Research paper

Study of the enzymatic activity inhibition on the saccharification of acid pretreated corn stover

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ABSTRACT

The inhibition of the enzymatic saccharification of acid pretreated corn stover (PCS) biomass due to several compounds either present in PCS or produced during saccharification has been studied. The prospective inhibitors tested were glucose ($\leq$110 g L^{-1}), celobiose ($\leq$24 g L^{-1}), xylose ($\leq$50 g L^{-1}), arabinose ($\leq$1.5 g L^{-1}), furfural ($\leq$2 g L^{-1}), hydroxymethylfurfural ($\leq$1 g L^{-1}), acetic acid ($\leq$4 g L^{-1}), and lignin ($\leq$50 g L^{-1}). Each of these compounds was added at three different concentrations, being the concentration intervals different according to standard maximum concentrations of such compounds in the reaction medium, previously measured and described in literature. In addition, these experiments were employed to evaluate the standard error present during the evaluation of the results obtained in the inhibition reactions. Those results show that significant inhibition was only detected for lignin (more than 25 g L^{-1}) and it was also appreciable for glucose at high concentrations (above 75 g L^{-1}), although it was not remarkable at medium concentrations (40 g L^{-1}). On the other hand, neither of the remaining compounds tested presented any significant inhibitory effect at the usual process concentration range.

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1. Introduction

Currently, lignocellulosic biomass has become one of the most important renewable sources for biofuels and chemicals production through its conversion to short-chained sugars [1,2]. Contrary to production of sugars employing food crops, which compete with food and animal feed crops [3], this new industrial technology involves the use of agroforestry waste, among other non-edible feedstock. In the latter years, an industrial process gaining increasing importance is the enzymatic hydrolysis of lignocellulosic biomass [4]. However, lignocellulosic biomass consists of cellulose surrounded by a hemicellulose-lignin matrix, being lignin recalcitrant to decomposition [4]. There are different pretreatment methods that can be used to facilitate enzymatic saccharification of lignocellulosic biomass (Table 1). Their purpose is to disrupt lignocellulosic biomass structure so as to yield several fractions of it and render it more accessible and reactive to enzymatic

degradation. However, pretreatments may produce several compounds able to act as inhibitors for the subsequent enzymatic hydrolysis [4–6].

This enzymatic degradation is carried out by a complex mixture of enzymes [7]. Although there are many different industrial enzyme cocktails, the main cellulase activities present in this kind of formulations are endoglucanases, exoglucanases, and β-glucosidases. It appears that these enzymes show interaction among them leading to a synergistic effect, for each of them creates substrates for the others or remove oligosaccharides and disaccharides that are inhibitors of another cellulases [3]. Moreover, the compounds released during pretreatment of lignocellulosic biomass may cause inhibitory effects to one or more of these enzymes or disrupt synergistic effects among the different enzymes present in enzymatic cocktail formulations [2].

Lignin has been found to be one of the most reported compounds in literature as an important inhibitor of enzymatic hydrolysis, jointly with its oligomers and monomers [5,8–11]. Lignin is a cross-linked aromatic polymer composed of hydroxyphenyl, guaiacyl, and syringyl units [11], which can interfere in the activity of cellulases. Although the mechanism of lignin-related inhibition

Table 1
Some pretreatment technologies used to enhance lignocellulosic biomass hydrolysis.

Pre-treatment	Lignocellulosic biomass	Reference
Electron beam	Switchgrass	[35]
Hydrothermal	Corncobs	[36]
Organosolv	Wheat straw	[37]
Diluted acid	Harding grass (*Phalarisaquatica*)	[38]
Diluted alkali	Rice straw	[39]
Concentrated acid	Alfalfa stems	[40]
Fenton and diluted alkali	Corn stover	[41]
Steam explosion	Vineyard pruning residues	[42]

still remains to be fully elucidated, several processes of this nature have been described, including lignin blocking enzymatic degradation by steric hindrances, soluble lignin phenolics acting as enzyme inhibitors, and improductive association of enzymes and lignin (non-specific union *via* hydrophobic bindings or electrostatic interactions) [6,8].

