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Inhibition Effects of Dilute-Acid Prehydrolysate of Corn Stover on Enzymatic Hydrolysis of Solka Floc

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Abstract Dilute-acid pretreatment liquor (PL) produced at NREL through a continuous screw-driven reactor was analyzed for sugars and other potential inhibitory components. Their inhibitory effects on enzymatic hydrolysis of Solka Floc were investigated. When the PL was mixed into the enzymatic hydrolysis reactor at 1:1 volume ratio, the glucan and xylan digestibility decreased by 63% and 90%, respectively. The tolerance level of the enzyme for each inhibitor was determined. Of the identified degradation components, acetic acid was found to be the strongest inhibitor for cellulase activity, as it decreased the glucan yield by 10% at 1 g/L. Among the sugars, cellobiose and glucose were found to be strong inhibitors to glucan hydrolysis, whereas xylose is a strong inhibitor to xylan hydrolysis. Xylo-oligomers inhibit xylan digestibility more strongly than the glucan digestibility. Inhibition by the PL was higher than that of the simulated mixture of the identifiable components. This indicates that some of the unidentified degradation components, originated mostly from lignin, are potent inhibitors to the cellulase enzyme. When the PL was added to a simultaneous saccharification and co-fermentation using *Escherichia coli* KO11, the bioprocess was severely inhibited showing no ethanol formation or cell growth.

Keywords Inhibition · Cellulase · Hydrolysate · Tolerance · Xylo-oligomers · Lignin · Dilute-acid pretreatment · Degradation · Phenolic · Enzymatic hydrolysis

Introduction

In the biomass structure, hemicellulose is covalently linked to cellulose and lignin, forming a rigid cellulose–hemicellulose–lignin matrix [1]. Lignin is a heterogeneous polymer whose main function in plants is to provide support, impermeability, and resistance to microbial and chemical attack [2, 3]. Hemicellulose and lignin cover and protect the cellulose from degradation by enzymes and chemicals [1]. Most pretreatment methods focus on removing part of these barriers, making biomass conducive to enzymatic degradation. Dilute-acid

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pretreatment is one of the most promising methods of pretreatment. In this method, a large part of hemicellulose is hydrolyzed into the liquid hydrolysate stream [4–6]. Cellulose and most of the lignin remain in the solid after dilute-acid treatment. In addition to sugars, the hydrolysate also contains degradation products in the form of phenolics, acids, and aldehydes. The sugars from hemicellulose (15–30% of original dry biomass [7, 8]) can be converted to ethanol by co-fermenting organisms which can use hexose sugars as well as pentose sugars for fermentation [9–12]. To effectively utilize the hemicellulose sugars, detoxification of the hemicellulose stream (pretreatment liquor) is necessary before it is introduced into the bioreactor.

The effects of the pretreatment liquor (PL) on various microorganisms used in bioconversion processes have been investigated extensively [1, 13–21]. Literature information is rather scant with regard to the extent of inhibition of the PL on the enzymatic hydrolysis reaction [22–24]. The composition and concentration of the degradation products varies with the type of feedstock, the chemistry, and the pretreatment process parameters such as temperature, time, pressure, pH, redox conditions, and presence of catalysts [1, 14]. Figure 1 is a schematic representation of degradation products formed during dilute-acid hydrolysis of biomass. Under low pH conditions, cellulose and hemicellulose degrade to their monomers which further degrade to aldehydes and acids. Pentoses form furfural while hexoses form hydroxymethylfurfural (HMF). Acetic acid is an initial product from the hydrolysis of hemicellulose, and lignin which are acetylated to some extent in the biomass [14, 24]. Carboxylic acids are products of further degradation of furfural and HMF. Lignin degradation gives many phenolic compounds such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde [14, 16, 24, 25]. The overall bioconversion process involves enzymatic hydrolysis and microbial conversion. The most common mode of process scheme is simultaneous saccharification and fermentation (SSF), or co-fermentation (SSCF) when conversion of hexoses and pentoses are applied. In either case, the mixture of PL and the pretreated solid substrate is to be processed by enzyme and consequently by microorganism. In the case of SSF or SSCF, the inhibitory effects of toxins are compounded as they deter the enzymatic hydrolysis reaction as well as the microbial

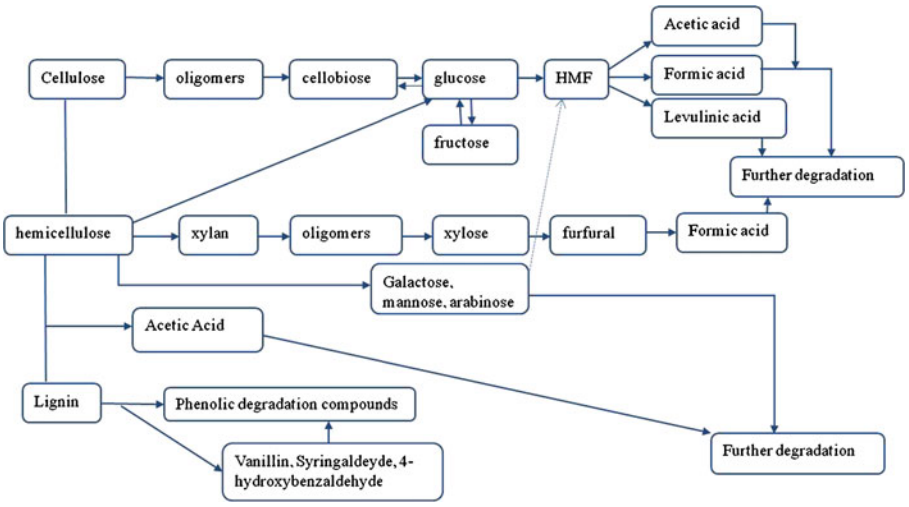

Fig. 1 Degradation products and their origin. Adapted from reference sources [14, 24–27, 34, 49, 50]

