SUSTAINABLE BIOPROCESSING OF VARIOUS BIOMASS FEEDSTOCKS: 2,3-BUTANEDIOL PRODUCTION USING NOVEL PRETREATMENT AND FERMENTATION

by

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B.Tech., Tribhuvan University, Dharan, Nepal, 2000
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

Abstract

Lignocellulosic biomass feedstocks are a sustainable resource required for rapid growth of bio-based industries. An integrated approach, including plant breeding, harvesting, handling, and conversion to fuels, chemicals and power, is required for the commercial viability of the lignocellulosic-based biorefineries. Optimization of conversion processes, including biomass pretreatment and hydrolysis, is a challenging task because of the distinct variations in composition and structure of biopolymers among biomass types. Efficient fermentation of biomass hydrolyzates comprising of different types of sugars is challenging. The purpose of this doctoral research was to evaluate and optimize the various processing steps in the entire the biomass value chain for efficient production of advanced biofuels and chemicals from diverse biomass feedstocks.

Our results showed that densification of bulky biomass by pelleting to better streamline the handling and logistic issues improved pretreatment and hydrolysis efficiencies. Alkali pretreatment was significantly more effective than acid pretreatment at same processing conditions for grass and hardwood. The ethanol-isopropanol mixture, and glycerol with 0.4% (w/v) sodium hydroxide were the promising organic solvent systems for the pretreatment of corn stover (grass), and poplar (hardwood), respectively. None of the pretreatment methods used in this study worked well for Douglas fir (softwood), which indicates a need to further optimize appropriate processing conditions, better solvent and catalyst for effective pretreatment of this biomass. The brown midrib (*bmr*) mutations improved the biomass quality as a feedstock for biochemicals production in some sorghum cultivars and *bmr* types, while adverse effects were observed in others. These results indicated that each potential sorghum cultivar should be separately evaluated for each type of *bmr* mutation to develop the best sorghum line as an energy crop. Development of an appropriate

biomass processing technology to generate separate cellulose and hemicellulose hydrolyzates is required for efficient 2,3-butanediol (BD) fermentation using a non-pathogenic bacterial strain, *Bacillus licheniformis* DSM 8785. This culture is significantly more efficient for BD fermentation in single sugar media than *Klebsiella oxytoca* ATCC 8724. Though *K. oxytoca* is a better culture reported so far for BD fermentation from diverse sugars media, but it is a biosafety level 2 organism, which limits its commercial potential.

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List of Abbreviations

AT: Atlas sorghum cultivar

BD: 2,3-butanediol

bmr: brown midrib

CAD: cinnamyl alcohol dehydrogenase;

COMT: caffeic acid *o*-methyl transferase;

DMSO: dimethyl sulfoxide

EH: Early Hegari sorghum cultivar

FCR: Folin-Ciocalteu Reagent

G: guaiacyl

GHG: greenhouse gas

H: *p*-hydroxyphenyl

HMF: hudroxymethylfurfural

HSQC: heteronuclear single quantum coherence

KC: Kansas Collier sorghum cultivar

LCA: life cycle assessment

LSD: least significant difference

NMR: Nuclear Magnetic Resonance

S: syringyl

SOC: soil organic carbon

SRWC: short-rotation woody crop

WSG: warm-seasons grass

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Dedication

I would like to dedicate this dissertation to my beloved mother Devi Guragain and father Prem Prasad Guragain.

Chapter 1 - Introduction: Background and Research Objectives

Background

Global biofuels and biochemical production scenario

Transportation fuels and climate change are topics of keen discussion in both the political and scientific communities throughout the world. A gradual decrease in global petroleum reservoirs leading to their depletion in near future requires a search for sustainable alternatives to fossil fuels to be a high priority. In addition, increasing conflict in most of oil-producing countries has further worsened the fuels crisis, particularly for non-oil-producing countries (Mousdale, 2008). The international energy crises in 1973 quadrupled United States (US) oil prices from $4.50 to $22.50 per barrel leading to huge damage to the US economy. This forced the US government to search for alternative sources of energy, including corn ethanol production (Klein-Marcuschamer & Blanch, 2015). Brazil also responded to the energy crises by developing a National Alcohol Program, called, "Proalcool" in 1975. Under this program, the Brazilian government extensively supported farmers to plant more sugarcane, investors to build more distilleries for sugarcane ethanol production, and automakers to design cars to run on 100% ethanol (Solomon et al., 2007). US and Brazil are still the main fuel ethanol producers in the world, and their major feedstocks are corn and sugarcane, respectively (Guragain et al., 2016). The US interest in bioethanol production to supplement gasoline slowly increased from 1980 to 2000, and the rapid growth was observed from 2000s as shown in **Figure 1.1**. Similarly, this trend was observed in the rest of the world. In 2014, 24.6 billion gallons fuel ethanol was produced globally, out of which, the US share was 58% of global production. Brazil was the second highest producer (25% of global production), followed by Europe (6%), China (3%) and Canada (2%) (Guragain et al., 2016).

US fuel ethanol production in 2014 represented only 4% of the national liquid transportation fuel demand, but it consumed almost 30% of total corn grown in US in that year (Klein-Marcuschamer & Blanch, 2015). This production is likely to increase 2.5 times by 2022 to meet the new target of current US Renewable Fuels Standards (RFS): 36 billion gallons per year (Ge et al. 2011; Kamireddy et al., 2013). Assuming same corn production, and same corn to ethanol conversion efficiency, at least 80% of the total US corn production will be required to meet the goal provided alternate feedstocks are not utilized. In addition, a number of platform and bulk chemicals, including 2,3-butanediol, should be produced from biomass to minimize dependency on petroleum-derived products. 2,3-butanediol is an important platform chemical used for the production of a number of high-value products, including foods, pharmaceuticals, fuels, polymers, and chemicals (Ge et al., 2011). The global demand for 2,3-butanediol is estimated around 32 million tons per year (Li et al., 2013). These data showed that current approaches to production of bio-based fuels and chemicals using food-based feedstocks are inadequate to replace petroleum products without affecting the global food supply; lignocellulosic biomass feedstocks are a sustainable and low-cost alternative to the current food-based feedstocks.

Use of abundantly available lignocellulosic biomass for bio-based fuels and chemicals production has a number of benefits. First, it helps reduce global food security issues by replacing food and feed materials for fuels production. Second, it is available in enough quantity for biofuels and biochemicals production to substantially replace petroleum-derived products (Nanda et al., 2015). Third, it helps to reduce greenhouse gas (GHG) emission if the excess biomass from agricultural and forest residues, and the perennial plants grown in degraded croplands are used as feedstocks without significant direct or indirect land use change (Fargione et al., 2008). Finally, it safeguards our huge investments in ethanol and other biorefinery industries from the shortage of

raw materials for these industries. It is because the existing grain and sugarcane-based bioprocessing technologies have several similar processing steps with the lignocellulosic-based bioprocessing technology. The major difference between these technologies is the release of sugars from biomass as shown in **Figure 1.2**. Therefore, current grain-based biorefineries can switch to lignocellulosic-based biorefining with minimum capital investment if appropriate biomass preprocessing technologies are developed (Hess et al., 2006).

Conversion of lignocellulose biomass to fuels and chemicals

Basically, there are two primary routes for the conversion of lignocellulosic biomass to biofuels and biochemicals: (1) biochemical conversion, and (2) thermochemical conversion, as shown in **Figure 1.3**. The biochemical conversion technologies utilize microorganisms or other biocatalysts to convert biomass to a number of biofuels and biochemicals, including bioethanol, biobutanol, 2,3-butanediol, biodiesel, and biogas. Thermochemical conversion technologies utilize heat and chemical catalysts to convert biomass to valuable secondary energy/fuel sources (Chew & Doshi, 2011; Menon & Rao, 2012). Each biomass conversion route has a number of inherent strengths and weaknesses; the appropriate conversion route is determined by many factors, including feedstock types, availability of technology, and energy requirements.

This doctoral research focuses on biochemical conversion route using fermentation as the conversion process to produces biofuels and biochemicals, including bioethanol and 2,3-butanediol. This route is also called the sugar platform route, in which lignocellulosic biomass is deconstructed using appropriate pretreatment method followed by enzymatic hydrolysis to release monomer sugars from carbohydrate polymers (cellulose and hemicellulose). The released sugars are fermented to desired biofuels or biochemicals using specific microbial culture (Dale & Ong,

2012). Integrated biorefinery comprises of four major core sections for the conversion of biomass to fuels and chemicals through sugar platform route: feedstocks handling and storage, pretreatment, hydrolysis, and fermentation, as shown in **Figure 1.4** (Ragauskas et al., 2014a). Each section of this biomass conversion process is associated with a number of challenges (Mousdale, 2008), among them pretreatment is the most energy-intensive single step that incurs 18% of total investment for cellulosic ethanol production (Yang & Wyman, 2008). Each section of biomass conversion via sugar platform route is briefly discussed below.

Biomass handling and storage

Efficient handling and transportation of lignocellulosic biomass is one of the major challenges for current biorefinery industry because of very low bulk density of these feedstocks, ranging from 50 to 130 kg/m^3, depending on biomass type, particle size and particle density (Sokhansanj & Turhollow, 2004). Hess et al. (2006) estimated that average baling and stacking cost for wheat and barley straw is around $23 per ton, and transportation cost around $11 per ton. It was also estimated that the actual cost would be even higher depending upon a number of factors, including transportation distance, local labor cost, and construction cost for handling and storage facilities. In addition, state regulation for maximum transport volume also restricts for loading transportation vehicle with enough biomass quantity (Hess et al., 2006). Densification of biomass in agricultural fields is, therefore, essential to better streamline the handling and logistics issues for biofuels and biochemicals production. Biomass pelleting is considered as one of the effective way to accomplish this task. The pelleting process resulted in 9 to 12-fold bulk density increase, and makes biomass as flowable as grain (Theerarattananoon et al., 2011). This leads to the utilization of grain handling equipment and storage facilities existing in current grain-based biorefineries to handle and store lignocellulosic biomass as well. Therefore, biomass pelleting

process helps in switching current biorefineries from grain-based processing technology to lignocellulosic-based processing technology without additional capital investment (Hess et al., 2003; Hess et al., 2006).

Biomass pretreatment

Efficient conversion of cellulose and hemicellulose of lignocellulosic biomass into fermentable sugars depends on effective modification of complex structure of plant cell wall – the process called deconstruction or pretreatment. The organization and interactions among the structural components of biomass (cellulose, hemicelluloses and lignin) make the plant cell wall recalcitrant to biological degradation. Various methods of pretreatment, including biological, physical, chemical and physiochemical are available today, and new methods are emerging to make the biomass deconstruction process more efficient and cost effective. The pretreatment process is reviewed in detail in Chapter 2.

Hydrolysis of pretreated biomass

The depolymerization of carbohydrates polymers, cellulose and hemicellulose, into their monomer units is called hydrolysis. Cellulose is a homopolysaccharide composed of glucose units linked by β 1,4-glycosidic bonds. The major part of cellulose is a highly crystallized structure, with some amorphous regions. In contrast, the hemicellulose is a heteropolymer sugar, which predominantly comprises of xylose, and small amount of glucose, mannose, arabinose, and other monomer sugars. The hemicellulose has a highly amorphous structure, and hence is easily hydrolyzed compared to cellulose (Hu et al., 2008). Depending upon the pretreatment methods and processing conditions, the carbohydrate polymers (mainly hemicellulose) is partially hydrolyzed during pretreatment process. The remaining hemicellulose and cellulose polymers are usually hydrolyzed using hemicellulase and cellulase enzymes, respectively. Synergistic reactions

of three type of cellulase enzymes, including endo-β-1, 4-glucanases, exo- β-1, 4-glucanases, and β-glucosidases, are required for efficient hydrolysis of cellulose polymers. Endo-glucanases randomly cut internal amorphous site of cellulose chain, generating oligosaccharides with various chain length. Exo-glucanases systematically cut the oligosaccharides chain, generated by endo-glucanases, to produce predominately cellobiose, and glucose as the major products. Exo-glucanases can also act on microcrystalline cellulose, by peeling cellulose chains from the microcrystalline structure, and making them accessible for endo-glucanases enzyme. Therefore, synergy among exo-glucanases and endo-glucanases is more important in crystalline region of cellulose compared to amorphous region. The β-glucosidases breakdown the cellobioses, generated by exo-glucosidase, into glucose units (Lynd et al., 2002; Zhang et al., 2007). The hydrolysis of xylan polymer of hemicellulose is accomplished by the group of enzymes, including, endo-1, 4- β-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase. Similarly, glucomannan polymer of hemicellulose is hydrolyzed by β -mannanase and α-mannosidase cleave glucomannan polymer (Kumar et al., 2008). Currently, robust enzymes cocktail comprising of all required enzymes for cellulose and hemicellulose hydrolysis are commercially available, such as Cellic CTec2 and Cellic HTec2 enzymes produced by Novozymes, Inc., Franklinton, NC, USA.

Fermentation of biomass hydrolyzates

The sugars released from biomass is finally fermented using specific microbial cultures depending upon the product of interest. For example, for the production of 2,3-butanediol, *Bacillus licheniformis, Klebsiella oxytoca,* and *Serratia marcescents* are some of the important microorganisms of interest (Jurchescu et al., 2013). Similarly, *Saccharomyces cerevisiae* and *Zymomonas mobilis* are utilized for ethanol production (Guragain et al., 2016).

Hydrolysis and fermentation are usually carried out separately one after another; such process is called Separate Hydrolysis and Fermentation (SHF). In many instances, both hydrolysis and fermentation processes are performed simultaneously in the same tank depending upon the processing technology and product of interest; such a process is called Simultaneous Saccharification and Fermentation (SSF). Many researchers reported that the SSF process is more cost effective than SHF. Tomas-Pejo et al. (2008) reported that SSF process for ethanol production reduced the production cost by 19% compared to SHF.

Global availability of lignocellulosic biomass

The global biomass production is estimated to be around 146 billion tons per year, and a very small part of it is currently being used for biofuels and biochemical production (Chew & Doshi, 2011). The estimated global production of four major crops residues - corn stover, rice straw, wheat straw, and sorghum stover in 2011 were 1413, 1084, 1056, and 81 million tons, respectively. Forest residues were estimated at 274 million tons in 2011, but are projected as the major feedstock for future biorefineries with estimated production of 6 billion tons per year by 2050. In addition, municipal solid waste is available in very large quantity, and can also be a potential feedstock for biorefinery operations; the estimated municipal solid waste in 2011 was 1.3 billion tons (Kurian et al., 2013).

The annual lignocellulosic biomass available in US and Canada is about 577 and 561 million tons, respectively. The large portion of the estimated US biomass was from agricultural residues (such as corn stover and wheat straw), and energy crops (such as switchgrass and hybrid poplar), whereas forest residues comprise the major portion of Canadian biomass (Gronowska et al., 2008). Brazil is the world's largest sugarcane producer; annual production of around 495 billion tons; therefore, the sugarcane bagasse is the main lignocellulosic residues in Brazil, which

is estimated at 186 million tons annually (Soccol et al., 2010). European Union set a low-carbon roadmap to reduce 80% of domestic GHG emission by 2050, taking 1990's emission as the base line. To achieve this goal, Sweden adopted energy crops (Willow) plantation, and targeted to replace 50% energy consumption by renewable energy by 2020. Austria produces more than 20 million tons of lignocellulosic biomass for energy production, and produces nearly 15% of its energy using this biomass waste (Nanda et al., 2015). Sub-Saharan Africa has the greatest potential for energy crop production because of the availability of land, labor and favorable climatic conditions. However, due to lack of proper agricultural development and appropriate national energy policies, only a few countries in these territory have exploited the biofuels production opportunities (Jumbe et al., 2009). Several hundred million tons of lignocellulosic biomass is annually produced in Asian Pacific, including crop residues in China, forest and agriculture residues in Korea, Palm tree residues in Malaysia, crops residues and sugarcane bagasse in India, and forest residues in Australia (Nanda et al., 2015).

Sustainability of biofuels and chemicals production from lignocellulosic biomass

The lignocellulosic-based biofuels and biochemical industries must address all three aspects of sustainability criteria: economic, environmental, and social factors (Ladanai & Vinterbäck, 2009). Life cycle assessment (LCA) of biofuels is extensively studied to evaluate their sustainability; however, LCA primarily focuses on environmental impact of biofuel with some effort for economic aspect, but little attention is given to social aspect. The functional units of LCA of biofuels from biomass are net energy balance and greenhouse gas (GHG) emission (Bonin & Lal, 2012). For sustainability of the bioeconomy, the biorefining program and feedstock production must be designed with the participation of local and rural communities to address their societal needs (Nanda et al., 2015). The major driving factors for growing interest in

lignocellulosic-based fuels and chemicals production are energy security, environmental concerns because of increasing GHG emission, economic development and job creation in rural areas (Ladanai & Vinterbäck, 2009). Currently, crop residues are the major feedstock for lignocellulosic-based biorefineries. Use of crop residues as energy feedstock is advantageous because of the dual use of land for both food and fuel production. However, these biomass feedstocks are only seasonally available, and excessive removal of crop residues from farm lands degrade soil quality, including soil organic carbon (SOC) pools, water transmission characteristics, soil structural stability and soil microbial activity (Blanco-Canqui, 2010). In addition, intensive fertilizer inputs in crop land to grow these crops results in increased emissions of nitrous oxide (N_2O), a highly potent GHG (Evers et al., 2013). Plantation of dedicated energy crops, including perennial warm-season grasses (such as switchgrass and miscanthus), and short-rotation woody crops (such poplar and Douglas fir), are sustainable supplements of crops residues for biorefineries. These energy crops require less nitrogen fertilizer, and can be grown in marginal and degraded land to avoid land competition for prime agricultural crops (Blanco-Canqui, 2010; Don et al., 2012).

Despite the hot debate regarding the sustainability of biofuels and biochemicals from lignocellulosic biomass, feedstocks availability will not be the key issue if highly efficient conversion technology is developed. It is because current global land use to grow biofuels feedstocks is only 25 million hectors, which is 0.19% of worlds' total land area (Ladanai & Vinterbäck, 2009). In addition, aquatic plants, such as algae, are another promising feedstock, which has the potential of producing 30 times more biofuels and biochemicals than terrestrial lignocellulosic biomass feedstocks. The algae plants can be fed with CO_2 or NO, and also help to reduce GHG concentrations in the atmosphere (Kurian et al., 2013). Therefore, development of efficient, cost effective, and environmentally friendly biomass technologies to utilize all

components of lignocellulosic biomass to produce fuels, chemical, and power is vital for transition of the current fossil-economy to a sustainable bioeconomy (Lopes, 2015). The key for success of petroleum industries is their fundamental understanding of high value applications of hydrocarbon feedstocks, and development of highly efficient conversion technologies because of their long history on research and development. The petroleum-derived chemicals' market is less than 10% of crude oil, but significantly contributes to the overall profitability of petrochemical industries (Bozell et al., 2014). A biorefinery concept analogue to that of the petroleum refinery concept must be developed and implemented for the sustainable future. Therefore, the smart biorefinery industry should focus on high-volume and low-value biofuels production to meet growing energy demand and low-volume and high-value bio-based chemicals production for the economic viability of the industry.

A number of high-value platform chemicals can be produced from lignocellulosic biomass, which are currently sourced from petroleum. The biomass-derived monomers sugars, including glucose, xylose, and arabinose, can be converted into 2-carbon (C_2) to 6-carbon (C_6) platform chemicals through biocatalytic conversion process. Some C_2 to C_6 platforms chemicals produced using microorganisms are listed below; these platform chemicals are used as important feedstocks to produce a number of high-value products, including fuels, pharmaceuticals, cosmetics, foods, solvents, and polymers (Jang et al., 2012).

C_2: Ethanol, and acetic acid

C_3: Propionic acid, lactic acid, 3-hydroxypropionic acid, isopropanol, 1,2-propanediol, and 1,3-propanodiol.

C_4: 2,3-Butanediol, 1,4-butanediol, 1-butanol, isobutanol, succinic acid, butyric acid, malic acid, fumaric acid, and putrescine

C_5: Itaconic acid, 3-hydroxyvalerate, 1-pentanol, 2-methyl-1-butanol, 3-methyl-1-butanol, xylitol, and cadaverine

C_6: Glucaric acid, anthracitic acid, phenol, catechol

Among these wide range of platform chemicals, this doctoral research study focuses on 2,3-butanediol production from lignocellulosic biomass-derived sugars. 2,3-Butaneidiol is a four-carbon diol, which can be converted into various compounds, and widely used in a number of application, including food, fuel, chemical, pharmaceutical , and polymer as shown in **Figure 1.5** (Celińska & Grajek, 2009; Ji et al., 2011; Li et al., 2013; Qi et al., 2014)**.** It exists in three stereoisomers: L-(+)-2,3-butanediol (S,S – dextrorotatory form), D-(-)-2,3-butanediol (R,R – Levorotatory form), and meso-2,3-butanediol (optically inactive form). A number of microbial species, including *Klebsiella, Enterobacter, Bacillus* and *Serratia* genera can produce BD from different feedstocks. Among them, *K. oxytoca, K. pneumoniae, E. aerogenes, B. licheniformis,* and *S. marcescents* are the promising microorganisms for efficient BD fermentation (Ji et al., 2011; Jurchescu et al., 2013).

Lignin valorization is another important research area for the sustainability of lignocellulosic-based biorefinery industries. Lignin has been considered a low value byproduct, and mainly combusted as boiler fuel, despite the great potentiality of the lignin products for a number of high-value applications to produce fuels, chemicals, fibers, and polymers (Ragauskas et al., 2014a). In addition, the biomass also contains a number of valuable phytochemicals, including terpenes and terpenoids, fats and waxes, phenolics, and alkaloids (Beatson, 2011). Efficient extraction and isolation of these extractives prior to pretreatment for biomass deconstruction, is vital for the sustainability of lignocellulosic-based biorefineries.

Research objectives

A wide range of lignocellulosic biomass feedstocks are available for the rapidly growing biorefinery industries. Sustainable production of biofuels and biochemicals requires an integrated approach, including plant breeding, harvesting and handling, and conversion of diverse biomass feedstocks to fuels, chemicals and power. Densification of biomass at the agricultural field is required to better streamline the handling and logistic issues of bulky feedstocks, such as crop residues and perennial grasses. The distinct variation on structure and composition of these biomass feedstocks demands separate pretreatment and hydrolysis strategies for each type of feedstock. Development of efficient fermentation process for the utilization of biomass-derived sugars, comprising glucose, xylose and other monomer sugars, is critical for any biorefinery. Therefore, the overall objective of this doctoral research was to evaluate and optimize various steps of biomass processing for efficient production of 2,3-butanediol from lignocellulosic-based feedstocks.

Specific objectives of this study are listed below, which are discussed in each chapter of this dissertation.

1. To review and evaluate some promising biomass pretreatment technologies (Chapter 2)
2. To evaluate effect of biomass densification by pelleting on pretreatment and hydrolysis of biomass (Chapter 3)
3. To develop appropriate biorefining strategies for multiple feedstocks based on acid and alkali pretreatment (Chapter 4)
4. To develop novel biomass pretreatment methods using alkaline organic solvents (Chapter 5)
5. To review and evaluate lignin composition and structure of lignin from various bioenergy crops (Chapter 6)

6. To evaluate lignin-specific sorghum mutants as a potential biomass feedstock for 2,3-butanediol production (Chapter 7)
7. To optimize 2,3-butanediol fermentation from biomass-derived sugars using *Klebsiella oxytoca* ATCC 8724 (Chapter 8)
8. To evaluate *Bacillus licheniformis* DSM 8785 bacteria for 2,3-butanediol fermentation from biomass-derived sugars (Chapter 9)
9. To provide effective conclusions and to propose future research on biomass processing (Chapter 10)

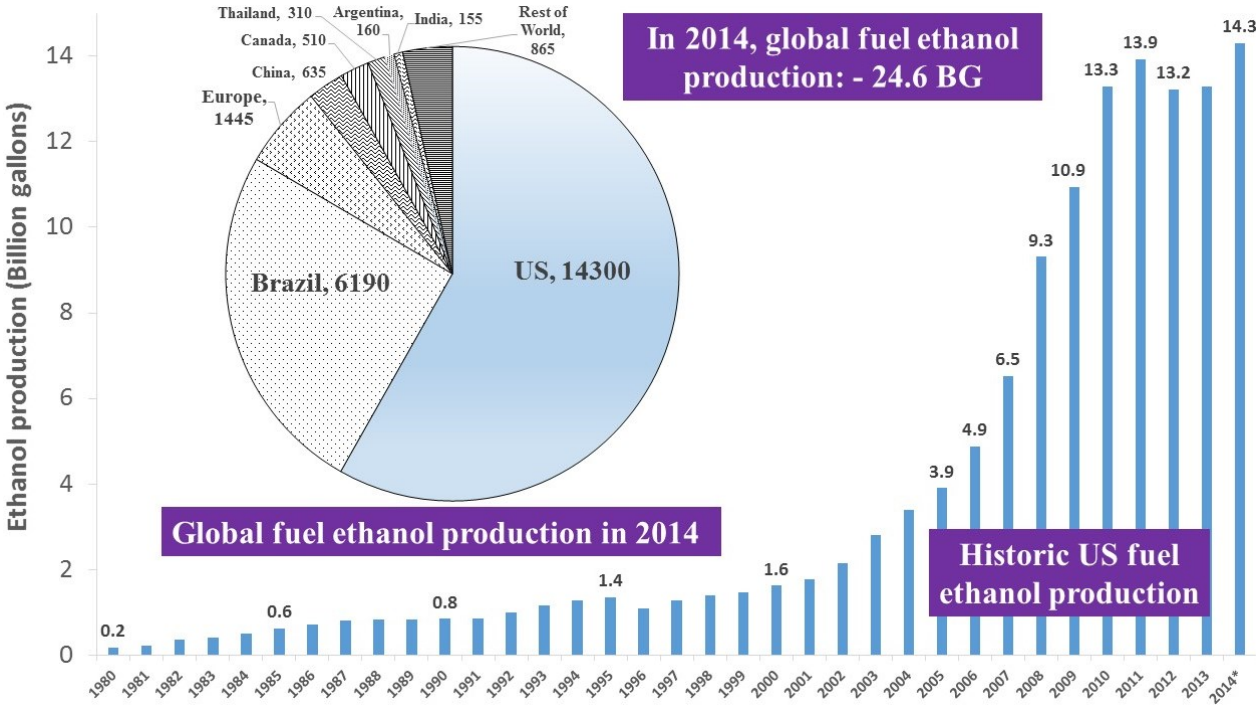

Figure 1.1 Fuel ethanol production current and past (Guragain et al., 2016)

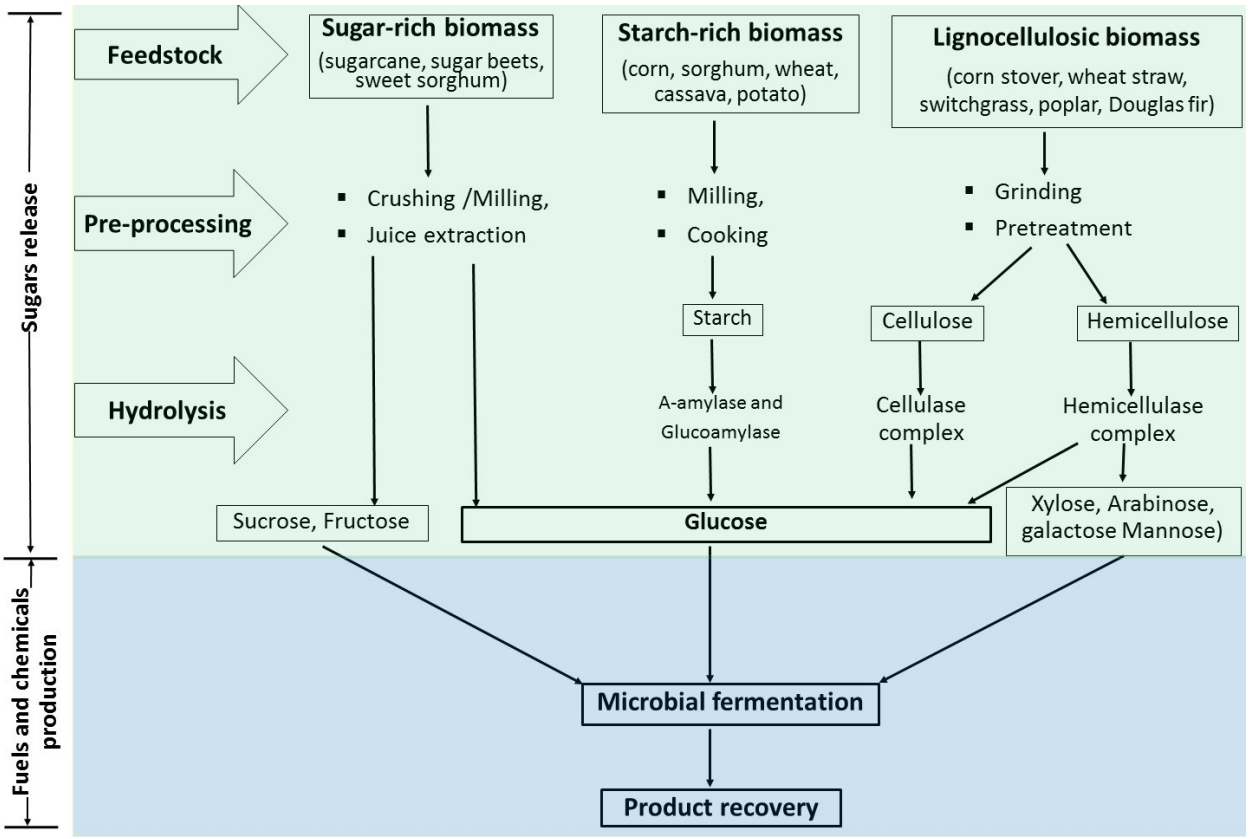

Figure 1.2 Biofuels and biochemicals production processes from diverse biomass feedstocks.

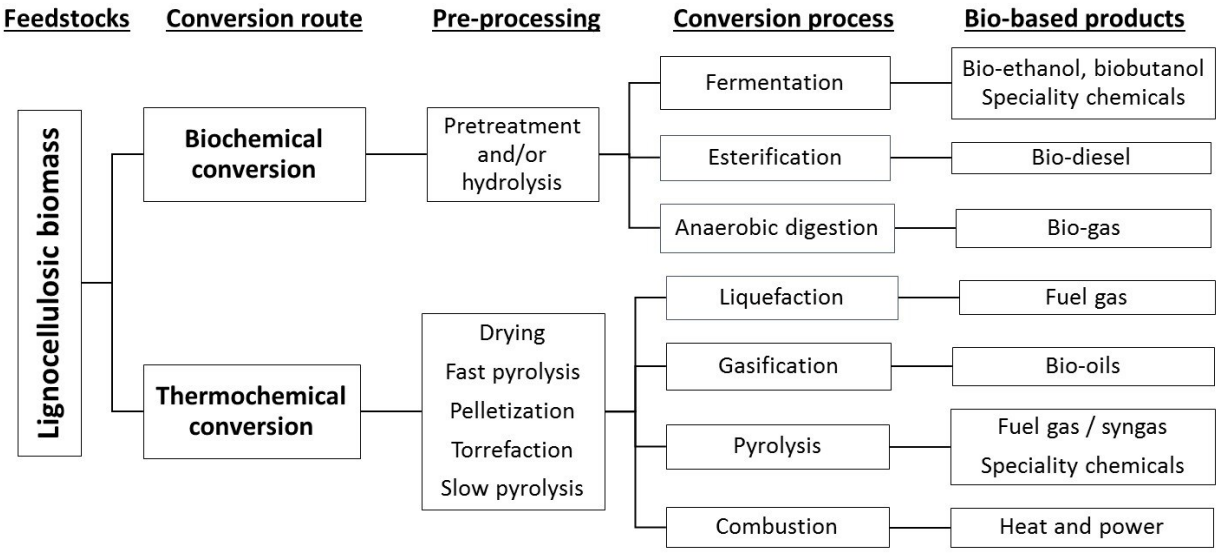

Figure 1.3 Conversion routes for lignocellulosic biomass feedstocks to bio-based products.

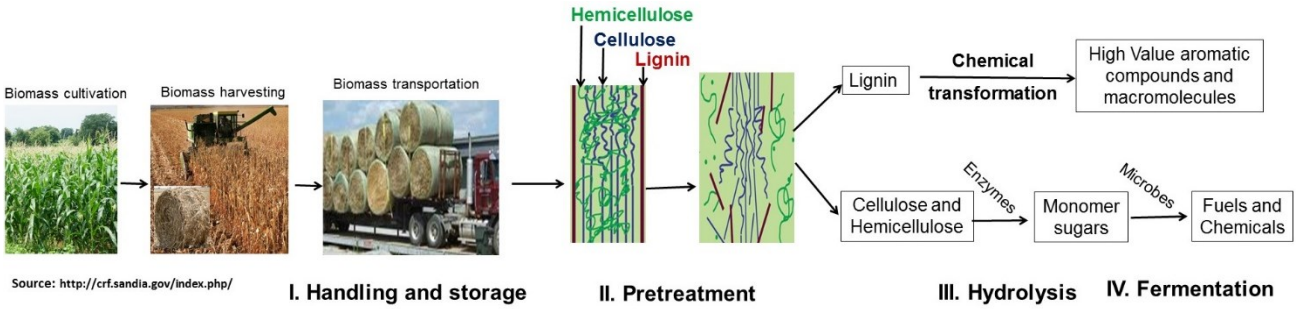

Figure 1.4 Integrated biorefinery to produce biofuels and biochemicals through sugar platform route.

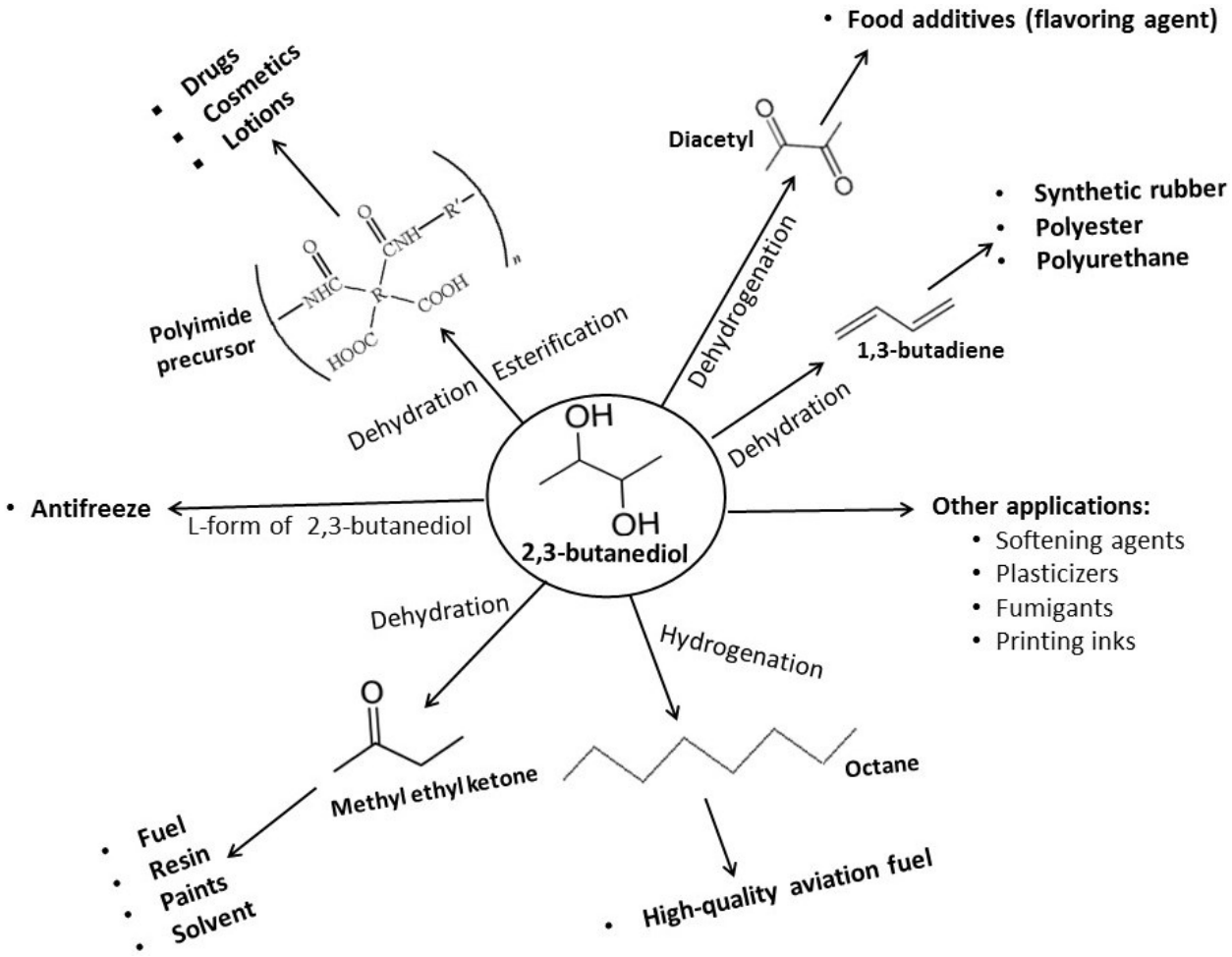

Figure 1.5 Some important derivatives of 2,3-butanediol, and their potential applications

(Celińska & Grajek, 2009; Ji et al., 2011; Li et al., 2013; Qi et al., 2014).

