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ORIGINAL ARTICLE

Effect of hemicellulose liquid phase on the enzymatic hydrolysis of autohydrolyzed *Eucalyptus globulus* wood

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Abstract In this work, *Eucalyptus globulus* wood was pretreated under non-isothermal autohydrolysis process at 210, 220, and 230 °C, obtaining a pretreated solid with high cellulose content and a hemicellulosic liquid phase (HLP) containing mainly xylose, acetic acid, furfural, xylooligosaccharides, and phenolic compounds. The maximum concentration of xylooligosaccharides (8.97 g/L) and phenolic compounds (2.66 g/L) was obtained at 210 and 230 °C, respectively. To evaluate the effect of HLP addition on the enzymatic hydrolysis using unwashed pretreated solid as substrate, different proportions of HLP were studied. Also, in order to use the whole slurry on enzymatic hydrolysis, the supplementation of xylanases was evaluated. Glucose concentration of 107.49 g/L (corresponding to 74.65 % of conversion) was obtained using pretreated solid at 220 °C liquid/solid ratio (LSR) of 4 g/g and enzyme solid ratio (ESR) of 25 FPU/g—without the addition of HLP. Thus, it was shown that the unwashed pretreated solids are susceptible to enzymatic hydrolysis contributing to reduce operational cost (water consumption). Additionally, the influence of the inhibitory compounds in the HLP was shown to affect the enzymatic hydrolysis. Results indicated that 82.52 g/L of glucose (59.37 % of conversion) was obtained, using 100 % of HLP at LSR of 4 g/g and ESR of 16 FPU/g at 210 °C of pretreated solid. However, a positive effect was shown on the enzymatic hydrolysis when the xylanases were added using 100 % of HLP, increasing to 35 and 27 % in the glucose production with respect to whole slurry without addition of xylanases.

Keywords Enzymatic hydrolysis · Hydrothermal process · Whole-slurry material · Hemicellulosic liquid phase · Biorefinery · Inhibitors

Abbreviations

CGC_{96}	Cellulose to glucose conversion at 96 h
CGC_{max}	Cellulose-to-glucose conversion predicted for an infinite reaction time (%)
CGC_t	Cellulose-to-glucose conversion achieved at time (%)
DP	Degree of polymerization
EGW	*Eucalyptus globulus* wood
ESR	Enzyme to solid ratio (FPU/g of solid)
FPU	Filter paper unit
G_{96h}	Glucose concentration at 96 h
Gn	Glucan of pre-treated solids (%) on dry basis
G_{pot}	Potential glucose (g/L)
G_t	Concentration of glucose achieved at time (g/L)
HLP	Hemicellulosic liquid phase
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
KL	Klason lignin on dry basis
LSR	Liquid to solid ratio (grams of liquid/gram of solid on dry basis)
R^2	Coefficient of determination
S_0	Severity
SY	Solid yield on dry basis
T	Enzymatic hydrolysis time (h)
$T_{(t)}$	Temperature at time (°C)
$t_{1/2}$	Time needed to reach $CGC_{max}/2$ (h)

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T_{MAX}	Minimum temperature achieved on a given hydrothermal treatment
T_{REF}	Reference temperature (°C)
UI	Unit international
P	Density of the hydrolysis enzymatic medium (g/L)

1 Introduction

The biorefinery concept is often considered for the production of fuels (i.e., bioethanol) and chemicals from lignocellulosic materials [1, 2]. *Eucalyptus globulus* wood (EGW) is an abundant lignocellulosic material in Portugal and Spain and is known to be a fast growing hardwood and made up by high cellulose content, limited lignin, and hemicelluloses (composed by acetyl-xylan). One promising technology is to convert these lignocellulosic materials to fermentable sugars using enzymes that are applied after a pretreatment. The pretreatment is required to alter the structural and chemical composition, improving the accessibility of the cellulose component to the action of hydrolytic enzymes. Autohydrolysis pretreatment or also called hydrothermal process is an eco-friendly process in which the lignocellulosic material is pretreated only with water at high temperatures [3, 4]. The autohydrolysis pretreatment fulfills with the philosophy of biorefinery, since that the first step is the depolymerization of hemicellulose into soluble products mainly in oligosaccharides, while the solids from this pretreatment are composed of cellulose and lignin, causing a re-localization of lignin to improve the enzymatic hydrolysis, additionally the solids after autohydrolysis pretreatment exhibited susceptibility to delignification with solvents [5, 6]. However, when the lignocellulosic material is pretreated under harsh conditions in autohydrolysis process, the biomass chemical components will degrade into by-products (furfural, hydroxymethylfurfural, acetic acid, and phenolic compounds) that become inhibitors for the subsequent processing (enzymatic hydrolysis and fermentation) [7, 8].

On the other hand, enzymatic hydrolysis that converts lignocellulosic materials to fermentable sugars may be the most complex step in this process due to the effects and interactions between enzyme and substrate such as crystallinity of cellulose, surface area, pore size, degree of polymerization (DP), and hemicellulose/lignin content. Furthermore, the soluble lignocellulosic components as phenolic compounds and hemicellulosic-oligomers are produced during the pretreatment affecting the enzymatic hydrolysis process [9].