activity. The effects of dilute-acid pretreatment hydrolysate on fermentation are well documented [13–15, 18, 26, 27]. At the present time, however, it is unclear to what extent they affect the cellulase enzyme reactions. This investigation was undertaken to identify the inhibitory components existing in the dilute-acid pretreatment liquor and to assess their effects on enzymatic hydrolysis. Solka Floc was chosen as the substrate in order to eliminate the effects of extraneous components in biomass focusing more on the carbohydrates, the main target of the cellulase enzyme. The upper limit to which these inhibitors can be tolerated by the enzymes, an index of inhibition, was among the main items of interest.

Materials and Methods

Substrates

Solka Floc (International Fiber Corporation, Urbana, OH, USA, cat. no. U064072) was used as the cellulose substrate. Its composition was determined to be 77.3% glucan and 22.8% xylan. Birchwood xylan was obtained from Sigma Aldrich (cat. no. 038K0751). It contained >99.9% xylan. Avicel PH-101 was obtained from Fluka (cat. no. 1344705), containing 97.7% glucan. Other chemicals used in this work were laboratory grade chemicals purchased from Sigma Aldrich. Corn Stover and Pretreated Corn Stover (PCS) were provided by the National Renewable Energy Laboratory (NREL, Golden, CO, USA).

Enzymes

Cellulase enzyme, Genencor Spezyme CP (lot no. 301-00348-257), was a kind gift from Genecor/Danisco, Palo Alto, CA, USA. The activity of Spezyme CP, as determined by NREL, was 59 filter paper units (FPU)/mL. Activity of β-glucosidase (Novozyme 188 from Novozymes, Inc, batch no. 018K0735) was 750 cellubiose units (CBU)/mL. Multifect Xylanase (protein content 42 mg/mL) was a kind gift of Genecor/Danisco, Palo Alto, CA, USA.

Microorganism

The microorganism used for the SSCF experiments was recombinant *Escherichia coli*, ATCC-55124 (KO11). This organism was grown in LB medium (Sigma, L-3152), which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol.

Analytical Methods

Sugars in liquid sample were determined by HPLC using a Biorad-HPX-87P column. The BioRad-HPX-87H column was used for measurement of organic acids, ethanol, and furans. Refractive index detector was used in the HPLC. Solid substrates were analyzed for carbohydrates and ash using NREL standard biomass analytical procedures NREL-TP-510-42618 to 42622. Moisture content of biomass was measured by an infrared moisture balance (Denver Instruments IR-30). Cellulase and beta-glucosidase activities were measured using NREL-TP-510-42628 using filter paper and cellobiose as substrates, respectively.

Quantification of Sugars and Oligomers in Liquid

All liquid samples from enzymatic hydrolysis and SSCF were first analyzed for composition using HPLC: HPX-87H column for aldehydes and acids and HPX-87P column for monomeric sugars. Oligomeric sugar concentration was obtained by secondary hydrolysis of the liquid according to the NREL standard biomass analytical procedure NREL-TP-510-42623. The secondary hydrolysis was performed with 4% sulfuric acid, at 121°C for 1 h. The lignin degradation products vanillin and syringaldehyde were measured using GC/MS (GC: Agilent 7890A; MS: Agilent 5975). DB-1701 (30 m×250 μm× 0.25 μm) column and the NIST mass spectral library were used in the GC.

Preparation of Hydrolysate, Oligomers, and Soluble Lignin

Corn stover pretreated by dilute-acid was obtained from NREL. The pretreated corn stover (PCS) was squeezed and vacuum filtered to obtain dilute-acid hydrolysate.

Xylo-oligomer solution used in this study was obtained by treating pure Birchwood xylan at 121 °C for 25 min to get an oligomer solution. This condition was selected since it gave the minimum amount of degradation products while solubilizing most of the solid xylan. Also, as an alternate method, pure Birchwood xylan was treated with Multifect Xylanase (enzyme loading 20 mg/g xylan) for a fixed time (30 min) as shown by Zhu [28]. After the set time, the liquid solution was placed in a boiling water bath for 10 min to ensure that the enzyme activity stopped. The resulting solutions had a mixture of xylose and xylo-oligomers, but no lignin or its degradation products.

Water-soluble lignin solution was prepared by extracting lignin from the PL using ethyl acetate. The solvent was evaporated and the solid remaining behind was dissolved in water at desired concentrations. Insoluble lignin was extracted from the PCS solids using sodium hydroxide and then precipitated from the alkaline solution by adding acid.

