Minimizing water consumption for biofuel and bioproduct conversion from lignocellulosic biomass

by

Jikai Zhao

B.S., Henan University of Technology, China, 2014
M.S., Henan University of Technology, China, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Carl and Melinda Helwig Department of Biological and Agricultural Engineering
Carl R. Ice College of Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Global demand for renewable biofuels and biochemicals has been promoting the development of biomass valorization technologies. However, the efforts have not led to its commercial realization. To unlock the recalcitrant biomass, pretreatment is an inevitably critical step. Unfortunately, excessive water washing of the pretreated biomass with wastewater discarding significantly causes water overconsumption and chemicals loss. The goal of this research was to minimize water consumption for biofuel and bioproduct conversion from biomass in an economically viable manner.

The first chapter aimed to answer three prevalent questions: (i) why is excessive water washing needed after biomass pretreatment? (ii) is the higher solid loading used for pretreatment, hydrolysis, and fermentation better? (iii) why are most of the proposed economically viable biorefineries still not commercialized? Regarding the reduction in water consumption and enhancement of sugar and ethanol concentrations, physicochemical and biological detoxification, black liquor recycling, fed-batch model, strain genetic engineering, and ethanol (first and second generations) integration approaches were critically discussed in terms of strengths and weaknesses. Collectively, biomass-to-ethanol commercialization necessitates a comprehensive understanding of economic, environmental, and policy perspectives. Technoeconomic analysis as an essential valuation for potential commercialization from laboratory scale is recommended, and it should be relaying on accurate experimental data rather than an overrated empirical assessment.

The second chapter evaluated the potential of acetic acid (HOAc)-sodium hydroxide (NaOH) integrated pretreatment of biomass to reduce water and chemicals consumption. Pretreatment effectiveness including morphology, crystallinity, and component recovery was elucidated. Results showed that HOAc and NaOH in the mixed filtrate were neutralized to achieve a pH of around 4.80 resulting in the alkali lignin precipitation. Lignin (46.01 and 48.38 g/kg-biomass for hemp and poplar, respectively) exhibiting comparable Fourier transform infrared spectroscopy (FTIR) characteristics with the commercial alkali lignin was recovered. Compared to sodium acetate buffer as a control, integrating HOAc and NaOH pretreated biomass and their mixed filtrate for enzymatic hydrolysis boosted total sugar concentration (hemp: 42.90 vs. 38.27 g/L; poplar: 43.18 vs. 38.76 g/L) without compromising glucose yield (hemp: 70.86 vs. 70.69%; poplar: 66.48 vs. 69.48 %) but improving xylose yield (hemp: 60.10 vs. 35.92%; poplar: 56.90 vs. 29.39 %).

The third chapter revealed the effects of post-washing [one-volume water (I-VW) or double-volume water (II-VW)] on pretreated biomass and enzymatic hydrolysis. Compared to I-VW, II-VW increased 3.76-6.80% of glucan content in NaOH pretreated biomass, diminished lignin recondensation, and heightened cellulose-related FTIR peak intensities, crystallinity index, and lignin removal. The pH of the mixed filtrate was around 4.80, precipitating the NaOH soluble lignin partially. Although II-VW had lower lignin recoveries than I-VW, their FTIR characteristics were equivalent to the commercial alkali lignin. Enzymatic hydrolysis at solid loadings of 2.5-10% (w/v) demonstrated that I-VW and II-VW had marginal variations in sugar concentration and conversion efficiency, indicating that I-VW is sufficient for post-washing pretreated biomass. Glucose concentration exhibited a quadratic correlation with solid loading and hemp biomass reached the maximum glucose (43.88 g/L) and total sugar (57.08 g/L) concentrations with I-VW.

The fourth chapter validated the potential of HOAc-NaOH integrated pretreatment for glucose, xylose, 5-hydroxymethylfurfural (HMF), furfural, and lignin production of four genotypes of industrial hemp biomass that were harvested from Haysville and Manhattan, KS. The integration process effectively rendered the pH of the integrated filtrate and slurry to approximately 4.80. The highest lignin recovery of 73.13 g/kg biomass was achieved by Rigel from Manhattan. FTIR spectrum showed that only lignin derived from Vega (Haysville) and Anka (Manhattan) was comparable to the commercial alkali lignin. Retaining monosaccharides (2.24-3.81 g/L) enhanced sugar concentrations (glucose: 40.40-45.71 g/L; xylose: 7.09-8.88 g/L) and conversion efficiencies (glucose: 71.19-77.71%; xylose: 45.42-52.03%). Besides, 0.79-1.25 g/L of HMF and 0.99-1.59 g/L of furfural coupling with 1.96-2.95% and 10.00-14.65% conversion efficiencies, respectively, were obtained in the final hydrolysate.

The fifth chapter performed three pretreatment scenarios (I: H_2SO_4 pretreatment with NaOH neutralization; II: NaOH pretreatment with H_2SO_4 neutralization; and III: parallel H_2SO_4 and NaOH pretreatments following their integration) with enzymatic hydrolysis for glucose, xylose, HMF, furfural, bioethanol production at high solid (15 and 25%, w/v) loading without solid-liquid separation and further detoxification. With an initial solid loading of 25% (w/v), scenario I reached the highest furfural (4.94 g/L) and HMF (2.82 g/L) concentrations, scenario II achieved the highest glucose (73.25%) and xylose (77.49%) yields, while scenario III displayed the highest sugar concentration (74.53 g/L). Only the hydrolysate from NaOH pretreatment and enzymatic hydrolysis with 10% initial solid loading can be efficiently fermented to ethanol (17.92 g/L) by the traditional yeast.

In summary, integrating acid and alkali pretreatment of biomass has great potential to reduce water consumption for multi-stream biofuel and bioproduct production. There is a lot of room for further research on upgrading sugar and lignin to high-value chemicals.

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Approved by:

Major Professor
Donghai Wang

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Global demand for renewable biofuels and biochemicals has been promoting the development of biomass valorization technologies. However, the efforts have not led to its commercial realization. To unlock the recalcitrant biomass, pretreatment is an inevitably critical step. Unfortunately, excessive water washing of the pretreated biomass with wastewater discarding significantly causes water overconsumption and chemicals loss. The goal of this research was to minimize water consumption for biofuel and bioproduct conversion from biomass in an economically viable manner.

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In summary, integrating acid and alkali pretreatment of biomass has great potential to reduce water consumption for multi-stream biofuel and bioproduct production. There is a lot of room for further research on upgrading sugar and lignin to high-value chemicals.

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Dedication

This dissertation is dedicated to my beloved wife, father, and mother for their unending love.

Chapter 1 - Conversion of lignocellulosic biomass to bioethanol and byproduct: challenges, solutions, and perspectives

1.1. Abstract

Global demand for renewable and sustainable biofuels promoted the considerable development of biomass-to-bioethanol valorization strategies. However, the efforts have not led to its large commercial realization in the United States yet. Herein, the objective of this chapter is to reveal the challenges such as abundant wastewater production that is discarded after biomass pretreatment, low solid loading used for biomass pretreatment, enzymatic hydrolysis, and fermentation, as well as overestimation and inconsistency in technoeconomic analysis (TEA). Regarding the reduction in water consumption and enhancement of sugar and ethanol concentrations, physicochemical and biological detoxification, black liquor recycling, fed-batch model, strain genetic engineering, and ethanol (first and second generations) integration approaches were critically discussed in terms of strengths and weaknesses. Collectively, biomass-to-ethanol commercialization necessitates a comprehensive understanding of economic, environmental, and policy perspectives. TEA as an essential evaluation for potential commercialization from laboratory scale is recommended and it should be relaying on accurate experimental data rather than an overrated empirical assessment.

1.2. Introduction

Global demand for coal and petroleum based energy sources has been resulting in the potential depletion of non-renewable fossil fuels (Keller et al., 2020; Martins et al., 2018; Wang et al., 2020). Exploring alternative bioresources with renewable and sustainable characteristics is an essential worldwide task to alleviate overdependence on fossil fuels and collateral environment issues (Ayer and Dias, 2018; Burnham et al., 2012; Gaete-Morales et al., 2019). The efforts had led to a gradual reduction in the share of coal in the total U.S. energy consumption in favor of emerging energy sources such as natural gas and nuclear for the past century (U.S. Energy Information Administration, 2021). Strikingly, in the last two decades, other renewable energy sources stood out and occupied approximately 11% of U.S. primary energy consumption in 2019, as it plays an important role in reducing greenhouse gas emissions (U.S. Energy Information Administration, 2019). Biomass-related resources including biomass waste, biofuels, and wood approximately accounted for 43% of renewable energy. Here, wood biomass is commercially used by power plants to generate electricity and steam via combustion with/without coal to reduce sulfur dioxide emissions. Counting its huge annual production, lignocellulosic biomass from agriculture (corn stover, wheat straw, sugarcane bagasse, etc.) and forestry (paper mill and sawmill discards) is typically underutilized (Limayem and Ricke, 2012; Wang et al., 2021).

In addition to combustion and gasification (Abuelnuor et al., 2014; Cruz et al., 2019; Hupa et al., 2017; Míguez et al., 2012; Nunes et al., 2016; Puig-Arnavat et al., 2010; Sansaniwal et al., 2017), lignocellulosic biomass has been considerably recognized as a competitive candidate for bioethanol production via chemical catalysis, enzymatic hydrolysis, and fermentation steps due to its substantial cellulose and hemicellulose components (Bhatia et al., 2020; Kumar et al., 2020; Tian et al., 2018). However, the recalcitrance of robust lignin necessitates physicochemical pretreatment required to render lignocellulosic biomass more amenable to enzymatic and microbial degradation (Limayem and Ricke, 2012; Zhao et al., 2020a). Regarding the mechanisms and effectiveness of pretreatment technologies, they can be found elsewhere (Bhatia et al., 2020; Elgharbawy et al., 2016; Kumar et al., 2020; Wang et al., 2021; Xu et al., 2016; Zhang et al., 2016). Despite half a century of efforts, the biomass-to-bioethanol commercial realization has stagnated confronting several technical and economic bottlenecks. From the production point of view, excessive water washing and anti-solvent addition after biomass pretreatment are commonly needed to remove inhibitory compounds such as derivatives from sugar [acetic acid, 5-hydroxymethylfurfural (HMF), and furfural] and lignin (the disrupted lignin units and phenols) degradation as well as residual chemicals (Arora et al., 2013; Jung and Kim, 2017; Lee and Park, 2016; Zhu et al., 2011). Strikingly, it is often accompanied by wastewater generation and subsequent discarding, resulting in chemical loss and environmental pollution. On the other hand, low solid loading for pretreatment and enzymatic hydrolysis has been generally applied to promote pretreatment technology with high sugar-to-bioethanol yield. From economic and environmental standpoints, maintaining high solid loading from pretreatment to fermentation is preferred to reduce water consumption and boost bioethanol concentration (Da Silva et al., 2020; Koppram et al., 2014; Modenbach and Nokes, 2012). However, it is particularly observed that the gradual decrease in sugar yield is often undesirable as it offsets the advantages of operating at high solids levels (Kristensen et al., 2009; Zhao et al., 2020a, 2021a). In terms of technoeconomic analysis (TEA) aspects, during process simulation post-pretreatment critical steps (such as excessive water washing and chemical recovery) are often neglected. Additionally, solid loading used for pretreatment, enzymatic hydrolysis, and fermentation is typically overestimated and higher sugar conversion performance is empirically assumed (Viswanathan, et al., 2021; Zang et al., 2020a).

The objectives of this chapter are to disclose the collateral challenges confronted for biomass to bioethanol and coproducts production in terms of experimental and TEA aspects and offer a consolidated source of information in connection with the latest advances from the laboratory to commercial exploration. Explicitly, following a detailed discussion of wastewater generation, solid loading bottleneck, and TEA controversy, the corresponding solutions and perspectives including physicochemical detoxification, black liquor recycling, engineered strain, fed-batch strategy, and ethanol [first generation (1G) and second generation (2G)] integration are critically discussed.

1.3. Technological challenges

1.3.1. Wastewater generation and discard

Although physicochemical pretreatments are able to remove most of the hemicellulose and partially solubilize the lignin enhancing the enzymatic accessibility to cellulose, the undesired derivatives from sugar and lignin degradation can also be released during biomass pretreatment. These derivatives include furans (furfural and HMF), organic acids (acetic acid, formic acid, and levulinic acid), pseudo-lignin, small lignin units, and phenolic compounds (Kim, 2018; Kumar et al., 2020; Meng and Ragauskas, 2017). Furthermore, the residual chemical reagents used for biomass pretreatment often contribute to inhibitory influences on enzymatic hydrolysis and microbial activities. In order to render the pretreated biomass amenable to ethanol bioconversion, the solid residues after solid-liquid separation are commonly washed extensively with water, whereas the resultant wastewater (liquid fraction) containing residual chemicals, derivatives, and sugars is often discarded (Table 1.1). For example, da Costa Nogueira et al. (2018) reported that washing (200 mL water/15g raw biomass per time) was conducted 12 times to achieve NaOH (2%, w/v) pretreated coconut fiber filtrate transparent and reach a neutral pH of this filtrate. Therefore, the post-washing process inevitably results in a large amount of water consumption and chemical loss. Herein, the inhibitory compounds appeared in the pretreated slurry can be roughly categorized into two groups: 1) HMF and furfural and 2) residual chemical reagents. The reasons for wastewater generation and discarding due to these two groups are elucidated as follows.

HMF and furfural are generally found in the acid pretreated slurry and derived from cellulose and hemicellulose degradation, respectively (Zhao et al., 2020b, 2020c, 2021a). On the basis of literature review, these molecules are not found to significantly inhibit enzymatic hydrolysis, apart from the work by Arora et al. (2013) reported that the addition of 2 or 5 mg/mL of furfural to the cellulose powder and enzyme substrate decreased glucose recovery by 5% and 9%, respectively. However, it has been widely recognized that they can negatively influence the microbial ethanol fermentation of the pretreated materials (Ask et al., 2013; Banerjee et al., 1981; Heer and Sauer, 2008; Horváth et al., 2001; Nigam, 2001; Oliva et al., 2006; Palmqvist and Hahn-Hägerdal, 2000a, 2000b; Sanchez and Bautista, 1988). In this regard, Roberto et al. (1991) demonstrated that furfural at 0.5 g/L had no significant effect on the cell growth of Scheffersomyces stipitis while furfural at 2 g/L was detrimental to cell growth. Similarly, Nigam (2001) found that the presence of furfural at 0.25 g/L was unable to limit the cell growth of Pichia stipitis and ethanol production from wheat straw hydrolysates while furfural at 1.5 g/L notably reduced ethanol yield and productivity by 90.4% and 85.1%, respectively.

With respect to their inhibitory mechanisms, Allen et al. (2010) illuminated that furfural triggered the accumulation of reactive oxygen species in Saccharomyces cerevisiae inducing cellular damage via the destruction of mitochondria and vacuole membranes as well as the actin cytoskeleton and nuclear chromatin. During fermentation, HMF and furfural can be metabolized by Saccharomyces cerevisiae into 5-hydroxymethyl furfuryl alcohol and furfuryl alcohol, respectively, indicating their similar inhibitory mechanisms (Palmqvist and Hahn-Hägerdal, 2000b). Compared to furfural, HMF was found to have less inhibitory to microbial activity due to its lower membrane permeability (Behera et al., 2014; Palmqvist and Hahn-Hägerdal, 2000b). In addition, weak organic acids such as acetic, formic, and levulinic acids can typically be produced from the dissociation of acetyl groups and furans during pretreatment (Behera et al., 2014; Kim, 2018; Xing et al., 2011). The individual addition of organic acids to the hydrolysis or fermentation system was noticed to be detrimental (Arora et al., 2013; Nigam, 2001), which could be attributed to the change in slurry pH inhibiting cell growth. Therefore, their negative effects may be mitigated via alkali neutralization, which can be seen in the report that H2SO4 pretreated slurry after ammonia conditioning could be used for enzymatic hydrolysis and fermentation directly (Humbird et al., 2011). In the case of lignin, it has been assessed that non-productive adsorption and steric hindrance are broadly known as the primary mechanism controlling lignin-enzyme interactions (Li and Zheng, 2017), depending on the molecular weight and structural characteristic of lignin (Li et al., 2018). Pseudo-lignin, formed from dilute acid pretreatment (Hu et al., 2012; Zhao et al., 2020b, 2021a), can also significantly retard cellulose hydrolysis (Kumar et al., 2013). Besides, the phenolic compounds are reported to be more poisonous than the previously-mentioned derivatives even at lower concentrations, due to their low molecular weight allowing them easily penetrate cell membranes and alter cell morphology

(Behera et al., 2014; Palmqvist and Hahn-Hägerdal, 2000a, 2000b). Even with solid-liquid separation after pretreatment, these degraded products are still partially adsorbed to the surface of the pretreated biomass, thus excessive water is usually used to remove them or reduce their recondensation (Rajan and Carrier, 2014).

Another reason for post-washing the pretreated biomass is to remove the residual chemical reagents used for biomass pretreatment. For dilute acid and alkali pretreatments, the pH of the pretreated slurry is commonly too severer to be used as a buffer solution for enzymatic hydrolysis and fermentation (Zhao et al., 2020b). The work by Frederick et al. (2014) highlighted that rinsing the 0.98% (v/v) H_2SO_4 pretreated biomass with 1.5 or 3 volumes of water reached glucose yields that were seven-folds higher than the unwashed treatment. They also mentioned that the H_2SO_4 pretreated biomass washed with 3 volumes of water created the highest ethanol yields (up to 0.43 g/g-glucose) and were significantly higher than those from the unwashed sample ($\leq$ 0.28 g/g-glucose) (Frederick et al., 2014). Based on the same enzymatic hydrolysis and fermentation conditions, it could be considered that the higher glucose and ethanol yields are highly associated with the removal of the residual H_2SO_4. In addition, Karuna et al. (2014) pretreated rice straw with NaOH and subsequently conditioned the pretreated slurry to a pH of 5-6 via extensive water washing or acidification with HCl then water washing. In this case, excessive post-washing removed the disrupted lignin and residual NaOH, while acidification (neutralization between HCl and NaOH) with post-washing precipitated the modified lignin on the surfaces of rice straw, therefore, the former showed higher enzymatic digestibility of rice straw than the latter (Karuna et al., 2014).

In terms of ionic liquid pretreatment, Ninomiya et al. (2015b) performed choline acetate (ChOAc) and 1-ethyl-3-methylimidazolium acetate (EmimOAc) pretreatments (0.5 g biomass/5.0 g ionic liquid) of bagasse powder with different water post-washing times (45 mL per time). It was found that cellulase and yeast were more sensitive to the residual EmimOAc concentrations in the pretreated biomass than ChOAc, based on their median effective concentrations (Ninomiya et al., 2015b). Besides, based on the original bagasse the overall ethanol yields after saccharification and co-fermentation of the pretreated bagasse with post-washing 5 times were only 54% for ChOAc and 22% for EmimOAc (Ninomiya et al., 2015a). This indicates that the residual ionic liquid in the pretreated biomass is dramatically detrimental to enzymatic and microbial activities, therefore, adequate water is crucial to remove the residual ionic liquid. Moreover, to remove the imidazole from the pretreated biomass, 2 volumes of distilled water and 3 volumes of ethanol (96%) were employed. However, the HCl used for lignin precipitation was found to deprotonate the imidazole (Morais et al., 2016). Therefore, even if the chemicals are recovered and recycled for biomass pretreatment, their functional integrity is unknown. Additionally, anti-solvent applied for chemical reagent removal from the pretreated biomass has a significant influence on glucose conversion efficiency (Ogura et al., 2014).

1.3.2. Solid loading bottleneck

The pursuit of high solid loading used for biomass pretreatment, enzymatic hydrolysis, and fermentation is excessively favored due to its envisioned lower water consumption, higher sugar and ethanol concentrations, and reduced capital costs (Da Silva et al., 2020; Koppram et al., 2014; Modenbach and Nokes, 2012). However, as regards the commercial promotion, it has to consider the "high-solids side effect". Here, the question of "whether the higher solid loading used for biomass pretreatment, enzymatic hydrolysis, and fermentation is better" was critically discussed as follows.

Conventionally, biomass pretreatment has been performed at lower solid loadings (≤10%) to efficiently fractionate biomass into cellulose-concentrated solid fraction and hemicellulose- and lignin-derived liquid fraction (Modenbach and Nokes, 2012). In keeping with the idea of reducing water consumption, high-solids loading (>10%) for biomass pretreatment was widely promoted. However, the undesired side effects such as weak pretreatment effectiveness, accumulation of inhibitory compounds, and high viscosity were often observed (Koppram et al., 2014; Modenbach and Nokes, 2012). In terms of pretreatment effectiveness, Xu et al., (2020) conducted the EmimOAc pretreatment of corn stover assisted with/without NaOH and aqueous ammonia (10%, v/v) at 36% (w/w) solid loading but performed the enzymatic hydrolysis of pretreated biomass at 1% (w/v) glucan loading after three times (10 mL/g biomass) of post-washing. In this study, only 60.65–64.82% of total glucose yields were obtained at higher enzyme loading (Nieves et al., 2016), indicating that the accessibility of cellulose to enzymes after pretreatment was relatively low. Besides, the increment of solid loading for biomass pretreatment significantly reduced xylan and lignin removal as reported by Chen et al. (2019) who carried out the ternary deep eutectic solvents pretreatment of switchgrass under 10-35% of solid loading.

The effects of solid loading used for pretreatment on the accumulation of inhibitory compounds in the pretreated slurry have not been investigated yet, but it can be inferred that an increase in solid loading would increase their concentrations (Koppram et al., 2014; Modenbach and Nokes, 2012). Therefore, the nominal reduction in water consumption may be offset by heavy water post-washing operation needed for removal of residual chemical reagents. Additionally, the resultant slurries with high solids tend to be super viscous in nature (Jørgensen et al., 2007), due to the water-biomass interaction (Da Silva et al., 2020). Viamajala et al. (2009)

reported that biomass size reduction can decrease slurry viscosity, but a large amount of energy is required for milling (Miao et al., 2011). Moreover, it has been demonstrated that feedstock pumpability could only be achieved at solid loading below 15% (Dãrãban et al., 2015). In this regard, Dãrãban et al., (2015) found that pumpable wood-based slurry containing 20% solids can be prepared using recycled biocrude as a carrier fluid, given that the particle sizes of biomass were smaller than 0.125 mm. However, this phenomenon has been typically overlooked, instead, excessive water was used to flush the slurry out (Chen et al., 2019; Li et al., 2013; Luterbacher et al., 2012; Wang et al., 2020; Xu et al., 2020). Based on the previous analysis, it might be debatable whether high solid loading for pretreatment can save water because of the subsequent challenges.

To reach higher sugar and ethanol concentrations, efficient and economic distillation (Koppram et al., 2014; Larsen et al., 2008; Zacchi and Axelsson, 1988) and enzymatic hydrolysis and fermentation at high solid loading are recommended. However, biomass has a low density and high hygroscopicity, its interaction with water at high solid loadings can induce water unavailability (Da Silva et al., 2020). Also, the gradually released lignin from the pretreated biomass acts as a spatial barrier and binds to the enzymes (Koppram et al., 2014; Kristensen et al., 2009; Modenbach and Nokes, 2012; Saini et al., 2016). Therefore, the decreases in sugar and ethanol yields might counteract the advantages of operating at high solids with higher product concentrations and lower water input (Kristensen et al., 2009; Zhao et al., 2020c). On this subject, Ioelovich and Morag (Ioelovich and Morag, 2012) confirmed that an increase of initial solid loading (50 to 200 g/L) of the H_2SO_4 and hypochlorite-alkaline pretreated switchgrass led to a diminution of enzymatic digestibility due to the complication of mass transfer of enzymes in the high viscous slurry and a constraining effect of the formed sugars. Additionally, Zhao et al.

(2021a) performed the enzymatic hydrolysis of liquid hot water, H_2SO_4, and NaOH pretreated corn stover and poplar wood at solid loadings of 2-15% (w/v) and observed that sugar yield almost exhibited a negative linear correlation with solid loading. This finding was in accordance with the works by Zheng et al. (2009) who reported that enzymatic digestibility of H_2SO_4 pretreated four saline crops decreased by 20% as solid loading increased from 4 to 12%, and by Nieves et al. (2016) who found that increasing solid loading of NaOH pretreated mango stem bark residues (15 to 21%) reduced sugar yield from 75.3 to 56.3%. However, there is one exception, where no significant variations in glucose and ethanol yields were observed with increasing solid loading from 10 to 30%, which is possibly attributed to the extremely high enzyme use (Nieves et al., 2016).

Is higher solid loading better for achieving high sugar or ethanol concentrations? Given that sugar and ethanol yields had a negative linear correlation with solid loading (Kristensen et al., 2009; Zhao et al., 2020c), the corresponding sugar and ethanol concentrations are supposed to have a quadratic relationship (parabola) with solid loading. In other words, the rising levels in sugar and ethanol concentrations gradually diminish as solid loading increases. If the focus point of the parabola was at 20% solid loading, the sugar and ethanol concentrations obtained from 15% solid loading with 90% yields would be the same as 30% solid loading with 45% yields. Therefore, an extra 15% biomass solids coupled with enzymes would be wasted. In this respect, Zhao et al., (2020a) studied the relationship between ethanol concentration and solid loading under three scenarios and achieved 76.92 g/L of ethanol with 56.36% ethanol yield when conducting fermentation at the focus point (31.0% solid loading) of a parabola. This finding also indicates that a random choice of higher solid loading to optimize ethanol production (higher enzymes and additives input) might be not desirable (Zhao et al., 2020c). Therefore, it is irrational to pursue high sugar or ethanol concentrations without paying attention to additional costs from enzymes, biomass, and pumping facilities (Gnansounou and Dauriat, 2010; Klein-Marcuschamer et al., 2012).

1.3.3. Technoeconomic analysis

A review of the literature shows the economic and environmental benefits from the multiple-stream valorization of lignocellulosic biomass to ethanol and coproducts (Boakye-Boaten et al., 2017; Gogar et al., 2021; Zang et al., 2020b; Zhao et al., 2015). Most studies demonstrated that the minimum selling price of ethanol obtained from the proposed strategies reached lower than \$2.50/gal (Da Silva et al., 2020; Gubicza et al., 2016; Huang et al., 2018; Humbird et al., 2011), whereas several works have confirmed or could be speculated that biomass-to-ethanol production is still unprofitable based on a discounted cash flow analysis (Klein-Marcuschamer et al., 2011; Nitzsche et al., 2016). The notable variations among studies are possibly credited to the differences in the overall methodology, especially the process simulation assumptions (Gnansounou and Dauriat, 2010). Although the directly explicit comparison between the studies is infeasible, this review tries to answer the question of "why are most of the proposed economically-viable biorefineries still not commercialized" from two perspectives: overestimation in empirical assumptions and inconsistencies among process simulation and experimental data.

High solid loading ($\geq$ 20%) used for pretreatment, enzymatic hydrolysis, and fermentation has been commonly assumed for TEA of biomass to ethanol with considerable sugar and ethanol yields ($\geq$ 90%). In this respect, Eggeman and Elander (2005) simulated 100% of glucose and xylose yields from enzymatic hydrolysis of H_2SO_4 pretreated corn stover at 20%

solid loading. On the basis of the previous National Renewable Energy Laboratory report (Aden et al., 2002), Huang et al. (2009) assumed that the H_2SO_4 pretreatment at 30% solid loading could reach 90% of glucose yield as enzymatic hydrolysis of hardwood, switchgrass, and corn stover at 20% solid loading. The lime pretreatment with 30% solid loading was assumed to achieve 4.5% (wt) of ethanol concentration from simultaneous saccharification and fermentation at 10% (wt) solid loading with 12 FPU/g-rice straw (Roy et al., 2012). Here, given a 100% glucose-to-ethanol yield at 10% solid loading, the lime pretreated rice straw should contain approximately 80% of glucan to reach 4.5% (wt) of ethanol, thus the assumption of ethanol yield is overestimated. This phenomenon was also observed in the work where 7-10% (w/w) of ethanol was supposed to be obtained after fermentation without mentioning solid loading and ethanol yield (Quintero et al., 2013). These empirical assumptions in terms of pretreatment effectiveness and sugar and ethanol yields have possibly been overestimated compared to the experimental results. This is strongly supported by the findings by Tao et al. (2011), who reported 65.5-80.9% of glucose, 30.8-78.4% of xylose, and 39.1-58.0 of ethanol yields from enzymatic hydrolysis of biomass at 20% solid loading. In this work, biomass was pretreated by six pretreatment methods (ammonia fiber expansion, dilute acid, lime, liquid hot water, soaking in aqueous ammonia, and sulfur dioxide-impregnated steam explosion) and washed by water until the pH of 5.0 (Tao et al., 2011). The overestimation phenomenon mentioned here is one of the reasons why most of the proposed economically-viable biorefineries are still not commercialized; although a limited number of studies on a representative basis are discussed. Therefore, there is no doubt that an overvalued conversion efficiency and ethanol yield will significantly increase the net income of biorefinery and thus reduce the minimum selling price of ethanol.

