Biomass pretreatment by metal oxides for reducing sugar degradation and water consumption in biofuel production

by

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B.S., Henan University of Technology, 2012
M.S., Henan University of Technology, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biological and Agricultural Engineering
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Abstract

Pretreatment is the first step of the three major steps (pretreatment, saccharification, and fermentation) for cellulosic ethanol production. The performance of pretreatment largely determines the performances of downstream saccharification and fermentation as well as whole economic feasibility for cellulosic ethanol production. Although dilute acid pretreatment has been industrialized and liquid hot water (LHW) pretreatment is considered as a green process due to no chemical use in the pretreatment step, both of them cause sugar degradation and inhibitor formation. The formation of inhibitors not only causes sugar loss but also inhibits downstream enzyme and yeast activities, especially during high-solids saccharification and fermentation, consequently lowering the final ethanol yield. The goal of this research was to develop a new pretreatment method to reduce sugar degradation, increase sugar recovery, reduce water usage for inhibitor removal, and eliminate the use of acid-resistant equipment.

Five metal oxides, Fe_2O_3, CuO, NiO, ZnO, and MgO, were investigated as catalysts to reduce sugar degradation and improve sugar recovery during corn stover pretreatment. LHW pretreatment was used as control. Among the five metal oxides, MgO was the most suitable catalyst for biomass pretreatment. The optimal pretreatment condition was 10% solids loading with 0.08 mol/L MgO at 190 °C for 40 min. Compared to LHW pretreatment, MgO pretreatment caused twice hemicellulose recovery and reduced pseudo-lignin formation with pretreatment slurry of neutral pH and trace amounts of furfural and 5-hydroxymethlfufural. Under the optimal pretreatment (as above) and saccharification (10% solids loading, 30/18 μL CTec3/NS22244/g treated biomass, 52 °C, and 72 h) conditions, the double hemicellulose recovery increased xylose yield by 20% and total sugar yield by 6% without sacrificing glucose yield.

Biomass slurry from MgO pretreatment was nearly neutral and free of furfural and 5-hydroxymethylfurfural, which allowed the direct integration of MgO-treated biomass and biomass liquor for enzymatic saccharification. Under the same saccharification condition (40/24 μL CTec3/NS22244/g treated biomass, 52 °C, and 72 h), MgO-treated corn stover with pretreatment liquor had a lower glucose yield (71 vs. 75%) but xylose yield was much higher than that from MgO-treated corn stover only (66 vs. 36%), resulting in no significant difference in total sugar concentration (57 vs. 58 g/L). Corn stover slurry with near-neutral pH and free of 5-hydroxymethylfurfural and furfural eliminated the need for washing and detoxification after pretreatment, lightening the burden for wastewater treatment.

Combination of MgO and ethanol was used to further enhance sugar recovery, reduce sugar degradation, and enhance enzymatic saccharification. The optimal pretreatment condition was 50% ethanol, 0.07 mol/L MgO, and 10% solid loading at 190 °C for 40 min. Under optimal condition, glucan was completely recovered along with 89.3% xylan recovery and 44.1% lignin removal. Corn stover pretreated by MgO and 50% ethanol achieved 75% glucan and 71% xylan conversions at the 10% solids loading and 30/18 μL CTec3/NS22244/g treated biomass. Under the same saccharification condition, corn stover pretreated by MgO and 30% ethanol had higher glucan and xylan conversions (80 and 78%). This result indicates that excessive xylan recovery from MgO and 50% ethanol pretreatment reduced enzymatic accessibility to cellulose and hemicellulose. When solids loading reached 16%, 74% glucan and 75% xylan conversions were obtained with glucose and xylose concentrations of 71 and 29 g/L. The total sugar concentration exceeded the 80 g/L minimum sugar concentration requirement for economic ethanol distillation. A 16%-solids loading largely reduced the poor mixing issue.

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Dedication

To my beloved family and friends.

Chapter 1 - Introduction

1.1. Problem statement

With the growing concerns regarding environmental pollution and climate change, the interest in developing green, environmentally-friendly, sustainable, and economical biofuels, such as bioethanol, bio-butanol, bio-diesel, bio-oil, etc., has been increasing recently (Alonso et al., 2012; Hu et al., 2018; Park et al., 2012). Bioethanol is a great alternative to gasoline derived from nonrenewable petroleum, thus has been attracting more and more attention due to its clean-burning nature and no NO_x and SO_x emission (Prasad et al., 2007). Survey results showed that more than 98% of gasoline in the United States is blended with ethanol to provide a series of flex fuels for different types of vehicles such as E85, E15, and E10 (Uria-Martinez et al., 2018). Currently, most of bioethanol is produced from cereal crops such as corn, wheat, and grain sorghum (Mohanty and Swain, 2019). Those starch-based crops have a high ethanol yield and fermentation efficiency; however, using large amounts of grains for bioethanol production will generate a competition against food and animal feed (Goswami and Choudhury, 2019). Thus, attention has been focused on seeking low-cost, nonfood, and readily available alternatives for bioethanol production.

Cellulosic biomass in nature, such as corn stover, wheat straw, switchgrass, sorghum stalk, miscanthus, big bluestem, poplar, and willow, has great potential to be used as a renewable resource for bioethanol production due to its renewability and availability at low cost (Brandt et al., 2013). Unlike that of starch-based crops, however, the use of cellulosic biomass for bioethanol production faces significant technical challenges due to its complex chemical structures (Balan, 2014). Figure 1.1 shows the major steps for production of cellulosic bioethanol. The success of cellulosic bioethanol as a promising fossil fuel alternative depends largely upon the physical and chemical properties of biomass, pretreatment methods, effective enzyme and fermentation

systems, and system process optimization. Pretreatment, enzymatic saccharification, and fermentation are the three major steps for bioethanol production from cellulosic biomass. Pretreatment of cellulosic biomass is crucial before proceeding to enzymatic saccharification and fermentation. The purpose of pretreatment is to break the lignin seal, to disrupt the crystalline structure of cellulose, and to increase surface area of cellulose, making the polysaccharides more susceptible to enzymatic saccharification. Steam explosion, dilute acid treatment, alkaline treatment, ammonia fiber explosion, liquid hot water (LHW), and supercritical CO_2 and SO_2 are the pretreatment methods often used (Fernandez-Bolaños et al., 2001; Kim and Hong, 2001; McMillan, 1994; Mosier et al., 2005; Sun and Cheng 2002; Taherzadeh and Niklasson, 2004; Teymouri et al., 2004; Van Walsum et al., 1996; Varga et al., 2004; Zheng et al., 1998). Although the above methods are available for biomass pretreatment, the major issues with the current pretreatment technologies are (1) high energy input, pretreatment accounts for more than 25% of the total energy input; (2) high sugar degradation, the sugar degradation generates a lot of toxic chemicals; (3) high water usage and wastewater disposal, a large amount of water has to be used to wash pretreated biomass before enzymatic saccharification; and (4) residual chemicals affecting downstream processing. Among these pretreatment methods, LHW pretreatment is considered as a green process without using chemicals in the pretreatment step (Yang and Wyman, 2008) and ethanol pretreatment is considered as a promising method that is capable of simultaneously producing the fermentable sugars and high-purity value-added lignin within a biorefinery (Jafari et al., 2016). However, both LHW and ethanol pretreatments also result in some sugar degradation, especially for sugars from hemicellulose, and generates numerous byproducts, such as acids and toxic furfural and 5-hydroxymethylfurfural (HMF) (Chandel et al., 2013; Huijgen et al., 2011; Pandey et al., 2014). These byproducts are inhibitors that reduce the efficiencies of the subsequent

enzymatic saccharification and fermentation (Klinke et al., 2004). The goal of this research was to address these issues by fundamentally studying the effects of metal oxides on biomass pretreatment.

1.2. Objectives of this research

This research employed metal oxides to pretreat cellulosic biomass for reducing sugar degradation, increasing fermentable sugars yield, and reducing water consumption in production of cellulosic bioethanol. A comprehensive investigation on metal oxides in biomass pretreatment will not only create a new method for biomass pretreatment, but also generate knowledge and provide practical guidance for cost-effective production of cellulosic bioethanol. The specific objectives include:

1) Investigating the effects of metal oxide pretreatment on fermentable sugar recoveries, sugar degradation, and pH value of biomass slurry;

2) Investigating the effects of metal oxide pretreatment on fermentable sugar yield of treated biomass only;

3 Investigating the effects of metal oxide pretreatment on fermentable sugar yield of treated biomass plus biomass liquor;

4) Investigating the effect of metal oxide-ethanol pretreatment on fermentable sugar recoveries, sugar degradation, and pH value of biomass slurry;

5) Investigating the effects of metal oxide-ethanol pretreatment on fermentable sugar yield of treated biomass.

1.3. Related current and previous research

1.3.1. Cellulosic biomass

Cellulosic biomass mainly consists of 38-50% cellulose, 23-32% hemicellulose, and 15-20% lignin (Figure 1.2) (Rao et al., 2010). Cellulose is a linear sugar polymer composed of glucose through β-1,4 linkages, thus can be used for bioethanol production. Generally, amorphous cellulose is easier to be decomposed than crystalline cellulose. Thus, more studies focus on reducing the degree of cellulose crystallinity through various pretreatments (see section 1.3.2). Hemicellulose is a short-branched sugar polymer composed of xylose, glucose, arabinose, and other minor sugars, thus can also be used for bioethanol production. In bioethanol production, however, the use of hemicellulose mainly consisting of xylose (pentose) is not as easy as that of cellulose consisting of glucose (hexose). Relevant reasons are discussed in detail in sections 1.3.3 and 1.3.4. Lignin is a polyphenol mainly consisting of *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol through ether bonds, carbon-carbon bonds, and ester bonds, which is located in the outer layer of plant cell wall to prevent cellulose and hemicellulose from being invaded by insects and microorganisms. It is lignin seal that restricts the accessibility of enzymes to cellulose and hemicellulose thus reduces the utilization efficiency of cellulosic biomass in bioethanol production. The most commonly investigated biomass for bioethanol production includes agricultural residues (e.g. corn stover, wheat straw, rice straw, and sorghum stalk), energy crops (e.g. switchgrass, miscanthus, and big bluestem), and woody materials (e.g. willow and poplar).

1.3.2. Biomass pretreatment

Pretreatment is usually conducted as the first step of the three major steps (pretreatment, saccharification, and fermentation) for cellulosic ethanol production in order to reduce the size of

biomass particles, break the lignin seal, disrupt the crystalline structure of cellulose, increase surface area of cellulose, and make the polysaccharides more susceptible to enzymatic saccharification (Mosier et al, 2005), which accounts for more than 25% of the total energy input (Humbird et al., 2011). Thus, the performance of pretreatment largely determines the performances of downstream saccharification and fermentation as well as whole economic feasibility for cellulosic ethanol production. Generally, pretreatment can be classified into five groups — mechanical pretreatment, physical pretreatment, chemical pretreatment, physicochemical pretreatment, and biological pretreatment. The advantages and disadvantages of each group are discussed in detail as follows.

1.3.2.1. Mechanical pretreatment

Mechanical pretreatment refers to mechanical milling, which is used to reduce the particle size of biomass. Previous studies showed that mechanical ball milling can reduce the long-range order of microfibrils, reduce the crystallinity and the degree of polymerization of cellulose, and increase the surface area, thus improving enzymatic saccharification efficiency and sugar yield (Hall et al., 2010; Koullas et al., 1992; Puri, 1984; Walker and Wilson, 1991; Zhao et al., 2006). However, mechanical milling is an energy-intensive process (Zheng et al., 2009). It is not economically viable to improve sugar yield by reducing the particle size as much as possible. Generally, to better handle biomass during physical, chemical, or biological pretreatment, mechanical pretreatment is used to chop biomass into a particle size that fits the pretreatment reactor. In industrial scale, biomass is usually milled into a particle size of ~4 mm. In lab scale, the reactor used is usually very small thus biomass is usually milled into a particle size of less than 1 mm to fit the minireactor.

1.3.2.2. Physical pretreatment

Physical pretreatment refers to ultrasound pretreatment, microwave pretreatment, and radiation pretreatment (Mosier et al., 2005). It's also an energy-intensive process and requires larger space (Mood et al., 2013). Physical pretreatment only cleaves partial chemical bonds among cellulose, hemicellulose, and lignin, thus hemicellulose decomposition and lignin removal are very limited. Generally, it is considered as an assisted means to favor following chemical pretreatment.

1.3.2.3. Chemical pretreatment

Among the four types of treatments, chemical treatment is widely regarded as the most powerful method to disrupt biomass structure and improve saccharification efficiency. Chemical treatment includes acid treatment, alkaline treatment, LHW treatment, organosolv treatment, ionic liquid treatment, and steam explosion treatment (Kim et al., 2016; Lau et al., 2009; Nakashima et al., 2011; Timung et al., 2015; Yat et al., 2008; Zhang et al., 2016). Among these chemical treatments, dilute sulfuric acid pretreatment is the only one that has been industrialized. However, dilute sulfuric acid pretreatment causes a large amount of sugar degradation and inhibitor formation, which inhibit enzyme activities and reduce saccharification efficiency of cellulose and hemicellulose. LHW pretreatment is considered as a promising method due to no chemical usage in the pretreatment step. However, LHW pretreatment has the same weakness as dilute sulfuric acid pretreatment, because acetic acid released from hemicellulose during pretreatment also causes sugar degradation and inhibitor formation (Chandel et al., 2013; Pandey et al., 2014). Although alkaline pretreatment performs well in delignification and cellulose swelling, most of hemicellulose still remains in the treated biomass, which has an inhibitory effect on the enzymatic

saccharification of cellulose. Also, the biomass liquor from alkaline pretreatment is so-called black liquor, which is hard to be used. Although organosolv pretreatment has a good performance in lignin removal and cellulose swelling, it also causes sugar degradation and inhibitor formation (Huijgen et al., 2011). The high cost of organic solvents is another reason that causes organosolv pretreatment not economically viable in cellulosic ethanol production. Ionic liquid performs well in separating cellulose, hemicellulose, and lignin. The regenerated cellulose after ionic liquid removal has a much lower crystallinity, improving saccharification efficiency of cellulose (Xu et al., 2016). However, residual ionic liquid in treated biomass has a large toxicity to enzyme. Also, high cost of ionic liquid limits its industrial application. Although steam explosion pretreatment can disrupt macro- and micro-structures of biomass, removed lignin and decomposed hemicellulose still remains in the treated biomass, which inhibit enzyme activities. Steam explosion pretreatment also causes the formation of inhibitors that inhibit enzymatic saccharification of cellulose (Mosier et al., 2005).

1.3.2.4. Biological pretreatment

Biological pretreatment is to use microorganisms, such as white-, brown-, and soft-rot fungi, to selectively degrade carbohydrates and lignin (Sindhu et al., 2016). White rot fungi have unique ligninolytic systems for delignification (Eriksson et al., 2012). The most commonly investigated white rot fungi are *Pleurotus ostreatus* (Taniguchi et al., 2005; Yu et al., 2009), *Ceriporiopsis subvermispora* (Wan and Li, 2010, 2012), *Coriolus versicolor* (Zhang et al., 2007a, 2007b), *Cyathus stercoreus* (Keller et al., 2003), and *Phanerochaete chrysosporium* (Bak et al., 2009; Keller et al., 2003; Sawada et al., 1995; Shi et al., 2009; Shrestha et al., 2008). Brown- and soft-rot fungi prefer to degrading cellulose but cause only little damage to lignin. White rot fungi

are studied more in biofuel field compared to the other two, because only cellulose and hemicellulose can be converted into bioethanol. Although lab- and pilot-scale studies have proved that biological pretreatment is able to successfully disrupt biomass structure and improve enzymatic saccharification efficiency and requires lower energy input than other pretreatments, its large-scale application in biofuel production is not economically feasible due to its low saccharification rate and long retention time (Cardona and Sanchez, 2007; Sun and Cheng, 2002; Tengerdy and Szakacs, 2003).

1.3.3. Enzymatic saccharification

Saccharification is the second step in the production of cellulosic bioethanol and can be classified into enzymatic and chemical saccharification. Although enzymatic saccharification is gradually replacing traditionally chemical saccharification in view of environmental pollution issues, it also faces some other problems such as expensive enzyme cost and reduction of enzyme activity caused by inhibitors such as furfural, 5-hydroxymethylfurfural (HMF), and lignin-derived aromatic compounds (Kristensen et al., 2009). Obviously, it is not advisable to compensate for the decrease in enzyme activity by increasing the loading of enzyme. To address these issues, some other efforts should be taken such as improving the activity of enzyme itself, improving the tolerance of enzyme to inhibitors by modification, and producing sugar-degradation-products-free biomass by appropriate pretreatment.

Generally, high-solids loading (>15%, w/w) is superior to low- (<10%, w/w) and moderate-solids (10–15%, w/w) loading due to its enhanced fermentable sugar concentration, subsequent high ethanol yield and titer, and reduced capital and energy input (Liu et al., 2014; Xu and Wang, 2017). However, one significant issue with high-solids enzymatic saccharification is

poor mixing, especially at the initial stage. Poor mixing reduces enzyme activities due to biomass absorption of free water, increases energy input due to the high-solids content, and may eventually deactivate enzymes due to the accumulation of inhibitors and residual lignin released from treated biomass (Modenbach and Nokes, 2013). To better handle high-solids loading during enzymatic saccharification, horizontal reactors with a better mixing capacity were attempted (Hodge et al., 2009; Roche et al., 2009a; Xu et al., 2017), and fed-batch loading was suggested (de Albuquerque Wanderley et al., 2013; Kuhad et al., 2010; Teymouri et al., 2005). However, the effects of accumulated inhibitors and residual lignin released from treated biomass on enzyme activities remain a challenging issue. In the ethanol industry, a minimum of 40 g/L of ethanol is generally required for economical ethanol distillation (Xu and Wang, 2017; Xu et al., 2016), which means that the concentration of fermentable sugars in saccharification solution should be more than 80 g/L (Modenbach and Nokes, 2013). Although many high-solids enzymatic saccharification methods had reached or exceeded this minimum requirement, their enzymatic saccharification efficiencies were not satisfactory (Caspeta et al., 2014; Jørgensen et al., 2007). Moreover, most previous studies focused only on the cellulose-to-glucose and glucose-to-ethanol conversions (Kristensen et al., 2009; Roche et al., 2009b). Most xylan was removed during pretreatment, and glucan was the only source of sugar used in subsequent fermentation for bioethanol production. Because of this, to reach the minimum requirement of sugar concentration, higher solids loading is usually required, creating poor mixing and enzyme inhibition issues.

1.3.4. Fermentation

Fermentation is the last step for cellulosic bioethanol production. The most commonly used microorganism for ethanol fermentation is yeast (e.g. Ethanol Red) due to its cheapness, easy

culture and storage, fast reproduction, and high ethanol tolerance (Leaf, 2017). Yeast can digest glucose only but not xylose. Thus, most previous studies focused only on the cellulose-to-glucose and glucose-to-ethanol conversions (Kristensen et al., 2009; Roche et al., 2009a). Most xylan was removed during pretreatment, and glucan was the only source of sugar used in subsequent fermentation for bioethanol production. In recent years, with advancement in enzyme complexes capable of simultaneously hydrolyzing glucan and xylan and engineered bacteria capable of co-fermenting glucose and xylose (Öhgren et al., 2006; Shen et al., 2012; Zhao et al., 2008), there is no need to completely remove xylan from biomass. Conversely, an approximate increase in xylan recovery during pretreatment could be a great option to improve sugar yield and concentration for bioethanol production. Integrating xylose-rich biomass liquor into enzymatic saccharification of treated biomass could be another great option to improve sugar yield and concentration for bioethanol production.

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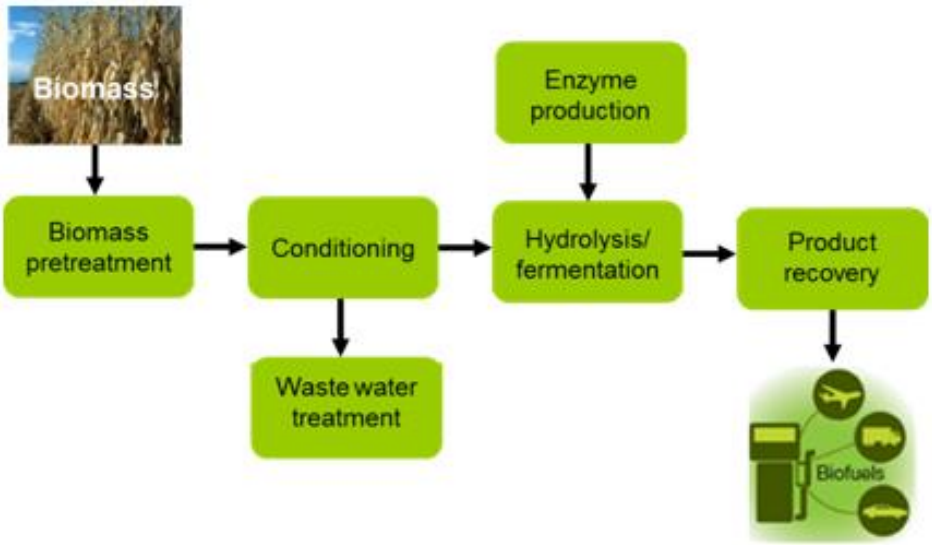

Figure 1.1 Major steps for cellulosic bioethanol production.

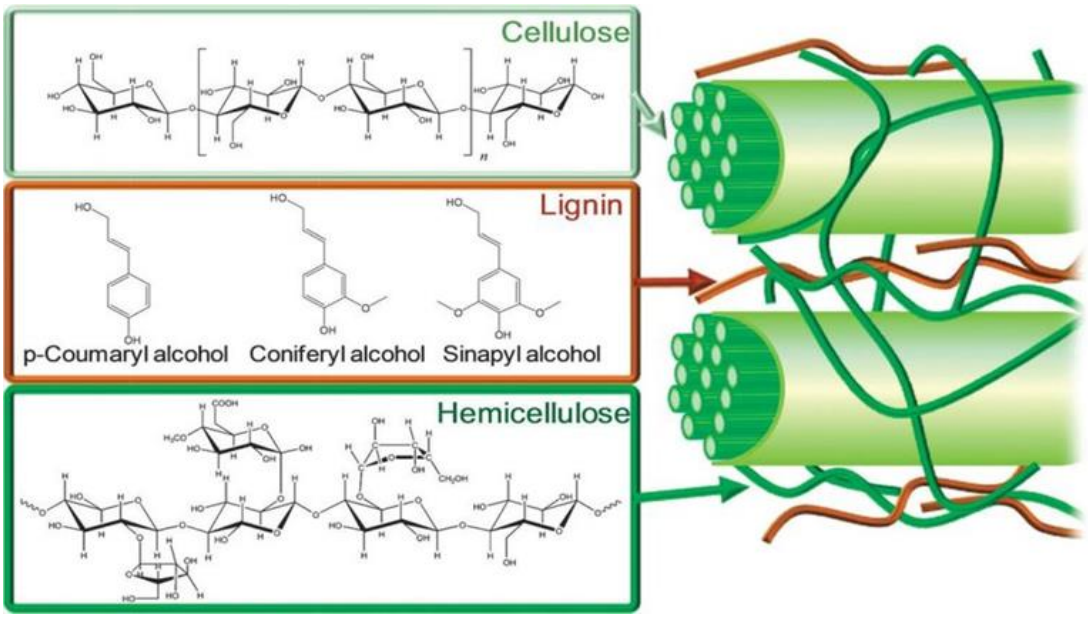

Figure 1.2 Structure of cellulosic biomass (Horvat, 2016)**.**

Chapter 2 - Effect of metal oxide pretreatment on sugar recovery and sugar degradation of corn stover

2.1. Abstract

The effects of five metal oxides, including Fe_2O_3, CuO, NiO, ZnO, and MgO, on corn stover pretreatment were studied as catalysts for sugar degradation mitigation in comparison to the control (liquid hot water pretreatment). MgO showed the superior pretreatment performance compared to other four metal oxides. The optimal pretreatment condition was the solid/liquid ratio of 1/10 with 0.08 mol/L MgO at 190 °C for 40 min. The glucan (99%) and fermentable xylose (86%) from MgO pretreatment was equivalent to those (98 and 84%) from liquid hot water pretreatment. MgO pretreatment reduced pseudo-lignin formation with pretreatment slurry of neutral pH and trace amounts of furfural and 5-hydroxymethlfufural. Thus, biomass liquor can be directly applied for downstream processing without any detoxification. In addition, expensive acid-resistant equipment could be eliminated due to the absence of acids.

2.2. Introduction

Due to depleting crude oil reserves and increasing global energy consumption, the increasing demand for low-cost and sustainable energy has led to the rapid development of biofuel production from lignocellulosic biomass, including agricultural crops and byproducts, herbaceous grasses, woody perennials, and forest residues (Manochio et al., 2017). Bioethanol, as an important transportation fuel alternative, has received much interest both from researchers and industries. The current commercial bioethanol production is derived from starch-rich substrates such as corn and grain sorghum (Mohr and Raman, 2013), and sugar-rich materials such as sugarcane and sugar beet (Manochio et al., 2017) due to the fact that starch or sugar-based crops exhibit high fermentation efficiency and ethanol yield. However, consuming a large number of cereal grains and sugar crops for bioethanol production may bring the competition against human food and animal feed (Naik et al., 2010). The "food vs. fuel" battle due to limited agricultural lands and accessible freshwater has been a challenging issue for further advance of biofuel sector. Instead, abundant lignocellulosic biomass presents a great future of a sustainable resource for bioethanol manufacturing (Brandt et al., 2013). In contrast to starch or sugar-based crops, lignocellulosic biomass fails to be directly converted into bioethanol via enzymatic saccharification and microbial fermentation because of its recalcitrant nature and complex chemical structures, which are mainly composed of intertwined cellulose, hemicellulose, and lignin components (Mosier et al., 2005; Pu et al., 2013). Pretreatment is imperative to disrupt biomass structure and improve the accessibility of enzymes to cellulose and hemicellulose (Kim et al., 2008).

The pretreatment is usually performed using various acids (inorganic and organic acids) (Kuglarz et al., 2018; Sahoo et al., 2018), bases (strong and weak alkalis) (Kang et al., 2018; Kim et al., 2016), liquid hot water (LHW) treatment (Wells et al., 2020; Yang et al., 2019), ammonia

steam explosion (Qi et al., 2018; Wei et al., 2018), ionic liquids (Alayoubi et al., 2020; Hou et al., 2019), organic solvents (Yu et al., 2018; Zhang et al., 2016), and physical methods (microwave and ultrasound) (Hassan et al., 2018); however, most of these pretreatment methods are still under the laboratory research stage, and not yet achieving the commercial application in the bioethanol industry.

LHW pretreatment, as a green and promising pretreatment method, has gained much attention due to its zero chemical addition during the pretreatment process in comparison to the industrialized dilute sulfuric acid pretreatment (Sahoo et al., 2018; Yang et al., 2019). However, these two dilute acid techniques have a major demerit in which added sulfuric acid and hemicellulose released acetic acid resulted in sugar degradation into furfural and 5-hydroxymethylfurfural (HMF) (Chandel et al., 2013; Pandey et al., 2014). Furfural and HMF are common furan aldehydes generated from dehydration of pentoses released mainly from hemicellulose, and hexoses released mainly from cellulose during pretreatment of lignocellulosic biomass (Chheda et al., 2007; Yang et al., 2012). The formation of furfural and HMF not only leads to the reduction of fermentable sugars, but also damages cell walls and cell membranes, consequently inhibiting activities of glycolytic enzymes and microorganisms, especially during high-solids loading enzymatic saccharification and microbial fermentation, which results in lower final ethanol yields (Klinke et al., 2004). A tremendous amount of water is required to detoxicate the inhibitors (residual acid, furfural, and HMF) produced during the pretreatment process. In addition, severe pretreatment conditions cause large amounts of acid, furfural, and HMF formation, which prohibits monosaccharides in the pretreatment liquor to be directly utilized for enzymatic saccharification and fermentation (Zhang et al., 2012). Moreover, expensive acid-resistant reactor is necessary because of the corrosiveness of sulfuric acid. Thus, developing a

novel pretreatment technique enabling the minimization of inhibitors formation, water consumption, and reactor corrosion, but the maximization of fermentable sugars, is highly desirable.

To achieve the goal, one possible way is to identify a catalyst capable of removing lignin with minimal sugar degradation via the neutralization of acids released from hemicellulose. Previous studies using metal chloride salts, such as $FeCl_3$, $CuCl_2$, and $MgCl_2$, have been proven to increase hemicellulose decomposition and lignin removal (Kamireddy et al., 2013; Kang et al., 2013; Loow et al., 2015). Metal ions have the capability to replace acids for biomass pretreatment and hydroxyl ions (OH^{-1}) have the capability to neutralize released acids. Thus, metal hydroxides, including metal ions and hydroxyl ions, are a suitable substrate to effectively break the recalcitrant structure of lignocellulosic biomass.

Metal hydroxides, as the simplest form of hydrates of metal oxides (Equation 2.1), are less stable than their corresponding metal oxides due to their easy dehydration and dissolution. Thus, in this work, corresponding metal oxides, such as Fe_2O_3, CuO, and MgO, were applied to pretreat biomass according to the demonstrated effect of metal salts on biomass pretreatment in previous publications (Kamireddy et al., 2013; Kang et al., 2013; Loow et al., 2015). In addition, ZnO, an amphoteric oxide, and NiO, a common catalyst for biomass pyrolysis, were incorporated into this comparative study. The effects of these five metal oxides on biomass pretreatment were studied by comparing sugar recoveries, lignin removal, sugar degradation, and biomass slurry pH.

$$2X(OH)_n \leftrightarrow X_2O_n + nH_2O \qquad (2.1)$$

Where X is the metal element and n is the chemical valence of the metal element.

2.3. Materials and methods

2.3.1. Materials

Glucose (purity > 99.5%, GC grade), xylose (purity > 99%, HPLC grade), and arabinose (purity > 99.5%, HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). Copper oxide (CuO, purity > 99%), iron oxide (Fe_2O_3, purity > 99%), magnesium oxide (MgO, purity > 96%), zinc oxide (ZnO, purity > 99%), nickel oxide (NiO, purity >99%), furfural (purity > 98%, ACS grade), HMF (purity > 97%, ACS grade), ultrapure water (HPLC grade), and 72% sulfuric acid (w/w) were purchased from Thermo Fisher Scientific Chemicals Inc. (Ward Hill, MA). Corn stover was harvested from the Kansas State University Research Farm (Manhattan, KS) and ground to < 1 mm particle size using a SM 2000 cutting mill (Retsch Inc. Newton, PA). The ground biomass was sealed in plastic bags with zippers and stored at room temperature before use.

2.3.2. Pretreatment with metal oxides

Five grams of ground corn stover, calculated amount of metal oxides, and 50 mL of deionized water were weighed into a 75 mL stainless steel reactor (Swagelok, Kansas City Valve & Fitting Co., KS) made of 316 L stainless steel with a 75 mL internal volume (38.1 mm outside diameter, 125 mm length, and 2.4 mm wall thickness). The reactor was shaken upside down for 2 min to completely hydrate biomass, and placed in a shaker at 45 ℃ for 1 h to facilitate metal oxides dispersing in water and touching biomass. To shorten the time that the reactor took to reach target temperatures, the reactor was heated in boiling water for 3 min. After that, the reactor was immediately submerged into a sandbath (Techne Inc., Princeton, NJ) set at target temperatures and hold for various reaction times. Once the reaction was complete, the reactor was immediately placed in ice water (approximately 5 ℃) to cease the biomass hydrolysis reaction. The pH value

of the biomass slurry was determined using a pH700 pH meter (Cole-Parmer, Vernon Hills, IL). The slurry was then filtrated with a Buchner funnel loaded with a filter paper (P8 grade, Fisherbrand) to separate the solids and liquids (biomass liquor). The solid fraction was washed with 180 mL of distilled water for detoxification, and then dried overnight at 45 °C and saved for further analysis. Washing water and biomass liquor were combined, diluted to 250 mL with deionized water, and frozen in a refrigerator until further analysis.