Enzymatic Hydrolysis

The enzymatic digestibility of substrate was determined according to the NREL standard biomass analytical procedure NREL-TP-510-42621. Screw capped 250-mL Erlenmeyer flasks were used as the hydrolysis reactors. Solka Floc was used as the pure cellulose substrate in most of the experiments. The solid loading applied was such that the glucan content was 1% (*w*/*v*) in the reactor. Enzyme loading used was 15 FPU and 30 CBU/g glucan. Antibiotics tetracycline and cyclohexamide were added into the reactor to maintain sterile conditions. The enzymatic digestibility tests were carried out at 50°C, and pH 4.8 using 0.05 M sodium citrate buffer in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 rpm. Desired concentration of inhibitors was added to the reaction mixture. When the inhibitor was acidic, the solution was first treated with calcium carbonate to increase the pH to 4.8. The pH of the enzymatic hydrolysis mixture was tested before and after the experiment to make sure that it remained constant. The total reaction volume was kept constant at 100 mL irrespective of whether inhibitors were added to the mixture. Hydrolysate samples were taken at 6, 12, 24, 48, and 72 h, and analyzed for glucose, xylose, and cellobiose. Total released glucose and cellobiose after 72 h of hydrolysis were used to calculate the enzymatic digestibility:

$$\text{Digestibility}\,(\%) = \frac{\text{Glucose released}\,(\text{g}) + 1.053 \times \text{Cellobiose released}\,(\text{g})}{1.111 \times \text{Glucan added}\,(\text{g})} \times 100$$

The xylan digestibility was also determined in a similar manner. For xylan digestibility, hydration factor of 1.136 instead of 1.111 was used in the above equation.

Simultaneous Saccharification and Co-Fermentation

The procedure for the fermentation test was based on the NREL standard analytical procedures (NREL-TP-510-4263). Erlenmeyer flasks (250 mL) were used as the bioreactors. They were operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37°C and 150 rpm with 100-mL working volume. Substrate loading was 3% (*w*/*v*). Acid-treated liquor was added to one set of reactors for comparison. The samples were sterilized by autoclaving (121°C for 30 min). SSCF of sample was carried out at 37°C and pH 6 (0.05 M sodium phosphate buffer). The cellulase enzyme loading was 15 FPU Spezyme CP supplemented with 30 CBU Novozyme-188/g-glucan. The ethanol yield was calculated as follows:

$$\text{Theoretical maximum ethanol yield}\,(\%) = \frac{\text{Ethanol produced}\,(\text{g})\,\text{in reactor}}{\text{Initial sugar added}\,(\text{g})\,\text{in reactor} \times 0.511} \times 100$$

Sugar is interpreted as glucose plus xylose in SSCF.

Tolerance of Enzyme to Specific Inhibitors (10% Tolerance)

Spezyme CP was also tested for tolerance by different inhibitor compounds identified in this study. The inhibitor to be tested was added at different concentrations in the range of 1 to 10 g/L in the enzymatic reaction mixture as explained earlier, and the digestibility tests were run in parallel with one control flask. The "10% Tolerance" (10PT) for an inhibitor was defined as the concentration of the inhibitor at which the glucan digestibility decreased by 10% compared to the control.

Results and Discussion

Composition of Hydrolysate

The composition of dilute-acid pretreatment liquor is shown in Table 1. The main sugar component in the hydrolysate is xylose, the main ingredient in hemicelluloses, which is easily hydrolyzed by dilute acid [1]. HMF and furfural were also present in the liquor indicating that there is degradation of the released sugars. Among the acids, acetic acid was the predominant component. The liquor also contains a large amount of unidentified compounds as seen in the HPLC and GC chromatograms in Fig. 2. Most of the unidentified components are believed to come from lignin and sugar degradation. The lignin degradation products identified and tested in this study were vanillin and syringaldehyde as these are known to significantly inhibit the enzymes and microorganisms [7, 13].

Fermentation Test (SSCF)

In the SSCF carried out with addition of 50:50 mixture of PL and buffer, there was no ethanol formation (Fig. 3). The sugars accumulated throughout the fermentation, whereas in the control run the sugar levels remained near zero throughout, which indicates that the

Table 1 Composition of pretreatment liquid

Component	Concentration (g/L)
Cellobiose	2.45
Glucose	21.80
Xylose	74.08
Galactose	12.35
Arabinose	11.48
Mannose	2.55
Oligomers	
Xylo-oligomers	17.39
Gluco-oligomers (DP>2)	2.82
Identifiable degradation products	
From carbohydrates	
HMF	2.00
Furfural	1.14
Acetate	13.23
Formate	3.73
From lignin	μg/L
Vanillin	22.65
Syringaldehyde	386.14