In addition to empirical assumptions, several studies have performed the TEA of biomass to ethanol and coproducts based on the experimental data. Strikingly, inconsistencies in critical operation steps and bioproduct yield between the cited references were found. For example, Zang et al. (2020a) reported that switchgrass pretreated by choline chloride:ethylene glycol (ChCl:EG) with 1% H_2SO_4 was washed by water-acetone mixture and subjected to enzymatic hydrolysis with adding water and enzymes directly, however, it was inconsistent with the original procedure, where the citrate was used as a buffer (Chen et al., 2018). It was also mentioned that after lignin precipitation the resulting liquor was evaporated at 70 °C in a convection oven to remove excess water to recycle ChCl:EG (Chen et al., 2018), whereas during process simulation of TEA the resultant liquor was directly reused for pretreatment (Zang et al., 2020a). Actually, the ChCl:EG concentration was seriously diluted due to the post-washing, even if some of it is recycled, it would result in abundant wastewater (containing ChCl:EG) accumulation for a continuous model. The residual ChCl:EG and H_2SO_4 in the pretreated biomass cannot be ignored because of their toxicity to enzymes and microbes as aforementioned (Ninomiya et al., 2015a, 2015b). Therefore, the absence of critical operation steps might lead to an overestimation of the proposed method. Moreover, Viswanathan et al. (2021) conducted the TEA of ethanol and biodiesel production from industrial hemp biomass with hydrothermal pretreatment at 50% solid loading and enzymatic hydrolysis subsequently. They mentioned that glucose and xylose yields were 85 and 80% respectively. However, according to the literature cited, two-step pretreatment and disk milling of the pretreated biomass were inevitably needed, and the sugar yields previously mentioned were obtained from 20% solid loading used for pretreatment and subsequent enzymatic hydrolysis (Wang et al., 2019). Under the same pretreatment conditions (50% solid loading coupled with disk milling), Cheng et al. (2020)

reported that only around 55% glucose and xylose yields were reached with adding very higher enzymes (50 FPU/g dry substrates). These findings indicate that process simulation conducted in this study essentially lacks experimental data support. Coincidentally, there was a similar case, in which an integrated strategy for 1,5-pentanediol and ethanol co-production was proposed based on the data from various sources (Huang et al., 2018). Based on the previous analysis, it is clear that TEA based on comprehensive experimental results and accurate reproducibility is needed since it is a way to promote the commercialization of biomass to ethanol and coproducts. Conversely, the inconsistent and oversimplified process simulation from laboratory to TEA and multiple pieces of data without foundational support would gravely mislead the perception of biomass-to-bioproducts commercialization.

1.4. Solutions and perspectives

1.4.1. Reducing water consumption

To render the dilute acid pretreated biomass and hydrolysate (liquid fraction) more acquiescent for microbial fermentation, a number of physicochemical [membrane filtration (Fayet et al., 2018; Nguyen et al., 2015), alkaline neutralization (Alriksson et al., 2006), ion exchange resin (Kumar et al., 2019, 2018; Vallejos et al., 2016), liquid-liquid extraction (Roque et al., 2019; Zhu et al., 2011), and activated charcoal adsorption (Lee and Park, 2016; Nguyen et al., 2019; Sarawan et al., 2019)] and biological [laccase treatment (Fang et al., 2015; Saravanakumar et al., 2016), microbial degradation (He et al., 2016; Singh et al., 2017), and engineered strain (Suo et al., 2019; Wang et al., 2018; Zhu et al., 2016)] detoxification methods have been investigated. A summary of these representative studies is presented in terms of strengths and drawbacks (Table 1.2). The comprehensive collection and comparison among studies in this respect can be found elsewhere (Bhatia et al., 2020; Kumar et al., 2020), but several crucial discoveries can be extracted from them: 1) alkali addition is unavoidably required to neutralize the residual acid in both pretreated biomass and hydrolysate before enzymatic hydrolysis and fermentation regardless of which methods are applied; 2) the cost of chemicals and enzymes for detoxification should be taken into consideration since the end product-ethanol is considerably sensitive to materials input (Gnansounou and Dauriat, 2010); 3) excessive pursuit of HMF and furfural removal efficiency and negligence of their subsequent recovery are undesirable because they are especially high-value platform molecules for biofuels and chemicals conversion (Davidson et al., 2021); and 4) almost all techniques only focus on the acid pretreated hydrolysate and ignore the residual inhibitors in the pretreated biomass. Concerning the simple operation and low capital investment, alkali neutralization and liquid-liquid extraction might be relatively preferable. It is possible for the former to find optimal conditions that resulted in an analogous fermentability comparing a synthetic sugar solution without inhibitors (Alriksson et al., 2006), whereas the latter is able to entirely extract HMF and furfural as high-value coproducts with the extraction solvent can be recycled to the system (Roque et al., 2019).

In the case of alkali pretreatment, the black liquor from the pretreated hydrolysate has typically been recycled for biomass pretreatment (Table 1.3). Seemingly, it is a promising strategy to reduce water and chemical consumption during biomass valorization. Several drawbacks make this method controversial: 1) the extra water and NaOH usually need to be replenished to the black liquor (Alencar et al., 2017; Chen et al., 2018; Li et al., 2015; Rocha et al., 2014; Wang et al., 2020); 2) the pretreated biomass is commonly subjected to excessive water post-washing and then combined with the fresh buffer before enzymatic hydrolysis and fermentation (Alencar et al., 2017; Chen et al., 2018; Goshadrou, 2019; Wang et al., 2016); and

3) the pretreatment effectiveness (e.g., lignin removal, sugar conversion, ethanol yield, etc.) often decreases as the number of recycling time increases (Alencar et al., 2017; Cha et al., 2016; Goshadrou, 2019; Li et al., 2015; Rocha et al., 2014; Wang et al., 2016). Based on the previous analysis, it is difficult for a single acid and alkali pretreatment to make up for their shortcomings in a comprehensive way. Furthermore, although organosolv, ionic liquid, and deep eutectic solvent pretreatments have attracted considerable attention to fractionate biomass, the efficient and practical strategies targeting the reduction in water (it is commonly used for washing the pretreated biomass as described in Table 1.1) consumption are not available in amount.

Concerning chemical cost, dilute acid and alkali pretreatment technologies are the most possible options for a commercial implementation. Dilute acid pretreatment has been favored by the NREL (Roque et al., 2019), where the acid pretreated corn stover slurry was assumed to be used for enzymatic hydrolysis directly without excessive post-washing after ammonia conditioning. It was reported that ethanol yields obtained from the unwashed NaOH pretreated sugarcane bagasse were significantly higher than those from the washed biomass with black liquor recycling times (Fan et al., 2020). Also, Wang et al. (2020) demonstrated the possibility of NaOH pretreated sugarcane bagasse without post-washing for direct enzymatic hydrolysis. Additionally, liquid-liquid extraction was proved its efficiency for furan recovery from the acid pretreated hydrolysate (Mittal et al., 2017; Rihko-Struckmann et al., 2017; Roque et al., 2019). Based on these findings and clues, an acid-base integrated biorefinery might have the potential to reduce water consumption and recover chemicals during biomass valorization. Herein, the biomass is assumed to be pretreated by acid and alkali parallelly (Zhao et al., 2021a). The furan in the resultant acid slurry is extracted by the extraction solvent (such as methyl isobutyl ketone) and purified as the extraction solvent is recycled. After solid and liquid separation, the filtrates from acid and alkali pretreatments are combined to precipitate the solubilized lignin and phenolic compounds, while their pretreated biomass is mixed for the neutralization reaction of residual acid and alkali as well (Zhao et al., 2021a). Even if a small amount of the inhibitory compounds is retained, the mixing process will dilute them twice as much. In addition, the degraded sugars can be retained and utilized by microbes. Therefore, integrating acid and alkali pretreated biomass and their mixed filtrate could hypothetically be used for enzymatic hydrolysis or fermentation without post-washing, pH adjusting, and new buffer addition, given the pHs of acid and alkali reagents are optimally designed.

1.4.2. Boosting sugar and ethanol concentrations

The fed-batch enzymatic hydrolysis and fermentation model has been commonly proposed to improve the concentration and conversion efficiency of fermentable sugar and ethanol. When hydrolysis and fermentation are conducted at high solid loading, enzymatic (catalytic rate) and microbial cell (cell growth and metabolite production) activity are significantly constrained (Koppram et al., 2014; Modenbach and Nokes, 2012). Underfeeding would induce starvation of cells, while overfeeding would accumulate the inhibitory compounds (Srivastava and Gupta, 2011). Besides, it was reported that the accumulation of high glucose concentration can result in an interruption in the enzyme biosynthesis of microbes, this catabolite repression can be conquered by applying fed-batch fermentation (Srivastava and Gupta, 2011). Indeed, compared to the batch model, the fed-batch model is able to increase water availability and decrease slurry viscosity, maintain enzymatic activity, and subsequently enhance ethanol concentration and yield (Table 1.4). In this regard, initial and final solid loading (Wanderley et al., 2013), solid feeding mode (Gupta et al., 2012; Hernández-Beltrán and Hernández-Escoto, 2018; Lu et al., 2013; Park et al., 2013; Wanderley et al., 2013; Zhang et al., 2010; Zhang and Zhu, 2017), enzyme adding strategy (Gupta et al., 2012; Zhang et al., 2010; Zhang and Zhu, 2017), and incubation condition (Lu et al., 2013) were essential factors to determine the consequential conversion performance. However, to make the fed-batch model commercially viable, some challenges need to be addressed: 1) plentiful water washing of the pretreated biomass tremendously leads to high water consumption; 2) enzymatic hydrolysis and fermentation durations are certainly extended; and 3) the fed-batch model can induce the accumulation of wastewater since the pretreated biomass is used for downstream, but the pretreated hydrolysate is retained.

Saccharomyces cerevisiae is the conventional microorganism employed to digest glucose for ethanol production due to its excellent tolerance and conversion efficiency (Liu et al., 2019). However, it is incapable of utilizing xylose as a carbon source for effective ethanol conversion (Sànchez Nogué and Karhumaa, 2015). Therefore, during fermentation of lignocellulosic hydrolysates xylose as the secondary abundant sugar is often overlooked and discarded (Liu et al., 2019; Sànchez Nogué and Karhumaa, 2015). In order to co-ferment glucose and xylose simultaneously, engineered strains have been widely developed and proved their robust scavenging capacity (Cadete et al., 2016; Casey et al., 2010; Gonçalves et al., 2014; Ishola et al., 2015; Kim et al., 2014; Ko et al., 2016; Lee et al., 2017; Li et al., 2016; Parreiras et al., 2014; Pereira et al., 2011; Romaní et al., 2015; Tsai et al., 2015; Vilela et al., 2015; Wang et al., 2015). The engineering mechanisms and strategies can be found in detail elsewhere (Kim et al., 2013; Kwak et al., 2019; Liu et al., 2019; Sànchez Nogué and Karhumaa, 2015). Based on the literature review, there are two concerns that need attention: 1) depositing unsatisfactory genetic elements into strain chromosomes is inevitably avoided via genomic incorporation of expression cassettes comprising heterologous genes, explicitly, drug resistance genes for selecting transformants may perhaps hinder further industrial application due to public health concerns (Tsai et al., 2015); and 2) xylose has been found to compete with glucose for metabolic pathway resulting in the inhibition of strain metabolism (Kim et al., 2013), given the complexity of such an environment the role of antibiotic resistance in engineered *Saccharomyces cerevisiae* is uncertain.

Integrating 1G starchy grain flour into cellulosic ethanol production offers a practical solution to boost ethanol concentration (Table 1.5). Certainly, it should be highlighted that more than 100 g/L of ethanol concentration can be achieved by the simple integration process (Chen et al., 2018; Xu et al., 2018). Compared to 1G ethanol production, 2G cellulosic ethanol production inevitably requires more capital input, therefore, we may need to seriously consider whether 1G ethanol manufacturers will be willing to sacrifice their interests to pursue the merging of 1G and 2G ethanol manufacture. Additionally, for continuous-model production, because the operation processes among starchy grain flour and biomass are not synchronized, the upstream and downstream material connection needs accurate design. Collectively, it is not advisable to chase only the conversion efficiency of biomass to ethanol, its real commercialization requires careful consideration from economic, environmental, and policy aspects-such as the generation and post-treatment of wastewater, the investment and recovery of chemical reagents, the investment and maintenance of related equipment, and the financial support of the government.

1.5. Conclusions

Recent developments in pretreatment strategies to unlock lignocellulosic biomass and convert it into ethanol and coproducts have demonstrated economic and environmental feasibility. However, a large amount of wastewater generated after pretreatment, low ethanol concentration, and overestimated economic analysis might stall this promising exploration. Herein, the common challenges and reasons in terms of biomass pretreatment (abundant water post-washing, discarded wastewater and chemicals, and low solid loading), enzymatic hydrolysis and fermentation (low sugar and ethanol concentrations, water unavailability, and high viscosity), and techno-economic analysis (overestimation in empirical assumptions and inconsistencies among process simulations and experimental data) were critically revealed and elucidated. Regarding the reduction in water consumption and boosting sugar and ethanol concentrations, several strategies such as physicochemical and biological detoxification, black liquor recycling, fed-batch model, genetic engineering, as well as 1G and 2G ethanol integration were illuminated with the respect of advantages and drawbacks. In summary, biomass-to-ethanol commercialization necessitates cautious consideration of economic, environmental, and policy perspectives. The sole pursuit of high sugar and ethanol conversion yields is unreasonable, and the associated costs of wastewater generation and treatment, equipment input and maintenance, and chemical reagents should all be taken into consideration. Technoeconomic analysis of the proposed biorefinery should rely on comprehensive and accurate experimental data to provide a valuable reference for commercialization.

Table 1.1 A summary of representative studies generating wastewater and its post-treatment under different biomass pretreatment conditions.

Biomass	Pretreatment condition	Post-treatment	Reference
Switchgrass	15% solid loading with liquid hot water at 200 °C for 5 min	The pretreated biomass is washed by 10 g of distilled water per gram of solids four times with the wastewater is discarded	(Larnaudie et al., 2019)
Empty palm fruit bunch	5–25% solid loading with liquid hot water at 160–210 °C for 0–60 min	The pretreated biomass is washed with an amount of water equivalent to 10 times the amount of biomass initially loaded to the reactor with the wastewater is discarded	(Cardona et al., 2018)
Acacia wood	5% solid loading with 0.05% H_2SO_4 at 200 ℃ for 5 min	The pretreated filtrate and 100 mL of washed deionized water are discarded	(Lee and Yu, 2020)
Bamboo	10% solid loading with 30% hydrogen peroxide/glacial HOAc (1:1, v/v) at 85 ℃ for 120 min	The pretreated biomass is washed by tap water with the wastewater is discarded	(Song et al., 2020)
Sugarcane biomass	2–8% solid loading with 1% (m/v) of H_2SO_4 at 121 ℃ for 20–60 min	The pretreated biomass is washed with distilled water until a pH close to 5.0 with the wastewater is discarded	(Santos et al., 2020)
Cashew apple bagasse	10% solid loading with HOAc (0–60 v/v) or H_2SO_4 (0–0.8 w/v) at 121 ℃ for 30–60 min	The pretreated biomass is washed eight times using 200 mL of tap water with the wastewater is discarded	(de Araújo Padilha et al., 2020)
Wheat straw	10% solid loading with 1–10% NaOH solutions at 190 ℃ for 240 min	Deionized water is used to wash the pretreated biomass and then discarded	(Tsegaye et al., 2019)
Prosopis juliflora biomass	Microwave irradiation power (270–450 W) for 3–5 min with 0.75–1.25% (w/v) NaOH solutions at liquid to solid ratio of 10–20 mL/g	The filtrate is washed with distilled water until the pH becomes neutral and then discarded	(Alexander et al., 2020)
Softwood pine	10% solid loading with 0–2% w/v NaOH at 100–180 ℃ for 1–5 h	The pretreated biomass is washed with distilled water until reaching pH 7 with the wastewater is discarded	(Safari et al., 2017)

Table 1.1 Continued.

Poplar biomass	13% solid loading with ethanol (60%, v/v) solution and 1.25% (w/w) of H_2SO_4 at 180 °C for 60 min	The pretreated biomass is washed with 60 °C aqueous ethanol (60%) with the washings are combined with the filtrate and poured into ~500 mL of deionized water.	(Meng et al., 2020)
Hybrid *Pennisetum*	Four organosolv (γ-valerolactone, tetrahydrofurfuryl alcohol, ethanol, and acetone) assisted by 0.05 mol/L H_2SO_4 with a liquid/solid ratio of 12:1 at 100 °C for 2 h	The pretreated biomass is first washed with an equal volume of the organic solvent at least three times to avoid lignin deposition and then washed to neutral with water as the wastewater is discarded	(Tan et al., 2019)
Spruce and oak sawdusts	2% solid loading with 1-ethyl-3-methylimidazolium acetate at 45 °C for 40 min	The pretreated biomass is precipitated by adding two times of ultrapure water and then centrifuged; it is thoroughly washed with ultrapure water as the wastewater is discarded	(Alayoubi et al., 2020)
Hornbeam and spruce wood	4% solid loading with biomass to 1-butyl-3-methylimidazolium chloride ratio of 1:4 at 50–150 °C for 0.5–2 h	The pretreated biomass is washed with distilled water to obtain 95–99% removal of the ionic liquids as the wastewater is discarded	(Dotsenko et al., 2018)
Wheat straw	10% solid loading with ChCl, guaiacol and $AlCl_3$ (molar ratio of 25:50:1) at 80–130 °C for 1 h	The pretreated biomass is washed with 200 mL of hot ethanol and excessive water stepwise as the wastewater is discarded	(Huang et al., 2021)
Hybrid *Pennisetum*	10% solid loading with $FeCl_3$ in ChCl/glycerol at 60–140 °C for 1–9 h	The pretreated slurry is washed with 50% acetone/water (100 mL) and then vacuum-filtered and re-washed with 50% acetone/water until the filtrate is colorless as the acetone is distilled from filtrate	(Wang et al., 2020)

Table 1.2 Different physicochemical and biological detoxification methods with their advantages and disadvantages.

Samples	Methods	Advantages	Disadvantages	Reference
H_2SO_4 pretreated corn stover hydrolysate[1]	Trialkylamine extraction	73.3% of HOAc, 45.7% of HMF, and 100% of furfural are removed	The concentration of hydrolysate and post-washing of pretreated biomass are needed	(Zhu et al., 2011)
Stepwisely liquid hot water and H_2SO_4 pretreated sugarcane bagasse hydrolysate	A sequence of treatments including $Ca(OH)_2$ neutralization, IR-120 resin, activated charcoal, and IRA-67 resin	Inhibitors such as HMF, furfural, HOAc, and formic acid are removed	Various chemicals [$Ca(OH)_2$, resin, and activated charcoal] input with tedious operation procedures increases production baseline cost	(Vallejos et al., 2016)
H_2SO_4 pretreated seaweed hydrolysate	Activated carbon, the overliming method with $Ca(OH)_2$, and the ion exchange method with polyethyleneimine	Activated carbon shows the best performance for HMF removal with simple operation	Ion exchange leads to a significant loss of fermentable sugars; higher energy is demanded to produce activated carbon	(Nguyen et al., 2019)
H_2SO_4 pretreated spruce hydrolysate	NH_4OH, NaOH, and $Ca(OH)_2$ neutralization	It is practical to operate with the mild optimal conditions (pH 9.0/60 °C for NH4OH; from pH 9.0/80 °C to pH 12.0/30 °C for NaOH treatment)	Sugars are partially lost; removal efficiency of phenols is relatively low; HMF and furfural removal highly depends on alkali concentration	(Alriksson et al., 2006)
H_2SO_4 pretreated sugarcane bagasse	Vacuum evaporation followed by liquid-liquid extraction using 1-butanol, isobutyl acetate, or methyl isobutyl ketone	Methyl isobutyl ketone leads to 69.0% of phenolics, 85.4% of acetic acid, and 100.0% of HMF and furfural removal	Vacuum evaporation increases energy input; organic solvents are costly and can be partially dissolved in the hydrolysate	(Roque et al., 2019)
Modeled hydrolysate	Ten nanofiltration and reverse osmosis membranes with low molecular weight cut-off	High rejection performances (97% for sugars and 80% for HMF and vanillin)	The operation process is costly; inhibitors can only be selectively removed; sugars are partially lost	(Nguyen et al., 2015)

[1] Hydrolysate was denoted as liquid fraction after pretreatment and solid-liquid separation.

Table 1.2 Continued.

Simulated hydrolysate	Activated charcoal in a fixed-bed column adsorption system	HMF, furfural, and phenolic compounds can be efficiently removed	The affinity of activated charcoal with sulfuric acid and acetic acid is weak; activated charcoal is costly	(Lee and Park, 2016)
HNO_3 pretreated corncob hydrolysate	Ion exchange resin	70% of nitrate salt, phenolic content, and HMF are removed	Pore diffusion is slow and required high processing time	(Kumar et al., 2018)
H_2SO_4 pretreated corn stover hydrolysate	Laccase treatment	84% of the phenolic compounds is removed	Laccase is costly and cannot be recycled; the treatment is time-consuming	(Fang et al., 2015)
H_2SO_4 pretreated sugarcane bagasse hydrolysate	The isolated bacterium (Bordetella sp. BTIITR) treatment	100% of furfural, 94% of HMF, and 82% of acetic acid are removed	The treatment is time consuming, as incubation took 16 h	(Singh et al., 2017)
Dry H_2SO_4 pretreated corn stover	Co-culture of xylose-utilizing and inhibitor-tolerant *Saccharomyces cerevisiae*	Ethanol yield and concentration are enhanced	Large amounts of sugars are left in the final slurry; complex operation procedures are needed	(Zhu et al., 2016)

Table 1.3 Recycling of black liquor from alkali pretreated biomass hydrolysate for biomass pretreatment.

Biomass	Initial pretreatment conditions	Post-treatment process	Pretreatment effectiveness and findings	Reference
Sugarcane bagasse	Steam explosion under 190 ℃ for 15 min, followed by NaOH (1%, w/v) delignification at 98–100 ℃ for 1 h with 5% solid loading	The black liquor is recycled as a delignification solution with the pH adjusted to 13; the pretreated biomass is washed with water until pH 6	NaOH can be recycled as black liquor but it is vital to keep the pH in the 12.6–13.3 range before each new delignification cycle	(Rocha et al., 2014)
Rice straw	10% solid loading with NaOH (0.5 M, pH=13.9) at 121 °C for 2 h	The black liquor is ultrafiltered by a ceramic membrane; the permeate is recycled as a delignification solution with its pH readjusted to 13.9; the membrane is cleaned at 50 °C with an aqueous NaOH solution of 1% (w/v) for 1 h and then washed with deionized water	Glucose yields from enzymatic hydrolysis at 10% solid loading with citrate buffer ranges from 40–50% as the black liquor is recycled four times	(Li et al., 2015)
Sugarcane bagasse	10% solid loading with NaOH (2%, W/V) at 80 °C for 2 h	The pretreated solid is washed by 600 mL water three times; the black liquor is added 0–1.5% (w/v) of NaOH and recycled for pretreatment	Enzymatic hydrolysis efficiency decreases as the recycling times of black liquor increase	(Wang et al., 2016)
Miscanthus sacchariflorus	Twin-screw extrusion NaOH (0.6 M) pretreatment at 100 °C	The black liquor is recycled for pretreatment; the pretreated biomass is used for enzymatic hydrolysis directly without details	Lignin removal and sugar yields decreases as recycling times increase	(Cha et al., 2016)

Table 1.3 Continued.

Corn stover	10% solid loading with H_2O_2 (7.5%, v/v) solution with pH of 11.58 at 25 °C for 1 h	The pretreated biomass is washed with distilled water until neutral pH; the black liquor is recycled for pretreatment	Lignin and hemicellulose removal and sugar yields decrease as recycling times increase	(Alencar et al., 2017)
Corn stover	3 kg solids with 0.165 kg sodium hydroxide pellets and 29.6 kg of tap water at 80 °C for 2 h	The black liquor is combined with makeup of water and 0.165 kg of sodium hydroxide; the pretreated biomass is washed with 30 kg of fresh water in the paddle reactor for 30min	The accumulation does not lower acetyl and lignin removal during pretreatment, resulting in comparable sugar yields in enzymatic hydrolysis	(Chen et al., 2018)
Cogongrass	10% solid loading with NaOH (2%, W/V) at 85 °C for 90 min	The pretreated biomass is washed with 300 mL water in three stages; the black liquor is diluted by wasted water and replenished with 1% (W/V) NaOH	Ethanol yield decreases from 90.8% (zero recycle) to 66.4% (tenth recycle) at 3% solid loading of enzymatic hydrolysis	(Goshadrou, 2019)
Sugarcane bagasse	10% solid loading with vacuum-assisted NaOH 2% (w/v) pretreatment at 121 °C for 1 h	The pretreated biomass is washed to neutral pH in hot deionized water; the black liquor is supplied by fresh deionized water and adjusted to pH 13.70	Glucose yields are not significantly different between pretreatment with fresh NaOH and recovered black liquor; ethanol yields obtained from the unwashed biomass are significantly higher than those from the washed biomass	(Fan et al., 2020)

Table 1.4 Fed-batch enzymatic hydrolysis or fermentation of the pretreated biomass.

Biomass	Pretreatment and post-conditioning	Enzymatic hydrolysis or fermentation conditions	Results and findings	Reference
Corncob	Two-stage: 1g solid/10 mL 2% H_2SO_4 at 121 °C for 45 min; the pretreated biomass is washed with tap water until neutral and then pretreated with 1g/6 mL 2% NaOH at 80 °C; the pretreated biomass is washed with tap water until neutral again	Simultaneous saccharification and fermentation is conducted with an initial 19% solid loading for a total of 96 h; another 6% is added in 3, 6, and 12 portions during the first 24 h, giving a final solid loading of 25%	Substrate supplementation every 4 h reaches the highest ethanol concentration of 84.7 g/L after 96 h corresponding to a 79% overall ethanol yield	(Zhang et al., 2010)
Prosopis juliflora wood	Delignification with 4% sodium chlorite at 80 °C for 30 min with the pretreated biomass is filtered and washed thoroughly till neutral pH	Enzymatic hydrolysis is performed with an initial solid loading of 5% for around 110 h; the equal amount of initial solids and half of the initial enzyme are added thrice after 24, 56, and 80 h to get a final solid loading of 200 g/L	Fed-batch achieves a higher final sugar concentration (127 g/L) and yield (around 64%) than batch (80.78 g/L and around 40%)	(Gupta et al., 2012)
Empty palm fruit bunch fibers	2 kg biomass with 8 L of 1 M NaOH at 121 °C for 15 min; the pretreated biomass is washed with flowing tap water to remove NaOH	Simultaneous saccharification and fermentation is carried out with an initial 10% solid loading for 95 h, with additional feedings of 50 g at 4, 8, 12, and 16 h, resulting in a final solid of 30%; extra enzyme is added together with extra solid biomass	An economical ethanol concentration (40 g/L) is achieved within 17 h, which further increases up to 62.5 g/L after 95 h with a 70.6% ethanol yield	(Park et al., 2013)

Table 1.4 Continued.

Sugarcane bagasse	Two-stage: steam-explosion at 200 °C for 7 min; after drying, the biomass is pretreated with 1% (w/v) NaOH at 100 °C for 1 h; the pretreated biomass is washed seven times by distilled water	Enzymatic hydrolysis is carried out at an initial of 8% (w/v) for 120 h with 1% solids are fed at 12, 24, or 48 h intervals; the resultant hydrolysates are fermented for 48 h	The fed-batch addition of biomass within 12-h periods leads to higher glucose and ethanol production	(Wanderley et al., 2013)
Sugarcane bagasse	Two-stage: 10% solid loading with 10% (w/v) H_2O_2 for 6 h; the pretreated biomass is washed to the neutral pH and then pretreated by 1% (w/w) NaOH at 80 °C for 1h with 5% solid loading; the pretreated biomass is washed to the neutral pH again	Simultaneous saccharification and fermentation is started with a 10% solid loading for 120 h under different enzyme and solid feeding modes	Ethanol concentrations of above 80 g/L are obtained at a solid loading of 30%; enzyme feeding mode is found to increase ethanol production and reduce enzyme loading	(Zhang and Zhu, 2017)

Table 1.5 Integrating starchy grain flour and pretreated biomass for ethanol co-production.

Pretreatment and integration process	Results and findings	Reference
Corn flour is saccharified by α-amylase and glucoamylase subsequently; corn stover is hydrothermally pretreated at 200 °C for 30 min; simultaneous saccharification and fermentation of corn flour hydrolysate and corn stover mixtures at ratios of 4:12, 8:8, 12:4, and 12:12 and the pretreated corn stover (16%) is used as a control	Ethanol concentration and ethanol yield decreases as the pretreated corn stover ratio increases; the highest ethanol concentration of 68.7 g/L, which is notably higher than 37.9 g/L from the control, is realized at the corn flour hydrolysate and corn stover ratio of 12:12 with the ethanol yield of 86.0%	(Mirfakhar et al., 2020)
Corn stover is pretreated by 2% (w/w) NaOH at 121 °C for 20 min or pretreated by 1% (w/ w) H_2SO_4 loading at 160 °C for 10 min at 10% solid loading and then hydrolyzed at solid loadings of 14–30%; corn flour is liquefied by α-amylase at solid loadings of 28–36%; the corn stover hydrolysate (0–30%) is mixed with the liquefied corn mash (0–30%) at different ratios to reach a final solid loading of 30%	The mixture of NaOH pretreated corn stover hydrolysate (10%) and corn flour (20%) achieves 92.30 g/L ethanol concentration; the mixture of H_2SO_4 pretreated corn stover and corn flour leads to 104.9 g/L ethanol concentration and 80.47% of ethanol yield	(Chen et al., 2018)
Corn stover is hydrothermally pretreated at 200 °C for 30 min or pretreated by 1% (w/v) H_2SO_4 at 140 °C for 60 min or pretreated by 1% (w/v) NaOH at 120 °C for 60 min and then hydrolyzed or fermented at solid loadings of 8 and 16%; corn flour is saccharified or fermented at a solid loading of 30%; the liquid fraction from them is combined with four scenarios	Co-fermentation of corn flour with hydrolysate from the saccharified corn stover boosts ethanol concentrations from 19.9 to 123.2 g/L with biomass loading of 8% and from 36.8 to 130.2 g/L with biomass loading of 16%, respectively,	(Xu et al., 2018)
Corn stover is pretreated by 1% (w/w) H_2SO_4 at 160 °C for 10 min and then hydrolyzed at the solid loadings of 10–30%; corn flour is liquefied by α-amylase; three corn flour to pretreated corn stover ratios (20%:10%, 10%:20%, and 5%:25%) are examined under five different integration flowcharts	Corn flour and corn stover hydrolysate are mixed at ratio of 20% to 10% for liquefaction followed by simultaneous saccharification and fermentation, the highest ethanol concentration is 99.3 g/L	(Yu et al., 2019)

Chapter 2 - Minimizing water consumption for sugar and lignin production via an acid-base integration process

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2.1. Abstract

Excessive post-washing of pretreated biomass leads to water and chemical loss. To address this issue, parallel HOAc and NaOH pretreatments of biomass followed by integration of their biomass and filtrate were investigated. Pretreatment effectiveness including morphology, crystallinity, and component recovery was elucidated. Results showed that HOAc and NaOH in the mixed filtrate were neutralized to achieve a pH of around 4.80 resulting in the alkali lignin precipitation. Lignin (46.01 and 48.38 g/kg-biomass for hemp and poplar, respectively) exhibiting comparable FTIR characteristics with the commercial alkali lignin was recovered. Compared to sodium acetate buffer as a control, integrating HOAc and NaOH pretreated biomass and their mixed filtrate for enzymatic hydrolysis boosted total sugar concentration (hemp: 42.90 vs. 38.27 g/L; poplar: 43.18 vs. 38.76 g/L) without compromising glucose yield (hemp: 70.86 vs. 70.69%; poplar: 66.48 vs. 69.48 %) but improving xylose yield (hemp: 60.10 vs. 35.92%; poplar: 56.90 vs. 29.39 %).

