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 (H₂) is a carbon free fuel expected to play a pivotal role in energy production in the future (Boodhun et al., 2017). Dark fermentative H₂ 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 H₂ 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 H₂ dark fermentation.

Thermophilic dark fermentation of TMP wastewater could be advantageous, as both biological polysaccharide hydrolysis (Elsharnouby et al., 2013) and H₂ 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 H₂ 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 H₂ yield (Dessi 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 H₂ 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) H₂. 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 H₂ 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 H₂ 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 H₂ 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 H₂ 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 N₂ 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 H₂ 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 CODₗₒₜ 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

Biofilm-coated activated carbon 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 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 from 42 to 59 °C. Nucleic acids extraction using a modified method from Griffiths et al. (2000), DNA inhibition, complementary DNA (cDNA) synthesis and sequencing (using an Illumina MiSeq platform) were performed as described previously (Dessì et al., 2018). Sequence analysis (1,395,864 sequences in total, 1,238,862 after quality check) was 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 by 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 was 5 mM H$_2$SO$_4$ and the flow rate was 0.6 mL min$^{-1}$. 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 Milli-Q$^\circ$ water at a flow rate of 0.6 mL min$^{-1}$ as the mobile phase. Furfural concentrations were measured by gas chromatography-mass spectrometry (GC-MS) according to Doddapaneni et al. (2018). Samples for HPLC and GC-MS analysis were filtered using 0.2 µm pore size filters. 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 PO₄³⁻-P were determined by the APHA standard procedures (APHA, 1998).

2.6 Calculations
Cumulative H₂ and CO₂ production was calculated according to Logan et al. (2002) and corrected for temperature according to the Arrhenius equation. The theoretical CODₜₒₜ was estimated from the sum of the compounds detected by HPLC, according to the following equation (Van Haandel and Van der Lubbe, 2012):

\[
\text{COD}_{\text{tot}} = \frac{8(4x+y-2z)}{(12x+y+16z)} \text{ g COD}_{\text{tot}} \text{ g}^{-1} \text{C}_x\text{H}_y\text{O}_z
\]

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 H₂ 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 H₂ yield at different temperatures (Figure 1; Table 2). The highest final H₂ yield of 3.6 (± 0.1) mmol H₂ g⁻¹ CODₜₒₜ was obtained in the batch cultures at 70 °C, in which H₂ production started within 24 h of incubation and remained stable after reaching the maximum (Figure 1). The maximum H₂ yield obtained at 65 °C
was comparable to the one obtained at 70 °C, but the produced H\textsubscript{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\textsubscript{2} produced was always partially (at 37, 42, 59 and 65 °C) or totally (at 48 and 55 °C) consumed. A negligible H\textsubscript{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\textsubscript{tot} removal and metabolite production at the various temperatures

Similarly to H\textsubscript{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\textsubscript{tot} L\textsuperscript{-1} at 37 °C to 0.75 (± 0.18) g COD\textsubscript{tot} L\textsuperscript{-1} at 55 °C, and then decreased stepwise to 0.07 (± 0.00) and 0.08 (± 0.01) g COD\textsubscript{tot} L\textsuperscript{-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\textsubscript{tot} L\textsuperscript{-1} at 70 °C to 0.19 (± 0.00) g COD\textsubscript{tot} L\textsuperscript{-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\textsubscript{tot} L\textsuperscript{-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$_{\text{tot}}$ 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$_{\text{tot}}$ L$^{-1}$ at 42 °C and 0.88 (± 0.06) g COD$_{\text{tot}}$ L$^{-1}$ at 74 °C (Table 3). The COD$_{\text{tot}}$ 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$_{\text{tot}}$ measured was comparable to the COD$_{\text{tot}}$ 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$_{\text{tot}}$ was about 0.20 g COD$_{\text{tot}}$ L$^{-1}$ at 37, 70 and 80 °C, and even higher at 74 °C (0.51 g COD$_{\text{tot}}$ 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.

