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Development of a sustainable bioprocess based on green technologies for xylitol production from corn cob

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ABSTRACT

In this work, a sustainable and environmental friendly strategy for the biotechnological production of xylitol was proposed and optimized. For this purpose, corn cob was hydrothermally pretreated at high solid loadings (25%) for an efficient solubilization of xylan in hemicellulose derived compounds, xylooligosaccharides and xylose. Xylose enriched streams were obtained from the enzymatic saccharification of the whole slurry (solid and liquid fraction) resulting from the autohydrolysis pretreatment. The xylitol production in a simultaneous saccharification and fermentation (SSF) process, by the recombinant *Saccharomyces cerevisiae* PE-2-GRE3 strain, was optimized using different enzyme and substrate (pretreated corn cob solid) loadings by an experimental design. This study demonstrated a significant effect of substrate loading on the production process achieving a maximal concentration of 47 g/L with 6.7 % of pretreated corn cob and 24 FPU/g of enzyme loading, with partial detoxification of the hydrolysate. Furthermore, the 1.42-fold increase in xylitol titer and 1.56-fold increase in productivity achieved in a SSF using an acetic acid free-hydrolysate evidenced the negative effect of acetic acid on the yeast-based xylitol production process. The combination of these green technologies and the optimization of the proposed strategy enhanced the overall xylitol production through the valorization of corn cob.

1. Introduction

The excessive dependence of non-renewable fossil resources and the need for climate change mitigation are the main driving forces for the development of novel technologies to produce high value chemicals from renewable resources. Lignocellulosic biomass, which includes plant-derived materials from wood and grass to agro-industrial residues, is the most abundant renewable feedstock and appears to be the most promising starting material for high value chemicals production (Bedő et al., 2019; Sheldon, 2018).

Xylitol is included within the twelve building blocks that can be produced from lignocellulosic sugars and subsequently converted to a number of valuable derivatives for food, pharmaceutical and chemistry industries (Bozell and Petersen, 2010; Cortivo et al., 2018; Dall Cortivo et al., 2020). It is a naturally occurring sugar alcohol that presents a sweetness profile similar to sucrose but with 40% less calories. In addition to its low-caloric content, exhibits other benefits especially antidiabetic and anti-cariogenic properties (Salli et al., 2019). Currently, xylitol is commercially produced by hydrogenation of xylose extracted from lignocellulosic biomass. In this production process, xylose-enriched hydrolysates are obtained through acid hydrolysis of hemicellulose and subsequent concentration. In spite of using an inexpensive and renewable raw material, is not environmental-friendly and requires large energy requirements. In this sense, the production of xylitol through microbial fermentation of sugars from renewable feedstocks has gained increasing interest (Dasgupta et al., 2017).

There is a wide range of xylose-fermenting yeasts able to produce xylitol as a by-product of xylose utilization pathway (Abdul Manaf et al., 2018). However, xylitol yields are limited by the use of xylose as carbon source for yeast growth and maintenance energy. To overcome this limitation, the expression of enzymes with xylose reductase activity in *Saccharomyces cerevisiae*, naturally incapable of xylose utilization, has shown to increase the conversion of xylose into xylitol close to the maximum theoretical yield (~100%), since the produced xylitol is not further metabolized (Baptista et al., 2018; Hallborn et al., 1991; Jo et al., 2015; Kogje and Ghosalkar, 2016). Moreover, the possibility of using robust *S. cerevisiae* strains, isolated from harsh environmental industrial conditions, with higher tolerance to the lignocellulosic-derived inhibitors represents another advantage for xylitol production in lignocellulose-based processes (Cunha et al., 2019; Pereira et al., 2014a,

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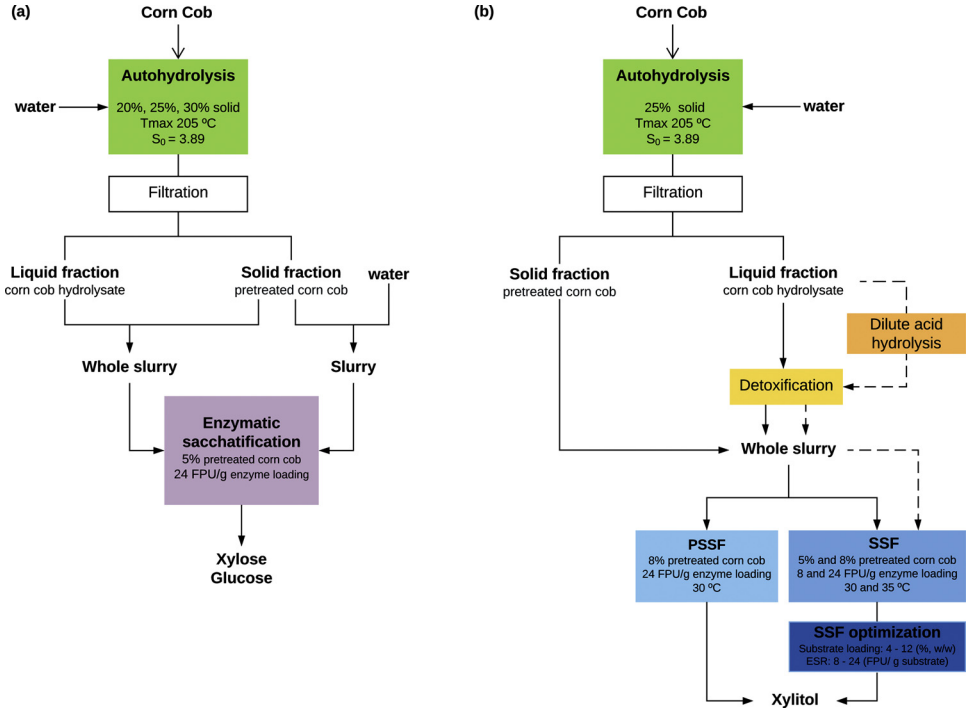

Fig. 1. Flowchart of experimental procedure for (a) evaluation of the effect of autohydrolysis pretreatment at different different solid loadings on enzymatic saccharification and (b) optimization of operational conditions and process configuration for xylitol production using whole slurry corn cob. Dotted lines refer to an optional strategy for xylitol production by the complete removal of acetic acid from hemicellulosic hydrolysate.

2014b). Considering this, the *S. cerevisiae* PE-2 industrial strain presenting innate capacity for xylitol accumulation (Romani et al., 2015), was recently engineered to overexpress an endogenous aldose reductase with xylose reductase activity (encoded by *GRE3* gene) and efficiently used as whole-cell biocatalyst for xylitol production (Baptista et al., 2018).