Currently, it is relevant to find alternatives in the conversion of these materials, which can significantly reduce capital-operational cost in the global bioethanol process. One way is the use of the whole slurry or slurries after pretreatment process without washing (water consumption) or detoxification, eliminating the solid–liquid separation step in the pretreatment area and reducing the operational costs, taking into account that in future, robust microorganism can ferment the sugars from whole slurry or slurries. Martín et al. [10] studied the water consumption in bioethanol plants and mentioned that it can be reduced by the use of different technologies. For this reason, the use of whole slurry or slurries after pretreatment is an important alternative to reduce water requirements and the production of wastewater. Agbor et al. [11] reported that a disadvantage of autohydrolysis pretreatment is the down-stream, due to the large volumes of water involved. Other important process that provides negative outcomes to the process of bioethanol, such as the massive freshwater usage and wastewater generation is the process of detoxification [12]. Nevertheless, an extensive detoxification step is not desirable due to the additional cost. The detoxification process is a high cost that can represent up to 22 % of the total cost of production of bioethanol [13].

Additionally, the supplement of hemicellulases can enhance the enzymatic hydrolysis yields [14]. According to García-Aparicio et al. [15], the xylan content after the hydrothermal pretreatment is low, however the xylanases addition increase the accessibility of cellulose, eliminating the hemicellulose redeposited on the solid pretreated. For all the abovementioned, the objective of the present work was to investigate the effect of whole slurry (100 % hemicellulosic liquid phase (HLP) plus unwashed autohydrolyzed pretreated solid), slurries (50 % HLP plus unwashed pretreated solid), unwashed pretreated solid, and the addition of xylanases (100 % HLP plus unwashed pretreated solid) on the enzymatic hydrolysis using autohydrolyzed EGW as substrate, operating at high solids (15–25 %), and corresponding liquid solid ratio (6–4 g/g).

2 Experimental

2.1 Raw material

EGW used in this study was kindly provided by a local pulp factory (ENCE, Pontevedra, Spain). EGW was milled to pass through 8-mm screen, and the resulting samples were dried and stored in a dark and dry place until use. The material composition was previously analyzed by Romaní et al. [16], containing (expressed in g/100 g of raw material, on dry basis) 44.7 % cellulose, 16 % xylan, 1.1 % arabinan, 3 % acetyl groups, 24.7 % Klason lignin (KL), 2.9 % extractives, and 0.23 % ash.

2.2 Autohydrolysis process

Distilled water and EGW were mixed in order to obtain a ratio 8:1 g of liquid/g of dry solid (on dry basis) and treated in a 3.75 l total volume stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA). The moisture content of EGW was analyzed by TAPPI standards (T-264-cm-97 method) and was considered as water in the material balances. The

reactor was operated in non-isothermal heating regimen [17] at 210, 220, and 230 °C or severity (S_0)=4.08, 4.38, and 4.67, respectively. The harshness of autohydrolysis process can be expressed in terms of the severity (S_0; see Table 1) defined as [18]:

$$S_0 = \log\left[\int_0^t \exp\left(\frac{T(t)-T_{REF}}{\omega}\right)dt\right] \quad (1)$$

where $T(t)$ stands for the time–temperature profile (including heating, non-isothermal period, and cooling) [17]. Calculations were made assuming the values usually employed in literature (T_{REF}=100 °C, ω=14.75 °C).

At the end of autohydrolysis process, the liquid and solid phases were separated by filtration. The autohydrolysis pretreated solids were not washed and these were used as substrate for enzymatic hydrolysis. Additionally, the HLP was added at different proportions (Fig. 1). For the chemical composition of pretreated solids after autohydrolysis process, the residue solids were washed with distilled water and analyzed as raw material. Whereas, the liquid fractions HLP were analyzed by high-performance liquid chromatography (HPLC; see below) and the samples were quantified for glucose, xylose, arabinose, 5-hydroxymethylfurfural (HMF), furfural, and acetic acid. A second aliquot of HLP was subjected to quantitative acid post-hydrolysis (with 4 % H_2SO_4 at 121 °C for 30 min) [17] and analyzed by HPLC. The increase in the concentrations of monosaccharides and acetic acid caused by post-hydrolysis measured the concentrations of oligomers and acetyl groups bound to oligosaccharides. The amounts of the total phenolic compounds of HLP were determined with the Folin–Ciocalteu method using as the standard caffeic acid [19].

