Assessment of hydrothermal pretreatment of various lignocellulosic biomass with CO₂ catalyst for enhanced methane and hydrogen production

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Abstract

Hydrothermal pretreatment of five lignocellulosic substrates (i.e. wheat straw, rice straw, biomass sorghum, corn stover and Douglas fir bark) were conducted in the presence of CO₂ as a catalyst. To maximize disintegration and conversion into bioenergy (methane and hydrogen), pretreatment temperatures and subsequent pressures varied with a range of 26-175°C, and 25-102 bars, respectively. Among lignin, cellulose and hemicelluloses, hydrothermal pretreatment caused the highest reduction (23-42%) in hemicelluloses while delignification was limited to only 0-12%. These reductions in structural integrity resulted in 20-30% faster hydrolysis rates during anaerobic digestion for the pretreated substrates of straws, sorghum, and corn stover while Douglas fir bark yielded 172% faster hydrolysis/digestion due to its highly refractory nature in the control. Furans and phenolic compounds formed in the pretreated hydrolyzates were below the inhibitory levels for methane and hydrogen production which had a range of 98 – 340 ml CH₄/ g volatile solids (VS) and 5 – 26 ml H₂/ g VS, respectively. Results indicated that hydrothermal pretreatment is able to accelerate the rate of biodegradation without generating high levels of inhibitory compounds while showing no discernible effect on ultimate biodegradation.

Keywords: anaerobic digestion, dark fermentation, straw, sorghum, corn stover, Douglas fir bark
1. Introduction

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

Anaerobic digestion and dark fermentation have been extensively studied for their ability to convert a wide variety of lignocellulosic biomass to methane (Sawatdeenarunat et al., 2015) and biohydrogen (Singh et al., 2015), respectively. However, the complex structure of lignocellulosic biomass, comprised of cellulose, hemicelluloses and lignin, does not provide easy access for the biodegradable organics in bioreactors. Such limited accessibility causes significantly lower methane/hydrogen yields than the theoretical estimations based on biomass compositional/structural features (Monlau et al., 2012a; Carrere et al., 2015). A review by Monlau et al. (2013a) states that the amount of lignin is the most important factor when determining the biodegradability of lignocellulosic biomass along with other factors, such as cellulose crystallinity and accessible surface area. Therefore, research has focused on various pretreatment technologies utilizing mechanical (Sharma et al., 1988; Palmowski and Muller, 2000), chemical (Sun et al., 2001; Zhu et al.,
2010; Monlau et al., 2013b), thermal (Kumar et al., 2009), and biological (Rouches et al., 2016) methods or combinations (Monlau et al., 2013c) to dissolve lignin (delignification), and to reduce cellulose crystallinity for increasing surface area and accessibility for better enzymatic hydrolysis/microbial degradation. Following pretreatment, chemical methods may have a high impact on downstream processes or the environment and limited chemical recovery potential which may require further pH neutralization before digestion. For example, high sodium may jeopardize digestate land application or inhibit methanogens in digesters (Antonopoulou and Lyberatos, 2013; Carrere et al., 2016). For enzymatic methods, typically, polysaccharides hiding under the lignin limit the enzymatic activity, therefore are at times combined with other types of pretreatment (Sun and Cheng, 2002). Furthermore, depending on the type/intensity of the pretreatment applied (i.e. dilute-acid, high temperature/pressure, steam explosion and thermo-alkaline pretreatments), some of the inhibitory by-products of pretreatment, such as furfural, 5-hydroxymethylfurfural (5-HMF) and phenolic compounds, to hydrogen and/or methane formers have also been reported (Palmqvist and Hahn-Hagerdal, 2000; Horn et al., 2011; Monlau et al., 2013c). Therefore, the chosen pretreatment method should reduce energy demand, minimize use of chemicals and formation of inhibitory by-products, and allow for reuse of co-products in a feasible biorefinery concept (Cherubini, 2010).

Hydrothermal pretreatment of lignocellulosic biomass at elevated temperatures/pressures (150 - 300°C, initial pressure of 0-60 bar, 2-40 min) has garnered consideration for the production of methane, hydrogen and bioethanol from lignocellulosic substrate as it eliminates chemical addition and corrosion resistant material requirements for hydrolysis reactors (Chandra et al., 2012; He et al., 2016). It has been traditionally applied in pulp
industries (Kubikova et al., 1996) and is considered more environmentally friendly compared to other methods with higher sugar recovery in a relatively short period of time and little to no inhibitor production (Kaparaju and Felby, 2010). It has been postulated that when optimized the results are comparable to dilute-acid pretreatment, but without chemical addition/post-neutralization. The use of CO$_2$ in hydrothermal pretreatment has been shown to further enhance hydrolysis of various types of biomass, such as Eucalyptus bark (Matsushita et al., 2010), corn stover (van Walsum and Shi, 2004), wheat straw (Relvas et al., 2015), and various polysaccharides (Miyazawa and Funazukuri, 2005). Carbonic acids generated in situ from water and added CO$_2$ can lower the pH of the solution and act as an environmentally friendly acid catalyst accelerating hydrolytic organic reactions at high-temperatures (Relvas et al., 2015). At the end of the pretreatment process, CO$_2$ can be easily removed by depressurizing the reactor to atmospheric pressure, avoiding the need of a subsequent treatment. The availability of CO$_2$ as a component in the fermentation processes (i.e. bioethanol or biogas) makes this a potentially cost-effective option for biomass pretreatment.

Although hydrothermal pretreatment with CO$_2$ pressurization is viewed as a promising technology, the existing literature is limited to the aforementioned studies focusing only on either the hydrolysis phase (van Walsum and Shi, 2004; Miyazawa and Funazukuri, 2005; Matsushita et al., 2010; Relvas et al., 2015), or enhancement of methane yield from sewage sludge (Spooner et al., 2007) and hydrogen yield from starch (Orozco et al., 2012). However, no sufficient insight has been provided on the levels/effect of inhibitory compounds on methane/hydrogen yields from common lignocellulosic biomass including energy crops. Therefore, the objective of this study was to evaluate the entire impact of
hydrothermal pretreatment with CO₂ pressurization not only on the compositional
structure of various lignocellulosic substrates but also on the enhancement of methane and
hydrogen yields in anaerobic digestion and dark fermentation, respectively. The yields
were estimated from batch biochemical methane potential (BMP) and biochemical
hydrogen potential (BHP) assays. Additionally, potentially inhibitory compounds after
pretreatment were quantified.

