Studies on the Cellulase Inhibition by Phenols from Hydrothermal Pretreated Soybean Wastes

by

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ABSTRACT

Biofuels can be generated through the processing of lignocellulosic plant material. Chemical and biological processes known as pretreatment are utilized to process plant material. Pretreatment contributes to the breakdown of the crystalline structure of lignocellulosic materials. A method of pretreatment is hydrothermal pretreatment with various acids and bases. This study addresses the effect of hydrothermal pretreatment with sodium hydroxide (NaOH) or hydrogen peroxide (H_2O_2) on enzymatic hydrolysis of soybean straw. Hydrothermal pretreatment of soybean straws (10% w/v) was carried out with either sodium hydroxide (1% v/v, NaOH) or hydrogen peroxide (1% v/v, H_2O_2) at 121°C for 60 min to evaluate the effect of water-soluble inhibitors (released during the pretreatment) on cellulolytic enzymes. The cellulose in pretreated solids (1% w/v glucan) was enzymatically hydrolyzed for 72 h with 25 mg enzyme protein/g glucan in the presence of either buffer or pretreated liquor (rich in phenols and lignin-derived molecules). The hydrolysis of NaOH treated solids in buffer gave a 57% cellulose conversion to glucose versus a 39% glucose yield from the H_2O_2 treated solids. When pretreated liquor was applied, NaOH and H_2O_2 treated solids had a 20% and 30% glucose yield, respectively, indicating the suppression of enzyme activity by non-productive bindings between enzyme proteins and inhibitors.

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1. INTRODUCTION

Lignocellulosic biomass is plant dry mass that is used in the production of biofuels (Raud et al., 2019). It is a plant material that is not edible by humans which makes it eco-friendly and an excellent choice to use for biofuel production. Lignocellulosic biomass is used because of its structural composition. Figure 1. shows the structural composition of lignocellulosic biomass (Hernández-Beltrán et al., 2019).

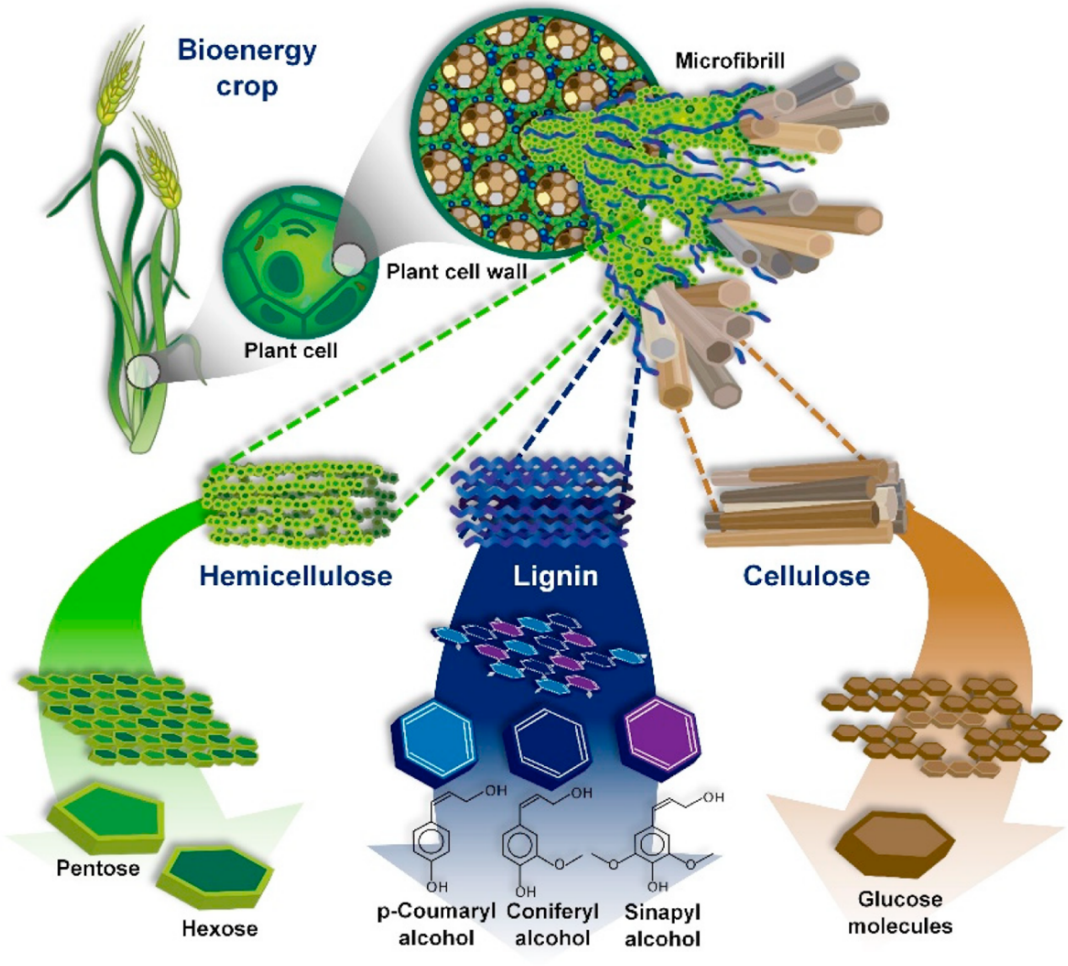

Figure 1. Structure of lignocellulosic biomass and its biopolymers; cellulose, hemicellulose, and lignin (Hernández-Beltrán et al., 2019).