Table 1 Composition of solid and liquid phase of pre-treated EGW

Severity S_o (−)	4.08	4.38	4.67
Solid yield (g/100 g r.m., on dry basis)	71.66	70.82	66.98
Non-volatile compounds in liquid phase (g/100 g r.m., on dry basis)	20.96	15.06	11.27
Solid phase composition (g/100 g pre-treated solid, on dry basis)			
Glucan	58.27	60.40	59.51
Xylan	0.93	0.32	0.00
Arabinan	0.00	0.00	0.00
Acetyl groups	0.25	0.73	0.90
Klason lignin	38.04	34.04	37.04
Liquid phase composition (g/L)			
Glucose	0.64	1.41	1.94
Xylose	8.85	7.14	2.10
Arabinose	0.18	0.05	0.06
Acetic acid	3.91	5.80	4.62
Hydroxymethylfurfural	0.26	0.95	1.40
Furfural	1.66	4.97	5.40
Glucooligosaccharides	1.15	0.99	1.04
Xylooligosaccharides	8.97	1.46	0.00
Arabinooligosaccharides	0.00	0.01	0.00
Acetyl groups	2.55	0.50	0.37
Phenolic compounds (g/L)	2.00	2.46	2.66

r.m. raw material

2.3 Enzyme

Commercially available enzyme solutions, cellulase (Celluclast 1.5 L), β-glucosidase (Novozyme 188) and endo-1, 4-β-xylanase (Shearzyme 500 L), were kindly supplied by Novozymes (Madrid, Spain). The enzyme activities of commercial concentrates were 44.7 filter paper unit per milliliter (FPU/mL) for Celluclast 1.5 L, 611.2 UI/mL for Novozyme 188, and 171 UI/mL for Shearzyme 500 L. The enzymatic activities were analyzed according to the standard analytical methods [20–22].

2.4 Enzymatic hydrolysis

The assays of enzymatic hydrolysis were carried out in 100-mL Erlenmeyer flask with a working volume of 40 mL at 48.5 °C in an orbital shaker (Model SI-600R, Jeio Tech, Korea) at 150 rpm using the unwashed autohydrolyzed pretreated solid as substrate at different liquid to solid ratios (LSR) and different loadings (enzyme to solid ratio, ESR) of cellulases, β-glucosidase, and xylanase (see Tables 2, 3, and 4). A 50-mM citrated buffer was used to maintain the pH at 4.8 and thymol as microbial preservative. Also, the necessary amount of HLP in different fractions was calculated and added to make the total volume of 40 mL and determine its effect on the enzymatic hydrolysis (Fig. 1). All determinations were performed in duplicate. Samples of 800 μl were withdrawn from the flasks at 0, 2, 5, 9, 24, 48, 72, and 96 h. Samples were immediately centrifuged at 3,421×*g* during 6 min. Glucose concentration was determined by HPLC (see below) [20].

2.5 Analysis of sugars, furans, and acetic acid by HPLC

The sugars, furans, and acetic acid were quantified by HPLC in a Jasco 880-PU intelligent pump (Tokyo, Japan) chromatograph equipped with a refractive index detector and a Metacarb 87 H (300×7.8 mm, Varian, USA) column. Chromatographic separation was performed under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.7 mL/min, and column temperature 60 °C [23].

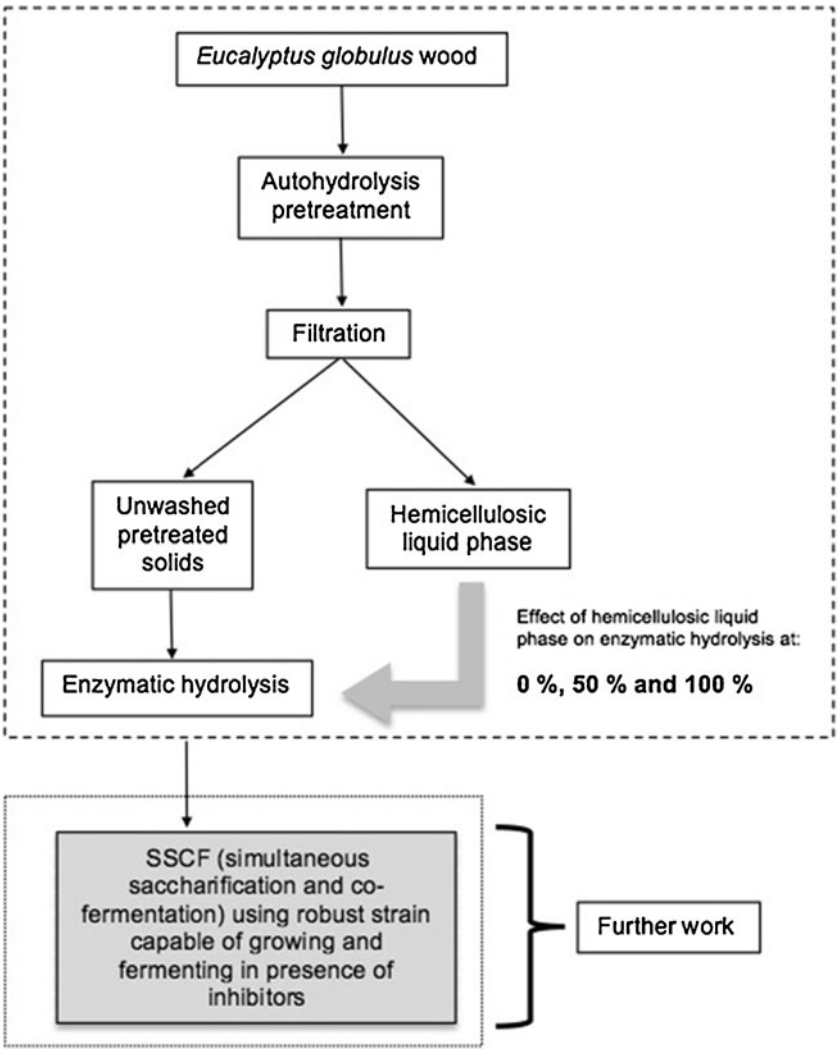

Fig. 1 Schematic representation of the *E. globulus* wood processing

2.6 Modeling of enzymatic hydrolysis using the hyperbolic empirical model

The kinetics of released glucose were fitted to the Holtzapple empirical equation [24]:

$$CGC_t = CGC_{\max} \cdot \frac{t}{t + t_{1/2}} \quad (2)$$

Table 2 Results of enzymatic hydrolysis using the 100 % of HLP at different conditions of pretreatment

		S_0=4.08		S_0=4.38		S_0=4.67	
LSR (g/g)[a]	ESR (FPU/g)	G_{96h} (g/L)	CGC_{96h} (%)	G_{96h} (g/L)	CGC_{96h} (%)	G_{96h} (g/L)	CGC_{96h} (%)
4	16	82.52	59.37	75.41	52.37	61.69	43.35
6	16	50.16	51.60	54.67	54.34	38.53	38.76

[a] Gram of liquid per gram of solid on dry basis

Table 3 Data concerning the enzymatic hydrolysis of whole slurry from autohydrolyzed *Eucalyptus globulus* wood: operational conditions and experimental results obtained

S_0 (−)	LSR (g/g)	ESR (FPU/g)	HLP (%)	G_{96h} (g/L)	CGC_{96h} (%)	$t_{1/2}$ (h)	CGC_{max} (%)
4.08	6	25	0	85.82	88.30	10.36	98.00
			50	74.60	76.75	12.04	85.00
			100	53.27	54.81	7.33	60.00
		16	0	74.38	76.48	9.69	86.24
			50	62.17	63.92	8.45	70.04
			100	45.16	46.43	6.74	52.03
4.38	6	25	0	89.64	89.10	5.22	97.00
			50	71.65	71.22	3.68	74.00
			100	61.92	61.55	3.10	66.00
	4		0	107.49	74.65	5.08	82.00
			50	90.19	62.63	3.36	65.00
			100	81.78	56.79	3.03	61.00

Table 4 Data concerning the enzymatic hydrolysis of whole slurry from autohydrolyzed *Eucalyptus globulus* wood with xylanase supplementation: operational conditions and experimental results obtained

S_0=4.08

LSR (g/g)	ESR (FPU/g)	Xylanase/cellulase (UI/FPU)	G_{96h} (g/L)	CGC_{96h} (%)	$t_{1/2}$ (h)	CGC_{max} (%)
6	25	2	62.14	63.93	9.66	75.00
6	25	4	67.29	69.23	9.67	79.00
6	25	6	67.87	69.82	9.95	79.00
6	16	4	60.87	62.63	10.20	70.00

where CGC_t is the cellulose-to-glucose conversion (%) achieved at time, CGC_{max} is the cellulose-to-glucose conversion (%) predicted for an infinite reaction time, t is the enzymatic hydrolysis time (h), and $t_{1/2}$ (h) is the time needed to reach $CGC=CGC_{max}/2$.

CGC_t is calculated as:

$$CGC_t = 100 \cdot \frac{G_t}{G_{pot}} \tag{3}$$

where G_t is the concentration of glucose (g/L) achieved at time t and G_{pot} is the potential glucose (g/L) that is calculated as:

$$G_{pot} = \frac{Gn}{100} \cdot \frac{180}{162} \cdot \frac{\rho}{LSR + 1 - \frac{KL}{100}} \tag{4}$$

where Gn is the glucan of pre-treated solids (g of glucan/100 g of pre-treated solid dry basis), 180/162 is the stoichiometric factor, ρ is the density of the hydrolysis enzymatic medium (average value, 1,005 g/L), LSR is the liquid to solid ratio (g of liquid/g of oven dry solid, depending on the experiment), and KL is the Klason lignin of pretreated solid (g of Klason lignin/100 g pre-treated solid on dry basis).

3 Results and discussion

3.1 Autohydrolysis process

The *E. globulus* wood was subjected to autohydrolysis process at maximal temperature (T_{MAX})=210, 220, and 230 °C or severity S_0=4.08, 4.38, and 4.67, respectively. The conditions of treatment were chosen based on reported data by Romaní et al. [25] in which the treatment was suitable for enhancing the susceptibility of *E. globulus* wood towards enzymatic hydrolysis for T_{MAX}>210 °C.