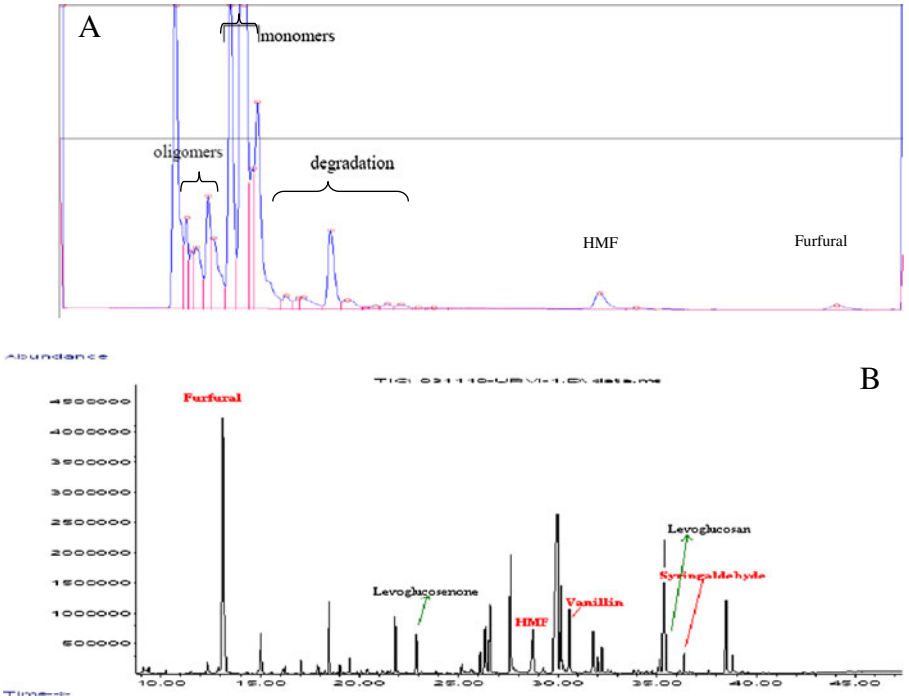

Fig. 2 **a** HPLC chromatogram and **b** GC/MS for dilute-acid hydrolysate

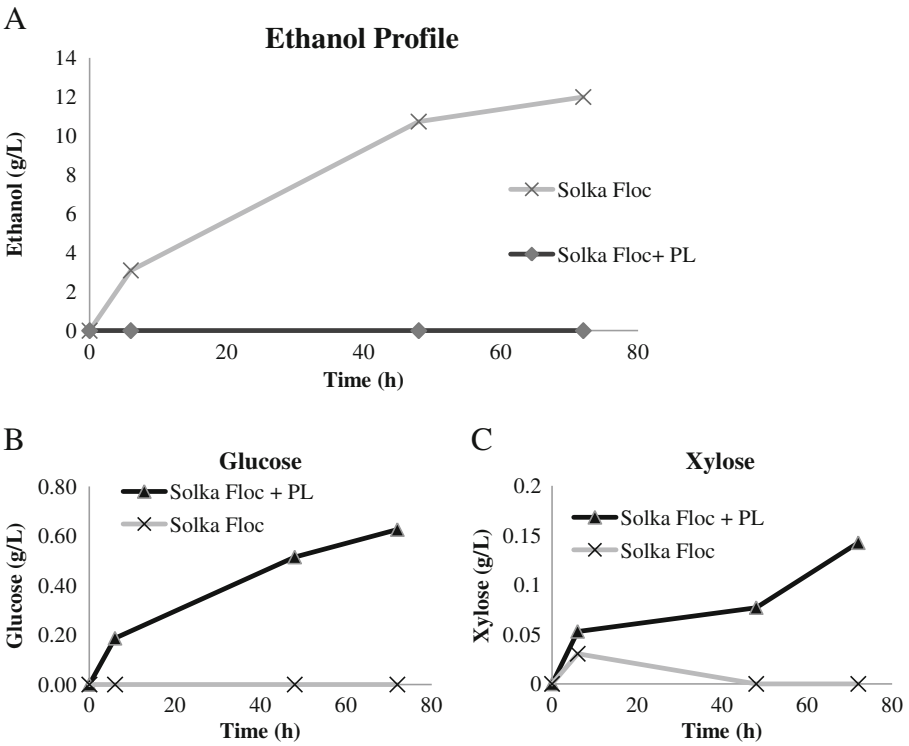

Fig. 3 Effect of dilute-acid hydrolysate (PL) on fermentation of Solka Floc. **a** Ethanol, **b** glucose, and **c** xylose profile. Enzyme loading: 15 FPU/g glucan, 30 CBU/g glucan; substrate: Solka Floc 1% glucan loading. Inhibitor: dilute-acid hydrolysate (PL) 50% (vol.%). Micro organism: *E. coli* KO11

organism is unable to take up any of the sugars during fermentation. This further indicates that the organism did not grow due to toxic effects of the hydrolysate, although the enzyme released sugars from cellulose. Obviously the inhibitory effects of the PL are more acute on the microbial process (both fermentation and cell growth) than the enzymatic hydrolysis reaction. Since the effects of the pretreatment hydrolysate on fermentation are well understood, the focus of this study was placed on the enzymatic hydrolysis.

Enzymatic Hydrolysis of the Pretreatment Liquor

The glucan and xylan digestibility profiles with and without addition of PL are shown Fig. 4. It is clearly seen that cellulase activity is significantly reduced by the presence of dilute-acid pretreatment liquor. Since the liquor was neutralized before hydrolysis, pH was not the cause of this drop. With 50% (*v*/*v*) mixture of PL and buffer solution, the 72-h digestibility of glucan in Solka Floc was reduced by 63% in comparison to the control run (no PL addition). The decrease in digestibility for the xylan fraction of the Solka Floc was even greater at 93%. This may be due to the fact that Spezyme CP has low xylanase activity and that the xylan in Solka Floc is a resilient fraction left behind during the manufacturing process.

The same tests were repeated applying different levels of enzyme loading. The results are shown in Fig. 5. The increased enzymatic loading has a positive effect on the digestibility, which is well-known [6]. A point to be noted here is that increase of enzyme

