flow rate of 0.6 mL min^{-1} as the mobile phase. 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 (TS), volatile solids (VS), total nitrogen and PO_4^{3-}-P were determined by the APHA standard procedures (APHA, 1998). Furfural concentrations were measured by gas chromatography – mass spectrometry (GC-MS) according to Doddapaneni et al. (Doddapaneni et al., 2018). Samples for HPLC and GC-MS analysis were filtered using 0.2 µm pore size filters.

2.6 Calculations

Cumulative H_2 and CO_2 production was calculated according to Logan et al. (Logan et al., 2002) and corrected for temperature according to the Arrhenius equation. The theoretical COD was estimated from the sum of the compounds detected by HPLC, according to the following equation:

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

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 H_2 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) mol H_2 g^{-1} COD_{tot} was obtained in the batch cultures at 70 °C, in which H_2 production started after a 24-h lag-time 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. H_2 production was negligible at both 74 and 80 °C (Figure 1), as well as in the negative controls (see additional file 1).

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 was in the range 5.7-6.1 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).

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). COD_{tot} removal efficiency was likely overestimated due to the adsorption of VFAs on the activated carbon: in the adsorption experiment (see Additional file

2), 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 by the sum of sugars and volatile fatty acids in the liquid phase after incubation in the temperature range 42-65 °C (Table 3). However, the difference between COD_{tot} measured and calculated 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}).

3.3 Effect of temperature on the active microbial community

Incubation temperature clearly impacted the composition of the active microbial community of both the FBR granules and the liquid medium growing for 111 h on TMP wastewater (Figure 3, Table 4). At 37 °C, *Clostridium* spp. 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* spp., 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* (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* 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* 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* was again the dominant active microorganism in both attached and suspended form, with a relative abundance of 88-89%. After incubation at 59 and 70 °C, *Caldanaerobius* was also found in both attached and suspended form with relative abundance > 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.

4. Discussion

4.1 Fermentation of TMP wastewater at different temperatures

H_2 production from TMP wastewater by the FBR biomass was observed at a wide temperature range of 37-70 °C (Figure 1). The highest final H_2 yield was obtained at 70 °C, which could be expected as the used inoculum was collected from an FBR operated at 70 °C (Dessì et al., 2018). The thermophilic active mixed microbial community previously enriched on xylose in the FBR was dominated by microorganisms closely related to *Thermoanaerobacterium thermosaccharolyticum* (Dessì et al., 2018). Changing of the substrate from xylose to TMP wastewater marginally impacted the active microbial community in the temperature range 59-70 °C, as most of the sequences obtained from the RNA samples matched *T. thermosaccharolyticum* (Table 4). *T. thermosaccharolyticum* is a cellulolytic bacterium able to hydrolyse both cellulose and hemicellulose, and produce H_2 from the resulting monosaccharides (Cao et al., 2014). A mixed culture dominated by *T. thermosaccharolyticum* has been shown to produce 7 mmol H_2 g^{-1} cellulose at 70 °C (Gadow et al., 2013), showing potential for the one-step conversion of lignocellulosic materials to H_2, avoiding a costly hydrolysis step. The highest yield of 3.6 (± 0.1) mmol H_2 mol^{-1} COD_{tot} supplied, or 4.9 mmol H_2 mol^{-1} COD_{tot} consumed, obtained in this study at 70°C (Table 2), is of the same order of magnitude compared to previous studies on thermophilic direct dark fermentation of industrial, sugar-containing wastewaters. For example, Xie et al. (Xie et al., 2014)

and Khongkliang et al. (Khongkliang et al., 2017) reported a yield of, respectively, 5.8 and 11.4 mmol H_2 mol^{-1} COD_{tot} by dark fermentation of starch wastewater at 55°C using a mixed culture dominated by *T. thermosaccharolyticum* and a pure *T. thermosaccharolyticum* culture.

