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Case Study

Multi-step approach to add value to corncob: Production of biomass-degrading enzymes, lignin and fermentable sugars

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ABSTRACT

This work presents an integrated and multi-step approach for the recovery and/or application of the lignocellulosic fractions from corncob in the production of high value added compounds as xylo-oligosaccharides, enzymes, fermentable sugars, and lignin in terms of biorefinery concept. For that, liquid hot water followed by enzymatic hydrolysis were used. Liquid hot water was performed using different residence times (10–50 min) and holding temperature (180–200 °C), corresponding to severities ($\log(R_0)$) of 3.36–4.64. The most severe conditions showed higher xylo-oligosaccharides extraction (maximum of 93%) into the hydrolysates and higher recovery of cellulose on pretreated solids (maximum of 65%). Subsequently, hydrolysates and solids were used in the production of xylanases and cellulases, respectively, as well as, pretreated solids were also subjected to enzymatic hydrolysis for the recovery of lignin and fermentable sugars from cellulose. Maximum glucose yield (100%) was achieved for solids pretreated at $\log(R_0)$ of 4.42 and 5% solid loading.

1. Introduction

The harvested production of cereals in the EU-28 was around 317 million tonnes in 2015. This represented about 12.5% of global cereal production (FAO, 2016). Common wheat and spelt, barley, grain maize and corn-cob-mix accounted for a high share (86% in 2015) of the cereals produced in the EU-28 (EUROSTAT, 2017). These harvests generate a large amount of lignocellulosic residues that mainly consist of cellulose (30%–50%), hemicellulose (15%–35%) and lignin (10%–20%) that are linked with each other (Michelin et al., 2015).

These lignocellulosic materials (LCM) are organized in a complex matrix that needs to be broken in order to isolate the lignocellulosic components. Biomass-degrading enzymes act on hydrolysis of the polymeric cellulose or hemicellulose into oligosaccharides and after in sugars, which can be fermented by microorganisms, or used as building blocks, for synthesis of fuel or chemicals. In general, these enzymes, i.e. cellulases and hemicellulases, consist of an enzymatic complex that works synergically to hydrolyze the different regions of cellulose and hemicellulose on lignocellulose, according to their specificity (Sweeney and Xu, 2012).

The enzymatic hydrolysis of cellulose has been shown to improve significantly with the removal of hemicellulose, suggesting that hemicellulose acts as a barrier to the hydrolysis of cellulose by cellulolytic enzymes (Yang et al., 2011). Thus, the pretreatment of LCM before hydrolysis is a prerequisite and it can be performed by different methods. Liquid hot water (LHW) pretreatment or autohydrolysis (hydrothermal processing) allows a high recovery of hemicelluloses as soluble saccharides, while both cellulose and lignin could be recovered in the solid phase as essentially non-degraded polymers. Furthermore, it has many technological and environmental benefits, mainly related to its non-catalyzed nature, as well as limited equipment corrosion problems, reduction of operational costs, and lower byproducts generation, such as furfural and hydroxymethylfurfural (Michelin et al., 2015; Michelin and Teixeira, 2016).

This work uses LHW pretreatment, enzymatic saccharification and fungal fermentation to convert corncob residues into valuable products in terms of biorefinery. For that, xylanases (endoxylanase and β-xylosidase) and cellulases (FP activity, β-glucosidase) were produced through the fungal fermentation using the hemicellulose hydrolysates and the solid fraction (rich in cellulose), respectively, obtained from LHW pretreatment. The use of pretreated lignocellulosic residues is an important strategy to improve the enzymatic production and to compete with commercial substrates, because of the lower production cost of the enzymes associated with these residues. Moreover, a step of

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enzymatic hydrolysis (using commercial enzymatic cocktails) was performed on the solid fraction for saccharification of cellulose and recovery of lignin that was evaluated regarding to its antioxidant potential.

2. Materials and methods

2.1. Materials

Corncob (CC) was kindly supplied by a farmer from Northern Portugal. The material was dried at 40 °C for 12 h, and after that it was cut into small chips (1–3 cm), milled using a knives mill to pass through a 1.0 mm screen, and stored at room temperature until use. Cellic® Ctec2 (a blend of cellulases and hemicellulases) and NS 22083 (xylanases) were kindly given by Novozymes (Bagsvaerd, Denmark). Whatman® filter paper grade 1 (Whatman International Ltd, England), Beechwood xylan, ρ-nitrophenyl-β-D-glucopyranoside and ρ-nitrophenyl-β-D-xylopyranoside, 1,1-diphenyl-2-picrylhydrazyl (DPPH), trolox and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Liquid hot water (LHW) pretreatment

LHW pretreatment of CC was carried out in 160 mL stainless steel cylinder reactors (4.0 cm internal diameter and 12.4 cm internal height), with a working volume of 50 mL). Milled CC samples of 1.0 mm and water were added into the closed and pressurized vessel at 10% (w/v) solids loading. The reactor was submerged in an oil bath with an open heating circulator (Julabo Labortechnik GmbH, Seelbath, Germany) with PID temperature control at 180, 190 and 200 °C and maintained for a certain residence time (10, 30 and 50 min). After that, the reactor was immediately cooled in an ice bath to quench the reaction. The pretreated slurry was vacuum filtered using Whatman® N° 1 filter paper to separate the liquid (hydrolysates) and solid fractions. The pH value of the hydrolysates was measured using a digital pH meter and after that it was stored frozen. Solids were washed with distilled water, and after that dried at 40 °C.

Intensity of pretreatments was measured in terms of severity index ($\log(R_0)$) calculated according to Eq. (1):

$$\log(R_0) = \log\left[t\exp\left(\frac{T-100}{14.75}\right)\right] \tag{1}$$

where t is the residence time (min), T is the holding temperature (°C), and 14.75 an empirical parameter related to activation energy and temperature.

2.3. Compositional biomass analysis

The chemical composition of CC (untreated and pretreated) was determined according to the standard Laboratory Analytical Procedures (LAPs) for biomass analysis provided by the US National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). Analyzed components were glucan, xylan, arabinan, acetyl groups, Klason lignin, and ashes. The hydrolysates of the pretreated CC were 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 post-hydrolysis with 4% sulfuric acid at 121 °C, during 60 min. The increase in the concentrations of monosaccharides (glucose, xylose and arabinose) and acetic acid caused by post-hydrolysis was considered a measure of the concentrations of oligomers and acetyl groups bound to oligosaccharides. These components were analyzed by HPLC as described below. All measurements were made in duplicate.