2. Materials and Methods

2.1. Lignocellulosic substrates

The substrates tested included wheat straw (WS), biomass sorghum (B140) (S), rice straw
(RS), corn stover (CS), and Douglas fir bark (DFB) containing different solids (Table 1) and
compositional structure (cellulose, hemicellulose and Klason lignin). Wheat straw
(Triticum aestivum), grown in France (latitude: 48°50´18´´N, longitude: 4°13´54.5´´E), was
first processed using a cutting mill. It was further sieved to have a particle size range of 400
µm - 1 mm. Sorghum (B140) was produced at a site (latitude: 43.6491994, longitude:
3.874161111) in Montpellier Lavalette (France) in 2012. It was milled to pass a 1mm
screen. Rice straw was provided by RIZ Camargue CANAVERE (a local farm in Saint-Gilles
Languedoc-Roussillon region, south of France). It was first cut by a mill equipped with a 6
mm sieve followed by a 1 mm sieve (SM200, Retsch, GE). Corn stover was provided by
INRA Versailles (Paris region, France). The sample was coarsely cut to less than 2 mm by
knife milling (SM200, Retsch, GE). Douglas fir bark was supplied by Brassac Industries
sawmill (Tarn region, France). It was a heterogeneous size material (chips of 5–20 cm
length and 1 mm–3 cm thickness) produced from the debarking of 50 years old Douglas fir
trees harvested in April 2013. Douglas fir bark chips were first dried in an oven at 40°C
overnight to reach a moisture content of 8.87%. Knife milling was then performed in a Retsch SM 100 system with a 6 mm and 2 mm size sieve at a speed of 1500 rpm.

2.2. Hydrothermal pretreatment

Hydrothermal pretreatment of substrates was conducted in a PARR 5500 High Pressure Compact Reactor equipped with mechanical mixer, heater, and controller. The reactor had an effective volume of 450 mL and was capable of achieving a maximum temperature and pressure of 350°C and 200 bar, respectively. Upon addition of substrate (15 g) and distilled water (300 g), the reactor was sealed and pressurized to the desired levels by a CO₂ line from a cylinder/regulator. After pressurization the CO₂ line was disconnected and the temperature/pressure increase in the reactor was recorded with respect to heating time while the reactor content was being mixed at 140 rpm. The PARR reactor controller allowed the pretreatment to be programmed based on different ramping rates (temperature increased per unit heating time). In this research, the heating duration was kept constant (30 min) and at the end of the 30 min, the heater and mixer were turned off and the vessel was immersed in an ice bath. When the temperature levels dropped below 40°C, the vessel was slowly depressurized to atmospheric levels by turning on a pressure release valve found on the reactor. The lid was opened and pretreated slurry was recovered. The solid fraction was separated from the liquid fraction (hydrolyzate) via a mesh sieve with 150 µm pore size (Figure 1). The use of the 150 µm sieve resulted in loss of 2.2 to 8.3% of TS in substrates pretreated based on mass balance. Separation of liquid from the solid fraction was necessary to conduct BHP assays on the solubilized sugars (main source of H₂ production) as well as to optimize BMP conditions separately for different fractions.
Pretreatments were applied in two separate stages. Stage I involved the pretreatment of wheat straw at a wide range of set temperature and CO$_2$ pressure combinations for preliminary screening. The pretreatment reactor was programmed to simulate four different scenarios with set temperature/initial CO$_2$ pressure/duration time combinations 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 min, respectively. Based on the preliminary results on substrate characterization and methane yields from Stage I, Stage II applied only the most intensive temperature/pressure combination (150°C/50 bar/30 min) for the remaining four substrates (Table 2). The observed (actual) maximum temperatures/pressures reached within 30 min of heating was quite substrate specific due to differences in the interstitial volume of the solids and therefore water absorption capacities of substrates affecting headspace pressure. Therefore, in all of the pretreatment runs, maximum temperature reached exceeded the set temperature due to common overshooting reported for Parr reactors without water or fan cooling. Table 2 lists the actual maximum temperatures/pressures observed for each substrate. The overshooting was higher (60%) at the low set-temperature (50°C) than that (17%) of the high set temperature (150°C) as these reactors are designed to reach temperatures up to 350°C in a short period of time.

2.3. Anaerobic inocula

2.3.1. Inoculum for BMP assay

The inoculum used for BMP assays was granular sludge from a mesophilic upflow anaerobic sludge blanket (UASB) reactor utilizing wastewater from a sugar factory in France. Prior to setting up the BMP assays, the inoculum was placed in a closed 5-L glass vessel, diluted 10 times with distilled water to total solids (TS) and volatile solids (VS)
concentrations of 1.24 ± 0.01 and 1.08 ± 0.02% (by wt.), respectively, and mixed to break
apart the granules under endogenous anaerobic conditions (35°C for 5-7 days) to reduce
non-specific biogas generation. The inoculum had a maximum specific methanogenic
activity of 33 ± 2 mL CH₄/g VS/d, as measured by degrading 1.3 ± 0.3 g/L of ethanol as
chemical oxygen demand (COD).

2.3.2. Inoculum for BHP assay

Among the three inocula tested (granular sludge described above, activated sludge,
municipal sludge digested under low pH conditions in a BMP bottle), the inoculum chosen
for BHP assays was the activated sludge taken from the aeration tank at the municipal
wastewater treatment plant (WWTP) in Narbonne (France). The decision was based on
rate/extent of H₂ yields from a preliminary BHP assay (in four replicates) conducted with
glucose (5 g COD/L) at a substrate to inoculum ratio (S/I) of 10 g COD/g VS at mesophilic
temperature (37°C). The activated sludge had a TS and VS concentration of 0.46 ± 0.00 and
0.33 ± 0.00% (by wt.), respectively, and achieved a maximum H₂ yield of 1.2 ± 0.2 mol
H₂/mol glucose within the first 39-40 hours while the granular sludge achieved a similar
yield (1.2 ± 0.1 mol H₂/mol glucose) only after 61 hours. The digested municipal sludge
reached a maximum yield of only 0.6 ± 0.2 mol H₂/mol glucose after 48 hours. Before the
addition to the BHP assays, all three inocula were thermally treated for 30 min in capped
glass tubes immersed in a water bath set at 90°C to inhibit the activity of methanogens.

