

1 **Assessment of hydrothermal pretreatment of various lignocellulosic biomass with**  
2 **CO<sub>2</sub> catalyst for enhanced methane and hydrogen production**

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1 **Abstract**

2 Hydrothermal pretreatment of five lignocellulosic substrates (i.e. wheat straw, rice straw,  
3 biomass sorghum, corn stover and Douglas fir bark) were conducted in the presence of CO<sub>2</sub>  
4 as a catalyst. To maximize disintegration and conversion into bioenergy (methane and  
5 hydrogen), pretreatment temperatures and subsequent pressures varied with a range of  
6 26-175°C, and 25-102 bars, respectively. Among lignin, cellulose and hemicelluloses,  
7 hydrothermal pretreatment caused the highest reduction (23-42%) in hemicelluloses while  
8 delignification was limited to only 0-12%. These reductions in structural integrity resulted  
9 in 20-30% faster hydrolysis rates during anaerobic digestion for the pretreated substrates  
10 of straws, sorghum, and corn stover while Douglas fir bark yielded 172% faster  
11 hydrolysis/digestion due to its highly refractory nature in the control. Furans and phenolic  
12 compounds formed in the pretreated hydrolyzates were below the inhibitory levels for  
13 methane and hydrogen production which had a range of 98 – 340 ml CH<sub>4</sub>/ g volatile solids  
14 (VS) and 5 – 26 ml H<sub>2</sub>/ g VS, respectively. Results indicated that hydrothermal  
15 pretreatment is able to accelerate the rate of biodegradation without generating high levels  
16 of inhibitory compounds while showing no discernible effect on ultimate biodegradation.

17

18 **Keywords:** anaerobic digestion, dark fermentation, straw, sorghum, corn stover, Douglas  
19 fir bark

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## 1 **1. Introduction**

2 As energy sustainability concerns are increasing globally, alternatives to renewable energy  
3 sources are being brought to the forefront. Among these renewable sources is the  
4 development of an energy efficient biomass-to-biofuel process which can replace fossil fuel  
5 use and reduce greenhouse gas emissions. In particular, lignocellulosic agricultural  
6 residues have a very significant unutilized energy potential. For example, lignocellulosic  
7 biomass is abundant all year round and often after the remaining portion is used as animal  
8 feed it is burnt in an open environment which can further cause environmental concerns  
9 (Palacios-Orueta et al., 2005; Bhatia et al., 2012). Another advantage of using  
10 lignocellulosic waste for bioenergy is that it does not compete with land use for food  
11 production.

12 Anaerobic digestion and dark fermentation have been extensively studied for their ability  
13 to convert a wide variety of lignocellulosic biomass to methane (Sawatdeenarunat et al.,  
14 2015) and biohydrogen (Singh et al., 2015), respectively. However, the complex structure  
15 of lignocellulosic biomass, comprised of cellulose, hemicelluloses and lignin, does not  
16 provide easy access for the biodegradable organics in bioreactors. Such limited  
17 accessibility causes significantly lower methane/hydrogen yields than the theoretical  
18 estimations based on biomass compositional/structural features (Monlau et al., 2012a;  
19 Carrere et al., 2015). A review by Monlau et al. (2013a) states that the amount of lignin is  
20 the most important factor when determining the biodegradability of lignocellulosic  
21 biomass along with other factors, such as cellulose crystallinity and accessible surface area.  
22 Therefore, research has focused on various pretreatment technologies utilizing mechanical  
23 (Sharma et al., 1988; Palmowski and Muller, 2000), chemical (Sun et al., 2001; Zhu et al.,

1 2010; Monlau et al., 2013b), thermal (Kumar et al., 2009), and biological (Rouches et al.,  
2 2016) methods or combinations (Monlau et al., 2013c) to dissolve lignin (delignification),  
3 and to reduce cellulose crystallinity for increasing surface area and accessibility for better  
4 enzymatic hydrolysis/microbial degradation. Following pretreatment, chemical methods  
5 may have a high impact on downstream processes or the environment and limited  
6 chemical recovery potential which may require further pH neutralization before digestion.  
7 For example, high sodium may jeopardize digestate land application or inhibit  
8 methanogens in digesters (Antonopoulou and Lyberatos, 2013; Carrere et al., 2016). For  
9 enzymatic methods, typically, polysaccharides hiding under the lignin limit the enzymatic  
10 activity, therefore are at times combined with other types of pretreatment (Sun and Cheng,  
11 2002). Furthermore, depending on the type/intensity of the pretreatment applied (i.e.  
12 dilute-acid, high temperature/pressure, steam explosion and thermo-alkaline  
13 pretreatments), some of the inhibitory by-products of pretreatment, such as furfural, 5-  
14 hydroxymethylfurfural (5-HMF) and phenolic compounds, to hydrogen and/or methane  
15 formers have also been reported (Palmqvist and Hahn-Hagerdal, 2000; Horn et al., 2011;  
16 Monlau et al., 2013c). Therefore, the chosen pretreatment method should reduce energy  
17 demand, minimize use of chemicals and formation of inhibitory by-products, and allow for  
18 reuse of co-products in a feasible biorefinery concept (Cherubini, 2010).

19 Hydrothermal pretreatment of lignocellulosic biomass at elevated temperatures/pressures  
20 (150 - 300°C, initial pressure of 0-60 bar, 2-40 min) has garnered consideration for the  
21 production of methane, hydrogen and bioethanol from lignocellulosic substrate as it  
22 eliminates chemical addition and corrosion resistant material requirements for hydrolysis  
23 reactors (Chandra et al., 2012; He et al., 2016). It has been traditionally applied in pulp

1 industries (Kubikova et al., 1996) and is considered more environmentally friendly  
2 compared to other methods with higher sugar recovery in a relatively short period of time  
3 and little to no inhibitor production (Kaparaju and Felby, 2010). It has been postulated that  
4 when optimized the results are comparable to dilute-acid pretreatment, but without  
5 chemical addition/post-neutralization. The use of CO<sub>2</sub> in hydrothermal pretreatment has  
6 been shown to further enhance hydrolysis of various types of biomass, such as Eucalyptus  
7 bark (Matsushita et al., 2010), corn stover (van Walsum and Shi, 2004), wheat straw  
8 (Relvas et al., 2015), and various polysaccharides (Miyazawa and Funazukuri, 2005).  
9 Carbonic acids generated in situ from water and added CO<sub>2</sub> can lower the pH of the solution  
10 and act as an environmentally friendly acid catalyst accelerating hydrolytic organic  
11 reactions at high-temperatures (Relvas et al., 2015). At the end of the pretreatment  
12 process, CO<sub>2</sub> can be easily removed by depressurizing the reactor to atmospheric pressure,  
13 avoiding the need of a subsequent treatment. The availability of CO<sub>2</sub> as a component in the  
14 fermentation processes (i.e. bioethanol or biogas) makes this a potentially cost-effective  
15 option for biomass pretreatment.

16 Although hydrothermal pretreatment with CO<sub>2</sub> pressurization is viewed as a promising  
17 technology, the existing literature is limited to the aforementioned studies focusing only on  
18 either the hydrolysis phase (van Walsum and Shi, 2004; Miyazawa and Funazukuri, 2005;  
19 Matsushita et al., 2010; Relvas et al., 2015), or enhancement of methane yield from sewage  
20 sludge (Spooner et al., 2007) and hydrogen yield from starch (Orozco et al., 2012).  
21 However, no sufficient insight has been provided on the levels/effect of inhibitory  
22 compounds on methane/hydrogen yields from common lignocellulosic biomass including  
23 energy crops. Therefore, the objective of this study was to evaluate the entire impact of

1 hydrothermal pretreatment with CO<sub>2</sub> pressurization not only on the compositional  
2 structure of various lignocellulosic substrates but also on the enhancement of methane and  
3 hydrogen yields in anaerobic digestion and dark fermentation, respectively. The yields  
4 were estimated from batch biochemical methane potential (BMP) and biochemical  
5 hydrogen potential (BHP) assays. Additionally, potentially inhibitory compounds after  
6 pretreatment were quantified.

## 7 **2. Materials and Methods**

### 8 **2.1. Lignocellulosic substrates**

9 The substrates tested included wheat straw (WS), biomass sorghum (B140) (S), rice straw  
10 (RS), corn stover (CS), and Douglas fir bark (DFB) containing different solids (Table 1) and  
11 compositional structure (cellulose, hemicellulose and Klason lignin). Wheat straw  
12 (*Triticum aestivum*), grown in France (latitude: 48°50'18''N, longitude: 4°13'54.5''E), was  
13 first processed using a cutting mill. It was further sieved to have a particle size range of 400  
14 µm - 1 mm. Sorghum (B140) was produced at a site (latitude: 43.6491994, longitude:  
15 3.874161111) in Montpellier Lavalette (France) in 2012. It was milled to pass a 1mm  
16 screen. Rice straw was provided by RIZ Camargue CANAVERE (a local farm in Saint-Gilles  
17 Languedoc-Roussillon region, south of France). It was first cut by a mill equipped with a 6  
18 mm sieve followed by a 1 mm sieve (SM200, Retsch, GE). Corn stover was provided by  
19 INRA Versailles (Paris region, France). The sample was coarsely cut to less than 2 mm by  
20 knife milling (SM200, Retsch, GE). Douglas fir bark was supplied by Brassac Industries  
21 sawmill (Tarn region, France). It was a heterogeneous size material (chips of 5–20 cm  
22 length and 1 mm–3 cm thickness) produced from the debarking of 50 years old Douglas fir  
23 trees harvested in April 2013. Douglas fir bark chips were first dried in an oven at 40°C

1 overnight to reach a moisture content of 8.87%. Knife milling was then performed in a  
2 Retsch SM 100 system with a 6 mm and 2 mm size sieve at a speed of 1500 rpm.

### 3 **2.2. Hydrothermal pretreatment**

4 Hydrothermal pretreatment of substrates was conducted in a PARR 5500 High Pressure  
5 Compact Reactor equipped with mechanical mixer, heater, and controller. The reactor had  
6 an effective volume of 450 mL and was capable of achieving a maximum temperature and  
7 pressure of 350°C and 200 bar, respectively. Upon addition of substrate (15 g) and distilled  
8 water (300 g), the reactor was sealed and pressurized to the desired levels by a CO<sub>2</sub> line  
9 from a cylinder/regulator. After pressurization the CO<sub>2</sub> line was disconnected and the  
10 temperature/pressure increase in the reactor was recorded with respect to heating time  
11 while the reactor content was being mixed at 140 rpm. The PARR reactor controller  
12 allowed the pretreatment to be programmed based on different ramping rates (temperature  
13 increased per unit heating time). In this research, the heating duration was kept constant  
14 (30 min) and at the end of the 30 min, the heater and mixer were turned off and the vessel  
15 was immersed in an ice bath. When the temperature levels dropped below 40°C, the vessel  
16 was slowly depressurized to atmospheric levels by turning on a pressure release valve  
17 found on the reactor. The lid was opened and pretreated slurry was recovered. The solid  
18 fraction was separated from the liquid fraction (hydrolyzate) via a mesh sieve with 150 µm  
19 pore size (Figure 1). The use of the 150 µm sieve resulted in loss of 2.2 to 8.3% of TS in  
20 substrates pretreated based on mass balance. Separation of liquid from the solid fraction  
21 was necessary to conduct BHP assays on the solubilized sugars (main source of H<sub>2</sub>  
22 production) as well as to optimize BMP conditions separately for different fractions.