Table 1 shows the composition of solid and liquid phase. The solid yield (SY) decreases when the severity increases (71.7±0.89–67.0±2.1 g of autohydrolyzed pretreated EGW/100 g of raw material on dry basis) these SY values are according with previously reported works in the same conditions [17, 25]. The solid phase of pretreated EGW is constituted mainly for glucan and lignin, the hemicelluloses (mainly constituted by xylan) was totally solubilized. The glucan varied in the range 58.3–60.4 g of glucan/100 g (on dry basis) of autohydrolyzed solid. The glucan increase could be correlated to the solubilization of hemicellulose components. Also, the remained xylan in the solid was <0.9 % (expressed as g of xylan/100 g of autohydrolyzed EGW, on dry basis). Figure 2 shows the recovery of main compounds in the solid phase. The average recovery of glucan was 92.7 % (measured as the percentage of glucan present in pretreated EGW with respect to glucan present in the corresponding amount of raw material on dry basis), revealing that the glucan was almost not affected by the autohydrolysis process at 210, 220, and 230 °C. However, the losses of cellulose were higher when the T_{MAX} increased. The cellulose recovery at 230 °C was 89 % in the pretreated solid, while a 5 % of cellulose present in raw material was solubilized in the liquid phase in form of sugars (glucose and glucooligosaccharides); these results show cellulose losses of 6 %. In a recent work, Ruiz et al. [5], reported that the cellulose presents degradation at temperatures >230 °C using hydrothermal process. With respect to the content of lignin in the autohydrolyzed EGW decreased (38.0 to 34.0 g of lignin/100 g of autohydrolyzed EGW, on dry basis) at 210 and 220 °C, respectively and increased (37.0 %) at 230 °C due to condensation reactions, these data are according with previous studies reported by Romaní et al. [25]. Carvalheiro et al. [26] reported that the solubilization of lignin is moderately low, revealing that autohydrolysis process does not interrelate with lignin. But also the fraction of solubilized lignin depends on the severity conditions and raw materials [5].

Autohydrolysis process caused a substantial fractionation of components including monosaccharides, oligosaccharides, acetyl groups, and degradation of sugars as furfural,

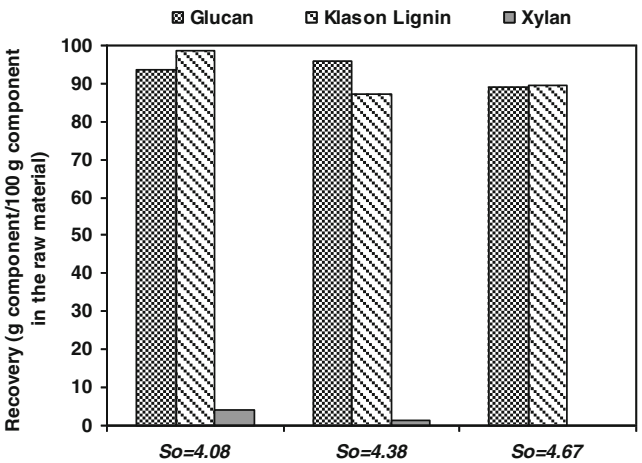

Fig. 2 Recovery of main components in the pretreated solid (on dry basis) after autohydrolysis process

hydroxymethylfurfural, and phenolic compounds. The concentrations (g/L) of the liquid phase components derived principally from *E. globulus* hemicellulose fractions by autohydrolysis at 210, 220, and 230 °C are shown in Table 1. In the mildest conditions (S_0=4.08), the main compounds were xylooligosaccharides (8.97 g/L) and xylose (8.85 g/L) (accounting for 82.4 % of xylan contained in wood). Under the conditions S_0=4.38 and 4.67, the xylose and xylooligosaccharide concentrations in liquid phase decreased with the severity, corresponding to 39.8 and 9.7 % of xylan in raw material, respectively. This behavior is due to the degradation of xylose into furfural. The harshest conditions led to the degradation of xylose, the lowest concentration (2.1 g/L) being achieved and yielding the highest concentration of furfural (5.4 g/L). The resulted liquors of treatments at 220 and 230 °C had a high concentration of inhibitor compounds, as furfural, HMF, and acetic acid (see Table 1). This behavior is in agreement with previous studies reported using hydrothermal processed wheat straw at 200 °C and 60 min (S_0= 4.72) [27]. The acetic acid reached a maximal value of 5.8 g/L at 220 °C. The total phenolic compounds were measured since they can have an inhibitor effect on the enzymatic hydrolysis and fermentation process [28]. The concentration of phenolic compounds varied in the range 2.0–2.66 g/L (S_0=4.08 and S_0=4.67, respectively). Amendola et al. [29] reported 2.54 g/L of the total phenol content in autohydrolysis process liquor using grape stalk as raw material at 180 °C for 30 min.