2.2. Introduction

Renewable and sustainable bioproducts obtained from lignocellulosic biomass have attracted considerable attention. To reduce the recalcitrance of biomass for the enzymatic and microbial attacks, tremendous thermochemical pretreatment methods, such as acid, alkali, liquid hot water, steam explosion, organosolv, ionic liquids, and eutectic solvents have been proposed (Elgharbawy et al., 2016; Wang et al., 2021; Zhao et al., 2020a; Zhuang et al., 2016). During pretreatment, the chemical bonds among lignin, cellulose, and hemicellulose can be disrupted, inducing the degradation and solubilization of sugar and lignin into the pretreated liquor (Limayem and Ricke, 2012; Wang et al., 2021; Zhao et al., 2020a). In general, after pretreatment, the slurry is filtered or centrifuged to separate the solid and liquid fractions; solid residues are washed with extensive water and then used for enzymatic hydrolysis with a fresh buffer solution (Ninomiya et al., 2015a; Yu et al., 2019). Whereas liquid fraction, including pretreated liquor and washed water, is often discarded due to its inhibitory compounds and severer pH (Santos et al., 2018; Song et al., 2020). This conventional process inevitably results in large amounts of water and chemicals consumption, especially in the case of acid and alkali pretreatments (Alexander et al., 2020; de Araújo Padilha et al., 2020; Lee and Yu, 2020; Safari et al., 2017; Santos et al., 2020; Sheng et al., 2020; Song et al., 2020; Wang et al., 2019).

In order to utilize the pretreated liquid fraction, various detoxification technologies, such as evaporation, membrane filtration, alkaline and microbial detoxification, ion exchange, and biochar adsorption, have been investigated to render biomass filtrate acquiescent to enzymatic hydrolysis and fermentation (Bhatia et al., 2020; Fayet et al., 2018; Kumar et al., 2018; Pan et al., 2019; Persson et al., 2002; Roque et al., 2019; Zhu et al., 2011). However, their commercial or industrial applications would confront vast challenges. For example, membrane filtration has been proved to separate the liquid fraction's chemical components according to their molecular weights, but high-molecular-weight sugars were retained in the retentate portion, and the liquid fraction containing chemicals and derivatives was discarded finally. In the case of acid and alkali pretreatments, the neutralization reaction for pH adjusting of the solid and liquid fractions using chemical reagents is needed, regardless of ion exchange or biochar adsorption applied for detoxification. For organosolv, ionic liquid, and eutectic solvent pretreatments, recovering these reagents via evaporation or anti-solvent washing of pretreated biomass and filtrate is inevitable before enzymatic hydrolysis (Chen et al., 2018; Ninomiya et al., 2015b; Procentese et al., 2015), which surges the baseline cost for biomass valorization. Therefore, an economical and practical process, which can use the pretreated liquid fraction, is highly preferred.

Acetic acid (HOAc) pretreatment can disintegrate hemicellulose mainly and cellulose partially into furfural and 5-hydroxymethylfurfural (HMF) (de Araújo Padilha et al., 2020; Kim et al., 2013; Wen et al., 2019), while sodium hydroxide (NaOH) pretreatment can degrade partial hemicellulose and lignin (Zhao et al., 2020a). However, liquid fraction from individual HOAc and NaOH pretreatment is unfeasible to be used for enzymatic hydrolysis directly due to its severer pH and high concentrations of inhibitors (furfural and HMF for HOAc; lignin complex for NaOH). If the filtrates from HOAc and NaOH pretreatments are combined, the resultant filtrate is supposed to be neutralized to form sodium acetate, resulting in the mitigation of pH (Wang et al., 2017). In addition, the NaOH soluble lignin can be partially precipitated out due to the pH change, and the degraded sugars were retained in the mixed filtrate. This would apply to their pretreated biomass as well. Therefore, this mixing process is supposed to be self-sufficient without extra water and chemical consumption.

The objective of this chapter is to verify whether this simple and practical integration process is applicable for industrial hemp and poplar biomass to produce lignin and sugar without wastewater generation and chemical detoxification. In the experiments, biophysical techniques including scanning electron microscope (SEM), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy were used to characterize the changes in structural properties of pretreated biomass without post-washing. Pretreatment effectiveness in terms of chemical compositions of solid and filtrate as well as component (glucan, xylan, solid, and lignin) recovery, were evaluated. In addition, integrating HOAc and NaOH pretreated biomass and their mixed filtrate for enzymatic hydrolysis was performed with the sodium acetate buffer used as a control. Collectively, this study provides, for the first time, a practical method to reduce water and chemicals consumption during the biomass valorization process.

2.3. Materials and method

2.3.1. Materials

Industrial hemp (Eletta Campana) biomass was obtained from the John C. Pair Horticultural Center (Haysville, KS) of Kansas State University in 2019, and its stem was remained and dried in an oven at 49 ℃ for 72 h after the branches and leaves were removed (Zhao et al., 2021a). Both hemp and poplar biomass were shredded by an SM 2000 cutting mill (Restsch Inc. Newton, PA) to reach a particle size lower than 2 mm and then stored in Ziploc bags before further use. Cellulase (Cellic® CTec3) and hemicellulose (NS22244) were kindly supplied by Novozymes (Franklinton, NC). Glacial HOAc and NaOH were bought from Thermo Fisher Scientific Chemicals Inc. (Ward Hill, MA). The commercial alkali lignin (370959-100G) was purchased from Sigma-Aldrich chemicals company (St. Louis, MO).

2.3.2. Biomass pretreatment

Five-gram biomass was added into the 75 mL stainless steel reactors (Swagelok, Kansas City Valve & Fitting Co., KS), followed by loading 50 mL HOAc (pH=2.36, around 1 mol/L) or NaOH (pH=12.71, around 0.05 mol/L) to form a solid/liquid ratio of 1/10 (w/v). The selected pHs of HOAc and NaOH reagents were based on the pre-experimental results to reach the pHs of final mixed filtrate and slurry (biomass and filtrate) around 4.80. After vortexing manually, these reactors were immediately submerged into a sandbath (Techne Inc., Princeton, NJ) coupled with continuous air blast at the temperature of 190 ℃ for 40 min. To quench the further reaction, these reactors were instantly cooled down using cold tap water after pretreatment. The pretreated slurry was then separated into a solid and liquid fraction using vacuum filtration with Whatman Grade 5 filter paper. Herein, the solid fraction was oven-dried at 49 ℃ overnight. The reason for oven-drying was to illustrate the pretreatment effectiveness, while for enzymatic hydrolysis HOAc and NaOH pretreated biomass can be mixed and loaded to flask directly. The filtrates obtained from HOAc and NaOH pretreatments were blended and kept the neutralization reaction for 20 min. After that, the precipitated lignin was filtered from the mixed filtrate and dried at 49 °C overnight for its recovery. Before and after mixing, the pH value of the filtrate was measured by a pH700 meter (Cole-Parmer, Vernon Hills, IL).

2.3.3. Structural characterization

Microstructural characteristics of raw and pretreated biomass were captured using the S-3500 SEM (Hitachinaka, Ibaraki, Japan). Concisely, biomass was mounted on conductive adhesive tapes and sprayed with a 4 nm thick metal film coat (40% palladium and 60% gold) before observation and image capture. Changes in chemical bonds of biomass before and after pretreatment were recognized using a 400 FTIR spectrophotometer (PerkinElmer Corp., Shelton, CT) armed with an RT-DLaTGS detector. Determination parameters including wavenumber range of 4000-400 cm^{-1}, 4 cm^{-1} resolution, 68-72 pressure intensity, and 32 scans per sample were employed. Besides, the areas of absorption of bands at 1437 and 898 cm^{-1} were measured for cellulose crystallinity index based on Eq. 2.1. XRD was also employed to reveal the change in the crystallinity of biomass using a Siemens D-5000 diffractometer (Bruker, Ettlingen, Germany) with Cu-K radiation generated at 40 kV and 40 mA. The diffraction degrees ranged from 5 to 40° (2θ) with a step rate of 0.1°/second. The crystallinity index (CrI) was calculated as shown in Eq. 2.2.

$$\mathrm{LOI} = \mathrm{A}_{1437}/\mathrm{A}_{898} \quad (2.1)$$

$$\mathrm{CrI} = (\mathrm{I}_{002} - \mathrm{I}_{\mathrm{am}})/\mathrm{I}_{002} \quad (2.2)$$

Where LOI is lateral order index and A is the absorbance value of the corresponding band; I_{002} is the maximum peak intensity of lattice diffraction angle (002) around 22°-23° (2θ) considered as cellulose crystalline I while I_{am} is the amorphous contribution at around 18°-19° (2θ).

2.3.4. Enzymatic hydrolysis

After pretreatment, the HOAc and NaOH pretreated biomass was mixed and then combined with their mixed filtrate for enzymatic hydrolysis with the sodium acetate buffer (50 mM, pH 4.8) as a control. When the biomass with filtrate or buffer was loaded in a 50 mL Erlenmeyer flask, the slurry pH was adjusted to 4.80 using 10 N HCl or 10 M NaOH. Here, it was found that the pH of slurry containing mixed biomass and filtrate was around 4.5. Enzymatic hydrolysis was initiated by pipetting 100 µL Cellic® CTec3/g biomass and 50 µL NS22244/g biomass (Zhao et al., 2021a). These flasks were incubated in an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) at 49 °C at 160 rpm for 72 h. After enzymatic hydrolysis, the slurry was subjected to centrifugation, and the supernatant of hydrolysate was subjected to glucose and xylose measurement. Sugar yields were evaluated as the percentage of monosaccharides in the hydrolysate compared with the amount of initial sugars in biomass, as shown in Eq. 2.3 and 2.4.

$$E_{(g)} = \frac{C_{(glucose)} \times V_{(mL)} \div 1000}{M_{(b)} \times C_{(glucan)} \div 0.9} \times 100\% \; (2.3)$$

$$E_{(x)} = \frac{C_{(xylose)} \times V_{(mL)} \div 1000}{M_{(b)} \times C_{(xylan)} \div 0.88} \times 100\% \; (2.4)$$

Where $E_{(g)}$ and $E_{(x)}$ are glucan-to-glucose and xylan-to-xylose yields, respectively; $C_{(glucose)}$ and $C_{(xylose)}$ are glucose and xylose concentrations (g/L) in the hydrolysate, respectively; $V_{(mL)}$ is the volume of hydrolysate; $C_{(glucan)}$ and $C_{(xylan)}$ are glucan and xylan contents in the biomass, respectively; 0.9 and 0.88 are the transformation factor of glucan-to-glucose and xylan-to-xylose, respectively.

2.3.5. Analytical procedures

Chemical compositions of raw and pretreated biomass and oligosaccharides in the filtrate were measured as the procedures from the National Renewable Energy Laboratory (Sluiter et al., 2008). In the case of the chemical composition of biomass, the extracted or pretreated biomass (~0.3000 g) was first degraded with 3 mL of 72% sulfuric acid in a 30 °C water bath for 1 h. The slurry was then diluted to a 4% acid concentration by adding 84 mL deionized water and autoclaved at 121 °C for 1 h. It was filtered by a pre-weighed filter crucible when cooling down to room temperature. 1.5 mL filtrate was pipetted for the acid-soluble lignin test, and 10 mL filtrate neutralized with 0.40 g calcium carbonate for 40 min was used for the sugar test. The solid residue in the crucible was then washed with 50 mL distilled water and dried overnight at 105 °C for lignin recovery. After that, the crucible was shifted into the Muffle furnace at 575 °C for ash recovery. For oligosaccharides in the filtrate, 10 mL of filtrate was loaded in a glass bottle, followed by adding 348 μL of 72% sulfuric acid to form a 4% acid concentration. Then, the filtrate was autoclaved at 121 °C for 1 h. Glucose, xylose, furfural, and HMF concentrations were determined by a 1260 high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA) under the following conditions: The separation column was an HPX-87H organic acid column (7.8×300 mm), and set at 60 °C; the mobile phase was 0.005 M H_2SO_4 buffer with a flow rate of 0.6 mL/min and the refractive index detector temperature was 45 °C (Zhao et al., 2020b).

2.3.6. Statistical analysis

Data were collected and calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA). The figures were created by Origin 2018 (OriginLab, Northampton, USA). The flowchart was drawn by SuperPro Designer software (Version 9, Intelligen Inc., Scotch Plains, NJ).

2.4. Results and discussion

2.4.1. Chemical composition of raw and pretreated biomass

Changes in principal components of biomass before and after pretreatment are shown in Table 2.1. In comparison to raw biomass, glucan content in the pretreated biomass (hemp: 47.11-

57.42%; poplar: 53.21-57.21%) increased, xylan content (hemp: 7.14-8.18%; poplar: 5.85-8.71%) reduced, lignin content enhanced in HOAc (32.37-34.65%) pretreated biomass but reduced in NaOH (8.35-9.29%) pretreated biomass (Table 2.1). Tracking compositional changes in the biomass often disclose differences in pretreatment mechanism (Zhao et al., 2020b). An increase in glucan content after pretreatment is ascribed to the solubilization of hemicellulose and extractives for HOAc pretreatment and lignin removal for NaOH pretreatment. Because hemicellulose is more susceptible to hydrogen ions, HOAc pretreated biomass had lesser xylan than those of NaOH pretreated biomass. The notable variation in lignin is ascribed to that acid shows limited solubility for unsealed lignin units, but alkali can dissolve the lignin matrix into the filtrate (Kim et al., 2016). It was also noticed that HOAc pretreated hemp and poplar had higher glucan contents than those of NaOH pretreated biomass. In contrast, Zhao et al. (2020a) reported that NaOH pretreated hemp with post-washing showed higher glucan content than sulfuric acid pretreated hemp under the same pretreatment conditions. The inconsistent would be attributed to no post-washing process which induced recondensation of degraded sugars, extractives, and lignin derivatives. It was particularly reflected by the finding that NaOH pretreated biomass contained higher other components than raw and HOAc pretreated biomass (Table 2.1). Herein, other constituents are assumed to be the degraded sugars, residual chemicals, and minerals. In other studies, de Araújo Padilha et al. (2020) reported that HOAc (60%, v/v) pretreatment (at 121 °C for 60 min) increased glucan content of biomass from 34.7 to 57.2% and decreased hemicellulose content from 17.6 to 15.5% via cloth filtration and eight-time tap water washing. Besides, Wang et al. (2019) found that HOAc (11 g/L) pretreatment (at 190 °C for 20 min) increased the glucan and lignin contents of biomass from 35.6 to 44.5% and 22.6 to 30.3%, respectively, but decreased hemicellulose content from 19.2 to 15.6% using vacuum filtration and fifteen-time tap water washing. However, there are rarely available studies conducting biomass pretreatment without post-washing.

2.4.2. Sugar, solid, and lignin recovery based on raw biomass

Pretreatment effectiveness in terms of sugar, solid, and lignin recovery of pretreated biomass without post-washing are exhibited in Figure 2.1. Glucan recovery of 92.22-100.49 g/100g was notably higher than xylan recovery of 22.30-38.75g/100g with NaOH pretreatment had higher xylan recovery than HOAc pretreatment (34.85-38.75g/100g vs.22.30-30.26g/100g) (Figure 2.1A). The phenomenon that several glucan recoveries higher than 100g/100g is possibly caused by the adsorption of oligomeric and monomeric glucose on the biomass surface during filtration and the overestimated solid recovery attributed to the residual chemicals. It was found that NaOH pretreatment offered slightly higher solid recovery (hemp: 78.51 g/100g; poplar: 74.47 g/100g) than HOAc (hemp: 70.21 g/100g; poplar: 71.06 g/100g) pretreatment (Figure 2.1B). It has been reported that derivatives (such as 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol) from cellulose and hemicellulose can be recondensed on the surface of pretreated biomass to form pseudo-lignin through polymerization (Hu et al., 2012; Kumar et al., 2013; Meng and Ragauskas, 2017; Zhao et al., 2021a). This was in accordance with the finding that HOAc pretreatment induced pseudo-lignin formation as reflected by lignin recoveries higher than 100 g/100g (Figure 2.1B). In the case of NaOH pretreatment, low lignin recovery (hemp: 41.01 g/100g; poplar: 29.86 g/100g) was reached (Figure 2.1B). No post-washing of the pretreated biomass could be responsible for the overestimated lignin recovery. In contrast, Wang et al. (2019) reported about 60 g/100g of solid, 75 g/100g of glucan, and 20 g/100g of xylan recoveries using HOAc (11 g/L) pretreatment (at 170 ℃ for 20 min) of switchgrass with five-times tap water (300 mL) washing. In addition, Zhao et al. (2020a) found that 1% alkali pretreatment (at 190 ℃ for 30 min) of hemp (Eletta Campana) biomass resulted in 46.5 g/100g of solid, 77.5 g/100g of glucan, and 24.5 g/100g of xylan and 32.1 g/100g of lignin recoveries after 4.5-fold water washing.

2.4.3. Structural characterization of raw and pretreated biomass

It might be the first study to report the effects of pretreatment on structural characteristics of pretreated biomass without post-washing (Figure 2.2). SEM was used for the morphological analysis, namely surface texture change, in the biomass from different experimental conditions. It was found that raw hemp and poplar biomass had highly impenetrable, inflexible, and intact fascicular structures (Figure 2.2). This indicates that the lignin complex is recalcitrant to enzymatic attack (Morais et al., 2016). After HOAc and NaOH pretreatments, biomass samples showed an increased surface roughness with some visible shallow gullies, small fibers, deep trenches, and irregular microporous protuberances formed (Figure 2.2). It should be highlighted that the small disrupted fibers (lignin units) were observed on the surface of HOAc pretreated biomass. Besides, hemp biomass exhibited a slight variation in morphological change regarding NaOH pretreatment compared to poplar biomass: the rough structures were presented on the external surface of NaOH pretreated hemp, while it was more smooth for NaOH pretreated poplar (Figure 2.2).

Quantitative determination of biomass crystallinity has been facing a huge technical challenge as biomass contains a spectrum of complex amorphous compounds, principally hemicellulose, and lignin, in addition to crystalline cellulose. Morais et al. (2016) concluded that XRD and FTIR methods can offer essential complementary data for revealing the transformation of biomass crystallinity. Therefore, XRD and FTIR determination of raw and pretreated biomass without post-washing were conducted (Figure 2.3). Strikingly, compared to raw biomass, HOAc and NaOH pretreatment promoted the increases in CrI of hemp from 52.38 to 70.48% and of poplar from 45.64 to 66.86%, which was established by the increasing intensity of I_{002} and the decreasing intensity of I_{am} (Table 2.2). Therefore, it leads to the conclusion that HOAc and NaOH pretreatments can enhance biomass crystallinity by removing hemicellulose and lignin, respectively (Zhao et al., 2020b). Besides, it was found that HOAc pretreated biomass showed higher CrI values than NaOH pretreated biomass, which was associated with the glucan content of their pretreated biomass (Table 2.1). Different from XRD, FTIR delivers information about the crystallinity of cellulose enclosed in the cellulose-rich material (Morais et al., 2016). The absorption band at 1437 cm^{-1} allocated to the CH_2 stretching is an intense signal categorized as cellulose I, while the absorption band at 898 cm^{-1} allocated to C−O−C stretching at β-(1,4)-glycosidic linkage is considered as cellulose II and tangled amorphous cellulose (Nelson and O’Connor, 1964). The LOI values of HOAc (hemp: 0.98; poplar: 0.82) and NaOH (hemp: 1.02; poplar: 0.96) pretreated biomass were higher than those of (0.77) raw biomass. However, LOI values of NaOH pretreated biomass were higher than those of HOAc pretreated biomass (Table 2.2).

FTIR spectra analysis in the wavenumber range of 400-4000 cm^{-1} was also performed to discern the fluctuations of chemical bonds in raw and HOAc and NaOH pretreated biomass (Figure 2.3B). It was observed that the intensities of cellulose-related peaks at 3329 cm^{-1} (1, O-H stretching of cellulose), 1091 cm^{-1} (6, C-O vibration of crystalline cellulose), 1051 cm^{-1} (7, C-O stretching of cellulose), and 1023 cm^{-1} (8, C-O-C pyranose ring skeletal vibration of cellulose) in HOAc and NaOH pretreated hemp and poplar biomass were more intense than raw biomass.

This is ascribed to the increasing glucan content in pretreated biomass (Table 2.1). While the intensities of lignin-related peaks at 1514 cm^{-1} (4, aromatic skeleton from lignin) and 1319 cm^{-1} [5, syringyl (S) and guaiacyl (G) lignin units] somewhat amplified in HOAc pretreated biomass but diminished in NaOH pretreated biomass (Figure 2.3B), which was in agreement with the change of lignin content in HOAc and NaOH pretreated biomass (Table 2.1).

2.4.4. pH and composition of filtrate before and after the mixing process

Table 2.3 shows the pH and composition of filtrate before and after the mixing process. Compared to the initial pHs of HOAc and NaOH reagents, the pHs of the pretreated filtrates were alleviated slightly due to the dilution by biomass, but they were still too harsher (HOAc: pH < 3; NaOH: pH > 12) to be used as a buffer solution. This result indicates that individual acid and alkali pretreated filtrates need extra chemical reagents for pH mitigation to meet the requirement of enzymes and microbes. Thus, the pretreated filtrate is generally discarded. The pH (2.87) of HOAc pretreated hemp filtrate was slightly higher than that (2.51) of HOAc pretreated poplar filtrate, which could be attributed to poplar having more acetic groups than hemp (indirectly revealed by xylan content). Besides, HOAc pretreated filtrate was transparent with an orange color, whereas NaOH pretreated filtrate was opaque and black. After mixing HOAc and NaOH pretreated filtrate, the pHs of the mixed filtrates reached around 4.5-4.8, indicating the neutralization reaction between the residual HOAc and NaOH in the filtrate occurred. The black NaOH pretreated filtrate became transparent due to the precipitation of lignin resulting in the formation of a brown filtrate.

Oligomeric (hemp: 0.92 g/L; poplar: 0.58 g/L) and monomeric (hemp: 1.26 g/L; poplar: 1.86 g/L) glucose concentrations in HOAc pretreated filtrate were higher than those (oligomeric:

0.69 g/L for hemp and 0.26 g/L for poplar; monomeric: 0.00 g/L for hemp and 0.49 g/L for poplar) of NaOH pretreated filtrate. High glucose concentrations in pretreated filtrate increased the degradation risk, evidenced by HMF formation (1.18 g/L for hemp and 0.69 g/L for poplar) in HOAc pretreated filtrate. Oligomeric xylose concentrations (3.67 g/L for hemp and 1.79 g/L for poplar) in HOAc pretreated filtrate were also higher than those (0.75 g/L for hemp and 0.82 g/L for poplar) of NaOH pretreated filtrate. Monomeric xylose concentrations (4.19 g/L for hemp and 9.27 g/L for poplar) in HOAc pretreated filtrate were also higher than those (0.75 g/L for hemp and 0.00 g/L for poplar) of NaOH pretreated filtrate. Large amounts of xylan decomposition during HOAc pretreatment induced furfural formation (3.08 g/L for hemp and 3.55 g/L for poplar). However, neither HMF nor furfural was undetectable in NaOH pretreated filtrate. In the case of HOAc pretreatment, initial HOAc and internal-released acids (such as HOAc and formic acid) accelerated sugar degradation. Whereas for NaOH pretreatment, the neutralization reaction between NaOH and released weak acids protected sugar from excessive decomposition (Zhao et al., 2020a, 2020b, 2020c). It was also found that xylose concentration in HOAc pretreated filtrate was higher than glucose because the former was more vulnerable to acid. However, this was inconsistent with the previous study, where Wang et al. (2019) reported higher oligomeric and monomeric glucose in the HOAc pretreated filtrate than those of xylose. After mixing HOAc and NaOH pretreated filtrate, the concentrations of inhibitors were alleviated compared to the HOAc pretreated filtrate: (1) HMF (hemp: 0.56 g/L; poplar: 0.39 g/L) and furfural (hemp: 1.31 g/L; poplar: 1.58 g/L) concentrations were diluted (Table 3); and (2) the solubilized lignin in the pretreated filtrate were precipitated out. Besides, total sugar of 6.95 g/L for hemp and 8.72 g/L for poplar remained in the mixed filtrate. Studies have reported that high concentrations of HMF and furfural had negative effects on enzymatic and microbial activities

(Baral and Shah, 2014; Fayet et al., 2018; Pan et al., 2019), and the lignin units can limit enzymatic functions through hydrophobic interaction (Li et al., 2018; Li and Zheng, 2017). In this work, a simple mixing process not only attenuated the inhibitory compounds but also offered the possibility of the mixed filtrate as a buffer directly for enzymatic hydrolysis of pretreated biomass.

2.4.5. Lignin recovery and FTIR characterization

Tremendous efforts have been devoted to enhancing the accessibility of biomass to enzymes and microorganisms, while lignin has rarely been recovered and characterized qualitatively. It is believed that the conversion of biomass-based lignin to polymeric materials offers potential values for biomass upgrades (Huang et al., 2020; Upton and Kasko, 2016). Lignin recovery based on raw biomass was calculated, and its FTIR characteristics were compared with commercial alkali lignin used as a control (Figure 2.3C and D). It was noticed that 46.01 g/kg and 48.38 g/kg lignin was recovered for hemp and poplar, respectively. The intensities of peaks at 2940 cm^{-1} [C-H symmetrical vibration of the methylene ($-CH_2$) group], 1592 cm^{-1} (the vibration of aromatic rings), 1506 cm^{-1} (the vibration of aromatic rings), 1461 cm^{-1} (the methoxyl C-H bending and C-C stretching in the aromatic skeleton), and 1420 cm^{-1} (the vibration of aromatic rings) of the recovered hemp and poplar lignin were equivalent to the commercial alkali lignin. This indicates that mixing HOAc and NaOH pretreated filtrate effectively maintained the integrity of solubilized lignin. Besides, the absorption peaks at 1159 cm^{-1} [p-hydroxy phenylpropane (H) unit], 1120 cm^{-1} (S unit), and 830 cm^{-1} (G unit) of typical HGS-lignin also retained consistent with the commercial alkali lignin. These results indicate that the lignin recovered from hemp and poplar biomass via the mixing process had almost comparable chemical structures with the commercial alkali lignin.

2.4.6. Sugar concentration and yield from enzymatic hydrolysis

The potential of mixed filtrate as a buffer for enzymatic hydrolysis of their pretreated biomass simultaneously was investigated with the sodium acetate buffer (50 mM, pH=4.8) used as a control. Sugar concentrations in the final hydrolysate and yields based on raw biomass after 72-h enzymatic hydrolysis are shown in Figure 2.4. There was no significant variation in glucose concentration between hemp-control (31.51 g/L) and hemp-mixed (31.59 g/L), while glucose concentration from poplar-mixed (31.13 g/L) was slightly lower than that (32.54 g/L) from control. However, xylose concentration presented the identical trend: 11.31 g/L for hemp-mixed and 12.05 g/L for poplar-mixed were significantly higher than 6.76 g/L for hemp-control and 6.23 g/L for poplar-control. Correspondingly, glucose yield (70.86%) from hemp-mixed showed no notable difference with that (70.69%) from hemp-control, whereas glucose yield (66.48%) from poplar-mixed was lower than that (69.48%) from poplar-control. Xylose yields from hemp-mixed (60.10%) and poplar-mixed (56.90) were almost two-fold higher than those from hemp-control (35.92%) and poplar-control (29.39%). Therefore, total sugar concentrations obtained from the mixed filtrate (hemp: 42.90 g/L; poplar: 43.18 g/L) were remarkably higher than those (hemp: 38.27 g/L; poplar: 38.76 g/L) from control. This indicates that integrating HOAc and NaOH pretreated biomass and their mixed filtrate for enzymatic hydrolysis could boost sugar concentration without compromising glucose yield but enhancing xylose yield. Besides, it can be observed that residual HMF and furfural in the mixed filtrate were not detrimental to biomass hydrolases, but if the final hydrolysate is supposed to be used for biofuel production via microbial digestion, extracting the high-value HMF and furfural from the mixed filtrate using methyl isobutyl ketone is recommended. On the other hand, if the final hydrolysate is used for other chemical production (such as furan) in situ through thermochemical conversion, it could be not needed to consider the effects of inhibitors. Overall, this practical integration process offers an alternative method to reduce water consumption and chemical loss during biomass valorization.

2.6. Conclusions

The potential of integrating HOAc and NaOH pretreated biomass and their mixed filtrates for enzymatic hydrolysis without post-washing was investigated. First, the residual HOAc and NaOH in the mixed filtrate formed sodium acetate buffer with a pH of ~4.80, promoting the precipitation of the solubilized lignin which showed comparable FTIR characteristics with the commercial alkali lignin. The utilization of degraded sugar in the mixed filtrate enhanced the total sugar concentration in the final hydrolysate without conceding glucose yield but boosting xylose yield. This practical mixing process not only reduced water and chemicals consumption but also improved sugar concentration.