1 Pretreatments were applied in two separate stages. Stage I involved the pretreatment of  
2 wheat straw at a wide range of set temperature and CO<sub>2</sub> pressure combinations for  
3 preliminary screening. The pretreatment reactor was programmed to simulate four  
4 different scenarios with set temperature/initial CO<sub>2</sub> pressure/duration time combinations  
5 of 25°C/50 bar/30 min, 50°C/50 bar/30 min, 150°C/10 bar/30 min, and 150°C/50 bar/30  
6 min, respectively. Based on the preliminary results on substrate characterization and  
7 methane yields from Stage I, Stage II applied only the most intensive temperature/pressure  
8 combination (150°C/50 bar/30 min) for the remaining four substrates (Table 2). The  
9 observed (actual) maximum temperatures/pressures reached within 30 min of heating  
10 was quite substrate specific due to differences in the interstitial volume of the solids and  
11 therefore water absorption capacities of substrates affecting headspace pressure.  
12 Therefore, in all of the pretreatment runs, maximum temperature reached exceeded the set  
13 temperature due to common overshooting reported for Parr reactors without water or fan  
14 cooling. Table 2 lists the actual maximum temperatures/pressures observed for each  
15 substrate. The overshooting was higher (60%) at the low set-temperature (50°C) than that  
16 (17%) of the high set temperature (150°C) as these reactors are designed to reach  
17 temperatures up to 350°C in a short period of time.

## 18 **2.3. Anaerobic inocula**

### 19 *2.3.1. Inoculum for BMP assay*

20 The inoculum used for BMP assays was granular sludge from a mesophilic upflow  
21 anaerobic sludge blanket (UASB) reactor utilizing wastewater from a sugar factory in  
22 France. Prior to setting up the BMP assays, the inoculum was placed in a closed 5-L glass  
23 vessel, diluted 10 times with distilled water to total solids (TS) and volatile solids (VS)



1 concentrations of  $1.24 \pm 0.01$  and  $1.08 \pm 0.02\%$  (by wt.), respectively, and mixed to break  
2 apart the granules under endogenous anaerobic conditions ( $35^{\circ}\text{C}$  for 5-7 days) to reduce  
3 non-specific biogas generation. The inoculum had a maximum specific methanogenic  
4 activity of  $33 \pm 2$  mL  $\text{CH}_4/\text{g VS/d}$ , as measured by degrading  $1.3 \pm 0.3$  g/L of ethanol as  
5 chemical oxygen demand (COD).

### 6 *2.3.2. Inoculum for BHP assay*

7 Among the three inocula tested (granular sludge described above, activated sludge,  
8 municipal sludge digested under low pH conditions in a BMP bottle), the inoculum chosen  
9 for BHP assays was the activated sludge taken from the aeration tank at the municipal  
10 wastewater treatment plant (WWTP) in Narbonne (France). The decision was based on  
11 rate/extent of  $\text{H}_2$  yields from a preliminary BHP assay (in four replicates) conducted with  
12 glucose (5 g COD/L) at a substrate to inoculum ratio (S/I) of 10 g COD/g VS at mesophilic  
13 temperature ( $37^{\circ}\text{C}$ ). The activated sludge had a TS and VS concentration of  $0.46 \pm 0.00$  and  
14  $0.33 \pm 0.00\%$  (by wt.), respectively, and achieved a maximum  $\text{H}_2$  yield of  $1.2 \pm 0.2$  mol  
15  $\text{H}_2/\text{mol}$  glucose within the first 39-40 hours while the granular sludge achieved a similar  
16 yield ( $1.2 \pm 0.1$  mol  $\text{H}_2/\text{mol}$  glucose) only after 61 hours. The digested municipal sludge  
17 reached a maximum yield of only  $0.6 \pm 0.2$  mol  $\text{H}_2/\text{mol}$  glucose after 48 hours. Before the  
18 addition to the BHP assays, all three inocula were thermally treated for 30 min in capped  
19 glass tubes immersed in a water bath set at  $90^{\circ}\text{C}$  to inhibit the activity of methanogens.

### 20 **2.4. BMP Assay Set-up**

21 A total of four sets of BMP assays were conducted concurrently to determine methane  
22 potential from liquid and solid fractions of pretreated substrates in both Stage I and II  
23 (Figure 1; Table 2). A total of 72 bottles (including pretreated, non-pretreated substrates

1 and blanks in triplicates) were operated, with solid and liquid fractions set-up in bottles  
2 with 600 mL (350 mL liquid) and 120 mL (80-84 mL liquid) total volumes, respectively.  
3 BMP assays with solid fractions contained total substrate concentration of 5 g VS/L and the  
4 amount of the substrate and granular inoculum added to each bottle was calculated  
5 considering S/I ratio of  $1 \frac{\text{g VS}}{\text{g VS}}$  which has been previously used for various lignocellulosic  
6 substrates (Sambusiti et al., 2012a; Monlau et al., 2013b). For the liquid fractions, a  
7 substrate concentration and S/I ratio in the bottles was 2.5 g COD/L and 0.5 g COD/g VS,  
8 respectively. Each assay contained: macroelements (NH<sub>4</sub>Cl, 286 mg/L; KH<sub>2</sub>PO<sub>4</sub>, 108 mg/L;  
9 MgCl<sub>2</sub>, 65 mg/L; CaCl<sub>2</sub>, 32 mg/L), oligoelements (FeCl<sub>2</sub>, 20 mg/L; CoCl<sub>2</sub>, 5 mg/L; MnCl<sub>2</sub>, 1  
10 mg/L; NiCl<sub>2</sub>, 1 mg/L; ZnCl<sub>2</sub>, 0.5 mg/L; H<sub>3</sub>BO<sub>3</sub>, 0.5 mg/L; Na<sub>2</sub>SeO<sub>3</sub>, 0.5 mg/L; CuCl<sub>2</sub>, 0.4 mg/L;  
11 Na<sub>2</sub>MoO<sub>4</sub>, 0.1 mg/L), and a bicarbonate buffer solution (NaHCO<sub>3</sub>, 2.6 g/L). Finally, the  
12 nitrogen gas was purged into each bottle to remove the residual oxygen and the bottles  
13 were sealed with septa/caps. The septa were then punctured to release excess N<sub>2</sub> pressure.  
14 The bottles were placed on a shaker (at 90 rpm) in a temperature controlled room at 37°C.  
15 Accumulated gas pressure in the bottles were measured with a digital manometer (LEO 2,  
16 Keller, Switzerland), while biogas composition was analyzed by a gas chromatograph (GC)  
17 every time excess pressure was released until bottles stopped producing biogas.

## 18 **2.5. BHP Assay Set-up**

19 Based on the biodegradation potential comparison among substrates from BMP, BHP  
20 assays excluded Douglas fir bark and assessed hydrogen yields and by-products of dark  
21 fermentation from liquid fraction of pretreated straws, sorghum and corn stover only at the  
22 most intensive condition (set temp:150°C, initial CO<sub>2</sub> pressure: 50 bar, 30 min). BHP assays  
23 included a total of 21 bottles set-up with hydrolyzes of pretreated substrates and control

1 (glucose) in 3-5 replicates (depending on the volume recovered after pretreatment).  
2 Bottles had total and liquid volume of 120 mL and 60 mL, respectively and the amount of  
3 the substrate and inoculum needed for each bottle was calculated considering a S/I ratio of  
4  $8.7 \frac{\text{g COD degradable}}{\text{g VS}}$ , where degradable COD was estimated as total sugars (i.e. summation of  
5 cellobiose, glucose, xylose, arabinose concentrations in Table 3) in hydrolyzates of  
6 pretreated substrates. Each BHP assay contained a dilution solution prepared with  
7 macroelements (NH<sub>4</sub>Cl, 0.8 g/L; KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/L), oligoelements (FeCl<sub>2</sub>, 1.5 g/L; H<sub>3</sub>BO<sub>3</sub>, 60  
8 mg/L; CoCl<sub>2</sub>, 25 mg/L; MnSO<sub>4</sub>, 117 mg/L; NiCl<sub>2</sub>, 25 mg/L; ZnCl<sub>2</sub>, 70 mg/L; CuCl<sub>2</sub>, 15 mg/L;  
9 Na<sub>2</sub>MoO<sub>4</sub>, 25 mg/L), 1.2 mL/L vitamin solution (mixture of biotin, cyanocobalamin,  
10 thiamine), and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at 19.52 g/L. Upon  
11 addition of substrate and dilution solution, pH of the bottle content was adjusted to 6 by  
12 adding drops of NaOH solution (32% by vol.). Then thermally treated inoculum (activated  
13 sludge) was added. Finally, the bottles were sealed with septa/caps and the nitrogen gas  
14 was purged to each bottle to remove residual oxygen. The bottles were then placed on a  
15 shaker (at 90 rpm) in a temperature controlled room at 37°C. Accumulated gas pressure in  
16 the bottles was measured with a digital manometer (LEO 2, Keller, Switzerland), while  
17 biogas composition was analyzed by a GC every time excess pressure was released until  
18 cumulative hydrogen yields plateaued and the first signs of hydrogen consumption  
19 (reduction in H<sub>2</sub> % in headspace) were observed. Upon termination of assays, metabolites  
20 of dark fermentation were quantified.

## 21 **2.6. Analytical procedures**

22 The TS/VS analysis of raw/pretreated substrates and inocula was done according to the  
23 Standard Methods, sections 2540 B and 2540 E, respectively (APHA, 2005). Quantification

1 of COD in liquid fractions of pretreated substrates were performed according to the closed  
2 reflux colorimetric method outlined by Standard Methods (APHA, 2005). Compositional  
3 analysis (i.e. cellulose, hemicelluloses and Klason lignin) on substrates were conducted  
4 using a strong hydrolysis method adapted from Sluiter et al. (2008). Raw or freeze-dried (-  
5 69°C, 0.21 Pa for 3 days) solid fraction of pretreated substrates (100 mg) were first  
6 hydrolyzed with H<sub>2</sub>SO<sub>4</sub> (72% by vol.) in capped/mixed test tubes (in triplicates) at 30°C for  
7 1 h, then diluted to reach a final acid concentration of H<sub>2</sub>SO<sub>4</sub> (4%) and kept at 120°C for 1 h.  
8 Upon cooling, the tube content was filtered via glass-fiber filters (0.45 μm) to separate  
9 insoluble residue, which was placed in a crucible and dried at 100°C for 24 h to yield  
10 Klason lignin content. The liquid fraction obtained after filtration was further filtered via  
11 0.2 μm syringe filters and analyzed by a high-performance liquid chromatograph (HPLC)  
12 equipped with a refractive index detector (Waters R410/Waters 2414) for structural  
13 carbohydrates (i.e. glucose, xylose, arabinose). Target compounds were separated by an  
14 Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) placed after a protective precolumn  
15 (Microguard cation H refill cartridges, Bio-Rad). The eluting solution was 4 mM H<sub>2</sub>SO<sub>4</sub>, and  
16 the flowrate, column/detector temperatures were 0.3 mL/min, 45°C, respectively. Cellulose  
17 and hemicellulose contents of lignocellulosic substrates were calculated as follows:

$$\text{Cellulose (\% VS)} = \frac{\text{Glucose (\% VS)}}{1.11} \quad (1)$$

$$\text{Hemicellulose (\% VS)} = \frac{[\text{Xylose (\% VS)} + \text{Arabinose (\% VS)}]}{1.13} \quad (2)$$

18 where 1.11 and 1.13 are the ratios of the molecular weights of glucose to glucan (180/162)  
19 and xylose/arabinose to xylan (150/132), respectively. Similarly, soluble sugars, inhibitory  
20 by-products of pretreatment (i.e. 5-HMF and furfural), as well as soluble metabolites of

1 BHP assays were also quantified by HPLC in liquid fractions filtered via 0.2 µm syringe  
2 filters. Poly-phenols were quantified by a colorimetric method at 735 nm by using Folin  
3 Ciocalteu reagent, Na<sub>2</sub>CO<sub>3</sub>, and gallic acid as standard (Cicco et al., 2009).