3.2 Enzymatic hydrolysis

3.2.1 Effect of autohydrolysis treatment on whole-slurry hydrolysis

Different experiments were performed to evaluate the effect of hemicellulosic liquid phase on enzymatic hydrolysis and the operational conditions are listed in the Table 2. Enzymatic hydrolysis were carried out using the whole slurry with the aim of studying the inhibitory loading of each treatment condition (S_0=4.08, 4.38, and 4.67). Table 2 summarized the main results of hydrolysis assays: the glucose concentration and the conversion at 96 h. The maximal concentration of glucose (82.5 g/L) was obtained in the mildest condition of autohydrolysis, corresponding to cellulose to glucose conversion (CGC)=59.4 % at liquid solid ratio=4 g/g and enzyme solid ratio (ESR)=16 FPU/g. The minimal concentration of glucose was 38.5 g/L (corresponded with a CGC=38.8 %), operating at LSR=6 g/g, and ESR=16 FPU/g of substrate, under the harshest conditions of pretreatment. The increase of pretreatment severity implies a decrease of glucose concentration and conversion, using the whole slurry. It can be seen a clear effect of inhibitory loading of HLP on enzymatic hydrolysis. Moreover, the hydrolysis conversion can be limited for the effect of high solids used in the assays. Hodge et al. [30] studied the contribution of soluble and insoluble solids on the enzymatic hydrolysis using corn stover pretreated with dilute acid and demonstrated that the high concentration of sugars were the primary cause of inhibition, also the inhibition by insoluble solids (mass transfer and insoluble lignin) and inhibition by soluble solids (acetic acid, furans, and phenolics) decreases of cellulose conversion. Shen et al. [31] reported that increasing the substrate from 2 to 16 % led to a significant reduction in glucan conversion. According to Kristensen et al. [32], providing high substrate concentrations throughout the conversion process is an important key for the economic viability of bioethanol production. In these days, to meet the economic requirements in the production of bioethanol, the final concentration of ethanol should be above 4 % (*w*/*w*) therefore the liquid to solid ratio of work should be <8 g/g [33]. An important factor to consider is that the use of HLP provides additional soluble solids due to the hemicellulose-derived compounds, increasing the solid loading in the enzymatic hydrolysis. The non-volatile compounds of HLP was 0.0235, 0.0181, and 0.0135 g/g for S_0=4.08, 4.38, and 4.67, respectively. Considering the soluble solids of HLP, the solid loading can increase between 2.35 and 1.35 %, depending on the experiment (see Table 2).

3.2.2 Effect of HLP percentage on enzymatic hydrolysis

On the basis of previous results, the autohydrolysis treatments selected to study the amount of HLP on enzymatic hydrolysis were carried out at T_{MAX}=210 and 220 °C. The enzymatic hydrolysis of EGW provided different results according to the different composition of the pretreated material at 210 and 220 °C (see Table 3). The HLP were added in the enzymatic assays in percentages of 0, 50, and 100 % of total liquid in the experiment. The 0 % of HLP means that the solid is added without washed, and without hemicellulosic liquid phase (see Fig. 1); the other conditions (50 and 100 % of HLP) also used the unwashed solid, however the liquid fraction was composed by different loadings of HLP. The highest glucose concentration at 210 °C was 85.82 g/L on the unwashed autohydrolyzed EGW (0 % HLP) for an enzyme loading of 25 FPU/g of substrate and liquid solid ratio of 6 g/g. Under these conditions, the CGC was also maximal with an 88.3 %.

The glucose concentration was reduced between 13.3 and 16.7 % using an enzyme loading of 16 FPU/g of substrate for each percentage of HLP used in the enzymatic hydrolysis. On the other hand, 53.2 g of glucose/L was obtained when the 100 % of HLP was added, corresponding to 54.8 % of cellulose–glucose conversion. The concentration of glucose augmented 37.9 % without HLP addition. To explain this behavior, Kumar and Wyman [34] showed that xylooligosaccharides strongly inhibit cellulase action. In a recent work, Zhang and Viikari [35] reported that the xylobiose and xylotriose were competitive inhibitors of cellobiohydrolase I, revealing the strong inhibition of cellulase by xylooligosaccharides.