Lignocellulosic biomass' major components are cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are useful because they are polymers made of long sugar chains that

can be broken down to generate sugars that can be used in biofuel production. Cellulose and hemicellulose play a central role in the production of renewable biofuels and biomaterials as an alternative energy source to replace fossil-based fuels (e.g., coal, petroleum, and natural gas). Currently, traditional fossil-based fuels support approximately 80% of the worldwide industrial/technological energy supply and need (Cárdenas et al., 2019). Lignocellulosic biomass can reduce the use of fossil-based energy resources to resolve the issues associated with these energy systems, such as limited energy resources, rapid climate change, and other environmental issues caused by the heavy dependence on fossil fuels. Lignocellulosic materials, such as agricultural and forestry wastes, field-grown grasses, crop residues, municipal wastes, wood, and other plant residues are regarded as competitive renewable energy sources due to their plentiful availability and their large-scale feasibility at low cost. Biofuels are a great eco-friendly alternative to the harmful use of nonrenewable fossil fuels as an energy source.

Lignocellulosic ethanol, for example, has been considered a potential renewable transportation biofuel for several decades. The U.S. and Brazil are the leading producers of biofuels (mainly ethanol) in the world. The U.S. produced 16.1 million gallons of ethanol fuel, and Brazil produced around 8.1 million gallons in 2018 (Renewable Fuels Association, 2021). However, lignocellulosic bioethanol has not been widely commercialized because of the difficulties associated with the complex structure of lignocellulose, technical challenges, and cost-efficiency. The physical structure of lignocellulose biomass (cellulose, hemicellulose, and lignin components) is constructed from carbon dioxide, water, and sunlight through photosynthesis. In particular, the lignin acts as a glue to bind with cellulose and hemicellulose, providing physical strength and inflexibility to the plant cell wall as well as protecting plant cells from foreign pathogens (Kim, 2018; Kim and Ku, 2018). Furthermore, the chemically stable aromatic structure

of lignin, crystallinity of cell wall carbohydrates, and the chemical linkages, such as hydrogen, ester, and ether bonds that exist between the cellulose-hemicellulose and cellulose-lignin, make the plant cell wall recalcitrant to chemical or biological decomposition. Thus, a pretreatment process is required to disrupt the highly sophisticated and complex structure of the plant cell wall and enhance the enzyme accessibility of cellulose for producing fermentable sugars.

The complex cellulose structure of lignocellulosic biomass often makes it difficult to produce biofuels efficiently, so the structure must be broken down. In order to hydrolyze the complex molecule to produce sugars for biofuel production, the lignocellulose must be pretreated to break down the macrostructures of the cellulose and lignin to release polysaccharides that can be hydrolyzed with enzymes or acids into monomeric sugar for biofuel production (Kim, 2018). Pretreatment is done to breakdown the molecules' crystalline structure, and it can be done in various ways such as physical-chemical treatment with hot water or steam explosion, physical through grinding or crushing, chemical with the use of organic solvents, and biological with the use of bacteria or fungi (Kim, 2018). During pretreatment of the lignocellulose biomass, the lignin begins to solubilize, and hemicellulose is removed (Mansfield et al., 1999). This is useful for enzyme hydrolysis because it exposes the cellulose more thus making it more accessible during the sugar conversion. However, the process releases lignocellulose-derived compounds such as phenolic compounds, organic acids, furan derivatives, and soluble sugars that inhibit enzymes during hydrolysis (Kim, 2018). The composition of lignocellulosic biomass and their potential inhibitors are summarized in Figure 2.

