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Research Article

Enhancing Prehydrolysates Fermentability by Adding Nucleophilic Amino Acids and Proteins in Biomass Pretreatment

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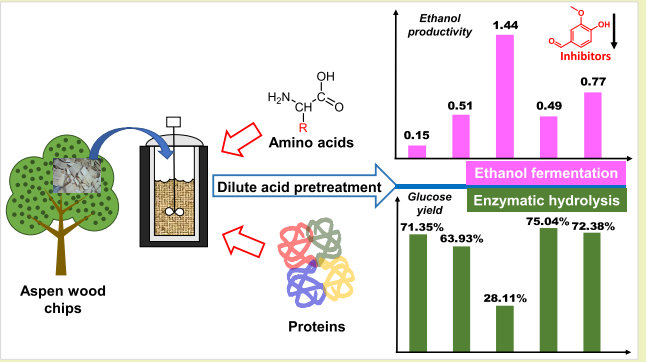
ABSTRACT: Dilute acid pretreatment produced a considerable amount of carbonyl compounds in the biomass prehydrolysates, which significantly inhibited the sequential microbial fermentation. To reduce the release of carbonyl inhibitors, a novel approach of pretreatment with amino acids and proteins has been developed to improve the fermentability of prehydrolysates. Four percent (w/w) of cysteine (Cys), histidine (His), soy protein isolate (SPI), and bovine serum albumin (BSA) was added into dilute acid pretreatment of aspen (DAPA). The resulted prehydrolysates were fermented by *Saccharomyces cerevisiae*, and the glucose consumption rate in the prehydrolysates was increased from 0.32 to 1.35, 3.22, 1.02, and 1.61 g/L/h, respectively. The pretreated substrates were applied to enzymatic hydrolysis. Unexpectedly, it was observed that 72 h hydrolysis yields of DAPA-Cys and DAPA-His decreased from 71.35% (DAPA) to 63.93% and 28.11%, respectively, while the 72 h hydrolysis yield of DAPA-SPI increased to 75.04%, and the 72 hydrolysis yield of DAPA-BSA did not change. The results showed that BSA was the most effective additive to enhance the prehydrolysate fermentability. It increased the ethanol productivity of prehydrolysates from 0.15 (without addition) to 0.77 g/L/h. The final yield was promoted from 0.05 to 0.44 g/g glucose. In addition, a total of 22 potential inhibitors in prehydrolysates have been identified and quantified by gas chromatography/mass spectrometry (GC/MS). The results showed that addition of histidine in pretreatment reduced inhibitors much more than addition of cysteine, SPI, and BSA. The results suggests that addition of protein decreases inhibitors by reaction with aldehydes/ketones and also by buffering and reducing pretreatment severity, which results in less inhibitors produced. The reduced severity also decreases the cellulose digestibility, especially with the addition of cysteine and histidine. Hence, the overall effect on yield and productivity is a trade-off between the positive effect of reducing inhibitors and the undesirable effect of less sugar release from the substrates.



KEYWORDS: *Pretreatment, Carbonyl inhibitors, Prehydrolysates, Detoxification, Ethanol fermentation*

INTRODUCTION

Lignocellulosic biomass could be a feedstock for production of alternative biofuels to reduce the dependence on petroleum-based liquid fuels.[1,2] Pretreatment is a necessary step to break down the recalcitrant structure and enhance subsequent enzymatic hydrolysis and microbial fermentation.[3,4] Dilute acid pretreatment has been widely applied to many types of biomass due to its low cost and simplicity. However, the acid hydrolysis process also produces various inhibitory compounds from the degradation of extractives, carbohydrates, and lignin in the biomass, which significantly limited biomass prehydrolysate fermentability and biofuel production.[5−7] Acidic resins, tannic acids, and terpene acids derived from biomass extractives are released into the prehydrolysates (liquid portion after pretreatment) during the acid hydrolysis process. Carbohydrates-degraded compounds, including furfural, hydroxymethylfurfural (HMF), and several types of acids (acetic, formic, and levulinic acid), have been considered as important indicators of prehydrolysate toxicity, and their inhibitory effects depend on the tested microbies.[8,9] The inhibitors derived from lignin are aromatic, polyaromatic, phenolic, and aldehydic compounds. These phenolic inhibitors degraded from lignin have been identified to be more toxic than extractives and carbohydrates-derived inhibitors.[10,11] Generation of inhibitors during biomass pretreatment has been suggested to affect the fermentation yield and productivity significantly.[12] Therefore, it is essential to alleviate the inhibitory effect and improve the fermentability of the biomass prehydrolysates.

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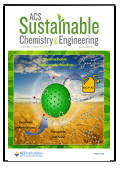

Numerous research studies have been concentrated on developing a cost-effective detoxification approach to minimize the toxicity and improve the fermentability of prehydrolysates.[13,14] Xie et al. examined the detoxification efficiency of 20 nucleophilic amino acids and found cysteine (Cys) and histidine (His) were the most effective detoxification regents which dramatically improved ethanol productivity and final yield.[15] The highly nucleophilic cysteine with a thiol group and histidine with an imidazole side chain can readily react with the electrophilic functional groups of carbonyl compounds and reduce the toxicity.[16] Although plenty of detoxification methods have been reported to minimize the toxicity of inhibitors, most are required to add another step with extra costs. Herein, it is critical to develop highly effective and cost-efficient detoxification approaches. Alriksson et al. in situ detoxified the enzymatic hydrolysates along with the fermentation process with the addition of dithionite and sulfite.[17] This in situ detoxification approach offered a new way to improve the hydrolysate fermentability without an additional detoxification step and achieved the desirable ethanol production. Similarly, sodium borohydride was used to detoxify the hydrolysates under mild conditions and increased the ethanol productivity and yield to 0.57g/L/h and 0.31 g/g, respectively.[18] A pretreatment process with new additives is another effective method to minimize the formation of inhibitors and improve the prehydrolysate fermentability. Zhu et al. developed a pretreatment method using sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) and reduce the formation of inhibitors.[19] It suggested that only 35% of total inhibitors (including furfural, HMF, and formic, acetic, and levulinic acids) had been generated in SPORL compared to the dilute acid pretreatment.[20]