4 Biogas compositions were conducted by measuring the H<sub>2</sub>S, CO<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub>, CH<sub>4</sub> percentage  
5 with a Perkin Elmer Clarus 480 GC and H<sub>2</sub>, CO<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub>, CH<sub>4</sub> percentage by a Clarus 580 GC,  
6 in BMP and BHP bottles, respectively. Both GCs were equipped with thermal conductivity  
7 detectors but used different carrier gasses (helium for GC 480, argon for GC 580). Volatile  
8 fatty acids (VFAs) in pretreated liquid fractions were measured by injecting filtered  
9 samples (0.2 µm) into the Clarus 580 GC equipped with an auto-sampler, flame ionization  
10 detector. Nitrogen was the carrier gas.

## 11 **2.7. Kinetic rate estimation**

12 Previous studies commonly used a first-order kinetic process to assess the advantage of  
13 different pretreatments on lignocellulosic and non-lignocellulosic substrates in terms of  
14 hydrolysis rate (Sambusiti et al., 2012a; Monlau et al., 2012b; Mirmohamadsadeghi et al.,  
15 2014; Hosseini Koupaie and Eskicioglu, 2016). In this study, all BMP assays generated  
16 biogas without any acute inhibition, therefore the first order kinetic constants were  
17 estimated by Equation (3) that does not take into account lag phase:

$$BMP_t = BMP_{t \rightarrow \infty} (1 - \exp(-k \cdot t)) \quad (3)$$

18 where  $BMP_t$  is cumulative specific methane yield (ml CH<sub>4</sub>/g VS<sub>added</sub>) at a given time,  $t$ ,  
19 calculated from Equation (4) below;  $BMP_{t \rightarrow \infty}$  is the ultimate specific methane yield (ml  
20 CH<sub>4</sub>/g VS<sub>added</sub>) obtained at the end of the assay;  $k$  is the first-order hydrolysis constant  
21 (1/d), and  $t$  is the digestion time (d).

$$BMP_t = (V_{CH_4,s,t} - V_{CH_4,blank,t})/VS_s \quad (4)$$

1 where  $(V_{CH_4,s,t} - V_{CH_4,blank,t})$  is the net methane volume (ml) obtained from the substrate only,  
 2 adjusted to the standard temperature (0°C) and pressure (1 atm) condition (STP);  $VS_s$  is the  
 3 mass of substrate VS in the bottle (g).

4 In order to model BHP assays from pretreated hydrolyzates with initial lag period, the  
 5 following modified Gompertz equation was used (Equation (5)):

$$H_t = P \exp \left\{ -\exp \left[ \frac{Rm \cdot e}{P} (\lambda - t) + 1 \right] \right\} \quad (5)$$

6 Where  $P$  is the maximum specific cumulative  $H_2$  production (ml  $H_2$ /g  $VS_{added}$ );  $Rm$  is the  
 7 maximum specific  $H_2$  production rate (ml  $H_2$ /g  $VS_{added}$ /h);  $\lambda$  is the lag phase (hr);  $t$  is the  
 8 fermentation time (hr); and  $e$  is exp (1).  $H_t$  represents cumulative specific  $H_2$  production  
 9 expressed as ml  $H_2$ /g  $VS_{added}$  at a given time ( $t$ ) at STP (0°C, 1 atm).

10 Parameter estimation was conducted by fitting the measured to predicted BMP and BHP  
 11 data and using the Microsoft Excel 2013 Solver function to estimate the values of  $k$ ,  $P$ ,  $Rm$ ,  
 12 and  $\lambda$ . The coefficient of determination,  $R^2$ , was used to evaluate the adequacy of fit.

### 13 **3. Results and Discussion**

#### 14 **3.1. Impact of pretreatment on chemical composition of substrates**

15 The cellulose, hemicellulose, and Klason lignin compositions of five substrates before and  
 16 after hydrothermal pretreatment in the presence of  $CO_2$  are presented in Figure 2. For raw  
 17 substrates, summation of cellulose, hemicellulose and lignin accounted for 93% (wheat  
 18 straw), 76% (sorghum), 86% (rice straw), 68% (corn stover), and 91% (Douglas fir bark)  
 19 of the initial VS, suggesting that sorghum and corn stover contained higher amount of  
 20 organics (i.e. proteins, lipids and different sugars), not quantified in the compositional

1 analysis than those of the other substrates. Sambusiti et al. (2013a) reported  $9 \pm 3\%$  and  
2  $1.8 \pm 0.3\%$  of initial VS protein and fat, respectively, for *Sorghum sudanense* hybrid while  
3 wheat straw had only  $4 \pm 1\%$  protein and  $0.9 \pm 0.8\%$  fat of the initial VS. Compared to  
4 ranges reported in the literature (Baker, 1973; Taherzadeh and Karimi, 2007; Cherubini,  
5 2010; Monlau et al., 2013a; Mirmohamadsadeghi et al., 2014), wheat straw (Lig: 26; Cell:  
6 38; Hem: 30% VS), rice straw (Lig: 20; Cell: 37; Hem: 29% VS), corn stover (Lig: 14; Cell:  
7 25; Hem: 18% VS), and Douglas fir bark (soft pinewood) (Lig: 28; Cell: 37; Hem: 25% VS)  
8 yielded typical compositional analysis results. However for sorghum, literature varies  
9 significantly based on the end-use of sorghum: biomass, forage, sorghum-sudangrass, and  
10 sweet. Sambusiti et al. (2013b) compared different sorghums (seed sorghum stalks,  
11 biomass sorghum, forage sorghum and three different sweet sorghums) and reported that  
12 lignin, cellulose, and hemicellulose ranged in 19-21%, 18-29%, and 19-26% TS,  
13 respectively. Interestingly, *Sorghum sudanense* hybrid grown in Lombardy region (Italy)  
14 had only 4% VS lignin, while cellulose (49% VS) and hemicellulose (35% VS) were  
15 significantly higher (Sambusiti et al., 2013b). Biomass 140 sorghum used in this study also  
16 resulted in lower Klason lignin and higher cellulose and hemicellulose (Lig: 16; Cell: 33;  
17 Hem: 27% VS) compared to biomass sorghum (Lig: 21; Cell: 23; Hem: 19% TS) in the study  
18 of Monlau et al. (2012a). The results, on the other hand, were closer to another study that  
19 compared 63 sweet sorghum collected worldwide and reported cellulose, hemicellulose,  
20 and lignin ranges of 28-37%, 26-33%, and 17-23% by TS, respectively (Li et al., 2014).  
21 During Stage I, hydrothermal pretreatment with CO<sub>2</sub> caused 4-18%, 8-11%, and 11-29%  
22 reductions (relative to non-pretreated wheat straw) in lignin, cellulose, and hemicellulose  
23 contents, respectively, with hemicelluloses being affected the most (Figure 2a). As

1 expected, the highest reduction in all three components was achieved at the most intensive  
2 pretreatment (175°C/66 bar/30 min). During Stage II, other substrates showed a similar  
3 behaviour with hemicellulose contents having the highest reductions, which were 28%,  
4 16%, 23%, and 42% for sorghum, rice straw, corn stover, and Douglas fir bark,  
5 respectively, while delignification was limited to 0-12% (Figures 2b-e). These results are in  
6 agreement with other hydrothermal pretreatment studies (with/without CO<sub>2</sub> catalysis)  
7 reporting that pretreatment can affect the hemicellulose the most while lignin, being the  
8 most inert component, stays relatively intact or is slightly affected (Kaparaju and Felby,  
9 2010; Relvas et al., 2015). When compared to other type of pretreatments, chemical  
10 pretreatments combined with low temperatures for 3-4 h appear to be more effective in  
11 delignification of lignocellulosic substrate than hydrothermal pretreatment. Thermo-  
12 alkaline pretreatment of wheat straw (Ca(OH)<sub>2</sub>, 85°C for 3 h) was reported to solubilize  
13 lignin by 14% (Chang et al., 1998), while 77% of delignification, 95% cellulose yield and  
14 44% of hemicellulose hydrolysis was reported for miscanthus pretreated with 12% NaOH,  
15 70°C for 4 h (de Vrije et al., 2002). Similarly, ammonia recycle percolation (ARP)  
16 pretreatment achieved 75-85% (total lignin), and 50-60% (hemicellulose) removal in corn  
17 stover (Kim and Lee, 2005) and 65-85% delignification of switchgrass (Iyer et al., 1996).

18 In this study, it was difficult to differentiate the effects of temperature and pressure for  
19 Stage I (wheat straw), as these two factors are not independent once the temperature  
20 reaches elevated levels. When the pairs of non-pretreated wheat straw vs. the least  
21 intensive pretreatment condition (25°C/50 bar/30 min) and two most intensive conditions  
22 (175°C/25 bar/30 min vs. 175°C/66 bar/30 min) were compared in an attempt to  
23 understand the pressure effect only, 12% and 18% decreases in hemicellulose were



1 observed for increases from atmospheric to 50 bars (at 25°C) and from 25 to 66 bars (at  
2 175°C), respectively. Temperature effect, on the other hand, was more pronounced with  
3 21% decrease in hemicellulose content when temperature increased from 80 to 175°C (at  
4 60-66 bars) but with no hemicellulose reduction after the increase from 26 to 80°C (at 50-  
5 60 bars) (i.e.  $26.2 \pm 1.1$  versus  $26.6 \pm 1.8\%$  hemicellulose in Figure 2a). When both  
6 temperature and pressure effects were combined, up to 29% reduction was observed in  
7 hemicellulose of non-pretreated wheat straw (Figure 2a).

## 8 **3.2. Impact of pretreatment on biomass biodegradability**

### 9 *3.2.1. Methane production*

10 The mesophilic BMP assays were monitored for 71-92 days for liquid fractions (Figures 3a  
11 and c) and 123-125 days for solid fractions (Figures 3b and d) of substrates. Non-  
12 pretreated substrates generated ultimate cumulative specific methane yields of  $256 \pm 18$ ,  
13  $340 \pm 2$ ,  $311 \pm 5$ ,  $336 \pm 11$ ,  $98 \pm 5$  ml CH<sub>4</sub>/g VS<sub>added</sub> for wheat straw, sorghum, rice straw,  
14 corn stover and Douglas fir bark, respectively. As expected, for raw lignocellulosic  
15 substrates, low lignin and high hemicellulose/cellulose levels for sorghum and corn stover  
16 (Figure 2) corresponded to higher methane yields than the other substrates with Douglas  
17 fir bark being the most difficult to degrade. These results coincide with the literature values  
18 of 204-285 ml CH<sub>4</sub>/g VS<sub>added</sub> for wheat straw (Menardo and Balsari, 2012; Sambusiti et al.,  
19 2013a), 260-362 ml CH<sub>4</sub>/g VS<sub>added</sub> for sorghum (Bauer et al., 2010; Sambusiti et al., 2013a),  
20 and 270-290 ml CH<sub>4</sub>/g VS<sub>added</sub> for rice straw (Lei et al., 2010), but higher than the yields of  
21 280 ml CH<sub>4</sub>/g VS<sub>added</sub> (Yu, 2010) and 440 ml biogas/g VS<sub>added</sub> (Qingming et al., 2005) for  
22 corn stalk/stover. The methane yield obtained for Douglas fir bark ( $98 \pm 5$  ml CH<sub>4</sub>/g  
23 VS<sub>added</sub>) was also higher than those reported in the literature for non-pretreated pine

1 residues (i.e. 5-39 ml CH<sub>4</sub>/g VS<sub>added</sub>) (Matsakas et al., 2015; Mirmohamadsadeghi et al.,  
2 2014).

