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A residue-free and effective corncob extrusion pretreatment for the enhancement of high solids loading enzymatic hydrolysis to produce sugars

Álvaro H.M. José [a], Esperidiana A.B. Moura [b], Durval Rodrigues Jr [c], Eduardo K. Kleingesinds [a], Rita C.L.B. Rodrigues [a,*]

[a] Department of Biotechnology, Engineering School of Lorena, University of São Paulo, 12602-810 Lorena, SP, Brazil
[b] Nuclear and Energy Research Institute, IPEN-CNEN/SP, 05508-000 São Paulo, SP, Brazil
[c] Department of Material Engineering, Engineering School of Lorena, University of São Paulo, 12612-550 Lorena, SP, Brazil

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ABSTRACT

To convert biomass into biofuel, pretreatment is the first stage required to de-structure lignocellulose – twin-screw extrusion is one of the viable pretreatment technologies. The enzymatic hydrolysis of corncobs pretreated with twin-screw extrusion to obtain sugar was evaluated. Corncob extrusion (115–130 °C; 14 rpm) was enhanced through the employment of additives (water and glycerol, 25:25, % w/w). By reproducing the response surface methodology (RSM) technique, the maximized glucose productivity (0.69 g L^{-1} h^{-1}) and conversion of cellulose to glucose (90.4 % w/w), as well as hemicellulose to xylose and arabinose (44.0 % w/w) were established with the dosage of the commercial enzymatic complex Cellic Ctec2 (32 FPU/$g_{dry\ lignocellulosic\ material}$) and solids loading (17.8 % w/w). Total sugar yield was of 471 kg (glucose 323 kg; xylose and arabinose 148 kg) for a dried corncob ton. Kinetic constants of the Michaelis-Menten model, V_{max} and K_m, for converting cellulose to glucose were of 6.00 % (w/w)/h and 22.59 $g_{cellulose}/L_{solution}$, respectively. A residue-free and effective corncob extrusion pretreatment enhanced high solids loading enzymatic hydrolysis to achieve a glucose-rich solution.

1. Introduction

Lignocellulosic materials have been extensively studied over the last three decades due to their increased capabilities of sugar and biofuel production. In addition to biofuels and sugars, other byproducts can be derived from these materials, such as chemicals, amino acids, nanocellulose and nanolignin, cellulose films, energy, etc. Thus, the concept currently referred to as *biorefinery* was created (Dou et al., 2017; Jin et al., 2018; Martínez-Ruano et al., 2018).

Costs required to obtain fuel from lignocellulosic materials are determined by multiple factors based on process sustainability, which range from conversion efficiency to biomass availability (Bozell, 2010). Among other agricultural residues, the use of corncob as lignocellulosic feedstock offers promising possibilities for the production of renewable energy. Corncob is one of the most abundant corn processing residues and corresponds to 11 % of the total vegetable mass (Carvalho, 1992). According to the Brazilian Institute of Geography and Statistics (IBGE), Brazil had a harvest of 88.17 million tons of corn in 2021 (IBGE, 2021). Therefore, corncob employed as lignocellulosic feedstock may be particularly suitable for the Brazilian agricultural scenario.

Lignocellulosic materials are composed of structural components (cellulose, hemicellulose, lignin, proteins, and inorganic molecules) and non-structural components, such as organic acids, non-structural sugars, and waxes (Gandam et al., 2022). The way lignocellulose is found in nature does not attract microorganisms that convert these polysaccharides into biomolecules with additional value. Regarding these facts, pretreatment is the first step when de-structuring lignocellulosic material and increasing the accessibility of its structure for enzymes (Liu et al., 2014). Biomass saccharification, or enzymatic hydrolysis of biomass, is the conversion process of cellulose to glucose or hemicellulose to xylose and other organic compounds. The conversion process is catalyzed by enzymes, cellulases, and hemicellulases, respectively (Pérez-Rodríguez et al., 2018; Zheng et al., 2015). A pool of enzymes is used for each enzymatic complex, which offers different cellulases: endocellulases, β-glucosidase, cellobiohydrolase, and others; the same process occurs for hemicellulases. Enzymatic digestibility of polysaccharides is affected by the variation of accessibility to enzymes, biomass loading, enzyme concentration, temperature, pH, etc (Chen

* Corresponding author.
E-mail address: ritaclb_rodrigues@usp.br (R.C.L.B. Rodrigues).

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et al., 2016). Pretreatment can be a physical, chemical, and biological process or a combination of all the previous ones. Twin-screw extrusion technology is commonly employed in the polymer and food industries and can be a viable method for pretreatment. However, for biomass to be properly extruded, employment of additives is required (glycerol, Tween 80, ethylene glycol, etc) to ease transport within the extruder, reducing equipment torque and effectively fibrillating the biomass cell walls (Ai et al., 2020; da Silva et al., 2013). Glycerol has a high boiling point (290 °C), which is fundamental when working with high-temperature processes such as extrusion and it is cheaper than other additives, resulting in better outcomes from extrusion when compared to Tween 80 or ethylene glycol (Moro et al., 2017). Extrusion, a thermophysical process, provides high shear force on one or two screws which get into contact with, and apply pressure on lignocellulosic biomass; hence, defibrillating the material (Konan et al., 2022), increasing its contact surface and enhancing enzyme accessibility during enzymatic hydrolysis process (Zheng and Rehmann, 2014). Despite the high rate of energy consumption that extruder machinery requires, the extrusion process may operate at high solids loading ($\geq$ 15.0 %) and is a continuous process (Duque et al., 2017). The thread design is a relevant factor for the material shearing step, as it may influence the amount of shear force inflicted on lignocellulosic biomass (Zheng et al., 2015). Several authors in laboratory literature have applied the twin-screw extrusion method associated with enzymatic hydrolysis to pretreat corncob and other biomasses over the last few years (Pérez-Rodríguez et al., 2018; Rehmann, 2015; Zhang et al., 2015).

Henceforth, this investigation evaluated sugar obtainment via the enzymatic hydrolysis of corncob pretreated by a pilot extruder design with intermeshed and co-rotational twin screws. The liquid fraction was researched through the extrusion pretreatment by varying the water and glycerol additive ratio. Extruded corncob, when in the best water and glycerol ratio among tested conditions, was hydrolyzed by the Cellic CTec2 enzymatic complex, associated (or not) to the Cellic HTec2 enzymatic complex. Then, the dosage of enzymes and the solids loading was optimized by response surface methodology (RSM) to obtain high values of effectiveness and conversion to sugars following previous steps. To further enhance the process, an evaluation of enzymatic hydrolysis kinetics was performed, using the conditions obtained through the optimization method that reduced the enzymatic hydrolysis reaction time.

2. Materials and methods

2.1. Preparation of corncob: extrusion pretreatment

The extrusion process was performed at the Chemistry and Environment Center of the Nuclear and Energy Research Institute (IPEN), São Paulo/Brazil. The process was carried out by a pilot twin-screw extruder (American Maplan Corporation-AMC) with intermeshed and possessed co-rotational movement screws. The biomass used was of *in natura* corncobs. A few tests with different particle sizes were performed and the particle size of 710 μm was chosen for further experimentation. Addition of glycerol to the corncob extrusion process was performed under three different conditions:

a) Extrusion of *in natura corncobs (CC) without the addition of additives (condition 1);*.
b) Extrusion of corncobs soaked in glycerol in the proportion of 50 % (w/w) (condition 2);.
c) Extrusion of corncobs soaked in different concentrations of water and glycerol additives, as mentioned next (conditions 3, 4, and 5). *In natura corncobs (CC) were soaked in an aqueous solution which contained different concentrations of glycerol (0 %, 10 %, and 50 % w/w), using a solid: liquid ratio of 1:1 (1 g of corncob in dry mass to 1 g of the glycerol solution in water). Thus, when corncob processing, the ratio (% w/w) of water:glycerol additives in the final product (soaked corncob) were 50:0 (condition 3); 45:5 (condition 4), and 25:25 (condition 5) named CC-0 %, CC-5 %, and CC-25 %, respectively. Following the twin-screw extrusion pretreatment, extruded corncobs were named ECC-0 %, ECC-5 %, and ECC-25 %, respectively.*

For all glycerol conditions tested, temperatures were maintained in the range of 115–120 °C within the feed zone and between 125 and 130 °C within compression and flow control zones. Screw speed was maintained at 14 rpm – Compression positively affects the development of shear forces and material flow within the extruder barrel. Cylinder temperature increases, when it comes to biomass processing, softening, by affecting flow pattern and residence time. Screw speed is responsible for developing the material's shear and average residence times.

