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Case Study

Liquid hot water pretreatment of multi feedstocks and enzymatic hydrolysis of solids obtained thereof

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HIGHLIGHTS

- Chemical composition showed different susceptibility of multi feedstocks to LHW.
- LHW improved thermal properties and structural characteristics of multi feedstocks.
- The crystallinity degree of multi feedstocks increased after LHW-pretreatment.
- Cellulose conversion to glucose rate was higher in brewers' spent grain and corn husk.

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ABSTRACT

Agricultural feedstocks (brewers' spent grain – BSG, corncob – CC, corn husk – CH, wheat straw – WS and Luffa sponge – LS) were pretreated by liquid hot water (LHW) in order to increase cellulose recovery and enzymatic saccharification. LHW-pretreatment resulted in hemicellulose solubilization, and solids enriched in cellulose. Chemical analysis showed different susceptibilities of the feedstocks to LHW-pretreatment and enzymatic hydrolysis. Pretreated feedstocks presented higher crystallinity (determined through X-ray diffraction) and thermal stability (determined through thermogravimetric analysis) than untreated feedstocks. SEM images confirmed the effect of LHW-pretreatment on structural changes. Moreover, enzymatic hydrolysis and cellulose conversion to glucose (CCG) were improved for pretreated feedstocks, with exception of LS. CCG (in relation to glucose potential on solids) followed the order: BSG > CH > WS > CC > LS. LHW-pretreatment showed to be a good technology to pretreat multi feedstocks and for improving the enzymatic hydrolysis of recalcitrant agricultural feedstocks to sugars, which can be further converted to ethanol-fuel and other value-added chemicals.

1. Introduction

Lignocellulosic materials (LCMs), including agricultural residues and agro-industrial by-products, are abundant alternative feedstocks for the production of value-added products. An efficient approach for LCM processing/utilization is the "biomass refinery" philosophy (Myerly et al., 1981), where biomass can be processed to obtain its constituents (i.e. cellulose, hemicelluloses, and lignin) in separate fractions for individual valorization; e.g. cellulose can be hydrolyzed to glucose that is easily fermented to ethanol-fuel and other compounds, and alternatively xylose from hemicellulose can be converted to xylitol.

However, the use of LCMs for this purpose presents some drawbacks such as their chemical composition that varies according to the feedstock, climate and soil conditions, and their robust structure. To overcome this barrier, the development of a biorefinery that can operate using several feedstocks, together with the use of green pretreatment technologies have been presented as a great challenge (Imman et al., 2013). In this sense, several technologies have been evaluated to pretreat LCMs. Liquid hot water (LHW), also known as autohydrolysis, is considered one of the most promising pretreatment strategies due to its environmental friendly feature, its high efficiency and low cost (Carvalheiro et al., 2005). It is a hydrothermal process that treats the LCM in a water-only media at high temperatures (160–240 °C) and pressure. Under these conditions, hydronium ions are generated *in situ* by ionization of water, leading to the release of acetic acid from hemicelluloses. This last one in turn auto-catalyzes the hydrolysis of hemicellulose, resulting in an increased accessibility to cellulose while avoiding the accumulation of inhibitory by-products (Imman et al., 2013; Han et al., 2015).

The amount of degradation products generated in LHW is largely driven by the severity (i.e. severity factor, log R_0) of the

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reaction, proposed by Overend and Chornet (1987). Garrote et al. (2003) suggested the use of relatively mild temperatures (between 181 and 196 °C) to avoid excessive depolymerization of the cellulose after the extraction of xylo-oligomers, xylose, and furfural derivatives. Moreover, Michelin et al. (2016) showed that a more severe LHW-pretreatment condition improved the hemicellulose removal, however, the use of this condition promoted the formation of inhibitors, leading to lower enzymatic efficiency during the conversion process of cellulose to glucose. Also, Imman et al. (2013) found that LHW-pretreatment at a severity factor (log R_0) ranging from 3.64 to 4.25 resulted in high levels of hemicellulose solubilization and marked improvement on enzymatic hydrolysis of the solid cellulose-enriched residues. Based on these, the condition of 190 °C for 30 min (log R_0 = 4.13) was chosen to this study, since it is considered a mild condition that fits on the desirable requirements for this pretreatment (i.e. high hemicellulose solubilization, and low cellulose depolymerization and inhibitor formation). Thus, the aim of this work was to study different feedstocks, namely brewerś spent grain, corncob, corn husk, wheat straw and Luffa sponge, using a common pretreatment technology (i.e. LHW) at a specific condition (i.e. 190 °C, 30 min) to compare the effect of LHW-pretreatment on the modification of physicochemical properties and the accessibility of pretreated feedstocks to enzymatic saccharification.

2. Materials and methods

2.1. Materials

Corncob (CC), corn husk (CH) and wheat straw (WS) were obtained from a local farmer in the North of Portugal. Brewer's spent grain (BSG) was kindly provided by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal). Luffa sponge (LS) was purchased in the local market (Braga, Portugal). These feedstocks were dried at 40 °C for 12 h. After that, they were ground and sieved to particles sizes from 1 to 5 mm, and stored at room temperature.