Other important group of compounds reported as inhibitors of enzymatic saccharification are those produced after several pretreatments like furan derivatives, such as furfural or hydromethylfurfural (HMF) [12]. Organic acids, such as acetic, formic and levulinic acid have also been cited as potential inhibitors, as well as other kind of compounds, such as some phenolic species (e.g. vanillin, syringaldehyde, tannic acid, etc.), which have been reported to act as inhibitors at concentrations higher than 5−10 g/L [6,12]. In addition, the presence of this type of compounds has also been accounted for in literature as potential inhibitors of fermentation of lignocellulosic biomass hydrolysate by yeasts [13].

Finally, other intermediate products of enzymatic degradation (known as oligosaccharides) have been deemed recently as a possible cause of the decrease of sugar production rate on the lignocellulosic biomass hydrolysis [14,15]. Inevitably, these inhibitory compounds are generated as intermediates in cellulose and hemicellulose hydrolysis, for the former of which final enzymes, such as β-glucosidases, β-mannosidases, and β-xylosidases are the main actors in the final hydrolysis to monosaccharides. Additionally, it is important to remark that the diverse inhibitory subtances could also influence negatively the enzymatic degradation due to synergistic effects, as recently reported by Arora et al. who gave evidence on the formic acid and furfural combined effect [16].

To overcome the reduction in productivity and the excessive concentration of enzymes in the saccharification processes, that render them economically unfeasible, studies have been focused on screening for new inhibitor-tolerant glucanases and β-glucosidases [17−20], genomic modifications of known enzymes [20,21], and process design to minimize inhibitory effects [22−24].

The aim of this work is to evaluate the possible inhibitory effect of different compounds (lignin, acids, and aldehydes) on the enzymatic saccharification of acid-treated corn stover (PCS) using each inhibitor at a time. Before determining any inhibitory effects, several enzymatic hydrolysis runs without the addition of possible inhibitors were performed to estimate experimental error. Thereafter, different compounds prone to be produced during pretreatment or by the saccharification process itself were added to PCS prior to enzymatic reaction. The intent of the latter experiment is to evaluate whether their presence led to a reduction in the productivity of the hydrolysis process, which was measured as glucose yield at three different reaction times.

2. Materials and Methods

2.1. Pretreated biomass properties

Pretreated corn stover (PCS) was provided by Abengoa. Corn stover was pretreated employing a dilute acid/steam explosion method (completed in York Pilot Plant, Nebraska, USA). The initial total solids content (T_S) was 0.37 $g_{solids} \cdot g_{total}^{-1}$, which was afterwards diluted to 0.20 $g_{solids} \cdot g_{total}^{-1}$ by adding de-ionized water before each experiment was carried out. The pH value of the PCS suspension was adjusted to a value of 6.50 using an ammonium solution (10% of NH_3 content). After this treatment, PCS was stored at −5 °C for a maximum time of 3 days.

The compositional analysis of the raw material of the enzymatic hydrolysis, after dilution and prior to enzymatic runs, was conducted using NREL methodology [25]. The concentration of glucanes, xylanes, arabinanes, and acetyl groups was determined by using a complete acid hydrolysis methodology, as specified by NREL [26,27]. The concentrations of the monomers were measured by HPLC, using an Agilent 1100 HPLC device with a Phenomenex Rezex-RHM column, employing 2.5 mM H_2SO_4 in Milli-Q water as mobile phase. Finally, the amount of extracts and insoluble solids were determined by NREL methods [28,29].

2.2. Enzymatic hydrolysis procedure

All hydrolysis experiments were realized in screwed plastic flasks. Initially, the mass content inside each flask was 90 g of PCS as described in Section 2.1. Flasks with PCS were heated in order to achieve operational temperature value (50 °C). Once this temperature was reached, the enzyme cocktail was inoculated into each flask, always at the same concentration. The enzymatic preparation employed was the industrial cocktail Zylase^R commercialized by Abengoa Biotechnology, which has an average activity of 90 ± 2.5 filter paper units (FPU) and 21 ± 1.4 activity units on cellobiose (CBU) per gram of solution, as specified by the supplier.

Experiments were performed in shaken flasks at an agitation speed of 125 rpm and an enzyme cocktail dosage of 15.5 $mg_{protein} \cdot g_{glucane}^{-1}$. It was verified with previous experiments that, under the agitation conditions employed, mass transfer is not the controlling step of the overall process rate. This in turn means that mass transfer resistance in an orbital shaker at such agitation speed is negligible, thus not controlling the overall saccharification process rate [30].