2.2. Evaluation of the impact of glycerol present in the extruded corncobs within the enzymatic hydrolysis step applying the following enzymatic complexes: Cellic CTec2 and Cellic HTec2

2.2.1. Enzyme complexes

The following commercial enzyme complexes donated by Novozymes A/S (Curitiba, PR) were employed in the enzymatic hydrolysis process: a) Cellic CTec2: a combination of potent cellulases, containing a high level of β-glucosidases, with operational stability within the temperature range from 45 to 50 °C and pH range from 5.0 to 5.5; b) Cellic HTec2: a combination of endoxylanase with high specificity for soluble hemicellulose, as well as also having cellulase.

2.2.2. Sanitizing extruded corncobs

The extruded corncob obtained by the best extrusion conditions was submitted to a sanitization process to remove glycerol. In a 125 mL Erlenmeyer flask, 5 g of extruded corncob (dry weight) were added to 50 mL of distilled water. The mixture was agitated for 5 min. A supernatant sample was collected and analyzed by High Performance Liquid Chromatography (HPLC) to verify the absence of glycerol. The washing process was repeated 3 times for each sample to remove all glycerol. Extruded corncob was filtered using a cellulose filter. The filtered washed or unwashed extruded corncobs were used to research the enzymatic hydrolysis process.

2.2.3. Enzymatic hydrolysis of the extruded corncob

Corncob enzymatic hydrolysis was performed in a 125 mL Erlenmeyer flask, according to the following requirements: 5 g of the extruded corncob obtained by the best extrusion conditions (washed or unwashed), 0.48 g of Tween 80, 0.33 g of sodium azide, enzyme loading of 0.4 g, which corresponds to 25.25 FPU/g_{ECC} (Kleingesinds et al., 2018). A tampon solution of sodium citrate (50 mM, pH 4.8) was added to reach a total solution with 50 g. Enzyme complexes applied were the Cellic CTec2 and Cellic HTec2. Tests were performed in two enzymatic conditions, the first, using 100 % (w/w) of Cellic CTec2 and the second, a mixture composed of 90 % (w/w) Cellic CTec2 and 10 % (w/w) Cellic HTec2, as recommended by the Manufacturer. Enzymatic hydrolysis was performed in a shaker at 50 °C for 96 h, with an agitation speed of 150 rpm. The agitation was sufficient enough to properly mix the solution and maintain the extruded corncob submerged and in touch with the solution. Then, the enzymatic hydrolyzate obtained was autoclaved at 111 °C for 5 min to deactivate the enzymes and was then centrifuged at 2000 × *g* for 20 min. The supernatant was collected and analyzed via high-performance liquid chromatography (HPLC). Two control assays were carried out; in one of them, there were no enzymes to detect the possible release of sugars present in each cob sample, and in the other, there was no cob present to verify the existence of sugars released in the medium by the enzymatic complex.

The enzymatic cellulose conversion was calculated by Eq. (1):

$$\eta = ((m_{glucose} * f_{hc}) + m_{cellobiose} * f_{hcb})/(m_{initial} * y_{ic}) * 100 \quad (1)$$

In which:
η: enzymatic conversion of cellulose (%);
$m_{glucose}$: mass of glucose present in the enzymatic hydrolyzate (g);
$m_{initial}$: dry mass of lignocellulosic material before the hydrolysis step;
y_{ic}: cellulose content in the lignocellulosic material;
f_{hc}: cellulose hydrolysis factor (corresponding to 0.9);
f_{hcb}: cellobiose hydrolysis factor (corresponding to 0.95).
The enzymatic conversion of hemicellulose was calculated by Eq. (2):

$$\eta = (m_{xylose} + m_{arabinose}) * f_{hh}/(m_{initial} * y_{ih}) * 100 \quad (2)$$

In which:
η = enzymatic conversion of hemicellulose (%);
m_{xylose}: mass of xylose present in the hydrolyzate (g);
$m_{arabinose}$: mass of arabinose present in the hydrolyzate (g);
$m_{initial}$: dry mass of lignocellulosic material before the hydrolysis step;
y_{ih}: hemicellulose content in lignocellulosic material;
f_{hh}: hemicellulose hydrolysis factor (corresponding to 0.88).

2.3. Response Surface Methodology (RSM)

The best conditions obtained for the extrusion process (Section 2.1) and enzyme complexes (Section 2.2) were employed to optimize the conversion of cellulose to glucose (y_1) and glucose productivity (y_2) by applying response surface methodology (RSM). Conduction of enzymatic hydrolysis was performed as described in Section 2.2, with various enzyme dosages and solids loading (Table 4). Tests were carried out according to a complete experimental design 2^2 with centered face and 4 repetitions in the central point (CP). Statistical analysis of the enzymatic hydrolysis was done using Response Surface Methodology (RSM), which analyzed 2 independent variables: solids loading (x_1) and enzyme dosage (x_2) as well as 2 response variables: cellulose conversion to glucose (y_1) and glucose productivity (y_2). The factors, levels and trials are shown in Table 4. Factor levels were coded according to Eq. (3).

$$X = (V_R - \sum V_R/2)/((V_{R2} - V_{R1})/2) \quad (3)$$

In which: X is the encoded value of the variable;
V_R is the real value of the variable;
$\sum V_R/2$ is the mean for the variable's actual values.
Response surfaces were made using the Statistica program, version 6.0. Optimization required to maximize response variables (cellulose conversion into glucose and productivity into glucose) was achieved through the Minitab Program, version 17.

2.4. Determination of kinetic constants of enzymatic hydrolysis reactions

Kinetics of most reactions catalyzed by enzymes follow the Michaelis-Menten model (Kari et al., 2017). Determination of kinetic constants (K_m and V_{max}) of the enzymatic hydrolysis reaction was carried out in the optimized conditions obtained through RSM methodology. During the enzymatic reaction, 15 samples were collected at regular time intervals and the reaction was carried out for 96 h. Kinetic constants (K_m and V_{max}) were determined by the Lineweaver-Burk graphical method, plotting 1/[S] as a function of $1/V_0$ (Shuler and Kargi, 2002). Maximum rate (V_{max}) was obtained in the linear region of the conversion of cellulose to glucose versus the reaction time curve.
The Michaelis-Menten Equation was used for the Michaelis constant (K_m) calculation (Eq. (4)).

$$V_o = V_{max} * S/(K_m + S) \quad (4)$$

In which:
V_o = Rate at a given time (% (w/w) h^{-1});
V_{max} = Maximum rate of the enzymatic hydrolysis process (% (w/w) h^{-1});
[S] = Glucose concentration at a given time (g L^{-1});
K_m = Michaelis-Menten constant (g L^{-1}).

2.5. Chemical analysis of raw and pretreated biomass

In natura corncobs (CC) and pretreated corncobs obtained by the best extrusion conditions were chemically characterized for their contents of cellulose, hemicellulose, lignin, ashes, and extractives based on the methodology followed in the Laboratory Procedures (LAP) adopted by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008).