Cellic® Ctec2 and NS 22083 were obtained from Novozymes (Bagsvaerd, Denmark). Whatman® filter paper grade 1 (Whatman International Ltd, England), Beechwood xylan (⩾90% xylose), ρ-nitrophenyl-β-D-glucopyranoside (⩾99% purity) and ρ-nitrophenyl β-D-xylopyranoside (⩾98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Liquid hot water pretreatment

Pretreatment was performed in a stainless steel cylindrical reactor (4.0 cm internal diameter and 12.4 cm internal height) with a working volume of 50 mL. The feedstocks were pretreated at 190 °C for 30 min (log R_0 = 4.13), while solids loading rate was fixed at 10% (w/v). The reactor was immersed in an oil bath, with an open heating circulator (Julabo Labortechnik GmbH, Seelbath, Germany) with PID temperature control and maintained for 30 min at 190 °C. After that, the reactor was immediately cooled in an ice bath to quench the reaction. The insoluble solids were separated from the liquid fraction (slurry) by filtration (filter paper) and then washed with three volumes of 100 mL distilled water and dried at 40 °C. The liquid fraction was stored until further use at −20 °C.

2.3. Compositional analysis

Aliquots of the solid material (untreated and pretreated feedstocks) were milled to particle size < 0.5 mm and subjected to chemical composition. Approximately, 0.3 g of material was hydrolyzed with 3 ml of 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by a quantitative post-hydrolysis with 4% sulfuric acid (adding 84 g Milli-Q water) at 121 °C during 60 min. The monosaccharide sugars (glucose, xylose and arabinose) and acetic acid contained in the hydrolysates were determined by HPLC. The solid material after hydrolysis was recovered by filtration and considered as Klason lignin after being dried at 105 °C. For the determination of ash content, the solid material was taken in a crucible and kept in a muffle furnace at 750 °C for 8 h.

The slurry from pretreated materials was analyzed for monomeric sugars, acetic acid, oligomeric sugars, acetyl groups and degradation products (5-hydroxymethyl-2-furaldehyde (HMF) and furfural). The oligomeric sugars were calculated after a quantitative posthydrolysis with 4% sulfuric acid at 121 °C, during 60 min. The increase of monosaccharides (glucose, xylose and arabinose) and acetic acid concentrations caused by posthydrolysis provided a measure of the concentrations of oligomers and acetyl groups bound to oligosaccharides. These components were analyzed by High-performance liquid chromatography (HPLC) as described below. All measurements were made in duplicate.

2.4. HPLC analysis

The samples were filtered through a 0.45 μm syringe filters and automatically injected (JASCO Intelligent Sampler AS 2057 Plus) through a Metacarb 87H column (300 × 7.8 mm, Varian, USA) preheated to 60 °C by a thermostatted column compartment (Chrompack Instruments AG, Neuheim, Switzerland). The mobile phase (0.005 M H_2SO_4 in Milli-Q water filtered through 0.2 μm Millipore® nylon filter and degassed) was pumped at a flow rate of 0.6 mL·min^{-1} through a JASCO 880 PU pump. Sugars and acetic acid were analyzed with a refractive index (RI) detector and furfural and hydroxymethylfurfural (HMF) with a UV detector.

2.5. Thermogravimetric analysis (TGA)

TGA was performed using a thermogravimetric analyzer 4000 (Perkin Elmer, Instrumentos de Laboratório e Científicos, Lda, Portugal). Approximately 10 mg of untreated and pretreated feedstocks were loaded in ceramic pan. TG scans were conducted in a temperature range from 20 °C to 600 °C at a heating rate of 20 °C·min^{-1}, under nitrogen atmosphere of 20 mL·min^{-1}. The weight loss of the samples was recorded as a function of temperature and characterized by a TG curve. The derivative thermogravimetric (DTG) curve was used to emphasize the temperature zone where each phenomenon occurred.

2.6. Crystallinity measurement

Crystallinity of untreated and pretreated feedstocks was measured by X-ray diffraction (XRD), using a Buker D8 Discover diffractometer equipped with Ni filtered Cu-K_β radiation source of 40 kV and 40 mA. Samples were scanned in the range of 5–40° (2θ), (10° min^{-1}), with a step size of 0.02° and step time of 1 s under room temperature. The crystallinity index (*CrI*) was determined according to Segal et al. (1959), using the following equation:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (1)$$

where I_{002} is the intensity of maximum diffraction of crystalline region (i.e., cellulose) at about 2θ = 22.5°, and I_{am} is the intensity of diffraction attributed to the amorphous region at about 2θ = 18°.

2.7. Scanning electron microscopy (SEM)

The morphology of the untreated and LHW-pretreated feedstocks was analyzed by scanning electron microscopy (SEM) using a NanoSEM – FEI Nova 200 (FEG/SEM) equipped with EDAX – Pegasus X4M (EDS/EBSD). Dry samples were affixed on aluminum stubs covered by carbon ribbon, and then the samples were coated with gold palladium and observed using a voltage of 10 kV in vacuum mode. Images of surfaces of native and pretreated LCMs were taken at magnification of 2500×.