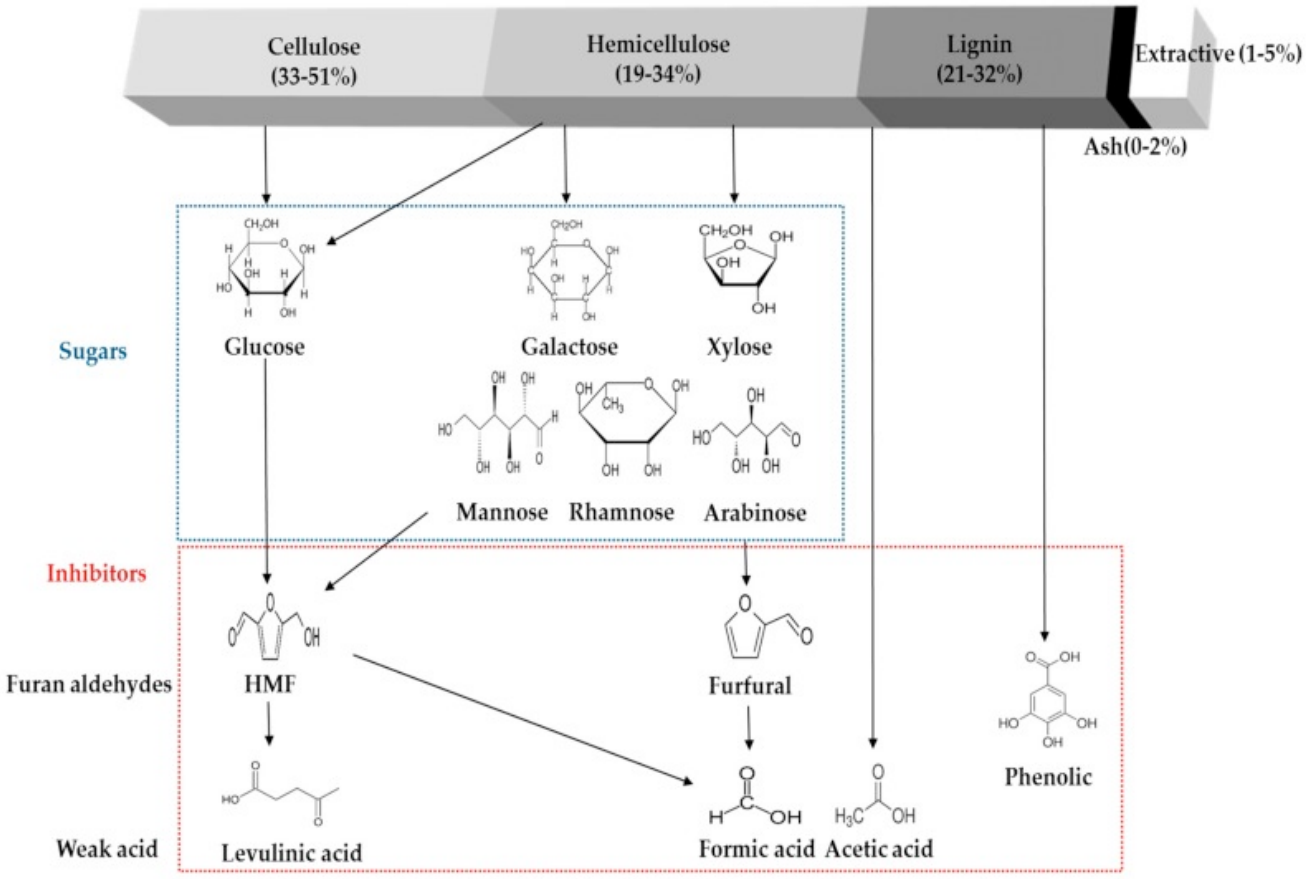

Figure 2. The average chemical composition of lignocellulosic materials and brief scheme of inhibitory compound formation (Kim, 2018).

The type and levels of the soluble enzyme and fermentation inhibitors generated during pretreatment depend on many factors: types of lignocellulose feedstocks, catalysts used in the pretreatment, pretreatment severity (temperature, duration, acidity), and solids loading (Kim et al., 2015). Regardless of the feedstock and pretreatment method applied, processing the lignocellulosic feedstock at a high concentration of solids (>15% dry solids) is a requirement to reduce the water consumption and downstream processing costs of lignocellulosic conversion. However, the levels of soluble inhibitors also increase with the solids loading in the pretreatment process. Thus, the increased inhibitory effects of the water-soluble fraction of the pretreated slurry may offset the benefit of high solids loading pretreatment in the subsequent enzymatic and fermentation processes

unless the potential adverse effects of those soluble inhibitors are avoided by applying a proper mitigation strategy. The enhanced accessibility of enzymes to high-severity pretreated cellulose could also be partially canceled by the increased presence of inhibitors released during the high-severity pretreatment. In addition, the inhibitory effects of soluble inhibitors are often synergistic, suggesting that the compounded inhibitory effects of the various soluble inhibitors are more significant than the combined incremental inhibition of each inhibitor (Alam et al., 2019). There is a multi-targeted and diversified approach to enzyme inhibition with various soluble inhibitors combined thus substantially increasing inhibitory effects on enzyme activity.

In addition, the inhibitory impact of soluble inhibitors will be even more amplified at low cellulase loadings, which is required to make the lignocellulosic bioprocessing economically attractive (Jönsson and Martín, 2016). Thus, developing an efficient mitigation strategy to overcome the inhibitory impacts of soluble inhibitors is a critical consideration in improving the economic feasibility of enzyme-based lignocellulose processing. For this study, biomass from soybean straw was utilized. Soybeans are useful for studying biofuel development because they are widely grown and substantial amounts of soybean waste are often produced (Voight, 2021). The large amounts of crop waste from soybeans can be beneficial in the sustainable production of biofuels. Table 1. shows the top sources of biomass fuel in the U.S. (Chisti, 2021).

Table 1. Comparison of some sources of biodiesel (Chisti, 2021).

Crop	Oil yield (L/ha)	Land area needed (M ha)[a]	% of existing US crop area[a]
Corn	172	1,540	846
Soybean	446	594	326
Canola	1,190	223	122
Jatropha	1,892	140	77
Coconut	2,689	99	54
Oil palm	5,950	45	24
Microalgae	136,900	2	1.1
Microalgae	58,700	4.5	2.5

[a] For meeting all transport fuel needs of the United States. M ha, million hectares.

Corn and soybean have the highest percentage of U.S. crop area, which shows that they both are the largest source of biomass in the U.S (Table 1). According to the United States Department of Agriculture, the annual Maryland soybean production in 2021 was 25,705 K bushels, equivalent to about 64 M ha (USDA, 2021). The widespread use of soybean makes it easily accessible and inexpensive to use and makes the use of soybean wastes a great option to use in this study.

The purpose of this study was to examine compositional and structural changes in lignocellulosic biomass of soybean wastes before and after hydrothermal pretreatment with H_2O_2 (10% w/v) and NaOH (10% w/v). Enzymatic hydrolysis of cellulose in the pretreated soybean solids (1% w/v) using the cellulolytic enzyme preparations was utilized to evaluate the impacts of the pretreatments on the changes in chemical composition and the subsequent cellulose conversion to glucose. Furthermore, the influence of soluble inhibitors (mainly phenolics), collected from NaOH or H_2O_2 pretreated soybean straw, on endo-glucanase and β-glucosidase was examined.

2. MATERIALS AND METHODS

2.1 Materials

The raw soybean wastes originated from a local Frederick farm where they were cultivated in 2019, and after harvest, they donated their soybean wastes. The soybean samples were ground by a Wiley mill and collected with a 0.595 mm (30-mesh) screen, and oven-dried until they had a moisture content of less than 10%. The Cellic Ctec2 (enzyme blend) was used for the enzymatic hydrolysis of the soybean samples, Cellulase from *T. reesei* was purchased from Sigma-Aldrich (St. Louis, MO), and Multipect pectinase was provided from Genencor, Danisco Division (Palo Alto, CA). Enzyme activities of a filter paper unit, endo-glucanase, β-glucosidase, and protein concentration were determined in the laboratory and summarized in Table 2.

