Integrated bioprocess to boost cellulosic bioethanol titers and yields

by

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B.S., South China Agricultural University, 2012
M.S., Kansas State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Among potential alternative liquid fuels, bioethanol is the widest utilized transportation fuels and mainly made from grains. Cellulosic biofuels provide environmental benefits not available from grain or sugar-based biofuels and are considered as a solid foundation to meet transportation fuels needs in a low-carbon economy, albeit with electrified vehicles and other technical advances. The objective of this research was to develop and optimize various bioprocessing units to boost cellulosic bioethanol titers and yields in order to accelerate the commercialization of cellulosic bioethanol production.

The results showed high-solids biomass bioconversion (12%, w/v) was inefficient in the laboratory rotary shaker. However, a horizontal reactor with good mixing was effective for high solids loading (20%, w/v), yielding 75 g/L of glucose. To achieve the minimal economical ethanol distillation requirement of 40 g/L, integrated bioprocesses were conducted to boost ethanol titers and yields through co-fermentation of starchy grain and cellulosic biomass. The maximum ethanol concentration (68.7 g/L) was achieved at the corn flour and hydrothermal-treated corn stover ratio of 12:12 using raw starch granular enzyme with the ethanol yield of 86.0%. Co-fermentation of starchy substrate with hydrolysate liquor from saccharified biomass was able to significantly enhance ethanol concentration and reduce energy cost for distillation without sacrificing ethanol yields. These results indicated integration of first and second generation ethanol production could significantly accelerate the commercialization of cellulosic biofuel production. Novel technology, modified simultaneous saccharification and fermentation, was firstly established to enhance ethanol titers and yields, which achieved high ethanol titers of 72.3 g/L at high biomass loadings of 30% (w/v) with 70.0% ethanol yield.

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Among potential alternative liquid fuels, bioethanol is considered as the widest utilized transportation fuels. Cellulosic biofuels provide environmental benefits not available from grain or sugar-based biofuels and are considered as a solid foundation to meet transportation fuels needs in a low-carbon economy, albeit with electrified vehicles and other technical advances. The objective of this doctoral research was to develop and optimize various bioprocessing units in order to accelerate the commercialization of cellulosic bioethanol production.

The results showed high-solids bioconversion (12%, w/v) was inefficient in the laboratory rotary shaker. However, using a horizontal reactor with good mixing is effective for high solids loading (20%, w/v), yielding 75 g/L of glucose. To achieve the minimal economical ethanol distillation requirement of 40 g/L, integrated bioprocesses were proposed to boost ethanol titers and yields using starchy grain and cellulosic biomass co-fermentation. The maximum ethanol concentration (68.7 g/L) was achieved at the corn flour and hydrothermal-treated corn stover ratio of 12:12 using raw starch granular enzyme with the ethanol yield of 86.0%. Instead of simply mixing the starchy grain with cellulosic biomass, four alternative integrated designs were proposed to boost cellulosic ethanol titers and yields. Co-fermentation of starchy substrate with hydrolysate liquor from saccharified biomass is able to significantly enhance ethanol concentration to reduce energy cost for distillation without sacrificing ethanol yields. These results indicated integration of first and second generation ethanol production could significantly accelerate the commercialization of cellulosic biofuel production. Novel technology, modified simultaneous saccharification and fermentation, was firstly proposed to enhance ethanol titers and yields, which achieved high ethanol titers of 72.3 g/L at high loadings of 30% (w/v) with yields of 70.0%.

Table of Contents

List of Figures

List of Tables

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Dedication

To my family

Chapter 1 - Introduction

1.1 Problem Statement and Objectives

As the global population expands and the number of vehicles around the world increases, the demand for transportation fuels is expected to increase rapidly [1-3]. In addition, limited crude oil reserves and environmental concerns to mitigate greenhouse gas (GHG) emissions have driven global research to explore alternatives to fossil fuels and renewable energy [19-21]. Among potential alternative liquid fuels, bioethanol is the widest utilized transportation fuels [1, 4-6]. Bioethanol is a renewable alternative fuel derived from various sustainable feedstocks such as sugar-based crops, starch-based crops, and lignocellulosic biomass [4, 6]. In addition, with an octane rating of 113, ethanol offers indispensable value as a clean, low-cost octane booster to resist engine knocking. Biotechnologies are mature to produce ethanol from sugar and starch-rich crops in large scales and continued to develop advanced cellulosic ethanol production [1, 6]. Currently, biotechnologies are mature enough to produce ethanol from starch-rich grains (1st generation) such as corn, wheat and sorghum in large scales and continue to advance development of cellulosic ethanol production [1, 3, 9]. However, commercial production of 2nd generation bioethanol from lignocellulosic biomass is still not economically feasible, mainly facing challenges of low ethanol titer, low ethanol yield, high enzyme cost, and high water usage [6, 8, 9, 14]. For cellulosic bioethanol production, it is challenging to enhance ethanol titer without sacrificing ethanol yield due to the complex and recalcitrant structure of lignocellulosic biomass [4, 11, 13, 14].

The goal of this research was to develop integrated strategies to boost cellulosic bioethanol titers and yields in order to accelerate the commercialization of cellulosic ethanol production. The specific objectives include 1) high-gravity bioconversion of lignocellulosic

biomass as a traditional method to enhance ethanol titers, 2) integrated 1st and 2nd generation bioethanol production to enhance ethanol titers and ethanol yields, and (3) modified simultaneous saccharification and fermentation (mSSF) process with decantation technology to boost cellulosic ethanol titers and yields.

Bioconversion of lignocellulosic biomass to ethanol includes pretreatment, enzymatic hydrolysis, fermentation, and distillation for ethanol recovery (Fig. 1). Well-developed pretreatments such as dilute acid pretreatment or hydrothermal pretreatment are often implemented to improve enzymatic digestibility of cellulose mainly through eliminating most hemicellulose intertwined with cellulose [2, 22-24]. Liquid hot water pretreatment is also an effective method to disrupt the microstructure of biomass and considered as a green process because of less waste disposal and less required post-treatment [22, 25, 26]. Detoxification of pretreated biomass is usually beneficial following enzymatic hydrolysis and ethanol fermentation as degraded products such as acetic acid, furfural, and hydroxymethyfurfural are generated in pretreatment hydrolysates [25, 26]. High ethanol concentration is necessary to reduce capital and energy costs as a minimal 40 g/L is required for economical ethanol distillation [7, 10, 27]. Common ways to achieve higher ethanol titers are to increase biomass loadings. However, there's a trade-off between ethanol yield and ethanol concentration due to mass transfer limitation and accumulated inhibitors [11, 13, 14, 16, 17]. Alternatively, integrating cereal grain into cellulosic ethanol production could boost ethanol titers and yields. Through preliminary tests, we have proved the co-fermentation process of cereal grain and cellulosic biomass is an effective approach to simultaneously boost ethanol titers and yields. Integration of first (cereal grain-based) and second (cellulosic biomass-based) generation biofuel production could reduce capital cost and accelerate the commercialization of cellulosic ethanol production.

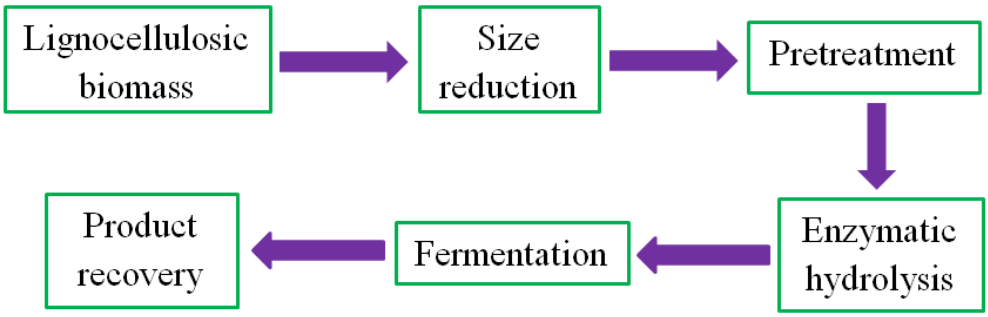

Fig. 1. Major processes for cellulosic bioethanol production.

In addition, a novel process, modified simultaneous saccharification and fermentation (mSSF) with decantation technology, which with combined the advantages of both separate hydrolysis and fermentation (SHF) and SSF would effectively enhance the ethanol titers and ethanol yields.

1.2 Related Current and Previous Research

1.2.1 Plant

Cereal crops such as corn, wheat, sorghum are viable feedstocks to produce bioethanol via starch fermentation. Starch, tiny white granules, can be isolated from various botanical sources such as cereal grains (including maize, wheat, rice, barley, sorghum, oat), roots or tubers (potato, sweet potato, cassava, arrowroots, yam), stems (sago palm), fruits like green banana, legume seeds (beans, peas, lentils) [28]. Currently, commercial production of starch is mainly isolated from maize, wheat, potatoes, cassava and waxy maize while other starch sources are commercially available in some areas [28]. In addition, agricultural residues represent the next potential feedstock to supplement bioethanol production. Lignocellulosic biomass is mainly

composed of cellulose, hemicellulose and lignin, in which the fractions of both cellulose and hemicellulose are carbohydrates and thus is a potential source of fermentable sugars.

1.2.2 Cellulose

Cellulose is a polymer of glucose with β-1,4 linkage and is found in both the crystalline and non-crystalline structure [29]. Properties of cellulose are highly linked to its degree of polymerization (DP), which represents the number of glucose monomers that make up one polymer molecule. Cellulose is insoluble in water and dilute acid solutions at room temperature, but in alkaline solutions, swelling of cellulose occurs and cellulose with low molecular weight (DP < 200) was dissolved [29, 30]. Cellulose is a hygroscopic material which can absorb 8 -14% moisture under normal atmospheric conditions.

1.2.3 Hemicellulose

Hemicellulose, usually consists of 20 to 30% of the biomass of annual and perennial plants, are viewed as important renewable resources of fermentable sugars [31]. Hemicelluloses isolated from agricultural residues could be potential sources of xylans, which are the linear β-1,4-linked xylopyranosyl main chains. Xylans are heteopolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units, which contain xylose, arabinose, glucuronic acid or its 4-O-ether, and acetic, ferulic and *p*-coumaric acids [31, 32]. The distribution pattern of side chains in heteroxylans which reflects the structure of the polymer chains has major influence on their solubility, interactions with other cell wall polymers, digestibility of enzymes, rheological properties, and other functional properties [31-33]. The extraction methods to isolate hemicellulose could also affect the functional properties. Hemicellulose has potential applications in producing biofilms and also in food uses as a

thickening agent or substitution for other commercial gums. Hemicelluloses show potentials as fermentation feedstock in the production of ethanol, acetone, butanol, and xylitol.

1.2.4 Lignin

Lignin is a complex polymer of phenylpropanoid units and its presence in the cell wall provides carbohydrate-lignin seal protection, thus, inhibiting enzymatic hydrolysis of fermentable sugars [10, 26, 34].

1.2.5 Starch

Starch, tiny white granules consisting of amylose and amylopectin, can be extracted from various botanical sources.

The physical and chemical properties of starch are strongly linked to their biological origin and growing environment of various plants. Particle size distribution is one of the main factors influence the physico-chemical properties. Potato starch is relatively large oval granule compared to other starch such as smaller polygonal granules of maize starch. Wheat starch has a unique bimodal particle size distribution while rice starch has its unique property of small particle size.

Starch mainly consists of relatively linear amylose and branched amylopectin. Amylose is a predominantly linear 1, 4-α-D-glucan linked, whereas side chains of amylopectin linked to the linear chains by 1, 6-α-D-glucan linkage at an interval of every 20 glucose units. Starch, is a semi-crystalline material containing alternating crystalline and amorphous regions. Amylose has a relatively large molar mass ($> 10^6$ g/mol) while amylopectin has a larger molar mass ($> 10^7$ g/mol). Amylopectin predominantly contributes to the crystalline region as their outer branches are hydrogen bonded together to generate double helices crystallites that are being disrupted during gelatinization while the amorphous regions of starch granules are mainly made up of

amylose and amylopectin branched points. Amylose and the branching points of amylopectin form the amorphous regions while the linear parts of amylopectin are mainly responsible for crystalline components in granular starch. Amylopectin is also believed to responsible for granule swelling as waxy starch usually swelled much more than normal starch.

1.2.6 Pretreatment

Cellulose in native biomass is difficult to digest by enzymes and its sugar yield is usually lower than 20%.[35] Pretreatment of biomass feedstocks was used to breakdown barriers and open cellulose to make it more accessible for further saccharification conversion. Numerous pretreatment methods have been developed to overcome the recalcitrant structure, such as ball mill, steam explosion, liquid hot water, dilute acid, lime, ammonia, organic solvent, and ionic liquid pretreatments. Challenges in the current pretreatment processes include incomplete separation of cellulose and lignin, which could reduce the subsequent enzymatic hydrolysis efficiency; formation of inhibitors that affect ethanol fermentation, such as acetic acid from hemicellulose, furans from sugar degradation and phenolic compounds from lignin composition; high usage of chemicals; energy-intensive processes; and high cost of waste disposal.

1.2.6.1 Mechanical pretreatment of biomass

Mechanical pretreatments includes chipping, grinding and milling, which reduce particle size and cellulose crystallinity but increase the specific surface area available for enzymatic hydrolysis [22, 36]. After chipping, particle size could be reduced to 10 to 30 mm, while milling or grinding could reach smaller size in the range of 0.2 to 2 mm [22, 37]. Vibratory ball milling was also powerful and efficient in reducing cellulose crystallinity of biomass [2, 37]. Mechanical refining could be conducted through three major behaviors, including cutting (size reduction), shearing (external fibrillation), and compression (internal fibrillation) [2]. The effectiveness of

mechanical pretreatment is highly dependent on the moisture content of the biomass. Knife mills and hammer mills are usually used to cut dry biomass, while ball mills and disk mills are suitable for dry and wet biomass. Ball mills can reduce particle size and cellulose crystallinity through shear and compressive force, but long processing time and high power usage make ball mills impractical for industrial application [2]. Disk mills are a continuous process and use shear force to disrupt the cell wall structure of biomass, but also have high power consumption. The major drawback of mechanical pretreatment is high energy input. Combined mechanical and other pretreatments are commonly used to reduce energy consumption and also improve the digestibility of treated biomass.

1.2.6.2 Biomass pretreatment at low pH

Acid pretreatments promote hydrolysis and improve fermentable sugar yield by removing hemicellulose during pretreatment and the most commonly used acid is sulfuric acid (H_2SO_4). Dilute sulfuric acid pretreatment showed great potential as a cost-effective pretreatment and had received extensive research attention over the years [22]. Reaction temperature in dilute acid pretreatment ranged from 140 to 160 °C while acid levels of sulfuric acid is in the range of 0.5 to 2% (w/w). The yield of reducing sugars through enzymatic hydrolysis is mainly influenced by acid concentration, reaction temperature, and reaction time. Acid hydrolysis releases oligosaccharides and monosaccharides but also results in the formation of degradation products such as aldehydes. In order to reduce the decomposition of the sugars, the hydrolysis time of biomasses should be strictly controlled.

Hydrothermal pretreatment is an eco-friendly process as only water is used as reaction medium without chemicals addition, which is usually processed at relatively high temperatures (140 – 220 °C) under mild acidic conditions [22, 38, 39]. At room temperature, the

corresponding pH of water is 7.0. However, as the temperature and pressure of saturated liquid water increases, pH of water decreases until reaching a minimum point of 5.6 at around 250 °C [22]. The concentration of hydronium ions increases as pH of water drops, which enhances the ability to catalyze acid reactions 25 times stronger than at room temperature [22]. During hydrothermal pretreatment, the hydronium ions released by water depolymerizes hemicellulose from plant cell wall to form acetic acids, improving enzymatic accessibility to cellulose and thus enzyme-catalyzed hydrolysis yields of fermentable glucose [39].

1.2.6.3 Biomass pretreatment at high pH

Alkaline pretreatment is conducted at lower temperature and pressure as compared to acid pretreatment. Alkali pretreatment can be carried out at ambient conditions, but it usually takes longer time in terms of days rather than hours or minutes and requires neutralization of the treated biomass slurry. Sodium hydroxide and calcium hydroxide are commonly used as reactants in alkali pretreatments. Alkali pretreatment is effective to remove the lignin fraction from biomass, thus exposing the remaining polysaccharides. The use of an alkali results in cellulose swelling and partial decrystallization of cellulose [5, 8, 36, 37]. Sodium hydroxide could effectively attack the lignin and hemicellulose linkage by cleavage of the ether and ester bonds in the lignin-carbohydrate complexes [2]. Sodium hydroxide can also effectively cleave the ester and carbon-to-carbon bonds in lignin molecules. Ammonia pretreatment has also received considerable research focus and was found extremely effective for herbaceous and agricultural residues, but not well suited for woody biomass [22]. Ammonia fiber explosion (AFEX) and ammonia recycle percolation (ARP) are widely studied in ammonia pretreatments. AFEX is a process of biomass exposed to liquid ammonia at high temperature and pressure for a period of time and suddenly released the pressure, while ARP is the process of aqueous ammonia

passing through biomass at elevated temperatures with recycling ammonia. Ammonia-based treatments of biomass have been studied extensively due to its easy recovery, non-corrosiveness and non-toxicity [2, 22]. Alkaline pretreatment is more effective for lignin removal as alkaline reagents interact with the lignin. The major effects of ammonia-based pretreatment include the removal or alteration of lignin, increased surface area and pore size, and decrystallization of cellulose structure [2]. In aqueous pretreatment of biomass, it could cause biomass swelling and result in significant morphological change. ARP employs a fixed bed reactor operated in the flow-through mode to remove soluble lignin portion. It is highly effectively for lignin removal (>80%) as it can minimize the re-polymerication and re-precipitation of soluble lignin onto the surface of biomass. In the AFEX process, biomass is reacted with liquefied anhydrous ammonia at elevated temperature (60 to 120 °C) and rapidly released into atmosphere after treatment. The lignocellulosic structure was disrupted once upon the expansion of the liquid ammonia trapped in the biomass, resulting in the reduction of cellulose crystallization, partial dissolution of hemicellulose and depolymerization of lignin [22]. The AFEX process has some disadvantages of high pressure requirement and high energy input for ammonia recovery.

1.2.6.4 Biomass pretreatment by ionic liquid

Ionic liquid pretreatment is an effective method to improve enzymatic digestibility [7, 40, 41]. The application of ionic liquids (salts composed of anions and cations) to biomass pretreatment has its unique properties as these solvents have low melting point and are fluid at room temperature. Their properties are defined by a broad range of anions and cations, of which the anion has a primary effect on water miscibility and the cation has a minor effect. The thermodynamic and physicochemical properties of the ionic liquid are determined by its specific combination of the anion and cation [41]. Ionic liquid pretreatment is suitable for a wide range of

biomass feedstocks and was proved to decrease the recalcitrant structure of biomass including efficient biomass dissolution, reduced cellulose crystallinity and lignin content in the pretreated biomass, and thus improving the enzymatic saccharification yields. Lignocellulosic biomass is completely or partial solubilized in ionic liquids at relatively high temperatures (>130 °C) and could be separated by adding an antisolvent such as water to the biomass and ionic liquid mixture.

1.2.6.5 Biomass pretreatment by organic solvent

Organosolv pretreatment is a process of using aqueous organic solvents as reaction medium to fractionate the major biomass components, of which cellulose and lignin are usually recovered as precipitated solid streams while hemicellulose and sugar degradation products are dissolved into a water-soluble fraction [42]. Orgonosolv pretreatment could obtain high-purity cellulose with negligible degradation. Organosolv pretreatment is able to obtain a clean lignin component from biomass, while the lignin is usually burned as an energy source in other pretreatment processes. High-quality lignin can be upgraded to high-value chemicals for many industrial applications, such as plasticizes for concrete construction, specific adhesives, resins for coating and high-performance brakes. Ethanol is a commonly used organic solvent and using acid or alkaline catalysts to assist in the delignification process. Other solvents and catalysts such as methanol, acetone, acetic acid, and formic acid were tested for organosolv pretreatment. A two-step process involving dilute-acid presoaking and aqueous-ethanol organosolv pretreatment of Miscanthus x giganteus yielded a solid residue with 95% initial glucan recovery, of which 98% was converted to glucose after 48 h of enzymatic saccharification, and 73% initial xylan recovery as well as 71% lignin recovery as ethanol organosolv lignin [43].

1.2.7 Hydrolysis

Hydrolysis of biomass includes acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis of biomass using aqueous acid solution such as sulfuric acid or through solid acid catalysts could break down the long glucose chains. Acid hydrolysis process is more technically mature, however they have greater environment and personal risks. Sugar yield is usually less than 60% by conventional acid-catalyzed processes. The Scholler process using 0.5 wt% sulfuric acid solution at 170 °C for 45 min was able to achieve 50% fermentable sugar yield. Solid acid catalysts for transformation of cellulose into glucose were also studied as it had advantages of easily separation and efficient solid catalysts recycling over aqueous acid hydrolysis. Enzymatic hydrolysis of biomass is superior to acid hydrolysis due to its good selectivity and high fermentable sugar yields.

1.2.8 Fermentation

Ethanol concentration and titer are interchangeable term in this dissertation. Enzymatic hydrolysis of pretreated biomass is effective at low solid loadings (<10%, w/v), but the conversion efficiency decreases markedly at higher solid loadings[11, 14]. From the economic and environmental standpoints, high-gravity bioconversion is superior to low-solid loadings as enhanced fermentable sugars and less water usage are preferred [7]. High ethanol concentration is necessary to reduce capital and energy costs as a minimum of 40 g/L is generally required for economical ethanol distillation [7, 12, 13, 17]. Fermentation of treated cellulosic biomass alone was not able to reach this goal and one common approach to achieve higher ethanol titers is to increase the amount of biomass loadings (> 8wt% glucan loading), so-called high-gravity processing. However, it is also known that fermentable sugars yield normally decreases as solids loadings increase due to the problems like mass transfer limitations or accumulated inhibitors by

degraded products that subsequently occur, which results in decreased fermentation yield [7, 11, 13, 14, 16, 17]. Jorgensen et al. [13] utilized a reactor system with excellent mixing capability that was able to liquefy and saccharify pretreated wheat straw up to 40% (w/w) solids loading and obtained the highest ethanol concentration of 48 g/L at the 35% (w/w) solids loading but with relatively low and unacceptable ethanol yield, less than 50%. Xue et al. also reported similar results of reduced biofuel yields due to accumulated recalcitrant oligosaccharides at high-solids loading [44]. Thus, it is challenging to effectively hydrolyze and ferment cellulosic biomass at high solids loading.

1.3 References

[1] Balat M, Balat H: Recent trends in global production and utilization of bio-ethanol fuel. Appl Energy 2009, 86(11):2273-2282.

[2] Kim SM, Dien BS, Singh V: Promise of combined hydrothermal/chemical and mechanical refining for pretreatment of woody and herbaceous biomass. Biotechnology for biofuels 2016, 9(1):97.

[3] Limayem A, Ricke SC: Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science 2012, 38(4):449-467.

[4] Aditiya H, Mahlia T, Chong W, Nur H, Sebayang A: Second generation bioethanol production: A critical review. Renewable and Sustainable Energy Reviews 2016, 66:631-653.

[5] Menon V, Rao M: Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. Progress in Energy and Combustion Science 2012, 38(4):522-550.

[6] Thangavelu SK, Ahmed AS, Ani FN: Review on bioethanol as alternative fuel for spark ignition engines. Renewable and Sustainable Energy Reviews 2016, 56:820-835.

[7] Xu F, Sun J, Konda NM, Shi J, Dutta T, Scown CD, Simmons BA, Singh S: Transforming biomass conversion with ionic liquids: process intensification and the development of a

high-gravity, one-pot process for the production of cellulosic ethanol. Energy & Environmental Science 2016, 9(3):1042-1049.

[8] Mohapatra S, Mishra C, Behera SS, Thatoi H: Application of pretreatment, fermentation and molecular techniques for enhancing bioethanol production from grass biomass–A review. Renewable and Sustainable Energy Reviews 2017, 78:1007-1032.

[9] Muktham R, Bhargava SK, Bankupalli S, Ball AS: A Review on 1st and 2nd Generation Bioethanol Production-Recent Progress. Journal of Sustainable Bioenergy Systems 2016, 6(03):72.

[10] Kim TH, Kim TH: Overview of technical barriers and implementation of cellulosic ethanol in the US. Energy 2014, 66:13-19.

[11] Hodge DB, Karim MN, Schell DJ, McMillan JD: Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. Bioresour Technol 2008, 99(18):8940-8948.

[12] Koppram R, Olsson L: Combined substrate, enzyme and yeast feed in simultaneous saccharification and fermentation allow bioethanol production from pretreated spruce biomass at high solids loadings. Biotechnology for biofuels 2014, 7(1):1.

[13] Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C: Liquefaction of lignocellulose at high-solids concentrations. Biotechnol Bioeng 2007, 96(5):862-870.

[14] Koppram R, Tomás-Pejó E, Xiros C, Olsson L: Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol 2014, 32(1):46-53.

[15] Liu Z, Qin L, Zhu J, Li B, Yuan Y: Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. Biotechnology for biofuels 2014, 7(1):1.

[16] Lu Y, Wang Y, Xu G, Chu J, Zhuang Y, Zhang S: Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Appl Biochem Biotechnol 2010, 160(2):360-369.

[17] Modenbach AA, Nokes SE: The use of high-solids loadings in biomass pretreatment—a review. Biotechnol Bioeng 2012, 109(6):1430-1442.

[18] Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM: Ethanol can contribute to energy and environmental goals. Science 2006, 311(5760):506-508.

[19] Gaurav N, Sivasankari S, Kiran G, Ninawe A, Selvin J: Utilization of bioresources for sustainable biofuels: A Review. Renewable and Sustainable Energy Reviews 2017, 73:205-214.

[20] Ghosh S, Chowdhury R, Bhattacharya P: Sustainability of cereal straws for the fermentative production of second generation biofuels: A review of the efficiency and economics of biochemical pretreatment processes. Appl Energy 2017, 198:284-298.

[21] Jiang Y, Xin F, Lu J, Dong W, Zhang W, Zhang M, Wu H, Ma J, Jiang M: State of the art review of biofuels production from lignocellulose by thermophilic bacteria. Bioresour Technol 2017

[22] Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M: Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005, 96(6):673-686.

[23] Xu F, Shi Y, Wu X, Theerarattananoon K, Staggenborg S, Wang D: Sulfuric acid pretreatment and enzymatic hydrolysis of photoperiod sensitive sorghum for ethanol production. Bioprocess and biosystems engineering 2011, 34(4):485-492.

[24] Zheng Y, Lee C, Yu C, Cheng Y, Zhang R, Jenkins BM, VanderGheynst JS: Dilute acid pretreatment and fermentation of sugar beet pulp to ethanol. Appl Energy 2013, 105:1-7.

[25] Kim Y, Kreke T, Ko JK, Ladisch MR: Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. Biotechnol Bioeng 2015, 112(4):677-687.

[26] Li H, Pu Y, Kumar R, Ragauskas AJ, Wyman CE: Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. Biotechnol Bioeng 2014, 111(3):485-492.

[27] Erdei B, Barta Z, Sipos B, Réczey K, Galbe M, Zacchi G: Ethanol production from mixtures of wheat straw and wheat meal. Biotechnology for Biofuels 2010, 3(1):1.

[28] Swinkels J: Composition and properties of commercial native starches. Starch-Stärke 1985, 37(1):1-5.

[29] Ioelovich M: Cellulose as a nanostructured polymer: a short review. BioResources 2008, 3(4):1403-1418.

[30] Badger P: Ethanol from cellulose: a general review. Trends in new crops and new uses 2002, 1:17-21.

[31] Scheller HV, Ulvskov P: Hemicelluloses. Annual review of plant biology 2010, 61:.

[32] Saha BC: Hemicellulose bioconversion. Journal of Industrial Microbiology and Biotechnology 2003, 30(5):279-291.

[33] Gáspár M, Kálmán G, Réczey K: Corn fiber as a raw material for hemicellulose and ethanol production. Process Biochemistry 2007, 42(7):1135-1139.