Proteins are large biomolecules consisting of one or more chains of nucleophilic amino acid residues, which could react with carbonyl inhibitors and work as potential detoxification reagents. It has been reported that soluble aggregated proteins such as bovine serum albumin (BSA) and casein could form an intermolecular cross-link with various aldehydes.[21] Similarly, ovalbumin, human gamma globulin, and soy protein isolate (SPI) could conjugate glutaraldehyde with their amino groups.[22,23] In addition, the side chains of cysteine, histidine, and lysine residues in BSA could react with unsaturated aldehydes (such as acrolein).[24] However, the effect of protein on biomass prehydrolysate detoxification has not been reported. Furthermore, addition of BSA could enhance enzymatic hydrolysis.[25,26] Yang et al. found that approximately a 10% improvement of the 72 h hydrolysis yield could be achieved with BSA treatment prior to enzymatic hydrolysis.[25] Also, addition of BSA increased the glucose yield of Avicel and corn stover by 76% and 40%, respectively, due to the enzyme-stabilizing effect.[26] SPI was also reported as an excellent additive to significantly reduce the cellulase loading and promote enzymatic hydrolysis by reducing the nonproductive binding of cellulolytic enzymes to lignin.[27] As a result, nucleophilic proteins, which hold great potential to react with carbonyl inhibitors, could be a promising additive to biomass pretreatment to promote prehydrolysate fermentability as well as enzymatic hydrolysis of pretreated substrates (residual solid materials).

The objective of this study is to enhance the prehydrolysates fermentability by adding nucleophilic amino acids and proteins in dilute acid pretreatment. It was hypothesized that both nucleophilic proteins and the hydrolyzed amino acids could react with carbonyl inhibitors and in situ detoxify the biomass prehydrolysates during the pretreatment processes. To verify our hypothesis, four nucleophiles including amino acids (cysteine and histidine) and commercial proteins (SPI and BSA) were added into the dilute acid pretreatment of aspen to improve the fermentability of prehydrolysates without an additional detoxification step. Gas chrmotography/mass spectrometry (GC/MS) was used to determine the major inhibitors concentration changes in the prehydrolysates. Enzymatic digestibility of the pretreated substrates was determined to evaluate the pretreatment efficiency. In addition, the detoxification efficiency of BSA on the prehydrolysate after pretreatment (without additives) was also investigated.

EXPERIMENTAL SECTION

Chemicals and Reagents. Aspen wood chips (*Populus tremuloides*) (6 mm × 38 mm) were kindly offered by the Wisconsin Rapids mill of Stora Enso North America. These wood chips were kept at 4 °C with vacuum until use. Glucose, mannose, galactose, xylose, arabinose, cysteine, histidine, acetic acid, 5-methylfurfural, levulinic acid, phenol, benzoic acid, guaiacylacetone, vanillin, syringaldehyde, and syringlacetone were purchased from Sigma-Aldrich (St. Louis, MO). Soy protein isolate (SPI) was obtained from Acros organics (Morris Plains, NJ). Calcium carbonate ($CaCO_3$) and bovine serum albumin (BSA) were products from Alfa Aesar (Ward Hill, MA). Formic acid, furfural, and hydroxymethylfurfural (HMF) were products from Fisher Scientific (Fair Lawn, NJ). Peptone was obtained from Research Products International (Prospect, IL). All chemical reagents were of analytical grade.

Dilute Acid Pretreatment of Aspen. The chemical composition of untreated aspen was 43.39% glucan, 16.40% xylan, 1.59% galactan, 1.19% arabinan, 1.67% mannan, 22.15% lignin, and 3.2% extractives. This analysis was carried out according to the NREL protocol.[28] Pretreatment was conducted in a Parr batch pressure vessel (model 4520 of 2.0 L of capacity) (Parr Instrument Co., Moline, IL). Briefly, aspen wood chips (80 g, dry weight) were soaked overnight in 560 mL of a 1% sulfuric acid (based on the dry weight of biomass) aqueous solution (cooking liquor, the liquid to solid ratio is 7:1) prior to the pretreatment. To reduce the release of inhibitors in prehydrolysates, 4% cysteine, histidine, SPI, and 1%, 2%, 3%, and 4% BSA (based on the dry weight of biomass) were added into the cooking liquor.[29] The additives were added into the cooking liquor and mixed before presoaking the biomass. All of the pretreatment processes were conducted at 170 °C for 60 min. After pretreatment, the stirred reactor would be quenched in a bucket with cold water. The slurry after pretreatment was separated into substrates and prehydrolysates by vacuum filtration. These prehydrolysates were stored at 4 °C and used for subsequent analyses and fermentation. The pretreated substrates were washed with 560 mL of water three times and collected by vacuum filtration.

Enzymatic Hydrolysis of Dilute Acid Pretreated Substrates. Commercial cellulase (Novozym 22C) was provided by Novozymes (Franklinton, NC) and applied for enzymatic hydrolysis. Novozym 22C (92 FPU/mL) is a hybrid of cellulase enzymes with high β-glucosidase activity (343 IU/mL), determined using *p*-nitrophenyl-β-glucoside as the substrate.[30] Each enzymatic hydrolysis experiment was conducted in a 250 mL flask with 2% glucan (w/v) in 50 mL of 50 mM sodium citrate buffer (pH 4.8). The loading of Novozym 22C was 10 FPU/g glucan. The flasks were placed on an incubator shaker (150 rpm), and the temperature was controlled at 50 °C for 72 h. The samples (0.5 mL) were intermittently collected from the hydrolysis solution (0, 3, 6, 12, 24, 48, and 72 h). The released glucose in the enzymatic hydrolysis was used to calculate the hydrolysis yield. The released glucose in the first 3 h of enzymatic hydrolysis was used to calculate the initial hydrolysis rate.