Table 2.1 Chemical composition of raw and pretreated biomass without post-washing.[1]

Sample	Glucan (%)	Xylan (%)	Lignin (%)	Others[2] (%)
Hemp-Raw	40.12 ± 0.12	16.56 ± 0.02	17.79 ± 0.04	25.53 ± 1.32
Hemp-HOAc	57.42 ± 1.16	7.14 ± 0.51	32.37 ± 0.42	3.07 ± 1.02
Hemp-NaOH	47.11 ± 2.48	8.18 ± 0.79	9.29 ± 0.36	35.41 ± 1.81
Poplar-Raw	42.15 ± 0.36	18.64 ± 0.14	21.29 ± 0.41	17.92 ± 1.17
Poplar-HOAc	57.21 ± 3.53	5.85 ± 0.80	34.65 ± 0.90	2.29 ± 2.74
Poplar-NaOH	53.21 ± 2.85	8.71 ± 0.84	8.53 ± 0.81	29.55 ± 1.31

[1] Solid composition was on a dry basis. Data: means ± standard deviations. [2] Others included residual chemical reagents, degraded sugars, extractives, and mineral substances.

Table 2.2 The crystallinity index of raw and pretreated biomass measured by XRD and FTIR.

Sample	XRD[1]			FTIR[2]		
	I_{002}	I_{am}	CrI[3](%)	A_{1437}	A_{898}	LOI[4]
Hemp-raw	2505	1193	52.38	2.69	3.49	0.77
Hemp-HOAc	3276	967	70.48	3.29	3.38	0.98
Hemp-NaOH	2960	962	67.50	2.64	2.59	1.02
Poplar-raw	2272	1235	45.64	2.05	2.66	0.77
Poplar-HOAc	3416	1132	66.86	4.52	5.53	0.82
Poplar-NaOH	2881	1020	64.60	2.23	2.33	0.96

[1] XRD: X-ray diffraction; [2] FTIR: Fourier transform infrared spectroscopy; [3] CrI: crystallinity index; [4] LOI: lateral order index.

Table 2.3 pH and composition (g/L) of HOAc and NaOH pretreated filtrate and their mixed filtrate.

Sample	pH	Oligo-glucose[1]	Mono-glucose[2]	HMF[3]	Oligo-xylose[1]	Mono-xylose[2]	Furfural
Hemp-HOAc	2.87	0.92	1.26	1.18	3.67	4.19	3.08
Hemp-NaOH	12.32	0.69	0.00	0.00	0.75	0.75	0.00
Hemp-Mixed	4.66	0.29	0.99	0.56	3.13	2.53	1.31
Poplar-HOAc	2.51	0.58	1.86	0.69	1.79	9.27	3.55
Poplar-NaOH	12.31	0.26	0.49	0.00	0.82	0.00	0.00
Poplar-Mixed	4.78	0.22	1.21	0.39	2.44	4.85	1.58

[1] Oligo: oligomeric; [2] Mono: monomeric; [3] HMF: 5-hydroxymethylfurfural.

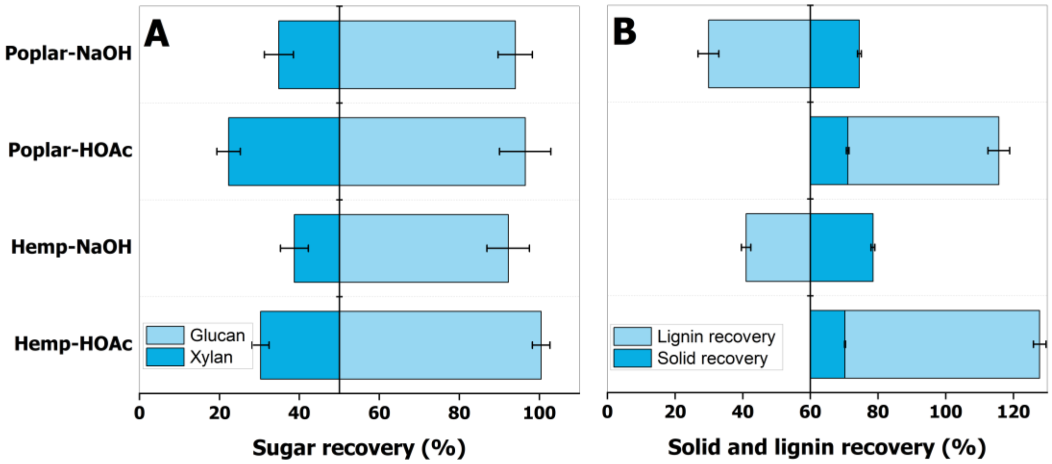

Figure 2.1 Glucan, xylan, solid, and lignin recoveries (g of component/100 g of component in the raw biomass) after pretreatment.

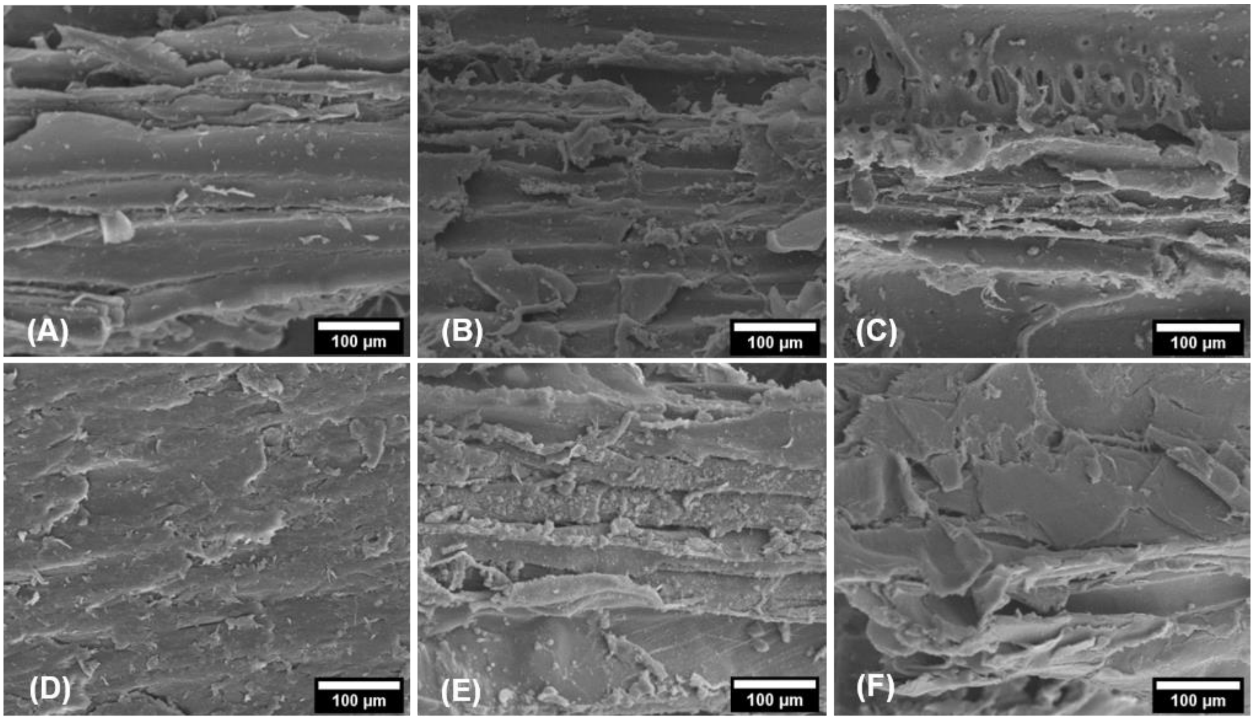

Figure 2.2 SEM images of raw and pretreated biomass without post-washing (A: hemp raw biomass; B: HOAc pretreated hemp biomass; C: NaOH pretreated hemp biomass; D: poplar raw biomass; E: HOAc pretreated poplar biomass; and F: NaOH pretreated poplar biomass).

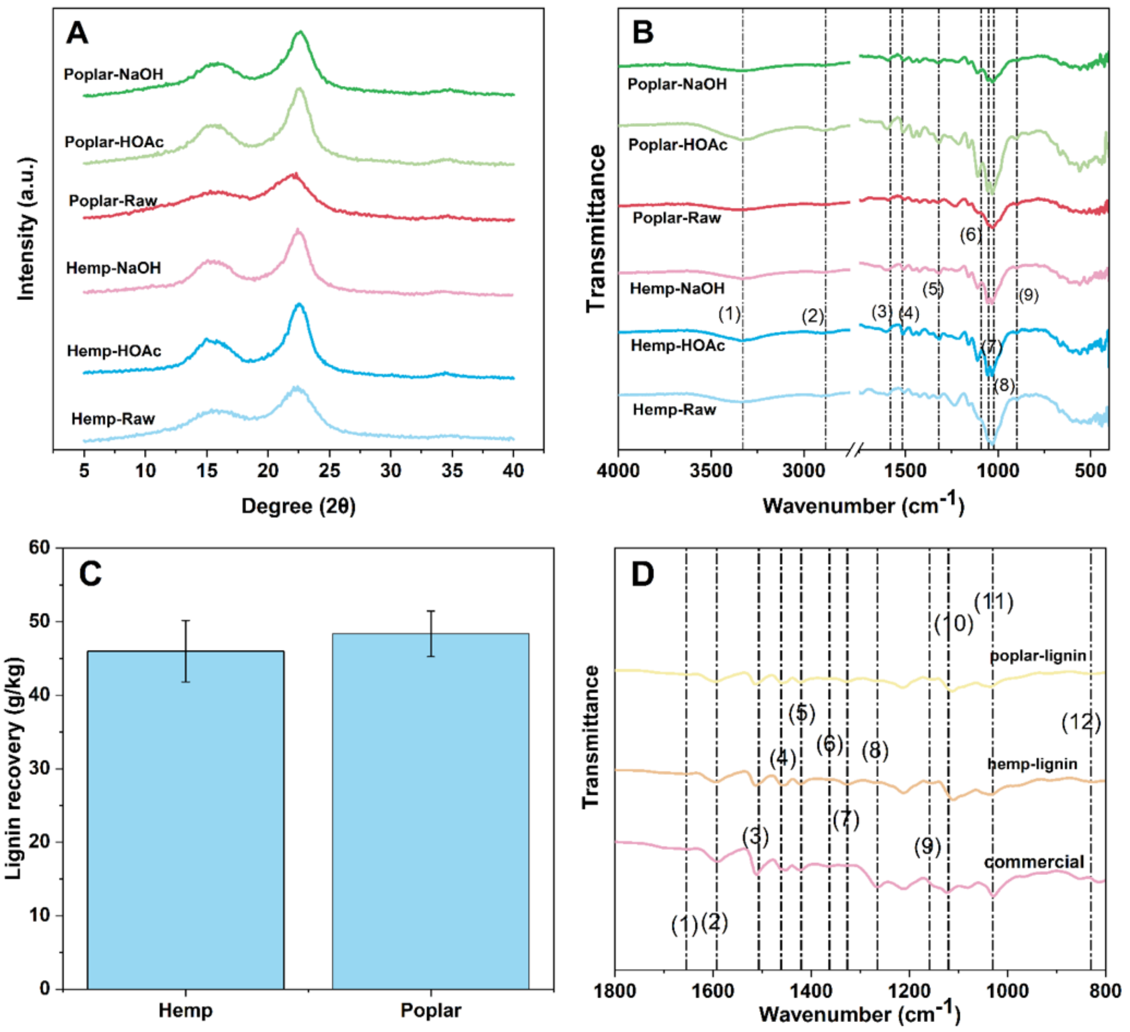

Figure 2.3 XRD (A) and FTIR (B) characterization of raw and pretreated biomass as well as lignin recovery based on raw biomass (C) and its FTIR characteristics (D) with the commercial alkali lignin used as a control.

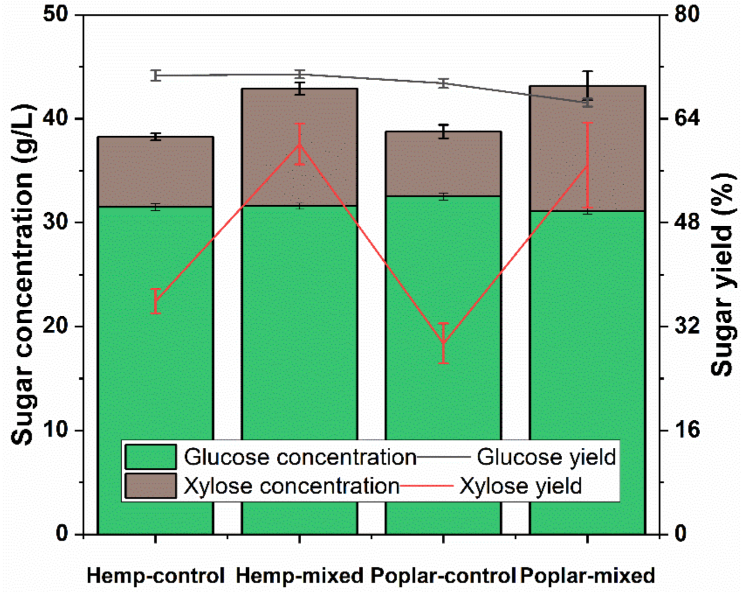

Figure 2.4 Sugar concentration (column) and yield (line) from enzymatic hydrolysis of integrating HOAc and NaOH pretreated biomass and their mixed filtrate with the sodium acetate buffer used as a control.

Chapter 3 - Insights into water washing impact on pretreatment and relationship between solid loading and sugar concentration

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3.1. Abstract

Effects of post-washing [one-volume water (I-VW) or double-volume water (II-VW)] on pretreated hemp and poplar biomass and enzymatic hydrolysis of the mixture of HOAc and NaOH pretreated biomass and their mixed filtrate were investigated. Compared to I-VW, II-VW increased 3.76-6.80% of glucan content in NaOH pretreated biomass, diminished lignin recondensation, and heightened cellulose-related FTIR peak intensities, crystallinity index, and lignin removal. The pH of the mixed filtrate was around 4.80, precipitating the NaOH soluble lignin partially. Although II-VW showed lower lignin recoveries than I-VW, their FTIR characteristics were equivalent to the commercial alkali lignin. Enzymatic hydrolysis at solid loadings of 2.5-10% (w/v) demonstrated that I-VW and II-VW had marginal variations in sugar concentration and conversion efficiency, indicating that I-VW is sufficient for post-washing pretreated biomass. Glucose concentration exhibited a quadratic correlation with solid loading and hemp biomass reached the maximum glucose (43.88 g/L) and total sugar (57.08 g/L) concentrations with I-VW.

3.2. Introduction

Physicochemical pretreatment is a crucial stage for biomass-to-bioenergy production to render cellulose and hemicellulose more accessible to enzymatic and microbial digestion. Dilute acid and alkali pretreatments have been highly preferred due to the low cost of chemical reagents and simple operating procedures (Kim et al., 2016; Solarte-Toro et al., 2019). However, extreme post-washing is typically applied to alleviate the pH and toxicity of pretreated biomass with the resulting filtrate being discarded finally (Li et al., 2016; Skiba et al., 2017; Sritrakul et al., 2017; Wen et al., 2019), which inevitably results in massive water consumption and chemical (such as acid, alkali, sugars, and lignin) loss. Therefore, it will increase the cost of production and the problem of environmental pollution.

For acid pretreatment, physical and chemical treatments such as membrane filtration, ion exchange, and biochar adsorption were used to make the pretreated filtrate or pre-hydrolysate amenable to microbial digestion (Fayet et al., 2018; Kumar et al., 2020; Lee and Park, 2016; Pan et al., 2019; Roque et al., 2019; Sarawan et al., 2019; Zhu et al., 2015). However, there are several huge challenges for the commercialization: (1) selectively separating low-molecular-weight derivatives [such as acetic acid (HOAc), furfural, and 5-hydroxymethylfurfural (HMF)] from high-molecular-weight sugars via membrane filtration is inefficient to conduct (Pan et al., 2019); (2) extra chemicals input is unavoidable for ion exchange and biochar adsorption (Bhatia et al., 2020; Kim, 2018; Kumar et al., 2020); and (3) concentrated alkali is required to neutralize the residual acids in the filtrate regardless of which strategies are applied. For alkali pretreatment, sequential recycling of black liquor for biomass pretreatment has been encouraged (Cha et al., 2016; Goshadrou, 2019; Li et al., 2015; Wang et al., 2016; Zhou et al., 2019). Seemingly, it has a great potential to reduce water consumption and chemical discharge, but the accumulation of inhibitors and dilution of alkali concentration in recycled black liquor can increase the difficulty of detoxification and reduce the pretreatment effectiveness. This has been evidenced by extreme post-washing by water and a progressive reduction in sugar yield (Rocha et al., 2014; Zhou et al., 2019). Also, the resulting black liquor was commonly abandoned (Alencar et al., 2017; Cha et al., 2016). In this regard, our previous study has demonstrated that directly hydrolyzing the mixture of HOAc and NaOH pretreated filtrate and biomass without post-washing of the pretreated biomass can significantly reduce water consumption. Additionally, the NaOH soluble lignin exhibiting the nearly identical features with the commercial alkali lignin can be recovered (Zhao et al., 2021a).

Solid loading is a critical factor affecting sugar conversion efficiency of enzymatic hydrolysis of pretreated biomass (Modenbach and Nokes, 2013). Low solid loading has been generally applied to promote pretreatment technology with higher sugar yield. However, maintaining high-solid enzymatic hydrolysis is highly preferred to reduce water and enzyme consumption (Chen and Liu, 2017). Whether the higher solid loading used for enzymatic hydrolysis is better? A previous study reported that glucose yield had a negatively linear correlation with solid loading (Kristensen et al., 2009). Therefore, the decrease in glucose yield can offset the advantages of enzymatic hydrolysis conducted at high solid loading. Exploring the turning point of solid loading can not only avoid a random choice of solid loading used for enzymatic hydrolysis but also reduce extra enzymes and chemicals input.

In this chapter, hemp and poplar biomass were first pretreated by HOAc and sodium hydroxide (NaOH) parallelly and washed by one-volume water (I-VW) or two-volume water (II-VW). After solid and liquid separation, their pretreated biomass and filtrate were combined, respectively. The mixing process is able to promote the neutralization reaction of residual HOAc and NaOH in the pretreated biomass and filtrate to form sodium acetate (Zhao et al., 2021a). The mixed filtrate was filtered to recover the NaOH soluble lignin partially and then used as a buffer for enzymatic hydrolysis of the mixed biomass. Effects of post-washing on the pretreated biomass were illuminated by scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR) in terms of microstructural characteristics, crystallinity, and chemical linkage, respectively. Compositional analysis of solid and filtrate as well as solid and sugar recoveries and lignin removal with different post-washing were offered. The recovered lignin was compared with the commercial alkali lignin. Moreover, the relationships between sugar concentration/conversion efficiency and solid loading were determined and can be used to boost sugar concentration.

3.3. Materials and method

3.3.1. Materials

Industrial hemp and poplar biomass were initially ground by an SM 2000 cutting mill (Restsch Inc. Newton, PA) and then pulverized to achieve particle sizes approximately lower than 2 mm (Zhao et al., 2021a). Cellulase (Cellic® CTec3, 516 mg protein/mL) and hemicellulose (NS22244, 266 mg protein/mL) were supplied by Novozymes (Franklinton, NC). The commercial alkali lignin (370959-100G) was bought from Sigma-Aldrich chemicals company (St. Louis, MO).

3.3.2. Biomass pretreatment

The pretreatment procedures and conditions can be found in our previous study (Zhao et al., 2021a). HOAc（pH = 2.36, around 1 mol/L) and NaOH (pH = 12.71, around 0.05 mol/L) reagents were applied for biomass pretreatment with a solid/solvent ratio of 5 g/50 mL at 190 ℃ for 40 min (Zhao et al., 2021a). After pretreatment, the reactors were quenched to room temperature using cold tap water. Herein, the vacuum filtration with Whatman Grade 5 filter paper was applied for solid and liquid separation of the pretreated slurry. The solid fraction was washed by I-VW or II-VW based on an initial pretreatment liquid volume and then dried at 49 ℃. The filtrates from HOAc and NaOH pretreatments were merged for 20 min to precipitate the NaOH soluble lignin and then subjected to vacuum filtration for lignin recovery. Before and after the mixing process, the pH values of filtrates were examined by an A211 Benchtop pH Meter.

3.3.3. Structural characterization

S-3500 SEM (Hitachinaka, Ibaraki, Japan) was used to detect the microstructural properties of raw and pretreated biomass. In brief, biomass was sequentially mounted and coated by conductive adhesive tapes and a four nm thick metal film, respectively, for image capturing (Zhao et al., 2021a). A 400 FTIR spectrophotometer (PerkinElmer Corp., Shelton, CT) coupled with an RT-DLaTGS detector was employed to identify chemical bonds and biomass crystallinity as the test parameters from our previous study (Zhao et al., 2020b). The intensity of peaks at 1437 and 898 cm^{-1} was calculated for the lateral order index (LOI) (Nelson and O’Connor, 1964). In contrast, the crystallinity index (CrI) obtained from a Siemens D-5000 diffractometer (Bruker, Ettlingen, Germany) was also used to reveal biomass crystallinity. The LOI and CrI values calculation can be found in our previous study (Zhao et al., 2021a).

3.3.4. Enzymatic hydrolysis

Enzymatic hydrolysis of the mixture of HOAc and NaOH pretreated biomass and their mixed filtrate was performed in the 50 mL Erlenmeyer flasks. In brief, the designed amounts of pre-mixed HOAc and NaOH pretreated biomass was added into flasks and followed by loading 20 mL mixed filtrate to form 2.5-10.0% (w/v) solid loadings. After adjusting the pHs of slurries to 4.80 by HCl (10 mol/L) and NaOH (10 mol/L), enzymes (100 μL cellulase/g biomass and 50 μL hemicellulase/g biomass) were added. Enzymatic hydrolysis was executed in an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) with 160 rpm at 49 °C. The supernatant of hydrolysate was pipetted for glucose and xylose examination after 72 h. Sugar conversion efficiencies were calculated as the amount of monosaccharides in the hydrolysate divided by the amount of potential monosaccharides from biomass and filtrate.

3.3.5. Analytical procedures

Compositional analyses of biomass and filtrate were conducted following the procedures from the National Renewable Energy Laboratory (Sluiter et al., 2008). The detailed descriptions can be found in our previous study (Zhao et al., 2021a). It should be mentioned that concentrations of monosaccharides (glucose and xylose) and inhibitory compounds (furfural, and HMF) in the filtrate and hydrolysate were measured quantitatively by a 1260 high performance liquid chromatography system (Agilent, Santa Clara, CA).

3.4. Results and discussion

3.4.1. Effect of washing on the composition of pretreated biomass

Principal components, including glucan, xylan, and lignin in raw and pretreated biomass, are shown in Figure 3.1. It was observed that post-washing inhibited the recondensation of the solubilized lignin to the surface of cellulose and hemicellulose, which was evidenced by that II-VW washed biomass had lower lignin contents than I-VW washed biomass [hemp: 31.63 vs. 33.13% for HOAc and 7.51 vs. 8.94% for NaOH (Figure 3.1A); poplar: 31.63 vs. 33.13% for HOAc and 7.51 vs. 8.94% for NaOH (Figure 3.1B)]. Besides, compared to NaOH pretreated and I-VW washed biomass, II-VW washed biomass showed higher glucan (hemp: 73.61 vs. 66.81%; poplar: 75.35 to 79.11%) and xylan (hemp: 12.20 vs. 11.34%; poplar: 13.92 to 13.00%) (Figure 3.1). These discoveries are in agreement with a previous study, where aqueous ammonia [10% solid loading, 180 ℃ for 30 min, 20% (w/w) ammonia] pretreated corn stover with post-washing (1 g biomass/20 ml water) exhibited higher glucan (63.7 vs. 51.4%) and xylan (19.2 vs. 17.8%) but lower lignin (5.4 vs. 7.5%) than unwashed corn stover (Qin et al., 2013). This could be attributed to the removal of solubilized extractives. In comparison, Karuna et al. (2014) reported that NaOH pretreated rice straw after acidification with HCl and washing (2.75 L water) had 45.5% of glucan and 11.6% of xylan, which were lesser than those (82.8% of glucan and 13.4% of xylan) of direct washed rice straw (without acidification). Toquero and Bolado (2014) demonstrated that wheat straw pretreated by NaOH and washed with distilled water in a solid/liquid ratio of 1:10 showed higher cellulose (56.84 vs. 50.67%) and hemicellulose (20.25 vs. 19.70%) than unwashed wheat straw. Liu and Chen (2017) found that corn stover washed with 75 ℃ deionized water under 10:1 (w/w) water/solid ratio showed notably higher glucan and lignin contents compared to unwashed corn stover. Additionally, it has been found that the composition of acid pretreated bamboo was highly associated with the post-extraction process in which phosphoric acid, urea, and ethanol were investigated (Huang et al., 2019). These results indicate that post-pretreatment can alter the chemical composition of pretreated biomass via removing the degraded compounds and extractives.

3.4.2. Effect of washing on sugar and solid recoveries and lignin removal

Glucan, xylan, and solid recoveries as well as lignin removal with different post-washing are presented in Figure 3.2. It was found that II-VW washing resulted in the reduction of glucan (2.98-7.36% for hemp and 1.55-6.67% for poplar) and xylan (0.97-3.93% for hemp and 0.1-6.09% for poplar) recoveries compared with I-VW washing (Figure 3.2A and B). This phenomenon was consistent with a finding that aqueous ammonia pretreatment and post-washing (1 g biomass/20 ml water) of corn stover reduced 1.6% of glucan and 7.7% of xylan recoveries relative to unwashed corn stover (Qin et al., 2013). It is due to that the decomposed sugars adsorbed on the surface of pretreated biomass was gradually removed, as reflected by the decreased solid recoveries [hemp: 65.31% of HOAc-I-VW vs. 64.05% of HOAc-II-VW and 59.93% of NaOH-I-VW vs. 50.38% of NaOH-II-VW (Figure 3.2C); poplar: 67.70% of HOAc-I-VW vs. 62.60% of HOAc-II-VW and 51.35% of NaOH-I-VW vs. 48.09% of NaOH-II-VW (Figure 3.2D)]. This is in accordance with the finding that mass yield showed a decreasing trend with more washes being carried out (da Costa Nogueira et al., 2018).

Herein, negative lignin removal was noticed for HOAc pretreatment, attributed to the pseudo-lignin formation from sugar-degraded derivatives (Zhao et al., 2021a), while NaOH pretreatment reached high lignin removal (Figure 3.2C and D). In comparison to I-VW, II-VW efficiently inhibited the recondensation and absorption of the disrupted lignin, evidenced by an increased lignin removal [hemp: -21.62% of HOAc-I-VW vs. -13.87% of HOAc-II-VW and 69.88% of NaOH-I-VW vs. 78.74% of NaOH- II-VW (Fig. 2C); poplar: -10.27% of HOAc-I-VW vs. -0.79% of HOAc- II-VW and 87.76% of NaOH-I-VW vs. 90.01% of NaOH-II-VW (Figure 3.2D)]. The enhancement of lignin removal might be attributed to the release of small lignin units from the pretreated biomass surface. In other studies, it was also found that post-washing reduced lignin recovery by 12.1-24.3%, depending on solid loading used for aqueous ammonia pretreatment of corn stover (Qin et al., 2013). Besides, Karuna et al. (2014) reported that pre-acidification of alkali pretreated slurry before post-washing induced lignin reconsolidation as reflected by lower lignin removal (63% for pre-acidified vs. 90% for directly washed).

3.4.3. Effect of washing on structural characterization of biomass

HOAc and NaOH pretreatments altered raw biomass from highly intact fascicular structures to an increased roughness structure (Figure 3.3). With the increment of post-washing from I-VW to II-VW, the small disrupted fibers gradually disappeared with an increase in reflective intensity on the surface of HOAc pretreated biomass. Herein, it was also found that hemp biomass exhibited a slight variation in morphological changes for NaOH pretreatment compared to poplar biomass: the regular structures were displayed on the external surface of hemp with II-VW, while the surface of poplar with II-VW was smoother. In our previous study (Zhao et al., 2020b), these structures in NaOH pretreated hemp biomass with 4.5-fold post-washing have also been observed.

In this study, both XRD diffractogram and FTIR spectrum of raw and pretreated biomass with different post-washing were investigated (Figure 3.4), as the CrI and LOI values are shown in Table 3.1. Strikingly, HOAc and NaOH pretreatments with post-washing promoted the increases in CrI of hemp from 52.38 to 77.29% and of poplar from 45.64 to 76.55% (Table 3.1), which was propelled by increasing intensity of I_{002} and decreasing intensity of I_{am}. As the post-washing water volume increased from I-VW to II-VW, CrI values of NaOH pretreated hemp and poplar biomass enhanced from 75.71 to 77.29% and from 73.47 to 76.55%, respectively, while CrI values decreased slightly for HOAc pretreated biomass (Table 3.1). This phenomenon for NaOH pretreatment was in line with significant enrichment in glucan content of NaOH pretreated biomass from I-VW to II-VW (Figure 3.1). The decrease in CrI for HOAc pretreatment was possibly related to the change in the degree of crystalline (I_{002}) and amorphous (I_{am}) zones. The LOI values of HOAc (hemp: 0.93-1.04; poplar: 0.82-0.89) and NaOH (hemp: 1.00-1.25; poplar: 0.82-0.1.32) pretreated biomass were higher than those of (0.77) raw biomass (Table 3.1). However, it should be highlighted that pretreated biomass washed by II-VW showed lower LOI values than those washed by I-VW, attributed to the increasing rate of A_{898} being significantly higher than that of A_{1437} (Table 3.1).