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 H₂ yield of 3.6 (± 0.1) mmol H₂ g⁻¹ COD<sub>tot</sub> supplied, or 4.9 mmol H₂ g⁻¹ COD<sub>tot</sub> consumed, was obtained at 70 °C (Table 2), which could be expected as the inoculum was collected from an FBR.
operated at 70 °C (Dessì et al., 2018). The H\textsubscript{2} yield obtained in this study 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. (2014) obtained 5.8 mmol H\textsubscript{2} g\textsuperscript{-1} COD\textsubscript{tot} from starch wastewater at 55°C by a mixed culture dominated by *T. thermosaccharolyticum*, whereas Khongkliang et al. (2017) obtained 11.4 mmol H\textsubscript{2} g\textsuperscript{-1} COD\textsubscript{tot} from starch wastewater by a pure culture of *T. thermosaccharolyticum*.

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 to 70 °C, as most of the sequences obtained from the RNA samples matched *T. thermosaccharolyticum* (Table 4). A mixed culture dominated by *T. thermosaccharolyticum* has been shown to produce 7 mmol H\textsubscript{2} g\textsuperscript{-1} cellulose at 70 °C (Gadow et al., 2013), showing potential for the one-step conversion of lignocellulosic materials to H\textsubscript{2}, avoiding a costly hydrolysis step. In fact, the genus *Thermoanaerobacterium* includes strains of cellulolytic microorganisms, such as some strains of *T. thermosaccharolyticum*, able to hydrolyse both cellulose and hemicellulose, and produce H\textsubscript{2} from the resulting monosaccharides (Cao et al., 2014). In this study, however, the microbial community analysis conducted at genus level does not allow to assess possible cellulolytic capabilities of the detected *Thermoanaerobacterium* sp.

Although the inoculum was enriched for dark fermentation at 70 °C, H\textsubscript{2} production at 70 °C 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* sp. 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 was attributed to either the decreased activity of *Thermoanaerobacterium* sp. 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, reaching a maximum yield of 3.2 (± 0.1) mmol H\(_2\) g\(^{-1}\) COD\(_{\text{tot}}\) supplied within 24 h (Figure 1). A maximum yield of only 0.9 mmol H\(_2\) g\(^{-1}\) COD\(_{\text{tot}}\) supplied was previously obtained at 37 °C from a paper mill wastewater (production process type and wastewater fraction used not specified) using heat treated digested sludge as inoculum (Marone et al., 2017). The H\(_2\) yields obtained in this study are also higher than those reported by Lucas et al. (2015) by mesophilic (37 °C) dark fermentation of cassava, dairy and citrus wastewaters, which produced 1.4, 1.7 and 1.3 mmol H\(_2\) g\(^{-1}\) COD\(_{\text{tot}}\) supplied, respectively. This confirms the potential of TMP wastewater for dark fermentation.