[34] Azadi P, Inderwildi OR, Farnood R, King DA: Liquid fuels, hydrogen and chemicals from lignin: A critical review. Renewable and Sustainable Energy Reviews 2013, 21:506-523.

[35] Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M: Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005, 96(6):673-686.

[36] Kumar P, Barrett DM, Delwiche MJ, Stroeve P: Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009, 48(8):3713-3729.

[37] Mood SH, Golfeshan AH, Tabatabaei M, Jouzani GS, Najafi GH, Gholami M, Ardjmand M: Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renewable and Sustainable Energy Reviews 2013, 27:77-93.

[38] Ruiz HA, Rodriguez-Jasso RM, Fernandes BD, Vicente AA, Teixeira JA: Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: a review. Renewable and Sustainable Energy Reviews 2013, 21:35-51.

[39] Pu Y, Hu F, Huang F, Davison BH, Ragauskas AJ: Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. Biotechnology for biofuels 2013, 6(1):1.

[40] Brandt A, Ray MJ, To TQ, Leak DJ, Murphy RJ, Welton T: Ionic liquid pretreatment of lignocellulosic biomass with ionic liquid–water mixtures. Green Chem 2011, 13(9):2489-2499.

[41] Shill K, Padmanabhan S, Xin Q, Prausnitz JM, Clark DS, Blanch HW: Ionic liquid pretreatment of cellulosic biomass: enzymatic hydrolysis and ionic liquid recycle. Biotechnol Bioeng 2011, 108(3):511-520.

[42] Zhang K, Pei Z, Wang D: Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: a review. Bioresour Technol 2016, 199:21-33.

[43] Brosse N, Sannigrahi P, Ragauskas A: Pretreatment of Miscanthus x giganteus using the ethanol organosolv process for ethanol production. Ind Eng Chem Res 2009, 48(18):8328-8334.

[44] Xue S, Uppugundla N, Bowman MJ, Cavalier D, Sousa LC, Dale BE, Balan V: Sugar loss and enzyme inhibition due to oligosaccharide accumulation during high solids-loading enzymatic hydrolysis. Biotechnology for biofuels 2015, 8(1):195.

Chapter 2 - High gravity enzymatic hydrolysis of hydrothermal and ultrasonic pretreated big bluestem with recycling prehydrolysate water

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2.1 Abstract

Enhancing sugar concentration and minimizing water consumption are key objectives for future cellulosic biofuel economics. To achieve those objectives, high-solids loading pretreatment and enzymatic hydrolysis (up to 20%, w/v) were studied. Big bluestem was selected and combined hydrothermal and ultrasonic treatment without chemicals addition was carried out in this study. Optimal high-solids loading pretreatment (16%, w/v) was identified in the ultrasonic reactor at 200 °C for 30 min. High-solids enzymatic hydrolysis (12%, w/v) was inefficient in the laboratory rotary shaker. However, using a horizontal reactor with good mixing is effective for high solids loading (20%, w/v), yielding 75 g/L of glucose. Minimum water to detoxify pretreated biomass while maintaining high sugar yields was 10 mL/g, which reduced by 50% as compared to the conventional washing process for steam-treated wheat straw. Recycling pretreatment liquor to treat the next batch of biomass proved to be feasible without affecting the sugar yields.

2.2 Introduction

Biofuel, a sustainable and renewable energy source, has a strong impact to relieve the burden in over-consumption of petroleum-based fuels and chemicals, consequently reducing global carbon dioxide (CO_2) emission and also dependence on foreign oil imports [1,2]. As the global population expands and the production of vehicle around the world increases, the demands to produce transportation fuels increase rapidly. Among numbers of potential alternative fuels, bioethanol is considered as the widest utilized transportation fuels [1,3]. Bioethanol is a renewable alternative fuel derived from various sustainable feedstocks such as sugar-based crops (sugarcane, sweet sorghum, sugar beet, etc.), starch-based crops (wheat, corn, cassava, grain sorghum, etc.), cellulosic biomass (agricultural residues such as corn stover or wheat straw, herbaceous biomass such as switchgrass, big bluestem, miscanthus, woody biomass such as polar, etc.).

Lignocellulosic biomass is considered as a desirable alternative to currently used petroleum-based energy sources due to its abundant availability and no competition with food [4]. Big bluestem (Andropogon gerardii), a dominant warm-season (C4) perennial native grass, consists as much as 80% of the plant biomass in the Midwestern grasslands of North America [5]. Although big bluestem is not commonly considered as a traditional cellulosic biomass such as switchgrass and corn stover, it offers many economic benefits, including more biomass yields, greater ability to grow with low input and increase ecosystem biodiversity [6,7].

The process of converting lignocellulosic biomass to ethanol mainly consists of pretreatment, hydrolysis, fermentation and distillation. Pretreatment is a critical process to break down the recalcitrant structure of cellulosic biomass in order to improve biomass enzymatic digestibility [8,9]. Hydrothermal pretreatment is an eco-friendly process as only water is used as

reaction medium without chemicals addition, which is usually processed at relatively high temperatures (140 – 220 °C) under mild acidic conditions [10, 11]. At room temperature, the corresponding pH of water is 7.0. However, as the temperature and pressure of saturated liquid water increases, pH of water decreases until reaching a minimum point of 5.6 at around 250 °C [9]. The concentration of hydronium ions increases as pH of water drops, which enhances the ability to catalyze acid reactions 25 times stronger than at room temperature [9]. During hydrothermal pretreatment, the hydronium ions released by water depolymerizes hemicellulose from plant cell wall to form acetic acids, improving enzymatic accessibility to cellulose and thus enzyme-catalyzed hydrolysis yields of fermentable glucose [10]. Ultrasonic pretreatment is a mechanical process equipped with heating source, in which biomass structure is disrupted by cavitational bubbles. During ultrasonic pretreatment, microbubbles are developed in local hotspots and then collapsed to release a huge amount of energy which may cause the melting of the crystalline structure of biomass as reactive radicals ($^{\bullet}HO$ and $^{\bullet}H$) can be formed at the moment of bubble collapse [12]. Compared with other thermochemical conversion technologies, such as acid and alkaline pretreatments, ultrasonic pretreatment is conducted without chemical agents and does not require severe conditions. Another advantage is that ultrasound-treated biomass can be converted into sugar and fermented to ethanol without additional pH adjustment and formation of inhibitors in chemical reactions during the pretreatment process.

High-solids loadings (≥15% , w/w) of pretreatment and hydrolysis processes towards lignocellulosic ethanol is superior to lower-solids loading, including concentrated fermentable sugars, potential enhanced ethanol titers and reduced capital and energy costs [13]. This process is environmentally friendly as it minimizes water usage and waste disposal. In order to meet the minimum ethanol concentration (>40 g/L) for industrial distillation process, at least 15% (w/w)

solids is required for enzymatic hydrolysis [13-16]. However, high-solid pretreatment and hydrolysis could result in poor mass transfer, increased slurry viscosity and increased inhibitors concentration [17].

High gravity enzymatic hydrolysis of high-solids pretreated lignocellulosic biomass envisions great potential in improving the process economics through enhanced fermentable sugars and ethanol yields [18]. Unfortunately, limited data are available for high loading pretreatment and enzymatic hydrolysis of lignocellulosic biomass. The objective of this research was to study the effect of high-solids loading pretreatment and enzymatic hydrolysis on sugar yields. Combined hydrothermal and ultrasonic treatment was used in this study. Furthermore, this study explored possibility of minimizing water usage and waste water disposal, including reduced water consumption for detoxification after pretreatment and recycling pretreatment liquor to treat next batch of biomass.

2.3 Materials and methods

2.3.1 Materials

Big bluestem, is a dominant warm-season (C4) perennial native grass planted mostly in the Midwestern grasslands of North America. One big bluestem ecotype (Fults population) which was harvested in Carbondale, IL in 2013 was used for this study. After grinding into < 1 mm particle size with a cutting mill (SM 2000, Retsch Inc., Newton, PA, U.S.), the sample with <7% moisture content was sealed in a plastic bag and stored at room temperature. The chemical composition of big bluestem was determined according to the National Renewable Energy Laboratory (NREL) procedure as shown in Table 1. In the NREL procedure, samples were first subject to sulfuric acid (72%) treatment for 60 min at 30 °C and then hydrolyzed by dilute acid (4%) at 121 °C for another 60 min. After acid hydrolysis, carbohydrate including cellulose and

hemicellulose was converted monosaccharide, which was measured by high-performance liquid chromatography (HPLC). Lignin consists of acid insoluble and acid soluble lignin. Acid insoluble lignin was weighed from the solid after oven heating overnight at 105 °C (the weight of acid insoluble lignin and ash) and then at 575°C for at least 6 hrs to measure the ash content. All chemicals used for this research were purchased from Sigma Chemical Co. (St. Louis, MO).

2.3.2 Hydrothermal and ultrasound pretreatment

The primary objective of pretreatment is to increase cell wall porosity and accessibility of plant cell wall surfaces to cellulolytic enzymes. Hydrothermal and ultrasonic pretreatment was carried out in a laboratory ultrasonic reactor (Ultrasonic High-Pressure Chemical Reactor, Columbia International). The stainless steel reactor has a total volume of 200 mL and is heated by an electric heater. The recommended maximum input volume is 150 mL as some space is left for slurry expansion. After weighted biomass samples were introduced into the reactor and the target temperature was set, the reactor was heated at a rate of round 4 °C min^{-1}. At the time of reaction temperature reached the target temperature, it was set as Time 0, so Time 30 min means after the reaction temperature reached the target temperature and maintained at this temperature for 30 min. Based on preliminary experimental results, reaction temperature ranged from 180°C to 200°C were able to achieve high fermentable sugar yields, thus, the reaction temperature from 180°C to 200°C was used for this study. Ultrasound treatment was applied based on determined process conditions and the ultrasonic pattern was set at 5 seconds on and then 5 seconds off. After the treatment was complete, the ultrasonic reactor was removed from the electric heater and placed into room temperature water to cool down to 50 °C within 5 min. The slurry was vacuum filtered using Whatman Paper (No. 4). Water insoluble solid was washed thoroughly with water and collected for composition analysis and enzymatic hydrolysis to evaluate the

pretreatment effect. Distilled water was used to wash the pretreated solids after pretreatment. The pretreated solids was not dried as drying will destroy the open pores generated by the pretreatment process and consequently result in lower sugar yields.

2.3.3 Enzymatic saccharification

Enzymatic saccharification of pretreated biomass to evaluate the pretreatment effect was carried out at 4% solid loadings (grams dry weight per 100 ml) in 50 mM sodium acetate buffer solution (pH 5) with addition of 0.2 g/L to prevent microbial contamination. An enzyme complex, Accellerase 1500 is an enzyme complex including cellulose and β-glucosidase [Endoglucanase activity: 2,200–2,800 CMCU/g (1 CMCU unit of activity liberates 1 lmol of reducing sugars, expressed as glucose equivalents) in 1 min under specific assay conditions of 50°C and pH 4.8]. Accellerase 1500, was generously provided by DuPont Industrial Biosciences (Rochester, NY, U.S.) and applied to this study at the recommended dosage (0.5 mL/g cellulose)[19]. Flasks were incubated at 50 °C in a rotary shaker (Model I2400, New Brunswick Scientific Inc., Edison, NJ, U.S.) with the speed of 140 rpm. Supernatants were extracted after 72-hr enzymatic hydrolysis to analyze sugar concentration by HPLC. All reactions were performed in duplicate. Glucose yield was calculated as follows:

$$\text{Glucose yield (\%)} = \frac{Released\ sugar\ amount}{Theoretical\ sugar\ amount\ in\ raw\ materials} * 100\%$$

2.3.4 Statistics

Analysis of variance and pairwise comparisons for the means using the Tukey adjustment were performed with SAS (SAS Institute, Inc., Cary, NC, USA). Means values and standard deviation from the duplicated experiments are reported.

2.4 Result and discussion

2.4.1 Biomass composition before and after treatment

Structural sugars and lignin consist of the major composition of herbaceous grasses. The chemical composition of big bluestem used in this study fell into the range of many varieties of big bluestem reported by Zhang et al. [20]. Raw big bluestem used in this study contained 36.6% cellulose, 30.6% hemicellulose and 18.2% lignin while the hemicellulose of treated biomass significantly reduced to 5.8%, consequently enhanced cellulose (58.6%) and lignin (31.2%) composition (Table 2.1). This is probably due to the acetic acids hydrolyzed from hemicellulose upon the liquid hot water treatment. Modified structure of biomass tremendously improved the enzymatic digestibility of cellulose as compared to untreated biomass. Hydrothermal pretreatment might alter the lignin position, but rarely reduced the lignin content of biomass, which was suspected to bind or deactivate the enzymes' accessibility to cellulose and as a cost of requiring adding more cellulolytic enzymes [21].

Table 2.1 Chemical composition of untreated and treated biomass used in this experiment.

Composition	Untreated big bluestem (%, db)	Treated big bluestem (%, db)[1]
Cellulose	36.6±0.51	58.6±0.65
Hemicellulose	30.6±0.56	5.8±0.58
Lignin	18.2±0.6	31.2±0.43
Ash	4.64±0.78	0.8±0.81

[1](The pretreatment condition was 200 °C and 30 min residence time). Values are means ±SD (standard deviation).

2.4.2 Effect of hydrothermal pretreatment on glucose yields

Hydrothermal pretreatment was applied to big bluestem using distilled water as medium at various temperatures without any chemicals addition. Different treatment conditions (180 °C, 200 °C, and 220 °C with residence time 0 or 30 min after reached target temperature) were carried out to test the hydrothermal effect on glucose yields of big bluestem via enzymatic hydrolysis. Both reaction temperature and residence time were found to have a significant effect on glucose yields. As shown in Fig. 2.1, hydrothermal pretreated samples at 180 °C achieved around 24 % glucose yield. As reaction temperature increased to 200 °C, the glucose yield increased to 31.8 %, further increasing reaction temperature to 220 °C resulting in enhanced glucose yield of 71.78%. Further, maintaining the reaction temperature for 30 min after reaching target temperature could benefit to additional increased glucose yields, increased from 24 to 38.7% for 180 °C, 31.8 to 71.01% for 200 °C, and 71.78 to 89.16% for 220 °C. Perez et al. [22] also showed that reaction temperature and time had a significant effect on sacchrification yields of hydrothermal pretreated wheat straw.

Combined with the enhanced kinetic energy in the slurry at high temperatures, the rate of depolymerization reactions that open the complex cell wall structure are significantly increased during hydrothermal pretreatment. Hemicellulose which is the most sensitive to relatively high temperatures among the three polymers of plant cell walls, a branched heterogeneous carbohydrate, consists mainly of xylose, arabinose, glucose and mannose that varies depending on plant resource. The acetyl group of hemicellulose is easily hydrolyzed into acetic acid upon heated, the acids dissolve into water and result in more acidic solution.

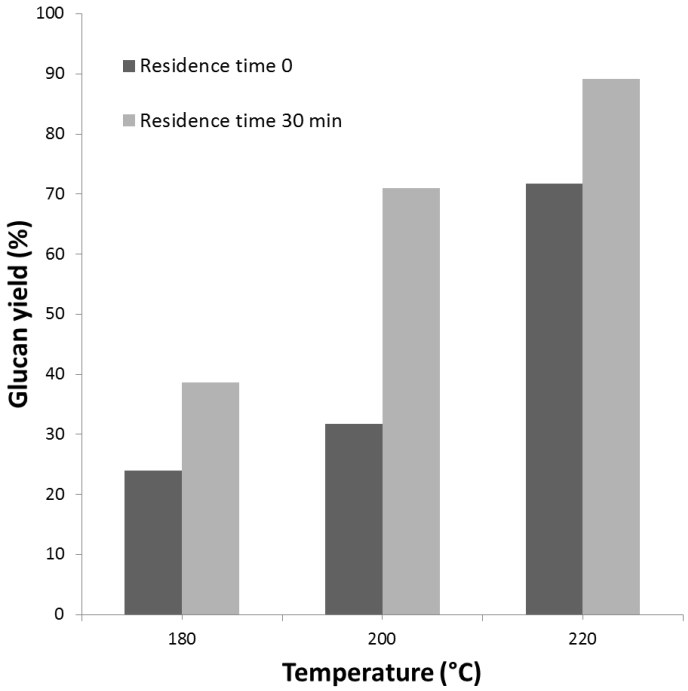

Fig. 2.1 Enzymatic hydrolyzed cellulose to glucose of hydrothermally treated big bluestem (8% solid loading). Values re the averages of the duplicated experiments and standard deviations were in the range of 0.19-0.79 of the mean.

2.4.3 Effect of combined ultrasound and hydrothermal pretreatment on glucose yields

Hydrothermal pretreatment is an effective and environmental-friendly process to improve the accessibility of enzymes to cellulose and subsequently the fermentation yield. Van Walsum et al. [23] reported that simultaneous saccharification and fermentation of hydrothermal pretreated sugarcane bagasse at 220 °C for 2 min achieved 90% ethanol yield at the enzyme loadings of 15 FPU/g. Ultrasonic pretreatment is also a clean technology to process the lignocellulosic biomass and it was combined with hydrothermal pretreatment to test their effect on glucose yields of big bluestem. As shown in Fig. 2.2, it is a clearly demonstrated that the combined thermal and ultrasonic reaction improved the glucose yields of big bluestem. However,

the combined reaction was not effective to increase glucose yield at high temperatures (i.e. 200 °C and 220 °C). It seems that hydrothermal pretreatment at higher temperatures may be strong enough to break down the recalcitrant structure to achieve high glucose yield (i.e. 89.16% for samples treated at 220 °C for 30 min). One hypothesis is that the particle size used in this study is relatively small (< 1 mm) and the microstructure of biomass is relatively easy to be disrupted so that hydrothermal pretreatment only is sufficient to gain high hydrolysis yield.

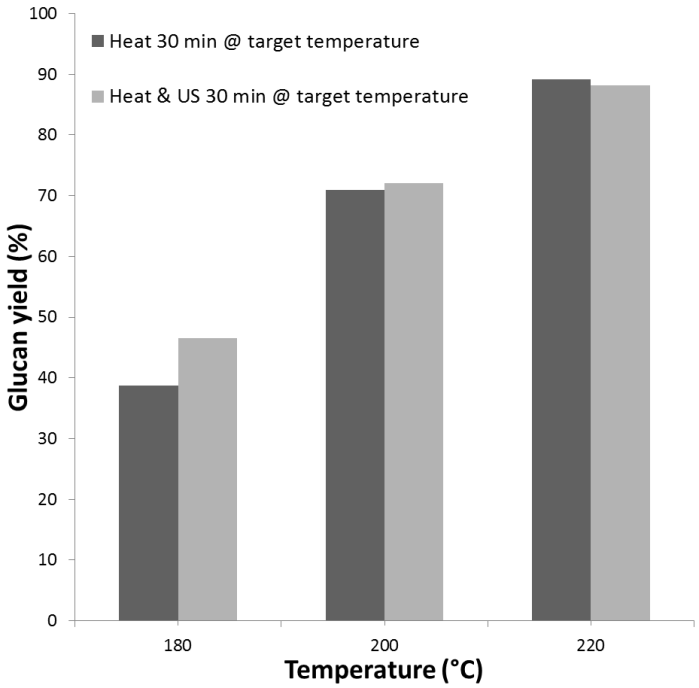

Fig. 2.2 Enzymatic hydrolyzed cellulose to glucose of combined ultrasonic and hydrothermal treated big bluestem (8% solid loading). Values re the averages of the duplicated experiments and standard deviations were in the range of 0.32-0.55 of the mean.

2.4.4 Effect of high-gravity hydrothermal pretreatment on glucose yields

Pretreatment occupies a significant portion of the total cost of the whole cellulosic bioethanol production process and water is critical to the pretreatment process by reducing the slurry viscosity and improving mass transfer and solvent diffusion [13]. Although advanced

pretreatment technology has been developed at relatively low-solids loadings (5-10% solids) and achieved high conversion yields of fermentable sugars, it is important to develop efficient and clean technologies at high solids concentration to lower the production cost. Pretreatment at high-solids loading could enhance sugars concentration and reduce water and energy consumption, which is considered as a more cost-effective process. However, high-gravity pretreatment induces some challenges such as limited mass transfer, increased slurry viscosity and accumulation of inhibitory compounds which have the potential to reduce the subsequent enzymatic hydrolysis and fermentation efficiency.

In this research, the solids loading was tested from 8 to 20% (w/v) and its enzymatic hydrolysis yield was investigated. Water washing was carried out to detoxify the pretreated biomass. As shown in Fig. 2.3, the glucose yield of pretreated biomass at 200 °C for 30 min increased as solids loading increased from 8 to 16% (w/v), however, as it further increased to 20% (w/v), the glucose yield reduced slightly as compared that of 16% solid content (84.13 % vs. 81.13%, w/v). More hemicellulose hydrolyzed during pretreatment at higher-solids loadings which resulted in lower pH could help explain the enhanced glucose yields. However, it seemed to be a threshold of optimum solids loading for the reactor used in this study as over-loading might result in poor mass transfer. Note, the pretreatment process was carried out without the aid of mixing and it may explain why the optimum solids loading was limited to 16% (w/v) as compared to other studies which reported much higher solids loading pretreatment (up to 30%, w/w) [24]. The following experiments were carried out using 16% (w/v) solids loading to pretreat biomass.

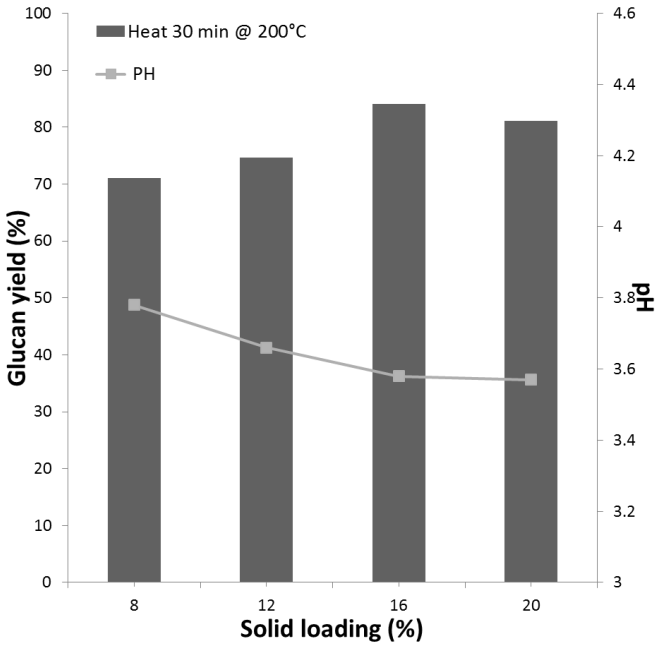

Fig. 2.3 Enzymatic hydrolyzed cellulose to glucose of hydrothermally treated big bluestem at various pretreatment solid loading (the pretreatment condition was to heat 30 min at 200 °C). Values re the averages of the duplicated experiments and standard deviations were in the range of 0.05-0.76 of the mean.

2.4.5 Effect of solids loading enzymatic hydrolysis on glucose yields

High gravity bioconversion offers benefits such as improved water usage efficiency of the process and lower industrial distillation costs [18,25]. By increasing the solid loadings, the final sugar concentration and consequently ethanol titer will be higher [14,17]. However, the mixing of biomass in laboratory rotary shaker is insufficient and generally limited to 10% solids loading [14]. Enzymatic hydrolysis at 4% (w/v) achieved 77.7% glucose yield (Fig. 2.4), but it reduced as solids loading increased. It was also confirmed by this study that glucose yield at 12% solids (w/v) was significantly less than at 4% solids (w/v) under laboratory rotary shaking.

Adequate mixing is required to obtain sufficient cellulolytic enzymes and biomass interaction to avoid areas with concentrated sugars which would inhibit the enzymes, especially

during the high-solids loading hydrolysis process [14, 26]. Advanced bioreactors are needed to ensure the sufficient mixing of substrate and enzymes, the low energy consumption and the low stress to the enzymes and yeast cells [25]. Enzymatic saccharification of higher solids loading (up to 20%, w/v) was tested using a horizontal reactor with mixing (Parr Instrument Co., Moline, IL). At the same solids loading (12%, w/v), the glucose yield from the horizontal reactor (85.6%) was significantly higher than laboratory rotary shaker (68.9%). Increasing the solids loading to 16 and 20% (w/v), the glucose yield reduced to 76.8 an 72%, respectively. However, the glucose concentration was increased from 58.4 g/L to 65.2 g/L and 74.5 g/L, respectively. The maximum glucose titer achieved in this study was 74.5 g/L at 20% (w/v) solids loading, which was slightly lower than proposed minimum glucose titer (80 g/L) for industrial distillation economics [16,17]. Further increase in solids loading has a potential to meet this requirement, but there's a trade-off between the glucose titer and glucose yield as the glucose yield gradually decreased as the solids loading increased while the glucose concentration generally increased as the solids concentration increased [14]. Instead, bovine serum albumin and other protein treatments prior to enzymatic hydrolysis were found to effectively reduce adsorption of cellulose and especially beta-glucosidase on lignin which subsequently improved the enzymatic digestibility of cellulose and potentially reduce the enzyme loadings [27]. In addition, optimized enzyme cocktails could be a feasible way to boost the glucose concentration as Accellerase 1500 supplemented with Novozyme 188 was shown to achieve the glucose concentration of above 80 g/L at solids loading of 20.3% [17].

The end-product inhibition of cellulolytic enzymes by concentrated sugars at the final stage of hydrolysis was believed to be one of the main reasons for reduced saccharification yields [18]. Thus, simultaneous saccharification and fermentation (SSF) could be performed to

overcome this problem. Another method to increase sugar yield is to adjust the feeding strategies, similar to gradual feeding pretreated biomass to surpass enzymatic saccharification limitations [15].

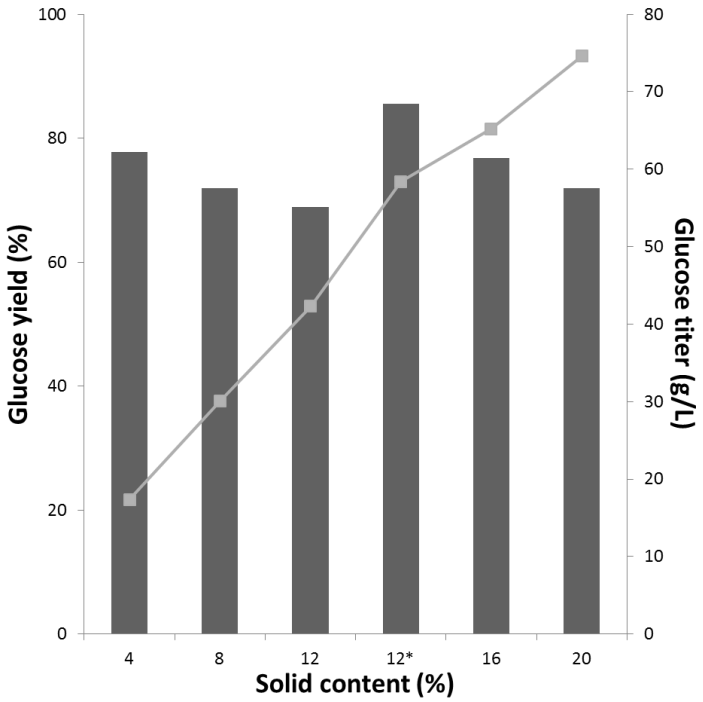

Fig. 2.4 Enzymatic hydrolysis of hydrothermally treated big bluestem at different solid loadings. (The line with dots represents the glucose titer; the pretreatment condition was to heat 30 min at 200 °C and water-washed at 10 mL/g after pretreatment; solid content of 4–12% was conducted with the laboratory rotary shaker while 12*–20% was conducted with the horizontal reactor). Values re the averages of the duplicated experiments and standard deviations were in the range of 0.05-0.82 of the mean.

2.4.6 Water consumption for detoxification after pretreatment

After pretreatment, water is usually required to wash the treated biomass to remove the inhibitors (such as acetic acid, furfural and hydroxymethylfurfural), especially after pretreatment at high-solids loadings, otherwise, they may affect the downstream processes such as enzymatic hydrolysis and subsequent fermentation [28]. The slurry containing 3.3 g/L acetic acid and 145 mg/L furfural was strong enough to inhibit the fermentation process [29].