Chapter 2 - Evaluation of promising lignocellulosic biomass pretreatment: A review

Abstract

Deconstruction of complex lignocellulosic biomass structure to overcome its recalcitrant nature for enzymatic hydrolysis of carbohydrate polymers is the primary objective of biomass pretreatment. It is the central unit operation that significantly affects the efficiency of all subsequent steps of bioprocessing of lignocellulosic biomass to produce fuels and chemicals. In addition, pretreatment is the most expensive single unit operation in the context of existing biomass conversion technologies *via* sugar platform route. The development of an efficient and cost effective biomass pretreatment method is critical for the fractionation of the each component of biomass without quality degradation for their high-value applications. Pretreatment methods are broadly divided into four major categories based on the mechanism of biomass deconstruction: physical, chemical, physico-chemical, and biological pretreatment methods. Optimization of a single pretreatment method for all types of feedstock is complicated due to the variations in the compositions and structures of lignin, cellulose, and hemicellulose among biomass types. Therefore, each type of biomass feedstock must be separately evaluated to design appropriate biomass deconstruction strategies. In this review chapter, we evaluated some of the most promising biomass pretreatment methods, including their mechanisms, advantages and disadvantages.

Keywords: Lignocellulosic biomass, cellulose, hemicellulose, lignin, pretreatment.

Introduction

The primary objective of pretreatment process is to deconstruct the complex biomass structure comprising lignin, hemicellulose, and cellulose so that each biopolymer can be

effectively utilized to produce fuels, chemicals and power. Each step of biomass processing, including pretreatment, hydrolysis and fermentation, have many challenges that need to be overcome for commercial viability of lignocellulosic-based biorefineries. Among these processes, pretreatment is considered the central unit operation, which significantly affect the effectiveness of all other subsequent steps of biomass processing (Sousa et al., 2009). Montague et al. (2002) estimated that if dilute acid pretreatment is used before enzymatic hydrolysis, the capital investment for pretreatment is 17% of the total capital investment ($ 19 million out of $ 113 million), and incurs the highest investment of a single step of bioethanol production from corn stover, other than procurement and installation of Boiler/Turbogenerator. Humbird et al. (2011) proposed an improved two-step acid pretreatment with some process modifications, and estimated that capital investment for biomass pretreatment is around 13% of total capital investment ($ 30 million out of $ 232 million). The percentage reduction in capital investment for pretreatment compared to the earlier report by Montague et al. (2002) was not due to the significant improvement in pretreatment technology, but due to an increase in capital cost for wastewater treatment. These results indicated that development of cost effective pretreatment method is vital for lignocellulose-based biorefineries. In addition, lignocellulosic-based biorefinery currently focuses on cellulose-derived sugars, and the lignin stream is combusted as boiler fuel, which is a very low-value application of the energy-rich biopolymer. Lignin comprises 40% of lignocellulosic biomass by energy even though it is only 15 to 30% by weight (Laskar et al., 2013). Therefore, lignin valorization is critical for the sustainability of biorefining industries, which in turn is related to the appropriate pretreatment method to extract good quality biomass lignin (Ragauskas et al., 2014a).

A number of biomass pretreatment methods are available, including physical, chemical, physico-chemical, and biological (Mood et al., 2013); some of them are as follow

1. **Physical methods**: Extrusion, ball milling, wet-disc milling, microwave pretreatment
2. **Chemical methods**: Acid pretreatment, alkali pretreatment, organosolv pretreatment, ozonolysis pretreatment
3. **Physico-chemical methods**: Steam explosion, ammonia fiber explosion, liquid hot water, carbon dioxide explosion, wet oxidation
4. **Biological methods**: White-rot fungi, brown-rot fungi, soft-rot fungi

Some of the promising pretreatment process is briefly discussed below.

Extrusion pretreatment

Extrusion cooking is one of the promising physical pretreatment process of lignocellulosic biomass for biofuels and biochemicals production (Zheng & Rehmann, 2014). It is widely used process in snack food industries. In this process, material is forced through a die with desired cross-section profile. When the material passes through the extruder, a number of unit operations, including heating, mixing and shearing, take place simultaneously resulting in physical and chemical alteration of the material. Finally, the material experiences an abrupt expansion while exiting the die (Zhan et al., 2006). The complex networks of biopolymers in lignocellulosic biomass is disrupted during extrusion process, thereby making the biomass susceptible for enzymatic hydrolysis without production of pretreatment-induced inhibitory compounds for subsequent biomass hydrolysis and fermentation (Karunanithy et al., 2008).

There are two type of extruders: single-screw extruder and twin-screw extruder, with three type of screw elements: (a) forward screws elements – transports bulk materials through different

pitches and lengths with minimum mixing and shearing effect; (b) kneading screw elements – very slowly conveys materials forward with high mixing and shearing effect through different stagger angles; and (c) reverse screw elements – pushes material backward with very high mixing and shearing effect (Zheng & Rehmann, 2014). Effective biomass pretreatment can be achieved by optimizing appropriate screw configuration, including pitches, lengths, stagger angles, positions and spaces. A batch-type kneader with combination of twin-screw elements is suitable for biomass pretreatment at large scale because of excellent temperature control, very high grinding, mixing and shearing forces, high throughputs, and scalability (Lee et al., 2010). In twin-screw extruders, two parallel screws with same length are fixed on a stationary barrel. The direction of the screw is either co-rotating or counter-rotating. The screw speed (in rpm) and the barrel temperature are the main factors to be optimized for a specific extruder and specific material to develop high shearing force for achieving maximum extrusion effect (Karunanithy & Muthukumarappan, 2010).

Poor flow of lignocellulosic material during extrusion process is one of the major challenges for extrusion pretreatment of lignocellulosic biomass. This frequently leads to burning of the substrate and even blocking of the die during extrusion (Yoo et al., 2011). This problem can be overcome by using high moisture content in the material (Lamsal et al., 2010; Yoo et al., 2011), 2010), by adding processing aid, like starch (Lamsal et al., 2010), or cellulose affinity additives, like ethylene glycol, glycerol, and dimethyl sulfoxide (Lee et al., 2009). Alternatively, the biomass is soaked with alkali solution (for example, sodium hydroxide) prior to feeding into extruder to overcome the poor flow of biomass during extrusion as well as to improve the delignification of biomass (Kang et al., 2013; Liu et al., 2013). However, inconsistent results are reported in literature for the extrusion pretreatment using cellulose affinity additives. For example, Lee et al. (2009) reported 62.4% cellulose conversion to glucose from Douglas fir using ethylene glycol as cellulose

affinity additives while the same additives did not help much for soybean hulls as reported by Yoo et al. (2011).

Advantages:

- Easy process monitoring and control
- No inhibitory compounds formation due to sugar degradation
- Adaptability for process modification
- Continuous and high throughput
- No need for washing of pretreated biomass if extrusion is carried out without chemical addition
- Can be combined with other methods of pretreatment for better results (Karunanithy, et al., 2014)

Disadvantages:

- Lack of data for economic analysis
- Energy intensive process
- Poor flow during continuous processing leading to burning of material (Yoo et al., 2011).

Acid pretreatment

Dilute acid pretreatment is the most extensively studied and widely used lignocellulosic biomass pretreatment. In this method biomass is mixed with dilute acid (<4%, v/v) and heated at desired time and temperature, ranging from few seconds to several minutes, and 140°C to 215°C, respectively (Agbor et al., 2011). Usually sulfuric acid is used; however, other organic acids, like formic or maleic acids were also found equally effective (Alvira, et al., 2010). National Renewable Energy Laboratory recently designed two-step dilute acid pretreatment method for corn stover to minimize sugar degradation during pretreatment (Humbird et al., 2011). In this method, biomass

slurry containing 18 mg sulfuric acid/dry g biomass is heated at 158°C for 5 min followed by second step heating at 130°C for 20-30 with 4.1 g additional acid/dry g biomass.

Advantage:

- High reaction rate to solubilize hemicellulose fraction of biomass thereby making cellulose fraction accessible for cellulase enzymes (Agbor et al., 2011; Alvira et al., 2010). Therefore, this method of deconstruction can be designed for biomass processing to generate separate hemicellulose hydrolyzates (after pretreatment) and cellulose hydrolyzates (after enzymatic hydrolysis).
- Cost saving for xylanase enzymes: Hemicellulose is extensively hydrolyzed during pretreatment depending upon the feedstock type and processing conditions; therefore, xylanase enzyme is not generally required for hydrolysis (Sousa et al., 2009).

Disadvantage:

- Inhibitors production, such as formation of furfural and hydroxymethylfurfural (HMF) from sugar degradation resulting in sugar loss as well as additional detoxification cost required to make the released sugars fermentable (Hu & Ragauskas, 2012).
- Need expensive stainless steel vessels due to corrosive nature of acid (Esteghlalian et al., 1997).
- Additional cost for alkali to neutralize acid after pretreatment
- Environmental concern due to excessive use of chemicals

Alkali pretreatment

Alkali pretreatment is another extensively studied and widely used lignocellulosic biomass pretreatment. Sodium hydroxide (NaOH), potassium hydroxide (KOH) and ammonium hydroxide (NH_4OH) are suitable for biomass pretreatment (Alvira et al., 2010). NaOH is considered as the

best alkali because it results in higher delignificaiton and deacetylation (Hu et al., 2008) as well as decrease in crystallinity of cellulose due to swelling of residual biomass during pretreatment (Alvira et al., 2010). Process is similar to acid pretreatment, but usually at lower temperature.

Advantage:

- Effective delignification (Hu et al., 2008)
- Lower sugar degradation products formation compared to dilute acid pretreatment due to the lower processing temperature; possible to pretreat at room temperature using longer time
- Lignin and other extractives can be separated before enzymatic hydrolysis without loss of carbohydrate; high possibility of getting reactive lignin for high value application (Ghaffar & Fan, 2014).

Disadvantage:

- Excessive phenolic compounds due to lignin degradation, which are potential inhibitors for enzymatic hydrolysis unless separated prior to enzymatic hydrolysis (Alvira et al., 2010).
- Additional cost for hemicellulose hydrolytic enzymes in addition to cellulase enzymes (Esteghlalian et al., 1997).
- Additional cost for acid to neutralize alkali after pretreatment
- Environment concern due to excessive use of chemicals

Organosolv pretreatment

Organosolv pretreatment is one of the promising biomass pretreatment methods, in which biomass is mixed with selected organic solvent with or without additional catalysis (acid or alkali), and heated for selected time and temperature. Various organic solvents or solvent mixtures can be used, including low boiling point solvents, such as ethanol, methanol and acetone, high boiling

point solvents, such as glycerol, ethylene glycol and tetrahydrofurfuryl alcohol, and other classes of organic solvents, such as organic acids, phenols, ketones and dimethyl sulfoxide (Zhao et al., 2009b). Ethanol organosolv pretreatment using acid catalyst is the most widely used method among the organosolv pretreatments. It is carried out at 90°C to 120°C for grasses and 155°C to 220° C for woods with processing time 25 min to 100 min, ethanol concentration 25% to 74% and catalyst concentration 0.83% to 1.67% (v/v) (Sousa et al., 2009). Catalyst addition is not required if processing temperature is high (>185°C) because organic acids released from biomass act as catalyst at that temperature (Duff & Murray, 1996). Organosolv pretreatment almost completely removes hemicellulose and extensively removes lignin, thereby leaving digestible cellulose residues (Zhao et al., 2009b). Studies on the change in cellulose crystallinity during organosolv pretreatment is limited. Ni and Van Heiningen (1997) reported that ethanol-water organosolv pretreatment leads to swelling of cellulose, and the effect is inversely related to ethanol concentration. Cellulose crystallinity is not an important factor for the digestibility of pretreated biomass with low residual lignin content if hydrolysis is carried out for sufficiently long periods (Zhu et al., 2008); however, productivity of sugar release is decreased.

Organosolv pretreatment using high boiling point alcohols, mostly polyhydroxy alcohols, is also gaining attraction because the process can be performed at atmospheric condition. Glycerol is one of the most extensively used high boiling point alcohols for the delignification of lignocellulosic biomass (Zhao et al., 2009b). Its high boiling point (290°C) favors biomass pretreatment at high temperature and atmospheric pressure, called atmospheric aqueous glycerol autocatalytic organosolv pretreatment (AAGAOP) (Sun & Chen, 2008). Use of low cost crude glycerol, a major byproduct of oleochemical industries, for biomass pretreatment is considered an attractive economic route for biofuels and biochemicals production (Guragain et al., 2011).

Oleochemical industries produce crude glycerol around 10% of total biodiesel production. High value application of crude glycerol for food, pharmaceutical and cosmetic use is economically infeasible due to the very expensive purification process (Sun & Chen, 2008). The rapid growth in global biodiesel production (Bournay et al., 2005) indicated that crude glycerol will be available even in larger quantity at lower cost in future. However, recycling of glycerol is very challenging and energy intensive.

One of the major drawbacks of organosolv pretreatment is loss of hemicellulose in the lignin stream, which is hard to recover as fermentable sugars due to presence of a number of inhibitory compounds, including phenolics. For the separation of all three major biopolymers (cellulose, hemicellulose and lignin) into separate streams, Hongzhang & Liying (2007) proposed a combination of steam explosion and ethanol organosolv pretreatment. In this method the biomass was first pretreated by steam explosion method to hydrolyze hemicellulose, followed by ethanol organosolv pretreatment to extract lignin. The residual cellulose is easily hydrolyzed using cellulase enzymes to monomers sugar. However, use of multiple steps results in increase in pretreatment cost. Diner & Fan (2012) developed a single step alkaline organosolv pretreatment method in which various amount of ammonia (2 to 20% of biomass) was added in biomass slurry in organic solvents and heated at desired time and temperature. This process led to extensive removal of lignin without significant loss of hemicellulose. The lignin-free carbohydrate polymers (cellulose and hemicellulose) was effectively hydrolyzed using enzymes.

Advantage:

- Extracted lignin is relatively of high purity, low molecular weight and sulfur free leading to high possibility for high value application of lignin (Agbor et al., 2011; Zhao et al., 2009b)

- Very selective pretreatment methods; all three biopolymers – cellulose, hemicellulose and lignin can be separated into different streams (Duff & Murray, 1996)
- It can be combined with other pretreatment processes for effective pretreatment of more recalcitrant biomass

Disadvantage:

- High cost of solvent: Recycling process is also energy intensive (Alvira et al., 2010). Additional solvent is required during washing to avoid lignin precipitation due to washing with water; this leads to further increase in solvent recovery cost (Zhao et al., 2009b) Formation of inhibitory compounds, such as furfural and HMF, due to sugar degradation when acid catalyst is used (Agbor et al., 2011)
- Residual solvent will be inhibitory for enzymatic hydrolysis and fermentative organisms (Sun & Cheng, 2002)
- Environmental and health concerns due to the use of volatile organic liquids at high temperature (Agbor et al., 2011)

Ionic liquid pretreatment

This is relatively new approach for biomass pretreatment, in which whole biomass is dissolved in selected ionic liquid and then carbohydrate polymers are precipitated by adding appropriate anti-solvents, thereby separating lignins and carbohydrates. Generally water is used as anti-solvent, but methanol and ethanol can also be used. The regenerated cellulose will have reduced crystallinity and hence will be more easily digestible (Mousdale, 2008). Ionic liquids possess the capability to form hydrogen bonds with cellulose due to the presences of anions, like chloride, acetate, formate, or alkylphosphonate at higher temperatures, leading to dissolution of cellulose (Q. Li et al., 2009). During the regeneration process, the water (anti-solvent) competes with ionic

liquids to form hydrogen-bonding with cellulose, leading to decrease in the solubility of cellulose in ionic liquids, and ultimately results in precipitation (Swatloski et al., 2002). The solvent properties of the ionic liquid can be adjusted by changing desired cation and /or anion (Illanes et al., 2012; Moniruzzaman et al., 2010).

Advantage:

- Ionic liquids are stable up to 300^{o}C and hence have minimum environmental impact due to extremely low volatility – green solvent (Mousdale, 2008)
- Possible to separate each of the biopolymers – cellulose, hemicellulose and lignin
- Ionic liquid with desirable properties can be synthesized

Disadvantage:

- Cost of ionic liquid is still very high
- Many ionic liquids are toxic to enzyme and fermenting organisms (Zhao et al., 2009a)
- Cost of recovery is expensive
- Very difficult to handle biomass slurry with ionic liquid during pretreatment because it becomes too viscous with increase in pretreatment temperature beyond 150°C (Guragain et al., 2011)

Steam explosion pretreatment

Steam explosion pretreatment is the most extensively studied physiochemical pretreatment process. In this process, the ground and preconditioned biomass is treated with saturated steam at high temperature (160 -290°C) and high pressure for a few seconds to several minutes before the pressure is explosively released (Agbor et al., 2011; Chen et al., 2005; Sousa et al., 2009). During this pretreatment process, hemicellulose is extensively hydrolyzed due to the formation of acetic

acid from the released acetyl groups present in the hemicellulose; additionally, water also acts as an acid at high temperature and further helps hemicellulose hydrolysis – a process also called autohydrolysis (Mosier et al., 2005). The chemical effects of hemicellulose hydrolysis is coupled with physical benefit of explosive decompression due to sudden pressure release leading to redistribution of lignin polymers as well as its partial removal from the material (Alvira et al., 2010). This method is more effective in hardwood and herbaceous biomass, but needs addition of acid catalyst for effective pretreatment of softwood due to the presence of lower amount of acetyl groups in softwood hemicellulose (Agbor et al., 2011; Sun & Cheng, 2002)

Advantage:

- No use of chemicals and hence no recycling and environmental cost
- Relatively less dilution of released hemicellulose
- High particle size biomass can be used, leading to energy saving for size reduction, which is one third of entire pretreatment process (Hamelinck et al., 2005)

Disadvantage:

- Incomplete destruction of lignin-carbohydrate complex that may lead to condensation and precipitation of soluble lignin and thereby reducing the hydrolysis efficiency of the pretreated biomass (Li & Gellerstedt, 2008)
- Use of high severity (around 270°C) is the best to enhance cellulose digestibility but it also leads to formation of sugar degradation inhibitory compounds – furfural and HMF (Wright, 1988).
- Weak acids and phenolic compounds generated during this process are also inhibitory for subsequent enzymatic hydrolysis and fermentation; these include acetic acid from acetyl groups present in hemicellulose, and formic and levulinic acids produced due to further

degradation of furfural and HMF. Phenolic compounds are also formed due to lignin breakdown (Alvira et al., 2010).

Ammonia fiber explosion pretreatment (AFEX)

The AFEX method is an alkaline physicochemical pretreatment process. Its processing method is similar to steam explosion, but operate at lower temperature. In this process, the biomass is mixed with liquid anhydrous ammonia (0.3 to 2 kg/kg dry biomass) maintained at 60 - 90°C and at pressure above 3 Mpa for 10 - 60 min. The biomass and ammonia mixture is then heated in a closed vessel under pressure to attain target temperature. After holding it for about 5 min at the desired temperature, the vent valve is rapidly opened to release pressure explosively (Sousa et al., 2009; Taherzadeh & Karimi, 2008). The sudden pressure release results in a rapid expansion of the ammonia gas leading to swelling and physical disruption of biopolymers as well as reduction of celluloase crystallinity. The volatile ammonia gas is recovered for reuse and the dried solid biomass is ready for enzymatic hydrolysis (Sendich et al., 2008). The AFEX method is very effective for herbaceous crops and agricultural residues, but relatively less effective for woody biomass (Wyman et al., 2005). AFEX is also considered as a feasible method for the pretreatment of herbaceous biomass to extract protein for animal feed along with sugar generation for biofuels production (Bals et al., 2007).

Advantage:

- No formation of inhibitory compounds like furfural and HMF from sugar degradation due to low temperature operation (Alvira et al., 2010; Taherzadeh & Karimi, 2008)
- High selectivity for delignification
- Easy for recycling due to volatile nature of ammonia; 99% ammonia recovery is possible
- Residual ammonia can serve as a nitrogen source for the organisms during fermentation

Disadvantage:

- Excess water requirement because the phenolic fragments of lignins must be washed to avoid inhibition during enzymatic hydrolysis and fermentation (Taherzadeh & Karimi, 2008)
- Ammonia recycling is very costly for commercial scale processing (Mosier et al., 2005)
- Inefficient for high lignin content biomass, such as softwood and newspapers
- Environmental concern for using this process in commercial scale

Liquid hot water (LHW) pretreatment

Different terminologies are used in literature to describe this process, including solvolysis, hydrothermolysis, aqueous fractionation, and aquasolv (Agbor et al., 2011). This process is comparable with dilute acid pretreatment without using acid (Taherzadeh & Karimi, 2008). In this process, biomass slurry in water is cooked at elevated temperature (160 -240°C) for various time periods, depending on biomass type, to solubilize hemicellulose fraction of biomass leading to cellulose enriched portion being more accessible for cellulase action (Agbor et al., 2011; Alvira et al., 2010). Mechanism of action of this process is similar to steam explosion for hemicellulose solubilization. Sometime potassium hydroxide (KOH) is used to control pH from 5 to 7 during LHW pretreatment and minimize inhibitory compound formation due to sugar degradation (Mosier et al., 2005).

Advantage:

- No use of additional chemicals
- No need to use expensive corrosive-resistant materials for pretreatment reactors
- Relatively large size particle can be used leading to energy saving for size reduction to fine particle.

- Possible to recover cellulose and hemicellulose separately
- Minimum formation of inhibitory compounds due to sugar degradation

Disadvantage:

- The xylose stream is of very low concentration and hence needs evaporation of water to get appropriate sugar concentration for fermentation – additional cost
- High cost since high pretreatment temperature is required
- Not suitable for high lignin content biomass

Biological pretreatment

Biological pretreatment involves use of microorganism to degrade biomass lignin, and make carbohydrate polymers susceptible for enzymatic hydrolysis. Among various organisms capable of producing enzymes to degrade lignin and carbohydrate polymers of biomass, white-rot, brown-rot, and soft-rot fungi are important (Zhao et al., 2012), with white-rot being the most effective for biomass pretreatment because of their enzymatic efficiency and economy. The brown-rot fungi degrade cellulose, whereas white-rot and soft-rot fungi degrade both lignin and cellulose (Agbor et al., 2011). The ligninolytic system of white rot fungi primarily consists of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Wang et al., 2008). Lip and MnP are heme containing glycoproteins and laccase is a muti-copper oxidase having four copper ions per enzyme molecule as metal clusters (Irshad & Asgher, 2013; Y. Kim et al., 2002). The most commonly used white rot fungi for lignin-degrading enzyme production is *Phynerochaete chrysosporium*, which produces multiple isoenzymes of lignin peroxidase and manganese peroxidase. Many other white rot fungi produce laccase in addition to lignin and manganese peroxidase in varying combination. Based on enzyme production patterns, the white rot fungi could be categorized into three groups. They are;

- Lignin-manganese peroxidase group – *P. chrysosporium* and *Phlebia radiate*
- Manganese peroxidase-laccase groups – *Dichomitus squalens* and *Rigidoporus lignosus*
- Lignin peroxidase-laccase group – *Phlebia ochraceofulva* and *Junghuhnia separabilima* (Hatakka, 1994)

Advantages:

- No inhibitory compounds are produced
- The process is environmental friendly

Disadvantages:

- Very slow process; residence time usually between 10 to 14 days (Agbor et al., 2011)
- Large space is required to perform the experiment
- Strict temperature control is required, leading to increased processing cost
- Cellulose crystallinity could not be reduced during pretreatment (Zhao et al., 2012)

Concluding remarks

Each pretreatment method is associated with some advantages as well as disadvantages, and hence one method cannot be the best choice for all type of biomass, including grass, hardwood and softwood. A distinct variation in biomass composition and structure within same category of biomass further complicates the pretreatment optimization process. Fundamental understanding of the mechanism of various pretreatment technologies and the composition of biomass feedstocks to understand the relationship between biomass composition and pretreatment method is critical to develop an appropriate pretreatment method for each type of biomass. In addition, combinations of two or more pretreatment method can be a better approach for specific biomass feedstocks to overcome the limitations of single pretreatment methods. For example, Karunanithy et al. (2014) reported that sequential extrusion and microwave pretreatment is promising for switchgrass and

big bluestem. Hongzhang & Liying (2007) proposed a combination of steam explosion and ethanol extraction for the fractionation of biopolymers from wheat straw. In this process, the hemicellulose sugars, comprising 86% xylose, was recovered using water extraction after steam explosion pretreatment, followed by lignin extraction using ethanol organosolv process for residual biomass. The cellulose-rich final biomass fraction can be hydrolyzed using cellulase enzymes to generate glucose. Similar process for the fractionation of eucalyptus wood was reported by Sun et al. (2014) using two-step process comprising hydrothermal pretreatment and alkali pretreatment. We proposed a slightly modified method using a three-step pretreatment in **Figure 10.1** for the fractionation of biomass extractives, hemicellulose hydrolyzate, lignin, and cellulose hydrolyzate. However, the increased pretreatment efficiencies due to the combination of more than one pretreatment method should weigh against the increased pretreatment cost. Therefore, economic assessment and environmental consideration of each pretreatment technology or combination of pretreatment technologies is equally critical, in addition to technical evaluation, to design appropriate biomass pretreatment strategies.

Despite dramatic advancement in analytical techniques, complete characterization of biopolymers, especially lignin, is still challenging, and our fundamental understanding of the actual mechanism of recalcitrance of biomass is still poor. Further research is needed to better understand the biomass structure and pretreatment mechanism to develop a novel pretreatment method specific to biomass feedstock for high-value applications of each biomass component, including extractives, lignin, hemicellulose, and cellulose.

Chapter 3 - Effect of biomass densification by pelleting process on pretreatment and hydrolysis efficiencies[1]

Abstract

Densification of bulky forages by pelleting reduces their transportation, handling, and storage costs. Because of high shearing force and frictional heating during the pelleting process, it is hypothesized that pelleting of lignocellulosic biomass could also partially deconstruct its complex structure and facilitate bioethanol production. In this study, pelleted wheat straw, corn stover, big bluestem, and sorghum stalks were evaluated for sugar and ethanol production, and compared with those of unpelleted biomass. Mass recovery after alkali pretreatment was 14, 11, 2, and 5% more, respectively, in unpelleted biomass samples. Lignin content reduced significantly more in pelleted samples for all types of biomass samples, except sorghum stalks. Volumetric productivity of sugar release during enzymatic hydrolysis was 23, 21, 20 and 12% higher, respectively, in pelleted forages; ethanol yield on the basis of released sugars did not differ significantly between pelleted and unpelleted samples. These results indicate that the pelleting process led to better enzymatic hydrolysis of pretreated biomass without affecting the quality of sugars for fermentation. However, overall yield of ethanol from the raw biomass was not significantly higher in pelleted biomass because of higher mass loss during pretreatment. In our study, we propose a schematic for complete utilization of various byproducts for enhanced economic viability.

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Keywords: lignocellulosic biomass, pelleting, biomass deconstruction, alkali pretreatment, enzymatic hydrolysis, ethanol fermentation.

Introduction

Global depletion of fossil fuel due to its excessive utilization poses a great threat of energy crises to the future world (Isarankura-Na-Ayudhya et al. 2007). Moreover, rising political conflict in the major oil-producing countries makes it difficult for non-oil producing countries to get a continuous supply of petroleum products (Balat & Balat, 2009). Bioethanol could be a supplement to gasoline, the major transportation fuel. First generation bioethanol production technology is now mature; it uses sugar and starchy food materials as feedstock. Furthermore, government supports for bioethanol production with subsidies and tax incentives made it economically viable in the context of existing petroleum prices (Kim & Dale, 2004; Mousdale, 2008). The major obstacles to its continuous future production include scarcity of raw materials to produce bioethanol in quantities large enough to meet the high demand for transportation fuel and global concern for food security (Demirbas, 2009). Other alternative energy sources such as hydroelectricity, solar, wind, and wave energy could fulfill only 4% or less of global energy demand by 2030 (Mousdale, 2008); therefore, utilization of lignocellulosic residues for the production of bioethanol is envisioned as a sustainable alternative to gasoline because these feedstocks are globally available in large quantities and are relatively inexpensive (Theerarattananoon et al., 2011). As a result, cellulosic ethanol production is a focus of research worldwide (Kim & Dale, 2004; A. Kumar et al., 2009). The United States government also prioritized the utilization of cellulosic biomass when it approved the Energy Independence and Security Act in 2007. This Act mandated production of 21 billion gallons of second-generation

biofuels by 2022 and stipulated that 16 billion gallons must be derived from lignocellulosic feedstocks (Dadi et al., 2007).

Exploitation of lignocellulosic biomass for commercial production of biofuel and bio-based chemicals faces a number of challenges (Mousdale, 2008). Development of effective biomass pretreatment (Sousa et al., 2009; Ren et al., 2009; Shi et al., 2009; Tomas-Pejo et al., 2008) and handling and storage of bulky feedstocks (Rijal et al., 2012; Theerarattananoon et al., 2011) are the major bottlenecks. Pretreatment is the process of disrupting the lignin-carbohydrate complex of plant cell walls to overcome the recalcitrance of biomass for enzymatic hydrolysis of carbohydrate polymers. The recalcitrance of biomass is due to the presence of a strong lignin layer barrier in the plant cell wall (Hu et al., 2008; Rijal et al., 2012; Zhao et al., 2009a). In addition, the cost of enzymes for hydrolysis of cellulose and hemicellulose (Brijwani et al., 2010) as well as the quality of sugars produced after hydrolysis (Sousa et al., 2009; Hu et al., 2008; H. Zhao et al., 2009a) are key impediments for the utilization of biomass at biorefineries. The latter problems could be addressed to a large extent by developing efficient and economically viable pretreatment methods. An ideal pretreatment process improves hydrolysis yield of sugars without affecting the quality of sugars for subsequent operations (Guragain et al., 2011; Q. Li et al., 2009).

Handling and transportation equipment available for biorefineries to utilize baled or ground biomass is severely limited due to biomass's low bulk density. An economic analysis report from Hess et al. (2006) for wheat and barley straw showed baling and stacking cost around \$23 ton^{-1} and transportation cost of \$11 ton^{-1}. These costs could be even higher depending on the transportation distance and labor, and construction costs for handling and storage facilities. One of the best alternatives to overcome these problems is densification of the light biomass by pelleting. Pelleting increases the bulk density of forages by 9- to 12-fold and makes them flowable

like grain (Rijal et al., 2012; Theerarattananoon et al., 2011), leading to significant reduction of transportation, handling, and storage costs. Pelleting also helps overcome the limitation of transportation of light biomass, because transportation regulations do not allow the transportation of any materials beyond the mandatory legal volume. Moreover, it encourages current grain-processing biorefineries to shift from grains to lignocellulosic feedstocks, because they could use their existing grain handling and storage facilities for pelleted biomass without additional capital investment (Hess et al., 2003; Hess et al., 2006).

High shearing force can be developed during the pelleting process by using an appropriately sized die in the pellet mill coupled with high temperature due to frictional heating. Therefore, the mechanism of mechanical and thermal processing of biomass in pelleting is similar to extrusion (Larsson et al., 2012). Because extrusion of lignocellulosic biomass disrupts its lignin-carbohydrate complex, it is considered one of the promising physical methods of biomass pretreatment for bioethanol production (De Vrije et al., 2002; Karunanithy et al., 2008; Zhan et al., 2006); therefore, it can be hypothesized that the pelleting process could also partially deconstruct the complex structure of lignocellulosic biomass. This can lead to some beneficial effects on subsequent steps of bioethanol production; however, the effect of biomass pelleting on various steps of bioethanol production, including pretreatment, hydrolysis, and fermentation, have not been investigated so far. Therefore, this study attempted to evaluate the effect of pelleting on various stages of bioethanol production using wheat straw, corn stover, big bluestem, and sorghum stalks, as feedstocks. Pelleted and unpelleted samples of each biomass type were ground to a specific particle size, pretreated by an alkali pretreatment method, and hydrolyzed using optimum concentration of cellulase complex and endoxylanase enzymes; released sugars were fermented using *Saccharomyces cerevisiae*. Effectiveness of the pelleting process was evaluated by

comparing pelleted and unpelleted biomass in terms of compositional change and mass recovery after alkali pretreatment, enzymatic hydrolysis yield of sugars, and fermentation yield of ethanol.

Materials and methods

Materials

The big bluestem was kindly donated by Star Seed in Beloit, Kansas, in the form of square bales (1.8 m × 1.2 m × 1.8 m) in January 2009. Wheat straw, corn stover, and photoperiod-sensitive sorghum stalks (Cultivar 'PS 1990', Sorghum Partners, New Deal, Texas) were harvested by the Kansas State University Agronomy Farm in 2008 and 2009. Wheat straw and corn stover were obtained in the form of square bales (1.8 m × 1.2 m × 1.8 m), and sorghum stalks was in the form of a round bale (1.83 m diameter). Particle size reduction of biomass was carried out in two steps. The baled forages were first chopped to approximately 20-cm length with a tub grinder (Model Haybuster H-1150 series, DaraTech Industries International Inc., Jamestown, ND). The chopped biomass samples were further ground in a hammer mill with a 3.2-mm screen opening (Model 18-7-300, Schuttle-Buffalo Hammer mill, Buffalo, NY). The ground samples were packed in sealed paper bags and stored at room temperature until further processing (Theerarattananoon et al., 2011).

Cellulase complex (Cellic CTec 2) and Endoxylanase (Cellic HTec 2) enzymes were procured from Novozymes Inc., USA. The *Saccharomyces cerevisiae* available at the Bioprocessing and Renewable Energy Laboratory of Kansas State University was first revived, then sub-cultured in yeast extract peptone dextrose (YPD) agar media. This yeast was used throughout the experiments.

Pelleting of biomass

Initial moisture contents of wheat straw, corn stover, big bluestem, and sorghum stalks were 7.38, 7.89, 7.45, and 9.05%, respectively. Moisture contents of all forages were increased to approximately 17% before feeding them into the pellet mill. Tap water was sprayed at a predetermined flow rate on the ground biomasses and mixed in a custom-built ribbon mixer. The water was used in place of steam to increase moisture content of forages to simulate on-farm pellet processing, where a boiler would not be available. Conditioned biomass samples were then fed into a pellet mill [California Pellet Mill (CPM) Master model series 2000, CPM Co., San Francisco, CA] using 6.35 mm × 44.45 mm die size. The exit temperature of biomass, which was fed at room temperature, was increased to 74 to 82^{0}C. Forced air was passed through the hot-pelleted biomass until it cooled to room temperature. The pelleted biomass samples were packed in sealed plastic bags and stored at room temperature until further processing (Theerarattananoon et al., 2011).

Pretreatment of biomass

Both pelleted and unpelleted biomass samples were ground with a Thomas-Wiley Laboratory Mill (Model 4) with a 1-mm sieve. Then, the specific sizes (177μm to 841μm) of these samples were separated by sieving the ground biomass samples in a shaker (W.S. Tyler, Model - RX 29, Serial - 25225, Year of Manufacture - 2005) using two sieves of size 20 Mesh (841μm) and 80 Mesh (177 μm). The size range was chosen based on the particle size required for composition analysis without further size separation (Sluiter et al., 2007); more than 90% of original ground material met the desired size range in all types of biomass. Alkali pretreatment of ground samples was carried out by mixing 30 g biomass in 300 ml 1% (W/V) sodium hydroxide. Biomass slurries were then autoclaved at 121^{o}C for 30 min. Pretreated samples were then

transferred onto the 200-mesh (74 μ m) sieve and washed with distilled water until the filtrate was clear and neutral to litmus paper. Residues were squeezed in muslin cloth and spread on trays. The samples were allowed to dry on the trays at room temperature for about 48 h until moisture content decreased to less than 10% (**Figure 3.1**). Weight of samples was taken before and after pretreatment to find percentage mass recovery during pretreatment. Dry samples were then packed in air-tight plastic bags.

Enzymatic hydrolysis

Optimization of biocatalysis

Optimum enzyme loading was determined by taking three different concentrations of enzymes: 1.5, 3.0, and 6.0% of pretreated biomass (W/W). Cellic CTec2 and Cellic HTec2 were taken in the ratio of 9:1 for each concentration as recommended by the enzyme supplier (Novozymes, Inc., USA). One gram of each sample and 10 ml citrate buffer (0.05 M, pH 4.8) were added to a 125-ml conical flask, and the calculated amount of enzymes was added for each concentration of enzyme loading. The flasks were incubated in a shaker at 50^{o}C and 150 rpm for 72 h. Samples (500 μl) were drawn every 12 h from each flask to measure hydrolysis yield of sugars. Total yield of glucose and xylose was measured to evaluate the optimum enzymes concentration.