2.8. Enzyme activity

Polysaccharides degrading activities were analyzed by the 3,5-dinitrosalisylic acid (DNS) method (Miller, 1959). The cellulase activity from Cellic Ctec2 was determined at 50 °C for 60 min, using filter paper as substrate (Ghose, 1987) and expressed as Filter Paper Unit (FPU). Xylanase activity from NS 22083 was determined at 50 °C for 15 min, using 1% (w/v) Beechwood xylan, as substrate. The international unit (IU) of enzymatic activity was defined as the amount of enzyme that releases 1 μmol of product per min under the assay conditions.

The β-glucosidase activity from Cellic Ctec2 and the β-xylosidase activity from NS 22083 were determined at 50 °C for 10 min, by monitoring the hydrolysis of 10 mM *ρ*-nitrophenol-β-D-glucopyranoside (PNP-glu) and *ρ*-nitrophenol-β-D-xylopyranoside (PNP-xyl), respectively. The reaction was stopped with 1 M sodium carbonate. The released product was quantified using *ρ*-nitrophenol as standard and the activity expressed as International Unit (IU). All substrates were suspended in 50 mM sodium citrate buffer, pH 4.8.

The protein content of the Cellic Ctec2 and NS 22083 enzymes was determined according to Lowry et al. (1951), using bovine serum albumin as standard. All assays were done in triplicate.

Cellic Ctec2 and NS 22083 presented 180 mg protein·mL^{-1} (160 FPU·mL^{-1} of cellulase and 2300 IU·mL^{-1} of β-glucosidase), and 200 mg protein·mL^{-1} (2800 IU·mL^{-1} of xylanase and 135 IU·mL^{-1} of β-xylosidase), respectively.

2.9. Enzymatic hydrolysis

Enzymatic hydrolysis (EH) experiments were performed using a commercial enzymes blend: Cellic Ctec2 and NS 22083. Supplementation with β-glucosidase was not necessary due to the high activity of this enzyme in Cellic Ctec2. Cellic Ctec2 has been considered the *state-of-the-art* enzyme, once it has proven to be effective on a wide variety of pretreated LCMs and at high solids concentration. Besides, it has shown a high conversion yield, tolerance to inhibitors, among others.

Although Cellic Ctec2 presents hemicellulase activity in its composition, NS 22083 xylanase was added to improve the cellulose saccharification, since previous results have shown that the addition of hemicellulases (i.e. NS 22083 xylanase) has improved the cellulose saccharification. Besides, according to Cellic Ctec2 Novozymes protocol, if the pretreated feedstock of interest contains an appreciable amount of hemicellulose, it is advised to combine Cellic CTec2 and Novozymes hemicellulases to boost the cellulose hydrolysis.

EH experiments of 1-mL total volume were carried out at 5% (w/v) pretreated solids loading rate, and 15 FPU·g^{-1} dry solids of Cellic Ctec2 (16.9 mg protein·g^{-1} solids) plus 15 IU·g^{-1} dry solids of NS 22083 xylanase (1.1 mg protein·g^{-1} solids), both dissolved in 50 mM sodium citrate buffer, pH 4.8. These conditions were selected based on previous works (Silva et al., 2010; Amores et al., 2013). Untreated feedstocks were used as control to analyze the efficiency of the LHW-pretreatment. EH runs were carried out in a thermostatically controlled orbital shaker at 350 rpm and 50 °C for 72 h. Samples were centrifuged at 14,000 rpm for 5 min and filtered through a 0.2 μm syringe filter. Released glucose was quantified by GOPOD format assay kit (Megazyme, Wicklow, Ireland). All runs were performed in triplicate.The cellulose conversion to glucose (CCG, %) from the studied feedstocks (g glucose per 100 g glucan) was calculated using the following equation:

$$CCG = C_G \times \frac{V}{m \times C_{Gn}} \times 100 \quad (2)$$

where C_G is the glucose concentration (g·L^{-1}) released in the EH assay, V is the volume (L) employed in the experiment, m is the mass of solids employed in the experiment and C_{Gn} is the glucan content of the solids employed in the experiment.

3. Results and discussion

3.1. Chemical composition of the raw feedstocks

The differences on physical properties and chemical characteristics of the feedstocks influence their use as LCM. In this context, it is important to determine their initial chemical composition and evaluate changes after pretreatment. Table 1 presents the chemical composition of the raw feedstocks in terms of their main constituents (i.e. cellulose, hemicellulose and lignin).

BSG is a residue that remains of the beer wort preparation process and consists of a complex mixture of barley grain husk, pericarp, and fragments of endosperm (Forssell et al., 2008). BSG presented the highest lignin content (20.4%), and the lowest cellulose content (16.5%) of the studied feedstocks. This chemical composition is in agreement with other published works (Carvalheiro et al., 2005; Mussatto and Roberto, 2005). It is important to highlight that its composition may vary according to the operating conditions used during the harvest, the barley variety, malting, and mashing conditions (Muthusamy, 2014). It is also, from the studied materials, the one that presents more unknown constituents (i.e. others, please see Table 1), which can be related with the presence of proteins and other polysaccharides. This is in agreement with other authors that have shown that BSG is rich in proteins. Carvalheiro et al. (2005) reported protein values of around 24%, while Mussatto and Roberto (2005) reported 15.25% and Pires et al. (2012) reported almost 40% of proteins on BSG.