Postdetoxification of Biomass Prehydrolysates with BSA. The pH of prehydrolysates from the dilute acid pretreatment without

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addition of additives was adjusted to 6 by adding $CaCO_3$. The glucose concentration in the prehydrolysates was adjusted to around 20 g/L by addition of pure glucose. The biomass prehydrolysates were detoxified in a 250 mL flask with BSA (0.2% w/v) at 20 and 60 °C for 2 h. Centrifugation at 7000 rpm for 10 min was applied to remove the precipitates. The liquid part was kept for yeast fermentation and inhibitor analysis. The detoxification treatments were performed in duplicate.

Yeast Fermentation of Dilute Acid Pretreatment Hydrolysates. Fleischmann's pure dry yeast, *Saccharomyces cerevisiae* was stored at 4 °C and used for ethanol fermentation. The strain was grown at 30 °C overnight in yeast extract peptone dextrose medium (YPD) containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. The concentration of yeast suspensions was determined by a ultraviolet−visible (UV) spectrophotometer at 600 nm.[31] A 2.0 g/L yeast inoculum (dry weight) was applied for ethanol fermentation. Batch fermentation took place in a 250 mL flask with 50 mL of prehydrolysates without any extra nutrients. The glucose concentration in prehydrolysates was brought to around 20 g/L with the addition of pure glucose. Moreover, the pH of prehydrolysates was adjusted to 6 by excessive $CaCO_3$. Then the solution was filtered to remove $CaCO_3$ and other impurities through the membrane filter (0.22 μm). All fermentation experiments were incubated at 30 °C and 150 rpm for 48 h in an incubator shaker (E24, New Brunswick Scientific). The samples (0.5 mL) were intermittently collected from the fermentation solution (0, 3, 6, 9, 12, 24, and 48 h). The volumetric ethanol productivity (Q_{EtOH}) was calculated based on the produced ethanol in the first 6 h of fermentation as described previously.[11] A 6 h glucose consumption was used to calculate the initial glucose consumption rate (R_G).

High-Performance Liquid Chromatography (HPLC) Analysis. The sugar concentrations were detected by an Agilent 1260 HPLC system equipped with a Bio-Rad HPX-87P column (300 × 7.8 mm) connected to a refractive index detector (RID-10A). Deionized water with a flow rate of 0.6 mL/min was applied as the mobile phase. The column temperature and detector temperature were kept at 80 and 45 °C, respectively. Formic acid, acetic acid, levulinic acid, furfural, HMF, and ethanol were determined with an Aminex HPX-87H column (300 × 7.8 mm) with a RID detector. The column was heated to 45 °C with 5.0 mM H_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis. The inhibitory compounds were extracted from the aspen prehydrolysates determined as described before.[6] Briefly, the inhibitors in 20 mL of prehydrolysate were extracted with 20 mL of dichloromethane (DCM) twice. About 10 g of anhydrous sodium sulfate was used to remove the residual water, 2 mL of the DCM extraction sample was collected, and concentrated with a nitrogen blowing concentrator (TurboVap II workstation). GC/MS chromatography was collected from an Agilent 7890B equipped with a mass-selective detector. A 30 m length, 0.25 mm internal diameter, and 0.25 μm thickness Ultra Alloy-5 column (Frontier-lab) were applied to obtain chromatographic separation of the samples. The electron ionization ion source was held at 70 eV and 250 °C. The molecular weight range of the mass spectra was set from 30 to 700. The oven temperature was kept at 60 °C and then increased to 105 °C (12 °C/min ramping, held for 2 min, 6 min solvent delay), to 160 °C (15 °C/min ramping, held for 2 min), and finally to 315 °C (10 °C/min ramping, held for 8 min). The total running time for the method was 40 min.

RESULTS AND DISCUSSION

Effect of Nucleophilic Additives in Biomass Pretreatment on Prehydrolysates Fermentability. Nucleophilic amino acids have been used to detoxify biomass hydrolysates for alcoholic fermentation.[15] To examine whether adding nucleophilic additives in pretreatment can enhance prehydrolysate fermentability, 4% of cysteine, histidine, soy protein isolate (SPI), and bovine serum albumin (BSA) were added in the dilute acid pretreatment of aspen. The prehydrolysates with spiked glucose were fermented by *S. cerevisiae* for 48 h, and the glucose consumption rates, ethanol productivity, final concentrations, and ethanol yields were determined (Figure 1).

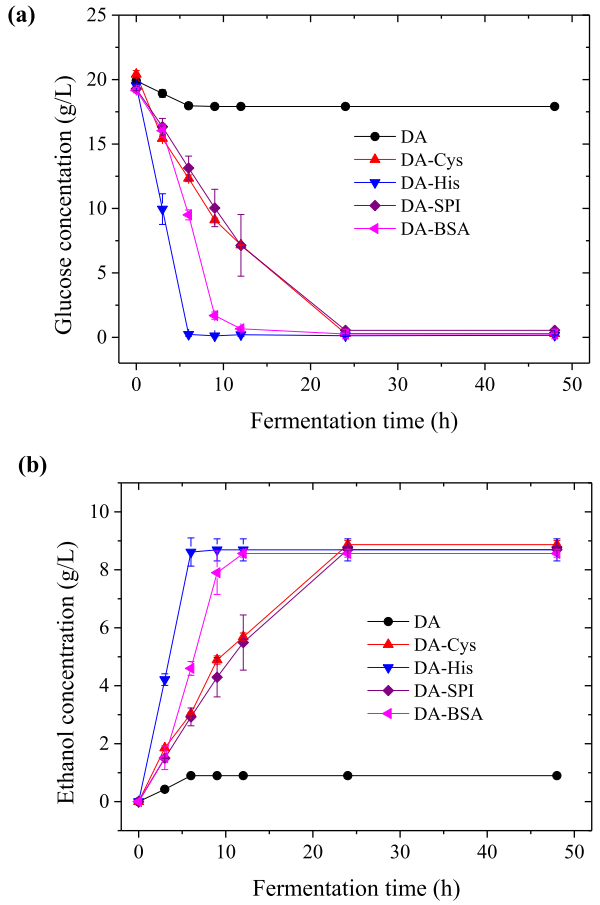

Figure 1. Fermentation of dilute acid prehydrolysates with different amino acids (4%) and proteins addition (4%): (a) glucose consumption; (b) ethanol production.