Hydrolysis of pretreated biomass

Enzymes loading of 6% was found optimal for the comparative analysis of different forages, as explained in section 2.4.1; therefore, the pretreated biomass samples were hydrolyzed using 5.4% Cellic CTec2 and 0.6% Cellic HTec2 enzymes. Solid loading was used at 5% with 2 g biomass in 40 ml citrate buffer in 125-ml flasks. The hydrolysis slurries then were incubated in a shaker at 50^{o}C and 150 rpm for 48 h. A 500-μl sample was drawn from each sample at 14 and

24 h, and the hydrolysis was stopped after 48 h by boiling the flasks in a water bath for 15 min. Yield of total sugars (glucose and xylose) was measured for all samples at 14, 24, and 48 h of hydrolysis. The supernatants of the final hydrolyzates were separated by centrifuging at 13,000 rpm (maximum g-force 20,400×g) for 15 min with a Sorvall Superspeed Angle Rotors (SS-34) centrifuge and preserved in a freezer at -20^{o}C until fermentation.

Fermentation

Inoculum preparation

The stock culture of *Saccharomyces cerevisiae* preserved at -80^{o}C at the Bioprocessing and Renewable Energy Laboratory at Kansas State University was first revived in YPD media that contained 20 g/l glucose, 20 g/l peptone, and 10 g/l yeast extract. The revived culture was then sub-cultured in YPD agar plate and refrigerated to use throughout the experiments. To prepare the inoculum, 50 ml YPD media was taken in a 250-ml flask and autoclaved 121^{o}C for 15 min. One loop of the culture from the agar plate was aseptically inoculated into the flask and incubated at 30 ^{o}C and 150 rpm for 24 h. The prepared inoculum was directly added to the fermentation medium at the rate of 10% (V/V) of fermentation medium (Guragain et al., 2011).

Fermentation of hydrolyzates

Two milliliters of supplementary nutrition solution and 25 ml of biomass hydrolysates were taken in 125-ml flasks. The nutrition solution was prepared in a citrate buffer so that when 2 ml solution was added to 25 ml hydrolyzates and 3 ml inoculum, the final medium would contain 10 g/l yeast extract, 5 g/l NH_4Cl, 1 g/L $MgSO_4$.$7H_2O$, and 2 g/l KH_2PO_4. Control flasks also were prepared by taking the same volume of synthetic medium containing 28 mg/ml glucose and 12 mg/ml xylose instead of biomass hydrolyzates. All the flasks were autoclaved at 121^{o}C for 15 min, and 3 ml inoculum was aseptically added to each flask. These flasks were then incubated in a

shaker at 30°C and 150 rpm for 24 h. Samples were drawn at 0, 6, 10, 15, and 24 h of fermentation to measure ethanol yield and sugar consumption (Guragain et al., 2011).

Analytical procedures

Moisture content of the raw and pretreated samples was determined with an electric moisture meter (IR35M-00015V1, Denver Instrument GmbH, Goettingen, Germany). Extractives, acid-soluble lignin, acid-insoluble lignin, glucan, and xylan were determined by following the protocol NREL/TP-510-42618 and NREL/TP-510-42619 (Sluiter et al., 2007; Sluiter et al., 2005). Glucose, xylose, and ethanol were measured using an HPLC instrument (Shimadzu Corporation, Japan) equipped with an LC-20AB pump, an SIL-20 AC auto sampler, an SPD-M 20A photodiode array detector, and a Phenomenex RPM-Monosaccharide Pb+ column (300 × 7.8 mm). Deionized water (prepared using Millipore, cat No – ZRQSVP030) was degassed by sonication (Ultrasonic cleaner, Fisher Scientific, Model FS 60) and used as mobile phase at a flow rate of 0.6 ml min^{-1}. The column oven and refractive index detector (RID-10A) were maintained at 80 °C and 65 °C, respectively. All experiments were carried out in triplicate and the data were statistically analyzed for least significant difference (LSD) using JMP software (SAS Inc., Cary, North Carolina).

Results and Discussion

Mass recovery after alkali pretreatment

Weights of biomass samples and their moisture contents were measured before and after alkali pretreatment to find the loss of mass from pretreatment and washing. Mass recovery was then calculated as the percentage of original mass. **Figure 3.2** shows that unpelleted samples of wheat straw, corn stover, big bluestem, and sorghum stalks had 14, 11, 2, and 5% more mass recovery, respectively, than pelleted biomass samples. These differences in mass recovery between pelleted and unpelleted samples were statistically significant at the 95% confidence level in all

biomass types, except in big bluestem. These differences in total mass loss during pretreatment led to statistically equal ethanol yield in pelleted and unpelleted biomass samples (see section "Overall mass balance") even though hydrolysis efficiency was better in pelleted biomass samples (see section "Enzymatic hydrolysis"). Variation of mass recovery among these samples was less in unpelleted samples than in pelleted samples.

Composition of raw and pretreated biomass

Table 3.1 shows that a significant amount of lignin was removed during alkali pretreatment in all types of biomass, which led to increased glucan and xylan content. The lignin content in these data is a sum of acid-soluble and acid-insoluble lignin. The change in glucan and xylan content was also due to loss of extractives during pretreatment (**Figure 3.3**). Lignin content of raw samples varied significantly among different types of biomass, but it was not significantly different between pelleted and unpelleted samples of the same biomass type at 95% confidence level. However, lignin content in pelleted samples was significantly lower than in unpelleted samples after alkali pretreatment in some biomass samples, indicating better delignification in pelleted samples. This effect was more significant in wheat straw and corn stover samples. Unpelleted wheat straw and corn stover had 19 and 24% more lignin, respectively, than pelleted samples after alkali pretreatment, but there was no significant difference in lignin content between pelleted and unpelleted samples for big bluestem and sorghum stalks. These data also show that the highest percentage of delignification was achieved in sorghum stalks and lowest in wheat straw. The lignin content of unpelleted sorghum stalks decreased from 26.2% to 8.1% during pretreatment and from 21.5% to 10.0% in unpelleted wheat straw samples. A similar trend was found for pelleted samples. Glucan and xylan contents did not differ significantly between pelleted and unpelleted samples

before or after pretreatment in all biomass types, except for glucan content in sorghum stalks after pretreatment.

Water-soluble extractives of biomass was determined by extracting the sample in distilled water using a Soxhlet extraction set (Wilmad LabGlass, Vineland, New Jersey, USA) for 8 to 12 h, until the siphoned solvent was clear. Similarly, alcohol-soluble extractives were determined using 95% ethanol as an extraction solvent. The total extractives were then calculated as the sum of water-soluble and alcohol-soluble extractives; these extractives are non-structural components of biomass, including non-structural sugars, nitrogenous materials, chlorophyll, and waxes, among other minor components (Sluiter et al., 2007). **Figure 3.3** shows that alkali pretreatment removed a significant amount of extractives from biomass. The percentage removal of these extractives varied from biomass to biomass. The highest proportion was removed in the sorghum stalks sample and was similar to delignification during pretreatment; however, for biomass samples other than sorghum stalks, total extractives content did not differ between pelleted and unpelleted biomasses in both raw and pretreated samples. This showed that difference in total mass loss between pelleted and unpelleted biomasses during pretreatment (**Figure 3.2**) were due to the difference in loss of structural carbohydrates and lignin.

Two important conclusions can be drawn from the compositional analysis of raw and pretreated samples and mass recovery during pretreatment. First, higher delignification and total mass loss in pelleted samples compared with unpelleted samples during alkali pretreatment indicate that the pelleting process led to partial deconstruction of lignocellulosic biomass. This result occurred because high shearing and mixing force coupled with heat development due to friction during the pelleting process disrupted the biomass structure (Theerarattananoon et al., 2012). However, the effect of pelleting differed significantly from biomass to biomass. Second,

alkali pretreatment was comparatively more effective for the removal of lignin and extractives in sorghum stalks than in other biomasses, thereby offsetting the positive effect of the pelleting process to improve the delignification of this forage during pretreatment. This shows that the maximum beneficial effect of the pelleting process could be realized in biomasses in which the selected pretreatment method is comparatively less effective for delignification. Because the effectiveness of a pretreatment method varies from biomass to biomass (Guragain et al., 2011; Mishima et al., 2008), the pelleting process must be separately optimized for each biomass type depending on the best pretreatment method for the specific biomass; however, comparative study of different pretreatment methods and pelleting conditions to evaluate the combined effect of pelleting and pretreatment on the delignification of biomass to improve enzymatic hydrolysis is outside the scope of this study.

Enzymatic hydrolysis of pretreated biomass

Initial experiments were carried out to optimize enzyme loading for the hydrolysis of pretreated biomass. An alkali-pretreated unpelleted wheat straw sample was hydrolyzed using three different loadings of an enzyme mixture containing Cellic CTec2 and Cellic HTec2 in the ratio of 9:1. The enzyme concentration of 6% (w/w) of pretreated biomass was found to be the optimal enzyme loading to maximize hydrolysis yield of total sugars (data not shown). For the comparative study of the unpelleted and pelleted biomass samples, this enzyme loading was taken for the hydrolysis of all biomass types without further individual optimization.

Figure 3.4 shows that the reaction rate of enzyme kinetics was significantly higher in pelleted biomass compared with unpelleted samples during the initial period of hydrolysis in all types of biomass. The yield of total sugars (glucose and xylose) increased linearly with hydrolysis time until 14 h, but no significant increment occurred thereafter. This result indicated that the first

14 h was the fastest reaction rate period of the enzymatic hydrolysis; therefore, the volumetric productivity of the enzyme kinetics was calculated for this period and expressed as gram of total sugars produced per liter per hour. Moreover, during optimization of enzyme loading, total sugars yield was almost constant after 48 h of hydrolysis until 72 h. The flattened curves of all types biomass after 14 h of hydrolysis in **Figure 3.4** also indicate the same result; that is, sugars yield would not increase after 48 h. Therefore, maximum hydrolysis yield of total sugars was calculated after 48 h of hydrolysis.

Table 3.2 shows that both productivity of enzymatic hydrolysis and maximum total sugars yield were significantly higher at 95% confidence level in pelleted samples compared with unpelleted samples in all types of biomass, except maximum sugars yield in sorghum stalks. The incremental productivity of enzymatic hydrolysis due to pelleting of biomass was almost double the incremental maximum sugars yield in all types of biomasses. Because productivity is the vital indicator in industrial application (Oberoi et al., 2010), the effect of the pelleting process is a highly significant factor for improving enzymatic hydrolysis yield of sugars from pretreated biomass. However, similar to effects on the delignification of biomass during pretreatment as discussed in section 3.2, pelleting effects varied significantly from biomass to biomass. The highest effect was observed in wheat straw, with 23.1% more productivity and 13.1% more maximum sugars yield in the pelleted sample than in the unpelleted sample. The lowest effect was found in sorghum stalks, in which productivity increased by 11.6% due to pelleting but the increase in maximum sugars yield was not statistically significant at 95% confidence level. These data also show that the highest delignification of biomass may not necessarily result in the highest enzymatic hydrolysis yield on the same biomass. As discussed in earlier section on the composition of biomass, the highest percentage of delignification was achieved in sorghum stalks, whereas the

highest productivity as well as maximum sugars yield was observed in corn stover. This is because enzymatic hydrolysis yield of sugars depends on the number of substrate-related factors in addition to total lignin content. Some of the important factors are degree of crystallinity and polymerization of cellulose, composition of biomass (Zhao et al., 2009a), type of lignin, and extent of side-chain branching of hemicellulose (Sousa et al., 2009).

Fermentation of hydrolyzates

Optimization of fermentation time was first conducted for all biomass types. The hydrolyzates were separated after enzymatic hydrolysis by centrifugation at 13,000 rpm (maximum g-force 20,400×g). One loop of *S. cerevisiae* culture was inoculated in 50 ml YPD media and incubated in a shaker at 30^{o}C for 24 h to prepare the inoculum. Three milliliters inoculum was added to 27 ml sterile hydrolyzates containing the required concentration of supplementary nutrients other than the carbon source in 125-ml flask. The flasks were then incubated in shaker at 30^{o}C for 24 h. Five hundred microliter samples were drawn at 0, 6, 10, 15, and 24 h of fermentation to measure sugars and ethanol, but analysis of samples beyond 10 h was not carried out because no additional sugar consumption occurred from 6 to 10 h (**Figure 3.5**). Glucose was consumed almost completely at 6 h of fermentation, and ethanol production reached the highest level at that time for the unpelleted wheat straw sample. The yeast culture could not utilize xylose, and ethanol concentration started to reduce after 6 h, probably due to consumption of ethanol by microbes in the absence of other consumable carbon sources (Guragain et al., 2011). A similar pattern of sugar consumption and ethanol production curves were found for other biomass samples (figures not shown); therefore, 6 h of fermentation time was selected as optimum for the comparative study of all biomass samples in subsequent experiments.

Ethanol yield in the samples was expressed as the percentage of ethanol yield in the control. The control samples were prepared by taking 28 mg/ml glucose and 12 mg/ml xylose in fermentation media, which was approximately equal to the average concentration of each sugar in the biomass hydrolyzates. Supplementary nutrients were added in the same concentration in samples and controls. **Table 3.3** shows that variation of ethanol yields among different types of biomass were statistically significant at 95% confidence level for some biomass types; however, ethanol yield of pelleted and unpelleted samples of the same type of biomass did not differ significantly. Similarly, volumetric productivity of ethanol fermentation was calculated for optimum fermentation time (6 h) and expressed as gram of ethanol produced per liter per hour. The productivity of ethanol from the biomass hydrolyzates was more than 1 in all samples, ranging from 1.4 to 1.7 gram per liter per hour, but differences in productivity among the different types of biomass samples were not statistically significant at the 95% confidence level, and neither were the differences between pelleted and unpelleted samples. Therefore the pelleting process did not have any effect on the quality of sugars produced after enzymatic hydrolysis of biomass.

Overall mass balance and proposed processes for the utilization of byproducts

In contrast to **Tables 3.2** and **Table 3.3**, **Table 3.4** indicates that overall ethanol yields on the basis of raw biomass were not significantly different between pelleted and unpelleted samples for all types of biomass; this is because of the loss of a significantly higher amount of biomass during pretreatment in pelleted samples than in unpelleted samples. The overall yield of ethanol was only 13 to 15 g per 100 g raw biomass due to losses and incomplete utilization of its various components in the following bioprocessing steps.

1. Loss of biomass during washing of pretreated sample (total mass in liquid fraction): - it accounted for 40% to 50% of initial biomass, which contained 38% to 50% lignin and remaining mainly hydrolyzed cellulose and hemicellulose.
2. Loss of biomass after hydrolysis of pretreated sample (Residue 1): - it contained mainly lignin and unhydrolyzed cellulose and hemicellulose.
3. Loss of xylose present in hydrolyzates: - it is because *S. cerevisiae*, the yeast used in this experiment, could not utilize xylose.
4. Loss of biomass in the form of carbon dioxide during fermentation: - 0.96 g carbon dioxide per g ethanol is produced (using reaction stoichiometry).

Adoption of appropriate technologies to utilize byproducts/wastes can minimize these losses and make bioprocessing of lignocellulosic residue economically viable, as proposed in **Figure 3.6**. The liquid fraction of the pretreated biomass contains a significant amount of hydrolyzed carbohydrate polymers, which can be a very good source for the production of single-cell protein for animal feed or enzyme production. Residue thereafter (Residue 2) mainly contains lignin that can be used for power generation. Similarly, the residue after hydrolysis of the pretreated sample (Residue 1) contains lignin and unhydrolyzed carbohydrate polymers, which also can be used for power generation. The xylose present in the hydrolyzates can be fermented to ethanol using xylose fermenting organisms such as genetically engineered *S. cerevisiae* and *Zymomonas mobilise* (Zhang & Lynd, 2010).

The ethanol fermentation process leads to production of a nearly saturated stream of carbon dioxide (Xu et al., 2010). Using reaction stoichiometry from glucose to ethanol and carbon dioxide, carbon dioxide is produced at the rate of 96% of ethanol on a mass basis. Such gas needs only dehydration and compression for purification. The total cost of capture of carbon dioxide is,

therefore, approximately $6 to $12 per ton carbon dioxide, which is much less than its total cost of production from the most modern coal-fired power plants (Dahowski & Dooley, 2008). Therefore, the carbon dioxide produced from ethanol fermentation plants is a potential alternative to other conventional sources such as anhydrous ammonia. The majority of carbon dioxide traditionally is used in food processing, carbonated beverages, and preservatives markets; however, the pure carbon dioxide from ethanol fermentation can be used in production of fuels, chemicals, and polymers, as well as in enhanced oil recovery industry (Xu et al., 2010).

If the byproducts of this experiment were utilized for the production of valuable products as proposed in **Figure 3.6**, benefits of the pelleting process could be enhanced for overall production of desired products from the same quantity of raw biomass, because pelleted samples contained a significantly higher amount of hydrolyzed sugars in the liquid fraction of the pretreated biomass, whereas the unpelleted samples contained a higher amount of unhydrolyzed carbohydrate polymers in the residue after hydrolysis of pretreated samples (Residue 1) for all types of biomass. The former residue is much easier to utilize for production of desirable products. If the proposed processes in **Figure 3.6** are not economically feasible in the context of existing technologies in biorefineries, the pelleting process would remain beneficial for ethanol production from forages. On the one hand, enzymes consumed for hydrolysis of pretreated biomass samples were significantly less in pelleted samples than unpelleted samples because of a lesser amount of pretreated biomass in pelleted samples, but ultimate ethanol production was equal in pelleted and unpelleted samples. On the other hand, pelleted biomasses could need milder pretreatment conditions than unpelleted biomasses to achieve similar mass loss during pretreatment and thereby similar enzymatic hydrolysis yield of sugars and fermentation yield of ethanol. Therefore, the

former option would reduce enzyme cost, whereas the latter would reduce cost of pretreatment, both of which are major costs of cellulosic ethanol production.

Conclusions

Pelleting of forages led to better delignification of biomass during alkali pretreatment; however, mass recovery after pretreatment was significantly lower in pelleted samples than in unpelleted samples. Volumetric productivity of sugars release during enzymatic hydrolysis of pretreated biomass was 23, 21, 20, and 12% higher in pelleted samples than in unpelleted samples for wheat straw, corn stover, big bluestem, and sorghum stalks, respectively. These results indicate that the complex structure of lignocellulosic biomass was partially disrupted during pelleting process and thereby made alkali-pretreated biomass samples more susceptible to hydrolytic enzyme activities in pelleted samples than in unpelleted samples. Moreover, the pelleting process did not negatively affect the quality of released sugars for fermentation. Nevertheless, the effect of pelleting was significantly different among different types of biomass; therefore, each biomass must be optimized separately to obtain maximum benefit of the pelleting process to reduce the cost of ethanol production from lignocellulosic residues. However, overall yield of ethanol from the raw biomass was not significantly higher in pelleted biomasses than unpelleted biomasses because of the higher amount of mass loss during pretreatment in pelleted samples. Our study showed that pelleted biomass needed less enzyme than unpelleted biomass to obtain the same amount of ethanol production from the same amount of feedstock. Development of appropriate processing technology is crucial to utilize valuable byproducts produced at different stages of bioethanol production to make the bioethanol industry economically viable and to maximize the benefits of the pelleting process.

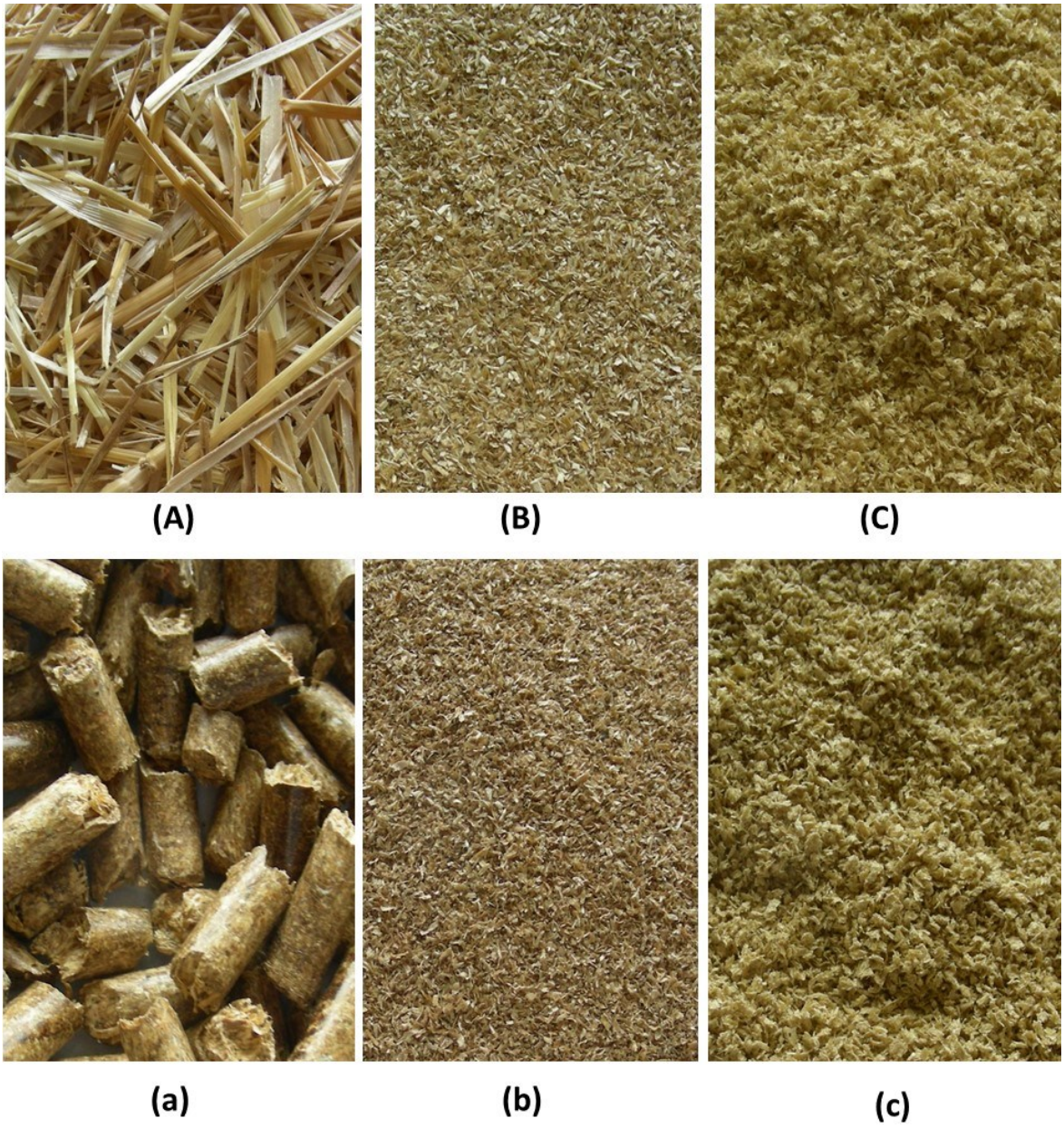


Figure 3.1 Pelleted and unpelleted wheat straw samples. (A) Unpelleted raw biomass, (B) unpelleted biomass after grinding, (C) unpelleted biomass after pretreatment, (a) pelleted raw biomass, (b) pelleted biomass after grinding, and (c) pelleted biomass after pretreatment.

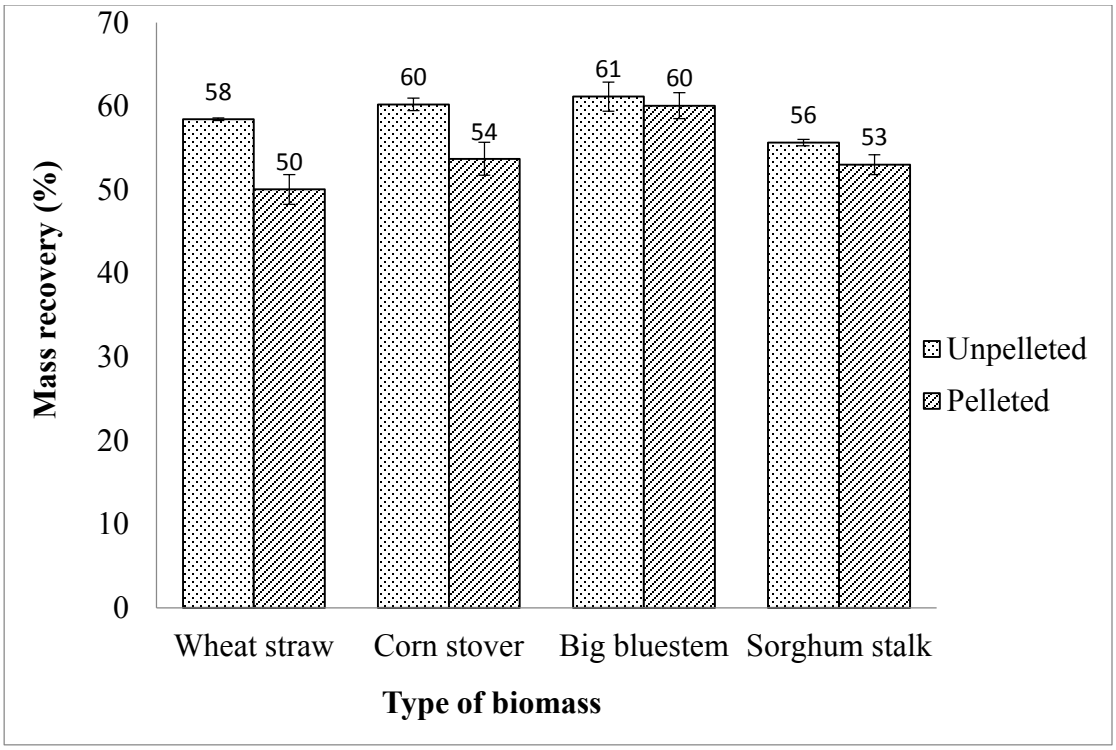


Figure 3.2 Mass recovery after alkali pretreatment. Recovery was calculated as the percentage (W/W) of raw biomass after autoclaving 30 g biomass in 300 ml 1% (W/W) NaOH at 121 ºC for 30 min. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

Table 3.1 Composition of raw and pretreated biomass.

Type of biomass		Glucan		Xylan		Lignin	
		Raw	Pretreated	Raw	Pretreated	Raw	Pretreated
Wheat	Unpelleted	48.2±1.9[a]	51.3±0.7[ad]	25.3±1.8[a]	29.7±0.5[a]	21.5±0.4[a]	10.0±0.2[ac]
straw	Pelleted	46.5±1.8[a]	52.6±1.0[ae]	24.1±1.6[a]	29.5±0.3[a]	21.6±0.7[a]	8.4±0.2[b]
Corn	Unpelleted	49.6±1.9[a]	53.9±1.0[bef]	25.1±0.7[a]	29.7±1.2[a]	23.7±0.2[b]	10.3±0.4[a]
stover	Pelleted	46.9±0.7[a]	55.1±0.3[b]	23.8±0.1[a]	29.0±0.6[a]	23.2±0.4[b]	8.3±0.3[b]
Big	Unpelleted	35.6±2.7[b]	49.6±0.7[cd]	20.2±1.7[b]	32.4±2.6[b]	21.9±1.1[a]	9.7±0.3[cd]
bluestem	Pelleted	35.4±2.1[b]	48.2±0.6[c]	20.0±1.1[b]	33.1±1.7[b]	22.4±0.6[a]	9.5±0.1[d]
Sorghum	Unpelleted	41.1±2.7[c]	52.8±0.7[af]	23.9±1.3[a]	27.6±1.2[a]	26.2±0.6[c]	8.1±0.4[b]
stalk	Pelleted	39.5±0.2[c]	48.5±2.1[c]	23.3± 0.9[a]	28.9±1.0[a]	25.6±0.6[c]	8.1±0.2[b]

Data are average values of triplicate experiment ± sample standard deviation.
Values with the same letters, in superscripts, within the same column are not significantly different from each at the $P<0.05$ level.

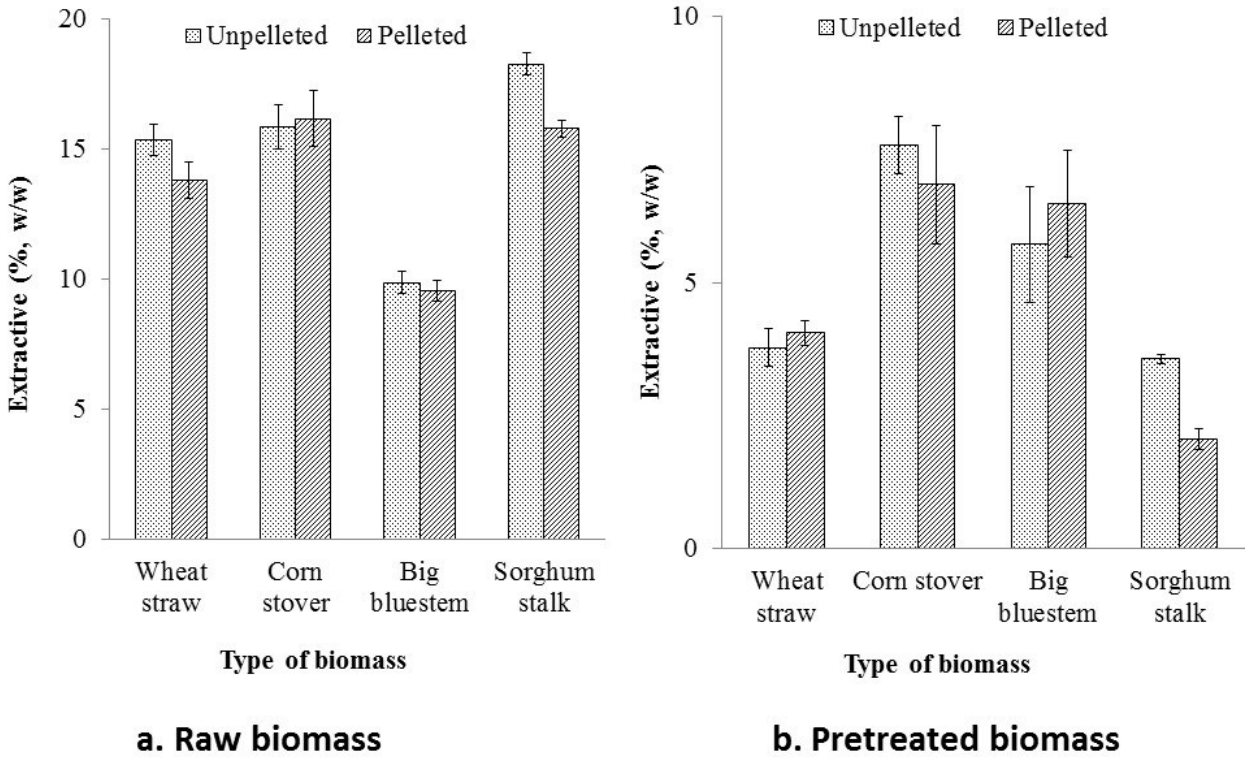


Figure 3.3 Extractives content in biomass. (a) Raw biomass and (b) pretreated biomass. Extractives are the sum of water-soluble and alcohol (95% ethanol)-soluble material extracted in Soxhlet apparatus for 10 to 12 h. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

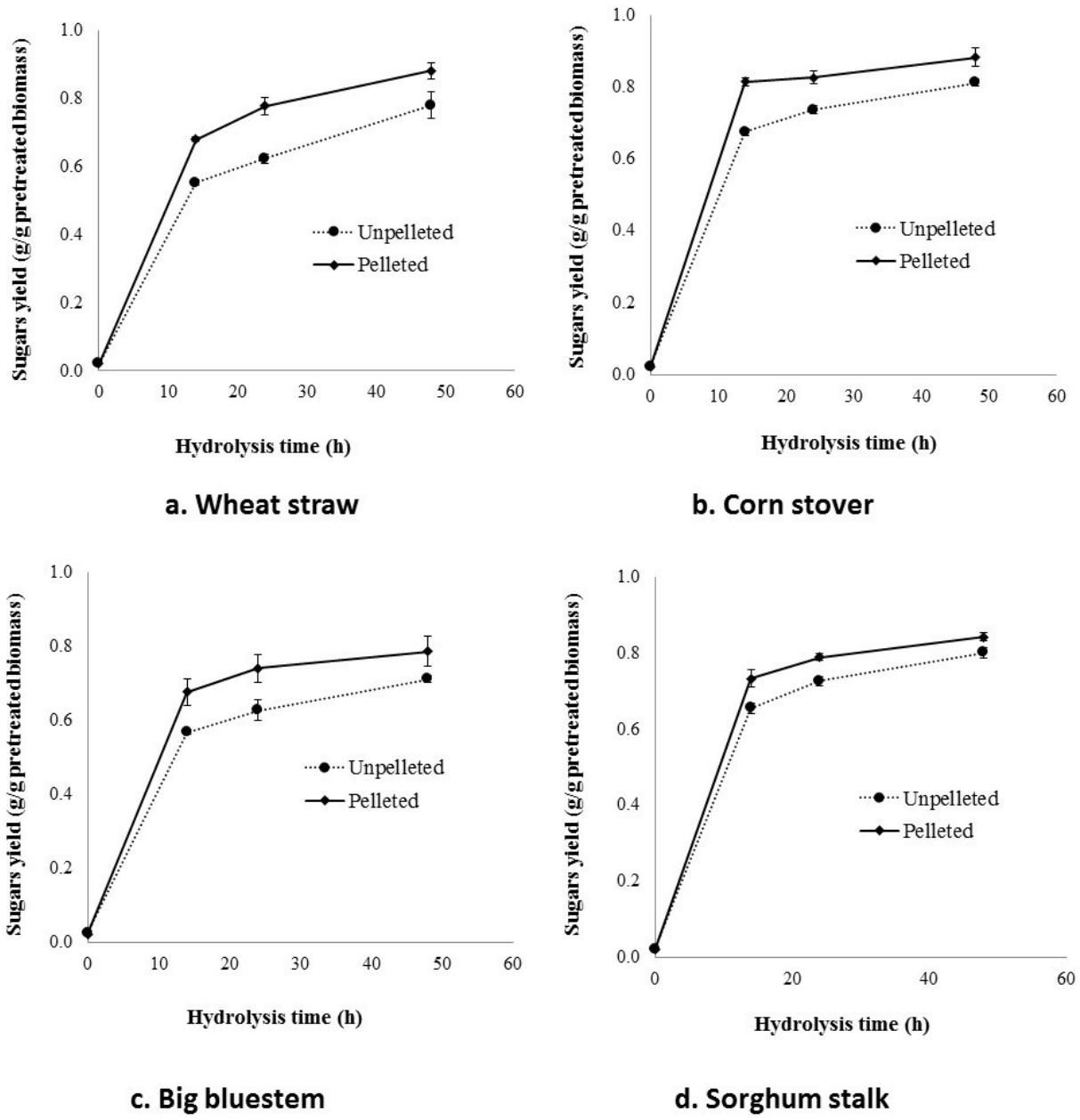


Figure 3.4 Sugar yield during hydrolysis of pretreated biomass. (a) Wheat straw, (b) corn stover, (c) big bluestem, and (d) sorghum stalks. Hydrolysis was carried out at 50ºC with 2% solid loading in citrate buffer with enzyme loading of 6% of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

Table 3.2 Volumetric productivity and maximum enzymatic hydrolysis yields of total sugars

Type of sample		*Productivity (g/l/h)	Increase in productivity due to pelleting (%)	**Maximum sugar yield (g/g pretreated biomass)	Increase in maximum sugar yield due to pelleting (%)
Wheat straw	Unpelleted	2.03 ± 0.03^{a}	23.1	0.78 ± 0.04^{a}	13.1
	Pelleted	2.50 ± 0.02^{b}		0.88 ± 0.02^{b}	
Corn stover	Unpelleted	2.48 ± 0.04^{b}	22.9	0.81 ± 0.01^{ad}	8.7
	Pelleted	2.99 ± 0.04^{c}		0.88 ± 0.03^{b}	
Big bluestem	Unpelleted	2.08 ± 0.02^{a}	19.8	0.71 ± 0.01^{c}	10.7
	Pelleted	2.49 ± 0.13^{b}		0.79 ± 0.04^{a}	
Sorghum stalk	Unpelleted	2.41 ± 0.06^{b}	11.6	0.80 ± 0.01^{ad}	5.2
	Pelleted	2.69 ± 0.08^{d}		0.84 ± 0.01^{bd}	

**Productivity was measured for first 14 h of hydrolysis and expressed as gram of total sugar produced per liter per hour.*

***Maximum hydrolysis yield of total sugar (glucose and xylose) was measured after 48 h of hydrolysis.*

Hydrolysis was carried out at 50°C with 2% solid loading in citrate buffer with enzyme loading of 6% of solid. Data are average values of triplicate experiment ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each at the $P<0.05$ level.