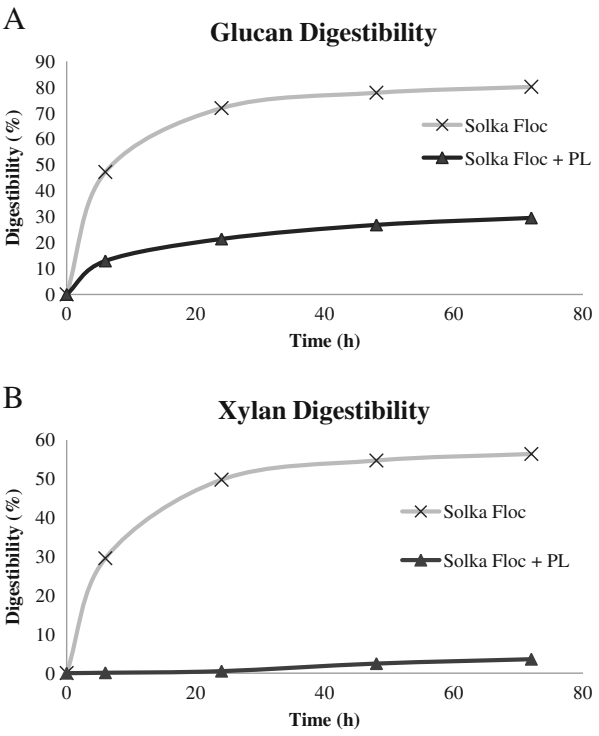

Fig. 4 **a** Glucan and **b** xylan digestibility of Solka Flock with and without PL. Enzyme loading: 15 FPU/g glucan, 30 CBU/g glucan. Substrate: Solka Floc 1% glucan loading. Inhibitor: dilute-acid hydrolysate (PL) 50% (vol.%)

loading tends to decrease the inhibition effects as the percent difference in glucan digestibility between the two runs reduced as the enzyme loading was increased. We used a moderate enzyme loading of 15 FPU/g glucan in most of the experiments in this study for comparison purpose, as this level has been adopted in many of the previous investigations [29].

Effects of Sugar Degradation Products

It is well-known that HMF and furfural are toxic to the microorganisms [18, 19, 30–32]. Various levels of HMF and furfural were applied to the enzymatic hydrolysis tests to assess

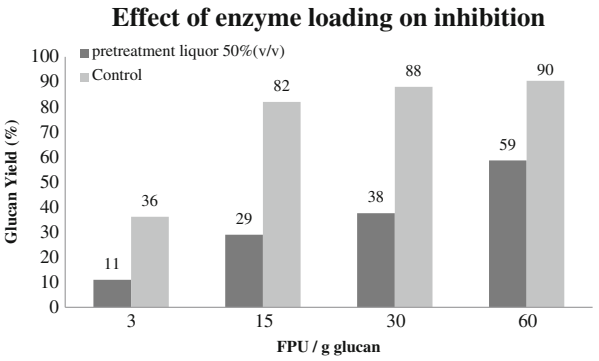

Fig. 5 Effect of enzyme loading on inhibition. Enzyme added: Spezyme CP: Novozyme 188 ratio 1 FPU: 2 CBU. Substrate: Solka Floc 1% glucan loading. Inhibitor: Dilute-acid hydrolysate (PL) 50% (vol. %)

Table 2 10% tolerance (10PT) of cellulase for various inhibitors

	Inhibitor concentration (10% tolerance) (g/L)	% Inhibition of cellulase activity (Spezyme CP)	Inhibitor concentration (10% tolerance) (g/L)	% Inhibition of xylanase activity (Spezyme CP)
Glucose	1	16	2	11
Cellobiose	1	21	1	21
Xylose	40	8	1	43
HMF	5	11	5	14
Furfural	10	9	10	9
Acetate	1	10	1	12
Formate	3.5	10	3.5	11
Vanillin	4	11	4	9
Syringaldehyde	5	9	5	10

their inhibitory effects. We find that they show significant inhibition, but only at concentrations above 5 g/L (Table 2). In the pretreatment hydrolysate used in this work, the concentrations of HMF and furfural found were much lower at 2 and 1.14 g/L respectively. The test data show that inhibition on cellulase activity by HMF and furfural at these concentrations is insignificant (Fig. 6). We note that the concentrations of HMF and furfural shown in Fig. 6 are half the actual concentration in the pretreatment liquor since it was diluted at 1:1 with buffer before it was subjected to hydrolysis test. Although the inhibitory effects of these compounds on the growth of microorganisms and enzyme activity are well recognized [20, 21], at the level existing in the pretreatment liquor, the inhibition on cellulase activity by each individual components is less than 5%. The maximum HMF tolerated by the enzyme such that the cellulase activity decreased by 10% is to be termed as "10% tolerance", abbreviated by 10PT in this paper. The 10% tolerance

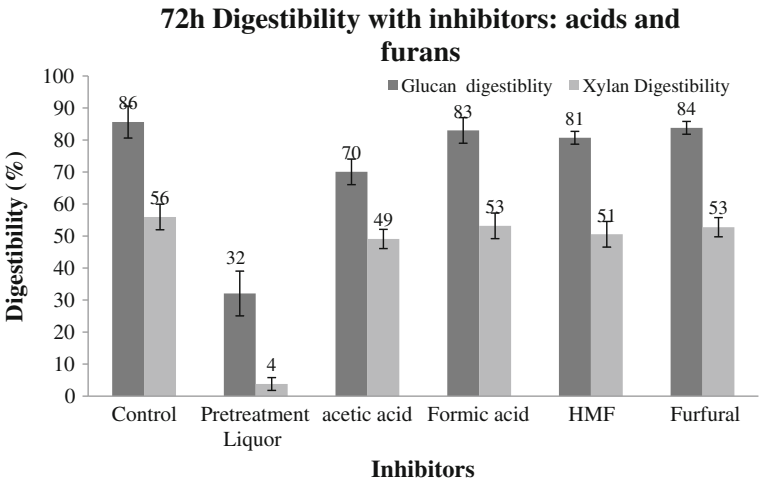

Fig. 6 Comparison of effect of individual components furfural, HMF, acetic acid, and pretreatment liquor. Enzyme loading: 15 FPU/g glucan, 30 CBU/g glucan. Substrate: Solka Floc 1% glucan loading. Inhibitors: dilute-acid hydrolysate 50% (vol.%); furfural, 0.57 g/L; HMF, 1 g/L; acetic acid, 6.62 g/L; and formic acid, 1.9 g/L

for HMF is approximately 5 g/L, since at this concentration of HMF, the glucan and xylan hydrolysis yields decreased by 11% and 14%, respectively. The same for furfural was determined to be 10 g/L where both glucan and xylan yields decreased by 9% (Table 2).