2.6. Determination of sugars, glycerol, acetic acid, and HMF

Samples for glycerol content (% w/w) were prepared by adding 0.20 g of dry-pretreated corncob obtained at the best extrusion conditions and 4.80 g of distilled water into a 15 mL Falcon tube. The tube was manually agitated every 5 min for 1 h. The sample was centrifuged (6000 rpm for 20 min) and the supernatant was then separated from the solids and diluted for glycerol quantification by HPLC. This procedure was then repeated 3 times in to remove and quantify all glycerol present in the sample. Samples were prepared in triplicate.
Concentrations of sugars (glucose, L-arabinose, and xylose), glycerol and acetic acid were determined by High-Performance Liquid Chromatography (HPLC). The equipment used was a Bio-Rad (Hercules, CA) Aminex HPX-87H column (300 × 7.8 mm) at 45 °C with H_2SO_4 (5 mM) as eluent and at a flow rate of 0.6 mL min^{-1}. A sample volume of 20 μL was used in each injection. The refractive index detector used to quantify the products was a Waters 410 model at 35 °C (Rodrigues et al., 2001). The diluted samples were filtered on Sep Pak C18 filters (Millipore) before injection. In addition, before application, the eluent was vacuum filtered on a cellulose ester membrane (0.45 μm pore, 47 mm diameter) (Millipore) and was degassed in an ultrasound bath (Thornton) for 25 min (Rodrigues et al., 2001).
Concentration of 5-hydroxymethylfurfural (HMF) in corncob enzymatic hydrolysates was determined by High-Performance Liquid Chromatography (HPLC) according to Rodrigues et al. (2001), observing the following conditions: Waters Resolve™ 5 μ Spherical C18 column (3.9 × 300 mm); column temperature, 25 °C; Waters 2487 ultraviolet detector at 276 nm wavelength; eluent, acetonitrile/water (1:8) solution with 1 % acetic acid; a volume of injected sample, 20 μL. Samples were adequately diluted and filtered through Swinnex filters with a cellulose ester HA membrane, 0.45 μm pore and 13 mm diameter (Millipore). In the eluent composition, deionized water was vacuum filtered using an HA membrane in cellulose ester, 0.45 μm pore, 0.47 mm in diameter (Millipore) – other components such as acetic acid and acetonitrile were added in appropriate proportions to properly filtered water. Then, the eluent was degassed in an ultrasonic bath (Thornton) for 15 min and left to rest for 10 min before being applied (Rodrigues et al., 2001).

2.7. Analysis by Scanning Electron Microscopy (SEM)

Solids were fixed on a sample holder with the aid of a carbon double-face conductive tape. For grounding, a 10 nm-layer of gold was deposited on the samples to increase the material's conductivity and then they were submitted to a Scanning Electron Microscope, model LEO1450VP. Analyses were performed in the backscattered-electrons mode and the micrographs were obtained with 100 × and 1000 × magnifications for *in natura* corncob (CC added with glycerol) and the pretreated corncob obtained by the best extrusion condition.

2.8. Crystallographic analysis by X-ray diffraction

X-ray diffraction enabled the crystallographic analysis of *in natura* corncob (CC) biomass and the pretreated corncob obtained by the best extrusion condition. A Panalytical diffractometer, model Empyrean,

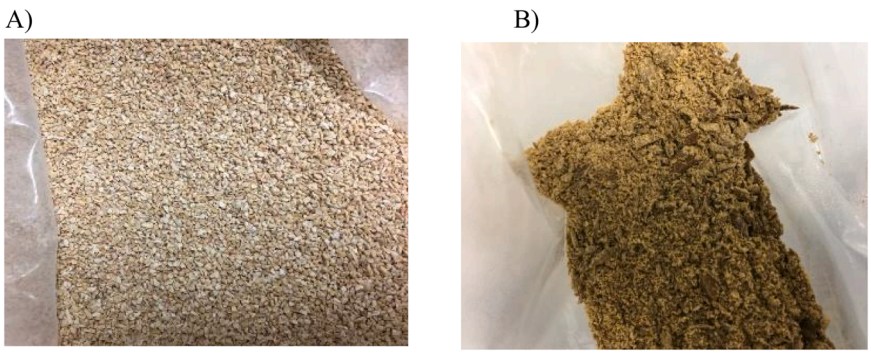

Fig. 1. A) *In natura* corncob (CC) and B) Extruded corncob with additives (water and glycerol) (ECC-25 %).

with Kα copper radiation, Ni filter, initial angle of 10°, final angle of 90°, angular pitch of 0.02°, exposure time per step of 80 s and a quarter-degree crevice was employed. Comparing the crystallinity of Avicel (100 % cellulose) with corncob, the crystallinity value obtained was of 39.46 %, according to Eq. (5) (Rodrigues et al., 2022; Mori, 2015):

$$I_c = (I_{002} - I_{101})/I_{002} * 100 \quad (5)$$

In which:

I_c: measured crystallinity index (%);

I_{002}: Peak intensity due to crystalline planes (002), with $2\theta = 22.4°$;

I_{101}: Intensity of the peak due to the crystalline planes (101), with $2\theta = 16.4°$.

The difference between the expected crystallinity I_{ce} and the experimentally obtained crystallinity I_c leads to the cellulose's decreased crystallinity (Dc), as indicated in Eqs. (6) and (7) (Mori, 2015):

$$I_{ce} = CQ_{celulose} * 39.46/CQ_{celulose\ in\ natura} \quad (6)$$

$$D_c = I_{ce} - I_c \quad (7)$$

In which:

I_{ce}: Expected crystallinity index (%);

$CQ_{cellulose}$: Chemical composition of the cellulose in corncob samples (% w/w);

$CQ_{cellulose\ in\ natura}$: Chemical composition of the cellulose of *in natura* corncob samples (% w/w);

D_c: Decreased crystallinity (%);

I_c: Crystalline index obtained experimentally (%).

3. Results and discussion

3.1. Twin-screw extrusion

3.1.1. Extrusion pretreatment of the corncob

The attempt to use the corncob without addition of any solvents (condition 1) caused blockages in equipment set at corncob higher feed rates. Thus, by reducing the feed rate, a heterogeneous material was obtained, probably due to lack of screw filling in the extruder and consequently application of a low shear force. Furthermore, the twin-screw extruder clogged and caused burnt points on the biomass.

Utilization of glycerol as an additive to the twin-screw extrusion (condition 2), led the process to face few challenges. Extruded material was almost intact, indicating that the concentration of glycerol (50 % m/m) may have reduced the shear effects inside the extruder. The material was retained inside the feed zone, probably due to the decreased friction force in this region caused by a high glycerol concentration.

The extrusion process of the corncobs was not efficient when observing conditions 3 and 4 (0 % and 5 % [w/w] glycerol, respectively). In these cases, the material left the extruder intermittently, pressurized and with a dark pigmentation. Furthermore, the extruder exit thread became clogged after a while.

Nonetheless, continuous and homogeneous processing was obtained by observing condition 5 (25 % w/w glycerol), which meant adding the water and glycerol additives in ratios (% w/w) of 25:25 to *in natura* corncob. This procedure increased the initial *in natura* corncob moisture content from 5.94 % to 27.25 %. After extrusion, corncob moisture reached values of 7.24 %, while glycerol concentration went from 21.44 % (w/w), to an increased value of 29.08 % (w/w), due to the escape flow of water present within corncobs. Therefore, water and glycerol favored thermal softening while under cylinder temperature, maintaining good shear speed, friction, and residence time.

Fig. 1 exhibits *in natura* corncob (CC) and extruded corncob (ECC-25 %) using 25 % (w/w) glycerol obtained through the extrusion process. It is possible to notice an alteration in the color, texture, and particle size after the twin-screw extrusion pretreatment.

Glycerol has a high boiling point (290 °C), which is fundamental to high-temperature processes, like extrusion, in which its cost as an additive can be reduced due to it being a by-product of biodiesel production and leads to better extrusion results when compared to Tween 80 or ethylene glycol (Moro et al., 2017). In addition, according to da Silva et al. (2013), additive glycerol, due to its affinity towards cellulose, can efficiently defibrillate the cellulosic walls of biomass, facilitating transportation along the extruder screw.