The extraction yield of xylo-oligosaccharides (Y_{XOS}) from feedstock xylan (gXOS/100 g xylan) was calculated according to Eq. (2):

$$Yield_{XOS} = \mathrm{XOS}\frac{V}{Xn_{FS}}100 \tag{2}$$

where XOS is the concentrations of xylo-oligosaccharides (g/L) on hydrolysates, V is the volume of LHW assay (mL) and Xn_{FS} is the percentage of xylan in feedstock material.

2.4. HPLC analysis

HPLC analysis of hydrolyzed samples was performed using a Metacarb 87H carbohydrate analysis column (300 × 7.8 mm, Varian, USA) at 60 °C. Sugars and acetic acid were analyzed with a refractive index (RI) detector and furfural and hydroxymethylfurfural (HMF) contents with a UV detector, both in a Jasco chromatograph. The mobile phase was 0.005 M H_2SO_4 in ultrapure water filtered through 0.45 μm nylon filter (Millipore) and degassed. The flow rate was 0.7 mL/min.

2.5. Microorganisms

The microorganisms used in this work were the fungal strains *Trichoderma reesei* MUM 97.53 and *Aspergillus niger* van Tieghem. The first one was provided by MUM (Micoteca da Universidade do Minho, Portugal); and the second one obtained from Department of Biology from FFCLRP/USP (Brazil). Stock cultures were propagated on PDA medium slants (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD, USA), at 30 °C for 1 week, and stored at 4 °C.

2.6. Cultivation conditions

Conidia from 7 day-old cultures from *Trichoderma reesei* MUM 97.53 and *Aspergillus niger* van Tieghem, with 1×10^9 spores per mL, were inoculated into 100 mL Erlenmeyer flasks containing 20 mL of the liquid medium described by Mandels & Weber (1969), pH 6.0. Pretreated solids (1%, w/v) or hydrolysates (100%, v/v) were used as carbon source. Untreated CC, xylan and avicel were used as control. The cultures were incubated at 30 °C, 100 rpm, for 6 days. After fermentations, the mycelia and residues were removed from fermentation media by vacuum filtration using Whatman® N° 1 filter paper. The filtrates were used as sources of crude extracellular cellulase or xylanase enzymes.

2.7. Enzyme activities

All enzymatic assays were performed using the procedure recommended by the International Union of Pure and Applied Chemistry (IUPAC). Cellulase activity was determined at 50 °C for 60 min, according to Ghose (1987), using filter paper Whatman N° 1 as substrate and expressed as Filter Paper Unit per mL (FPU/mL). Xylanase activity was determined at 60 °C for 20 min, using birchwood xylan as substrate and expressed as International Unit per mL (IU/mL). Release of reducing sugars from both polysaccharides was analyzed by DNS (Miller, 1959), using glucose or xylose, respectively, as standard.

β-Glucosidase activity was determined at 50 °C for 10 min by monitoring the hydrolysis of *p*-nitrophenol-β-D-glucopyranoside (PNP-glu), and β-xylosidase activity was determined at 70 °C for 15 min through the hydrolysis of *p*-nitrophenol-β-D-xyloopyranoside (PNP-xyl). The released ρ-nitrophenolate was estimated with 1 M sodium carbonate, using ρ-nitrophenol as standard and expressed as International Unit (IU) per mL. All substrates were suspended in 50 mM sodium citrate buffer, pH 4.8.

Cellic® Ctec2 presented 160 FPU/mL of cellulase and 2300 IU/mL of β-glucosidase; and the NS 22083 contained 2800 IU/mL of xylanase and 135 IU/mL of β-xylosidase, respectively.