*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* spp. 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 non-inoculated control incubation at 37 °C (Figure S1 in Supplementary Material) suggests that *Clostridium* sp. did not proliferate in the absence of the inoculum.
In this study, no H\textsubscript{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 active species. This was attributed to the source of inoculum used, as species within the \textit{Thermoanaerobacterium} genus, such as \textit{T. thermosaccharolyticum}, may be inhibited by temperatures higher than 70 °C (Ren et al., 2008). Gadow et al. (2013) obtained H\textsubscript{2} production from cellulose by a mixed microflora from a sewage sludge digester even at 75 and 80 °C. However, H\textsubscript{2} production at such high temperatures was attributed to \textit{Thermoanaerobacter tengcongensis} (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 \textit{Thermoanaerobacterium}, at a concentration over 1 g L\textsuperscript{-1} (Cao et al., 2010). However, the TMP process is conducted at temperatures below 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\textsubscript{2} production occurred in all the incubations at temperatures lower than 70 °C (Figure 1), probably due to the activity of homoacetogenic bacteria. Homooacetogenesis, in which 4 moles of H\textsubscript{2} and 2 mol of CO\textsubscript{2} are consumed per mol of acetate produced, often occurs in batch H\textsubscript{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\textsubscript{2} seems to be consumed faster under thermophilic (from 48 to 65 °C) as compared to mesophilic (37 °C) conditions (Figure 1), suggesting that homoacetogenic microorganisms were mainly thermophiles or moderate thermophiles. The CO\textsubscript{2} concentration in the batch incubations did not decrease as expected in case of homoacetogenesis (Figure S3 in Supplementary Material). However, this could be explained considering that CO\textsubscript{2} production may occur also through non-hydrogenic pathways, mainly the...
ethanol production pathway (Figure 2). In the non-inoculated control incubations, \( \text{CO}_2 \) was also detected, together with acetate, at both 55 and 70 °C, where \( \text{H}_2 \) production was not observed (Figure S1 in Supplementary Material). This suggests that non-hydrogenic, \( \text{CO}_2 \) producing pathways other than ethanol production could have occurred as well.

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 ranging from 55 to 60 °C (Drake et al., 2006). *Clostridium* spp. have also 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. (2003).

### 4.2 COD\(_{\text{tot}}\) balance and metabolite production

The COD\(_{\text{tot}}\) 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\(_{\text{tot}}\) concentration decrease. The COD\(_{\text{tot}}\) removal efficiency during the incubations was 69 to 80% regardless the incubation temperature (Table 3). It is in line with the COD\(_{\text{tot}}\) 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 to 40% of the COD\(_{\text{tot}}\) (Sharma and Li, 2010). This was likely due to the adsorption of VFAs on the activated carbon (Figure S2 in Supplementary Material), which caused an overestimation of the COD\(_{\text{tot}}\) removal. However, it should be noted that the adsorption experiment (Figure S2 in 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 H₂ 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
decrease of 5 °C may already result in a decreased efficiency due to H2 consumption by homoacetogenic bacteria. However, H2 production at 70 °C can be quickly restored in case of failure of the temperature control. In fact, H2 production was detected at 70 °C within only 24 h (Figure 1) with a thermophilic inoculum previously stored at 4 °C for one week.

Despite the surprisingly high CODtot removal efficiency of 69 to 80 % obtained in this study (Table 3), dark fermentation of TMP wastewater resulted in the generation of an effluent containing 0.5 to 1.0 g CODtot L⁻¹ (Table 3), mainly in the form of VFAs, thus requiring further treatment prior to discharge to the environment. 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 H2 production by photofermentation or microbial electrolysis cells, methane production by anaerobic digestion, and bioplastics or electricity production using microbial fuel cells (for reviews, see Ghimire et al., 2015 and Bundhoo, 2017).

5. Conclusions

Hydrogen was produced by dark fermentation from TMP wastewater at a wide range of temperatures (37 to 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 H2 production and effectively repressed the activity of homoacetogenic bacteria. Therefore, considering 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 a shift of only a few degrees may decrease the H2 yield.

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.

References


Gadow, S.I., Jiang, H., Hojo, T., Li, Y.-Y., 2013. Cellulosic hydrogen production and microbial