Reducing the amount of water used for detoxification could increase water usage efficiency and reduce the waste water disposal, which is an essential way to reduce the overall production cost. However, a lot of published studies didn't provide the exact amount of water used to wash the biomass after pretreatment. Our goal is to quantify the minimum amount of water necessary to wash the hydrothermally pretreated biomass without affecting enzymatic hydrolysis and subsequent fermentation yield. For unwashed biomass (16%, w/v) pretreated at 200 °C for 30 min, glucose yield (76.87%) was significantly lower than those from washed biomass (Fig. 2.5). For washed biomass, the glucose yield increased to 78.22% and 83.2% when washing water consumed per unit biomass were 5 mL/g and 10 ml/g, respectively. Further increasing amount of washing water did not significantly enhance the glucose yield. Therefore, we recommended that based on this hydrothermal pretreatment condition, 10 mL/g (washing water per unit biomass) is sufficient to achieve high glucose yield after enzymatic hydrolysis. As compared to the claimed water per unit biomass at 20 kg/kg in a conventional washing process for steam-treated wheat straw and 40 kg/kg for dilute acid treated grass, the consumed washing water after pretreatment in this study was reduced by 50 and 75%, respectively [20,30]. Another study reported using deionized water at water-to- solid ratio of 15:1 to wash steam-explosion pretreated corn stover [17]. The following experiments were carried out using 10 mL/g to wash pretreated biomass.

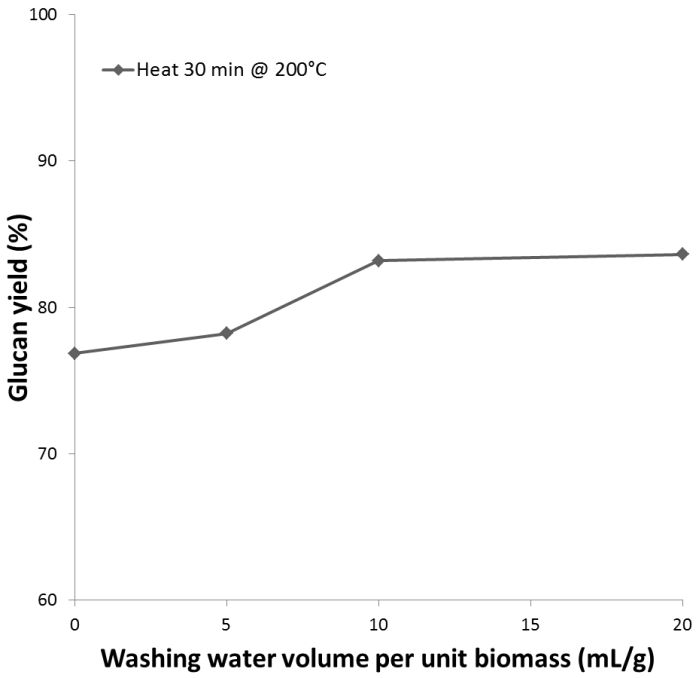

Fig. 2.5 Enzymatic hydrolyzed cellulose to glucose of water washed big bluestem after hydrothermal pretreatment (the pretreatment condition was to heat 30 min at 200 °C and16% solid loading). Values re the averages of the duplicated experiments and standard deviations were in the range of 0.17-0.71 of the mean.

2.4.7 Effect of recycling pretreatment liquor on glucose yields

Most of current biomass conversion process just focuses on the recovery of glucose from cellulose instead of total fermentable sugars, and the sugars from hemicellulose may lose during disposal of pretreatment liquors. Petersen et al. [24] showed that pretreatment liquors could be used to soak and soften the biomass before pretreatment process. In this study, we used pretreatment liquor as recycling water for biomass treatment to reduce water usage and save post-waste water treatment cost. The result showed that slight increment in glucose yield (85.76 vs. 83.2%) was obtained when using previous pretreatment liquor to treat subsequent batch of biomass (Fig. 2.6). This result indicates that there has an advantage of using use recycling water for biomass pretreatment. In addition, pretreatment liquor could be utilized for yeast propagation

to improve its adaption and fermentation performance. The sugars present in pretreatment liquor can be used as carbon source for cell growth which reduces the sugar consumption from cellulose, thereby, it may increase ethanol fermentation efficiency and ethanol yield.

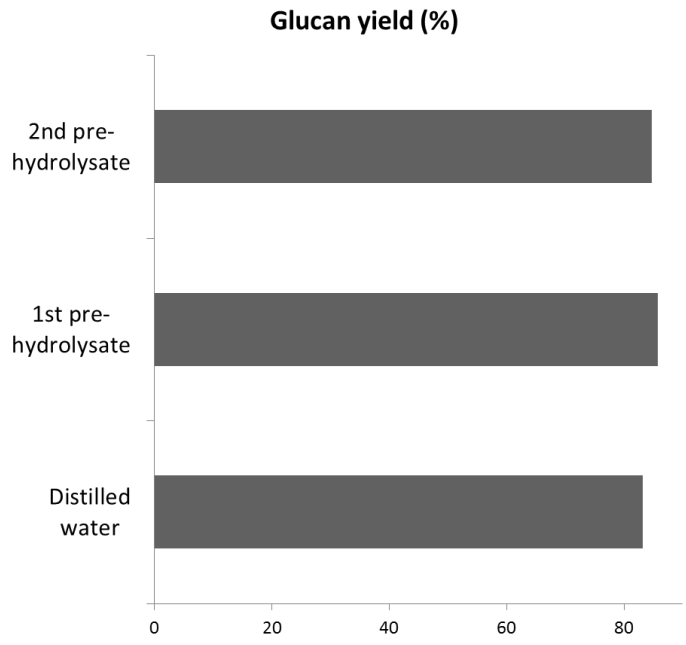

Fig. 2.6 Enzymatic hydrolyzed cellulose to glucose of hydrothermally treated big bluestem using pretreatment liquors. (The pretreatment condition was to heat 30 min at 200 °C and water washed at10 mL/g after pretreatment and 16% solid loading). Values re the averages of the duplicated experiments and standard deviations were in the range of 0.21-0.64 of the mean.

2.5 Conclusions

Producing concentrated sugars and reducing water usage are critical elements to commercialize cellulosic ethanol production. Hydrothermal pretreatment at higher temperatures was strong enough to disrupt the recalcitrant structure and increase enzymatic digestibility of cellulose while the addition of ultrasound to hydrothermal pretreatment can significantly improve sugar yields at low temperature treatment. High gravity pretreatment brings benefits such as enhanced sugars concentration and reduced water and energy consumption, meanwhile

facing the challenges of poor mass transfer, high slurry viscosity and accumulated inhibitory compounds. The mixing capability of reactor is critical to enzymatic hydrolysis. With a laboratory rotary shaker, only 69% of glucose yield was achieved at 12% solids loading (w/v), while a horizontal reactor with good mixing achieved 86% of glucose yield. Even at 20% solids loading, 72% glucose yield and around 75 g/L glucose titer were achieved with a horizontal reactor. The minimum water necessary to wash the hydrothermally pretreated biomass reduced by 50 and 75% as compared to steam-treated wheat straw and dilute acid treated grass, respectively, without sacrificing enzymatic hydrolysis and subsequent fermentation. Recycling prehydrolysate water was found to be a potential alternative approach to save water usage and reduce wastewater disposal.

2.6 References

[1] Balat M, Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review, Energy Convers. Manage. 52 (2011) 858-875.

[2] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ,Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T., The path forward for biofuels and biomaterials, Science 311 (2006) 484-489.

[3] Baeyens J, Kang Q, Appels L, Dewil R, Lv Y, Tan T., Challenges and opportunities in improving the production of bio-ethanol, Prog. Energy Combust. Sci. 47 (2015) 60-88.

[4] Kim TH, Kim TH., Overview of technical barriers and implementation of cellulosic ethanol in the US, Energy 66 (2014) 13-19.

[5] Zhang K, Johnson L, Prasad PV, Pei Z, Wang D., Big bluestem as a bioenergy crop: A review, Renew. Sustain. Energy Rev. 52 (2015) 740-756.

[6] Adler PR, Grosso SJD, Parton WJ, Life-cycle assessment of net greenhouse-gas flux for bioenergy cropping systems, Ecol. Appl. 17 (2007) 675-691.

[7] Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM., Ethanol can contribute to energy and environmental goals, Science 311 (2006) 506-508.

[8] Kumar P, Barrett DM, Delwiche MJ, Stroeve P., Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production, Ind. Eng. Chem. Res. 48 (2009) 3713-3729.

[9] Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M., Features of promising technologies for pretreatment of lignocellulosic biomass, Bioresour. Technol. 96 (2005) 673-686.

[10] Pu Y, Hu F, Huang F, Davison BH, Ragauskas AJ, Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments, Biotechnol. Biofuels 6 (2013) 1.

[11] Ruiz HA, Rodríguez-Jasso MA, Fernandes BD, Vicente AA, Teixeira JA, Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: A review. Renew. Sustain. Energy Rev. 21(2013) 35-51.

[12] Luo J, Fang Z, Smith RL., Ultrasound-enhanced conversion of biomass to biofuels, Prog. Energy Combust. Sci. 41(. 2014) 56-93.

[13] Modenbach AA, Nokes SE., The use of high-solids loadings in biomass pretreatment—a review, Biotechnol. Bioeng. 109 (2012) 1430-1442.

[14] Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C., Liquefaction of lignocellulose at high-solids concentrations, Biotechnol. Bioeng. 96 (2007) 862-870.

[15] Koppram R, Olsson L., Combined substrate, enzyme and yeast feed in simultaneous saccharification and fermentation allow bioethanol production from pretreated spruce biomass at high solids loadings, Biotechnol. Biofuels 7 (2014) 1.

[16] Xu F, Sun J, Konda NM, Shi J, Dutta T, Scown CD, Simmons BA, Singh S., Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol, Energy Environ. Sci. 9 (2016) 1042-1049.

[17] Liu Z, Qin L, Zhu J, Li B, Yuan Y., Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature, Biotechnol. Biofuels 7 (2014) 1.

[18] Koppram R, Tomás-Pejó E, Xiros C, Olsson L, Lignocellulosic ethanol production at high-gravity: challenges and perspectives, Trends Biotechnol. 32 (2014) 46-53.

[19] Xu F, Shi Y, Wu X, Theerarattananoon K, Staggenborg S, Wang D., Sulfuric acid pretreatment and enzymatic hydrolysis of photoperiod sensitive sorghum for ethanol production, Bioprocess Biosyst. Eng. 34 (2011) 485-492.

[20] Zhang K, Johnson L, Prasad PV, Pei Z, Yuan W, Wang D., Comparison of big bluestem with other native grasses: Chemical composition and biofuel yield, Energy 83 (2015) 358-365.

[21] Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C., Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment, Biotechnol. Biofuels 3 (2010) 1.

[22] Pérez JA, González A, Oliva JM, Ballesteros I, Manzanares P., Effect of process variables on liquid hot water pretreatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor, J Chem. Technol. and Biotechnol. 82 (2007) 929-938.

[23] Van Walsum GP, Allen SG, Spencer MJ, Laser MS, Antal Jr MJ, Lynd LR., Conversion of lignocellulosics pretreated with liquid hot water to ethanol, Biotechnol. Fuels Chem. Symp. Humana Press (1996) p157-170.

[24] Petersen MØ, Larsen J, Thomsen MH, Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals, Biomass Bioenerg. 33 (2009) 834-840.

[25] Zhang J, Chu D, Huang J, Yu Z, Dai G, Bao J., Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor, Biotechnol. Bioeng. 105 (2010) 718-728.

[26] Kadić A, Palmqvist B, Lidén G., Effects of agitation on particle-size distribution and enzymatic hydrolysis of pretreated spruce and giant reed, Biotechnol. Biofuels 7 (2014) 1.

[27] Yang B, Wyman CE., BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates, Biotechnol. Bioeng. 94 (2006) 611-617.

[28] Hodge DB, Karim MN, Schell DJ, McMillan JD., Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose, Bioresour. Technol. 99 (2008) 8940-8948.

[29] Lu Y, Wang Y, Xu G, Chu J, Zhuang Y, Zhang S., Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass, Appl. Biochem. Biotechnol. 160 (2010) 360-369.

[30] Vera RM, Bura R, Gustafson R., Synergistic effects of mixing hybrid poplar and wheat straw biomass for bioconversion processes, Biotechnol. Biofuels 8 (2015) 1.

Chapter 3 - Integrating starchy substrate into cellulosic ethanol production to boost ethanol titers and yields

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3.1 Abstract

Starchy grains as current major feedstocks for bioethanol production are competing with food supply; therefore, lignocellulosic biomass was pursued as an alternative feedstock for bioethanol production due to its high availability and low price. Commercial production of second-generation bioethanol from lignocellulosic biomass is still under development as significant challenges of low fermentation efficiency, low ethanol titers, high enzyme cost, and high water usage remain to be addressed. In this research, sugar-rich substrates such as starchy grains were integrated into cellulosic ethanol production, which could boost ethanol titers and ethanol yields. The substrates with various ratios of corn flour and hydrothermal treated corn stover (4:12; 8:8; 12:4; 12:12) were evaluated on the ethanol concentration and ethanol yield via simultaneous saccharification and fermentation. Ethanol concentration and ethanol yield decreased with increasing amounts of treated corn stover in the mixtures. The maximum ethanol concentration (68.7 g/L) was achieved at the corn flour and corn stover ratio of 12:12 using raw starch granular enzyme with the ethanol yield of 86.0%, whereas the maximum ethanol yield was obtained at the corn flour and corn stover ratio of 12:4 as it contained higher amounts of corn flour. All the ethanol concentrations from various mixtures of corn flour and corn stover were higher than 37.9 g/L from the control with 100% of treated corn stover (16%, w/v).

Saccharification and fermentation processes were optimized to reduce energy cost and the optimized process was able to complete ethanol fermentation within 48 h.

Keywords: Starch, Biomass, Pretreatment, Co-fermentation, Ethanol yield, Ethanol concentration

3.2 Introduction

Bioethanol used to fuel vehicles can reduce our dependence on fossil fuels and net greenhouse gas emissions [1, 2]. Biotechnologies are mature enough to produce ethanol from starch-rich grains in large scales and continue to advance development of cellulosic ethanol production. Currently, commercial production of second-generation bioethanol from lignocellulosic biomass is still not economically feasible, mainly facing challenges of high enzyme cost, high water usage, low ethanol titer and low ethanol yield [3, 4]. Enhancing ethanol titer without sacrificing ethanol yield challenges the production of cellulosic ethanol due to the complex and recalcitrant structure of lignocellulosic biomass [5].

To effectively convert lignocellulosic biomass into biofuel, pretreatment is usually required to disrupt the complicated interconnected structure of cellulose, hemicellulose and lignin [6-8]. Raw lignocellulosic biomass such as agricultural residues usually contains 25-35% cellulose, 20-30% hemicellulose and 25-35% lignin [1, 9], in which hemicellulose is more likely to depolymerize upon heat or acidic conditions, while cellulose and lignin are more resistant to thermal decomposition [7-10]. Well-developed pretreatments such as dilute acid pretreatment or hydrothermal pretreatment are often implemented to improve enzymatic digestibility of cellulose mainly through eliminating most hemicellulose intertwined with cellulose [7, 8]. Consequently, the amount of cellulose in the pretreated biomass could be increased to around 50-60% as

compared to raw materials, which would have the potential of producing concentrated sugars and higher ethanol titers [11, 12]. Liquid hot water pretreatment is also an effective method to disrupt the microstructure of biomass and considered as a green process because of less waste disposal and less required post-treatment [13, 14]. Detoxification of pretreated biomass is usually beneficial following enzymatic hydrolysis and ethanol fermentation as degraded products such as acetic acid, furfural and hydroxymethyfurfural are generated in pretreatment hydrolysates [13, 14]. Multiple products through fermentation of acetone pretreated sweet sorghum bagasse (SSB) at 180 °C for 60 min were able to obtain 78g butanol, 35g acetone, 12g ethanol, 28g acetic acid, and 6g butyric acid per kg of SSB input [15].

High gravity enzymatic hydrolysis of pretreated biomass is superior to low-solids loadings as enhanced fermentable sugars and less water consumption are preferred from the economic and environmental standpoints [16]. High ethanol concentration is necessary to reduce capital and energy costs as a minimum of 40 g/L is generally required for economical ethanol distillation [4]. Fermentation of treated cellulosic biomass alone was not able to reach this goal and one common approach to achieve higher ethanol titers is to increase the amount of biomass loadings (> 8wt% glucan loading), so-called high-gravity processing. However, it is also known that fermentable sugars yield normally decreases as solids loadings increase due to the problems like mass transfer limitations or accumulated inhibitors by degraded products that subsequently occur, which results in decreased fermentation yield. Jorgensen et al. [17] utilized a reactor system with excellent mixing capability that was able to liquefy and saccharify pretreated wheat straw up to 40% (w/w) solids loading and obtained the highest ethanol concentration of 48 g/L at the 35% (w/w) solids loading but with relatively low and unacceptable ethanol yield, less than

50%. Thus, it is challenging to effectively hydrolyze and ferment cellulosic biomass at high solids loading.

Grains such as corn, sorghum and wheat usually have starch content of more than 70%, with much more fermentable sugars than cellulosic biomass. In addition, large-scale ethanol production from starch-rich or sugar-rich substrates is well established around the world, including corn grain-based bioethanol production in the U.S. and sugarcane-based ethanol production in Brazil [18]. Cereal crops for biofuel production lead to argument of the increasing food prices and security of food supply, but biofuels derived from cellulosic biomass such as agricultural residues provide positive impacts and without competing with human food [19]. Integration of first and second generation biofuel production could reduce the capital cost and accelerate the production of cellulosic ethanol [20-22]. Integrated first and second generation ethanol production was mainly applied to sugarcane as byproduct of abundant sugarcane bagasse was available after sugar juice extraction at the plant [21, 22]. In addition, co-fermentation of cellulosic biomass and grains was utilized to generate fermentative hydrogen [20] and for efficient pentose utilization in acetone-butanol-ethanol production [23]. Co-fermentation of biomass and grains or sugar juice was able to increase fermentable sugar concentration [24], consequently enhance ethanol concentration which could lower the downstream distillation cost [4].

Corn and corn stover were selected as representative feedstocks in this study. Corn flour was treated separately with two different hydrolyzing enzymes to generate fermentable sugars with or without the step of starch gelatinization. To the best of our knowledge, this is the first paper to evaluate the raw starch granular enzyme for co-fermentation of biomass and grain mixtures. Liquid hot water was used to pretreat corn stover without any additions of chemicals.

Mixtures of pre-saccharified corn slurry and hydrothermally pretreated corn stover at various ratios were subjected to simultaneous saccharification and fermentation (SSF) at high solid loadings and their effects on ethanol titers and ethanol yields were evaluated. The objective of this study was to utilize more cellulosic biomass but less starchy substrate in the co-fermentation process. With the addition of starch, the final ethanol concentration was able to meet the minimum of 40 g/L which is generally required for economical ethanol distillation [4]. High solid loadings (up to 24%, including 12% biomass and 12% grain) were tested in this study. Further experiments were carried out to optimize the processing conditions to achieve high ethanol titers and high ethanol yields.

3.3 Materials and methods

3.3.1 Materials

Corn grain and corn stover were harvested at a local farm (Manhattan, KS). After grinding into <1 mm particle size with a cutting mill (SM 2000, Retsch Inc., Newton, PA, USA), corn stover was sealed in a plastic bag and stored at room temperature. Corn grain was ground into flour using a UDY cyclone sample mill with a 1.0 mm screen (Fort Collins, CO). The starch content of corn grain (73%, db) was analyzed following the Megazyme assay procedure with Total Starch assay kit (K-TSTA, Megazyme, Bray, Ireland) according to AACC Approved Method 76-13.01 [25]. Corn stover was subjected to warm water (50°C) extraction to generate water insoluble solids (WIS). The chemical composition of corn stover was determined according to the National Renewable Energy Laboratory (NREL) procedure as shown in Table 1 [26]. In the NREL procedure, corn stover was first subjected to warm water (50 °C) extraction to generate water insoluble solids (WIS), then samples were treated with sulfuric acid (72%) at 30 °C for 60 min and hydrolyzed by dilute acid (4%) at 121 °C for another 60 min. After acid

hydrolysis, carbohydrates including cellulose and hemicellulose were converted to monosaccharide, which was measured by high-performance liquid chromatography (HPLC). Lignin consists of acid insoluble and acid soluble lignin. Acid insoluble lignin was weighed from the solid after oven heating overnight at 105 °C (the weight of acid insoluble lignin and ash) and then at 575°C for at least 6 h to measure the ash content. All chemicals used for this research were purchased from Sigma Chemical Co. (St. Louis, MO).

3.3.2 Hydrothermal pretreatment

The primary objective of pretreatment is to open the recalcitrant structure and increase enzymatic accessibility to plant cell wall surfaces. Hydrothermal pretreatment was carried out in a Parr reactor (Parr Instrument Co., Moline, IL). The stainless steel reactor has a total volume of 1 L and is heated by an electric heater. The recommended maximum input volume is 750 mL as some space is reserved for slurry expansion. After weighted biomass samples were introduced into the reactor and the target temperature was set, the reactor was heated at a rate of approximately 6 °C min^{-1}. At the time reaction temperature reached the target temperature (200°C), it was maintained at this temperature for 30 min. After the treatment was complete, the reactor was removed from the electric heater and placed into room temperature water to cool down to 50 °C within 5 min. Then the slurry was vacuum filtered using Whatman Paper (No. 4). Pretreated biomass was washed thoroughly with water and collected for composition analysis and enzymatic hydrolysis to evaluate the pretreatment effect.

3.3.3 Enzymes

Liquefying enzyme, α-amylase (Liquozyme; Novozymes) and saccharifying enzyme, glucoamylase (Spirizyme® Fuel; Novozymes) were used to hydrolyze starch into glucose

monomers. A comparative study using granular starch hydrolyzing enzyme (STARGEN^{TM} 002; Genencor) was carried out without the need for starch gelatinization or liquefaction. Enzyme dosage was applied as recommended by the manufacturer without further optimization. Cellulolytic enzymes Accellerase 1500, was generously provided by DuPont Industrial Biosciences (Rochester, NY, USA) and applied to this study at the recommended dosage (0.5 mL/g cellulose).

3.3.4 Enzymatic saccharification

In Case 1, weighted corn flour was poured into clean 250 mL flasks and mixed with preheated (60 to 70°C) broth (containing 1.0g KH_2PO_4 and 200 µL high-temperature α-amylase, Liquozyme, 240 KNU/g, ≈ 1.26 g/mL, per liter) into each flask (20 µL of Liquozyme per flask). The flasks were transferred to a rotary water bath with shaking speed of 180 rpm and 70°C for liquefaction. The temperature of the water bath was raised to 90°C in 35 to 40 min and then kept for 90 min. The pH of the mashes was adjusted to around 4.2 with 2N HCL after liquefied mashes were cooled to room temperature (25°C). Next, 100 µL of Spirizyme (750 AUG/g, ≈ 1.15 g/mL) was added into each flask and sealed for enzymatic saccharification. Flasks were incubated at 50°C placed in a rotary shaker (Model I2400, New Brunswick Scientific Inc., Edison, NJ, USA) with the speed of 150 rpm. Supernatants were extracted by filtration after 24 and 48-hr enzymatic hydrolysis to analyze glucose concentration by HPLC. All reactions were performed in duplicate. Glucose yield was calculated as follows:

$$\text{Glucose yield (\%)} = \frac{Released\ sugar\ amount}{Theoretical\ sugar\ amount\ in\ raw\ materials} * 100\%$$

In Case 2, weighted corn flour was poured into clean 250 mL flasks and the pH of the mashes was adjusted to around 4.2 with 2N HCL after distilled water addition. Granular starch

hydrolyzing enzyme (STARGEN^{TM} 002; Genencor) was added into each flask. This enzyme is distinctly different from enzymes applied in Case 1 as it can attack the whole starch granules without the cooking step. Flasks were incubated at 50°C placed in a rotary shaker (Model I2400, New Brunswick Scientific Inc., Edison, NJ, USA) with the speed of 150 rpm. Supernatants were extracted by filtration after 6 and 24-hr enzymatic hydrolysis to analyze glucose concentration by HPLC. Glucose yields were compared to that of Case 1 from conventional liquefaction and saccharification enzyme system with a cooking step.

3.3.5 Simultaneous saccharification and fermentation

After enzymatic hydrolysis of corn flour, hydrothermally pretreated corn stovers were added to each flask according to calculated weight. Various mixtures of saccharified corn flour and pretreated corn stover (referred as Mixture A, B, and C) were used as substrates in SSF with a total water insoluble solids (WIS) content of 16% (represents 16 g solids per 100 mL liquor). The WIS ratios of corn flour and corn stover mixtures were 4:12 (Mixture A), 8:8 (Mixture B), 12:4 (Mixture C), and pure pretreated corn stover (16% WIS) was used as control. Mixture 12 and 12* represented the ratio of corn flour to corn stover was 12:12. SSF experiments were performed using Accellerase 1500, and applied at the recommended dosage (0.5 mL/g cellulose). Yeast (Red Star Ethanol Red, Lasaffre, Milwaukee, WI, USA), *S. cerevisiae,* was activated before inoculation by weighing 1.0 g of active dry yeast into 19 mL of culture broth (containing 20 g of glucose, 5.0 g of peptone, 3.0 g of yeast extracts, 1.0 g of KH_2PO_4, and 0.5 g of $MgSO_4 \bullet 7H_20$ per liter and autoclaved at 121°C for 20 min) and incubating at 38°C at 200 rpm for 30 min. The activated yeast culture had a cell concentration of around 10^9 cells/mL of broth. The simultaneous saccharification and fermentation process started when 1.0 mL of activated yeast culture and 0.3 g of yeast extract were added into each flask and sealed with an S-shaped

airlock. Fermentation was conducted at 38°C in a rotary shaker operating at 150 rpm for 72 h. The fermentation process was monitored by measuring the weight changes of each flask as CO_2 escaped during fermentation [27]. Supernatants were extracted by filtration after 24, 48 and 72 h SSF to analyze ethanol concentration by HPLC. One gram of starch or cellulose could be hydrolyzed into 1.11 g of glucose and that 1 g of glucose could stoichiometrically be converted into 0.511 g of ethanol during fermentation.

$$\text{Ethanol fermentation efficiency (\%)} = \frac{Actual\ ethanol\ released}{Theoretical\ ethanol\ release} * 100\%$$

3.3.6 Experimental design

The experimental procedure is shown in Fig. 1. Various mixtures of corn flour and corn stover were tested in this study. In Case 1, corn flour was liquefied by α-amylase and then saccharified with glucoamylase for 48 hr. Then the entire slurry was subjected to SSF with the addition of hydrothermally pretreated corn stover. In contrast, granular starch hydrolyzing enzyme was used in Case 2 without the liquefaction step and corn flour mash was saccharified for 24 hr. Then the following step of SSF was the same as Case 1 mentioned above. Case 3 was designed to optimize the processes of Case 1 without the pre-saccharification step.