2.4. BMP Assay Set-up

A total of four sets of BMP assays were conducted concurrently to determine methane
potential from liquid and solid fractions of pretreated substrates in both Stage I and II
(Figure 1; Table 2). A total of 72 bottles (including pretreated, non-pretreated substrates
and blanks in triplicates) were operated, with solid and liquid fractions set-up in bottles with 600 mL (350 mL liquid) and 120 mL (80-84 mL liquid) total volumes, respectively. BMP assays with solid fractions contained total substrate concentration of 5 g VS/L and the amount of the substrate and granular inoculum added to each bottle was calculated considering S/I ratio of $\frac{1 \text{ g VS}}{\text{g VS}}$ which has been previously used for various lignocellulosic substrates (Sambusiti et al., 2012a; Monlau et al., 2013b). For the liquid fractions, a substrate concentration and S/I ratio in the bottles was 2.5 g COD/L and 0.5 g COD/g VS, respectively. Each assay contained: macroelements (NH$_4$Cl, 286 mg/L; KH$_2$PO$_4$, 108 mg/L; MgCl$_2$, 65 mg/L; CaCl$_2$, 32 mg/L), oligoelements (FeCl$_2$, 20 mg/L; CoCl$_2$, 5 mg/L; MnCl$_2$, 1 mg/L; NiCl$_2$, 1 mg/L; ZnCl$_2$, 0.5 mg/L; H$_3$BO$_3$, 0.5 mg/L; Na$_2$SeO$_3$, 0.5 mg/L; CuCl$_2$, 0.4 mg/L; Na$_2$MoO$_4$, 0.1 mg/L), and a bicarbonate buffer solution (NaHCO$_3$, 2.6 g/L). Finally, the nitrogen gas was purged into each bottle to remove the residual oxygen and the bottles were sealed with septa/caps. The septa were then punctured to release excess N$_2$ pressure. The bottles were placed on a shaker (at 90 rpm) in a temperature controlled room at 37°C. Accumulated gas pressure in the bottles were measured with a digital manometer (LEO 2, Keller, Switzerland), while biogas composition was analyzed by a gas chromatograph (GC) every time excess pressure was released until bottles stopped producing biogas.

**2.5. BHP Assay Set-up**

Based on the biodegradation potential comparison among substrates from BMP, BHP assays excluded Douglas fir bark and assessed hydrogen yields and by-products of dark fermentation from liquid fraction of pretreated straws, sorghum and corn stover only at the most intensive condition (set temp: 150°C, initial CO$_2$ pressure: 50 bar, 30 min). BHP assays included a total of 21 bottles set-up with hydrolyzes of pretreated substrates and control
(glucose) in 3-5 replicates (depending on the volume recovered after pretreatment).

Bottles had total and liquid volume of 120 mL and 60 mL, respectively and the amount of the substrate and inoculum needed for each bottle was calculated considering a S/I ratio of $\frac{8.7 \text{ g COD degradable}}{\text{g VS}}$, where degradable COD was estimated as total sugars (i.e. summation of cellobiose, glucose, xylose, arabinose concentrations in Table 3) in hydrolyzates of pretreated substrates. Each BHP assay contained a dilution solution prepared with macroelements (NH$_4$Cl, 0.8 g/L; KH$_2$PO$_4$, 0.5 g/L), oligoelements (FeCl$_2$, 1.5 g/L; H$_3$BO$_3$, 60 mg/L; CoCl$_2$, 25 mg/L; MnSO$_4$, 117 mg/L; NiCl$_2$, 25 mg/L; ZnCl$_2$, 70 mg/L; CuCl$_2$, 15 mg/L; Na$_2$MoO$_4$, 25 mg/L), 1.2 mL/L vitamin solution (mixture of biotin, cyanocobalamin, thiamine), and 2-(N-morpholino)ethanesulfonic acid (MES) buffer at 19.52 g/L. Upon addition of substrate and dilution solution, pH of the bottle content was adjusted to 6 by adding drops of NaOH solution (32% by vol.). Then thermally treated inoculum (activated sludge) was added. Finally, the bottles were sealed with septa/caps and the nitrogen gas was purged to each bottle to remove residual oxygen. The bottles were then placed on a shaker (at 90 rpm) in a temperature controlled room at 37°C. Accumulated gas pressure in the bottles was measured with a digital manometer (LEO 2, Keller, Switzerland), while biogas composition was analyzed by a GC every time excess pressure was released until cumulative hydrogen yields plateaued and the first signs of hydrogen consumption (reduction in H$_2$ % in headspace) were observed. Upon termination of assays, metabolites of dark fermentation were quantified.

2.6. Analytical procedures

The TS/VS analysis of raw/pretreated substrates and inocula was done according to the Standard Methods, sections 2540 B and 2540 E, respectively (APHA, 2005). Quantification
of COD in liquid fractions of pretreated substrates were performed according to the closed reflux colorimetric method outlined by Standard Methods (APHA, 2005). Compositional analysis (i.e. cellulose, hemicelluloses and Klason lignin) on substrates were conducted using a strong hydrolysis method adapted from Sluiter et al. (2008). Raw or freeze-dried (−69°C, 0.21 Pa for 3 days) solid fraction of pretreated substrates (100 mg) were first hydrolyzed with H₂SO₄ (72% by vol.) in capped/mixed test tubes (in triplicates) at 30°C for 1 h, then diluted to reach a final acid concentration of H₂SO₄ (4%) and kept at 120°C for 1 h. Upon cooling, the tube content was filtered via glass-fiber filters (0.45 µm) to separate insoluble residue, which was placed in a crucible and dried at 100°C for 24 h to yield Klason lignin content. The liquid fraction obtained after filtration was further filtered via 0.2 µm syringe filters and analyzed by a high-performance liquid chromatograph (HPLC) equipped with a refractive index detector (Waters R410/Waters 2414) for structural carbohydrates (i.e. glucose, xylose, arabinose). Target compounds were separated by an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) placed after a protective precolumn (Microguard cation H refill cartridges, Bio-Rad). The eluting solution was 4 mM H₂SO₄, and the flowrate, column/detector temperatures were 0.3 mL/min, 45°C, respectively. Cellulose and hemicellulose contents of lignocellulosic substrates were calculated as follows:

\[
Cellulose \ (\% \ VS) = \frac{Glucose \ (\% \ VS)}{1.11}
\]