2.3.3. Analytical procedures

Monosaccharides, oligosaccharides, furfural, and HMF in biomass liquor were analyzed according to the National Renewable Energy Laboratory (NREL) laboratory analytical procedure (LAP) "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples" (Sluiter et al., 2008a). Chemical composition of untreated and treated biomass was analyzed according to the NREL LAP "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008b).

Monosaccharides, furfural, and HMF in biomass liquor and structural carbohydrates in treated biomass were determined by high-performance liquid chromatography (HPLC) with the following conditions: the injection volume of 20 μL; an Aminex HPX-87H ion exclusion column (7.8 × 300 mm, Bio-Rad) as the separation unit; HPLC-grade water containing 0.005 M sulfuric acid as the mobile phase; the flow rate of 0.6 mL/min; the column temperature of 60 °C and the RID temperature of 45 °C. Data were processed and analyzed using OpenLAB CDS C.01.05 ChemStation (Agilent, Santa Clara, CA). Oligosaccharides in biomass liquor were calculated based on total saccharides in biomass liquor after autoclaving minus corresponding monosaccharides in biomass liquor before autoclaving.

To analyze the total saccharides in biomass liquor, 10 mL of each biomass liquor was autoclaved with 4% sulfuric acid (w/w) for 1 h at 121 °C to convert oligosaccharides into monosaccharides. Sugar standards with known concentrations were also autoclaved under the same treatment to compensate the sugar loss during autoclaving.

2.3.4. Statistical analyses

All experiments were performed at least duplicate. All data were reported as the mean ± standard deviation (SD). Data were analyzed using SPSS software for Windows (version rel. 16.0, SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when the *p* value was < 0.05.

2.4. Results and discussion

2.4.1. Effect of metal oxide types on sugar recoveries

Inorganic salts pretreatment has recently attracted interest to treat various types of lignocellulosic biomass, which exhibit the capability of hydrolyzing hemicelluloses into monomeric and oligomeric sugars in biomass liquor with a large amount of xylose whereas reserving a cellulose-rich solid substrate that is highly digestible in downstream processing to produce glucose (Loow et al., 2015). Monovalent alkali metal oxides and divalent alkaline earth metal oxides other than MgO were not used because alkali metal oxides can react with water to form strong alkali solutions, which is equivalent to alkali pretreatments. BeO is a kind of rank poison. CaO can react with water to form the corresponding precipitate-$Ca(OH)_2$,which is equivalent to lime pretreatment. Other alkaline earth metal oxides are not commonly used. Four other commonly used metal oxides were selected as catalysts to treat corn stover, including one

trivalent transition metal oxide Fe_2O_3, and three divalent transition metal oxides CuO, NiO, and ZnO. To maintain the same amounts of metal ions with the assumption of all five metal oxides completely dissolving in water, the concentrations of CuO, NiO, ZnO, and MgO applied in pretreatment were 0.08 mol/L, except for the applied dosage of 0.04 mol/L for Fe_2O_3. All other reaction conditions (190 °C, 40 min, and solid/liquid ratio of 1/10 (5 g of corn stover dissolved in 50 mL of distilled water)) remained the same so that metal oxide type was the only major factor influencing the experimental results. Data on the pretreatment of corn stover with different metal oxides are presented in Figure 2.1 with LHW pretreatment as the control.

LHW pretreatment caused a significant pH reduction (4.13) as shown in Figure 2.1A, due to weak acids released from hemicellulose and monosaccharide degradation. Compared to LHW pretreatment, the pH values of Fe_2O_3, CuO, and NiO treated biomass slurries were in the similar range of 3.89 to 4.13, which indicates these three metal oxides rarely interacted with released weak acids during pretreatment. In contrast, among the five metal oxides, only ZnO and MgO, especially MgO, exhibited the best capability of acid neutralization during pretreatment. The neutral pH value of MgO-treated biomass slurry (6.87) demonstrates that acids such as acetic acid, levulinic acid, and formic acid released from hemicellulose and monosaccharide degradation during pretreatment were completely neutralized by MgO.

Among the tested five metal oxides, the most potential total xylose loss (40%) occurred in ZnO pretreatment, whereas Fe_2O_3, CuO, MgO, and NiO pretreatments caused a mild potential total xylose loss of 8-13%, compared to the similar xylose loss (10%) by LHW pretreatment as shown in Figure 2.1B. Compared to LHW pretreatment, Fe_2O_3, CuO, and NiO pretreatments didn't cause significant changes ($p > 0.05$) in the amounts of monomeric (9-14% vs. 10%) and oligomeric (46-51% vs. 49%) xylose and furfural (5.8-8.0% vs. 6.6%) in the liquid fraction or remaining xylan

(21-25% vs. 25%) in the solid fraction. These results indicate that Fe_2O_3, CuO, and NiO addition had no influence on improving xylose recovery and mitigating xylose degradation, which also further confirmed that they cannot react with weak acids released during pretreatment. Although MgO also caused a reduction in potential total xylose (13%), the amount of fermentable xylose (excluding furfural) remained equivalent to that of LHW pretreatment (86 vs. 84%). Only a trace amount of furfural (0.3%) in the liquid fraction was detected after MgO pretreatment, which was due to the high amount of residual xylan in the solid fraction (51%), the small amount of monomeric xylose in the liquid fraction (2.3%), and the near absence of acids in the liquid fraction (pH of 6.87).

Figure 2.1C shows that the amounts of potential total glucose with Fe_2O_3, CuO, NiO, ZnO, and MgO pretreatments were similar to that with LHW pretreatment. In comparison to LHW pretreatment, Fe_2O_3, CuO, NiO, and ZnO pretreatments generated similar amounts of monomeric (1.1-1.3% vs. 0.8%) and oligomeric (4.0-4.5% vs. 4.8%) glucose and HMF (0.5-0.8% vs. 0.6%) in the liquid fraction and similar amounts of remaining glucan (97-100% vs. 98%) in the solid fraction, which further confirmed that these metal oxides cannot react with or only partially reacted with weak acids released during pretreatment. Compared to LHW pretreatment, MgO pretreatment showed a 1.4% higher glucan recovery and 1.8% less oligomeric glucose, thus reducing glucose degradation and resulting in less HMF formation (0.2 vs. 0.6%).

Based on the above analysis, MgO demonstrated the best overall performance for corn stover pretreatment, followed by ZnO, whereas Fe_2O_3, NiO, and CuO pretreatments didn't show significant effects for sugar recoveries. This is mainly attributed to the pH values of starting and complete precipitation of metal ions at a given concentration. Table 2.1 shows the pH values of starting and complete precipitation of the five metal ions at 0.08 mol/L and 25 °C. The pHc (pH

value of solution when metal ions completely precipitate) of Fe^{3+} was 2.82, which is less than the pH value (3.5-4.5) of the LHW treated biomass slurry, indicating that $Fe(OH)_3$ and Fe_2O_3 cannot react with weak acids released during pretreatment. Although the pHs (pH value of solution when metal ions start to precipitate) of Cu^{2+} (4.89), Ni^{2+} (6.92), and Zn^{2+} (6.29) were higher than the pH value of the LHW treated biomass slurry, the deactivation resulted in CuO and NiO hardly and ZnO only partially reacted with released weak acids. The pHs of Mg^{2+} (8.92) indicates that MgO and $Mg(OH)_2$ as moderately strong bases have the capability to completely react with weak acids, which agrees with the experimental results. Therefore, MgO was used for subsequent experiments to compare with LHW as the control.

2.4.2. Effect of reaction time on sugar recoveries

With the need to reduce processing cycle and energy consumption, the effect of reaction time on the pretreatment of corn stover was investigated with other conditions remaining unchanged (temperature at 190 °C, MgO concentration of 0.08 mol/L, and solid to liquid ratio of 1/10). Data on the pretreatment of corn stover with different pretreatment times are presented in Figures 2.2 and 2.3.

The biomass slurry pH from MgO pretreatment decreased from 7.33 to 5.68 (Figure 2.2A) and the biomass slurry pH from LHW pretreatment decreased from 4.23 to 3.93 (Figure 2.3A) as reaction time increased from 30 to 60 min, which was attributed to the gradual release of acids from hemicellulose. The biomass slurry pH after MgO pretreatment for 40 min remained nearly neutral. Though the reaction time extended to 50 min, the pH value was still around 6, which indicates that most of weak acids released during pretreatment were neutralized by MgO.

The amount of potential total xylose from MgO pretreatment decreased from 99 to 75% (Figure 2.2B) and the amount of potential total xylose from LHW pretreatment decreased from 98 to 69% (Figure 2.3B) as reaction time increased from 30 to 60 min. With the extension of pretreatment time, xylan recovery from MgO pretreatment decreased as well as oligomeric xylose in the liquid fraction but monomeric xylose and furfural in the liquid fraction increased (Figure 2.2B). LHW pretreatment had the same trends (Figure 2.3B) as MgO pretreatment with the increase of reaction time. Under each same reaction time, MgO pretreatment had a higher xylan recovery (46-68% vs. 15-32%) and less monomeric (1.9-2.4% vs. 7.3-14.6%) and oligomeric (26-33% vs. 24-54%) xylose in the liquid fraction than LHW pretreatment, thus resulting in less furfural formation (0-1.0% vs. 3.9-16.0%).

The amounts of potential total glucose with MgO and LHW pretreatments decreased by less than 3% (Figures 2.2C and 2.3C) when pretreatment time extended from 30 to 40 min, which might be a result from the degradation of a small amount of glucose released from low structural strength hemicellulose, and there was no significant change ($p > 0.05$) when reaction time extended from 40 to 60 min, which is due to the recalcitrant structure of cellulose. Under each same reaction time, MgO pretreatment had a 1-5% higher glucan recovery and 2-3% less glucose in the liquid fraction than LHW pretreatment, which was also ascribed to the absence of acids.

Based on the above analysis, the optimal MgO reaction time for corn stover pretreatment was 40 min.

2.4.3. Effect of MgO concentration on sugar recoveries

Insufficient MgO concentration could lead to incomplete neutralization of released acids and reduced sugar recoveries. In contrast, excessive MgO concentration incurs unnecessary cost and

may cause plugged filters due to unreacted MgO. Thus, the effect of MgO concentration on corn stover pretreatment was investigated with other conditions remaining unchanged (190 °C for 40 min with a solid to liquid ratio of 1/10). Figure 2.4 shows the effects of MgO concentration on corn stover pretreatment.

As MgO concentration increased from 0 to 0.08 mol/L, the biomass slurry pH was closer to but less than 7.0 (Figure 2.4A) due to a small amount of unreacted acids still present in the liquid fraction. When MgO concentration increased to 0.10 mol/L, the biomass slurry pH was higher than 7.0, due to the complete neutralization of acids by MgO and the partial hydrolysis of $Mg(CH_3COO)_2$.

The amount of potential total xylose did not change significantly (~1%) ($p > 0.05$) as MgO concentration increased from 0.06 to 0.08 mol/L (Figure 2.4B), but the amount of xylan in the solid fraction increased by 7%. In addition, the amount of oligomeric xylose in the liquid fraction reduced by 5%, thus reducing the probability of xylose degradation for furfural formation. When MgO concentration increased to 0.10 mol/L, the amount of potential total xylose (92%) was significantly higher ($p < 0.05$) than that (86-87%) with the previous two MgO concentrations and similar to that (90%) with LHW. Compared to LHW pretreatment, MgO pretreatment enhanced xylan recovery by ~2 times (44-56% vs. 25%) and reduced monomeric (1.9-2.9% vs. 10.0%) and oligomeric (32-37% vs. 49%) xylose in the liquid fraction, thus reducing furfural formation (0-1.0% vs. 6.6%).

Figure 2.4C shows the effect of MgO concentration on glucose recovery. With the increase of MgO concentration, there were no significant changes ($p > 0.05$) in glucan recovery and oligomeric and monomeric glucose in the liquid fraction as well as HMF, which is due to the recalcitrant structure of cellulose.

According to the previous analysis regarding the effects of MgO concentration on sugar recoveries and glucose, xylose, HMF, and furfural formations in the liquid fraction, 0.08 mol/L MgO was selected for the following reaction temperature study.

2.4.4. Effect of reaction temperature on sugar recoveries

Low reaction temperature will not only reduce pretreatment effect but also extend the processing cycle, whereas high reaction temperature will not only cause more energy cost and sugar degradation but also require high-pressure tolerant reactor due to excessive pressure build-up, consequently increasing operation cost. Thus, the effect of reaction temperature on corn stover pretreatment was studied with other reaction conditions remaining unchanged (0.08 mol/L MgO, 40 min, and solid/liquid ratio of 1/10). The findings are presented in Figures 2.5 and 2.6.

As temperature increased from 170 to 210 °C, especially from 190 to 210 °C, the biomass slurry pH from MgO pretreatment decreased from 7.84 to 4.57 (Figure 2.5A) and the biomass slurry pH from LHW pretreatment decreased from 4.58 to 3.36 (Figure 2.6A), due to increased sugar degradation, especially xylose degradation (Figures 2.5B and 2.6B). The generated weak acids exceeded the neutralization capacity of MgO and resulted in pH values closer to that with LHW pretreatment. As reaction temperature increased from 170 to 210 °C, the amount of potential total glucose with MgO pretreatment decreased and the amount of potential total glucose with LHW pretreatment slightly increased and then decreased (Figures 2.5C and 2.6C). Therefore, 190 ^{o}C was considered as an optimum temperature for MgO pretreatment.

2.4.5. Composition comparison of untreated and treated corn stover

Table 2.2 shows the composition of untreated, metal oxide-treated, and LHW-treated corn stover. Compared to untreated corn stover, treated corn stover had a higher cellulose content but a lower hemicellulose content, which is because most of hemicellulose with a weak structural strength was decomposed during pretreatment and most of cellulose with a recalcitrant structural strength still remained in the solid fraction. Solid recoveries from Fe_2O_3, NiO, CuO, and ZnO pretreatments were higher than that from LHW pretreatment, which is a result of the untreated metal oxides in the solid fraction. Lignin recoveries from Fe_2O_3, NiO, CuO, ZnO, and LHW pretreatments were more than 100%, which is due to the formation of carbohydrate-derived-pseudo-lignin (Hu et al., 2012; Kumar et al., 2013). Compared to LHW pretreatment, MgO pretreatment reduced the formation of pseudo-lignin. With LHW pretreatment as the reference, Fe_2O_3, NiO, CuO, and ZnO pretreatments had a lignin removal increase of -4.4-0.6%, which indicates that these metal oxide pretreatments had no difference in lignin removal with LHW pretreatment. However, compared to LHW pretreatment, MgO pretreatment increased lignin removal and the removal increased with the increase of MgO concentration.

2.5. Conclusions

Metal oxides demonstrate great potential for biomass pretreatment. MgO pretreatment has significant advantages over LHW pretreatment: 1) MgO can neutralize released acids, avoiding the need for acid-resistant equipment and saving cost; 2) Neutralization of released acids also reduces monosaccharide degradation and inhibitor formation, largely saving water uses for inhibitor removal; 3) MgO reduced the pseudo-lignin formation; and 4) Near-neutral liquid fractions with trace amounts of furfural and HMF render the water-washing step unnecessary and

allow the direct use of biomass slurries for saccharification and fermentation, which will be explored in our following chapters.

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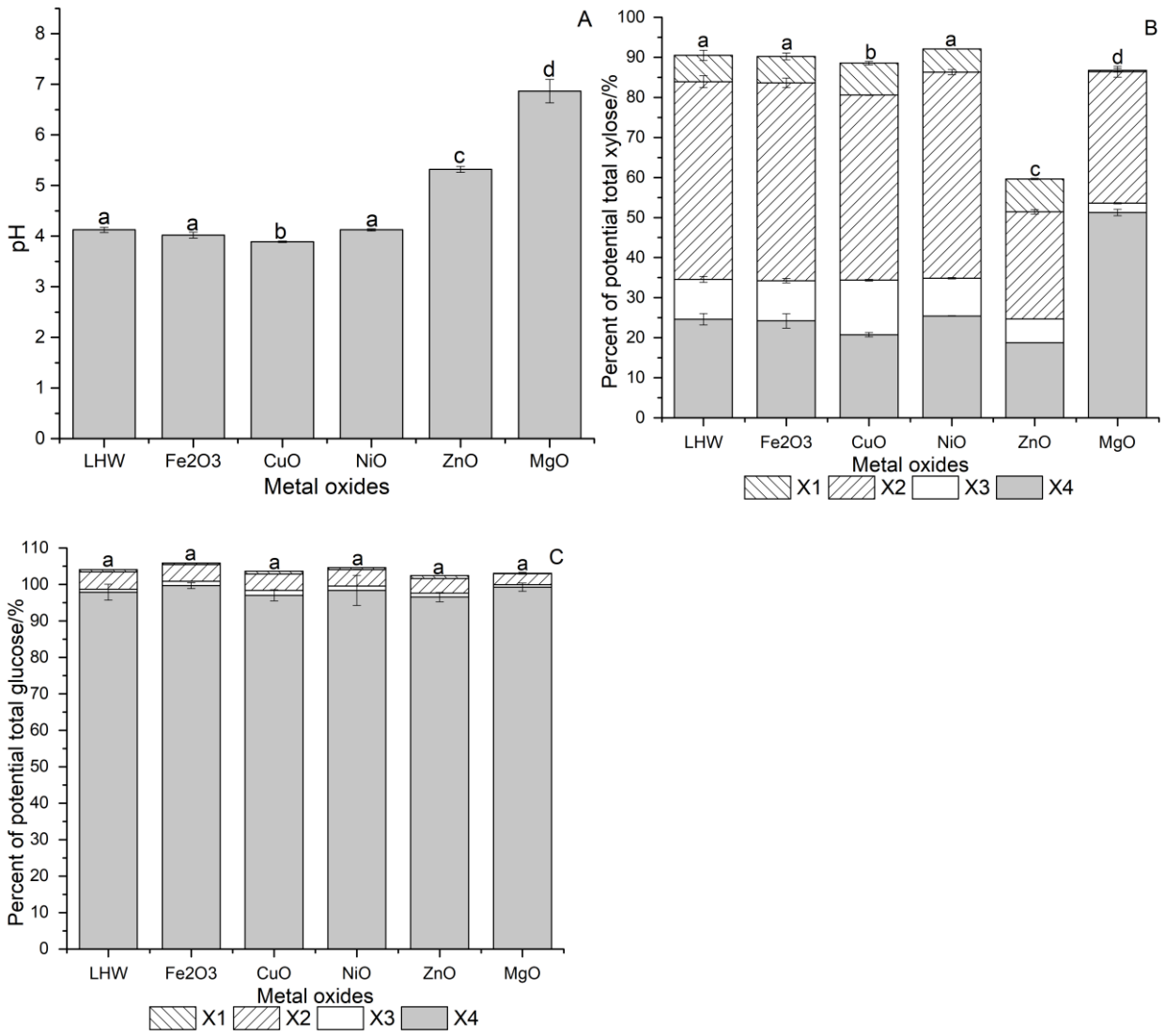

Figure 2.1 The effects of metal oxides on sugar recoveries. (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.)

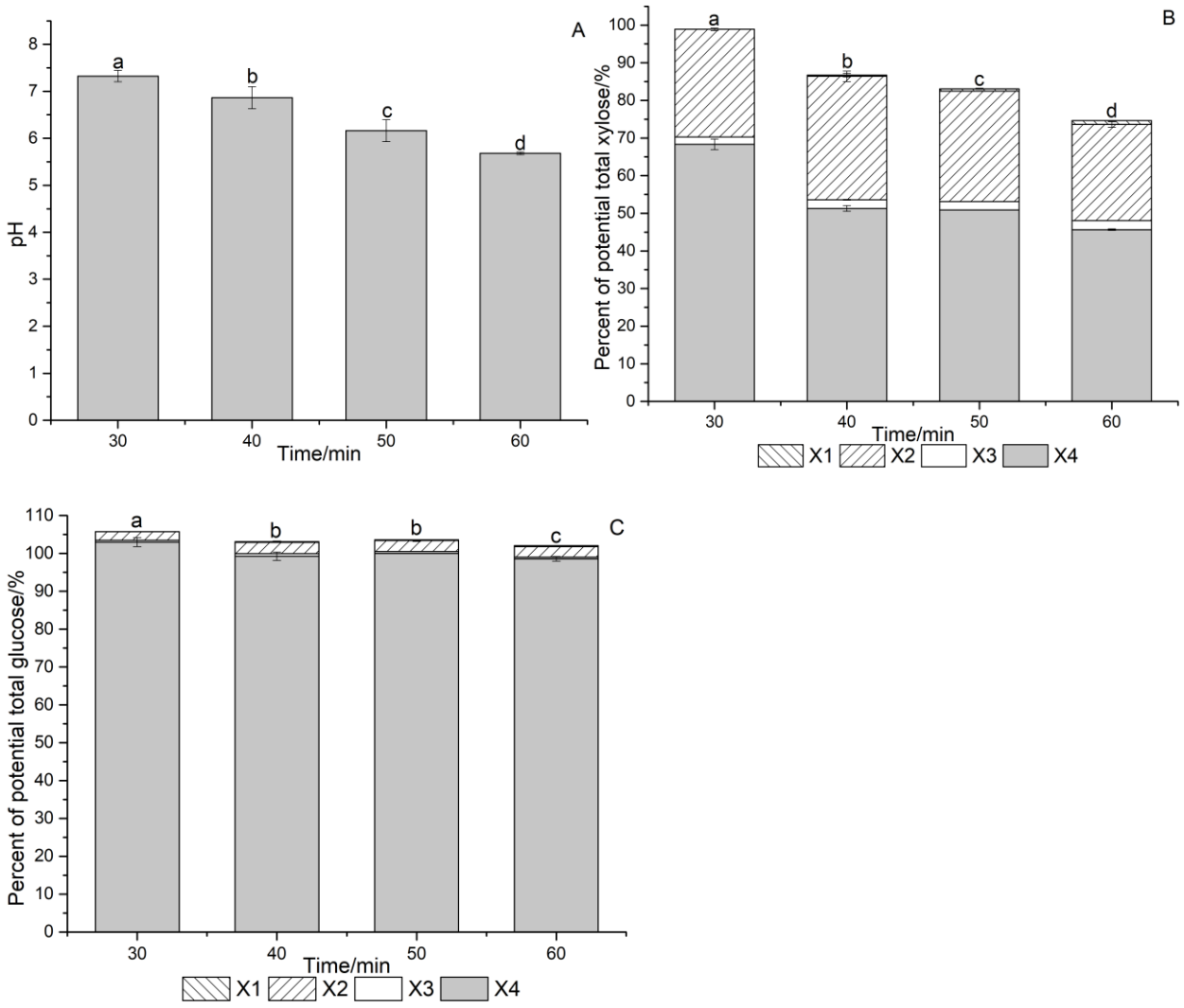

Figure 2.2 The effects of reaction time on sugar recoveries with MgO pretreatment. (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.)

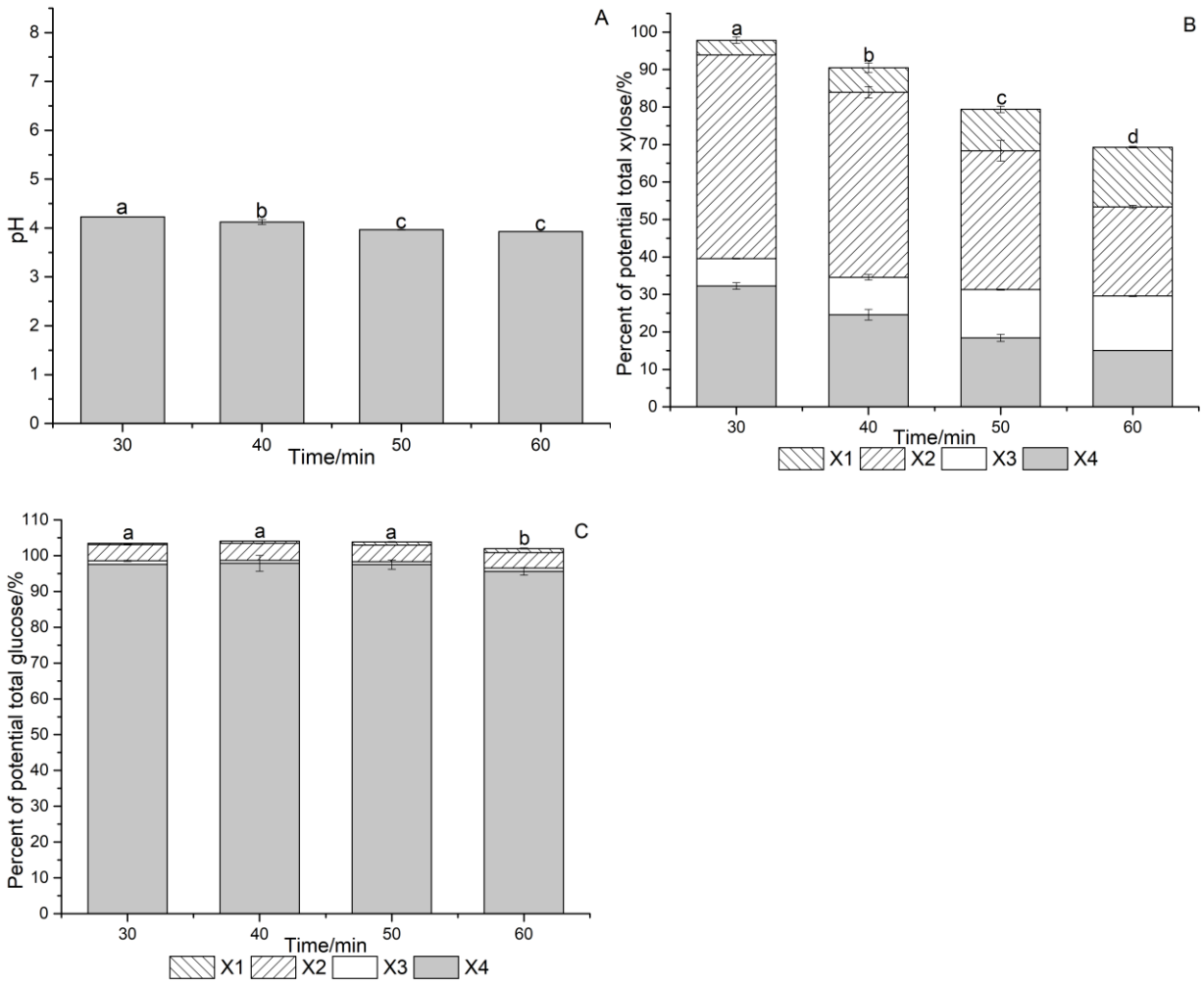

Figure 2.3 The effects of reaction time on sugar recoveries with liquid hot water pretreatment. (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.)

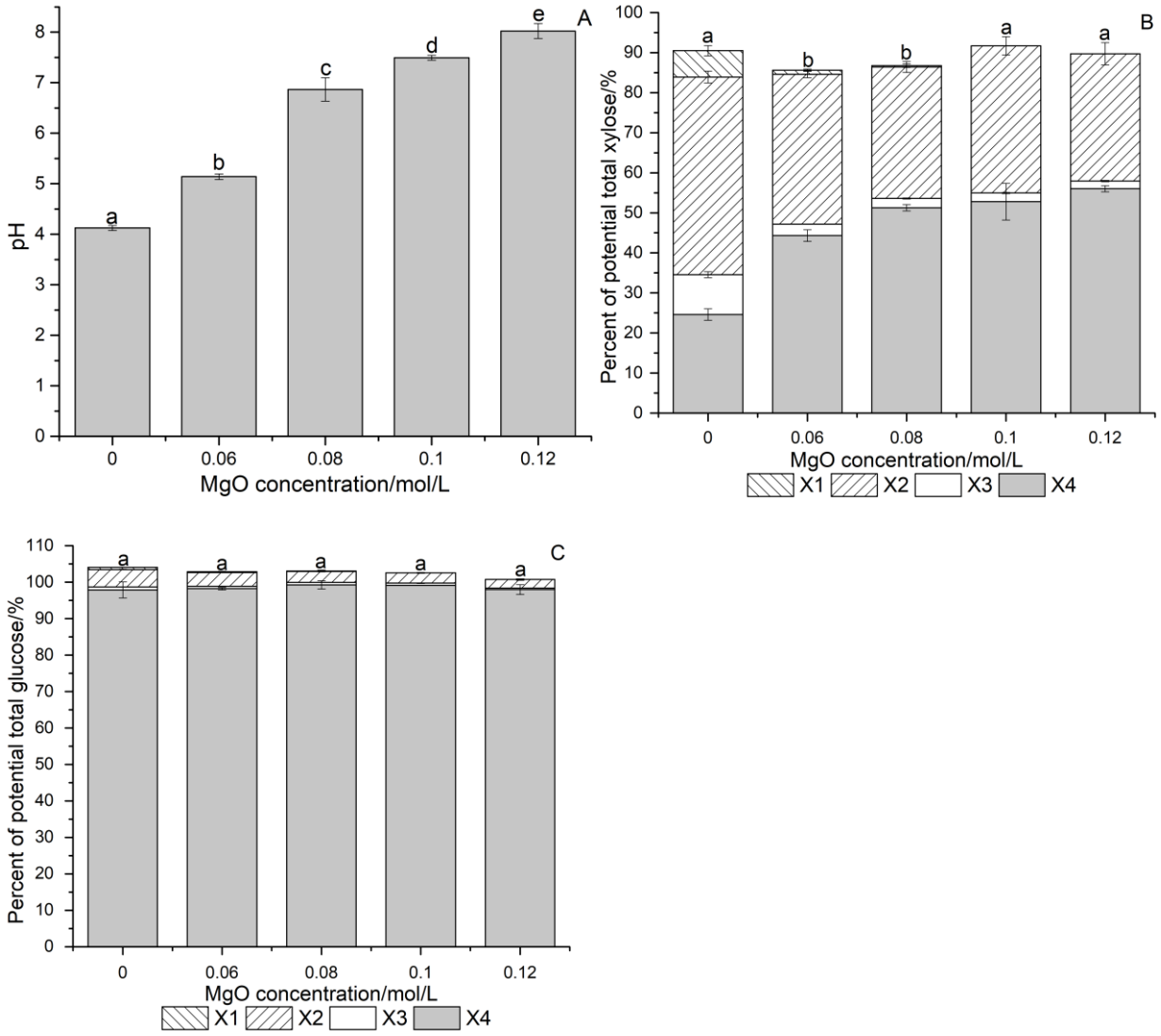

Figure 2.4 The effects of MgO concentration on sugar recoveries. (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.)