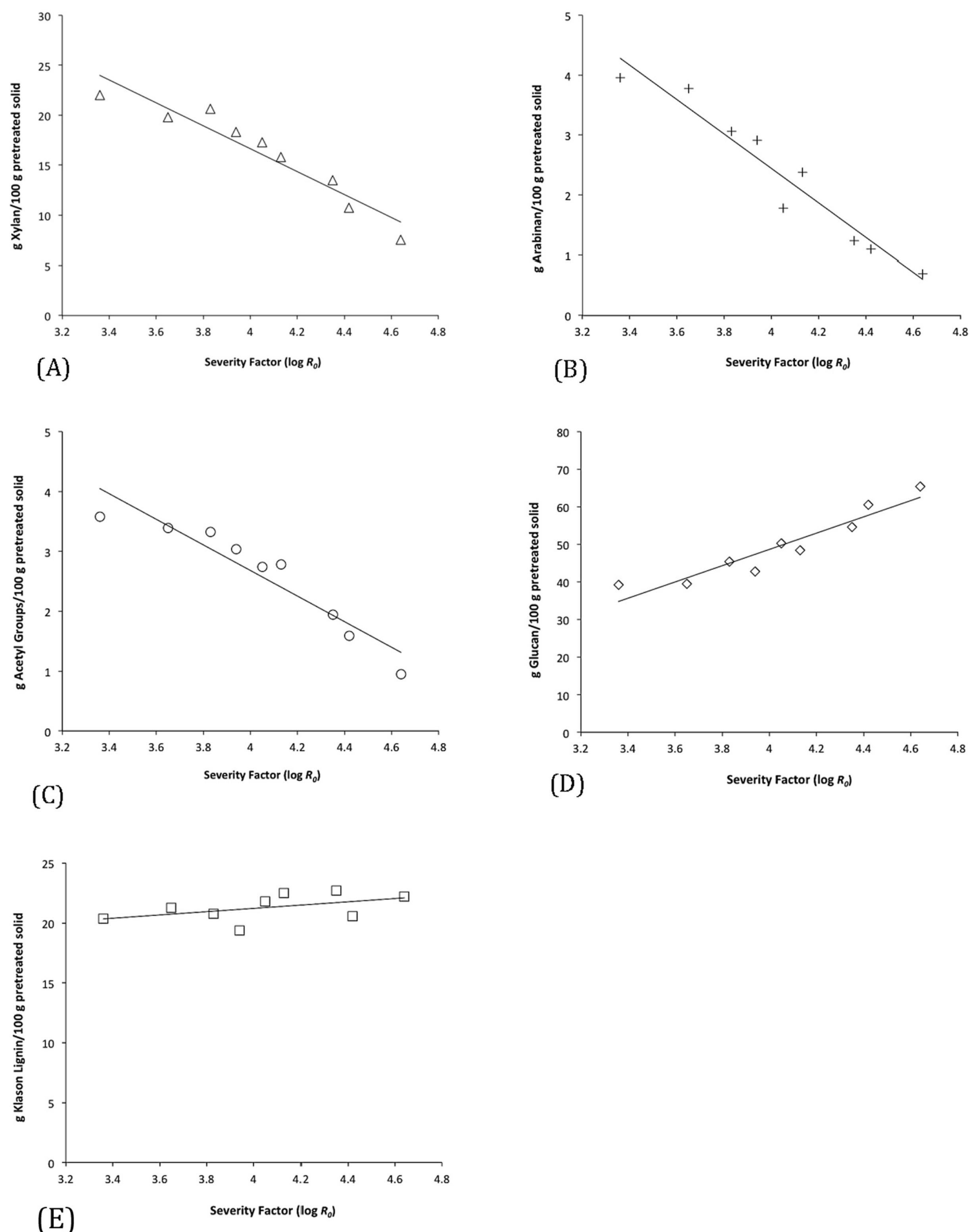

Fig. 1. Relationship between severity factor of liquid hot water and percentage of recovered components (g/100 g pretreated material) in the solid fraction: (A) xylan; (B) arabinan; (C) acetyl groups; (D) glucan, and (E) Klason lignin.

2.8. Enzymatic hydrolysis

Enzymatic hydrolysis (EH) experiments on the pretreated solids were performed using the commercial enzymes: Cellic® Ctec2 and NS 22083. EH experiments of 1 mL total volume were carried out at 5% (w/v) and 10% (w/v) pretreated solids loading (LHW pretreated solids at severities of log(R_0) of 4.42 and 4.64), and a blend of 15 FPU/g dry solids of Cellic® Ctec2 and 15 IU/g dry solids of NS 22083, both dissolved in 50 mM sodium citrate buffer, pH 4.8. EH runs were carried out in a thermostatically controlled orbital shaker at 350 rpm and 50 °C for 12, 24, 48 and 72 h. Samples were centrifuged at 14,000 rpm for 5 min and filtered through a 0.2 µm syringe filter for HPLC analysis, described previously, using glucose and cellobiose as standard. All runs were performed in triplicate.

The glucose yield (%) was calculated according to Eq. (3):

$$\text{Glucose yield}(\%) = \frac{[\text{glucose}] + 1.053[\text{cellobiose}]}{1.111f\,[\text{biomass}]}100 \tag{3}$$

where [glucose] is the glucose concentration (g/L), [cellobiose] is the cellobiose concentration (g/L), [biomass] is the dry biomass concentration at the beginning of the enzymatic saccharification (g/L), f is the cellulose fraction in dry biomass (g/g), 1.111 is the factor that converts cellulose to equivalent glucose and the factor 1.053 converts cellobiose to equivalent glucose.

2.9. DPPH free radical scavenging

The DPPH scavenging activity of lignin was determined according to the slightly modified method of Blois (1958) that is based on its ability to act as free radical scavenger.

Commercial antioxidants like trolox, and butylated hydroxytoluene (BHT) were used as reference. Briefly, 150 mM solution of DPPH was prepared and diluted in ethanol to get an absorbance of 0.700 at 517 nm. A volume of 200 µL of this solution was added to 25 µL of lignin samples dispersed in 60% ethanol at different concentrations (0.05–1 mg/mL). These solutions were incubated in the dark for 30 min at room temperature. The decrease of the solution absorbance, due to proton donating activity, was measured using a UV–Vis spectrophotometer against a control sample (200 µL of DPPH mixed with 25 µL of ethanol).

The percentage of radical scavenging activity (RSA) was calculated using the Eq. (4):

$$\text{RSA}(\%) = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] \times 100 \tag{4}$$

where $A_{control}$ is the absorbance of the control sample and A_{sample} is the absorbance of the lignin sample. The IC_{50} (half maximal inhibitory concentration) value was calculated as the concentration of the compounds that causes 50% reduction in the DPPH color (also referred as inhibition). All experiments were carried out in triplicate.

3. Results and discussion

3.1. Chemical composition of untreated and LHW pretreated corncob

The composition of the feedstock and its transformation during the pretreatment are the main focus in the lignocellulosic biomass conversion process (Liu and Chen 2015). The composition of untreated CC biomass used in this work (as a percentage by dry weight) was: 37.95% cellulose (measured as glucan), 23.74% xylan, 4.13% arabinan, 3.65% acetyl groups, 19.09% lignin and 0.77% ashes. The difference includes non-analyzed components of minor importance for this study, such as extractives or acid-soluble lignin.

The total sugar content was about 65%, being glucan and xylan the main polysaccharides presented. Like other crop-based residues, xylan in CC is analogous to hardwood xylan and consists of a β-D-(1,4)-linked xylopyranosyl backbone, substituted with arabinose, acetyl groups, and xylose. This chemical composition was similar to some previously reported values in the literature (Garrote et al., 2007; Michelin et al., 2012a; Xie et al., 2014; Michelin and Teixeira 2016). On the other hand, some authors reported corncob presenting 40.38%–42.6% cellulose, 39.04%–46% hemicellulose, and 7.26%–7.56% lignin (Boonsombuti et al., 2013; Boonchuay et al., 2014). These results reflect the difference in corncob biomass itself, which depend on the climate and the agro-technical conditions.

Fig. 1 shows the recovery of xylan, arabinan, acetyl groups, glucan, and Klason lignin (acid-insoluble lignin) as a function of the severity factor after LHW pretreatment. As expected, the fraction of dissolved solids mainly corresponded to hemicellulose solubilization and depolymerization, once LHW mainly affects hemicellulose components; and a decrease in the hemicellulose content of the pretreated CC was observed with the increase of both holding temperature and residence time (Fig. 1A–C). The results also showed that most of the hemicellulose in CC biomass was removed at high pretreatment severity. Within hemicellulose, acetyl groups are the first to be hydrolyzed, followed by arabinan and xylan. This component (acetyl) of the side chains of the xylan backbone facilitates the autohydrolysis effect in LHW (Michelin et al., 2015).

In contrast to hemicellulose components, glucan content increased in the pretreated solids, being the highest glucan content (65.4%) obtained at 200 °C for 50 min, i.e. in the pretreatment of higher severity (Fig. 1D). This behavior is related to the solubilization of hemicellulose components that was higher in the more severe pretreatment conditions, since cellulose suffers little modification in LHW pretreatments. These results are in good agreement with previous reports for similar feedstock, such as rye straw (Gullón et al., 2010), sugarcane bagasse (Vallejos et al., 2015) and corn stover (Liu and Chen, 2015).