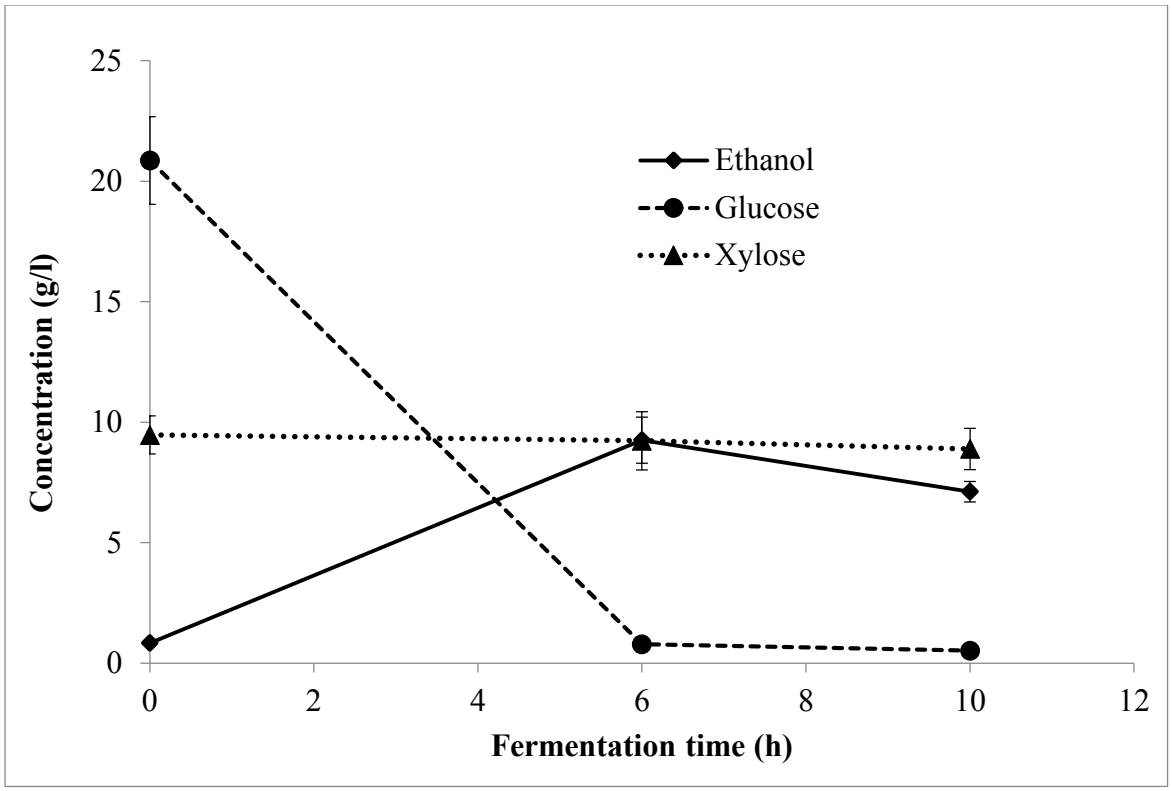


Figure 3.5 Fermentation of hydrolyzates of unpelleted wheat straw using *Saccharomyces cerevisiae.* Data are average values of triplicate experiments, and error bars represent sample standard deviation.

Table 3.3 Ethanol yield and volumetric productivity during fermentation of biomass hydrolyzed using *S. cerevisiae*

Type of biomass		*Ethanol yield (%)	**Productivity (g/l/h)
Wheat straw	Unpelleted	93 ± 9^{ab}	1.41 ± 0.16^{a}
	Pelleted	88 ± 7^{a}	1.66 ± 0.12^{a}
Corn stover	Unpelleted	91 ± 7^{ab}	1.51 ± 0.07^{a}
	Pelleted	87 ± 3^{a}	1.65 ± 0.04^{a}
Big bluestem	Unpelleted	95 ± 5^{ab}	1.59 ± 0.21^{a}
	Pelleted	99 ± 4^{b}	1.73 ± 0.20^{a}
Sorghum stalk	Unpelleted	100 ± 2^{b}	1.65 ± 0.08^{a}
	Pelleted	100 ± 5^{b}	1.71 ± 0.37^{a}

**Ethanol yield was gram of ethanol produced per gram glucose consumed and expressed as the percentage of ethanol yield in the control, the sample containing pure glucose and xylose.*
***Productivity was measured for first 6 h of fermentation at 30 ^{o}C and expressed as gram of ethanol produced per liter per hour.*
Data are average values of triplicate experiment ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each at the $P<0.05$ level.

Table 3.4 Overall mass balance during biomass processing, on the basis of 100 g initial raw biomass

Type of biomass		Initial mass (lignin) (g)	Pretreated mass (lignin) (g)	Total mass (lignin) in *liquid fraction (g)	Hydrolyzed sugars		Residue 1** (g)	Ethanol (g)
					Glucose (g)	Xylose (g)		
Wheat straw	Unpelleted	100 (21)	58 (6)	42 (16)	31	15	18	13^{ab}
	Pelleted	100 (22)	50 (4)	50 (17)	32	13	11	13^{a}
Corn stover	Unpelleted	100 (24)	60 (6)	40 (17)	35	14	16	14^{bc}
	Pelleted	100 (23)	54 (4)	46 (19)	34	13	11	13^{abc}
Big bluestem	Unpelleted	100 (22)	61 (6)	39 (16)	31	13	22	14^{abc}
	Pelleted	100 (22)	60 (6)	40 (17)	34	14	18	15^{c}
Sorghum stalk	Unpelleted	100 (26)	56 (4)	44 (22)	31	13	16	13^{abc}
	Pelleted	100 (26)	53 (4)	47 (21)	31	13	13	14^{abc}

**Liquid fraction is the filtrate during washing of pretreated biomass.*
***Residue 1 is the mass left after hydrolysis of pretreated biomass, which principally contained lignin and unhydrolyzed cellulose and hemicellulose. It was calculated using following formula.*
Residue 1 = Pretreated mass – (glucose in hydrolyzates × 0.9 + Xylose in hydrolyzates × 0.88)
Values with the same letters, in superscripts, within the same column are not significantly different from each at the $P<0.05$ level.

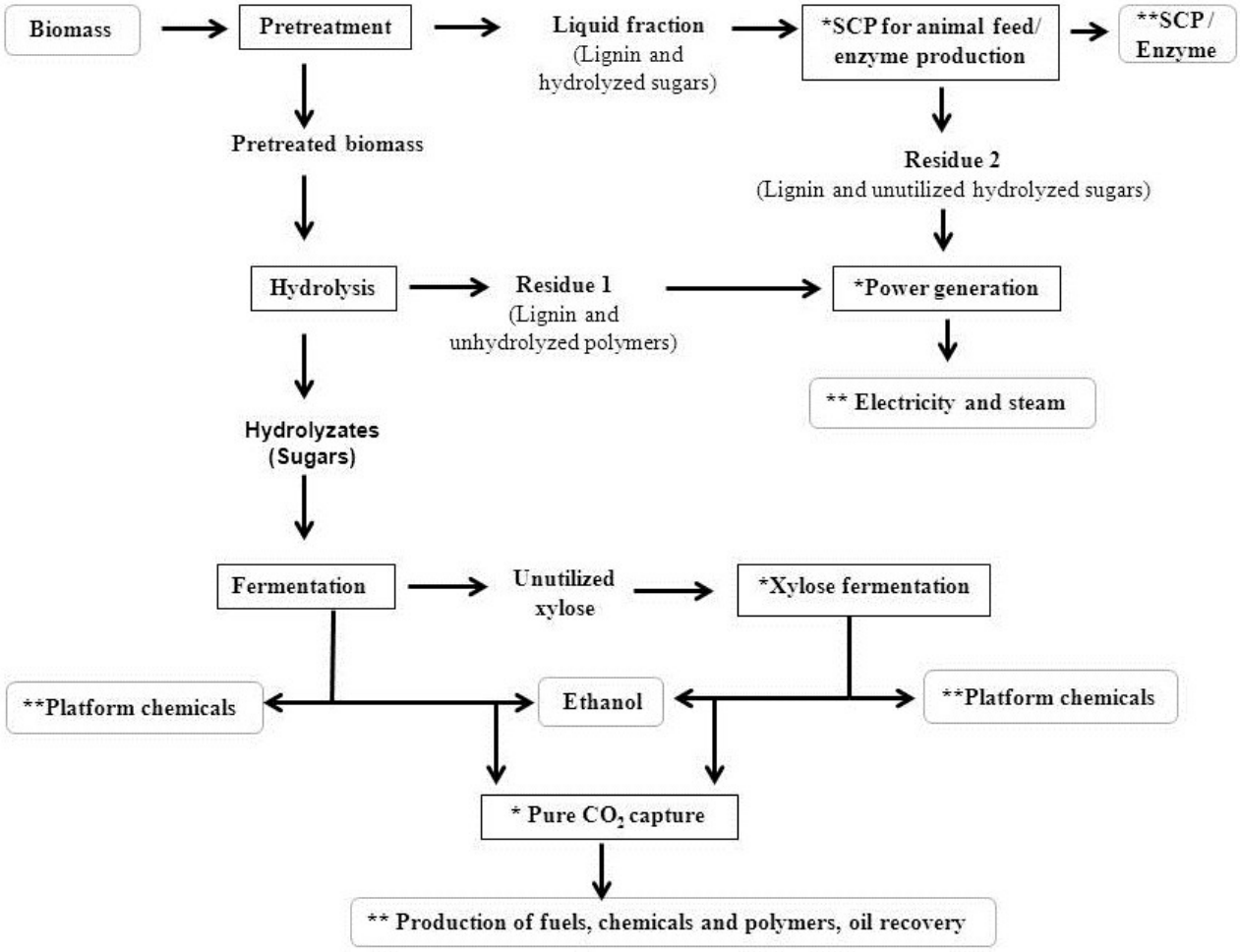


Figure 3.6 Schematic diagram for complete utilization of lignocellulosic biomass.

*Proposed process to utilize byproducts.

**Products obtained from the proposed process.

SCP = Single-cell protein

CO_2 = Carbon dioxide gas

Chapter 4 - Appropriate biorefining strategies for multiple feedstocks based on acid and alkali pretreatment

Abstract

Efficient utilization of a wide range of feedstocks requires appropriate biorefining strategies based on pretreatment methods. This study showed that alkali pretreatment was significantly more effective than acid pretreatment at identical processing conditions for grass and hardwood biomass samples; but, both methods were ineffective for softwood biomass. Separate glucose and xylose streams can be obtained for efficient fermentation from acid-pretreated biomass; nevertheless, need for more severe processing conditions to achieve effective pretreatment necessitates an additional detoxification step. High sugars concentration (10.6%, w/v) in hydrolyzates was obtained from alkali-pretreated biomass using optimum solids loading of 17.5% (w/v), which opens up an opportunity to produce high concentrations of biofuels and biochemicals in fermentation broth at reduced downstream processing costs. We propose a schematic for innovative biorefining strategies based on established pretreatment methods for different types of feedstocks. This information is very pertinent for choosing the appropriate processing methods and for setting up large-scale biorefineries utilizing multiple feedstocks.

Keywords: Lignocellulosic biomass; pretreatment; hydrolysis; fermentation.

Introduction

The rapid increase in global biofuel and biochemicals production in the last decade is considered as an important achievement for energy security and climate change mitigation. United State (US) bioethanol production increased from 3 billion gallons in 2003 to 13 billion gallons in 2013, which accounted for 57% of global production (Guragain et al., 2016). US bioethanol

industries consumed 30% of national corn grown in 2013 to produce 13 billion gallons of ethanol, which represented approximately 4% of national transportation fuel demands. The nation has set its goal of producing 36 billion gallons of transportation fuel per year from renewable resources by 2022 (Klein-Marcuschamer & Blanch, 2015). Assuming the same corn production, and the same conversion efficiency from corn to ethanol, more than 80% of US corn will be consumed to meet its goal of producing 36 billion gallons of transportation fuel by 2022 if alternative feedstocks are not exploited. In addition, a number of platform and bulk chemicals should be produced via sustainable alternative routes, including from biomass feedstocks, to minimize dependency on petroleum-derived products. About 80% of current global power consumption is sourced from petroleum (Dale & Ong, 2012). The world's finite petroleum resources have been rapidly depleting due to increased energy consumption, especially in developing countries. Still, power consumption per capita in most developing countries is much less than United Nations Human Development Index (HDI) standards: 4 kilowatt per capita. In order to achieve this power consumption for 7 billion world population, about 28 terawatt total power is required, which is almost double the current global power consumption of 15 terawatt (Dale & Ong, 2012). Exploitation of abundantly available lignocellulosic biomass for fuels and chemicals production is one of the promising alternatives to address a number of these global issues, including energy security, environmental concerns, and rural economic development (Ragauskas et al., 2014a).

Use of lignocellulosic biomass for biofuels and biochemicals production is associated with a number of opportunities as well as challenges. The beauty of the lignocellulosic biomass is its unique components, including carbohydrate polymers (cellulose and hemicellulose), lignin, and extractives, which can be used for a wide range of biofuels and biochemicals production (Beatson, 2011; Werpy et al., 2004). Primarily, there are two routes for biofuels production from

lignocellulosic biomass: (1) thermochemical platform – use of heat and chemical catalysts to produce fuels, and (2) sugar platform – a biochemical process to release sugars from biomass, which are subsequently converted to fuels and chemicals using microbial and/or chemical catalysts (Dale & Ong, 2012). This study focused on a sugar platform route, which is comprised of four major core sections: feedstock handling and storage, pretreatment, enzymatic hydrolysis, and sugar fermentation to desired biofuels and biochemicals (Cheng & Timilsina, 2011; Zhang et al., 2015). Each section in this route is associated with a number of challenges, but the greatest challenge is the need for an effective pretreatment process prior to hydrolysis of carbohydrate polymers to separate the strong outer lignin layer (Guragain et al., 2011; Tomas-Pejo et al., 2008); the pretreatment is the most challenging step for the thermochemical platform route as well (Dale & Ong, 2012). The biomass pretreatment methods are broadly classified into biological, physical, chemical and physico-chemical process (Sarkar et al. 2012). Dilute acid and alkali are the most extensively studied chemical pretreatment methods. Cellulose in lignocellulosic biomass will be accessible for cellulase enzyme after acid pretreatment due to hydrolysis of hemicellulose whereas it happens after alkali pretreatment due to removal of the lignin polymer (Leu & Zhu, 2013). The huge variations in composition and structure of biopolymers among different types of feedstocks (Guragain et al., 2015) further complicated the optimization of pretreatment process; therefore, pretreatment processes must be separately optimized for each biomass feedstock.

Currently, crops residues, such as sorghum stalks and corn stover, are the most widely used feedstocks in lignocellulosic-based biorefineries. Dual use of land for both food and fuel is the main advantage of using crop residues as energy feedstocks. However, these feedstocks are only seasonally available. Besides, excessive removal of crop residues from farm lands and intensive fertilizer use to grow these crops degrade soil quality and increase greenhouse gas emission. In

addition, the cultivation of a monoculture crop in the large area for biofuels and biochemicals production deteriorates the local biodiversity (Mathews, 2009). Crop rotation, and planting dedicated energy crops are sustainable approaches to maintain soil quality and supply sufficient amount of feedstocks for energy industries; some of dedicated energy crops include perennial warm-seasons grasses (such as switchgrass and miscanthus), and short-rotation woody crops (such poplar and Douglas fir) (Blanco-Canqui, 2010; Fazio & Barbanti, 2014; Mola-Yudego et al., 2014). Therefore, modern biorefineries must be capable of utilizing a wide range of biomass resources to operate their plants at full capacity throughout the year, and separate biorefining strategies must be developed for each type of feedstock. Studies comparing biorefining strategies for different types of biomass feedstocks based on pretreatment methods is limited. In this study, three crops resides (sorghum stalks, *brm* sorghum stalks, and corn stover), one perennial grass (switchgrass), one hardwood (poplar), and one softwood (Douglas fir) were compared for acid and alkali pretreatment at the same pretreatment severity, including acid/alkali concentration, solids loading, time, and processing temperature. Pretreatment effectiveness was evaluated based on the sugars released during enzymatic hydrolysis of pretreated biomass, and inhibitory compounds produced and sugar lost during pretreatment. In addition, solids loading during enzymatic hydrolysis was optimized to get high sugars concentration in hydrolyzates for efficient fermentation and thereby reduced product recovery cost. Finally, a schematic for biorefining strategies based on acid and alkali pretreatment methods was proposed for different types of biomass feedstocks.

Materials and methods

Materials

Switchgrass, *bmr* (brown midrib) sorghum stalks (*bmr*12 mutant of forage sorghum, GW8528) and corn stover was obtained from the Kansas State University Agronomy Farm (Manhattan, Kansas). Regular sorghum stalks (the ground biomass) was obtained from Mesa Reduction Engineering & Processing Inc. (Auburn, New York); the sorghum was cultivated in Texas A&M University (College Station, Texas). Chopped (2 to 5 cm long) wild type poplar sample was provided by Edenspace, Inc. (Manhattan, Kansas). Ground Douglas fir sample was kindly provided by Dr. Michael Wolcott, Washington State University (Pullman, Washington). Novozymes, Inc. (Franklinton, North Carolina) provided Cellic CTec2 and Cellic HTec2 enzymes for biomass hydrolysis.

Sample preparation

The biomass samples were first chopped into 5 to 10 cm long pieces, and then ground using a Thomas-Wiley Laboratory Mill (Model 4) fitted with a 2-mm sieve. The ground biomass samples were sieved in a shaker (W.S. Tyler, Model – RX 29, Serial – 25225) fitted with two sieves with size 20 mesh (841 μm) and 80 mesh (177 μm) to get a specific particle size. The sorghum stalks and Douglas fir samples were directly sieved to get the same cut size because these samples were received in ground form. The size range of biomass was chosen based on the particle size required for biomass composition analysis without further size separation (Sluiter et al., 2007). The prepared samples were packed in sealed paper bags and stored at room temperature until further processing.

Optimization of biomass pretreatment

One sample from each type of the biomass samples (grass, hardwood and softwood) were selected for the optimization of alkali pretreatment; the selected samples were sorghum stalks (grass), poplar (hardwood) and Douglas fir (softwood). Wang et al. (2010) reported that 0.75% (w/v) sodium hydroxide (NaOH) solution at 121ºC is the optimum for the pretreatment of Coastal Bermuda grass, whereas Cao et al. (2012) reported that 2% (w/v) NaOH solution at 121ºC is effective for the pretreatment of sweet sorghum stalks. Seven different NaOH concentrations, from 0.5% to 2.0% (w/v), were taken for the optimization of sorghum stalks pretreatment. Higher NaOH concentration is required for the pretreatment of woody biomass (Rawat et al., 2013; Salehian et al., 2013); five different NaOH concentrations, from 1% to 8% (w/v), and from 2% to 10% (w/v), were taken for the pretreatment of poplar and Douglas fir, respectively. Twenty grams of ground biomass sample was mixed with 200 ml alkali solution for each concentration in a 500-ml Erlenmeyer flask and autoclaved at 121°C for 30 min. The biomass slurry was then filtered using a 200-mesh (74 μm) sieve. Approximately 15 ml filtrate was collected to measure sugars and inhibitors produced during pretreatment, and solids residue was washed with excess distilled water until the filtrate was clear and neutral to litmus paper. The pretreated samples were then dried overnight at 45°C and hydrolyzed as explained in section 2.5. The released sugars were measured to determine the optimum alkali concentration for pretreatment of each type of biomass.

Pretreatment of biomass

The optimum NaOH concentrations for pretreatment of sorghum and poplar were 1% and 2% (w/v), respectively; however, alkali pretreatment at 121ºC using upto10% (w/v) alkali concentration did not work well for Douglas fir (section 3.2). Therefore, 1% alkali concentration was used for the pretreatment of grasses (switchgrass, sorghum stalks, *bmr* sorghum stalks and

corn stover) and 2% alkali concentration was used for hardwood (poplar). Acid pretreatment was carried out by taking sulfuric acid (H_2SO_4) concentration equal to the optimized alkali concentration for each type of biomass, without separate optimization. However, our preliminary work on wheat straw pretreatment using 1% H_2SO_4 (v/v) at 121ºC for 30 min did not work well – total sugars released during enzymatic hydrolysis of pretreated wheat straw was less than 10% of biomass (unpublished data). Therefore, acid pretreatment for grasses were carried out at 140ºC for 40 min using 1% H_2SO_4 (Lloyd & Wyman, 2005) for the comparison of acid and alkali pretreatment. For softwood (Douglas fir), no significant improvement on hydrolysis efficiency was observed by increasing NaOH concentration from 2 to 10% (w/v) (section 3.2); therefore, 2% H_2SO_4 (v/v) and 2% NaOH (w/v) concentrations were used for the comparative evaluation of acid and alkali pretreatments, respectively, for Douglas fir. Twenty grams of ground biomass sample was mixed with 200 ml alkali/acid solution at the selected concentration in a 500-ml Erlenmeyer flask and pretreated at selected time and temperature, washed and dried as explained in section 2.3. Parr Pressure Reactor (Model 4523) was used for the pretreatment at 140ºC, and autoclave was used for the pretreatment at 121ºC.

Hydrolysis of pretreated biomass

Two grams of pretreated biomass was mixed with 40 ml citrate buffer (4.8 pH and 0.05 M) in a 125-ml conical flask with a screw cap, and 94 µl and 10 µl Cellic CTec2 and Cellic HTec2 enzymes, respectively, were added in each flask. The flasks were incubated in a temperature-controlled shaker (Innova 4300, New Brunswick Scientific, New Jersey, USA) at 50ºC and 150 rpm for 48 h. About 500 µl samples were drawn at 12, 24, and 48 h of hydrolysis from each flask to measure released monomer sugars. The final hydrolyzates were separated by centrifuging the

biomass slurry at 13,000 rpm (maximum g-force 20,400×g) for 15 min (Guragain et al., 2013), and used to produce biochemicals (2,3-butanediol); the fermentation data is not reported here.

Analytical procedures

An electric moisture meter (IR35M-00015V1, Denver Instrument GmbH, Goettingen, Germany) was used for moisture content determination in raw and pretreated biomass. The NREL standard protocols (Sluiter et al., 2005; Sluiter et al., 2007) were followed for biomass composition analysis, including extractives, lignin, glucan, xylan, and arabinan. Monomer sugars, including glucose, xylose, sucrose fructose, and arabinose were measured using High Performance Liquid Chromatography (HPLC). The HPLC instrument (Shimadzu Corporation, Japan) was equipped with an LC-20AB pump, an SIL-20 AC auto sampler, an SPD-M 20A photodiode array detector, and a Phenomenex RCM-Monosaccharide Ca^{+} column (300 × 7.8 mm). Flow rate of mobile phase (deionized water) was 0.6 ml min^{-1}. The column oven and refractive index detector (RID-10A) were maintained at 80°C and 65°C, respectively. The pretreatment-induced inhibitors, like hydroxymethylfurfural (HMF), furfural, acetic acid, lactic acid, and formic acid, were measure using ROA organic acid column (300 × 7.8 mm)., both RID and PDA (Photodiode Array)-UV detectors were used in the same HPLC system. A 0.005 N sulfuric acid in deionized water was used as mobile phase at the rate of 1.0 ml min^{-1}.

A modified Folin-Ciocalteu Reagent (FCR) method (Amendola et al., 2012) was used for the determination of total phenolics. The FCR method modification was done to draw standard curves because the initial experiments showed that the standard curve using only one phenolic (gallic acid) did not work well for the samples containing two or more phenolics. A standard curve using five phenolics was found to be a good estimation of total phenolics in samples containing known concentration of several phenolic compounds; the five phenolics used for standard mixtures

were vanillic acid, catechol, gallic acid, guaiacol and vanillin. The method in brief: 0.1 ml sample (neutralized and diluted, if required) was mixed with 5 ml FCR in a 15-ml centrifuge tube, and mixed for 5 min. A 3.5 ml sodium carbonate solution (11.5%, w/v) was added and mixed well. A similar process was followed for blank preparation using 0.1 ml deionized water instead of the sample. The mixture was incubated at 40°C for 1 h, then cooled to room temperature and the absorbance was taken at 745 nm. Concentration of total phenolics was determined using the previously drawn standard curve.

All the experiments were performed in triplicates, and data were statistically analyzed for significance test. The least significant difference (LSD) test was carried out at 95% confidence level ($P < 0.05$) using JMP software (SAS Institute Inc., Cary, North Carolina, United States).

Results and Discussion

Composition of biomass

Figure 4.1 shows that composition of biomass feedstocks significantly varied not only among softwood (Douglas fir), hardwood (poplar) and grass, but also among different grasses: switchgrass, sorghum stalks, *bmr* sorghum stalks and corn stover. Douglas fir had the highest lignin content (29% of biomass), followed by poplar (21%) and sorghum stalks (20%). The lowest lignin was found in *bmr* sorghum stalks (10%), indicating that the *bmr* mutation led to around 50% reduction in lignin content in sorghum biomass; however, the wild type sorghum used in this experiment was not the same parent line into which the *bmr* gene was introduced. Poplar had the highest glucan content (40%) followed by Douglas fir (37%) and switchgrass (35%). The highest total carbohydrate (glucan + xylan) content was found in switchgrass (64%) because of the highest amount of xylan (29%) in this biomass. Sorghum stalks and corn stover had statistically equal amounts of glucan (around 30%) and xylan (around 19%), but it was not true for lignin and

extractives contents in these biomass samples. The *bmr* sorghum stalks had the lowest amount of carbohydrate (46%), in addition to the lowest lignin content, but it had exceptionally high amount of extractives (40%). The biomass extractives were not characterized for this *bmr* sorghum sample; however, our earlier research (manuscript in review) showed that around 50% of *bmr* sorghum extractives were non-structural sugars (sucrose, glucose and fructose). These non-structural sugars can be extracted in hot water prior to biomass pretreatment and used as fermentable sugars, and the remaining biomass can be pretreated using a relatively less energy-intensive pretreatment method. This indicated that *bmr* sorghum stalks could be the most promising feedstock for biofuels and biochemicals production because of the possibility of getting maximum amount of fermentable sugars from this biomass with reduced pretreatment cost. The extractives components in woody biomass was very low compared to the grasses.

Optimization of alkali concentration for biomass pretreatment

Sorghum stalks: - Increasing NaOH concentration for pretreatment from 0.5% to 1.25% (w/v) resulted in a gradual increase in sugar released during hydrolysis from 54% to 75% of pretreated biomass weight, but beyond 1.25% of NaOH conferred no additional benefits (**Figure 4.2a**). On the other hand, the increase in NaOH concentration during pretreatment resulted in an increase in biomass loss as a result of delignification as well as partial hemicellulose hydrolysis. Total mass loss during pretreatment was 45%, 48%, 52%, 55%, and 56% of raw biomass using 0.5%, 0.75%, 1.0%, 1.25%, and 1.5 % of NaOH concentrations, respectively. This led to decreased overall sugar yield from biomass at higher NaOH concentration. Based on the raw biomass weight, the highest sugar yield (36% of biomass) was obtained from the sample pretreated with 1.0% NaOH. Biomass pretreatments were also performed at higher NaOH concentrations (1.75% and 2.0%). However, data are shown here only up to 1.5% because total sugars yield (gram per gram biomass) during

hydrolysis were statistically equal at 95% confidence level for all biomass samples pretreated with 1.5%, 1.75%, and 2.0% of NaOH concentrations for both pretreated and raw biomass weight basis. Total sugars loss, and phenolics, acetic acid and formic acid formation during pretreatment gradually increased by increasing NaOH concentration; however, hydromethylfurfural (HMF) and furfural were produced less than 0.01% of biomass for all NaOH concentrations (unpublished data). This results showed that 1% NaOH (w/v) was the optimum alkali concentration for the pretreatment of sorghum to achieve maximize sugar release during hydrolysis, and minimize the sugar loss and inhibitors formation during pretreatment. The 1% (w/v) NaOH concentration was taken as the optimum for the pretreatment of other grass samples as well, including switchgrass, *bmr* sorghum and corn stover, without optimization for each biomass.

Poplar: - Figure 4.2b shows that increasing NaOH concentration for pretreatment from 1% to 2% (w/v) resulted in an increase in sugar release during hydrolysis from 52% to 58% of pretreated biomass, but increase beyond 2% led to decreased sugar yield. Total biomass loss during pretreatment was equal in both 1% and 2% NaOH pretreatment (27% of raw biomass), but pretreatments with 4%, 6%, and 8% NaOH resulted in 32%, 37%, and 39% of biomass loss, respectively. Such increased biomass loss with increase in NaOH concentration led to further reduced sugars yield based on raw biomass weight at higher NaOH concentrations. The highest sugar yield during hydrolysis was obtained with 2% NaOH pretreatment in both pretreated biomass weight basis (58% of pretreated biomass) and raw biomass weight basis (43% of raw biomass). Similar to sorghum stalks, total sugar loss and phenolics, acetic acid and formic acid formation during pretreatment gradually increased by increasing NaOH concentration; however, HMF and furfural were produced less than 0.01% of biomass for all NaOH concentrations (unpublished

data). Based on these results, 2% NaOH (w/v) was considered as the optimum alkali concentration for the pretreatment of poplar.

Douglas fir: - **Figure 4.2c** shows that sugars yield during hydrolysis of alkali pretreated Douglas fir was very low (<5% of pretreated biomass) even at 10% (w/v) NaOH concentration. These results indicated that aqueous alkali pretreatment at 121°C is not effective for the deconstruction of softwood biomass.

Comparison of alkali and acid pretreatment

Figure 4.3 shows that alkali pretreatment led to much higher total sugars yield during enzymatic hydrolysis of pretreated biomass than acid pretreatment at the same pretreatment conditions; acid pretreatment in grasses was carried out even at higher temperature and longer time (140°C for 40 min) compared with alkali pretreatment (121°C for 30 min). The effectiveness of each pretreatment method significantly varied among the biomass samples. Based on the pretreated biomass weight (**Figure 4.3a**), the highest sugar release during hydrolysis was obtained from corn stover (83%), followed by *bmr* sorghum stalks (78%), sorghum stalks (75%), switchgrass (65%), and poplar (58%) for alkali pretreatment. Acid pretreatment was the most effective for switchgrass and *bmr* sorghum stalks, followed by sorghum stalks, corn stover and poplar. Both acid and alkali pretreatment methods at the conditions used in this experiment did not work for Douglas fir; total sugars released during hydrolysis of pretreated Douglas fir was less than 5% of biomass.

Various biomass components were removed during pretreatment depending upon the pretreatment method and biomass types, leading to a significant reduction in total sugar yield based on raw biomass weight than that of pretreated biomass weight. Alkali and acid pretreatment significantly remove lignin and hemicellulose, respectively, from lignocellulosic biomass (Leu &

Zhu, 2013). In addition, most of the biomass extractives are removed during pretreatment. Total mass loss with different biomass feedstocks differed significantly in both pretreatment methods. Total mass loss from switchgrass, sorghum stalks, *bmr* sorghum stalks, corn stover, poplar and Douglas fir during alkali pretreatment was 38%, 42%, 58%, 50%, 26%, and 22%, respectively, and 54%, 39%, 68%, 54%, 26%, and 21%, respectively, during acid pretreatment. Such variation on biomass loss during pretreatment among different type of biomass samples led to significant variation on relative sugar yield when the raw biomass (**Figure 4.3b**) and pretreated biomass (**Figure 4.3a**) were used as base materials for sugar yield calculation. The greatest change on the relative sugars yield among these biomass samples was observed in *bmr* sorghum stalks and poplar. Total sugar yield from alkali pretreated *bmr* sorghum stalks was 34% more than poplar based on pretreated biomass weight, but it was 24% less than poplar based on raw biomass weight. Similarly, total sugar yield from acid pretreated *bmr* sorghum stalks was 85% more than poplar based on pretreated biomass weight, but it was 19% less than poplar based on raw biomass weight. Such changes on relative sugar yields based on pretreated and raw biomass weight was as a result of higher proportion of extractives in *brm* sorghum stalks (40%) than poplar (5%), and thereby leading to the higher mass loss during pretreatment in *brm* sorghum stalks than poplar . If the non-structural sugars present in *bmr* sorghum stalks was extracted prior to pretreatment and added to the total sugars yield, the sugars yield from *bmr* sorghum stalks should be higher than poplar in raw biomass weight basis. Therefore, non-structural sugars recovery prior to pretreatment is vital for the biomass with high extractives content, like *bmr* sorghum stalks.

Alkali pretreatment results in effective delignification of lignocellulosic biomass leading to increased accessibility of enzyme for hydrolysis of carbohydrate polymers; therefore, it is generally considered that biomass with lower lignin content can be more effectively hydrolyzed

after alkali pretreatment (Alvira et al., 2010). In this study, a weak negative correlation ($R^2 = 0.15$) between lignin content in raw biomass in extractive-free basis and sugars released during hydrolysis of pretreated biomass was observed. For example, switchgrass and corn stover had statistically equal (at 95% confidence level) lignin content both in gross composition as well as extractive-free basis; but total sugars released during hydrolysis of alkali pretreated corn stover was 28% more than that of switchgrass (**Figure 4.3a**). Similarly, the *bmr* sorghum stalks had the lowest amount of lignin content, but hydrolysis efficiency of this biomass after alkali pretreatment was less than that of corn stover. These results indicated that low lignin content in biomass does not necessarily improve hydrolysis efficiency of alkali pretreated biomass (Guragain et al., 2014). In fact, the composition and structure of lignin as well as crystallinity of cellulose also affect sugar yield (Chang & Holtzapple, 2000; Wen et al., 2013). Lignin is a heterogeneous polyphenolic polymer containing three types of monomer units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). These lignin units are linked by carbon-carbon, ester, or ether linkages (Guragain et al., 2015). In addition, lignin in herbaceous plant also contains a considerable amount of *p*-coumarate and ferulate monomers, which significantly affect the type of linkages in lignin monomers (Pu et al., 2011). The pretreatment efficiency is better for biomass containing higher S/G ratio, and higher amount of ester and ether inter-unit linkages in biomass lignin, whereas high carbon-carbon linkages decrease pretreatment efficiency (Studer et al., 2011). Additionally, variation in crystallinity of cellulose among biomass types could be another significant factor that led to unequal sugars released during hydrolysis of pretreated biomass (Chang & Holtzapple, 2000).

The efficiency of acid pretreatment (**Figure 4.3a**) had a good positive correlation ($R^2 = 0.86$) with xylan content in raw biomass on an extractive-free basis. Xylan content on extractive-free

basis in switchgrass, sorghum stalks, *bmr* sorghum stalks, corn stover, and poplar were 34%, 21%, 32%, 25%, and 23%, respectively, and total sugars released during hydrolysis of pretreated biomass was 40%, 26%, 40%, 24%, and 22%, respectively. This indicated that biomass with higher xylan content on an extractive-free basis is more favorable for dilute acid pretreatment, and the variation in the xylan structure for various biomass types is less than that for lignin structure. However, the acid pretreatment of poplar was carried out at higher acid concentration, lower temperature, and less time than other biomass samples: 121°C for 30 min with 2% (v/v) H_2SO_4 for poplar and 140°C for 40 min with 1% (v/v) H_2SO_4 for other biomass samples. The correlation would be even stronger ($R^2 = 0.97$) for the biomass samples other than sorghum stalks. The slight deviation of results in some biomass, including sorghum stalks, might be due to the variation on the slow and fast reaction portion in xylan as well as reactivity of acetyl group present in the xylan polymer. It is postulated that the xylan polymer has two zones, called fast and slow reactive zones, due to the physical accessibility of a portion of the xylan polymer to the hydrolytic reagent, but not due to gross difference in their chemical structure (Hu & Ragauskas, 2012). Higher pretreatment temperature (>200ºC) is required for the biomass with high proportion of slow reactive zone to depolymerize xylan in acid pretreatment. In addition, accessibility of acetyl groups present in xylan polymers to the hydrolytic reagent significantly affects subsequent reactions. The hydrolyzed acetyl group becomes an *in situ* source of acetic acid and thereby helps further depolymerization of xylan polymers, but if the acetyl esters covalently linked to the xylan backbone are released as esterified xylo-oligosaccharides, it will be a stronger inhibitor to cellulase enzyme than pure xylo-oligosaccharides due to the steric hindrances of the acetyl groups (Hu & Ragauskas, 2012).

Sugar loss and inhibitory compounds produced during pretreatment

Depending on the pretreatment method and the severity of the process, biomass hemicellulose is partially hydrolyzed to monomer sugars and acetic acid; the sugars can be further degraded to a number of toxic compounds, including furfural, HMF, levulinic acid, and formic acid. In addition, various degradation products of lignin, including phenolic compounds, are also formed during pretreatment (Siqueira & Reginatto, 2015). These compounds are considered as toxins because they inhibit sugar-fermenting microbes (Feldman et al., 2015).