Changes in chemical bonds of raw and pretreated biomass with post-washing by I-VW or II-VW were also discerned by FTIR spectra (Figure 3.4C and D). It was noticed that the intensities of cellulose-related peaks at 3329 cm^{-1} assigned to O-H stretching, 1091 cm^{-1} assigned to C-O vibration, 1051 cm^{-1} assigned to C-O stretching, and 1023 cm^{-1} assigned to C-O-C pyranose ring skeletal vibration in pretreated biomass with post-washing by I-VW and II-VW were more intense than raw biomass. Whereas the intensities of lignin-related peaks at 1514 cm^{-1} assigned to the aromatic skeleton and 1319 cm^{-1} assigned to syringyl and guaiacyl lignin units amplified slightly in HOAc pretreated biomass but diminished in NaOH pretreated biomass.

Towards post-washing by I-VW or II-VW, only the intensities of cellulose-related peaks at 1091 and 1051 cm^{-1} in NaOH pretreated biomass increased.

3.4.4. Effect of washing on composition and pH of the filtrate

Dilute acid and alkali pretreatments often induce inhibitor formation and cause severer pH of pretreated biomass and filtrate compared to buffer, therefore, rinsing pretreated biomass with adequate water has been commonly performed before enzymatic hydrolysis as the filtrate is discarded subsequently (da Costa Nogueira et al., 2018; Liu and Chen, 2017; Toquero and Bolado, 2014; Zhang et al., 2017). For example, da Costa Nogueira et al. (2018) reported that twelve-time washes (one time: 200 mL water/15g raw biomass) were needed to render the filtrate transparent and its pH around neutral for NaOH (2%, w/v) pretreated coconut fiber. However, this extensive post-washing with filtrate being abandoned would face tech-economic challenges when scaling up into commercial applications. In this work, little water [one-two times (I-VW and II-VW) of the initial liquid volume used for pretreatment] was used to wash the HOAc and NaOH pretreated biomass, and these filtrates were mixed and used as a buffer to reduce water consumption and chemical loss.

It was found that pHs of II-VW washed filtrates were slightly mitigated comparing those of I-VW washed filtrates but still harsher than buffer (pH=4.8) solution (Table 3.2), indicating that these filtrates are unable to be applied as a buffer for enzymatic hydrolysis directly. This result was in accordance with the finding that change in pH value of HOAc pretreated corncob after one volume of water washing was negligible and the corresponding sugar conversion efficiency only reached 16.35% after 72-h enzymatic hydrolysis (Zhang et al., 2017). Thus, extra acid or alkali was often added to neutralize both biomass and filtrate (Kim, 2018). In terms of filtrate composition, filtrates from HOAc pretreatment showed higher monomeric glucose (hemp: 0.69 g/L of I-VW and 0.46 g/L of II-VW; poplar: 1.10 g/L of I-VW and 0.78 g/L of II-VW) and xylose (hemp: 2.35 g/L of I-VW and 1.71 g/L of II-VW; poplar: 5.24 g/L of I-VW and 3.62 g/L of II-VW) concentrations than those from NaOH pretreatment, resulting in high HMF and furfural production. It was also found that HOAc-derived filtrate was composed of higher total sugar (hemp: 4.24-5.09 vs. 2.22-3.18 g/L; poplar: 4.99-7.21 vs. 1.96-3.07 g/L) than those from NaOH-derived filtrate. As compared with I-VW, II-VW showed lower sugar concentrations in HOAc and NaOH pretreated filtrate and lower HMF and furfural concentrations in HOAc pretreated filtrate. In contrast, Toquero and Bolado (2014) found that filtrate from dilute HCl (1.5%, w/w) pretreated wheat straw with post-washing (solid/liquid ratio of 1/10) had no HMF and furfural whereas that without post-washing contained 0.04 g/L of HMF and 0.84 g/L of furfural. Although no furans (HMF and furfural) were detected in NaOH-derived filtrate, high concentrations of lignin-derived phenolic compounds were reported in alkali pretreated liquor (da Costa Nogueira et al., 2018; Toquero and Bolado, 2014), inhibiting enzymatic and microbial activity.

In order to use filtrate, I-VW and II-VW filtrates from HOAc pretreatment were mixed with those from NaOH pretreatment, respectively. As expected, the pHs of the mixed filtrates, ranging from 4.60 to 4.72, were mitigated to form sodium acetate via the neutralization reaction of residual HOAc and NaOH. Meanwhile, the soluble lignin in the NaOH pretreated filtrates was partially precipitated out with the filtrate turned transparent after vacuum filtration (Table 3.3). HMF (hemp: 0.34 g/L of mixed-I-VW and 0.20 g/L of mixed-II-VW; poplar: 0.28 g/L of mixed-I-VW and 0.16 g/L of mixed-II-VW) and furfural (hemp: 0.90 g/L of mixed-I-VW and 0.49 g/L of mixed-II-VW; poplar: 0.96 g/L of mixed-I-VW and 0.64 g/L of mixed-II-VW) concentrations derived from HOAc pretreatment were attenuated as compared with those from HOAc pretreatment (Table 3.2).

3.4.5. Effect of washing on recovery and FTIR characterization of Lignin

As the increment of post-washing from I-VW to II-VW, lignin recoveries decreased from 38.48 to 35.69 g/kg hemp biomass and 44.69 to 39.23 g/kg poplar biomass, respectively (Figure 3.5A), which can be specialized to the gradual removal of lignin-derived phenolic compounds. The conversion of biomass-based lignin to polymeric materials may offer potential values for biomass upgrades (Huang et al., 2020). The FTIR spectrum of the isolated lignin fraction was compared with commercial alkali lignin (Figure 3.5B). It is visible that the lignin from hemp and poplar biomass had similar peak intensities at 1654 cm^{-1}, 1592 cm^{-1}, 1506 cm^{-1}, 1420 cm^{-1}, 1461 cm^{-1}, 1362 cm^{-1}, 1326 cm^{-1}, 1265 cm^{-1}, 1159 cm^{-1}, 1030 cm^{-1}, and 830 cm^{-1} with the commercial alkali lignin. In addition, the recovered lignin exhibited weaker peak intensity at 1265 cm^{-1} and stronger peak intensity at 1120 cm^{-1} than the commercial alkali lignin. However, comparing the FTIR spectrum obtained from post-washing of I-VW and II-VW, there were no prominent variations. The results obtained in the present work lead to the conclusion that lignin from mixed HOAc and NaOH pretreated filtrate had almost comparable chemical structures with the commercial alkali lignin.

3.4.6. Enzymatic hydrolysis of combined biomass and filtrate

Enzymatic hydrolysis of combining HOAc and NaOH biomass and their mixed filtrate was explored at solid loadings of 2.5-10% (Figure 3.6). It was observed that glucose (hemp: 14.20-38.04 g/L for I-VW and 15.13-39.11 g/L for II-VW; poplar: 14.28-38.19 g/L for I-VW and

15.15-39.44 g/L for II-VW) and xylose (hemp: 4.70-10.70 g/L for I-VW and 4.29-10.28 g/L for II-VW; poplar: 5.40-11.47 g/L for I-VW and 4.66-10.67 g/L for II-VW) concentrations increased significantly with solid loading increased from 2.5 to 10%. However, the differences in glucose concentration reduced gradually as solid loading increased, whereas no similar trends were found in xylose concentration. For instance, regarding hemp I-VW, the variation (11.40 g/L) in glucose concentration between 2.5 and 5.0% was more significant than those (7.23 g/L) between 5.0 and 7.5% and (5.21 g/L) between 7.5 and 10.0% (Figure 3.6A). This is accompanied by the reduction of glucose conversion efficiencies (hemp: 77.97-53.93% for I-VW and 81.18-53..43% for II-VW; poplar: 75.80-52.40% for I-VW and 77.69-51.87% for II-VW) (Table 3.3). This trend would be mainly ascribed to the accumulated lignin limiting enzymatic activities and the reduced water availability slowing down mass transfer efficiency (Chen and Liu, 2017; Kristensen et al., 2009; Modenbach and Nokes, 2012, 2013). It has to mention that no significant decreases in xylose conversion efficiencies with solid loading increasing except hemp-II-VW (Table 3.3). This may be because xylan is in the outermost part of the biomass and is highly accessible to hemicellulase. In contrast, Ramachandriya et al. (2013) found no notable changes in glucose conversion efficiencies from enzymatic hydrolysis of acid bisulfite pretreated Eastern redcedar with a solid loading of 2 to 16%. However, this finding is inconsistent with other studies that sugar conversion efficiencies decreased gradually with the increment of solid loading used for enzymatic hydrolysis (Cara et al., 2007; Lu et al., 2010; Xue et al., 2015).

Compared with I-VW, II-VW increased glucose concentrations (0.93-1.71 g/L for hemp and 0.68-1.25 g/L for poplar) but decreased xylose concentrations (0.25-0.45 g/L for hemp and 0.70-0.90 g/L for poplar). It was noticed that glucose conversion efficiencies showed different trends depending on biomass type and solid loading: for hemp, II-VW increased glucose conversion efficiencies at 2.5 (3.21%) and 5.0% (2.35%) of solid loading but decreased at 7.5 (-0.37%) and 10% (-0.50%) of solid loading; for poplar, II-VW increased glucose conversion efficiency at 2.5% (1.89%) of solid loading and decreased at 5.0 (-1.05%), 7.5 (-1.30%) and 10% (-0.53%) of solid loading (Table 3.3). The reduced conversion efficiency from II-VW is possibly due to the relative attenuation of cellulase/glucan dose (II-VW washed biomass had higher glucan content than I-VW washed biomass) and the diluted potential glucose in the mixed filtrate (II-VW had lower glucose concentrations than I-VW). However, II-VW showed a significant increase in xylose conversion efficiency (hemp: 4.5-13.0%; poplar: 4.0-7.7%) compared with I-VW (Table 3.3). The enhanced xylose conversion efficiencies with reduced xylose concentrations from II-VW were related to its low potential xylose concentrations in the mixed filtrate. These results suggest that post-washing by I-VW is sufficient for pretreated biomass without compromising notable sugar concentrations and yields relative to II-VW.

3.4.6. Relationship between glucose concentration and solid loading

It might be the first report to illuminate the response of glucose concentration to solid loading, although a previous study by Kristensen et al. (2009) revealed that glucose conversion efficiency showed a negatively linear correlation with solid loading attributed to inhibition of cellulase adsorption to cellulose. Here, similar decreased trends of glucan-to-glucose conversion with the increment of solid loading among scenarios were presented (Table 3.4). It indicates that when comparing pretreatment methods and improved processes with other studies, solid loading used for enzymatic hydrolysis should be taken into consideration. As expected, the corresponding glucose concentration exhibited a quadratic correlation with solid loading (Table 3.4). Based on that, the focus points (solid loading as an axis of symmetry) of these four scenarios were obtained. These findings suggest that the linear decrease in glucose conversion efficiency and parabolic trend of glucose concentration with solid loading can offset the advantages of enzymatic hydrolysis at high solids levels. Besides, enzymatic hydrolysis at the focus point (solid loading) would statistically reach the highest point (glucose concentration) of a parabola. Thus, enzymatic hydrolysis of mixed biomass and filtrate at the focus points for four scenarios was conducted (Table 3.4). It was observed that glucose concentrations and conversion efficiencies were higher than those from the regression. The maximum glucose concentrations of 43.88 g/L for hemp washed by I-VW and 42.71 g/L for poplar washed by II-VW were achieved. In addition, total sugar concentrations of 57.08 g/L for hemp washed by I-VW, 55.91 g/L for hemp washed by II-VW, 53.62 g/L for poplar by washed I-VW, and 54.25 g/L for poplar washed by II-VW were accomplished at the focus points (Table 3.4).

3.6. Conclusions

Effects of post-washing pretreated biomass on pretreatment effectiveness were illuminated. Post-washing was able to alter the composition of pretreated biomass through removing extractives and lignin units, increasing glucan content, lignin removal, and CrI. Mixing HOAc and NaOH pretreated filtrates led to the precipitation of solubilized lignin. Lignin recovery decreased with post-washing level increased from I-VW to II-VW, but their FTIR characteristics were still comparable with the commercial alkali lignin. I-VW and II-VW exhibited marginal differences in sugar concentration and conversion efficiency, therefore, post-washing of I-VW might be sufficient for the pretreated biomass. Glucose concentration exhibited a quadratic correlation with solid loading.

Table 3.1 The crystallinity of raw and pretreated biomass with post-washing by I-VW or II-VW.

Sample	XRD[1]			FTIR[2]		
	I_{002}	I_{am}	CrI[3] (%)	A_{1437}	A_{898}	LOI[4]
Hemp-raw biomass	2505	1193	52.38	2.69	3.49	0.77
Hemp-HOAc-I-VW	2921	935	67.99	2.64	2.53	1.04
Hemp-HOAc-II-VW	3332	1076	67.71	3.24	3.47	0.93
Hemp-NaOH-I-VW	2919	709	75.71	4.20	3.36	1.25
Hemp-NaOH-II-VW	3408	774	77.29	6.57	6.57	1.00
Poplar-raw biomass	2272	1235	45.64	2.05	2.66	0.77
Poplar-HOAc-I-VW	3678	1107	69.90	3.06	3.43	0.89
Poplar-HOAc-II-VW	3484	1070	69.29	3.28	3.72	0.88
Poplar-NaOH-I-VW	3091	820	73.47	1.99	1.51	1.32
Poplar-NaOH-II-VW	3749	879	76.55	2.34	2.86	0.82

[1]XRD: X-ray diffraction; [2]FTIR: Fourier transform infrared spectroscopy; [3]CrI: crystallinity index; [4]LOI: lateral order index.

Table 3.2 pH and chemical composition of HOAc and NaOH pretreated filtrates and their mixed filtrates with post-washing by I-VW or II-VW.

Sample	pH	Glucose (g/L)	Xylose (g/L)	Oligo-glucose (g/L)	Oligo-xylose (g/L)	HMF[1] (g/L)	Furfural (g/L)
Hemp-HOAc-I-VW	3.03	0.69	2.35	0.47	1.58	0.65	1.92
Hemp-HOAc-II-VW	3.05	0.46	1.71	0.39	1.69	0.44	1.17
Hemp-NaOH-I-VW	12.43	0.27	0.00	0.13	2.78	0.00	0.00
Hemp-NaOH-II-VW	12.34	0.17	0.33	0.16	1.56	0.00	0.00
Hemp-mixed-I-VW	4.66	0.54	1.41	0.24	1.78	0.34	0.90
Hemp-mixed-II-VW	4.64	0.36	0.98	0.09	1.04	0.20	0.49
Poplar-HOAc-I-VW	2.80	1.10	5.24	0.26	0.60	0.42	2.15
Poplar-HOAc-II-VW	2.85	0.78	3.62	0.19	0.41	0.28	1.52
Poplar-NaOH-I-VW	12.42	0.00	0.00	0.24	2.83	0.00	0.00
Poplar-NaOH-II-VW	12.21	0.00	0.00	0.19	1.77	0.00	0.00
Poplar-mixed-I-VW	4.72	0.38	1.54	0.45	2.45	0.28	0.96
Poplar-mixed-II-VW	4.60	0.26	1.05	0.39	1.70	0.16	0.64

[1] HMF: hydroxymethylfurfural.

Table 3.3 Sugar conversion efficiencies (%) of combining HOAc and NaOH pretreated biomass and their mixed filtrate at different solid loading.

Solid loading	Hemp-I-VW		Hemp-I-VW		Poplar-I-VW		Poplar-I-VW	
	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose
2.5%	77.97 ± 0.11	85.53 ± 1.66	81.18 ± 0.87	98.56 ± 0.43	75.80 ± 0.07	87.36 ± 0.32	77.69 ± 0.12	95.02 ± 0.19
5.0%	71.81 ± 0.07	85.48 ± 0.00	74.15 ± 0.00	96.09 ± 0.27	73.47 ± 0.01	90.39 ± 0.27	72.42 ± 0.11	94.39 ± 0.19
7.5%	61.83 ± 0.04	87.55 ± 0.55	61.46 ± 0.09	93.27 ± 0.24	61.44 ± 0.19	89.47 ± 0.12	60.14 ± 0.01	94.96 ± 0.51
10.0%	53.93 ± 0.06	86.10 ± 0.18	53.43 ± 0.05	90.55 ± 0.36	52.40 ± 0.12	89.87 ± 0.30	51.87 ± 0.06	93.87 ± 0.74

Table 3.4 The relationship between glucose concentration/conversion efficiency and solid loading with post-washing by I-VW or II-VW.

Scenario	Conversion efficiency/solid loading		Concentration/solid loading			Hydrolysis at the focus point	
	Linear	R^2	Non-linear	R^2	Focus point	Glucose (g/L)	Xylose (g/L)
Hemp I-VW	$Y= -3.282*X + 86.91$	0.99	$Y= 0.235 + 6.249*X - 0.248*X^2$	1.00	12.60	43.88	13.20
Hemp II-VW	$Y= -3.838*X + 91.55$	0.99	$Y= 0.630 + 6.578*X - 0.275*X^2$	1.00	11.95	43.78	12.13
Poplar I-VW	$Y= -3.289*X + 86.33$	0.95	$Y= -1.678 + 7.295*X - 0.339*X^2$	1.00	10.96	41.27	12.35
Poplar II-VW	$Y= -3.590*X + 87.97$	0.98	$Y= -0.154+ 6.967*X - 0.303*X^2$	1.00	11.50	42.71	11.53

Y_1: glucose conversion efficiency; Y_2: glucose concentration; X: solid loading.

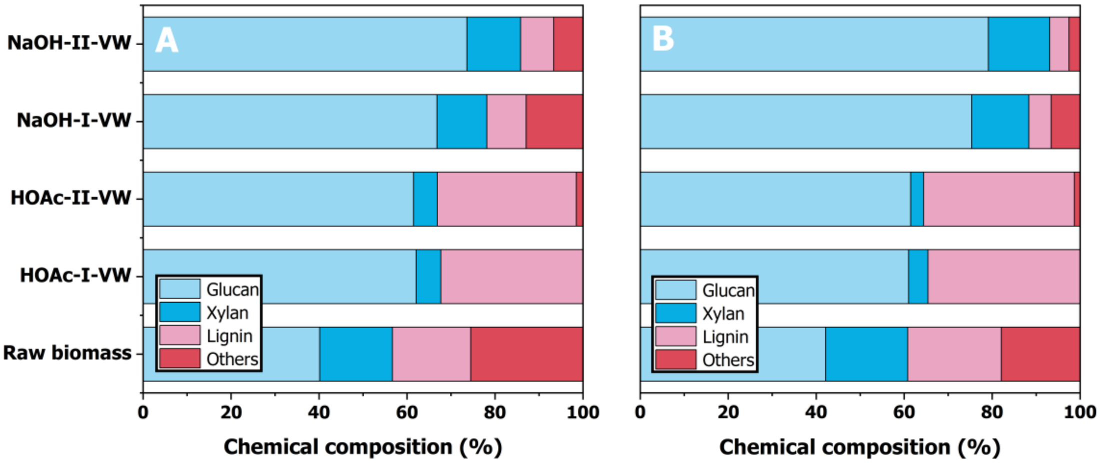

Figure 3.1 Chemical composition of raw and pretreated hemp (A) and poplar (B) biomass with post-washing by I-VW or II-VW.

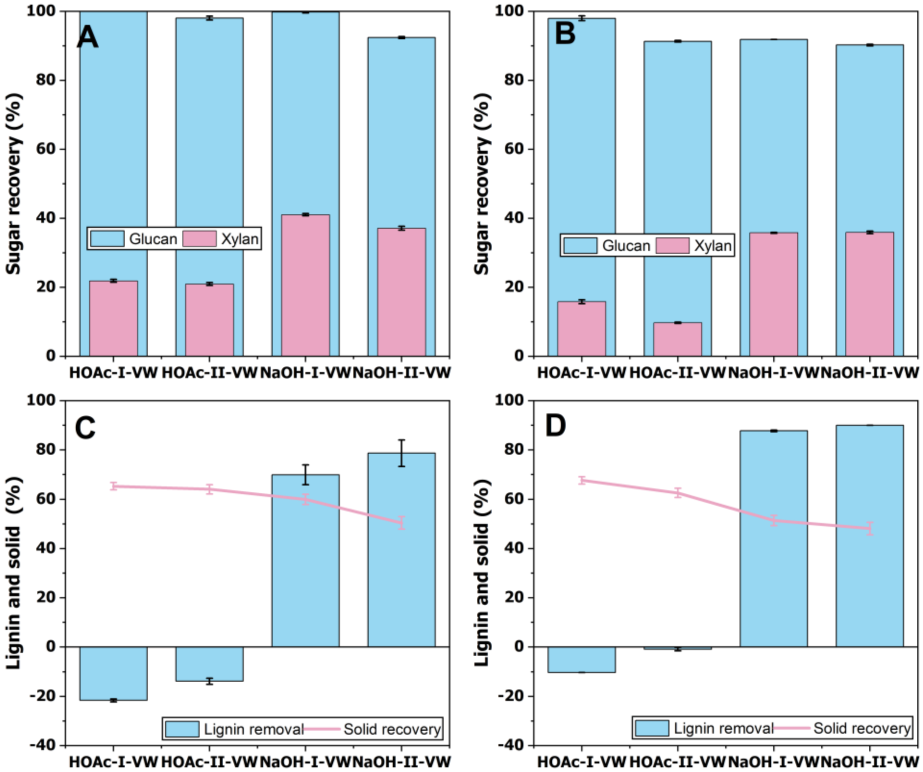

Figure 3.2 Sugar and solid recoveries and lignin removal of HOAc and NaOH pretreated hemp (A and C) and poplar (B and D) biomass with post-washing by I-VW or II-VW.

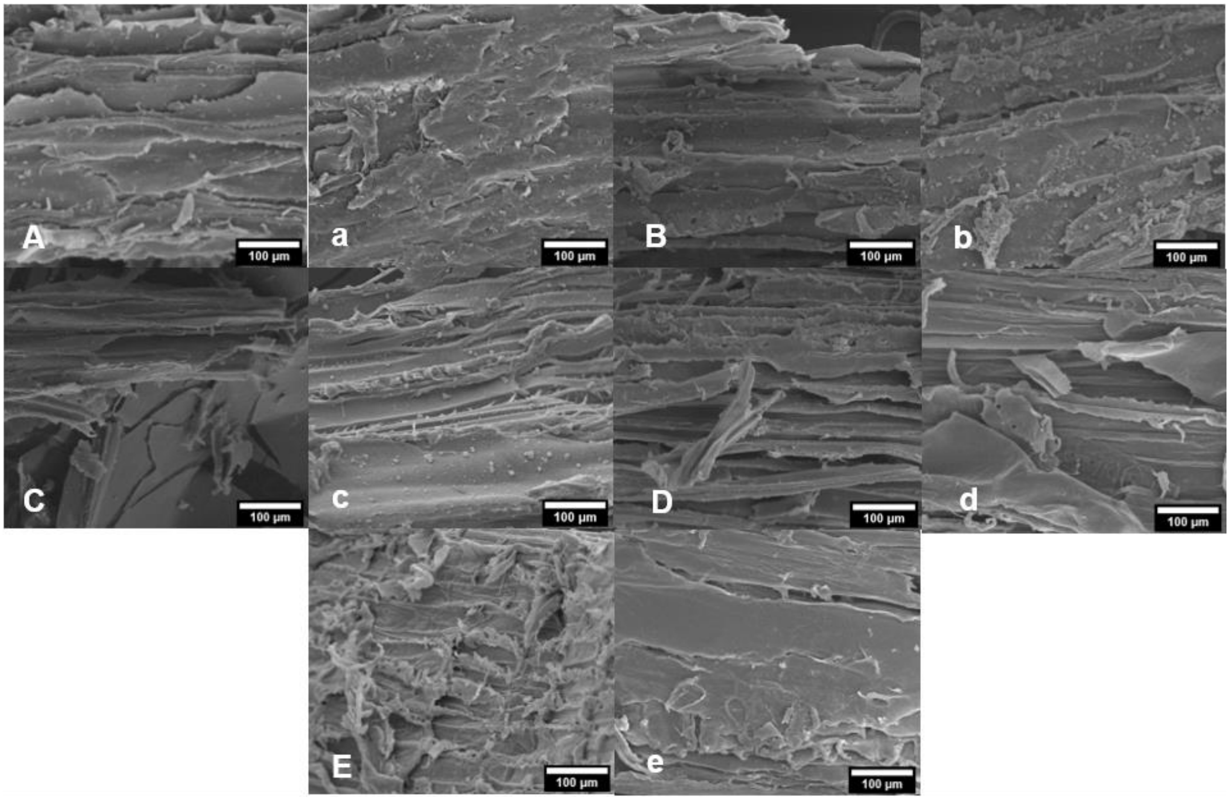

Figure 3.3 SEM images of raw and pretreated hemp (capital letter) and poplar (lowercase) biomass with post-washing by I-VW or II-VW (A and a: raw biomass; B and b: HOAc-I-VW; C and c: HOAc- II-VW; D and d: NaOH-I-VW; E and e: NaOH-II-VW).

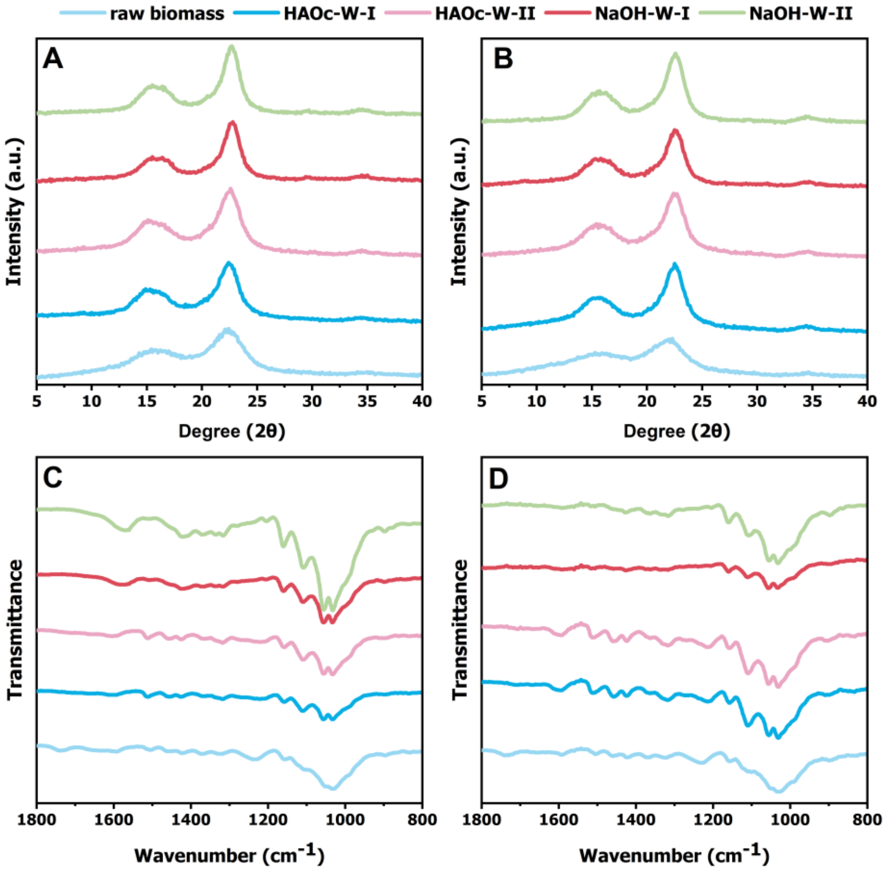

Figure 3.4 XRD and FTIR characterization of raw and pretreated hemp (A and C) and poplar (B and D) biomass with post-washing by I-VW or II-VW.

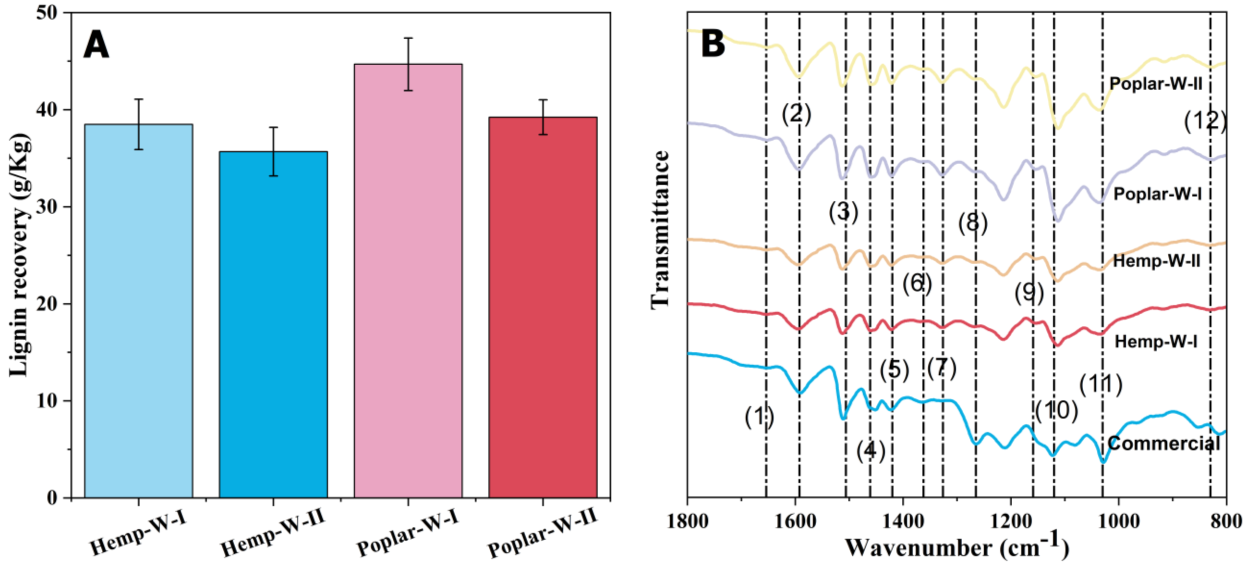

Figure 3.5 Lignin recovery (A) and its FTIR characteristics (B) after post-washing by I-VW or II-VW with the commercial alkali lignin used as a control.