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

where 1.11 and 1.13 are the ratios of the molecular weights of glucose to glucan (180/162) and xylose/arabinose to xylan (150/132), respectively. Similarly, soluble sugars, inhibitory by-products of pretreatment (i.e. 5-HMF and furfural), as well as soluble metabolites of
BHP assays were also quantified by HPLC in liquid fractions filtered via 0.2 µm syringe filters. Poly-phenols were quantified by a colorimetric method at 735 nm by using Folin Ciocalteu reagent, Na$_2$CO$_3$, and gallic acid as standard (Cicco et al., 2009).

Biogas compositions were conducted by measuring the H$_2$S, CO$_2$, O$_2$, N$_2$, CH$_4$ percentage with a Perkin Elmer Clarus 480 GC and H$_2$, CO$_2$, O$_2$, N$_2$, CH$_4$ percentage by a Clarus 580 GC, in BMP and BHP bottles, respectively. Both GCs were equipped with thermal conductivity detectors but used different carrier gasses (helium for GC 480, argon for GC 580). Volatile fatty acids (VFAs) in pretreated liquid fractions were measured by injecting filtered samples (0.2 µm) into the Clarus 580 GC equipped with an auto-sampler, flame ionization detector. Nitrogen was the carrier gas.

2.7. Kinetic rate estimation

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

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

where $BMP_t$ is cumulative specific methane yield (ml CH$_4$/g VS$_{added}$) at a given time, $t$, calculated from Equation (4) below; $BMP_{t\to\infty}$ is the ultimate specific methane yield (ml CH$_4$/g VS$_{added}$) obtained at the end of the assay; $k$ is the first-order hydrolysis constant (1/d), and $t$ is the digestion time (d).
\[ BMP_t = \left( V_{CH4,s,t} - V_{CH4,blank,t} \right) / VS \]  

where \( V_{CH4,s,t} - V_{CH4,blank,t} \) is the net methane volume (ml) obtained from the substrate only, adjusted to the standard temperature (0°C) and pressure (1 atm) condition (STP); \( VS \) is the mass of substrate VS in the bottle (g).

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

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

Where \( P \) is the maximum specific cumulative \( H_2 \) production (ml \( H_2/g \) VS<sub>added</sub>); \( Rm \) is the maximum specific \( H_2 \) production rate (ml \( H_2/g \) VS<sub>added</sub>/h); \( \lambda \) is the lag phase (hr); \( t \) is the fermentation time (hr); and \( e \) is \( \exp (1) \). \( H_t \) represents cumulative specific \( H_2 \) production expressed as ml \( H_2/g \) VS<sub>added</sub> at a given time (\( t \)) at STP (0°C, 1 atm).

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

3. Results and Discussion

3.1. Impact of pretreatment on chemical composition of substrates

The cellulose, hemicellulose, and Klason lignin compositions of five substrates before and after hydrothermal pretreatment in the presence of \( CO_2 \) are presented in Figure 2. For raw substrates, summation of cellulose, hemicellulose and lignin accounted for 93% (wheat straw), 76% (sorghum), 86% (rice straw), 68% (corn stover), and 91% (Douglas fir bark) of the initial VS, suggesting that sorghum and corn stover contained higher amount of organics (i.e. proteins, lipids and different sugars), not quantified in the compositional
analysis than those of the other substrates. Sambusiti et al. (2013a) reported 9 ± 3% and
1.8 ± 0.3% of initial VS protein and fat, respectively, for *Sorghum sudanense* hybrid while
wheat straw had only 4 ± 1% protein and 0.9 ± 0.8% fat of the initial VS. Compared to
ranges reported in the literature (Baker, 1973; Taherzadeh and Karimi, 2007; Cherubini,
2010; Monlau et al., 2013a; Mirmohamadsadeghi et al., 2014), wheat straw (Lig: 26; Cell:
38; Hem: 30% VS), rice straw (Lig: 20; Cell: 37; Hem: 29% VS), corn stover (Lig: 14; Cell:
25; Hem: 18% VS), and Douglas fir bark (soft pinewood) (Lig: 28; Cell: 37; Hem: 25% VS)
yielded typical compositional analysis results. However for sorghum, literature varies
significantly based on the end-use of sorghum: biomass, forage, sorghum-sudangrass, and
sweet. Sambusiti et al. (2013b) compared different sorghums (seed sorghum stalks,
biomass sorghum, forage sorghum and three different sweet sorghums) and reported that
lignin, cellulose, and hemicellulose ranged in 19-21%, 18-29%, and 19-26% TS,
respectively. Interestingly, *Sorghum sudanense* hybrid grown in Lombardy region (Italy)
had only 4% VS lignin, while cellulose (49% VS) and hemicellulose (35% VS) were
significantly higher (Sambusiti et al., 2013b). Biomass 140 sorghum used in this study also
resulted in lower Klason lignin and higher cellulose and hemicellulose (Lig: 16; Cell: 33;
Hem: 27% VS) compared to biomass sorghum (Lig: 21; Cell: 23; Hem: 19% TS) in the study
of Monlau et al. (2012a). The results, on the other hand, were closer to another study that
compared 63 sweet sorghum collected worldwide and reported cellulose, hemicellulose,
and lignin ranges of 28-37%, 26-33%, and 17-23% by TS, respectively (Li et al., 2014).