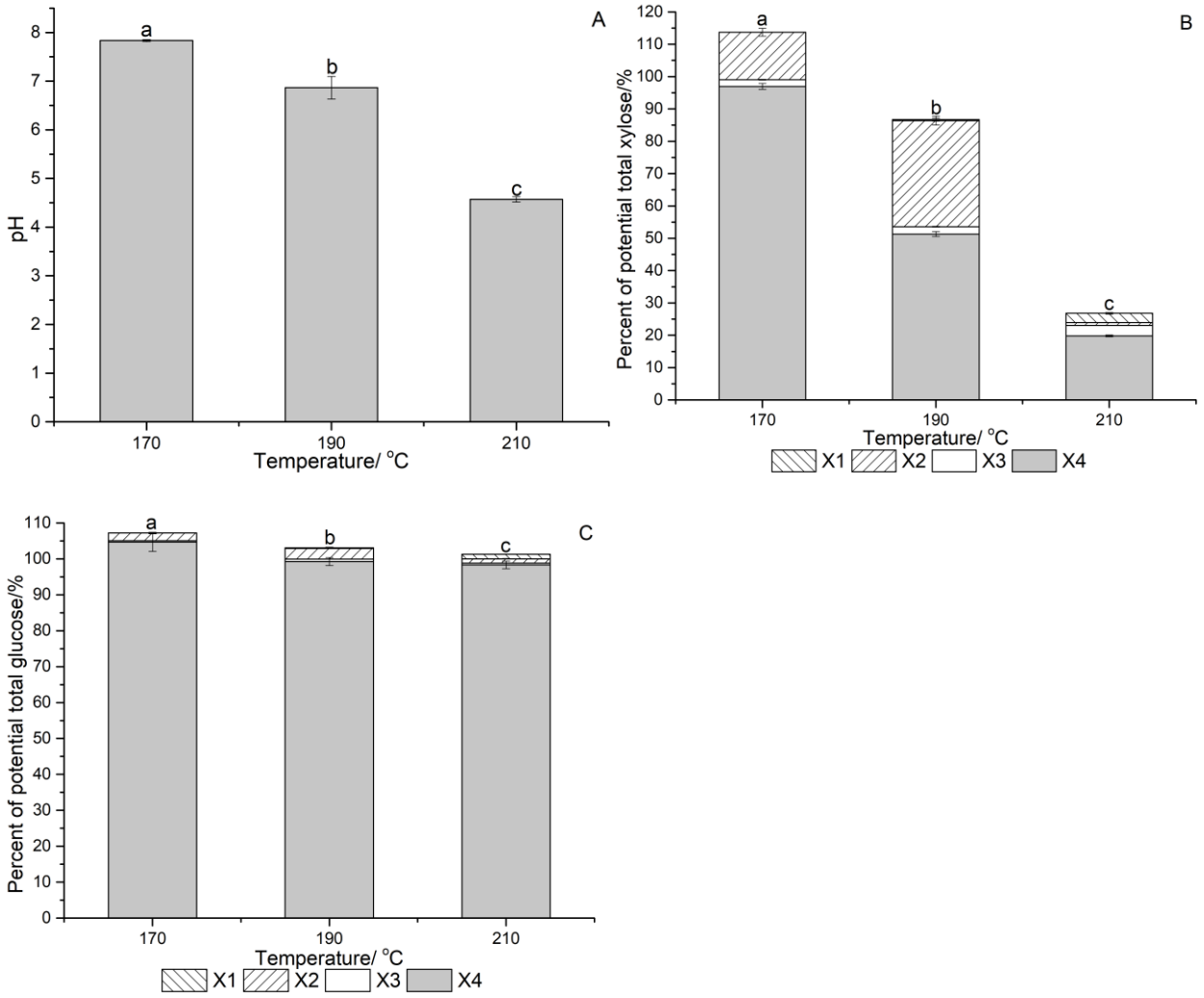

Figure 2.5 The effects of reaction temperature on sugar recoveries with MgO pretreatment. (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.)

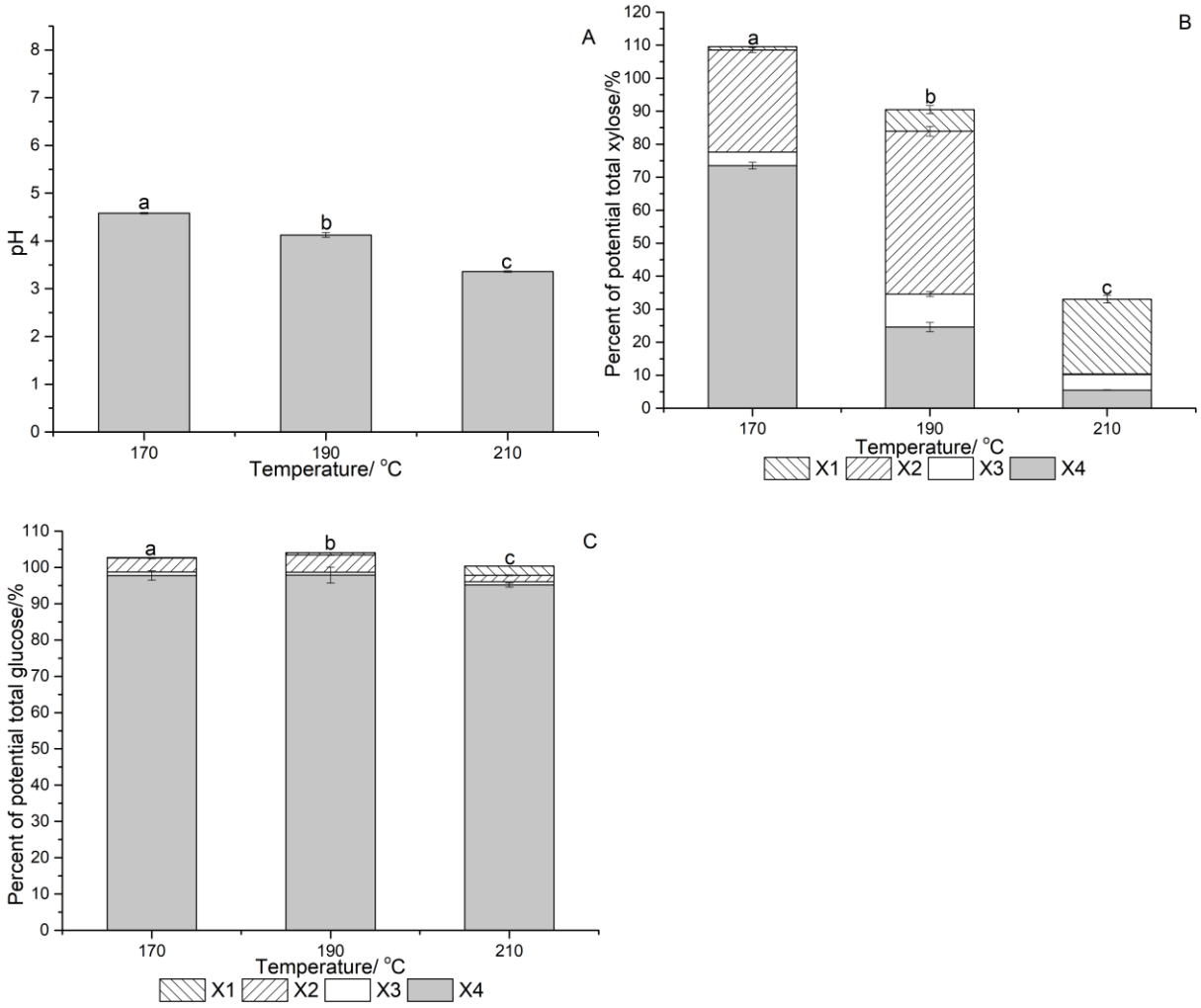

Figure 2.6 The effects of reaction temperature on sugar recoveries with liquid hot water pretreatment. (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.)

Table 2.1 The pH_s and pH_c of Fe^{3+}, Cu^{2+}, Ni^{2+}, Ni^{2+}, and Mg^{2+}.

Metal ions	Ksp (Haynes, 2014)	Initial concentration (mol/L)	pH_s	pH_c
Fe^{3+}	2.79×10^{-39}	0.08	1.51	2.82
Cu^{2+}	4.80×10^{-20}	0.08	4.89	6.84
Ni^{2+}	5.48×10^{-16}	0.08	6.92	8.87
Zn^{2+}	3.00×10^{-17}	0.08	6.29	8.23
Mg^{2+}	5.61×10^{-12}	0.08	8.92	10.87

$$M(OH)_n \leftrightarrow M^{n+} + nOH^-$$

$$pH_s = 14 + \log \sqrt[n]{\frac{Ksp}{C_i}}$$

$$pH_c = 14 + \log \sqrt[n]{\frac{Ksp}{C_r}}$$

M is the metal element; n is the chemical valence of the metal element; pH_s is the pH value of solution when M^{n+} starts to precipitate; pH_c is the pH value of solution when M^{n+} completely precipitates; K_{sp} is the solubility product constant at 25 °C and is defined for equilibrium between a solid and its respective ions in a solution; C_i is the initial concentration of M^{n+}; and C_r is 10^{-5} mol/L, which is defined for the residual concentration of M^{n+} after the complete precipitation of M^{n+}.

Table 2.2 Composition of untreated and treated corn stover.

Corn stover[1]	Cellulose (%)	Hemicellulose[2] (%)	Lignin (%)	Solid recovery (%)	Lignin recovery (%)	Lignin removal increase[3] (%)
Untreated	36.8±0.29	21.9±0.04	15.1±0.40			
Liquid hot water treated	57.6±0.96	8.1±0.50	29.9±0.22	62.5±0.36	123.7±1.61	0
0.04 mol/L Fe_2O_3 treated	53.8±0.37	7.3±0.44	27.4±0.05	68.2±1.02	123.9±0.33	-0.2
0.08 mol/L CuO treated	52.9±1.00	6.4±0.18	28.7±0.23	67.5±0.22	128.1±1.45	-4.4
0.08 mol/L NiO treated	53.3±1.96	7.7±0.01	27.4±0.06	67.8±0.33	123.1±0.33	0.6
0.08 mol/L ZnO treated	54.3±0.41	5.9±0.03	28.8±0.45	65.4±0.37	124.8±1.26	-1.1
0.06 mol/L MgO treated	56.0±0.84	14.0±0.34	26.0±0.25	64.6±0.75	111.3±2.35	12.4
0.08 mol/L MgO treated	55.4±0.42	16.0±0.31	23.8±0.77	66.0±0.23	104.0±3.75	19.7
0.10 mol/L MgO treated	54.4±1.55	16.4±1.12	22.3±0.97	67.1±1.94	98.8±1.45	24.8
0.12 mol/L MgO treated	52.4±0.76	17.2±0.28	21.7±0.15	68.8±0.03	98.6±0.62	25.1

[1] Temperature and time for all pretreatments are 190 °C and 40 min, respectively.

[2] Hemicellulose includes xylan and arabinan.

[3] Lignin removal increase is calculated using liquid hot water pretreatment as the reference.

Chapter 3 - Magnesium oxide pretreatment to achieve high fermentable sugar concentration and yield from corn stover

3.1. Abstract

MgO pretreatment was used to enhance the fermentable sugars derived from corn stover compared with liquid how water pretreatment as control. Compared to control, MgO pretreatment achieved double hemicellulose recovery (49 vs. 25%). Double hemicellulose recovery enhanced xylose yield by 20% and total sugar yield by 6% without sacrificing glucose yield (76 vs. 77%) under the optimal conditions (reaction condition: 10% biomass loading, 0.08 mol/L MgO, 190 °C, and 40 min; saccharification condition: treated biomass loading of 10 g/100 mL, enzyme dosages of 30 µL CTec3 and 18 µL NS22244/g treated biomass, 52 °C, and 72 h). A total sugar concentration of 58 g/L was achieved under the optimal reaction conditions. Both SEM and FTIR analyses show that MgO effectively disrupted the recalcitrant structures of biomass and enlarged the exposed surface area of carbohydrates, thus boosting the enzymatic saccharification efficiency and fermentable sugars.

3.2. Introduction

The demand to develop sustainable and renewable energy is urgent with the rising global environmental issues and the rapid depletion of traditional fossil fuels. Biofuels, especially bioethanol, is regarded as an environmentally-friendly and renewable energy and can be used to substitute the unsustainable fossil derived gasoline (Stacey et al., 2016). First generation ethanol production using cereal crops, such as corn, wheat, and sorghum, or sugar-rich crops, such as sugarcane and sugar beet, has been commercially produced owing to the high starch or sugar-to-ethanol conversion efficiency. The highly mature biotechnologies for starch-derived ethanol production have also been advanced with efficient processes and low-cost enzymes (Mohanty and Swain, 2019); however, the competition of biofuel industry against food and feed industry may become severe due to the overuse of starchy grains for biofuel production (Xu et al., 2011). Robust growth in human population and fast increase in demand for animal feed will further intensify the "food vs. fuel" competition. Therefore, researchers are seeking new pathways to produce second generation biofuels obtained from non-food crops, perennial grasses, and agricultural and food wastes. Lignocellulosic biomass, as a renewable and widely-available biomass resource, is a highly potential solution to substitute cereal crops for ethanol production, and has already received a lot of research and industrial attention (Hassan et al., 2018; Moreno et al., 2017). However, great challenge for lignocellulosic ethanol production remains to overcome due to the inherent complex structure of lignocellulosic biomass, which forms strong native recalcitrance and blocks the enzymatic accessibility to carbohydrates, thus causing a low conversion efficiency with a glucose yield of approximately 20% without any pretreatment process (Mosier et al., 2005). To improve the enzymatic digestibility of lignocellulosic biomass for cellulosic ethanol production, an efficient pretreatment is generally required to untie the structural seal from lignin and hemicellulose, reduce

the cellulose crystallinity, limit the production of inhibitory products, and increase the porosity of lignocellulosic biomass (Mahmood et al., 2019).

Various biomass pretreatment technologies have been explored such as physical, chemical, physicochemical, biological, and combined pretreatments (Hassan et al., 2018; Hou et al., 2019; Kang et al., 2018; Sahoo et al., 2018; Wei et al., 2018; Wells et al., 2020; Yu et al., 2018), among which dilute sulfuric acid method has been industrialized (Sahoo et al., 2018) and liquid hot water (LHW) pretreatment has also gained much attention due to zero chemical addition in the pretreatment step (Wells et al., 2020). However, both of the two techniques cause sugar degradation and inhibitor formation due to the presence of added sulfuric acid and/or released acetic acid from hemicellulose. The production of inhibitory products by acids will seriously reduce the efficiency of downstream processes, such as enzymatic saccharification of carbohydrate-to-sugar, and microbial fermentation of sugar-to-ethanol (Pandey et al., 2014). The findings from Chapter 2 have indicated that magnesium oxide (MgO) functions an effective catalyst and shows the capability for completely neutralizing the acetic acid released from hemicellulose, thus avoiding furfural and HMF formation in the pretreatment slurry. Compared to LHW pretreatment, MgO pretreatment improves cellulose and hemicellulose recoveries, especially hemicellulose recovery, which would increase fermentable sugars during enzymatic saccharification.

The improvement in both cellulose and hemicellulose recoveries after MgO pretreatment results in enhanced amounts of initial sugars in treated biomass for subsequent enzymatic saccharification; however, it can’t guarantee increased sugar yield because sugar yield is determined by both the percentage of initial sugars in treated biomass used for enzymatic saccharification and the sugar conversion efficiency (Equation 3.1). The degree in biomass

recalcitrance disruption via pretreatment technology decides the enzymatic accessibility to carbohydrates, eventually determining the conversion of lignocellulosic biomass to biofuel (Liu et al., 2009). Thus, the effectiveness and feasibility of MgO pretreatment on enhancing the enzymatic saccharification of cellulose-to-glucose and hemicellulose-to-xylose need to be further studied as demonstrated in Figure 3.1. In order to achieve this objective, the enzymatic saccharification efficiencies of MgO-treated corn stover were investigated through comparing the yields and conversions of sugars (glucose, xylose, and total sugar). The macro- and microstructural modifications of corn stover before and after pretreatment were also examed by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM).

$$\text{Sugar yield} = \frac{sugar\ recovery\ from\ pretretament \times sugar\ conversion\ during\ enzymatic\ hydrolysis}{the\ amount\ of\ initial\ sugars\ in\ biomass\ used\ for\ pretreatment} \times 100\% \quad (3.1)$$

3.3. Materials and methods

3.3.1. Materials

MgO with a purity of >96.0% and Pierce™ BCA Protein Assay Kit were obtained from Fisher (Ward Hill, MA). Enzymes CTec3 and NS22244 were freely supplied by Novozymes (Franklinton, NC). Protein contents of CTec3 and NS22244 were measured using the Pierce™ BCA Protein Assay Kit. Protein contents of CTec3 and NS22244 were 516 and 266 mg protein/mL, respectively. Corn stover was collected from the Agricultural Trial Base (Kansas State University, Manhattan, KS) and milled to a particle size below 1 mm before use.

3.3.2. Pretreatment

Pretreatment process is as described in Chapter 2. Briefly, five grams of ground corn stover (10% solid loading), calculated amount of MgO (0.06-0.12 mol/L), and 50 mL of deionized water

were weighed into a 75 mL stainless steel reactor. The reactor was shaken upside down for 2 min to completely hydrate biomass, placed in a shaker at 45 ℃ for 1 h to facilitate metal oxides dispersing in water and touching biomass, and heated in boiling water for 3 min in order to reduce the time that the reactor took from room temperature to target temperatures. After that, the reactor was immediately transferred into a sandbath (Techne Inc., Princeton, NJ) set at target temperatures (170-210 ℃) and hold for various reaction times (30-60 min). Once the reaction was complete, the reactor was immediately placed to ice water (approximately 5 ℃) to cease the biomass hydrolysis reaction. The slurry was then filtrated with a Buchner funnel loaded with a filter paper (P8 grade, Fisherbrand) to separate the solids and liquids (biomass liquor). The solid fraction was washed with 180 mL of deionized water for detoxification, and then dried overnight at 45 ℃ and saved for future analysis. Washing water and biomass liquor were combined, and diluted to 250 mL with deionized water, and frozen in a refrigerator until further analysis.

3.3.3. Enzymatic saccharification

The calculated amount of MgO-treated corn stover was placed in a 125 mL flask, followed by the addition of calculated volume of sodium acetate buffer (50 mM, pH 5.0). To avoid the sugar loss caused by microbial contamination during enzymatic saccharification, sodium azide was added as a bacteriostatic agent with a loading of 0.02% (w/v). After that, the calculated volumes of CTec3 and NS22244 were loaded. The optimal protein ratio of CTec3 and NS22244 was 10 to 3 (Figure A.1), which corresponded the volume ratio of 10 to 6. The slurry was hydrolyzed enzymatically at 52 ℃ and 140 rpm for 72 h. During enzymatic saccharification, 80 μL of biomass slurry was sampled periodically from each flask to monitor sugar concentrations.

Sugar conversion efficiency was used to investigate the effects of solid loadings and enzyme dosages on enzymatic saccharification of treated biomass. The cellulose conversion efficiency was equal to the amount of glucose in slurries after enzymatic saccharification divided by the amount of total original glucose (1.11 times of the cellulose amount) in treated biomass before enzymatic saccharification. The hemicellulose conversion efficiency was the same as that of cellulose. The specific equations were as followings:

$$E_c = \frac{V \times C_g}{1.11 \times m \times A_g} \times 100\% \qquad (3.2)$$

$$E_h = \frac{V \times C_x}{1.14 \times m \times A_x} \times 100\% \qquad (3.3)$$

where E_c and E_h are the enzymatic conversion efficiencies of cellulose and hemicellulose of treated biomass (%), respectively; C_g and C_x are the concentrations of glucose and xylose in saccharification slurry determined by HPLC (g/mL); m is the dry weight of treated biomass used for enzymatic saccharification (g); A_g and A_x are the glucan and xylan contents in treated biomass (%), respectively; 1.11 and 1.14 are the conversion factors of glucan-to-glucose and xylan-to-xylose, respectively; and V is the volume of saccharification solution (mL).

The effects of pretreatment on the sugar yields as received biomass were determined by following formulas:

$$Y_g = \frac{R_b \times E_c \times A_g}{A_{g'}} \times 100\% \qquad (3.4)$$

$$Y_x = \frac{R_b \times E_h \times A_x}{A_{x'}} \times 100\% \qquad (3.5)$$

where Y_g and Y_x are the glucose and xylose yields (%) as received biomass, respectively; R_b is the treated biomass recovery (%); and $A_{g'}$ and $A_{x'}$ are the glucan and xylan contents in raw biomass (%), respectively.

3.3.4. HPLC analysis

Monomeric and oligomeric sugars and inhibitors in biomass liquor and composition of pretreated biomass were analyzed according to the procedures recommended by NREL (Sluiter et al., 2008a, 2008b). Concentrations of sugars, furfural, and HMF were measured by a 1200 HPLC system (Agilent, Santa Clara, CA). The separation unit was an HPX-87H organic acid column (7.8 × 300 mm) purchased from the Bio-Rad (Hercules, CA) and set at 60 °C. The temperature of refractive index detector was set at 45 °C. The mobile phase was 0.005 M sulfuric acid water and set at a flow rate of 0.6 mL/min.

3.3.5. FTIR analysis

The changes in chemical structures of corn stover during pretreatment were analyzed by a 400 FTIR/FT-NIR spectrophotometer (PerkinElmer Corp., Shelton, CT). The FTIR spectra of samples were measured under following conditions: scattering mode, 32 scans, 4 cm^{-1} resolution, and 400-4000 cm^{-1} wavenumber range.

3.3.6. SEM images

The morphological modification of treated biomass was characterized with a scanning electron microscope (S-3500 SEM) and an absorbed electron detector (Hitachinaka, Lbaraki, Japan). Samples were mounted on conductive adhesive tapes, coated with a 4 nm thick metal mixture of 40% palladium and 60% gold by spraying. After that, prepared samples were observed on SEM and corresponding images were captured.

3.3.7. Statistics

Experiments were conducted at least duplicate. All data were reported as the mean ± standard deviation (SD). Data were analyzed statistically using SAS Studio (SAS Institute Inc., Cary, NC). Differences were considered statistically significant when the *p* value was < 0.05.

3.4. Results and discussion

3.4.1. Composition of raw and MgO-treated corn stover

Composition of raw and MgO-treated corn stover was shown in Table 3.1. Raw corn stover had 36.8% cellulose and 21.9% hemicellulose. MgO-treated corn stover had 56.3% cellulose and 15.2% hemicellulose, which are insignificantly different from those (55.4% cellulose and 16.0% hemicellulose, Table 2.1) in MgO-treated corn stover used for pretreatment performance investigation (Chapter 2). These two batches of MgO-treated corn stover also differed insignificantly in other components such as glucan recovery (99 vs. 100%), xylan recovery (51 vs. 49%), monomeric glucose (0.7 vs. 0.6%) and xylose (2.3 vs. 2.4%) in liquor, oligomeric glucose (3.0 vs. 3.3%) and xylose (33 vs. 36%) in liquor, furfural (0.32 vs. 0.58%), HMF (0.15 vs. 0.15%), and slurry pH (6.87 vs. 6.77) (Figure 3.2).

3.4.2. Effect of reaction temperature on enzymatic saccharification of corn stover

Insufficient reaction temperature may result in poor pretreatment performance and increase the reaction cycle as well as reduce the enzymatic accessibility to carbohydrates, resulting in a low conversion efficiency. In contrast, excessive reaction temperature not only increases the energy cost and inhibitor formation, also reduces the sugar recoveries, resulting in a low final sugar yield. Thus, the effects of reaction temperature on the enzymatic saccharification of corn stover were

investigated with other reaction factors remaining constant (reaction condition: 0.08 mol/L MgO, 40 min, and 10% biomass loading; saccharification condition: treated biomass loading of 1 g/100 mL, enzyme dosages of 50 μL CTec3 and 30 μL NS22244/g treated biomass, saccharification temperature of 52 ℃, and 72 h). The effects of reaction temperature on the enzymatic saccharification of corn stover are presented in Figure 3.3A.

The glucose yield as received biomass increased from 54 to 73% as reaction temperature increased from 170 to 210 ℃, indicating that high reaction temperature can more effectively disrupt the rigid biomass structures, and increase the enzymatic accessibility to cellulose. However, the xylose yield as received biomass decreased from 41 to 6% as reaction temperature increased from 170 to 210 ℃, indicating that severe reaction temperature resulted in significant hemicellulose decomposition due to its weak structural strength. To obtain the optimal reaction temperature, the total sugar yield as received biomass, including glucose and xylose, was calculated to evaluate the effectiveness of reaction temperature. The total sugar yield of 58% was obtained at the reaction temperature of 190 ℃, which was significantly higher than that at 170 (50%) and 210 ℃ (50%). Therefore, 190 ℃ was selected as reaction temperature for the following studies.

3.4.3. Effect of reaction time on enzymatic saccharification of corn stover

To save the energy cost and reduce the processing cycle, the effects of reaction time on the enzymatic saccharification of corn stover were investigated in subsequent conditions (reaction condition: 10% biomass loading, 0.08 mol/L MgO, and reaction temperature of 190 ℃; saccharification condition: treated biomass loading of 1 g/100 mL, enzyme dosages of 50 μL

CTec3 and 30 μL NS22244/g treated biomass, saccharification temperature of 52 °C, and 72 h). Results are shown in Figure 3.3B.

As reaction time increased from 30 to 40 min, the glucose yield as received biomass increased significantly ($p < 0.05$) from 67 to 70%, but the xylose yield as received biomass decreased from 38 to 34%, thus the total sugar yield as received biomass remaining unchanged. This demonstrates the initial reaction time extension resulted in the disruption of more biomass structures and increased enzymatic accessibility to carbohydrates. With the reaction time increasing from 40 to 60 min, the glucose, xylose, and total sugar yields as received biomass all reduced (70 to 66%, 34 to 24%, and 58 to 51%), indicating that further extension of reaction time might result in cellulose and hemicellulose degradation, especially hemicellulose degradation (Results in Chapter 2). Therefore, 40 min was selected as reaction time for further experiments.

3.4.4. Effect of MgO concentration on enzymatic saccharification of corn stover

MgO concentration is not only the critical factor for complete neutralization of acids released during pretreatment but also highly related to downstream processes. Therefore, the effects of MgO concentration on the enzymatic saccharification of corn stover were investigated in subsequent conditions (reaction condition: 10% biomass loading, reaction temperature of 190 °C, and 40 min; saccharification condition: treated biomass loading of 1 g/100 mL, enzyme dosages of 50 μL CTec3 and 30 μL NS22244/g treated biomass, saccharification temperature of 52 °C, and saccharification time of 72 h). Results are shown in Figure 3.3C.

With MgO concentration increasing from 0.06 to 0.12 mol/L, glucose, xylose, and total sugar yields as received biomass increased from 65 to 80%, 27 to 42%, and 52 to 67%, respectively, which was mainly because increased MgO concentration improved lignin removal and

hemicellulose recovery (Results in Chapter 2). This agrees with the previous publication (Park et al., 2010), in which Mg was recognized as an effective catalyst to delignify the lignocellulosic biomass. Given that the biomass slurry pH with 0.10 mol/L MgO was greater than 7 due to the partial dissociation of formed $Mg(CH_3COO)_2$ (Results in Chapter 2), 0.08 mol/L MgO concentration was selected for following experiments. In addition, compared to LHW pretreatment (0 mol/L MgO concentration), the pretreatment with 0.08 mol/L MgO concentration had a lower glucose yield (70 vs. 75%) but a higher xylose yield (34 vs. 14%) as received biomass, resulting in a higher total sugar yield (58 vs. 53%) as received biomass (Figure 3.3C).

3.4.5. Effect of solid/liquid ratio on enzymatic saccharification of corn stover

The treated biomass loading is highly related to not only chemicals addition and water consumption used for buffer preparation but also mass and heat transfers that impact enzymatic saccharification efficiency. Therefore, the effects of treated biomass loading on enzymatic saccharification of corn stover were investigated with other factors remaining constant (reaction condition: 10% biomass loading, 0.08 mol/L MgO, reaction temperature of 190 °C, and 40 min; saccharification condition: enzyme dosages of 30 μL CTec3 and 18 μL NS22244/g treated biomass, saccharification temperature of 52 °C, and saccharification time of 72 h). Results are shown in Figure 3.4.

In Figure 3.4A, glucose concentration increased significantly ($p < 0.05$) as treated biomass loading increased. Significant difference ($p > 0.05$) was not found in glucan conversion among treated biomass loadings of 3 to 6 g/100 mL (Figure 3.4C), demonstrating that mass transfer efficiency and enzyme activities were not significantly impacted by the treated biomass loading of below 6 g/100 mL under the evaluated conditions. The glucan conversion efficiency reduced from

78 to 75% with the treated biomass loading increasing from 6 to 10 g/100 mL, showing that further increased biomass loading not only limited the mass transfer efficiency but also increased the amount of lignin released from treated biomass to saccharification slurry (Huang et al., 2015), thus resulting in lower enzyme activities and glucan conversion efficiency. However, the glucose concentration enhanced significantly from 29 to 46 g/L as treated biomass loading further increased from 6 to 10 g/100 mL. The xylose concentration and xylan conversion efficiency (Figure 3.4B and D) had similar trends. The xylan conversion efficiency reduced from 75 to 73%, whereas the xylose concentration enhanced from 7 to 12 g/L as treated biomass loading increased from 6 to 10 g/100 mL. In addition, the mixing issues arose when the treated biomass loading for enzymatic saccharification was 12 g or more/100 mL, which resulted in a poor mass transfer efficiency due to the adsorption of most free water by biomass and the limited mixing capability of incubator shaker used in this work (Cara et al., 2007). Therefore, the treated biomass loading of 10 g/100 mL was selected for the following experiments.

3.4.6. Effect of enzyme dosage on enzymatic saccharification of corn stover

Expensive enzyme cost is one of the major obstacle limiting the commercialization of cellulosic biofuels. To reduce the enzyme cost, the effects of enzyme dosage on enzymatic saccharification of corn stover were investigated with other factors remaining constant (reaction condition: 10% biomass loading, 0.08 mol/L MgO, reaction temperature of 190 °C, and 40 min; saccharification condition: treated biomass loading of 10 g/100 mL, saccharification temperature of 52 °C, and saccharification time of 72 h). Results are shown in Figure 3.5.

As CTec3/NS22244 dosage increased from 20/12 to 40/24 μL/g treated biomass, both glucose concentration (39 to 46 g/L) and glucan conversion efficiency (63 to 75%) enhanced as well as

xylose concentration (10 to 12 g/L) and xylan conversion efficiency (62 to 73%). The glucose concentration and glucan conversion efficiency didn't increase significantly with the CTec3/NS22244 dosage increasing from 30/18 to 40/24 μL/g treated biomass, demonstrating that the CTec3/NS22244 dosage of 30/18 μL/g treated biomass was sufficient to overcome the reduced enzyme activities caused by lignin derived phenol compounds in treated biomass and consequently obtain a high glucose concentration and glucan conversion efficiency. The similar trends were found in xylose concentration and xylan conversion efficiency. Based on comprehensive consideration, the CTec3/NS22244 dosage of 30/18 μL/g treated biomass was selected for enzymatic saccharification of MgO-treated corn stover. Under the selected conditions above (reaction condition: 0.08 mol/L MgO, 190 °C for 40 min with 10% biomass loading; saccharification condition: treated biomass loading of 10 g/100 mL and enzyme dosages of 30 μL CTec3 and 18 μL NS22244/g treated biomass at 52 °C for 72 h), the total fermentable sugar (glucose and xylose) concentration of 58 g/L was obtained after enzymatic saccharification.

3.4.7. Comparison of sugar yields of MgO- and LHW-treated corn stover

To compare the sugar yields of MgO- and LHW-treated corn stover, the enzymatic saccharification of 0.08 mol/L MgO- and LHW-treated corn stover was investigated with other factors remaining constant (reaction condition: 10% biomass loading, reaction temperature of 190 °C, and reaction time of 40 min; saccharification condition: treated biomass loading of 10 g/100 mL, enzyme dosages of 30 μL CTec3 and 18 μL NS22244/g treated biomass, saccharification temperature of 52 °C, and saccharification time of 72 h). The sugar yields of 0.08 mol/L MgO- and LHW-treated corn stover are shown in Figure 3.6.

Similar glucose yields (76 vs. 77%) as received biomass were achieved from MgO- and LHW-treated corn stover. However, the xylose yield of MgO-treated corn stover was more than double that of LHW-treated corn stover (36 vs. 16%), leading to a higher total sugar yield (62 vs. 55%) as received biomass. In addition, the near-neutral biomass slurry with trace amounts of furfural and HMF (Results in Chapter 2) may enable ethanol plants to directly apply biomass slurry for enzymatic saccharification without acid neutralization and water-washing or detoxification, which will not only largely save water consumption but also significantly enhance the total sugar yield in ethanol production, especially xylose yield, due to most hemicellulose degrading to monomeric and oligomeric xyloses and dissolving in biomass liquor during pretreatment process (Results in Chapter 2). This hypothesis is under further investigation and will be presented in subsequent chapters.

3.4.8. Modification of chemical structures of corn stover

To detect the modification of chemical structures of corn stover after MgO pretreatment, FTIR was applied in the study (Figure 3.7). The peak in the range of 3330-3340 cm^{-1} is owing to OH stretching, and the peak intensity reduced significantly after MgO pretreatment, demonstrating the breakage of hydrogen bonds inter-connecting hemicellulose, cellulose, and lignin together (Kumar et al., 2009). This is due to the weak bond strength of hydrogen bond (Xu et al., 2018). The peak in the range of 2900-2920 cm^{-1} is derived from C-H stretching (He et al., 2008), and the reduction of peak intensity shows that MgO pretreatment caused the partial disruption of the methyl and methylene portions in cellulose. The complicated fingerprint region at 900-1800 cm^{-1} usually indicates more inside information on the structural modification of carbohydrates and lignin (Corredor et al., 2009). The disappearance of the peak in the range of 1720-1730 cm^{-1} proves

MgO pretreatment disrupted uronic ester and acetyl groups on hemicellulose (Windeison et al., 2007). The intensities of the three peaks in the ranges of 1600-1610, 1510-1520, and 1315-1320 cm^{-1} which are ascribed to aromatic skeletal vibration, carbonyl stretching, and C-O and C=C vibrations of lignin side chains (Sun et al., 2005) changed, demonstrating that aliphatic side chains of lignin linked with carbohydrates were cleaved after MgO pretreatment. The disappearance of the peak in the range of 1240-1230 cm^{-1} after MgO pretreatment demonstrates the shift of esters and solubilization of phenolics in treated corn stover (Sene et al., 1994).