Effect of Acids

In the PL, acetate is of the highest concentration at 13 g/L (Table 1). Acetate is generated from hemicellulose-lignin composite structure which contains acetyl groups. It may also be formed from further degradation of HMF and furfural in acidic conditions [14, 33, 34]. Formate and levulinate are produced by degradation of HMF and furfural. We found that the inhibitory effect of the acetate is the highest amongst the three acids of acetate, formate, and levulinate (levulinic acid data not shown). The extent of specific inhibition (grams per liter basis) of the acids is in the order of: acetate>formate>levulinate. The global inhibition is by far the highest for acetate since its concentration is also the highest in the PL. We have determined the 10PT against the acetic acid to be 1 g/L. With 5 g/L concentration in the PL, acetate becomes indeed a very strong inhibitor to enzymatic hydrolysis. It appears to play a major role by reducing glucan hydrolysis yield by more than 50% (Fig. 6). Formate is a weaker inhibitor than acetate, but with the concentration higher than 5 g/L in the PL, it decreased the glucan hydrolysis yield by about 20%. Levulinic acid is also known to be inhibitory to the enzyme and microorganisms [14, 24], but its concentration in the hydrolysate was much lower than the other two acids. The 10PT for acetic acid was 1 g/L, at which the glucan and xylan yields decreased by 10% and 12%. The 10PT for formate was estimated to be 3.5 g/L and at this concentration the glucan digestibility was reduced by 10% and xylan digestibility by 11% (Table 2).

Effects of Soluble Lignin and Lignin Degradation Products

The PL contains various lignin degradation products and water-soluble lignin. Syringaldehyde and vanillin are among the identified components. The concentration of these two components in the PL was found to be less than 1 g/L. At this level, their inhibition on glucan or xylan digestibility was insignificant (<1%, <5% respectively). The 10PT for syringaldehyde was estimated to be 5 g/L, as the yield for glucan and xylan decreased by 9% and 19%, respectively, and at 4 g/L, by 10% and 9% with vanillin. The PL also contains large number unidentified phenolic compounds with wide range of molecular weight (MW) [7, 13] which originate from lignin. Some of these are breakdown products of solid lignin whose MW became low enough to be water soluble. Although not individually identified, the lignin breakdown products are believed to impose strong inhibition to cellulase. The soluble lignin in the PL was extracted using ethyl acetate and then dissolved in water to desired concentrations. This water-soluble lignin was mixed with pure substrates (filter paper and cellobiose) to determine the reduction in cellulase and beta-glucosidase activities in presence of the lignin (Fig. 7). At a concentration of 3 g/L, water-soluble lignin decreased the cellulase activity by approximately 10%.

The lignin remaining in solid biomass after pretreatment is known to bind with cellulase enzyme, a phenomenon known as “nonproductive binding” [35, 36]. In this mode of action, the enzyme is irreversibly bound, essentially deactivating the enzyme. This is different from inhibition, which is a reversible interference to the enzyme reaction. It has been reported that lignin also forms a complex with cellulase enzyme (lignin–cellulase complex) [35, 37]. The 10PT for water-insoluble lignin tolerated by the enzymes was determined to be 5 g/L where the cellulase activity decreased by more than 15%. To remove this inhibition, surfactants and other long-chain compounds are used. Yang et al. found that surfactants

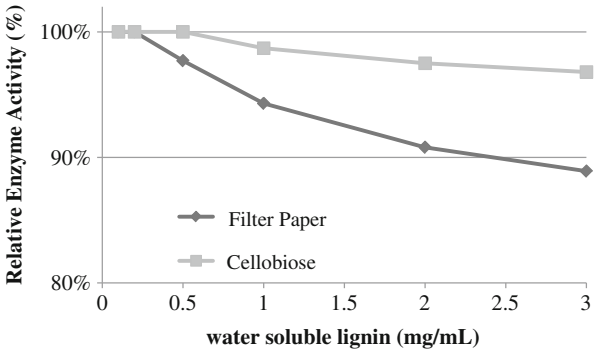

Fig. 7 Effect of water-soluble lignin on enzyme activity. Substrates: filter paper (filter-paper-units-per-milliliter activity, FPU/mL) and cellobiose (cellobiose units per milliliter, CBU/mL)

adsorb to lignin and thus prevent nonproductive binding of enzymes to lignin [36]. Errikson et al. proposed that surfactants form a mycelial structure around lignin by hydrophobic interactions and block the lignin molecules from adsorbing to the enzyme [35].