Among lignocellulosic biomass, corn cob is potentially the most favorable feedstock for xylitol production due its high xylan content (Xu et al., 2017). Nevertheless, the main challenge of corn cob processing, like other lignocellulosic materials, is the requirement of pretreatment technologies to break down its recalcitrant structure and to obtain xylose enriched hemicellulosic hydrolysates (Morales et al., 2018). Hydrothermal pretreatment (also known as autohydrolysis) represents an environmental friendly alternative to dilute acid hydrolysis, the most common pretreatment to solubilize the hemicellulosic fraction in lignocellulose-based xylitol production processes (Abdul Manaf et al., 2018; Hernandéz-Peréz et al., 2019). The autohydrolysis method, using water as reaction media, yields a liquid fraction mainly composed by xylooligosaccharides (XOS) and increases cellulose accessibility to enzymatic hydrolysis (González-García et al., 2018; Romaní et al., 2010; Ruiz et al., 2020). The hemicellulosic derived compounds (oligosaccharides) can be hydrolysed by acid or enzymes. Enzymatic hydrolysis of XOS offers several advantages compared to acid hydrolysis since it occurs at milder operational conditions with less inhibitory compounds formation and does not require neutralization procedures before fermentation (Hu et al., 2016). Given the limited research on enzymatic hydrolysis of XOS and also in the valorization of whole-slurry (containing both cellulose and xylooligosaccharides polysaccharides in solid and liquid fractions, respectively) in presence of lignocellulose-derived inhibitors, (Hu et al., 2016; Romaní et al., 2014b) the main goal of this work is the development a high effective strategy using green technologies (autohydrolysis, enzymatic saccharification and fermentation) for xylitol production from corn cob.

2. Materials and Methods

2.1. Raw material and autohydrolysis pretreatment

Corn cob was milled to a particle size less than 8 mm, homogenized and submitted to autohydrolysis. The raw material was mixed with water at different solid loadings: 20, 25 and 30 g of corn cob solid dry weight per 100 g of water, and heated to 205 °C in a 2 L stainless steel reactor (Parr Instruments Company) equipped with Parr PDI temperature controller. Temperature and time of autohydrolysis was correlated using the following equation, which allows the determination of severity factor (R_0) expressed as severity ($S_0 = \log R_0$) as follows:

$$S_0 = \log\left[\int_0^{t_{max}} \frac{T(t) - T_{ref}}{\omega} \cdot dt + \int_{t_{max}}^{t_f} \frac{T'(t) - T_{ref}}{\omega} \cdot dt\right] \quad (1)$$

where, t_{max} and t_f refers to the time (min) required to achieve the maximum temperature and the t_0 is referred to time of the heating-cooling profiles (limited by T_{ref}), respectively, while T(t) and T'(t) correspond to the temperature profiles for the stages of heating and cooling. T_{ref} is the reference temperature (373.15 K) and ω is an empirical parameter related to the activation energy, set to 14.75 K for corn cob.

After treatment, liquid and solid fractions were separated by filtration and solid fraction (pretreated corn cob) was recovered and washed for Solid Yield (SY) determination. Chemical composition of corn cob and pretreated corn cob was analyzed following NREL protocols (NREL/TP-510-42618-42622-4218). The concentrations of sugars, acetic acid and furan compounds were measured by HPLC. For determination of oligosaccharides and acetyl groups, one aliquot of hydrolysate was submitted to an analytical hydrolysis (4 % w/w H_2SO_4 at 121 °C for 20 min).

2.2. Enzymatic saccharification of pretreated corn cob

Enzymatic saccharification of pretreated corn cob was carried out at 45 °C, 150 rpm in an orbital shaker using 5% of pretreated corn cob and 24 FPU/g of enzyme loading for 96 h. Commercial enzyme preparation used in these assays was Cellic CTec2 (kindly supplied by Novozymes,

Bagsvaerd, Denmark). Cellulase and hemicellulase activities of Cellic CTec2 were 122 FPU/mL and 9764 U/mL, determined following the procedures previously described (Bailey et al., 1992; Ghose, 1987). Enzymatic saccharifications were carried out using pretreated corn cob as substrate in water (named slurry) and pretreated corn cob in hydrolysate (named whole slurry) as shown in Fig. 1a. Glucose and xylose concentrations were analyzed by HPLC. Glucose (GY) and xylose yield (XY) were calculated following the equations:

$$GY\ (\%) = \frac{G_t - G_{t0}}{G_{POT}} 100 \quad (2)$$

where, Gt is the glucose concentration (g/L) achieved at time *t* and Gt_0 is the glucose concentration at the beginning of the experiments; whereas G_{POT} represents the potential glucose concentration that was calculated as:

$$G_{POT} = Bf\frac{180}{162} \quad (3)$$

where, B is dry corn cob biomass concentration (g/L), f is glucan fraction in dry biomass (g per g) and 180/162 is the stoichiometric factor that converts glucan to equivalent glucose.

$$XY\ (\%) = \frac{X_t - X_{t0}}{X_{POT}} 100 \quad (4)$$

where, Xt is the xylose concentration (g/L) achieved at time *t* and Xt_0 is the xylose concentration at the beginning of the experiments, whereas X_{POT} represents the potential xylose concentration that was calculated as:

$$X_{POT} = Bf\frac{150}{132} + XOS \quad (5)$$

where, B is dry corn cob concentration (g/L), f is xylan fraction in dry biomass (g per g) 150/132 is the stoichiometric factor that converts xylan to equivalent xylose, and XOS is xylooligosaccharides concentration measured as xylose equivalent in g/L present in the hydrolysate (XOS were only considered for potential xylose for the enzymatic saccharification of whole slurry).

2.3. Yeast strain and inoculum

The yeast strain used in this work was the yeast strain *Saccharomyces cerevisiae* PE-2, isolated from 1st generation bioethanol plants in Brazil, (Basso et al., 2008; Pereira et al., 2014a, 2014b; Pereira et al., 2011, 2010) overexpressing the endogenous *GRE3* gene, *S. cerevisiae* PE-2-GRE3 (Baptista et al., 2018). Yeast strain was maintained at 4 °C on YPD plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) supplemented with 200 mg/L of geneticin (G418). Yeast cells for inoculation were grown overnight at 30 °C and 200 rpm in YPD medium supplemented with 150 mg/L of G418. The cell suspension was collected by centrifugation for 5 min at 3000 rpm, 4 °C and suspended in 0.9% (w/v) sodium chloride solution. The fermentation experiments were conducted with a cellular concentration of 11 g and 22 g fresh yeast/L corresponding to 5 g and 10 g of dry yeast/L, respectively.

2.4. Preparation of corn cob hydrolysate: detoxification, neutralization and sterilization

Corn cob hydrolysate and corn cob hydrolysate after dilute acid hydrolysis (0.5% w/w of H_2SO_4 for 165 min at 125 °C) (Rivas et al., 2006) were submitted to ion exchange detoxification to remove acetic acid, as previously described (Rodríguez-López et al., 2012). Briefly, corn cob hydrolysates were mixed with Amberlite IR-120 cationic resin (in H^+ form) at a mass ratio of 10 g cationic resin per gram of hydrolysate for 1 h with agitation. Cationic resin was recovered by filtration and the hydrolysate was treated for 2 h under agitation with Mto-Dowex M43 anionic resin (in OH^- form) at a mass ratio of 20 g anionic resin per gram of acetic acid present in the hydrolysate. The resulted acid-hydrolysed corn cob hydrolysate was neutralized with $CaCO_3$ until pH 5 and the pH of corn cob hydrolysate was adjusted with NaOH or HCl solutions. Both hydrolysates were sterilized by filtration (0.2 µm) and added to solid fraction (sterilized at 121 °C for 20 min) to obtain the whole-slurry used for xylitol production.