Acid-insoluble lignin content in the pretreated solids ranged from 19.4% to 22.7%, increasing to a higher holding temperature and residence time. However, the solubilization of lignin was relatively low demonstrating that hydrothermal pretreatment does not significantly change lignin structure; the most important effect in the pretreated solids is the re-localization of the lignin (Fig. 1E).

3.2. Chemical composition of hydrolysates from LHW pretreatment

LHW pretreatments were carried out under several operational conditions aiming a maximal extraction of xylo-oligosaccharide and a more accessible pretreated biomass. A variety of both non-volatile components, including monosaccharides (xylose, arabinose and glucose) and oligosaccharides (xylo-oligosaccharides – XOS, arabino-oligosaccharides – ArOS, gluco-oligosaccharides – GOS, and acetyl groups linked to oligosaccharides – AcOS), and volatile components, mainly acetic acid, furfural and HMF appeared into the hydrolysates.

Fig. 2 shows the XOS extraction yield and sugar-degradation products (HMF and furfural) recovery, as well as the pH, as a function of the severity factor. XOS were the major components of hydrolysates obtained for all the analyzed conditions. The concentration of XOS increased for higher severity values, and reached a maximum at $\log(R_0)$ of 4.42 (Fig. 2A). The maximal extraction yield of XOS was 93 g XOS/100 g xylan. For higher values of the pretreatment severity factor, the hydrolytic degradation proceeds, and the XOS of high molecular weight are converted into oligomers of low molecular weight, xylose and furfural in subsequent reactions. Therefore, at $\log(R_0)$ of 4.64, the extraction yield of XOS decreased, explained by the conversion/degradation of XOS to xylose and subsequently to furfural.

Table 1 presents the concentration of the hydrolysates components (g/L), i.e. xylose, arabinose, glucose and acetic acid bound in oligomers as well as the free monomers, in all studied conditions. As mentioned previously LHW pretreatment mainly solubilized the xylan into XOS (22.1 g/L at $\log(R_0)$ of 4.42 and 16.8 g/L at $\log(R_0)$ of 4.64) and small amounts of xylose, which were detected into the hydrolysates (1.1 g/L at $\log(R_0)$ of 4.42 and 2.7 g/L at $\log(R_0)$ of 4.64). In the most severe condition, it is clear the high degradation of XOS into xylose and furfural (Fig. 2B).

Regarding the low amounts of glucose (glucose bound to oligomers as well as the free glucose) detected into the hydrolysates, it is important to highlight that this glucose could be derived from hemicellulose or from depolymerized cellulose, indicating in the last case, a limited hydrolysis of the cellulose under the experimental conditions used. In general, GOS concentration increased for higher holding temperatures and residence times, while glucose concentrations decreased. Maximum of 1.4 g/L GOS and 0.13 g/L glucose at $\log(R_0)$ of 4.64 (Table 1) were detected. HMF concentration also increased with the severity of the pretreatment conditions being the maximal

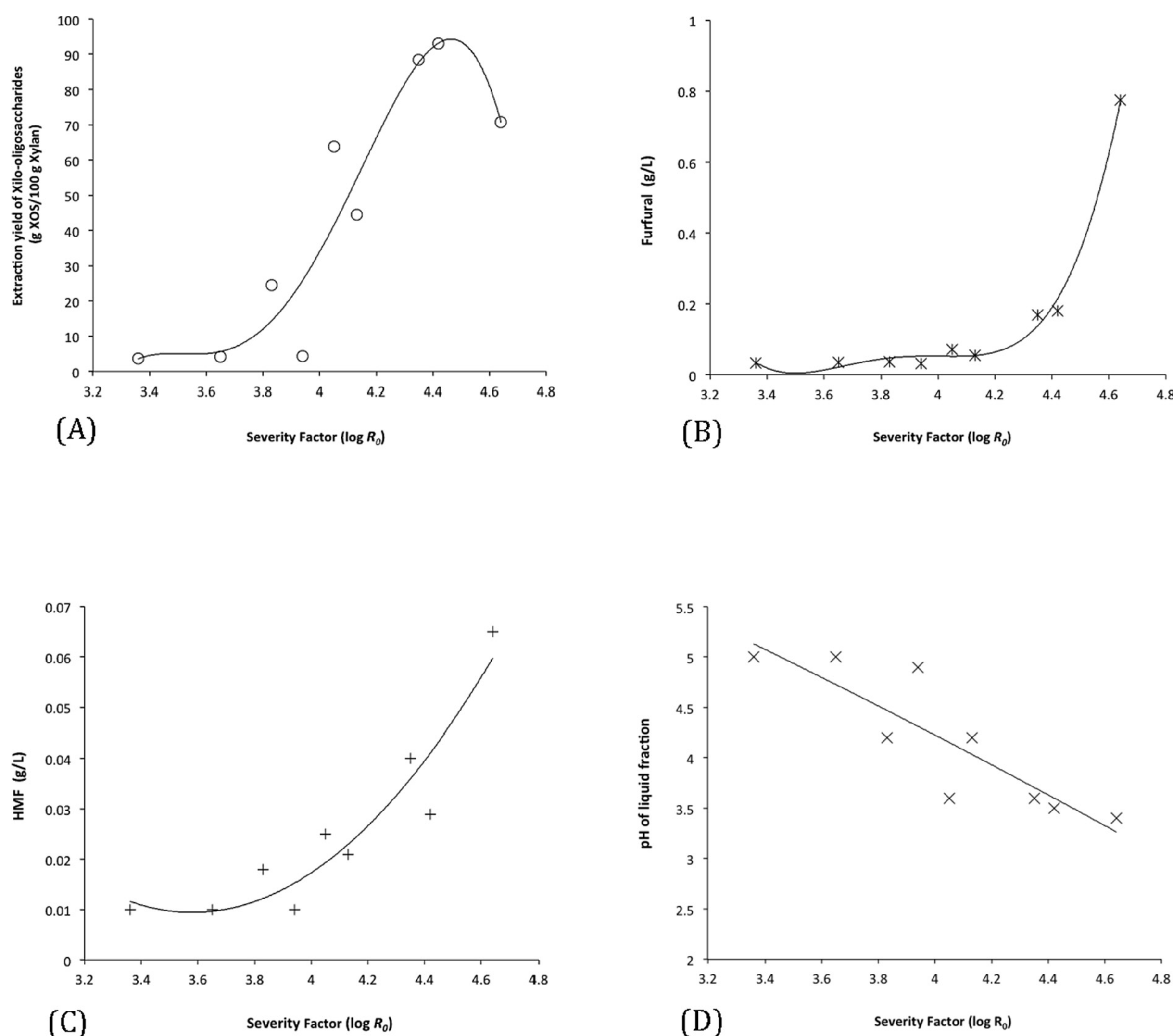

Fig. 2. Effect of liquid hot water on the extraction of XOS, formation of degradation products and pH of hydrolysates in the liquid fraction: (A) XOS extraction yield; (B) furfural; (C) hydroxymethylfurfural (HMF), and (D) pH of hydrolysates.

concentration obtained of 0.07 g/L (Fig. 2C).