Although the inoculum was enriched for dark fermentation at 70 °C, H_2 production occurred only after 24 h of incubation (Figure 1). This is probably due to the handling of the inoculum, which was stored at 4 °C for one week prior to being used for this experiment. Changes in gene expression and DNA replication were shown to occur in *Thermoanaerobacter tengcongensis* as response to a cold shock (Liu et al., 2014), as could be the case for the *Thermoanaerobacterium* sp. dominating the active microbial community of the inoculum used in this study. Although *Thermoanaerobacterium* was the most abundant microorganism (relative abundance close to 90%) in both the attached and suspended microbial community at both 59 and 70 °C, its relative abundance was lower at 65 °C (Figure 3). The same phenomenon was observed in the FBR from where the inoculum originated (Dessì et al., 2018), and attributed to either the decreased activity of *Thermoanaerobacterium* or to the increased activity of competing microorganisms at 65 °C.

Despite the inoculum was enriched for thermophilic dark fermentation, H_2 was already produced after 12 h of incubation at 37 °C (Figure 1). *Clostridium* sp. proliferated at 37 °C accounting for more than 80% of both the attached and suspended active microbial community at the end of the batch incubation (Figure 3). It is plausible that *Clostridium* sp. were present in the parent activated sludge but inactive in the FBR operated at 70 °C (Dessì et al., 2018). In fact, *Clostridium* sp. produce spores to survive under harsh conditions, and are able to restore their metabolic activity after desporulation as soon as the environmental conditions become more favourable (Li and Fang, 2007). *Clostridium* sp. cells might also have been present in the TMP wastewater, which was not sterilised. However, the absence of H_2 and CO_2 in the abiotic negative control at 37 °C (see Additional file 1) suggests that *Clostidium* sp. did not proliferate in absence of the inoculum.

In this study, no H_2 was produced at 74 or 80 °C (Figure 1) and the RNA concentration was too low to allow sequencing analysis, suggesting a lack of abundant quantity of active species. This was attributed to the source of inoculum used, as bacteria of the *Thermoanaerobacterium* genus, such as *T. thermosaccharolyticum*, may be inhibited by temperatures higher than 70 °C (Ren et al., 2008). Gadow et al. (Gadow et al., 2013) obtained H_2 production from cellulose by a mixed microflora from a sewage sludge digester even at 75 and 80 °C. However, H_2 production at such high temperatures was attributed to *Thermoanaerobacter tengcongensins* (Gadow et al., 2013), which was not part of the active microbial community in this study. Some degradation products of hemicellulose such as furfural or hydromethylfurfural may inhibit fermentative microorganisms (Jönsson et al., 2013), including *Thermoanaerobacter*, at a concentration over 1 g L^{-1} (Cao et al., 2010). However, the TMP process is conducted at a temperature < 120 °C, which is too low to produce such high concentrations of these inhibitory compounds (Baêta et al., 2017). In fact, the concentration of furfural in the TMP wastewater used in this study was below the detection limit of the GC-MS (Table 1).

A decrease in the cumulative H_2 production occurred in all the incubations at temperatures lower than 70 °C (Figure 1), probably due to the activity of homoacetogenic bacteria. Homoacetogenesis, in which 4 moles of H_2 and 2 mol of CO_2 are consumed per mol of acetate produced, often occurs in batch H_2 production experiments within the first 80 h of incubation, especially under mesophilic conditions (for a review, see Saady, 2013). However, in this study, H_2 seems to be consumed faster under thermophilic (from 48 to 65 °C) rather than mesophilic (37 °C) conditions (Figure 1), suggesting that homoacetogenic microorganisms were mainly thermophiles or moderate thermophiles. The CO_2 concentration in the batch incubations did not decrease as expected in case of homoacetogenesis (see Additional file 3). However, this could be explained considering that CO_2 production may occur also through non-hydrogenic pathways, such as the ethanol pathway (Figure

2). CO_2 was also detected in the abiotic negative controls at both 55 and 70 °C, in which H_2 production was not observed (see Additional file 1).

Homoacetogens are among the most phylogenetically diverse functional groups of bacteria (Drake et al., 2006). Among the thermophiles, *Moorella thermoacetica*, which accounted for 5% of the suspended active community at 55 °C and 6% of the attached active community at 65 °C (Figure 3), is a known homoacetogenic bacterium with an optimum growth temperature of 55-60 °C (Drake et al., 2006). Also *Clostridium* sp. have been previously found in thermophilic fermentative reactors and associated with homoacetogenesis (Ryan et al., 2008). It is plausible that the shift to autotrophic metabolism (e.g. homoacetogenesis) occurred after substrate depletion, as suggested by Oh et al. (Oh et al., 2003).