3.1.2. Chemical composition analysis

Glycerol concentration of extruded corncob (ECC-25 %) was 28.7 %. To analyze the alteration in the concentration of the fractions of the corncob before and after the extrusion process, a factor of 1.4 was applied to the fractions present in the ECC-25 %. Chemical characterization of both *in natura* (CC) and extruded corncob (ECC-25 %) are shown in Table 1.

Table 1
Chemical composition of *in natura* corncobs (CC) and corncobs after extrusion pretreatment with glycerol (ECC-25 %).

Sample	Cellulose % (w/w)	Hemicellulose % (w/w)	Lignin % (w/w)	Ash % (w/w)	Extractives % (w/w)	Total % (w/w)
CC	36.15 ± 0.64[b]	41.07 ± 0.82	16.70 ± 2.80	1.54 ± 0.37	5.51 ± 0.05	100.97 ± 3.03
ECC-25 %[a]	36.72 ± 0.24	37.51 ± 0.09	16.66 ± 1.33	2.24 ± 0.78	6.59 ± 0.78	102.02 ± 2.34

[a] Chemical composition of extruded corncobs with glycerol additives (25 % m/m) after correction for mass presence of glycerol in extractives (mass recovery value used in the correction was 1.4).

[b] The text is overlaping, maybe change the length of the column width

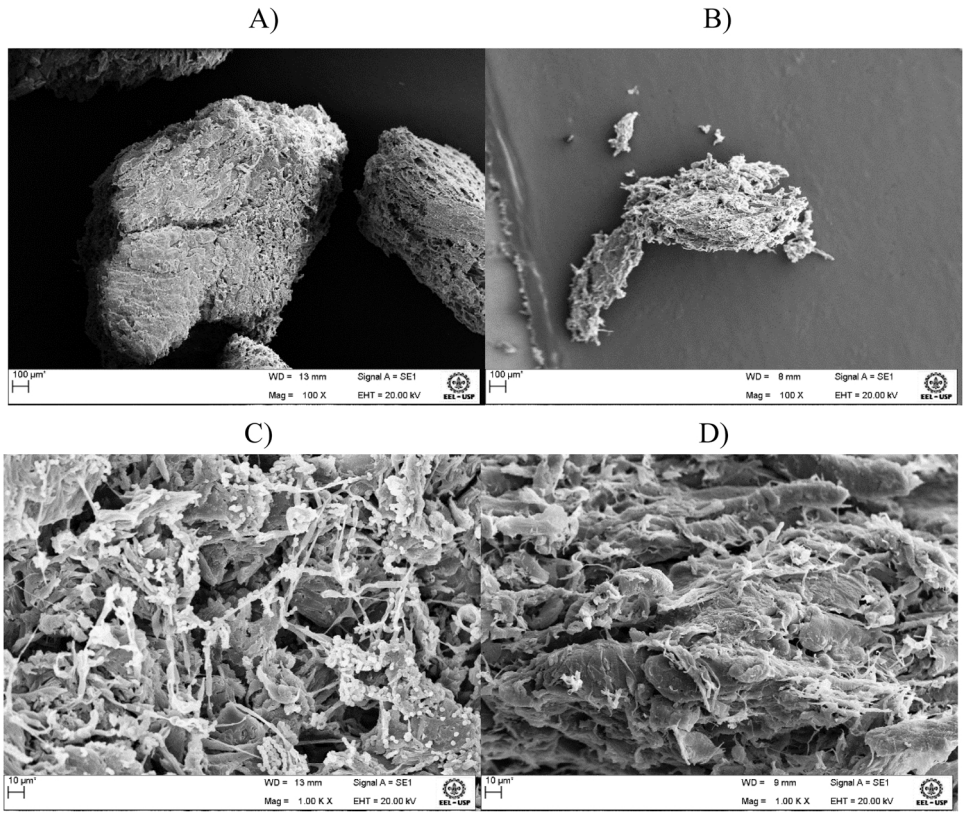

Fig. 2. Scanning electron microscopy (SEM) analysis of extruded and *in natura* corncobs: A) *in natura* corncob (CC-25 %), 100 × magnification; B) extruded corncob, 100 × magnification (ECC-25 %); C) *in natura* corncob (CC-25 %), 1000 × magnification; D) extruded corncob, 1000 × magnification (ECC-25 %).

In natura corncob (CC) had a chemical composition like the one presented in other literature papers, showing 35–45 % cellulose, 35–42 % hemicelluloses, and 5–15 % lignin (Anwar et al., 2014). As shown in Table 1, composition of the extruded corncob fractions has not deviated significantly after the extrusion process, except for hemicellulose, which presented a slight reduction of 8.5 %. This fact is probably due to the exposure of the acetic acid present in the hemicellulose to elevated temperatures, which could initiate an acid hydrolysis reaction within the corncob.

Low ash content in the CC (1.54 % ± 0.37) and ECC-25 % (2.24 % ± 0.78, Table 1) represents an advantage for corncob use in bioconversion processes when compared to other lignocellulosic biomasses, such as rice straw and wheat straw, which have ash contents of 17.5 % and 11.0 %, respectively (Pandey et al., 2000); since a lower ash concentration means a greater concentration of other material components.

According to Alvira et al. (2010), extrusion does not change the chemical composition of biomass; however, it increases the accessibility enzymes have to cellulose, amplifying the biomass surface area and defibrillating cellulose fibers, thus reducing the fibers' degree of polymerization and crystallization.

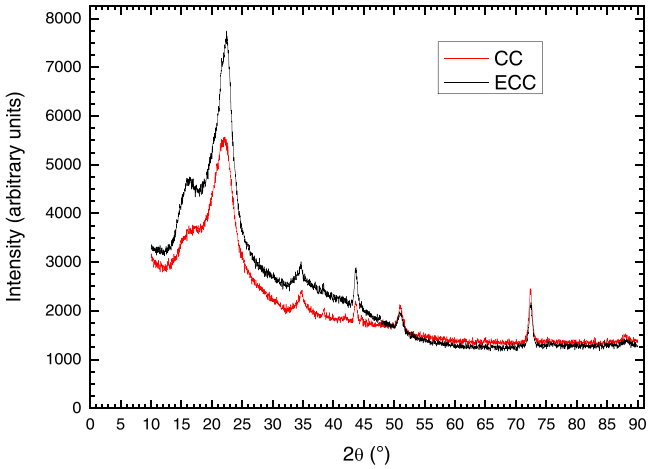

Fig. 3. X-ray diffractograms of *in natura* corncobs with added glycerol (CC-25 %), and extruded corncobs (ECC-25 %).

3.1.3. Scanning electron microscopy (SEM)

Fig. 2 shows the micrographs obtained for *in natura* corncob (CC-25 %) and extruded corncob – condition 5 (ECC-25 %).

After extrusion, corncob presented a fibrillar structure with tiny irregular pores on the surface, which may have been caused due to the evaporation of water in the biomass. Furthermore, a reduction in the length of cellulose fibers could be observed.

According to Zheng et al. (2016), pretreatment by extrusion increases the homogeneity of the biomass and provides shear and heat through its structure. Water inside the particles can evaporate due to sudden pressure reduction at the end of the extrusion process, resulting in a porous structure that can be more permeable to enzymatic action, enhancing the efficiency of enzymatic hydrolysis (Zheng et al., 2016).

In addition to the extrusion process altering biomass structure, glycerol is an additive with an affinity for cellulose and can effectively fibrillate the biomass cell wall at sub-micro or nanoscales; therefore, opening up the cell wall structure to improved enzymatic accessibility and decreasing the extrusion torque (J.-W. Lee et al., 2009).