Effect of Sugars

Inhibition experiments were carried out for sugars found in the PL, namely, glucose, cellobiose, xylose, and xylose oligomers. The experiments were carried out for each individual sugar. The sugars were added to the hydrolysis reactor to such that the concentration is the same as those in the 50:50 hydrolysate–buffer mixture. The results summarized in Fig. 8a show the 72-h digestibility with and without addition of individual sugar components. The 72-h digestibility of glucan in Solka Floc was affected strongly by cellobiose and glucose. On the other hand, the digestibility of xylan was strongly affected by xylose. We also note that the degree of inhibition by three sugars combined (glucose+cellobiose+xylose) is far less than the addition of the inhibition by each component. This reaffirms the established concept that the cellulose enzymes are inhibited by the reaction intermediates (cellobiose) as well as the end-products (glucose, xylose) [38, 39]. The product inhibition is specific to the enzyme; a product inhibits mainly the enzyme by which it was produced. The data in Fig. 8a also show that the inhibition can also go the other way; although the degree of inhibition is much lower, xylose inhibits glucan hydrolysis and vice versa. It appears that the various non-cellulolytic enzymes existing in "cellulase" are affected by the sugars released from hemicellulose, inhibiting the hydrolysis of hemicellulose fraction (a barrier impeding accessibility of the enzyme to cellulose), then eventually the glucan hydrolysis. This concurs with findings of previous research along these lines that glucose and cellobiose are the major inhibitors to cellulase and beta glucosidase, whereas xylose is a minor inhibitor [40–43]. According to Xiao et al. glucose inhibits exo-glucanase as well as β-glucosidase. Xylose, mannose, and galactose also have a significant inhibitory effect, but only on the initial cellulase activity [44]. This subject was further investigated to see if hydrolysis of pure glucan is inhibited by xylose, which is not a product of glucanase. The experimental results are shown in Fig. 8b, where the profiles of Avicel hydrolysis with and without xylose addition are compared. An interesting point here is that although Avicel does not contain any xylan (97.7% glucan), glucan hydrolysis was inhibited by xylose, but only at the early phase of the reaction up to 24 h. The final 72-h digestibility was not affected significantly. Even though xylose is not a natural substrate to cellulase, it seems to interfere with glucan hydrolysis perhaps acting as a competitive inhibitor to active sites of certain cellulase enzyme component(s). The extent of inhibition then gradually

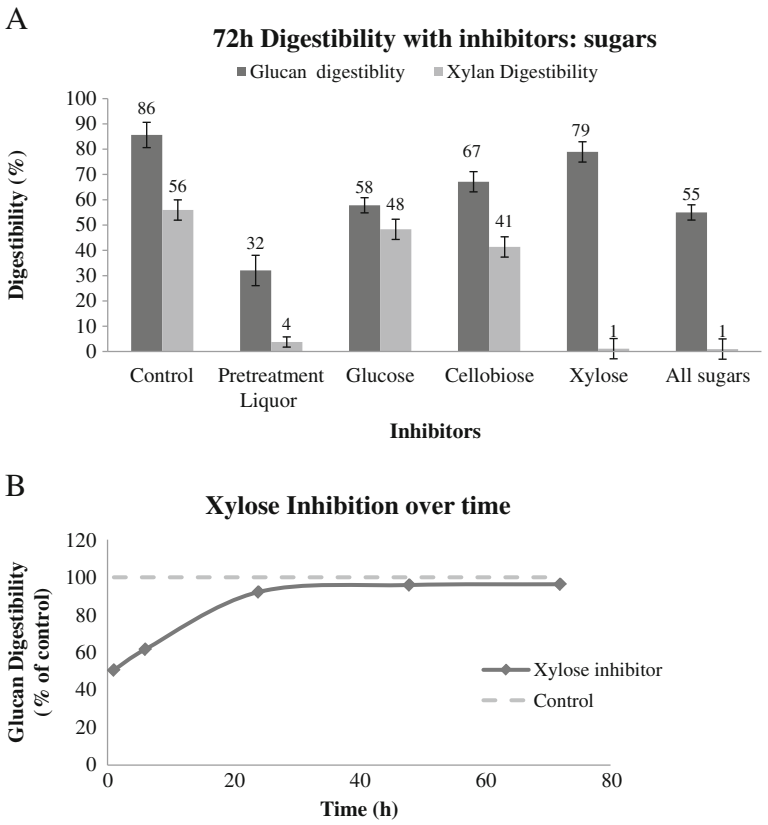

Fig. 8 **a** Comparison of effect of individual components main sugars: glucose, xylose and cellobiose and sugar mixture. Enzyme loading. 15 FPU/g glucan; 30 CBU/g glucan. Substrate: Solka Floc 1% glucan loading. Inhibitors: glucose 11 g/L, xylose 37 g/L, cellobiose 1.23 g/L. All sugars as found in 50 mL dilute-acid hydrolysate. **b** Xylose inhibition over time plotted as Percentage of control. Xylose decreases initial glucan yield by around 50% while final yield is almost the same as control. Avicel 97.7% cellulose+10 g/L xylose

diminishes as glucose, the main inhibitor, is accumulated, which is in agreement with Liao et al. [45].

Inhibition by xylo-oligomers is shown in Fig. 9. The data indicate that the oligomers severely inhibit hydrolysis of xylan, but only slightly the hydrolysis of glucan at the concentration seen in the PL. It was also found that oligomers did not disappear after 72 h of enzymatic hydrolysis. This indicates that the cellulase enzyme does not hydrolyze the oligomers effectively. Addition of external xylanase (Multifect Xylanase, Genencor-Danisco) increased conversion of xylo-oligomers, but at the end of 72 h there were still about 20% oligomers left unhydrolyzed. As significant as the inhibition by sugars to the cellulase enzyme is, this issue can be resolved by adoption of SSF or SSCF bioprocess scheme [12, 46].