2.5. Pre-saccharification and Simultaneous Saccharification and Fermentation (PSSF and SSF) assays of corn cob whole slurry

Simultaneous saccharification and fermentation (SSF) and Pre-saccharification and simultaneous saccharification and fermentation (PSSF) assays of whole-slurry were carried out in Erlenmeyer flasks at 30 °C and/or 35 °C in an orbital shaker at 200 rpm. For PSSF, an enzymatic saccharification step of whole slurry was carried out for 24 h using 8 % solids at 8 or 24 FPU/g at 45 °C and 200 rpm. After this step, temperature was decreased up to 35 °C for cell inoculation. For determination of operational conditions, SSF assays were carried out using 5 and/or 8 % of solids (pretreated corn cob). To evaluate the effect of acetic acid SSF assays were carried out using 6.76 % of solid, 24 FPU/g at 35 °C and corn cob hydrolysate with or without diluted acid posthydrolysis. Corn cob hydrolysate medium was supplemented with 20 g/L of peptone and 10 g/L of yeast extract.

2.6. Experimental design of Simultaneous Saccharification and Fermentation of whole slurry

Simultaneous saccharification and fermentation (SSF) process conditions were evaluated and optimized following a full factorial design (2 factors with two replicates of the central point, 10 total experiments). The independent variables evaluated were: solid loading of pretreated corn cob or x_1 (ranged between 4-12 % w/w) and enzyme to substrate ratio (ESR) or x_2 (ranged between 8-24 FPU/g). Dependent variables were correlated with the independent variables by empirical models, following the equation:

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij}x_i + \sum_{i=1}^{2}\ldots\sum_{k\geq i}^{2} b_{ikj}x_i x_k \quad (6)$$

where y_j (j = 1 to 3) is the dependent variable; x_i or x_k (i or k: 1 to 2, k ≥ i) are the normalized, independent variables and $b_{0j}\ldots b_{ikj}$ are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel, Microsoft Office 365 ProPlus).

2.7. Determination of fermentation parameters

Xylitol yield (Y_{XL}) and productivity (Q_{pt}) were calculated as follows:

$$Y_{XL} = \frac{[XL]_t}{[X_{POT}]} \cdot 100 \quad (7)$$

where, XL is the concentration of xylitol at time *t*, X_{POT} is the potential xylitol considering the xylose, xylooligosaccharides and the xylan present in the SSF.

$$Qp_t = \frac{[XL]_t}{t} \quad (8)$$

where [XL] is xylitol concentration at time *t* divided by time *t*.

2.8. Analytical methods

Samples from saccharification and fermentation assays, chemical characterization and autohydrolysis treatment (including solid and hydrolysate) of corn cob were analyzed for quantification of sugars

(glucose, xylose, arabinose), acetic acid, xylitol, furfural, hydroxymethylfurfural (HMF) and ethanol by HPLC using a BioRad Aminex HPX-87H (300 x 7.8 mm) column, at 60 °C, and 0.005 M sulfuric acid as eluent in a flow rate 0.6 mL/min. The peaks corresponding to sugars, acetic acid, xylitol and ethanol were detected using a Knauer-IR intelligent refractive index detector, whereas furfural and HMF were detected using a Knauer-UV detector set at 280 nm.

3. Results and discussion

3.1. Autohydrolysis pretreatment for corn cob processing: effect of solid loading

The xylitol production from lignocellulosic biomass depends on the fractionation pretreatment used to obtain xylose for the bioconversion process (Dasgupta et al., 2017; Hernandéz-Peréz et al., 2019). In this study, the hydrothermal pretreatment of corn cob at high solid content (between 20% and 30%) was evaluated in order to maximize xylan solubilization and recovery the hemicellulose derived compounds, especially xylose and xylooligosaccharides. The biomass processing strategy proposed for xylitol production is shown in Fig. 1b.

Corn cob was chemically analyzed and its composition (based on three replicates) was: 28.8% ± 1.63 of glucan, 29.6% ± 1.88 g of xylan, 22.9% ± 0.30 g of Klason lignin, 3.4% g ± 0.83 of arabinan and 2.0% ± 0.04 g of acetyl groups per 100 g of dry weight.

The pretreatment severity was based on previous works that have shown that the use of 12 g of corn cob per 100 g of water lead to maximum concentration of xylooligosaccharides (Garrote et al., 2008; Rivas et al., 2006). In order to reduce the water consumption in the process and increase the xylose concentration in the liquid fraction (hydrolysate), the solid loading of corn cob was evaluated in the range of 20 to 30 g of corn cob per 100 g of water at T_{max} of 205 °C (S_0 = 3.89) (Baptista et al., 2018). The use of high-solid loadings in the pretreatment minimizes the water consumption and reduces the energy required for heating, improving the economic and environmental sustainability of the process (Modenbach and Nokes, 2012; Jesus et al., 2017). Nevertheless, increased solid concentrations could negatively affect the process efficiency by insufficient mixing, limitations of heat and mass transfer and also by increasing the concentration of inhibitor compounds in the hydrolysate. Chemical composition of solid and liquid fractions after pretreatment is shown in Table 1. The recovery of glucan and lignin in the solid phase varied in the range of 88.9-97.4 g of glucan/100 g of glucan and 60.2-71.7 g of lignin/100 g of lignin in raw material, respectively. Chemical composition of liquid fraction (Table 1) showed that the increase of solid loading up to 25% in the pretreatment resulted in the highest concentration of released xylooligosaccharides (35.75 g/L). Therefore, under this condition, 51.4 % of xylan (measured as sum of xylose and xylooligosaccharides) was recovered in the liquid fraction, corresponding to 43.3 g/L of potential xylose that may be used as substrate for xylitol production. For a solid loading of 30%, the concentration of XOS was lower (31.9 g/L) due to dehydration of xylose to furfural (4.3 g/L). Furthermore, this condition resulted in higher concentration (3.65 g/L) of acetic acid, a degradation compound generated *in situ* during pretreatment that acts as catalyst for the hemicellulose hydrolysis (Garrote et al., 2017) and could be directly related to a higher degradation of xylose into furfural comparing to the conditions using 20 and 25 % of solid loading. The effectiveness of pretreatments at high solid loadings (> 15 %) has been demonstrated in several strategies, such as the process developed by Inbicon AS (Denmark) using hydrated wheat straw with recycled condensate or the wet explosion pretreatment of lobelly pine (Xiros et al., 2014). Similar solid loadings (20 and 25 %) were also tested for hydrothermal treatment of brewer´s spent grain, generating higher oligosaccharides concentration using 25 % of solid loading in the pretreatment (Pinheiro et al., 2019). In addition, presoaked wheat straw was maintained at temperatures between 195-205 °C and residence time in the range of 6-12 min by injection of stream, resulting in a concentration of solids in the reactor between 23% and 28% (w/w), with the correspondent whole slurries being used for ethanol production by SSF (Jørgensen et al., 2007).