In some experiments, different amounts of potential inhibitors were added prior to enzyme inoculation. These compounds and the concentrations employed in the different runs are shown in Table 2. The inhibitor concentration interval studied in each case depends on the final concentration regularly obtained in a typical enzymatic hydrolysis experiment. Such standard inhibitor concentration intervals are given in Table 3.

Potential inhibitor compounds employed in this work were commercial substances, whose purities and suppliers are as follows: Glucose (99%, Panreac-AppliChem, Darmstadt, Germany), xylose (99%, Sigma-Aldrich, Saint Louis MO, USA), arabinose (98%, Sigma-Aldrich, Saint Louis MO, USA), cellobiose (99%, Fluka-Sigma-Aldrich, UK), furfural (98%, Sigma-Aldrich, Saint Louis MO, USA), 5-hydroxymethylfurfural −HMF- (99%, Sigma-Aldrich, Saint Louis MO,USA), and acetic acid (100%, Panreac-Applichem, Darmstadt, Germany). Lignin was provided by INIA-CIFOR, obtained from a wheat straw organosolv liquor (170 °C, 90 min treatment time, 30% w/w ethanol/water) subjected to acid precipitation. In the hydrolysis experiments, several samples were withdrawn throughout the course of the reaction. Liquid fraction of the samples was separated from solid fraction by centrifugation. After separation, the liquid

Table 2
Experiments carried out: inhibitors added at different concentrations.

Run	Inhibitor	Concentration added (g·L^{-1})
1	N/A	N/A
2		
3		
4		
5		
6		
7	Glucose	40.00
8		75.00
9		110.00
10	Celobiose	6.00
11		12.00
12		24.00
13	Xylose	25.00
14		40.00
15		50.00
16	Arabinose	0.50
17		1.00
18		1.50
19	Furfural	0.50
20		1.00
21		2.00
22	HMF	0.25
23		0.50
24		1.00
25	Lignin	25.00
26		50.00
27	Acetic acid	4.00[a]
28		4.00[b]

[a] pH was adjusted before acetic acid dosification.
[b] pH was adjusted after acetic acid dosification.

total potential glucane content (g$_{glucane}$·g$_{total}^{-1}$) and T_S is the fraction (w/w) of solids in the initial PCS. Water insoluble solids were evaluated employing NREL methodology [28]. Finally, 1.11 is the potential conversion factor from glucane to glucose.

3. RESULTS and DISCUSSION

3.1. Pre-treated cornstover composition and average enzymatic reaction behaviour

The initial compositions of PCS employed in all saccharification experiments were as follows: 39.7, 9.1, 0.7, 0.9, and 23.8% w/w of glucose, xylose, arabinose, acetyl groups, and lignin, respectively. The amount of extractive compounds was 21.3% w/w.

In order to evaluate the influence of possible inhibitors on enzymatic saccharification of lignocellulosic biomass (PCS), it is necessary to know, firstly, the average behaviour of the enzymatic hydrolysis without the addition of any potential inhibitor. For that purpose, six runs were performed under the same operating conditions, identified as runs 1 through 6 in Table 2.

Measuring glucose concentration, conversion (X) was calculated with equation (1) at different reaction times. Then, an average X value was estimated for each reaction time, together with its standard deviation. Results achieved (both experimental and averages) are given in Fig. 1, showing that the experimental conversion values at each time are very similar. Therefore, there are not large deviations from the average value (lower than 10% at 0.5 conversion value), and it can be inferred that such average conversions describe the enzymatic saccharification behaviour prop-

Table 3
Concentration values of different compounds at final reaction time in control runs, without added prospective inhibitors (runs 1 to 6).

Compound	Average concentration (g·L^{-1})	Standard deviation (g·L^{-1})
Glucose	60.0	10.0
Xylose	22.0	2.0
Celobiose	1.38	0.01
Arabinose	3.01	0.04
Acetic acid	4.54	0.03
Furfural	1.41	0.01
Hydroximethylfurfural	0.364	0.001

phase was filtrated and then refrigerated prior to analysis.

### 2.3. Samples analysis and calculations

The concentrations of the compounds (glucose, xylose, arabinose, acetic acid, furfural and HMF) in the samples were determined from the liquid phase with a HPLC technique, utilizing an Agilent 1100 HPLC with a PhenomenexRezex-RHM column, employing 2.5 mM H_2SO_4 solution as the mobile phase. Column temperature was fixed at 80 °C.