3.4.9. Surface properties of corn stover

To detect the macrostructural modification of surface properties of corn stover after MgO pretreatment, SEM was used. Results (Figure 3.8) showed that the surface of raw corn stover was highly fibrillar, smooth, and intact; however, MgO-treated corn stover had a smaller particle size and broken cell structure, demonstrating the disruption of silicified waxy surface and the more exposed internal cell structure. Therefore, MgO pretreatment enlarged the porosity of corn stover and the external surface area, which would benefit the enzymatic accessibility to carbohydrates during enzymatic saccharification.

3.5. Conclusions

MgO pretreatment effectively improved hemicellulose and cellulose recoveries and minimized sugar degradation compared with LHW pretreatment. Enhanced hemicellulose recovery from MgO pretreatment improved xylose yield by 20% as received biomass without sacrificing glucose yield, therefore enhancing the total sugar yield by 6%. A total sugar concentration of 58 g/L was achieved under the optimal conditions. Both SEM and FTIR analyses

proved MgO pretreatment effectively disrupted the recalcitrant structures of biomass and enlarged the exposed surface area of cellulose, consequently enhancing enzymatic saccharification efficiency.

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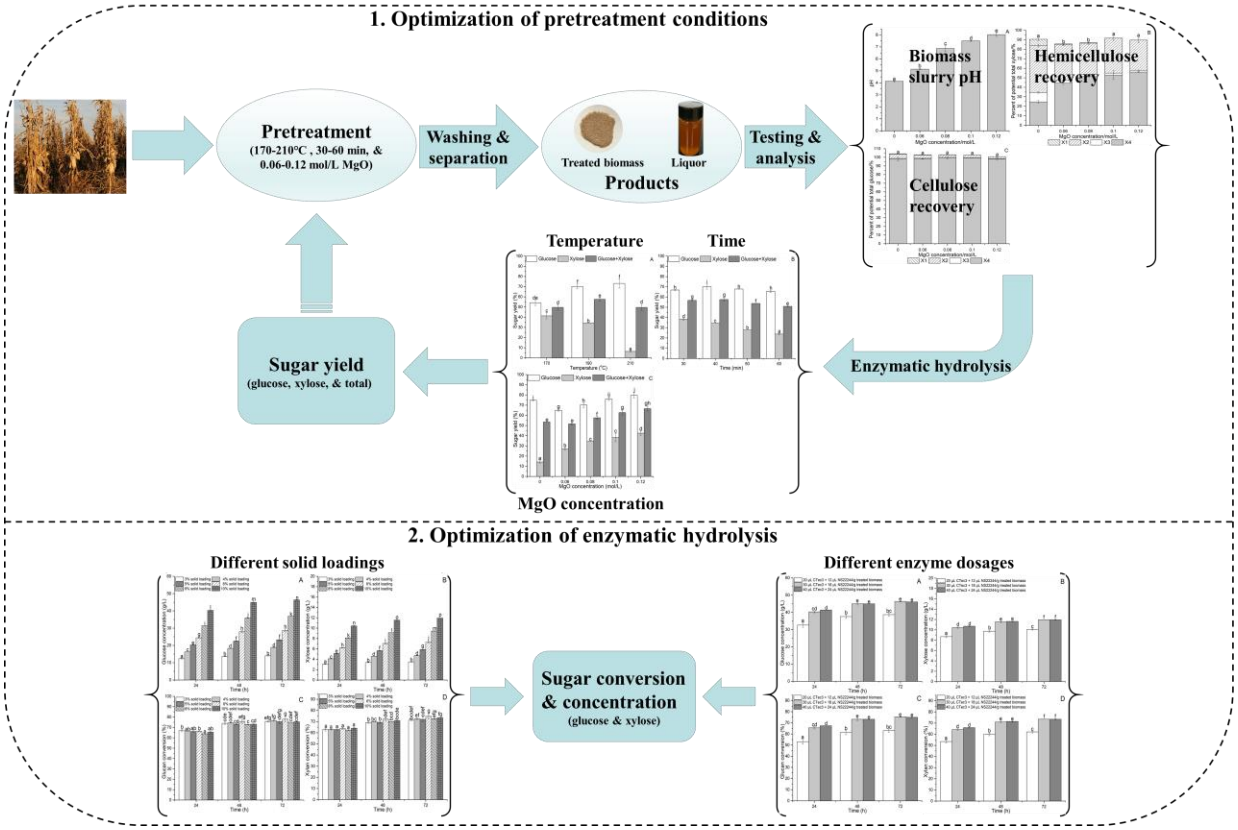

Figure 3.1 The process flow diagram of this study.

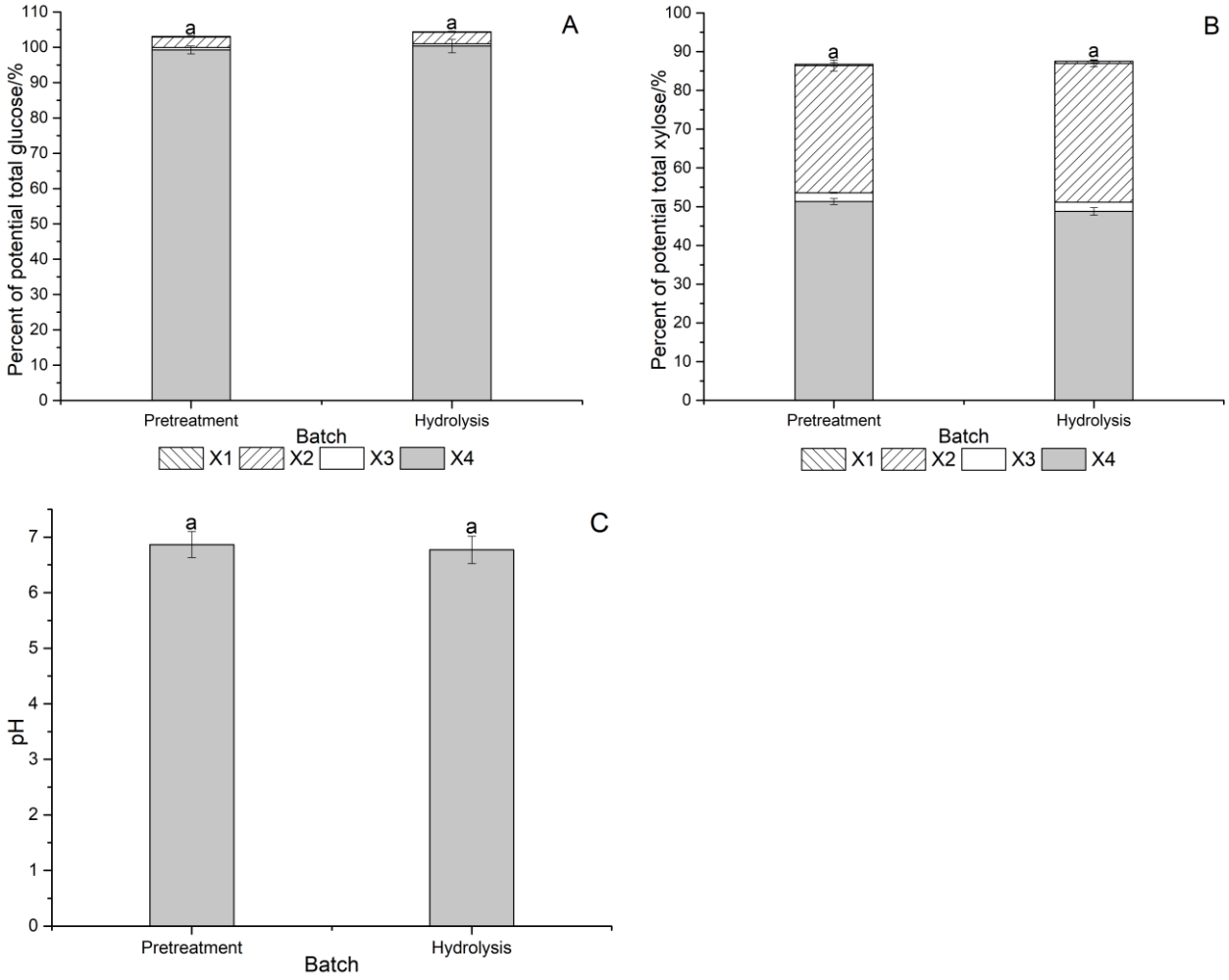

Figure 3.2 Chemical composition comparison between the batch of MgO-treated corn stover used for enzymatic saccharification investigation and the batch of MgO-treated corn stover used for pretreatment performance investigation (X1 is furfural or HMF in the liquid fraction; X2 is oligomeric xylose or glucose in the liquid fraction; X3 is monomeric xylose or glucose in the liquid fraction; X4 is remaining xylan or glucan in the solid fraction; all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.).

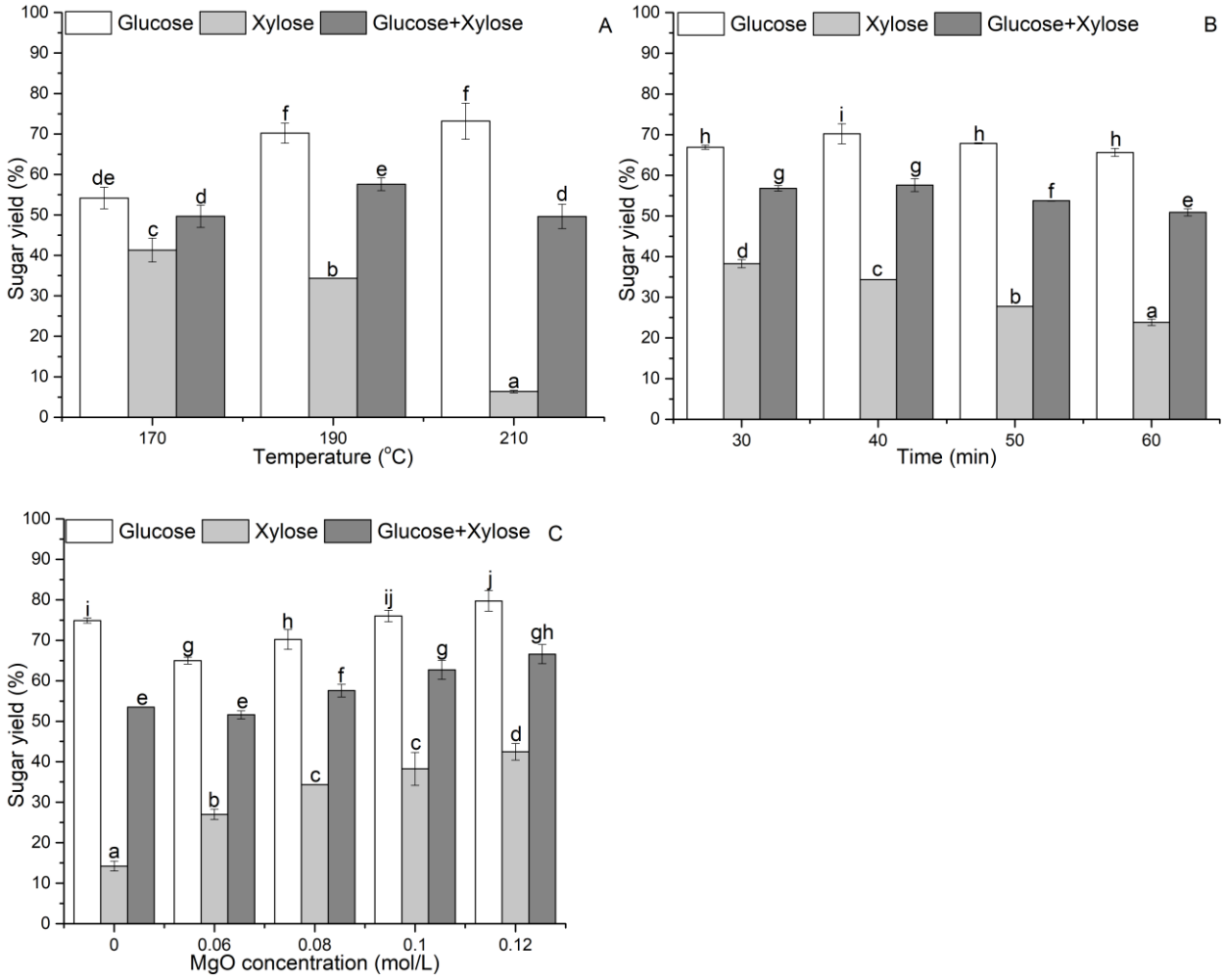

Figure 3.3 Effects of reaction temperature (A), reaction time (B), and MgO concentration (C) on enzymatic saccharification of MgO-treated corn stover (compositions of corn stover under different MgO pretreatments were listed in Table B.1).

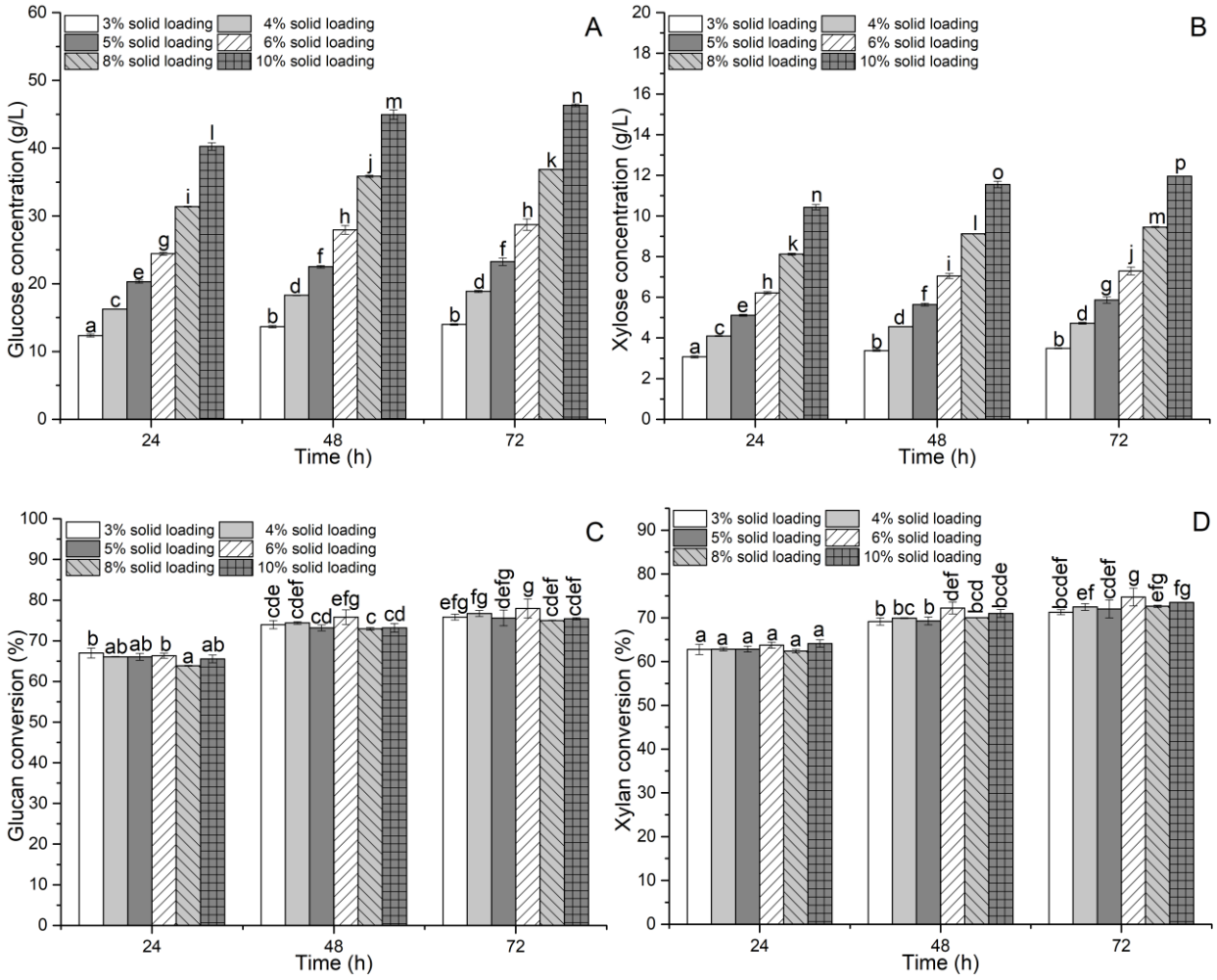

Figure 3.4 Effect of solid/liquid ratio on enzymatic saccharification of MgO-treated corn stover (MgO-treated corn stover had 56.3% cellulose and 15.2% hemicellulose).

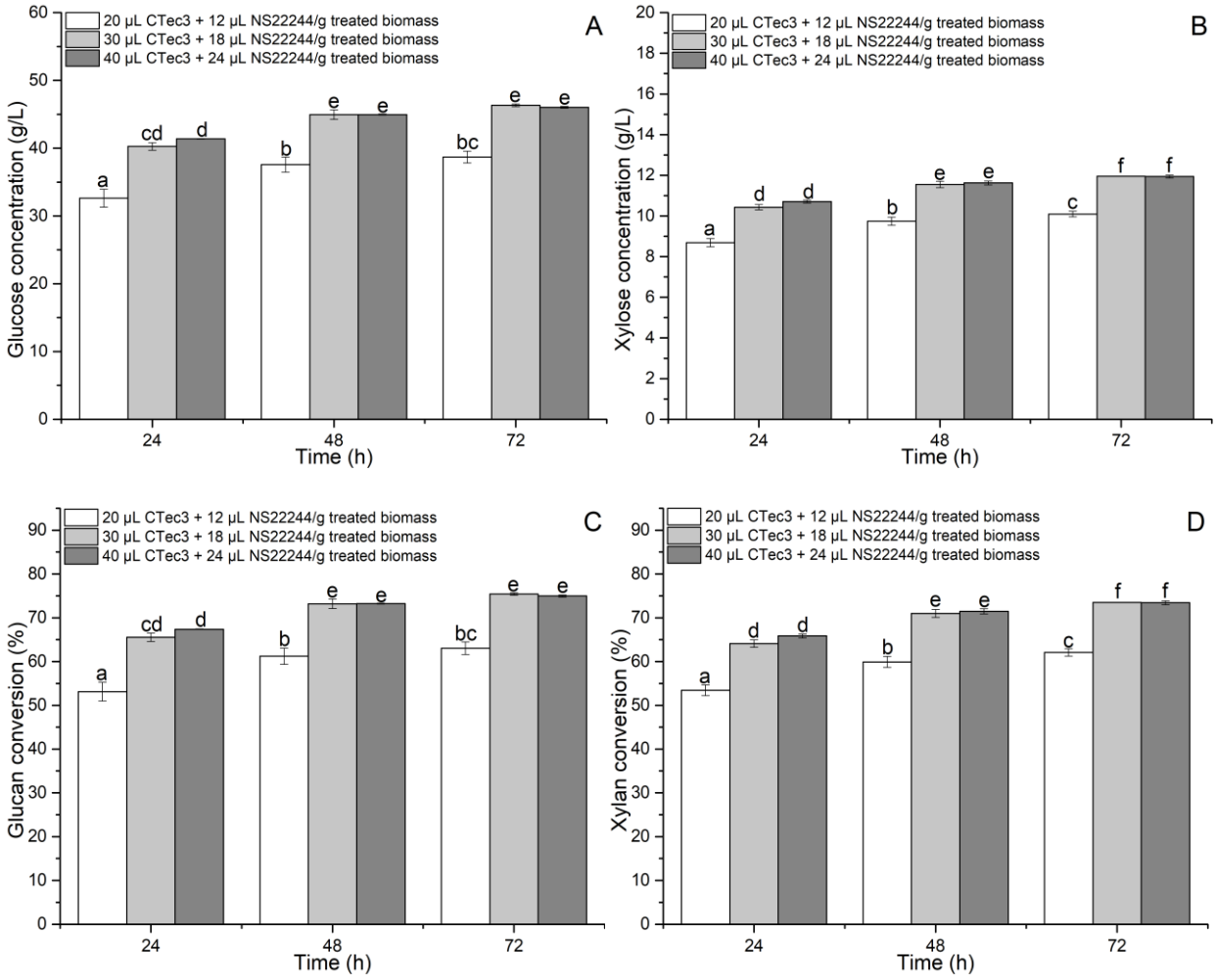

Figure 3.5 Effect of enzyme dosage on enzymatic saccharification of MgO-treated corn stover (MgO-treated corn stover had 56.3% cellulose and 15.2% hemicellulose).

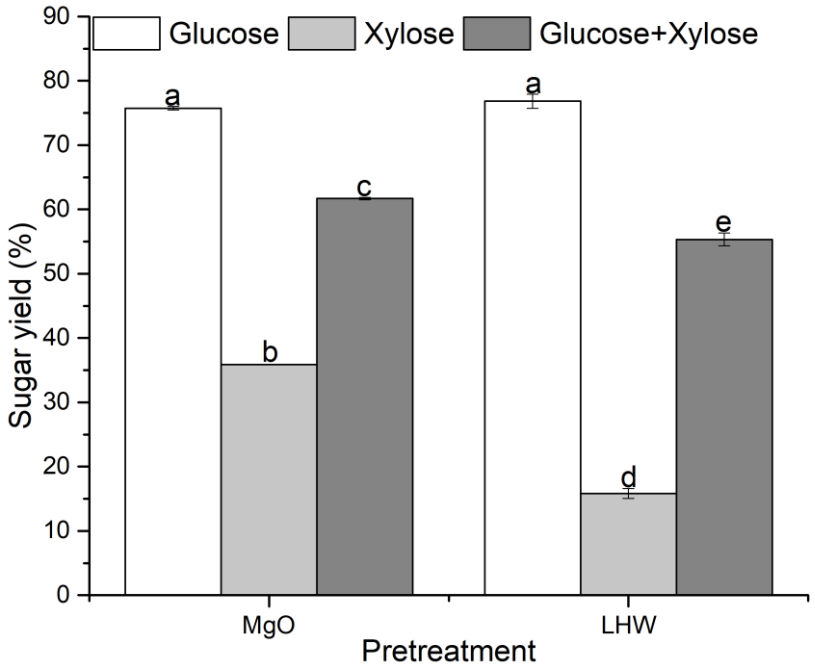

Figure 3.6 Comparison of sugar yields of MgO- and LHW-treated corn stover (MgO-treated corn stover had 56.3% cellulose and 15.2% hemicellulose; and LHW-treated biomass had 57.6% cellulose and 8.1% hemicellulose).

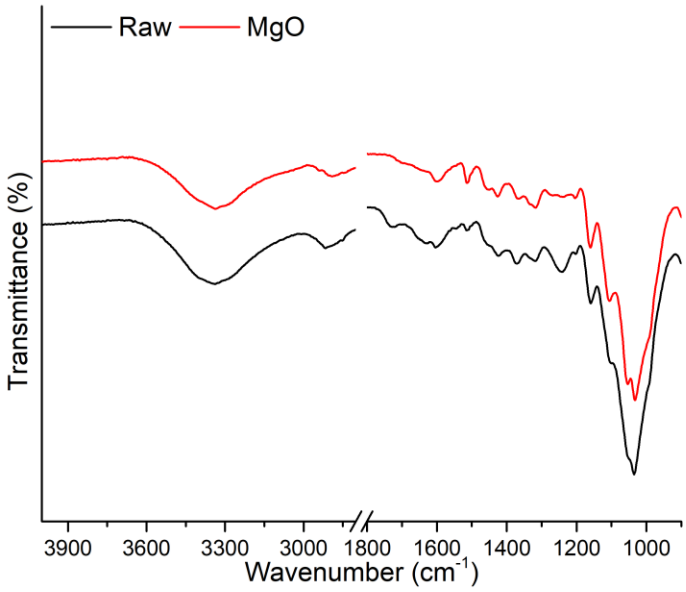

Figure 3.7 FTIR spectra of corn stover before and after MgO pretreatment.

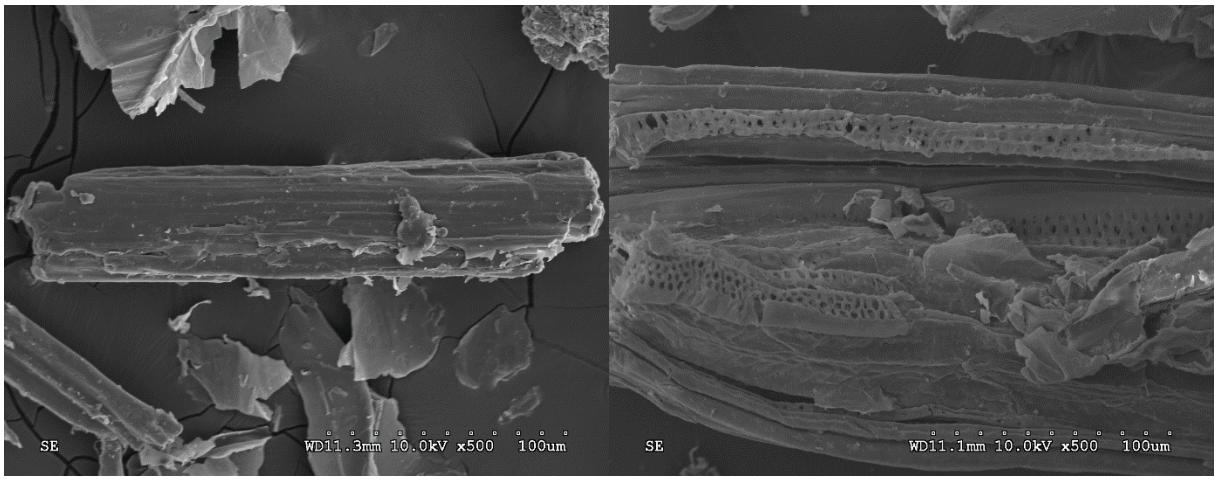

Figure 3.8 SEM of corn stover before (left) and after (right) MgO pretreatment.

Table 3.1 Composition of raw and MgO-treated corn stover.

Corn stover	Cellulose (%)	Hemicellulose[2] (%)
Raw	36.8±0.29a[3]	21.9±0.04a
MgO-treated[1]	56.3±1.07b	15.2±0.34b

[1] MgO pretreatment conditions are 10% solid loading, 0.08 mol/L MgO, 190 °C, and 40 min.
[2] Hemicellulose includes xylan and arabinan.
[3] In each column, values with different letters are significantly different at $p < 0.05$.

Chapter 4 - High fermentable sugar yield through integration of magnesium oxide-treated corn stover and pretreatment liquor without washing and detoxification

4.1. Abstract

The objective of this research was to boost fermentable sugar yield and concentration through integration of pretreatment liquor into enzymatic saccharification of MgO-treated corn stover as well as to simplify the bioconversion process. Results showed that enzymatic saccharification of MgO-treated corn stover with pretreatment liquor had a lower glucose yield (71 vs. 75%) but xylose yield was much higher than that from MgO-treated corn stover only (66 vs. 36%), resulting in no significant difference in total sugar concentration (57 vs. 58 g/L). Corn stover slurry from MgO pretreatment with near-neutral pH had only a trace amount of furfural and 5-hydroxymethylfurfural, and was used directly for enzymatic saccharification, eliminating the need for washing and detoxification and lightening the burden for wastewater treatment. Additionally, using surfactant Tween 80 can effectively reduce the binding of lignin to enzyme, increasing glucose and xylose yields by 8 and 10% and sugar concentration by 7 g/L.

4.2. Introduction

Substituting fossil fuels with biofuels can mitigate environmental pollution and climate change (Ho et al., 2019). Currently, more than 98% of the gasoline used in the United States is blended with bioethanol to generate a series of flex fuels such as E85, E15, and E10 for different vehicles (DOE, 2020). More than 95% of ethanol produced in the United States is from corn ethanol, whereas cellulosic ethanol accounts for less than 1% (RFA, 2017). This is because using lignocellulosic biomass for biofuel production still faces technical challenges, including the low coefficient of utilization of both cellulose and hemicellulose due to the chemical structural seal caused by lignin in the outer layer of the biomass cell wall (Ponnusamy et al. 2019). To address this issue, pretreatment is usually required as the first step of cellulosic ethanol production to dismantle the structural seal of lignin and expose more cellulose and hemicellulose to enzymes for saccharification (Kumar et al., 2009). In recent years, various pretreatment methods for lignocellulosic biomass have been developed, such as acid (Kuglarz et al., 2018; Sahoo et al., 2018), alkali (Kang et al., 2018; Tran et al., 2020), liquid hot water (LHW) (Yang et al., 2019), ammonia fiber explosion (Sousa et al., 2019), ionic liquid (Sundstrom et al., 2018), organic solvent (Yu et al., 2018), and physical assisted pretreatments (Bussemaker and Zhang, 2013; Ma et al., 2009), but most of these techniques are still at the laboratory stage. Only the dilute sulfuric acid pretreatment method has been applied in the industrial production of cellulosic ethanol.

The LHW pretreatment method has received much attention due to its zero addition of chemicals in the pretreatment process compared to the industrialized dilute sulfuric acid pretreatment method (Sahoo et al., 2018; Yang et al., 2019). However, these two pretreatment methods have common defects. First, both added sulfuric acid and hemicellulose-derived acetic acid degrade monosaccharides and generate inhibitors such as furfural and 5-

hydroxymethylfurfural (HMF), resulting in fermentable sugar loss (van der Pol et al., 2015). In addition, to reduce energy input, water usage, and operating costs, high-solids loading saccharification is being attempted to utilize both cellulose and hemicellulose for ethanol production. Unfortunately, high-solids loading saccharification makes the inhibitory effect of inhibitors on enzyme activities more severe due to the accumulation of inhibitors and residual lignin released from treated biomass (Caspeta et al., 2014; Roche et al., 2009). Second, to reduce the inhibitory effect, a large amount of water must be used to wash the treated biomass (Figure 4.1A) prior to enzymatic saccharification (Tao et al., 2011; Zheng et al., 2012), which will increase cost and generate a large amount of wastewater and increase the burden of wastewater treatment (Figure 4.2A). Third, liquor from biomass pretreatment contains a significant amount of fermentable sugars, especially xylose (i.e. pretreatment liquor contains approximately 30% of the total xylose (Kumar et al., 2009)) that cannot be utilized directly for saccharification and fermentation due to the presence of degradation products such as acetic acid, furfural, and HMF (Humbird et al., 2011). Thus, detoxification is required for pretreatment liquor prior to enzymatic saccharification (Figure 4.1A and B) (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000).

The studies in Chapter 2 and 3 revealed that MgO pretreatment is a promising approach to solve the above-mentioned issues. MgO functions to completely neutralize the acetic acid released from hemicellulose during pretreatment, which can reduce sugar degradation and result in the biomass slurry nearly neutral in pH. Water-washing is no longer needed (Figure 4.1C), which greatly reduces downstream water usage and wastewater treatment (Figure 4.2B). Also, biomass liquor from MgO pretreatment can be used directly for cellulosic ethanol production because of the absence of inhibitory products such as acetic acid, furfural, and HMF. Therefore, MgO-treated

biomass slurry may need only a simple pH adjustment (Figure 4.2B) prior to saccharification and fermentation. In addition, magnesium acetate ($Mg(CH_3COO)_2$) generated from the neutralization of acetic acid by MgO is a buffer salt and dissolves in biomass slurry after pretreatment, which can reduce the chemical consumption for buffer preparation for enzymatic saccharification. Therefore, MgO pretreatment demonstrates great potential to largely simplify the biomass conversion process and save capital costs (Figure 4.1C and 4.2B), which completely meets the green and environmentally-friendly concept of creating more benefits and values with simple processing procedures and low capital costs.