The results showed four additives enhanced the prehydrolysates fermentability significantly. Pretreatment with cysteine and histidine enabled glucose completely to be consumed at 24 and 6 h, respectively. Their final ethanol concentration reached 8.87 and 8.69 g/L, respectively. Similarly, pretreatment with SPI and BSA enabled glucose to be consumed at 24 and 12 h, respectively. Their final ethanol concentration reached 8.70 and 8.56 g/L, respectively. The prehydrolysates from pretreatment without additives (control) only generated 0.90 g/L ethanol, and most glucose was not consumed at 48 h.

Specifically, addition of cysteine, histidine, SPI, and BSA increased the glucose consumption rate from 0.32 ± 0.01 (control) to 1.35 ± 0.00, 3.22 ± 0.00, 1.02 ± 0.09, and 1.61 ± 0.07 g/L/h, respectively. Correspondingly, addition of cysteine, histidine, SPI, and BSA increased the volumetric ethanol productivity from 0.15 ± 0.00 to 0.51 ± 0.01, 1.44 ± 0.06, 0.49 ± 0.04, and 0.77 ± 0.03 g/L/h, respectively. The results indicated that these four additives could in situ detoxify the prehydrolysates in dilute acid pretreatment. Histidine appeared to be the most effective one, and BSA was the second best. This agreed with previous reports on the detoxification of biomass prehydrolysates with amino acids, where cysteine and histidine were able to detoxify prehydrolysates for ethanol

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fermentation,[15] although previous reports showed cysteine was the most effective in hydrolysates detoxification. This difference probably was due to the better buffering capacities of histidine under the pretreatment conditions. The pH in the dilute acid pretreatment is around 1.8. The pK_{a1} of histidine and cysteine are 1.82 and 1.96, respectively. The closer value between the pK_a of histidine and the pretreatment pH results in a higher buffer capacity, which could result in a lower inhibitor content in the pretreatment with histidine. It also should be noted that different temperatures in the pretreatment could result in different reactions in detoxification when it was compared with previous amino acids detoxification at 60 °C. The degradation of carbohydrates and lignin can generate reducing sugars and carbonyl compounds, which can react with amino acids or peptides (from protein) through Maillard reaction at high temperature (>150 °C).[32] Previously, *N*-acetyl cysteine has been used to detoxify 2-hydroxyethyl methacrylate in dental biomaterial development.[33] They found the nucleophilic thiol group reacted with double bonds in 2-hydroxyethyl methacrylate and generated an adduct. Thus, the reaction detoxified the 2-hydroxyethyl methacrylate. Another study reported that Schiff base formation was the main reaction in detoxifying carbonyl aldehydes with nucleophilic amino acids.[15] The imidazole side chain of histidine can readily react with aldehydes and detoxify its toxicity.[34] Histidine-containing peptides have been used to detoxify aldehyde compounds in biological cells (as aldehyde scavengers).[35] BSA has been reported as a nucleophilic protein to react with unsaturated aldehydes (such as acrolein) through its side chains of cysteine, histidine, and lysine residues.[24] BSA protein with a total of 607 amino acids contains 35 cysteines (6.2%), 17 histidines (3.8%), and 60 lysines (12.8%).[36] Previously, the ε-amino group of lysine has been demonstrated to detoxify biomass hydrolysates in yeast fermentation.[15] Soy protein typically has 15 types of amino acids, which contain 2.3% histidine and 6.3% lysine and no cysteine.[37] The higher percentage of nucleophilic amino acids in BSA probably was the reason for its better detoxification efficiency in biomass pretreatment than soy protein. It should be noted that amino acids can be generated from protein by acid hydrolysis.[36] As a result, both nucleophilic proteins and the hydrolyzed amino acids can react with carbonyl inhibitors and detoxify the biomass prehydrolysates.

This new approach can also be applied in some other pretreatment strategies, such hot water, steam expansion, and organosolv pretreatment. For example, hot water pretreatment also generates acid prehydrolysates with a considerable amount of inhibitors. Addition of nucleophilic amino acids and proteins can still react with these carbonyl inhibitors and reduce the toxicity of the prehydrolysates.

Effect of Nucleophilic Additives in Biomass Pretreatment on Substrates Digestibility. Although nucleophilic amino acids and protein increased the prehydrolysates fermentability significantly, their effects on pretreated substrates digestibility were also essential. To assess whether the addition of amino acids and proteins in pretreatment can increase or decrease the substrates digestibility, the dilute acid pretreated aspen substrates (DAPA-Cys, DAPA-His, DAPA-SPI, and DAPA-BSA) from the pretreatment with cysteine, histidine, SPI and BSA were enzymatically hydrolyzed at 50 °C for 72 h with 10 FPU/g glucan of Novozym 22C (Figure 2). Their 72 hydrolysis yields were compared with the control substrates (DAPA). Unexpectedly, it was observed that 72 h