Micrographs of the CC-25 % (Fig. 2, A, and C) and the ECC-25 % (Fig. 2, B, and D) show globular structures, which can be attributed to glycerol and water adhered to the surface of these materials. However, after the extrusion process, globules were mostly located on the inside of the biomass and with relatively smaller sizes than *in natura* material. The ECC-25% was used for further steps.

Table 2
Crystallinity of *in natura* corncobs (CC) and after the corncob extrusion pretreatment (ECC-25 %).

Samples	Crystallinity index (I_c) (%)	Expected crystallinity index (I_{ce}) (%)	Decreased crystallinity (D_c) (%)
CC	32,75	–	–
ECC-25 %	38,68	40,08	1,4

3.1.4. Crystallographic analysis by X-ray diffraction (XRD)

Corncob structures *in natura* (CC-25 %) and extruded corncobs (ECC-25 %) were analyzed for their crystallinity. The crystallinity index of a lignocellulosic material measures the relative amount of crystalline cellulose present in the overall solid. Crystallinity index is used to determine the crystallinity of the cellulose once the determination of such index by XRD presents only relative values for the crystalline and amorphous regions (Park et al., 2010). Crystalline structure of cellulose can be altered by different pretreatments, with a rupture in the cellulose fibril g L^{-1} chains of its inter and intramolecular hydrogen bonds (Park et al., 2010).

Results of X-ray diffractometry (Fig. 3) show that corncobs *in natura* (CC-25 %) and extruded (ECC-25 %) have typical allomorphic characteristics of cellulose I and cellulose II at $2\theta = 22.4°$ and $2\theta = 16.4°$, respectively. After extrusion pretreatment, intensities of all their peaks had increased (Fig. 3). For both corncobs, CC-25 % and ECC-25 %, the crystalline peak ($2\theta = 22.4°$) predominated over the amorphous peak ($2\theta = 16.4°$) (Kumar et al., 2010).

Crystallinity indexes I_c obtained are 32.72 % and 38.68 % for the CC-25 % and ECC-25 %, respectively, evidencing an increase of the crystallinity caused by the removal of one of the amorphous components - the hemicelluloses – consistent with the chemical analysis of the corncobs shown in Table 2, in which an 8.5 % removal of hemicellulose was observed. According to Eq. (3), the calculated I_{ce} for ECC-25% was 40.08, probably due to a low removal of its amorphous part, evidenced by a slight decrease (1.4 %) in its crystallinity after the extrusion pretreatment.

Increased crystallinity of the material corresponds to the more significant presence of crystalline cellulose, which is the most difficult to be enzymatically hydrolyzed and provides increased stiffness for the biomass structure. However, the relationship between the crystallinity index of extruded biomass and its corresponding enzymatic hydrolysis rate is not well understood. Biomasses with a high crystallinity index may not negatively affect the rate of enzymatic hydrolysis (da Silva et al., 2013).

3.2. Enzymatic hydrolysis

3.2.1. Influence of glycerol and different enzymes

Corncobs *in natura* (CC-25 %), as well as the ones pretreated by extrusion (ECC-25 %) before (UW) and after (W) the sanitation step, were enzymatically hydrolyzed using the enzyme complexes: Cellic Ctec2 and Cellic Htec2, which, according to the Manufacturer, should be used in the proportion of 90–10 %, respectively, or only Cellic Ctec2 (Table 3).

3.2.1.1. Cellic Ctec2 and Cellic Htec2. For the sanitized extruded corncob (ECC-25 %-W), cellulose conversion to glucose and hemicellulose conversion to xylose and arabinose were of 63.2 % and 95.8 %, respectively. These values were slightly higher for the unwashed extruded corncob (ECC-25 %-UW), 67.4 % for cellulose conversion and approximately 100 % for hemicellulose conversion while using both enzymes (Cellic Ctec2 and Htec2). Furthermore, glucose released to the reaction medium was higher for ECC-25 %-UW with both enzymes (25.8 g L^{-1}) than for ECC-25 %-W (24.6 g L^{-1}). In these cases, concentrations of xylose and arabinose were of 45.1 g L^{-1} and 41.9 g L^{-1}, respectively (Table 3).

It should be noted that control tests were carried out to verify the presence of sugars both in the unwashed extruded material and in the enzymatic complex itself; in these cases, tests were carried out without enzymes and corncobs, respectively. These tests showed no detectable sugars in the enzymatic complex nor adsorbed on the corncobs pretreated by extrusion.

For extruded corncobs (ECC-25 %), indifferent to biomass sanitation done to remove glycerol, the use of both enzymes' complexes (Cellic Ctec2 and Cellic Htec2) improved conversion of hemicellulose to xylose and arabinose (Table 3), and, consequently, a more significant amount of xylose and arabinose released may have interfered with the conversion of cellulose to glucose. Five carbons of sugars can inhibit the action of cellulases present in the Cellic CTec2 (Kumar and Wyman, 2009; Mes-Hartree and Saddler, 1983). According to Zheng et al. (2016), removing the hemicellulose fraction is one of the pretreatment objectives once xylose interferes with cellulase enzymatic activity.

3.2.1.2. Cellic Ctec2. To verify if xylose release to the reaction medium could be hindering the conversion of cellulose to glucose, experiments were carried out applying only the enzymatic complex Cellic Ctec2 and unwashed corncobs: *in natura* (CC-25 %-UW) and extruded (ECC-25 %-UW, in which less glucose was released by using Cellic Ctec2 and Cellic Htec2) (Table 3).

For sanitized extruded corncob (ECC-25 %-W), Cellic Ctec2 enzyme complex favored cellulose conversion to glucose (94.0 % w/w) by reducing (45.66 %) the hemicellulose conversion to xylose and arabinose when compared to the application of Cellic Ctec2 and Cellic Htec2

Table 3
Conversion of cellulose and hemicellulose into sugars during enzymatic hydrolysis of *in natura* corncobs (CC-25 %) and after pretreatment by extrusion (ECC-25 %) before (UW) and after (W) sanitation using enzymatic complexes Cellic Ctec2 and Cellic Htec2 or only Cellic Ctec2 (Novozymes).

Samples	Cellobiose (g L^{-1})	Glucose (g L^{-1})	Xylose + Arabinose (g L^{-1})	Conversion of cellulose into glucose % (w/w)	Conversion of hemicellulose into xylose + arabinose % (w/w)
CC-25 %-UW (Ctec2/Htec2)[a]	0[b].00	20.4	12.1	51.0	24.8
CC-25 %-UW (Ctec2)	0.00	17.30	11.31	43.21	23.11
ECC-25 %-W (Ctec2/Htec2)[a]	2.4 ± 0.1	24.6 ± 0.2	41.9 ± 0.5	63.2 ± 1.6	95.8 ± 2.7
ECC-25 %-UW (Ctec2/Htec2)[a]	2.3 ± 0.0	25.8 ± 0.0	45.1 ± 1.0	67.4 ± 0.1	101.9 ± 2.1
ECC-25 %-UW (Ctec2)	1.8 ± 0.1	36.6 ± 3.7	23.7 ± 1.2	94.0 ± 3.0	55.4 ± 3.0

[a] Cellic CTec2 (90 %w/w) and Cellic HTec2 (10 %w/w) ratio according to Novozymes.
[b] Same problem with table 1, text is ovelaping. The width of the columns should be changed

Table 4
Real and coded values for a complete 22 factorial design with centered face (4 points) and 4 repetitions at the center point for the control variables: enzyme dosage (x1) and solids loading (x2) and the response variables: conversion of cellulose to glucose (y1) and glucose productivity (y2).