Despite being an attractive strategy to obtain higher sugar concentration, the use of high solid loading in the pretreatment generates higher amounts of degradation compounds, such as furfural, hydroxymethylfurfural and acetic acid (Jørgensen et al., 2007). Among these compounds that have inhibitory effects on enzymatic and fermentation processes, acetic acid (measured as sum of acetic acid and acetyl groups) was the major product in the hydrolysates, varying in the range of 4.57 to 9.02 g/L, raising with the increase of solid loading (Pino et al., 2019).

Table 1
Chemical composition of solid and liquid fractions obtained from corn cob processing by autohydrolysis at Severity of 3.89 using high solid loading.

Solid loading (g of corn cob per 100 g of water)	20	25	30
Solid yield (g of autohydrolysed corn cob/100 g of corn cob)	57.7	60.0	57.7
Autohydrolysed corn cob composition (g of component/100 g of pretreated corn cob)			
Glucan	48.6 ± 0.6	43.8 ± 0.6	44.4 ± 0.1
Xylan	16.8 ± 0.1	17.0 ± 0.2	18.3 ± 0.3
Arabinan	1.21 ± 0.10	1.29 ± 0.11	1.21 ± 0.02
Acetyl groups	0.53 ± 0.01	ND[a]	ND
Klason Lignin	19.4 ± 0.6	22.2 ± 0.2	21.2 ± 0.1
Liquid phase composition (g/L)			
Glucose	0.73 ± 0.04	0.75 ± 0.06	0.71 ± 0.04
Xylose	2.87 ± 0.14	7.52 ± 0.32	8.80 ± 0.44
Arabinose	1.31 ± 0.07	2.28 ± 0.04	2.09 ± 0.1
Acetic acid	1.56 ± 0.08	2.26 ± 0.15	3.65 ± 0.18
Hydroxymethylfurfural (HMF)	0.44 ± 0.02	0.26 ± 0.01	0.75 ± 0.04
Furfural (F)	1.34 ± 0.07	1.29 ± 0.06	4.30 ± 0.21
Glucooligosaccharides (GOS)	1.28 ± 0.3	3.15 ± 0.05	2.31 ± 0.01
Xylooligosaccharides (XOS)	25.1 ± 1.4	35.75 ± 1.10	31.9 ± 0.08
Arabinooligosaccharides (ArOS)	1.05 ± 0.22	ND[a]	0.50 ± 0.03

[a] ND not detected.

3.2. Enzymatic hydrolysis of corn cob slurry and whole slurry

Considering that hemicellulose and lignin derived compounds present in the hydrolysate (i.e., xylooligosaccharides and phenolic compounds) could inhibit the enzyme activities, reducing the saccharification yield (Romaní et al., 2014a, 2014b), the enzymatic hydrolysis of both fractions resulting from biomass pretreatment were evaluated by enzymatic saccharification of slurry (pretreated corn cob and water) and the whole-slurry (pretreated corn cob and hydrolysate).

As expected, the glucose concentration and glucose yield were higher in the enzymatic saccharification assays of slurries (Fig. 2, Table 2) comparing with the results obtained from whole-slurries (Fig. 3, Table 2), showing a clear effect of hemicellulosic hydrolysates on cellulose saccharification. The negative effect oligosaccharides on cellulose saccharification was also demonstrated by the high glucose yield (99%) achieved with the hydrolysate containing lower oligosaccharides content (pretreatment with 20% of solid loading) in comparison with the glucose yields (< 76%) achieved in the saccharifications of whole-slurries obtained from autohydrolysis using higher solid loadings (25 and 30%). This effect was also described by Oliveira et al. (2018), which reported 25% less glucose production for the enzymatic saccharification of eucalyptus whole-slurry.

In terms of xylose yield, the xylose concentration was inferior in the hydrolysate containing lower amount of xylooligosaccharides, resulting in a final xylose concentration of 36.8 g/L (98% of xylose yield). The enzymatic saccharification of whole slurries obtained from the autohydrolysis with 25% and 30% of solid loading resulted in equivalent xylose concentrations (48.8 and 48.4 g/L, respectively) but the highest

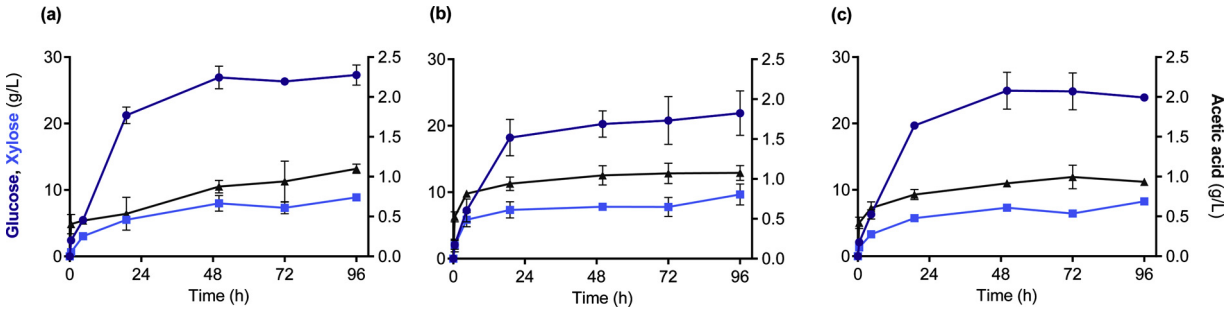

Fig. 2. Enzymatic saccharification of slurry using 5% of pretreated corn cob from autohydrolysis at (a) 20%, (b) 25% (c) 30% of solid loading. Profiles of glucose, xylose and acetic acid concentrations.

Table 2
Operational conditions used in the enzymatic saccharification of 5% pretreated corn cob using 24 FPU/g and main results (glucose concentration and yield and xylose concentration and yield) obtained at 96 h.

