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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 (≤110 g L^{-1}), celobiose (≤24 g L^{-1}), xylose (≤50 g L^{-1}), arabinose (≤1.5 g L^{-1}), furfural (≤2 g L^{-1}), hydroxymethylfurfural (≤1 g L^{-1}), acetic acid (≤4 g L^{-1}), and lignin (≤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.

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

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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