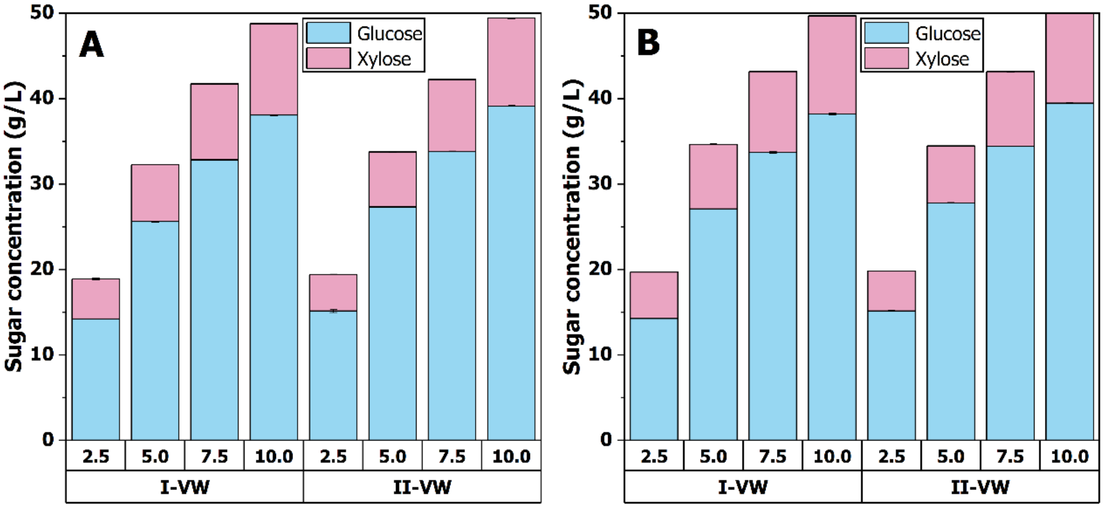

Figure 3.6 Sugar (glucose and xylose) concentration obtained from enzymatic hydrolysis of combining HOAc and NaOH pretreated biomass and their mixed filtrate at the solid loading of 2.5-10% with post-washing by I-VW or II-VW.

Chapter 4 - Lignin, sugar, and furan production of four genotypes of industrial hemp biomass harvested from two planting locations

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4.1. Abstract

The objective of this study was to validate the potential of an acid-base integrated process for sugars, furans, and lignin production of four genotypes of industrial hemp (*Cannabis sativa L.*) biomass that were harvested from two planting locations (Haysville and Manhattan, KS). Results showed that genotype, planting location, and their interaction had notable influences on biomass composition and its conversion to bioproducts but exhibited different trends. Glucan content of biomass from Haysville, ranging from 47.29-50.05%, were higher than those of 42.49-48.38% from Manhattan with the lowest being Vega (Manhattan) and the highest being Hlukouskii (Haysville). Xylan and lignin contents in all the hemp genotypes were 11.70-13.88% and 10.45-15.14%, respectively. The integration process effectively rendered the pH of the integrated filtrate and slurry to approximately 4.80. The highest lignin recovery of 73.13 g/kg biomass was achieved by Rigel from Manhattan. Fourier transform infrared spectroscopy (FTIR) characterization showed that only lignin derived from Vega (Haysville) and Anka (Manhattan) was comparable to the commercial alkali lignin. Retaining monosaccharides (2.24-3.81 g/L) enhanced sugar concentrations (glucose: 40.40-45.71 g/L; xylose: 7.09-8.88 g/L) and conversion efficiencies (glucose: 71.19-77.71%; xylose: 45.42-52.03%). Besides, furans including 0.79-1.25 g/L of hydroxymethylfurfural (HMF) and 0.99-1.59 g/L of furfural coupling with 1.96-2.95% and 10.00-14.65% conversion efficiencies, respectively, were obtained in the final hydrolysate.

Biomass from Haysville produced relatively higher glucose concentrations than those from Manhattan. Based on mass balance, the most productive genotype was Rigel.

4.2. Introduction

In past decades, the legislative limitation of industrial hemp (*Cannabis sativa L.*) cultivation and processing in Western Europe, the USA, and Canada, to inhibit its abuse and illegal utilization for drug production (Cherney and Small, 2016; Fike, 2016), remarkably weakened its economic importance. Therefore, industrial hemp is often recognized as an underdeveloped and underutilized crop. Currently, the USA federal restriction on industrial hemp cultivation is unlocked by the 2018 US farm bill that approved its cultivation with a delta-9 tetrahydrocannabinol content (THC) below 0.3% in 46 states (Adesso et al., 2019). Governing changes are leading to a resurgent commercial exploration of this crop. The chemical composition of hemp seed is of great interest, attributed to the potential for food and nutraceutical and pharmaceutical applications (Farinon et al., 2020; Wang and Xiong, 2019; Xu et al., 2021). Industrial hemp stover, either as a by-product of hemp grain production or as a primary product, could serve an important role as a biochemical and biofuel feedstock (Zhao et al., 2020a).

Compared to other lignocellulosic biomass such as wheat straw, corn stover, and sorghum biomass, industrial hemp biomass has been reported to contain higher cellulose content. Thus, it has the potential to be a competitive candidate for biomass-to-bioproducts production (Zhao et al., 2020a). To render industrial hemp biomass amenable to enzymatic hydrolysis, large numbers of pretreatment strategies such as acid, alkali, liquid hot water, steam, organosolv, and Microwave-assisted ionic liquids have been investigated by many research groups (de Vega and Ligero, 2017; George et al., 2015; Kamireddy et al., 2013; Kreuger et al., 2011; Kuglarz et al., 2014; Väisänen et al., 2019; Viswanathan et al., 2020; Xie et al., 2017; Zhao et al., 2020a). In this regard, excessive post-pretreatment washing was widely discerned to moderate the severity of pH and inhibitory compounds in the pretreated biomass and filtrate (Das et al., 2020; Kreuger et al., 2011; Kuglarz et al., 2014; Semhaoui et al., 2018). This process generates abundant wastewater, which inevitably inhibits commercial exploration. Therefore, reducing water and chemical consumption is highly needed for industrial hemp biomass valorization. To address this issue, our previous study proved that integrating acid and alkali pretreated biomass and their mixed filtrate for enzymatic hydrolysis was performed well without compromising sugar yield (Zhao et al., 2021a). The neutralization of residual acid and alkali in the biomass and filtrate can be achieved simultaneously during the integration process (Zhao et al., 2021a). Also, the dissolved lignin in the alkaline filtrate can be recovered. The post-washing and chemical disposal procedures can be avoided after pretreatment. Therefore, the major advantage of the acid-base integration process is the feasibility to minimize water and chemical overconsumption. On the other hand, Viswanathan, et al. (2021) investigated the economic perspectives of ethanol and biodiesel coproduction from industrial hemp biomass (genotype of 19m96136) and found that minimum selling prices of biodiesel ranged from $1.09 to $4.88/L when the ethanol selling price was $0.62/L depending on the lipid content. Das et al. (2020) reported that dilute acid pretreatment and enzymatic hydrolysis predicted practical ethanol yields of 264.98−344.47 L/dry ton hemp stems with the highest ethanol yield of 344.85 L/dry ton hemp stems from the Futura 75 genotype. These studies indicate that industrial hemp biomass has the potential to be used for biofuel production but the variation among genotypes and the effect of the growing environment should be considered.

The effects of agronomical practices, genotypes, and cultivation sites on the nutritional, phytochemical, and antioxidant properties of hempseed and pant growth performances and biomass yields have been considerably explored (Ascrizzi et al., 2019; Campiglia et al., 2017;

Cappelletto et al., 2001; García-Tejero et al., 2019; Irakli et al., 2019; Pagnani et al., 2018; Scheliga et al., 2018; Sraka et al., 2019; Struik et al., 2000). However, few studies have been targeted at the impacts of genotype and environmental factors on the bioconversion of industrial hemp biomass to lignin, sugar, and furan. In this respect, Das et al. (2020) compared the potential of eleven industrial hemp biomass for biofuel and bioproducts production through dilute sulfuric acid pretreatment coupled with theoretical simulation and found that dual-purpose genotypes had advantages over fiber-only Genotypes in terms of potential per hectare gross profit. Additionally, Zhao et al. (2020b) evaluated four varieties of industrial hemp biomass to illuminate their variation for bioethanol production through liquid hot water, sulfuric acid, and NaOH pretreatments. The two studies demonstrated the influence of genotypes on biomass valorization, but environmental factors such as planting location remain unanswered.

In this chapter, the integrated HOAc and NaOH pretreatments were applied to four dual-purpose industrial hemp genotypes grown at two planting locations. The objective of this research was to demonstrate the effects of genotype and planting location on industrial hemp biomass valorization using an integrated biomass pretreatment method. Thus, effects of genotype, planting location, and their interaction on the chemical composition of biomass and filtrate as well as chemical (glucose, xylose, HMF, furfural, and lignin) conversion performances were elucidated. The recovered lignin was also compared with commercial alkali lignin. Additionally, relationships between biomass composition and its conversion performances to bioproducts were demonstrated. This study offers essential information to understand the effects of genotype and planting location on biomass-to-chemicals production and promote industrial exploration with limited water and chemical consumption.

4.3. Materials and method

4.3.1. Materials

Stover remaining after threshing to remove grain from whole-plant samples of four dual-purpose genotypes of industrial hemp (Anka, Rigel, Vega, and Hlukouskii 51) that were harvested from two planting locations (Haysville and Manhattan, KS) were used for this study. Field management details can be found in Griffin et al. (2020). The detailed weather data in Haysville and Manhattan for May-September of 2020 are shown in Figure 4.1. Growing conditions were generally favorable for hemp growth at both locations, but the Haysville location had a shallower, more coarse soil [Canadian-Waldeck fine sandy loam (coarse-loamy, mixed, superactive, thermic Udic-Fluvaquentic Haplustoll)], compared to the Manhattan location [Wymore silty clay loam (fine, smectitic, mesic Aquertic Argiudoll)]. Overall, from May to September, the average relative humidity was 70.44% for Manhattan and 72.36% for Haysville, the total precipitation was 498.58 mm for Manhattan and 410.19 mm for Haysville, and the average of solar radiation was 20.80 MJ/m^2 for Manhattan and 20.57 MJ/m^2 for Haysville. Supplemental irrigation was applied during a dry period in June, soon after planting at Haysville to facilitate stand establishment and early growth. Substantial rainfall events in the first half of August likely minimized the negative impacts of a dry period in the second half of the month at both locations. Vegetative growth was essentially complete at that point, so the dry period likely had a minimal impact on biomass accumulation and composition but may have affected seed fill and composition at both locations.

The small branches and leaves of received hemp biomass were removed from the stems, and only the stems were used for analysis. Stems were sequentially ground by SM 2000 cutting mill (Restsch Inc. Newton, PA) and kitchen mill (Blendtec Residential, Orem, UT) for size reduction (< 2 mm). After grinding, the samples were stored in Ziploc bags at room temperature before further use. Alkali lignin (370959-100G) was purchased from Sigma-Aldrich chemicals company (St. Louis, MO). Cellic® CTec3 (cellulase, 516 mg/mL) and NS22244 (hemicellulase, 266 mg/mL) were obtained from Novozymes (Franklinton, NC).

4.3.2. Biomass pretreatment

Biomass pretreatment was carried out in a sandbath (Techne Inc., Princeton, NJ) with continuous air blast. Four-gram biomass was loaded into the 75 mL stainless steel reactors (Swagelok, Kansas City Valve & Fitting Co., KS), followed by adding 40 mL HOAc (pH=2.25) or NaOH (pH=13.46) reagents. The selected pHs of these reagents were based on the results of the pre-experiment. After manual mingling, these reactors were instantly submerged into the sandbath at 190 ℃. When pretreatment time reached 40 min, these reactors were quenched by cold tap water to avoid further reaction. The pretreated slurry was separated into solid and filtrate by vacuum filtration coupled with Whatman filter paper. The pH values were measured in situ by an Orion Star™ A211 Benchtop pH Meter (Thermo Fisher Scientific Inc., Waltham, MA). It must be mentioned that HOAc and NaOH pretreated biomass were not subjected to post-washing.

4.3.3. Enzymatic hydrolysis

After pretreatment, HOAc and NaOH pretreated filtrates were mixed with slow shaking to precipitate the lignin and then subjected to vacuum filtration. Whereas HOAc pretreated biomass was directly mixed with NaOH pretreated biomass in the 250 mL Erlenmeyer flasks and then added the above-integrated filtrate. To verify the neutralization of residual HOAc and

NaOH in solid residues, the pH of the integrated slurry was examined. Enzymatic hydrolysis was initiated by pipetting 100 µL cellulase/g biomass and 50 µL hemicellulase/g biomass (Zhao et al., 2021a). These flasks were transferred into an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) at 49 °C with 160 rpm for 72 h. Herein, no pH adjusting for the integrated slurry was carried out.

4.3.4. Analytical procedures

The chemical composition of raw industrial hemp biomass was measured according to the National Renewable Energy Laboratory (Sluiter et al., 2008). The extracted biomass (~ 0.3000 g) was mixed with 3 mL of 72% sulfuric acid and stirred in a 30 °C water bath for 60 min. After that, 84 mL of deionized water was added to reduce the sulfuric acid concentration to 4%, and the sealed flask was then autoclaved at 121 °C for 60 min. The flask was cooled down at room temperature and filtered by a pre-weighed filter crucible. The 1.5 mL filtrate was pipetted for acid-soluble lignin (ASL) test, and 10 mL filtrate was first neutralized with 0.40 g calcium carbonate for 40 min and filtered for glucose and xylose determination. Finally, solid residue in the crucible was washed with at least 50 mL distilled water and dried overnight at 105 °C for acid-insoluble lignin (AIL) test. After that, the crucible was shifted into the Muffle furnace at 575 °C for the ash test. Monosaccharides (glucose and xylose), furfural, and HMF in the filtrate were determined by a 1260 high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA). The measurement parameters were: an HPX-87H organic acid column (7.8×300 mm) was a separation column and set at 60 °C; 0.005 M sulfuric acid was mobile phase buffer with a flow rate of 0.6 mL/min; the refractive index detector temperature was set at 45 °C (Zhao et al., 2021a). Chemical bonds of raw hemp biomass were identified by a 400 FTIR spectrophotometer (PerkinElmer Corp., Shelton, CT) equipped with a room temperature deuterated lanthanum α alanine doped triGlycine sulphate (RT-DLαTGS) detector. Background scanning was carried out for calibration before determination. The parameters such as 4000-400 cm^{-1} wavenumber range, 4 cm^{-1} resolution, 68-71 pressure intensity, and 32 scans per sample were applied (Zhao et al., 2020d). Besides, glucose, xylose, HMF, and furfural conversion efficiencies were calculated as shown in Eqs (4.1) to (4.4), while glucose, xylose, HMF, and furfural yields based on the initial raw biomass weight were calculated as shown in Eqs (4.5) to (4.8). These equations were referred to our previous studies (Zhao et al., 2020b, 2021a).

$$E_{(g)} = \frac{C_{(glucose)} \times V_{(mL)} \div 1000}{M_{(b)} \times C_{(glucan)} \div 0.9} \times 100\% \quad (4.1)$$

$$E_{(x)} = \frac{C_{(xylose)} \times V_{(mL)} \div 1000}{M_{(b)} \times C_{(xylan)} \div 0.88} \times 100\% \quad (4.2)$$

$$E_{(HMF)} = \frac{C_{(HMF)} \times V_{(mL)} \div 1000 \div MM_{(HMF)} \times MM_{(glucose)}}{M_{(b)} \times C_{(glucan)} \div 0.9} \times 100\% \quad (4.3)$$

$$E_{(furfural)} = \frac{C_{(furfural)} \times V_{(mL)} \div 1000 \div MM_{(furfural)} \times MM_{(xylose)}}{M_{(b)} \times C_{(xylan)} \div 0.88} \times 100\% \quad (4.4)$$

$$Y_{(g)} = \frac{C_{(glucose)} \times V_{(mL)} \div 1000}{M_{(b)}} \quad (4.5)$$

$$Y_{(x)} = \frac{C_{(xylose)} \times V_{(mL)} \div 1000}{M_{(b)}} \quad (4.6)$$

$$Y_{(HMF)} = \frac{C_{(HMF)} \times V_{(mL)} \div 1000}{M_{(b)}} \quad (4.7)$$

$$Y_{(furfural)} = \frac{C_{(furfural)} \times V_{(mL)} \div 1000}{M_{(b)}} \quad (4.8)$$

$E_{(g)}$, $E_{(x)}$, $E_{(HMF)}$, and $E_{(furfural)}$ are glucan-to-glucose, xylan-to-xylose, glucose-to-HMF, and xylose-to-furfural conversion efficiencies, respectively. $Y_{(g)}$, $Y_{(x)}$, $Y_{(HMF)}$, and $Y_{(furfural)}$ are glucose, xylose, HMF, and furfural yields, respectively. $C_{(glucose)}$, $C_{(xylose)}$, $C_{(HMF)}$, and $C_{(furfural)}$ are glucose, xylose, HMF, and furfural concentrations (g/L) in the slurry, respectively. $M_{(b)}$ and $V_{(mL)}$ are dry-basis solid weight and slurry volume, respectively. $C_{(glucan)}$ and $C_{(xylan)}$ are glucan and xylan contents in raw hemp biomass before pretreatment, respectively. $MM_{(HMF)}$, $MM_{(glucose)}$, $MM_{(furfural)}$, and $MM_{(xylose)}$ are the molar mass of HMF, glucose, furfural, and xylose, respectively. 0.9 and 0.88 are the transformation factor of glucan-to-glucose and xylan-to-xylose, respectively.

4.3.5. Statistical analysis

In order to elucidate the effects of genotype, planting location, and their interaction on the chemical composition of biomass and filtrate as well as chemical conversion performances, multivariate analysis of variance (ANOVA) was performed using IBM SPSS Statistics Version 27.0 (Armonk, NY, IBM Corp). In this regard, the genotype and planting location were treated as fixed factors, other variables such as glucose concentration and conversion efficiency were recognized as dependent variables. Means for significant difference were identified using the least significant difference method.

4.4. Results and discussion

4.4.1. Chemical composition of industrial hemp biomass

Effects of genotype, planting location, and their interaction on the chemical composition of industrial hemp biomass are summarized in Table 4.1. It is clear that genotype played a significant role in determining glucan, xylan, ASL, and extractives, whereas planting location had significant effects on glucan and lignin components. This is comparable with the finding that planting location had significant effects on the chemical compositions of big bluestem (Zhang et al., 2012). However, it is unexpected that the interaction between genotype and planting location had a significant influence only on extractives (Table 4.1). Glucan content of biomass from Haysville ranged from 47.29-50.05%, which were relatively higher than those (42.49-48.38%) from Manhattan (Figure 4.2A). Vega from Manhattan exhibited the lowest glucan content of 42.49%, while Hlukouskii from Haysville showed the highest glucan content of 50.05%. It can be recognized that roughly compared to grain straws (31-39%) (Tian et al., 2018) and corn stover (31.0-41.2%) (Zhao et al., 2020a), industrial hemp biomass obtained from this work was composed of higher glucan content. Xylan content for all the genotypes fell in the range of 11.70-13.88% with the lowest xylan content being Hlukouskii from Haysville and the highest xylan content being Rigel from Manhattan (Figure 4.2A). Total carbohydrates (glucan and xylan) accounted for 56-63%. These results are comparable to the previous studies, where different genotypes of industrial hemp biomass were investigated (Xu et al., 2016; Zhao et al., 2020a). The lignin content of industrial hemp biomass has been reported to differ dramatically from 15 to 30%, depending on the genotypes (Xu et al., 2016). In this work, it was noticed that there was a slight variation in ASL (0.47-0.50%) and AIL (9.96-14.67%) lignin content among the biomass samples, with the total lignin content ranging from 10.45 to 15.14%. Extractives of four industrial hemp biomass ranged from 11.63 to 18.36% (Figure 4.2A). In contrast, Das et al. (2020) reported that eleven industrial hemp biomass genotypes contained 43.8-50.1% of glucan, 11.6-14.2% of xylan, 15.4-29.4% of lignin, and 5.5-11.9% of extractives. Additionally, the differences in chemical bonds among the four industrial hemp biomass harvested at Haysville and Manhattan were characterized by FTIR spectrum (Figure 4.2B). It was found that peaks at 1590 cm^{-1} (C=C stretching from aromatic skeletal vibration) and 1514 cm^{-1} (aromatic skeleton of lignin) in Rigel from Manhattan were slightly more intense than biomass from other genotypes and locations (Figure 4.2B). It is consistent with the relatively higher lignin content (Figure 4.2A). It was also noticed that other peaks showed no visible variations among the biomass samples (Figure 4.2B).

4.4.2. pH and chemical composition of filtrate

Due to the severer pH of acid and alkali pretreated filtrate than the buffer, adequate water washing followed by direct wastewater discarding was generally observed (de Vega and Ligero, 2017; Pagnani et al., 2018; Xie et al., 2017). For example, a previous study reported that 160 mL water/g raw biomass was required to clarify the waste liquor from NaOH and hydrothermal pretreatment of coconut fiber and reduce their pH to neutral (da Costa Nogueira et al., 2018). Herein, mixing the HOAc and NaOH pretreated filtrate was performed to utilize it as a buffer.

It was found that genotype, planting location, and interaction between genotype and planting location had significant effects on the pH of filtrate (Table 4.1). The initial pHs for HOAc and NaOH reagents used for biomass pretreatment were 2.25 and 13.46, respectively. After pretreatment, the pretreated filtrate showed slight variations in terms of pH, ranging from 2.91 to 2.99 (HOAc) and 12.96 to 13.15 (NaOH) for biomass from Haysville and from 3.03 to 3.08 (HOAc) and 13.05 to 13.20 (NaOH) for biomass from Manhattan (Figure 4.3). The slight variation in pH is attributed to the heterogeneous distribution of hemicellulose among different biomass samples (Zhao et al., 2020b). Compared to the starting pH of reagents, the mitigation of pH in the filtrate is also attributed to the dilution caused by adding biomass. As mentioned above, the resultant filtrate with the extreme pH is unable to be utilized for efficient enzymatic hydrolysis. However, the pH severity was attenuated through the integration of HOAc and NaOH pretreated filtrate (Figure 4.3), as the pHs of the integrated filtrate were 4.78-4.82 for biomass from Haysville and 4.79-4.92 for biomass from Manhattan, indicating that a neutralization reaction was performed between acids and NaOH in the filtrate. Furthermore, the black liquor obtained from NaOH pretreatment turned into a brown color, when it was mixed with HOAc pretreated filtrate with the precipitation of lignin. In comparison to the complicated and high-cost poly(ether)sulfone-based ultrafiltration membrane filter (Kim et al., 2020), this simple integration process might be more feasible to alleviate the pH of filtrate and utilize the residual chemicals.

The monosaccharides derived from HOAc and NaOH pretreatments of four genotypes of hemp biomass in the filtrate were tracked by HPLC (Figure 4.4). A comparison of sugar concentrations in the pretreated filtrate often discloses the variances in the biomass recalcitrance (Zhao et al., 2021a). For HOAc pretreatment, the highest and lowest glucose concentration of 1.77 g/L and 0.77 g/L was generated by Rigel from Haysville and Vega from Manhattan, respectively, and the highest and lowest xylose concentration of 4.82 g/L and 2.16 g/L was obtained by Hlukouskii and Rigel from Manhattan, respectively (Figure 4.4B). In the case of NaOH pretreatment, no glucose was found in the filtrate for Anka from Haysville or Anka and Vega from Manhattan. Carbohydrates of biomass are more susceptible to confront the pretreatment with acid rather than NaOH reagent (Zhao et al., 2020b), which is in agreement with the finding that HOAc pretreatment showed 0.16-1.12 g/L of glucose and 1.43-4.41 g/L of xylose higher than NaOH pretreatment. Based on the high sugar concentrations in the filtrate, direct disposal can certainly cause sugar loss. The final glucose and xylose concentrations in the integrated filtrate ranged from 0.84 to 1.64 g/L and 1.31 to 2.55 g/L, respectively, depending on the genotype and planting location of industrial hemp biomass.

Due to the inhibitory compounds, physical, chemical, and biological technologies including membrane filtration, neutralization reaction, and activated charcoal adsorption have been promoted to render biomass filtrate amenable to enzymes and microbes (Kumar et al., 2020). Furfural and HMF, decomposed from hemicellulose and cellulose, respectively, are mainly recognized to inhibit microbial growth. Unlocked lignin units were informed to absorb on the surfaces of cellulose and hemicellulose tightly to circumvent enzymatic accessibility (Li et al., 2018). Also, lignin-derived phenolic compounds can bring a severe inhibitory effect on enzymatic hydrolysis (Chen et al., 2020) and lead to incompatibility of biological membranes (Jung and Kim, 2017). It was discerned that HMF and furfural in HOAc pretreated filtrate ranged from 1.69 to 2.41 g/L and from 2.51 to 3.68 g/L, respectively (Figure 4.4). The highest HMF and furfural concentration was acquired by Hlukouskii and Rigel from Haysville, and the lowest HMF and furfural concentration was obtained by Anka and Hlukouskii from Manhattan. However, NaOH pretreatment prompted no HMF and furfural formation in the filtrate (Figure 4.4). After integration, the concentrations of sugar-derived inhibitors that originated from the HOAc pretreated filtrate were relieved: HMF and furfural were in the range of 0.71-0.95 g/L and 0.64-1.25 g/L, respectively (Figure 4.4). These results indicate that this simple integration approach is multipurpose to associate the strengths of acid-base pretreatments and to solve the bottleneck of inhibitors in a practical path.

4.4.3. Lignin recovery and FTIR characteristic

Tremendous attention has been attracted to reduce the resistance of biomass enhancing its accessibility to enzymes and microorganisms, while additional byproducts, especially lignin, have rarely been recovered qualitatively (Huang et al., 2020). However, the conversion of biomass-based lignin to polymeric materials might offer potential values for biomass upgrades (Upton and Kasko, 2016). In this work, the lignin was precipitated after integrating HOAc and NaOH pretreated filtrate and then subjected to vacuum filtration and oven drying. Lignin recoveries based on raw biomass and its FTIR characteristics are compared with commercial alkali lignin as a control (Figure 4.5). It was found that planting location and interaction between genotype and planting location showed significant effects on lignin recovery (Table 4.2). Industrial hemp biomass could be differentiated in terms of its lignin recovery: 50.63-57.50 g lignin per kg biomass from Haysville, 56.88-73.13 g lignin per kg biomass from Manhattan, and Rigel being the highest from Manhattan (Figure 4.5A).

The FTIR spectrum of the isolated lignin fraction was compared with commercial alkali lignin. For biomass from Haysville, only Vega showed similar intensities of peaks at 1592 and 1506 cm^{-1} (assigned to the vibration of aromatic rings), 1461 cm^{-1} (assigned to the methoxyl C-H bending and C-C stretching in the aromatic skeleton), 1265 cm^{-1} (assigned to the aromatic C-O stretching of syringyl units and/or condensed guaiacyl units), 1120 cm^{-1} (assigned to the syringyl units), 1030 cm^{-1} (assigned to C-OH and C-O-C stretching of the side groups and glycosidic bonds), and 830 cm^{-1} (assigned to the guaiacyl units) with the commercial alkali lignin (Figure 4.5B). For biomass from Manhattan, only Anka exhibited analogous intensities of peaks at 1592, 1506, 1120, 1030, and 830 cm^{-1} compared to the commercial alkali lignin (Figure 4.5B). These results indicate that lignin from Vega (Haysville) and Anka (Manhattan) might have comparable chemical structures with the commercial alkali lignin.

4.4.4. Enzymatic hydrolysis of integrated biomass and filtrate

Rinsing pretreated biomass with adequate water, followed by adding a fresh buffer solution (sodium acetate, citrate, and phosphate), has been typically applied for enzymatic hydrolysis. This conventional process inevitably causes solid-liquid imbalance, especially resulting in the accumulation of wastewater. Herein, we emphasize that HOAc and NaOH pretreated solid biomass were first mixed without post-washing, and the integrated filtrate was loaded and homogeneously stirred without pH adjusting. As expected, the neutralization reaction also happened between the HOAc and NaOH pretreated biomass, evidenced by the final slurry pHs ranging from 4.60 to 4.65 for biomass from Haysville and from 4.65 to 4.70 for biomass from Manhattan (Figure 4.6A). Thus, the resulting pH is suitable for enzymatic hydrolysis without pH adjusting and new buffer solution addition, avoiding wastewater discarding and extra chemical consumption. Besides, planting location and interaction between genotype and planting location had significant influences on the pH of the integrated slurry (Table 4.1).

After enzymatic hydrolysis, the slurry was subjected to centrifugal separation to segregate solid from hydrolysates. The chemical concentrations were quantitatively determined by HPLC (Figure 4.6B), and their conversion efficiencies and yields were calculated as shown in Table 4.3. It was observed that genotype, planting location and their interaction exhibited significant impacts on chemical conversion performances (Table 4.2). Industrial hemp biomass from Haysville reached glucose concentrations of 41.97-45.71 g/L, which were slightly higher than those from Manhattan (40.40-44.38 g/L) (Figure 4.6B), but there was no similar trend found for glucan-to-glucose conversion efficiencies: 72.97-75.33% and 71.19-77.71% for industrial hemp biomass from Haysville and Manhattan, respectively (Table 4.3). The highest glucose concentration and conversion efficiency were achieved by Hlukouskii (45.71 g/L) from Haysville and Vega (77.71%) from Manhattan, respectively. In terms of xylose, the integration process attained xylose concentrations of 7.09-8.88 g/L (Figure 4.6B), with their conversion efficiencies of 45.42-52.03% (Table 4.3). The highest xylose concentration and conversion efficiency were achieved by Rigel (8.88 g/L) from Manhattan and Hlukouskii (52.03%) from Haysville, respectively. In contrast, Das et al. (2020) reported that dilute sulfuric acid pretreatment of eleven industrial hemp biomass only reached 30% of xylose conversion efficiencies (maximum potential based on raw biomass). On the other hand, HMF (0.79-1.25 g/L) and furfural (0.99-1.59 g/L) were remained in the final hydrolysates (Figure 4.4B), corresponding with 1.96-2.95% (maximum potential based on glucose-to-HMF) and 10.00-14.65% (maximum potential based on xylose-to-furfural) conversion efficiencies, respectively (Table 4.3). Regarding the conversion performance based on raw biomass, sugar concentration and conversion efficiencies obtained in the present work were superior to those in the previous report (Zhao et al., 2020a), where industrial hemp biomass pretreated by steam, sulfuric acid, or NaOH was subjected to remarkable water washing and drying, followed by enzymatic hydrolysis with new buffer solution under low (7.5% <) solid loading. The chemical yields (g/kg biomass) based on the raw biomass were also summarized in Table 4.3. Given 1.0 kg of raw dried biomass used for HOAc (500 g) and NaOH (500 g) pretreatments, 366.85-418.89 g of glucose, 64.04-80.84 g of xylose, 7.14-11.46 g of HMF, and 8.94-14.53 g of furfural could be obtained, in addition to 50.63-73.13 g of lignin. Among the four genotypes harvested from Haysville and Manhattan, the most and least productive genotype is Rigel and Vega, respectively (Table 4.3).