Table 2. Profile of enzyme activity

	FPU[a] (IU/mL)	EG[b] (IU/mL)	β-G[c] (IU/mL)	Protein (mg/mL)
Cellic CTec 2 (Cellulase blend)	29.0	229.9	6470.0	127.0
Cellulase from *T. reesei*	13.8	119.3	147.2	137.0
Multipect pectinase	4.2	326.0	176.0	82.3

[a] Filter paper unit (FPU mL^{-1})
[b] Endoglucanase is defined as the amount of enzyme required to release 1 μmol of 4-nitrophenol with assay reagent
[c] β-glucosidase with respect to p-NPG (para-nitrophenyl glucoside)

2.2 NaOH and H_2O_2 pretreatment

The soybean samples (10 g dry weight biomass) in 100 mL of hydrogen peroxide or sodium hydroxide (10% w/v) in duplicates were pretreated at 121℃ for 60 minutes (severity factor: 2.40, determined by the severity equation that measures the intensity or severity of reaction conditions

to change the structure of lignocellulosic biomass to make the cellulose more available to the enzyme) in the autoclave. The pretreated slurry was separated into solids and liquid via a vacuum filter with a Whatman No.1 filter paper. The filtered solids were rinsed with 100 mL of distilled water at room temperature to eliminate the remaining soluble inhibitors on the solids. The washing procedure was repeated in triplicate and oven-dried at 50 ℃ overnight for further use. The pH of separated solid-free liquor was adjusted to 4.8 and kept at 4 ℃ for further analysis. The soybeans samples before and after pretreatment were captured and presented in Figure 3.

Figure 3. (A) Raw soybean straw, (B) H_2O_2 pretreated, and (C) NaOH pretreated soybean straw solids. Hydrothermal pretreatment decreased the particle size and the chemical composition of raw material.

2.3 Compositional analysis

The chemical composition of soybean straw before and after pretreatment was determined by the National Renewable Energy Laboratory (NREL) analytical procedures (Kim et al., 2021, Ázar et al., 2020). The soluble inhibitors and total phenolic compounds in vacuum filtered liquor were measured using HPLC analysis (Kim et al., 2016) and Folin-Ciocalteu colorimetry method (Kim et al., 2021), respectively.

2.4 Enzymatic hydrolysis of pretreated soybean straw solids

The NaOH or H_2O_2 pretreated straw solids (1% w/v, glucan) were mixed with a total of 10 mL of vacuum filtered liquor combined with cellulolytic enzyme preparation (25 mg protein/g glucan) in a 50 mL flask and hydrolyzed in a shaking incubator at 50 ℃ for 72 h. Lignin-free cellulose, Solka Floc (pure cellulose and hemicellulose) was used for control tests in the presence of 50 mM sodium citrate buffer (pH 4.8) or vacuum filtered liquor. The liquid samples were appropriated at timed periods, and glucose concentration was quantified using a GOPOD colorimetric assay kit (Megazyme, Wicklow, Ireland). The remaining cellulolytic activities were identified by a CellG5 assay method for endo-glucanase (Megazyme, Wicklow, Ireland) and hydrolysis of *para*-nitro-phenyl β-glucoside for β-glucosidase, respectively (Dien et al., 2008, Ázar et al., 2020). The experiment was performed in duplicate.

2.5 Analytical Assays

Cellulolytic activities in Cellic Ctec2 were determined with 1% carboxyl methyl cellulose sodium salt (CMC, Sigma-Aldrich, St. Louis, MO) and 10 mM *p*-nitrophenyl-β-D-glucopyranoside (pNG, Sigma-Aldrich, St. Louis, MO) as substrates for endo-glucanase and β-glucosidase, respectively (Ázar et al., 2020). One unit of enzyme activity refers to the enzyme amount that releases 1 micromole of the specified substrate per min under described protocol conditions (Ázar et al., 2020). The glucose content of hydrolyzed samples was quantified using a D-glucose GOPOD-format assay kit (Megazyme, Wicklow, Ireland).

3. RESULTS & DISCUSSION

The overarching goal for this project is a continuous and systematic approach to evaluating enzyme inhibition/deactivation with the goal to maximize cellulose conversion to sugars that can be fermented to ethanol and other valuable molecules. Briefly, soybean straw was pretreated with either NaOH or H_2O_2 (10% w/v, dry basis) followed by separation of the liquor and solids. The pretreated soybean-derived liquor (rich in phenolics) was applied during the enzymatic hydrolysis of pretreated solids (1% w/v) to test the effect of inhibitors on cellulose conversion to glucose. Lignin-free cellulose (Solka Floc) was used for the control test in the presence of 50 mM citrate buffer (pH 4.8). The experimental design is outlined in Figure 4.