Trials	Enzyme dosage (FPU/g) x_1	ECC-25 %-UW % (w/w) x_2	x_1	x_2	Cellulose conversion to glucose % (w/w) y_1	Glucose productivity (g L^{-1} h^{-1}) y_2
1	11.00	7.50	-1	-1	84.68	0.270
2	31.00	7.50	+1	-1	95.59	0.305
3	11.00	20.50	-1	+1	64.99	0.571
4	31.00	20.50	+1	+1	79.98	0.696
5	11.00	14.00	-1	0	69.43	0.414
6	31.00	14.00	+1	0	93.75	0.558
7	21.00	7.50	0	-1	87.53	0.279
8	21.00	20.50	0	+1	70.39	0.615
CP1	21.00	14.00	0	0	96.65	0.576
CP2	21.00	14.00	0	0	90.96	0.543
CP3	21.00	14.00	0	0	95.09	0.565
CP4	21.00	14.00	0	0	95.00	0.566

simultaneously (Table 3).

For *in natura*, unwashed corncob (CC-25%-UW), the released sugars (cellobiose, glucose and xylose + arabinose) and hemicellulose conversion to xylose and arabinose were similar after enzymatic hydrolysis by simultaneously applying Cellic Ctec2 and Cellic Htec2 or only Cellic Ctec2 (Table 3). However, cellulose conversion to glucose for CC-25 %-UW was favored (18.12 %) by using Cellic Ctec2 and Cellic Htec2 simultaneously (Table 3).

Extrusion reduced the size of the fibers, which may have led to the increase in saccharification regarding *in natura* biomass due to a greater contact area between enzymes and cellulose; thus, facilitating enzymatic action. These results for ECC-25 % were found even with a slight decrease in the crystallinity (1.4 %) of pretreated material and without addition of strong chemicals to the overall extrusion process. Similar results were obtained by Y. Zhang et al. (2012) when pretreating corn husks in a twin-screw extruder, as well as by S.-H. Lee et al. (2009), whom mentioned the increased surface area as a more important factor than the reduction of the crystallinity index when regarding improvements to the saccharification of the biomass. Deconstruction of the cell wall observed by SEM as well as the increase in surface area were considered responsible for the improvements in enzymatic saccharification in a research led by Y. Zhang et al. (2012).

The following experiments were carried out with extruded and unwashed corncobs (ECC-25 %-UW), since glycerol did not interfere in the hydrolysis process and enzymatic complex Cellic Ctec2 improved glucose rich-solution.

3.2.2. Response surface methodology

Enzymatic hydrolysis is an expensive part of the process, increasing costs and making several processes unfeasible (Rai et al., 2019; Yang et al., 2018). To reduce future operating costs and increase glucose production, enzyme dosage, and solids loading of the ECC-25 %-UW were analyzed. Optimal enzyme dosage and solids concentration were searched for; and the cellulose conversion to glucose and glucose productivity were used as response variables. Table 4 shows results obtained by the response surface methodology applied to enzymatic hydrolysis of extruded corncob.

As shown in Table 4, higher enzyme loading leads to higher cellulose conversion and glucose productivity. Even though higher values of solids loading increase glucose productivity, using fewer solids might increase the cellulose conversion. As expected, the highest cellulose conversion (95.6 % [w/w]) was obtained by maximizing the enzymes loading (31 FPU/g) and minimizing solids loading (7.5 % [w/w]). However, to obtain the highest productivity (0.696 g L^{-1} h^{-1}), maximizing enzymes and solids loading is necessary. Intermediate results were obtained by central points, which corroborate the aforementioned hypothesis.

Therefore, to achieve the highest productivity (around 0.6 g L^{-1} h^{-1}) and cellulose conversion to glucose (higher than 90 % [w/w]), it is necessary to use an intermediate value between the two control variables, which is discussed next. It is also known that solids loading inferior to 15 % (w/w) is not economically viable for biomass enzymatic saccharification (Jørgensen et al., 2007).

3.2.3. Process optimization

The following equations describe the conversion of cellulose to glucose (Eq. (8)) and glucose productivity (Eq. (9)). Such equations contain the coded value for the two variables: enzyme dosage (x_1) and solids loading (x_2). Reparameterized models predicted these equations as a function of the coded variables, which contained only statistically significant terms.

$$y_1 = 90.1467 + 8.37x_1 - 8.74x_2 - 9.62x_2^2 \quad (8)$$

$$y_2 = 0.5375 + 0.0506x_1 + 0.1716x_2 - 0.081x_2^2 \quad (9)$$

Eq. (8) implies that a higher enzyme dosage (x_1) results in greater cellulose saccharification. However, an increased number of solids loading harms cellulose saccharification, as both linear and quadratic constants for solids loading factor (x_2) are negative.

Eq. (9) shows that both linear variables, x_1 and x_2, showed a positive signal, implying that a higher solid loading ratio and enzymatic dosage increased glucose productivity. The negative value of the constant multiplying the quadratic x_2 term does not influence the glucose productivity significantly, as the constant multiplying the linear x_2 factor is higher and positive.

The optimal region for the cellulose conversion can be obtained by using 29–31 FPU/g of enzymes and 10.1–14 % (w/w) of solids loading. Enzyme dosage from 19 to 31 FPU/g and solids loading from 16.6 % to 20.5 % (w/w) can maximize glucose productivity at a range of high values.

Fig. 4 shows response surfaces obtained using conditions proposed for enzymatic hydrolysis of extruded corncob.

By analyzing the response surfaces, one can observe regions with higher cellulose conversion to glucose and glucose yield. Regarding cellulose to glucose conversion, the optimal region is achieved at an enzyme dosage higher than 28 FPU/g, while at values below 18 % (w/w) for solids loading. Optimal region for glucose productivity can be found for enzyme dosages higher than 20 FPU/g with solids loading greater than 16 % (w/w).

Response surface analysis methodology assisted in the determination of the optimal conditions for independent variables (enzyme dosage and solids loading) regarding the enzymatic hydrolysis of extruded corncobs. Using the MINITAB Software, the conversion of cellulose to glucose (> 90 % [w/w]) and maximum glucose productivity were stipulated as optimization parameters.

Optimized values for the independent variables, enzyme dosage (x_1) and solids loading (x_2), are, respectively, 31 FPU/g and 17.8 % (w/w), which corresponds to the coded values of + 1.00 and 0.64, respectively.

Experimental procedures performed at optimized conditions were suggested by statistic optimization software. Predicted results calculated for the response variables, cellulose conversion to glucose (y_1) and productivity of glucose (y_2), were of 90.12 ± 3.39 % (w/w) and 0.66 ± 0.02 g L^{-1} h^{-1}, respectively. Experimental results were of 91.84 % (w/w) and 0.69 g L^{-1} h^{-1}, respectively. Further experimental results obtained for cellulose conversion to glucose and glucose productivity were smaller than 5% of the predicted results. Therefore, it is reasonable to say that the model obtained faithfully represents the enzymatic hydrolysis of extruded corncobs.

Table 5 shows the partial composition of the corncob hydrolysate obtained under optimum conditions of the enzymatic hydrolysis process