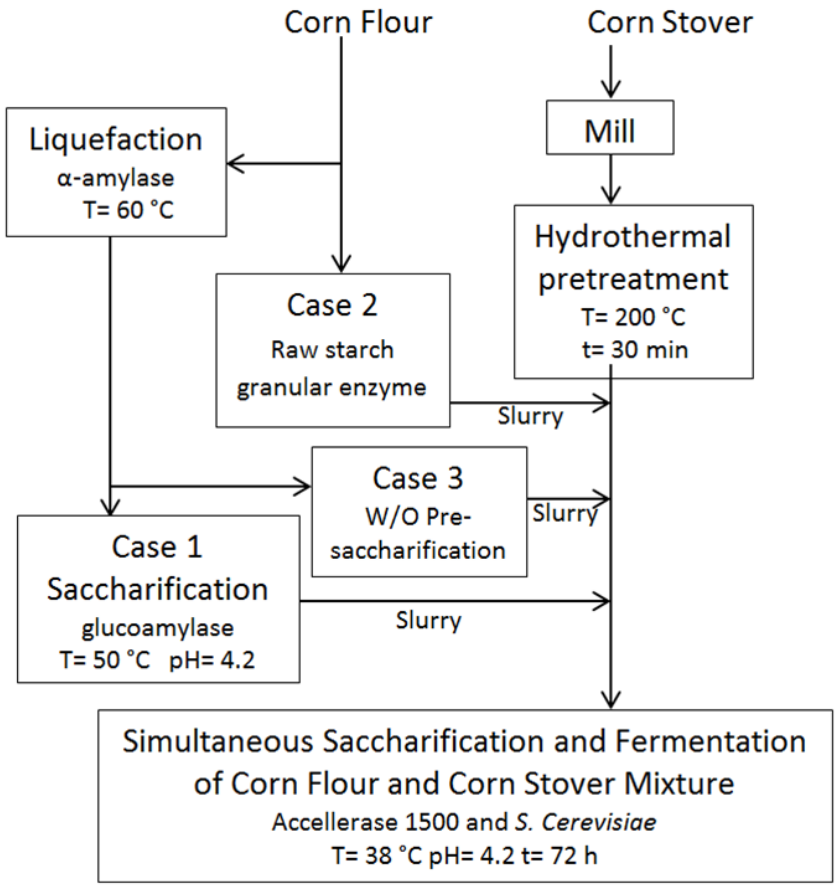

Fig. 3.1 Flow chart of experimental designs

3.3.7 Statistics

Analysis of variance and pairwise comparisons for the means using the Tukey adjustment were performed with SAS (SAS Institute, Inc., Cary, NC, USA). Means values from the duplicated experiments are reported.

3.4 Results and discussions

3.4.1 Hydrothermal pretreatment of corn stover

Agricultural residues, such as corn stover, mainly consist of cellulose, hemicellulose and lignin. The chemical composition of raw corn stover used in this study contained 33.8% cellulose, 25.7% hemicellulose and 16.8% lignin while the hemicellulose of hydrothermally

treated biomass significantly reduced to 5.4%, consequently enhanced cellulose (50.5%) and lignin (32.0%) composition (Table 3.1). This is likely due to weak acids like acetic acid hydrolyzed from hemicellulose as a result of the high temperature water treatment (200 °C for 30 min). Biomass solid recovery after treatment was 61.3% as most water soluble solids were dissolved and most hemicellulose was decomposed, meanwhile relatively high cellulose recovery (91.5%) was achieved in this study. Modified structure of biomass opened more accessible pathways to cellulolytic enzymes as compared to untreated biomass. Hydrothermal pretreatment could relocate the lignin component, but failed to decrease the lignin content of biomass, which had a negative effect on the enzymatic digestibility of cellulose as lignin could bind enzymes and consequently more cellulolytic enzymes might be needed [28-30].

Table 3.1 Chemical composition of untreated and treated biomass used in this experiment.

Composition	Cellulose (%, db[c])	Hemicellulose (%, db)	Lignin (%, db)	Mass recovery (%)	Cellulose recovery (%)
Untreated corn stover[a]	33.8±0.45	25.7±0.55	16.8±0.4		
Treated corn stover[b]	50.5±0.16	5.4±0.02	32.0±0.5	61.3±0.8	91.5±0.5

[a] Numbers do not sum to 100% since other minor components, such as ash, are not included.

[b] The pretreatment condition was 200 °C and 30 min residence time.

[c] db means dry basis.

3.4.2 Enzymatic saccharification of starch in the corn flour and corn stover mixtures

In Case 1, corn flour was cooked for starch gelatinization and followed by starch liquefaction using thermostable α-amylase. Then with the addition of glucoamylase, the liquefied slurry was cleaved to glucose and the viscosity of the slurry was significantly reduced after

enzymatic saccharification [31]. This saccharification process is widely applied in corn-based ethanol production as the rapid glucose release could ensure fast fermentation and reliable ethanol production [32]. Saccharification of starchy substrate is usually faster and typically completed within 48 h as shown in Fig. 3.2a. However, saccharification and fermentation of cellulosic biomass requires longer time due to different glycosidic linkages as shown in Fig. 3.2b. In Fig. 3.2a, Mixtures A, B, and C, containing 4 g, 8 g, and 12 g corn flour, respectively, were completely saccharified within 24 h and leveled off to 48 h. Further experiments could be carried out to minimize the saccharification time. Fig. 3.2 shows that glucose concentration increased as the amount of corn flour content in the slurry increased, ranging from 64.1 to 166.8 g/L, whereas Fig. 3.3 shows that glucose yield of 24-h saccharification decreased as corn four content increased, ranging from 98.6 to 85.7% due to higher viscosity at high solid loadings [31].

In Case 2, corn flour was liquefied and saccharified by raw starch granular enzyme without the cooking process, which could reduce unit operation costs and lower energy input per unit ethanol production. Shorter saccharification of corn flour (6 and 24 h, Fig. 3.4a) was conducted in order to save processing time and consequently improve processing efficiency. Mixture A with 4 g corn flour and 12 g biomass was completely saccharified within 6 h while longer time was needed to complete the saccharification process for Mixtures B and C with high corn flour content, as shown in Fig. 3.4a, glucose concentration continued to increase after 6-h sacchrification. Glucose concentrations after 24-h saccharification were 60.2, 94.5, and 122.1 g/L in Mixture A, B, and C, respectively, which were lower than that of Case 1. Saccharification yield followed the same trend as Case 1 that saccharification yield decreased as the amount of corn four in the slurry increased, ranging from 96.4 to 78.3% (Fig. 3.5), which was less than that of Case 1. It also indicated that saccharification process by granular starch hydrolyzing enzyme

was much slower as compared to using α-amylase and glucoamylase in Case 1. The results were consistent with another study applying granular starch hydrolyzing enzyme to hydrolyze grain sorghum and sweet sorghum juice mixture [33].

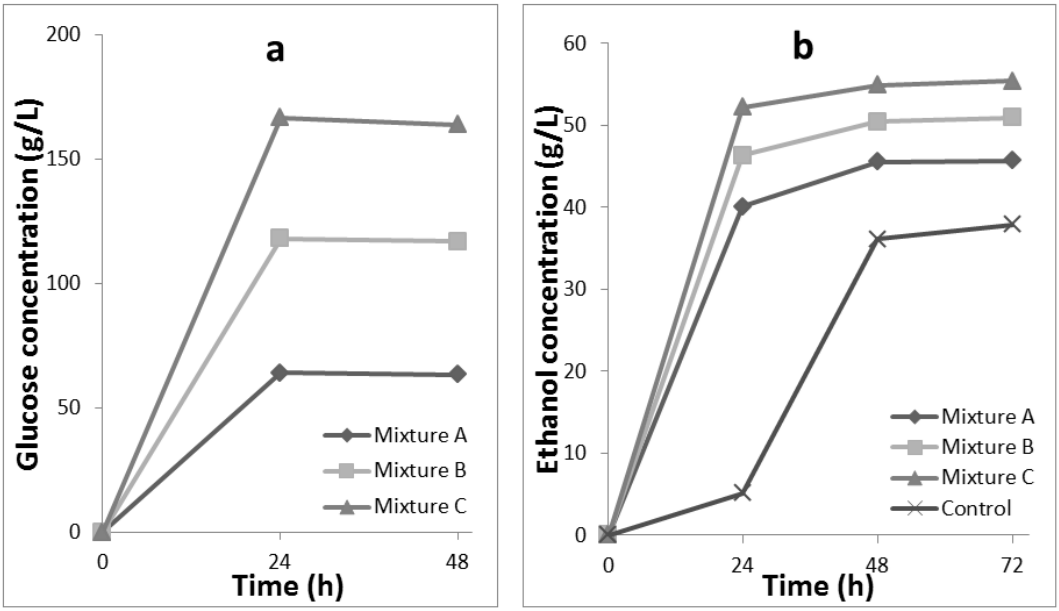

Fig. 3.2 (a) saccharification of corn flour and (b) simultaneous saccharification and fermentation of pre-saccharified corn flour and pretreated corn stover (using α-amylase + glucoamylase in Case 1). Ratios of corn flour and corn stover mixture: A (4:12); B (8:8); C (12:4); Control (0:16).

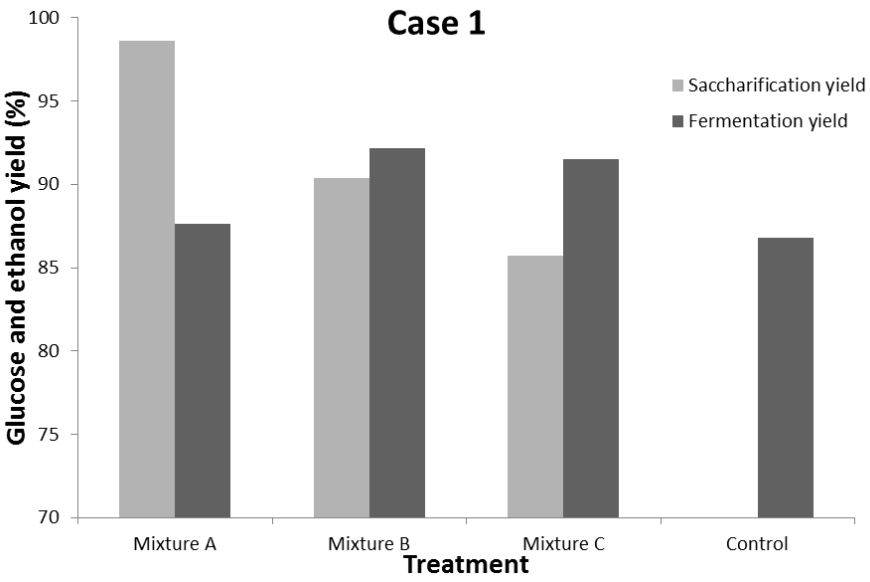

Fig. 3.3 Saccharification yield of corn flour and simultaneous saccharification and fermentation of pre-saccharified corn flour and pretreated corn stover (using α-amylase + glucoamylase in Case 1). Ratios of corn flour and corn stover mixture: A (4:12); B (8:8); C (12:4); Control (0:16).

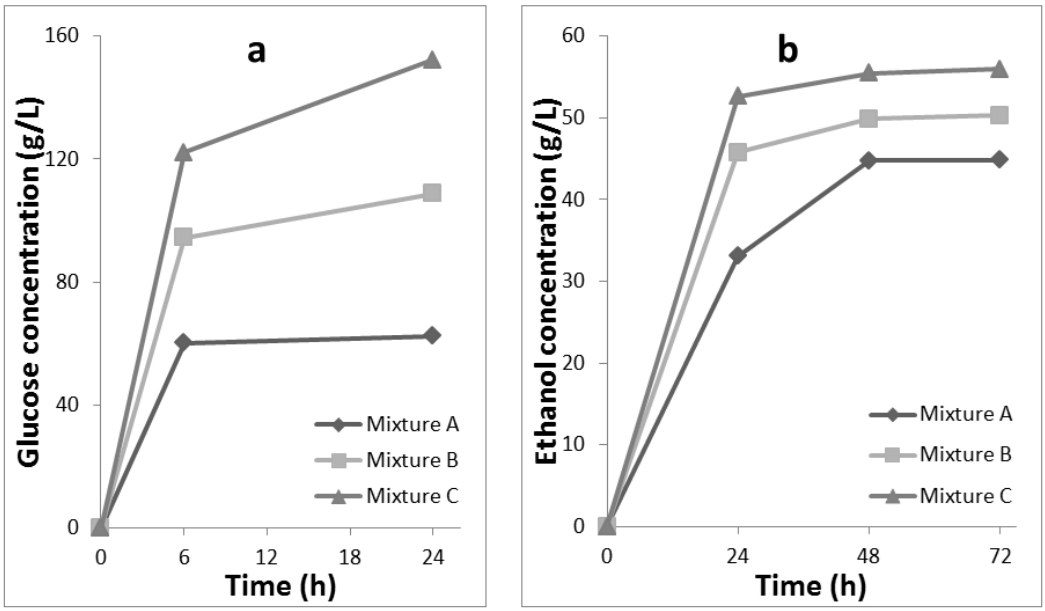

Fig. 3.4 (a) saccharification of corn flour and (b) simultaneous saccharification and fermentation of pre-saccharified corn flour and pretreated corn stover (using granular starch hydrolyzing enzyme in Case 2). Ratios of corn flour and corn stover mixture: A (4:12); B (8:8); C (12:4); Control (0:16).

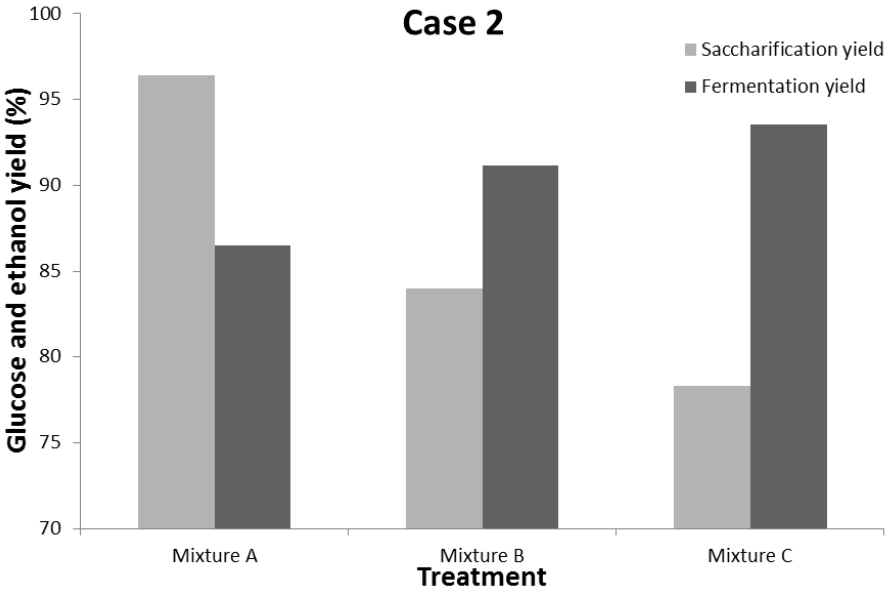

Fig. 3.5 Saccharification yield of corn flour and simultaneous saccharification and fermentation of pre-saccharified corn flour and pretreated corn stover (using granular starch hydrolyzing enzyme in Case 2). Ratios of corn flour and corn stover mixture: A (4:12); B (8:8); C (12:4); Control (0:16).

3.4.3 Simultaneous saccharification and fermentation of corn flour and corn stover mixture

For simultaneous saccharification and fermentation (SSF), hydrothermal pretreated corn stover, enzyme Accellerase 1500 and activated yeast culture were added to pre-saccharified corn flour slurry. The mixtures with different ratios of corn flour and corn stover were fermented for ethanol production. Pure hydrothermal pretreated corn stover (16%, w/v), referred as control, showed slow saccharification in first 24 h with a fermentation rate of only 0.2 g/L/h, while from 24 to 48 h, the fermentation process accelerated with the rate of 1.3 g/L/h and continued to ferment in the last 24 h (Fig. 3.2b). The final ethanol concentration after 72-h fermentation was 37.9 g/L, which is slightly lower than the economical ethanol distillation requirement of 40 g/L. However, for Mixtures A, B, and C of Case 1 (Fig. 3.2b), with the exiting glucose from enzymatic saccharification of corn flour, the fermentation rates in the first 24 h were much higher (1.7 to 2.2 g/L/h) than that of the control. The fermentation process became slower from 24 to 72 h fermentation and ethanol yield increment was not significant from 48 to 72 h. Final ethanol titers of Mixtures A, B, and C slurry were 45.7, 50.5, and 55.4 g/L, respectively, which were significantly higher than the control sample (37.9 g/L). The final ethanol concentration increased as corn flour content increased, whereas it decreased as the corn stover content increased. This occurred mainly due to higher sugar content in corn flour compared to corn stover (73% starch in corn vs. 51% cellulose in pretreated corn stover) and also likely because starch was relatively easy to hydrolyze by enzymes compared to cellulose with β-glycosidic bonds and ethanol conversion efficiency from starch alone was above 90% of theoretical yield [34].

In Case 2 as shown in Fig. 3.4b, the ethanol fermentation process followed the same trend as Case 1 with the fermentation rates of 1.4 to 2.2 g/L/h in the first 24 h and the final ethanol titers reached 44.8, 50.3, and 56.0 g/L for Mixtures A, B, and C, respectively, which were significantly higher than that of the control sample (37.9 g/L). Although the saccharification process by raw starch granular enzyme of Case 2 was slower than that of Case 1 (data not shown), the final ethanol concentrations after SSF of corn flour and corn stover mixtures in Case 1 and 2 were comparable (Table 3.2). This result demonstrated the advantages of utilizing raw granular starch enzyme which eliminated the cooking process, while achieving higher ethanol yield than that from conventional ethanol production, thus, reduced energy consumption and capital input can be obtained [35].

The SSF yield of the control experiment, pure hydrothermal pretreated corn stover (16%, w/v), was 86.8%. The maximum fermentation yield (92.2%) was obtained from Mixture B of Case1 as compared with 87.7% for Mixture A and 91.5% for Mixture C (Fig. 3.3). For Case 2, the maximum fermentation yield of 93.5% was obtained from Mixture C, which had the highest corn flour content (12%) (Fig. 3.5). To consider the optimal ratio of corn flour and cellulosic biomass and with the focus on cellulosic biomass-based ethanol production, the Mixture A (4:12) and Mixture B (8:8) were more favorable in future industrial application as more cellulosic biomass was used and the final ethanol titer was above the minimal distillation requirement (40 g/L) [4]. Particularly, the higher fermentation yield from Mixture B was worthy to further explore and can be potentially applied in commercial ethanol production, which would be discussed in the next section.

In addition, higher solid content was tested to further boost ethanol titer while maintaining high ethanol yield. The ratio of corn flour to corn stover (12:12), referred as Mixture

12 was tested using raw starch granular enzyme from Case 2. As shown in Fig. 3.6, glucose concentration soared rapidly to 140.5 g/L during the first 24-h enzymatic saccharification of corn flour slurry and then dropped to 16.5 g/L after 24-h fermentation, while ethanol concentration increased from 0 to 50.1 g/L. Significantly higher final ethanol titer (65.2 g/L) was achieved from the Mixture 12 (12: 12) as compared with the ethanol titer of 50.3 g/L in Mixture B (8: 8) (Table 3.2), whereas ethanol fermentation efficiency decreased from 91.2% to 84.8% as total solid content increased from 16% (Mixture B) to 24% (Mixture 12) [17].

Table 3.2 Simultaneous saccharification and fermentation ethanol yield (%) and final concentration (g/L) of corn flour and corn stover mixture.

	Case 1		Case 2		Case 3	
Samples	Efficiency (%)	Ethanol (g/L)	Efficiency (%)	Ethanol (g/L)	Efficiency (%)	Ethanol (g/L)
Control[1]	86.8a[2]	37.9a				
Mixture A	87.6a	45.7b	86.5a	44.8a		
Mixture B	92.2b	50.5c	91.2b	50.3b	93.8	50.7
Mixture C	91.5b	55.4d	93.5b	56.0c		
Mixture 12			84.8a	65.2d		
Mixture 12*			86.0a	68.7e		

[1]Mixtures of corn flour and corn stover were: A (4:12); B (8:8); C (12:4); Control (0:16); Mixture 12 and 12* represented the ratio of corn flour to corn stover was 12:12.
[2]Column means with the same letter are not significantly different at the 0.05 level.

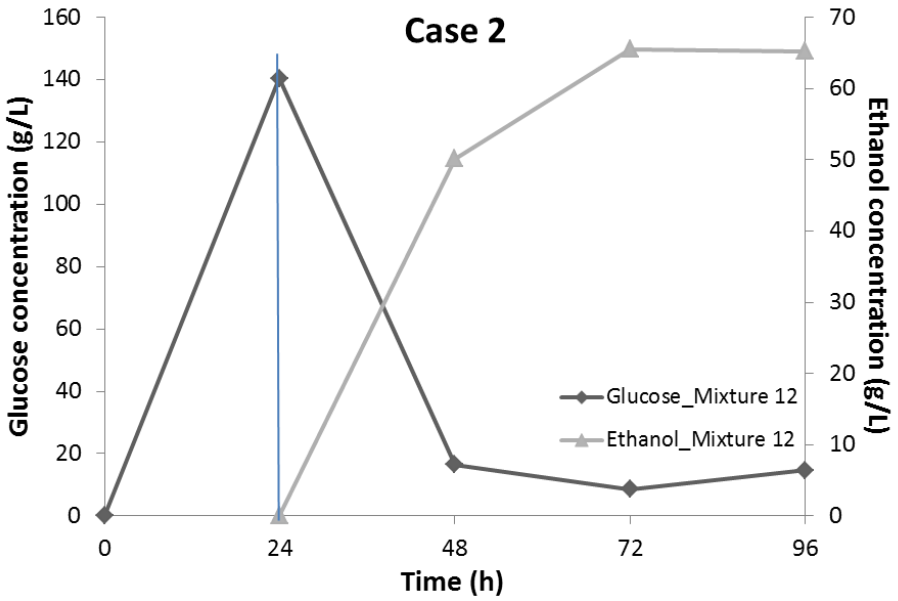

Fig. 3.6 Simultaneous saccharification and fermentation of pre-saccharified corn flour and pretreated corn stover mixture (Mixture 12 means the ratio of corn flour to corn stover was 12:12; using granular starch hydrolyzing enzyme in Case 2). Straight line indicates the addition of pretreated corn stover and activated yeast culture, which also represents the starting point of simultaneous saccharification and fermentation of the mixture.

3.4.4 Optimization of saccharification and fermentation processes

As shown in Fig. 3.1 of Case 1, corn flour was first liquefied by α-amylase and then saccharified by glucoamylase before subjecting to the fermentation process with the addition of pretreated corn stover. To optimize the process, SSF of mixtures of liquefied corn slurry and pretreated corn stover were tested without the pre-saccharification process. Results showed that the same fermentation rate of 1.9 g/L/h in the first 24 h and final ethanol titer of 50.7 after 72-h fermentation were achieved in Case 3 (Fig. 3.7), which were comparable to those in Case 1. The SSF process was complete within 48 h (Fig. 3.7). This simplified SSF process using liquefied corn slurry and pretreated corn stover had advantages of reduced processing time and energy saving [35].

Raw starch granular enzyme requires reaction temperature above 48 °C to improve hydration and increase saccharification rate than that of simultaneous saccharification and fermentation at 38 °C. For the mixture of corn flour and corn stover, the higher reaction temperature also promoted the liquefaction and saccharification of pretreated corn stover by cellulolytic enzymes. Because of this, the mixture of corn flour and pretreated corn stover was first subjected to enzymatic saccharification at 50 °C for 6 h with the addition of corresponding enzymes before lowering the temperature to 38 °C, then initiating the SSF process for 72 h. Slurry of Mixture 12* (12 g corn flour and 12 g pretreated corn stover) was tested following the procedure mentioned above and the result is shown in Fig. 3.8. After the first 6-h saccharification, significant amounts of glucose were released from the corn and corn stover mixture, reaching the concentration of 69.3 g/L, and continued to increase to 78.2 g/L at 24 h. Ethanol fermentation rate was slow during the first 24 h and a majority of ethanol was generated during the second 24 h, reaching the ethanol concentration of 67.6 g/L at 48 h, then slightly increasing to 68.7 g/L at 72 h. Ethanol fermentation efficiency (86.0%) and ethanol titer (68.7 g/l) obtained from Mixture 12* were higher than 84.8% and 65.2 g/L from Mixture 12 (Table 3.2).

Similar experiments of co-fermentation were conducted by Erdei et al. [18] using mixtures of wheat straw and wheat meal at low solid loading (5%) while our study was carried out at high solid loading (up to 24% solid loading) and achieved much higher ethanol concentration of 68.7 g/L. Co-fermentation of lignocellulosic residues from commercial furfural production and corn kernels was found to significantly increase ethanol concentration and achieved ethanol titer of 73.1 g/L with the mixtures of 7.5% furfural residues and 14.5% corn

kernels, of which corn starch significantly contributed to the enhanced ethanol concentration [24].

Integration of first and second generation ethanol production could facilitate the introduction of second-generation ethanol production from lignocellulosic biomass. A positive net value was reported in the techno-economic analysis of integrated ethanol production from grain and straw with the fermentation residues used for biogas generation [36]. Integrating the well-established sucrose-to-ethanol techniques with the enzymatic hydrolysis of lignocellulosic biomass such as the exiting sugarcane bagasse and leaves showed great potential to reduce the minimum ethanol selling price (MSEP) through improved plant energy efficiency and heat integration [37]. Co-fermentation of cellulosic biomass with starchy substrate offers an efficient and novel approach to significantly enhance low-concentrated cellulosic ethanol which will encounter in the bioprocessing of cellulosic biomass alone. Enhanced ethanol concentration could lower the downstream distillation cost, which would accelerate the commercialization of cellulosic ethanol production. In addition, co-fermentation of cellulosic biomass with cereal grains such as corn or sorghum can be easily adopted by the existing grain-based ethanol industry [38, 39].

Mass balance of Mixture 12* was conducted and shown in Fig. 9 as an example. Twelve gram, dry basis (db) corn flour and 12 g (db) treated corn stover were added into a 250 mL flask with 0.1 mL Stargen^{TM} 002 and 3.03 mL Accellerase 1500. Calculated amount of water (58.48 mL) was also added to make liquor volume of 100 mL and the whole slurry was saccharified at 50 °C in a rotary shaker with the speed of 150 rpm. Then the SSF process with the addition of 1 mL activated yeast culture and 0.3 g yeast extract was conducted at 38 °C. After 72 h fermentation, 7.23 g ethanol was obtained, equivalent to 86% theoretical ethanol yield.

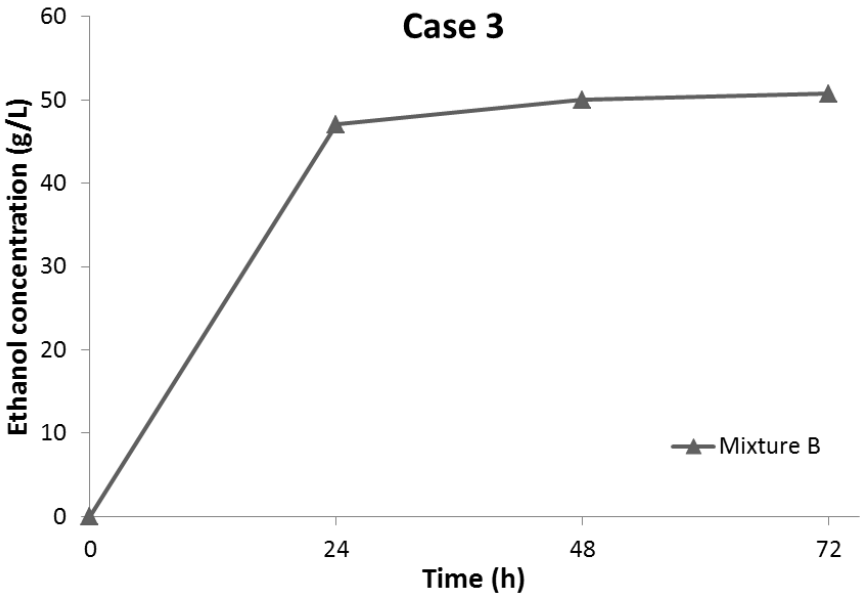

Fig. 3.7 Simultaneous saccharification and fermentation of liquefied corn flour and pretreated corn stover (using α-amylase + glucoamylase in Case 1). The ratio of corn flour to corn stover was 8:8 in Mixture B.