Operational Conditions		Main Results			
Substrate	Solid loading in autohydrolysis (%, w/w)	Glucose concentration (g/L)	Glucose Yield (%)	Xylose concentration (g/L)	Xylose Yield (%)
Slurry	20	27.3	101.1	8.9	92.9
Whole-Slurry		28.8	99.4	36.8	97.8
Slurry	25	21.9	90.0	9.7	100.0
Whole Slurry		23.1	72.3	48.8	90.5
Slurry	30	23.9	97.0	8.3	79.4
Whole Slurry		21.5	75.0	48.4	93.5

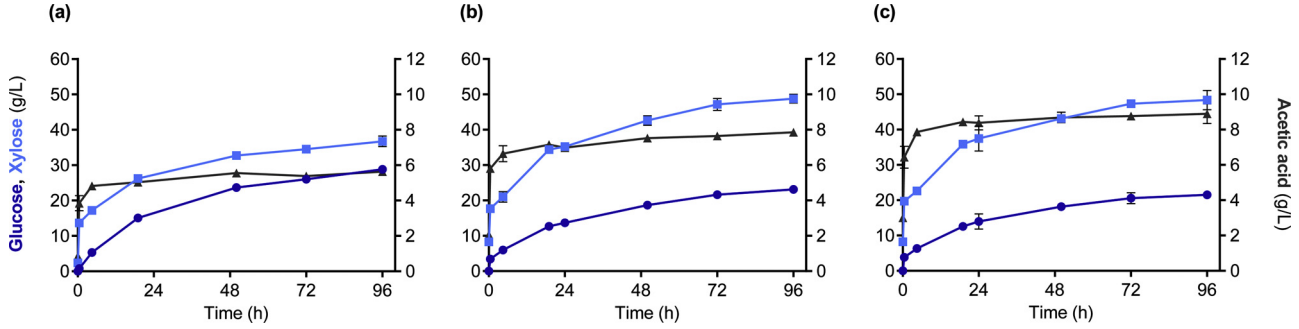

Fig. 3. Enzymatic saccharification of whole-slurry using 5 % of pretreated corn cob from autohydrolysis at (a) 20%, (b) 25% (c) 30% of solid loading. Profiles of glucose, xylose and acetic acid concentrations.

Table 3
Operational conditions (temperature, substrate and enzyme loading) of simultaneous saccharification and fermentation (SSF) and pre-saccharification and simultaneous saccharification and fermentation (PSSF) and main results obtained (xylitol concentration, yield and productivity).

	Operational conditions			Results				
Run	Temperature (ºC)	Substrate loading (%, w/w)	Enzyme loading (FPU/g)	X_{pot} [a] (g/L)	Xf [b] (g/L)	Xylitol max (g/L)	Xylitol Yield (%)	Qp_{max} [c] (g/L·h)
SSF_{30}	30	5	24	52.93	11.92	48.69	91.99	0.336
SSF_{35}	35	5	24	52.93	6.41	51.73	97.73	0.357
$PSSF_1$	45ºC; 35 °C	8	8	58.72	14.84	32.36	55.10	0.193
$PSSF_2$	45ºC; 35 °C	8	24	58.72	11.94	37.96	64.64	0.226
SSF_1	35	8	8	58.72	4.46	39.00	66.42	0.271
SSF_2	35	8	24	58.72	8.22	44.38	75.57	0.308

[a] X_{pot} potencial xylose, calculated considering the sum of xylose concentration in the t_0 of SSF or PSSF with the xylose produced from xylan and XOS saccharification.
[b] X_f xylose concentration in the t_f.
[c] Q_{max} maximal productivity, calculated when xylitol was maximum.

xylose yields (> 95%) were achieved using whole slurry obtained from the autohydrolysis with 20% of solid loading. A similar result (21 g/L of xylose corresponding to 93% of xylose yield) was observed with a hydrolysate obtained from industrial wheat straw processing (Ibicon and Beta-Renewable).(Hu et al., 2016)

The enzymatic cocktail used in the whole slurries assays (Fig. 3) also hydrolysed acetyl groups present in the hemicellulosic hydrolysate and pretreated solid corn cob, achieving a maximal concentration of acetic acid of 8.9 g/L (Fig. 3c). In fact, weak acids such as acetic acid may inhibit the cell growth or increase the fermentation lag phase, affecting the fermentation performance in the subsequent step of xylose to xylitol bioconversion (Cunha et al., 2019, 2018; Palmqvist and Hahn-

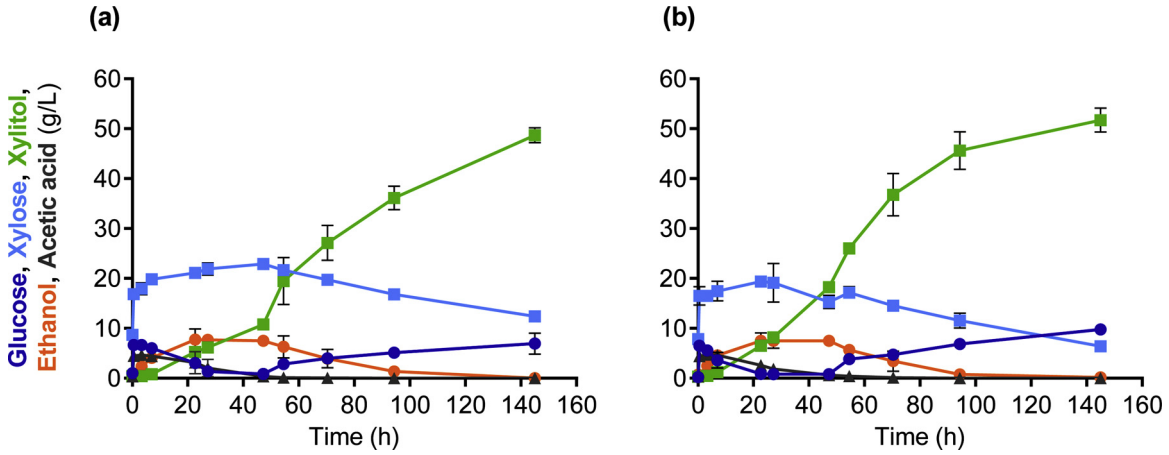

Fig. 4. Xylitol production from corn cob whole slurry by simultaneous saccharification and fermentation (SSF) process using 5% of solid and 24 FPU/g at (a) 30 °C and (b) 35 °C.