3 During Stage I, hydrothermal pretreatment with CO<sub>2</sub> decreased the specific methane yields  
4 of solid fractions of wheat straw by 1-8% (Figure 3b) due to solubilization of biomass  
5 organic matter into the liquid fraction (water), as evidenced by the liquid phase TS/VS,  
6 COD, VFAs, and soluble sugars increasing with the intensity of pretreatment (Table 3).

7 During Stage II, after releasing of sugars from solid into the liquid phase, similar behavior  
8 was observed for the sugar-rich substrates of sorghum and corn stover (Table 3) resulting  
9 in a 11% decrease of specific methane yields after hydrothermal pretreatments over their  
10 respective controls (Figure 3d). The sugars solubilized in the liquid phase led to the highest  
11 specific methane yield (222 ± 14 ml CH<sub>4</sub>/g COD<sub>added</sub>) for wheat straw after exposure to the  
12 most intensive pretreatment conditions (175°C/66 bars/30 min) of Stage I (Figure 3a).

13 For Stage II, pretreated (166°C/76 bars/30 min) sorghum achieved the highest yield (275 ±  
14 14 ml CH<sub>4</sub>/g COD<sub>added</sub>) of the liquid hydrolysate, followed by the pretreated (175°C/102  
15 bars/30 min) corn stover (260 ± 14 ml CH<sub>4</sub>/g COD<sub>added</sub>) (Figure 3c). Despite the low  
16 methane yield obtained from the solid fraction of raw and pretreated Douglas fir (Figure  
17 3d), the hydrothermally solubilized organics into the liquid phase achieved a similar  
18 biodegradability level (253 ± 5 ml CH<sub>4</sub>/g COD<sub>added</sub>) as the other substrates. Although  
19 pretreated hydrolyzates did not reach to the theoretical maximum methane yield of 350  
20 ml/g COD<sub>added</sub> based on the COD equivalence of methane (Droste, 1997), the pretreatment  
21 conditions tested did not create any acute or chronic inhibition on the granular sludge  
22 used. Although, pH of liquid fractions of Douglas fir, corn stover, and sorghum were quite

1 acidic (3.51-4.85; Table 3), the addition of a buffer solution prevented acidification in the  
2 bottles and methane production started from Day 2.

### 3 *3.2.2. Hydrogen production*

4 The mesophilic BHP assays were monitored for 48 hours for the control (glucose), 68 hours  
5 for liquid fractions of sorghum and corn stover, and 112-119 hours for wheat and rice  
6 straws. The assay termination times were determined based on reaching the maximum  
7 hydrogen yield and the first decrease in hydrogen percentage (consumption) in bottle  
8 headspace. For the heat treated (90°C, 30 min) inoculum (activated sludge from Narbonne  
9 WWTP), the lag phases of H<sub>2</sub> production were 15, 24, 39, and 42 hours for control,  
10 sorghum, corn stover, and wheat/rice straws, respectively.

11 Among BHP assays, the ultimate specific hydrogen yield for the control (135 ± 25 ml H<sub>2</sub>/g  
12 COD<sub>added</sub>; 1.2 ± 0.2 mol H<sub>2</sub>/mol glucose<sub>added</sub>) was the highest, followed by the hydrolyzates  
13 of hydrothermally pretreated sorghum (55 ± 5 ml H<sub>2</sub>/g COD<sub>added</sub>), corn stover (52 ± 6 ml  
14 H<sub>2</sub>/g COD<sub>added</sub>), wheat straw (32 ± 4 ml H<sub>2</sub>/g COD<sub>added</sub>), and rice straw (26 ± 2 ml H<sub>2</sub>/g  
15 COD<sub>added</sub>). The maximum hydrogen yield of 1.2 ± 0.2 mol H<sub>2</sub>/mol glucose from the control  
16 BHP was comparable to previous studies. Kawagoshi et al. (2005) reported 1.4 mol H<sub>2</sub>/mol  
17 glucose (pH: 6.5-7, 20 g glucose/L) while Davila-Vazquez et al. (2008) observed 1.46 mol  
18 H<sub>2</sub>/mol glucose (pH: 7.5, 5 g glucose/L). In other studies, lower yields were obtained (0.96  
19 to 1.17 mol H<sub>2</sub>/mol glucose by Salerno et al., 2006, and 1.75 mol H<sub>2</sub>/mol glucose at pH: 6.0,  
20 10 g glucose/L by Zheng and Yu, 2005).

21 In this study, pretreated (175°C/66 bars/30 min) wheat straw hydrolyzate achieved 287 ±  
22 32 ml H<sub>2</sub>/g sugars<sub>added</sub> at mesophilic (37°C) temperature, which is close to the maximum  
23 yield of 318 ± 5 ml H<sub>2</sub>/g sugars of wheat straw hydrolyzate (at 5% by vol.) obtained from a

1 three-step pilot-scale hydrothermal pretreatment (presoaking at 80°C, extraction of  
2 hemicelluloses at 170-180°C, and improved enzymatic cellulose conversion at 195°C)  
3 under extreme thermophilic conditions (70°C) (Kongian et al., 2010). This value  
4 corresponds to  $4.7 \pm 0.3$  ml H<sub>2</sub>/g VS<sub>initial</sub> (yield normalized based on initial straw VS in the  
5 HTP reactor) and is close to the lower end of the range reported for untreated wheat straw  
6 ( $5.18$ - $10.52$  ml H<sub>2</sub>/g VS<sub>initial</sub>) and lower than the values reported for wheat straw  
7 fermentation with enzyme addition ( $11.06$ - $19.63$  ml H<sub>2</sub>/g VS<sub>initial</sub>) (Quemeneur et al.,  
8 2012b). Similarly, the pretreated (165°C/76 bars/30 min) rice straw yield of  $4.6 \pm 0.5$  ml  
9 H<sub>2</sub>/g TS<sub>initial</sub> was lower than the literature value of un-pretreated rice straw of 25 ml H<sub>2</sub>/g  
10 TS<sub>initial</sub> (Chen et al. 2012; Guo et al., 2014). In the literature, unpretreated corn stover/stalk  
11 yield is low (3 ml H<sub>2</sub>/g VS<sub>initial</sub>), but can be increased up to 57, 64, and 150 ml H<sub>2</sub>/g VS<sub>initial</sub>  
12 after pretreatments with 0.5% NaOH, high pressure steam at 1.6 MPa/5 min, and 0.2%  
13 HCl/30 min boiling, respectively (Zhang et al., 2007; Lu et al., 2009). The HTP pretreated  
14 (175°C/102 bars/30 min) corn stover yield of  $17.1 \pm 5.4$  ml H<sub>2</sub>/g TS<sub>initial</sub> again falls into the  
15 lower end of this range. Finally, a maximum yield of 10.4 ml H<sub>2</sub>/g sorghum was reported for  
16 sweet sorghum liquid extract (obtained by mixing milled sorghum with tap water at 30°C  
17 for 1 h) (Antonopoulou et al., 2008), comparable to the hydrothermally pretreated  
18 (166°C/90 bars/30 min) sorghum B140 ( $18.5 \pm 1.7$  ml H<sub>2</sub>/g sorghum;  $21.1 \pm 1.9$  ml H<sub>2</sub>/g  
19 TS<sub>initial</sub>) in this study. Given the aforementioned variability with sorghum, literature has a  
20 wider range of data ( $9.7$ - $64$  ml H<sub>2</sub>/g TS<sub>initial</sub>) from different types of sorghum (Monlau et al.,  
21 2012a; Guo et al., 2014). Overall, when compared to literature, the results indicate that HTP  
22 pretreatments (165-175°C/66-102 bar/30 min) applied to various substrates did not  
23 achieve discernable improvements in the extent of hydrogen production. However,

1 observed yields are substrate, pretreatment and BHP assay condition specific, therefore,  
2 direct comparisons are not easy to make.

3 The results also indicated that poly-phenols (0.18 – 0.86 g/l), and 5-HMF (0.01-0.05 g/l) in  
4 BHP bottles were below the inhibitory levels (furans and phenolic compounds of 1 g/L)  
5 previously observed (Quemeneur et al., 2012a) and did not create any acute (extensive lag  
6 phase) or chronic inhibition to hydrogen formers in BHP assays at the S/I ratio of 8.7 g COD  
7 degradable/g VS. Maximum pretreatment temperature reached (175°C) was also below the  
8 levels (220-230°C) of furfural formation during steam explosion of lignocellulosic biomass  
9 (Horn et al., 2011). In addition, dilute acid pretreatment of sunflower stovers combined  
10 with heat (170°C+4% HCl for 1 h) yielded higher inhibitory levels of furfural (1.15 g/L), 5-  
11 HFM (0.13 g/L) in hydrolyzate at lower temperatures than 220-230°C (Monlau et al.,  
12 2013c). Some studies also mentioned a synergistic inhibition effect of co-existence of by-  
13 products (furfural, 5-HMF, phenols), although individually compounds were lower than the  
14 reported toxicity thresholds (Mussatto and Roberto, 2004).

15 After BHP assays were terminated at their highest point of cumulative hydrogen yield,  
16 soluble metabolites were quantified in order to understand the fermentation pathways.  
17 The results, presented in Figure 4, suggested that in all the bottles set up with pretreated  
18 hydrolyzate, acetate and butyrate were the main metabolites indicating that hydrogen  
19 production was mainly from the typical acetate-butyrate pathway for dark fermentation of  
20 carbohydrates (Monlau et al., 2013c; Chandrasekhar et al., 2015). Furthermore, ethanol  
21 was also detected in the control BHP with glucose (5 g COD/l) and BHP with sorghum at  
22 higher levels (0.7-0.9 g/l) than the other assays (0.1-0.2 g/l), suggesting a more

1 pronounced population shift to solvent (non-hydrogen) production such as ethanol from  
2 hydrogen production in these BHP assays than the others. Metabolite results in Figure 4  
3 also suggest that readily biodegradable sugars quantified in hydrolyzate samples (Table 3)  
4 were all consumed.