The objective of this research was to boost fermentable sugar yield and concentration through integration of pretreatment liquor into enzymatic saccharification of MgO-treated corn stover as well as to simplify the bioconversion process. To achieve this goal, enzymatic saccharification of both MgO-treated corn stover only and MgO-treated corn stover with pretreatment liquor were investigated using saccharification efficiency and sugar yield as evaluation criteria.

4.3. Materials and methods

4.3.1. Chemicals and materials

Magnesium oxide (MgO, purity >96.0%) and Tween 80 (purity >99.0%) were purchased from Fisher (Ward Hill, MA). Enzymes CTec3 and NS22244 were provided by Novozymes (Franklinton, NC). Protein contents of CTec3 and NS22244 were 516 and 266 mg protein/mL, respectively. Corn stover was harvest from the Agricultural Trial Base (Kansas State University, Manhattan, KS) and milled to a particle size below 1 mm before use.

4.3.2. Biomass pretreatment

Pretreatment was conducted as previously described in Chapter 2. Corn stover with a 10% solids loading and 0.08 mol/L MgO was treated at 190 °C for 40 min. The resulting biomass slurry was partitioned into treated biomass (filter cake) and pretreatment liquor using a Buchner funnel loaded with a filter paper (P8 grade, Fisherbrand). The solids and liquor were processed with the two following pathways:

Case 1: Filter cake was washed with 180 mL of distilled water and dried at 45 °C overnight prior to composition analysis and enzymatic saccharification. Washing water and pretreatment liquor were combined, diluted to 250 mL in a 250 mL volumetric flask, and frozen in a refrigerator until analysis.

Case 2: Pretreatment liquor was adjusted to pH 5.0 and re-slurried with filter cake for enzymatic saccharification.

4.3.3. Enzymatic saccharification

Enzymatic saccharification was conducted as previously described in Chapter 3. The calculated amount of treated biomass in Case 1 or Case 2 was loaded in a 125 mL flask, followed by the addition of calculated volume of sodium acetate buffer (50 mM, pH 5.0) for Case 1 or pH-adjusted pretreatment liquor (pH 5.0) for Case 2. Sodium azide (0.02%, w/v) was added to avoid microbial contamination. After that, the calculated volume of CTec3 and NS22244 was loaded. The volume ratio of CTec3 and NS22244 loadings was 10 to 6. The slurry was hydrolyzed enzymatically in an I2400 incubator (New Brunswick Science Inc. Edison, NJ) at 52 °C and 140 rpm for 72 h. During enzymatic saccharification, 80 μL of slurry was sampled periodically from

each flask up to 72 h. The sampled slurries were filtered into 300 μL autosampler vials using 0.22 μm membranes before HPLC analysis.

The saccharification conversions of glucan and xylan were calculated as follows:

$$E_g = \frac{V \times C_g}{1.11 \times m \times A_g + C_{g'} \times V_l} \times 100\% \quad (1)$$

$$E_x = \frac{V \times C_x}{1.14 \times m \times A_x + C_{x'} \times V_l} \times 100\% \quad (2)$$

where E_g and E_x are the enzymatic saccharification conversions of glucan and xylan (%), respectively; C_g and C_x are the concentrations of glucose and xylose after saccharification (g/mL), respectively; $C_{g'}$ and $C_{x'}$ are the concentrations of potential glucose and xylose (monomeric plus oligomeric sugars) in pretreatment liquor determined by HPLC (g/mL), respectively; m is the dry weight of treated biomass used for enzymatic saccharification (g); A_g and A_x are the amounts of glucan and xylan in treated biomass (%), respectively; 1.11 and 1.14 are the conversion factors of glucan to glucose and xylan to xylose, respectively; V_l is the volume of pretreatment liquor used for enzymatic saccharification (mL); and V is the volume of saccharification solution (mL).

Sugar yields as received biomass in Case 1 were calculated using the same equations as described in Chapter 3.

Sugar yields of treated biomass as received biomass in Case 2 were calculated using the same equations in Case 1. Sugar yields of pretreatment liquor as received biomass in Case 2 were calculated using the following formulas:

$$Y_{gl} = \frac{V_0 \times C_{g'} \times E_g}{m_0 \times A_{g'}} \times 100\% \quad (5)$$

$$Y_{xl} = \frac{V_0 \times C_{x'} \times E_x}{m_0 \times A_{x'}} \times 100\% \quad (6)$$

$$Y_{gt} = Y_g + Y_{gl} \quad (7)$$

$$Y_{xt} = Y_x + Y_{xl} \quad (8)$$

where Y_{gt} and Y_{xt} are the total glucose and xylose yields in Case 2, respectively (%); Y_g and Y_x are the glucose and xylose yields of treated biomass as received biomass in Case 2, respectively (%); Y_{gl} and Y_{xl} are the glucose and xylose yields of pretreatment liquor as received biomass in Case 2, respectively (%); $A_{g'}$ and $A_{x'}$ are the glucan and xylan contents in raw biomass, respectively (%); m_0 is the dry weight of raw biomass used for MgO pretreatment (g); and V_0 is the volume of pretreatment liquor from MgO pretreatment (mL).

4.3.4. Analytical procedures

Sugars in pretreatment liquor and structural carbohydrates in biomass were analyzed following the standard methods developed by the Natural Renewable Energy Laboratory (Sluiter et al., 2008a, 2008b). Sugar concentration was measured by a 1200 HPLC system (Agilent, Santa Clara, CA). The separation unit was an HPX-87H organic acid column (7.8 × 300 mm) purchased from the Bio-Rad (Hercules, CA) and set at 60 °C. The temperature of the refractive index detector was set at 45 °C. The mobile phase was 0.005 M sulfuric acid water and set at a flow rate of 0.6 mL/min.

4.3.5. Statistical analyses

All experiments were repeated at least twice. Statistical analysis of data was conducted using SAS (SAS Institute Inc., Cary, NC). Means were analyzed using Duncan with the *p* value of 0.05 as the cutoff for significance.

4.4. Results and discussion

4.4.1. Chemical compositions of corn stover before and after MgO pretreatment

Raw corn stover contained 36.8% cellulose and 21.9% hemicellulose (Table 4.1). After MgO pretreatment (treated corn stover was not washed), hemicellulose content decreased to 16.9%, which was attributed to acetic acid and sugar release because of the weak structural strength of hemicellulose. Cellulose content in treated corn stover without water washing increased to 49.0%. Compared to MgO-treated corn stover with water washing, MgO-treated corn stover without water washing had a lower cellulose content (49.0 vs. 56.3%) but a higher hemicellulose content (16.9 vs. 15.2%). This indicates that part of degraded cellulose and hemicellulose, especially hemicellulose, remained in the solid fraction when water washing was not applied, which was reflected by the higher xylan recovery (62.4 vs. 48.8%) and the lower oligomeric xylose (24.5 vs. 35.8%) in liquor (Table 4.2). Glucose concentration in pretreatment liquor was 1.52 g/L (Table 4.1), which accounted for 2.5 % of the total glucose in raw biomass (Table 4.2). Xylose concentration in pretreatment liquor was 7.83 g/L, which accounted for 26.2% of the total xylose in raw biomass. In addition, furfural (0.13%) and HMF (0.01%) derived from monosaccharide degradation were minimal in pretreatment liquor, which is attributed to the absence of released acids. Such a neutral, xylose-rich, and furfural-and-HMF-trace pretreatment liquor can be integrated into enzymatic saccharification of MgO-treated corn stover without washing and detoxification.

4.4.2. Effect of solids loading on enzymatic saccharification of MgO-treated corn stover with pretreatment liquor

The effect of solids loading on the enzymatic saccharification of MgO-treated corn stover with pretreatment liquor was investigated with an enzyme loading of 30 μL CTec3 and 18 μL NS22244/g treated biomass (Figure 4.3). As solids loading increased from 8 to 10%, glucose yields of neither treated biomass (64 vs. 63%, Figure 4.3A) nor pretreatment liquor (1.5 vs. 1.5%, Figure 4.3B) changed significantly, thus the change of total glucose yield (66 vs. 65%, Figure 4.3C) was not significant. As solids loading increased from 8 to 10%, xylose yields of treated biomass and pretreatment liquor had the same trends as glucose yields of treated biomass and pretreatment liquor; total xylose yield also had the same trend as total glucose yield. The slight decrease of glucose and xylose yields is because the increase of solids loading reduces the rate of mass transfer. As solids loading increased from 8 to 10%, however, both glucose and xylose concentrations increased from 28 to 34 g/L and 17 to 19 g/L (Figure 4.3D), respectively, resulting in a final total sugar concentration of up to 52 g/L at a 10% solids loading.

4.4.3. Effect of enzyme loading on enzymatic saccharification of MgO-treated corn stover with pretreatment liquor

The high cost of enzyme makes it necessary to investigate the effect of enzyme loading on enzymatic saccharification to reduce enzyme cost. The effect of CTec3/NS22244 loading on enzymatic saccharification of MgO-treated corn stover and pretreatment liquor was studied with a 10% solids loading (Figure 4.4). As CTec3/NS22244 loading increased from 30/18 to 50/30 μL/g biomass, glucose and xylose yields of treated biomass increased from 63 to 73% and 46 to 50% (Figure 4.4A), respectively, and glucose and xylose yields of pretreatment liquor increased from

1.5 to 1.7% and 17 to 18% (Figure 4.4B), respectively, resulting in increases of total glucose and xylose yields from 65 to 75% and 63 to 68% (Figure 4.4C), respectively. As CTec3/NS22244 loading increased from 30/18 to 50/30 μL/g biomass, glucose and xylose concentrations increased from 34 to 39 g/L and 19 to 20 g/L, respectively, and the total sugar concentration increased from 52 to 59 g/L (Figure 4.4D). In addition, as CTec3/NS22244 loading increased from 40/24 to 50/30 μL/g biomass, the increase in glucose yields of both treated biomass (69 to 73%) and pretreatment liquor (1.6 to 1.7%) were significantly less than when CTec3/NS22244 loading increased from 30/18 to 40/24 μL/g biomass (63 to 69% and 1.5 to 1.6%, Figure 4.4A and B). Similar trends were observed in xylose yields of both treated biomass and pretreatment liquor (Figure 4.4A and B), total glucose and xylose yields (Figure 4.4C), and total sugar concentrations (Figure 4.4D). Thus, a CTec3/NS22244 loading of 40/24 μL/g biomass was selected for enzymatic saccharification of MgO-treated corn stover with pretreatment liquor.

4.4.4. Comparison of enzymatic saccharification of MgO-treated corn stover only and MgO-treated corn stover with pretreatment liquor

To investigate the effects of integrating pretreatment liquor into the enzymatic saccharification system of MgO-treated corn stover on sugar yield and concentration, enzymatic saccharification of MgO-treated corn stover only (Case 1) and enzymatic saccharification of MgO-treated corn stover with pretreatment liquor (Case 2) were conducted with a solids loading of 10% and CTec3/NS22244 loading of 40/24 μL/g biomass (Figure 4.5). The total glucose yield in Case 2 (71%) was lower than that in Case 1 (75%) (Figure 4.5A), which is because dissolved lignin compounds in pretreatment liquor were also introduced into the saccharification system with the addition of pretreatment liquor. However, the total xylose yield (66%) in Case 2 was much higher

than that in Case 1 (36%) (Figure 4.5B), which was attributed to the addition of pretreatment liquor in Case 2. Case 1 and Case 2 had equivalent total sugar concentrations (58 vs. 57 g/L) (Figure 4.5C).

4.4.5. Effect of Tween 80 loading on enzymatic saccharification of MgO-treated corn stover with pretreatment liquor

Tween 80 is a surfactant that can reduce the binding of lignin to enzyme, functioning to maintain enzyme activity (Jin et al., 2010; Kaar and Holtzapple, 1998; Liu et al., 2014; Sun and Cheng, 2002). In this study, the effect of Tween 80 loading (0.075, 0.15, and 0.3 g/g treated biomass) on the improvement of enzyme activities was studied with a solids loading of 10% and CTec3/NS22244 loading of 40/24 μL/g treated biomass (Figure 4.6). As Tween 80 loading increased from 0 to 0.075 g/g treated biomass, glucose and xylose yields of treated biomass increased from 69 to 77% and 49 to 52% (Figure 4.6A), respectively; glucose and xylose yields of pretreatment liquor increased from 1.6 to 1.8% and 17 to 19% (Figure 4.6B), respectively; and the total glucose and xylose yields increased from 71 to 79% and 66 to 71% (Figure 4.6C), respectively. No significant increase in glucose yields of treated biomass and pretreatment liquor was observed when Tween 80 loading increased from 0.075 to 0.3 g/g treated biomass, but xylose yields of treated biomass and pretreatment liquor increased. As Tween 80 loading increased from 0 to 0.3 g/g treated biomass, glucose and xylose concentrations increased from 37 to 41 g/L and 20 to 23 g/L (Figure 4.6D), respectively, increasing total sugar concentration from 57 to 64 g/L (Figure 4.6D).

4.5. Conclusions

Corn stover liquor with near neutral pH and a trace amount of furfural and HMF from MgO pretreatment was directly introduced into enzymatic saccharification of treated corn stover without washing and detoxification, simplifying bioconversion processes and reducing downstream wastewater treatment. With 10% solids loading and 40 µL CTec3 plus 24 µL NS22244/g treated biomass, the addition of pretreatment liquor decreased glucose yield by 4% but increased xylose yield by 30%, resulting in an equivalent total sugar concentration. Tween 80 effectively reduced the binding of lignin to enzyme and increased glucose and xylose yields by 8 and 10%, respectively, and total sugar concentration by 7 g/L.

4.6. References

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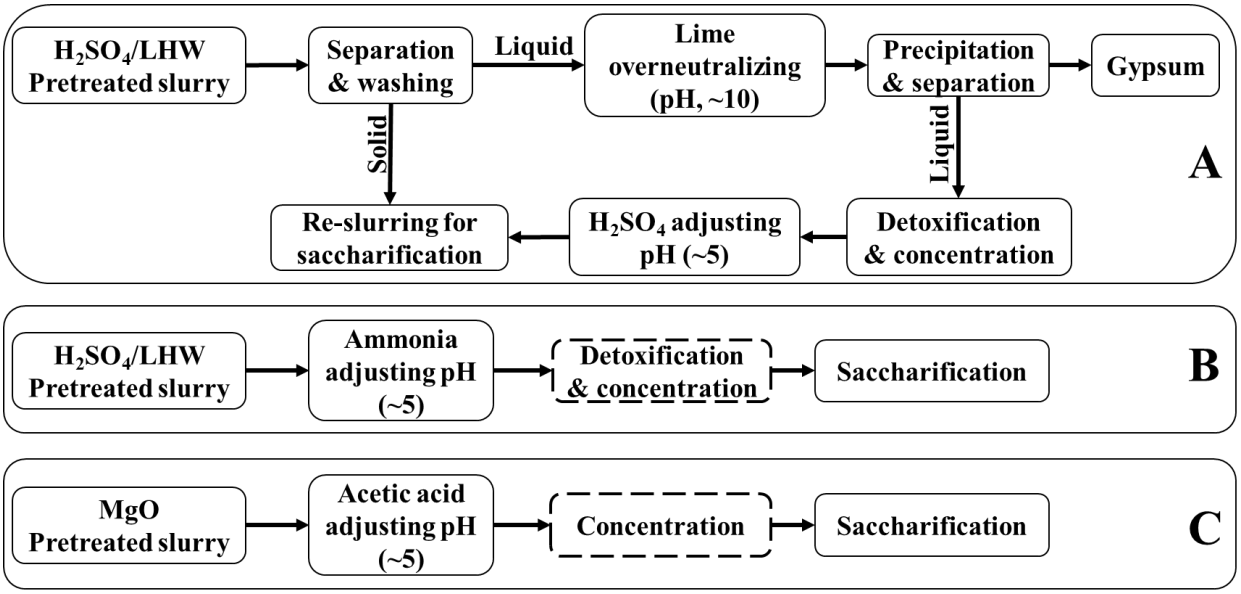

Figure 4.1 Schematic diagram of biomass slurry conditioning.

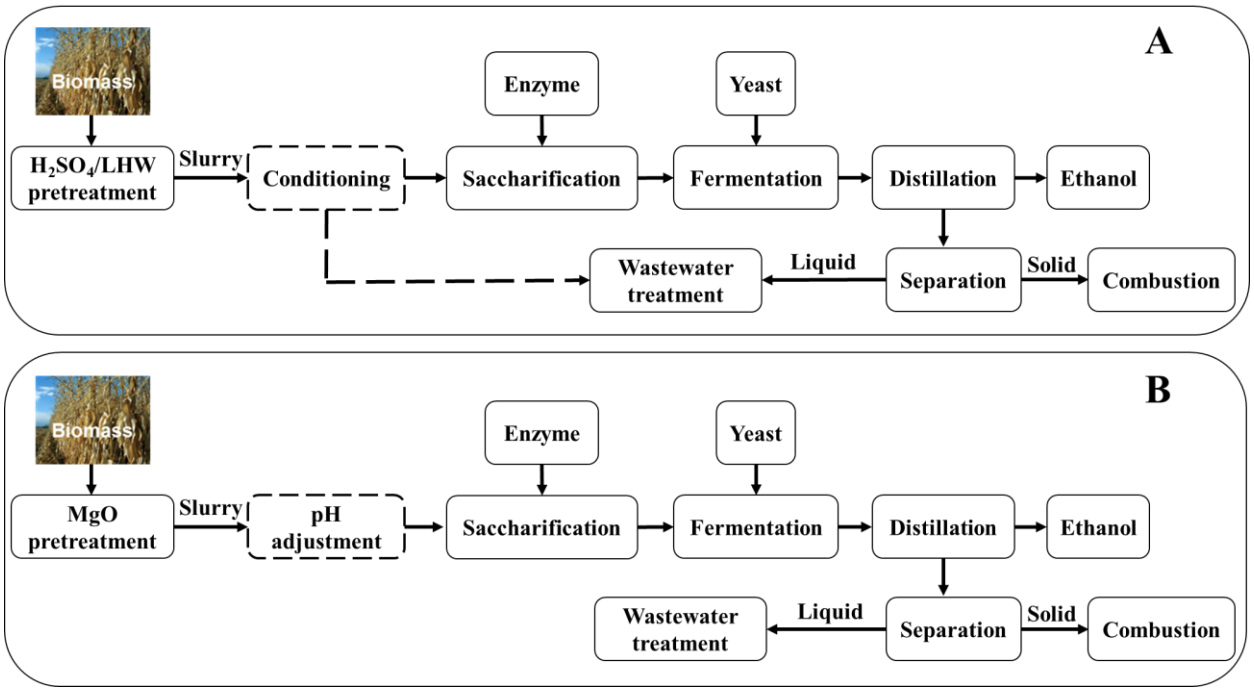

Figure 4.2 Schematic diagram of ethanol production.

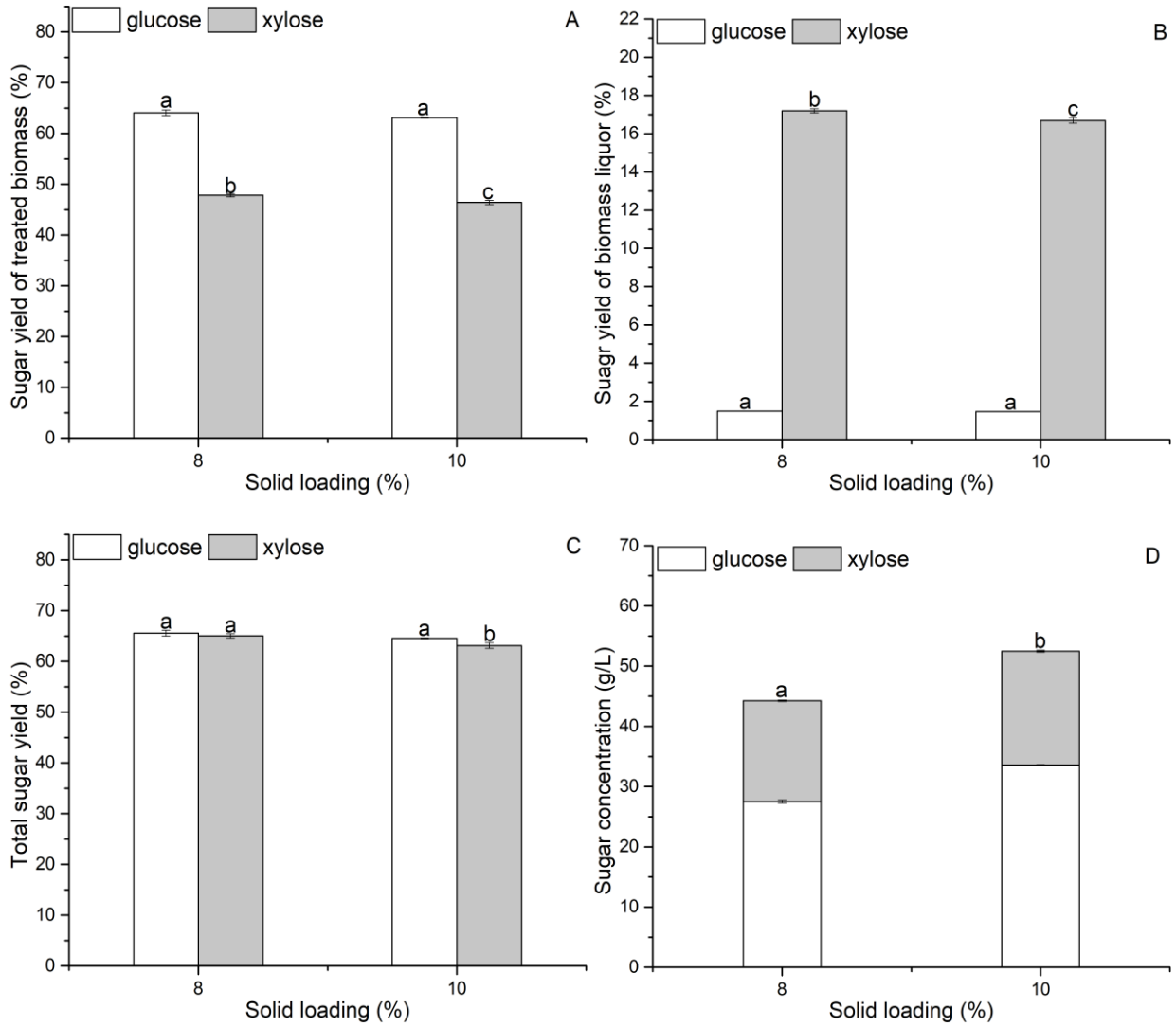

Figure 4.3 Effect of solids loading on enzymatic saccharification of MgO-treated corn stover with pretreatment liquor (MgO-treated corn stover without water washing had 49.0% cellulose and 16.9% hemicellulose).

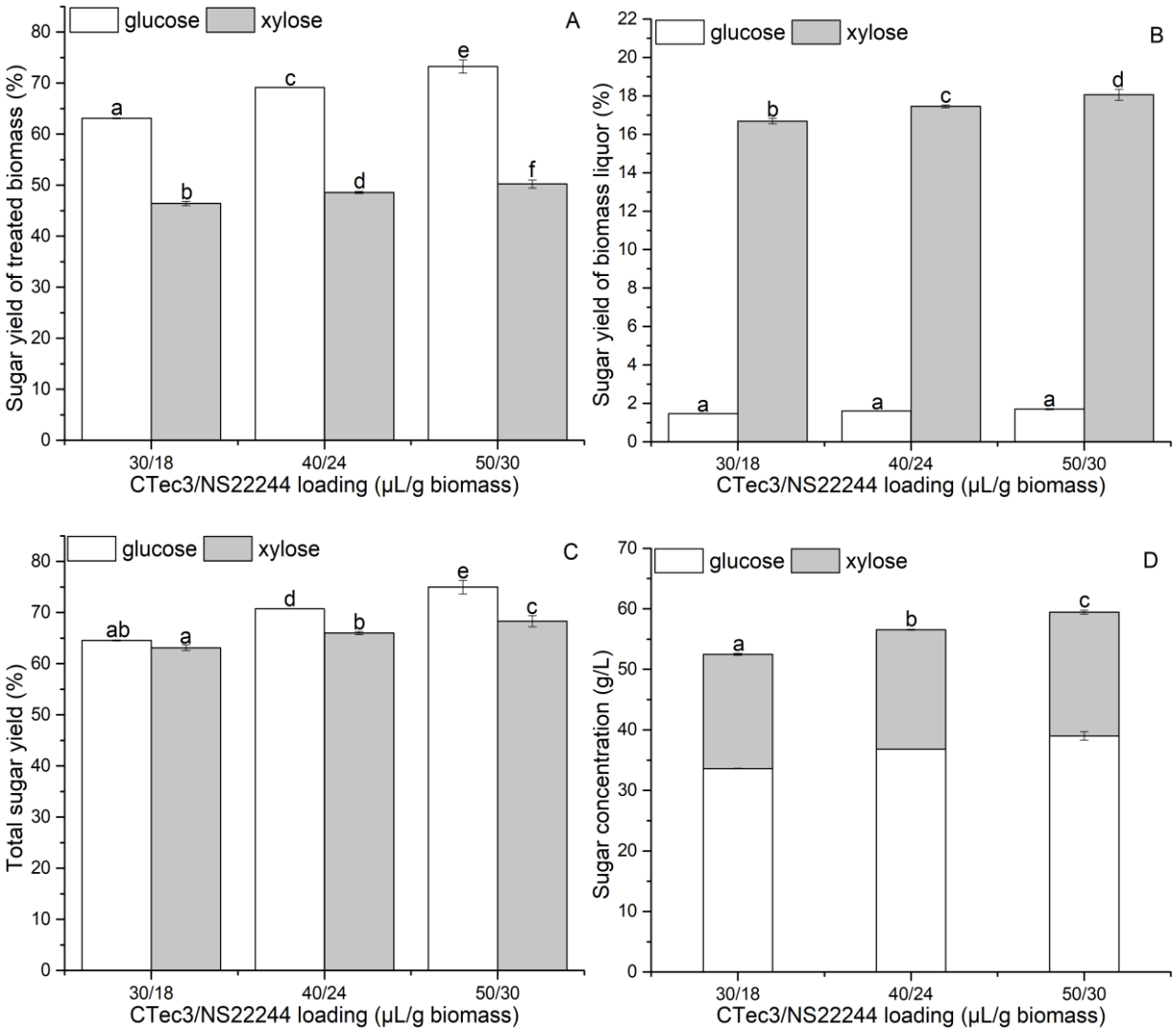

Figure 4.4 Effect of enzyme loading on enzymatic saccharification of MgO-treated corn stover with pretreatment liquor (MgO-treated corn stover without water washing had 49.0% cellulose and 16.9% hemicellulose).

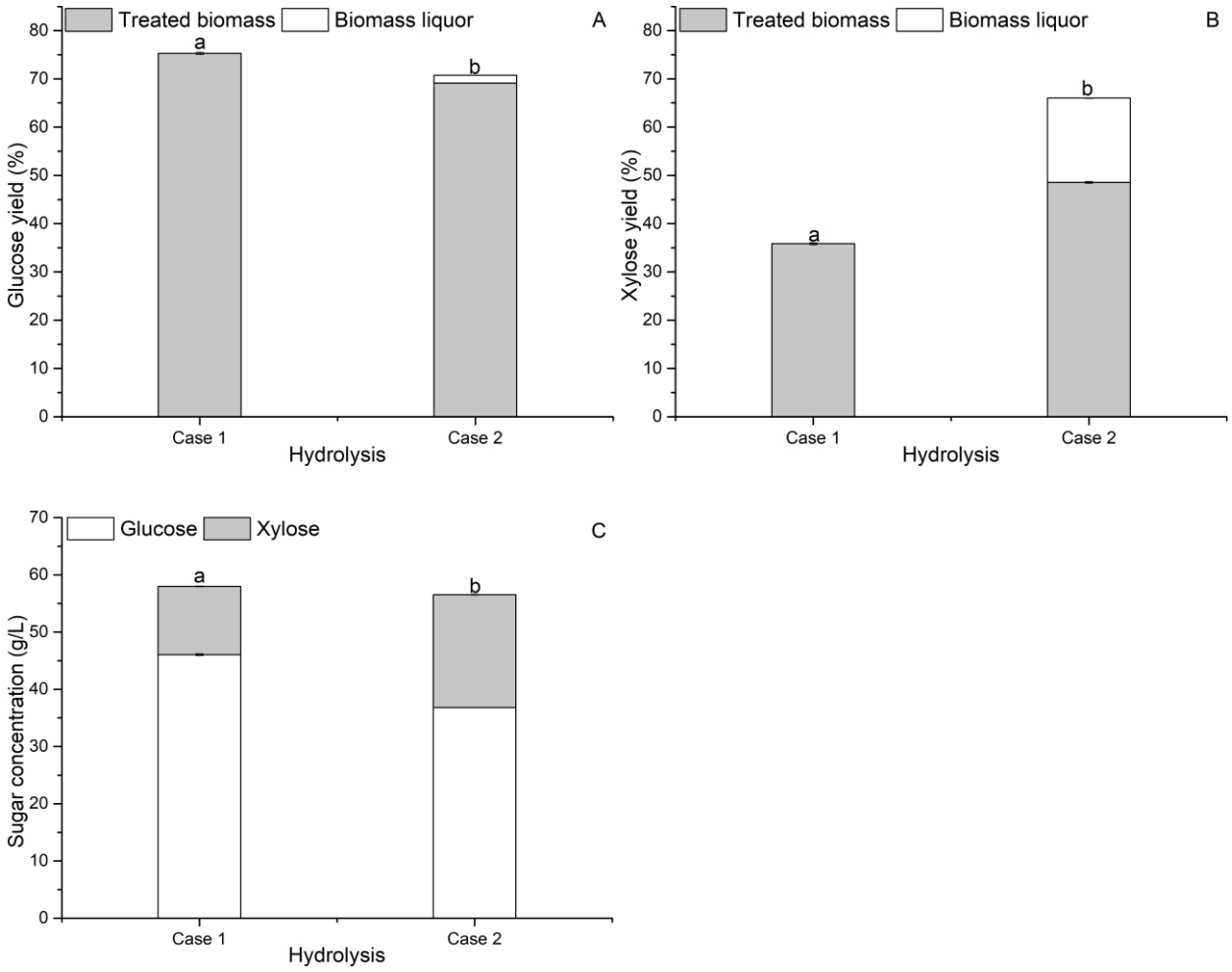

Figure 4.5 Comparison of enzymatic saccharification of MgO-treated corn stover only (Case 1) and MgO-treated corn stover with pretreatment liquor (Case 2) (MgO-treated corn stover with water washing (Case 1) had 56.3% cellulose and 15.2% hemicellulose; MgO-treated corn stover without water washing (Case 2) had 49.0% cellulose and 16.9% hemicellulose).