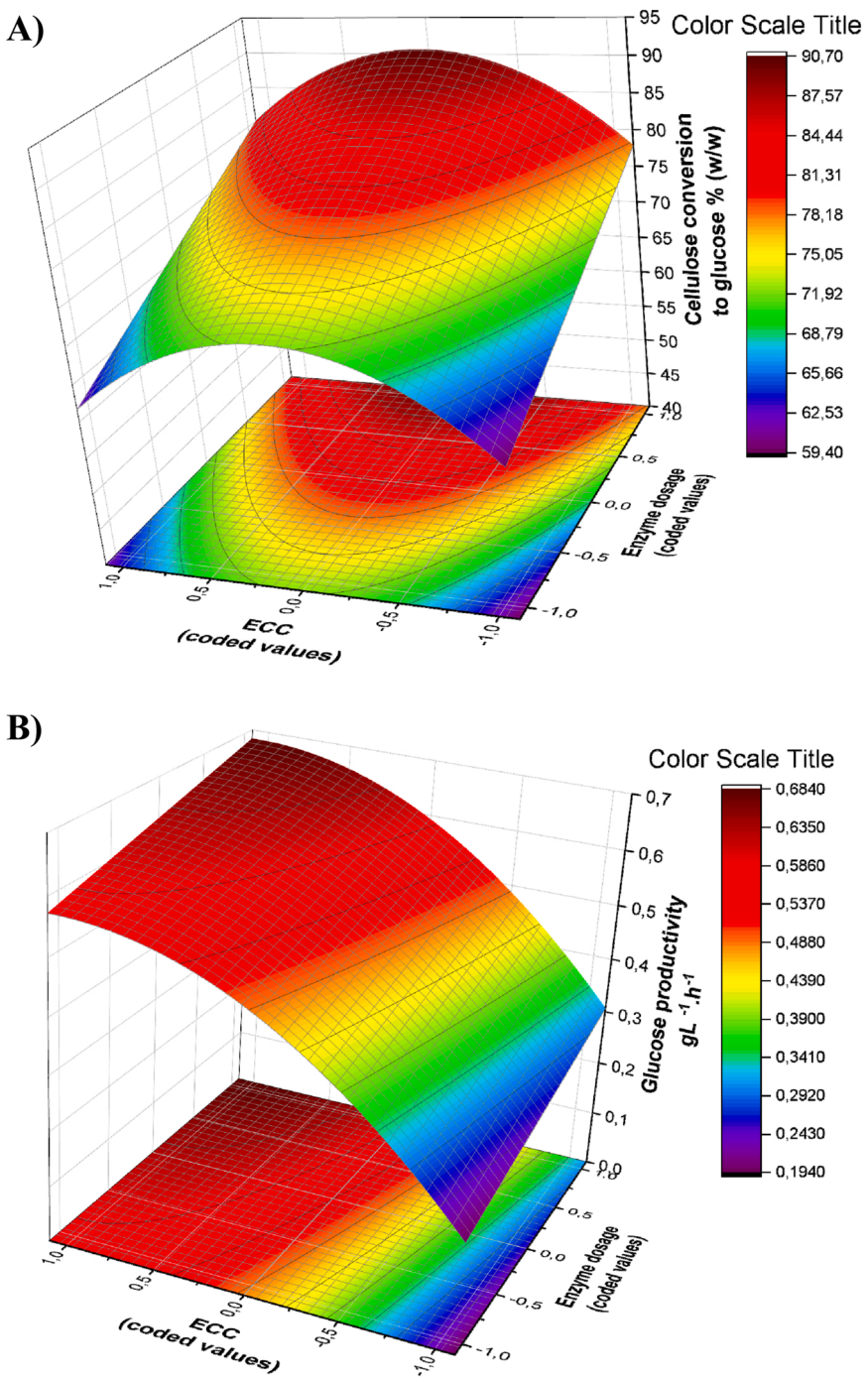

Fig. 4. A) Response surfaces and contour curves for: A) conversion of cellulose to glucose (%); B) glucose productivity (g L^{-1} h^{-1}), both as a function of coded values for enzyme dosage (x1) and solids loading (x2).

Table 5
Partial composition of corncob hydrolysates obtained under the optimal conditions of the enzymatic hydrolysis process.

Sample	Glucose (g L^{-1})	Cellobiose (g L^{-1})	Xylose + Arabinose (g L^{-1})	Acetic acid (g L^{-1})	Glycerol (g L^{-1})	HMF (g L^{-1})
Optimized condition	57.84	2.904	26.597	0.35	75.984	0.027

of corncob pretreated by extrusion. Under these conditions, the corncob hydrolysate, in addition to containing glucose (57.84 g L^{-1}), showed a high concentration of xylose (26.597 g L^{-1}), which corresponded to a xylose mass yield of 41.58 %. The hydrolysate also had small concentrations of cellobiose (2.904 g L^{-1}).

The extrusion process has a significant advantage: the non-formation of fermentation inhibitors. However, in the corncob hydrolysate, low concentrations of acetic acid (0.35 g L^{-1}) and 5-hydroxymethylfurfural (HMF) (0.027 g L^{-1}) were found (Table 5), which were probably formed during the enzyme deactivation after enzymatic hydrolysis (autoclaving

Table 6
Monomeric sugars obtained from corncobs pretreated by extrusion and after enzymatic hydrolysis under optimized conditions set by response surface methodology (RSM) as well as non-optimized conditions (ECC-25 %-UW, Ctec2).

Sample	Glucose		Xylose + Arabinose		Total sugars***	
	g L^{-1}*	g g^{-1}**	g L^{-1}*	g.g^{-1}**	g L^{-1}*	g g^{-1}**
Optimized condition	57.84	0.32	26.60	0.14	84.44	0.47
ECC–25 %-UW	36.57	0.32	23.71	0.21	60.27	0.54

g L^{-1}* = grams of sugars per liter of enzymatic hydrolysis solution.
g g^{-1}** = grams of sugars per gram of dry lignocellulosic material.
Total sugar*** = refers to the sum of glucose + xylose + arabinose.

at 111 °C for 15 min). However, low concentration of inhibitors has no impact on fermentation processes (Bondesson and Galbe, 2016; Duque et al., 2017; Uppugundla et al., 2014). The extrusion process only produces fermentation inhibitors in cases where extrusion is carried out in association with other pretreatments, in addition to also depending on temperatures applied (Singh et al., 2014). Geberekidan et al. (2019) used 25.00 % (w/w) (40 % higher than 17.8 % [w/w]) of solids loading at the enzymatic hydrolysis of the corn stover pretreated with acid, obtaining a medium containing similar amounts of sugars, 57.8 g L^{-1} of glucose and 15.6 g L^{-1} of xylose, but higher values for cellobiose (13.4 g L^{-1}) and acetic acid (1.2 g $L^{-1}$of acetic acid).

The high concentration of glycerol (75.984 g L^{-1}) in the hydrolysate is due to the fact that the extruded corncob was not sanitized; although, its presence did not interfere with the saccharification step (data aforementioned).

Table 6 shows the concentration of monomeric sugars obtained from corncobs pretreated by extrusion and after enzymatic hydrolysis under optimized and non-optimized conditions (ECC-25 %-UW, Ctec2) via response surface analysis methodology.

Optimized condition favored the obtainment of soluble sugars (g L^{-1}) by 40 %. Thus, it was possible to obtain a solution richer in soluble sugars (84.44 g L^{-1}) (Table 6), using the same medium volume. In this condition, higher solids load (17.8 % w/w) was used than in the non-optimized condition (10 % m/m) (ECC-25 %-UW, Cellic Ctec2); therefore, there was an increase of 58.20 % and 12.19 % in glucose and xylose and arabinose concentrations, respectively (Table 6).

Using RSM analysis, it was possible to increase the solid concentration, keeping the yield from the amount of glucose per gram of dry corn practically constant (~ 0.325 w/w) with a reduction (30.10 %) in the yield of the amount of xylose and arabinose per gram of dry corncob. As a result, the yield in total sugars per gram of dry corncob (0.471 w/w) was reduced by 12.78 % when compared to the condition not optimized by RSM.

These results showed that for the optimized condition (dosage of enzymes of 32 FPU/g dry lignocellulosic material and solids loading of 17.8 % w/w), it was possible to obtain 471 kg of total sugars (323 kg of glucose and 148 kg of xylose and arabinose) for each ton of corncob on a dry basis.

For this optimized condition, enzymatic conversion of cellulose to glucose was 90.12 % and the enzymatic conversion of hemicellulose to xylose and arabinose was 44.0 %. Thus, to improve total sugar yield, it is necessary to optimize hemicellulose conversion.

Cellulose accessibility can be increased mainly through efficient biomass pretreatments, which lead to partial removal of cell wall components, e.g., lignin or xylan from the lignocellulose matrix (Mosier et al., 2005). To increase the overall sugar yield from biomass, pentoses - mainly xylose - need to be recovered along with glucose. Depending on pretreatment conditions, xylose can be recovered partially from the pretreatment liquor and partially after the enzymatic hydrolysis of pretreated biomass (Mosier et al., 2005).