4.2 COD_{tot} balance and metabolite production

The initial COD_{tot} measured in the beginning of the incubations (Table 3) was 15% lower than the value obtained while characterizing the TMP wastewater (Table 1). Apparently, some biological or non-biological reaction occurred while storing the TMP wastewater at 4 °C before the experiment, resulting in a slight COD_{tot} concentration decrease. The COD_{tot} removal efficiency during the incubations was 69-80% regardless the incubation temperature (Table 3), which is in line with the COD_{tot} removal from anaerobic digestion of pulp and paper wastewater reported in the literature (Meyer and Edwards, 2014), but higher than expected for dark fermentation which usually removes only 30-40% of the COD_{tot} (Sharma and Li, 2010). This was due to the adsorption of VFAs on the activated carbon (see Additional file 2), which caused an overestimation of the COD_{tot} removal. However, it should be noted that the adsorption experiment (see Additional file 2) 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 produced in the FBR from where the inoculum originated (Dessì et al., 2018).

In the temperature range 42-65 °C, more than 85% of the residual COD_{tot} was detected as acetate, butyrate or ethanol by HPLC analysis (Table 3). However, 30-37% 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 incubations at 74 °C. At 74 and 80 °C, most of the undetected COD_{tot} is likely constituted by polysaccharides such as cellulose, which were not degraded due to the lack of bacterial activity at such high temperatures. CO_2 was also not produced at 74 and 80 °C (see Additional file 3), supporting this conclusion. VFAs can be released from lignocellulosic materials 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 37-70 °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 37-55 °C, and then decreased stepwise for 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 < 24 hours are typically used for dark fermentation of wastewater (Lin et al., 2012). Therefore, based on the results obtained (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, due to the high dilution of TMP wastewater, bioreactors retaining high active biomass content, such as FBRs or upflow anaerobic sludge bioreactors (UASBs), would enable higher organic loading and conversion rates than suspended biomass bioreactors (Koskinen et al., 2006). Therefore, dark fermentation of TMP in attached biomass bioreactors at 70 °C is recommended (Figure 1). A proper insulation and temperature control are nevertheless necessary to keep accurately 70 °C in the bioreactor, as a decrease of 5 °C may already result in a decreased efficiency due to H_2 consumption by homoacetogenic bacteria. However, H_2 production at 70 °C can be quickly restored in case of failure of the temperature control. In fact, H_2 production was detected at 70 °C in only 24 h (Figure 1) with a thermophilic inoculum previously stored at 4 °C for one week.

Despite the surprisingly high COD removal efficiency of 69-80 % obtained in this study (Table 3), dark fermentation of TMP wastewater resulted in the generation of an effluent containing 0.5 – 1.0 g COD_{tot} L^{-1} (Table 3), mainly in the form of VFAs, thus requiring further treatment prior to be discharged. Such effluent can be either treated by a traditional activated sludge plant, or further valorised by producing energy or high value chemicals. Promising strategies for the valorisation of dark fermentation effluents include further H_2 production by photofermentation or microbial electrolysis cells, methane production by anaerobic digestion, production of bioplastics or electricity production using microbial fuel cells (for reviews, see Ghimire et al., 2015 and Bundhoo, 2017).

5. Conclusions

H_2 was produced by dark fermentation from TMP wastewater at a wide range of temperatures (37-70 °C) using a mixed microbial community enriched on xylose at thermophilic conditions. An operation temperature of 70 °C was the most favourable for dark fermentative H_2 production and effectively repressed activity of homoacetogenic bacteria. Therefore, considering also that TMP wastewater is produced at elevated temperature, dark fermentation at 70 °C may be a cost-effective approach for the treatment and valorisation of this wastewater. However, temperature must be efficiently controlled, as even a shift of a few degrees may decrease the H_2 yield.

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References

Abreu, A.A., Karakashev, D., Angelidaki, I., Sousa, D.Z., Alves, M.M., 2012. Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. Biotechnol. Biofuels 5, 6.

