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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):

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

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):

$$Yield_{XOS} = \mathrm{XOS}\frac{V}{Xn_{FS}}100 \quad (2)$$

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.