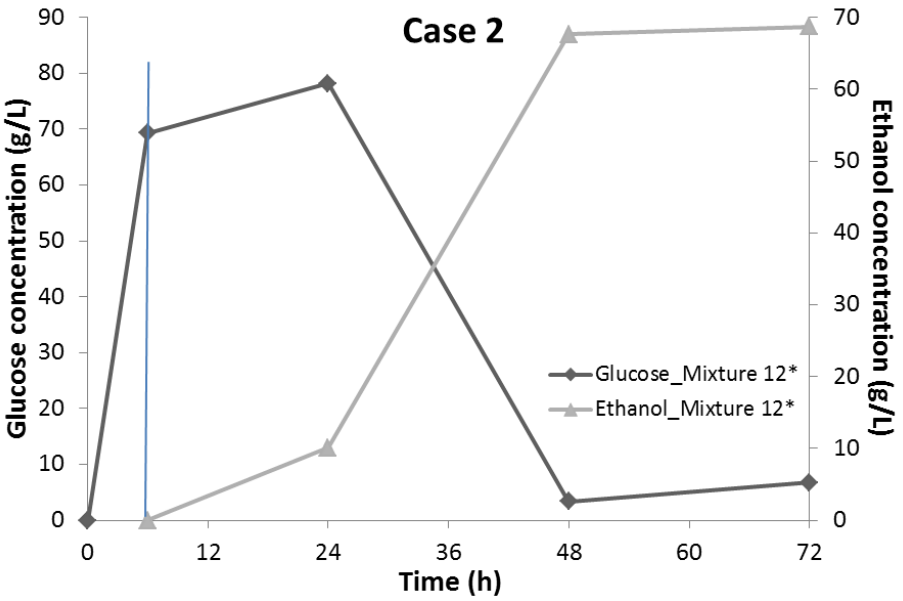

Fig. 3.8 Simultaneous saccharification and fermentation of corn flour and pretreated corn stover mixture (Mixture 12* means the ratio of corn flour to corn stover was 12:12; using granular starch hydrolyzing enzyme in Case 2). Mixture of 12 g corn flour and 12 g corn stover was added to a 250 mL flask with 100 mL liquors from Time 0 and saccharified at 50 °C for 6 h. Straight line indicates activated yeast culture, which also represents the starting point of simultaneous saccharification and fermentation of the mixture.

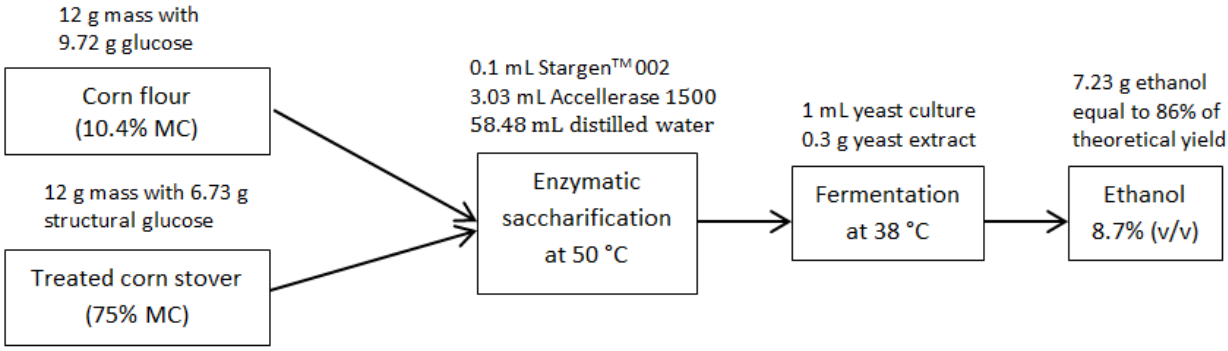

Fig. 3.9 Mass balance of ethanol production using corn flour and hydrothermal treated corn stover mixture.

3.5 Conclusions

Producing concentrated sugars and reducing water usage are key elements to accelerate cellulosic ethanol commercialization. However, fermentation of cellulosic biomass alone usually resulted in low ethanol titer and low ethanol yield as demonstrated in this study that only 37.9 g/L of final ethanol concentration was obtained even with high solids loading of 16% (w/v). In contrast, with the addition of starchy substrate into cellulosic ethanol production, final ethanol concentration was significantly improved, reaching final ethanol titers of 44.8, 50.3, and 56.0 g/L from the Mixtures A, B, and C, respectively. The ethanol yield from mixtures of corn flour and hydrothermal treated corn stover was higher than that of pure corn stover and also increased as increasing amounts of corn flour were added. Ethanol yields of co-fermentative of biomass and grain mixtures using raw granular starch enzyme without the cooking process were comparable to conventional ethanol production using α-amylase and glucoamylase, which could save capital and energy cost. The highest ethanol concentration (68.7 g/L) was obtained from Mixture 12* with the ethanol fermentation efficiency of 86.0%, although the highest ethanol fermentation efficiency was obtained from Mixture C as it contained a higher amount of corn flour. SSF of corn flour and treated corn stover mixtures were optimized to improve fermentation efficiency and to complete ethanol fermentation within 48 h. Future experiments could be conducted to test the mixture with even higher solids content by using fermenter with powerful mixing capability.

References

[1] Limayem A, Ricke SC. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog Energy and Combust Sci 2012;38:449-467.

[2] Sharma B, Birrell S, Miguez FE. Spatial modeling framework for bioethanol plant siting and biofuel production potential in the US. Appl Energy 2017;191:75-86.

[3] Menon V, Rao M. Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. Prog Energy and Combust Sci 2012;38:522-550.

[4] Xu F, Sun J, Konda NM, Shi J, Dutta T, Scown CD, Simmons BA, Singh S. Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. Energy Environ Sci 2016;9:1042-1049.

[5] Singh S, Khanna S, Moholkar VS, Goyal A. Screening and optimization of pretreatments for Parthenium hysterophorus as feedstock for alcoholic biofuels. Appl Energy 2014;129:195-206.

[6] Jin Y, Yang N, Tong Q, Jin Z, Xu X. Intensification of sodium hydroxide pretreatment of corn stalk using magnetic field in a fluidic system. Bioresour Technol 2016;220:1-7.

[7] Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005;96:673-686.

[8] Zheng Y, Lee C, Yu C, Cheng YS, Zhang R, Jenkins BM, VanderGheynst JS. Dilute acid pretreatment and fermentation of sugar beet pulp to ethanol. Appl Energy 2013;105:1-7.

[9] Ghosh S, Chowdhury R, Bhattacharya P. Sustainability of cereal straws for the fermentative production of second generation biofuels: A review of the efficiency and economics of biochemical pretreatment processes. Appl Energy 2016.

[10] Ko JK, Kim Y, Ximenes E, Ladisch MR. Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose. Biotechnol Bioeng 2015;112:252-262.

[11] Yu Q, Zhuang X, Yuan Z, Wang Q, Qi W, Wang W, Zhang Y, Xu J, Xu H. Two-step liquid hot water pretreatment of Eucalyptus grandis to enhance sugar recovery and enzymatic digestibility of cellulose. Bioresour Technol 2010;101:4895-4899.

[12] Thomsen MH, Thygesen A, Thomsen AB. Hydrothermal treatment of wheat straw at pilot plant scale using a three-step reactor system aiming at high hemicellulose recovery, high cellulose digestibility and low lignin hydrolysis. Bioresour Technol 2008;99:4221-4228.

[13] Kim Y, Kreke T, Ko JK, Ladisch MR. Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. Biotechnol Bioeng 2015;112:677-687.

[14] Li H, Pu Y, Kumar R, Ragauskas AJ, Wyman CE. Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. Biotechnol Bioeng 2014;111:485-492.

[15] Jafari Y, Amiri H, Karimi K. Acetone pretreatment for improvement of acetone, butanol, and ethanol production from sweet sorghum bagasse. Appl Energy 2016;168:216-25.

[16] Liu Z, Qin L, Zhu J, Li B, Yuan Y. Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. Biotechnol Biofuels 2014;7:1.

[17] Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C. Liquefaction of lignocellulose at high-solids concentrations. Biotechnol Bioeng 2007;96:862-870.

[18] Erdei B, Barta Z, Sipos B, Réczey K, Galbe M, Zacchi G. Ethanol production from mixtures of wheat straw and wheat meal. Biotechnol Biofuels 2010;3:1.

[19] Cobuloglu HI, Büyüktahtakın İE. Food vs. biofuel: An optimization approach to the spatio-temporal analysis of land-use competition and environmental impacts. Appl Energy 2015;140:418-34.

[20] Panagiotopoulos IA, Bakker RR, de Vrije T, Claassen PA, Koukios EG. Integration of first and second generation biofuels: fermentative hydrogen production from wheat grain and straw. Bioresour Technol. 2013;128:345-50.

[21] Dias MO, Cavalett O, Filhob RM, Bonomi A. Integrated first and second generation ethanol production from sugarcane. Chem Eng Trans. 2014;37:445-450.

[22] Dias MO, Junqueira TL, Cavalett O, Cunha MP, Jesus CD, Rossell CE, Maciel Filho R, Bonomi A. Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash. Bioresour Technol. 2012;103:152-61.

[23] Yang M, Kuittinen S, Zhang J, Vepsäläinen J, Keinänen M, Pappinen A. Co-fermentation of hemicellulose and starch from barley straw and grain for efficient pentoses utilization in acetone–butanol–ethanol production. Bioresour Technol. 2015;179:128-35.

[24] Tang Y, Zhao D, Cristhian C, Jiang J. Simultaneous saccharification and cofermentation of lignocellulosic residues from commercial furfural production and corn kernels using different nutrient media. Biotechnol Biofuels. 2011;4:1.

[25] AACC International. Approved Methods of Analysis, 11th Ed. Methods 02-52.01, 44-15.02A, and 76-13.01. 2010. Available online only. AACCI: St. Paul, MN.

[26] Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. Determination of structural carbohydrates and lignin in biomass. NREL/TP-510-42618. Golden, Colo.: National Renewable Energy Laboratory. 2008.

[27] Wu X, Maier A, Wu T, Li N, Pedersen JR, Wang D. Evaluation of Nebraska Waxy Sorghum Hybrids for Ethanol Production. Cereal Chem 2013;90:198-203.

[28] Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C. Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. Biotechnol Biofuels 2010;3:1.

[29] Nguyen TY, Cai CM, Kumar R, Wyman CE. Co-solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass. ChemSusChem 2015;8(10):1716-1725.

[30] Jin W, Chen L, Hu M, Sun D, Li A, Li Y, Hu Z, Zhou S, Tu Y, Xia T, Wang Y. Tween-80 is effective for enhancing steam-exploded biomass enzymatic saccharification and ethanol production by specifically lessening cellulase absorption with lignin in common reed. Appl Energy 2016;175:82-90.

[31] Wu X, Zhao R, Bean SR, Seib PA, McLaren JS, Madl RL, Tuinstra M, Lenz MC, Wang D. Factors impacting ethanol production from grain sorghum in the dry-grind process. Cereal Chem 2007;84(2):130-136.

[32] Wu X, Zhao R, Wang D, Bean SR, Seib PA, Tuinstra MR, Campbell M, O'brien A. Effects of amylose, corn protein, and corn fiber contents on production of ethanol from starch-rich media. Cereal Chem. 2006;83(5):569-575.

[33] Appiah-Nkansah NB, Saul K, Rooney WL, Wang D. Adding sweet sorghum juice into current dry-grind ethanol process for improving ethanol yields and water efficiency. Int J Agric Biol Eng. 2015;8(2):97.

[34] Ai Y, Medic J, Jiang H, Wang D, Jane JL. Starch characterization and ethanol production of sorghum. J Agric Food chem. 2011;59(13):7385-7392.

[35] Wang D, Bean S, McLaren J, Seib P, Madl R, Tuinstra M, Shi Y, Lenz M, Wu X, Zhao R. Grain sorghum is a viable feedstock for ethanol production. J Ind Microbiol Biotechnol. 2008;35(5):313-320.

[36] Joelsson E, Erdei B, Galbe M, Wallberg O. Techno-economic evaluation of integrated first- and second-generation ethanol production from grain and straw. Biotechnol Biofuels 2016;9(1):1.

[37] Macrelli S, Mogensen J, Zacchi G. Techno-economic evaluation of 2nd generation bioethanol production from sugar cane bagasse and leaves integrated with the sugar-based ethanol process. Biotechnol Biofuels 2012;5(1):1.

[38] Rohowsky B, Häßler T, Gladis A, Remmele E, Schieder D, Faulstich M. Feasibility of simultaneous saccharification and juice co-fermentation on hydrothermal pretreated sweet sorghum bagasse for ethanol production. Appl Energy 2013;102:211-9.

[39] Dias MO, Junqueira TL, Cavalett O, Pavanello LG, Cunha MP, Jesus CD, Maciel Filho R, Bonomi A. Biorefineries for the production of first and second generation ethanol and electricity from sugarcane. Appl energy 2013;109:72-8.

Chapter 4 - Integrated bioethanol production to boost low-concentrated cellulosic ethanol without sacrificing ethanol yield

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4.1 Abstract

Four integrated designs were proposed to boost cellulosic ethanol titer and yield. Results indicated co-fermentation of corn flour with hydrolysate liquor from saccharified corn stover was the best integration scheme and able to boost ethanol titers from 19.9 to 123.2 g/L with biomass loading of 8% and from 36.8 to 130.2 g/L with biomass loadings of 16%, respectively, while meeting the minimal ethanol distillation requirement of 40 g/L and achieving high ethanol yields of above 90%. These results indicated integration of first and second generation ethanol production could significantly accelerate the commercialization of cellulosic biofuel production. Co-fermentation of starchy substrate with hydrolysate liquor from saccharified biomass is able to significantly enhance ethanol concentration to reduce energy cost for distillation without sacrificing ethanol yields. This novel method could be extended to any pretreatment of biomass from low to high pH pretreatment as demonstrated in this study.

Keywords: Starch; lignocellulosic biomass; pretreatment; co-fermentation; high ethanol concentration

4.2 Introduction

Limited crude oil reserves and environmental concerns to mitigate greenhouse gas emissions have driven global research to explore alternatives to fossil fuels and renewable energy [1]. Bioethanol is one of the solutions and has been used to fuel vehicles, which could reduce our reliance on fossil fuels, meanwhile reducing the net greenhouse gas emissions [2]. First generation ethanol production from starchy grain and sugar-rich crops has been commercialized at large scales while second generation cellulosic ethanol production has not yet been fully commercialized [3].

Lignocellulosic plant is the most abundant and renewable biomass with substantial worldwide production, including agricultural residues such as corn stover and wheat straw, forestry wastes such as wood chips, dedicated energy crops such as switchgrass, and organic municipal solid waste, which makes it an indispensable feedstock for the production of commercialized biofuels and renewable chemicals [4, 5].

The majority of current global ethanol production is derived from starch-based crops (e.g. corn, wheat, and sorghum) or sucrose-rich materials (e.g. sugarcane, sugar beet) as they can be efficiently hydrolyzed to fermentable sugars, and directly used for fermentation [3]. However, cellulosic ethanol production is not economically viable, and it is limited by biomass recalcitrance due to the complex intertwined structure of cellulose, hemicellulose and lignin which inhibits enzyme accessibility [6, 7]. A key challenge for cellulosic ethanol commercialization is the low ethanol titer and low fermentation efficiency [8, 9]. Achieving high cellulosic ethanol titers and ethanol yields is still under development.

Through the biological conversion pathway, pretreatment, enzymatic hydrolysis and fermentation are the three major steps for ethanol production from lignocellulosic biomass [7,

10]. To effectively convert lignocellulosic biomass into biofuels, pretreatment is usually required to break the lignin seal, disrupt the crystalline structure of cellulose and to increase surface area of the cellulose, rendering the polysaccharides more susceptible to enzyme hydrolysis [11, 12]. Raw lignocellulosic biomass like agricultural residues usually contains 25-35% cellulose, 20-30% hemicellulose and 25-35% lignin [2], in which hemicellulose is relatively easy to decompose upon subjection to heat or acidic conditions, while cellulose and lignin are more resistant to thermal decomposition [12-14]. Various pretreatment methods, including dilute acid pretreatment, hydrothermal pretreatment and alkaline pretreatment, were studied to improve the enzymatic saccharification efficiency of lignocellulosic biomass [12]. Dilute acid and hydrothermal pretreatments are well developed and applied to improve enzymatic digestibility of cellulose by eliminating most hemicellulose linked with cellulose [12]. Consequently, the cellulose content of treated biomass could be greatly increased to approximately 50-60% and be highly exposed to enzymes [14]. Hydrothermal pretreatment is an effective method to disrupt the microstructure of biomass and is considered as an environmentally-friendly process because it requires less waste disposal and less post-treatment as compared to acid and alkaline pretreatments [15, 16]. Detoxification of treated biomass is essential to the subsequent enzymatic hydrolysis and fermentation processes as degraded products such as acetic acid, furfural and hydroxymethyfurfural, are generated in pretreatment hydrolysates, which could deactivate the enzyme and even kill microorganism [17-20].

High gravity bioconversion (> 15% biomass loading) is superior to low-solid loadings as enhanced fermentable sugars and less water usage are preferred from the economic and environmental standpoints [21, 22]. High ethanol titer has the advantage of reducing capital and energy costs as a minimum ethanol concentration of 40 g/L is generally required for economical

ethanol distillation [9, 23]. One common way to obtain higher ethanol titers is to increase the amount of biomass loadings (> 8% glucan loading), so-called high-gravity processing [22, 24, 25]. However, fermentation efficiency usually decreases as biomass loading increases due to limited mass transfer or accumulated inhibitors of degraded products [26]. Jorgensen et al. (2007) used a reactor system with sufficient mixing capability that was able to saccharify high solid loadings of treated wheat straw (up to 40% , w/w) and achieved the highest ethanol concentration of 48 g/L at the 35% (w/w) solid loadings but with relatively less-promising ethanol yield of less than 50%. Their results proved that fermentation of cellulosic biomass at high solid loadings was a great challenge. Xue et al. also reported similar results of reduced biofuel yields due to accumulated recalcitrant oligosaccharides at high-solids loading [20].

The integration of first and second generation biofuel production originated from the sugarcane industry as byproduct of excess sugarcane bagasse is available after sugar juice extraction at the processing facility [27, 28]. In a similar way, integration of cellulosic ethanol production with existing starch-based ethanol facilities could reduce the capital cost and accelerate its commercial production if some key challenges could be solved. Grains such as corn, sorghum and wheat usually have a high starch content (>70%), and have much more fermentable sugars than cellulosic biomass [29, 30]. In addition, large-scale ethanol productions from starch-rich grains are well-established in the USA [8, 31, 32]. Limited research has been conducted in the co-fermentation of cellulosic biomass and grains to produce bioethanol. Yang et al. (2015) studied co-fermentation of hemicellulose and grain to efficiently utilize pentose in acetone-butanol-ethanol production. Co-fermentation of cellulosic biomass and grains was also utilized to generate fermentative hydrogen [31].

Here, we propose integrated designs (integration of first and second generation ethanol production) to boost low cellulosic ethanol concentration without sacrificing ethanol yields. Two completely separate ethanol production processes could be optimized individually and combined together via liquid transfer. The integration process would result in accumulated sugars and consequently enhanced ethanol titers, thus, the downstream distillation cost would be reduced. To the best of our knowledge, this is the first paper to study the co-fermentation of starchy grains with hydrolysate liquor separated from saccharified biomass instead of the whole saccharified biomass slurry, which could help to reduce solid loadings during fermentation, and increase mass and heat transfer and enzyme activities. Corn and corn stover were selected as representative feedstocks in this study. Various pretreatment methods, including hydrothermal, acid, and alkaline, were applied to improve the enzymatic digestibility of biomass. Co-fermentation of corn flour and treated corn stover mixture was conducted according to the proposed integrated designs (Fig. 4.1) and the final ethanol concentration and ethanol yield were evaluated. The performances of two distinct enzymes for starch hydrolysis (amylase and glucoamylase, and raw starch granular enzyme) were also compared in the co-fermentation process.

4.3 Materials and methods

4.3.1 Materials

Corn grain and corn stover were harvested at the Kansas State University Research farm (Manhattan, KS). After grinding into <1 mm particle size with a cutting mill (SM 2000, Retsch Inc., Newton, PA, USA), corn stover was sealed in a plastic bag and stored at room temperature. Corn grain was ground into corn flour using a UDY cyclone sample mill with a 1.0 mm screen (Fort Collins, CO). The starch content of corn grain (73%, db) was analyzed following the Megazyme assay procedure with Total Starch assay kit (K-TSTA, Megazyme,

Bray, Ireland) according to AACC Approved Method 76-13.01. The chemical composition of corn stover was determined according to the National Renewable Energy Laboratory (NREL) procedures as shown in Table 4.1 [33]. In the NREL procedure, biomass was treated with sulfuric acid (72%) at 30 °C for 60 min and hydrolyzed by dilute acid (4%) at 121 °C for another 60 min. After acid hydrolysis, carbohydrates including cellulose and hemicellulose were converted into monosaccharide, which was measured by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) equipped with an RCM monosaccharide column (300×7.8 mm) (Phenomenex, Torrance, CA) and a refractive index detector (RID10A, Shimadzu, Kyoto, Japan). The mobile phase was 0.6 mL · min^{-1} of double-distilled water, and the oven temperature was 80℃. Lignin consists of acid insoluble and acid soluble lignin. Acid soluble lignin was measured using a UV-Visible spectrophotometer. Acid insoluble lignin was weighed from the solid after oven heating overnight at 105 °C (the weight of acid insoluble lignin and ash) and then at 575°C for at least 6 h to measure the ash content. All chemicals used for this research were purchased from the Sigma-Aldrich Co. (St. Louis, MO).

4.3.2 Hydrothermal pretreatment

The primary objective of pretreatment is to open the recalcitrant structure and increase enzymatic accessibility to plant cell wall surfaces. Hydrothermal pretreatment was carried out in a Parr reactor (Parr Instrument Co., Moline, IL). The stainless steel reactor vessel has a total volume of 1 L and is heated by an electric heater. The recommended maximum input volume is 750 mL as some space is reserved for slurry expansion. After biomass samples were introduced into the reactor and the target temperature was set, the reactor was heated at a rate of approximately 6 °C min^{-1}. At the time when reaction temperature reached the target temperature

(200°C), the reactor was maintained at this temperature for 30 min. After the treatment was complete, the reactor was removed from the electric heater and placed into room temperature water to cool down to 50 °C within 5 min. The slurry was then vacuum filtered using Whatman Paper (No. 4). Pretreated biomass was washed thoroughly with water and collected for composition analysis and enzymatic hydrolysis to evaluate the pretreatment effect. All pretreatment experiments were conducted at solid loadings of 16%.

4.3.3 Acid and alkaline pretreatment

Acid and alkaline pretreatments were carried out using the same Parr reactor. In acid pretreatment of biomass, sulfuric acid (1%, w/v) was used as the reaction medium and reaction temperature was maintained at 140 °C for a period of 60 min. In alkaline pretreatment of biomass, sodium hydroxide (1%, w/v) was used as the reaction medium and reaction temperature was maintained at 120 °C for a period of 60 min. After the treatment was complete, the reactor was removed from the electric heater and placed into room temperature water to cool down to 50 °C within 5 min. Then the slurry was vacuum filtered using Whatman Paper (No. 4). Pretreated biomass was washed thoroughly with water and collected for composition analysis and enzymatic hydrolysis to evaluate the pretreatment effect. All pretreatment experiments were conducted at 16% solid loadings.

4.3.4 Enzymatic saccharification

4.3.4.1 Enzymatic preparation

Liquefying enzyme, α-amylase (Liquozyme) and saccharifying enzyme, glucoamylase (Spirizyme® Fuel) were generously provided by Novozymes (Franklinton, NC, USA) for hydrolyzing starch into glucose monomers. Comparative study using raw starch granular enzyme

(STARGEN™ 002; DuPont Industrial Biosciences, NC, USA) was carried out without the requirement of starch gelatinization or liquefaction. Enzyme dosage described in the following section was applied as recommended by the manufacturer without further optimization. Cellulolytic enzymes Accellerase 1500, was generously provided by DuPont Industrial Biosciences (Rochester, NY, USA) and applied to this study at the recommended dosage (0.5 mL/g cellulose).

4.3.4.2 Enzymatic hydrolysis

In enzymatic hydrolysis of corn flour, weighted corn flour was poured into clean 250 mL flasks and mixed with preheated (60 to 70°C) broth (containing 1.0g KH_2PO_4 and 200 µL high-temperature α-amylase, Liquozyme, 240 KNU/g, ≈ 1.26 g/mL, per liter) into each flask (20 µL of Liquozyme per flask). The flasks were transferred to a rotary water bath with shaking speed of 180 rpm and 70°C for liquefaction. The temperature of the water bath was raised to 90°C in 35 to 40 min and then kept for 90 min. The pH of the mashes was adjusted to around 4.2 with 2N HCL after liquefied mashes were cooled to room temperature (25°C). Next, 100 µL of Spirizyme (750 AUG/g, ≈ 1.15 g/mL) was added into each flask and sealed for enzymatic saccharification.

Another enzyme treatment using raw starch granular enzyme was conducted to hydrolyze starch as compared to the enzymes mentioned above. The procedures were modified as the cooking process could be eliminated. Weighted corn flour was poured into clean 250 mL flasks and the pH of the mashes was adjusted to around 4.2 with 2M HCL. Raw starch granular enzyme (100 µL) was added into each flask. This enzyme is distinctly different from those enzymes applied above as it can attack the whole starch granules without the cooking step. Flasks were sealed and incubated at 50°C placed in a rotary shaker (Model I2400, New Brunswick Scientific

Inc., Edison, NJ, USA) with the speed of 150 rpm for 6 h. Next, 1.0 mL of activated yeast culture and 0.3 g of yeast extract were added into each flask and sealed with an S-shaped airlock to initiate the simultaneous saccharification and fermentation process conducted at 30°C in a rotary shaker operating at 150 rpm for 72 h. The fermentation process was monitored by measuring the weight changes of each flask as CO_2 escaped during fermentation [34]. The following analytical process was the same as mentioned above. All reactions were performed in duplicate.

In enzymatic hydrolysis of treated corn stover, water insoluble solids (8 and 16%, w/v) was added to flasks to perform enzymatic hydrolysis using Accellerase 1500, and applied at the recommended dosage (0.5 mL/g cellulose). Flasks were incubated at 50°C placed in a rotary shaker (Model I2400, New Brunswick Scientific Inc., Edison, NJ, USA) at the speed of 150 rpm. Supernatants were extracted by filtration after 72-h enzymatic hydrolysis and stored for further experiments at 4°C. Glucose concentration was measured by HPLC. All reactions were performed in duplicate. Glucose yield was calculated as follows:

$$\text{Glucose yield (\%)} = \frac{Released\ sugar\ amount}{Theoretical\ sugar\ amount\ in\ raw\ materials} * 100\%$$

4.3.5 Simultaneous saccharification and fermentation

Activated yeast culture (1.0mL) and yeast extract (0.3 g) were added into each flask with enzymes, which was sealed with an S-shaped airlock to initiate the SSF process conducted at 38°C in a rotary shaker operating at 150 rpm for 72 h. Yeast (Red Star Ethanol Red, Lasaffre, Milwaukee, WI, USA), *S. cerevisiae*, was activated before inoculation by weighing 1.0 g of active dry yeast into 19 mL of culture broth (containing 20 g of glucose, 5.0 g of peptone, 3.0 g of yeast extracts, 1.0 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_20$ per liter and autoclaved at 121°C for 20 min) and incubating at 38°C at 200 rpm for 30 min. The activated yeast culture had a cell concentration of approximately 10^9 cells/mL of broth. Supernatants were extracted by filtration

after 72-h fermentation and stored for further experiments at 4°C. Ethanol concentration was measured by HPLC. All reactions were performed in duplicate. Ethanol yield was calculated as follows:

Ethanol fermentation efficiency (%) $= \frac{Actual\ ethanol\ released}{Theoretical\ ethanol\ release} * 100\%$

4.3.6 Experimental design

Four integrated designs of first and second generation ethanol production are shown in Fig. 1. In design A and B, corn flour (30%) was subjected to EH and SSF, respectively, then the liquid portion was separated and mixed with pretreated corn stover to perform co-fermentation process. Alternatively, in design C and D, pretreated corn stover was subjected to EH and SSF, respectively, and then the liquid part was separated and mixed with corn flour to perform co-fermentation process.

4.3.7 Statistics

Analysis of variance and pairwise comparisons for the means using the Tukey adjustment were performed with SAS (SAS Institute, Inc., Cary, NC, USA). Means values from the duplicated experiments are reported.