During Stage I, hydrothermal pretreatment with CO$_2$ caused 4-18%, 8-11%, and 11-29%
reductions (relative to non-pretreated wheat straw) in lignin, cellulose, and hemicellulose
contents, respectively, with hemicelluloses being affected the most (Figure 2a). As
expected, the highest reduction in all three components was achieved at the most intensive pretreatment (175°C/66 bar/30 min). During Stage II, other substrates showed a similar behaviour with hemicellulose contents having the highest reductions, which were 28%, 16%, 23%, and 42% for sorghum, rice straw, corn stover, and Douglas fir bark, respectively, while delignification was limited to 0-12% (Figures 2b-e). These results are in agreement with other hydrothermal pretreatment studies (with/without CO₂ catalysis) reporting that pretreatment can affect the hemicellulose the most while lignin, being the most inert component, stays relatively intact or is slightly affected (Kaparaju and Felby, 2010; Relvas et al., 2015). When compared to other type of pretreatments, chemical pretreatments combined with low temperatures for 3-4 h appear to be more effective in delignification of lignocellulosic substrate than hydrothermal pretreatment. Thermo-alkaline pretreatment of wheat straw (Ca(OH)₂, 85°C for 3 h) was reported to solubilize lignin by 14% (Chang et al., 1998), while 77% of delignification, 95% cellulose yield and 44% of hemicellulose hydrolysis was reported for miscanthus pretreated with 12% NaOH, 70°C for 4 h (de Vrije et al., 2002). Similarly, ammonia recycle percolation (ARP) pretreatment achieved 75-85% (total lignin), and 50-60% (hemicellulose) removal in corn stover (Kim and Lee, 2005) and 65-85% delignification of switchgrass (Iyer et al., 1996).

In this study, it was difficult to differentiate the effects of temperature and pressure for Stage I (wheat straw), as these two factors are not independent once the temperature reaches elevated levels. When the pairs of non-pretreated wheat straw vs. the least intensive pretreatment condition (25°C/50 bar/30 min) and two most intensive conditions (175°C/25 bar/30 min vs. 175°C/66 bar/30 min) were compared in an attempt to understand the pressure effect only, 12% and 18% decreases in hemicellulose were
observed for increases from atmospheric to 50 bars (at 25°C) and from 25 to 66 bars (at 175°C), respectively. Temperature effect, on the other hand, was more pronounced with 21% decrease in hemicellulose content when temperature increased from 80 to 175°C (at 60-66 bars) but with no hemicellulose reduction after the increase from 26 to 80°C (at 50-60 bars) (i.e. 26.2 ± 1.1 versus 26.6 ± 1.8% hemicellulose in Figure 2a). When both temperature and pressure effects were combined, up to 29% reduction was observed in hemicellulose of non-pretreated wheat straw (Figure 2a).

3.2. Impact of pretreatment on biomass biodegradability

3.2.1. Methane production

The mesophilic BMP assays were monitored for 71-92 days for liquid fractions (Figures 3a and c) and 123-125 days for solid fractions (Figures 3b and d) of substrates. Non-pretreated substrates generated ultimate cumulative specific methane yields of 256 ± 18, 340 ± 2, 311 ± 5, 336 ± 11, 98 ± 5 ml CH₄/g VS added for wheat straw, sorghum, rice straw, corn stover and Douglas fir bark, respectively. As expected, for raw lignocellulosic substrates, low lignin and high hemicellulose/cellulose levels for sorghum and corn stover (Figure 2) corresponded to higher methane yields than the other substrates with Douglas fir bark being the most difficult to degrade. These results coincide with the literature values of 204-285 ml CH₄/g VS added for wheat straw (Menardo and Balsari, 2012; Sambusiti et al., 2013a), 260-362 ml CH₄/g VS added for sorghum (Bauer et al., 2010; Sambusiti et al., 2013a), and 270-290 ml CH₄/g VS added for rice straw (Lei et al., 2010), but higher than the yields of 280 ml CH₄/g VS added (Yu, 2010) and 440 ml biogas/g VS added (Qingming et al., 2005) for corn stalk/stover. The methane yield obtained for Douglas fir bark (98 ± 5 ml CH₄/g VS added) was also higher than those reported in the literature for non-pretreated pine
residues (i.e. 5-39 ml CH\textsubscript{4}/g VS\textsubscript{added}) (Matsakas et al., 2015; Mirmohamadsadeghi et al., 2014).

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

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

For Stage II, pretreated (166°C/76 bars/30 min) sorghum achieved the highest yield (275 ± 14 ml CH\textsubscript{4}/g COD\textsubscript{added}) of the liquid hydrolysate, followed by the pretreated (175°C/102 bars/30 min) corn stover (260 ± 14 ml CH\textsubscript{4}/g COD\textsubscript{added}) (Figure 3c). Despite the low methane yield obtained from the solid fraction of raw and pretreated Douglas fir (Figure 3d), the hydrothermally solubilized organics into the liquid phase achieved a similar biodegradability level (253 ± 5 ml CH\textsubscript{4}/g COD\textsubscript{added}) as the other substrates. Although pretreated hydrolyzates did not reach to the theoretical maximum methane yield of 350 ml/g COD\textsubscript{added} based on the COD equivalence of methane (Droste, 1997), the pretreatment conditions tested did not create any acute or chronic inhibition on the granular sludge used. Although, pH of liquid fractions of Douglas fir, corn stover, and sorghum were quite
acidic (3.51-4.85; Table 3), the addition of a buffer solution prevented acidification in the bottles and methane production started from Day 2.

3.2.2. Hydrogen production

The mesophilic BHP assays were monitored for 48 hours for the control (glucose), 68 hours for liquid fractions of sorghum and corn stover, and 112-119 hours for wheat and rice straws. The assay termination times were determined based on reaching the maximum hydrogen yield and the first decrease in hydrogen percentage (consumption) in bottle headspace. For the heat treated (90°C, 30 min) inoculum (activated sludge from Narbonne WWTP), the lag phases of H₂ production were 15, 24, 39, and 42 hours for control, sorghum, corn stover, and wheat/rice straws, respectively.