The results were described as glucose conversion achieved with time in each experiment of enzymatic saccharification. This conversion was evaluated following the equation:

$$X = \frac{(1 - WIS_t) \cdot C_{Gt} - (1 - WIS_0) \cdot C_{G0}}{G' \cdot 1.11 \cdot T_S - (1 - WIS_0) \cdot C_{G0}}$$ (1)

where X is the cellulose conversion into glucose, WIS_t is the water insoluble solids at a given time (g$_{WIS}$·g$_{total}^{-1}$), C_{Gt} (g$_{glu}$·g$_{total}^{-1}$) is the amount of glucose (glu) at a given time, WIS_0 is the water insoluble solids at initial time, C_{G0} is the glucose concentration at initial time, G' is the glucane concentration in the initial PCS, expressed as the

erly. As a consequence, these results can be used as reference for
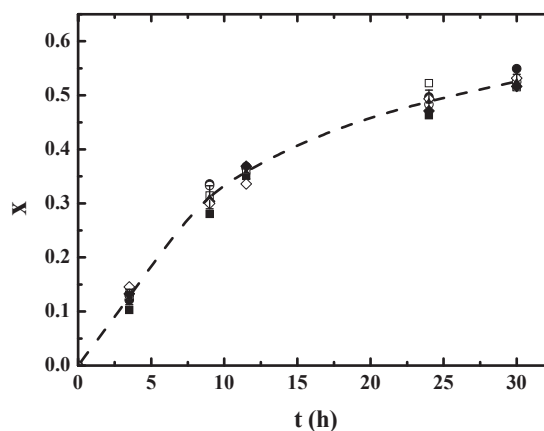
Fig. 1. Glucose conversion vs. time for experiments 1 (solid circles), 2 (hollow circles), 3 (solid squares), 4 (hollow squares), 5 (solid diamond), 6 (e hollow diamond), and average evolution of runs 1 to 6 (solid triangles and dashed line) with standard deviations.

experiments carried out with the addition of potential inhibitors added in different concentrations.

The maximum concentration of each potential inhibitor can be determined by obtaining the final concentration of these compounds for each control experiment (runs 1 to 6). The final average concentration and the standard deviation for those potential inhibitors are shown in Table 3. Although potential inhibitors studied in this work have been previously described as inhibitors of the enzyme activity on the biomass enzymatic saccharification, the aim of this work is to evaluate the effect of these compounds within the concentration interval given in Table 2, given that these values are achieved at standard operational conditions.

For lignin and acetic acid inhibition studies, several strategies were devised and tested. The initial amount of lignin was decided on the basis of the composition obtained as a result of PCS analysis. Initial concentration of acetic acid was set according to that obtained as final average value on the standard enzymatic hydrolysis (4 g L^{-1}). However, as pH dramatically decreases after acetic acid addition, two different runs were carried out: one adjusting pH prior to enzymatic reaction and another without such correction; in this way, the effect of pH and acetate concentration were taken into account separately.

3.2. Effect of different inhibitors on enzymatic saccharification

Results achieved in experiments 7 through 28 are shown in Figs. 2 and 3. In the former figure, results of the runs carried out adding glucose, celobiose, xylose, and arabinose at several concentrations can be seen. Moreover, in Fig. 3, results of the experiments performed with the addition of different initial amounts of furfural, HMF, lignin, and acetic acid are shown.