### 5 3.2.3. Biodegradation kinetics

6 Kinetic parameter estimation results are tabulated for raw and hydrothermally pretreated  
7 substrates in Table 4 for BMP assays and in Tables 5 for hydrolyzate BHP assays. For  
8 comparison with raw substrates, specific BMP results from liquid and solid phases of  
9 pretreated substrates were added together (based on VS distribution between liquid and  
10 solid fractions after pretreatment) prior to parameter estimation analysis. As it can be seen  
11 in Table 4, the first-order kinetics was successful in predicting specific cumulative BMP's  
12 with the squared correlation coefficient,  $R^2$ , generally close to unity, being greater than  
13 0.97. For visual observation, predicted BMPs were also plotted along with the observed  
14 values (Supplementary data; Figure S1). It is important to emphasize that improvements by  
15 hydrothermal pretreatment was in terms of hydrolysis rate rather than the extent of the  
16 methane production for all the substrates except Douglas fir bark. For wheat/rice straws,  
17 sorghum and corn stover, hydrothermal pretreatments (165-175°C/66-102 bars/30 min)  
18 with CO<sub>2</sub> addition increased the hydrolysis constant by 20-30% (relative to controls).  
19 However, for Douglas fir bark, both the rate as well as the extent of digestion was  
20 significantly enhanced by the pretreatment with 172% faster hydrolysis rate compared to  
21 its control (Table 4), as the control digester was challenged as a result of the highly  
22 refractory nature of this biomass. In general, these increases in  $k$  constant represent lower  
23 improvements compared to thermochemical pretreatments, which can be as high as 65%

1 and 163% for sorghum and wheat straw, respectively, at a pretreatment combination of  
2 10% NaOH, 40°C for 24 h (Sambusiti et al., 2012b). Similarly, the modified Gompertz  
3 equation represented the measured BHP data successfully with  $R^2$  values higher than 0.92  
4 (Table 5; Figure S2).

#### 5 *3.2.4. Energy yield from possible digestion scenarios*

6 For the pretreatment conditions with available data on both CH<sub>4</sub> and H<sub>2</sub> yields, the total  
7 produced energy from two possible configurations (one-stage CH<sub>4</sub> and two-stage H<sub>2</sub>/CH<sub>4</sub>)  
8 are compared in Table 6. In the one-stage CH<sub>4</sub> option, both liquid and solid fractions  
9 contribute to CH<sub>4</sub> generation, while in the two-stage H<sub>2</sub>/CH<sub>4</sub>, liquid fraction is sent to dark  
10 fermentation first for H<sub>2</sub> generation and then metabolites of dark fermentation is treated  
11 for CH<sub>4</sub> production along with the solid fraction. CH<sub>4</sub> potential from metabolites (Fig. 4)  
12 was calculated based on their theoretical methane potential. As it can be seen from Table 6,  
13 the rest of the pretreatment conditions achieved similar energy yields between the two  
14 scenarios except for the sorghum with high H<sub>2</sub> potential from readily biodegradable sugars  
15 and CH<sub>4</sub> potential from dark fermentation by-products giving an advantage to the two-  
16 stage H<sub>2</sub>/CH<sub>4</sub>. Although the hydrogen yields from the first-stage were low compared to  
17 literature, total energy obtained from both configurations are in the range reported for  
18 similar substrates compiled in a review (Monlau et al., 2013c).

### 19 **Conclusions**

20 Hydrothermal pretreatment (26-175°C, 25-102 bars, with CO<sub>2</sub> as catalyst) of various  
21 lignocellulosic substrates decreased the hemicellulose content by 23-42% while  
22 delignification was limited to 0-12%. The pretreatment was able to accelerate the rate of  
23 biodegradation by 20-172% without generating high levels of inhibitory compounds. There

1 was no discernable enhancement in the ultimate methane or hydrogen yield observed  
2 except for the most refractory biomass (Douglas fir bark). Between two possible reactor  
3 configurations after pretreatment (one-stage CH<sub>4</sub> and two-stage H<sub>2</sub>/CH<sub>4</sub>), straws and corn  
4 stover achieved similar energy yields (9.5-11.7 MJ/kg) while sorghum with high sugars/H<sub>2</sub>  
5 in a two-stage H<sub>2</sub>/CH<sub>4</sub> achieved 41% higher energy yield than the one-stage CH<sub>4</sub> process.  
6 Compared to other lignocellulosic biomass pretreatment techniques, hydrothermal  
7 pretreatment with CO<sub>2</sub> pressurization requires only heat energy and CO<sub>2</sub>, which is easily  
8 available on fermentation or biogas plants and is easily released by depressurization at the  
9 end of the pretreatment. One of its considerable advantages is the low level of inhibitory  
10 substances produced. Its industrial application may be relevant in the case of refractory  
11 biomass such as Douglas fir bark, allowing the use of some substrates whose adaptation to  
12 biogas plants would not have been possible without such pretreatment. Further work  
13 should thus optimize these pretreatment conditions in the case of refractory biomass, and  
14 carry out a life cycle analysis to assess environmental impacts in comparison to more  
15 conventional pretreatment techniques.

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**Table 1. Solid content of lignocellulosic substrates after milling**

Substrate	Milling size (mm)	Total solids (TS) (% by wt.)	Volatile solids (VS)	VS/TS*100 (%)
Wheat straw	1	93.5 (0.1) <sup>a</sup>	89.4 (0.1)	95.6 (0.0)
Sorghum (B 140)	1	87.8 (0.3)	81.1 (1.1)	92.5 (1.4)
Rice straw	1	91.5 (0.4)	77.5 (0.1)	84.6 (0.5)
Corn stover	2	90.1 (0.5)	84.0 (0.6)	93.2 (0.3)
Douglas fir bark	2	91.13 (0.23)	91.07 (0.13)	99.9 (0.4)

<sup>a</sup>Data represent arithmetic mean and standard deviation of triplicates

**Table 2. Hydrothermal pretreatment conditions for lignocellulosic substrates**

Substrate	Sample code	Maximum observed		Heating time (min) <sup>c</sup>	Initial CO <sub>2</sub> pressure (bar) <sup>d</sup>
		T (°C) <sup>a</sup>	P (bar) <sup>b</sup>		
<i>Stage I</i>					
Wheat straw	WS (26C/50b)	26	50	30	50
Wheat straw	WS (80C/60b)	80	60	30	50
Wheat straw	WS (175C/25b)	175	25	30	10
Wheat straw	WS (175C/66b)	175	66	30	50
<i>Stage II</i>					
Sorghum (B 140)	S (166C/90b)	166	90	30	50
Rice straw	RS (165C/76b)	165	76	30	50
Corn stover	CS (175C/102b)	175	102	30	50
Douglas fir bark	DFB (171C/86b)	171	86	30	50

<sup>a</sup>Maximum temperature reached during 30 minutes of pretreatment

<sup>b</sup>Maximum pressure reached during 30 minutes of pretreatment

<sup>c</sup>Total exposure time to pretreatment

<sup>d</sup>CO<sub>2</sub> gas was used to pressurize the pretreatment reactor initially, and was disconnected during the pretreatment .

**Table 3. Characterization of liquid fraction of hydrothermally pretreated substrates<sup>a</sup>**

Parameter	WS (26C/50b)	WS (80C/60b)	WS (175C/25b)	WS (175C/66b)	S (166C/90b)	RS (165C/76b)	CS (175C/102b)	DFB (171C/86b)
Volume recovered after pretreatment (ml)	209	208	204	214	220	220	220	228
pH (-)	5.83	6.00	5.53	5.08	4.85	6.44	4.83	3.51
COD (g/l)	4.9 (0.1) <sup>b</sup>	5.5 (0.1)	10.3 (0.1)	14.7 (0.3)	24.0 (0.5)	10.4 (0.2)	20.0 (0.5)	10.2 (0.4)
COD <sub>sugars</sub> (g/l) <sup>c</sup>	0.2 (0.0)	0.0 (0.0)	0.9 (0.1)	1.1 (0.0)	9.7 (0.0)	1.2 (0.1)	8.8 (0.0)	1.6 (0.2)
TS (g/l)	3.8 (0.0)	4.8 (0.0)	8.2 (0.0)	11.3 (0.0)	18.5 (0.3)	10.9 (0.1)	16.3 (0.2)	8.1 (0.2)
VS (g/l)	2.6 (0.0)	3.4 (0.0)	6.5 (0.0)	9.4 (0.1)	15.7 (0.0)	6.6 (0.0)	13.7 (0.2)	7.9 (0.3)
Poly-phenols (mg/l)	236 (3)	285 (2)	831 (4)	960 (6)	835 (31)	571 (8)	1029 (31)	538 (7)
5-HMF (g/l)	nd*	nd	nd	nd	0.11 (0.01)	0.06 (0.00)	0.06 (0.00)	nd
Furfural (g/l)	nd	nd	nd	nd	nd	nd	nd	nd
Total VFAs (mg/l) <sup>d</sup>	206 (8)	204 (4)	602 (3)	806 (13)	672 (0)	69 (1)	842 (14)	371 (6)
Cellobiose (g/l)	nd	nd	nd	nd	0.6 (0.0)	nd	0.5 (0.0)	0.4 (0.0)
Glucose (g/l)	nd	nd	nd	nd	3.4 (0.1)	0.4 (0.0)	3.1 (0.0)	0.1 (0.0)
Xylose (g/l)	0.1 (0.0)	nd	0.6 (0.0)	0.7 (0.0)	4.4 (1.1)	0.4 (0.0)	4.0 (0.0)	0.5 (0.0)
Arabinose (g/l)	0.1 (0.0)	nd	0.2 (0.0)	0.4 (0.0)	0.8 (0.0)	0.3 (0.0)	0.8 (0.0)	0.6 (0.1)
Acetate (g/l)	0.1 (0.0)	1.4 (0.0)	3.1 (0.3)	3.2 (0.1)	nd	nd	nd	nd
Lactate (g/l)	nd	0.6 (0.0)	nd	nd	nd	nd	nd	nd

<sup>a</sup>WS: wheat straw; S: sorghum; RS: rice straw; CS: corn stover; DFB: Douglas fir bark; 5-HMF: 5-hydroxymethylfurfural

<sup>b</sup>Data represent arithmetic mean and standard deviation of triplicates for COD, poly-phenols, VFAs and duplicates for TS/VS and sugar analyses

<sup>c</sup>Calculated from COD equivalency of sugars in the liquid phase

<sup>d</sup>Total volatile fatty acids (VFAs) as summation of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric acids

\*nd: not detected



**Table 4. Methane potential and first-order kinetics of raw and pretreated substrates (total fraction)<sup>a</sup>**

Conditions	BMP measured		BMP predicted (ml CH <sub>4</sub> /g VS <sub>added</sub> )	<i>k</i> (1/d)	R <sup>2</sup>	<i>k</i> increase (%)
	(ml CH <sub>4</sub> /g VS <sub>added</sub> )	% from liquid fraction				
Raw WS	256 (18) <sup>b</sup>	-	252 (9)	0.067 (0.002)	0.99 (0.00)	-
WS (175C/66b)	269 (3)	20 (1)	259 (3)	0.082 (0.010)	0.99 (0.01)	22
Raw S	340 (2)	-	318 (2)	0.090 (0.002)	0.99 (0.00)	-
S (166C/90b)	338 (4)	36 (2)	321 (5)	0.114 (0.001)	0.99 (0.01)	27
Raw RS	311 (5)	-	304 (12)	0.071 (0.002)	0.99 (0.00)	-
RS (165C/76b)	319 (6)	13 (1)	300 (5)	0.093 (0.010)	0.99 (0.01)	30
Raw CS	336 (11)	-	336 (11)	0.090 (0.004)	0.99 (0.00)	-
CS (175C/102b)	318 (11)	28 (1)	300 (10)	0.108 (0.002)	0.99 (0.01)	20
Raw DFB	98 (5)	-	96 (9)	0.021 (0.001)	0.99 (0.00)	-
DFB (171C/86b)	136 (3)	32 (1)	124 (1)	0.056 (0.005)	0.97 (0.03)	172

<sup>a</sup>WS: wheat straw; S: sorghum; RS: rice straw; CS: corn stover; DFB: Douglas fir bark,