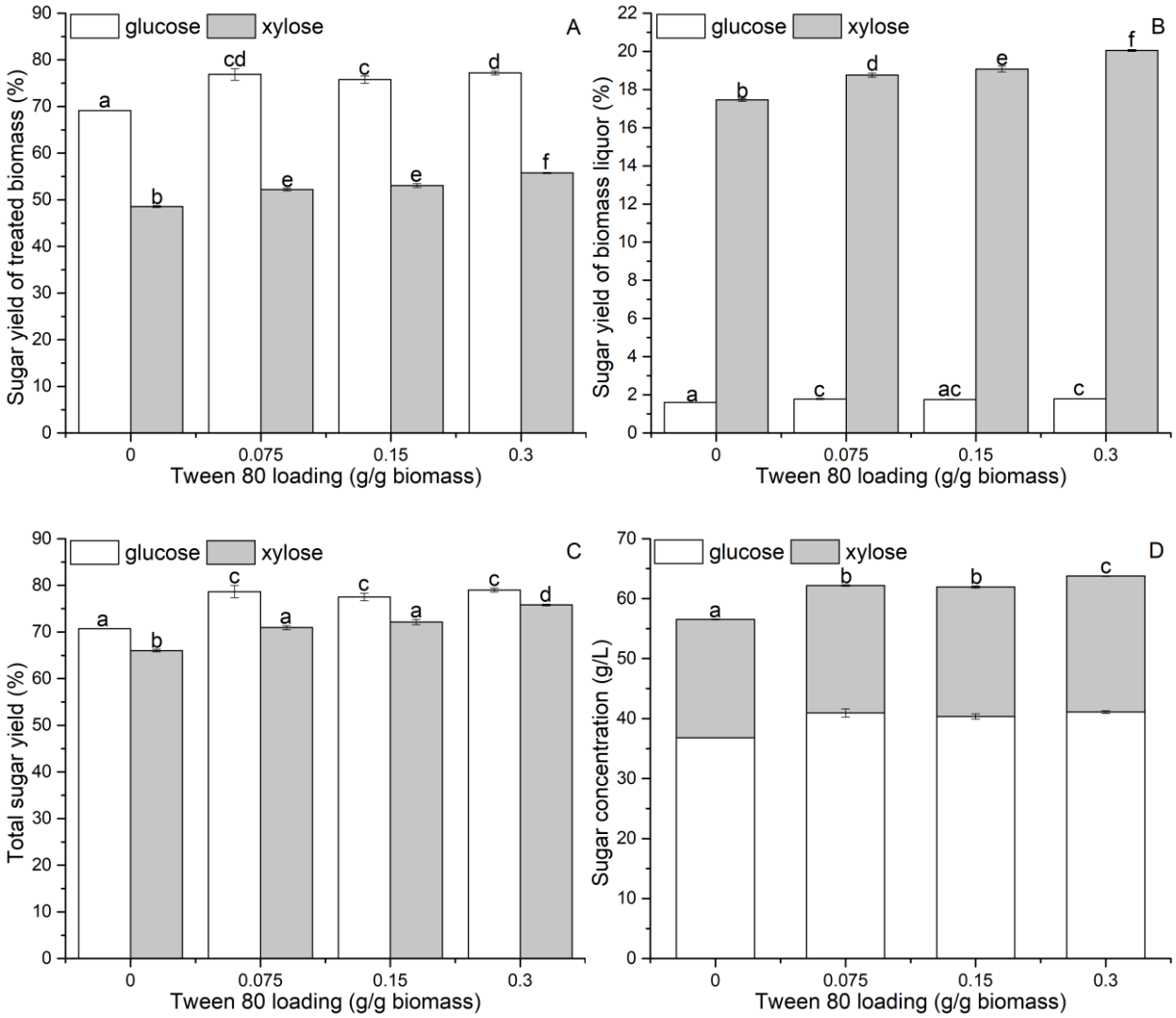

Figure 4.6 Effect of Tween 80 on enzymatic saccharification of MgO-treated corn stover with pretreatment liquor (MgO-treated corn stover without water washing had 49.0% cellulose and 16.9% hemicellulose).

Table 4.1 Chemical composition of raw and MgO-treated corn stover[1].

Corn stover	Solids		Liquor		
	Cellulose (%, db[4])	Hemicellulose[5] (%, db)	Glucose (g/L)	Xylose (g/L)	pH
Raw	36.8±0.29a[6]	21.9±0.04a			
MgO-treated1[2]	56.3±1.07b	15.2±0.34b			6.77±0.25a
MgO-treated2[3]	49.0±0.24c	16.9±0.12c	1.52±0.00	7.83±0.03	6.86±0.16a

[1] Data are presented in mean plus and minus standard deviation.
[2] MgO pretreatment condition was 10% solids loading with 0.08 mol/L MgO at 190 °C for 40 min. Treated corn stover was washed with 180 mL of distilled water.
[3] MgO pretreatment condition was 10% solids loading with 0.08 mol/L MgO at 190 °C for 40 min. Treated corn stover was not washed.
[4] db = dry basis.
[5] Hemicellulose includes xylan and arabinan.
[6] In each column, means with different letters are significantly different at $p < 0.05$.

Table 2. Composition comparison of MgO-treated corn stover with and without water washing[1].

Corn stover	Potential glucose (%)				Potential xylose (%)			
	Glucan	Monomeric glucose	Oligomeric glucose	HMF	Xylan	Monomeric xylose	Oligomeric xylose	Furfural
MgO-treated1[2]	100.3±1.90a[4]	0.64±0.01a	3.3±0.02a	0.15±0.17a	48.8±0.98a	2.4±0.02a	35.8±0.81a	0.58±0.38a
MgO-treated2[3]	102.0±0.50a	0.31±0.01b	2.2±0.00b	0.01±0.01b	62.4±0.25b	1.7±0.05b	24.5±0.16b	0.13±0.02b

[1] All components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.

[2] MgO pretreatment condition was 10% solids loading with 0.08 mol/L MgO at 190 °C for 40 min. Treated corn stover was washed with 180 mL of distilled water.

[3] MgO pretreatment condition was 10% solids loading with 0.08 mol/L MgO at 190 °C for 40 min. Treated corn stover was not washed.

[4] In each column, means with different letters are significantly different at $p < 0.05$.

Chapter 5 - Effect of magnesium oxide-ethanol pretreatment on lignin removal and enzymatic saccharification of corn stover

5.1. Abstract

MgO-ethanol pretreatment on corn stover was investigated to enhance sugar recovery, reduce sugar degradation, and enhance enzymatic saccharification by improving lignin removal and reducing inhibitor formation. MgO as an effective catalyst and Lewis base is capable to neutralize the acids released from hemicellulose during pretreatment, reduce monosaccharide degradation and inhibitor formation, and enhance lignin removal. The optimal pretreatment condition was 50% ethanol, 0.07 mol/L MgO, and 10% solid loading at 190 °C for 40 min. Under optimal condition, glucan was completely recovered along with 89.3% xylan recovery and 44.1% lignin removal. Total sugar yield of 72.4% as received biomass after enzymatic saccharification was achieved with 78.3% glucose and 61.7% xylose yields. The biomass liquor with near-neutral pH and free of furfural and 5-hydroxymethylfurfural can be used directly for downstream enzymatic saccharification. Therefore, the bioconversion process can be largely streamlined to produce fermentable sugars.

5.2. Introduction

With the increasing concerns regarding environmental pollution and climate change, the research on green, environmentally-friendly, and economical biofuels is critical to our sustainable economic development (Ali and Akbar, 2020; Goel and Sharma, 2019). Bioethanol is a great alternative to gasoline derived from nonrenewable petroleum, and about 15 billion gallons of fuel ethanol were consumed in the United States in 2018 (EIA, 2020). Survey results showed that more than 98% of gasoline in the United States is blended with ethanol to provide a series of flex fuels for different types of vehicles such as E85, E15, and E10 (Uria-Martinez et al., 2018). Based on the type of feedstocks, bioethanol is classified into starch-based ethanol (usually refers to corn and grain sorghum starch in the United States) and cellulosic ethanol (Du et al., 2018). Currently, most of ethanol is produced from starch-based crops and the share of cellulosic ethanol accounts for less than 1% (RFA, 2019). This is mainly because starch can be easily hydrolyzed to glucose by enzymes and the production techniques for starch ethanol have been well established. However, cellulosic ethanol still faces some technical challenges and the massive industrialization of cellulosic ethanol is not available yet (Li et al., 2018). The major challenge is that the structural seal of biomass restricts the acceptability of enzymes to cellulose and hemicellulose, resulting in a poor bioconversion efficiency (Zheng et al., 2009).

To solve the above issue, pretreatment is usually needed as the first step of cellulosic ethanol production. The purpose of pretreatment is to break the lignin seal, disrupt the crystalline structure of cellulose, and increase the surface area of cellulose, making the polysaccharides more susceptible to enzymatic saccharification (Agbor, et al., 2011; Zhu and Pan, 2010). However, biomass pretreatment generates some degradation products, such as 5-hydroxymethylfurfural (HMF), furfural, acetic acid, and phenolic compounds (Jönsson and Martín, 2016; Luo et al.,

2002), which not only reduces the availability of glucan and xylan for enzymatic saccharification but also inhibits their conversions to glucose and xylose. In addition, previous studies found that lignin-derived phenolic compounds are the main cause for enzyme deactivation during saccharification. Therefore, lignin removal is also critical to biomass saccharification (Kim et al., 2011).

Organosolv pretreatment is a promising method that is capable of simultaneously generating fermentable sugars and high-purity lignin (Jafari et al., 2016). Although various organic solvents have been attempted to pretreat the cellulosic biomass (Zhao et al., 2009), only those derived from renewable sources are suitable for cellulosic biofuel production (Huijgen et al., 2011). Ethanol as the most commonly used solvent has been extensively in the organosolv pretreatment study because of its non-toxicity, cheapness, easy recovery, and readily compensation in ethanol plant (Pan et al., 2005, 2006; Zhang et al., 2016). In addition, aqueous ethanol is usually used in organosolv pretreatment because aqueous ethanol has a better performance in lignin removal than pure ethanol, which also saves the production cost (Jafari et al., 2016). However, during aqueous ethanol pretreatment, acidic acid released from hemicellulose degradation decreases the pH of biomass slurry, which causes the monosaccharide degradation (Huijgen et al., 2011). In Chapter 2, five metal oxides (Fe_2O_3, ZnO, CuO, NiO, and MgO) were investigated and MgO was the most effective Lewis base and catalyst to completely neutralize the acetic acid released from hemicellulose during liquid hot water (LHW) pretreatment. MgO pretreatment can largely reduce the degradation of sugar monomers and the washing water consumption, and eliminate the detoxification to biomass liquor. However, MgO pretreatment is weak in lignin removal.

In this research, MgO was also used as an additive to solve the sugar degradation issue during pretreatment. Aqueous ethanol as solvent instead of water only was used to improve lignin removal during pretreatment. The effects of MgO-ethanol on corn stover pretreatment and subsequent enzymatic saccharification were studied by comparing sugar recovery, sugar degradation, lignin removal, pH of biomass slurry, efficiency of enzymatic saccharification, and total sugar yield. The effect of MgO-ethanol pretreatment on the micro- and macro-structural changes was visualized using Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM).

5.3. Materials and methods

5.3.1. Chemicals and materials

Corn stover was supplied by the Kansas State University Agricultural Trial Base (Manhattan, KS). Ground corn stover with <1 mm particle size was used in this study. Ethanol (200 proof, ACS grade) and MgO powder (96% purity) were obtained from Fisher Scientific (Ward Hill, MA). Enzymes CTec3 and NS22244 were generously supplied by Novozymes (Franklinton, NC). Protein contents of CTec3 and NS22244 were 516 and 266 mg protein/mL, respectively.

5.3.2. Biomass pretreatment

The pretreatment process is similar to the descriptions in Chapter 2. 10% solid loading (5 g of milled corn stover dissolved in 50 mL of aqueous ethanol), and designated loading of MgO (0.06-0.12 mol/L) were loaded into a reactor with a 75 mL working volume. The reactor was shaken upside down for 2 min to completely hydrate biomass, and placed in a shaker at 45 °C for

1 h to facilitate metal oxides dispersing in water and touching biomass. To shorten the time that the reactor took to reach target temperatures, the reactor was heated in boiling water for 3 min. After that, the reactor was drowned into a SLB-2 fluidized sandbath (Techne Inc., Princeton, NJ) set at 170-210 °C for 30-60 min. After reaching the set reaction time, the reaction was immediately terminated by rapidly submerging the reactor in ice water. After pH measurement, biomass slurry was partitioned into the treated biomass and biomass liquor by filtration using a Buchner funnel loaded with a filter paper (P8 grade, Fisherbrand). The solid was washed three times (60 mL each time) with the same concentration of aqueous ethanol and then dried at 45 °C overnight for following use. Finally, the biomass liquor and washing liquor were merged together, diluted to 250 mL with the same concentration of aqueous ethanol, and placed in a freezer for further analysis and use.

5.3.3. Enzymatic saccharification

The enzymatic saccharification is similar to the descriptions in Chapter 3. Treated biomass was enzymatically hydrolyzed in sodium acetate buffer (50 mM, pH 5.0) with the solid loading of 1% and the CTec3/NS22244 loading of 50/30 μL/g treated biomass. 0.02% (w/v) sodium azide was added into saccharification solutions to eliminate the microbial growth. After 72 h of saccharification at 52 °C and 140 rpm, sugar concentration was determined by high performance liquid chromatography (HPLC).

Sugar (glucose, xylose, and total) yields as received biomass and glucan and xylan conversions were calculated using the same equations used in Chapter 3.

5.3.4. HPLC analysis

Degradation products and sugars in biomass liquor and structural carbohydrates in biomass were analyzed following the NREL standard methods proposed by Sluiter et al. (2008a,b).

A 1200 HPLC system (Agilent, Santa Clara, CA) was employed to detect sugars and degradation products of interest. Components were separated in an HPX-87H organic acid column (7.8 × 300 mm, Bio-Rad, Hercules, CA) at 60 °C with 0.005 mol/L sulfuric acid as the elution solvent, and then detected by a refractive index detector at 45 °C. The flow rate of the elution solvent was 0.6 mL/min. A series of concentrations of sugar and inhibitor standards were measured to build the standard curves for calculation of the concentrations of sugars and degradation products in the real samples.

5.3.5. FTIR analysis

The 400 FTIR spectrophotometer (PerkinElmer Corp., Shelton, CT) was used to visualize the microstructure of corn stover before and after pretreatment. FTIR spectra were measured in the wavenumber range of 400-4000 cm^{-1} in a scattering mode with a resolution of 4 cm^{-1} and a total scans of 32.

5.3.6. SEM images

Effect of MgO-ethanol pretreatment on corn stover surface changes was visualized by an S-3500 SEM (Hitachinaka, lbaraki, Japan). Samples were mounted on specimen stubs with conductive adhesive tapes, coated by spraying the palladium-gold mixture (2:3) with a final metal thickness of 4 nm. Coated samples were put in the chamber of SEM and then observed under vacuum. Images of all samples were captured at the magnification of 500.

5.3.7. Statistics

All experiments were repeated at least twice. Statistical analysis of data were conducted using SAS (SAS Institute Inc., Cary, NC) with the *p* value of 0.05 as the cutoff for significance.

5.4. Results and discussion

5.4.1. Effect of ethanol concentration on sugar recoveries and lignin removal

The organic solvent concentration in the organic solvent-water mixture is a major factor affecting lignin removal and hemicellulose recovery in organolsolv pretreatment (Huijgen et al., 2010; Wildschut et al., 2013). Therefore, the effect of ethanol concentration from 30 to 70% was tested with other parameters keeping constant (10% solid loading, 190 °C, and 40 min) (Figure 5.1).

Both monomeric and oligomeric glucoses in biomass liquor decreased by 0.4 and 2.5%, respectively, as ethanol concentration increased from 30 to 70% (Figure 5.1A), indicating that cellulose degradation decreased as ethanol concentration increased (Amiri and Karimi, 2015; Pan et al., 2006). This was also confirmed by the increase of glucan recovery by 4.3% as ethanol concentration increased from 30 to 70%. It was also found that the further increase of ethanol concentration from 50 to 70% didn't significantly increase glucan recovery ($p > 0.05$).

Xylan recovery increased by 37.0% but both monomeric and oligomeric xyloses in biomass liquor decreased by 1.4 and 30.5% as ethanol concentration increased from 30 to 70% (Figure 5.1B), which also indicates that hemicellulose degradation decreased as ethanol concentration increased (Amiri and Karimi, 2015; Pan et al., 2006). In addition, inhibitors furfural and HMF formation also decreased as ethanol concentration increased from 30 to 70%, which was attributed

to the increase of biomass slurry pH (Figure 5.1C) due to less acid generated during pretreatment (Results in Chapter 2). This result indicates that the increase of ethanol concentration reduced the ratio of water, thereby reducing the dissociation of released acetic acid (concentration of released acetic acid in this study was ~0.09 mol/L) (Wang et al., 2017).

Lignin removal increased from 11.7 to 33.3% as ethanol concentration increased from 30 to 70%, which was mainly due to the increase of acid insoluble lignin removal (Figure 5.1D). It was also found that the further increase of ethanol concentration from 50 to 70% didn't significantly increase lignin removal ($p > 0.05$). Therefore, 50% ethanol concentration was selected as optimal concentration for the subsequent experiments.

5.4.2. Effect of MgO concentration on sugar recoveries and lignin removal

To reduce or avoid the monosaccharide degradation caused by acetic acid released during pretreatment, MgO was applied as a Lewis base to neutralize the acetic acid, eventually forming $Mg(CH_3COO)_2$. The MgO concentration has a significant effect on the thoroughness of neutralization. Also, Mg^{2+} dissociated from $Mg(CH_3COO)_2$ benefits the lignin removal (Results in Chapter 2). Therefore, the impact of MgO concentration (0.06-0.08 mol/L) was investigated with other parameters remaining constant (10% solid loading, 50% ethanol concentration, 190 °C, and 40 min) (Figure 5.2). The pretreatment with zero MgO addition was used as control.

Compared to control, MgO pretreatment increased glucan recovery and reduced oligomeric and monomeric glucoses in biomass liquor (Figure 5.2A). However, with the increase of MgO concentration, glucan recovery increased only by 1.9% and glucose in liquor decreased only by 0.3%, which is due to recalcitrant lignocellulosic structure.

Compared to control, MgO concentration within 0.07 mol/L increased xylan recovery by 9.4-11.4% and decreased xylose in biomass liquor by 7.4-7.9%, resulting in an increase of total fermentable xylose by 1.5-4.1%, which is attributed to that the increase of biomass slurry pH mitigated sugar degradation and furfural formation in biomass liquor (Figure 5.2B and C). The increase of MgO concentration from 0.07 to 0.08 mol/L further increased xylan recovery by 2.5% and decreased xylose in biomass liquor by 2.3%, which reduced the risk of xylose degradation. In addition, the higher MgO concentration (> 0.07 mol/L) increased biomass slurry pH higher than 7 (Figure 5.2C), which will increase the burden of the biomass liquor treatment.

Lignin removal, especially acid insoluble lignin removal, increased from 39.4 to 44.1% as MgO concentration increased from 0.06 to 0.07 mol/L (Figure 5.2D). This is because during pretreatment, MgO reacted with released acetic acid to form water soluble $Mg(CH_3COO)_2$. Mg^{2+} has unoccupied orbitals in its outermost (third) electron shell thus has electrophilicity. Mg^{2+} might attack the oxygen atoms on ether and ester bonds of lignin, resulting in the breakage of ether and ester bonds. Further increase of MgO concentration from 0.07 to 0.08 mol/L didn't bring a significant increase in lignin removal. Thus, MgO concentration of 0.07 mol/L was selected as optimal concentration for following optimization of reaction temperature and time.

Biomass slurry from MgO-ethanol pretreatment with neutral pH and without furfural and HMF formation allows eliminating washing and detoxification steps in industrial application. In addition, it's not necessary to remove $Mg(CH_3COO)_2$ in biomass liquor because it can be used as a buffer salt when biomass liquor is used directly for enzymatic saccharification and fermentation of treated biomass.

5.4.3. Effect of reaction temperature on sugar recoveries and lignin removal

Reaction temperature is critical in biomass pretreatment because it directly influences energy input, pretreatment efficiency, and production cycle. Therefore, the effect of pretreatment temperature was studied in the temperature range from 170 to 210 °C with other pretreatment parameters keeping constant (10% solid loading, 50% ethanol concentration, 0.07 mol/L MgO, and 40 min) (Figure 5.3).

The increase of temperature from 170 to 210 °C decreased glucan recovery only by 1.9% and increased glucose in biomass liquor only by 0.3% (Figure 5.3A), which is due to recalcitrant lignocellulosic structure. However, reaction temperature significantly affected xylan recovery and inhibitor formation. As reaction temperature increased from 170 to 210 °C, xylan recovery decreased by 48% but xylose in liquor and furfural increased by 6.5 and 1.3%, respectively (Figure 5.3B). In addition, xylan was prone to be degraded compared to glucan especially at high temperature, which is due to hemicellulose has a weaker structural strength than cellulose.

pH values of biomass slurries treated at 170 and 190 °C remained close to 7 (Figure 5.3C), which indicates that MgO completely neutralized the released weak acids at the two reaction temperatures. As reaction temperature further increased to 210 °C, more sugar degradation occurred, especially xylose degradation (Figure 5.3B). The amount of generated weak acids exceeded the amount of acids that can be neutralized by MgO, therefore resulting in a pH value close to that of control (without MgO addition).

Lignin removal, especially acid insoluble lignin removal, significantly increased from 15.8 to 44.1 and 68.5%, respectively, when reaction temperature increased from 170 to 190 and 210 °C (Figure 5.3D). Moreover, the temperature increment from 170 to 190 °C had a larger influence on

lignin removal than the temperature increment from 190 to 210 °C. Therefore, reaction temperature of 190 °C was selected for the optimization of reaction time.

5.4.4. Effect of reaction time on sugar recoveries and lignin removal

The impact of reaction time was studied from 30 to 60 min with other pretreatment parameters keeping constant (10% solid loading, 50% ethanol concentration, 0.07 mol/L MgO, and 190 °C). Results are showed in Figure 5.4.

As reaction time increased from 30 to 60 min, glucan recovery reduced about 2.7% and glucose in liquor changed only by 0.4% (Figure 5.4A). However, xylan recovery significantly reduced from 95.5 to 83.6% as reaction time increased from 30 to 60 min (Figure 5.4B). Xylose in liquor increased initially and then decreased at 40 min. Figure 5.4C shows that insufficient reaction time (30 min) caused only a partial neutralization of released weak acids, whereas excessive reaction time (50-60 min) caused more sugar degradation, resulting in a lower pH (Figure 5.4C). Lignin removal enhanced from 35.4 to 53.0% when reaction time increased from 30 to 60 min (Figure 5.4D). Therefore, reaction time of 40 min was selected as an optimum time for MgO-ethanol pretreatment.

Based on the above analyses, optimal condition for corn stover pretreatment in this work was 50% ethanol, 0.07 mol/L MgO, 10% solid loading, and 190 °C for 40 min. Under the optimal condition, 53.6% cellulose, 27.0% hemicellulose, and 12.0% lignin were obtained in treated biomass (Table 5.1). In this research, lab-scale biomass pretreatment was conducted using a mini-reactor without mixing function. To ensure good mass and heat transfer, only 10% solid loading was tested during pretreatment. It is believed that if the amplification test is performed with better

mixing function, high solid loading can be applied, which will improve pretreatment performance and reduce processing cost.

5.4.5. Chemical structures

FTIR was applied to characterize the microstructural modification of corn stover before and after pretreatment (Figure 5.5). The peak intensity at 3340-3330 cm^{-1} corresponding to OH stretching decreased after pretreatment, which indicates that hydrogen bonds linked between carbohydrates and lignin were cleaved (Kumar et al., 2009). Furthermore, it also indicates the weak strength of hydrogen bond (Xu et al., 2018). The peak intensity at 2920-2890 cm^{-1} corresponding to C-H stretching had no significant modification after pretreatment, which is because hydrogen bond is the major chemical linkage between cellulose and hemicellulose, therefore no impact on the C-H of cellulose (He et al., 2008). The complex fingerprint region (1800-900 cm^{-1}) usually reveals more structural information regarding carbohydrates and lignin (Corredor et al., 2009). The peak at 1730-1720 cm^{-1} corresponding to the acetyl and uronic ester stretching on hemicellulose branches disappeared after pretreatment, indicating these two groups were disrupted (Windeisen et al., 2007). The three peaks at 1325-1310, 1525-1510, and 1610-1590 cm^{-1} corresponding to the C-O and C=C vibrations, C=O stretching, and aromatic skeletal vibration of lignin side chains (Pandey, 1999; Sun et al., 2005) showed a reduced intensity, demonstrating the breakage of lignin side chains connected with cellulose and hemicellulose during pretreatment. The peak intensity at 1245-1235 cm^{-1} after pretreatment also decreased (Guo et al., 2008; Sene et al., 1994).

5.4.6. Surface features

To visualize the surface features of corn stover before and after pretreatment, SEM was applied in the work. Results (Figure 5.6) showed that raw corn stover had a smooth and intact surface, in comparison to treated corn stover with increased porosity and more exposed surface area due to the effective disruption of structural seal. MgO-ethanol pretreatment resulted in lighter corn stover in color than raw corn stover due to the removal of lignin. Thus, the pretreatment by MgO-ethanol would enhance the enzymatic accessibility to cellulose and hemicellulose, thus enhancing enzymatic saccharification efficiency.

5.4.7. Effect of ethanol treatment with and without MgO on enzymatic saccharification

Figure 5.7 shows the comparison of enzymatic saccharification of corn stover treated by 50% ethanol with and without MgO. Compared to 50% ethanol-treated corn stover with a 70.8% glucose yield, 49.5% xylose yield, and 63.3% total sugar yield, corn stover treated by 50% ethanol with MgO had higher glucose (78.3%), xylose (61.7%), and total sugar (72.4%) yields. In addition, compared with corn stover treated by 50% ethanol only, corn stover treated by 50% ethanol with MgO yielded higher glucan (76.3 vs. 70.1%) and xylan (69.0 vs. 63.5%) conversion efficiencies. This was mainly because the combination of MgO additive and ethanol increased the lignin removal (Figure 5.2D), thus improving enzymatic saccharification efficiency and sugar yield. In addition, corn stover treated by 50% ethanol with MgO had higher glucose, xylose, and total sugar yields than corn stover treated by LHW (78.3 vs. 76.8%, 61.7 vs. 15.8%, and 72.4 vs. 55.3%) or LHW with MgO (78.3 vs. 75.7%, 61.7 vs. 35.9%, and 72.4 vs. 61.7%) (Results in Chapter 3). This is mainly because ethanol largely increased lignin removal and MgO reduced the sugar degradation

and inhibitor formation, thus improving the sugar recovery during pretreatment and enhancing the enzymatic saccharification.

5.5. Conclusions

Aqueous ethanol is an efficient solvent mixture for lignin removal during biomass pretreatment. MgO is an effective Lewis base to neutralize the acids released during pretreatment, reducing sugar loss, inhibitor formation, and washing water usage. Combination of MgO and ethanol enhances lignin removal and cellulose and hemicellulose recoveries, improving sugar yield during enzymatic saccharification. Neutral biomass slurry without inhibitors will simplify the process for the isolation of high-purity value-added lignin and sugar recovery in biomass liquor, which is our ongoing research and will be presented in our future paper. The recovery and cyclic use of ethanol is also considered to make the pretreatment more economic.

5.6. References

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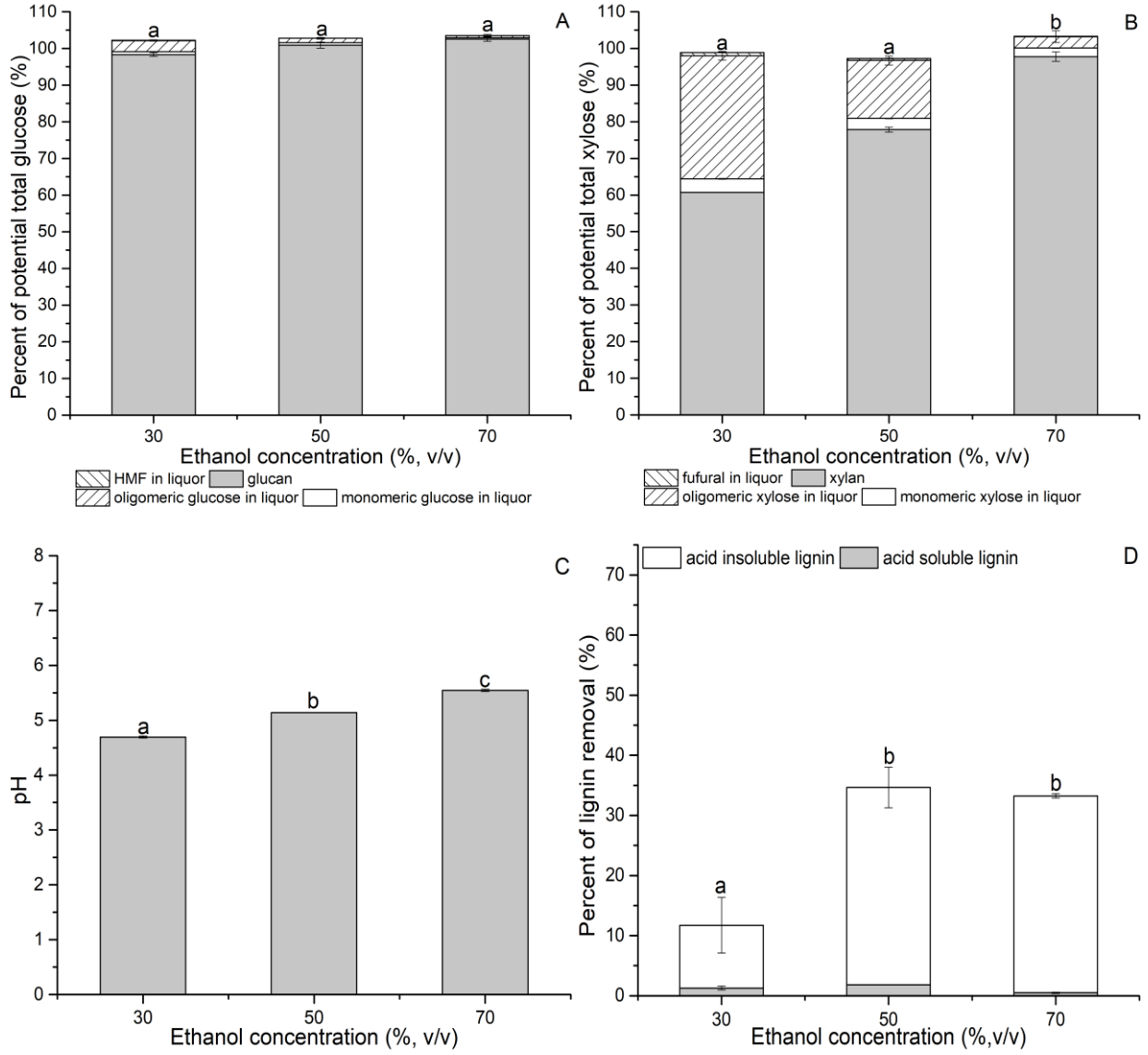

Figure 5.1 Effect of ethanol concentration on sugar recoveries and lignin removal (all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.).

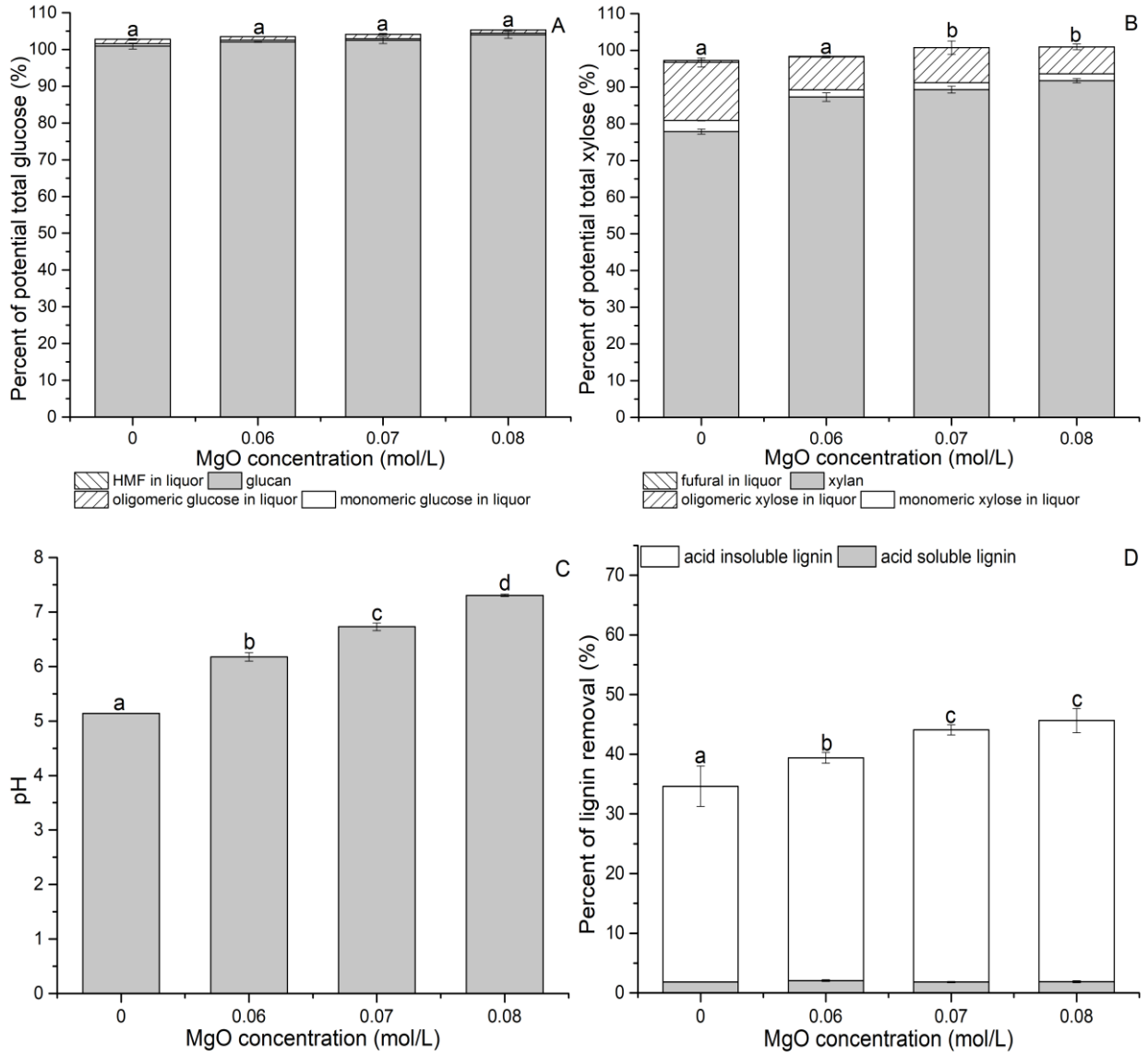

Figure 5.2 Effect of MgO concentration on sugar recoveries and lignin removal (all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.).