APHA, 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC.

Ashrafi, O., Yerushalmi, L., Haghighat, F., 2015. Wastewater treatment in the pulp-and-paper industry: A review of treatment processes and the associated greenhouse gas emission. J. Environ. Manage. 158, 146–157.

Badiei, M., Jahim, J.M., Anuar, N., Abdullah, S.R.S., 2011. Effect of hydraulic retention time on biohydrogen production from palm oil mill effluent in anaerobic sequencing batch reactor. Int. J. Hydrogen Energy 36, 5912–5919.

Baêta, B.E.L., Cordeiro, P.H. de M., Passos, F., Gurgel, V.A.L., de Aquino, S.F., Fdz-Polanco, F., 2017. Steam explosion pretreatment improved the biomethanization of coffee husks. Bioresour. Technol. 245, 66–72.

Boodhun, B.S.F., Mudhoo, A., Kumar, G., Kim, S.-H., Lin, C.-Y., 2017. Research perspectives on constraints, prospects and opportunities in biohydrogen production. Int. J. Hydrogen Energy 42, 27471–27481.

Bundhoo, Z.M.A., 2017. Coupling dark fermentation with biochemical or bioelectrochemical systems for enhanced bio-energy production: A review. Int. J. Hydrogen Energy 42, 26667–26686.

Cao, G.-L., Zhao, L., Wang, A.-J., Wang, Z.-Y., Ren, N.-Q., 2014. Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. Biotechnol. Biofuels 7, 82.

Cao, G., Ren, N., Wang, A., Guo, W., Xu, J., Liu, B., 2010. Effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium*

thermosaccharolyticum W16. Int. J. Hydrogen Energy 35, 13475–13480.

De Vrieze, J., Regueiro, L., Props, R., Vilchez-Vargas, R., Jáuregui, R., Pieper, D.H., Lema, J.M., Carballa, M., 2016. Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. Biotechnol. Biofuels 9, 244.

Dessì, P., Lakaniemi, A.-M., Lens, P.N.L., 2017. Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70 °C. Water Res. 115, 120–129.

Dessì, P., Porca, E., Waters, N.R., Lakaniemi, A.-M., Collins, G., Lens, P.N.L., 2018. Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: Biohydrogen production and active microbial community. Int. J. Hydrogen Energy 43, 5473–5485.

Doddapaneni, T.R.K.C., Jain, R., Praveenkumar, R., Rintala, J., Romar, H., Konttinen, J., 2018. Adsorption of furfural from torrefaction condensate using torrefied biomass. Chem. Eng. J. 334, 558–568.

Drake, H.L., Küsel, K., Matthies, C., 2006. Acetogenic Prokaryotes, in: Springer (Ed.), The Prokaryotes. New York, pp. 354–420.

Eker, S., Sarp, M., 2017. Hydrogen gas production from waste paper by dark fermentation: Effects of initial substrate and biomass concentrations. Int. J. Hydrogen Energy 42, 2562–2568.

Ekstrand, E.-M., Larsson, M., Truong, X.-B., Cardell, L., Borgström, Y., Björn, A., Ejlertsson, J., Svensson, B.H., Nilsson, F., Karlsson, A., 2013. Methane potentials of the Swedish pulp and paper industry – A screening of wastewater effluents. Appl. Energy 112, 507–517.

Elsharnouby, O., Hafez, H., Nakhla, G., El Naggar, M.H., 2013. A critical literature review on biohydrogen production by pure cultures. Int. J. Hydrogen Energy 38, 4945–4966.

Gadow, S.I., Jiang, H., Hojo, T., Li, Y.-Y., 2013. Cellulosic hydrogen production and microbial community characterization in hyper-thermophilic continuous bioreactor. Int. J. Hydrogen Energy 38, 7259–7267.

Gao, W.J., Han, M.N., Xu, C.C., Liao, B.Q., Hong, Y., Cumin, J., Dagnew, M., 2016. Performance of submerged anaerobic membrane bioreactor for thermomechanical pulping wastewater

treatment. J. Water Process Eng. 13, 70–78.

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Appl. Energy 144, 73–95.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66, 5488–5491.