Considering the effect of adding glucose at time zero, it can be said that the lowest glucose concentration added (40 g L^{-1}) does not affect the conversion reached at any reaction time. However, with higher concentrations of initial glucose added (75 and 110 g L^{-1}), glucose yield during enzymatic inhibition clearly decreases. Therefore, it can be stated that medium to high concentrations of glucose inhibit glucan enzymatic hydrolysis. Nevertheless, those concentration levels are not reached in a standard enzymatic saccharification, except at long reaction time and with an exceptionally pretreated lignocellulosic biomass. Hence, it is possible to uphold that glucose does not exert high inhibitory control over enzymatic reaction in average conditions, in this case. However, recent works in the literature pointed out that one of the enzymes employed for lignocellulosic biomass hydrolysis (β-glucosidase), which is always present in enzymatic cocktail
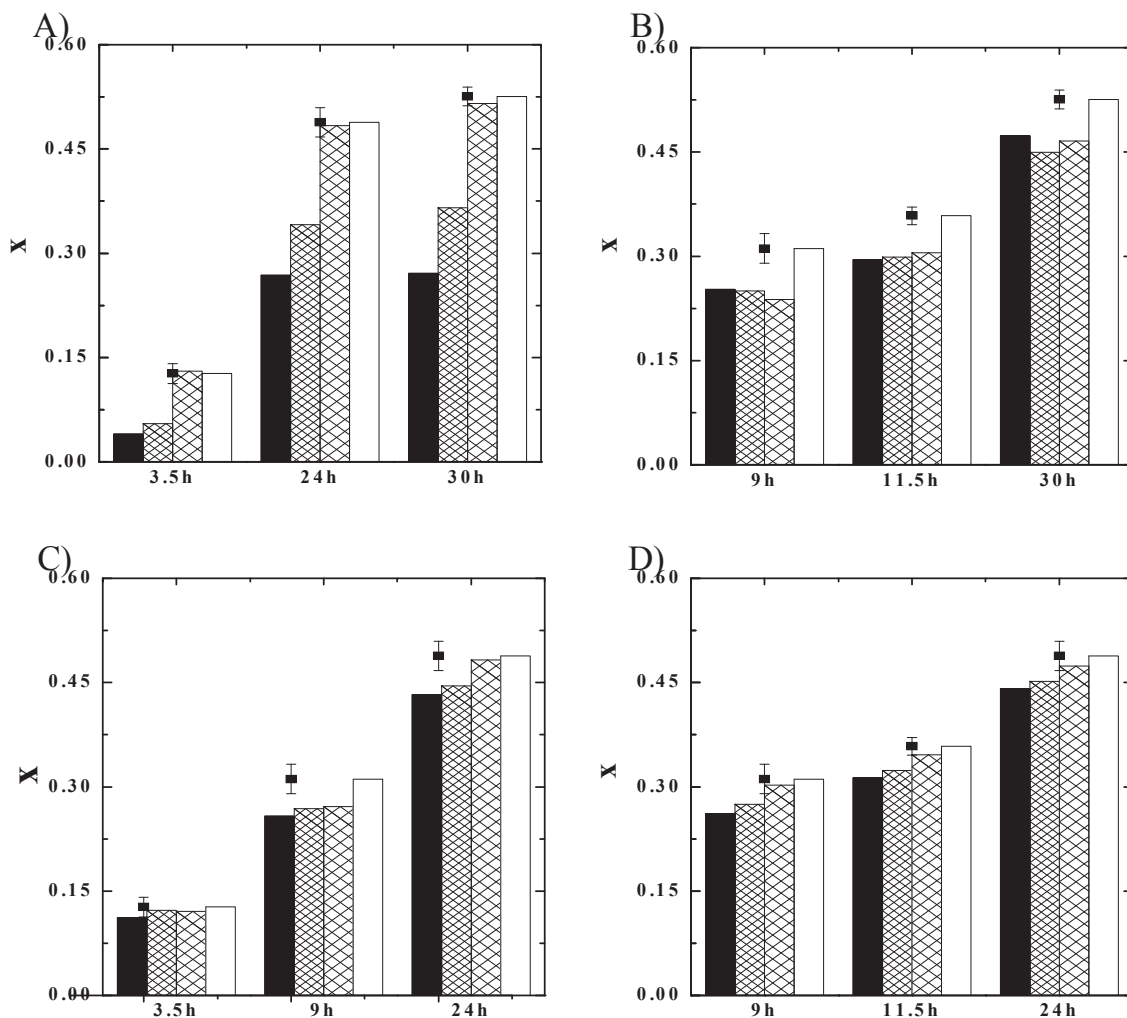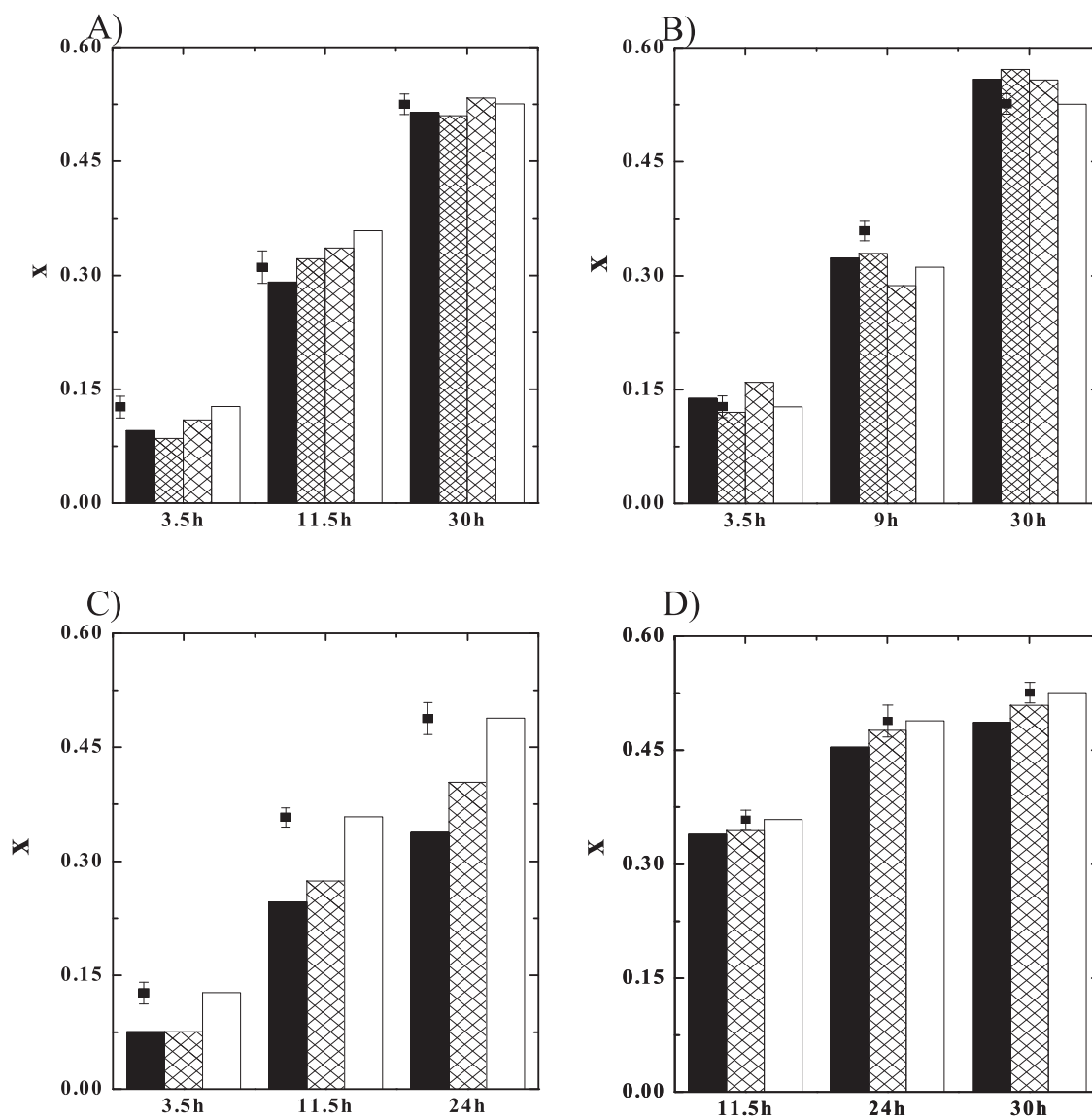
Fig. 2. Glucose conversion at several saccharification times A) glucose (runs 7, 8 and 9); B) cellobiose (runs 10, 11 and 12); C) xylose (runs 13, 14 and 15); D) arabinose (runs 16, 17 and 18). Comparison with average results in runs 1 to 6, without inhibitors (white bars and black solid squares with the standard deviation intervals). Black bars corresponds to the highest level of inhibitor; narrow-striped bars to medium values for inhibitor concentration, and wide-striped bars for the lowest concentration of the inhibitor.