Combined Effects

The overall inhibition effects by groups of known inhibitors on glucan and xylan digestibility are summarized in Fig. 10. Inhibition by each group and by all the known

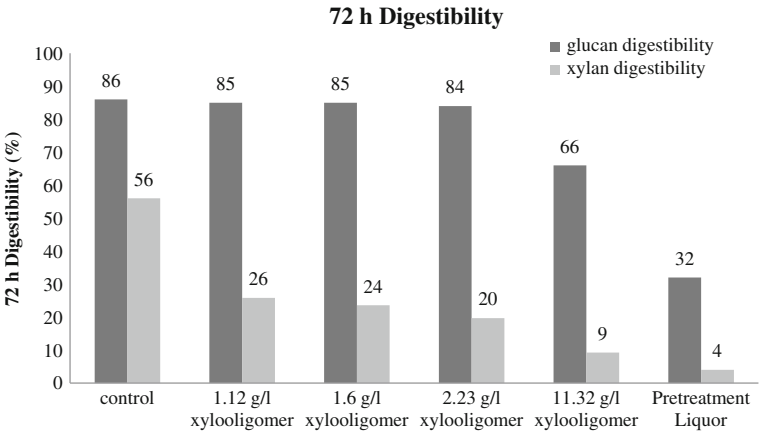

Fig. 9 Effect of xylo-oligomers on the glucan and xylan digestibility of Solka Floc. Substrate: Solka Floc 1% glucan loading. Inhibitor: xylo-oligomers

inhibitor groups combined were investigated to assess the overall inhibition effects and to see if there is any interaction between the different groups of inhibitors. With all of the known inhibitors combined, the cellulase activity decreased by 42% (86% down to 50%). An important point here is that the overall inhibition on glucan hydrolysis by PL (63%) is much higher than that by mixture of all known inhibitors combined. This indicates that the unidentified inhibitors, mostly phenolic compounds generated by lignin degradation, also act as major inhibitors comparable to any of the known inhibitor groups as suggested by Palmqvist et al. [17]. The inhibition effects by each group are not necessarily additive. The inhibitor groups interact with each other as the combined inhibitory effects were found to be

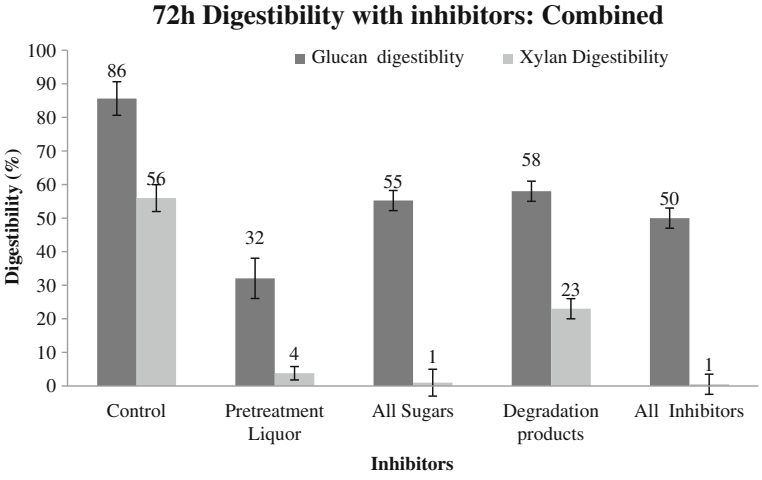

Fig. 10 Comparison of effect of inhibitor groups: sugars, degradation products, and all inhibitors. Enzyme loading. 15 FPU/g glucan; 30 CBU/g glucan. Substrate: Solka Floc 1% glucan loading. Inhibitors: all inhibitors=all compounds from Table 1 as found in 50 mL dilute-acid hydrolysate; degradation products= carbohydrate degradation products (acids and furans) from Table 1

different from addition of the individual inhibition. The sugar group decreased the glucan digestibility by 33% and xylan digestibility by 98%, which was more than the decrease seen by any of the inhibitor groups. The reason for this is unclear. Perhaps the sugars had an interaction, increasing the overall inhibitory effect. Similarly, adding all degradation products together showed a higher inhibition as compared to any one compound alone. The inhibition mechanism for these inhibitors is poorly understood at this time. Various reaction schemes have been proposed for describing inhibition. Some models assume competitive inhibition for certain type of inhibitors while some assume non-competitive inhibition schemes [24, 47, 48]. In these studies however the inhibition (interference) and deactivation (irreversible loss of activity) were not distinguished. This investigation was restricted to the gross effects of inhibitors and deactivators that exist in the PL of corn stover on the overall terminal digestibility.

Conclusion

Potential inhibitors existing in the dilute-acid pretreatment liquor (PL) of corn stover were identified and their inhibition effects on enzymatic hydrolysis of Solka Floc were investigated. The inhibitors were grouped into sugar/lignin degradation products, sugars, and unknowns. The relative inhibition effects of each group were determined using 72-h enzymatic hydrolysis yield of glucan and xylan as the index. Inhibition by the dilute-acid PL was found to be higher than that by simulated mixture of individual compounds. The inhibition effects by individual groups are not additive. Of the individual inhibitors studied, cellobiose, glucose, and acetate were identified as the major inhibitors to glucan digestibility. The 10% tolerance level (10PT) of these inhibitors was in the vicinity of 1 g/L. The unidentified inhibitors also play a significant role in the overall inhibition. Xylose and xylo-oligomers inhibit xylanase activity and initial glucanase activity in Spezyme CP. For all the known inhibitors existing in the PL, at the given concentrations, the order of inhibitory strength is: phenolic lignin derivatives>sugars>organic acids>furans.

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