<sup>b</sup>Data represent arithmetic mean and standard deviation of triplicate bottles

**Table 5. Hydrogen potential and Modified Gompertz equation parameters of pretreated substrates (liquid fraction)<sup>a</sup>**

Conditions	BHP measured (ml H <sub>2</sub> /g VS <sub>added</sub> )	BHP predicted (ml H <sub>2</sub> /g VS <sub>added</sub> )	P (ml H <sub>2</sub> /g VS <sub>added</sub> )	Rm (ml H <sub>2</sub> /g VS <sub>added</sub> /h)	λ (h)	R <sup>2</sup>
WS (175C/66b)	55 (6) <sup>b</sup>	55 (6)	56 (5)	4.7 (1.3)	38 (3)	1.00 (0.00)
S (166C/90b)	94 (9)	84 (7)	84 (15)	42.5 (13.6)	29 (7)	0.95 (0.06)
RS (165C/76b)	39 (3)	40 (1)	45 (2)	1.3 (0.2)	27 (9)	0.98 (0.02)
CS (175C/102b)	84 (9)	74 (18)	94 (44)	23.0 (16.2)	35 (4)	0.92 (0.09)
Control (glucose)	143 (28) <sup>c</sup>	143 (27) <sup>c</sup>	143 (27) <sup>c</sup>	3.2 (4.7) <sup>d</sup>	14 (5)	1.00 (0.00)
Control (glucose)	1.2 (0.2) <sup>e</sup>	1.2 (0.2) <sup>e</sup>	1.2 (0.2) <sup>e</sup>	0.2 (0.1) <sup>f</sup>	14 (1)	1.00 (0.00)

<sup>a</sup>WS: wheat straw; S: sorghum; RS: rice straw; CS: corn stover,

<sup>b</sup>Data represent arithmetic mean and standard deviation of 3-5 replicate bottles,

<sup>c</sup>ml H<sub>2</sub>/g glucose added,

<sup>d</sup>ml H<sub>2</sub>/g glucose/h,

<sup>e</sup>mol H<sub>2</sub>/mol initial glucose,

<sup>f</sup>mol H<sub>2</sub>/mol initial glucose/h.

**Table 6. Energy yield from possible two bioreactor options<sup>a</sup>**

Conditions	One-stage CH <sub>4</sub>		Two-stage H <sub>2</sub> /CH <sub>4</sub>				
			Stage one (H <sub>2</sub> )		Stage two (CH <sub>4</sub> )		Total
	ml CH <sub>4</sub> /g TS <sub>initial</sub>	MJ/kg TS <sub>initial</sub> <sup>c</sup>	ml H <sub>2</sub> /g TS <sub>initial</sub>	MJ/kg TS <sub>initial</sub> <sup>d</sup>	ml CH <sub>4</sub> /g TS <sub>initial</sub>	MJ/kg TS <sub>initial</sub> <sup>c</sup>	MJ/kg TS <sub>initial</sub>
WS (175C/66b)	252 (3) <sup>b</sup>	<b>10.1 (0.1)</b>	4.4 (0.3)	0.05 (0.00)	234 (4)	9.3 (0.1)	<b>9.4 (0.1)</b>
S (166C/90b)	313 (5)	<b>12.5 (0.2)</b>	21.1 (11.9)	0.23 (0.02)	438 (8)	17.4 (0.3)	<b>17.6 (0.3)</b>
RS (165C/76b)	277 (6)	<b>11.0 (0.2)</b>	4.6 (0.5)	0.05 (0.01)	263 (5)	10.5 (0.2)	<b>10.5 (0.2)</b>
CS (175C/102b)	295 (9)	<b>11.7 (0.4)</b>	17.1 (5.4)	0.18 (0.06)	262 (6)	10.4 (0.2)	<b>10.6 (0.2)</b>

<sup>a</sup>WS: wheat straw; S: sorghum; RS: rice straw; CS: corn stover,

<sup>b</sup>Data represent arithmetic mean and standard deviation of 3-5 replicate bottles,