formulations, shows a decrease in its activity even in the presence of concentrations as low as 1 mM or 2 g L^{-1} of glucose. On the contrary, other enzymes with similar activity even show activation in the presence of such monosaccharide at concentrations as high as 10 mM or 18 L^{-1} or are affected only at very high concentrations of their main product –near 1 M or 180 g L^{-1} [19,21,31]. The concentration of glucose checked in this work is similar to the latter limit, and even so inhibition was hardly detected. Moreover, β-glucosidase is still able to release glucose at significant yields when the concentration of glucose initially added was lower than 40 g L^{-1}. In conclusion, it can be stated that β-glucosidases present in the cocktail under study are scarcely affected by their product (low inhibition), although no activation of the enzymes due to this sugar is observed. This effect was detected in enzymes from several metagenomes at concentrations as high as 90–100 g L^{-1} of added glucose at zero time [19].

In the case of the other monosaccharides studied in this work, results in Fig. 2 prove that, even for the highest added concentration of these compounds (xylose and arabinose), only a slight lessening on cellulose conversion into glucose is observed. Therefore, for the case under study, xylose and arabinose have low inhibitory effects on enzymatic saccharification, at least for the concentrations of potential inhibitor studied in this work. Furthermore, concentration of both sugars only increase slightly during enzymatic saccharification. Both compounds are generated during corn stover pretreatment, due to hemicellulose degradation [3].

Finally, according to the results given in Fig. 2, it can be said that cellobiose addition has no negative influence on enzymatic saccharification. According to literature, there are several works claiming for inhibition of endoglucanases and exoglucanases by the presence of large concentrations of cellobiose [32], although there is a significant lack of recent studies on the effect of cellobiose on enzymatic saccharification. Reports and papers published in the recent years have demonstrated that the combination of different enzymes present in cellulolytic cocktails (as employed for experiments shown in these works) generate a synergistic effect that reduces inhibitory effects on cellulase reactions [33]. At the same

Fig. 3. Glucose conversion at different saccharification times for added A) Furfural (runs 19, 20 and 21); B) HMF(runs 22, 23 and 24); C) Lignin (runs 25 and 26); D) acetic acid (runs 27 and 28). Solid black bars correspond to the highest level of added inhibitor; narrow- and wide-striped bars to medium and low inhibitor levels and white bars to for no addition of inhibitors at time zero (as well as the solid squares with the standard deviation intervals).

time, although enzymes from *Trichoderma* and *Aspergillus* are well-known for being inhibited by oligo-, di-, and mono-saccharides, the number of microorganisms excreting cellulases (mainly β-glucosidases) with low inhibition, no inhibition or even activation by glucose and cellobiose is increasing in the last years [19,21]. In any case, cellobiose is more prone to affect exoglucanases and endoglucanases, whilst the action of β-glucosidases could be more affected by glucose. The latter enzymes are usually more active than glucanases, and cellobiose and cellooligosaccharides are rapidly removed by β-glucosidases.

In Fig. 3, the effect of the addition of aldehydes (furfural and HMF), lignin, and acetic acid is shown. As can be seen, HMF has no effect on cellulose conversion during enzymatic hydrolysis of PCS, at the concentration levels studied in this work. For furfural, only a slight decrease in conversion at medium saccharification time is evident, so inhibition, if present, is not significant. However, due to PCS carbohydrate composition, high concentrations of furfural (a product due to xylose dehydration during biomass pretreatment) are not to be expected.

Curiously, acetic acid acts as a mild inhibitor, when added initially, with or without a neutralisation step prior to the enzyme addition (similar results). This result is not in line with the results reported in the literature [6,12,34]. Once again, it can be explained by two different factors: the low concentrations of these compounds used (though similar to those used in previous studies), and the resistance of the enzymes used in this work with respect to this potential inhibitor.

Nevertheless, as can also be seen in Fig. 3, the addition of lignin has an important inhibitory effect that is reflected on final glucose conversion achieved. Similar results have been observed by other authors [8–10]. Therefore, according to the results achieved in this work, it is possible to say that lignin affects negatively the final glucose yield in the case of the enzymatic cocktail (Zylase^R) employed. However, because lignin composition and polymerisation degree can be very different, depending on its origin, further studies are necessary so as to establish relationships between lignin amount and physicochemical properties and the decrease of final concentration of glucose achieved after enzymatic hydrolysis.

Finally, this work deals only with independent inhibitory effect of the different substances evaluated. It would be desirable in further works to study the synergistic effect between the different potential inhibitors studied.

4. Conclusions

According to the results previously described, high contents of lignin and glucose have a significant inhibitory effect on the activity of the enzyme cocktail herein studied, with the final glucose yield decreasing as the addition of the initial concentration of these compounds increases. Other inhibitory compounds studied in this work (cellobiose, xylose, arabinose, furfural, HMF, and acetic acid) only have a slight effect on cellulose to glucose enzymatic conversion, at least at the concentration levels studied. However, it is likely that the synergistic effect of mixtures of these compounds could have a significant negative impact on enzymatic saccharification.

Acknowledgements

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