Hay, J.X.W., Wu, T.Y., Juan, J.C., Jahim, J.M., 2015. Improved biohydrogen production and treatment of pulp and paper mill effluent through ultrasonication pretreatment of wastewater. Energy Convers. Manag. 106, 576–583.

Jung-Yeol, L., Chen, X.-J., Lee, E.-J., Min, K.-S., 2012. Effects of pH and carbon sources on biohydrogen production by co-culture of *Clostridium butyricum* and *Rhodobacter sphaeroides*. J. Microbiol. Biotechnol. 22, 400–406.

Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels 6, 16.

Karadag, D., Puhakka, J.A., 2010. Effect of changing temperature on anaerobic hydrogen production and microbial community composition in an open-mixed culture bioreactor. Int. J. Hydrogen Energy 35, 10954–10959.

Khongkliang, P., Kongjan, P., Utarapichat, B., Reungsang, A., O-Thong, S., 2017. Continuous hydrogen production from cassava starch processing wastewater by two-stage thermophilic dark fermentation and microbial electrolysis. Int. J. Hydrogen Energy 42, 27584–27592.

Kinnunen, V., Ylä-Outinen, A., Rintala, J., 2015. Mesophilic anaerobic digestion of pulp and paper industry biosludge–long-term reactor performance and effects of thermal pretreatment. Water Res. 87, 105–111.

Koskinen, P.E.P., Kaksonen, A.H., Puhakka, J.A., 2006. The Relationship Between Instability of H_2 Production and Compositions of Bacterial Communities Within a Dark Fermentation

Fluidized-Bed Bioreactor. Biotechnol. Bioeng. 97, 742–758.

Kumar, G., Sivagurunathan, P., Sen, B., Mudhoo, A., Davila-Vazquez, G., Wang, G., Kim, S.-H., 2017. Research and development perspectives of lignocellulose-based biohydrogen production. Int. Biodeterior. Biodegradation 119, 225–238.

Kumar, M., Gayen, K., Saini, S., 2013. Role of extracellular cues to trigger the metabolic phase shifting from acidogenesis to solventogenesis in *Clostridium acetobutylicum*. Bioresour. Technol. 138, 55–62.

Lakshmidevi, R., Muthukumar, K., 2010. Enzymatic saccharification and fermentation of paper and pulp industry effluent for biohydrogen production. Int. J. Hydrogen Energy 35, 3389–3400.

Li, C., Fang, H.H.P., 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit. Rev. Environ. Sci. Technol. 37, 1–39.

Lin, C.-Y., Lay, C.-H., Sen, B., Chu, C.-Y., Kumar, G., Chen, C.-C., Chang, J.-S., 2012. Fermentative hydrogen production from wastewaters: A review and prognosis. Int. J. Hydrogen Energy 37, 15632–15642.

Liu, B., Zhang, Y., Zhang, W., 2014. RNA-seq-based analysis of cold shock response in *Thermoanaerobacter tengcongensis*, a bacterium harboring a single cold shock protein encoding gene. PLoS One 9, 3.

Logan, B.E., Oh, S.-E., Kim, I.S., Van Ginkel, S., 2002. Biological hydrogen production measured in batch anaerobic respirometers. Environ. Sci. Technol. 36, 2530–2535.

Machani, M., Nourelfath, M., D'Amours, S., 2014. A mathematically-based framework for evaluating the technical and economic potential of integrating bioenergy production within pulp and paper mills. Biomass and Bioenergy 63, 126–139.

Meyer, T., Edwards, E.A., 2014. Anaerobic digestion of pulp and paper mill wastewater and sludge. Water Res. 65, 321–349.

Moncada B., J., Aristizábal M., V., Cardona A., C.A., 2016. Design strategies for sustainable biorefineries. Biochem. Eng. J. 116, 122–134. https://doi.org/10.1016/j.bej.2016.06.009

Nissilä, M.E., Li, Y.-C., Wu, S.-Y., Lin, C.-Y., Puhakka, J.A., 2012. Hydrogenic and methanogenic fermentation of birch and conifer pulps. Appl. Energy 100, 58–65.

Oh, S.-E., Van Ginkel, S., Logan, B.E., 2003. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environ. Sci. Technol. 37, 5186–90.