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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids</td>
<td>3771 ± 10</td>
</tr>
<tr>
<td>Volatile solids</td>
<td>2452 ± 8</td>
</tr>
<tr>
<td>Total COD</td>
<td>3352 ± 82</td>
</tr>
<tr>
<td>Soluble COD</td>
<td>3289 ± 54</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Total PO(_4^3-)P</td>
<td>2.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>Furfural</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Glucose</td>
<td>43 (± 2)</td>
</tr>
<tr>
<td>Xylose</td>
<td>38 (± 0)</td>
</tr>
</tbody>
</table>
Table 2 - Maximum and final hydrogen yield obtained from batch incubation of thermomechanical pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>H₂ yield (mmol H₂ g⁻¹ CODₜot supplied)</th>
<th>CODₜot consumed</th>
<th>Time required for maximum H₂ yield (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum</td>
<td>Final</td>
<td>Final</td>
</tr>
<tr>
<td>37</td>
<td>3.2 (± 0.1)</td>
<td>1.4 (± 0.1)</td>
<td>1.9 (± 0.2)</td>
</tr>
<tr>
<td>42a</td>
<td>1.5</td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td>48</td>
<td>0.6 (± 0.1)</td>
<td>0.1 (± 0.0)</td>
<td>0.1 (± 0.0)</td>
</tr>
<tr>
<td>55</td>
<td>0.4 (± 0.1)</td>
<td>0.0 (± 0.0)</td>
<td>0.0 (± 0.0)</td>
</tr>
<tr>
<td>59</td>
<td>1.7 (± 0.8)</td>
<td>0.6 (± 0.3)</td>
<td>0.9 (± 0.5)</td>
</tr>
<tr>
<td>65</td>
<td>3.7 (± 0.4)</td>
<td>1.8 (± 0.2)</td>
<td>2.6 (± 0.3)</td>
</tr>
<tr>
<td>70</td>
<td>3.6 (± 0.1)</td>
<td>3.6 (± 0.1)</td>
<td>4.9 (± 0.4)</td>
</tr>
<tr>
<td>74</td>
<td>0.1 (± 0.0)</td>
<td>0.1 (± 0.0)</td>
<td>0.2 (± 0.0)</td>
</tr>
<tr>
<td>80</td>
<td>0.0 (± 0.0)</td>
<td>0.0 (± 0.0)</td>
<td>0.0 (± 0.0)</td>
</tr>
</tbody>
</table>

a Hydrogen was produced only in one of the duplicate tubes;
b Not applicable.
Table 3 - COD$_{\text{tot}}$ balances after incubation of thermomechanical pulping wastewater at various temperatures (from 37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum

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

$^a$ Data obtained by measurement according to the standard procedure; the initial COD$_{\text{tot}}$ was 2.86 g L$^{-1}$;

$^b$ Data obtained by the sum of the COD$_{\text{tot}}$ equivalents (Eq. 1) of organic compounds measured in the liquid phase;

$^c$ Calculated from COD$_{\text{tot}}$ measured.
**Table 4** - Association of the six most abundant 16S rRNA gene sequences to species collected in the GenBank

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus and speciesa</th>
<th>Accession number</th>
<th>Matching sequenceb</th>
<th>Similarity (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thermoanaerobacteraceae</em></td>
<td><em>Thermoanaerobacterium</em> thermostosaccharolyticum</td>
<td>JX984971</td>
<td>474-765</td>
<td>99</td>
</tr>
<tr>
<td><em>Clostridiaceae</em></td>
<td><em>Clostridium</em> sp.</td>
<td>AY548785</td>
<td>450-741</td>
<td>99</td>
</tr>
<tr>
<td><em>Bacillaceae</em></td>
<td><em>Bacillus coagulans</em></td>
<td>MF373392</td>
<td>512-803</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>Calditerricola</em> yamamurae</td>
<td>NR_112684</td>
<td>529-820</td>
<td>92</td>
</tr>
<tr>
<td><em>Thermoanaerobacteraceae</em></td>
<td><em>Caldanaerobius</em> sp.</td>
<td>LC127102</td>
<td>482-773</td>
<td>99</td>
</tr>
<tr>
<td><em>Thermoanaerobacteraceae</em></td>
<td><em>Moorella thermoacetica</em></td>
<td>CP017237</td>
<td>145404-145695</td>
<td>100</td>
</tr>
</tbody>
</table>

* Closest cultured species in GenBank;

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

* Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest cultured species in GenBank.
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\textsubscript{tot} L\textsuperscript{-1} was removed through dark fermentation, and equally distributing the remaining 1.71 g COD\textsubscript{tot} L\textsuperscript{-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.