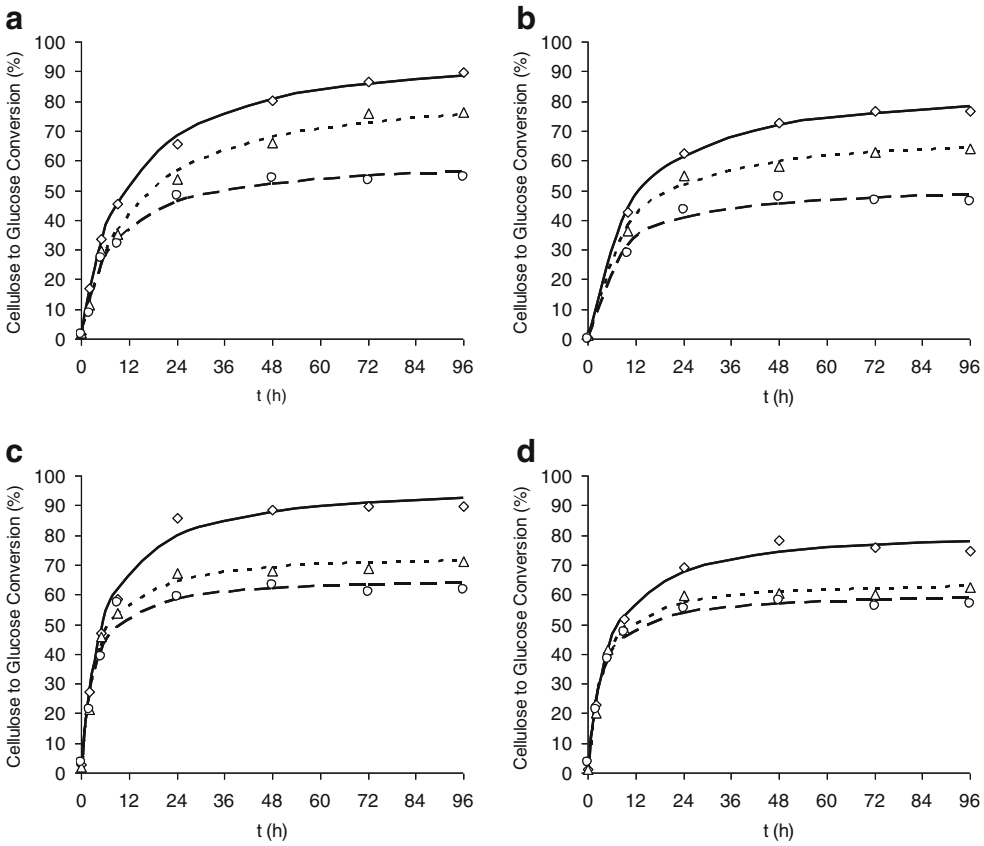

Fig. 3 Cellulose to glucose conversion (*CGC*) using different proportions of HLP (0, 50, and 100 %) on enzymatic hydrolysis: **a** autohydrolyzed EGW (S_o=4.08; LSR=6 g/g, and ESR=25 FPU/g). **b** Autohydrolyzed EGW (S_o=4.08; LSR=6 g/g, and ESR=16 FPU/g). **c** Autohydrolyzed EGW (S_o=4.37; LSR=6 g/g, and ESR=25 FPU/g). **d** Autohydrolyzed EGW (S_o=4.37; LSR=4 g/g, and ESR=25 FPU/g). *Symbols*: CGC experimental with 0 % (*diamonds*), 50 % (*triangles*), and 100 % (*circles*) of HLP. *Lines*: CGC calculated with 0 % (*black line*), 50 % (*dotted line*), and 100 % (*dashed line*) of HLP

Qing et al. [36] showed that the xylooligosaccharides were more inhibitory than xylan, or xylose in terms of a decreased initial hydrolysis rate. In addition, they reported no inhibition of xylooligosaccharides on the β-glucosidase enzymes.

For 220 °C, the highest glucose concentration was 107.5 g/L using an enzyme loading of 25 FPU/g of substrate without addition of HLP. As is mentioned above, the lowest glucose concentration was 61.9 g/L with 100 % of HLP. The effect of HPL from 220 °C also inhibited the enzymes action. The xylooligosaccharides content was lower compared with the treatment at 210 °C, so the inhibitory effect can be due to the high concentration of phenolic compounds (2.46 g/L), furfural (4.97 g/L), and acetic acid (5.08 g/L), this effect has also been reported by Hodge et al. [30]. Kim et al. [37] reported that phenolic compounds and xylooligosaccharides were found to be the most important causes of decreased cellulase activity.

In order to use the whole slurry on enzymatic hydrolysis, the addition of xylanases was studied. The inhibition caused by xylooligosaccharides can be reduced or overcome using a xylanase supplementation [38]. The experiments were performed with HLP from treatment at 210 °C, since the xylooligosaccharides concentration was higher (8.97 g/L). Table 4 shows the operational conditions and the main results of enzymatic hydrolysis: glucose concentration (G_{96h}) and cellulose to glucose conversion (CGC_{96}). A dosage of 2, 4 and 6 UI xylanase/FPU cellulase was supplemented. The highest concentration of glucose was 67.87 g/L (68.2 % of CGC_{96}). The supplementation of xylanase caused a positive effect in the glucose production, increasing from 45.15 to 60.87 g/L (under conditions LSR=6 g/g and ESR=16 FPU/g of substrate) and from 53.27 to 67.87 g/L (LSR=6 g/g and ESR=25 FPU/g of substrate) without and with xylanase, respectively. The enhancement correspond to an increase 35 and 27 %, respectively, with respect to whole slurry

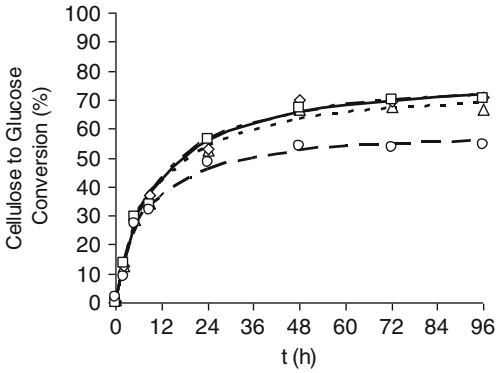

Fig. 4 Cellulose to glucose conversion of autohydrolyzed EGW using the 100 % of HLP with and without xylanase supplementation (S_0=4.08; LSR=6 g/g, and ESR=25 FPU/g). *Symbols*: CGC experimental without (*diamond*); with 2 UI (*triangle*); with 4 UI (*circle*); with 6 UI (*square*) xylanase/FPU of cellulase. *Lines*: CGC calculated without (*dashed line*); with 2 UI (*dotted line*); with 4 UI (*dashed dotted line*); and with 6 UI (*black line*) xylanase/FPU of cellulase