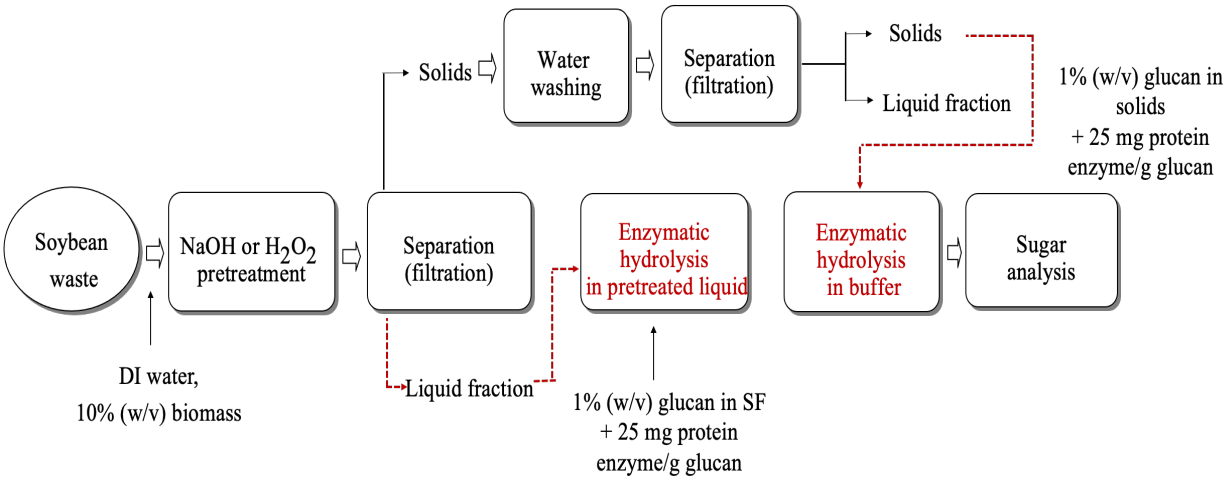

Figure 4. The schematic diagram for the enzymatic hydrolysis of hydrothermally pretreated soybean wastes.

3.1 Impact of pretreatment on the chemical composition of soybean straw

To interpret the change of cellulose/hemicellulose/lignin components during the pretreatment, the chemical composition of pretreated solids was determined and summarized in Table 3. The NaOH soybean straw pretreatment was effective in increasing the glucan component (from 27.7% to 39.7%) by solubilizing a portion of hemicellulose and/or some lignin content

although the hemicellulose component unexpectedly increased slightly. Similarly, solubilization of the xylan fraction (the major component of hemicellulose) resulted in increasing glucan composition in the H_2O_2 pretreated soybean straw solids, which was 5.9% lower than those from the NaOH solids. The increased percentage of cellulose is expected in the pretreated samples because pretreatment is required to break up the complex structure of lignocellulosic biomass. A higher percentage of cellulose means a higher sugar yield as there is more cellulose that can be converted into glucose. However, the lignin component also slightly increased in the H_2O_2 pretreated samples likely due to some lignin reaggregation. A higher extent of lignin in the pretreated solids would be reflected by plant species, pretreatment type (e.g., hydrothermal, liquid hot water, dilute acid), and severity factor (temperature and time). For instance, Ko et al. (2015) found a high solubilization/decomposition of lignin in hardwood achieved during liquid hot water (LHW) pretreatment between 170-180°C, but simply increasing the temperature was not efficient because re-deposition of the lignin content on the hardwood surface was observed above 180°C. Similarly, Trevorah et al. (2018) demonstrated that lignin solubilization in *Eucalyptus* via γ-Valerolactone (GVL) pretreatment was enhanced by increasing the temperature from 120 to 150°C but less lignin removal was obtained at 180°C. These findings are consistent with the current work for pretreatment at 121°C for 30 min to minimize the generation of inhibitors and to avoid the re-aggregation and re-deposition of lignin onto the biomass.

The liquid fraction of the pretreated soybean straw contained inhibitory molecules (mainly phenolics, Table 3B). The NaOH pretreatment produced a higher concentration of total phenols (2038.2 mg/mL) than from the H_2O_2 pretreated soybean straw sample (622.5 mg/mL), and these originated from some ester groups linked to hemicellulose and lignin degradation. The NaOH pretreatment was more effective by generating more cellulose content than the H_2O_2 pretreatment

(39.7% vs. 33.9%) while more phenolic compounds were generated than those from the H_2O_2 pretreated liquor. The higher percentage of inhibitors can potentially result in lower sugar yields by hindering/inhibiting the enzyme thus affecting the efficiency of the pretreatment and enzyme activity during hydrolysis.

Table 3. Compositional analysis of soybean waste samples before and after pretreatment by either NaOH or H_2O_2.

(A)	Composition (% by dry weight basis)		
Composition (%)	As received	NaOH pretreated	H_2O_2 pretreated
Cellulose	27.7 ± 1.5	39.7 ± 1.0	33.8 ± 0.2
Hemicellulose	13.0 ± 0.9	14.8 ± 0.8	14.7 ± 0.2
Lignin	26.9 ± 0.1	25.2 ± 0.4	29.8 ± 0.1
Solid recovery (%)	-	57.8	78.8
(B) Soluble inhibitors		Composition in vacuum filtrate	
[b] Total phenols (mg/L)		2038.2	622.5

3.2 Enzymatic hydrolysis of pretreated soybean straw solids

To verify the effect of cellulolytic enzymes on soybean hydrolysis, three different types of enzymes (Cellulase Blend, Multifect pectinase originating from *Aspergillus niger*, and Cellulase from *Tricodermar. reesei*) were evaluated, and Cellulase Blend enzyme was selected for further assays with higher endo-glucanase and β-glucosidase activity (Table 2). Pretreated and washed soybean straw solids of 0.1 g dry glucan/10 mL pretreated liquid (NaOH or H_2O_2) were hydrolyzed using enzyme preparation (25 mg enzyme protein/g glucan) at 50°C for 7 hr in an orbital shaker. Hydrolysis yields of control run with lignin-free pure cellulose (Solka Floc) in buffer solution had the highest glucose conversion yield of 75% while when the pretreated liquid was applied instead of buffer solution, the conversion was decreased to 39% with NaOH and 42% with H_2O_2 pretreated

liquid, respectively. This suggests that there is enzyme inhibition by non-productive bindings between enzyme proteins and inhibitors which results in less cellulose being converted to glucose. Tests with pretreated solids in the presence of buffer under similar conditions generated 57% and 39% for the NaOH and H_2O_2 pretreated solids, respectively. It is possible that the higher cellulose and lower lignin contents in the NaOH pretreated solids were capable of increasing cellulose conversion yield than H_2O_2 pretreated solids. As expected, the lowest conversion yields were observed when the pretreated solids were hydrolyzed in the NaOH (20% cellulose conversion) and H_2O_2 (30% cellulose conversion) pretreated liquid, respectively (Figure 5). Higher phenolic concentration in the NaOH pretreated liquid decreased hydrolysis efficiency, compared to the H_2O_2 pretreated liquid.