Owen, W.F., Stuckey, D.C., Healy Jr., J.B., Young, L.Y., McCarty, P.L., 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res. 13, 485–492.

Pokhrel, D., Viraraghavan, T., 2004. Treatment of pulp and paper mill wastewater—A review. Sci. Total Environ. 333, 37–58.

Ren, N., Cao, G., Wang, A., Lee, D., Guo, W., Zhu, Y., 2008. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. Int. J. Hydrogen Energy 33, 6124–6132.

Rintala, J.A., Lepistö, S.S., 1992. Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C. Water Res. 26, 1297–1305.

Rintala, J.A., Puhakka, J.A., 1994. Anaerobic treatment in pulp- and paper-mill waste management: A review. Bioresour. Technol. 47, 1–18.

Ryan, P., Forbes, C., Colleran, E., 2008. Investigation of the diversity of homoacetogenic bacteria in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene. Water Sci. Technol. 57, 675–680.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38, 13172–13191.

Sharma, Y., Li, B., 2010. Optimizing energy harvest in wastewater treatment by combining anaerobic hydrogen producing biofermentor (HPB) and microbial fuel cell (MFC). Int. J. Hydrogen Energy 35, 3789–3797.

Toppinen, A., Pätäri, S., Tuppura, A., Jantunen, A., 2017. The European pulp and paper industry in transition to a bio-economy: A Delphi study. Futures 88, 1–14.

Veluchamy, C., Kalamdhad, A.S., 2017. Enhancement of hydrolysis of lignocellulose waste pulp

and paper mill sludge through different heating processes on thermal pretreatment. J. Clean. Prod. 168, 219–226.

Verhaart, M.R.A., Bielen, A.A.M., Van der Oost, J., Stams, A.J.M., Kengen, S.W.M., 2010. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: Mechanisms for reductant disposal. Environ. Technol. 31, 993–1003.

Xie, L., Dong, N., Wang, L., Zhou, Q., 2014. Thermophilic hydrogen production from starch wastewater using two-phase sequencing batch fermentation coupled with UASB methanogenic effluent recycling. Int. J. Hydrogen Energy 39, 20942–20949.

Figures

Figure 1 – H_2 yield from batch incubation of TMP wastewater at various temperatures (37-80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.

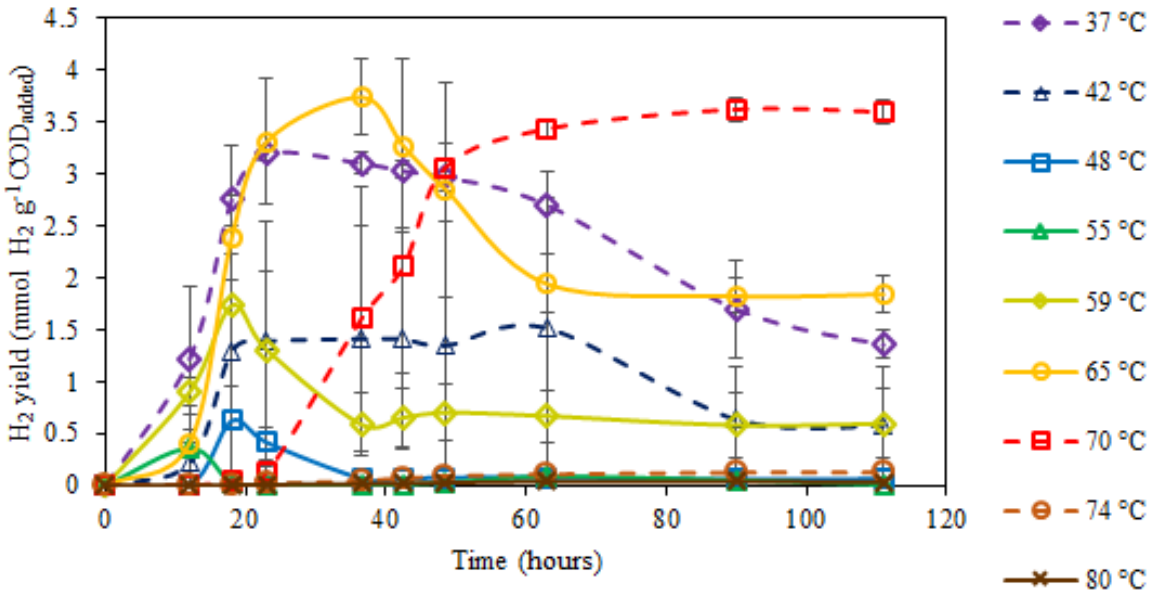

Figure 2 – Composition and pH of the liquid phase after 111 h of incubation of TMP wastewater at various temperatures (37-80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.