It is important to highlight that the achieved furfural and HMF concentrations (maximal of 0.78 g/L and 0.07 g/L) is considered quite low compared with other pretreatments, such as dilute acid pretreatment (Wang et al., 2011). The generation of low concentrations of degradation compounds is a characteristic of LHW pretreatment and an advantage regarding to other processes. The formation of degradation compounds (inhibitors) is related to the pH of the process, and it has been suggested to control the pH between 4 and 7 to avoid the formation of degradation products, since at this pH range the hemicellulosic sugars are retained in oligomeric form and monomers formation is minimized (Mosier et al., 2005). In this study the pH ranged from 3.4 to 5.0 (Fig. 2D), and as expected the higher formation of inhibitor products was observed in the lowest pH.

The low formation of furfural and HMF is important, once concentrations higher than 1 g/L HMF are reported as inhibitory to microbial metabolism (Olsson and Hahn-Hagerdal 1996; Michelin et al., 2012a). Taherzadeh et al. (2000) reported 70% and 89% inhibition of

Table 1
Corncob hydrolysates composition obtained from LHW-pretreatment.

Operational Conditions (log R_0)	3.36	3.65	3.83	3.94	4.05	4.13	4.35	4.42	4.64
Oligosaccharides									
Xylo-oligosaccharides (g/L)	0.86	0.98	5.83	1.02	15.15	10.57	20.98	22.08	16.80
Gluco-oligosaccharides (g/L)	0.43	0.53	0.80	0.47	1.02	0.80	1.30	1.40	1.42
Arabino-oligosaccharides (g/L)	0.04	0.06	0.58	0.06	0.95	0.65	1.28	1.10	0.52
Acetyl groups-oligosaccharides (g/L)	0.30	0.33	0.90	0.46	1.50	1.05	2.03	2.15	1.98
Monosaccharides									
Glucose (g/L)	0.23	0.18	0.20	0.19	0.18	0.17	0.13	0.14	0.13
Xylose (g/L)	0.25	0.18	0.26	0.19	0.45	0.32	0.96	1.14	2.70
Arabinose (g/L)	0.01	0.05	0.56	0.06	0.92	0.77	0.92	1.02	0.85
Acetic acid (g/L)	0.10	0.12	0.38	0.15	0.69	0.53	0.83	0.98	1.13

Saccharomyces cerevisiae growth on 4 g/L HMF and furfural, respectively, and the presence of both furfural (2 g/L) and HMF (2 g/L) completely inhibited the growth of this microorganism.

Therefore, the generation of fermentation inhibiting compounds was quite low, which is an advantage for the use of hydrolysates for fermentation purposes as already demonstrated in previous works (Michelin et al., 2012a,b).

3.3. Production of value-added compounds

Pretreatment has often been useful for improving the digestibility of lignocellulosic materials and for facilitating access to microbial attack. The hemicellulose removal by LHW is known to disrupt the material structure possibly increasing porosity, and a solid residue with improved enzymatic digestibility is expected. In fact, xylan solubilization is generally considered the main factor to improve EH, although in some cases lignin disruption/removal can also be relevant for further EH. In this context, LHW pretreatment was used to obtain a well-adapted substrate for EH and fermentation. Thus, the solubilized and depolymerized hemicellulose fraction from LHW (hydrolysates) was used as substrate to produce xylanases and the solid fraction enriched in cellulose and also lignin on production of cellulases. On the other hand, solids from two selected conditions of LHW pretreatment (log(R_0) of 4.42 and 4.64) were used as substrate for enzymatic saccharification of cellulose and recovery of fermentable sugars (i.e. glucose) and lignin. A schematic representation of the CC pretreatment by liquid hot water (1st processing step) and production of value-added compounds (products) after enzymatic saccharification and fermentation (2nd processing step) is presented on Fig. 3.

3.3.1. Production of hemicellulases from LHW hydrolysates by Aspergillus niger

In order to improve the production of biomass-degrading enzymes, the hydrolysates from LHW pretreatment rich in XOS were used as substrate to produce hemicellulases, i.e. xylanases and β-xylosidases, by *A. niger* – a recognized producer of xylanolytic enzymes, aiming to obtain a well-adapted substrate for the production of these enzymes. Fig. 4 presents the enzyme production obtained on cultures of *A. niger*.

The production of xylanase (endoxylanase) was higher using hydrolysates obtained at log(R_0) of 4.13 (Fig. 4A), that contained 10.6 g/L XOS (Table 1). In this condition was obtained approximately a value of 10 IU/mL that was 13.4% higher than the production for commercial xylan, and 25.3% higher than untreated CC. These results showed that a well-adapted substrate for the production of this enzyme was achieved, probably due to the more accessible substrate (XOS) for the microbial attack than xylan and untreated CC. Although other fractions presented higher XOS content (Table 1), the highest xylanase production in the fraction of log(R_0) of 4.13 can be related to the lower amount of inhibitory compounds, such as furfural, HMF and acetic acid, as well as phenolic compounds that could be present (Michelin et al., 2016).

In the case of the β-xylosidase production, similar enzymatic levels were obtained for all fractions/conditions (Fig. 4B), which ranged from 1.5 to 1.9 IU/mL. The highest production corresponded to 16.7% and 24.2% of those from commercial xylan and untreated CC, respectively. Michelin et al. (2012a) reported an improvement of 15%–35% on enzymatic production in relation to commercial xylan, by using a mixture of untreated CC and hydrolysates from LHW pretreatment at 200 °C for 30 min. Maximum of 15.2 IU/mL and 12.1 IU/mL of xylanase and 0.6 IU/mL and 1.1 IU/mL of β-xylosidase were obtained in cultures of *A. terricola* and *A. ochraceus*, respectively. Michelin et al. (2012 b) explored the inclusion of a mixture of 1% (w/v) wheat bran and 10% (v/v) hydrolysates of wheat straw pretreated by LHW at 200 °C for 15 min and achieved a xylanase production 20% higher than commercial xylan and 42% higher than untreated wheat straw.