Table 4.1 shows that acid pretreatment resulted in much higher total sugar loss during pretreatment in all biomass samples than alkali pretreatment due to hydrolysis of hemicellulose. Alkali pretreatment produced significantly higher amounts of phenolics than acid pretreatment in all biomass samples, except corn stover, due to the degradation of biomass lignin. No specific trend was observed between acid and alkali pretreatment among these biomass samples for acetic acid production, that is, acid pretreatment produced higher acetic acid levels than alkali pretreatment in some biomass samples, but the opposite was true for others. This was because the acetic acid is produced from acyle/acetyl group present in both hemicellulose and lignin. Biomass lignin is partially acylated at γ-carbon of lignin monomer and linked to hemicellulose backbone; the proportion of acylated lignin varies from biomass to biomass (Guragain et al., 2015). Alkali pretreatment of poplar produced exceptionally very high amounts of acetic acid (12.2% of biomass), in which sugar released was only 3.4% of biomass, formic acid production was 3.1 % of biomass, and very low HMF and furfural (less than 0.01% of biomass). This result shows that only a small portion of hemicellulose was hydrolyzed during pretreatment of poplar and hence the major proportion of the acetic acid was produced due to the deacetylation of lignin, indicating that the poplar lignin was more extensively acylated/acetylated compared to other biomass samples.

This result was consistent with the fact that acetylation predominantly occurs at syringyl lignin units (Del Río et al., 2007), and the poplar has higher proportion of syringyl units; S/G ratio is as high as 3 (Studer et al., 2011), indicating high acylation/acetylation in poplar lignin. Formic acid was 0.3 to 2.4% of biomass; similar to acetic acid, no specific trend was observed between acid and alkali pretreatment among these biomass samples for formic acid production.

Small amounts of HMF and furfural (0.3% to 2.1% of biomass) were produced in acid pretreatment of grass samples, in which pretreatment was carried out at 140ºC using 1% (v/v) H_2SO_4. Very low amounts of these toxins (less than 0.01% of biomass) were produced in the remaining pretreatment methods, including acid pretreatment at 121ºC using 2% (v/v) H_2SO_4 (poplar and Douglas fir) and alkali pretreatment at 121ºC using 1% (w/v) NaOH (all grass samples) or 2% (w/v) NaOH (poplar and Douglas fir) did not significantly degrade sugars to HMF and furfural.

Comparison of potassium hydroxide and sodium hydroxide for alkali pretreatment

NaOH is the most extensively used alkali for biomass pretreatment, but potassium hydroxide (KOH) was also reported as an effective alkali for the pretreatment of grasses, such as switchgrass and rice straw (Ong et al., 2010; Sharma et al., 2013). Above results (**Figure 4.3**) showed that alkali pretreatment was better than acid pretreatment to maximize sugar release during hydrolysis; our next objective was to compare the effectiveness of two alkalis - NaOH and KOH, for biomass pretreatment using sorghum stalks as a feedstock. **Figure 4.4a** shows that equal molar (0.25 M) concentration of NaOH alone or mixture of NaOH and KOH were significantly more effective than KOH alone to release sugars from pretreated sorghum stalks. NaOH also led to production of higher amount of toxic materials and more sugar loss during pretreatment (**Figure 4.4b**). However, total sugars loss was very low (2.3% of biomass at the most) and the phenolics, the major inhibitory

compound produced during pretreatment, can be separated from pretreated biomass by filtration and used for high value applications (Kleinert & Barth, 2008). Mixture of NaOH and KOH at 0.125 M concentration each was as effective as NaOH alone at 0.25 M concentration, but NaOH alone is preferred over the mixture because a higher amount of KOH is required than NaOH to get the same molar concentration, leading to higher cost of alkali.

Optimization of solids loading during hydrolysis

Alkali pretreatment using NaOH was found promising to maximize sugar release during hydrolysis of pretreated biomass. Nonetheless, all above results were based on biomass hydrolysis using 5% (w/v) solids loading; the maximum total sugars in hydrolyzates was around 4% or 40 g/L. In order to reduce downstream processing cost, maximum possible concentration of product of interest is desired in the fermentation broth, which in turn requires higher sugars concentration in hydrolyzates. In a separate study, we found that 90 g/L total sugars is optimum to maximize yield (g per g sugars) and concentration (g/L) of 2,3-butanediol in fermentation broth using *Klebsiella oxytoca* (unpublished data). 2,3-butanediol is a valuable platform chemical used for production of a number of high value products, including methyl ethyl ketone, synthetic rubber and plastic, antifreeze, drugs, cosmetics, and food additives ((Ji et al., 2011; L. Li et al., 2013). **Figure 4.5** shows that by increasing solids loading in hydrolysis from 5% to 17.5% (w/v), the total sugar concentration in hydrolyzates increased from 37 g/L to 106 g/L, but sugars yields reduced from 73% to 60% (w/w) of pretreated biomass. The increase beyond 17.5% solids loading led to a further decrease in sugars yield without increase in sugar concentration in hydrolyzates. Therefore, 17.5% solids loading in hydrolysis is the optimum to maximize sugar concentration in biomass hydrolyzates. Based on the optimum sugars concentration for 2,3-butanediol production using *Klebsiella oxytoca* (90 g/L), the 15% solids loading in hydrolysis is the best option; 15%

solids loading produced 94 g/L total sugar in hydrolyzates with sugars yield of 63% of pretreated biomass.

Development of appropriate biorefining strategies for acid and alkali pretreatments

The biorefinery concept is analogous to the current petroleum-refinery concept, in which biomass conversion processes are integrated to utilize all biomass components for their high value applications to produce fuels, chemicals, and power in a similar way that multiple fuels and products are produced from petroleum (Menon & Rao, 2012). Current lignocellulosic-based biorefineries have focused on sugars derived from carbohydrate polymers. Lignin has been considered as a low value byproduct, and meanly combusted as boiler fuel, despite the great potential of the lignin products for a number of high-value applications to produce fuels, chemicals, fibers, and polymers (Kong et al., 2013; Ragauskas et al., 2014a). In addition to primary plant's metabolites (cellulose, hemicelluloses and lignin) and non-structural sugars (sucrose, glucose, and fructose), biomass also contains a number of valuable phytochemicals (plant's secondary metabolites), including terpenes and terpenoids, fats and waxes, phenolics, and alkaloids (Beatson, 2011). Efficient extraction and isolation of these extractives, and utilization of lignin for higher value applications is vital for the commercial viability of lignocellulosic-based biorefineries.

Figure 4.6 shows a schematic diagram for the development of appropriate biorefining strategies based on the feedstock type for acid and alkali pretreatment methods. Some biomass such as *bmr* sorghum contains around 15 to 25% non-structural sugars, including sucrose, glucose, and fructose. These sugars must be extracted from biomass before pretreatment otherwise the sugars can be degraded into furan derivatives during pretreatment. In addition, other inhibitory compounds produced during pretreatment, including phenolics, render the sugars present in the

pretreatment slurry useful as fermentable sugars. However, this process is not necessary for biomass with trace amounts of non-structural sugars such as poplar. In addition, appropriate extraction and isolation processes are to be developed for the extraction of valuable phytochemicals from biomass feedstock prior to biomass pretreatment.

After extraction of non-structural sugars and high value extractives, separate strategies should be used for the utilization of all biomass components using acid and alkali pretreatments. This study showed that acid pretreatment is much less efficient than alkali pretreatment under identical processing conditions. However, appropriate acid concentration and pretreatment temperature can be used to maximize hemicellulose hydrolysis during pretreatment and cellulose hydrolysis in subsequent enzymatic process (Lloyd & Wyman, 2005) for biomass feedstock containing high hemicellulose content and moderate amounts of lignin. The hemicellulose hydrolyzates obtained after acid pretreatment must be detoxified to get rid of pretreatment-induced inhibitory compounds to generate a clean sugar stream for fermentation, which incurs an additional processing cost. However, such process offers an opportunity of getting separate glucose and xylose streams for efficient fermentation because the bacteria with capability of utilizing both glucose and xylose sugars (such as *Klebsiella oxytoca*) have reduced fermentation efficiency in mixed sugar media compared to single sugar media. Such reduction was because of the catabolic repression of xylose utilization until the glucose is completely depleted in mixed sugars medium (Ji et al., 2011). In addition, residual solids after hydrolysis of acid pretreated biomass contains intact biomass lignin, which can be used for high value composite material production (Ragauskas et al., 2014a). In the alkali pretreatment process, the biomass lignin is depolymerized to smaller lignin units, which can be extracted and used for production of high value phenolic compounds (Kleinert & Barth, 2008).

The carbohydrate-rich pretreated biomass is then enzymatically hydrolyzed to produce clean sugars for biofuels and biochemicals production.

Conclusions

Effectiveness of acid and alkali pretreatment differed significantly among different biomass types, which indicates that an appropriate pretreatment process must be optimized for each biomass feedstock. Acid process requires harsher conditions compared to alkali for effective pretreatment; however, acid process can generate separate glucose and xylose streams for efficient sugar utilization during fermentation. Our study indicated that 17.5% (w/v) solids loading during hydrolysis of alkali pretreated biomass is optimum to maximize sugars concentration in hydrolyzates; the high sugars concentration (10.6%, w/v) obtained at this loading is advantageous to achieve high product titer and productivity for any bioprocess with concomitant reduced downstream processing costs. We finally proposed a schematic for innovative biorefining strategies for different types of biomass based on acid and alkali pretreatment methods for the sustainable bioprocessing of multiple feedstocks.

Table 4.1 Sugar loss and inhibitors production during acid and alkali pretreatment

Biomass types	Catalyst	Total sugars and inhibitors production (%, g/g biomass)					
		Sugars	Phenolics	Acetic acid	Formic acid	HMF	Furfural
Douglas fir	Alkali	0.4 ± 0.0^{g}	3.5 ± 0.1^{b}	3.1 ± 0.0^{def}	2.2 ± 0.0^{b}	< 0.01	< 0.01
	Acid	12.2 ± 0.9^{d}	0.1 ± 0.0^{I}	1.5 ± 0.1^{f}	0.3 ± 0.0^{e}	< 0.01	< 0.01
Poplar	Alkali	3.4 ± 0.2^{ef}	1.8 ± 0.3^{e}	12.2 ± 2.8^{a}	3.1 ± 1.1^{a}	< 0.01	< 0.01
	Acid	12.6 ± 0.6^{cd}	0.4 ± 0.0^{h}	4.5 ± 0.1^{bcd}	2.4 ± 0.2^{b}	< 0.01	< 0.01
Switch-grass	Alkali	1.4 ± 0.1^{fg}	3.0 ± 0.1^{c}	4.5 ± 0.5^{bcd}	0.5 ± 0.0^{de}	< 0.01	< 0.01
	Acid	24.5 ± 2.7^{a}	0.5 ± 0.1^{h}	4.5 ± 0.6^{bcd}	1.0 ± 0.2^{cd}	0.2 ± 0.0^{c}	2.1 ± 0.2
Sorghum Stalk	Alkali	1.4 ± 0.7^{fg}	3.9 ± 0.2^{a}	3.7 ± 0.1^{cdef}	0.5 ± 0.0^{de}	< 0.01	< 0.01
	Acid	17.1 ± 0.9^{b}	1.3 ± 0.1^{f}	2.3 ± 0.1^{def}	0.3 ± 0.0^{e}	0.1 ± 0.0^{c}	0.3 ± 0.0
bmr Sorghum stalk	Alkali	14.7 ± 2.4^{bc}	2.9 ± 0.4^{c}	4.1 ± 0.6^{cde}	0.5 ± 0.1^{de}	< 0.01	< 0.01
	Acid	25.2 ± 2.8^{a}	1.1 ± 0.1^{g}	6.0 ± 0.7^{bc}	1.4 ± 0.3^{c}	2.0 ± 0.4^{a}	1.0 ± 0.2
Corn stover	Alkali	5.1 ± 1.4^{e}	1.8 ± 0.1^{f}	1.9 ± 0.5^{ef}	0.3 ± 0.1^{e}	< 0.01	< 0.01
	Acid	25.7 ± 0.2^{a}	2.5 ± 0.1^{d}	6.6 ± 0.1^{b}	2.2 ± 0.1^{b}	1.2 ± 0.0^{b}	1.5 ± 0.0

HFM = Hydroxymethylfurfural. Acid pretreatment was carried out at 121°c for 30 min with 10% solid loading in 2% (v/v) sulfuric acid (H_2SO_4) for Douglas fir and poplar, and at 140°c for 40 min with 10% solid loading in 1% (v/v) H_2SO_4 for remaining biomass samples, and alkali pretreatment at 121°c for 30 min with 10% solid loading in 1% (w/v) sodium hydroxide. Data are average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each other at the $P < 0.05$ level.

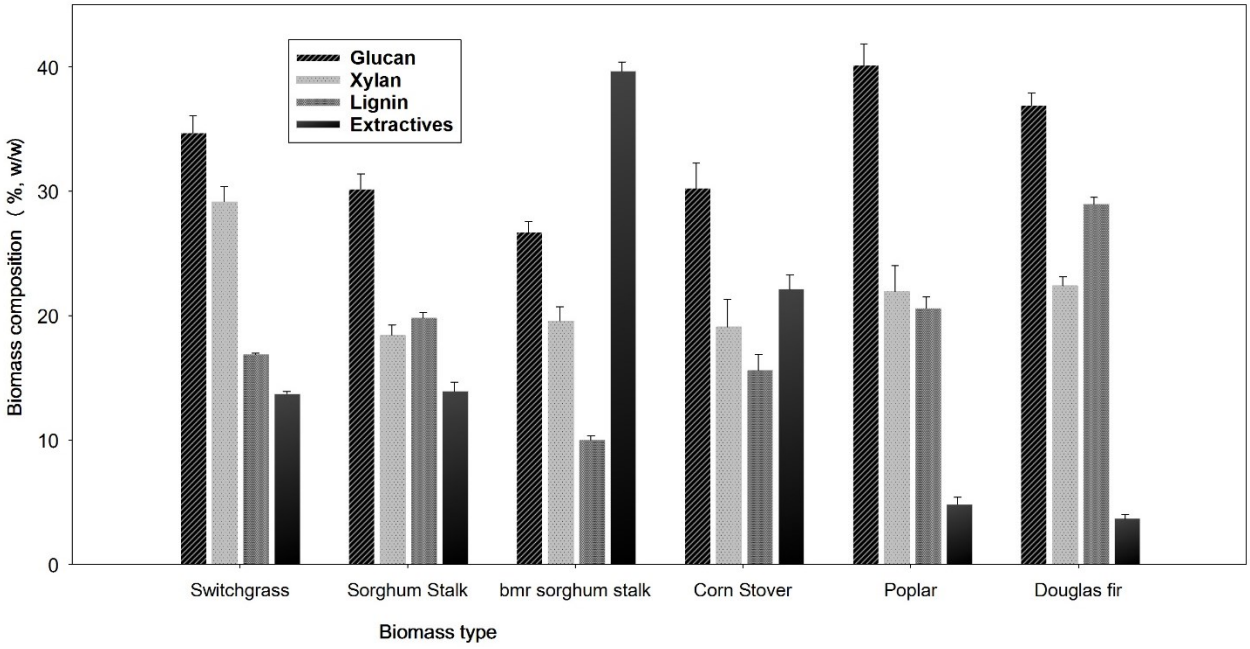


Figure 4.1 Composition of various biomass feedstocks. *bmr* = brown midrib sorghum mutant. Extractives = sum of water-soluble and 95% ethanol-soluble extractives. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

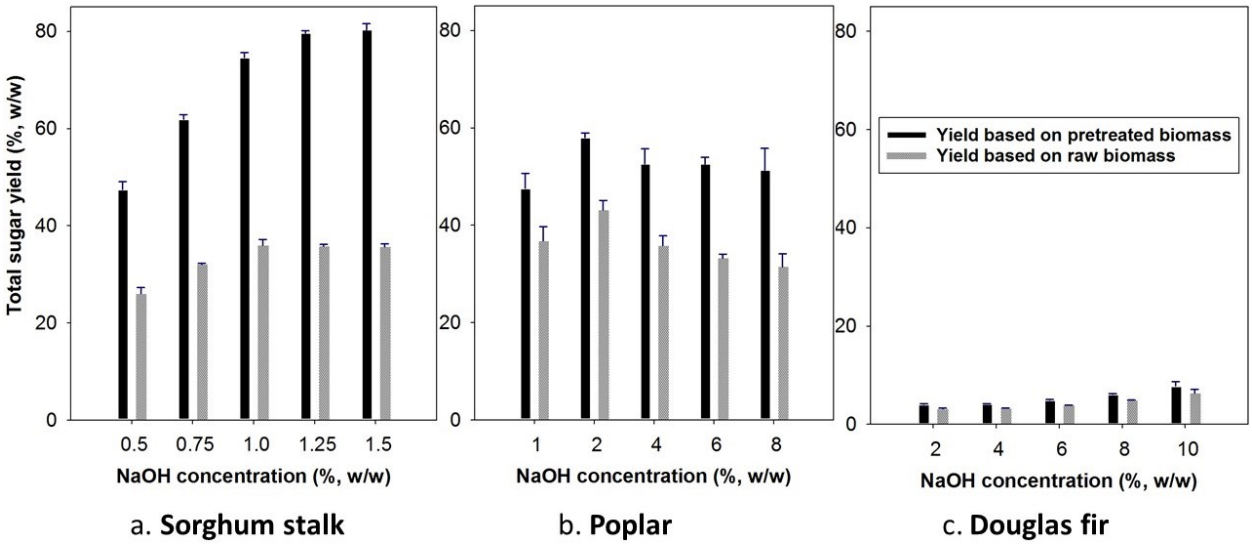
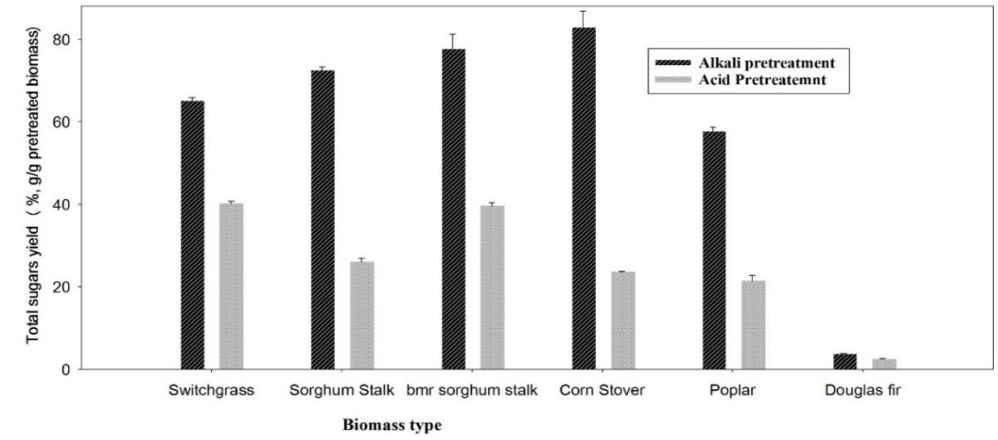
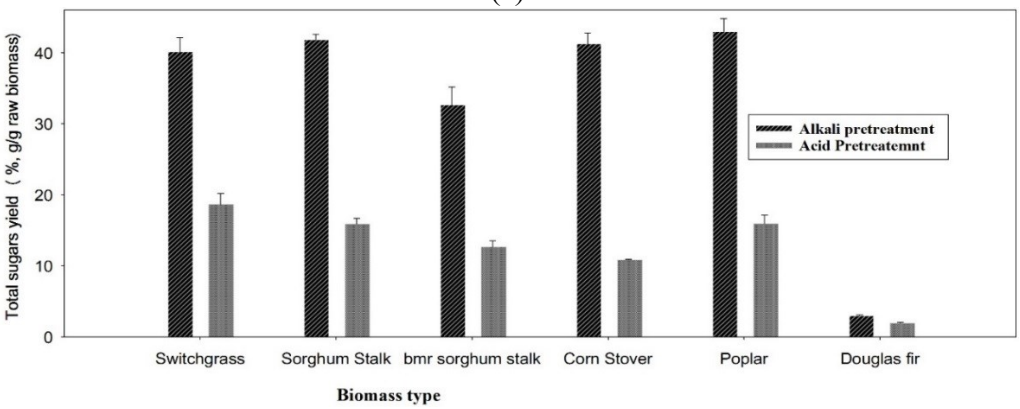


Figure 4.2 Total sugars released during hydrolysis of pretreated biomass at various concentration of sodium hydroxide (NaOH). Pretreatment was carried out at 121°C for 30 min with 10% (w/v) solid loading in NaOH solution, followed by hydrolysis at 50°C for 48 h with 5% (w/v) solid loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation



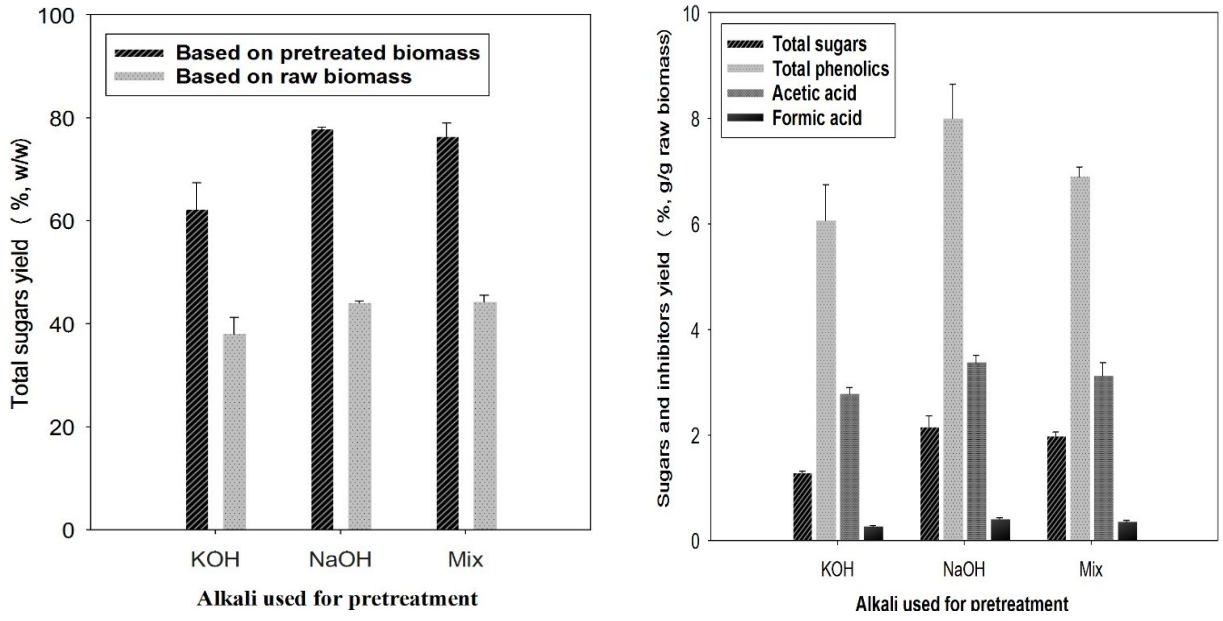
(a)



(b)

Figure 4.3 Comparison of acid and alkali pretreatment for various biomass feedstocks in terms of total sugars yield during enzymatic hydrolysis – (a) Based on pretreated biomass weight and (b) based on raw biomass weight. Acid pretreatment was carried out at 121°C for 30 min with 10% solid loading in 2% (v/v) sulfuric acid (H_2SO_4) for woody biomass (Douglas fir and poplar), and at 140°c for 40 min with 10% solid loading in 1% (v/v) H_2SO_4 for grasses (switchgrass, sorghum stalks, *bmr* sorghum stalks, and corn stover). Alkali pretreatment was carried out at 121°C for 30 min with 10% solid loading using 1 and 2% (w/v) sodium hydroxide for grasses and woody biomass respectively. Hydrolysis was carried out at 50°C with 5% solid loading in citrate buffer (4.8 pH and 0.05 M) with enzyme loading of 6% (w/w) of solid for all

samples. Data are average values of triplicate experiments, and error bars represent sample standard deviation.



(a) (b)

Figure 4.4 Comparative evaluation of potassium hydroxide (KOH) and sodium hydroxide (NaOH) pretreatment for sorghum stalks in terms of (a) total sugars yield during enzymatic hydrolysis and (b) Sugar lost and inhibitors produced during pretreatment. Pretreatment was carried out at 121°c for 30 with 10% solid loading using 0.25 M for single alkali or 0.125 M each for mixed alkali. Hydrolysis was carried out at 50°C with 5% solid loading in citrate buffer (4.8 pH and 0.05 M) with enzyme loading of 6% (w/v) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

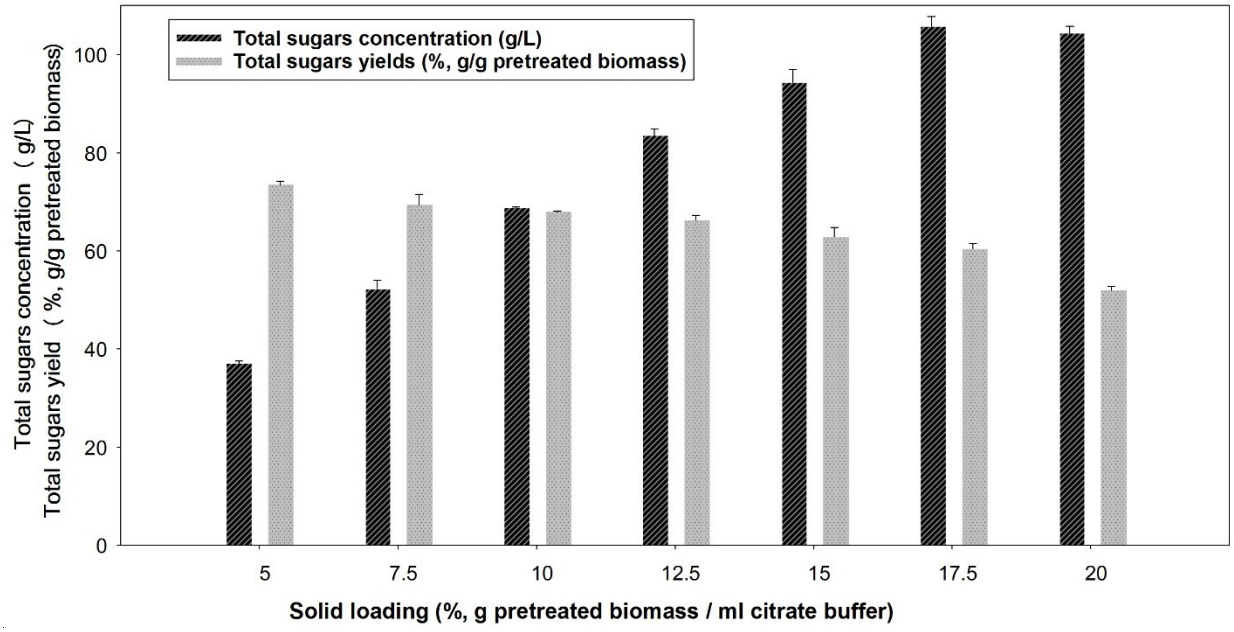


Figure 4.5 Evaluation of hydrolysis efficiency of pretreated sorghum stalks at various solid loading. Pretreatment was carried out at 121°c for 30 with 10% solid loading using 1% (w/v) sodium hydroxide, and hydrolysis was carried out at 50°C with 2% solid loading in citrate buffer with enzyme loading of 6% of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

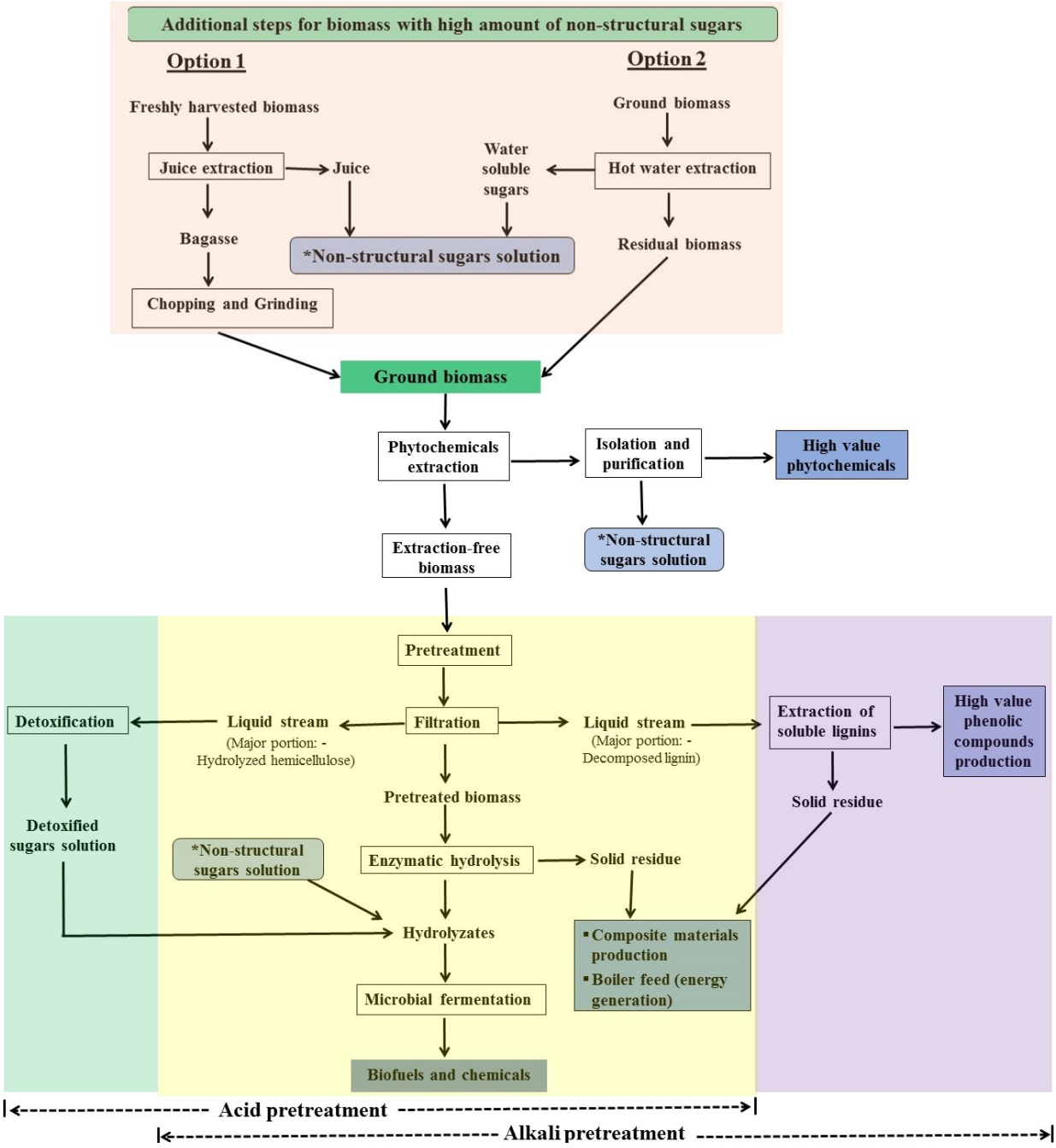


Figure 4.6 Schematic diagram of appropriate biorefining strategies using acid and alkali pretreatment

Chapter 5 - Novel biomass pretreatment using alkaline organic solvents: A green approach for biomass fractionation, and 2,3-butanediol production

Abstract

Valorization of each component of lignocellulosic biomass is critical for sustainability of biorefinery industries. Current biorefineries are confined to ethanol-centric processes, and focus only on the carbohydrate-derived sugar using energy-intensive pretreatment methods, leading to deteriorated liquid quality for high value applications. Organosolv fractionation is an effective method to improve hydrolysis efficiency of cellulose, and extract a good quality lignin stream; however, hemicellulose recovery is challenging if an acid catalyst is used. An alkali catalyst in the organosolv process, therefore, could be a promising alternative approach. We evaluated various organic solvents (glycerol, 2,3-butanediol, dimethyl sulfoxide, ethanol, butanol, isopropanol, acetonitrile, and water) for pretreatment of different biomass feedstocks, including corn stover (grass), poplar (hardwood) and Douglas fir (softwood) using sodium hydroxide as a catalyst. Results showed that an ethanol and isopropanol mixture led to 18% more sugar released per gram of biomass than the control (conventional aqueous alkali pretreatment) for corn stover; a mixture of ethanol, butanol and water was the next most effective solvent. For pretreatment of poplar biomass, glycerol and 2,3-butaneidol were the most efficient solvents; glycerol pretreatment offers further process improvement opportunities. The organic solvents used in this experiment were not effective for Douglas fir. The quality of released sugar were statistically equal to that of synthetic sugar for 2,3-butanediol fermentation using *Klebsiella oxytoca*. This study opened up a promising

route for high value application for all biomass components. Further research is needed to characterize the extracted lignin for quality evaluation.

Keywords: Organosolv, catalyst, pretreatment, fermentation, organic solvent

Introduction

Rapid growth in fuel ethanol production in last decade (Guragain et al., 2016) indicates that non-food-based materials, including lignocellulosic residues, must be utilized to meet the huge feedstock demand for biorefineries. Lignocellulosic biomass feedstocks are a sustainable resources for biofuels and biochemicals production to replace their petroleum-derived counterparts (Casler et al., 2009; Hu & Ragauskas, 2012). Development of integrated biorefineries is critical for the commercial viability of biorefining industries (Bozell et al., 2014). The recently developed biorefinery concept is analogous to that of petroleum-refinery concept, in which biomass conversion processes are integrated to utilize all biomass components to produce fuels, chemicals, and power, in a similar way to how multiple fuels and products are produced from petroleum (Menon & Rao, 2012). Lignocellulosic biomass is mainly composed of lignin (15 to 30%) and carbohydrates (50 to 70%) that include cellulose, hemicellulose, and other non-structural sugars. In addition, the biomass contains valuable secondary metabolites, such as phenolics, alkaloids, terpenes, terpenoids, fats and waxes. All of the components of biomass can be converted to high value products through various bioprocessing routes (Beatson, 2011; Werpy et al., 2004). Therefore, the robust biorefinery industry should focus on high-volume, low-value biofuel production to meet growing energy demand and low-volume, high-value bio-based chemicals production for the economic viability of the industry (Bozell et al., 2014). The petroleum-derived

chemicals market is less than 10% of crude oil consumption, but significantly contributes toward the overall profitability of petrochemical industries (Bozell et al., 2014; Marshall, 2007).

Current lignocellulosic-based biorefineries are overwhelmingly confined to the release of sugar from carbohydrate polymers for ethanol production. The default biomass conversion process is the use of energy-intensive biomass deconstruction processes to make carbohydrate polymers susceptible for enzymatic hydrolysis to monomer sugars, which are then fermented to ethanol (Guragain et al., 2014). The strong lignin barrier in lignocellulosic biomass is considered a major obstacle, making the biomass deconstruction process expensive (Hu & Ragauskas, 2012; Tomas-Pejo et al., 2008). Biomass lignin is, therefore, recognized only for its negative impact on biochemical conversion (Dien et al., 2009; Sattler et al., 2012), and little attention is given to the quality of extracted lignin because the lignin stream is usually combusted as boiler fuel, despite its great potential for applications in bulk and specialty chemical production (Yuan et al., 2011a). The carbon to oxygen ratio in lignin is almost double that found in carbohydrates (about 2:1 in lignin and 1:1 in carbohydrates); therefore, lignin comprises 40% of lignocellulosic biomass energy even though it is only 15 to 30% by weight (Laskar et al., 2013). Lignin valorization is a daunting challenge as well as a great opportunity for design of a profitable lignocellulosic-based biorefinery. The distinct variation of composition and structure of lignin among different biomass types (Guragain et al., 2015) further complicates the lignin valorization process. In addition, extraction of a number of valuable biomass extractives prior to delignification is equally important. A schematic representation of the conventional ethanol-centric lignocellulosic-based process and a new concept of biorefinery is illustrated in **Figure 5.1**.

Lignin valorization is critical for the sustainability of biorefining industries; however, it cannot be done at the expense of effective carbohydrate polymer utilization (Ragauskas et al.,

2014a). Thus, appropriate pretreatment methods must be developed to extract high quality lignin with improved hydrolysis efficiency of the residual carbohydrate polymers. Organosolv pretreatment is considered one of the promising methods to achieve this goal. Lignin extracted using the organosolv process is sulfur-free, rich in functional components, including phenolics, relatively more homogeneous, and includes minimum contamination of residual carbohydrate (Bozell et al., 2014; Ragauskas et al., 2014a). In the organosolv pretreatment method, biomass is mixed in selected organic solvents, with or without catalysts (acid or alkali) addition, and heated for selected time and temperature. A diverse range of organic solvents can be used in this process. These include low boiling point solvents, such as ethanol, methanol and acetone; and high boiling point solvents, such as glycerol, ethylene glycol and tetrahydrofurfuryl alcohol. Other classes of organic solvents, such as organic acids, phenols, ketones and dimethyl sulfoxide can also be used (Thring et al., 1990; Zhao et al., 2009b). Ethanol organosolv pretreatment using acid catalyst is the most widely used method among the organosolv pretreatments (Sousa et al., 2009). Such a process almost completely removes hemicellulose and extensively removes lignin from biomass, thereby leaving digestible cellulose residues (Zhao et al., 2009b). Organosolv pretreatment using high boiling point alcohols, mostly polyhydroxy alcohols, is also gaining attention since the process can be performed at atmospheric conditions (Sun & Chen, 2008). Use of low cost crude glycerol, a major byproduct of oleochemical industries, for biomass pretreatment is also considered an attractive economic route for biofuels and biochemical production (Guragain et al., 2011). Oleochemical industries produce crude glycerol at a rate of around 10% of total biodiesel production. The purification process to recover high quality glycerol from such crude glycerol is very expensive, and therefore, economically infeasible to use in food, pharmaceutical and cosmetic applications (Sun & Chen, 2008).