CC and CH are byproducts from corn grain production. While CC exhibits a highly fibrillar, ordered and rigid structure with a relatively smooth surface (Zheng et al., 2014), CH presents cellulose fibers with a structure considered too short and/or weak (Reddy

Table 1
Chemical composition of the raw feedstocks, expressed as percentage by dry material weight.

Components	Composition (%)				
	Brewers' spent grain	Corn husk	Corncob	Wheat straw	Luffa sponge
Cellulose[a]	16.50	32.50	35.75	34.00	55.00
Hemicellulose					
Xylan	16.75	21.10	22.40	16.30	13.60
Arabinan	8.80	6.30	4.85	4.75	1.65
Acetyl group	0.75	3.00	3.45	2.10	0.15
Klason lignin	20.40	15.50	18.50	20.20	14.20
Ashes	2.10	2.00	0.80	2.55	1.10
Others[b]	34.70	19.60	14.25	20.10	14.30

[a] Estimated from glucan content.
[b] Calculated by difference (includes non-analyzed components, considered of minor importance for this study, such as extractives, protein or acid-soluble lignin).

and Yang, 2007). These LCMs are considered a potential feedstock for cellulosic ethanol production due to their low lignin and high carbohydrate contents. These were confirmed by the obtained results, with CC presenting 35.8% cellulose, 30.7% hemicellulose and a lignin content of 18.5%, while CH presented 32.5% cellulose, 30.4% hemicellulose and 15.5% lignin. These values are in agreement with other reports (Nabarlatz et al., 2007; Michelin et al., 2012; Shankarappa and Geeta, 2013). CC and CH presented the highest hemicellulose contents among all studied feedstocks, while the highest cellulose content (55.0%) and the lowest lignin content (14.2%) were found for LS. This sponge gourd, fruit of *Luffa cylindrica*, has a fibrous vascular system like a multi directional network, which is composed of fibrils joined with natural resinous materials of plant tissue (Ghali et al., 2009). According to Akgul et al. (2013), LS cellulose content varies from 55% to 90%, the hemicellulose content ranges between 8% and 22%, and the lignin content is within 10 and 23%.

Wheat straw is one of the most abundant agricultural feedstocks, presenting a low commercial value. Most of it is used for cattle feed or considered waste (Zahoor and Tu, 2014). Its chemical composition may vary according to the wheat variety and the culture conditions. Studied WS presented 34.0% cellulose, 23.2% hemicellulose and 20.2% lignin (Table 1). Carvalheiro et al. (2009) and Han et al. (2015) reported similar chemical composition for wheat straw.

The differences between these feedstocks make them useful for the comparison of the effects of LHW-pretreatment on their compositions.

3.2. Effect of LHW on studied feedstocks

The composition of the feedstock and its transformation after the pretreatment are the main focus of the LCM conversion process (Liu and Chen, 2015). Table 2 shows the amount of cellulose, hemicellulose, and Klason lignin recovery on pretreated feedstocks. As expected, LHW removed a large fraction of hemicellulose from raw feedstocks, confirmed by the observed decrease in its content in the solid fractions. The dissolved and/or degraded hemicellulose can also be confirmed through the hemicellulose content in the hydrolysates (Table 2). BSG and CC presented a hemicellulose extraction yield of 64.1 and 74.1%, respectively (based on xylan solubilization), while for CH and WS, the obtained values were 42.0 and 39.3%, respectively. LS presented the lowest hemicellulose extraction yield (18.4%).

On the other hand, the cellulose and lignin contents increased after the pretreatment, as a result of the removal of hemicelluloses. This behavior is typical for hydrothermal processes and has been reported by Gullón et al. (2010) and Liu and Chen (2015) for rye straw and corn stover, respectively.

Regarding hydrolysates constituents, low amounts of glucose (glucose bound to oligomers as well as free glucose) were detected. It is important to highlight that glucose could be derived from hemicellulose or from a small part of the cellulose that was depolymerized; indicating in the last case, a limited hydrolysis of the cellulose fraction under the experimental pretreatment condition. This is a desirable feature of the studied process, which seeks a selective fractionation of the raw material. Other feature of LHW-pretreatment process was the release of oligosaccharides on hydrolysates (Table 2). The release of more oligosaccharides than monosaccharides could be related to the pH of the sample. Pretreatments at low pH hydrolyze most of hemicellulose into monomers, whereas pretreatments at nearly neutral pH produce mostly oligosaccharides with some monomers (Mosier et al., 2005). Besides, low levels of sugar degradation products, such as furfural and HMF, were detected on hydrolysates of all feedstocks, which is in agreement with a previous report (Mosier et al., 2005). Furfural

Table 2
Chemical compositions of solids and hydrolysates of the feedstocks after the LHW-pretreatment.