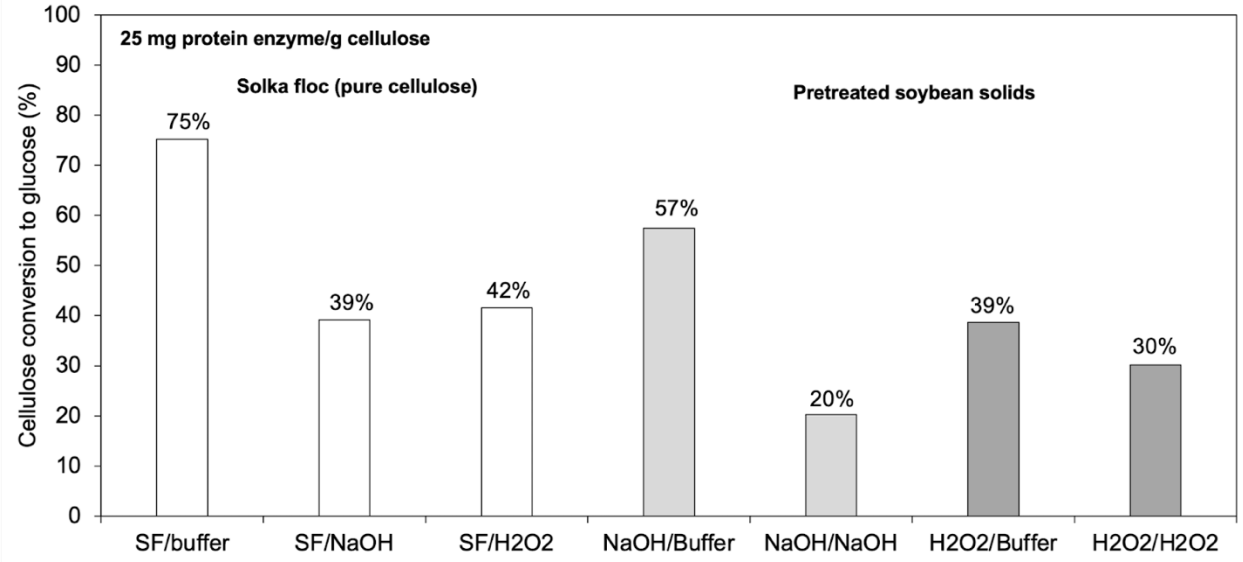

Figure 5. The cellulose conversion to glucose after the enzymatic hydrolysis of H_2O_2 and NaOH pretreated soybean solids and Solka Floc samples with 25 mg protein enzyme/g cellulose in 10 mL of 50 mM citrate buffer (pH 4.8) or pretreated H_2O_2 and NaOH liquid. After the enzymatic hydrolysis, the remaining endo-glucanase and β-glucosidase enzyme activity of the Solka Floc and H_2O_2 or NaOH pretreated samples was measured.

The overall results indicate that NaOH pretreatment was capable of more chemical composition changes in soybean straw than those from H_2O_2 pretreatment, and it also showed that subsequent hydrolysis with cellulolytic enzyme preparation was sufficient in converting cellulose fractions in the solids to glucose, to provide an 18% higher yield when the solids were hydrolyzed without soluble inhibitors (Figure 5, NaOH/buffer vs. H_2O_2/buffer). Besides enzyme activities, non-productive bindings with undesirable molecules (e.g., lignin-derived by-products including phenolics) can cause enzyme inhibition. Solubilization and degradation of soybean straw during pretreatment facilitates the formation of inhibitors/deactivators, mainly phenols in this work, which would impede the catalytic action of enzymes for cellulose hydrolysis.

3.3 Remaining cellulolytic enzyme activity after hydrolysis

Pretreatment type and severity factors affect the lignocellulosic composition (lignin-cellulose-hemicellulose) and impact the production and dispersal of inhibitors. Increasing the pretreatment severity factor (intensity or severeness through the increase of pressure, temperature, acidity, etc) affect the disruption of chemical linkages between cellulose-hemicellulose and cellulose-lignin, and it causes re-deposition/re-aggregation of solubilized lignin on the biomass' surface and an increase in inhibitors (Kim et al., 2021). It is known that inhibitors are the molecules generated from pretreatment, and in particular, phenols (lignin-derived aromatic alcohols) can significantly reduce the action of the enzymes by hindering their accessibility to the targeted cellulose. Qin and colleagues (2016) investigated the inhibitory effect of vanillin, a major lignin-degraded phenolic molecule, on the lignin-free, Avicel (pure cellulose) hydrolysis such that the glucose conversion was decreased from 53% to 26% when hydrolysis of Avicel was conducted in the presence of vanillin at 10 mg/mL. The effect of enzyme loading was also addressed and showed that increasing enzyme loading could partially alleviate the enzyme inhibition and improve the

conversion yield; however, raising inhibitor concentration was more severe to the cellulose conversion and inhibition degree (Qin et al. 2016). Another study of phenolic acids demonstrated that the hydrolysis of 1% (w/v) Solka Floc at 25 mg cellulase protein/g glucan in the presence of either citrate buffer or LHW pretreatment liquor gave 92% and 40% glucose conversion, respectively, but when the enzyme loading was decreased to 1 mg protein/g glucan, about 77% and 30% conversion rates were observed in buffer and pretreatment liquid, respectively (Ximenes et al. 2011). This shows that the decrease in enzyme loading resulted in a substantial increase in enzyme inhibition and decreased glucose conversion.