Aiming to decrease the cost of enzymes used to hydrolyze biomass, the hydrolysates from the LHW pretreatment of sugarcane bagasse were used in the growth medium of *Aspergillus awamori* by Paredes et al. (2015). The hydrolysates containing 23.7 g/L xylan was diluted and used as carbon source for fungal enzyme production. In a growth medium containing 5 g/L xylan, the fungus produced 46 U/mL of xylanase and 0.24 U/mL of β-xylosidase. The xylanase production was higher than that achieved in the present study, but the β-xylosidase production was lower; this difference can be related to the heterogeneity of the substrate, as well to the potential of each fungus to produce the enzyme.

Anyway, the use of pretreated agro-residues to improve xylanase production is an important approach to add value to these materials as well as to make xylanases production more feasible, since the cost of purified xylan is very high.

3.3.2. Production of cellulases from LHW solids by Trichoderma reesei

For cellulases production, the LHW solid fractions (rich in cellulose,

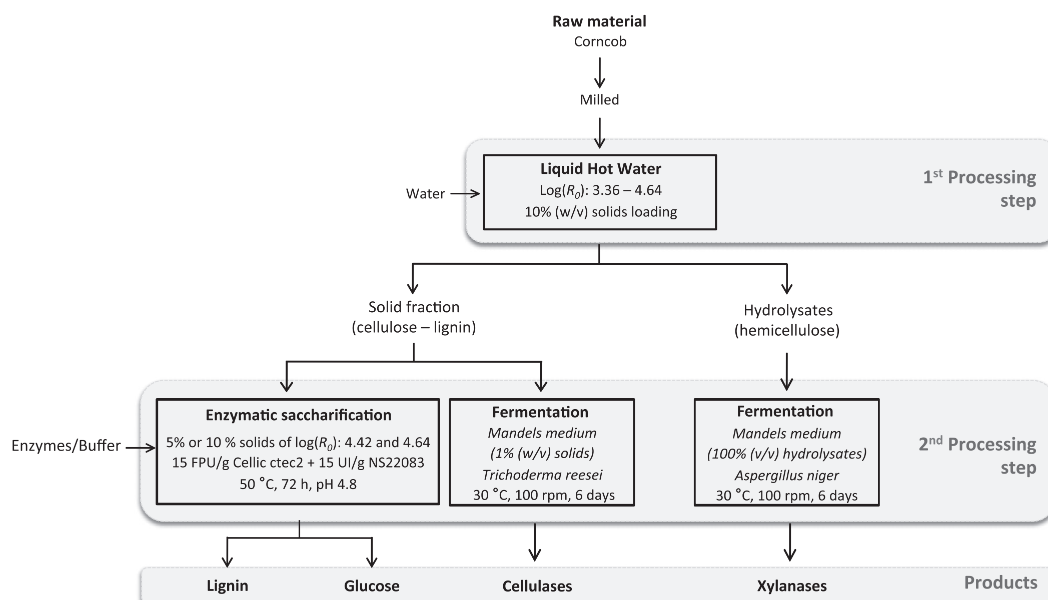

Fig. 3. Schematic representation of the liquid hot water process and enzymatic saccharification for recovery of value-added products.

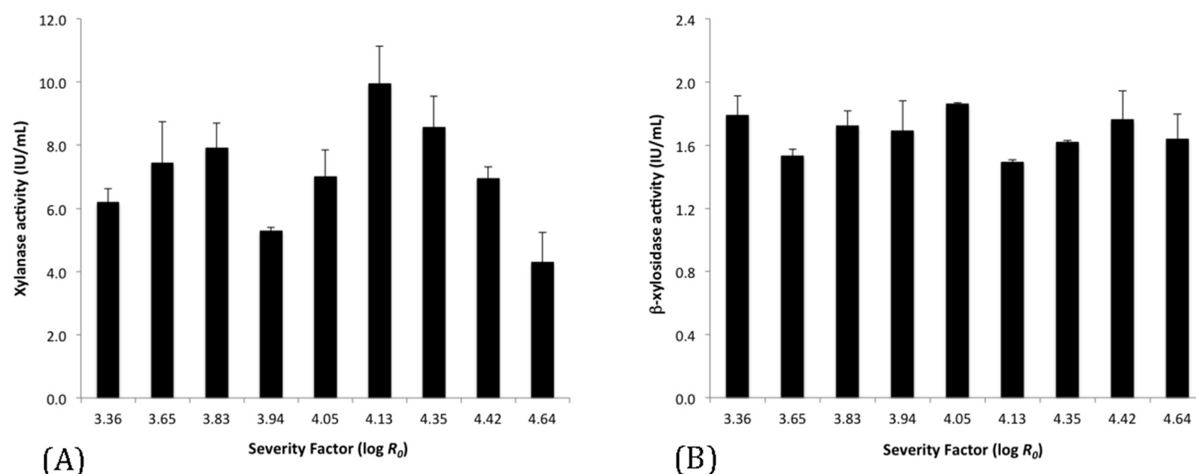

Fig. 4. Xylanase (A) and β-xylosidase (B) productions by *A. niger* using LHW hydrolysates from corncob as carbon source obtained by different pretreatment severities (log(R_0)). Microorganism was cultivated in Mandels medium at 30 °C, 100 rpm, for 6 days. Error bars represent standard deviation.

but also lignin) were used as substrate for cultivation of *T. reesei*, and two activities were evaluated: total cellulase (measured as filter paper activity) and β-glucosidase.

Regarding to cellulase activity, in general, it was observed an increase in the cellulase production with the increase of the pretreatment severity (Fig. 5A), being the highest cellulase production (measured as filter paper activity) by *T. reesei* verified on extracts using pretreated solids at log(R_0) of 4.42 and 4.64 (around 3.5 FPU/mL). This result can be explained by the high content of cellulose in the solid fraction, once in the highest severities was verified the highest cellulose content (60%–65%). This production was 20% higher than untreated CC and only 11.25% of this production was verified when avicel was used as substrate.

Regarding to β-glucosidase activity, 3.85 IU/mL were detected on extracts using pretreated solids at log(R_0) of 4.42 (Fig. 5B). This enzymatic production was also 20% higher than untreated CC and corresponded to only 5% of the enzyme production using avicel as substrate.