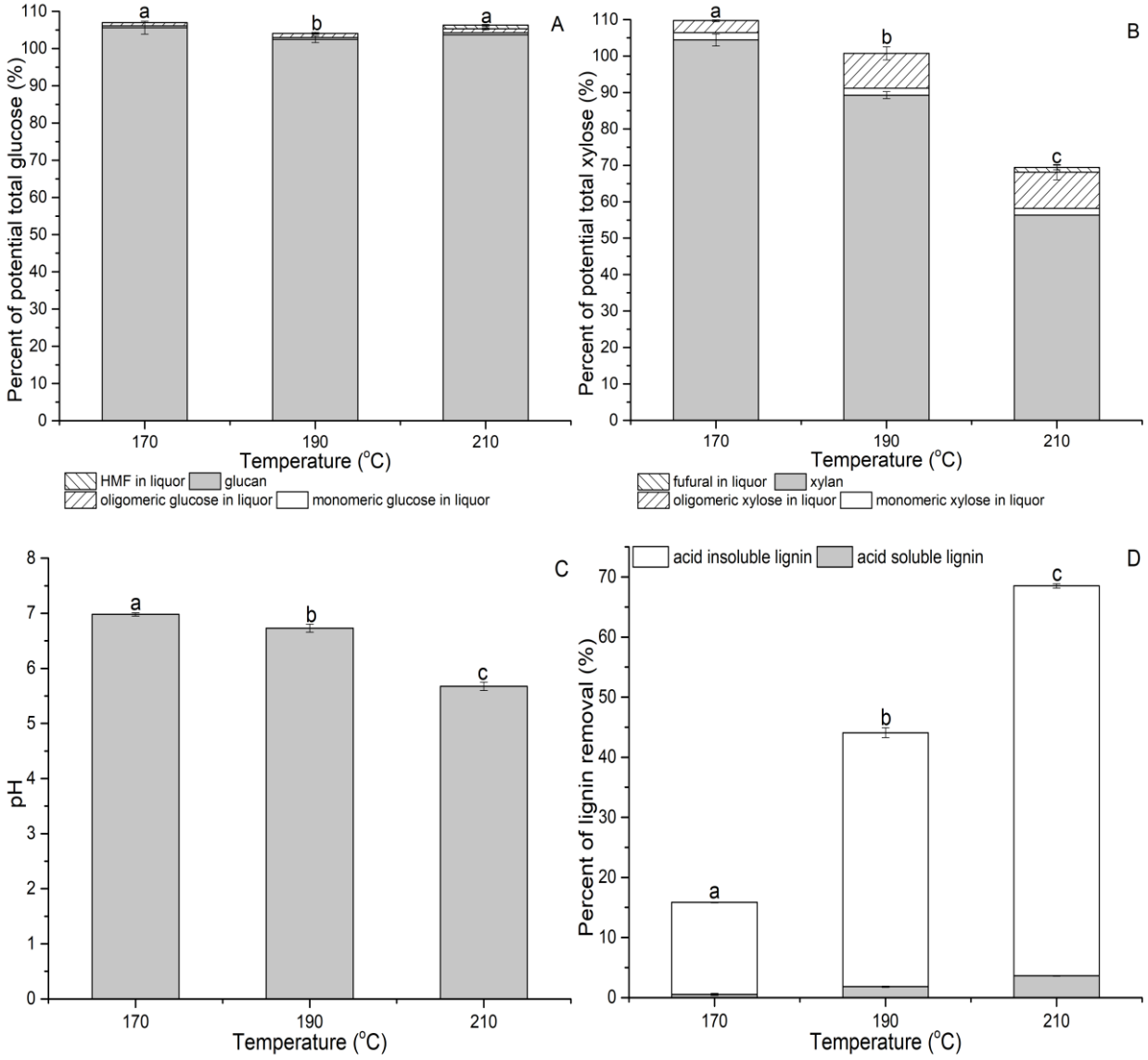

Figure 5.3 Effect of reaction temperature on sugar recoveries and lignin removal (all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.).

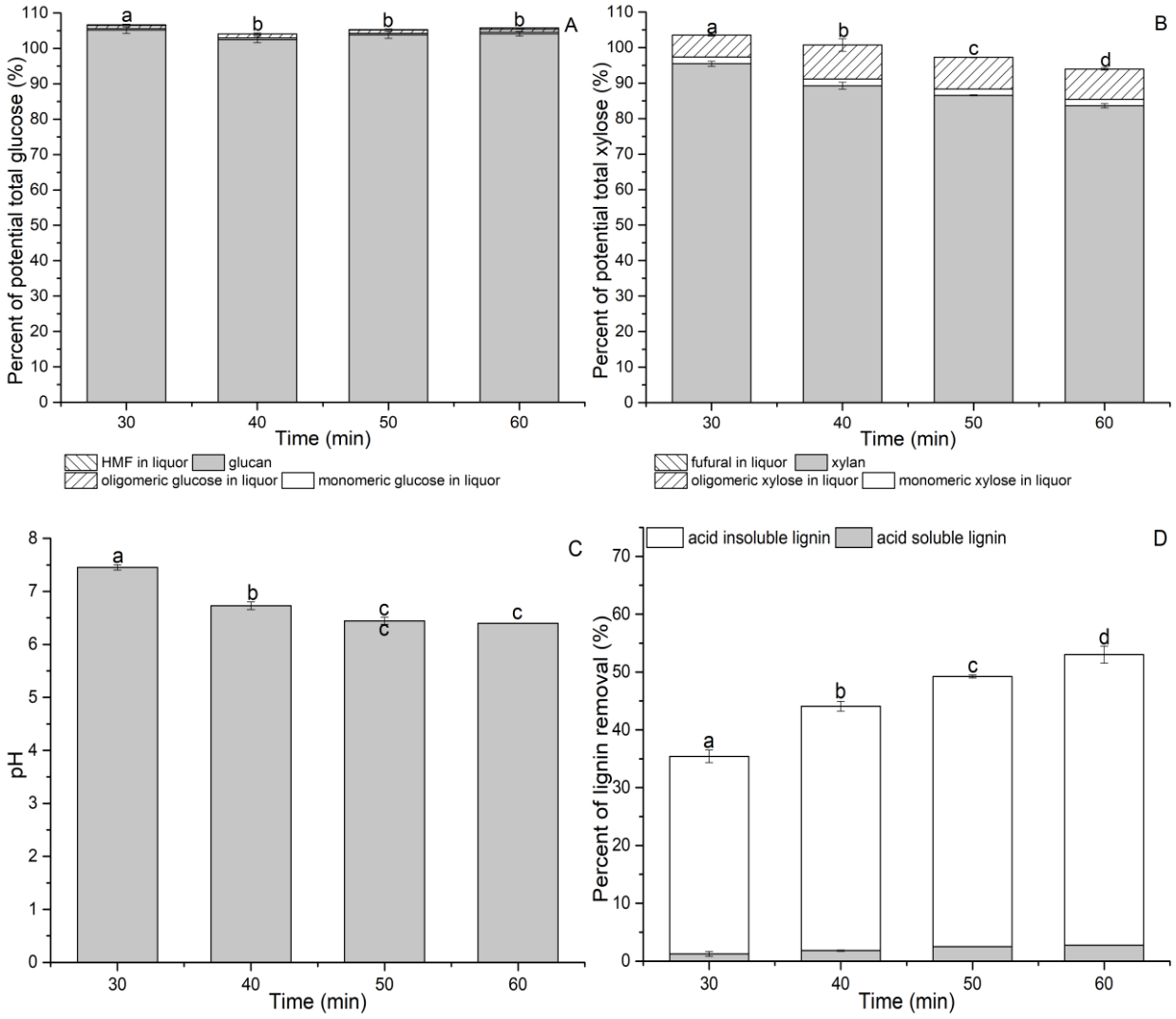

Figure 5.4 Effect of reaction time on sugar recoveries and lignin removal (all components were calculated on the basis of structural sugars in raw corn stover and free glucose (1.4%) and xylose (1.7%) in raw corn stover were not taken into account.).

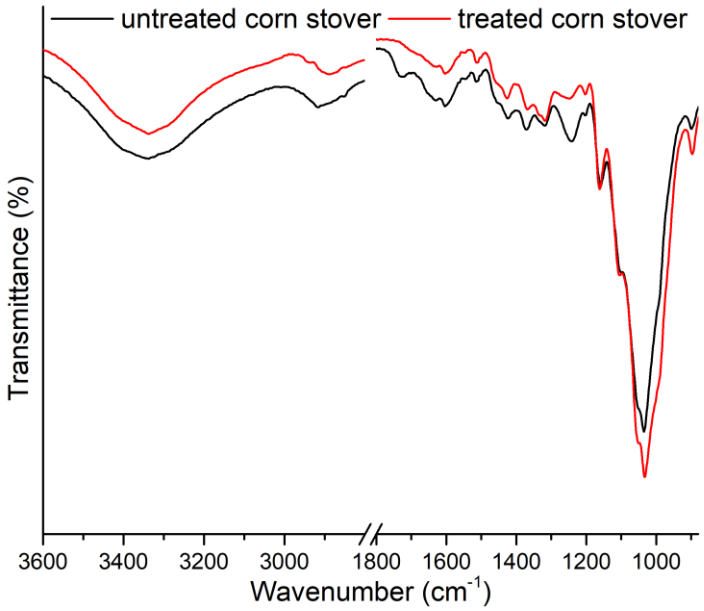

Figure 5.5 FTIR spectra of corn stover before and after MgO-ethanol pretreatment.

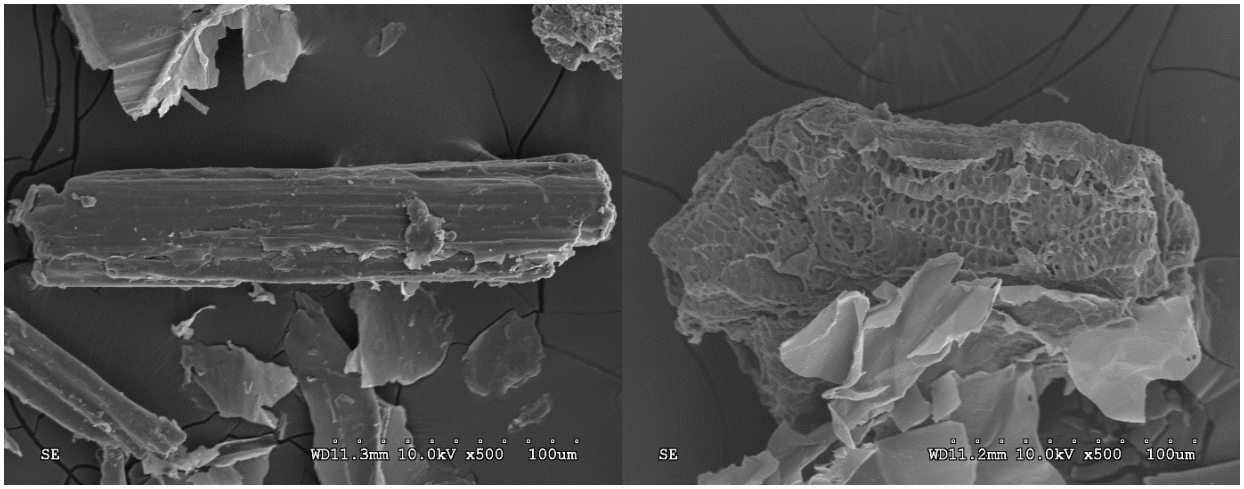

Figure 5.6 SEM of corn stover before (left) and after (right) MgO-ethanol pretreatment.

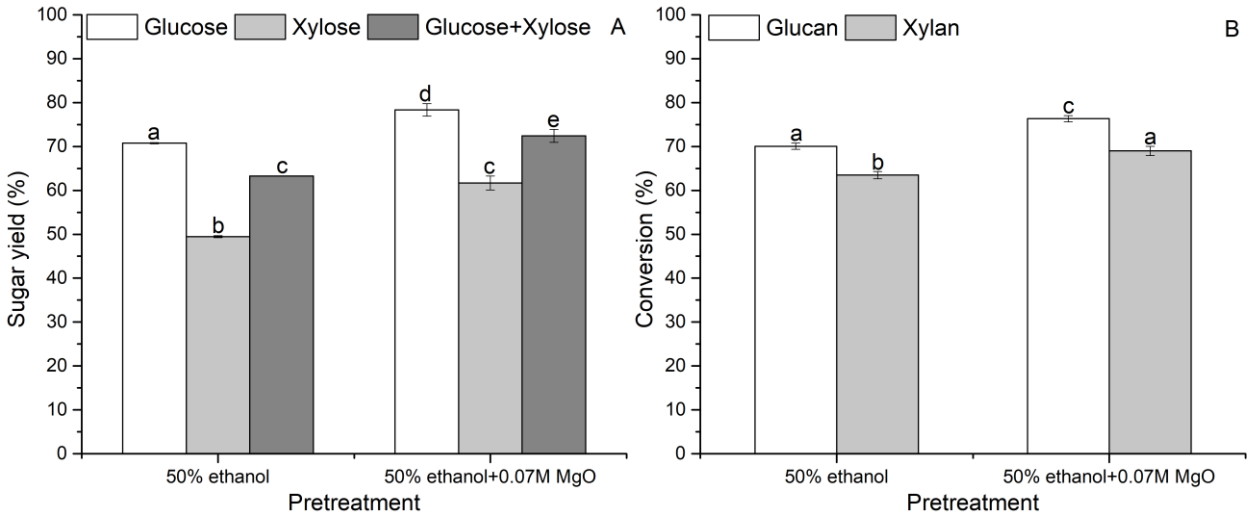

Figure 5.7 Enzymatic saccharification of corn stover pretreated by 50% ethanol with and without MgO.

Table 5.1 Chemical composition of untreated and treated corn stover[1].

Corn stover	Cellulose (%, db[4])	Hemicellulose[3] (%, db)	Lignin (%, db)	Solid recovery (%, db)
Raw	36.8±0.29a[5]	21.9±0.04a	15.1±0.40a	
Treated[2]	53.6±0.54b	27.0±0.33b	12.0±0.14b	70.4±0.10

[1] Results are presented in mean plus and minus standard deviation.
[2] Treatment condition was 50% ethanol and 0.07 mol/L MgO at 190 °C for 40 min with 10% solid loading.
[3] Hemicellulose includes xylan and arabinan.
[4] db means dry basis.
[5] In each column, means with different letters are significantly different at $p < 0.05$.

Chapter 6 - Boosting fermentable sugar yield and concentration through high-solids saccharification and high xylan recovery from magnesium oxide-ethanol treated corn stover

6.1. Abstract

MgO-ethanol pretreatment and high-solids saccharification were used to boost sugar yields and concentrations during saccharification. Corn stover pretreated by MgO and 50% ethanol achieved 75% glucan and 71% xylan conversions at the 10% solids loading and 30/18 μL CTec3/NS22244/g treated biomass. Under the same saccharification condition, corn stover pretreated by MgO and 30% ethanol had higher glucan and xylan conversions (80 and 78%). This result indicates that excessive xylan recovery from MgO and 50% ethanol pretreatment reduced enzymatic accessibility to cellulose and hemicellulose. When solids loading reached 16%, 74% glucan and 75% xylan conversions were obtained with glucose and xylose concentrations of 71 and 29 g/L. A 16%-solids loading largely reduced the poor mixing issue. The addition of Tween 80 effectively reduced the binding of lignin with enzymes, glucan and xylan conversions increased to 76 and 82%, respectively, and sugar concentration increased to 104 g/L.

6.2. Introduction

To reduce the dependency on petroleum-based transportation fuels, significant efforts have been made to produce renewable and sustainable biofuels (Ramos et al., 2016). Among different biofuels, bioethanol has been largely produced and used due to its clean-burning nature and zero nitrogen oxide and sulfur oxide emission (Elfasakhany, 2017). Currently, more than 95% of the annual ethanol supply was produced from starch-based raw materials, while cellulosic ethanol only has a market share of less than 1% (RFA, 2019). This reality is mainly attributed to that the techniques for starch ethanol production are well established; however, cellulosic ethanol production still faces significant technical challenges, which prevents the large-scale production of cellulosic ethanol (Liu et al., 2019). These challenges include biomass pretreatment, enzymatic saccharification, and fermentation/co-fermentation (Sun and Cheng, 2002).

Among various pretreatment methods, dilute sulfuric acid method has been industrialized, and liquid hot water (LHW) method is also attracted due to no chemical addition in the pretreatment process (Zhuang et al., 2016). However, the addition of sulfuric acid and the disassociation of acetic acid (HAc) released from hemicellulose cause degradation of monosaccharides (e.g. arabinose, xylose, glucose, etc.), generating a significant amount of inhibitors such as furfural, 5-hydroxymethylfurfural (HMF), and levulinic and formic acids, which significantly affect the downstream enzymatic saccharification and ethanol fermentation (Luo et al., 2002). In addition, both dilute acid and LHW methods are relatively weak in lignin removal. The residual lignin released from the treated biomass can irreversibly bind to enzymes, causing reduced enzyme activities or even enzyme inactivation during enzymatic saccharification (Kim et al., 2011; Zhai et al., 2018). To solve this issue, organic solvents derived from renewable sources, such as ethanol, acetone, methanol, and butanol, have been used for biomass pretreatment to

harness their excellent performance in lignin removal (Huijgen et al., 2011; Jafari et al., 2016; Zhang et al., 2016; Zhao et al., 2009). Among these organic solvents, ethanol is the most suitable one used for organosolv pretreatment due to its non-toxicity, low cost, easy recovery, and readily compensation in ethanol plant (Pan et al., 2005, 2006). In addition, aqueous ethanol usually performs better in lignin removal than pure ethanol (Jafari et al., 2016). However, HAc released during aqueous ethanol pretreatment reduces the pH of biomass slurry, consequently causing monosaccharides degradation (Huijgen et al., 2011). In Chapter 2 and 5, MgO was found to be a powerful Lewis base to neutralize the released HAc during LHW and ethanol pretreatments, largely reduce the monosaccharide degradation and washing water consumption as well as eliminate the need for biomass detoxification. Thus, MgO-ethanol pretreatment has great potential to produce sugar-degradation-products-free biomass for downstream saccharification and fermentation.

Regarding biomass saccharification, high-solids loading (> 15%, w/w) is superior to low- (< 10%, w/w) and moderate-solids (10~15%, w/w) loadings due to its enhanced fermentable sugar concentration, subsequent high ethanol yield and titer, and reduced capital and energy input (Chen et al., 2017; Weiss et al., 2019). However, one significant issue with high-solids enzymatic saccharification is poor mixing, especially at the initial stage. Poor mixing reduces enzyme activities due to biomass absorption of free water, and may deactivate enzymes due to the accumulation of inhibitors and residual lignin released from treated biomass (Weiss et al., 2019). To better handle high-solids loading during enzymatic saccharification, both horizontal reactors and fed-batch loading with better mixing capacity were attempted (de Albuquerque Wanderley et al., 2013; Roche et al., 2009a). However, the accumulated inhibitors and residual lignin released from treated biomass are still significant issues affecting enzymatic saccharification. In Chapter 3,

using MgO for biomass pretreatment reduced inhibitor formation, thus achieving higher sugar yields with a saccharification efficiency compatible to LHW-treated biomass at the same solids loading.

For ethanol industry, a minimum of 40 g/L of ethanol is generally required for economical ethanol distillation (Xu et al., 2016), which means that the fermentable sugars in fermentation broth should be higher than 80 g/L. Although high-solids enzymatic saccharification methods can achieve or exceed this minimum requirement, their enzymatic saccharification efficiencies were not satisfactory (Caspeta et al., 2014; Jørgensen et al., 2007). In addition, most previous studies focused only on the cellulose-to-glucose and glucose-to-ethanol conversions (Kristensen et al., 2009; Rocche et al., 2009b). Most xylan was removed during pretreatment, and glucan was the only source of sugar used in subsequent ethanol fermentation. Because of this, to achieve the minimum requirement of sugar concentration, higher solids loading is usually required, creating poor mixing and enzyme inhibition issues.

With advanced enzyme complexes and engineered bacteria, it is possible to simultaneously hydrolyze glucan and xylan and co-ferment glucose and xylose (Öhgren et al., 2006; Shen et al., 2012; Zhao et al., 2008), there is no need to completely remove xylan from biomass. Conversely, an appropriate increase in xylan recovery during biomass pretreatment could be a great option to achieve the minimum sugar concentration requirement (80 g/L) under a relatively low high-solids loading (e.g. 16 or 18%). In this case, the poor mixing and the inhibition of enzyme activities due to the excessive high-solids loading could be largely eased. Our previous studies found that MgO is a powerful Lewis base to neutralize the HAc (acid is a critical factor that causes the xylan decomposition) released from hemicellulose during pretreatment, increasing xylan recovery (Results in Chapter 2 and 5). Thus, biomass from MgO-ethanol pretreatment could have the ability

to achieve the minimum sugar concentration requirement at a relatively low high-solids loading (e.g. 16%) during saccharification.

In this research, the effects of solids loading (low: 6 and 8%; moderate: 10, 12, and 14%; and high: 16%) on enzymatic saccharification of MgO-ethanol treated corn stover were studied. Sugar yield and saccharification conversion efficiency were used as evaluation criteria. In addition, optimal enzyme loading was investigated as well as the addition of Tween 80 with function of improving enzyme activities.

6.3. Materials and methods

6.3.1. Materials

MgO powder (96% purity), ethanol (200 proof, ACS grade), and Tween 80 (99% purity), were obtained from Fisher (Ward Hill, MA). Enzymes CTec3 and NS22244 were generous gifts from Novozymes (Franklinton, NC). Protein contents of CTec3 and NS22244 were 516 and 266 mg protein/mL, respectively. Milled corn stover with <1 mm particle size (Agricultural Trial Base, Kansas State University, Manhattan, KS) was used in this work.

6.3.2. MgO-ethanol pretreatment

The process is similar to that described in Chapter 5. 10% solids loading (five grams of milled corn stover dissolved in 50 mL of aqueous ethanol) and designated amount of MgO were weighed into a reactor with a 75 mL internal volume. The reactor was shaken upside down for 2 min to completely hydrate biomass, and placed in a shaker at 45 °C for 1 h to facilitate metal oxides dispersing in water and touching biomass. To shorten the time that the reactor took to reach

target temperatures, the reactor was heated in boiling water for 3 min. The reactor was then submerged into a SLB-2 fluidized sandbath (Techne Inc., Princeton, NJ) set at 170-210 °C for 30-60 min. Once the set reaction time was reached, the reactor was rapidly cooled in ice water to immediately stop the hydrolysis reaction. After pH measurement, the biomass slurry was partitioned into treated biomass and biomass liquor by filtration using a Buchner funnel loaded with a filter paper (P8 grade, Fisherbrand). The solids were washed three times (60 mL each time) with the same concentration of aqueous ethanol and then dried at 45 °C overnight for following use. Finally, the biomass liquor and washing liquor were merged, diluted to 250 mL with the same concentration of aqueous ethanol, and placed in a freezer until further analysis and use.

Considering biomass with 30% ethanol and 0.075 mol/L MgO treatment was used for saccharification of moderate- and high-solids loading (sections 3.3-3.6), the required amount of the treated biomass was large. Multiple experiments under the same pretreatment condition had to be conducted, followed by multiple solids and liquids separation. To reduce the time used for separation, biomass slurries were combined and centrifuged to separate solids and liquids, and then liquids were filtered using a filter paper (P8 grade, Fisherbrand) to collect the residual solids.

6.3.3. Enzymatic saccharification

The enzymatic saccharification process is same as that described in Chapter 3. The designated amount of treated corn stover was loaded in a 125 mL flask, followed by the addition of a designated volume of sodium acetate buffer (50 mM, pH 5.0). To avoid sugar loss caused by microbial contamination during saccharification, sodium azide was added as a bacteriostatic agent with a loading of 0.02% (w/v). After that, the designated volume of CTec3 and NS22244 was loaded. The volume ratio of CTec3 and NS22244 loadings was 10 to 6. The slurry was hydrolyzed

enzymatically at 52 °C with 140 rpm agitation for 96 h. During enzymatic saccharification, 80 μL of slurry was periodically sampled from each flask to detect sugar concentrations.

Conversion efficiencies of glucan and xylan were computed using the following formulas:

$$E_c = \frac{V \times C_g}{1.11 \times m \times A_g} \times 100\% \quad (1)$$

$$E_h = \frac{V \times C_x}{1.14 \times m \times A_x} \times 100\% \quad (2)$$

where E_c and E_h are the conversion efficiencies of glucan and xylan in pretreated biomass (%), respectively; C_g and C_x are the concentrations of glucose and xylose in saccharification solution determined by HPLC (g/mL); m is the dry weight of pretreated biomass used for enzymatic saccharification (g); A_g and A_x are the glucan and xylan contents in pretreated biomass (%), respectively; 1.11 and 1.14 are the conversion factors of glucan-to-glucose and xylan-to-xylose, respectively; and V is the volume of saccharification solution (mL).

Sugar yields as received biomass were calculated using following formulas:

$$Y_g = \frac{R_b \times E_c \times A_g}{A_{g'}} \times 100\% \quad (3)$$

$$Y_x = \frac{R_b \times E_h \times A_x}{A_{x'}} \times 100\% \quad (4)$$

where Y_g and Y_x are the glucose and xylose yields (%) as received biomass, respectively; R_b is the biomass recovery from pretreatment (%); and $A_{g'}$ and $A_{x'}$ are the glucan and xylan contents in raw biomass (%), respectively.

6.3.4. HPLC analysis

Composition of treated biomass and biomass liquor was analyzed according to the National Renewable Energy Laboratory procedures (Sluiter et al., 2008a, b). A 1200 HPLC system (Agilent,

Santa Clara, CA) was employed to determine sugar concentrations with an HPX-87H organic acid column (7.8 × 300 mm) (Bio-Rad, Hercules, CA) as separation unit, a refractive index detector as detection unit, and 0.005 M sulfuric acid water as elution solvent. The temperatures of separation and detection units were set at 60 and 45 °C, respectively. The flow rate of the elution solvent was 0.6 mL/min. A series of concentrations of sugar standards were measured to build the standard curves in order to compute the concentrations of sugars in the real samples.

6.3.5. Statistics

All experiments were performed at least in duplicate. SAS (SAS Institute Inc., Cary, NC) was employed to statistically analyze data with the *p*-value of 0.05 as the cutoff for significance.

6.4. Results and discussion

6.4.1. Effects of ethanol concentration, MgO loading, reaction temperature, and reaction time on low-solids enzymatic saccharification of corn stover

To investigate the effects of pretreatment factors (ethanol concentration, MgO loading, reaction temperature, and reaction time) on enzymatic saccharification of corn stover, a low-solids loading of 1% and CTec3/NS22244 loading of 50/30 μL/g treated biomass were selected to minimize the interferences of poor mixing and accumulated inhibitors and lignin residues. Relevant data are listed in Figure 6.1.

As ethanol concentration increased from 30 to 50 and 70%, glucose yield gradually reduced from 79 to 71 and 56%, and xylose yield initially enhanced from 44 to 49% and then dropped to 48% (Figure 6.1A), which indicates appropriate xylan recovery can enhance xylose yield, while excessive xylan recovery (98% from 70% ethanol pretreatment) hinders the enzymatic

accessibility into internal cellulose and hemicellulose. Therefore, 50% ethanol was selected for optimization of subsequent pretreatment factors.

As MgO loading increased from 0.06 to 0.08 mol/L, glucose and xylose yields enhanced by 14 and 11%, respectively, consequently enhancing total sugar yield by 13% (Figure 6.1B). The increments of glucose and xylose yields when MgO loading increased from 0.06 to 0.07 mol/L were more than when MgO loading increased from 0.07 to 0.08 mol/L. Additionally, considering biomass slurry with 0.08 mol/L MgO had a pH value of more than 7 (Results in Chapter 5), 0.07 mol/L MgO loading was selected for subsequent parameter optimization.

Corn stover treated at 190 °C achieved a higher xylose yield (62%) than those (47 and 49%) treated at 170 and 210 °C (Figure 6.1C). As temperature increased from 170 to 210 °C, glucose yield increased from 57 to 99%. This is because cellulose is more resistant to high temperature due to its recalcitrant structure, whereas hemicellulose is easily decomposed at high temperature due to its weak structural strength. Additionally, considering temperature of 210 °C causes xylose degradation, furfural formation, and reduced slurry pH, 190 °C was selected for subsequent parameter optimization.

As reaction time increased from 30 to 60 min, glucose yield increased from 72 to 86%, and xylose yield initially increased from 59 to 64% and then decreased to 63% at 60 min (Figure 6.1D). Low glucose and xylose yields with the reaction time of 30 min is because insufficient reaction time causes excessive xylan recovery and less lignin removal. Additionally, considering reaction time of more than 40 min causes more sugar degradation and reduced slurry pH, reaction time of 40 min was selected for corn stover pretreatment in this work.

Under the optimal pretreatment conditions (0.07 mol/L MgO, 50% ethanol, 190 °C, and 40 min), glucose and xylose yields of pretreated corn stover were 78 and 62%, respectively.

6.4.2. Effects of low- and moderate-solids loading on enzymatic saccharification of corn stover treated by MgO and 50% ethanol

The effects of low- (6 and 8%) and moderate-solids (10%) loading on enzymatic saccharification of corn stover treated by MgO (0.07 mol/L) and 50% ethanol were investigated with a CTec3/NS22244 loading of 30/18 μL/g treated biomass (Figure 6.2). As solids loading increased from 6 to 8%, glucan conversion (80%) remained unchanged and xylan conversion increased by only 1%, but glucose and xylose concentrations increased from 28 and 12 g/L to 37 and 17 g/L, respectively. This demonstrates that the increase in low-solids loading has a larger effect on glucose and xylose concentrations than glucan and xylan conversions. When solids loading reached 10%, glucan and xylan conversions decreased to 75 and 71%, respectively; however, glucose and xylose concentrations further enhanced to 44 and 20 g/L, respectively.

Composition analysis results (Table 6.1) showed that compared to raw corn stover, corn stover treated by MgO and 50% ethanol had higher cellulose (53.4 vs. 36.8%) and hemicellulose (27.1 vs. 21.9%) contents and a lower xylan/glucan ratio (51 vs. 60%). However, 27.1% hemicellulose content in MgO-ethanol treated corn stover is equivalent to 90% xylan recovery (Results in Chapter 5), which indicates that most of the hemicellulose was not decomposed during pretreatment and still remained as it was in the raw corn stover. This could reduce the enzymatic accessibility into internal cellulose and hemicellulose. Compared to corn stover treated by MgO and 50% ethanol, corn stover treated by MgO and 30% ethanol had a similar cellulose content (54.4 vs. 53.4%) but a lower hemicellulose content (23.0 vs. 27.1%), thus having a lower xylan/glucan ratio (42 vs. 51%). 23.0% hemicellulose content in MgO-ethanol treated corn stover is equivalent to 76% xylan recovery. Therefore, compared to corn stover treated MgO and 50%

ethanol, corn stover treated by MgO and 30% ethanol could have higher saccharification efficiencies and sugar yields due to the appropriate xylan recovery.