<sup>c</sup>Energy yield: 1NI CH<sub>4</sub> = 39790 J

<sup>d</sup>Energy yield: 1NI H<sub>2</sub> = 10780 J

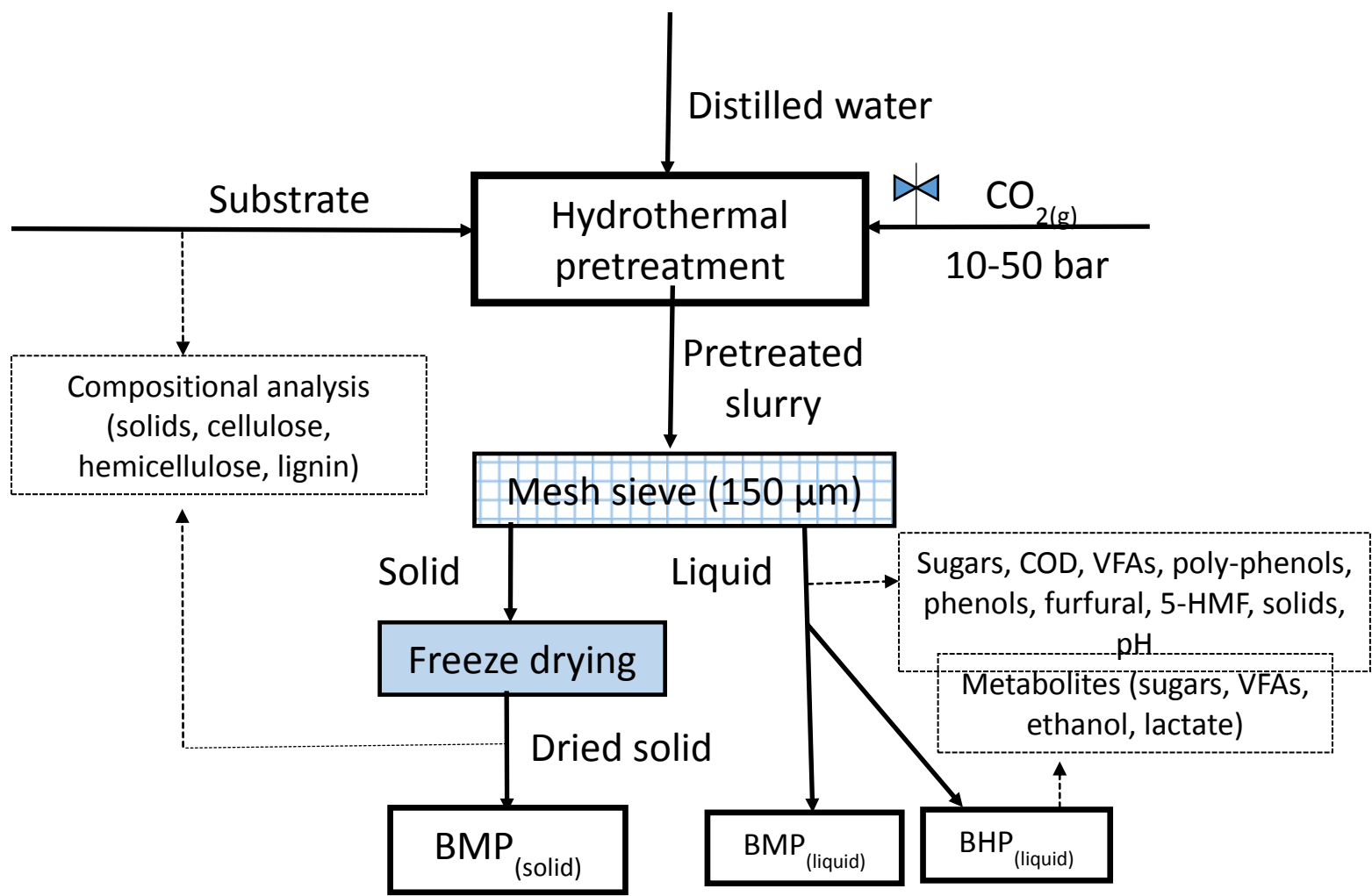
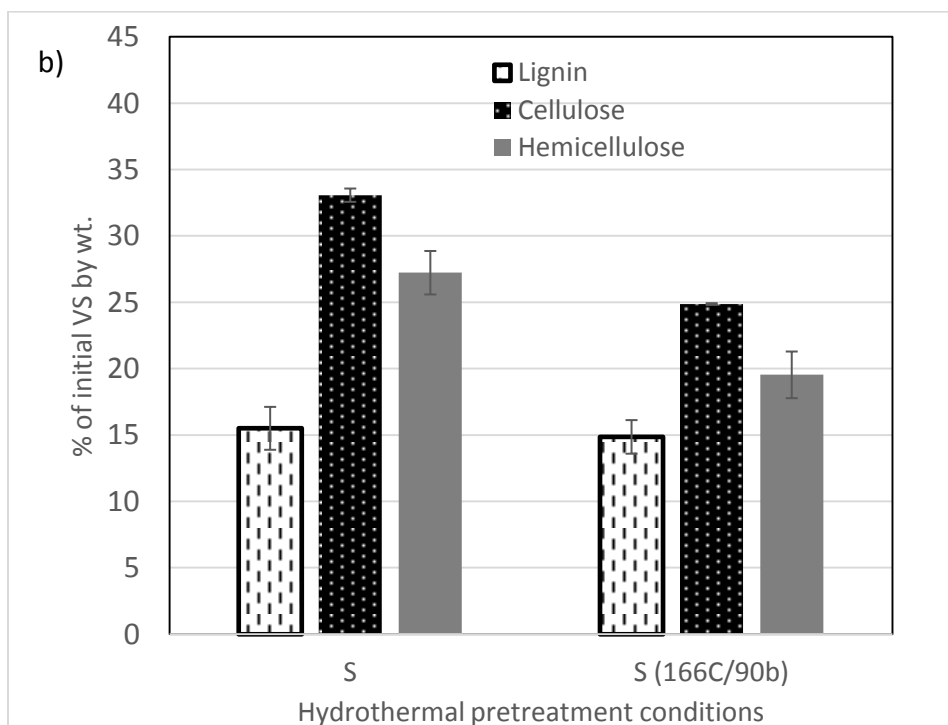
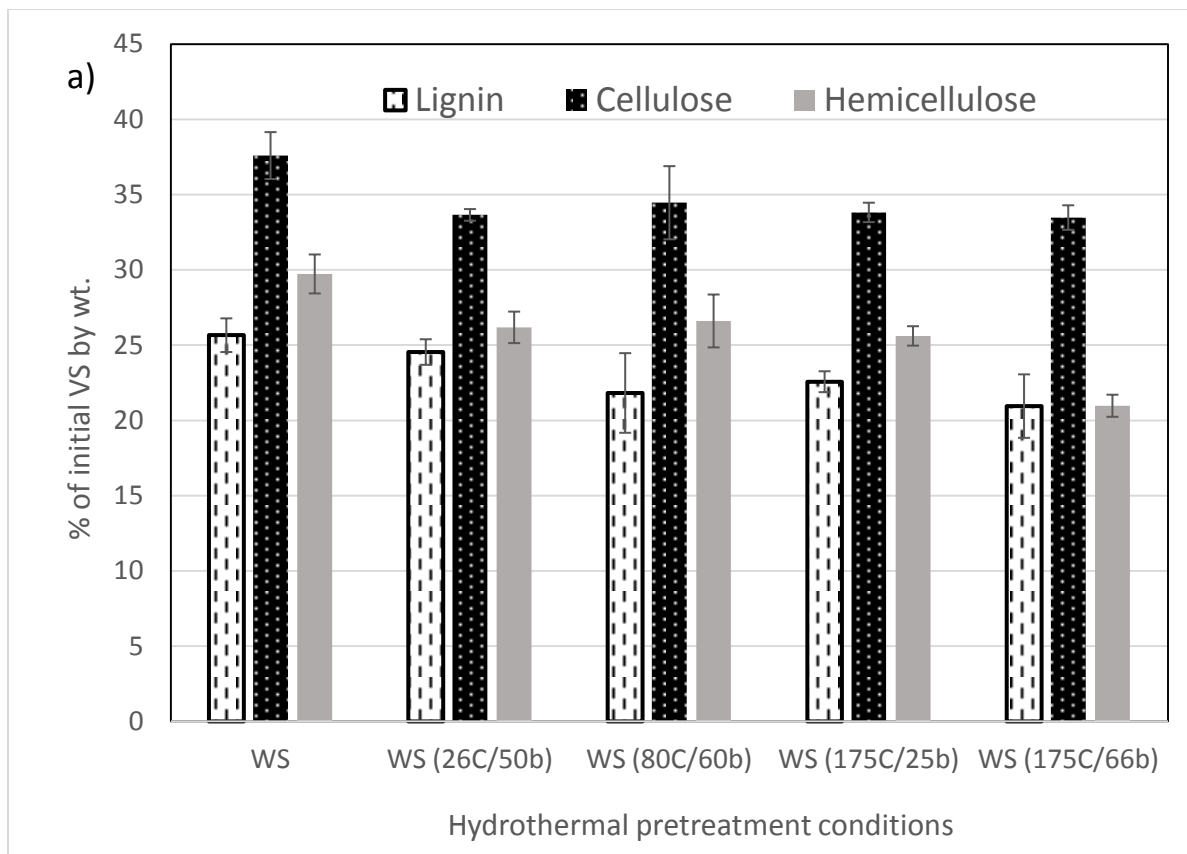
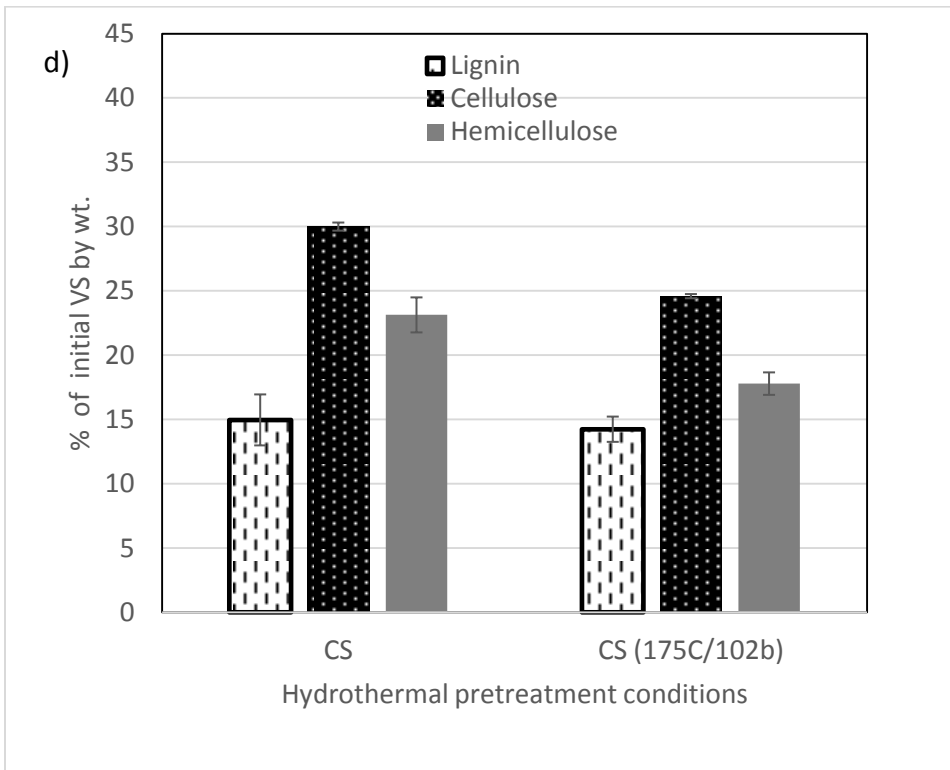
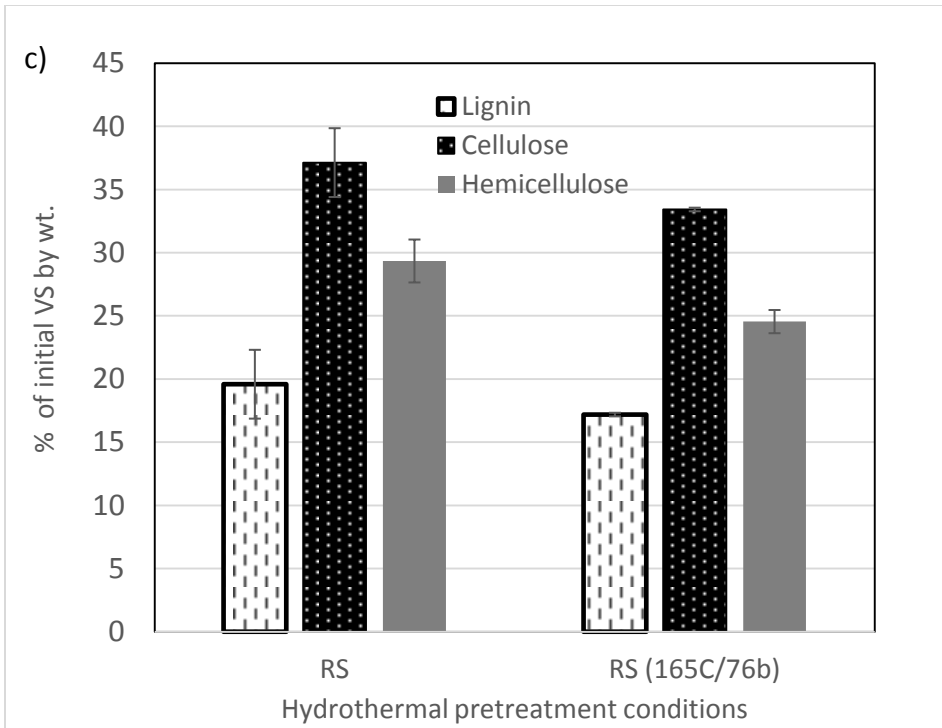
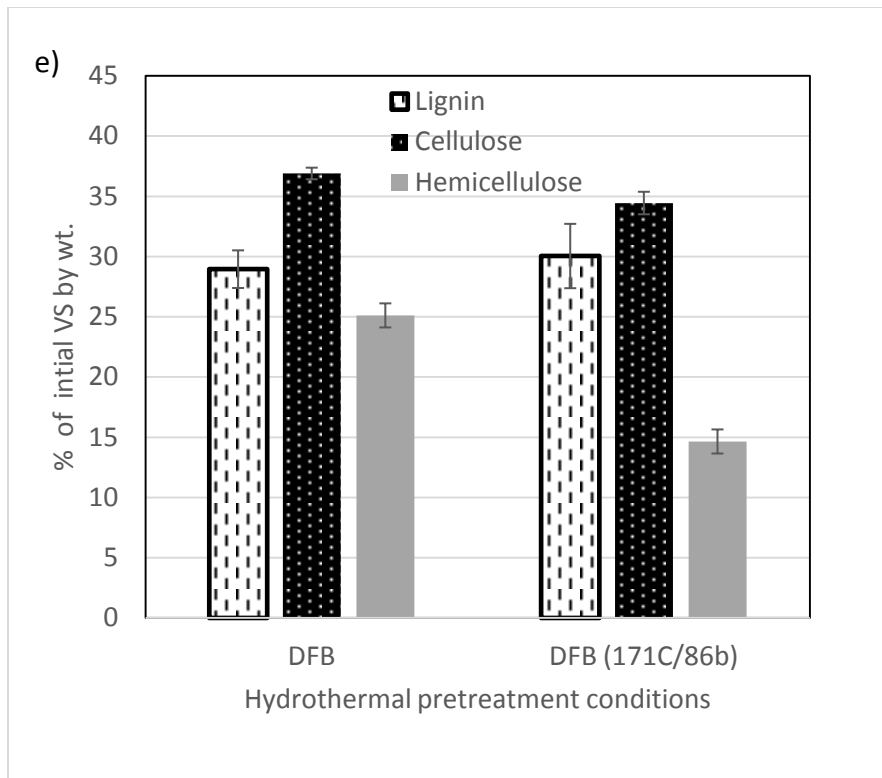