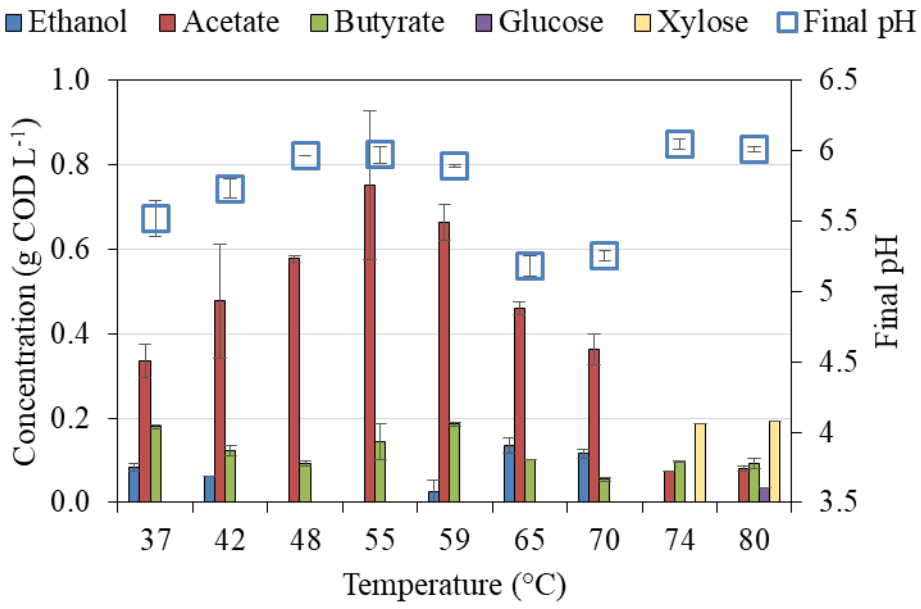

Figure 3 – Relative abundance of the active genera resulting from MiSeq sequencing of the partial 16S rRNA (transcribed to 16S cDNA) on microbiological samples obtained from the FBR granules (attached) and from the liquid medium (suspended) after batch incubation with TMP wastewater at various temperatures (37-70 °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.