4.4.5. Relationship between biomass composition and chemical conversion performance

To our knowledge, this is the first report on studying the relationship between chemical composition (glucan, xylan, and lignin) of raw biomass and the concentration and conversion efficiency of hydrolysates (glucose, xylose, HMF, and furfural), although a previous study by Xu et al. (2016) reported that glucan and lignin contents in the ionic liquid pretreated rice straw had a positive linear correlation and negative linear correlation with sugar digestibility. Herein, it was observed that glucan content showed a positive linear correlation (r = 0.81) with glucose concentration (Figure 4.7A), xylan content showed a positive linear correlation (r = 0.72) with furfural concentration (Figure 4.7B), and lignin content showed a negative linear correlation (r = -0.73) with HMF concentration (Figure 4.7C). Das et al. (2020) found no obvious correlation between lignin content and sugar conversion efficiencies among the eleven hemp biomass that had notable variation in lignin contents. Besides, glucan content had a moderate correlation (r = 0.65) with HMF concentration; lignin content had a moderate correlation (r = 0.67) with HMF conversion efficiency; glucose conversion efficiency had a moderate correlation (r = -0.58) with furfural concentration and conversion efficiency; xylose conversion efficiency had moderate correlation with glucose concentration (r = 0.60), xylose concentration (r = 0.53), and HMF concentration (r = 0.54); and glucose concentration had a moderate correlation (r = 0.61) with HMF concentration (Fig. 7D). This relatively weak correlation between the composition of raw industrial hemp biomass and chemical conversion parameters indicates that non-quantitative factors are playing a role in the biomass-to-chemicals conversion interdependently.

4.6. Conclusions

Parallel HOAc and NaOH pretreatments with solid and liquid integration were demonstrated to produce glucose, xylose, HMF, furfural, and lignin without washing and wastewater discarding. Genotypes and planting locations had significant effects on bioproduct conversion performances. Industrial hemp biomass harvested from Haysville had relatively higher glucan contents (47.29-50.05%) than those (42.49-48.38%) from Manhattan. Integrating HOAc and NaOH integrated filtrate reached the pH of 4.78-4.92, precipitating the soluble lignin (50.63-73.13 g/kg biomass). The recovered lignin from Vega (Haysville) and Anka (Manhattan) showed almost comparable FTIR characteristics with the commercial alkali lignin. Combining HOAc and NaOH pretreated biomass and their mixed filtrate for enzymatic hydrolysis achieved 40.40-45.71 g/L of glucose and 7.09-8.88 g/L of xylose. Besides, high-value HMF (0.79-1.25 g/L) and furfural (0.99-1.59 g/L) remained in the final hydrolysate. The higher glucose (71.19-77.71%) and xylose (45.42-52.03%) conversion efficiencies validate the potential of this integrated process to reduce water and chemical consumption. Based on mass balance, the most and least productive genotypes were Rigel and Vega, respectively. Glucan and xylan contents in the biomass showed a positive linear correlation with glucose and furfural concentration, respectively; whereas lignin content showed a negative linear correlation with HMF concentration. Additionally, increasing the solid loading of biomass and screening of low-cost acidic catalysts for pretreatment could be new perspectives for further explorations.

Table 4.1 Mean squares from multi-factor analysis of variance for the effects of genotype, planting location, and their interaction between genotype and planting location on the chemical composition of raw biomass and pH of pretreated and integrated filtrate.

Source of variation	df	Chemical composition of biomass (%)					pH of filtrates		
		Glucan	Xylan	ASL[1]	AIL[2]	Extractives	HOAc-treated	NaOH-treated	Integrated slurry
Genotype	3	13.08*	2.16**	0.00	5.92**	23.64**	0.00**	0.03**	0.00
Planting location	1	36.60**	0.20	0.00*	7.84**	0.90	0.05**	0.04**	0.01**
Genotype x location	3	2.63	0.27	0.00	1.12	5.21**	0.00**	0.01**	0.00*

[1]ASL: acid-soluble lignin; [2]AIL: acid-insoluble lignin. Integrated slurry refers to the mixture of pretreated biomass and filtrate. * and ** are statistically significant at $P < 0.05$ and $P < 0.01$ probability, respectively.

Table 4.2 Mean squares from multi-factor analysis of variance for the effects of genotype, planting location, and their interaction between genotype and planting location on the concentration and conversion efficiency of chemicals obtained from simultaneous enzymatic hydrolysis of integrated biomass and filtrate.

Source of variation	df	Lignin recovery	Chemical concentration (g/L)				Conversion efficiency (%)			
			Glucose	Xylose	HMF[1]	Furfural	Glucose	Xylose	HMF	Furfural
Genotype	3	21.00	11.64**	0.74**	0.01	0.16**	10.62**	14.23**	0.03	7.51**
Planting location	1	172.20*	12.92**	0.10**	0.20**	0.09*	6.23**	0.48	0.65**	10.61**
Genotype x location	3	113.17*	2.30**	0.46**	0.03	0.02	8.12**	18.80**	0.18**	1.33

[1] HMF: hydroxymethylfurfural. * and ** are statistically significant at $P < 0.05$ and $P < 0.01$ probability, respectively.

Table 4.3 The conversion efficiency (%, based on original component) and yield (g/kg-biomass) of chemicals obtained from simultaneous enzymatic hydrolysis of integrated biomass and filtrate.

Biomass	Conversion efficiency (%)				Yield (g/kg biomass)			
	Glucose	Xylose	HMF[1]	Furfural	Glucose	Xylose	HMF	Furfural
				Haysville				
Anka	73.40 ± 0.25b	45.42 ± 0.18a	2.38 ± 0.15ab	14.65 ± 1.01b	405.28 ± 1.37c	70.47 ± 0.27b	9.19 ± 0.59bc	14.53 ± 1.01b
Rigel	74.10 ± 0.18bc	47.05 ± 0.15a	2.61 ± 0.04bc	14.52 ± 0.33b	405.91 ± 1.00c	71.08 ± 0.23b	10.00 ± 0.14cd	14.01 ± 0.32b
Vega	72.97 ± 0.13b	45.87 ± 0.21a	2.39 ± 0.16abc	12.22 ± 1.23ab	383.37 ± 0.69b	69.20 ± 0.32b	8.82 ± 0.59abc	11.78 ± 1.19ab
Hlukouskii	75.33 ± 0.19cd	52.03 ± 0.97b	2.95 ± 0.02c	11.14 ± 0.27ab	418.89 ± 1.05d	69.20 ± 1.28b	11.46 ± 0.09d	9.49 ± 0.23a
				Manhattan				
Anka	71.19 ± 0.05a	46.20 ± 0.09a	2.13 ± 0.03ab	12.88 ± 0.57ab	370.51 ± 0.27a	68.91 ± 0.14b	7.76 ± 0.09ab	12.33 ± 0.55ab
Rigel	75.12 ± 0.77cd	51.26 ± 0.32b	2.18 ± 0.08ab	11.31 ± 0.59ab	403.84 ± 4.14c	80.84 ± 0.50c	8.23 ± 0.32abc	11.42 ± 0.59ab
Vega	77.71 ± 0.00e	45.66 ± 0.15a	2.43 ± 0.14abc	11.73 ± 0.18ab	366.85 ± 0.00a	70.15 ± 0.23b	7.99 ± 0.45abc	11.53 ± 0.18ab
Hlukouskii	76.77 ± 0.06de	45.85 ± 0.00a	1.96 ± 0.02a	10.00 ± 0.20a	396.76 ± 0.32c	64.04 ± 0.00a	7.14 ± 0.09a	8.94 ± 0.18a

[1] HMF: hydroxymethylfurfural. Data: means ± standard deviations. In each column, means with different letters are significantly different at $P < 0.05$.

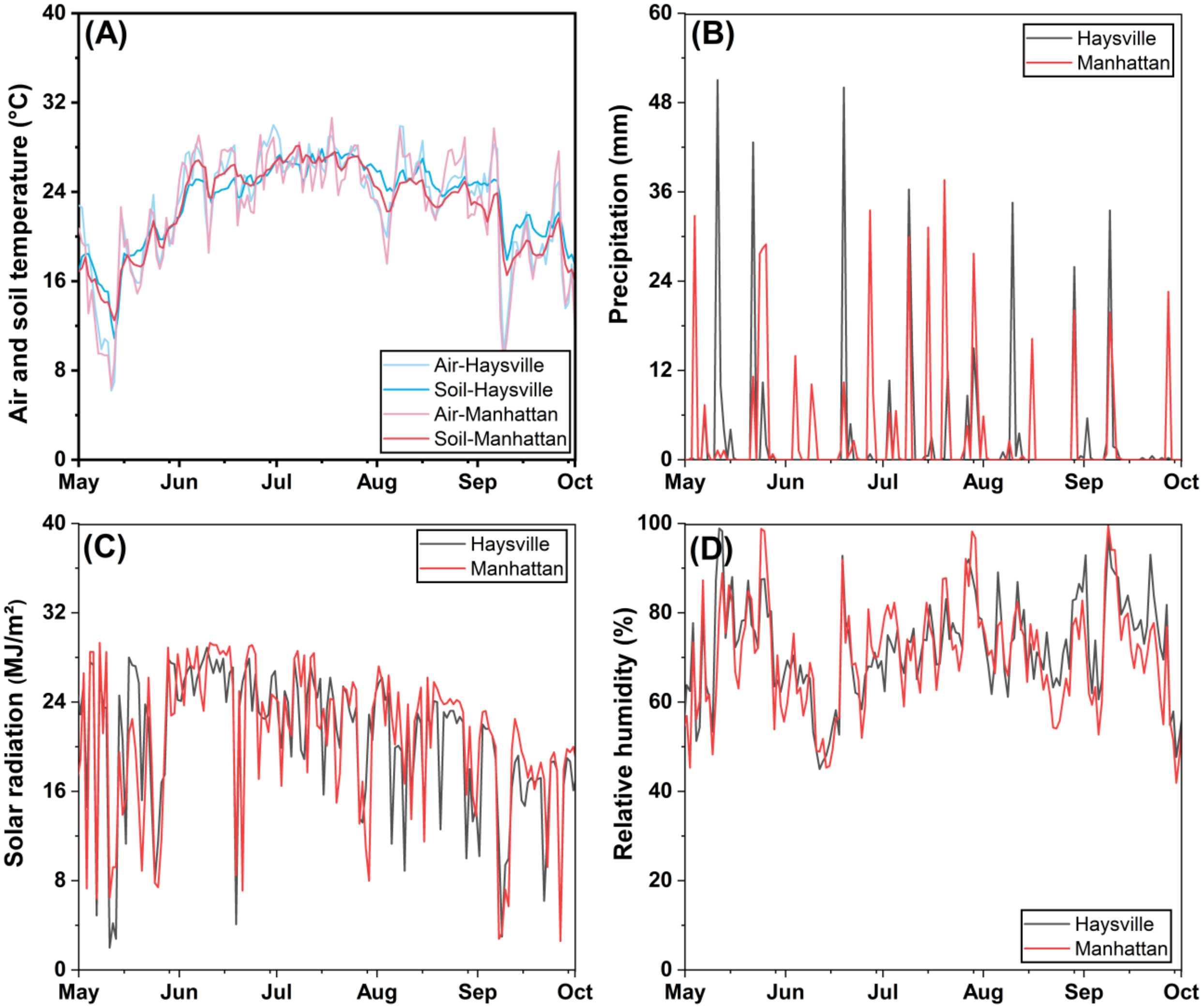

Figure 4.1 Detailed weather data from May 1st to October 1st (Kansas Mesonet, 2020: Kansas Mesonet Historical Data. Accessed 12 March 2020, http://mesonet.k-state.edu/weather/historical) at Haysville and Manhattan where four genotypes of industrial hemp were cultivated.

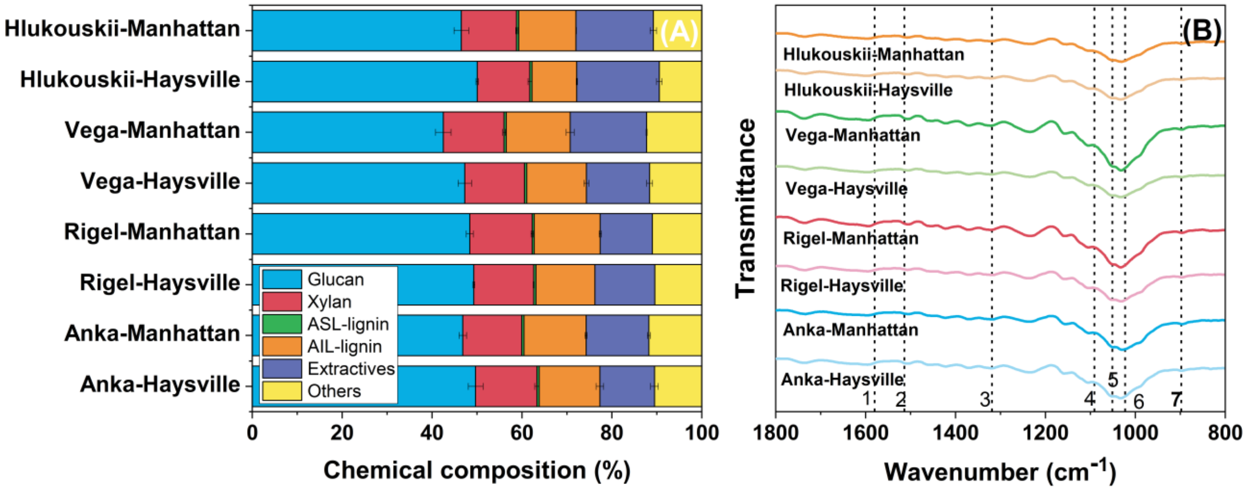

Figure 4.2 Chemical composition (A) and FTIR spectrum (B) of four genotypes of industrial hemp biomass that were harvested at Haysville and Manhattan (ASL: acid soluble lignin; AIL: acid insoluble lignin).

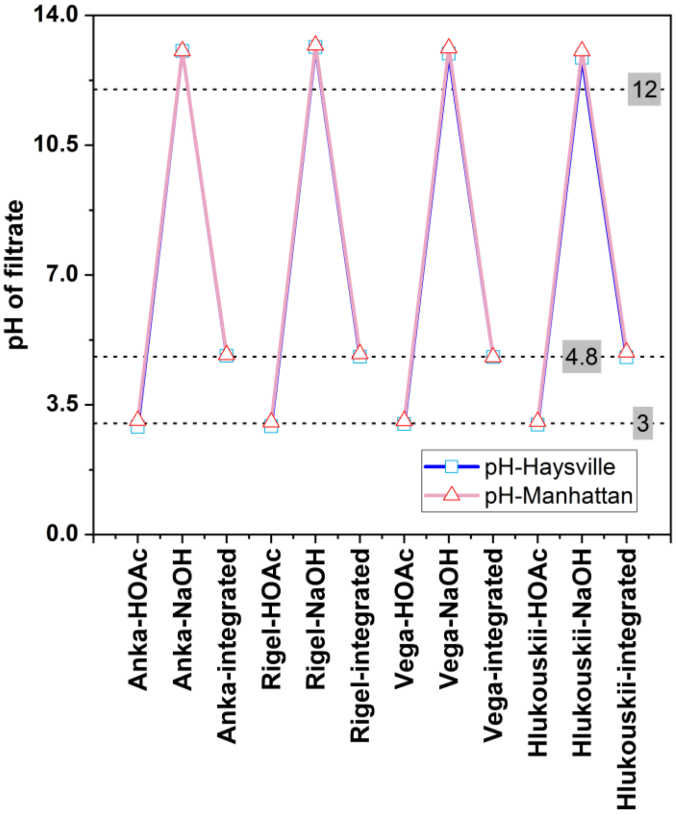

Figure 4.3 The pH of acetic acid (HOAc) and sodium hydroxide (NaOH) pretreated filtrate before and after the integration process.

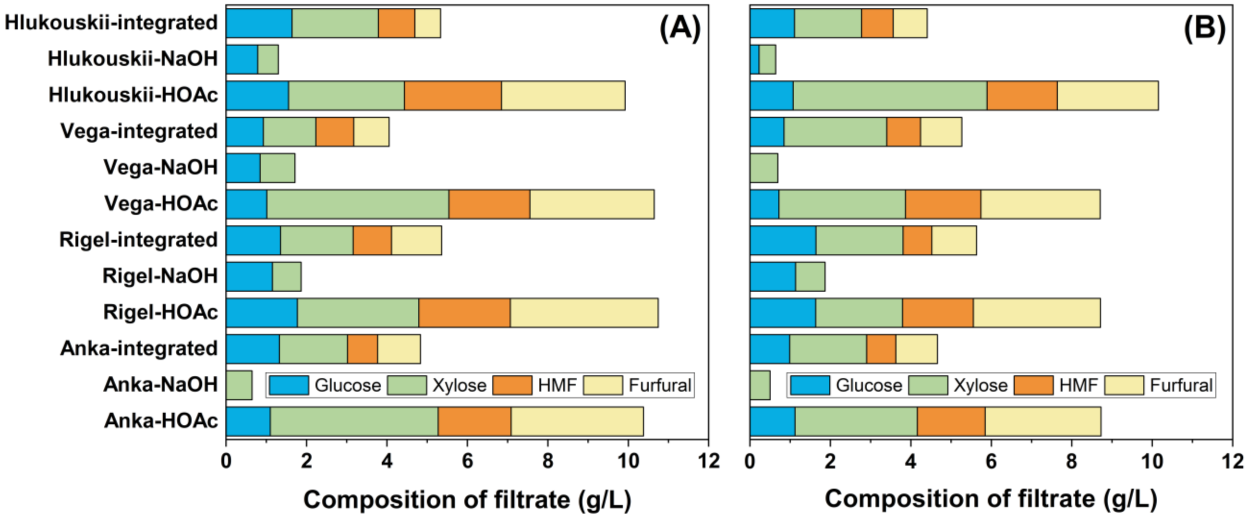

Figure 4.4 Glucose, xylose, hydroxymethylfurfural (HMF), and furfural concentrations in HOAc and NaOH pretreated filtrate before and after the integration process (A: Haysville; B: Manhattan).

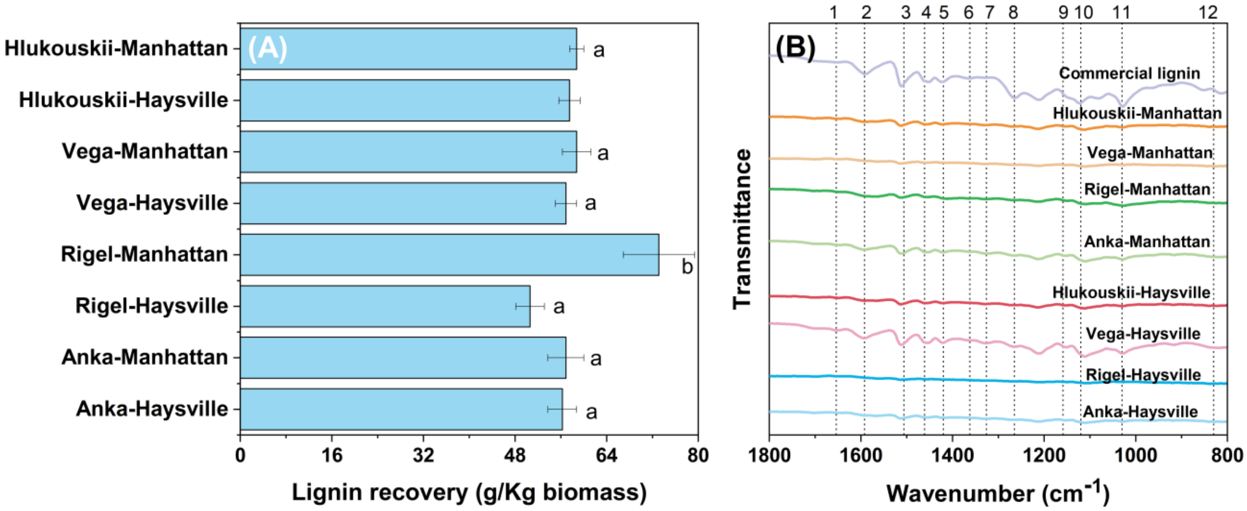

Figure 4.5 Lignin recovery (A) and Fourier transform infrared spectroscopy characterization (B) with the commercial alkali lignin used as a control. The mean difference is significant at $P < 0.05$.

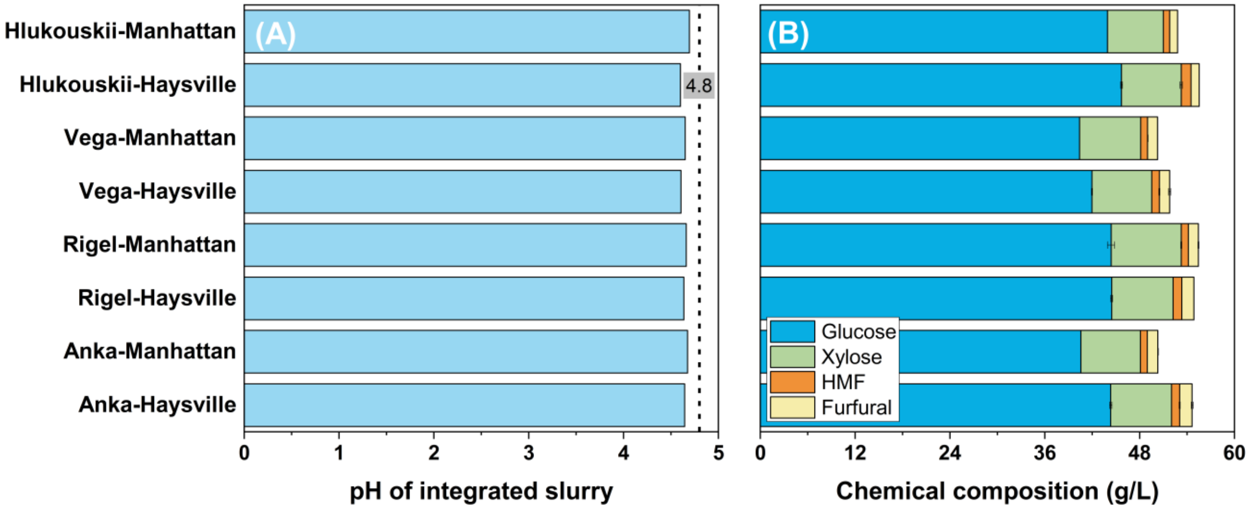

Figure 4.6 The pH of integrated slurry including pretreated biomass and filtrate (A) and concentrations of glucose, xylose, hydroxymethylfurfural (HMF), and furfural in the hydrolysate after 72-h enzymatic hydrolysis (B).

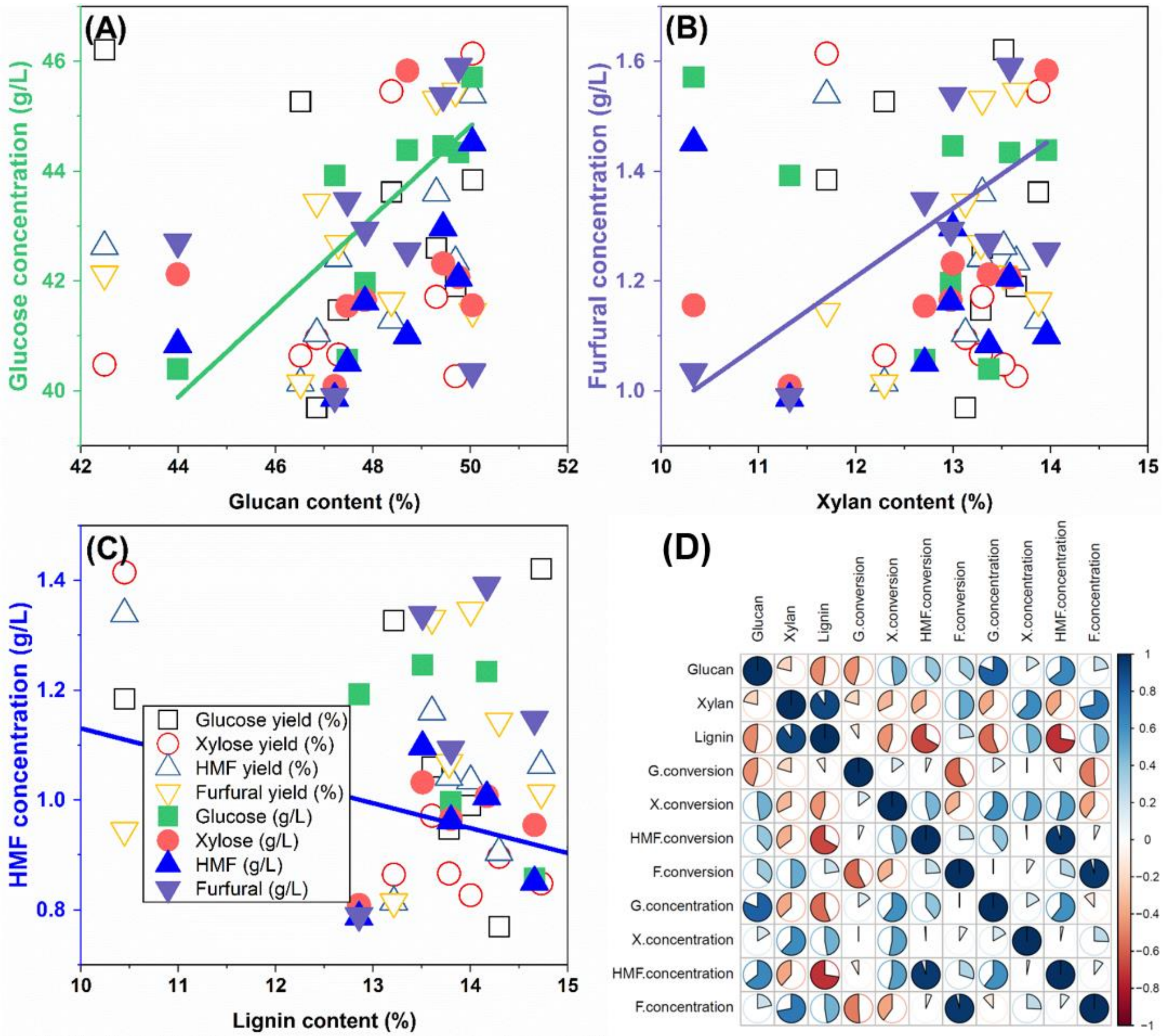

Figure 4.7 The linear relationships between composition (A: glucan; B: xylan; and C: lignin) of raw industrial hemp biomass and chemical conversion performances as well as correlation coefficients among these parameters (D). The abbreviations are denoted as: G is glucose, X is xylose, HMF is hydroxymethylfurfural, and F is furfural.

Chapter 5 - Sugar, furan, and bioethanol production under high biomass loading without solid-liquid separation

5.1. Abstract

Three pretreatment scenarios (I: H_2SO_4 pretreatment with NaOH neutralization; II: NaOH pretreatment with H_2SO_4 neutralization; and III: parallel H_2SO_4 and NaOH pretreatments following their integration) with enzymatic hydrolysis were performed for glucose, xylose, 5-hydroxymethylfurfural (HMF), and furfural production at high solid (15 and 25%, w/v) loading without solid-liquid separation and further detoxification. With an initial solid loading of 25% (w/v), scenario I reached the highest furfural (4.94 g/L) and HMF (2.82 g/L) concentrations, scenario II achieved the highest glucose (73.25%) and xylose (77.49%) yields, while scenario III displayed the highest sugar concentration (74.53 g/L). Only the hydrolysate from NaOH pretreatment and enzymatic hydrolysis with 10% initial solid loading can be efficiently fermented to ethanol (17.92 g/L) by the traditional yeast.

5.2. Introduction

Bioproducts derived from lignocellulosic biomass have a great potential to replace fossil-based chemicals and energy materials. To unlock the recalcitrant biomass, thermochemical pretreatment is an inevitably critical step (Elgharbawy et al., 2016; Haghighi Mood et al., 2013; Kim et al., 2016; Xu et al., 2020; Zhuang et al., 2016). During pretreatment, cellulose, hemicellulose, and lignin can be partially degraded and solubilized into the liquid fraction (Cocero et al., 2018; Elgharbawy et al., 2016; Haghighi Mood et al., 2013; Kim et al., 2016; Singh et al., 2015; Usmani et al., 2020; Wang et al., 2021; Xu et al., 2020; Zhao et al., 2009; Zhuang et al., 2016). The pretreated solid fraction is often separated from the liquid fraction and washed with excessive water and then mixed with a fresh buffer solution for enzymatic hydrolysis, while the resultant liquid fraction is often discarded (Zhao et al., 2021a, 2021b). This conventional process induces water overconsumption and chemicals loss. The main reason for wastewater disposal is that derivatives (such as acids, furans, and phenols) and residual chemicals (such as acids, alkalis, ionic liquids, organic solvents, and deep eutectic solvents) from initial pretreatment reagents are detrimental to enzymes and strains (Bhatia et al., 2020; Kumar et al., 2020; Sivagurunathan et al., 2017). For high-cost solvents (such as ionic liquids, organic solvents, and deep eutectic solvents) used for pretreatment, it is cost-effective to recover them from the liquid fraction. In this respect, bioproduct minimum selling price (MSP) was reported to be sensitive to the recycling rates of solvents (Klein-Marcuschamer et al., 2011; Zang et al., 2020b). Regarding the economic aspects, low-cost dilute sulfuric acid (H_2SO_4) pretreatment is highly recommended by the National Renewable Energy Laboratory for commercial development (Humbird et al., 2011).