without addition of xylanases on enzymatic hydrolysis. These results are according with Alvira et al. [39] that reported an improvement in the enzymatic hydrolysis of steam exploded wheat straw (210 °C for 2.5 min) when the xylanases were supplemented, with an increase of 40 % with respect to non-supplementation of xylanase, reaching a 67 % of cellulose–glucose conversion. Moreover, they suggest that the xylanases acts not only on the insoluble hemicellulosic fraction but also on the soluble xylooligomers present in the slurry, which may have an important inhibitory effect. Várnai et al. [40] showed that the supplementation of xylanolytic and mannanolytic enzymes acted synergistically on the enhanced the hydrolysis of cellulose and concluding that are crucial to obtain high yields.

3.2.3 Enzymatic hydrolysis kinetics

Figure 3 shows the cellulose conversion profiles obtained when the two substrates (210 and 220 °C) were hydrolyzed under conditions of experiment shown in Table 3. Figure 3a and c shows the effect of pretreatment on enzymatic hydrolysis. The maximum conversion of cellulose was achieved within the 48 h, although an increase of 9 % was obtained in the experiment with a 50 % of HLP (see Fig. 3a) between 48 and 96 h. The harshest treatment (220 °C) achieved the 80 % of conversion for t=24 h using the unwashed solid, while for substrate from treatment at 210 °C, the CGC was 65 % at t=24 h. An increase in the severity of the pretreatment conditions allowed faster hydrolysis using the unwashed solid. However, the HLP addition in 50 and 100 % strongly inhibited the cellulase action. The HLP from mildest treatment (210 °C) on enzymatic hydrolysis showed a gradual decrease of conversion as function of HLP percentage. An increase of enzyme loading (16 to 25 FPU/g) caused an improvement of 12, 13, and 8 % of conversion in enzymatic hydrolysis using 0, 50, and 100 % of HLP (Fig. 3a and b). The experiments carried out at LSR=6 g/g (Fig. 3c) improved the cellulose to glucose conversion in 14, 9, and 5 % using 0, 50, and 100 % of HLP, respectively, compared with enzymatic hydrolysis carried out at LSR=4 g/g (Fig. 3d).

The curves of glucose representation showed a typical pattern (see Fig. 3), the data of cellulose to glucose conversion/time of each experiment were fitted to the Eq. 2 [24]. The values of variables CGC_{MAX} and $t_{1/2}$ were shown in the Tables 3 and 4. The CGC_{MAX} increased with the ESR, the CGC_{MAX} decreased with the percentage of HLP supplemented and with the severity of pretreatment. The average of $t_{1/2}$ for S_0=4.08 was 9.1 h and for S_0=4.37 was 3.91 h. In general for this study, for given conditions of enzymatic hydrolysis, the values of $t_{1/2}$ decreased with the hardness of pretreatment. Though, in the hydrolysis of substrates from the harshest conditions the CGC_{MAX} was <65 % using the 50 and 100 % of HLP. In the Fig. 3, it also can be seen a good correlation of calculated and experimental data of enzymatic hydrolysis and consequently the R^2 values were in the range 0.987–0.998.

Figure 4 shows the released glucose of experiments listed in Table 4. Moreover, the differences of enzymatic hydrolysis with and without xylanases can be observed. A higher xylanase supplementation did not cause a significant improvement on cellulose to glucose conversion (see Fig. 4). The kinetic of hydrolysis were similar within the 10 h. An increase in the CGC=17 % was obtained when the xylanases were supplemented at enzymatic hydrolysis t=48 h. The employed xylanases also allowed the complete hydrolysis of xylooligosaccharides into xylose (12.5–18.45 g/L). Lin et al. [41] reported an increase the yields of glucose and xylose using the cellulases combined with xylanase. Tabka et al. [42] showed the synergistic effect in the enzymatic hydrolysis of wheat straw (pretreated with diluted acid followed by steam explosion) when cellulase, xylanase, and feruloyl esterase were mixed obtained the highest released of glucose. Moreover, the percentage of released glucose was increased from 37 to 51.4 % with the increase of temperature [42]. Several studies have proven the effect of xylanases, since the xylanases increases the accessibility of cellulase to cellulose by removing the hemicelluloses barrier [41, 43].

4 Conclusions

In summary, it was shown that autohydrolysis is an effective pretreatment for improving the enzymatic susceptibility of unwashed pretreated EGW contributing to reduce operational cost (water consumption). Moreover, when 100 % of the HLP is added to the enzymatic hydrolysis a strong inhibition was observed. However, a positive effect was shown on the enzymatic hydrolysis when the xylanases were added. Nevertheless, additional research is needed to determine the main operational effects on enzymatic hydrolysis using whole slurry after autohydrolysis pretreatment and in a further work, the use of a robust strain capable of growing and fermenting in the presence of inhibitors.

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