Among BHP assays, the ultimate specific hydrogen yield for the control (135 ± 25 ml H₂/g COD<sub>added</sub>; 1.2 ± 0.2 mol H₂/mol glucose<sub>added</sub>) was the highest, followed by the hydrolyzates of hydrothermally pretreated sorghum (55 ± 5 ml H₂/g COD<sub>added</sub>), corn stover (52 ± 6 ml H₂/g COD<sub>added</sub>), wheat straw (32 ± 4 ml H₂/g COD<sub>added</sub>), and rice straw (26 ± 2 ml H₂/g COD<sub>added</sub>). The maximum hydrogen yield of 1.2 ± 0.2 mol H₂/mol glucose from the control BHP was comparable to previous studies. Kawagoshi et al. (2005) reported 1.4 mol H₂/mol glucose (pH: 6.5-7, 20 g glucose/L) while Davila-Vazquez et al. (2008) observed 1.46 mol H₂/mol glucose (pH: 7.5, 5 g glucose/L). In other studies, lower yields were obtained (0.96 to 1.17 mol H₂/mol glucose by Salerno et al., 2006, and 1.75 mol H₂/mol glucose at pH: 6.0, 10 g glucose/L by Zheng and Yu, 2005).

In this study, pretreated (175°C/66 bars/30 min) wheat straw hydrolyzate achieved 287 ± 32 ml H₂/g sugars<sub>added</sub> at mesophilic (37°C) temperature, which is close to the maximum yield of 318 ± 5 ml H₂/g sugars of wheat straw hydrolyzate (at 5% by vol.) obtained from a
three-step pilot-scale hydrothermal pretreatment (presoaking at 80°C, extraction of hemicelluloses at 170-180°C, and improved enzymatic cellulose conversion at 195°C) under extreme thermophilic conditions (70°C) (Kongian et al., 2010). This value corresponds to 4.7 ± 0.3 ml H₂/g VS_initial (yield normalized based on initial straw VS in the HTP reactor) and is close to the lower end of the range reported for untreated wheat straw (5.18-10.52 ml H₂/g VS_initial) and lower than the values reported for wheat straw fermentation with enzyme addition (11.06-19.63 ml H₂/g VS_initial) (Quemeneur et al., 2012b). Similarly, the pretreated (165°C/76 bars/30 min) rice straw yield of 4.6 ± 0.5 ml H₂/g TS_initial was lower than the literature value of un-pretreated rice straw of 25 ml H₂/g TS_initial (Chen et al. 2012; Guo et al., 2014). In the literature, unpretreated corn stover/stalk yield is low (3 ml H₂/g VS_initial), but can be increased up to 57, 64, and 150 ml H₂/g VS_initial after pretreatments with 0.5% NaOH, high pressure stream at 1.6 MPa/5 min, and 0.2% HCl/30 min boiling, respectively (Zhang et al., 2007; Lu et al., 2009). The HTP pretreated (175°C/102 bars/30 min) corn stover yield of 17.1 ± 5.4 ml H₂/g TS_initial again falls into the lower end of this range. Finally, a maximum yield of 10.4 ml H₂/g sorghum was reported for sweet sorghum liquid extract (obtained by mixing milled sorghum with tap water at 30°C for 1 h) (Antonopoulou et al., 2008), comparable to the hydrothermally pretreated (166°C/90 bars/30 min) sorghum B140 (18.5 ± 1.7 ml H₂/g sorghum; 21.1 ± 1.9 ml H₂/g TS_initial) in this study. Given the aforementioned variability with sorghum, literature has a wider range of data (9.7-64 ml H₂/g TS_initial) from different types of sorghum (Monlau et al., 2012a; Guo et al., 2014). Overall, when compared to literature, the results indicate that HTP pretreatments (165-175°C/66-102 bar/30 min) applied to various substrates did not achieve discernable improvements in the extent of hydrogen production. However,
observed yields are substrate, pretreatment and BHP assay condition specific, therefore, direct comparisons are not easy to make.

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

After BHP assays were terminated at their highest point of cumulative hydrogen yield, soluble metabolites were quantified in order to understand the fermentation pathways. The results, presented in Figure 4, suggested that in all the bottles set up with pretreated hydrolyzate, acetate and butyrate were the main metabolites indicating that hydrogen production was mainly from the typical acetate-butyrate pathway for dark fermentation of carbohydrates (Monlau et al., 2013c; Chandrasekhar et al., 2015). Furthermore, ethanol was also detected in the control BHP with glucose (5 g COD/l) and BHP with sorghum at higher levels (0.7-0.9 g/l) than the other assays (0.1-0.2 g/l), suggesting a more
pronounced population shift to solvent (non-hydrogen) production such as ethanol from hydrogen production in these BHP assays than the others. Metabolite results in Figure 4 also suggest that readily biodegradable sugars quantified in hydrolyzate samples (Table 3) were all consumed.

3.2.3. Biodegradation kinetics

Kinetic parameter estimation results are tabulated for raw and hydrothermally pretreated substrates in Table 4 for BMP assays and in Tables 5 for hydrolyzate BHP assays. For comparison with raw substrates, specific BMP results from liquid and solid phases of pretreated substrates were added together (based on VS distribution between liquid and solid fractions after pretreatment) prior to parameter estimation analysis. As it can be seen in Table 4, the first-order kinetics was successful in predicting specific cumulative BMP’s with the squared correlation coefficient, $R^2$, generally close to unity, being greater than 0.97. For visual observation, predicted BMPs were also plotted along with the observed values (Supplementary data; Figure S1). It is important to emphasize that improvements by hydrothermal pretreatment was in terms of hydrolysis rate rather than the extent of the methane production for all the substrates except Douglas fir bark. For wheat/rice straws, sorghum and corn stover, hydrothermal pretreatments (165-175°C/66-102 bars/30 min) with CO$_2$ addition increased the hydrolysis constant by 20-30% (relative to controls). However, for Douglas fir bark, both the rate as well as the extent of digestion was significantly enhanced by the pretreatment with 172% faster hydrolysis rate compared to its control (Table 4), as the control digester was challenged as a result of the highly refractory nature of this biomass. In general, these increases in $k$ constant represent lower improvements compared to thermochemical pretreatments, which can be as high as 65%
and 163% for sorghum and wheat straw, respectively, at a pretreatment combination of 10% NaOH, 40°C for 24 h (Sambusiti et al., 2012b). Similarly, the modified Gompertz equation represented the measured BHP data successfully with $R^2$ values higher than 0.92 (Table 5; Figure S2).