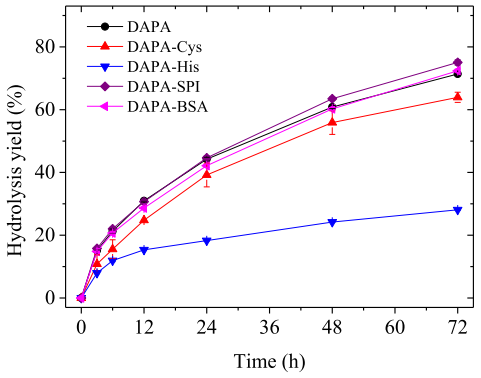

Figure 2. Time course of enzymatic hydrolysis of dilute acid pretreated biomass with amino acids and proteins addition.

hydrolysis yields of DAPA-Cys and DAPA-His decreased from 71.35% (DAPA) to 63.93% and 28.11%, respectively. The 72 h hydrolysis yield of DAPA-SPI increased to 75.04%, and the 72 h hydrolysis of yield (72.38%) of DAPA-BSA did not change, as compared to the control. Similarly, the initial hydrolysis rates of DAPA-Cys (0.80 g/L/h) and DAPA-His (0.58 g/L/h) were much lower than that from the control (DAPA, 1.12 g/L/h); the initial hydrolysis rates of DAPA-SPI and DAPA-BSA were comparable to the control. Although the residual lignin content (Table 1) in all pretreated substrates was the same around 28.4%, the xylan contents from DAPA-Cys (7.63%) and DAPA-His (9.74%) were much higher than that in DAPA (3.22%). The xylan contents of DAPA-SPI and DAPA-BSA were 4.85% and 4.96%, respectively. This indicated that the additives affected the residual xylan content but did not affect the amount of residual lignin. Most likely, the additives in dilute acid pretreatment did not assist in solubilizing lignin and in delignification, but the buffering capacity of cysteine and histidine reduced the pH and severity of dilute acid pretreatment, which resulted in a higher xylan content. A similar observation has been reported on dilute acid pretreated canary grass, in which they found dilute acid pretreatment (with 1% and 2% sulfuric acid) resulted in the same amount (13.4%) of residual lignin but much higher residual hemicellulose (mainly xylan) with a lower sulfuric acid (5.94% vs 2.53%).[38] In addition, the nucleophilic additives mostly reacted with electrophiles (reducing sugars and carbonyl compounds) in pretreatment. In our study, the residual xylan in DAPA-His was 3-fold higher than that in DAPA, which resulted in 2-fold lower of its initial hydrolysate rate than that from DAPA. A strong correlation between xylan content and the initial hydrolysis rate has been observed (r^2 = 0.91). This indicated that higher residual xylan limit the initial enzymatic hydrolysis. A similar observation has been reported on the role of residual xylan in organosolv pretreated loblolly pine.[39] Xylan (1.67 g/L) has been shown to reduce the initial hydrolysis rate of Avicel by 34.5%.[40] Xylooligomers have been reported to inhibit the initial hydrolysis rate of Solka Floc by 40%.[41] In this study, the pretreated substrates have been washed, and the presence of xylooligomers was less likely. It should be noted that the buffering capacity reduced pretreatment severity and also decreased the inhibitors levels in the prehydrolysates. On the basis of the decrease of acetic acid concentration, it can be estimated that the reduced severity by SPI and BSA contributed to a 32% reduction of inhibitors in DA-SPI and DA-BSA; addition of BSA and SPI contributed to 68% for mitigation of inhibitors through nucleophilic reactions.

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Table 1. Chemical Compositions of Raw Material and Dilute Acid Pretreated Substrates

biomass	glucan	xylan	galactan	arabinan	mannan	acid-insoluble lignin	acid-soluble lignin
raw material	43.39 ± 0.50	16.40 ± 0.12	1.59 ± 0.01	1.19 ± 0.23	1.67 ± 0.26	21.20 ± 0.79	2.47 ± 0.04
DAPA	60.43 ± 0.18	3.22 ± 0.28	0.32 ± 0.03	0.61 ± 0.58	1.36 ± 0.24	28.39 ± 0.46	2.39 ± 0.08
DAPA-Cys	57.57 ± 0.02	7.63 ± 0.11	0.28 ± 0.21	0.51 ± 0.05	1.24 ± 0.05	28.39 ± 0.21	2.27 ± 0.02
DAPA-His	55.54 ± 0.05	9.74 ± 0.21	0.56 ± 0.02	0.45 ± 0.02	2.01 ± 0.01	28.47 ± 0.05	2.36 ± 0.01
DAPA-SPI	58.57 ± 0.01	4.85 ± 0.21	0.45 ± 0.07	0.54 ± 0.02	1.46 ± 0.08	28.37 ± 0.40	2.18 ± 0.04
DAPA-BSA	56.72 ± 0.18	4.96 ± 0.18	0.51 ± 0.02	0.52 ± 0.01	1.49 ± 0.07	28.26 ± 0.01	2.38 ± 0.05

Table 2. Sugars and Inhibitors Concentrations in the Prehydrolysates

	sugar concentration (g/L)							
prehydrolysates	glucose	xylose	galactose	arabinose	mannose	formic acid	acetic acid	levulinic acid
DAPA	2.83 ± 0.09	18.44 ± 0.28	1.04 ± 0.02	0.55 ± 0.03	1.95 ± 0.12	1.03 ± 0.01	5.73 ± 0.25	0.56 ± 0.01
DAPA-Cys	0.95 ± 0.02	7.29 ± 0.05	0.32 ± 0.05	0.51 ± 0.02	0.89 ± 0.05	0.46 ± 0.04	3.13 ± 0.09	0.36 ± 0.01
DAPA-His	0.43 ± 0.06	2.39 ± 0.08	0	0	0.25 ± 0.03	0.56 ± 0.05	2.59 ± 0.06	0.36 ± 0.02
DAPA-SPI	1.84 ± 0.20	13.47 ± 0.26	0.56 ± 0.01	0.15 ± 0.02	1.46 ± 0.08	0.47 ± 0.02	3.85 ± 0.15	0.36 ± 0.02
DAPA-BSA	1.64 ± 0.07	12.40 ± 0.07	0.51 ± 0.01	0.15 ± 0.04	1.43 ± 0.13	0.51 ± 0.04	3.89 ± 0.23	0.38 ± 0.12