Imman et al. (2018) made a scientific review of corncob enzymatic

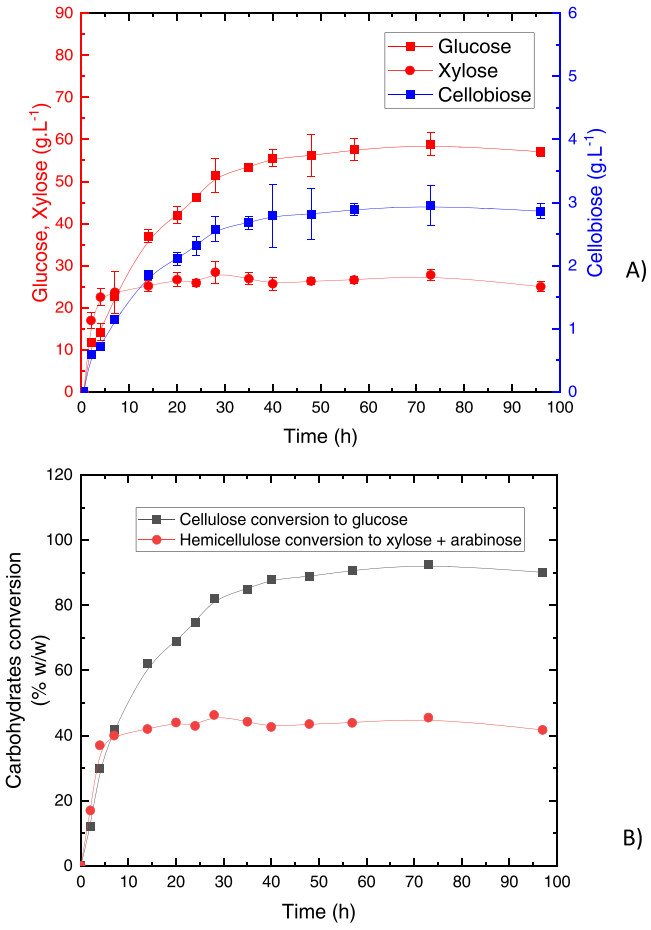

Fig. 5. A) Sugar concentration profile (glucose (■), xylose plus arabinose (●) and cellobiose (■); B) Conversion of cellulose to glucose (■) and hemicellulose to xylose (●), obtained during enzymatic hydrolysis of extruded corncobs (ECC-25 %-UW) under conditions established by response surface methodology (RSM).

hydrolysis using different pretreatment methods, obtaining a maximum of 86.0 % (w/w) of glucose recovery. In the works of Fan et al. (2014), cellulose conversion to glucose of 85.3 % (w/w) was achieved; however, corn starch was pretreated by the addition of acid, high enzymatic dosing (~ 50 FPU/g $_{\text{lignocellulosic material}}$) and solids loading of 15 % (w/w). Kleingesinds et al. (2018) studied the impact of using Tween 80 in both corncob pretreatment and saccharification, reaching 80.54 % (w/w) of glucose recovery and 70.66 % (w/w) of xylose recovery.

3.2.4. *Kinetics study*

Fig. 5A shows the sugar concentration profiles (glucose and xylose) obtained during enzymatic hydrolysis of extruded corncob (ECC-25 %-UW) via an enzyme dosing of 31 FPU/g) and solids loading of 17.8 % (w/w) previously established by the response surface methodology (RSM) to maximize the conversion of cellulose to glucose and glucose productivity.

The highest concentrations of glucose (57.84 g L^{-1}) and cellobiose (2.90 g L^{-1}) were obtained at 57 h of the enzymatic reaction, while maximum concentration of xylose (26.59 g L^{-1}) was achieved at 20 h of the enzymatic reaction. For each respective reaction time, glucose recovery was of 90.84 % (w/w) and the xylose + arabinose recovery was of 44.0 % (w/w) (Fig. 5B). These conversion values can be considered satisfactory, as similar values are obtained when extrusion is associated with a chemical treatment, such as alkali or acid (Procentese et al., 2015).

S. Zhang et al. (2012) obtained similar values for the conversion of

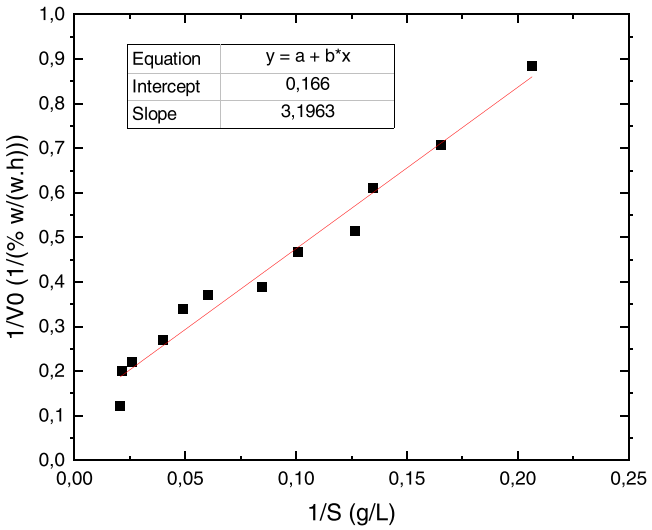

Fig. 6. Lineweaver-Burk graph ($1/V_0$ versus $1/[S]$), for the conversion of cellulose to glucose.

cellulose to glucose (86.8 % [w/w]) and the conversion of hemicellulose to xylose (50.5 % [w/w]) using similar enzymatic hydrolysis conditions for corn stovers (solids loading of 18 % w/w) pretreated by double-screw extruders with the addition of alkali. However, Coimbra et al. (2016) obtained, in addition to high conversion of cellulose to glucose (80 % w/w), a high conversion of hemicellulose to xylose (90 % w/w), using similar conditions of enzymatic hydrolysis for wheat straw pretreated by alkaline extrusion.

Kinetics of most enzyme-catalyzed reactions follow the Michaelis-Menten model. Kinetic constants (K_m and V_{max}) were determined using Lineweaver-Burk's graphical method (Shuler and Kargi, 2002), plotting $1/S$ as a function of $1/V_0$ (Fig. 6).

V_{max} is the limit for which the reaction rate tends to (V_0) whilst the substrate [S] concentration tends to infinity. K_m is the substrate concentration at which the value of the reaction rate (V_0) is half the value of V_{max}. These constants were calculated using Eq. 1.

Maximum rate (V_{max}) obtained was 9.62 % (w/w) h^{-1} of hemicellulose conversion to xylose plus arabinose, a value greater than the maximum rate for conversion of cellulose to glucose, which was 6.00 % (w/w) h^{-1}. The K_m obtained for converting cellulose to glucose was 22.59 g of cellulose per liter of solution (g L^{-1}).

Eckard et al. (2011) obtained similar values of K_m (21.29 g L^{-1}) and maximum rate of enzymatic hydrolysis (5.3 % w/w) in their kinetic study of the enzymatic hydrolysis of corn stover pretreated by extrusion and polyethylene glycol. With these values of kinetic constants, they obtained cellulose conversions into glucose of up to 98 % (w/w) (Eckard et al., 2011).

4. Conclusion

The advantages observed imply that the use of a pilot twin-screw extrusion as a pretreatment method for corncobs without the association of any strong chemical pretreatments leads to a residue-free pretreatment with no waste generation. This single pretreatment with twin-screw extrusion combined with the enzymatic hydrolysis with the enzymatic complex Cellic Ctec 2 achieved a high efficiency of cellulose-to-glucose conversion by employing higher solids loading and providing a sugar solution with high concentration for further fermentation. However, further research needs to be carried out to recover the hemicellulose and lignin lost in the extrusion-pretreatment corncobs.

CRediT authorship contribution statement

The authors confirm responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation. All authors reviewed the results and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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