One of the major drawbacks of organosolv pretreatment using an acid catalyst is loss of hemicellulose in the lignin stream. The hydrolyzed hemicellulose from the lignin stream is hard to recover as fermentable sugar due to the presence of a number of inhibitory compounds, including phenolics. Diner & Fan (2012) proposed an alkaline organosolv pretreatment method using ammonia (2 to 20% of biomass) as catalyst and ethanol (40 % to 70% in water) as solvent. This process led to extensive removal of lignin without significant loss of hemicellulose. The lignin-free carbohydrate polymers (cellulose and hemicellulose) were effectively hydrolyzed using enzymes. However, high cost for ammonia recovery (Mosier et al., 2005) and environmental issues for using ammonia in commercial scale production are the main concerns for using ammonia as the catalyst. Therefore, an alkaline organic solvent using sodium hydroxide (NaOH) catalyst could be a promising alternative approach. The delignification mechanism in organosolv pretreatment involves breakage of β-O-4 inter-unit linkages in lignin, and lignin-carbohydrate bonds followed by lignin solubilization in organic solvents (Ragauskas et al., 2014a); the process could be further improved by NaOH addition (Alvira et al., 2010). In addition, during acid-catalyzed delignification process, the simultaneous lignin repolymerization reaction by lignin-lignin condensation is induced due to the formation of new carbon-carbon linkages between side chain (especially β-position) of one lignin unit with aromatic ring (especially 5- or 6-position) with another lignin unit (Balakshin et al., 2003). The lignin condensation results in formation of low-reactive lignin for potential high value applications and reduced hydrolysis efficiency of residual carbohydrate polymers (Li & Gellerstedt, 2008). The addition of NaOH (0.2%, w/v) or 2-naphthol (0.16%, w/v) significantly reduces lignin-lignin condensation by reacting with lignin side chains of depolymerized lignin units and forming stable low molecular lignin units; such changes are

beneficial for lignin valorization as the extracted lignin will contain homogeneous lignin oligomers (Li & Gellerstedt, 2008).

In this study, various organic solvents, including glycerol, 2,3-butanediol, dimethyl sulfoxide, ethanol, butanol, isopropanol, and acetonitrile, were assessed for the effective pretreatment of different types of biomass feedstocks, including corn stover (grass), poplar (hardwood) and Douglas fir (softwood), using NaOH as the catalyst. The objectives of this research were to test the hypotheses: (a) alkaline organic solvent pretreatment is effective to improve hydrolysis efficiency of pretreated biomass; (b) biomass type significantly affects the effectiveness of alkaline organic solvent pretreatment; and (c) the quality of released sugar during hydrolysis of pretreated biomass is good for biofuel and biochemical production. The quality of biomass hydrolyzates was evaluated and compared with synthetic sugar solutions for 2,3-butanediol fermentation using *Klebsiella oxytoca* bacteria. 2,3-butanediol is an important platform chemical possessing diverse industrial applications for the production of a number of high-value products, including foods, pharmaceuticals, fuel, rubber, and chemicals (Xin et al., 2015).

Materials and methods

Materials

Three different types of biomass representing grass (corn stover), hardwood (poplar) and softwood (Douglas fir) were used in this study. These biomass samples were obtained from the Kansas State University Agronomy Farm (Manhattan, Kansas), Edenspace Inc. (Manhattan, Kansas), and Dr. Michael Wolcott, Washington State University (Pullman, Washington), respectively. Novozymes, Inc., Franklinton, North Carolina provided Cellic CTec2 and Cellic HTec2 enzymes for biomass hydrolysis. *Klebsiella oxytoca* ATCC 8724 was obtained from

American Type Culture Collection (Manassas, Virginia, USA). The culture was revived in nutrient broth medium, and stock culture tubes were prepared in 15% glycerol media comprising 0.5 ml each of revived culture and 30% (v/v) sterilized glycerol. The stock cultures were stored at -80°C until used for inoculum preparation. Organic solvents, including dimethyl sulfoxide (DMSO), 2,3-butanediol, glycerol, ethanol, butanol, isopropanol, and acetonitrile, were procured from Thermo Fisher Scientific and were used as received.

Biomass sample preparation

Corn stover and poplar samples were ground using a Thomas-Wiley Laboratory Mill (Model 4) fitted with a 2-mm sieve. The ground biomass samples were sieved in a shaker (W.S. Tyler, Model – RX 29, Serial – 25225) fitted with two sieves, size 20 mesh (841 μm) and 80 mesh (177 μm) to get a specific particle size range (177 μm to 841 μm). The Douglas fir samples were directly sieved to get the same particle size because these samples were received in ground form. The size range of biomass was chosen based on the particle size required for biomass composition analysis without further size separation (Sluiter et al., 2007). The ground samples were packed in sealed paper bags and stored at room temperature until further processing.

Biomass pretreatment

The optimized aqueous alkali pretreatment was used as the control pretreatment experiment for comparative evaluation of effectiveness of various alkaline organic solvent pretreatment methods. Our earlier research (Guragain et al., 2013) showed that 1% (w/v) sodium hydroxide (NaOH) pretreatment using 10% solids loading (30 g biomass in 300 ml alkali solution) at 121°C for 30 min was effective for the pretreatment of grasses, including corn stover. These pretreatment conditions were taken as the optimum control pretreatment method for corn stover. For poplar

biomass pretreatment, NaOH concentration was optimized keeping all other parameters same as that for corn stover pretreatment. Rawat et al. (2013) reported that 2.8% NaOH concentration using biomass to alkali solution ratio of 1:8 at 94°C for 60 min was optimum for poplar biomass pretreatment. Five different NaOH concentrations ranging from 1% to 6% (w/v) were used for the optimization study of poplar biomass pretreatment. A 20 g ground biomass sample (dry weight basis) was mixed with 200 ml alkali solution for each concentration in 500-ml flask, and autoclaved at 121°C for 30 min. The biomass slurry was allowed to cool to room temperature and filtered using a 200-mesh (74 μm) sieve. The residual solids were washed with excess distilled water until the filtrate was clear and neutral to litmus paper. Pretreated samples were then dried overnight at 45°C and hydrolyzed as explained below ("Biomass hydrolysis" sub-section). The released sugar content was measured to determine optimum NaOH concentration required for poplar biomass pretreatment. Optimized control pretreatment conditions for poplar were also used as the control for Douglas fir biomass pretreatment.

The alkaline organic solvent pretreatment process was optimized for processing temperature (75 to 170°C) and catalyst (NaOH) concentration (0 to 0.4%, w/v) using corn stover as feedstock. The maximum pretreatment temperature of 170°C was selected for high boiling point (bp) solvents (DMSO: bp- 189°C, Glycerol: bp- 290°C, and 2,3-butanediol: bp- 177°C), to perform the process at atmospheric pressure. Among the low boiling point solvents, ethanol (bp- 78°C) pretreatment was evaluated at 75°C and 170°C (under pressure). A 20 g ground biomass sample (dry weight basis) was mixed with 200 ml organic solvent with calculated amount of NaOH in 500-ml two-neck flask; NaOH was first dissolved in 1 ml water before mixing with the organic solvent. A condenser was inserted into one neck of the flask, and a thermometer was inserted into the other neck. The flasks were refluxed for two hours using a heater (Electrothermal House,

Rochford, Great Britain, Model: EMEA30500/CEX1) to achieve desired temperature. Magnetic stirrer was used to stir the sample during heating. For the ethanol pretreatment at 170°C, the sample was heated in a Parr Pressure Reactor (Model 4523). After heating the biomass slurry for desired time and temperature, it was washed, dried and hydrolyzed following the same processes used for the control pretreatment. The same pretreatment conditions optimized for ethanol were used for all other low boiling solvents, including acetonitrile, isopropanol, butanol, and water. The optimized conditions used for corn stover were also used for poplar and Douglas fir and no separate optimizations were performed for those biomass samples.

Finally, all the biomass samples were pretreated using control as well as alkaline organic solvent methods at optimized conditions. The optimized conditions for different biomass feedstocks and pretreatment solvents are summarized in **Table 5.1**. Pretreated biomass was used for enzymatic hydrolysis after washing with excess water and drying overnight at 45°C.

Biomass hydrolysis

Two grams of pretreated biomass was mixed with 40 ml citrate buffer (4.8 pH and 0.05 M) in a 125-ml conical flask with a screw cap, and 94 µl and 10 µl Cellic CTec2 and Cellic HTec2 enzymes, respectively, were added to each flask. The flasks were incubated in an incubator -shaker (Innova 4300, New Brunswick Scientific, New Jersey, USA) at 50°C and 150 rpm. Samples of approximately 500 µl were drawn every 12 h, for 71 h, from each flask to measure released monomer sugars. The final hydrolyzates were separated by centrifuging the biomass slurry at 13,000 rpm (maximum g-force 20,400×g) for 15 min and stored -20° C until used for fermentation (Guragain et al., 2013).

Fermentation of biomass hydrolyzates

The growth curve for *Klebsiella oxytoca* bacteria in shake-flask fermentation showed that the culture has a very slow growth (lag phase) for the first 4 hours, followed by exponential growth (log phase) between 4 to 10 hours, and finally very slow or almost no growth after 10 hours (unpublished data). Therefore, 7 h incubation was considered the optimum time for inoculum preparation. To prepare inoculum, 0.3 ml stock culture was added to 100 ml sterilized nutrient broth medium in a 1000-ml flask and incubated in a temperature-controlled shaker (Innova 4300, New Brunswick Scientific, NJ, USA) at 37°C and 200 rpm for 7 h.

Initial fermentation experiments were carried out using synthetic sugar solutions with different concentration of glucose and xylose to study sugar utilization and 2,3-butanediol production efficiency of the culture, *K. oxytoca*. Then, the biomass hydrolyzates prepared from different biomass feedstocks and pretreatment methods were evaluated for fermentability of the biomass-derived sugar. Fermentation media was prepared by adding 2 ml filter sterilized (Millipore, 0.22μm) concentrated nutrient solution to 16 ml sterilized (autoclaved at 121°C for 15 min) biomass hydrolyzates (or sugar solution) in 250-ml flasks. The nutrient solution was prepared 10 times more concentrated than the recommended concentration for each nutrient. The recommended nutrients (per liter): 2 g monopotassium phosphate, 10.5 g dipotassium phosphate, 3.3 g diammonium phosphate, 6.6 g ammonium sulfate, 0.25 g magnesium sulfate heptahydrate, 0.05 g ferrous sulfate heptahydrate, 0.001 g zinc sulfate heptahydrate, 0.001 g manganese (II) sulfate monohydrate, 0.01 g calcium chloride dihydrate, and 0.05 g ethylenediaminetetraacetic acid (Jansen et al., 1984). Control sample containing glucose, xylose and arabinose in the concentration similar to biomass hydrolyzates (the average concentration of biomass hydrolyzates) was also prepared along with biomass hydrolyzates. Freshly prepared 2 ml inoculum was added to

each flask and incubated at 37°C and 200 rpm. About 500 µl samples were drawn at different time intervals from each flask to measure residual sugar and 2,3-butanediol produced until almost all sugars were consumed.

Analytical methods

Monomer sugars (glucose, xylose and arabinose), and fermentation products/byproducts including, 2,3-butanediol, acetoin, and glycerol were measured using HPLC. The HPLC instrument (Shimadzu Corporation, Japan) was equipped with an LC-20AB pump, an SIL-20 AC auto sampler, an SPD-M 20A photodiode array detector, and a Phenomenex RCM-Monosaccharide Ca+ column (300 × 7.8 mm). Flow rate of mobile phase (deionized water) was 0.6 ml min−1. The column oven and refractive index detector (RID-10A) were maintained at 80°C and 65°C, respectively. Other fermentation byproducts, such as acetic acid, lactic acid, formic acid, and ethanol, were measure using ROA organic acid column (150 × 7.8 mm), using both RID and PDA (Photodiode Array)-UV detectors for the analysis. A 0.005 N sulfuric acid in deionized water was used as mobile phase with the same flow rate of 0.6 ml/min

Statistical method

All experiments, including pretreatment, hydrolysis and fermentation, were carried out in triplicate. The data were statistically analyzed by the least significant difference (LSD) test at 95% confidence level ($P < 0.05$) using JMP software (SAS Institute Inc., Cary, North Carolina, United States).

Results and discussion

Composition of biomass

Figure 5.2 shows that the chemical composition of the three biomass samples: corn stover (grass), poplar (hardwood) and Douglas fir (softwood), were significantly different from one another. Poplar had the highest total carbohydrate polymers (40.2% glucan, 22.0% xylan and 3.8% arabinan), followed by Douglas fir (36.9% glucan, 22.4% xylan and 2.2% arabinan), and corn stover (30.3% glucan, 19.1% xylan and 3.2% arabinan). Douglas fir had the highest lignin content (29.0%), which was 1.4 and 1.9 times more than the poplar and corn stover, respectively. Corn stover contained an exceptionally high amount of extractives (22.1%) compared to woody biomass samples (poplar: 4.8% and Douglas fir: 3.8%). Extractives were the sum of water-soluble and alcohol (95%)-soluble extractives; corn stover had 18.9% water soluble extractives and 3.2% alcohol soluble extractives. The water-soluble extractives include non-structural sugars, nitrogenous material and other inorganic materials. Alcohol-soluble extractives include waxes, chlorophylls and other minor components (Sluiter et al., 2005). The extractives were not characterized in this study; however, our previous study on grass samples showed that approximately 50% of water soluble extractives were non-structural sugars, including glucose, sucrose and fructose (unpublished data). This study showed that the corn stover could have as high as 10% non-structural sugars, hence separate strategies are required to recover these sugars prior to pretreatment. Presence of a number of pretreatment-induced inhibitory compounds in the pretreatment liquid limits use of these sugars as fermentable sugars unless an appropriate detoxification process is used (Chandel et al., 2013). If non-structural sugars in corn stover were also included in this study, total carbohydrate in all biomass samples would be 62 to 67%, indicating fairly equal amount of total carbohydrates.

Optimization of pretreatment methods

Aqueous alkali pretreatment was used as control in this study to evaluate the effectiveness of alkaline organic solvent pretreatment. **Figure 5.3** shows that 2% (w/v) sodium hydroxide (NaOH) pretreatment resulted in the highest sugar yield per gram raw poplar biomass during enzymatic hydrolysis. The lower NaOH concentration (1%, w/v) led to incomplete hydrolysis of carbohydrate polymers due to higher amount of residual lignin in the pretreated biomass. The higher NaOH concentration (3%, w/v or more) led to sugar loss during pretreatment due to partial hydrolysis of carbohydrate polymers (especially hemicellulose), and thereby reduced overall sugar yield per gram raw biomass; 6% (w/v) NaOH led to the least overall sugar yield. Therefore, 2% (w/v) NaOH was the optimum alkali concentration for poplar biomass pretreatment at 121°C for 30 min using 10% (w/v) solids loading. Same pretreatment conditions were used as optimum for Douglas fir. For corn stover, 1% (w/v) NaOH concentration at the same processing conditions was taken as optimum based on our earlier research (Guragain et al., 2013).

For the optimization of alkaline organic solvent pretreatment, dimethyl sulfoxide (DMSO) solvent was initially evaluated for processing temperatures between 80°C to 170°C, and NaOH concentration 0 to 0.4% (w/v) while using corn stover as feedstock. **Figure 5.4** shows that the DMSO pretreatment at 80°C with NaOH up to 0.4% was not effective and total sugar released was only 15.4 g/g pretreated biomass. Pretreatment at 125°C using 0.4% (w/v) NaOH significantly improved saccharification efficiency leading to total sugar released of 51.2 g/g pretreated biomass; however, the yield was much lower than control pretreatment (81.7 g/g pretreated biomass). Total sugar yield for DMSO pretreatment at 170°C without addition of NaOH was 39.1 g/g pretreated biomass; the yield gradually increased with increase in NaOH concentration. The highest total sugar yield (89.7 g/g pretreated biomass) was obtained using 0.4% NaOH concentration at 170°C,

which is the only condition we achieved in this study for DMSO pretreatment that led to sugar being released more than control; all other pretreatment conditions were much less efficient than control pretreatment. Two other high boiling point solvents (glycerol and 2,3-butanediol) were also evaluated for pretreatment of corn stover at 170°C using 0 and 0.4% (w/v) NaOH concentration. Results showed that 2,3-butanediol pretreatment without alkali addition did not work effectively, and total sugar released was only 15.5 g/g pretreated biomass, but the sugar yield was increased to 73.8 g/g for the biomass pretreated with 0.4% alkali addition. Similarly, for glycerol pretreatment, 0.4% alkali addition led to double the sugar released compared to without alkali addition. Therefore, 170°C pretreatment temperature and 0.4% NaOH concentration were taken as optimum for all high boiling point solvents, including DMSO, 2,3-butanediol and glycerol. Glycerol has a much higher boiling point (290°C) and hence pretreatment could be carried out at higher temperature for atmospheric pressure processing; however, in this study, for comparison of pretreatment efficiency of organic solvents, the maximum processing temperature of high boiling point solvents was set at 170°C based on the boiling point of 2,3-butanediol (177°C).

Among the various low boiling point organic solvents, ethanol's pretreatment efficiency was evaluated at 75°C (refluxed at atmospheric pressure) and 170°C (heated in a Pressure Reactor); ethanol is the most common solvent for organosolv pretreatment (Zhao et al., 2009b). Ethanol pretreatment was carried out without NaOH and with 0.4% (w/v) NaOH for both temperatures; however, pretreatment without NaOH addition led to very low sugar released (less than 0.20 g/g pretreated biomass) and hence was not reported here. The 0.4% NaOH addition in ethanol pretreatment at 75°C led to sugar release of 0.61 g/g pretreated biomass, which is slightly more than sugar released in pretreatment at 170°C using same NaOH concentration; however the

difference in sugar yields was not statistically significant at 95% confidence level. Therefore, 75°C pretreatment temperature with 0.4% NaOH concentration was considered as the optimum pretreatment condition for ethanol. Same pretreatment condition was considered as optimum for all other low boiling point solvents, including isopropanol, butanol, acetonitrile and water, without further optimization for each of these solvents.

Comparison of organic solvents for the pretreatment of corn stover

The scatter plots in **Figure 5.5** shows that alkaline DMSO pretreatment led to the highest total sugar yield per gram pretreated biomass, and it is the only pretreatment solvent that released significantly more sugar yield than control. Acetonitrile was the least efficient pretreatment solvent, followed by butanol. Ethanol, water, E:IP (mixture of ethanol and isopropanol) and E:A:W (mixture of ethanol, acetonitrile, and water) led to statistically equal amounts of sugar released at 95% confidence level. Similarly, 2,3-butanediol, glycerol and E:B:W (mixture of ethanol, butanol, and water) were statistically equal in solvent efficiency. The mixture of ethanol, butanol and water was significantly more effective than each of the solvents alone, which indicated that each alcohol selectively breaks different linkages in lignin polymers and hence the mixture of alcohols is more efficient than any single alcohol (Küçük, 2005). Addition of water helped change polarity of solvent mixture, and thereby might have beneficial effect on solvent properties and the solubility of lignin oligomers. Additionally, the mixture of ethanol, butanol and water was significantly more efficient than the mixture of ethanol, acetonitrile, and water, indicating that butanol addition in an ethanol and water mixture led to a better pretreatment solvent compared to that of acetonitrile addition. This is consistent with results of butanol and acetonitrile's pretreatment efficiency as a single solvent. Pretreatment efficiency of equal mixtures of ethanol and water, and butanol and water were also evaluated; but these solvent mixtures were less efficient than single solvents to

improve hydrolysis efficiency (data not shown here). These variations on the results might also be due to variation of polarity of this solvent leading to more desirable polarity in one solvent mixture than the other; the relative polarities of water, acetonitrile, ethanol, butanol, and isopropanol are 9.0, 5.8, 5.2, 4.0, and 3.9, respectively.

Mass loss during pretreatment significantly differed among alkaline organic solvents and control methods, which led to different total sugar yield based on raw biomass weight (**Figure 5.5 - vertical bars**) compared to pretreated biomass weight (**Figure 5.5 - scatter plots**). The mass loss during pretreatment includes extractives, degraded lignin and hydrolyzed carbohydrate polymers (cellulose and hemicellulose). **Figure 5.5** (**scatter plots**) shows that the DMSO pretreatment was the best method based on sugar yield per gram pretreated biomass, but it was not true based on sugar yield per gram raw biomass, as shown in **Figure 5.5** (**vertical bar**). The reduced yield based on raw biomass weight was because of 64.3% total mass loss during DMSO pretreatment, indicating that a significant amount of carbohydrate polymers were hydrolyzed during pretreatment. Based on the raw biomass weight, equal mixtures of ethanol and isopropanol (E:IP) was the best solvent, which led to sugar yield of 49.8 g/g raw biomass; the mass loss during pretreatment was 21.6% in this solvent. Alkaline solvents 2,3-butanediol, ethanol, and E:A:W were not significantly different than control for sugar yield during enzymatic hydrolysis based on raw biomass weight, but this was not true based on pretreated biomass weight. These results indicated a significantly lower biomass loss in these organic solvents during pretreatment compared to control.

The sugar released during pretreatment cannot be used as fermentable sugar for biofuels and biochemicals production due to the presence of a number of inhibitory compounds in the pretreatment liquid. Therefore, sugar yield based on raw biomass weight is more relevant to

calculate amount of sugar obtained from a specific amount of biomass. However, if an appropriate sugar recovery and detoxification method is developed to recover good quality sugar from pretreatment liquid, sugars released during both pretreatment and hydrolysis would be fermentable sugar. In such situation, sugar yield based on pretreated biomass weight is more relevant because this reflects hydrolysis efficiency of the pretreated biomass.

Comparison of organic solvents for the pretreatment of poplar

Figure 5.6 (**scatter plot**) shows that all higher boiling point solvents were significantly more efficient than control, and all lower boiling point solvents or solvent mixtures were significantly less efficient than control for poplar biomass pretreatment. Total sugar yield based on pretreated biomass weight in glycerol, 2,3-butanediol and DMSO pretreatment were 18, 12 and 6% more than control pretreatment, respectively. Similar to corn stover, acetonitrile was the least efficient solvent. The mixture of ethanol, butanol and water was not significantly more efficient than each solvent alone; the solvent mixture was significantly more efficient than any solvent alone in corn stover pretreatment.

Similar to corn stover, variation on mass loss during pretreatment among different pretreatment solvents resulted in different sugar yield based on raw biomass weight (**Figure 5.6 - vertical bars**) compared to pretreated biomass weight (**Figure 5.6 - scatter plots**). However, the change in relative efficiency of different pretreatment solvents based on pretreated biomass and raw biomass weight for poplar biomass was not the same as that determined for corn stover biomass. For corn stover, DMSO was the best solvent based on pretreated biomass weight, and mixture of ethanol and isopropanol was the best solvent based on raw biomass weight; but glycerol was the best solvent for poplar based on both pretreated biomass and raw biomass weight, followed by 2,3-butanediol. Total sugar yield based on raw biomass weight was statistically equal for

glycerol and control for poplar; however, glycerol is the more promising pretreatment solvent for poplar for several reasons. First, the total sugar yield was 18% more than control based on pretreated biomass weight. Second, five times less NaOH was used in glycerol pretreatment than control; NaOH concentration in control and organic solvents were 2% and 0.4% (w/v), respectively (**Table 5.1**). Third, autoclave conditions were used for control method whereas the sample was heated at atmospheric pressure in glycerol method. Finally, the glycerol pretreatment can be further improved by processing at higher temperature and atmospheric pressure; current experiment was carried out at 170°C while boiling point of glycerol is 290°C. Our previous experiments (Guragain et al., 2011) showed that glycerol pretreatment at 230°C without NaOH addition was efficient for the pretreatment of wheat straw and water hyacinth, an aquatic plant. The use of low boiling point solvents (ethanol, butanol, isopropanol, and acetonitrile) is not beneficial for the pretreatment of poplar because sugar yield in these solvents was statistically equal to that of water for both pretreated biomass and raw biomass weight basis.

Comparison of organic solvents for pretreatment of Douglas fir

Figure 5.7 shows that none of the alkaline organic solvent pretreatment methods worked well for Douglas fir, including control. Sugar yields were less than 8% (w/w) in all methods for pretreated as well as raw biomass weight basis. Pretreatment using all organic solvents used for corn stover and poplar were evaluated for Douglas fir, but none of these solvents were effective (data not shown here). Additionally, the control pretreatment was carried out using up to 10% (w/v) NaOH at the same pretreatment conditions, but could not find any significant improvement in pretreatment efficiency. These results showed that both aqueous alkali and alkaline organic solvent methods at the conditions used in this study did not work for softwood. Further research is

required using better solvents and catalysts or more severe pretreatment conditions (higher temperature and longer time) to identify an effective combination for Douglas fir pretreatment.

Above results showed that the effectiveness of alkaline organic solvent pretreatment methods depend on both solvent type as well as biomass type. Therefore, each type of biomass must be separately evaluated to find the best pretreatment solvent specific to the biomass. In this study, mixture of ethanol and isopropanol was found promising for corn stover (grass) whereas glycerol was promising for poplar (hardwood) for improved hydrolysis efficiency of pretreated biomass. Further research is required to characterize the structure of extracted lignin to evaluate its quality for high value applications.

Evaluation of quality of released sugar for 2,3-butanediol fermentation

Initial experiments were carried out for 2,3-butanediol fermentation using *Klebsiella oxytoca* in synthetic sugar media containing different concentration of glucose and xylose: the major sugars in biomass hydrolyzates. **Figure 5.8** shows that the *K. oxytoca* is capable of utilizing both glucose and xylose; however, it preferably utilizes glucose first, followed by xylose. The lack of simultaneous utilization of glucose and xylose was because of the catabolic hindrance of xylose utilization in the media until complete depletion of glucose - the process is called carbon catabolite repression (Ji et al., 2011). The 2,3-butanediol yield in the media with total sugar concentration of 25 g/L, 60 g/L and 90 g/L were 0.20 g/g, 0.35 g/g and 0.38 g/g sugar, respectively; theoretical maximum 2,3-butanediol yield is 0.50 g/g sugar (Jansen et al., 1984). These results indicated that at lower sugar concentration, a significantly larger portion of sugar was used for bacterial cell growth and byproduct formation. This was because at the initial stage of fermentation using *K. oxytoca*, maximum bacterial cell growth and organic acids (lactic and acetic acid) formation took place, and 2,3-butanadiol formation initiated only after pH of the fermentation medium dropped

below 6 (unpublished data). Therefore, higher total sugar concentrations in fermentation media is beneficial for 2,3-butaneidol fermentation; but, the 2,3-butanediol productivity significantly decreased at sugar concentration beyond 90 g/L (unpublished data).

Biomass hydrolyzates prepared from different biomass type and pretreatment methods were evaluated for fermentability of the biomass-derived sugar to produce 2,3-butanediol using *K. oxytoca*. Control sample was prepared using synthetic sugar solution containing glucose, xylose and arabinose with the concentration similar to that in biomass hydrolyzates (the average concentration of biomass hydrolyzates). **Figure 5.9** shows that DMSO pretreated corn stover hydrolyzates yielded significantly higher 2,3-butanediol (0.33 g/g sugar) than control (0.28 g/g sugar), and mixture of ethanol and isopropanol pretreated poplar hydrolyzates yielded significantly lower 2,3-butanediol (0.24 g/g sugar) than control at 95% confidence level. This difference in 2,3-butaneiol yields was due to the difference in initial sugar concentration in these hydrolyzates. All other biomass hydrolyzates did not differ from control for 2,3-butanediol yield at 95% confidence level. The 2,3-butanediol yield was very low in all biomass hydrolyzates and control: 0.24 g/g to 0.33 g/g sugar, which is 48% to 66% of theoretical maximum yield. This lower yield was consistent with synthetic sugar fermentation (**Figure 5.8**), which was because of lower total sugar concentration in biomass hydrolyzates, and a significant portion of sugar was used for cell growth and byproduct formation. Further research work is needed to evaluate the quality of biomass hydrolyzates with high solids loading during enzymatic hydrolysis to release high sugar concentration in hydrolyzates and thereby improve 2,3-butanediol yield per gram total sugar. In addition, such a process is beneficial to reduce downstream processing cost because of high 2,3-butanediol titer in fermentation broth.

Conclusions

A mixture of ethanol and isopropanol with sodium hydroxide catalyst was a promising pretreatment solvent for corn stover, followed by mixture of ethanol, butanol and water solvent. Glycerol was the most promising solvent for poplar biomass pretreatment. None of the solvents used in this study were effective for Douglas fir pretreatment under process conditions evaluated. These results indicated that effectiveness of alkaline organic solvent pretreatment depend on both solvent and biomass type. The quality of biomass-derived sugar was statistically equal to synthetic sugar for 2,3-butanediol fermentation using *Klebsiella oxytoca*; but high sugar concentrations in biomass hydrolyzates is required to improve 2,3-butaneidol yield. This study opened up a new route for biomass fractionation for the valorization of each biomass component. Further research on the characterization of extracted lignin structure is required to evaluate the lignin quality for high value applications.

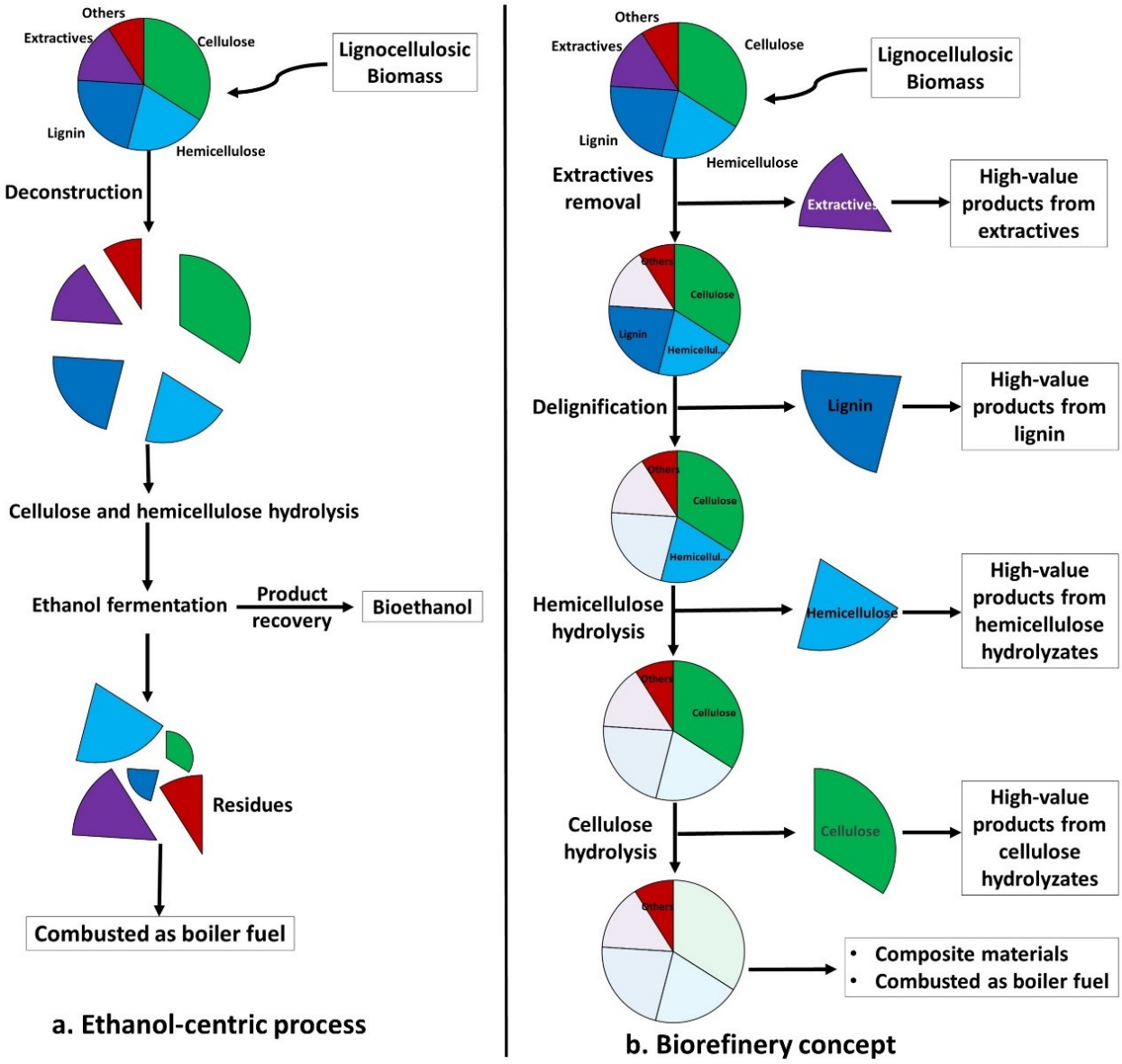


Figure 5.1 A schematic representation of conventional ethanol-centric lignocellulosic-based process and new concept of biorefinery.

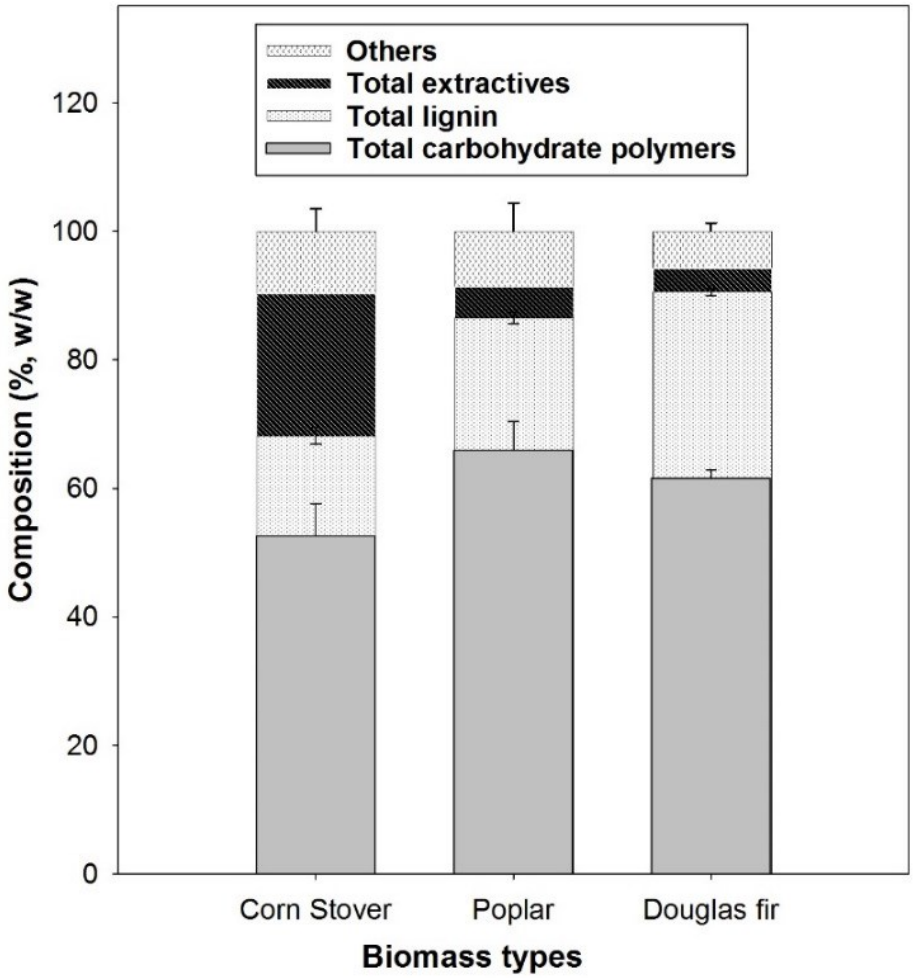


Figure 5.2 Composition of biomass feedstocks

Table 5.1 Optimized pretreatment conditons for different biomass and pretreatment

Biomass	Pretreatment solvent	Pretreatment conditions		
		Temp. (°C)	NaOH conc. (%, w/v)	Process
Corn stover	Control*	121	1.0	Autoclaved
	High boiling point solvents**	170	0.4	Reflx. atm.
	Low boiling point solvents***	75 - 80	0.4	Reflx. atm.
Poplar and Douglas fir	Control*	121	2.0	Autoclaved
	High boiling point solvents**	170	0.4	Reflx. atm.
	Low boiling point solvents***	75 - 80	0.4	Reflx. atm.