3.2.4. Energy yield from possible digestion scenarios

For the pretreatment conditions with available data on both CH$_4$ and H$_2$ yields, the total produced energy from two possible configurations (one-stage CH$_4$ and two-stage H$_2$/CH$_4$) are compared in Table 6. In the one-stage CH$_4$ option, both liquid and solid fractions contribute to CH$_4$ generation, while in the two-stage H$_2$/CH$_4$, liquid fraction is sent to dark fermentation first for H$_2$ generation and then metabolites of dark fermentation is treated for CH$_4$ production along with the solid fraction. CH$_4$ potential from metabolites (Fig. 4) was calculated based on their theoretical methane potential. As it can be seen from Table 6, the rest of the pretreatment conditions achieved similar energy yields between the two scenarios except for the sorghum with high H$_2$ potential from readily biodegradable sugars and CH$_4$ potential from dark fermentation by-products giving an advantage to the two-stage H$_2$/CH$_4$. Although the hydrogen yields from the first-stage were low compared to literature, total energy obtained from both configurations are in the range reported for similar substrates compiled in a review (Monlau et al., 2013c).

Conclusions

Hydrothermal pretreatment (26-175°C, 25-102 bars, with CO$_2$ as catalyst) of various lignocellulosic substrates decreased the hemicellulose content by 23-42% while delignification was limited to 0-12%. The pretreatment was able to accelerate the rate of biodegradation by 20-172% without generating high levels of inhibitory compounds. There
was no discernable enhancement in the ultimate methane or hydrogen yield observed except for the most refractory biomass (Douglas fir bark). Between two possible reactor configurations after pretreatment (one-stage CH₄ and two-stage H₂/CH₄), straws and corn stover achieved similar energy yields (9.5-11.7 MJ/kg) while sorghum with high sugars/H₂ in a two-stage H₂/CH₄ achieved 41% higher energy yield than the one-stage CH₄ process. Compared to other lignocellulosic biomass pretreatment techniques, hydrothermal pretreatment with CO₂ pressurization requires only heat energy and CO₂, which is easily available on fermentation or biogas plants and is easily released by depressurization at the end of the pretreatment. One of its considerable advantages is the low level of inhibitory substances produced. Its industrial application may be relevant in the case of refractory biomass such as Douglas fir bark, allowing the use of some substrates whose adaptation to biogas plants would not have been possible without such pretreatment. Further work should thus optimize these pretreatment conditions in the case of refractory biomass, and carry out a life cycle analysis to assess environmental impacts in comparison to more conventional pretreatment techniques.

Acknowledgements
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References


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

*Data represent arithmetic mean and standard deviation of triplicates
Table 2. Hydrothermal pretreatment conditions for lignocellulosic substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sample code</th>
<th>Maximum observed</th>
<th>Heating time (min)</th>
<th>Initial CO₂ pressure (bar)</th>
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<tr>
<td></td>
<td></td>
<td>T (°C)</td>
<td>P (bar)</td>
<td></td>
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<tr>
<td><strong>Stage I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>WS (26C/50b)</td>
<td>26</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>WS (80C/60b)</td>
<td>80</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>WS (175C/25b)</td>
<td>175</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>WS (175C/66b)</td>
<td>175</td>
<td>66</td>
<td>30</td>
</tr>
<tr>
<td><strong>Stage II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorghum (B 140)</td>
<td>S (166C/90b)</td>
<td>166</td>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>Rice straw</td>
<td>RS (165C/76b)</td>
<td>165</td>
<td>76</td>
<td>30</td>
</tr>
<tr>
<td>Corn stover</td>
<td>CS (175C/102b)</td>
<td>175</td>
<td>102</td>
<td>30</td>
</tr>
<tr>
<td>Douglas fir bark</td>
<td>DFB (171C/86b)</td>
<td>171</td>
<td>86</td>
<td>30</td>
</tr>
</tbody>
</table>

*a* Maximum temperature reached during 30 minutes of pretreatment  
*b* Maximum pressure reached during 30 minutes of pretreatment  
*c* Total exposure time to pretreatment  
*d* CO₂ 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

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

\(^a\)WS: wheat straw; S: sorghum; RS: rice straw; CS: corn stover; DFB: Douglas fir bark; 5-HMF: 5-hydroxymethylfurfural

\(^b\)Data represent arithmetic mean and standard deviation of triplicates for COD, poly-phenols, VFAs and duplicates for TS/VS and sugar analyses

\(^c\)Calculated from COD equivalency of sugars in the liquid phase

\(^d\)Total volatile fatty acids (VFAs) as summation of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric acids

\(^*\)nd: not detected
<table>
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<tr>
<th>Conditions</th>
<th>BMP measured (ml CH₄/g VS&lt;sub&gt;added&lt;/sub&gt;)</th>
<th>% from liquid fraction</th>
<th>BMP predicted (ml CH₄/g VS&lt;sub&gt;added&lt;/sub&gt;)</th>
<th>k (1/d)</th>
<th>R²</th>
<th>k increase (%)</th>
</tr>
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<td>Raw WS</td>
<td>256 (18)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>252 (9)</td>
<td>0.067 (0.002)</td>
<td>0.99 (0.00)</td>
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<tr>
<td>WS (175C/66b)</td>
<td>269 (3)</td>
<td>20 (1)</td>
<td>259 (3)</td>
<td>0.082 (0.010)</td>
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<tr>
<td>Raw S</td>
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<td>-</td>
<td>318 (2)</td>
<td>0.090 (0.002)</td>
<td>0.99 (0.00)</td>
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<tr>
<td>S (166C/90b)</td>
<td>338 (4)</td>
<td>36 (2)</td>
<td>321 (5)</td>
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<tr>
<td>Raw RS</td>
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<td>-</td>
<td>304 (12)</td>
<td>0.071 (0.002)</td>
<td>0.99 (0.00)</td>
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<tr>
<td>RS (165C/76b)</td>
<td>319 (6)</td>
<td>13 (1)</td>
<td>300 (5)</td>
<td>0.093 (0.010)</td>
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<tr>
<td>Raw CS</td>
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<td>-</td>
<td>336 (11)</td>
<td>0.090 (0.004)</td>
<td>0.99 (0.00)</td>
<td>-</td>
</tr>
<tr>
<td>CS (175C/102b)</td>
<td>318 (11)</td>
<td>28 (1)</td>
<td>300 (10)</td>
<td>0.108 (0.002)</td>
<td>0.99 (0.01)</td>
<td>20</td>
</tr>
<tr>
<td>Raw DFB</td>
<td>98 (5)</td>
<td>-</td>
<td>96 (9)</td>
<td>0.021 (0.001)</td>
<td>0.99 (0.00)</td>
<td>-</td>
</tr>
<tr>
<td>DFB (171C/86b)</td>
<td>136 (3)</td>
<td>32 (1)</td>
<td>124 (1)</td>
<td>0.056 (0.005)</td>
<td>0.97 (0.03)</td>
<td>172</td>
</tr>
</tbody>
</table>