While pretreatment liquid was separated and potential soluble inhibitors in the solid fractions were washed away with distilled water, further investigations were conducted to evaluate the remaining enzyme activities. The control tests with Solka Floc have relatively higher remaining endo-glucanase activity in buffer (30%), NaOH liquor (32%), and H_2O_2 liquor (50%), respectively (Figure 6A). While the β-glucosidase activity for Solka Floc hydrolysis was 10% (buffer), 15% (NaOH pretreated liquid), and 10% (H_2O_2 pretreated liquid), respectively (Figure 6B). The remaining endo-glucanase activity after hydrolysis for the NaOH pretreated soybean straw solids in buffer was about 18%, and 15% in NaOH pretreated liquid (Figure 6C). It is expected that the NaOH pretreated solids in the buffer would have less enzyme activity than in NaOH pretreated liquid because there are no inhibitors in the buffer (but in the pretreated liquid) although the difference is small. Similarly, the remaining endo-glucanase in the H_2O_2 pretreated solids in buffer was about 40%, which was 10% higher than those from the H_2O_2 pretreated liquid (Figure 6C). On the other hand, the remaining β-glucosidase activity for the NaOH pretreated solids in buffer was about 25%; however, almost half of the enzyme activity (12%) was observed in the NaOH pretreated liquid (Figure 6D). The present β-glucosidase activity in the H_2O_2 pretreated solids in

buffer was about 1%, and 14% in H_2O_2 pretreated liquid. It is expected to be less enzyme activity in H_2O_2 solid in the buffer because the H_2O_2 pretreated liquid has inhibitors that inhibit the enzyme activity, so more enzyme activity is required for hydrolysis thus more activity is present after hydrolysis than in H_2O_2 with buffer (Figure 6D).

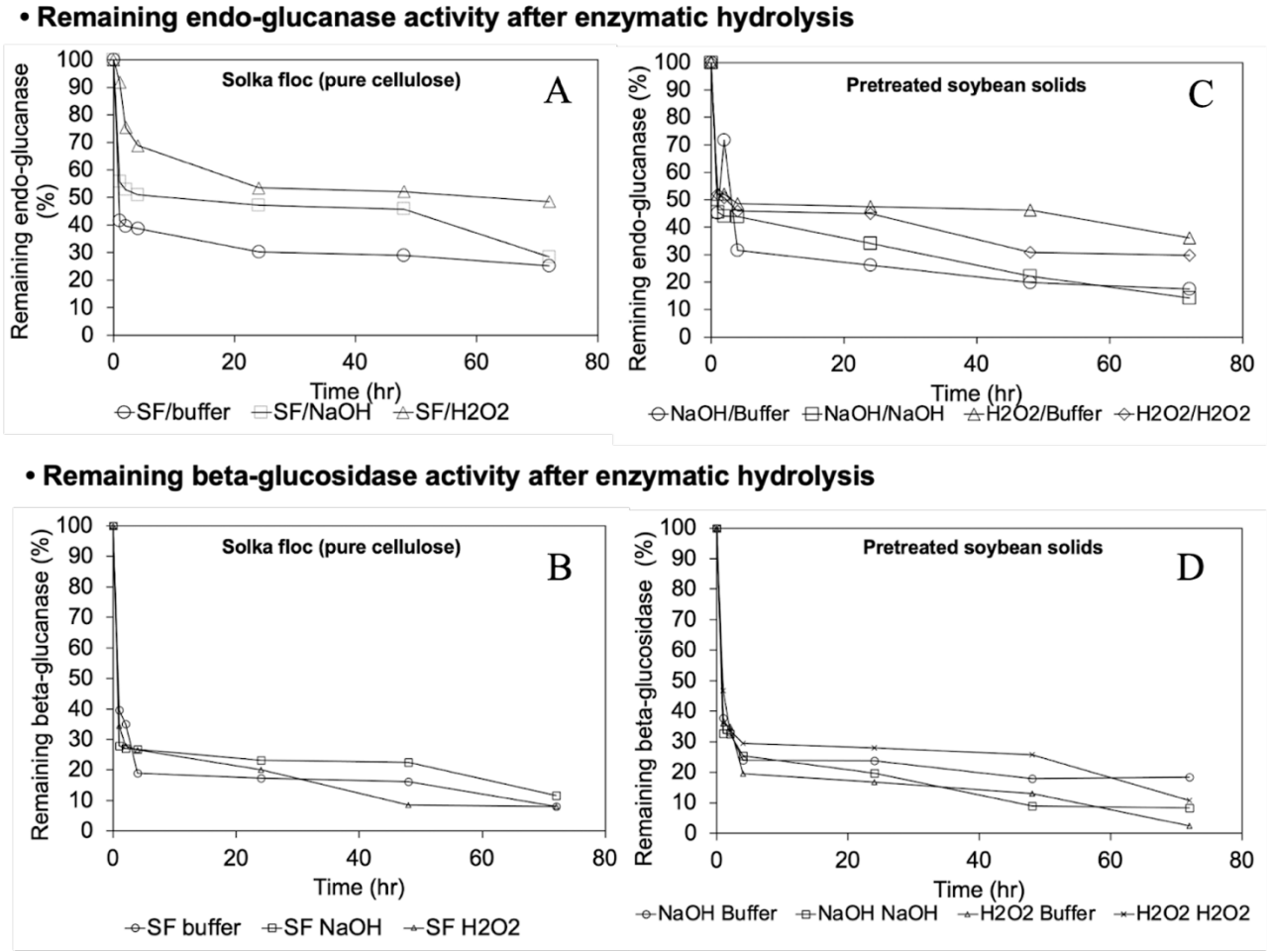

Figure 6. The remaining endo-glucanase and β-glucosidase enzyme activity of the Solka Floc and H_2O_2 or NaOH pretreated samples was measured using seven sets of the samples in a shaking incubator at 50°C for 72 hrs with Cellic Ctec2 cellulose enzyme. The liquid was extracted from the samples at set intervals, filtered using a syringe, and analyzed with a CellG5 assay reagent kit.