Components	Composition				
	Brewers' spent grain	Corn husk	Corncob	Wheat straw	Luffa sponge
Pretreated solids (%)					
Cellulose[a]	26.55	42.75	50.00	52.15	70.80
Hemicellulose					
Xylan	7.50	16.00	14.40	11.60	9.85
Arabinan	2.75	2.65	2.15	1.60	0.10
Acetyl group	0.25	1.85	2.15	1.55	n.d.
Klason lignin	32.60	18.55	20.00	25.20	16.65
Others[b]	30.35	18.20	10.30	7.90	2.60
Hydrolysates (g/L)					
Oligosaccharides					
Gluco-oligosaccharides	5.65	1.65	3.10	1.50	0.70
Xylo-oligosaccharides	10.10	8.10	15.50	6.00	2.45
Arabino-oligosaccharides	3.55	1.85	1.90	0.75	0.45
Acetyl groups-oligosaccharides	0.15	1.30	1.80	0.50	n.d
Monosaccharides					
Glucose	0.11	0.40	0.45	0.15	0.10
Xylose	0.63	0.76	1.10	0.40	0.05
Arabinose	2.97	0.90	1.50	1.65	0.10
Acetic acid	0.26	0.78	0.85	0.65	0.15
Degradation products					
HMF	0.07	0.05	0.08	0.07	0.03
Furfural	0.17	0.12	0.22	0.12	0.05
Hemicellulose extraction yield (%)[c]	64.06	42.00	74.11	39.26	18.38

n.d.: Not detected.
[a] Estimated from glucan content.
[b] Calculated by difference.
[c] Based on xylan solubilization.

obtained from the dehydration of pentoses released from hemicelluloses was found as the major degradation product (maximum of 0.22 $g \cdot L^{-1}$), while hydroxymethylfurfural (HMF) obtained from the dehydration of hexoses, especially glucose, was detected as the lowest degradation product (maximum of 0.08 $g \cdot L^{-1}$). Michelin et al. (2016) reported an increase in inhibitors production (i.e. 4.8 $g \cdot L^{-1}$ of furfural at 200 °C *versus* 0.5 $g \cdot L^{-1}$ at 180 °C, both for 30 min) using a more severe LHW-condition to pretreat sugarcane bagasse.

Other authors used similar conditions to our study and showed that LHW-pretreatment at 195 °C during 20 min led to a composition of 57.5% glucan, 3.8% hemicellulose and 23.2% lignin on pretreated WS solids (Perez et al., 2008), which is a very low hemicellulose content in relation to our study. On the other hand, Carvalheiro et al. (2004) reported that BSG pretreated by LHW at 190 °C during 20 min presented a composition of 25.5% glucan, 6% hemicellulose, and a higher lignin content (54.3%) on solid fraction. They also reported 6.75 $g \cdot L^{-1}$ XOS and 5.17 $g \cdot L^{-1}$ xylose, besides a high concentration of degradation products. These differences can be related to heterogeneity of the LCMs, as discussed previously, or even to specific conditions of each study (e.g. Carvalheiro et al. (2004) used the liquid/solid ratio of 8 $g \cdot g^{-1}$).

3.3. Effect of pretreatment on thermochemical properties of the feedstocks

The thermochemical properties of the untreated and pretreated feedstocks were evaluated by TGA. Fig. 1 shows the TG and DTG profiles of the studied feedstocks. Three weight loss stages were observed to all materials, with exception of LS (please see DTG, Fig. 1E). In the first stage, which ranged from 30 to 150 °C, occurs the moisture loss by evaporation (less than 10% of weight loss). After that stage, some components start to break down chemically,

namely hemicellulose and lignin. It is known that hemicellulose decomposes at temperatures ranged between 190 and 380 °C. Lignin decomposition occurs on a very wide interval. It starts at low temperatures (~170 °C) where the mass loss is low, and can extend up to more than 600 °C. Lignin is different from hemicellulose and cellulose, because it is composed of three kinds of benzene-propane units, being heavily cross-linked and having very high molecular weight, thus presenting a broad range of decomposition (Yang et al., 2006; Poletto et al., 2014). By other side, the cellulose decomposes in a narrow range between 280 and 400 °C (Popescu et al., 2011).

TG and DTG profiles clearly showed (Fig. 1) increased thermal stability for the LHW-pretreated materials in relation to the untreated materials, evidenced by the gradually higher decomposition temperature (please see TG curve, Fig. 1). This is attributed to the removal of hemicellulose on pretreated material, since it has a random amorphous structure, which is easily hydrolyzed. In contrast, the cellulose is a very long polymer of glucose units, and its crystalline regions increase the thermal stability of lignocellulosic fibers (Yang et al., 2006; Poletto et al., 2014). Regarding the cellulose thermal stability, the LCMs presented different decomposition temperatures. The decomposition temperature for pretreated- BSG and CH were 371.7 and 378.4 °C, respectively, while for CC, WS and LS these values were higher (383.3, 389.1 and 397.5 °C, respectively). Results showed a higher thermal stability of some studied materials and gave an indication of the stiffness of each structure.