Table 3. Effects of Amino Acids and Proteins Addition on Inhibitors Removal of Prehydrolysates

				inhibitor concentration (mg/L)				
GC peak	compound name	RT[a] (min)	*m/z*	DA	DA-Cys	DA-His	DA-SPI	DA-BSA
	furan derivatives			4567.4	2545.4	2020.7	2516.7	2501.9
1	furfural	6.794	96	2957.6	1706.7	1860.3	1207.9	1447.7
2	2-acetylfuran	8.106	110	4.9[c]	10.0[c]	NA	NA	NA
4	5-methylfurfural	9.211	110	63.3	51.4	40.8	25.7	30.4
6	2,5-furandicarboxyaldehyde	11.893	124	60.9[c]	15.4[c]	11.6[c]	1.5[c]	2.5[c]
8	hydroxymethylfurfural	14.502	126	1480.7	715.2	108.0	1281.6	1021.3
	aiphatic derivatives			20.6	4.5	NA	NA	NA
3	2,5-hexandeione	8.415	114	20.6[c]	4.5[c]	NA	NA	NA
	aromatic monomers			552.4	381.3	131.1	261.8	245
5	phenol	9.550	94	6.7	NA	5.1	NA	2.9
7	benzoic acid	13.441	122	8.0	NA	8.6	4.7	4.7
9	3,4,5-trihydroxybenzaldehyde	16.093	154	2.8[e]	4.3[e]	5.2[e]	2.2[e]	NA
10	vanillin	16.874	152	55.5	39.2	24.4	41.8	39.6
11	homovanillin	17.685	166	15.4[d]	2.6[d]	6.0[d]	3.6[d]	NA
12	guaiacylacetone	18.628	180	5.4	11.6	2.9	NA	2.0
13	1-(4-hydroxy-3-methoxyphenyl)propane-1,2-dione	19.335	194	9.1[d]	11.4[d]	3.6[d]	5.8[d]	5.4[d]
14	syringaldehyde	20.323	182	104.4	51.8	39.1	79.2	74.3
15	hydroxypropiovanillone	20.617	196	5.9[d]	NA	0.9[d]	1.0[d]	NA
16	homosyringaldehyde	20.735	196	35.3[e]	21.0[e]	0.8[e]	NA	NA
17	1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propan-2-one	21.192	196	53.5[d]	56.0[d]	10.0[d]	42.5[d]	38.8[d]
18	syringylacetone	21.501	210	58.2	78.9	12.8	17.6	13.2
19	(2*E*)-4-(4-hydroxy-3-methoxyphenyl)but-2-enal	21.678	192	5.1[d]	0.3[d]	NA	3.7[d]	2.5[d]
20	1-(4-hydroxy-3,5-dimethoxyphenyl)propane-1,2-dione	22.047	224	26.2[e]	20.6[e]	5.3[e]	13.1[e]	12.1[e]
21	2-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-one	23.211	226	5.6[e]	NA	NA	3.0[e]	12.7[e]
22	2-hydroxy-1-syringyl-ethanone	23.815	226	155.9[e]	83.6[e]	6.4[e]	42.0[e]	36.8[e]

[a]RT, retention time. [b]Inhibitor residual was calculated based on the intergradation area of each compound. [c]Concentration was determined by calibration of hydroxymethylfurfural. [d]Concentration was determined by calibration of vanillin. [e]Concentration was determined by calibration of syringaldehyde.

Similarly, the reduced severity by cysteine and histidine contributed to a 45% and 55% reduction of inhibitors, respectively. They contributed to 55% and 45% for the mitigation of inhibitors through nucleophilic reactions. Pretreatment with SPI slightly increased the 72 h hydrolysis yield, and pretreatment with BSA did not change the hydrolysis yield. However, pretreatment with cysteine and histidine reduced the 72 h hydrolysis yield significantly. The benefits of improving the fermentability by cysteine and histidine were unlikely to overcome its loss of digestibility.

Unexpectedly, addition of histidine significantly reduced the substrates digestibility. Histidine is one kind of amino acid with two functional groups, α-amino group and imidazole group. Both groups were beneficial to react with carbonyl compounds in the prehydrolysates. However, these groups might react with lignin fragments and link them together to generate the repolymerized lignin in pretreatment. Because these groups are highly nucleophilic with multiple substitutions and act as a crossing agent for lignin fragments. Pielhop et al. reported that

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addition of amines into the spruce autohydrolysis pretreatment reduced the enzymatic cellulose digestibility.[29]

However, considering both the prehydrolysate fermentability and the substrates digestibility, SPI and BSA are potentially better additives to enhance the prehydrolysate fermentability without sacrificing the negative effects on enzymatic hydrolysis. In further experiments, we examined the inhibitors contents in the prehydrolysates with the addition if nucleophilic additives. We also expect the enzymatically hydrolyzed sugar stream from substrates can be easily fermented with detoxified prehydrolysates together.

Effect of Nucleophilic Additives in Biomass Pretreatment on Inhibitors Concentration. To investigate the effects of the additives on inhibitors concentration in the prehydrolysates, HPLC and GC-MS were used to determine the sugars, aliphatic acids, and potential inhibitors in the biomass prehydrolysates (Tables 2 and 3). The results showed that addition of nucleophilic additives significantly reduced the concentration of released glucose and xylose. Specifically, the glucose and xylose concentrations in cysteine-assisted pretreatment (DA-Cys) decreased by 3-fold from 2.83 and 18.44 g/L (control) to 0.95 and 7.29 g/L, respectively. The glucose and xylose from DA-His prehydrolysate was 7-fold lower than those in the control. For the prehydrolysate from DA-SPI and DA-BSA, the glucose and xylose dropped similarly by 1.5-fold. The reduction of sugar contents could result from the Maillard reaction between the amino acids (or proteins with amino groups) and reducing sugars in the biomass prehydrolysate.[42,43] Addition of amino acids or proteins could also reduce the pretreatment severity due to their buffering capacity, which in turn reduced the released glucose and xylose due to lower pretreatment severity. Histidine buffer has been used in biological systems,[44] and most proteins with positively charged amino groups and negatively charged carboxyl groups have been suggested to function as buffers in biological cells. The higher residual xylan in DAPA-Cys, DAPA-His, DAPA-SPI, and DAPA-BSA also indicated the decrease of pretreatment severity (Table 1). In addition, the formic, acetic, and levulinic acids in all of the prehydrolysates dropped by 35−55% as compared to the control.