Temp. = temperature, NaOH = sodium hydroxide, conc. = concentration
*Control = aqueous alkali
**High boiling point solvents = Dimethyl sulfoxide, glycerol, and 2,3-butanediol
***Low boiling point solvents = Ethanol, isopropanol, butanol, acetonitrile, water, and mixture of these solvents
Reflx. atm. = Refluxed at atmospheric pressure

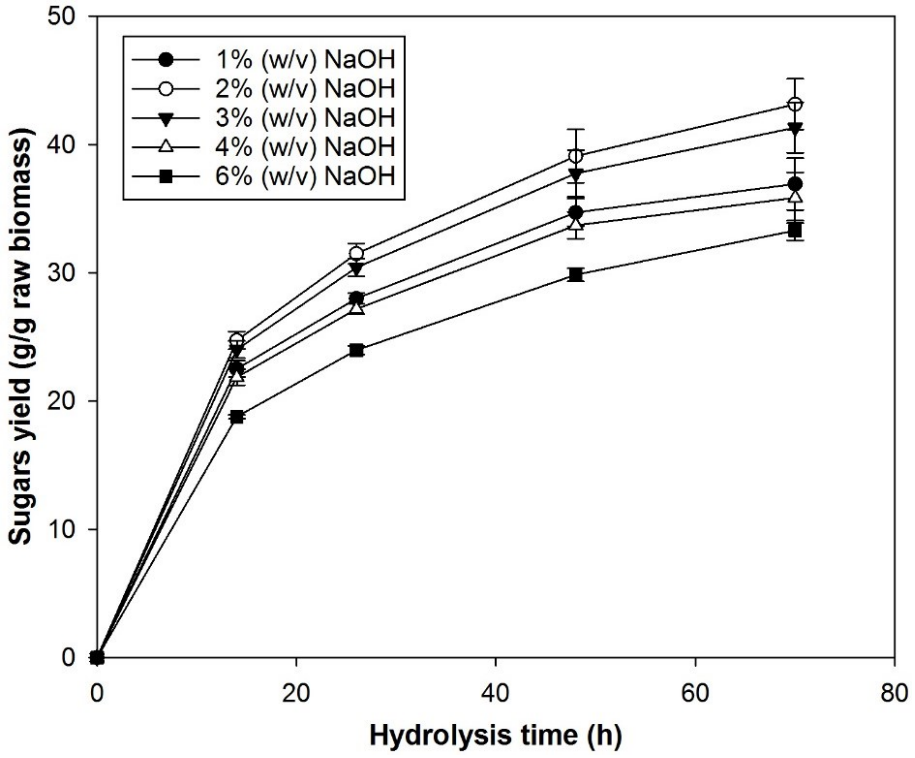


Figure 5.3 Total sugars released during hydrolysis of pretreated poplar biomass at various concentration of sodium hydroxide (NaOH). Pretreatment was carried out at 121°C for 30 min with 10% (w/v) solid loading in NaOH solution, followed by hydrolysis at 50°C for 48 h with 5% (w/v) solid loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation

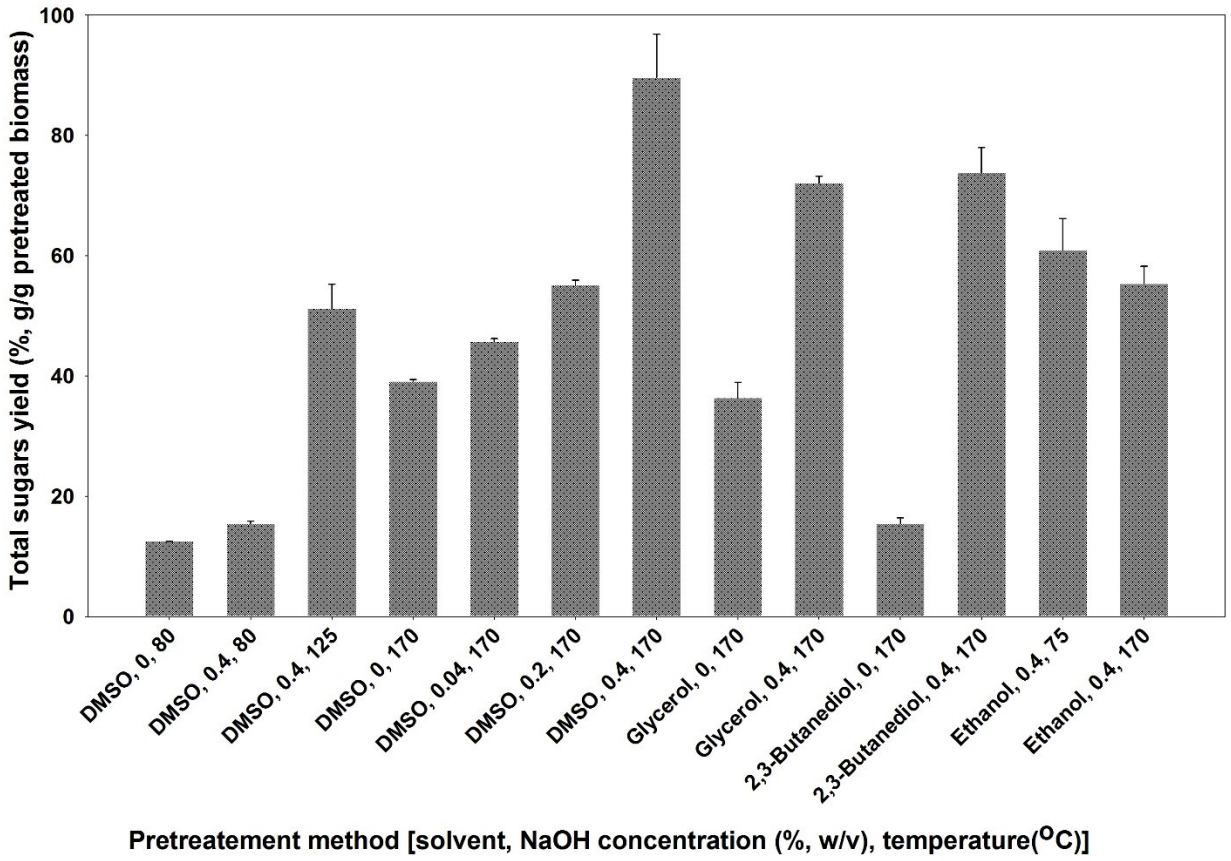


Figure 5.4 Total sugars released during hydrolysis of pretreated corn stover biomass. Pretreatment was carried out in various organic solvent with different concentration of sodium hydroxide (NaOH) and processing temperature using 10% solid loading. Hydrolysis was carried out at 50°C for 48 h with 5% (w/v) solid loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation

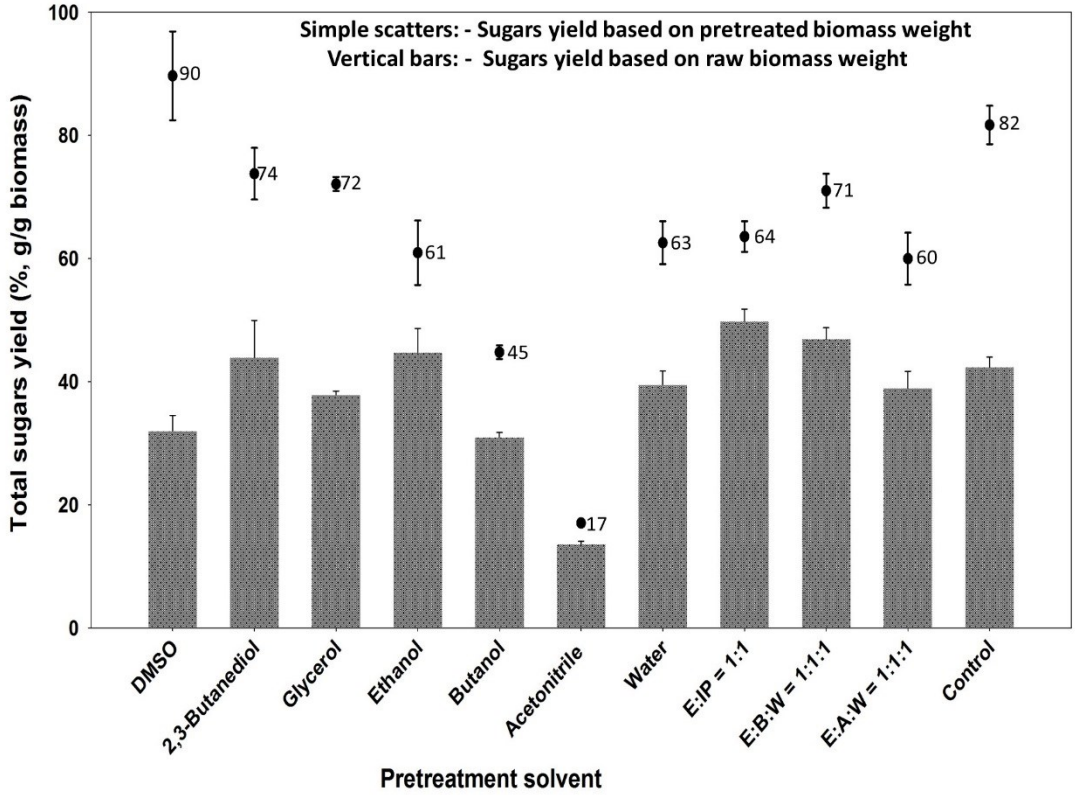


Figure 5.5 Total sugars released during hydrolysis of pretreated corn stover biomass using various organic solvents. DMSO = Dimethyl sulfoxide, E:IP = equal mixture, by volume, of ethanol and isopropanol, E:B:W = equal mixture, by volume, of ethanol, butanol and water, E:A:W = equal mixture, by volume, of ethanol, acetonitrile and water, Control = 1% (w/v) sodium hydroxide (NaOH). Pretreatment was carried out by reflexing at 170°C for DMSO, 2,3-butanediol and glycerol, and at 75 to 80°C for rest of solvents using biomass to solvent ratio 1:10 (w/v) and 0.4% (w/v) NaOH. Control pretreatment was carried out in autoclave at 121°C for 30 min using same biomass to solvent ratio. Hydrolysis was carried out at 50°C for 48 h with 5% (w/v) solids loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

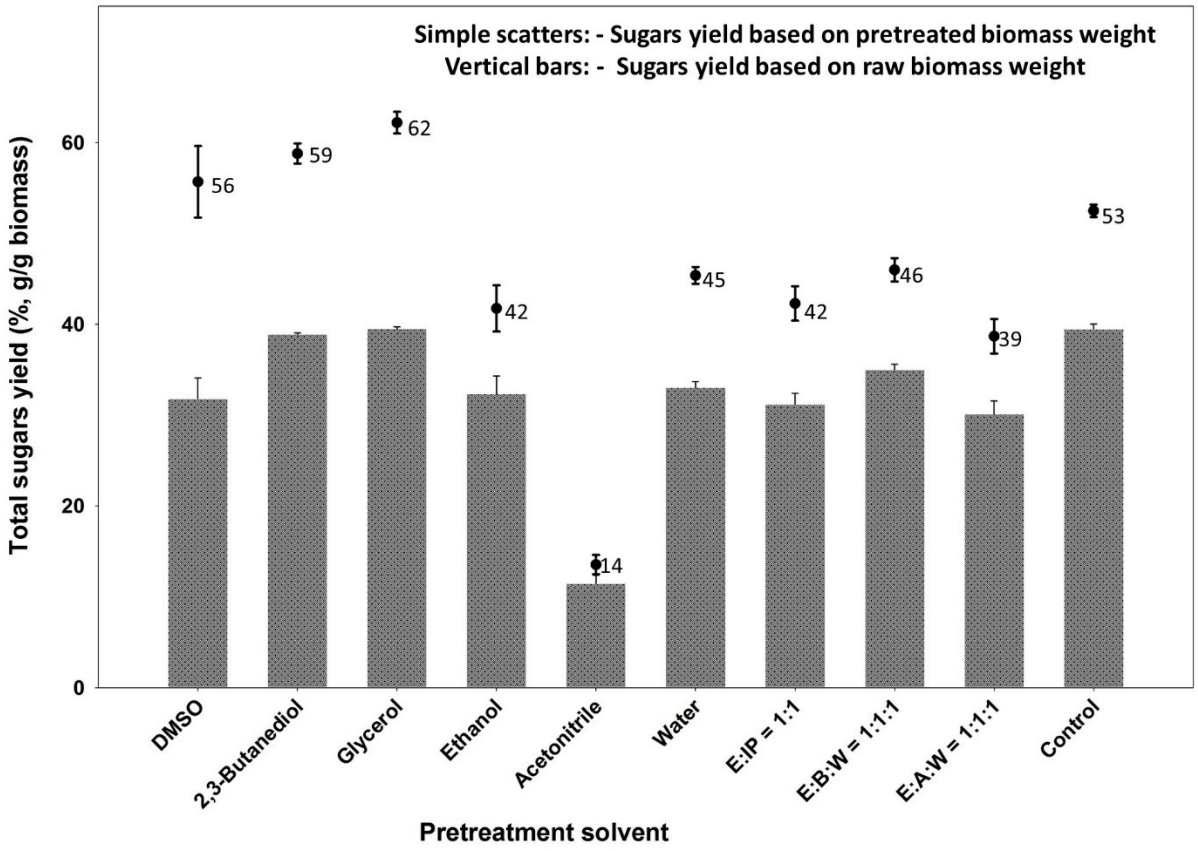


Figure 5.6 Total sugars released during hydrolysis of pretreated poplar biomass using various organic solvents. DMSO = Dimethyl sulfoxide, E:IP = equal mixture, by volume, of ethanol and isopropanol, E:B:W = equal mixture, by volume, of ethanol, butanol and water, E:A:W = equal mixture, by volume, of ethanol, acetonitrile and water, Control = 2% (w/v) sodium hydroxide (NaOH). Pretreatment was carried out by reflexing at 170°C for DMSO, 2,3-butanediol and glycerol, and at 75 to 80°C for rest of solvents using biomass to solvent ratio 1:10 (w/v) and 0.4% (w/v) NaOH. Control pretreatment was carried out in autoclave at 121°C for 30 min using same biomass to solvent ratio. Hydrolysis was carried out at 50°C for 48 h with 5% (w/v) solids loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

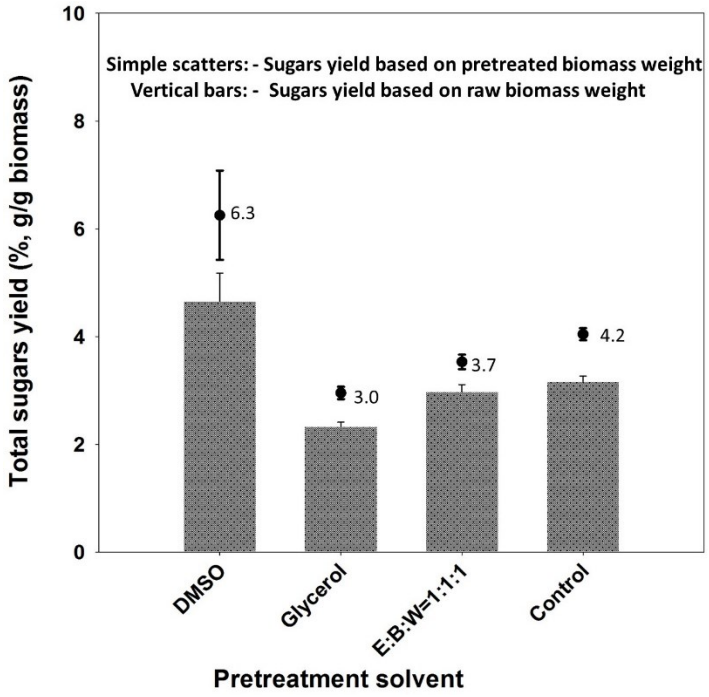


Figure 5.7 Total sugars released during hydrolysis of pretreated Douglas fir biomass using various organic solvents. DMSO = Dimethyl sulfoxide, E = Ethanol, IO= Isopropanol, B = Butanol, W = Water, and A = Acetonitrile. Pretreatment was carried out by reflexing at 170°C for DMSO, 2,3-butnediol and glycerol, and at 75 to 80°C for rest of solvents using biomass to solvent ratio 1:10 (w/v); 0.4% (w/v) sodium hydroxide was added in all solvents. Control pretreatment was carried out in autoclave at 121°C for 30 min using same ratio of biomass to 2% (w/v) NaOH solution. Hydrolysis was carried out at 50°C for 48 h with 5% (w/v) solids loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

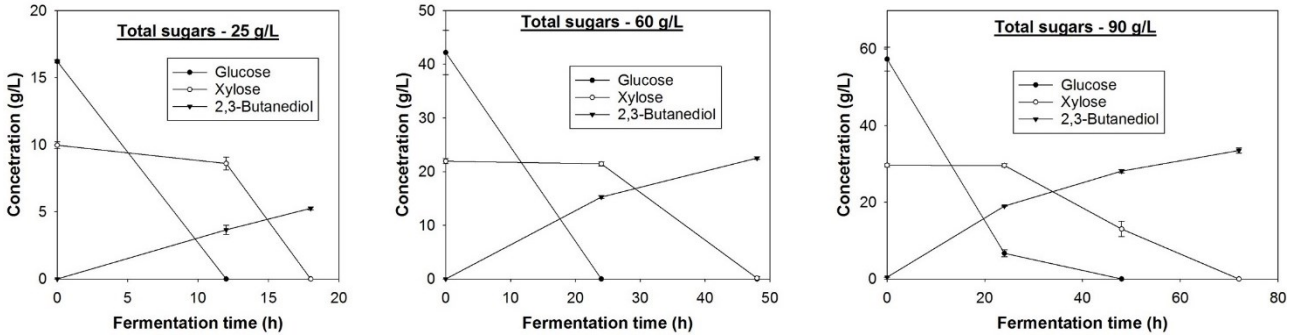


Figure 5.8 2,3-Butanediol fermentation using various concertation of synthetic sugars solutions. Fermentation was carried out at 37°C and 200 rpm using *Klebsiella oxytoca* ATCC 8724. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

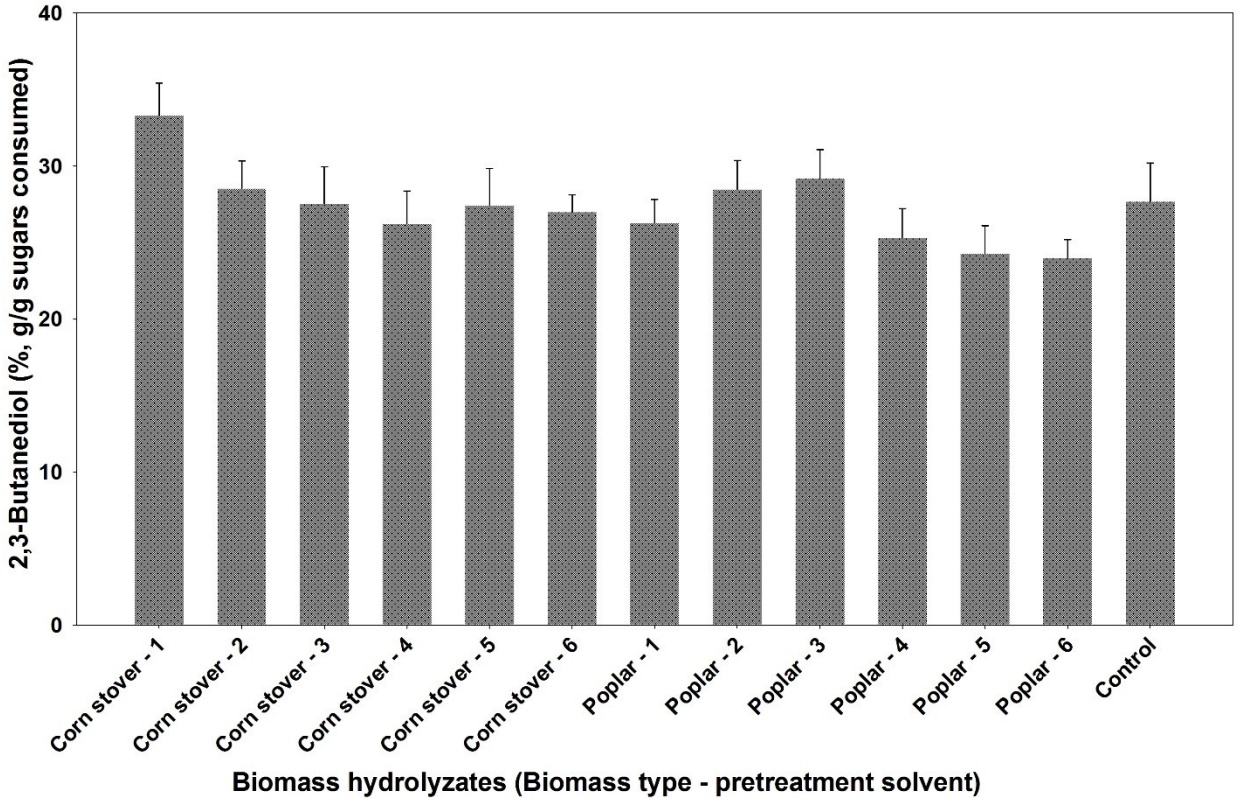


Figure 5.9 2,3-Butanediol fermentation using biomass hydrolyzates from different feedstocks pretreated by various alkaline organic solvents. Solvents: 1 = dimethyl sulfoxide, 2= 2,3-butanediol, 3= Glycerol, 4 = ethanol, 5 = equal mixture (by volume) of ethanol, butanol and water, 6 = equal mixture (by volume) of ethanol and isopropanol. Fermentation was carried out at 37°C and 200 rpm using *Klebsiella oxytoca* ATCC 8724. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

Chapter 6 - Lignin composition and structure of various bioenergy Crops: A review[2]

Abstract

Lignin provides structural support, a mechanical barrier against microbial infestation and facilitates movement of water inside plant systems. It is the second most abundant natural polymer in the terrestrial environment and possesses unique routes for the production of bulk and specialty chemicals with aromatic/phenolic skeletons. The commercial applications of lignin are limited and it is often recognized for its negative impact on the biochemical conversion of lignocellulosic biomass to fuels and chemicals. Understanding of the structure of lignin monomers and their interactions among themselves, as well as with carbohydrate polymers in biomass, is vital for the development of innovative biomass deconstruction processes and thereby valorization of all biopolymers of lignocellulosic residues, including lignin. In this paper, we review the major energy crops and their lignin structure, as well as the recent developments in biomass lignin characterization, with special focus on 1D and 2D Nuclear Magnetic Resonance (NMR) techniques.

Keywords: Lignin, Bioenergy crops, Nuclear Magnetic Resonance, Lignin isolation, Inter-unit linkages

Introduction

Lignin is the second most abundant natural polymer in the terrestrial environment (Davin & Lewis, 2005; Martínez et al., 2008). It provides structural support, and a mechanical barrier against microbial infestation. Lignin accelerates water movement within plant systems (Wagner et

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al., 2011). However, the lignins of bioenergy crops are often recognized for their negative impact in the biochemical conversion of lignocellulosic biomass to fuels and chemicals. Current biorefineries focus primarily on the cellulose and hemicellulose-derived sugars using energy-intensive pretreatment methods to remove lignins (Guragain et al., 2011). Such extremely severe pretreatment processes lead to considerable modification of the native lignin structure and, therefore, loss of potential for economic value-addition to the lignin. The extracted lignin stream is usually treated as low-value byproducts and burned as fuel, despite its great number of potential high-value applications (Yuan et al., 2011a).

The unique structure and composition along with abundant availability of lignin presents opportunities for the production of bulk and specialty chemicals. Lignin is the only viable renewable resource with an aromatic/phenolic skeleton for the production of high value aromatic compounds, including benzene, xylene, and cyclohexane, among many others. In addition, lignin is envisioned as a potential resource for the production of high value macromolecules, such as carbon fibers, polymer modifiers, adhesives, and resins (Yuan et al., 2011a; Zakzeski et al., 2010). Similarly, other products derived from lignin include mixed alcohols, dimethyl ether, green diesel, bio-oil, and naphthenic/aromatic/oxygenated fuel additives (Yuan et al., 2011a).

The major roadblock to utilize biomass lignin for high-value application is the recovery of a high quality lignin stream during biochemical conversion of lignocellulosic biomass (Yuan et al., 2011a). The heterogeneous structure of lignin (Yuan et al., 2011a), as well as variation in its composition between plant species (Grabber et al., 2004) and environmental conditions (Boerjan et al., 2003) further complicate the process. Effectiveness of the pretreatment process depends on both amount and structural composition of lignin in the feedstock (Guragain et al., 2014; Guragain et al., 2013; Wen et al., 2013). Therefore, a comprehensive understanding of lignin and evaluation

of its structural changes during pretreatment processes is critical for mapping out potential conversion processes for commercial viability of biorefineries due to additional benefits from lignin-based high value co-products (Wen et al., 2013).

The analytical methods for the characterization of lignin are broadly classified as destructive and nondestructive methods. The most common destructive methods include derivatization followed by reductive cleavage (DFRC), thioacidolysis, and nitrobenzene oxidation. The nondestructive methods include UV spectroscopy, Interference microscopy, Fourier Transform Infra-Red (FTIR) spectroscopy, Raman spectroscopy, and Nuclear Magnetic Resonance (NMR) techniques (Wen et al., 2013). Among these methods, two-dimensional (2D) NMR spectroscopy is the most powerful tool for complete characterization of lignin biomass (Pu et al., 2011; Wen et al., 2013). The inverse detection NMR techniques, such as two-dimensional heteronuclear single quantum coherence (^{13}C-^{1}H 2D-HSQC) spectroscopy further improve the sensitivity and hence are most widely used for structural characterization of lignin. However, comprehensive published work on the lignin structure of bioenergy crops is scanty so far.

The objective of this paper is to present an up-to-date compendium of the published literature on major energy crops and their lignin structure, as well as the recent development in analytical methods for the characterization of lignin, with special focus on Nuclear Magnetic Resonance (NMR) techniques. We hope that the paper will be a milestone for future research to evaluate structural changes of lignin in bioenergy plants during pretreatment, and thereby to develop an innovative biomass pretreatment method for the valorization of all biopolymers of lignocellulosic residues, including lignin.

Energy crops:

It is projected that the future demand for biomass will be significantly increased because of global focus on bio-economy. Therefore, availability of energy crops to meet the demand as well as their implications on soil and the environment is gaining an unprecedented interest globally (Blanco-Canqui, 2010). At present, lignocellulosic feedstock for production of biofuels and bio-based chemicals is mainly derived from crop residues, such as corn stover and wheat straw. However, excessive removal of crop residues can adversely affect soil quality, including soil organic carbon (SOC) pools, water transmission characteristics, soil structural stability and soil microbial activity (Evers et al., 2013). Moreover, intensive fertilizer inputs into the farming of these crops lead to increase in emissions of nitrous oxide (N_2O), a potent greenhouse gas (GHG) (Mathews, 2009). Nitrous oxide and methane have 298 and 25 times, respectively, more potential than carbon dioxide (CO_2) for global warming (Don et al., 2012). Therefore, all of these gases must be taken into consideration while evaluating the effect of bioenergy in climate change. In contrast, the dedicated energy crops, including perennial warm-seasons grasses (WSGs) and short-rotation woody crops, (SRWCs) possess a number of benefits over annual crop residues to reduce adverse effects on soil quality and environment. Therefore, these crops could be sustainable alternatives of lignocellulosic feedstock for energy industries in the long run (Blanco-Canqui, 2010). The WSGs such as switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus* spp.) and big bluestem (*Andropogon gerardii*), and SRWC such as poplar (*Populus* species), eucalyptus (*Eucalyptus* species) and willow (*Salix* species) need low-maintenance, reduce net GHGs emissions, grow rapidly and present high mass yields, reduce water and wind erosion, and sequester SOC. These plants emit much less N_2O as compared with annual crops due to lower requirement for nitrogen fertilizer. Moreover, the dedicated bioenergy crops can grow in marginal,

degraded, and abandoned lands to avoid competition for land for prime agricultural crops (Blanco-Canqui, 2010; Don et al., 2012; Evers et al., 2013).

Millions of hectares of potential arable land are available in sub-Saharan Africa, South and Central America, and many other parts of the world which are not presently under cultivation. These lands could be used for the production of dedicated bioenergy crops (Mathews, 2009). We also need to rethink the concept of marginal land for the cultivation of dedicated energy crops; the marginal land is not only the area with low soil fertility for grain production, but also the arable land not suitable for food and fodder production due to safety reasons. For example, land near industrial plants, high traffic highways and municipal waste dumping areas could be very fertile for plant growth, but food and fodder cannot be grown in these lands; plantations of appropriate dedicated energy crops could possibly be the best option. Therefore, appropriate regulatory guidelines must be set up for crop rotations and straw cutting height for annual crops, and planting of dedicated energy crops must be promoted simultaneously in appropriate lands to ensure soil quality and environmental conditions. If these strategies are effectively implemented, bioenergy production will be seen as complementary to food and feed production by resolving the widespread fear of food and fuel conflict (Ceotto & Candilo, 2011; Mathews, 2009). However, in-depth localized research is needed for the selection of appropriate plant species for agronomic management practices that maximize yields and at the same time reduce the pretreatment and other bioprocessing costs (Zegada-Lizarazu et al., 2010).

Lignin biosynthesis and structure

Unlike hemicellulose and cellulose, lignin lacks stereo-regularity (Martinez et al., 2004; Ralph et al., 2004). It is a heterogeneous polyphenolic biopolymer of plant cell walls formed by the radical polymerization of three *p*-hydroxycinnamyl alcohol precursors, called monolignols,

and related compounds (Martínez et al., 2008; Pu et al., 2011; Ralph et al., 2004). Three monolignols are *p*-coumaryl (4-hydroxycinnamyl) alcohol (**1**), coniferyl (4-hydroxy-3-methoxycinnamyl) alcohol (**2**), and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohol (**3**). These monolignols give rise to *p*-hydroxyphenyl (H) lignin units (**4**), guiacyl (G) lignin units (**5**) and syringyl (S) lignin units (**6**), respectively, (**Figure 6.1**), linked by several types of carbon-carbon or ether bonds(Del Río et al., 2012; Martínez et al., 2008).

Monolignols biosynthesis consists of several intermediate metabolism steps through a long sequence of reactions. Broadly, the whole biosynthesis process is divided into three pathways that occur one after another, as shown in **Figure 6.2**.

a. Shikimate pathway: produce phenylalanine starting from phosphoenolpyruvate and erythrose 4-phosphate.
b. Phenylpropanoid pathway: series of metabolic reactions to convert phenylalanine to cinnamoyl CoAs, the precursors of phenolic compounds.
c. Monolignol specific pathway: series of metabolic reactions to convert cinnamoyl CoAs to different monolignols (Castellanos-Hernandez et al., 2011; Vanholme et al., 2013).

Significant progress has been achieved to understand lignin chemistry and biosynthesis. However, characterization of many enzymes and reactions in the lignin biosynthetic pathway is still not completed and new discoveries are still ongoing, indicating the need for revision of current lignin biosynthesis pathway models (Fraser & Chapple, 2011; Vanholme et al., 2013).

In addition to H, S and G monolignol units, grass lignin structure also contains the *p*-hydroxycinnamates: *p*-coumarates (**7**) and ferulates (**8**) (**Figure 6.3**). Most of the *p*-coumarates are acylated at the γ-position of the lignin side chain, whereas ferulates acylate cell wall polysaccharides and participate in both polysaccharide−polysaccharide and lignin−polysaccharide

cross-coupling reactions (Del Río et al., 2012). Similarly, the monolignols, especially S-units, are also acylated up to 60% in non-woody lignins (**9**) (Del Río et al., 2012; Martínez et al., 2008). After the biosynthesis of the monolignols, they are transported to the cell wall; the mechanism of transport is still not clearly understood. Then, the lignin polymerization occurs via oxidative radical coupling between two phenoxy radicals at specific positions, corresponding to their resonant forms leading to different ether and carbon-carbon linkages. The major inter-unit linkages of lignin monomers are β-*O*-4: arylglycerol-β-ether dimer (**10**, **11**), β-5: phenylcoumaran (**12**), β–β': resinols (**13**), (α, β)-diaryl ether (**14**), 5-5'/4-*O*-β': dibenzodioxin (**15**) and β–1': spirodienone (**16**), as shown in **Figure 6.4** (Vanholme et al. 2010; Wen et al., 2013). Finally, the lignin deposition takes place, especially during secondary thickening of the cell wall. There are three layers in the secondary cell wall: outer, middle and inner layers. In general, the majority of lignins deposit on the secondary cell wall after cellulose and hemicellulose deposition in the inner layer (Boerjan et al., 2003). The lignin monomers form covalent linkages with xylose molecules of the hemicellulose polymer; there are three possible linkages between lignin and hemicellulose: ester (**17**), ether (**18**) and glycosidic (**19**), as shown in **Figure 6.5** (Bussemaker & Zhang, 2013).

Some major bioenergy crops and their lignin structure

The total lignin content and proportion of various monomer units of lignin in cell walls vary among plant species, as well as due to the environmental conditions where the plants are grown (Boerjan et al., 2003; Grabber et al., 2004; Yuan et al., 2013). Typically, softwood, hardwood and grasses contain 27 – 33%, 18 – 25% and 17 – 24% lignin (on dry weight basis), respectively (Laskar et al., 2013). The hardwood lignin contains S and G units in various proportions, softwood lignins contain mainly G units with small amount of H units, and grass lignin contains all three units (Del Río et al., 2012). The H units in grasses are the lowest among

the three lignin units, but their proportion is much higher than in hardwood and softwood (Yan, Hu, Pu, Charles Brummer, & Ragauskas, 2010). Here we briefly discuss the three types of bioenergy crops: annual crops (wheat straw and corn stover), perennial warm-season grasses (switchgrass and miscanthus), and short-rotation woody crops (poplar) and their lignin structure.

Wheat straw

Wheat straw is one of the most widely available and relatively inexpensive feedstocks for advanced biofuels and biochemical production (Talebnia et al., 2010). Wheat is cultivated in more than 115 countries and it is one of the world's major crops. China is the worlds' largest wheat producer with 18% of the total global production; however, Ireland has the highest yield of wheat production, 7.7 million kg per hectare (Kim & Dale, 2004). It is estimated that 1.3 to 1.4 kg wheat straw is produced per kg wheat grain (Del Río et al., 2012), therefore, an annual production of around 680 billion kg wheat (an estimation in 2011) would make available more than 900 billion kg wheat straw each year. A large portion of the wheat straw produced annually is inefficiently utilized by burning (Del Río et al., 2012), leading to the production of notorious gases like CO and N_2O in air (Blanco-Canqui, 2010). Depending on the soil quality and crop rotation, a fraction of the wheat straw must be left on the field to maintain soil organic matter (Kim & Dale, 2004). According to an estimate, at least 430 billion kg wheat straw is globally available each year for biofuels and chemicals production that can produce about 120 billion liters of bioethanol plus $698x10^{15}$ J steam by burning the residual material (Talebnia et al., 2010); higher value application of lignin residues would be an additional benefit.

Similar to other grass lignin, wheat straw lignin also contains all three types of monolignol units (H, G and S) in significant amounts; however, the proportions of these subunits are reported differently in various publications (Buranov & Mazza, 2008; Del Río et al., 2012). The recent

report by (Del Río et al., 2012) seems relatively more accurate because they investigated the lignin structure by a combination of various analytical techniques and resolved a number of interfering and unassigned signals due to various compounds, including *p*-hydroxycinnamates (*p*-coumarates and ferulates) and the flavone tricin. The authors reported a strong predominance of G unit; the ratio of H, G and S was 6:64:30. Wheat straw lignin is partially acylated (about 10%) at the γ-position of the side chain carbon, predominately with acetate and very rarely with *p*-coumarate. In contrast to other grasses in which acylation has been found mainly in the S unit of lignin, the acylation of wheat straw lignin was much higher in the G unit (12%) than the S unit (1%) (Buranov & Mazza, 2008; Del Río et al., 2012). The main inter-unit linkage of wheat straw lignin is β-*O*-4' ether linkages (about 75%), followed by pheylcoumarans (about 11%) and very small amount of other linkages, including resinols, dibenzodioxoncins, α, β-daryl ether, and spirodienones (Del Río et al., 2012; Xu et al., 2006). Del Río et al. (2012) also reported for the first time that the flavone tricin is present in wheat straw lignin; it is etherified with G units of the lignin. Zeng et al. (2013) reported up to 8.0 units of tricin in wheat straw lignin per aromatic ring.