LHW-pretreated materials showed a higher weight loss in the cellulose region (3rd stage) and a lower weight loss in the hemicellulose region (2nd stage) than untreated materials (see DTG curve, Fig. 1). This behavior indicates the removal of hemicellulose from

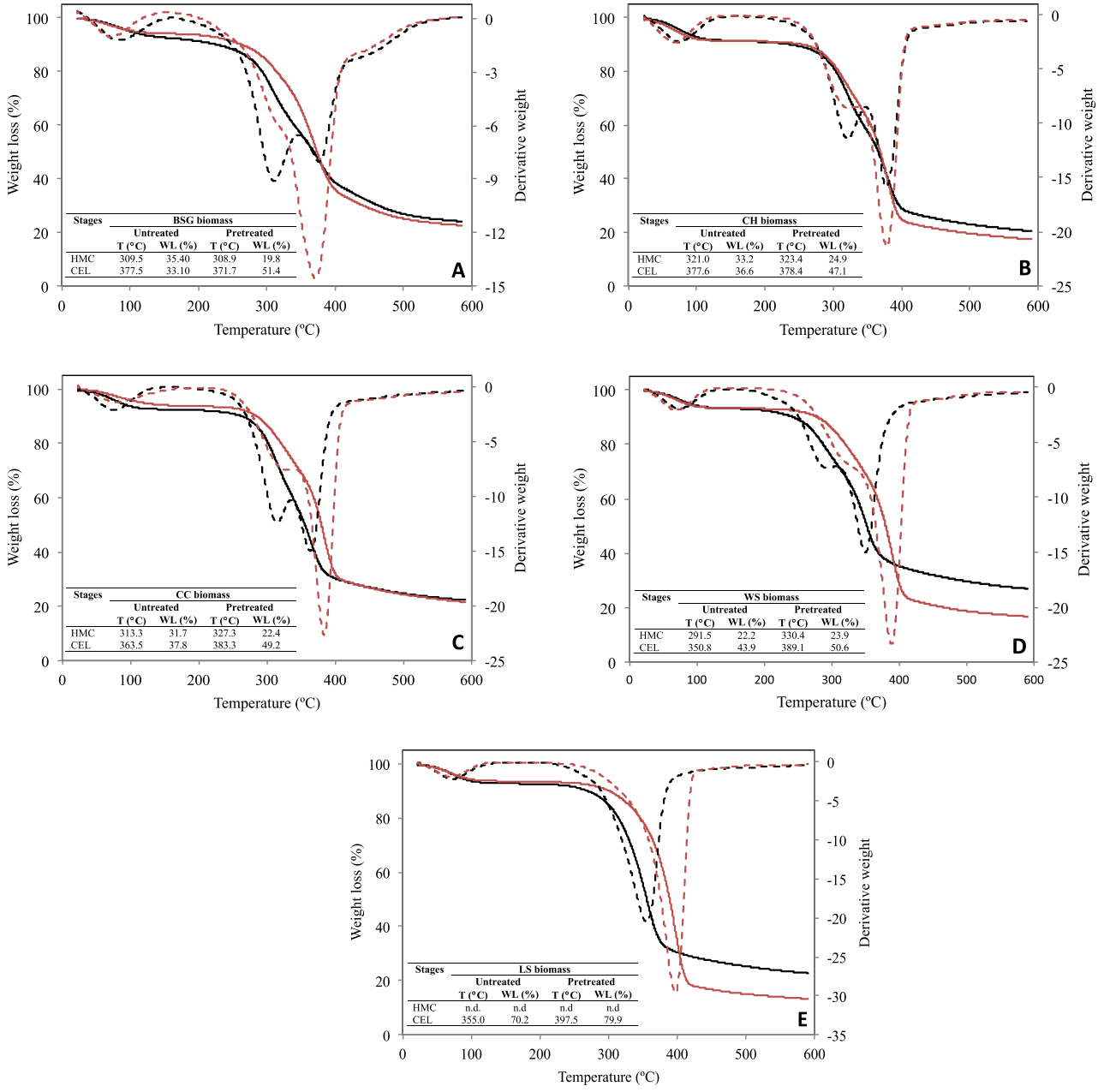

Fig. 1. TG (solid lines) and DTG (dotted lines) curves of untreated (black lines) and LHW-pretreated (red lines) feedstocks: BSG (A), CH (B), CC (C), WS (D) and LS (E). The inserted tables contain data regarding to temperature at maximum rate of decomposition (T) and the percentage of weight loss (WL) for the stages relative to cellulose (CEL) and hemicellulose (HMC) degradations. Legend: n.d. – not detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the LHW-pretreated materials, which is consistent with the hydrothermal pretreatment and the compositional analysis presented in Tables 1 and 2. The estimated temperature of maximum decomposition rate and the percentage of weight loss of each untreated and LHW-pretreated materials are presented in Fig. 1 in the inserted Table.

As mentioned above for LS, it was not possible to discriminate the thermal degradation stages of hemicellulose and cellulose (see DTG curve, Fig. 1E), probably due the large amount of cellulose in this feedstock when compared to hemicellulose.

These results confirmed the thermal depolymerization of hemicelluloses with the pretreatment process and showed the susceptibility of each feedstocks to thermal degradation, since each of three major components (cellulose, lignin and hemicelluloses) of the feedstocks has its own properties with respect to thermal degradation, which are based on polymer composition and its three-dimensional structure (Popescu et al., 2011).

3.4. Effect of pretreatment on cellulose crystallinity

The X-ray diffraction (XRD) is typically used to assess the crystallinity degree of bio-based materials. Among the analyzed feedstocks, cellulose is considered the only crystalline component (although cellulose has a portion of imperfect crystallites that contribute to the amorphous content in LCMs), whereas hemicellulose and lignin are considered amorphous (Popescu et al., 2011).