Other authors have used pretreated residues on production of biomass-degrading enzymes. Salihu et al. (2015) studied eleven agricultural residues for the cellulolytic enzyme production by *A. niger* and showed the effect of pretreatments (i.e. alkali, acid and oxidative pretreatments) on the improvement of enzyme production. Results showed a maximum production of cellulolytic enzyme on alkali pretreatment of soybean hulls with 6.2 FPU/g cellulase and 5.7 U/g of β-glucosidase. Pensupa et al. (2013) reported a solid-state fermentation of *A. niger* to produce cellulolytic enzymes by using wheat straw pretreated by different technologies. Around 10 FPU/g cellulase activity was obtained using wheat straw pretreated with dilute acid, and by the addition of yeast extracts and minerals that significantly improved the cellulase production to 24 FPU/g, showing the importance of the optimization of the enzyme production assays.

Zhang et al. (2012) also studied the effect of acid and alkali pretreatments on lignocellulosic materials to produce cellulase/xylanase by *T. reesei* Rut C-30. Three feedstocks (switchgrass, corn stover, and anaerobically digested manure fiber) were evaluated and cultures with alkali pretreated materials showed better results than the acid pretreated samples. Therefore, the type of pretreatment has shown to influence the cellulase production, since pretreatment influences the chemical composition of the recovered material.

Considering the high cost of the enzymes, the use of lignocellulosic residues for enzyme production is an important strategy to produce enzymes at lower costs and to add value to these residues. This is because most of the costs of enzymes production are related to the high costs of the substrates. On the other hand, the high lignocellulose content in some agricultural residues, such as CC, makes them an attractive source for the production of biomass-degrading enzymes by microorganisms. The use of pretreated biomass can further contribute for the improvement of the enzyme production due to a more accessible substrate (less recalcitrant biomass).

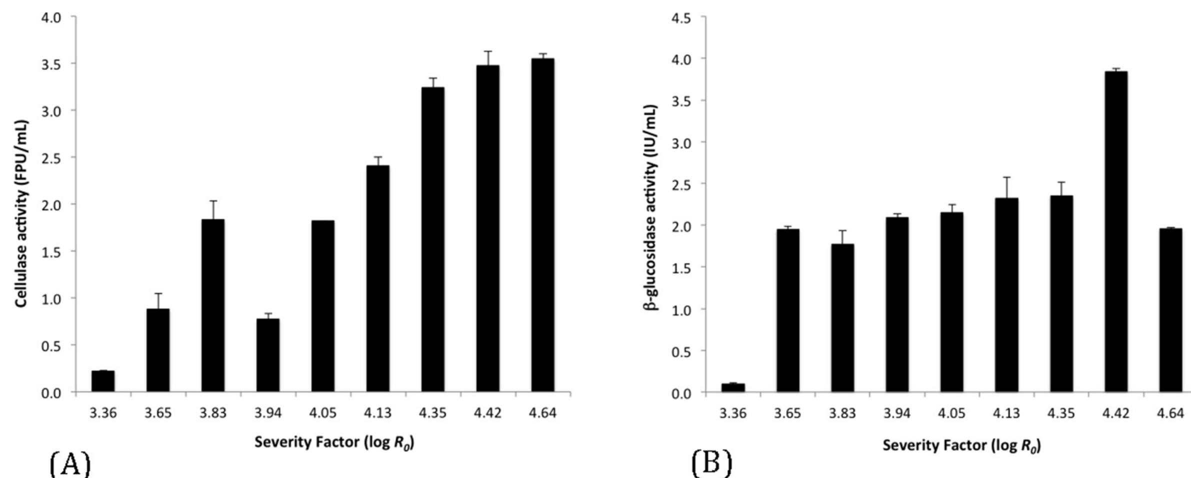

Fig. 5. Cellulase (A) and β-glucosidase (B) productions by *T. reesei* using LHW solids from corncob as carbon source obtained by different pretreatment severities (log(R_0)). Microorganism was cultivated in Mandels medium at 30 °C, 100 rpm, for 6 days. Error bars represent standard deviation.

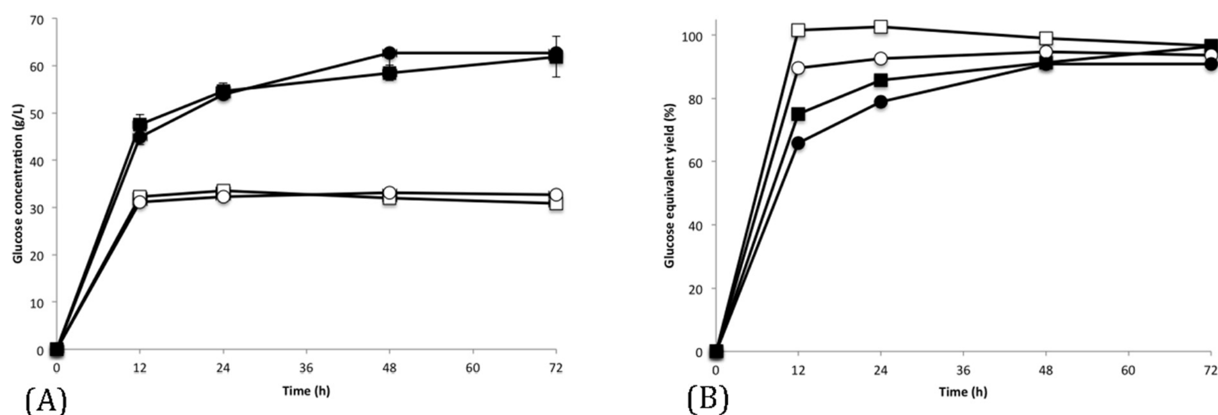

Fig. 6. Time course of glucose concentration (g/L) (A) and glucose yield (%) (B) of LHW pretreated corncob at $\log(R_0)$ of 4.42 and 4.64. Experiments were carried out in duplicate, using 5% or 10% solid loadings (w/v), 15 FPU/g dry solids of Cellic® Ctec2 plus 15 IU/g dry solids of NS 22083, at 50 °C. Symbols: $\log(R_0)$ of 4.42 and 5% solids (–□–); $\log(R_0)$ of 4.42 and 10% solids (–■–); $\log(R_0)$ of 4.64 and 5% solids (–○–); $\log(R_0)$ of 4.64 and 10% solids (–●–). Error bars represent standard deviation.

3.3.3. Production of fermentable sugars

The enzymatic hydrolysis (EH) step was performed using the pretreated solid fraction from two LHW pretreatment conditions: $\log(R_0)$ of 4.42 and 4.64. A mixture of two commercial enzymes was used in this study, 15 FPU/g dry solids of Cellic® Ctec2 and 15 IU/g dry solids of NS 22083, aiming to boost the cellulose conversion to glucose.