Figure 1. Schematic of experimental procedure

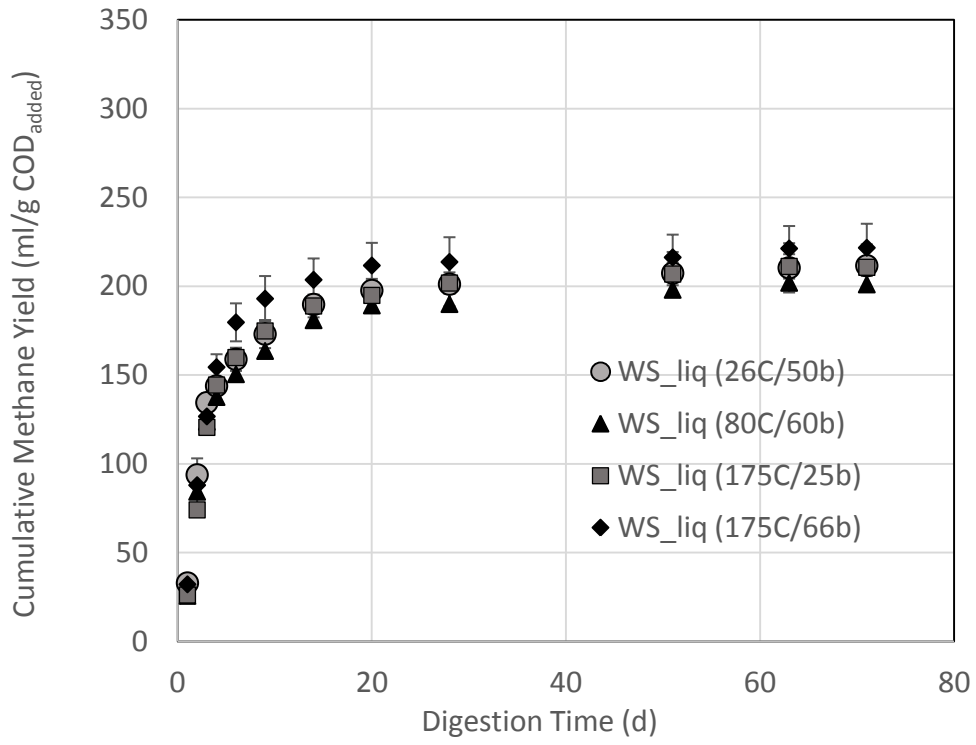




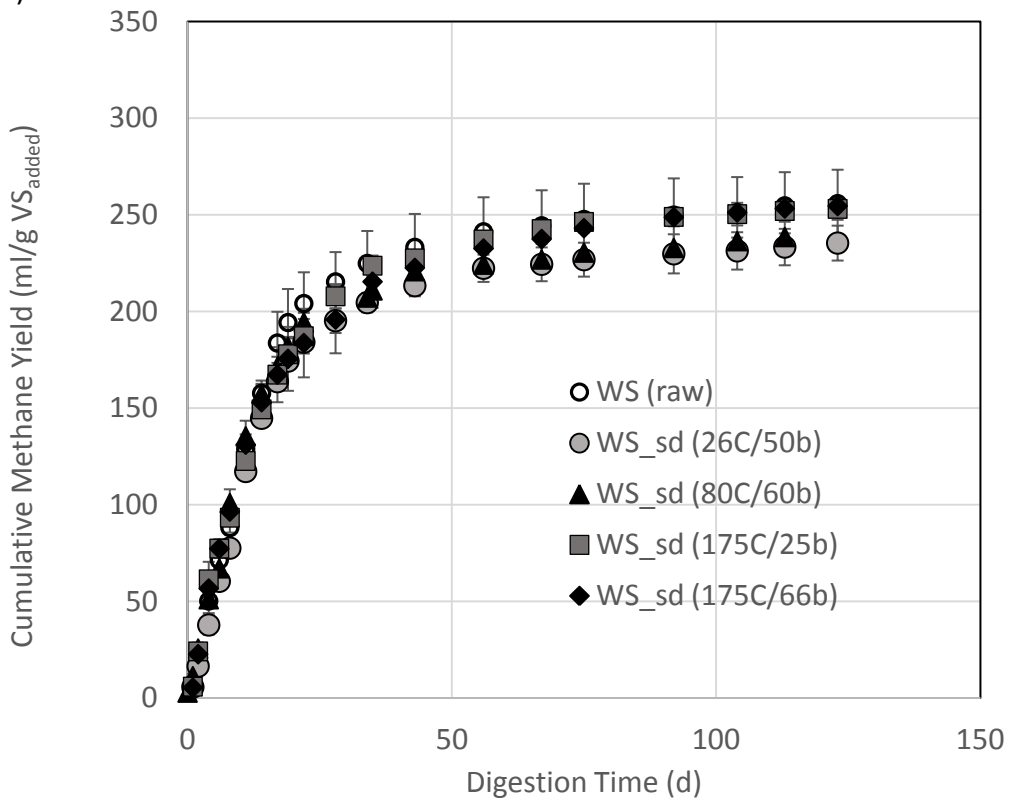


**Figure 2. Biochemical composition of raw and hydrothermal pretreated; a) wheat straw (WS), b) sorghum (S), c) rice straw (RS), d) corn stover (CS), and e) Douglas fir bark (DFB). Values represent average and error bars represent standard deviation of triplicates.**

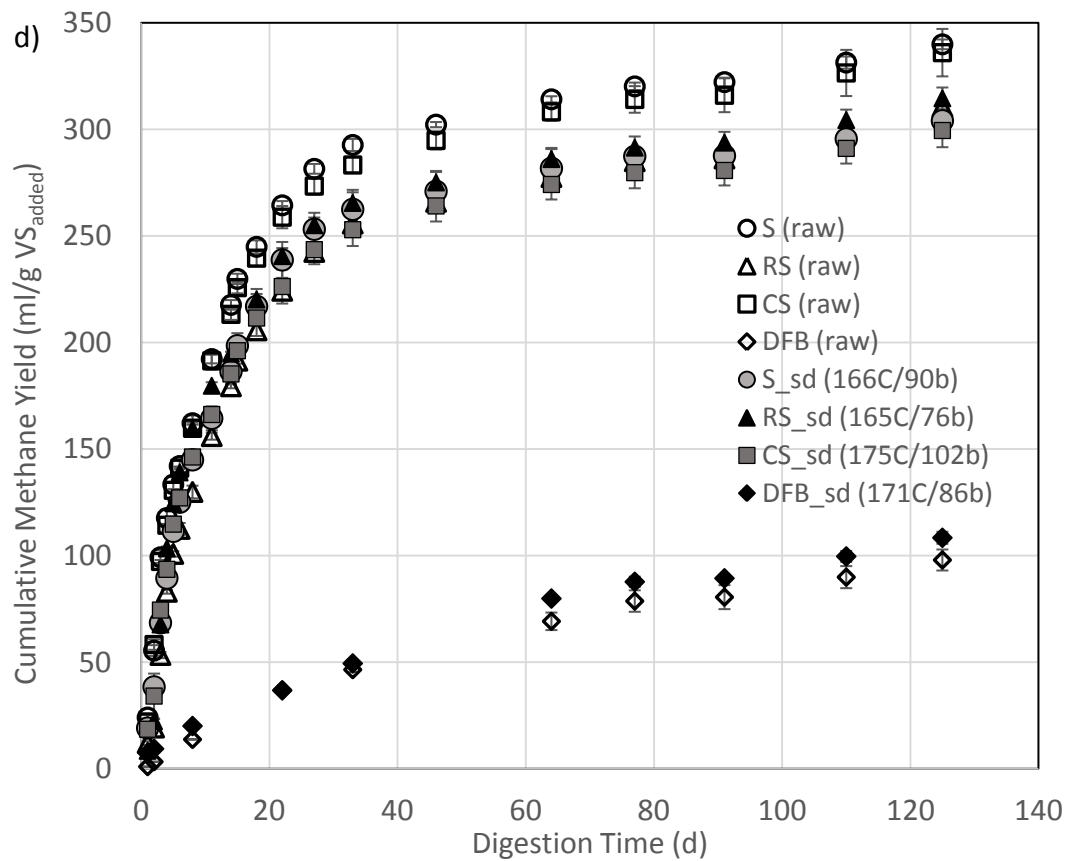
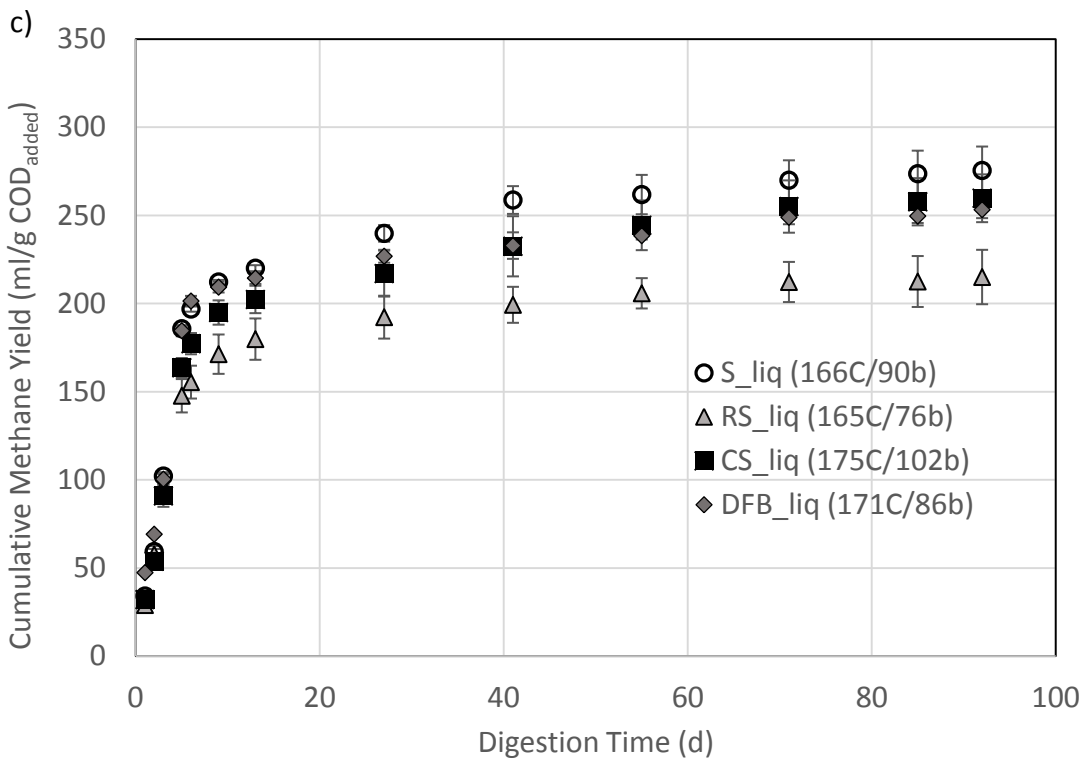
a)



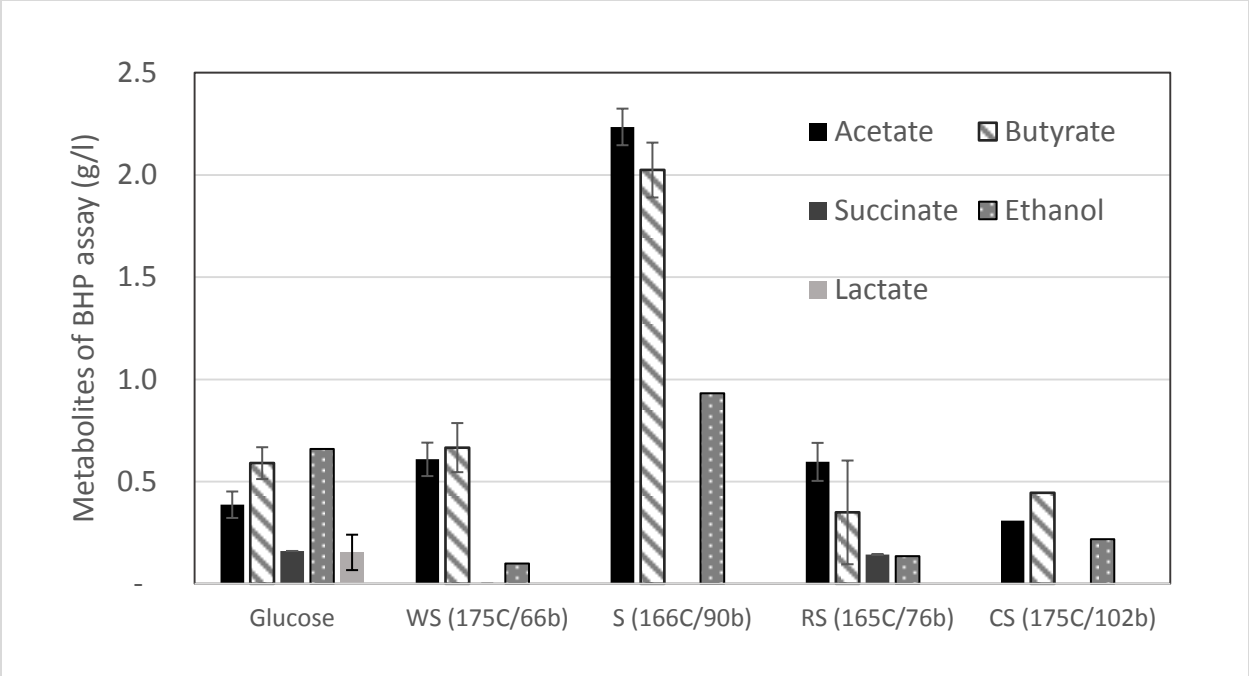
b)







**Figure 3. Specific cumulative methane production from (a) liquid (liq) fraction, (b) solid (sd) fraction of pretreated wheat straw, (c) liquid fraction, (d) solid fraction of pretreated sorghum (S), rice straw (RS), corn stalk (CS), and Douglas fir bark (DFB). Values represent average and error bars represent standard deviation of three replicates.**



**Figure 4. Metabolites determined at the end of BHP assays (point of maximum hydrogen yield). Values represent average and error bars represent standard deviation of 4-8 replicates.**