All studied materials (with exception of untreated BSG) presented a diffractogram typical of cellulose I structure (Fig. 1S), with the crystalline and amorphous peaks at 22.5° and 18°, respectively (Oh et al., 2005). According to Popescu et al. (2007), most of LCMs present a large amount of cellulose and in this case the diffraction pattern is not influenced by other components. However, in the case of BSG, the lignin and hemicellulose components are strongly present along with the cellulose, as can be confirmed through the chemical composition of BSG presented in Table 1, which influenced the XRD pattern. This is agreement with the XRD pattern presented by Ferraz et al. (2013) for the same material (i.e. BSG). Pretreated BSG presented the lowest *CrI* value (38.20%).

CrI values increased for the pretreated materials when compared to untreated samples. Generally, the removal of hemicellulose and the lignin re-localization as result of condensation reactions under severe pretreatment conditions lead to an increase of the *CrI* (Xiao et al., 2011). In this case, the increase of *CrI* values was probably caused by the removal of amorphous parts (i.e. hemicellulose) after the LHW-pretreatment. They followed the order: LS > WS > CC > CH > BSG. *CrI* increased 10.8% (67.88% *versus* 76.14%) and 11.2% (56.25% *versus* 63.41%) for pretreated- LS and WS, respectively, while for CC and CH the *CrI* increased 21.4% (41.41% *versus* 52.74%) and 22.5% (40.35% *versus* 52.11%), respectively, in relation to untreated materials. From these results, it is clear the influence of the cellulose content in *CrI* values, being the samples with higher cellulose content (Tables 1 and 2), the ones with higher values of *CrI*.

Other authors verified an increase in *CrI* values after the pretreatments, mainly due the removal of hemicellulose and lignin (Xiao et al., 2011; Boonsombuti et al., 2013; Wanitwattanarumlug et al., 2012). However, some authors have also observed a decrease in *CrI* values, explained by the changes on cellulose crystalline structure from cellulose I to II (with peaks at 11.6° and 20.3°), as observed for ionic liquid pretreatment (Cruz et al., 2013). Wanitwattanarumlug et al. (2012) obtained *CrI* of 24.5% for untreated corncob, and after KOH alkali-pretreatment the *CrI* increased up to 57.3%. On other hand, Sahare et al. (2012) obtained *CrI* of 39.16% for untreated corncob that decreased to 15.36% after NaOH alkali-pretreatment. Barman et al. (2012) studied alkali pretreatment on wheat straw and obtained *CrI* of 53.3% for untreated wheat straw; however, the *CrI* increased up to 60.3% with pretreatment at 1.5% NaOH and decreased to 52.5% with pretreatment at 2% NaOH, suggesting changes on cellulose crystalline structure.

3.5. Structural analysis by SEM

SEM was performed to evaluate microstructural changes in the feedstocks after LHW-pretreatment. SEM images of untreated feedstocks showed a smooth and flat surface, and no fiber bundles were observed. After LHW-pretreatment, the surface morphologies changed significantly and showed a less compact structure than untreated feedstocks (Fig. 2S).

The fiber bundles of pretreated BSG was badly damaged and showed an arrangement with many deep longitudinal cracks. The fractured fiber bundles may have contributed to the increase of rough and fresh surfaces, which may have increased the accessibility of cellulase. Pretreated CH showed a more porous surface than other materials, apparently decreasing the rough external surface, and expanding the external surface area. These are in agreement with the EH results (please see Section 3.6 Enzymatic Hydrolysis).

Pretreated- CC and WS showed similar structural changes on their surfaces, i.e. the structures have become looser. However, the EH was more efficient on WS than CC (please see Section 3.6 Enzymatic Hydrolysis). This behavior can be related with the more rigid inner of CC, which poses an obstacle for the access of cellulase to cellulose. Some structural changes were also observed on the surface of pretreated LS, showing the effect of LHW-pretreatment on this LCM.

3.6. Enzymatic hydrolysis (EH)

The solid fraction of the LCMs obtained after the LHW-pretreatment can be used for the production of some value-added products, such as ethanol fuel. However, the conversion of LCM to suitable fermentation substrates requires a step of enzymatic hydrolysis of cellulose to get a high yield of fermentable sugars.

Cellulase enzymes usually catalyze the enzymatic hydrolysis process. However, the hemicelluloses have been often described as one of the important physical barriers to enzymatic hydrolysis of cellulose, which act by blocking the enzyme access to the cellulose surface. On the other hand, it has also been reported that hemicelluloses, particularly in the form of oligomers, are strong inhibitors of cellulase activity, presenting a more inhibitory effect than xylan and xylose (Qing et al., 2010; Qing and Wyman, 2011). To overcome the negative effects of residual hemicellulose on enzymatic hydrolysis of the studied feedstocks, Cellic Ctec2 was supplemented with NS 22083 to boost the cellulose saccharification by removal of the remaining hemicellulose, enlarging the contact area between the cellulose and the enzyme and/or through conversion of xylan and xylo-oligomers to the less inhibitory xylose.