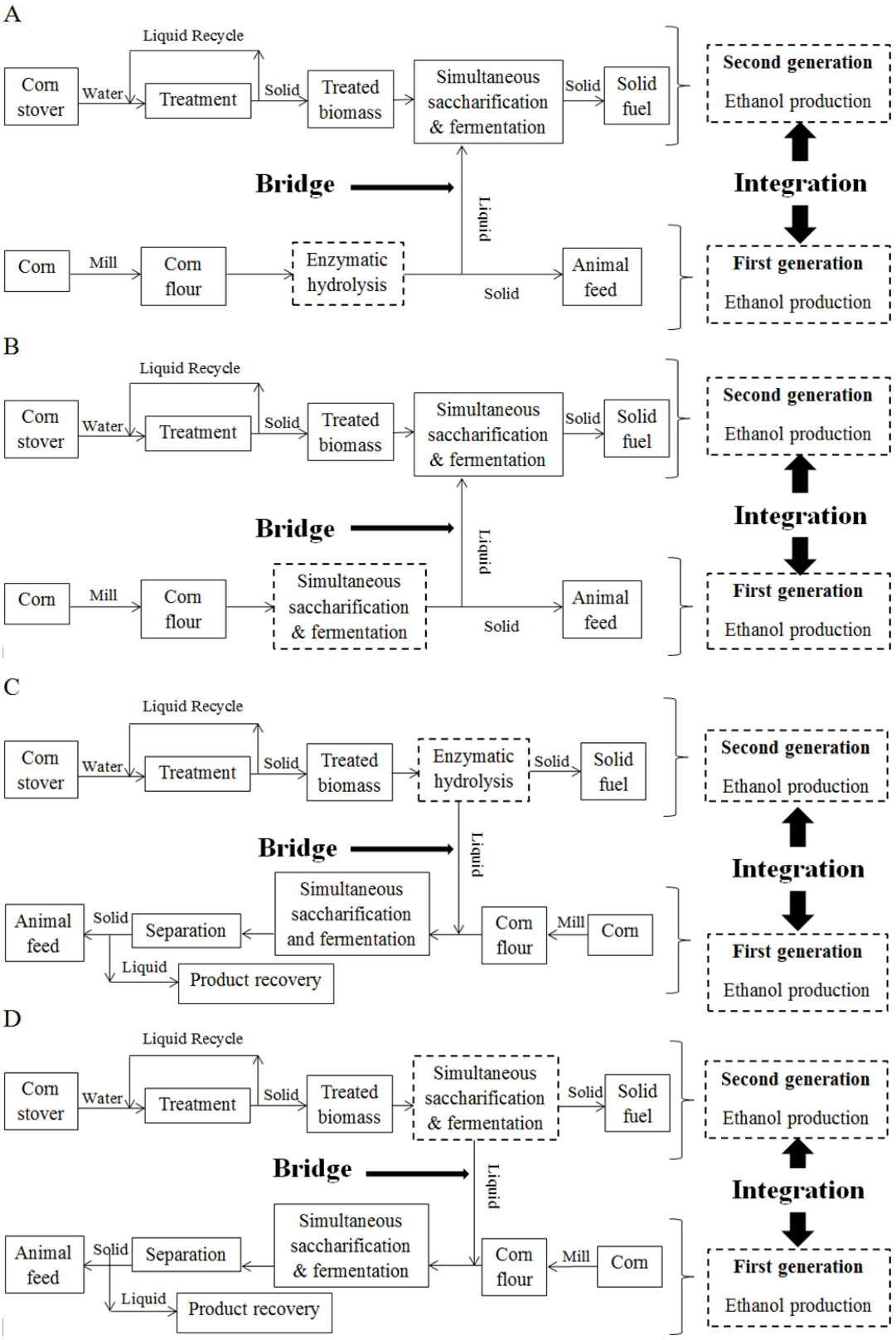

Fig. 4.1 Flow charts of integrated design.

4.4 Results and discussions

4.4.1 Composition change of corn stover

Corn stover as a major agricultural residue consists mainly of cellulose, hemicellulose and lignin. The chemical composition of raw corn stover used in this study contained 33.8% cellulose, 25.7% hemicellulose and 16.8% lignin while the hemicellulose of hydrothermally treated biomass significantly reduced to 5.4%, consequently enhanced cellulose (50.5%) and lignin (32.0%) composition (Table 4.1). This is due to weak acids like acetic acid hydrolyzed from the acetyl group of hemicellulose upon hot water treatment (200 °C for 30 min). Similar effects of treated biomass were observed on acid treated corn stover with reduced hemicellulose content of 6.8% and enhanced cellulose content of 43.4%. In contrast, the lignin content of alkaline treated corn stover was significantly reduced from 16.8 to 9.9% and as a result, the cellulose content was increased to 40.9%.

Table 4.1 Chemical composition of untreated and treated corn stover.

Composition	Cellulose (%, db)	Hemicellulose (%, db)	Lignin (%, db)
Raw corn stover[1]	33.8±0.5a[5]	25.7±0.6a	16.8±0.4a
Hydrothermal-treated corn stover[2]	50.5±0.2b	5.4±0.02b	32.0±0.5b
Acid-treated corn stover[3]	43.4±0.1c	6.8±0.6b	24.3±0.2c
Alkaline-treated corn stover[4]	40.9±0.7c	20.4±0.8c	9.9±0.2d

[1] Numbers do not sum to 100% since other minor components, such as ash, are not included.
[2] Pretreatment condition was 200 °C for 30 min with 16% solid loadings.
[3] Pretreatment condition was 140 °C for 60 min with 1% (w/v) NaOH and 16% solid loadings.
[4] Pretreatment condition was 120 °C for 60 min with 1% (w/v) H_2SO_4 and 16% solid loadings.
[5]Column means with the same letter are not significantly different at the 0.05 level.

4.4.2 Biomass saccharification and fermentation efficiency: low vs. high solid loadings

High gravity bioprocessing is superior to low solid loadings as it offers benefits such as improved water efficiency of the process and reduced ethanol distillation cost [21, 24]. It is a common way to increase the solid loadings so that the fermentable sugar concentration and consequently ethanol titer will be higher. However, sufficient mixing is required to obtain adequate enzymes and cellulose interaction to avoid areas with concentrated sugars which would inhibit the activity of enzymes, especially during the high-solids loading hydrolysis process [21]. There's a trade-off between the glucose concentration and glucose yield. In general, glucose concentration increases as the solids loading increases, but glucose yield decreases as the solid loading increases [25]. Glucose concentration of hydrothermal treated corn stover increased from 42.0 to 77.1 g/L as the solid loadings increased from 8 to16% (w/v). However, glucose yield reduced from 92.7 to 85.1% as the solid loadings increased (Table 4.2). Subsequently, ethanol titer and ethanol yield followed the same trend. Higher ethanol titer (36.8 g/L) was achieved at the higher solid loadings of 16% (w/v), but ethanol yield was reduced to 79.6% as compared to 86.0% at the solid loadings of 8% (w/v). Thus, increasing biomass loading alone is not an economically feasible way to enhance ethanol concentration as fermentation efficiency decreased.

Even at high solid loading of 16% (w/v), the ethanol concentration obtained was only 36.8 g/L, which was still lower than the minimum ethanol concentration of 40 g/L required for economical distillation process [8, 23]. Low ethanol titer is a key challenge that limits cellulosic ethanol commercialization [9]. Here, we propose integrated designs as a novel solution to enhance cellulosic ethanol concentration without sacrificing ethanol yields (Fig. 4.1).

Table 4.2 Hydrothermal-treated corn stover sacchrification and fermentation yields at low and high solid loadings.

Biomass loading (%)	Glucose titer (g/L)	Glucose yield (%)	Ethanol titer (g/L)	Ethanol yield (%)
8	42.0±0.1a[1]	92.7±0.3a	19.9±0.3a	86.0±1.2a
16	77.1±0.9b	85.1±0.9b	36.8±0.6b	79.6±1.2b

[1]Column means with the same letter are not significantly different at the 0.05 level.

4.4.3 Integrated designs of first and second generation ethanol production

Four integrated schemes of first and second generation ethanol production were proposed in order to boost low-concentrated cellulosic ethanol because 40 g/L is the minimal ethanol concentration required for economical distillation. Ethanol concentration of only 19.9 g/L was obtained at the biomass loading of 8% (w/v), however, with the integrated processes; ethanol concentration was significantly improved and met the distillation requirement as shown in Table 4.3. The high ethanol concentration of integrated designs was mainly contributed from highly fermentable starch in corn flour. In both design A and B, significant amounts of residual glucose were observed after co-fermentation due to the inhibitory effect of high sugar content in saccharified corn flour or high ethanol concentration of fermented corn flour. This phenomenon was also reported in the co-fermentaion of wheat straw and wheat meal mixtures [35]. High moisture content of pretreated corn stover (around 60 to 70%) would lower the sugar concentration when it mixed with hydrolysate liquor separated from saccharified corn flour. This was another main cause of lower ethanol concentration in design A and B as compared to design C and D (Table 4.3). Among four integrated designs, design C achieved the highest ethanol concentration of 123.2 g/L. Therefore, this design was further investigated and discussed in the following session with higher biomass loading (16%, w/v), various pretreated biomass, and two distinct starch hydrolyzing enzymes.

Table 4.3 Co-fermentation of corn flour (30%, w/v) and hydrothermal treated corn stover (8%, w/v) mixtures after 72 hr.

Integrated design	Ethanol concentration (g/L)	Glucose residues (g/L)
A	82.54±2.5a[1]	18.37±1.8
B	66.58±3.8b	39.72±2.6
C	123.2±1.8c	N/O
D	120.2±2.3c	N/O

[1]Column means with the same letter are not significantly different at the 0.05 level.

4.4.4 Co-fermentation of corn flour with juice extracted from saccharification of pretreated corn stover

Hydrothermal treated corn stover was first subjected to enzymatic saccharification at two water insoluble solid loadings (8 and 16%, w/v) according to design C. After saccharification, hydrolysate liquor was separated by filtration and mixed with corn flour to perform SSF process. Fermentation with 30% corn loading was selected to represent industrially-relevant processing conditions [36]. Two enzyme treatments (starch enzymes including α-amylase and glucoamylase vs. granular starch enzyme) for starch hydrolysis were also compared in this high-gravity fermentation.

After enzymatic saccharification of treated corn stover, glucose concentration of 77.1 g/L was obtained at 16% solid loadings and 42.0 g/L at 8% solid loadings (Table 4.2). Hydrolysate liquor (77.1 g/L and 42.0 g/L) was mixed with corn flour (30% corn flour) to perform high-gravity SSF and the fermentation performance is shown in Fig. 4.2. In the first 24-h fermentation, the ethanol level was much higher at corn flour mixed juice extracted from low biomass loading (8%). The fermentation process accelerated from 24 to 48 h and continued throughout the last 24 h. The performance of granular starch enzyme in high-gravity ethanol fermentation was not as good as using α-amylase and glucoamylase (Fig. 4.2 and Table 4.4). This was probably because granular starch enzyme for starch hydrolysis was not effective at high

solid loadings (30%). The maximal ethanol yield (130.2 g/L) was achieved at corn flour mixed with corn stover hydrolysate liquor separated from high solid loading (16%) using α-amylase and glucoamylase. As shown in Table 4, it can be noticed that there are some residual glucose from a corn flour mixed with juice extracted from high solid loading (16%) after 72-h fermentation. This was probably due to the relative high ethanol concentration (16.5% v/v). With this method, low cellulosic ethanol can be significantly enhanced and able to meet the minimum ethanol concentration of 40 g/L required for economical distillation process [9].

Erdei et al. had conducted similar co-fermentation studies of steam-pretreated but unwashed wheat straw with saccharified wheat meal and obtained the highest ethanol concentration of about 60 g/L, which was much lower than the results in this study (130.2 g/L) [35]. As we know, cellulose is a hygroscopic material which can absorb 8 -14% moisture under normal atmospheric conditions [37]. After hydrothermal treatment, lignocellulosic biomass may contain as high as 60% moisture content even after separation using centrifuge [38]. Adding the wet treated biomass to saccharified wheat meal as demonstrated by Erdei et al. would dilute the sugar concentration of saccharified wheat meal and consequently reduce the ethanol concentration after co-fermentation. In contrast, co-fermentation of starchy substrate with hydrolysate liquor separated from saccharification of treated biomass is able to greatly enhance ethanol concentration.

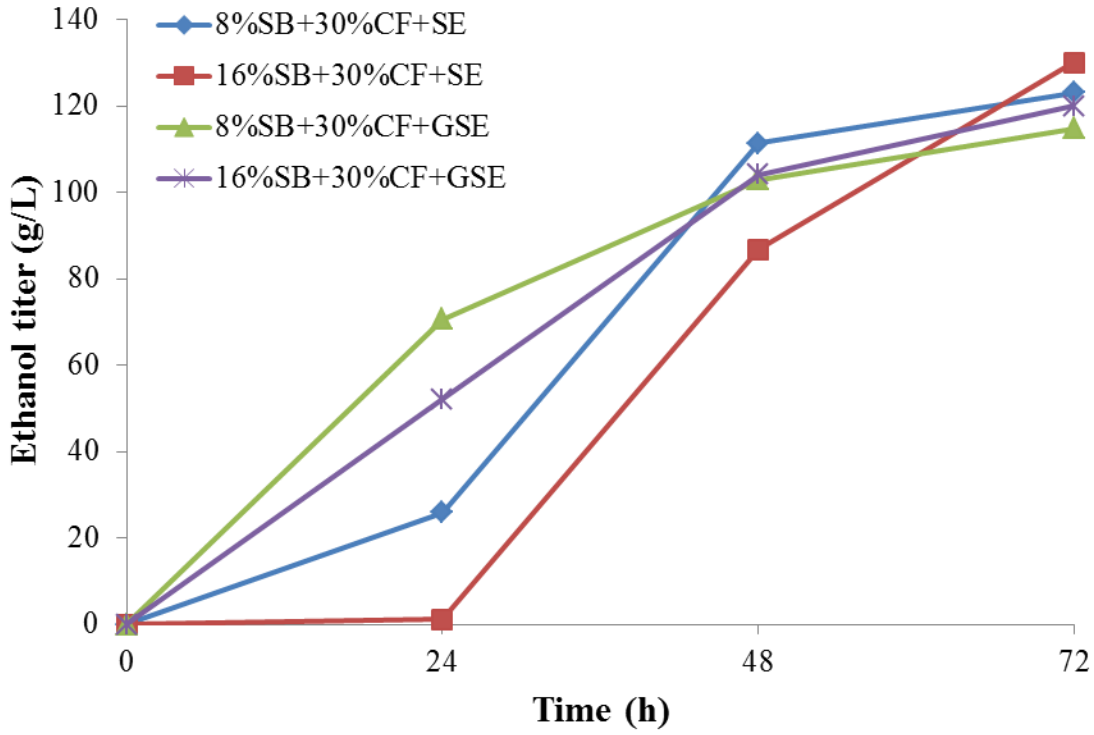

Fig. 4.2 Simultaneous saccharification and fermentation of corn flour (30%) and juice extracted from saccharification of hydrothermal treated corn stover. SB is saccharified biomass; CF is corn flour; SE is starch enzyme (amylase and glucoamylase), and GSE is granular starch enzyme.

The integration of first and second generation biofuel production is critical to accelerate the commercialization of cellulosic biofuel production and improve the overall process economics [23, 39]. Liquid extraction from saccharification of pretreated biomass functions as a bridge to connect the first and second generation bioethanol production and this unique method was proved in this study to significantly increase cellulosic ethanol concentration without sacrificing ethanol yield as compared to traditional method of increasing biomass loadings, consequently reducing downstream distillation cost. In addition, two separate ethanol production processes could be optimized independently and combined via the bridge mentioned above to boost ethanol concentration. In this study, hydrolysate liquor separated from saccharified biomass were co-fermented with 30% (w/v) corn flour, which is normal solid loading for current

dry-grind ethanol fermentation. Another advantage of integrated process was that lower amount of corn flour could be applied to co-Fermentaion process since the final ethanol concentration was high enough to meet the distillation requirement and indeed the lower amount of corn flour could minimize certain problems of high viscosity and enhance mass or heat transfer.

Co-fermentation of corn flour with hydrolysate liquor separated from saccharification of treated corn stover to boost ethanol titers was also tested on various pretreated biomass from low to high pH pretreatment to compare with hydrothermal treatment. After saccharification of acid and alkaline treated corn stover at 8% biomass loading, hydrolysate liquor was separated by filtration and mixed with corn flour (30% solid loadings) to perform SSF process. After enzymatic saccharification of treated corn stover at 8% solid loadings, glucose concentrations of 38.2 and 35.9 g/L were obtained from acid and alkaline treated corn stover, respectively, which is below the minimum ethanol concentration of 40 g/L required for economical distillation process if fermented to ethanol [9]. However, with the method of co-fermentation of corn flour (30% solid loadings) mixed with hydrolysate liquor, ethanol titers were significantly improved and reached the levels of 114.5 and 111.6 g/L for acid and alkaline treated corn stover, respectively (Table 4.5). The ethanol concentration from acid and alkaline treated biomass are lower than that from hydrothermal treated biomass (123.2 g/L) but it is still significant higher than 40 g/L required for industrial distillation. In addition, this result indicates that the co-fermentation technology also could be applied to other pretreated biomass.

Table 4.4 Fermentation yields of corn flour (30%) with extracted juice.

Substrates/Enzymes	Ethanol concentration (g/L)	Fermentation efficiency (%)	Glucose residues (g/L)
30% CF +SE	106.3±3.0a[1]	90.2±0.5a	N/O
8% SB +30% CF +SE	123.2±3.1b	96.5±0.7b	N/O
16% SB +30% CF+SE	130.2±2.9c	90.3±0.4a	11.5±0.3
30% CF +GSE	95.6±2.7a	81.1±1.2a	15.3±0.6
8% SB +30% CF +GSE	114.9±1.4b	93.4±0.4b	0.8±0.1
16% SB +30% CF +GSE	120.0±2.8c	83.9±1.3c	7.7±0.2

[1]Column means with the same letter are not significantly different at the 0.05 level.

SB is saccharified biomass; CF is corn flour; SE is starch enzyme (amylase and glucoamylase); GSE is granular starch enzyme; and N/O means below the detection limit.

Table 4.5 Glucose yield after saccharification of treated corn stover and ethanol yield after co-fermentation of corn flour (30%) mixed with extracted juice.

Sample	Glucose titer (g/L)	Ethanol titer (g/L)
Hydrothermal-treated corn stover	42.0±0.1a[1]	123.2±3.1a
Acid-treated corn stover	38.2±0.5b	114.5±0.7b
Alkaline-treated corn stover	35.9±0.2c	111.6±0.4b

[1]Column means with the same letter are not significantly different at the 0.05 level.

Enzymatic saccharification of treated corn stover was conducted at 8% (w/v) solid loadings

4.5 Conclusions

Co-fermentation of corn flour with hydrolysate liquor from saccharification of hydrothermal-treated corn stover boosted ethanol titer from 19.9 to 123.2 g/L and from 36.8 to 130.2 g/L when solid loading increased from 8 to 16%, respectively, which could significantly reduce the energy cost for ethanol distillation. In addition, the co-fermentation of corn flour with hydrolysate liquor from saccharification of low biomass loading (8%) reached ethanol

concentration of 123.2 g/L. This method could substitute the traditional method of increasing biomass loading to enhance ethanol concentration and be used as a novel solution to boost low-concentrated cellulosic ethanol without sacrificing ethanol yields.

References

[1] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ,Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T: The path forward for biofuels and biomaterials. Science 2006, 311(5760):484-489.

[2] Limayem A, Ricke SC: Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science 2012, 38(4):449-467.

[3] Nigam PS, Singh A: Production of liquid biofuels from renewable resources. Progress in energy and combustion science 2011, 37(1):52-68.

[4] Gupta A, Verma JP: Sustainable bio-ethanol production from agro-residues: a review. Renewable and Sustainable Energy Reviews 2015, 41:550-567.

[5] Sun J, Konda NM, Shi J, Parthasarathi R, Dutta T, Xu F, Scown CD, Simmons BA, Singh S: CO 2 enabled process integration for the production of cellulosic ethanol using bionic liquids. Energy & Environmental Science 2016, 9(9):2822-2834.

[6] Nguyen TY, Cai CM, Osman O, Kumar R, Wyman CE: CELF pretreatment of corn stover boosts ethanol titers and yields from high solids SSF with low enzyme loadings. Green Chem 2016, 18(6):1581-1589.

[7] Zhang K, Pei Z, Wang D: Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: a review. Bioresour Technol 2016, 199:21-33.

[8] Erdei B, Barta Z, Sipos B, Réczey K, Galbe M, Zacchi G: Ethanol production from mixtures of wheat straw and wheat meal. Biotechnology for Biofuels 2010, 3(1):16.

[9] Xu F, Sun J, Konda NM, Shi J, Dutta T, Scown CD, Simmons BA, Singh S: Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. Energy & Environmental Science 2016, 9(3):1042-1049.

[10] Zhang Z, Harrison MD, Rackemann DW, Doherty WO, O'Hara IM: Organosolv pretreatment of plant biomass for enhanced enzymatic saccharification. Green Chem 2016, 18(2):360-381.

[11] Jin Y, Yang N, Tong Q, Jin Z, Xu X: Intensification of sodium hydroxide pretreatment of corn stalk using magnetic field in a fluidic system. Bioresour Technol 2016, 220:1-7.

[12] Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M: Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005, 96(6):673-686.

[13] Ko JK, Kim Y, Ximenes E, Ladisch MR: Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose. Biotechnol Bioeng 2015, 112(2):252-262.

[14] Yu Q, Zhuang X, Yuan Z, Wang Q, Qi W, Wang W, Zhang Y, Xu J, Xu H: Two-step liquid hot water pretreatment of Eucalyptus grandis to enhance sugar recovery and enzymatic digestibility of cellulose. Bioresour Technol 2010, 101(13):4895-4899.

[15] Li H, Pu Y, Kumar R, Ragauskas AJ, Wyman CE: Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. Biotechnol Bioeng 2014, 111(3):485-492.

[16] Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C: Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. Biotechnology for biofuels 2010, 3(1):27.

[17] Jönsson LJ, Alriksson B, Nilvebrant N: Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnology for biofuels 2013, 6(1):16.

[18] Kont R, Kurašin M, Teugjas H, Väljamäe P: Strong cellulase inhibitors from the hydrothermal pretreatment of wheat straw. Biotechnology for biofuels 2013, 6(1):135.

[19] Wang Z, Zhu M, Li M, Wang J, Wei Q, Sun R: Comprehensive evaluation of the liquid fraction during the hydrothermal treatment of rapeseed straw. Biotechnology for biofuels 2016, 9(1):142.

[20] Xue S, Uppugundla N, Bowman MJ, Cavalier D, Sousa LC, Dale BE, Balan V: Sugar loss and enzyme inhibition due to oligosaccharide accumulation during high solids-loading enzymatic hydrolysis. Biotechnology for biofuels 2015, 8(1):195.

[21] Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C: Liquefaction of lignocellulose at high-solids concentrations. Biotechnol Bioeng 2007, 96(5):862-870.

[22] Koppram R, Olsson L: Combined substrate, enzyme and yeast feed in simultaneous saccharification and fermentation allow bioethanol production from pretreated spruce biomass at high solids loadings. Biotechnology for biofuels 2014, 7(1):54.

[23] Xu Y, Wang D: Integrating starchy substrate into cellulosic ethanol production to boost ethanol titers and yields. Appl Energy 2017, 195:196-203.

[24] Koppram R, Tomás-Pejó E, Xiros C, Olsson L: Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol 2014, 32(1):46-53.

[25] Liu Z, Qin L, Zhu J, Li B, Yuan Y: Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. Biotechnology for biofuels 2014, 7(1):167.

[26] Lu Y, Wang Y, Xu G, Chu J, Zhuang Y, Zhang S: Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Appl Biochem Biotechnol 2010, 160(2):360-369.

[27] Dias MO, Junqueira TL, Cavalett O, Cunha MP, Jesus CD, Rossell CE, Maciel Filho R, Bonomi A: Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash. Bioresour Technol 2012, 103(1):152-161.

[28] Dias M, Cavalett O, Filhob R, Bonomi A: Integrated first and second generation ethanol production from sugarcane. Chemical Engineering Transactions 2014, :.

[29] Ai Y, Medic J, Jiang H, Wang D, Jane J: Starch characterization and ethanol production of sorghum. J Agric Food Chem 2011, 59(13):7385-7392.

[30] Wang D, Bean S, McLaren J, Seib P, Madl R, Tuinstra M, Shi Y, Lenz M, Wu X, Zhao R: Grain sorghum is a viable feedstock for ethanol production. J Ind Microbiol Biotechnol 2008, 35(5):313-320.

[31] Panagiotopoulos I, Bakker R, de Vrije T, Claassen P, Koukios E: Integration of first and second generation biofuels: fermentative hydrogen production from wheat grain and straw. Bioresour Technol 2013, 128:345-350.

[32] Yang M, Kuittinen S, Zhang J, Vepsäläinen J, Keinänen M, Pappinen A: Co-fermentation of hemicellulose and starch from barley straw and grain for efficient pentoses utilization in acetone–butanol–ethanol production. Bioresour Technol 2015, 179:128-135.

[33] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D: Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure 2008, 1617:1-16.

[34] Wu X, Maier A, Wu T, Li N, Pedersen JR, Wang D: Evaluation of Nebraska waxy sorghum hybrids for ethanol production. Cereal Chem 2013, 90(3):198-203.

[35] Erdei B, Hancz D, Galbe M, Zacchi G: SSF of steam-pretreated wheat straw with the addition of saccharified or fermented wheat meal in integrated bioethanol production. Biotechnology for biofuels 2013, 6(1):169.

[36] Yan S, Wu X, Faubion J, Bean SR, Cai L, Shi Y, Sun XS, Wang D: Ethanol-production performance of ozone-treated tannin grain sorghum flour. Cereal Chem 2012, 89(1):30-37.

[37] Rinaldi R, Schüth F: Acid hydrolysis of cellulose as the entry point into biorefinery schemes. ChemSusChem 2009, 2(12):1096-1107.

[38] Erdei B, Frankó B, Galbe M, Zacchi G: Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw. Biotechnology for biofuels 2012, 5(1):12.

[39] Rohowsky B, Häßler T, Gladis A, Remmele E, Schieder D, Faulstich M: Feasibility of simultaneous saccharification and juice co-fermentation on hydrothermal pretreated sweet sorghum bagasse for ethanol production. Appl Energy 2013, 102:211-219.

Chapter 5 - Modified simultaneous saccharification and fermentation to enhance bioethanol titers and yields

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5.1 Abstract

To maintain our society's sustainability with respect to people, prosperity, and the planet, we must produce liquid transportation fuels such as bioethanol on the renewable basis at a competitive price to petroleum-based fuels. The major challenges to commercialize cellulosic biofuels are low fermentation efficiency, low ethanol titer, and lack of technology to fully utilize the byproduct from bioconversion process such as lignin which has been underutilized. To overcome these technical barriers, we have proposed a novel design to fully utilize each component of lignocellulosic biomass for biofuels and bio-chemicals production, which involves green technologies such as hydrothermal and organosolv pretreatments to produce a cellulose-rich solid with good recovery of clean lignin after solvent recycling for improvement of plant protein-based adhesives as well as xylose remained in the aqueous phase for furfural upgradation. The focus of this study, as a part of the whole biorefinery concept, is to develop modified simultaneous saccharification and fermentation (mSSF) to enhance ethanol titers and yields, which combines the advantages of both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) via unique decantation process. The mSSF achieved higher ethanol concentration of 58.5 g/L and ethanol yield of 83.5% as compared to the traditional SSF process (49.9 g/L and 71.1%) at the biomass loadings of 20% (w/v). The mSSF also enabled higher ethanol titers of 72.3 g/L at higher loadings of 30% (w/v) with yields of

70.0%. As compared to published high-gravity fermentation, ethanol concentration of 72.3 g/L achieved in this study was the highest one in the lab-scale process, which proved that the proposed mSSF was an effective process to increase ethanol titers without sacrificing ethanol yields. The improved ethanol titers and yields would significantly lower the distillation cost and accelerate the commercialization of cellulosic biofuel production.