Low solid loading (≤ 10%, w/v) for biomass pretreatment and enzymatic hydrolysis has been widely used to promote pretreatment technology with the expected higher sugar yield (Zhao et al., 2020a). However, from an economic standpoint, maintaining high solid loading is favored due to its envisioned lower water consumption and higher sugar concentration (Da Silva et al., 2020; Humbird et al., 2010; Modenbach and Nokes, 2012). A review of the literature shows that solid loading used for pretreatment can reach up to 200% (w/v), but enzymatic hydrolysis of the pretreated biomass was commonly performed under 10% (w/v) solid loading to promote the pretreatment technology (Chen et al., 2018; Dong et al., 2019; Luterbacher et al., 2010; Wang et al., 2018). Seemingly, these studies have great potential to diminish water consumption, but the phenomenon of excessive water washing and wastewater discarding after pretreatment would contradict the original idea of reducing water consumption. Nevertheless, the side effects such as weak pretreatment effectiveness, inhibitor accumulation, and high slurry viscosity were also observed (Da Silva et al., 2020; Koppram et al., 2014; Modenbach and Nokes, 2012). Batch and fed-batch models have been applied to prompt enzymatic hydrolysis over 50% (w/v) solid loading (Chen and Liu, 2017; Cheng et al., 2020; Raj and Krishnan, 2019; Sui et al., 2021; Xing et al., 2016; Ying et al., 2021). In this regard, biomass-water and lignin-enzyme interactions often result in low sugar yield and extend the hydrolysis time, which is recognized as "high-solid side effect" (Cheng et al., 2020; Da Silva et al., 2020; Koppram et al., 2014; Modenbach and Nokes, 2012; Weiss et al., 2019). Herein, the question, whether the higher solid loading used for biomass pretreatment and enzymatic hydrolysis is better, should be taken into consideration. Additionally, guaranteeing mass balance is a prerequisite for continuous industrial production. Therefore, in keeping with the objective of reducing water consumption and production cost, sustaining high solid loading from pretreatment to enzymatic hydrolysis without solid-liquid separation is required. However, few studies have been experimentally investigated this approach.

To reduce water consumption and chemical loss, three pretreatment methods were experimentally designed and implemented under high solid loading without post-washing, wastewater discarding, and new buffer addition. Pretreatment effectiveness was elucidated by biophysical techniques including scanning electron microscope (SEM), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy as well as compositional analysis. The potential of hydrolysate for yeast fermentation was also illustrated.

5.3. Materials and method

5.3.1. Materials

Industrial hemp biomass was harvested from Kansas State University Agricultural Farm (Manhattan, KS). After cutting off branches and leaves, stems were dried at 49 °C for 72 h and then sequentially pulverized by an SM 2000 cutting mill (Restsch Inc. Newton, PA) and kitchen mill (Blendtec Residential, Orem, UT) to achieve size (< 2 mm) reduction (Zhao et al., 2021a, 2021b). Cellulase (Cellic® CTec3) and hemicellulase (NS22244) were provided by Novozymes (Franklinton, NC). The chemicals were purchased from Thermo Fisher Scientific Chemicals Inc. (Ward Hill, MA).

5.3.2. Biomass pretreatment

4.5 or 7.5 g of biomass was poured into the 75 mL stainless steel reactor (Swagelok, Kansas City Valve & Fitting Co., KS), followed by filling 30 mL H_2SO_4 (pH=1.12, around 0.08 mol/L) or sodium hydroxide (NaOH, pH=13.53, around 0.34 mol/L) to form 15% or 25% (w/v) solid loading. It should be noted that the highest solid loading in the present laboratory conditions was limited to 25% (w/v) according to the pre-experimental results. These reactors were instantly immersed into a pre-heated 190 ℃ sandbath (Techne Inc., Princeton, NJ) coupled with continuous air blast for 40 min (Zhao et al., 2021a, 2021b). After pretreatment, cold tap water was used to quench the further reaction. Herein, 15 or 7.5 mL of water was poured into the reactor to wash out the pretreated biomass because the limited availability of liquid posed a significant challenge for slurry collection after pretreatment. For single H_2SO_4 or NaOH pretreatment, the pH of the pretreated slurry was adjusted by 10% (w/w) H_2SO_4 or 10 mol/L NaOH to 4.50, while for combined pretreatment the slurries from H_2SO_4 and NaOH pretreatments were mixed to reach the pH around 4.50 (Zhao et al., 2021b). To elucidate the pretreatment effectiveness, the slurry was then filtered into a solid and liquid fraction using vacuum filtration with Whatman filter paper (Zhao et al., 2021a). The solid fraction was oven-dried at 49 ℃ overnight for compositional and structural evaluation, whereas the liquid fraction was ultra-filtered for compositional analysis.

5.3.3. Enzymatic hydrolysis and fermentation

100 µL Cellic CTec3/g biomass and 50 µL NS22244/g biomass were added to the slurry without solid-liquid separation and further detoxification. These flasks were incubated in an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) at 49 °C with an agitation speed of 150 rpm for 72 h (Zhao et al., 2020b, 2020d). During hydrolysis, 80 µL of supernatant was pipetted every 12 h from each flask for sugar determination. Because the extra water was used to collect the pretreated biomass, the final solid loading of initial biomass relative to solvent was 10% or 20% (w/v). Additionally, the fermentation potential of hydrolysate from different

pretreatment processes was explored. The activated yeast (Ethanol Red, Lesaffre, Milwaukee, WI, USA) culture was firstly prepared following our previous study (Zhao et al., 2020b). The flask containing 10 mL of hydrolysate was inoculated about 5×10^8 cells and then incubated at 37 °C in an orbital shaker (I2400 Incubator Shaker, New Brunswick, USA) with 150 rpm for 72 h. After fermentation, 80 μL of supernatant was sampled for compositional measurement.

5.3.4. Structural characterization and analytical procedures

Changes in microstructure, crystallinity, and chemical bond of biomass before and after pretreatment were determined by S-3500 Scanning Electron Microscope (Hitachinaka, Ibaraki, Japan), Siemens D-5000 Diffractometer (Bruker, Ettlingen, Germany) with Cu-K radiation, and Fourier Transform Infrared Spectroscopy, respectively. In this regard, the parameters and operational details can be found in the previous studies (Zhao et al., 2021a, 2021b). Chemical composition of the solid fraction was measured following the procedures from the National Renewable Energy Laboratory (Sluiter et al., 2008). The concentrations of glucose, xylose, furfural, and HMF in the liquid fraction and hydrolysate were tested by a 1260 high-performance liquid chromatography system (Agilent, Santa Clara, CA) coupled with an HPX-87H organic acid separation column (7.8×300 mm). The temperature of the column and refractive index detector was set at 60 and 45 °C, respectively. 5 mM H_2SO_4 buffer with a flow rate of 0.6 mL/min was used as the mobile phase.

5.3.5. Statistical analysis

In order to elucidate the effects of genotype, planting location, and their interaction on the chemical composition of biomass and filtrate as well as chemical conversion performances, multivariate analysis of variance (ANOVA) was performed using IBM SPSS Statistics Version 27.0 (Armonk, NY, IBM Corp). In this regard, the genotype and planting location were treated as fixed factors, other variables such as glucose concentration and conversion efficiency were recognized as dependent variables. Means for significant difference were identified using the least significant difference method.

5.4. Results and discussion

5.4.1. Composition of solid and liquid fraction

Table 5.1 shows the principal chemical composition of raw and pretreated biomass from different pretreatment conditions. Compared to raw biomass, glucan (56.35-64.45%) and lignin (acid-soluble lignin: 1.18-1.59%; acid-insoluble lignin: 18.56-35.48%) contents increased in the pretreated biomass, while xylan (2.41-11.09%) and other (2.88-7.80%) contents decreased. An increase in glucan and decrease in xylan contents of biomass after pretreatment is in agreement with the previous study where hemp and poplar biomass were pretreated by acetic acid or NaOH at solid loading of 10% (w/v) without post-washing and pH adjusting (Zhao et al., 2021a). The relative enhancement of lignin in H_2SO_4 pretreated biomass is because H_2SO_4 pretreatment can induce the pseudo-lignin formation and marginally solubilize lignin (Hu et al., 2012; Shinde et al., 2018). For NaOH and combined pretreatments, the increase of lignin is possibly attributed to the recondensation of the solubilized lignin which is triggered by pH adjusting. This phenomenon can be verified by the previous finding that NaOH pretreated rice straw followed by acidification with hydrochloric acid showed higher lignin content and lower lignin removal than that without acidification (Karuna et al., 2014). It was also noticed that H_2SO_4 can catalyze degradation of hemicellulose more than NaOH (2.41-3.31% vs. 10.27-11.09%). As pretreatment solid loading increased from 15% to 25% (w/v), glucan in pretreated biomass decreased while lignin increased. This trend is in accordance with the report that glucan decreased from 51.40 to 36.20% and lignin increased from 7.50 to 10.60% in aqueous ammonia pretreated corn stover without washing as the pretreatment solid loading increased from 10 to 25% (w/w) (Qin et al., 2013).

Table 5.2 shows the concentrations of monosaccharides and derivatives in the liquid fraction from different pretreatment conditions. H_2SO_4 pretreatment significantly disrupted more glucan and xylan into the liquid fraction than NaOH pretreatment, which can be reflected by the higher glucose (7.85-16.53 g/L) and xylose (5.51-7.03 g/L) concentrations. Correspondingly, high monosaccharides availability is often accompanied by their further degradation with HMF (1.69-2.82 g/L) and furfural (3.36-4.94 g/L) formation. When the H_2SO_4 and NaOH pretreated slurries were combined, the concentrations of sugar (glucose and xylose) and its derivatives (HMF and furfural) in the liquid fraction were almost halved compared to H_2SO_4 pretreatment. As pretreatment solid loading increased from 15% to 25% (w/v), monosaccharides and derivatives were approximately doubled. Toquero and Bolado (2014) conducted four different pretreatments of wheat straw with 10% (w/w) solid loading and reported 0.67-2.26 g cellulose and 0.81-16.49 g hemicellulose/100 g raw material in the liquid fraction. However, the liquid fraction was not used for enzymatic hydrolysis and fermentation (Toquero and Bolado, 2014). If these high-value furans and sugars in the liquid fraction are discarded in industrial production, there would be a huge economic loss (Zhao et al., 2021a, 2021b).

5.4.2. Structural characterization of raw and pretreated biomass

Microstructural, crystalline, and chemical bond properties of raw and pretreated biomass are characterized in Figure 5.1. SEM is indirectly able to reflect the pretreatment mechanism by tracking the morphological change of biomass (Zhao et al., 2020b). It was observed that pretreatment amplified surface unevenness compared to raw intact lamellar structures (Figure 5.1A-G). The small particles on the surface of pretreated biomass might be the agglomerated lignin units which became insoluble after pH adjusting (Zhao et al., 2021b). H_2SO_4 pretreated biomass showed irregular micropores (Figure 5.1B and C) that could derive from the decomposition of hemicellulose (Zhao et al., 2020b). Whereas NaOH pretreatment altered the sealed skeleton of biomass due to the solubilization of lignin (Figure 5.1D and E).

The XRD spectrum and CrI values of raw and pretreated biomass are shown in Figure 5.1H. Pretreatment increased the CrI values of biomass from 58.92 to 76.20%, and the CrI values from solid loading of 15% (73.23-76.20%) were higher than those from solid loading of 25% (69.34-74.69%). These were attributed to the increase in glucan content (Zhao et al., 2020b, 2021a, 2021b). Under the same solid loading, NaOH pretreated biomass displayed higher CrI values than H_2SO_4 pretreated biomass, indicating that H_2SO_4 significantly altered the cellulose. FTIR spectra showed that glucan [1091 cm^{-1} (C-O vibration), 1051 cm^{-1} (C-O stretching), and 1023 cm^{-1} (C-O-C pyranose ring skeletal vibration)] and lignin [1514 cm^{-1} (aromatic skeleton) and 1319 cm^{-1} (syringyl and guaiacyl lignin units)] related peak intensity in pretreated biomass were stronger than raw biomass (Figure 5.1I). This was in agreement with the change of glucan and lignin contents in biomass (Zhao et al., 2021a).

5.4.3. Sugar concentration and yield from enzymatic hydrolysis

Sugar concentrations and yields from enzymatic hydrolysis of the pretreated slurry without solid-liquid separation and detoxification at different solid loadings are presented in Figure 5.2. Regardless of which pretreatment methods were applied, increasing solid loading significantly enhanced the final glucose (52.66-56.59 g/L vs. 36.76-38.74 g/L) and xylose (14.06-17.98 g/L vs. 6.27-10.56 g/L) concentrations, but the corresponding glucose yields (88.93-94.16% vs. 69.32-73.25%) notably decreased. However, xylose yield showed a different trend: it increased for H_2SO_4 and combined pretreatments (Figure 5.2A and C) but decreased for NaOH pretreatment (Figure 5.2B). Generally, as solid loading increases, the sugar conversion efficiency gradually decreases due to the low accessibility of enzymes and the cross-linking of lignin and enzymes (Zhao et al., 2021b, 2020c; Zheng et al., 2021). Surprisingly, xylose yields for H_2SO_4 and combined pretreatments increased, which is possibly ascribed to that 25% of solid loading used for pretreatment has a relatively less percentage of xylan degradation than 15% of solid loading. This can be indirectly reflected by the higher xylan content in their pretreated biomass (Table 5.2). Overall, the highest total sugar concentration (glucose: 56.59 g/L; xylose: 17.94 g/L) and yield (10% of solid loading: glucose of 94.16% and xylose of 81.56%; 20% of solid loading: glucose of 73.25% and xylose of 77.49%) were obtained from combined pretreatment (Figure 5.2C) and NaOH pretreatment (Figure 5.2B), respectively. Because in this work sugar yield was calculated based on the initial biomass, high sugar yield derived from NaOH pretreatment was associated with its lower sugar degradation during pretreatment (Zhao et al., 2020b, 2020d). These findings also indicate that calculating sugar yield based on the pretreated biomass would result in overestimating the effect of pretreatment because the monosaccharides derived from glucan and xylan in the liquid fraction are often discarded.

5.4.4. The potential of hydrolysate for ethanol fermentation

The possibility of hydrolysate from different hydrolysis conditions for ethanol fermentation by the traditional yeast was examined (Figure 5.2D). Several studies have assumed the feasibility of H_2SO_4 pretreated slurry for ethanol fermentation after pH adjusting without solid-liquid separation and detoxification (Aden and Foust, 2009; Humbird et al., 2011; Zhao et al., 2015). However, the present study showed that only the hydrolysate from NaOH pretreatment and enzymatic hydrolysis with 10% initial solid loading can be efficiently fermented to ethanol (17.92 g/L) by the traditional yeast. The fatal damage of hydrolysate to yeast can be attributed to inhibitory compounds such as HMF, furfural, and phenols (Kim, 2018; Kumar et al., 2020). It has been reported that methyl isobutyl ketone (MIBK) has excellent furan extraction properties than other water-immiscible organic solvents in the biphasic system (Mittal et al., 2017; Zhang et al., 2013). However, if MIBK is used for furan extraction from hydrolysate in this work, it will be difficult to recover it completely from the aqueous phase because of its solubility in water (1.9g/100 mL at 20 °C) and boiling point (117 °C). In addition, a high ratio of MIBK to the aqueous phase is required to achieve the efficient partition coefficient, which would make it economically unfeasible to distill low-concentration HMF (boiling point of 114-116 °C) and furfural (boiling point of 162 °C) from MIBK. Although the pathway of converting sugar to ethanol in this work has encountered technological obstacles, monosaccharides (glucose and xylose) in the hydrolysate can be used as important intermediates for the conversion of high-value chemicals (Chheda et al., 2007; Gogar et al., 2021; Mittal et al., 2017; Motagamwala et al., 2019; Zhu et al., 2020).

5.6. Conclusions

Three scenarios were experimentally implemented for monosaccharides and furans production at high solid loading without solid-liquid separation and detoxification. Increasing pretreatment solid loading boosted the concentration of derivatives but relatively reduced sugar decomposition per unit. Scenario I achieved the highest furan production but the lowest sugar concentrations. Scenario II reached the highest sugar yield without furan formation. Scenario III showed the highest sugar concentration with a moderate concentration of furan. Only the hydrolysate from NaOH pretreatment and enzymatic hydrolysis at initial 10% solid loading was able to be efficiently fermented to ethanol by the traditional yeast.

Table 5.1 Chemical composition (%) of solid fraction after pretreatment, pH adjusting, and solid-liquid separation.[1]

Biomass sample	Glucan	Xylan	Acid soluble lignin	Acid insoluble lignin	Others[2]
Raw biomass	43.12 ± 0.41	13.61 ± 0.06	0.01 ± 0.00	14.40 ± 0.29	28.86 ± 0.76
H_2SO_4 (15%)[3]	64.45 ± 0.02	2.41 ± 0.02	1.59 ± 0.01	28.67 ± 0.02	2.88 ± 0.00
H_2SO_4 (25%)	56.35 ± 0.04	3.31 ± 0.08	1.46 ± 0.02	35.48 ± 0.03	3.41 ± 0.04
NaOH (15%)	61.37 ± 0.04	11.09 ± 0.04	1.18 ± 0.01	18.56 ± 0.01	7.80 ± 0.01
NaOH (25%)	60.74 ± 0.04	10.27 ± 0.01	1.45 ± 0.02	19.27 ± 0.01	7.27 ± 0.05
Combined (15%)	62.44 ± 0.11	7.70 ± 0.01	1.23 ± 0.03	24.60 ± 0.05	4.04 ± 0.13
Combined (25%)	57.37 ± 0.07	8.03 ± 0.02	1.39 ± 0.01	27.68 ± 0.04	5.53 ± 0.09

[1] Solid composition was on a dry basis. Data: means ± standard deviations. Solid-liquid separation was conducted to determine the pretreatment effectiveness, whereas this process was not needed for enzymatic hydrolysis. [2] Other components included residual chemical reagents, degraded sugars, extractives, and mineral substances. [3] It represented the pretreatment solid loading (w/v).

Table 5.2 Chemical composition (g/L) of liquid fraction after pretreatment, pH adjusting, and solid-liquid separation.[1]

Sample	Glucose	HMF[2]	Xylose	Furfural
H_2SO_4 (15%)[3]	7.85 ± 0.06	1.69 ± 0.02	5.51 ± 0.01	3.36 ± 0.03
H_2SO_4 (25%)	16.53 ± 0.02	2.82 ± 0.06	7.03 ± 0.01	4.94 ± 0.12
NaOH (15%)	0.25 ± 0.12	nd[2]	0.64 ± 0.00	nd
NaOH (25%)	0.52 ± 0.02	nd	1.28 ± 0.00	nd
Combined (15%)	4.49 ± 0.00	0.77 ± 0.01	3.01 ± 0.00	1.62 ± 0.03
Combined (25%)	7.59 ± 0.00	1.17 ± 0.02	5.72 ± 0.02	2.23 ± 0.05

[1] Data: means ± standard deviations. [2] HMF: 5-hydroxymethylfurfural. nd: not detected. [3] It represents the pretreatment solid loading (w/v).

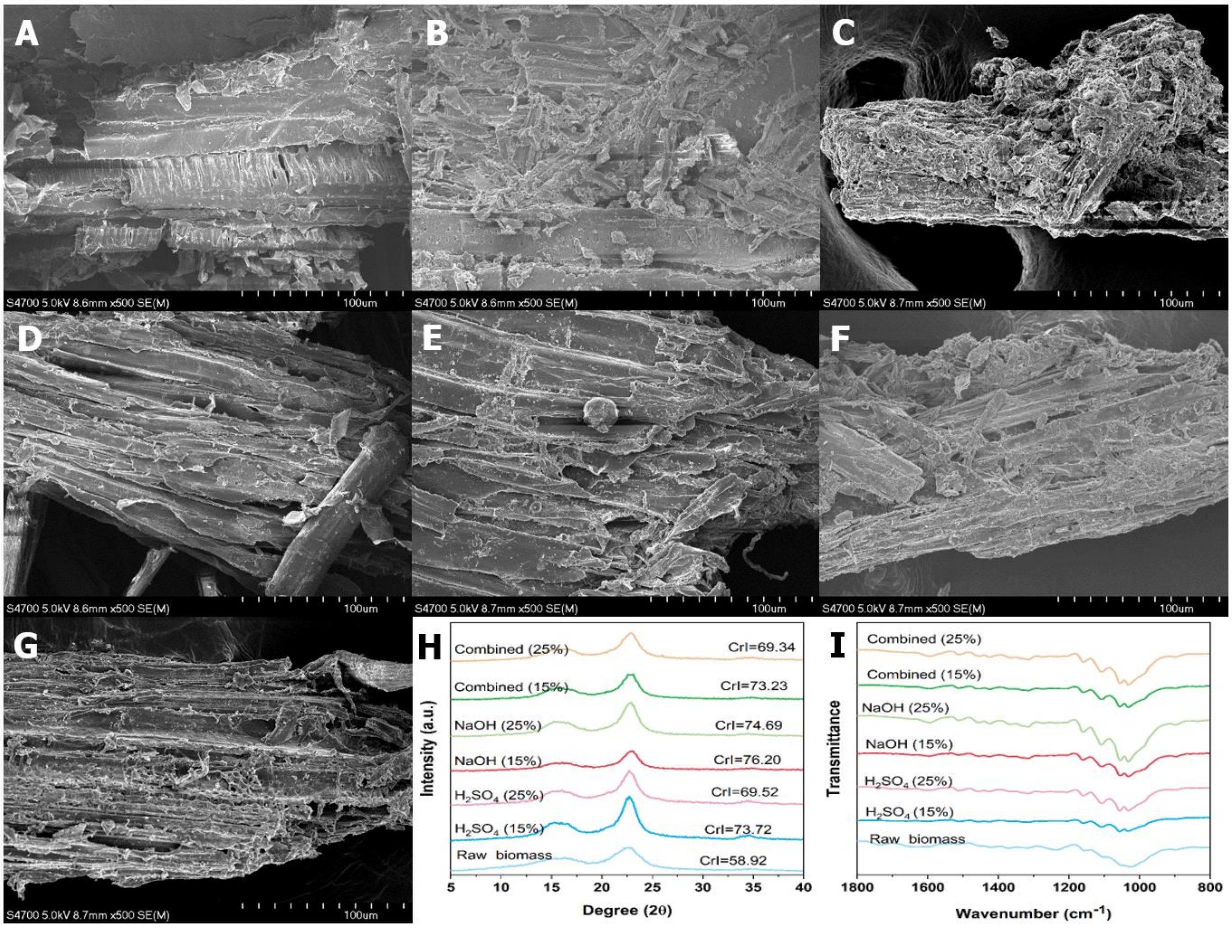

Figure 5.1 SEM of raw (A) and pretreated biomass (B: H_2SO_4-15%; C: H_2SO_4-25%; D: NaOH-15%; E: NaOH-25%; F: Combined-15%; G: Combined-25%) as well as their XRD (H) and FTIR (I) properties.

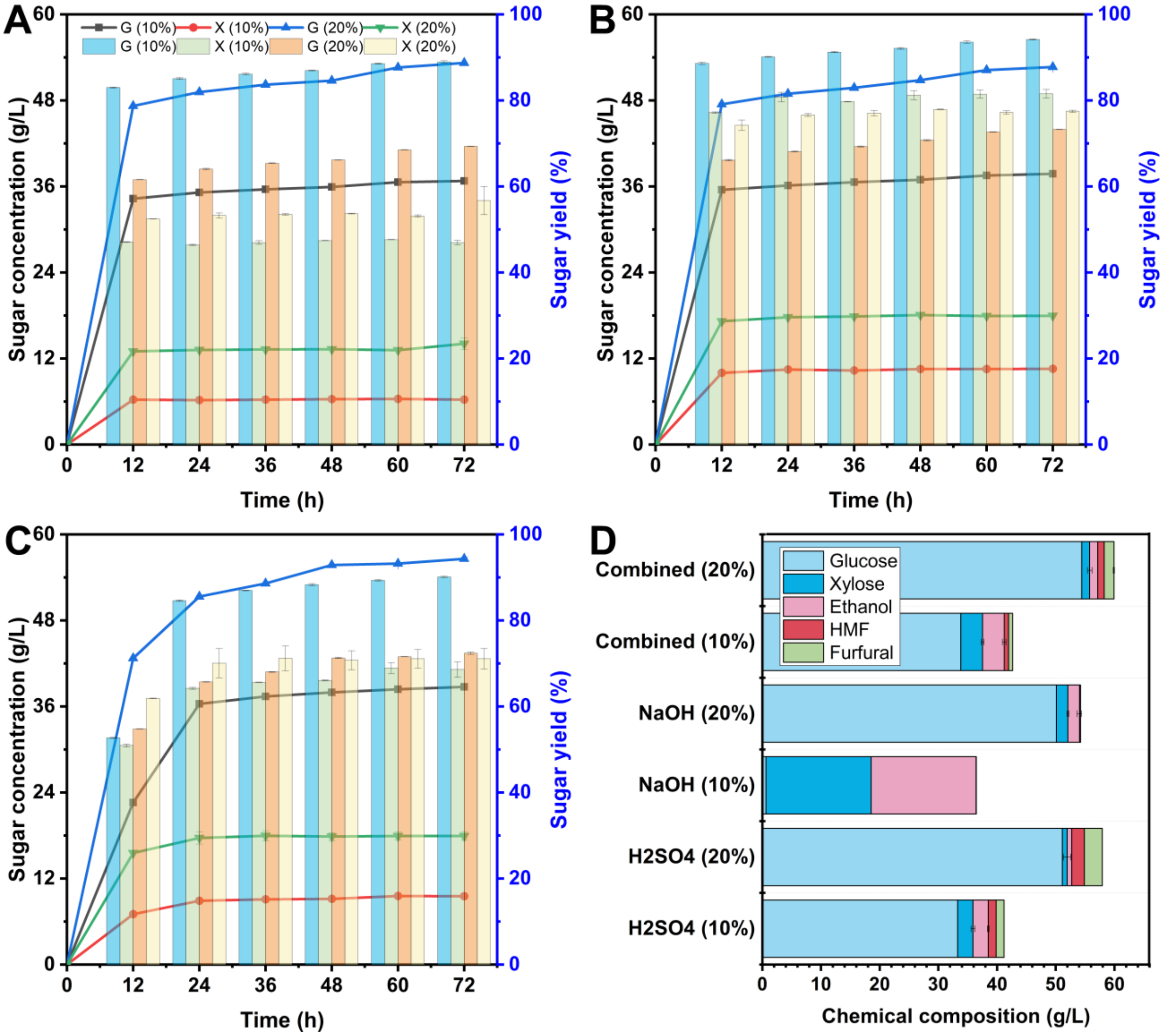

Figure 5.2 Glucose and xylose concentrations (line) and total yields (column) from enzymatic hydrolysis of H_2SO_4 (A), NaOH (B), and combined pretreated (C) biomass at different solid loadings as well as the compositional profile (D) of the hydrolysate after 72-h fermentation with the traditional yeast.

Chapter 6 - Conclusions and future work

6.1. Conclusions

Integrating acid and alkali pretreatment of lignocellulosic biomass demonstrates its potential to reduce water and chemical consumption for biofuel and bioproduct conversion. The advantages of this integration strategy include: 1) As long as the concentrations of acid and alkali reagents are properly adjusted, the whole process including pretreatment and enzymatic hydrolysis does not require extensive washing and the addition of other chemicals; 2) Combinating acid and alkali pretreated solid and liquid fraction promotes the neutralization of residual regents inducing lignin precipitation; 3) The recovered lignin shows comparable characteristics to commercial alkali lignin; 4) This integration method can be still applied to different biomass sources. The disadvantages of this integration strategy include: 1) Solid biomass loading used for pretreatment is hard to exceed 25% (w/v); 2) The trace amount of derivatives such as phenols and furans in the integrated slurry are toxic to yeast.

6.2. Future work

Based on findings from this project, there are several avenues that are worthy to be explored. One of the topics is to apply different filtration methods (ultrafiltration, nanofiltration, and diafiltration) to remove inhibitory compounds from the hydrolyzed slurry. The resultant sugars can be used for biofuel fermentation by yeast and biochemical conversion by thermochemical catalysis. Another topic is to expand the electrochemical applications of biomass-and lignin-derived graphene and graphene-like carbon aerogels in the field of energy storage and conversion. This would permit an investigation into the potential cross-linkages of various synthesis routes. The reason for looking at chemical synthesis is that, by integrating them into cellulosic ethanol biorefinery, the economic viability would be improved. Additionally, it is necessary to investigate the synthesis of biomass-based materials. First, agricultural biomass might be a biodegradable and eco-friendly alternative for polymer-based food packaging. Cellulose nanocrystals and micro- and nano-fibrillated cellulose have drawn great interest and attention towards the development of novel bio-based films and composites for food products. Hemicellulose-derived materials and packaging are other attractive forms of bio-based materials owing to their film-forming capacity and low permeability to oxygen. Lignin has more commonly been used as a hydrophobic coating or filler in bio-based materials. Therefore cellulose, hemicellulose, and lignin-derived waterproof coating can be investigated by combining the modified soy protein isolate to replace the traditional petroleum wax-based coatings. Second, agricultural biomass itself is a good hydrophilic and hydrophobic material, so it has great potential to create disposable meal boxes if adding green and natural adhesives such as soy protein isolate and lignin. Third, biomass-derived porous carbonaceous materials and their composites as absorbents for cationic and anionic dyes and wastewater decontamination.

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