Table 3 shows the cellulose conversion to glucose (determined by Eq. (2)) of untreated and LHW-pretreated materials. Results show a remarkable positive effect of LHW-pretreatment on enzymatic digestibility of pretreated materials in relation to untreated materials. The positive effect of this pretreatment was expected, since it has been reported that LHW causes disruption of cellulose and hemicellulose association due to the solubilization of this latter, thus increasing the accessibility of the enzymes to the pretreated solids (Alvira et al., 2010). However, the LHW-pretreatment condition was not efficient on improving the enzymatic hydrolysis of LS. This behavior is probably related to highly fibrous and rigid structure of LS, which hampers the accessibility of cellulase to cellulose, as well as the high crystallinity of LS (please see Fig. 1S).

Table 3
Cellulose conversion to glucose of untreated and LHW-pretreated solids.

Feedstocks	Cellulose conversion to glucose (%)	
	Untreated	Pretreated
Brewers' spent grain	36.97 ± 1.76	76.08 ± 0.85
Corn husk	31.82 ± 3.49	63.30 ± 2.45
Corncob	43.19 ± 2.64	47.68 ± 4.52
Wheat straw	41.35 ± 3.97	59.94 ± 3.65
Luffa sponge	48.00 ± 2.10	36.10 ± 1.77

Enzymatic hydrolysis was performed with 5% (w/v) solids loading, and 15 FPU.g^{-1} dry solids of Cellic Ctec2 plus 15 UI.g^{-1} dry solids of NS 22083, both dissolved in 50 mM sodium citrate buffer, pH 4.8. Runs were carried out in a thermostatically controlled orbital shaker at 350 rpm and 50 °C for 72 h.

Untreated- LS and CC, which presented the highest cellulose contents (see Table 1), had the highest cellulose conversion to glucose, 43% and 48% respectively, among the untreated feedstocks. However, the cellulose conversion on pretreated CC was only 9.5% higher than untreated material. So as to LS, this behavior can be related to highly fibrillar and rigid structure of the inner of CC that influences the availability of cellulose and thus the enzymatic hydrolysis, combined with the high *CrI*.

The highest cellulose conversion to glucose (in relation to glucose potential in the solids) was obtained from the pretreated BSG (about 76%), making it the most effective feedstock to achieve high yields of ethanol when taking into account all potentially fermentable glucose, coming from pretreatment and enzymatic hydrolysis steps. This conversion was 51% higher than untreated BSG. Although this material presented low cellulose content in relation to other studied feedstocks, the pretreatment condition was more effective in the disruption of its lignocellulosic structure, favoring the enzymatic action.

Amores et al. (2013) achieved EH yields (using 15 FPU·g^{-1} dry solids and 5% (w/v) solids load) of 60% using steam-explosion pretreated sugarcane bagasse (at 200 °C for 5 min). In similar EH condition, Cara et al. (2007) obtained EH yields of 70.3% and 60.2% from olive tree pruning biomass pretreated by LHW and steam-explosion, respectively. On the other hand, Radhakumari et al. (2014) obtained a maximal conversion of 58% carbohydrates using dilute acid hydrolysis to pretreat extractive-free and de-oiled karanja seed cake, an inedible feedstock residue.

LHW has been extensively studied due to the less degradation of sugars and the lower production of inhibitors compared to other pretreatments, such as acid hydrolysis, and because it is considered a green technology since no chemical is used to pretreat the biomass. However, it has high-energy requirements (high energy/water input). On the other hand, new technologies, such as ionic liquid, have been explored. Shill et al. (2011) achieved 80% and 50% cellulose conversion to glucose, using 20 FPU.g^{-1} cellulose of Celluclast plus β-glucosidase, from corn stover pretreated with ionic liquid and AFEX, respectively, showing conversion values in the range of this work. Being, in such cases, the major disadvantages, the high cost of ionic liquid/ammonia and the need for solvent recovery/recycle.

As commented above, another factor influencing the enzymatic hydrolysis rate is the crystallinity of cellulose (Alvira et al., 2010), which is mainly supported by the results obtained for pretreated-BSG and LS; i.e. the lowest *CrI* led to the highest cellulose conversion and the highest *CrI* led to the lowest cellulose conversion. It has been demonstrated that cellulase attack is principally initiated in the more easily accessible amorphous portion of cellulose, which is readily degraded before hydrolyzing slowly the less accessible crystalline portion (Sahare et al., 2012). Hall et al. (2010) showed a continuous decrease in the hydrolysis rate for higher crystallinity values, confirming that cellulose samples are less amenable to enzymatic hydrolysis for higher degrees of crystallinity.

Pretreated CH also presented good rates of cellulose conversion to glucose (approximately 65%), being almost 50% higher than untreated CH. In the case of pretreated WS, around 60% cellulose was converted to glucose, which was 31% higher than untreated WS. These results can also be related with cellulose content, structure and *CrI* of the LCMs.

4. Conclusions

This work elucidates the remarkable effect of LHW-pretreatment on the removal of hemicellulose and the improvement of enzymatic saccharification in different LCMs. CC and LS presented low improvements on enzymatic hydrolysis rates (maximal increase of approximately 10% to CC) after the pretreatment, while BSG, CH and WS had an increase from 30% to 50%. The susceptibility of each studied feedstock to autohydrolysis and enzymatic degradation was explained by chemical composition, crystallinity and physical properties of feedstocks. This work gave new insights concerning the potential of LHW to improve overall saccharification of multi feedstocks, and as a common technology for flexible biorefineries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.06.018.

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