6.4.3. Comparison of enzymatic saccharification of corn stover treated by MgO-ethanol at 30 and 50% ethanol concentrations

Enzymatic saccharification of corn stover treated by MgO-ethanol at 30 and 50% ethanol concentrations was compared at the solids loading of 10% and CTec3/NS22244 loading of 30/18 μL/g treated biomass (Figure 6.3). The glucan and xylan conversion efficiencies of corn stover treated by MgO and 30% ethanol were 80 and 78%, respectively, which are higher than those (75 and 71%) for corn stover treated by MgO and 50% ethanol. Also, corn stover treated by MgO and 30% ethanol achieved higher glucose and similar xylose concentrations (48 and 19 g/L) than corn stover treated by MgO and 50% ethanol (44 and 20 g/L), thus reaching a significantly higher total sugar concentration of 67 g/L. These results confirm the abovementioned hypothesis that excessive xylan recovery could inhibit saccharification conversion efficiency. Corn stover treated by MgO and 30% ethanol had a lower xylan/glucan ratio (42 vs. 51%) but a higher lignin/glucan ratio (32 vs. 24%) in comparison with corn stover treated by MgO and 50% ethanol. This indicates that the inhibitory effect of excessive xylan recovery on saccharification conversion efficiency was stronger than the promoting effect of lignin removal on saccharification conversion efficiency; thus glucan and xylan conversions and final glucose and xylose concentrations were lower in the corn stover treated by MgO and 50% ethanol. Thus, it was concluded that only appropriate hemicellulose recovery would create higher saccharification conversion efficiencies and sugar concentrations.

6.4.4. Effects of moderate- and high-solids loading on enzymatic saccharification of corn stover treated by MgO and 30% ethanol

The effects of moderate- (10, 12, and 14%) and high-solids (16%) loading on enzymatic saccharification of corn stover treated by MgO and 30% ethanol were investigated with a CTec3/NS22244 loading of 30/18 μL/g treated biomass (Figure 6.4). As solids loading increased from 10 to 14%, glucan conversion reduced from 80 to 77% and xylan conversion kept unchanged (78%), but glucose and xylose concentrations enhanced from 48 and 19 g/L to 65 and 26 g/L, respectively. In addition, the increase in moderate-solids loading had a stronger effect on glucose and xylose concentrations than glucan and xylan conversion efficiencies. When solids loading reached 16%, glucan and xylan conversions of 74 and 75% were achieved with the glucose, xylose, and total sugar concentrations of 71, 29, and 100 g/L, respectively. The total sugar concentration is higher than the 80 g/L minimum sugar concentration required for economic ethanol distillation. Furthermore, when solids loading increased from 14 to 16%, sugar conversion decreased only slightly, whereas sugar concentration increased significantly.

6.4.5. Effect of enzyme loading on high-solids enzymatic saccharification of corn stover treated by MgO and 30% ethanol

The effect of enzyme loading on enzymatic saccharification of corn stover treated by MgO and 30% ethanol was investigated at the solids loading of 16% (Figure 6.5). As CTec3/NS22244 loading increased from 20/12 to 30/18 μL/g treated biomass, glucan and xylan conversion efficiencies enhanced from 59 and 64% to 74 and 75%, respectively, and glucose and xylose concentrations enhanced from 56 and 25 g/L to 71 and 29 g/L, respectively. However, the further increase of CTec3/NS22244 loading from 30/18 to 40/24 μL/g treated biomass neither

significantly enhanced glucan and xylan conversions nor glucose and xylose concentrations, which could be because high xylose content inhibited the enzyme activities as previous reports (Dutta and Chakraborty, 2016; Kothari and Lee, 2011; Kumar and Wyman, 2009).

6.4.6. Effects of Tween 80 loading on high-solids enzymatic saccharification of corn stover treated by MgO and 30% ethanol

Tween 80 is an additive that can reduce the binding of lignin with enzyme (Jin et al., 2010; Kaar and Holtzapple, 1998; Liu et al., 2014; Sun and Cheng, 2002), thus reducing the number of deactivated enzymes. Tween 80 with loadings of 0.075, 0.15, and 0.3 g/g treated biomass was used to investigate the improvement of enzyme activities at the solids loading of 16% and CTec3/NS22244 loading of 30/18 μL/g treated biomass (Figure 6.6). Results indicated that the increase of Tween 80 loading had a significant effect on both glucan (74 to 76%) and xylan (75 to 82%) conversion efficiencies, especially xylan conversion efficiency, thus increasing both glucose (71 to 73 g/L) and xylose (29 to 31 g/L) concentrations. Total sugar concentration enhanced from 100 to 104 g/L, which exceeded the 80 g/L minimum sugar concentration requirement for economic ethanol distillation. The increment of saccharification conversion efficiencies and sugar concentrations as Tween 80 loading increased from 0.075 to 0.3 g/g treated biomass were less than that when Tween 80 loading increased from 0 to 0.075 g/g treated biomass. In addition, it seems like that the glucan and xylan conversions were relative “lower” than those in previous reports (less than 5% solids loading for enzymatic saccharification) using other pretreatment methods (Huijgen, et al., 2010, 2011; Jafari et al., 2016; Ludwig et al., 2014; Ramos et al., 2015), which is due to the difference of biomass compositions caused by different pretreatment methods as well as the different enzymatic saccharification conditions such as solids loading and enzyme loading.

It’s not advisable if saccharification performance is evaluated only using sugar conversion efficiency. This is because solids loading at too low level usually promotes a high sugar conversion but with a low sugar concentration, which is not economically viable for industrial application. Therefore, both sugar conversion efficiency and sugar concentration should be considered to evaluate overall saccharification performance.

6.5. Conclusions

The increase of ethanol concentration in MgO-ethanol pretreatment benefits both lignin removal and xylan recovery. However, increased lignin removal and xylan recovery do not guarantee high sugar conversions and concentrations, which is because excessive xylan recovery (e.g. 90% in biomass treated by MgO and 50% ethanol) hinders the enzymatic accessibility to internal cellulose and hemicellulose. Only an appropriate xylan recovery or xylan/glucan ratio could boost sugar conversion efficiencies and concentrations. In addition, the process only requires a 16%-solids loading, which reduces the poor mixing issue caused by higher solids loading.

6.6. References

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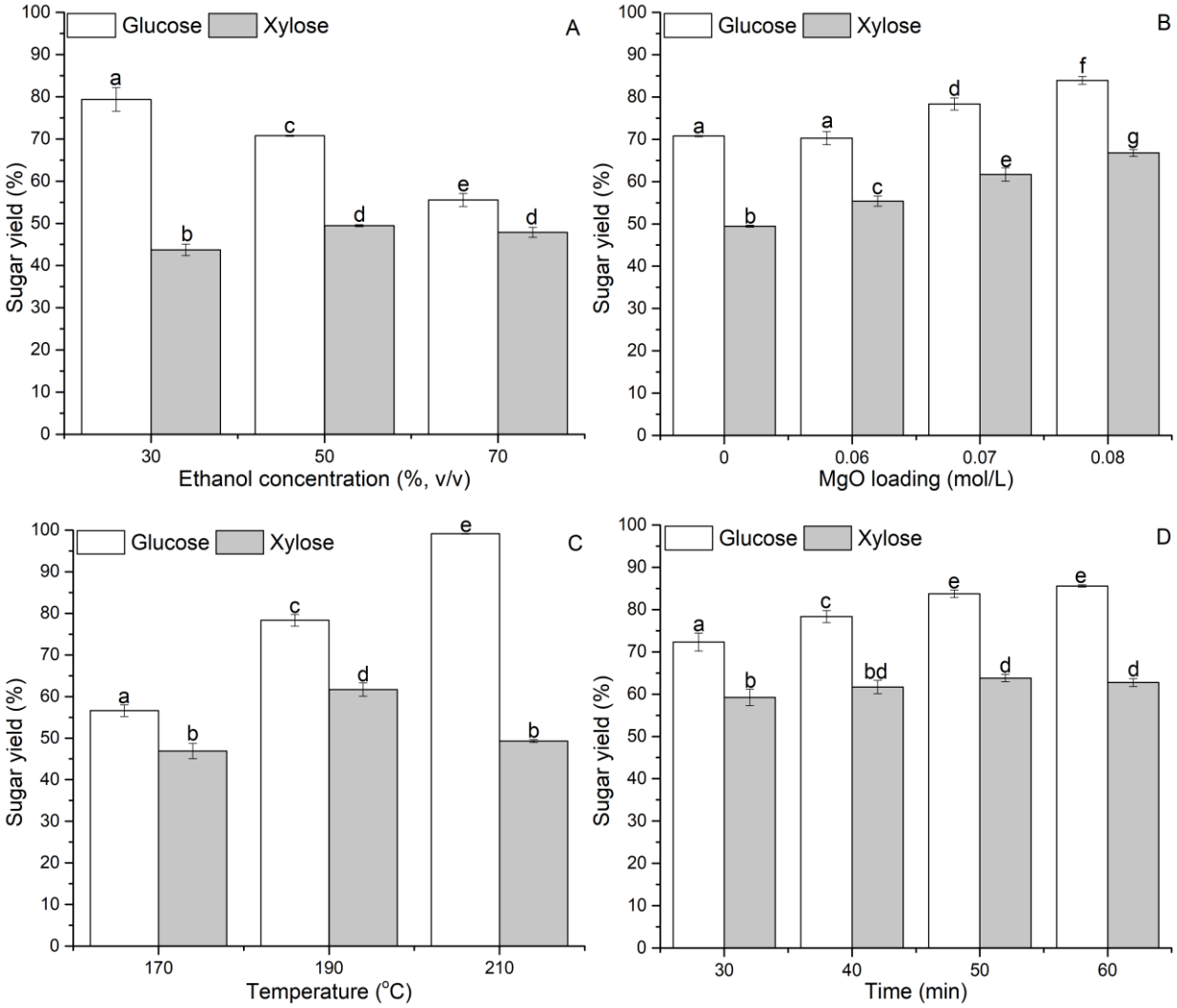

Figure 6.1 Effects of ethanol concentration, MgO loading, reaction temperature, and reaction time on low-solids loading enzymatic saccharification of corn stover (compositions of corn stover under different MgO-ethanol pretreatments were listed in Table B.2; saccharification conditions: 1% solids loading, 50/30 µL CTec3/NS22244/g treated biomass, 52 °C, 140 rpm, and 72 h).

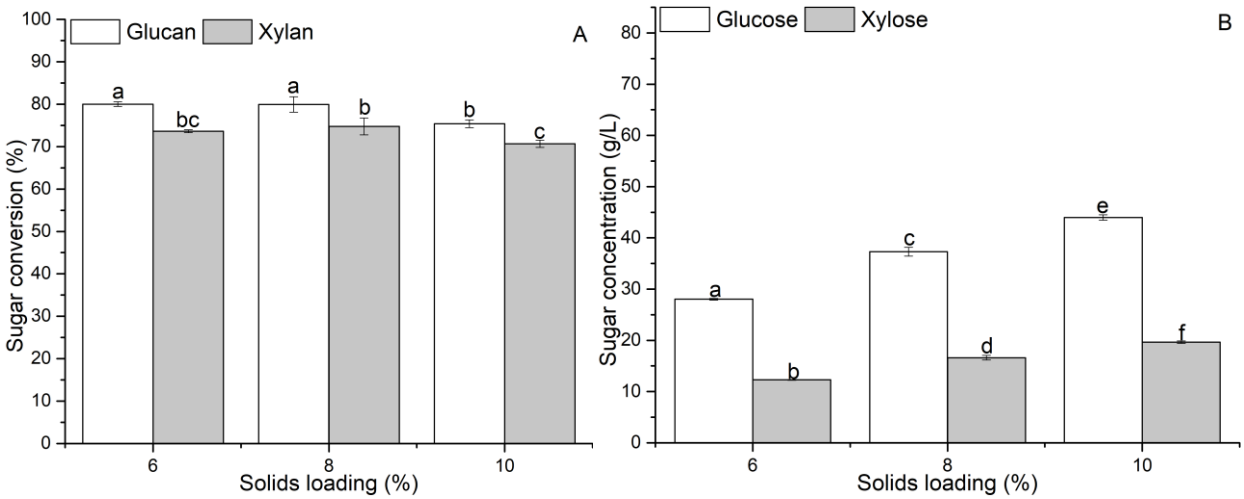

Figure 6.2 Effects of low- and moderate-solids loading on enzymatic saccharification of corn stover treated by MgO and 50% ethanol (Pretreatment conditions: 50% ethanol, 0.07 mol/L MgO, 190 °C, and 40 min; saccharification conditions: 6-10% solids loading, 30/18 µL CTec3/NS22244/g treated biomass, 52 °C, 140 rpm, and 96 h).

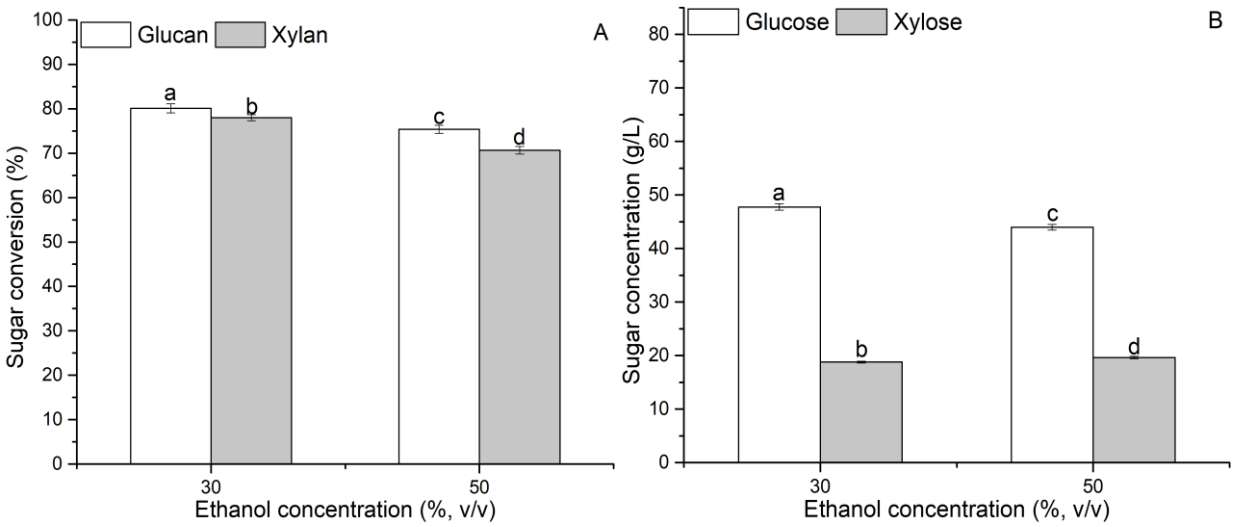

Figure 6.3 Comparison of enzymatic saccharification of corn stover treated by MgO and ethanol (30% vs. 50%) (Pretreatment conditions: 30% ethanol and 0.075 mol/L MgO or 50% ethanol and 0.07 mol/L MgO, 190 °C, and 40 min; saccharification conditions: 10% solids loading, 30/18 µL CTec3/NS22244/g treated biomass, 52 °C, 140 rpm, and 96 h).

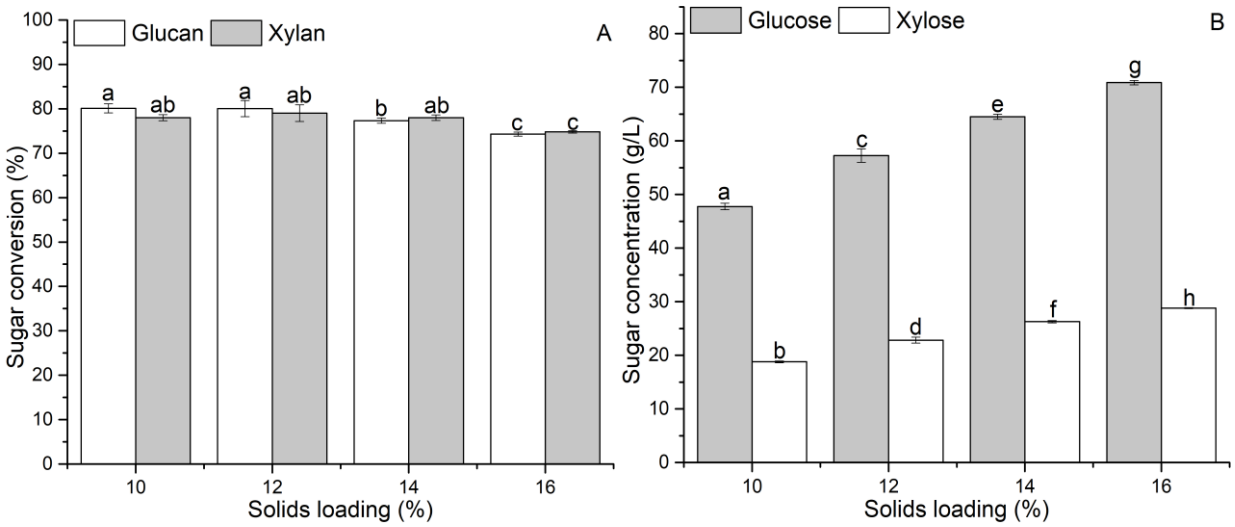

Figure 6.4 Effects of moderate- and high-solids loading on enzymatic saccharification of corn stover treated by MgO and 30% ethanol (Pretreatment conditions: 30% ethanol and 0.075 mol/L MgO, 190 °C, and 40 min; saccharification conditions: 10-16% solids loading, 30/18 µL CTec3/NS22244/g treated biomass, 52 °C, 140 rpm, and 96 h).

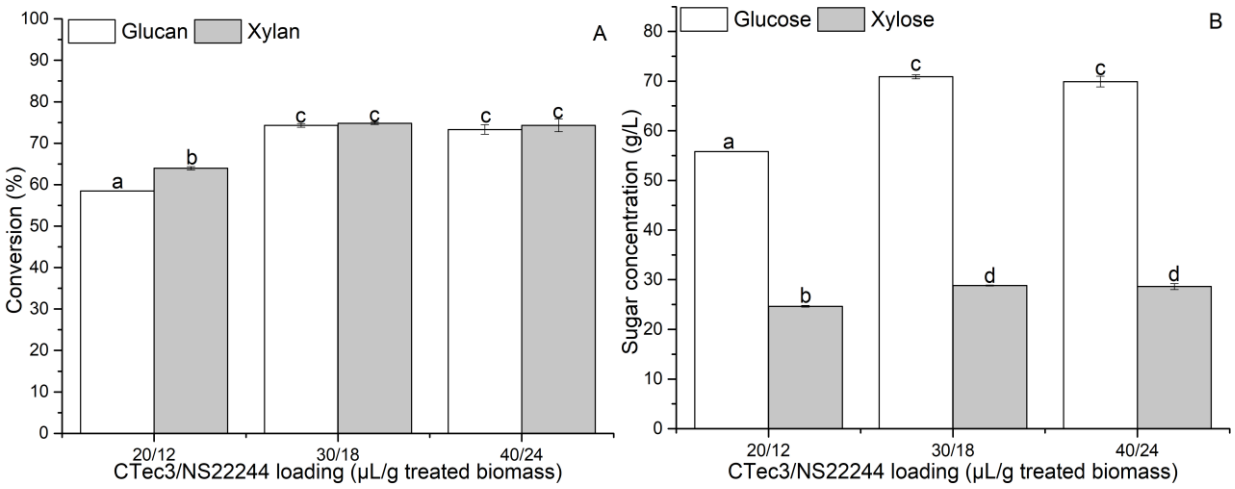

Figure 6.5 Effect of enzyme loading on high-solids loading enzymatic saccharification of corn stover treated by MgO and 30% ethanol (Pretreatment conditions: 30% ethanol and 0.075 mol/L MgO, 190 °C, and 40 min; saccharification conditions: 16% solids loading, 20/12-40/24 μL CTec3/NS22244/g treated biomass, 52 °C, 140 rpm, and 96 h).

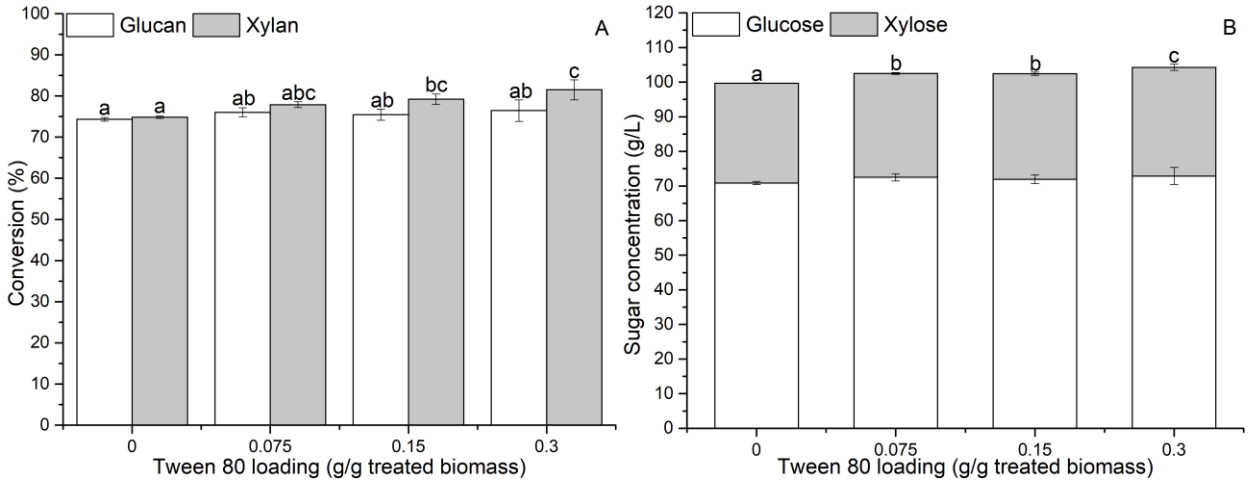

Figure 6.6 Effect of Tween 80 loading on high-solids loading enzymatic saccharification of corn stover treated by MgO and 30% ethanol (Pretreatment conditions: 30% ethanol and 0.075 mol/L MgO, 190 °C, and 40 min; saccharification conditions: 16% solids loading, 30/18 µL CTec3/NS22244/g treated biomass, 0.075-0.3 g Tween 80/g treated biomass, 52 °C, 140 rpm, and 96 h).

Table 6.1 Composition analysis of treated and untreated corn stover[1].

Corn stover	Cellulose (%, db[4])	Hemicellulose[5] (%, db)	Lignin (%, db)	Solids recovery (%, db)	Xylan/glucan ratio[6] (%)	Lignin/glucan ratio (%)	Liquor pH
Untreated	36.8±0.29a[7]	21.9±0.04a	15.1±0.40a		60	41	
Treated1[2]	54.4±0.01b	23.0±0.13b	17.5±0.02b	69.5±0.60	42	32	6.91±0.12
Treated2[3]	53.4±0.15c	27.1±0.04c	12.8±0.49c	70.9±0.58	51	24	6.90±0.02

[1] Data are presented in mean ± standard deviation.

[2] Treated 1 was 10% solids loading, 30% ethanol, 0.075 mol/L MgO, 190 °C, and 40 min.

[3] Treated 2 was 10% solids loading, 50% ethanol, 0.07 mol/L MgO, 190 °C, and 40 min.

[4] db = dry basis.

[5] Hemicellulose includes xylan and arabinan.

[6] Xylan/glucan ratio = $\frac{\text{Hemicellulose percentage in biomass}}{\text{Cellulose percentage in biomass}} \times 100\%$

[7] In each column, means with different letters are significantly different at $p < 0.05$.

Chapter 7 - Conclusions and future work

7.1. Conclusions

Metal oxides have great potential for biomass pretreatment. MgO pretreatment has significant advantages over LHW pretreatment:

1) MgO effectively interacted with acetic acid released from hemicellulose during pretreatment, leaving biomass slurry neutral;

2) Neutralization of released acids largely reduced sugar degradation and inhibitor (furfural and HMF) formation, eliminating the need for solids washing and detoxification;

3) MgO pretreatment effectively enhanced hemicellulose and cellulose recoveries, improving saccharification efficiency and sugar yield;

4) Neutral and furfural-and-HMF-free biomass slurry allowed the direct integration of treated biomass and biomass liquor for saccharification without solids washing and detoxification, largely reducing water usage and wastewater treatment as well as simplifying bioconversion processes.

Combination of MgO and ethanol for biomass pretreatment has significant advantages over ethanol only for biomass pretreatment:

1) Sugar degradation and inhibitor formation were largely reduced;

2) Combination of MgO and ethanol further enhanced lignin removal and cellulose and hemicellulose recoveries;

3) Increased lignin removal and sugar recovery allowed saccharification to be conducted at a relatively low high-solids loading (16%) to achieve high sugar yield and concentration, thus reducing the poor mixing issue.

7.2. Future work

Based on findings from this project, there are some recommendations for future research:

1) Different batches of corn stover required slightly different MgO pretreatment conditions, which is because the amount of acetic acid in different batches varied depending on crop varieties and growth environments. Thus, it's necessary to investigate the association between biomass types and MgO pretreatment;

2) MgO effectively interacted with released acids during pretreatment, which may reduce the condensation of lignin functional groups and increase the valorization of lignin. Thus, it's necessary to investigate the activity of lignin from MgO-treated biomass;

3) This project focused on the effects of MgO pretreatment on low-free-sugar biomass. It's necessary to investigate the effects of MgO pretreatment on moderate-free-sugar biomass in order to fundamentally understand the fitness of MgO pretreatment to different types of biomass.

Appendix A - Enzymatic saccharification

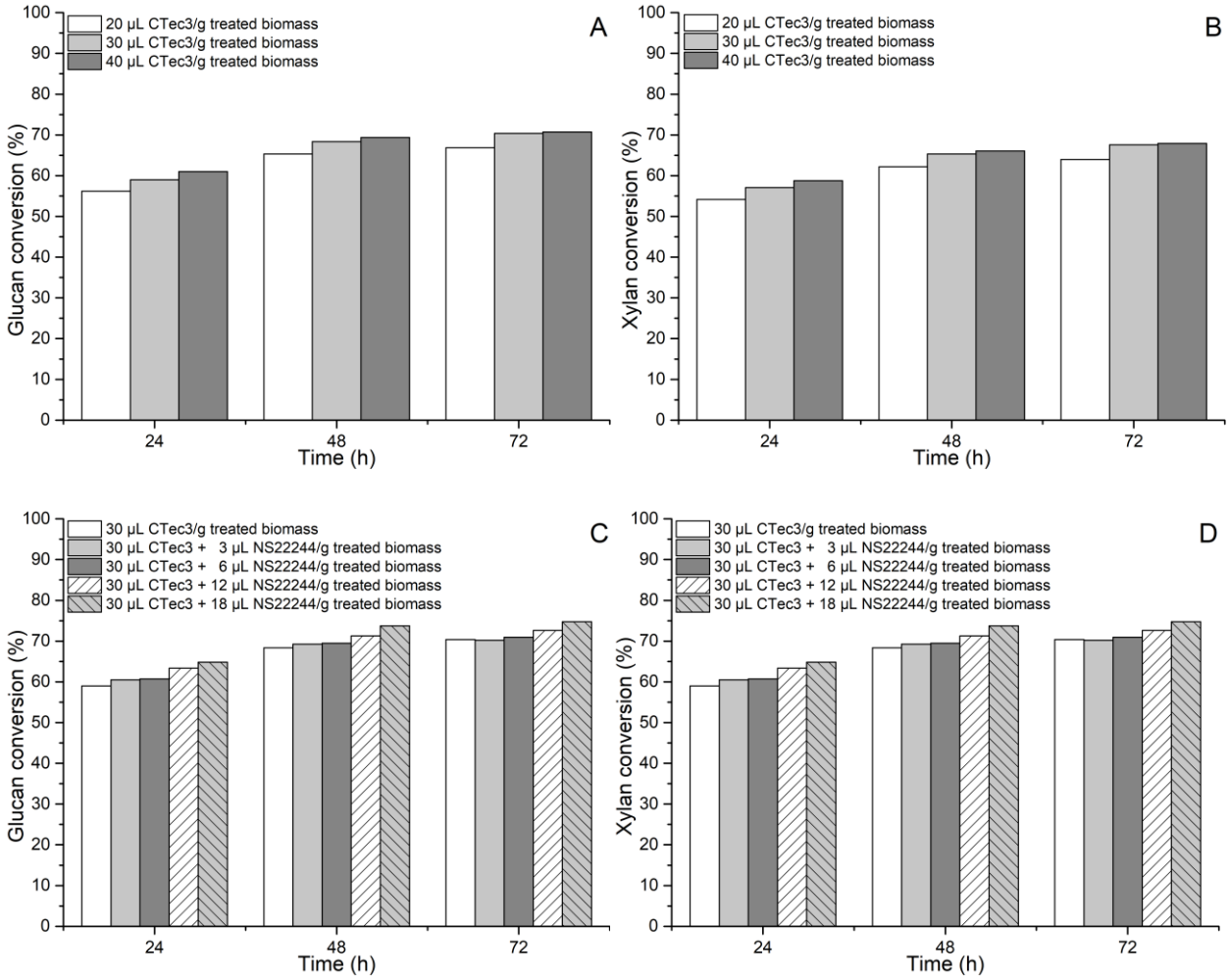

Figure A.1 Effect of CTec3 and NS22244 ratio on enzymatic saccharification of MgO-treated corn stover (MgO-treated corn stover had 56.3% cellulose and 15.2% hemicellulose; solids loading of 6% was used for hydrolysis; and the experiments were conducted without replicates).

Appendix B - Chemical composition

Table B.1 Chemical composition of corn stover under different MgO pretreatments.

MgO pretreatments			Solid recovery (%)	Cellulose (%)	Hemicellulose[1] (%)
MgO concentration (mol/L)	Temperature (°C)	Time (min)			
0	190	40	62.5±0.36	57.6±0.96	8.1±0.50
0.06	190	40	64.6±0.75	56.0±0.84	14.0±0.34
0.08	190	40	66.0±0.23	55.4±0.42	16.0±0.31
0.10	190	40	67.1±1.94	54.4±1.55	16.4±1.12
0.12	190	40	68.8±0.03	52.4±0.76	17.2±0.28
0.08	170	40	80.4±1.68	47.9±0.20	26.4±0.27
0.08	210	40	57.4±0.66	63.1±0.05	7.0±0.17
0.08	190	30	68.6±1.05	55.2±0.20	20.9±0.09
0.08	190	50	63.2±0.58	58.2±0.49	16.4±0.03
0.08	190	60	61.6±0.52	58.9±0.15	15.0±0.20

[1] Hemicellulose includes xylan and arabinan.

Table B.2 Chemical composition of corn stover under different MgO-ethanol pretreatments.

MgO-ethanol pretreatments				Solid recovery (%)	Cellulose (%)	Hemicellulose[1] (%)
Ethanol concentration (%, v/v)	MgO loading (mol/L)	Temperature (°C)	Time (min)			
30		190	40	65.8±0.93	55.0±0.48	19.1±0.26
50		190	40	66.8±0.23	55.6±0.65	24.1±0.14
70		190	40	74.5±0.31	50.7±0.12	27.9±0.28
50	0.06	190	40	70.2±0.80	53.5±0.67	26.2±0.11
50	0.07	190	40	70.4±0.10	53.6±0.54	27.0±0.33
50	0.08	190	40	71.0±0.82	53.9±1.10	27.6±0.43
50	0.07	170	40	80.1±0.88	48.5±0.27	28.6±0.08
50	0.07	210	40	55.8±0.42	68.4±0.55	20.2±0.14
50	0.07	190	30	73.2±0.85	52.9±0.13	27.9±0.13
50	0.07	190	50	67.6±0.24	56.5±0.34	27.0±0.11
50	0.07	190	60	65.6±0.45	58.4±0.03	26.6±0.47

[1] Hemicellulose includes xylan and arabinan.