Keywords: Lignocellulosic biomass; pretreatment; SSF; SHF; high ethanol concentration

5.2 Introduction

Limited resources of crude oil and environmental concerns for mitigating greenhouse gas emissions have driven global research to explore renewable and sustainable biofuels and bio-chemicals. As the global population expands and the number of vehicles around the world increases, the demand for transportation fuels is expected to increase rapidly [1]. Among potential alternative liquid fuels, bioethanol is considered as the widest utilized transportation fuels [1]. Bioethanol is a renewable alternative fuel derived from various sustainable feedstocks such as sugar-based crops, starch-based crops, and lignocellulosic biomass. Biotechnologies are mature to produce ethanol from sugar and starch-rich crops in large scales and continued to develop advanced cellulosic ethanol production [2]. Currently, commercial production of bioethanol from lignocellulosic biomass is still not economically feasible, mainly facing the technical barriers of low ethanol yield and low ethanol concentration, high enzyme cost, and high water consumption [3, 4].

Lignocellulosic biomass is the most abundant and renewable resource with the sustainable worldwide production, including agricultural residues, forestry wastes, dedicated energy crops, and organic municipal solid waste, which represents an indispensable feedstock for the production of commercialized biofuels and renewable chemicals [5, 6]. Lignocellulosic

biomass is mainly composed of cellulose, hemicellulose, and lignin, in which the fractions of both cellulose and hemicellulose are polysaccharides and thus is a potential source of fermentable sugars [7]. For the biological conversion pathway, a typical process is usually required to sufficiently convert structural sugars of plant cell wall to bioethanol: pretreatment, enzymatic hydrolysis, fermentation, and distillation. Cellulose in native biomass is difficult to digest by enzymes and its sugar yield is usually lower than 20% [8]. Pretreatment of biomass feedstocks was used to breakdown the structural barrier and make cellulose more accessible for subsequent saccharification process. Numerous pretreatment methods have been developed to overcome the recalcitrant structure, such as super-size reduction mill, steam explosion, liquid hot water (LHW), dilute acid, lime, ammonia, organic solvent, and ionic liquid pretreatments [9-12]. Challenges in the current leading pretreatment processes include incomplete separation of cellulose and lignin, which could reduce subsequent enzymatic hydrolysis efficiency; formation of inhibitors that affect ethanol fermentation, such as acetic acid from hemicellulose, furans from sugar degradation and phenolic compounds from lignin decomposition; low-concentrated fermentable sugars; low fermentation efficiency at high solids loading; high usage of chemicals and energy-intensive processes and also high cost of waste disposal [8, 13].

Hydrothermal pretreatment is an environmentally friendly process as water is used as a reaction medium without any addition of chemicals, which is processed at relatively high temperatures (140–220°C) under mild acidic conditions. Hot water cleaves hemiacetal linkages and liberates acetic acids during pretreatment, which facilitates the breakage of ether linkages in biomass [8]. LHW pretreatment is more effective on agricultural residues and hardwood, but not efficient for softwood species. LHW pretreatment of wheat straw achieved 80% hemicellulose-derived sugar recovery and 91% enzymatic hydrolysis of cellulose [14]. LHW pretreatment has

the capability of handling large particle size as the particles are usually broken apart during treatment, therefore, LHW pretreatment reduces the energy cost for particle reduction. The drawback of pretreatments such as hydrothermal pretreatment and acid pretreatment is that significant amount of lignin was retained in the pretreated biomass, which was found to bind enzymes and consequently increase enzymes costs [15].

Organosolv pretreatment is a process of using aqueous organic solvents as reaction medium to fractionate the major biomass components, of which cellulose and lignin are usually recovered as precipitated solid streams while hemicellulose and sugar degradation products are dissolved into a water-soluble fraction. Organosolv pretreatment is able to obtain a clean lignin component from biomass, while the lignin is usually burned as an energy source in other pretreatment processes [16]. Ethanol is a commonly used organic solvent and using acid or alkaline catalysts to assist in the delignification process. A two-step process involving dilute-acid presoaking and aqueous-ethanol organosolv pretreatment of Miscanthus x giganteus yielded a solid residue with 95% initial glucan recovery, of which 98% was converted to glucose after 48 h of enzymatic saccharification, and 73% initial xylan recovery as well as 71% lignin recovery as ethanol organosolv lignin [17].

Achieving high ethanol concentration is as critical as obtaining high ethanol conversion efficiency because the minimum ethanol concentration of 40 g/L is required for economical ethanol distillation [4]. Traditional method to reach high ethanol concentration is to increase the amount of biomass loadings (>16wt%), so-called high-gravity processing. However, ethanol yields usually decrease as biomass loadings increase due to poor mass transfer and accumulated inhibitors [18, 19]. An advanced bioreactor capable of handling high solids loading is needed to

ensure sufficient mixing of substrate and enzymes, low energy input and low stress to enzymes and yeast cells [20].

Enzymatic saccharification of treated biomass conducted separately from the fermentation step is referred to as separate hydrolysis and fermentation (SHF), while enzymatic saccharification of cellulose performed in the presence of the fermentative microorganism is referred to as simultaneous saccharification and fermentation (SSF) [8]. SSF is superior to SHF in terms of reduced amount of reactors and low risk of contamination, however, yeast recycling is very difficult when using SSF, in addition, the optimal temperatures for enzymes (50 °C) and yeasts (30 °C) are different, which indicates the conditions used in SSF cannot be optimal for both enzymes and yeast [21]. We proposed a new design of modified SSF (mSSF) with a focus on enhancing ethanol concentration as a part of the whole biorefinery process are shown in Fig. 5.1. Details of mSSF scheme are also shown in Fig. 5.1. Treated biomass is first subjected to SHF in one large-volume fermenter at the optimal conditions for enzymes and then the saccharified liquor (low-concentrated sugars) is decanted to small-volume fermenters with the addition of another set of treated biomass to perform SSF. SSF as a traditional method to produce ethanol will be conducted and compared to our proposed design [22].

In the proposed biorefinery concept, the hemicellulose component is hydrolyzed by hot water and used to generate furfural, which has been successfully produced in a commercial scale [23, 24], thus, conversion of hemicellulose to furfural is not the focus of this study. Lignin extracted through organosolv treatment exhibits high purity and more active functional groups [16]. Soy protein adhesives (SPA) have shown great potentials to replace petroleum-derived phenol-formaldehyde or urea-formaldehyde resins commonly used for wood adhesives, while water resistance of SPA can’t compete with phenol formaldehyde and isocyanate-based

adhesives for exterior applications [25, 26]. In contrast, lignin has an aromatic and cross-linked structure and can react with soy protein to form protein-lignin polymer which improves the wet adhesive strength due to the hydrophobic property of lignin [27-29]. As a part of the whole biorefinery process, solvent-extracted lignin will be reacted with soy protein to form protein-lignin polymers as bio-adhesive and the adhesive performance of protein-lignin adhesives will be evaluated, particularly the wet strength as compared to protein only and commercial Kraft lignin in separate subsequent studies.

To the best of our knowledge, we are the first to propose this novel integrated process (mSSF) to enhance ethanol concentration without sacrificing ethanol yield at the time of writing. Thus, for this study, we aim to optimize hydrothermal and organosolv pretreatment conditions for achieving the highest ethanol yield and test the integrated process for enhancing ethanol concentration.

5.3 Materials and methods

5.3.1 Materials

Switchgrass was harvested at the Kansas State University Research farm (Manhattan, KS). After grinding into <1 mm particle size with a cutting mill (SM 2000, Retsch Inc., Newton, PA, USA), the switchgrass samples was sealed in a plastic bag and stored at room temperature. The chemical composition of switchgrass was determined according to the National Renewable Energy Laboratory (NREL) procedure as shown in Table 1 [30]. In the NREL procedure, switchgrass was first subjected to warm water and ethanol extraction, and then samples were treated with sulfuric acid (72%) at 30 °C for 60 min and hydrolyzed by dilute acid (4%) at 121 °C for another 60 min. After acid hydrolysis, carbohydrates including cellulose and hemicellulose were converted to monosaccharide, which was measured by high-performance

liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) equipped with an RCM monosaccharide column (300×7.8 mm) (Phenomenex, Torrance, CA) and a refractive index detector (RID10A, Shimadzu, Kyoto, Japan). The mobile phase was 0.6 mL · min^{-1} of double-distilled water, and the oven temperature was 80℃. Lignin consists of acid insoluble and acid soluble lignin. Acid insoluble lignin was weighed from the solid after oven heating overnight at 105 °C (the weight of acid insoluble lignin and ash) and then at 575°C for at least 6 h to measure the ash content. All chemicals used for this research were purchased from Sigma Chemical Co. (St. Louis, MO).

5.3.2 Hydrothermal pretreatment

The primary objective of pretreatment is to open the recalcitrant structure and increase enzymatic accessibility to plant cell wall surfaces. Hydrothermal pretreatment was carried out in a Parr reactor (Parr Instrument Co., Moline, IL). The stainless steel reactor with the helical mixer has a total volume of 1 L and is heated by an electric heater. The recommended maximum input volume is 750 mL as some space is reserved for slurry expansion. After weighted biomass samples were introduced into the reactor and the target temperature was set, the reactor was heated at a rate of approximately 6 °C min -1. At the time reaction temperature reached the target temperature (200°C), it was maintained at this temperature for 30 min [31]. After the treatment was complete, the reactor was removed from the electric heater and placed into room temperature water to cool down to 50 °C within 5 min. Then the slurry was vacuum filtered using Whatman Paper (No. 4). Pretreated biomass was washed thoroughly with water and collected for composition analysis and enzymatic hydrolysis to evaluate the pretreatment performance. All hydrothermal pretreatment experiments were conducted at 16% solid loadings.

5.3.3 Organosolv pretreatment

Organosolv pretreatment was carried out using the same Parr reactor. Aqueous ethanol (80%, v/v) was used as reaction medium and reaction temperature was maintained at 170 °C for a period of 60 min [17, 32, 33]. After the treatment was complete, the reactor was removed from the electric heater and placed into room temperature water to cool down to 50 °C within 5 min. Then the slurry was vacuum filtered using Whatman Paper (No. 4). Pretreated biomass was washed thoroughly with water and collected for composition analysis and enzymatic hydrolysis to evaluate the pretreatment performance. All Organosolv pretreatment experiments were conducted at 10% solid loadings.

5.3.4 Enzymatic saccharification

The pretreated biomass was added to flasks to perform enzymatic hydrolysis using Accellerase 1500 provided by DuPont Industrial Biosciences (Rochester, NY, USA), and applied at the recommended dosage (0.5 mL/g cellulose) [34]. Accellerase 1500 is an enzyme complex including cellulose and b-glucosidase (Endoglucanase activity: 2,200–2,900 CMC U/g (1 CMC U unit of activity liberates 1 lmol of reducing sugars, expressed as glucose equivalents) in 1 min under specific assay conditions of 50 °C and pH 4.8). Flasks were incubated at 50°C placed in a rotary shaker (Model I2400, New Brunswick Scientific Inc., Edison, NJ, USA) with the speed of 150 rpm. Supernatants were extracted by filtration after 72-h enzymatic hydrolysis and stored for further experiments at 4°C. Glucose concentration was measured by HPLC. All experiments were performed in duplicate. Glucose yield was calculated as follows:

$$\text{Glucose yield (\%)} = \frac{Released\ sugar\ amount}{Theoretical\ sugar\ amount\ in\ raw\ materials} * 100\%$$

5.3.5 Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) of treated biomass was used as control. The pretreated biomass was added to flasks using Accellerase 1500, and applied at the recommended dosage (0.5 mL/g cellulose). 1.0 mL of activated yeast culture and 0.3 g of yeast extract were added into each flask and sealed with an S-shaped airlock to initiate the SSF process conducted at 38°C in a rotary shaker operating at 150 rpm for 72 h. Yeast (Red Star Ethanol Red, Lasaffre, Milwaukee, WI, USA), *Saccharomyces cerevisiae*, was activated before inoculation by weighing 1.0 g of active dry yeast into 19 mL of culture broth (containing 20 g of glucose, 5.0 g of peptone, 3.0 g of yeast extracts, 1.0 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter and autoclaved at 121°C for 20 min) and incubating at 38°C at 200 rpm for 30 min. The activated yeast culture had a cell concentration of around 10^9 cells/mL of broth. Supernatants were extracted by filtration after 72-h fermentation and stored for further experiments at 4°C. Ethanol concentration was measured by HPLC. All experiments were performed in duplicate.

$$\text{Ethanol fermentation efficiency (\%)} = \frac{Actual\ ethanol\ released}{Theoretical\ ethanol\ release} * 100\%$$

5.3.6 FT-IR analysis

The FTIR spectra of corn stover before and after pretreatment were measured in a scattering mode. Each spectrum was averaged with 32 scans at a resolution of 4 cm^{-1} in the wavenumber range of 400-4000 cm^{-1}. OMNIC 6.1a software (PerkinElmer Corp., Shelton, CT, USA) was used to determine peak positions and intensities.

5.3.7 Experimental design

A biorefinery concept of fuels and chemicals production from biomass is shown in Fig. 5.1. Biomass was first treated by hydrothermal pretreatment to release hemicellulose

component for furfural production, following by organosolv pretreatment to extract lignin component. Then treated biomass is subjected to the modified SSF (mSSF) process to perform enzymatic hydrolysis in a large fermenter and subsequently the saccharified liquor (low-concentrated sugars) is decanted to small-volume fermenters with the addition of another set of treated biomass to perform SSF. Solvent-extracted lignin as proposed in this study will be used to react with soy protein to form protein-lignin polymers as bio-adhesive. After fermentation, the broth was distilled to evaporate ethanol for product recovery and solid residues could be used as solid fuel to generate heat and energy needed for the whole process.

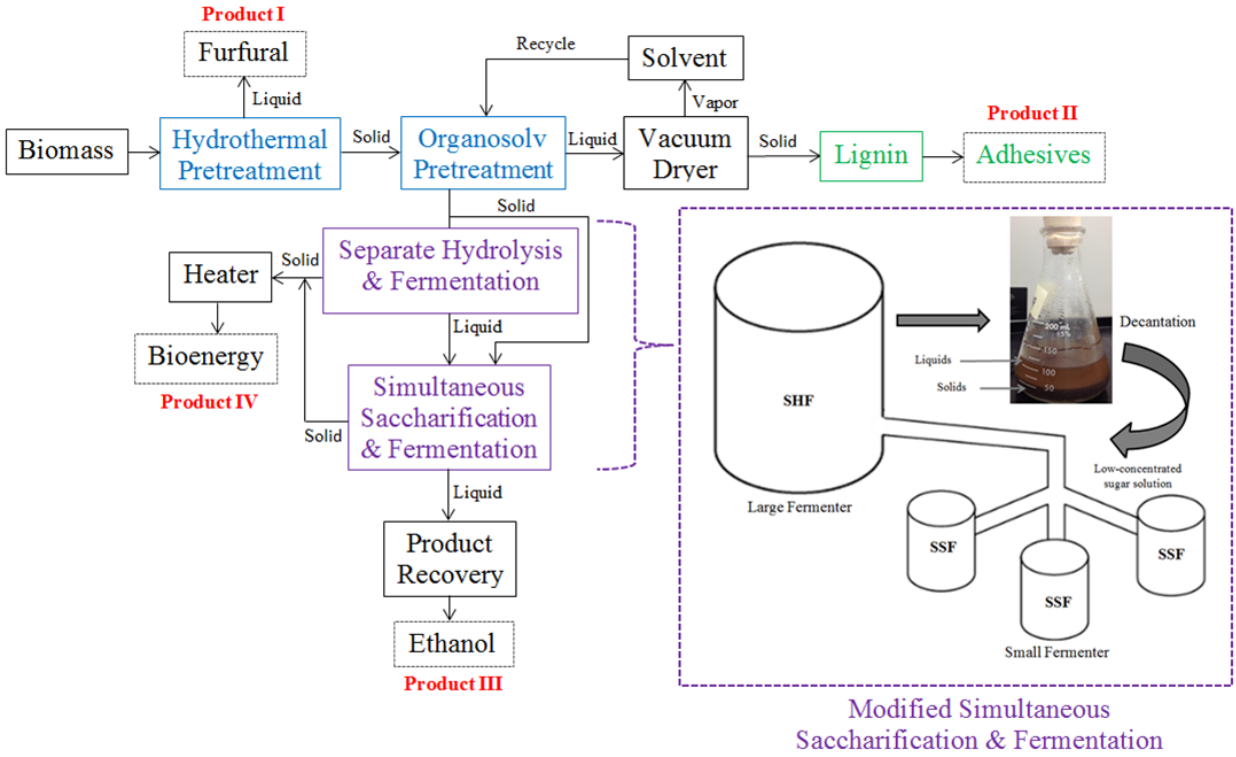

Fig. 5.1 Biorefinery concept with modified simultaneous saccharification and fermentation (mSSF). The first part (blue) focuses on the pretreatment optimization to achieve maximal ethanol yield; the second part (purple) aims to enhance ethanol concentration via decantation technology, separate hydrolysis and fermentation (SHF), and SSF; and the last part (green) represents lignin extraction and its application for bio-adhesives.

5.3.8 Statistics

Analysis of variance and pairwise comparisons for the means using the Tukey adjustment were performed with SAS (SAS Institute, Inc., Cary, NC, USA). Means values and standard deviations from the duplicated experiments are reported.

5.4 Results and discussion

5.4.1 Compositional changes of switchgrass

Energy crop, such as switchgrass, mainly consist of cellulose, hemicellulose and lignin. The chemical composition of raw switchgrass used in this study contains 35.4% cellulose, 26.9% hemicellulose, and 17.0% lignin while the hemicellulose in hydrothermally treated biomass tremendously reduced to 5.9%, consequently increased cellulose content to 51.8% and lignin content to 35.8% (Table 5.1). This is likely due to weak acids such as acetic acid released from acetyl groups attached to hemicellulose as a result of hot water treatment (200 °C for 30 min). Structure modification by hydrothermal pretreatment could reduce biomass recalcitrance and make it more accessible to cellulolytic enzymes as compared to untreated biomass [35]. Hydrothermal pretreatment could relocate the lignin component, and generate carbohydrate-derived pseudo-lignin, which might retard cellulose bioconversion as lignin could bind enzymes and consequently more cellulolytic enzymes might be needed [36, 37]. The disadvantage of hydrothermal pretreatment is that significant amount of lignin was retained in the pretreated biomass, and mostly used as low-valued heating source [15]. In addition, valorization of byproduct lignin plays a significant role in the commercialization of cellulosic biofuels. After ethanol pretreatment, the cellulose content was further increased to 65.2%, whereas lignin content reduced significantly from 35.8% to 19.8% and slight reduction of hemicellulose content was achieved. Enhanced cellulose content of hydrothermal and ethanol treated biomass would

result in increased amount of fermentable sugar and consequently higher ethanol concentration, which would lower the downstream distillation cost [4].

Table 5.1 Chemical composition changes of switchgrass after treatment.

Composition	Cellulose (%, db)	Hemicellulose (%, db)	Lignin (%, db)	Lignin recovery (%)
Raw switchgrass	35.4±0.2	26.9±0.3	17.0±0.2	100
Hydrothermal-treated switchgrass[a]	51.8±0.5	5.9±1.2	35.8±0.4	133±1.5
Hydrothermal and ethanol-treated switchgrass[b]	65.2±0.2	4.7±0.2	19.8±0.9	48.7±1.2

[a]Pretreatment condition was 200 °C for 30 min;
[b]Pretreatment condition was 170 °C for 60 min at ethanol concentration of 80% (v/v).

Hydrothermal and dilute acid pretreatments usually result in significant degradation of hemicellulose to soluble furans and insoluble products, which were found to highly interact with residual lignin component to form pseudo-lignin complexes [37]. Xylan derived-pseudo-lignin was formed at moderate severe pretreatment conditions and the resultant insoluble degradation products can significantly inhibit cellulose hydrolysis [37]. Raw switchgrass used in this study contains 17% lignin while the lignin content of hydrothermal treated biomass increased to 35.8% with the lignin recovery of 133% (Table 5.1). This could be due to the above mentioned pseudo-lignin products generated during the hot water pretreatment. Followed by ethanol treatment, significant amounts of lignin were dissolved in ethanol solvent, leading to residual lignin recovery of only 48.7% (Table 5.1). Organic solvent extraction process was an efficient tool to utilize the byproduct lignin and consequently enhance fermentable sugar content of treated biomass, which would eventually improve the overall process economics [38-40].

5.4.2 Glucose yield and concentration of hydrothermal and organosolv treated switchgrass

Enzymatic hydrolysis yield of hydrothermal treated switchgrass in this study significantly increased to 78.4% at 8% (w/v) biomass loadings as compared to untreated biomass. With the following ethanol treatment, glucose yield further increased to 87.9% at the same biomass loadings (Fig. 5.2). Meanwhile, enzymatic hydrolysis of hydrothermal and organosolv treated sample improved glucose concentration from 35.8 g/L to 50.4 g/L as compared to hydrothermal treated only sample (Fig. 5.2). Therefore, ethanol pretreatment not only utilized the lignin byproduct which hydrothermal treatment failed to remove, but also improved the bioconversion of cellulose and enhanced the fermentable sugar content.

Hydrothermal pretreatment is an effective method to improve the digestibility of biomass. At room temperature, the corresponding pH of de-ionized water is 7.0, while as the temperature and pressure of saturated liquid water increases, pH of de-ionized water decreases to a minimum point of 5.6 at approximately 250 °C [8]. During LHW pretreatment, the hydronium ions released from water decompose hemicellulose fractions of plant cell wall to generate acetic acids which assists in the structure disruption and thus enzyme-catalyzed hydrolysis of fermentable sugars. LHW pretreatment had been applied to remove up to 80% of the hemicellulose and subsequently enhance enzymatic digestibility of treated biomass such as corn fiber and sugarcane bagasse [3].

Lignin is a complex and cross-linked polymer of phenolic monomers, which consists of aliphatic and aromatic compounds. Organic solvent as an effective agent to dissolve lignin could improve the bioconversion of lignocellulosic biomass. Ethanol is a commonly used organic solvent and using acid or alkaline catalysts to assist in the delignification process. High

cellulose-to-glucose conversion yield of 97% within 48h was achieved in the ethanol organosolv–treated Lodgepole pine killed by mountain pine beetle [33]. Other solvents and catalysts such as tetrahydrofuran, methanol, acetone, acetic acid, and formic acid were tested for organosolv pretreatment. Tetrahydrofuran is a water miscible solvent with low boiling point of 66°C. Tetrahydrofuran is also a low viscosity solvent which can be derived from biomass through upgradation of furfural [16]. A novel pretreatment using tetrahydrofuran as a solvent obtained 95% theoretical yield of fermentable sugars from corn stover while using low enzyme loading at only 2 $mg_{enzyme}\ g^{-1}$ (Accellerase1500, DuPont Industrial Biosciences, Palo Alto, CA) based on the mass of glucan in the raw material [15].

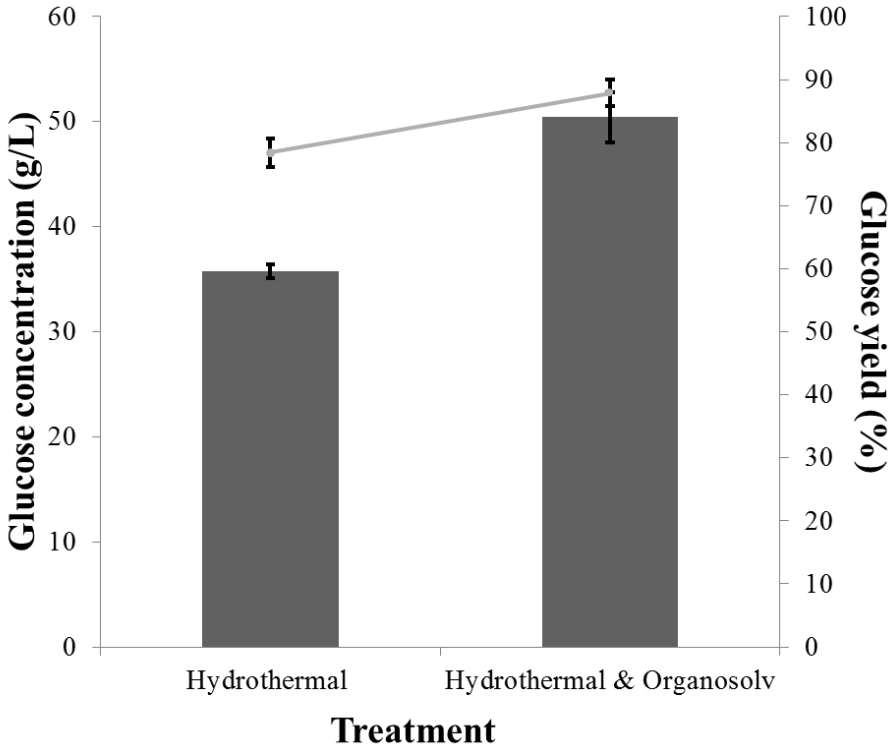

Fig. 5.2 Glucose yield and concentration of treated switchgrass. Column represents glucose concentration and line represents glucose yield. Error bar represents one standard deviation.

5.4.3 High-gravity fermentation of hydrothermal and organosolv treated switchgrass via mSSF

High-gravity bioconversion provides benefits such as improved water-use efficiency and reduced distillation costs as increased ethanol concentration due to high biomass loadings [31, 41]. High ethanol concentration is desired to reduce capital and energy costs and a minimum of 40 g/L is generally required for economical ethanol distillation [4]. One common approach to achieve higher ethanol titers is to increase the biomass loadings (> 8wt% glucan loading), so-called high-gravity bioconversion. However, it is also known that ethanol yield usually decreases as solid loadings increase due to the problems such as mass transfer limitations or accumulated inhibitors by degraded products that subsequently occur, which results in reduced fermentation efficiency [31]. As found in this study (Fig. 5.3), ethanol titer increased from 58.5 to 72.3 g/L as the biomass loading increased from 20 to 30% (w/v), however, ethanol yield decreased from 83.5 to 70.0% as increasing biomass loadings.

A novel process technology of mSSF was proposed in this study with the goal of increasing ethanol titers without sacrificing ethanol yields. The mSSF process was designed as the integration of SHF in one large-volume fermenter and SSF in several small-volume fermenters (Fig. 5.1). For instance, at laboratory scale, first of all, 30 g biomass is loaded into a large-volume flask containing 300 mL buffer solution to perform enzymatic saccharification according to the SHF process which is at the optimal condition for the enzymes (50 °C). Then once the saccharification is complete, the liquid portion containing fermentable sugars is transferred equivalently into 3 small-volume flasks (each contains 100 mL liquid) to conduct the SSF process (30 °C) with the addition of another set of biomass (10 g). Overall, a total of 20% (w/v) biomass loading is fermented in the mSSF process, consisting of two separate 10% (w/v)

biomass loading processes to result in increased ethanol titers as well as high ethanol yield. Likewise, a total of 30% (w/v) biomass loading is fermented in the mSSF process which consists of two separate 15% (w/v) biomass loading processes. Results showed that the ethanol titers of 58.5 g/L and 72.3 g/L were obtained at the biomass loadings of 20% and 30% (w/v), respectively (Fig. 5.3). Meanwhile, high ethanol yields of 83.5% and 70.0% were achieved at the biomass loadings of 20% and 30% (w/v), respectively. As compared to published high-gravity fermentation, ethanol concentration of 72.3 g/L achieved in this study was the highest one in the lab-scale process (Table 5.2), which proved that the proposed mSSF was an effective process to increase ethanol titers without sacrificing ethanol yields. With more advanced bioreactor capable of handling high solids loading, further increment of ethanol concentration can be expected.