For potential inhibitors, the compounds were extracted by dichloromethane from different prehydrolysates and determined by GC/MS (Table 3 and Figure S1). TIC-GC/MS chromatograms of the prehydrolysates with different additives were compared in Figure S1. In total, about 22 potential inhibitory compounds from the prehydrolysate have been identified and quantified, which include furfural, 5-methylfurfural, hydroxymethylfurfural (HMF), vanillin, syringaldehyde, homosyringaldehyde, and syrngylacetone. Table 3 shows that DAPA-His selectively removed certain carbonyl inhibitors to a much greater extent compared to the other amino acid and protein treatments. This could be caused by the lower inhibitors generated in the pretreatment and the Michael addition reaction. As discussed previously, the higher buffering capacity of histidine in pretreatment condition could result in a lower inhibitors content. Moreover, the imidazole side chain of histidine has been suggested to react with α,β-unsaturated aldehydes.[45] In this study, it is possible that the imidazole groups of histidine selectively reacted with phenolic aldehydes (peaks 20 and 22) at low pH and detoxified the prehydrolysates. The corresponding fragment patterns and chemical structures of all of the inhibitors are shown in the Supporting Information, Figures S1−S23. The results showed that addition of cysteine, histidine, SPI, and BSA significantly reduced the inhibitors concentration in the prehydrolysates. Specifically, the furfural concentration in the prehydrolysates of DA-Cys, DA-His, DA-SPI, and DA-BSA decreased by 42%, 37%, 59%, and 51%, respectively. 5-Methylfurfural (in DA-Cys, DA-His, DA-SPI, and DA-BSA) decreased by 19%, 35.5%, 59%, and 52%, respectively. The HMF concentration (in DA-Cys, DA-His, DA-SPI, and DA-BSA) dropped by 51%, 93%, 86%, and 69%, respectively. Vanillin (in DA-Cys, DA-His, DA-SPI, and DA-BSA) decreased by 29%, 56%, 25%, and 29%, respectively. Syringaldehyde (in DA-Cys, DA-His, DA-SPI, and DA-BSA) dropped by 50%, 63%, 24%, and 29% respectively. The results showed that addition of histidine reduced inhibitors much more than cysteine, SPI, and BSA. This corresponds with its best improvement of the glucose consumption rate in prehydrolysates fermentation. Although vanillin and HMF were not strong inhibitors, they have been suggested to be important indicators for the prehydrolysates toxicity. The released monomers (glucose and xylose) were converted to furfural and HMF; the lower amount of glucose and xylose resulted in lower furfural and HMF in the prehydrolysates. The results from this study suggest that addition of protein decreases inhibitors by reaction with aldehydes/ketones and also by buffering and reducing the pretreatment severity, which results in less inhibitors produced. The reduced severity also decreases the cellulose digestibility, especially with the addition of cysteine and histidine. Hence, the overall effect on yield and productivity is a trade-off between the positive effect of reducing inhibitors and the undesirable effect of less sugar release from the substrates.

Effects of BSA Concentration in Biomass Pretreatment on Prehydrolysates Fermentability. To assess whether a lower BSA concentration can be used to improve prehydrolysates fermentability, different amounts of BSA (1%, 2%, 3%, and 4% w/w) were added into the dilute acid pretreatment of aspen (Figure 3). The results showed that the

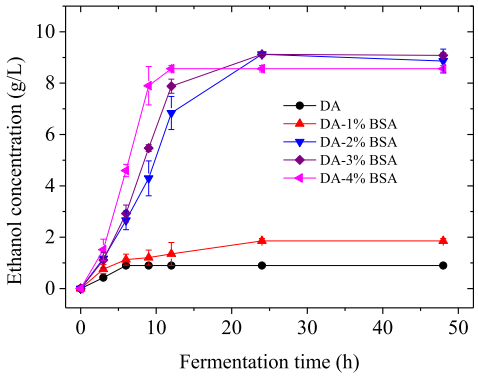

Figure 3. Effects of BSA concentrations on ethanol production of prehydrolysates.

BSA concentration more than 2% enhanced the prehydrolysates fermentability significantly. Specifically, the prehydrolysates from 1% of BSA were barely fermentable, its final ethanol concentration was only 1.86 g/L at 48 h, while the prehydrolysates from 2% and 3% BSA produced 8.86 and 9.08 g/L ethanol, respectively. The fermentation time was significantly short for the prehydrolysate with 4% BSA, and the glucose can be consumed within 12 h. This indicated that the fermentability of prehydrolysate was associated with the

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amount of BSA added into the pretreatment. In addition, it was observed that the volumetric ethanol productivity was higher with the higher BSA concentration. The volumetric ethanol productivity increased from 0.19 ± 0.02 (DA-1% BSA) to 0.44 ± 0.04 (DA-2% BSA), 0.49 ± 0.04 (DA-3% BSA), and 0.77 ± 0.03 g/L/h (DA-4% BSA). Similarly, the glucose consumption rate increased from 0.56 to 1.12, 1.22, and 1.61 g/L/h, respectively. The results indicated that 2% BSA was acceptable to make the prehydrolysates fermentable. As for substrates digestibility, the 72 h hydrolysis yield was similar and their yields were 70% (DA-1% BSA), 71% (DA-2% BSA), 72% (DA-3% BSA), and 74% (DA-4% BSA). It should be noted that the required BSA addition in the pretreatment can be further "tuned" and optimized based on the carbonyl content in the prehydrolysates. It should be noted that the SPI is relatively cheap and can be used in the industrial process.