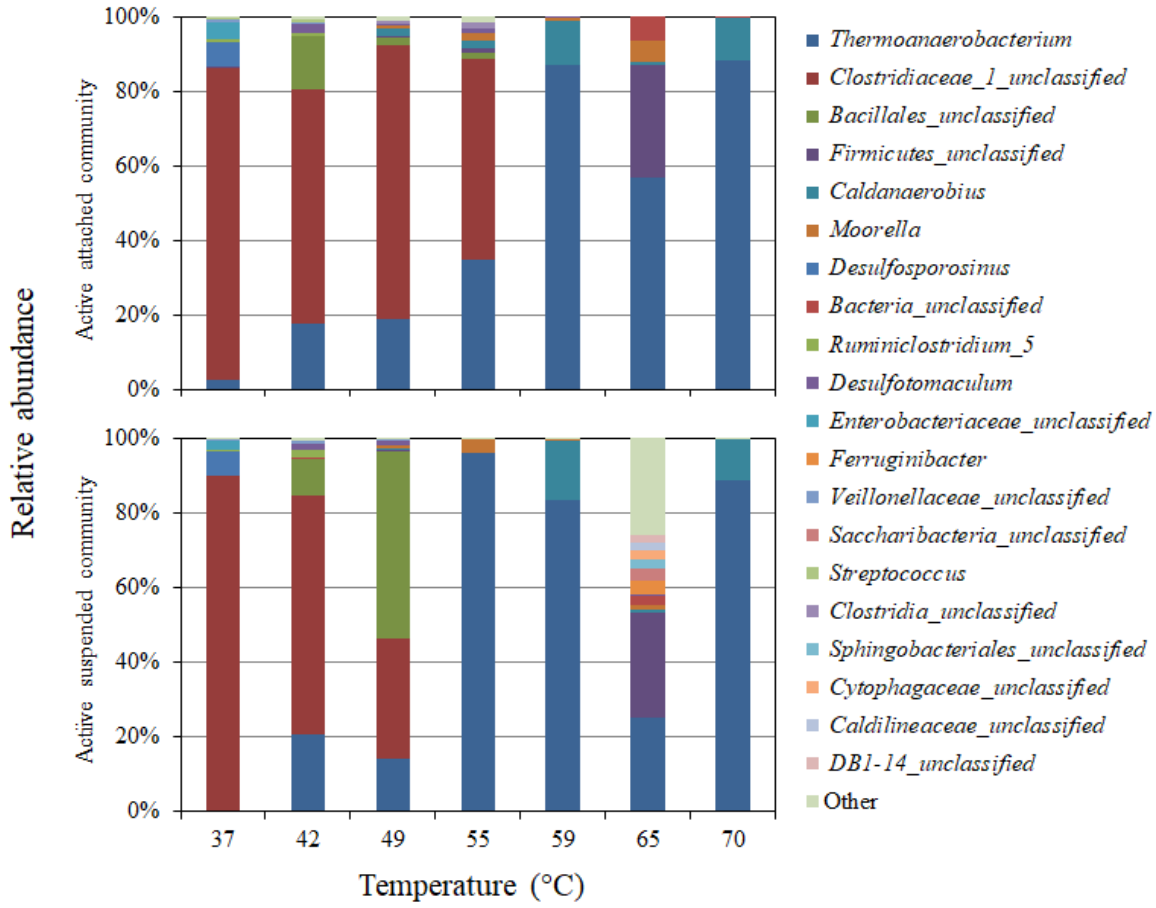

Table 1 - Composition of the thermomechanical pulping wastewater used in this study

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

Table 2 - Maximum and final H_2 yield obtained from TMP wastewater at the various temperatures

Temperature (°C)	**H_2 yield (mol H_2 g^{-1} COD_{tot} supplied)**		**H_2 yield (mol H_2 g^{-1} COD_{tot} consumed)**	**Lag time[a] (h)**
	Maximum	Final	Final	
37	3.2 (± 0.1)	1.4 (± 0.1)	1.9 (± 0.2)	23
42[b]	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.[c]
80	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	n.a.

[a] Time required to reach the maximum H_2 yield;

[b] H_2 was produced only in one of the duplicate tubes;

[c] Not applicable.

Table 3 - COD_{tot} balances after incubation of TMP wastewater with FBR granules at various temperatures

Temperature (°C)	**Final COD_{tot} measured[a] (g L^{-1})**	**Final COD_{tot} calculated[b] (g L^{-1})**	**Difference (measured – calculated)**	**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

[a] Data obtained by measurement according to the standard procedure; the initial COD_{tot} was 2.86 g L^{-1}

[b] Data obtained by the sum of the COD_{tot} equivalents of organic compounds measured in the liquid phase

[c] Calculated from measured COD_{tot}

Table 4 - Association of the six most abundant 16S rRNA gene sequences to species collected in 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

[a] Closest cultured species in GenBank

[b] Section of the 16S rRNA gene (in bp) matching the sequence obtained by MiSeq analysis

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

Supporting material

Additional file 1 – CO_2 yield profiles (a) and acetate yield after 111 h of incubation (b) obtained in the abiotic batch incubation of thermomechanical pulping (TMP) wastewater at 37, 55 and 70 °C. H_2 was not detected at any of the temperatures tested. Error bars refer to the standard deviations between the duplicates.

Additional file 2 – VFA adsorption 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 L^{-1} was removed through dark fermentation, and equally distributing the remaining 1.71 g COD L^{-1} between acetate and butyrate. Error bars refer to the standard deviations of the duplicates.

Additional file 3 – CO_2 yield from batch incubation of TMP wastewater with the dark fermentative microbial community at various temperatures (37-80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.