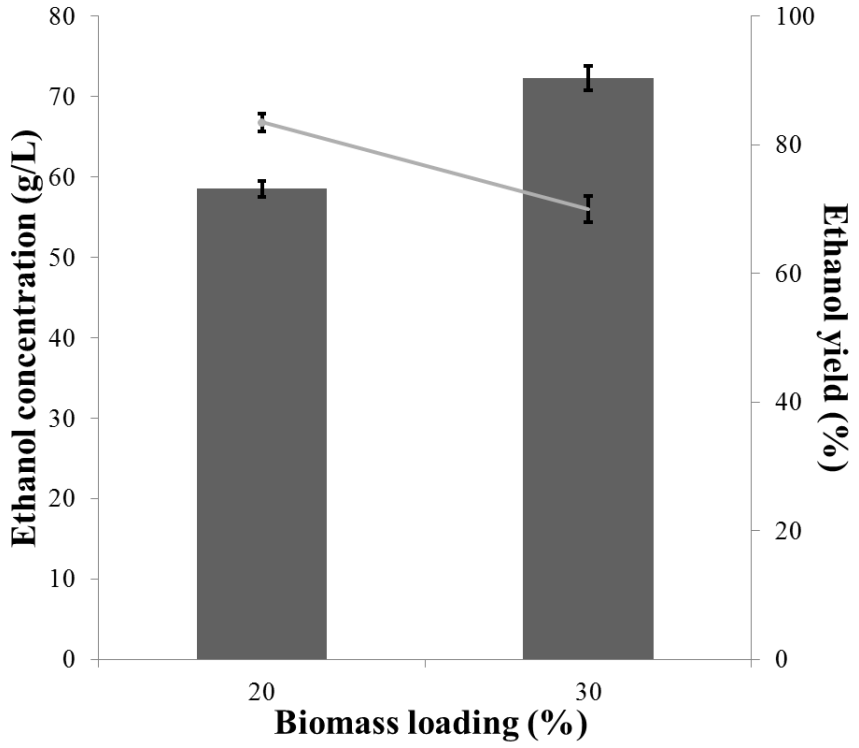

Fig. 5.3 Ethanol yield and concentration of treated switchgrass. Column represents glucose concentration and line represents glucose yield. Error bar represents one standard deviation.

Table 5.2 Comparisons of high-gravity fermentation.

Material	Pretreatment	Solid loading (%)	Fermentation Process*	Ethanol Yield (%)	Ethanol Titer (g/L)	Reference
Switchgrass	Hydrothermal & ethanol	20	mSSF	83.5	58.5	this study
Switchgrass	Hydrothermal & ethanol	30	mSSF	70.0	72.3	this study
Corn stover	Steam explosion	20	SSF	77.2	59.8	[18]
Corn stover	Ionic liquids	34.2	Fed-batch SSF	74.8	41.1	[4]
Eucalyptus	Autohydrolysis	15.6	SSF	95.0	50.2	[19]
Spruce	Steam explosion with acids	20	Fed-batch SSF	53.0	40.0	[42]

*SSF: simultaneous saccharification and fermentation; mSSF: modified simultaneous saccharification and fermentation; Fed-batch SSF: fed-batch simultaneous saccharification and fermentation.

5.4.4 SSF vs. modified SSF

Especially at high solids loadings, the mixing challenges and problems such as poor mass transfer and accumulated inhibitor will become more severe. mSSF process as an effective strategy can minimize those issues as solid residues are separated and only liquid with hydrolyzed sugars transferred into next fermenters, thus, the sugar concentration will be doubled, whereas solid loadings remain low for high ethanol yields as compared to the traditional SSF process. It was also demonstrated in this study that mSSF enabled higher ethanol concentration of (58.5 g/L) with the ethanol yield of 83.5% as compared to the SSF process (49.9 g/L and 71.1%) at the biomass loadings of 20% (w/v) (Fig. 5.4). It proved that mSSF as a novel processing technology proposed in this study can function as effectively as the commonly

recognized fed-batch SSF process and even slight improvement could be obtained. At higher solid loadings, the superior performance of mSSF process could be more obvious.

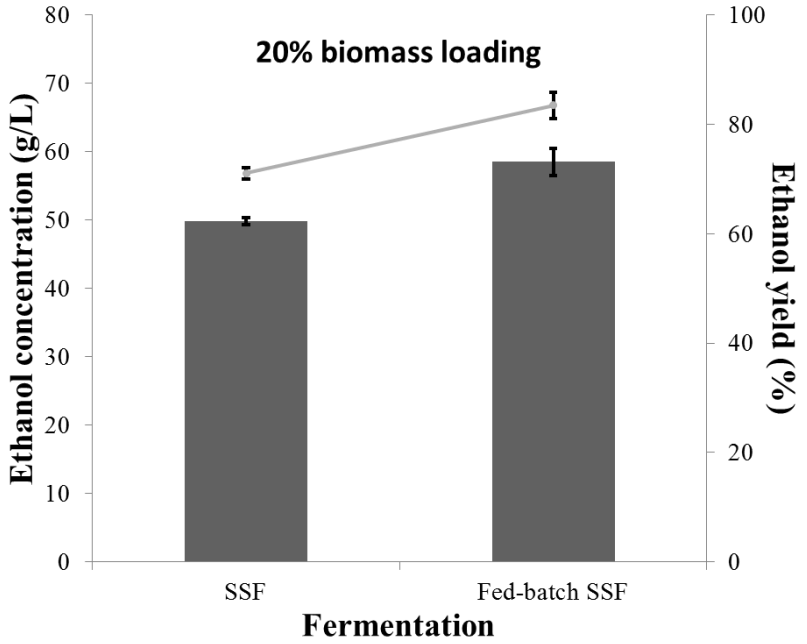

Fig. 5.4 Simultaneous saccharification and fermentation of treated switchgrass. Column represents glucose concentration and line represents glucose yield. Error bar represents one standard deviation.

5.4.5 Modified SSF vs. Hybrid SSF

SSF is a widely used process for production of ethanol from lignocellulose. The major benefits of performing the enzymatic hydrolysis together with the fermentation, instead of in a separate step after the hydrolysis, are the reduced end-product inhibition of the enzymatic hydrolysis, and the reduced equipment costs. mSSF with pre-hydrolysis, referred as hybrid SSF, was believed to improve the SSF performance as the pre-hydrolysis was conducted at favorable conditions (temperature and pH) for the enzymes [43].

Detailed process description of mSSF was mentioned in the above section, hybrid SSF was the same procedure as mSSF except inclusion of the pre-hydrolysis at 50 °C for 6 h before the SSF process at 30 °C initiated. Comparisons between mSSF and hybrid SSF were conducted

at two biomass loadings of 20% and 30% (w/v). No significant differences in ethanol concentrations between mSSF and hybrid SSF (58.5 vs. 58.7 g/L) were observed at the 20% (w/v) biomass loading (Fig. 5.5). In contrast, slight improvement of ethanol concentration (75.3 g/L) via hybrid SSF was achieved at the 30% (w/v) biomass loading as compared to mSSF (Fig. 5.5). This was probably due to different biomass loadings, at the lab-scale process of this study, issues such as poor mass transfer and accumulated inhibitor occur at relatively high biomass loadings, e.g. higher than 20% biomass loadings.

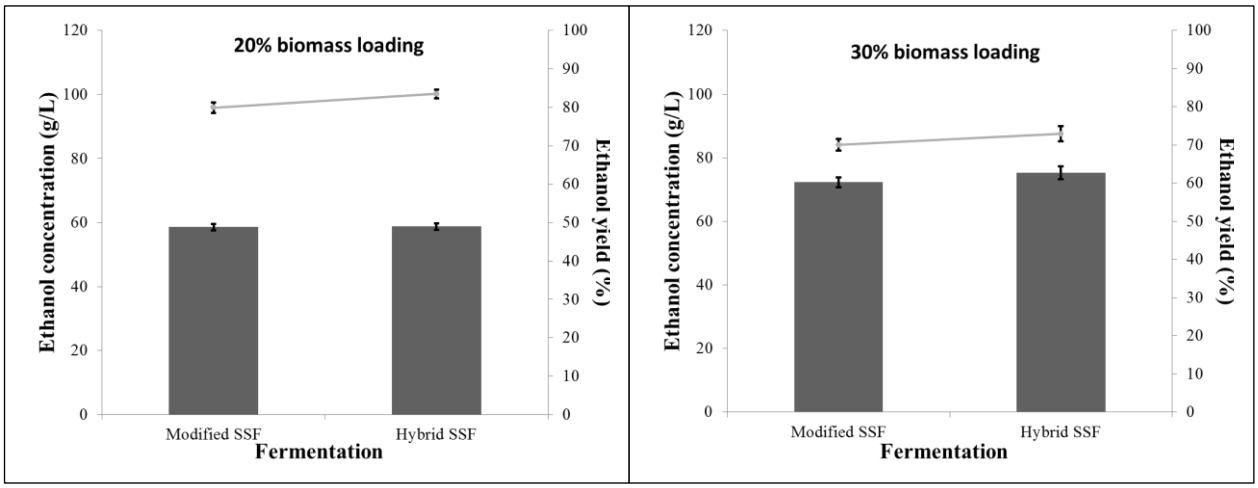

Fig. 5.5 Modified and hybrid simultaneous saccharification and fermentation. Column represents glucose concentration and line represents glucose yield. Error bar represents one standard deviation.

5.4.6 FT-IR Analysis

FT-IR analysis was used to investigate the chemical structure change during pretreatment (Fig. 5.6). The region of 4000-1800 cm^{-1} has only two major bands, which corresponds the O-H and C-H groups, respectively. The peak near 3337-3330 cm^{-1} represents free OH stretching, and the peak intensity had a significant increase after the pretreatment, indicating that hydrogen bonds between cellulose and hemicellulose as well as cellulose and lignin were disrupted and more OH of cellulose became exposed in the form of free state [44].

This is in accordance with our expectation because the hydrogen bond is a special intra- and intermolecular force, different from the covalent bond which needs a high-energy intensity to disrupt, and is easy to be disrupted with an intermediate- or low- energy intensity. The band position at 2917-2900 cm^{-1} is attributed to C-H stretching [45], and the peak almost had no changes after the LHW pretreatment. This is mainly because hydrogen bonds are the major linkages between hemicellulose and cellulose, thus no affecting the C-H of cellulose. However, the peak had an increased intensity after the ethanol pretreatment, indicating that cellulose and lignin were connected by covalent bonds such as ester and ether bonds etc., and the methyl and methylene portions of cellulose were exposed after the removal of lignin through the ethanol pretreatment. Compared to the two peaks discussed above, the peaks in fingerprint region (1800-900 cm^{-1}) are complex and a result of various vibration modes in carbohydrates and lignin, which is the focus of our attention. The peak at 1731 cm^{-1} represents the acetyl and uronic ester groups connected to branched chains of hemicellulose [45, 46], and disappeared after the LHW pretreatment, indicating the acetyl and uronic groups were cleaved from hemicellulose. The pH value of sugar liquor after the LHW pretreatment decreased by approximately 3.5, further proving the removal of the acetyl and uronic groups from hemicellulose. Compared to raw and LHW pretreated biomass, the ethanol pretreated biomass had an increased peak intensity in the 1602 cm^{-1} band position assigned to the aromatic skeletal vibration and C=O stretching of lignin[47]. The peak intensity in the 1513 cm^{-1} and 1315 cm^{-1} positions assigned respectively to the C=C and C-O vibrations of lignin side chains also increased [48, 49]. The increase of all three peak intensity indicated that the splitting of lignin aliphatic side chains connected with cellulose and hemicellulose occurred after the pretreatment. The peak in the 1232 cm^{-1} assigned to C-O-H deformation and C-O stretching of phenolics disappeared after the pretreatment,

indicating that the solubilization of phenolics and remotion of esters from biomass [50]. The increase of the peak intensity in 1200 cm^{-1} indicated an increased contribution from 2^{nd} OH group, which was accompanied by the development of doublet peaks at 1056 and 1031 cm^{-1} assigned to the aliphatic OH group [51]. The peak in 1158 cm^{-1} attributed to the antisymmetric C-O-C and β-1,4-glycosyl linkage appeared in the raw and pretreated biomass [52]. The peak in the 896 cm^{-1} is the characteristic of β-glycosidic linkages between the sugar units [53].

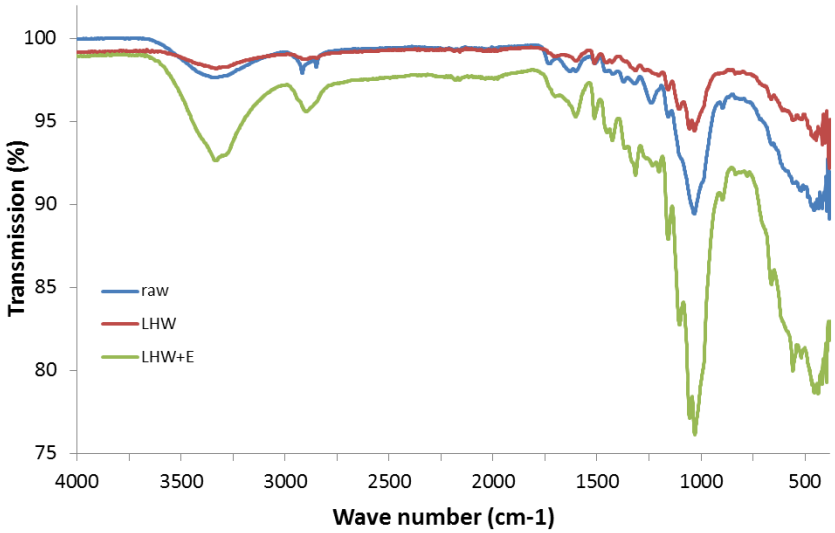

Fig. 5.6 Structural changes in treated materials. LHW represents liquid hot water treatment; LHW+E represents liquid hot water and ethanol treatment. (This part was conducted by co-author of this paper, Jun Li.)

5.5 Conclusions

Fully utilizing each component of lignocellulosic biomass for biofuels and bio-chemicals production is key element towards successful commercialization of cellulosic biofuels. The proposed novel biorefining concept utilizes hemicellulose for furfural production and enables high ethanol titer and high ethanol fermentation efficiency as well as utilization of solvent-extracted lignin for value added product such as bio-based adhesive. A new design of mSSF with the core of enhancing ethanol concentration was proposed as a part of the whole

biorefinery process. An ethanol titer of 72.3 g/L (equivalent to an overall yield of 70.0%) was obtained using mSSF of hydrothermal and organosolv treated switchgrass at biomass loading of 30% (w/v), which was the highest among the published results to the extent of our knowledge. mSSF with decantation process enabled the bioconversion process at high solid loadings. As a result, achieved high ethanol concentration will lower the downstream distillation cost for product recovery. The results in this study indicated high ethanol titer and ethanol yield as well as byproduct utilization through the proposed biorefining process could significantly accelerate the commercialization of cellulosic biofuel production.

5.6 References

[1] Kim SM, Dien BS, Singh V. Promise of combined hydrothermal/chemical and mechanical refining for pretreatment of woody and herbaceous biomass. Biotech Biofuels 2016; 9(1):97.

[2] Limayem A, Ricke SC. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog Energy Combustion Sci 2012; 38(4):449-467.

[3] Menon V, Rao M. Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. Prog Energy Combustion Sci 2012; 38(4):522-550.

[4] Xu F, Sun J, Konda NM, Shi J, Dutta T, Scown CD, Simmons BA, Singh S. Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. Energy Environ Sci 2016; 9(3):1042-1049.

[5] Zhang K, Johnson L, Prasad PV, Pei Z, Wang D. Big bluestem as a bioenergy crop: A review. Renew Sust Energy Rev 2015; 52:740-756.

[6] Mansouri A, Rihani R, Laoufi AN, Özkan M. Production of bioethanol from a mixture of agricultural feedstocks: Biofuels characterization. Fuel 2016; 185:612-621.

[7] Balat M, Balat H. Recent trends in global production and utilization of bio-ethanol fuel. Appl Energy 2009; 86(11):2273-2282.

[8] Mosier N, Wyman C, Dale B, Elander R, Lee Y, Holtzapple M, Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005; 96(6):673-686.

[9] Alvira P, Tomás-Pejó E, Ballesteros M, Negro M. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 2010; 101(13):4851-4861.

[10] Yu M, Li J, Chang S, Zhang L, Mao Y, Cui T, Yan Z, Luo C, Li S. Bioethanol production using the sodium hydroxide pretreated sweet sorghum bagasse without washing. Fuel 2016; 175:20-25.

[11] Smuga-Kogut M, Zgórska K, Kogut T, Kukiełka K, Wojdalski J, Kupczyk A, Dróżdż B, Wielewska I. The use of ionic liquid pretreatment of rye straw for bioethanol production. Fuel 2017; 191:266-274.

[12] Wang M, Zhou D, Wang Y, Wei S, Yang W, Kuang M, Ma L, Fang D, Xu S, Du S. Bioethanol production from cotton stalk: A comparative study of various pretreatments. Fuel 2016; 184:527-532.

[13] Tomás-Pejó E, Fermoso J, Herrador E, Hernando H, Jiménez-Sánchez S, Ballesteros M, González-Fernández C, Serrano D. Valorization of steam-exploded wheat straw through a biorefinery approach: Bioethanol and bio-oil co-production. Fuel 2017; 199:403-412.

[14] Pérez JA, González A, Oliva JM, Ballesteros I, Manzanares P. Effect of process variables on liquid hot water pretreatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor. J Chem Technol Biotechnol 2007; 82(10):929-938.

[15] Nguyen TY, Cai CM, Kumar R, Wyman CE. Co-solvent Pretreatment Reduces Costly Enzyme Requirements for High Sugar and Ethanol Yields from Lignocellulosic Biomass. ChemSusChem 2015; 8(10):1716-1725.

[16] Zhang Z, Harrison MD, Rackemann DW, Doherty WO, O'Hara IM. Organosolv pretreatment of plant biomass for enhanced enzymatic saccharification. Green Chem 2016; 18(2):360-381.

[17] Brosse N, Sannigrahi P, Ragauskas A. Pretreatment of Miscanthus x giganteus using the ethanol organosolv process for ethanol production. Ind Eng Chem Res 2009; 48(18):8328-8334.

[18] Liu Z, Qin L, Zhu J, Li B, Yuan Y. Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. Biotech Biofuels 2014; 7(1):167.

[19] Romaní A, Ruiz HA, Pereira FB, Teixeira JA, Domingues L. Integrated approach for effective bioethanol production using whole slurry from autohydrolyzed Eucalyptus globulus wood at high-solid loadings. Fuel 2014; 135:482-491.

[20] Zhang J, Chu D, Huang J, Yu Z, Dai G, Bao J. Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor. Biotechnol Bioeng 2010; 105(4):718-728.

[21] Öhgren K, Bura R, Lesnicki G, Saddler J, Zacchi G. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochem 2007; 42(5):834-839.

[22] Zhang M, Wang F, Su R, Qi W, He Z. Ethanol production from high dry matter corncob using fed-batch simultaneous saccharification and fermentation after combined pretreatment. Bioresour Technol 2010; 101(13):4959-4964.

[23] Tang Y, Zhao D, Cristhian C, Jiang J. Simultaneous saccharification and cofermentation of lignocellulosic residues from commercial furfural production and corn kernels using different nutrient media. Biotech Biofuels 2011; 4(1):22.

[24] Cai CM, Nagane N, Kumar R, Wyman CE. Coupling metal halides with a co-solvent to produce furfural and 5-HMF at high yields directly from lignocellulosic biomass as an integrated biofuels strategy. Green Chem 2014; 16(8):3819-3829.

[25] Qi G, Li N, Wang D, Sun XS. Adhesion and physicochemical properties of soy protein modified by sodium bisulfite. J Am Oil Chem Soc 2013; 90(12):1917-1926.

[26] Qi G, Li N, Wang D, Sun XS. Physicochemical properties of soy protein adhesives modified by 2-octen-1-ylsuccinic anhydride. Ind Crop Prod 2013; 46:165-172.

[27] Luo J, Luo J, Yuan C, Zhang W, Li J, Gao Q, Chen H. An eco-friendly wood adhesive from soy protein and lignin: performance properties. RSC Advances 2015; 5(122):100849-100855.

[28] Liu W, Jiang H, Yu H. Thermochemical conversion of lignin to functional materials: a review and future directions. Green Chem 2015; 17(11):4888-4907.

[29] Sen S, Patil S, Argyropoulos DS. Thermal properties of lignin in copolymers, blends, and composites: a review. Green Chem 2015; 17(11):4862-4887.

[30] Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. Determination of Structural Carbohydrates and Lignin in Biomass. National Renewable Energy Laboratory Technical Report NREL/ 2011.

[31] Xu Y, Wang D. Integrating starchy substrate into cellulosic ethanol production to boost ethanol titers and yields. Appl Energy 2017; 195:196-203.

[32] Pan X, Gilkes N, Kadla J, Pye K, Saka S, Gregg D, Ehara K, Xie D, Lam D, Saddler J. Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: Optimization of process yields. Biotechnol Bioeng 2006; 94(5):851-861.

[33] Pan X, Xie D, Yu RW, Lam D, Saddler JN. Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: fractionation and process optimization. Ind Eng Chem Res 2007; 46(8):2609-2617.

[34] Xu F, Shi Y, Wu X, Theerarattananoon K, Staggenborg S, Wang D. Sulfuric acid pretreatment and enzymatic hydrolysis of photoperiod sensitive sorghum for ethanol production. Biopro Biosyst Eng 2011; 34(4):485-492.

[35] Zhang T, Kumar R, Tsai Y, Elander RT, Wyman CE. Xylose yields and relationship to combined severity for dilute acid post-hydrolysis of xylooligomers from hydrothermal pretreatment of corn stover. Green Chem 2015; 17(1):394-403.

[36] Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C. Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. Biotechnology for biofuels 2010; 3(1):27.

[37] Kumar R, Hu F, Sannigrahi P, Jung S, Ragauskas AJ, Wyman CE. Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. Biotechnol Bioeng 2013; 110(3):737-753.

[38] Korotkova E, Pranovich A, Wärnå J, Salmi T, Murzin DY, Willför S. Lignin isolation from spruce wood with low concentration aqueous alkali at high temperature and pressure: influence of hot-water pre-extraction. Green Chem 2015; 17(11):5058-5068.

[39] Smith MD, Mostofian B, Cheng X, Petridis L, Cai CM, Wyman CE, Smith JC. Cosolvent pretreatment in cellulosic biofuel production: effect of tetrahydrofuran-water on lignin structure and dynamics. Green Chem 2016; 18(5):1268-1277.

[40] Nguyen TY, Cai CM, Osman O, Kumar R, Wyman CE. CELF pretreatment of corn stover boosts ethanol titers and yields from high solids SSF with low enzyme loadings. Green Chem 2016; 18(6):1581-1589.

[41] Koppram R, Tomás-Pejó E, Xiros C, Olsson L. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol 2014; 32(1):46-53.

[42] Koppram R, Olsson L. Combined substrate, enzyme and yeast feed in simultaneous saccharification and fermentation allow bioethanol production from pretreated spruce biomass at high solids loadings. Biotech Biofuels 2014; 7(1):54.

[43] Olofsson K, Bertilsson M, Lidén G. A short review on SSF–an interesting process option for ethanol production from lignocellulosic feedstocks. Biotech Biofuels 2008; 1(1):7.

[44] Kumar R, Mago G, Balan V, Wyman CE. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresour Technol 2009; 100(17):3948-3962.

[45] He Y, Pang Y, Liu Y, Li X, Wang K. Physicochemical characterization of rice straw pretreated with sodium hydroxide in the solid state for enhancing biogas production. Energy Fuels 2008; 22(4):2775-2781.

[46] Windeisen E, Strobel C, Wegener G. Chemical changes during the production of thermo-treated beech wood. Wood Sci Technol 2007; 41(6):523-536.

[47] Sun X, Xu F, Sun R, Fowler P, Baird M. Characteristics of degraded cellulose obtained from steam-exploded wheat straw. Carbohydr Res 2005; 340(1):97-106.

[48] Pandey K. A study of chemical structure of soft and hardwood and wood polymers by FTIR spectroscopy. J Appl Polym Sci 1999; 71(12):1969-1975.

[49] Stewart D, Wilson H, Hendra P, Morrison I. Fourier-transform infrared and Raman spectroscopic study of biochemical and chemical treatments of oak wood (Quercus rubra) and barley (Hordeum vulgare) straw. J Agric Food Chem 1995; 43(8):2219-2225.

[50] Sene C, McCann MC, Wilson RH, Grinter R: Fourier-Transform Raman and Fourier-Transform Infrared Spectroscopy (An Investigation of Five Higher Plant Cell Walls and Their Components). Plant Physiol 1994; 106(4):1623-1631.

[51] Guo G, Chen W, Chen W, Men L, Hwang W. Characterization of dilute acid pretreatment of silvergrass for ethanol production. Bioresour Technol 2008; 99(14):6046-6053.

[52] Liu R, Yu H, Huang Y. Structure and morphology of cellulose in wheat straw. Cellulose 2005; 12(1):25-34.

[53] Geng Z, Sun R, Sun X, Lu Q. Comparative study of hemicelluloses released during two-stage treatments with acidic organosolv and alkaline peroxide from Caligonum monogoliacum and Tamarix spp. Polym Degrad Stab 2003; 80(2):315-325.

Chapter 6 - Conclusions and future work

6.1 Conclusions

Integration of cellulosic ethanol production and existing starch-based ethanol facilities could accelerate the commercialization of lignocellulosic ethanol production. High gravity bioconversion is superior to low-solid loadings but fermentation efficiency usually decreases as increasing biomass loadings due to limited mass transfer or accumulated inhibitors of degraded products. Fermentation of cellulosic biomass alone usually resulted in low ethanol titer and low ethanol yield as demonstrated in this study that only 37.9 g/L of final ethanol concentration was obtained even with high solids loading of 16% (w/v). The highest ethanol concentration (68.7 g/L) was achieved at the corn flour and corn stover ratio of 12:12 using raw starch granular enzyme with the ethanol yield of 86.0%. Alternatively, co-fermentation of starchy substrate with juice extracted from saccharification of treated biomass was able to significantly enhance ethanol concentration without scarifying ethanol yields. Co-fermentation of corn flour mixed with juice extracted from enzymatic saccharification of hydrothermal treated corn stover boosted the ethanol titer from 19.9 to 123.2 g/L and from 36.8 to 130.2 g/L at the saccharification solid loadings of 8 and 16%, respectively, which could significantly reduce the energy cost of ethanol distillation. In addition, Low concentrated glucose (42.0 g/L) at the low solid loadings of 8 % but high sugar yield (92.7%) was able to meet the minimum ethanol concentration of 40 g/L required for economical distillation process after co-fermentation with corn flour as proved in this study to reach ethanol concentration of 123.2 g/L. This novel method could be further extended to any treated biomass from low to high pH pretreatment as demonstrated in this study.

The major challenges to commercialize cellulosic biofuels are low fermentation efficiency, low ethanol titer, and lack of technology to utilize the byproduct lignin. A novel

process was designed to fully utilize lignocellulosic biomass for biofuels and bio-chemicals production, which involves green technology such as hydrothermal and organosolv pretreatments to produce cellulose-rich solids with good recovery of clean lignin for improvement of plant protein-based adhesives as well as xylose for furfural upgradation. Modified simultaneous saccharification and fermentation (mSSF) was developed as part of the whole biorefinery process, which combines the advantages of both separate hydrolysis and fermentation and SSF via unique decantation technologies. The mSSF processing technology enabled to achieve the highest cellulosic ethanol titer of 75.3 g/L (73.0%, yield) at biomass loadings of 30% (w/v) among the published results to the extent of our knowledge.

6.2 Future Work

(1) High gravity bioprocessing, including pretreatment, hydrolysis and fermentation, is a trend to produce high concentrated product with reducing water usage. However, subsequent issues such as poor mass transfer, high slurry viscosity and accumulated inhibitory compounds remain to be addressed. Alternative pretreatment or effective bio-detoxification methods are needed to minimize the generation of degradation products. Advanced bioreactor with superior agitation is also needed to improve the mass transfer and reduce biomass precipitation.

(2) Integration of first and second generation feedstocks is a cost-effective and energy-saving method to boost cellulosic ethanol titers and yields. The quality of fermentation residues is remained to further examination, including protein, fiber and carbohydrate contents.

(3) A bio-refinery concept to utilize each component of biomass is desired to improve the economics. Lignin, as a fermentation by-product, still remains a major challenge and opportunity which needs further research to explore its usage, especially its subunits such as phenol compounds can be used as building block for many chemicals.