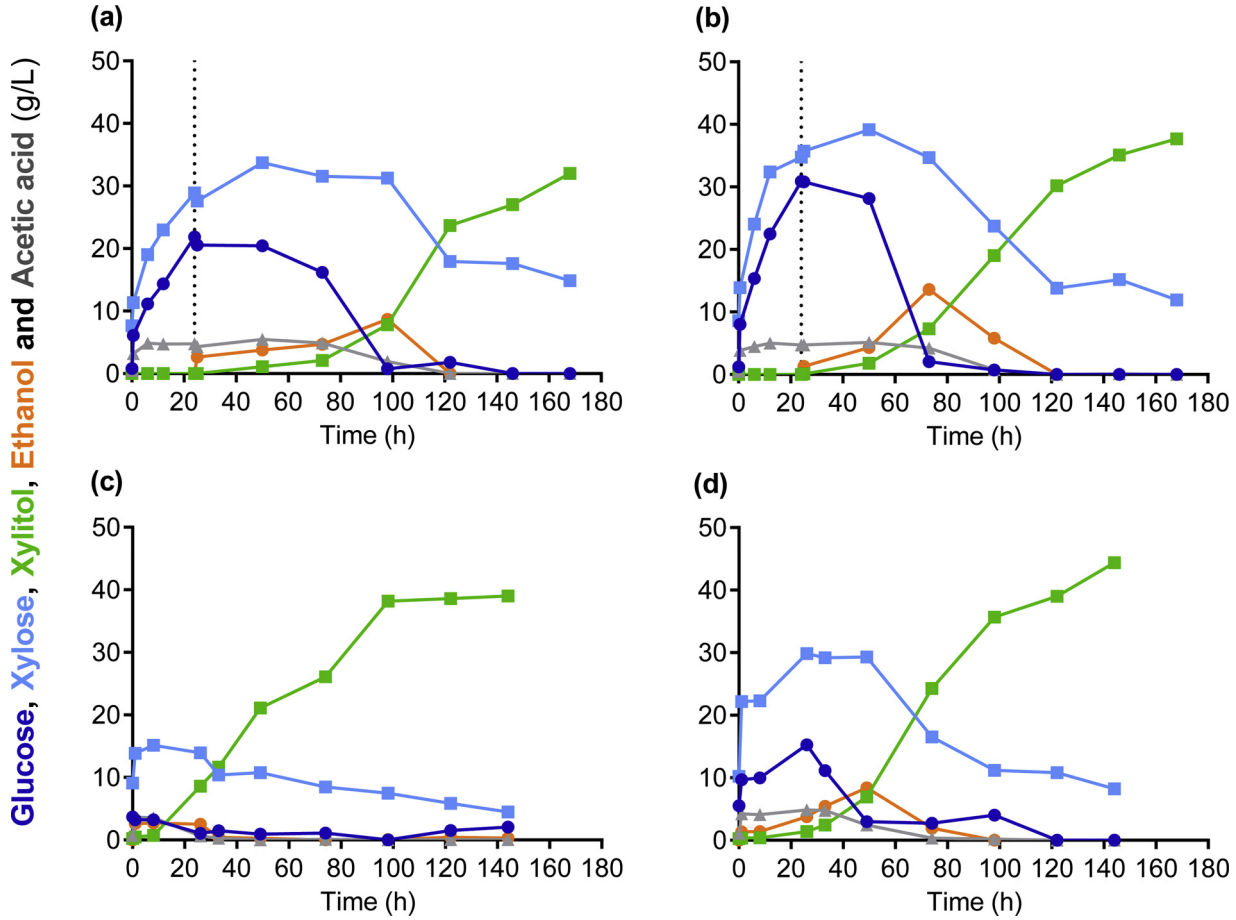

Fig. 5. Xylitol production from corn cob whole slurry by pre-saccharification simultaneous saccharification and fermentation (PSSF) and simultaneous saccharification and fermentation (SSF) using 8% of solid. (a) $PSSF_1$ with 8 FPU/g and (b) $PSSF_2$ with 24 FPU/g of enzyme loading. (c) SSF_1 with 8 FPU/g and (d) SSF_2 with 24 FPU/g of enzyme loading. The dotted lines indicate the yeast inoculation time.

Hägerdal, 2000). Considering the results obtained from the enzymatic saccharification of whole-slurries, the autohydrolysis with 25% of corn cob showed to be more advantageous in terms of xylooligosaccharide conversion and xylose concentration. Therefore, this operational condition was selected for the xylose to xylitol bioconversion process. In addition, the hydrolysate was detoxified by anion exchange for a complete removal of free acid acetic, reducing the final concentration after enzymatic saccharification from 7.9 g/L (Fig. 3b) to 5.6 g/L.

3.3. Determination of operational conditions for xylitol production using whole slurry corn cob

The whole-cell bioconversion process for the production of xylitol involves the xylose transportation into the yeast cell and the conversion into xylitol by the aldose reductase encoded by the *GRE3* gene. The yeast *S. cerevisiae* is a non-xylose-utilizing organism and therefore the recombinant PE-2-GRE3 strain need to be supplied with a carbon source to regenerate co-factors and ensure maintenance energy generation

Table 4
Operational conditions (expressed in terms of dimensional and dimensionless independent variables) of simultaneous saccharification and fermentation (SSF) assays and experimental results obtained (xylose concentration, yield and productivity) for dependent variables y_1 to y_3.

Run	x_1	x_2	Substrate loading (%, w/w)	ESR (FPU/ g substrate)	Final xylitol concentration (g/L) or y_1	Xylitol yield (%) or y_2	Productivity (g/L·h) or y_3
1	−1	−1	4	8	22.8	45.6	0.19
2	0	−1	8	8	39.0	66.9	0.27
3	1	−1	12	8	24.6	37.0	0.17
4	−1	0	4	16	16.0	32.0	0.11
5	0	0	8	16	40.3	69.0	0.28
6	0	0	8	16	43.7	75.02	0.31
7	1	0	12	16	11.1	16.7	0.08
8	−1	1	4	24	40.4	80.6	0.28
9	0	1	8	24	40.0	68.6	0.28
10	1	1	12	24	12.3	18.6	0.09

Table 5
Regression coefficients, values and significance (based on a t-test).

Response variable	Xylitol concentration		Xylitol yield		Xylitol productivity	
	coefficient	P value	coefficient	P value	coefficient	P value
b_0	38.63	0.0003	64.76	0.001	0.26	0.0007
b_1	−5.20	0.073	−14.33	0.039	−0.04	0.091
b_2	1.78	0.455	3.05	0.553	0.002	0.924
b_{12}	−7.45	0.047	−13.37	0.082	−0.04	0.129
b_{11}	−21.71	0.003	−33.20	0.012	−0.14	0.010
b_{22}	6.44	0.135	10.27	0.247	0.04	0.227
R^2	0.93		0.94		0.88	
Adjusted-R^2	0.85		0.77		0.79	
F	11.0		6.9		6.0	
Significance level	98 %		96 %		95 %	

(Hallborn et al., 1991). The glucan-enriched solid phase (obtained from corn cob autohydrolysis) can be efficiently hydrolysed by enzymes, providing glucose for cell metabolism during the bioconversion of xylose (from corn cob hydrolysate) into xylitol (Baptista et al., 2018).

Considering that SSF process efficiency is strongly affected by the temperature, preliminary SSF experiments were performed at 30 and 35 °C in order to evaluate its influence on xylitol production process (Table 3). As seen in Fig. 4, the use of 5% of pretreated corn cob and 24 FPU/g of enzyme loading allows the release of both xylose and glucose from XOS and cellulose hydrolysis and subsequent utilization of xylose for xylitol production and glucose for cell metabolism. The increasing concentration of xylitol and the simultaneous accumulation of xylose in medium suggest that enzymatic hydrolysis occurs faster than the bioconversion process. After glucose depletion, the yeast started to utilize the earlier produced ethanol by switching metabolism from glycolysis to aerobic utilization of ethanol, which prevents the competitive inhibition of xylose uptake by glucose and might be involved with the increased xylitol conversion rate, observed during the ethanol consumption phase. Despite the similar trends in fermentation profiles, the SSF performed at 35 °C resulted in a higher xylitol concentration (51.7 g/L) and the maximal productivity was about 1.3-fold higher (0.52 g/L·h at 70 h) compared to 30 °C (0.39 g/L·h at 70 h). In this sense, the subsequent SSF experiments were performed at 35 °C.