BSA Postdetoxification of Biomass Prehydrolysates. To test whether BSA can detoxify the biomass prehydrolysates or only reduce the pretreatment severity, the dilute acid prehydrolysates were detoxified with 0.2% (w/v) BSA at 20 and 60 °C for 2 h (pH 6.0) (Figure 4). The results showed the

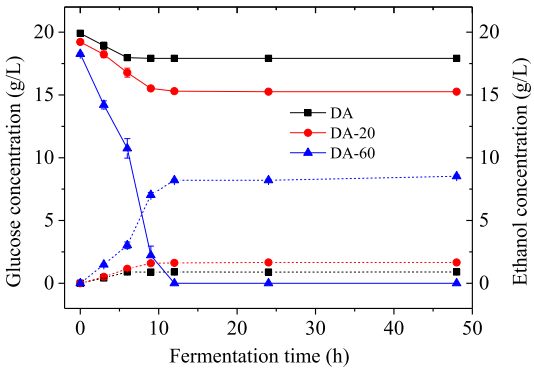

Figure 4. Effect of detoxification temperatures on prehydrolysate fermentability in yeast fermentation.

high-temperature detoxification (60 °C) made the prehydrolysates fermentable, while the low temperature did not. Specifically, the volumetric ethanol productivity increased from 0.15 ± 0.00 (control) to 0.19 ± 0.01 and 0.50 ± 0.03 g/L/h for the prehydrolysates detoxified at 20 and 60 °C, respectively. The glucose consumption rate increased from 0.32 (control) to 0.41 (DA-20) and 1.25 g/L/h (DA-60), respectively. The final ethanol concentration reached 1.66 (DA-20) and 8.52 g/L (DA-60), respectively. The results indicated BSA could also detoxify the prehydrolysates, and the detoxification process was dependent on temperature.

In addition, BSA detoxification at 60 °C removed significant amounts of inhibitors in the prehydrolysates (Table S1). Furfural was reduced by 74% from 2957.6 to 769.2 mg/L. HMF was decreased by 41% from 1480.7 to 867.7 mg/L. Similarly, vanillin was reduced by 53% from 55.5 to 26.2 mg/L. Syringaldehyde was reduced by 57% from 104.4 to 44.5 mg/L. Syringlacetone was reduced by 42% from 58.2 to 33.6 mg/L. 1-(4-Hydroxy-3,5-dimethoxyphenyl)propane-1,2-dione was reduced by 77% from 26.2 to 6.0 mg/L. The results suggested that a high temperature promoted the detoxification reaction between BSA and carbonyl compounds. Similar observations have been reported on cysteine and glycine detoxification of biomass hydrolysate in which a higher detoxification temperature resulted in higher fermentation rates and yields.[15] The reaction products between amino acids and model inhibitors have been also reported in this study. A Schiff base (thiazolidine carboxylic acid) was formed by the condensation of carbonyl aldehyde and nucleophilic amino acid. Although adding proteins and amino acids after pretreatment also could detoxify prehydrolysates for yeast fermentation, the additional detoxification step was required. However, we added proteins and amino acids in the pretreatment which could in situ detoxify the prehydrolysates in dilute acid pretreatment without performing any additional detoxification step. This approach could be potentially cost effective.

CONCLUSION

Addition of nucleophilic cysteine, histidine, SPI, and BSA in the dilute acid pretreatment reduced the release of carbonyl inhibitors and improved the fermentability of prehydrolysates significantly. Addition of cysteine, histidine, SPI, and BSA increased the volumetric ethanol productivity from 0.15 ± 0.00 to 0.51 ± 0.01, 1.44 ± 0.06, 0.49 ± 0.04, and 0.77 ± 0.03 g/L/h, respectively. The results indicated that these four additives could in situ detoxify the prehydrolysates in dilute acid pretreatment. Histidine appeared to be the most effective one. It was observed that 72 h hydrolysis yields of DAPA-Cys and DAPA-His decreased from 71.35% (DAPA) to 63.93% and 28.11%, respectively. The 72 h hydrolysis yields of DAPA-SPI increased to 75.04%, and the hydrolysis yield (72.38%) of DAPA-BSA did not change much. A strong correlation between xylan content and the initial hydrolysis rate has been observed (r^2 = 0.91). This indicated that a higher residual xylan limits the initial enzymatic hydrolysis. The furfural concentration in the prehydrolysates decreased by 37−59%. 5-Methylfurfural decreased by 19−59%. The results showed that addition of histidine reduced inhibitors much more than cysteine, SPI, and BSA. The results suggested that addition of nucleophilic proteins (especially BSA and SPI) in biomass pretreatment could be a potential cost-effective approach to reduce the toxicity of prehydrolysates in microbial fermentation. Addition of nucleophilic proteins decreased inhibitors by reaction with carbonyl compounds and also by buffering and reducing pretreatment severity, which results in less inhibitors produced. The reduced severity also decreased the digestibility of pretreated substrates, especially with the addition of cysteine and histidine. Hence, the overall effect of the addition of protein in pretreatment is a trade-off between the positive effect of reducing inhibitors and the undesirable effect of decreasing digestibility.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c01086.

TIC-GC/MS chromatograms of the prehydrolysates with different additives; GC/MS spectra of carbonyl inhibitors; structures of the identified carbonyl inhibitors from aspen prehydrolysate by GC/MS; effects of detoxification temperatures on inhibitors removal of prehydrolysates (PDF)

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Notes

The authors declare the following competing financial interest(s): The provisional patent application from this study has been filed by the authors and University of Cincinnati.

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