These results are consistent with published literature. One study found that the cellulose crystallinity decreased and reducing sugar production increased in water hyacinth pretreated with

sodium hydroxide and hydrogen peroxide (Yan et al., 2015). There was more sugar produced and a reduction in the crystalline structure of the biomass because pretreatment condenses the macrostructure of cellulose and hydrolyses it to sugar monomers. Similarly, another study found that pretreatment of sugarcane bagasse with hydrogen peroxide and sodium hydroxide significantly increased the cellulose content and sugar yields after enzymatic hydrolysis (Meléndez-Hernández et al., 2021). This shows the importance of pretreatment in biofuel production because the complex structure of the lignocellulosic structure must be processed for sugar production to occur. Furthermore, research with soybean wastes and other pretreatment methods show similar results. A study with modified steam explosion pretreatment and dilute acid found high sugar yields after hydrolysis (Corredor et al., 2008). The cellulose and hemicellulose material were solubilized and available for hydrolysis after pretreatment. Although the study found sufficient yields with the use of modified steam explosion pretreatment, it may not be viable as it is more expensive than the cost-efficient acid and base pretreatment of this study. Another study found increased porosity and specific surface area with soybean residues pretreated with a high-pressure homogenization method and 6% HCl and NaOH (Li et al., 2019). The processing and breakdown of the lignocellulose are necessary to convert cellulose to sugars.

Inhibitors released after pretreatment play an important role in impacting the glucose yield after enzyme hydrolysis. A study on reducing inhibitors during enzyme hydrolysis found that by using bioabatement and hemicellulase to remove inhibitory compounds after liquid hot water (LHW) pretreatment, the glucose yields were 20% higher than samples that were not treated (Cao et al., 2015). Utilizing methods to reduce inhibitors after pretreatment helps increase sugar yields. Similarly, another study utilized LHW pretreatment and biological detoxification with fungus to eliminate soluble inhibitors and found that cellulose conversion was 73% higher in biodetoxified

samples than in undetoxified samples (Kim et al., 2016). Cellulose conversion can be enhanced through additives that block inhibition. An experiment with enzyme hydrolysis on acid pretreated hemp waste found that adding bovine serum albumin, a lignin-blocking additive, lead to a glucose yield increase of 72% (Kim et al., 2021). Enzymes can avoid binding to the lignin-derived molecule that is not a productive binding. Moreover, even in instances where biomass is converted to sugar without utilizing pretreatments, inhibitors play an important role in impacting the yield. One study converted cellulose in corn pericarp without pretreatment and found a 98% conversion during enzyme hydrolysis with a phenol tolerant enzyme and only 16% conversion with an enzyme that is less tolerant to phenols (Kim et al., 2017). Cellulase enzyme activity is interrupted by highly inhibitory phenols that are released when the corn is ground to a fine powder for enzyme hydrolysis. It is important to note that corn pericarp lignin component is less than 5% whereas the soybean waste of this study's lignin component is five times higher (higher lignin leads to the formation of more inhibitors) thus the use of the phenol tolerant enzyme, cellulase blend, was not as effective as the phenol tolerant enzyme used in the corn pericarp hydrolysis. Although hydrolysis may be viable without pretreatment for biomasses, enzymes are still impacted by inhibition that must be mitigated for efficient conversion yields.

Furthermore, the soluble compounds released during pretreatment that inhibit enzymes during hydrolysis can be influenced by the pretreatment reaction conditions. A study examines steam explosion pretreatment reaction conditions' effect on the amount of inhibitors and found that increasing pretreatment severities resulted in increased amounts of phenolics and other inhibitors that reduced the sugar yields by 5-26% (Brethauer et al, 2020). There are the maximum amount of inhibitors under certain conditions that hinders the efficiency of enzyme hydrolysis; plant species, pretreatment type, and the experimental process would determine the optimal

conditions for hydrolysis of different lignocellulosic biomass. Although some studies found sufficient yields with pretreatments for certain plant species that may not be suitable for soybean wastes.

4. CONCLUSION

Soybean straw, which contains high cellulose and hemicellulose content, is a potential alternative for lignocellulosic conversion to sugar for biofuel production and renewable resources. Volatile compounds and soluble inhibitors from hydrothermal pretreatment with NaOH or H_2O_2 could be eliminated by extensive washing with distilled water; however, the remaining insoluble lignin-derived inhibitors in vacuum filtered liquid hamper the cellulolytic hydrolysis and decrease the sugar yield. The water-soluble inhibitors released during the pretreatment process primarily include phenolic acids, which negatively impact the cellulose conversion to glucose. Cellulase inhibition by phenols is important to study because it can be used to understand ways to maximize glucose yields during pretreatment and hydrolysis. This study confirms that hydrothermal pretreatment of soybean straws (10% w/v) carried out with 1% v/v NaOH at the conditions of 121°C for 60 min pretreatment was more efficient at lignin decomposition than the H_2O_2 pretreatment as there was higher glucose yield. However, the NaOH pretreatment did result in a higher percentage of inhibitors than the H_2O_2 pretreatment which likely resulted due to the higher solubilization of lignin.

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