In addition, as SSF processes require an equilibrium between the optimum temperature for enzymatic hydrolysis and for yeast fermentation (Olofsson et al., 2008; Tomás-Pejó et al., 2014), the effect on xylitol production of a saccharification before the SSF process (PSSF – pre-saccharification and simultaneous saccharification and fermentation) was investigated (Fig. 5). The pre-saccharification step was performed at optimum temperature for enzymatic hydrolysis, using 8 FPU/g ($PSSF_1$) and 24 FPU/g ($PSSF_2$) of enzyme loading and 8% of pretreated corn cob. SSF assays without pre-saccharification were performed for comparison (Table 3)

As seen in Fig. 5a, in the first 24 h of saccharification 28.9 g/L of xylose and 21.8 g/L of glucose were released from the whole slurry by using 8 FPU /g ($PSSF_1$). After yeast inoculation, the glucose released from hydrolysis was entirely consumed and the ethanol produced was subsequently re-assimilated. However, the xylitol concentration (32 g/L) and xylitol productivity (0.19 g/L·h) achieved in $PSSF_1$ were lower compared to SSF_1 (Fig. 5c) that resulted in 39 g/L of xylitol and 0.27 g/L·h of productivity. In $PSSF_2$, the utilization of 24 FPU/g increased the initial concentration of xylose and glucose to 34.7 and 30.9 g/L, respectively (Fig. 5b). This higher initial availability of sugars did not lead to higher xylitol production in comparison to the SSF_2 (Fig. 5d) that was conducted without pre-saccharification (37.9 g/L, 0.22 g/L·h vs 44 g/L, 0.30 g/L·h). In fact, the catabolite repression caused by high glucose concentrations has been recognized for long as the main factor for xylose transport inhibition in yeast, since glucose and xylose uptake occurs by facilitated diffusion through the same transport system that present low affinity for xylose (Hamacher et al., 2002; Subtil and Boles, 2012). As the pre-saccharification, under the evaluated conditions, was found to have a negative effect on the maximal xylitol concentration and productivity, the following experiments were performed under SSF conditions.

3.4. Optimization of Xylitol production by SSF process: Experimental design

Considering the results obtained in preliminary assays, the SSF strategy at 35 °C was selected for optimization of xylitol production using an experimental design. For that, pretreated corn cob loading (x_1) and enzyme to substrate ratio-ESR (x_2) were selected as independent variables and the dependent variables were xylitol production at the end of SSF process (y_1), xylitol yield (y_2) and productivity (y_3). Table 4 includes the experimental matrix (dimensional and normalized, dimensionless independent variables) and dependent variables. Time course of SSF experiments (run 1-10) can be seen in Figure S1 included in Supporting information.

In spite of the removal of acetic acid from the pretreated corn cob hydrolysate by ion exchange, the detoxification process only removes the acetic acid released from the autohydrolysis pretreatment and during the subsequent whole slurry enzymatic saccharification, more acetic acid is produced as a result of hydrolysis of acetyl groups linked

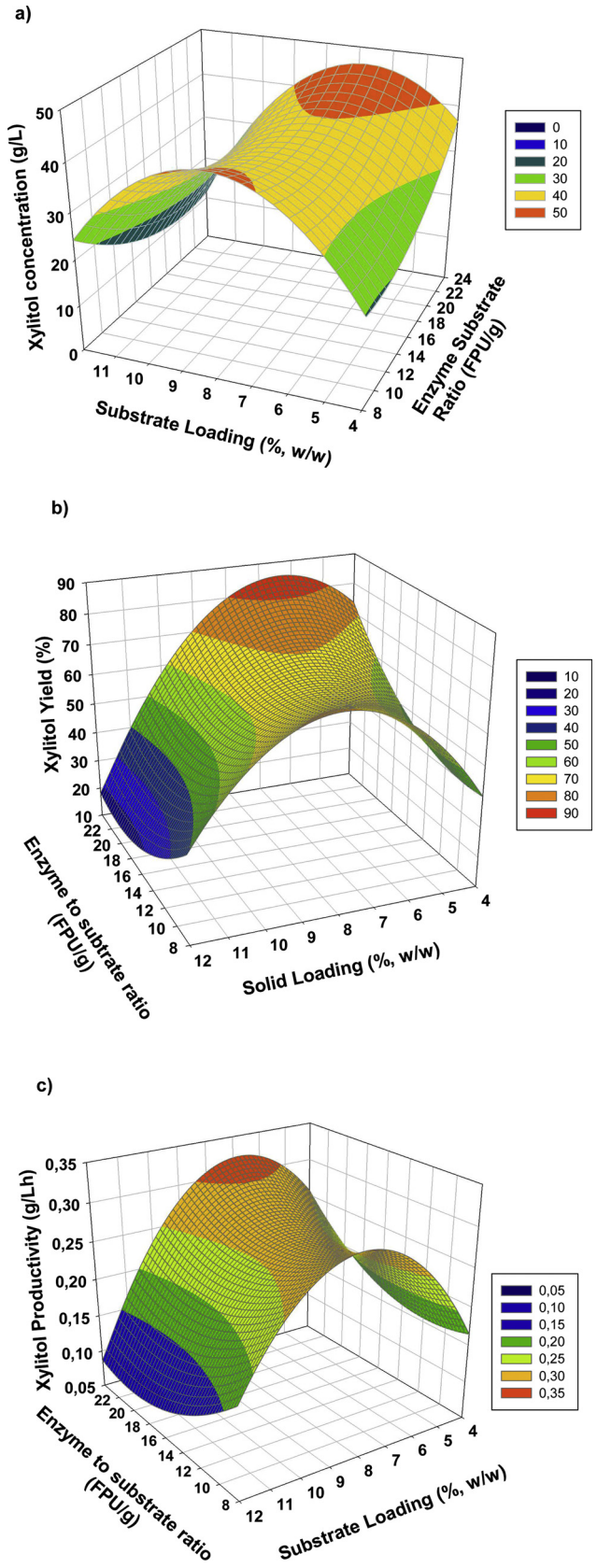

Fig. 6. Response surface for fitted for (a) xylitol concentration (g/L), (b) xylitol yield (%) and (c) xylitol productivity (g/L·h).

to xylooligosaccharides. In the first hours of SSF experiments the concentrations of this compound achieved an average concentration of 4.3 g/L, which could explain the longer lag phases, affecting the overall productivity (Figure S1). The maximal xylitol concentrations (> 40 g/L) were attained with the high enzyme loading (run 5, 6, 8 and 9) and substrate loadings (pretreated corn cob) lower than 8% (Table 4). Whereas, the highest xylitol yield (81%) was obtained with 4% of solid and 24 FPU/g of ESR (run 8). For a correct interpretation of the results, the experimental variables were correlated according to Equation (4). The fitting parameters were included in Table 5. The regression coefficients, the correspondent statistical significance (based in the Student´s t test) and the significance of the model (based on Fisher´s F parameter) measure the correlation and significance of the developed model for xylitol production by SSF. As seen in Table 5, linear and quadratic terms for variable x_1 (substrate loading) and combination of substrate loading and ESR (x_2) were significant ($P < 0.05$; $P < 0.1$). The coefficient R^2 of model was > 0.9 for xylitol concentration and yield, and only 0.88 for xylitol productivity.