Fig. 6 shows the cellulose conversion to glucose (determined by Eq. (3)) of LHW pretreated material. Similar concentration of glucose was recovered from both LHW pretreated solids ($\log(R_0)$ of 4.42 and 4.64), and more than 30 g/L of glucose was recovered from pretreated solids at 5% solids loading, while 62 g/L of glucose concentration was recovered from pretreated solids at 10% solids loading (Fig. 6A). From these concentrations, glucose yields higher than 65% were observed in the first 12 h with 10% solids at $\log(R_0)$ of 4.64; being the maximal glucose yield (100%) verified in the first 12 h with 5% solids at $\log(R_0)$ of 4.42 (Fig. 6B).

Rodrigues et al. (2015) also achieved a high glucose yield (> 98%) during 96 h hydrolysis (corresponding to > 65 g/L glucose) of steam pretreated wheat straw, using an enzyme loading of 10 FPU/g cellulose (Cellic® CTec2) and a biomass concentration of 12% (w/v) on a dry weight basis. In previous work, Michelin and Teixeira (2016) achieved almost 50% of glucose yield after 72 h hydrolysis using 5% solids and 15 FPU/g solids, but with CC pretreated at $\log(R_0)$ of 4.13. Domínguez et al. (2017) also verified that autohydrolyzed solids pretreated at low severities (< 3.99) presented a low glucose yield, reaching values < 50% after 120 h EH. On the other hand, 100% of glucose yield (27.5 g/L) was achieved when Paulownia wood was pretreated at severity of 4.82. In this experiment they used a cellulase to substrate ratio of 20 FPU/g, solids loadings of 4.8%, and Novozyme 188 and Celluclast 1.5 L enzymes (Domínguez et al., 2017).

Ruiz et al. (2012) obtained a maximum extent of the enzymatic conversion of cellulose to glucose of 90.9% at 96 h and 85% at 72 h using LHW pretreated wheat straw as raw material. However, they used 40 FPU/g cellulose of Celluclast 1.5 L plus 60 IU/g Novozyme 188 and 1% of cellulose concentration. Hongdan et al. (2013) used 2% of LHW pretreated sugarcane bagasse and 20 FPU/g substrate and the maximum glucose yield in enzyme hydrolysate was 37.27 g per 100 g raw material at 200 °C for 20 min, representing 90.13% of glucose in the sugarcane bagasse.

Other examples include Fockink et al. (2015) that studied the cellulose conversion to glucose using 5% (w/v) solids and 11.5 FPU/g dry substrate (85 mg/g dry substrate) of Cellic® Ctec2 and obtained < 60% and < 50% cellulose conversion on alkali pretreated cotton gin dust and alkali pretreated cotton gin waste, respectively, after 72 h EH.

3.3.4. Recovery of lignin

EH of the LHW pretreated solids resulted in the recovery of four lignin fractions: EHL1 and EHL2 recovered from pretreated solids from LHW at $\log(R_0)$ of 4.42 and 5% or 10% solids, respectively; EHL3 and EHL4 recovered from pretreated solids from LHW at $\log(R_0)$ of 4.64 and 5% or 10% solids, respectively.

Fig. 7 shows the DPPH scavenging activity of the EHL samples. The DPPH inhibitory effect increased with the increment of lignin concentrations and the results show that all EHL possessed antioxidant activity. The antioxidant activity in terms of percentage of inhibition of the DPPH radicals at 1 mg/mL achieved values of 69%, 71%, 70% and 60% for the EHL1, EHL2, EHL3 and EHL4, respectively, against 93% and 96% inhibition for the commercial antioxidants butylated hydroxytoluene (BHT) and trolox, respectively.

IC_{50} of the EHL samples were obtained varying lignin concentration from 0.05 to 1 mg/mL. EHL samples had IC_{50} values ranging from 0.5 to 0.75 mg/mL, while the commercial antioxidants BHT and trolox had values of 0.2 mg/mL and 0.07 mg/mL, respectively, showing the higher antioxidant potential of the commercial antioxidants. Nevertheless, the EHL2 presented an IC_{50} value (0.5 mg/mL) in the same range of BHT. Lu et al. (2012) studied different organosolv pulping methods to extract lignin from *Acanthopanax senticosus* residue, being the lignin from acetic acid–water method the most effective free radical scavengers, with an IC_{50} of 0.66 mg/mL; this value is in agreement with the values obtained in this work.

Thus, results showed the potential of using lignin obtained from sequential LHW/EH process as natural antioxidant compound. Further work should be performed exploring their use in cosmetic, pharmaceutical and food industry to ensure their safety and applicability.

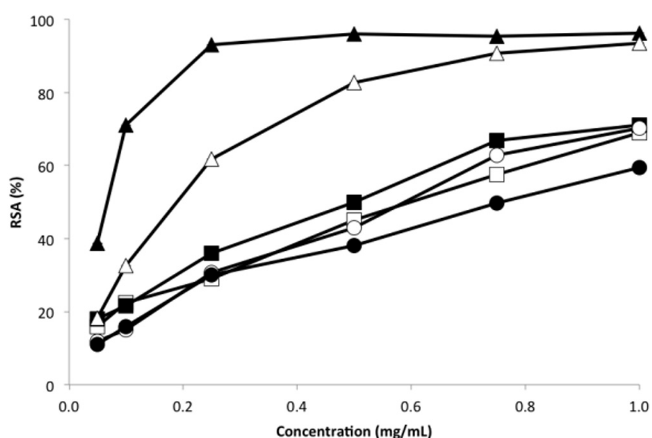

Fig. 7. Scavenging activity of EHL samples compared with commercial antioxidants for DPPH radical. Symbols: EHL1 (–□–); EHL2 (–■–); EHL3 (–○–); EHL4 (–●–); trolox (–▲–); BHT (–△–). Error bars represent standard deviation.

4. Conclusions

An evaluation of LHW pretreatment on the production of biomass-degrading enzymes, fermentable sugars and recovery of lignin was carried out using corncob. High recovery of hemicellulose-derived compounds (as xylo-oligosaccharides) was obtained into the hydrolysates (22 g/L at $\log(R_0)$ of 4.42). On pretreated solids, maximum of 65% cellulose and 22% lignin were recovered at $\log(R_0)$ of 4.64. Hydrolysates and pretreated solids improved the production of xylanases by *A. niger* and cellulases by *T. reesei*, respectively. On the other hand, EH of the pretreated solids recovered at least 65% fermentable sugars (achieving 100% glucose yield with 5% solid loading) and lignin with antioxidant activity (IC_{50}: 0.5–0.75 mg/mL).

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