<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>
<thead>
<tr>
<th>Conditions</th>
<th>BHP measured (ml H₂/g VS&lt;sub&gt;added&lt;/sub&gt;)</th>
<th>BHP predicted (ml H₂/g VS&lt;sub&gt;added&lt;/sub&gt;)</th>
<th>P (ml H₂/g VS&lt;sub&gt;added&lt;/sub&gt;)</th>
<th>R&lt;sub&gt;m&lt;/sub&gt; (ml H₂/g VS&lt;sub&gt;added/h&lt;/sub&gt;)</th>
<th>λ (h)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS (175C/66b)</td>
<td>55 (6)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55 (6)</td>
<td>56 (5)</td>
<td>4.7 (1.3)</td>
<td>38 (3)</td>
<td>1.00 (0.00)</td>
</tr>
<tr>
<td>S (166C/90b)</td>
<td>94 (9)</td>
<td>84 (7)</td>
<td>84 (15)</td>
<td>42.5 (13.6)</td>
<td>29 (7)</td>
<td>0.95 (0.06)</td>
</tr>
<tr>
<td>RS (165C/76b)</td>
<td>39 (3)</td>
<td>40 (1)</td>
<td>45 (2)</td>
<td>1.3 (0.2)</td>
<td>27 (9)</td>
<td>0.98 (0.02)</td>
</tr>
<tr>
<td>CS (175C/102b)</td>
<td>84 (9)</td>
<td>74 (18)</td>
<td>94 (44)</td>
<td>23.0 (16.2)</td>
<td>35 (4)</td>
<td>0.92 (0.09)</td>
</tr>
<tr>
<td>Control (glucose)</td>
<td>143 (28)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>143 (27)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>143 (27)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2 (4.7)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14 (5)</td>
<td>1.00 (0.00)</td>
</tr>
<tr>
<td>Control (glucose)</td>
<td>1.2 (0.2)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.2 (0.2)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.2 (0.2)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.2 (0.1)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>14 (1)</td>
<td>1.00 (0.00)</td>
</tr>
</tbody>
</table>

<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 H₂/g glucose added,
<sup>d</sup>ml H₂/g glucose/h,
<sup>e</sup>mol H₂/mol initial glucose,
<sup>f</sup>mol H₂/mol initial glucose/h.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>One-stage CH(_4)</th>
<th></th>
<th></th>
<th>Two-stage H(_2)/CH(_4)</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml CH(<em>4)/g TS(</em>{\text{initial}})</td>
<td>MJ/kg TS(_{\text{initial}})</td>
<td>ml H(<em>2)/g TS(</em>{\text{initial}})</td>
<td>MJ/kg TS(_{\text{initial}})</td>
<td>ml CH(<em>4)/g TS(</em>{\text{initial}})</td>
<td>MJ/kg TS(_{\text{initial}})</td>
<td></td>
</tr>
<tr>
<td>WS (175C/66b)</td>
<td>252 (3)(^c)</td>
<td>10.1 (0.1)</td>
<td>4.4 (0.3)</td>
<td>0.05 (0.00)</td>
<td>234 (4)</td>
<td>9.3 (0.1)</td>
<td>9.4 (0.1)</td>
</tr>
<tr>
<td>S (166C/90b)</td>
<td>313 (5)</td>
<td>12.5 (0.2)</td>
<td>21.1 (11.9)</td>
<td>0.23 (0.02)</td>
<td>438 (8)</td>
<td>17.4 (0.3)</td>
<td>17.6 (0.3)</td>
</tr>
<tr>
<td>RS (165C/76b)</td>
<td>277 (6)</td>
<td>11.0 (0.2)</td>
<td>4.6 (0.5)</td>
<td>0.05 (0.01)</td>
<td>263 (5)</td>
<td>10.5 (0.2)</td>
<td>10.5 (0.2)</td>
</tr>
<tr>
<td>CS (175C/102b)</td>
<td>295 (9)</td>
<td>11.7 (0.4)</td>
<td>17.1 (5.4)</td>
<td>0.18 (0.06)</td>
<td>262 (6)</td>
<td>10.4 (0.2)</td>
<td>10.6 (0.2)</td>
</tr>
</tbody>
</table>

\(^a\)WS: wheat straw; S: sorghum; RS: rice straw; CS: corn stover.
\(^b\)Data represent arithmetic mean and standard deviation of 3-5 replicate bottles.
\(^c\)Energy yield: 1Nl CH\(_4\) = 39790 J
\(^d\)Energy yield: 1Nl H\(_2\) = 10780 J
Figure 1. Schematic of experimental procedure
Hydrothermal pretreatment conditions

a) Lignin, Cellulose, Hemicellulose

% of initial VS by wt.

b) Lignin, Cellulose, Hemicellulose

% of initial VS by wt.
Hydrothermal pretreatment conditions

- **c)** RS and RS (165C/76b)
  - Lignin, Cellulose, Hemicellulose
  - % of initial VS by wt.

- **d)** CS and CS (175C/102b)
  - Lignin, Cellulose, Hemicellulose
  - % of initial VS by wt.
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.
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.