The representation of the effect of independent variables on response variables were evaluated using a response surface model (Fig. 6). Although the use of high ESR improved xylitol production, this variable was not significant in the proposed model. Substrate loading was the variable with the more significant impact on xylitol production, yield and productivity, showing a clear optimum with a substrate loading of 6.8% at highest ESR (24 FPU/g). Under these conditions, xylitol yield was higher than 80%. On the other hand, productivities were lower than 0.32 g/L·h, showing that the enzymatic saccharification of XOS and glucan could be limiting step of the process. The influence of solid loading on xylitol production could be related with the glucose catabolite repression, indicating that lower glucan concentration in the SSF is advantageous for xylitol production. According to the model, the optimum condition to maximize xylitol yield and productivity was 6.76% of substrate loading (w/w) and ESR of 24 FPU/g. In order to validate this prediction, an additional SSF experiment was carried out under these conditions (Fig. 7a), resulting in a concentration of xylitol of 42.9 g/L (at 144 h) and xylitol productivity of 0.30 g/L·h, with a corresponding error of 7.66 and 6.66 %, respectively. These results verified the suitability of the model for predicting the experimental observations.

3.5. Acid hydrolysis of hydrolysate for xylitol production by SSF

As mentioned before, acetic acid can severely affect the fermentation performance of the yeast and decrease its xylitol production capacity. Considering this negative effect, the corn cob hydrolysate, composed by xylooligosaccharides linked to acetyl groups, was submitted to an acid posthydrolysis for depolymerization and deacetylation of xylooligosaccharides to yield free xylose and acetic acid. The acetic acid was completely removed from the resulting acid-hydrolysed hydrolysate by ion exchange detoxification and used in a SSF, under the previously optimized conditions. The recombinant strain tested in detoxified acid-hydrolysed liquor showed a superior fermentative capacity (Fig. 7b) converting xylose to xylitol considerably faster and producing 1.56-fold more xylitol (67.03 g/L) in comparing to SSF using enzymatic-hydrolysed autohydrolysis liquor (Fig. 7a). Additionally, as this process uses the yeast cells as whole-cell biocatalysts, the inoculum was increased up to 22 g wet cells/L to maximize the bioconversion of whole-slurry corn cob into xylitol (Fig. 7c). In fact, the increase of biocatalyst concentration resulted in higher xylitol concentration (71.7 g/L), clearly improving the volumetric productivity at 48 h (0.83 g/L·h compared with 0.65 g/L·h obtained with 11 g/L of inoculum) and xylitol yield (94.6% in comparison with 84.4 %).

This evaluation showed a strong negative effect of acetic acid on yeast performance and revealed a clear advantage in using an acetic-acid deprived hydrolysate for an improved xylitol production. The inhibitory effect of this compound can be in part overcome by removing

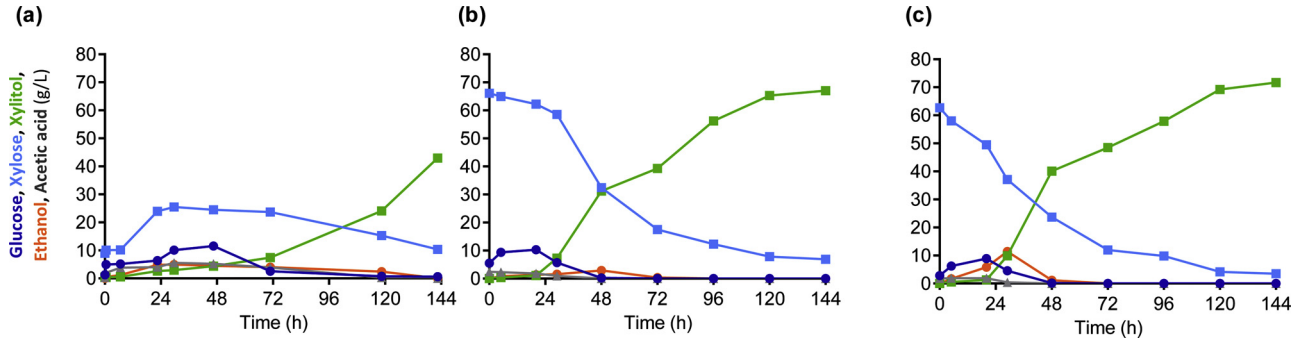

Fig. 7. Simultaneous saccharification and fermentation of corn cob whole slurry under optimal conditions (6.76 % of substrate loading and 24 FPU/g) using 11 g/L of inoculum and (a) enzymatic hydrolysed hydrolysate; (b) acid-hydrolysed hydrolysate and (c) 22 g/L of inoculum and acid-hydrolysed hydrolysate.

acid from the broth during the fermentation or through the development of metabolic engineering strategies, such as the overexpression of genes involved in acetic acid tolerance (Cunha et al., 2018; Weier et al., 1992). Moreover, the highest xylitol concentration obtained in this work (Fig. 7c) can be favorably compared to xylitol production reported in literature using concentrated hemicellulosic hydrolysates (Cheng et al., 2009; Kogje and Ghosalkar, 2017; Tada et al., 2012). Therefore, the use of high solid loadings in the pretreatment, approach followed in this work, is effective and avoids hydrolysate concentration steps, reducing time and cost of operation.

4. Conclusions

This work showed the feasibility of using high solid loadings in the hydrothermal pretreatment to obtain hydrolysates highly enriched in hemicellulose derived compounds (mainly xylooligosaccharides and xylose) suitable for the enzymatic hydrolysis and xylitol bioconversion, avoiding the need for costly evaporation steps. An experimental design was conducted to optimize the xylitol production by the recombinant *S. cerevisiae* strain. In addition, the absence of acetic acid resulted in the corn cob hydrolysate led to a further improved xylitol productivity and resulted in 72 g/L of xylitol, which represent the highest titer reported in *S. cerevisiae* using lignocellulosic biomass. The results obtained in here demonstrate an efficient and sustainable xylitol production, applying green technologies (autohydrolysis and saccharification and fermentation) for an integrated valorization of lignocellulosic biomass.

CRediT authorship contribution statement

Sara L. Baptista: Conceptualization, Writing - original draft, Validation, Writing - review & editing, Formal analysis. **Luís C. Carvalho:** Validation, Formal analysis. **Aloia Romaní:** Conceptualization, Writing - original draft, Validation, Writing - review & editing, Funding acquisition. **Lucília Domingues:** Conceptualization, Validation, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112867.

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