Corn stover

Corn stover is one of the major agricultural residues for biofuels and biochemical production. About 520 billion Kg of dry corn is produced each year globally; the USA alone produces 40% of it. Assuming 1 kg corn stover per kg of corn grain (on dry basis) and that 60% of the corn stover must be left on the field to maintain soil organic matter, more than 200 billion Kg dry corn stover is available per year globally for bio-based chemicals production (Kim & Dale, 2004).

Corn stover lignin contains all three types of monolignol units, as well as significant amounts of *p*-coumarate and ferulate. The β–*O*-4' is the major inter-unit linkages, followed by β-

5', β-β' and β-1' linkages (Fox & McDonald, 2010; Lei Mingliu et al., 2013; M. Li et al., 2012; Min et al., 2014). Li et al. (2012) reported an S/G ratio of 1.31 for hybrid corn stover, whereas it was only 0.24 for inbred brown midrib corn stover. Min *et al*. (Min et al., 2014) most recently used 2D ^{1}H-^{13}C HSQC NMR and alkaline nitrobenzene oxidation to characterize corn stover lignin. They reported that various fractions of the plants, including stem, cob and leaf, significantly varied in proportion of monolignol units; however, the proportion of inter-unit linkages was reported to be fairly similar. The ratio of S:G:H was 13:10:2, 15:10:5 and 7:10:3 in stem, cob and leaf, respectively. The inter-unit linkages β –*O*-4', β-5', β-β' and β-1' formed 60%, 27%, 10%, and 3%, respectively in the stem. The percentages of these linkages were very similar in cob and leaf. *p*-Coumarate and ferulate were reported significantly lower in leaf than in stem and cobs; 8%, 9% and 3% of total lignin in stem, cob and leaf, respectively (Min et al., 2014).

Switchgrass

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial C_4 grass found throughout North America and Europe. It is considered as one of the potential dedicated energy crops because of its number of desirable characteristics: high productivity (13.5 to 17.9 Mg ha^{-1}), low nutrient requirements, efficient water utilization, excellent resistance to pests and diseases, low production cost and adaption to marginal lands. The extensive root system of switchgrass also helps reduce soil erosion and increase sequestration of soil organic carbon (Bransby et al., 2002; Samson & Omielan, 1992). Switchgrass removes nitrogen and phosphorus from agricultural runoff. It can be used for surface water quality improvement as well (David & Ragauskas, 2010; McLaughlin et al., 2002). Hu *et al*. (Hu et al., 2010) found that the S/G ratio in lignin from four different cultivars of switchgrass (Alamo, Kanlow, GA992 and GA993) was comparable; but it varied significantly among leaves, internodes and nodes of the plant. The average S/G ratio was 0.68, 0.60 and 0.46 in

internodes, nodes and leaves, respectively; the average value for the whole plant was 0.52. The cultivar GA992 is an intercross between Alamo and Kanlow, and the cultivar GA993 is derived from Alamo by recurrent selection (David & Ragauskas, 2010). The average acetyl group in these four cultivars of switchgrass was 0.16 per aromatic ring and the average ratio of three monolignol units (G:S:H) was 42:32:26. The GSH complex lignin polymer also contains 0.20 ferulic acid and *p*-coumaric acid per aromatic ring on average (Yan et al., 2010). A different ratio of monolignol units (G:S:H 51:41:8) was reported for Alamo switchgrass by other researchers (Yuan et al., 2011b). Significant variation in the S/G ratio was reported in Alamo switchgrass lignin under different growth environments: 0.48, 0.40 and 0.46 for plants grown under field, growth chamber and greenhouse conditions (Mann et al., 2009). The major inter-unit linkage in switchgrass is the β-*O*-4 aryl ether linkage with trace amounts of other linkages, including resinol (β- β'), phenylcoumarin (β-5'), and spirodienone (β-1') unit (David & Ragauskas, 2010; Samuel et al., 2010).

Miscanthus

The genus *Miscanthus* belongs to the *Poaceae* family and comprises about 15 species of perennial grasses. This genus is originally from tropical and subtropical regions of Africa and South Asia; however, different species are found in temperate zones of Asia as well (Villaverde et al., 2010). These grasses are considered as dedicated energy crops because of their fast growth, resistance to disease, high productivity, and a long productive life of at least 10 to 15 years (Villaverde et al., 2009). Since these grasses have C_4 photosynthetic metabolism, they have a high carbon dioxide fixation rate and lower requirements of water and nitrogen than other C_3 plants (Hage et al., 2009). Among the various species of *Miscanthus*, the hybrid species between *M. sinensis* and *M. sacchariflorus*, called *Miscanthus x giganteus* (MxG), is the most extensively

studied as a potential feedstock for fuel and chemical production. *Miscanthus x giganteus* can grow more than 3.5 m in a single growth season, producing about 25 tons of biomass per hectare (Villaverde et al., 2010).

Milled wood lignin (MWL) of MxG contains a high proportion of β-*O*-4' ether linkages (up to 93% of all linkages and 0.41 linkages per aromatic ring) and a small proportion of condensed linkages: 4% resinol (β-β'), 3% phenylcoumaran (β-5') and less than 0.5% spirodienone (β-1') (Hage et al., 2009; Villaverde et al., 2009). It was estimated that 46% of γ-carbon are acylated with a *p*-coumarate and/or acetate group (Villaverde et al., 2009). The high acylation at C_γ is considered one of the main reasons for the low proportion of double tetrahydrofuran rings from resinol, because a β-β homo-coupling of nonacetylated monolignols is required to form the resinol (22), as shown in Scheme 5 (Del Río et al., 2007). The MxG predominately contains guaiacyl (G) monolignol units followed by syringyl (S) units and small amount of hydroxyphenyl (H) units, with a ratio of G, S and H of 54:44:4 (Hage et al., 2009).

Poplar

The genus *Populus*, a native to the northern hemisphere, comprises up to 35 species with considerable genetic diversity within the genus. The genus has a number of common names, such as poplar, cottonwood and aspen. The hybrid poplars are the fastest-growing plants in North America and hence considered as a potential feedstock for the production of biofuels, pulp and paper, as well as a number of other bio-based products. These are classified as short-rotation woody crops and cultivated in economically marginal croplands. The breeding to produce hybrid poplars generally focuses on five species: *Populus balsamifera* (balsam poplar), *P. deltoids* (eastern cottonwood), *P. trichocarpa* (western black cottonwood), *P. nigra* (European black poplar) and *P. maximowiczii* (Asian black poplar); the first three species are native to North

America. The productivity of hybrid poplar is estimated to be 14 Mg $ha^{-1}year^{-1}$, which is comparable with the productivity of swithchgrass, but higher than corn stover (8.4 Mg $ha^{-1}year^{-1}$) and wheat straw (6 Mg $ha^{-1}year^{-1}$) (Sannigrahi et al., 2010). A wide variation in the S/G ratio of poplars is reported ranging from 0.7 to 2.2; however, the majority of reports are in the range of 1.3 to 2.2 (Sannigrahi et al., 2010). Bose et al., (2009) reported a linear inverse relation ($R^2 = 0.85$) between S/G ratio and total lignin content in 13 poplar species: a S/G ratio from 1.01 to 1.68 and corresponding lignin content from 28 to 16.5%. The poplar lignin contains only trace amounts of H units; Robinson and Mansfield (2009) reported an average S:G:H ratio of 104 hybrid poplars as 68:32:0.02. The main interunit linkages in MWL of *P. tomentosa* Carr. were reported as β-*O*-4' aryl ether (83.2%), followed by β-β' resinol (12.7%), β-5' phenylcoumaran (2.6%) and β-1' spirodienones (1.4%) (Yuan et al., 2011a; Yuan et al., 2011b).

A brief history on lignin chemistry and technology

Anselme Payen, a chemical manufacturer in France, first discovered cellulose and lignin in 1838 as separate components of wood (McCarthy & Islam, 1999). Thousands of scientific papers and patents, and hundreds of books have been published concerning the chemistry of lignin to date. Several analytical techniques have been developed to characterize lignin biomass. Extensive investigation was performed concerning the structure and properties of wood lignin in the 20^{th} century due to rapid development of paper production from wood. Since the early 1990s, investigation of the lignin of herbaceous crops has also increased dramatically due to the growing interest in biofuel and biobased chemicals from lignocellulosic residues (Buranov & Mazza, 2008; Wen et al., 2013).

McCarthy and Islam (McCarthy & Islam, 1999) divided the history of the development of scientific literature and technology in lignin into seven arbitrary periods starting from its discovery in 1838 to 1998.

1838 to 1874: The existence of lignin in biomass was established and the sulfite-pulping process was developed and patented for the production of cellulose fibers from wood by dissolving lignin in hot aqueous sulfurous acid solution. The aromatic nature of lignin was assumed from the research during this period (McCarthy & Islam, 1999).

1875 to1899: Based on the elemental analysis at that time, the structure of lignin was assumed to be related to coniferyl aldehyde. The Kraft pulping process was developed and patented for delignification of wood using a hot aqueous solution of sodium hydroxide and sodium sulfide (McCarthy & Islam, 1999).

1900 to 1924: Lignin structural studies were undertaken extensively. Elemental and functional group (C, H, O and OCH_3) analysis of lignin showed that it is made up of coniferyl alcohol type units bonded together by ester linkages. An analytical method to determine Klason lignin was developed during this time. Industrial application of sulfite and Kraft pulping processes were started (McCarthy & Islam, 1999). Two ethanol plants were established during World War I that used wood saccharification. The ethanol yield of these plants was up to 20 gallons per ton dry sawdust; however, these plants were not financially successful and closed after a few years (Tomlinson, 1948).

1925 to 1949: Substantial results confirmed the aromatic nature as well as the phenylpropane skeleton of lignin. The research areas were expanded to biosynthesis and biodegradation studies on lignin; preparation of synthetic lignins *in vitro* was also initiated (McCarthy & Islam, 1999).

1950 to 1974: New analytical tools and procedures like acidolysis, mild hydrolysis, and permanganate oxidation were developed and several existing methodologies were improved to understand the linkages between lignin units and, thereby, lignin structure. Spectroscopy procedures (like UV absorbance) were popular during the 1950s for lignin analysis. The thioacidolysis procedure developed during the 1960s also helped to identify further the lignin structure. Industrial production of lignin products was initiated during this time (McCarthy & Islam, 1999). Characterization of lignin using 1D ^{1}H NMR and ^{13}C NMR was initiated in 1964 and 1974, respectively (Ludemann & Nimz, 1974a; Ludemann & Nimz, 1974b; Ludwig et al., 1964; McCarthy & Islam, 1999; Ralph & Landucci, 2010).

1975 to 1989: During this period, studies on lignin structure were continued to confirm the earlier accepted concepts. NMR spectroscopy was extensively used in this regard; ^{19}F-NMR and ^{31}P-NMR studies were also initiated to investigate and confirm lignin structure. Delignification and bleaching technology significantly improved; anthraquinone was started to be added to improve the alkaline pulping process. Significant research focus was given to find new uses of industrial lignin (Holton & Chapman, 1977; McCarthy & Islam, 1999).

1990 to 1998: Significant advances were made during this time in lignin chemistry and biosynthesis. Several studies in this field evidenced that lignin formation is a defined ordered process rather than a random one. Gene manipulation became possible giving rise to engineered plants with reduced lignin content to increase cellulose content (McCarthy & Islam, 1999).

Recent development in lignin biomass characterization by 1D and 2D NMR spectroscopy

Over the last two decades, several analytical techniques have been advanced for the characterization of lignin chemically and structurally, including its functional groups, monolignol ratio and inter-unit linkages (Lu & Ralph, 2011; Mansfield et al., 2012; Pu et al., 2011; Ralph & Landucci, 2010; Wen et al., 2013). Recently characterization of lignin by analyzing its molecular fragments after selective chemical treatment in destructive methods have been displaced and/or supplemented by nondestructive methods (Lu & Ralph, 2011; Pu et al., 2011). Nuclear magnetic resonance (NMR) spectroscopy has been widely used as a non-destructive technique in structural characterization as well as in the study of the structural transformation of lignin polymers. This methodology has provided structural and quantitative analysis of the sub-structural motifs within the overall polymer. Recent advances in NMR methodology, particularly inverse detection techniques and two dimensional heteronuclear correlation spectroscopy, along with availability of cryogenic NMR probes, have facilitated structural analysis of lignin in isolated and whole cell wall states (*in situ*) (Kim et al., 2008; Kim & Ralph, 2010; Lu & Ralph, 2011; Mansfield et al., 2012; Ralph & Landucci, 2010; Wen et al., 2013). Furthermore, the two dimensional heteronuclear single quantum coherence (2D ^{13}C-^{1}H HSQC) experiments using inverse detection methodology not only solved the problem of proton signals overlapping, but also have improved the signal to noise sensitivity by a factor of 31.6 (Wen et al., 2013) These developments have reduced the long acquisition times of previously used natural abundance 1D carbon detected NMR experiments in lignin characterization, making possible the acquisition of 2D ^{13}C-^{1}H HSQC spectra of adequate quality for most analysis, from whole cell walls in less than 20 minutes (Kim & Ralph, 2010).

2D ^{13}C-^{1}H HSQC NMR experiments have been successfully performed for evaluating the lignin structure of different cultivars and determining structural changes during pretreatments of switchgrasses (Samuel et al., 2010; Samuel et al., 2011; Yan et al., 2010). The carbon chemical shift (57-61 ppm) assignment for the C_γ resonance from β-*O*-4 ether linkages without a C_α=O group suggested a significant amount (0.42 per aromatic ring on average) of ether linkage in switchgrass lignin (Mann et al., 2009; Samuel et al., 2010). Samuel et al. (2010) used 2D NMR methodology to understand the effect of pretreatment on switchgrass lignin structure. ^{13}C NMR analysis of the ball milled lignin before and after dilute acid pretreatment suggested that guaiacyl and syringyl units were the main components of switchgrass lignin with limited amounts of *p*-hydroxyphenyl, which was also confirmed by 2D ^{13}C-^{1}H HSQC NMR experiments. The pretreatment caused the syringyl units to decrease by 10% while guaiacyl units increased by 7%, as reported by the quantitative 1D ^{13}C NMR studies. However, 2D ^{13}C-^{1}H HSQC correlation peaks did not find any difference between the inter unit linkages despite decreases in the syringyl units. Overall, ball milled lignin β-*O*-4 linkages decreased by 36% due to the sulfuric acid pretreatment. Bozell et al. (2011) also confirmed that acid pretreatment decreased the β-*O*-4 linkages.

2D NMR methodology has also been extensively used to elucidate the mechanism of lignin breakdown during biomass pretreatment (Pu et al., 2011). Hage et al. (2009) characterized milled wood lignin (MWL) and ethanol organosolv lignin (EOL) from *Miscanthus* using quantitative ^{13}C NMR and ^{31}P NMR to study the mechanism of lignin breakdown during organosolv pretreatment. Zeng et al. (2013) recently developed a new combinatorial approach for structural analysis of wheat straw lignin by correlating quantitative 1D ^{13}C NMR and 2D ^{13}C-^{1}H HSQC experimental results. In this technique, well dispersed NMR spectra of 2D ^{13}C-^{1}H HSQC serves as an internal standard to measure the integral values obtained from the quantitative 1D ^{13}C-NMR spectra. In

this approach it is assumed that the level of aromatic and aliphatic resonance intensities for various lignin structures in 2D ^{13}C-^{1}H HSQC spectra and quantitative 1D ^{13}C NMR spectra are comparable and compatible with each other (Del Río et al., 2012; Hage et al., 2009). These studies also showed that the carbon and proton NMR resonances of various lignin inter-unit linkages significantly differ from each other. These authors showed that the chemical shift values (δ_C/δ_H ppm) of various inter-unit linkages in the 2D ^{13}C-^{1}H HSQC spectra obtained for wheat straw lignin significantly differ from each other (**Table 6.1**). Furthermore the relative abundance of these inter-unit linkages was expressed as the quantitative number of each structure per aromatic ring by taking the integral value of the carbon chemical resonances at 102.0-160.0 ppm in the quantitative 1D ^{13}C NMR as reference. The carbon signals resonating in the range of 82.5-88.0 ppm includes ether linkages (β-*O*-4) as well as other carbon-carbon linkages (β-5 and β- β') and its integral value was 0.45 per aromatic ring in MWL. On the other hand, the carbon signal resonating in the range of 53.0-54.0 ppm includes only carbon-carbon linkages; its integral value was 0.04 per aromatic ring MWL. These results suggested that the MWL lignin contained large amounts of β-*O*-4 structure (0.41 per aromatic ring), which was reduced by more than 50% in EOL, indicating the extensive cleavage of ether linkages during organosolv pretreatment. Additionally, the integral values of the peaks for acetate carbonyl [O<u>C</u>(=O)CH_3] at 171 ppm and for cinnamaldehyde carbonyl (Ar-CH=CH-<u>C</u>HO) at 194 ppm were observed as 0.02 and 0.06 per aromatic ring, respectively, in MWL, but these NMR peaks were absent in EOL indicating complete hydrolysis of acetate and cinnamaldehyde groups during organosolv pretreatment due to the presence of acid catalyst. Moreover, significantly higher amount of aromatic carbon-carbon linkage (C-C) signals resonating in the range of 124.0-142.0 ppm and lower amount of aromatic C-H signal resonating in the range of

102.0-124.0 ppm in EOL than MWL indicated re-polymerization by a lignin-lignin condensation reaction (Li & Gellerstedt, 2008).

In ^{31}P NMR studies, both MWL and EOL samples were phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane (TMDP) and analyzed by quantitative ^{31}P NMR, using cyclohexanol as an internal standard. Using TMDP, hydroxyl groups from aliphatic, phenolic and carboxylic acid groups are phosphorylated with NMR active ^{31}P nuclei (Pu et al., 2011). Concentration of the hydroxyl functional group (mmol g^{-1}) in the sample was calculated based on OH content of the internal standard and the integral peak area. The number of functional groups per C9 unit of lignin monomer was calculated taking elemental analysis data of the samples. These results indicated that phenolic carbon resonance (136.0 – 144.0 ppm) intensity increased from 0.28 per C_9 in MWL to 0.50 per C_9 in EOL, indicating scission of β-*O*-4 linkages during organosolv pretreatment. Carboxylic acid resonance at 134.0 – 135.7 ppm also increased from 0.02 to 0.04 per C_9, indicating cleavage of some ester bonds. However, when OH groups from each type of monolignin unit were separately analyzed, *p*-hydroxyphenyl carbon resonance at 137.2 – 138.1 ppm gave contrary results; that is, they remained almost equal in MWL and EOL. This showed that formation of *p*-hydroxyphenyl OH due to cleavage of β-*O*-4 bonds was compensated by hydrolysis of *p*-coumaryl ester residues. Additionally, aliphatic OH at 145.5 – 150.0 ppm reduced from 0.67 to 0.19 per C_9, probably due to acid-catalyzed elimination reaction.

NMR data have also been used to quantify lignin composition (notably, the syringyl: guaiacyl: *p*-hydroxyphenyl ratio) in isolated as well as *in situ* forms from lignocellulosic materials. Thus NMR profiling has provided the best tool for the detailed structural study of lignin bioenergy crops (Ragauskas et al., 2014). Chemometric analysis using 2D ^{13}C-^{1}H NMR finger print region

of lignocellulosic samples has allowed a secondary screening for selecting biomass lines and for optimizing biomass processing and conversion efficiencies (Samuel et al., 2011).

Isolation of lignin biomass for NMR analysis

Isolation of native lignin from plant cell walls prior to NMR analysis is a crucial step for structural characterization of the lignin biomass, and in recent years there have been major advances in lignin biomass solvation for solution-state NMR studies (Holton & Chapman, 1977; Ralph & Landucci, 2010). Several isolation methods have been developed over the past decades; milled wood lignin (MWL) and cellulolytic enzyme lignin (CEL) are the most representing methods (Wen et al., 2013). The lignin is extracted in aqueous dioxane (96%) from ball-milled wood using the MWL method, while cellulolytic enzymes are used to remove most of the carbohydrate polymers before extracting lignin with aqueous dioxane in CEL (Holtman et al., 2004). Wu & Argyropoulos (2003) proposed further improvement in the lignin isolation technique, which is called the enzymatic/acidolysis lignin (EAL) method. In this, the residual carbohydrate after cellulolytic enzyme treatment is removed by acidified dioxane. Recently, *in-situ* characterization of lignin became possible due to new developments in high-resolution NMR instrumentation coupled with suitable gelling or dissolution solvents (Ralph & Landucci, 2010; Wen et al., 2013). Lu and Ralph (2003) have characterized the whole plant cell wall components without lignin fractionation/isolation using a solution-state 2D ^{13}C-^{1}H HSQC NMR methodology. They developed a two solvent system of dimethyl sulfoxide (DMSO) and either tetrabutylammonium fluoride (TBAF) or *N*-methylimidazole (NMI) to dissolve balled-milled plant cell walls, followed by acetylation using acetic anhydride making the sample fully soluble in NMR solvents like either chloroform or DMSO leading to intact lignin structure analysis. The dissolution process was later modified for characterization of the whole plant cell wall without

derivatization (acetylation) using different solvent systems, such as deuterated DMSO and NMI (4:1, v/v) (Yelle et al., 2008), deuterated DMSO (Kim et al., 2008) or deuterated DMSO and pyridine (4:1, v/v) (Kim and Ralph, 2010); these development in the solvent systems led to the determination of natural acylation in lignin. Now, it is possible to monitor *in situ* structural changes on different components of lignocellulosic biomass, including lignin, during pretreatment and hydrolysis stages of biomass processing for the production of fuels and chemicals by NMR spectroscopy (Wen et al., 2013). Furthermore, analysis of the generated NMR data is currently aided by the availability of the NMR Database of Lignin and Cell Wall Model Compounds (Huang, Singh, & Ragauskas, 2011; Marita et al., 1999; Rahimi et al., 2013; Ralph & Landucci, 2010; S. Ralph et al., 2004), which provides a cross platform unified source for the ^{1}H and ^{13}C chemical shift assignments of lignin model compounds.

Future out-look

Utilization of the lignin component of biomass has several daunting challenges as well as great opportunities for the commercial success of modern integrated biorefineries. Lignin is the major contributor to biomass recalcitrance, thereby resulting in higher costs for lignocellulosic biomass conversion to fuels and chemicals. At the same time, lignin is the most abundantly available resource to produce several high value specialty chemicals with pertinent aromatic skeletons. Globally, several studies are focused on the development of novel biomass deconstruction methods as well as valorization of the underutilized lignin stream. However, future efforts need to be dedicated to elucidate the complex structure and uncertain reactivity of the lignin polymer, and development of experimental techniques that would allow detailed and high-throughput analysis of lignin samples in the industrial setting. In our review, we have specifically highlighted the lignin structure characterization of important bioenergy

feedstocks, which will provide the necessary theoretical framework for bioprocess engineers to design broad-spectrum biorefineries. 1D ^{13}C NMR and 2D ^{13}C-^{1}H HSQC NMR methodology is envisioned as one of the most potential approaches to achieve this goal due to the recent development in instrumentation, sample preparation and the compendium of structural information currently available for the characterization of lignin biomass. Therefore, these methods will be extensively applied by researchers involved in biomass conversion to advanced fuels and chemicals in the future.

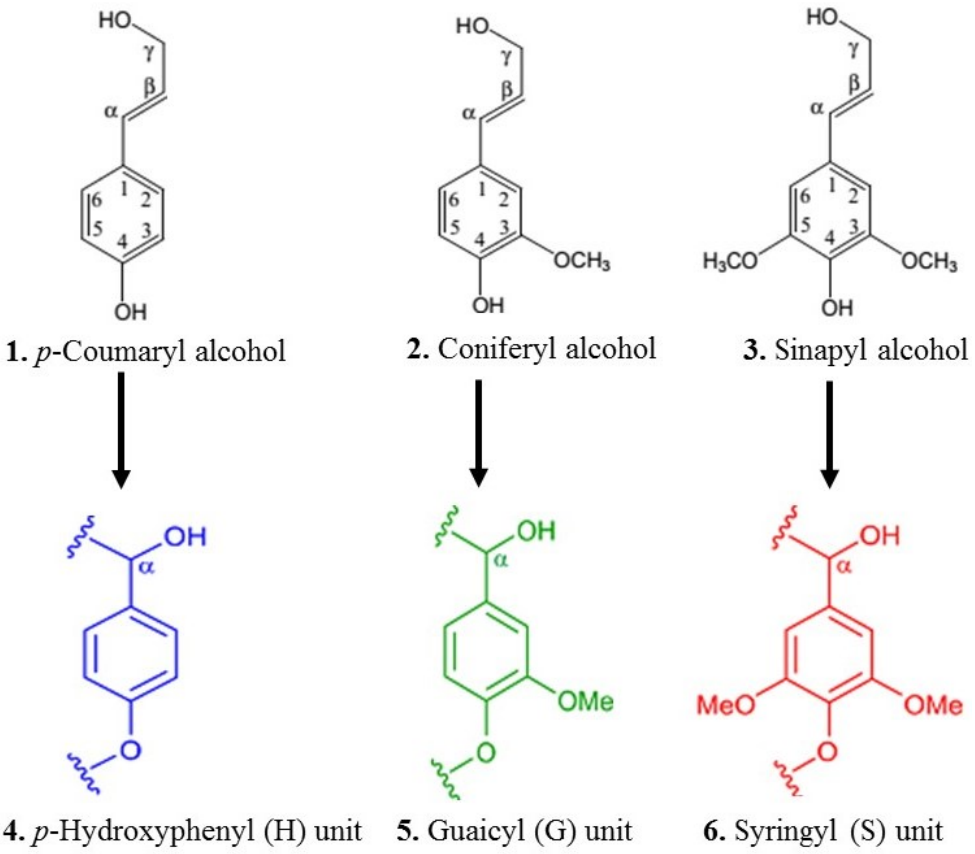


Figure 6.1 Monolignin units of lignin biomass (Martinez et al., 2008)**.**

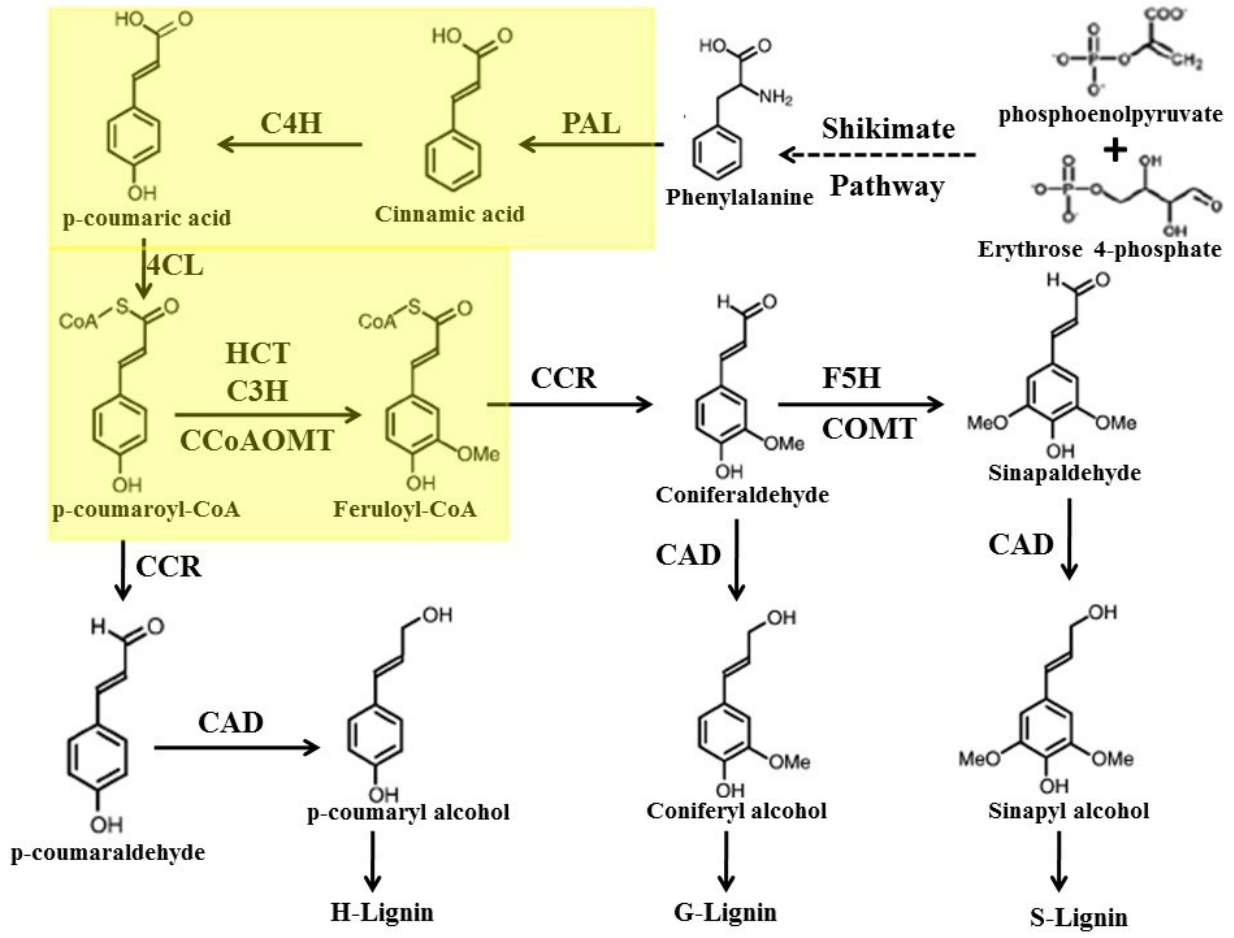
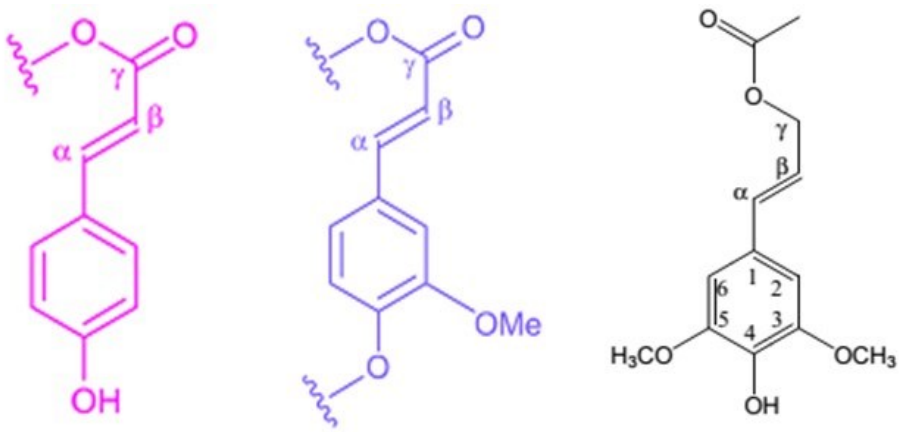
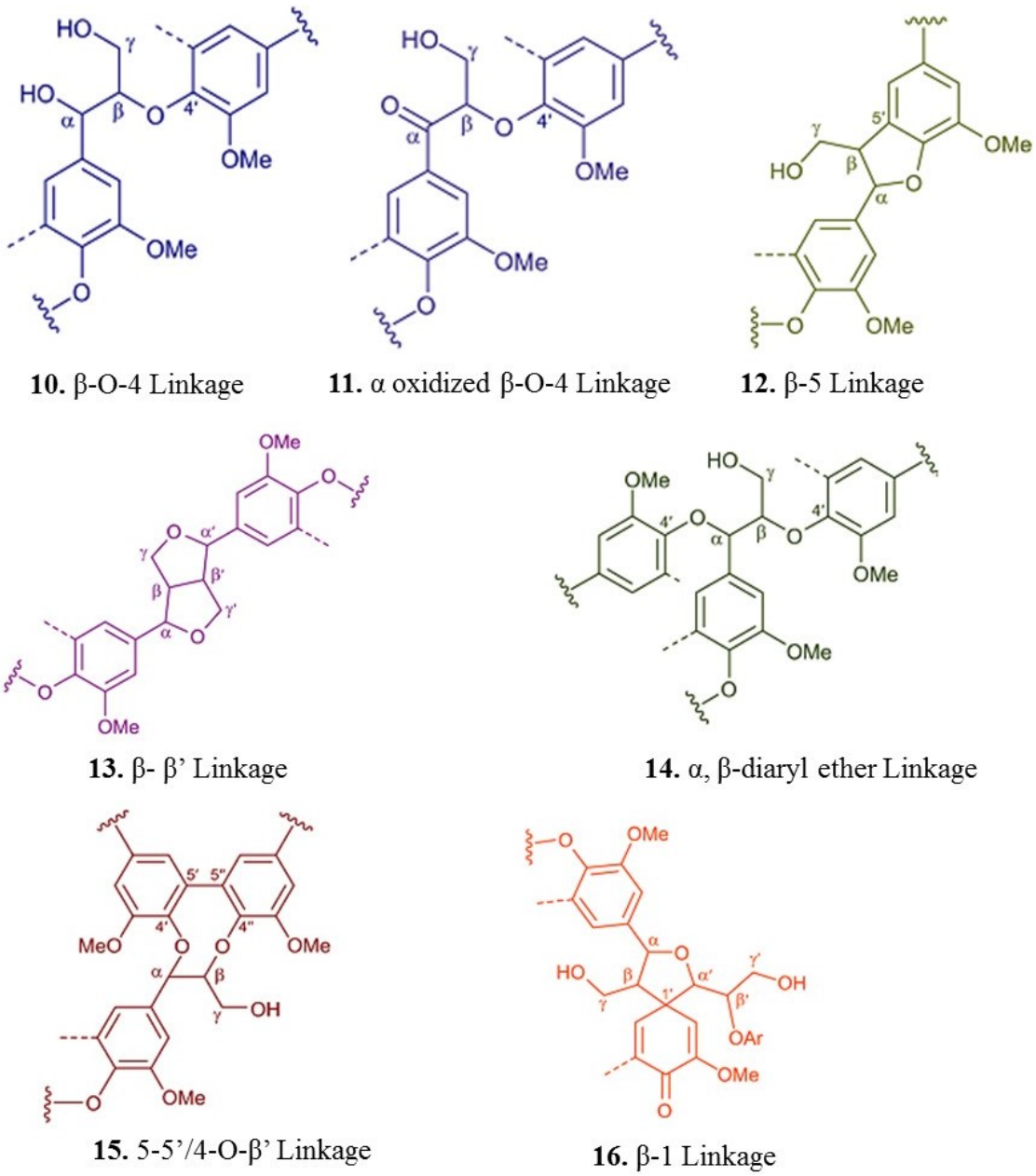


Figure 6.2 The main monolignol biosynthetic pathways; Shikimate pathway followed by phenylpropanoid pathway (shaded part) and monolignol-specific pathway. PAL= phenylalanine ammonia-lyase, C4H= cinnamate 4-hydroxylase, 4CL= 4-coumarate: CoA ligase, HCT= *p*-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase, C3H= *p*-coumarate 3-hydroxylase, CCoAOMT= caffeoyl-CoA O-methyltransferase, CCR= cinnamoyl-CoA reductase, F5H= ferulate 5-hydroxylase, COMT= caffic acid *O*-methyltransferase, CAD= cinnamyl alcohol dehydrogenase (Fraser & Chapple, 2011; Vanholme et al., 2010).



7. *p*-Coumarate **8.** Ferulate **9.** Sinapyl acetate

Figure 6.3 Acylated monomers of grass lignin (Martinez et al., 2008; Del Rio et al., 2012)**.**



10. β-O-4 Linkage **11.** α oxidized β-O-4 Linkage **12.** β-5 Linkage

13. β- β' Linkage **14.** α, β-diaryl ether Linkage

15. 5-5'/4-O-β' Linkage **16.** β-1 Linkage

Figure 6.4 Major inter-unit linkages of plant lignin (Del Rio et al., 2012)**.**

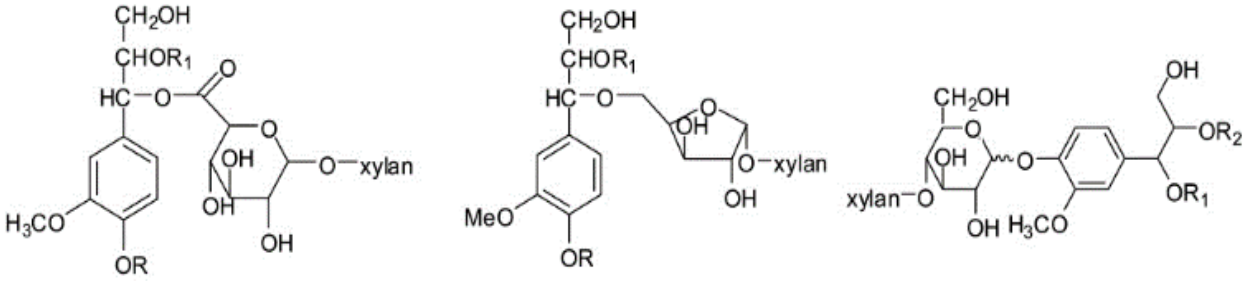


Figure 6.5 Lignin-carbohydrate linkages in lignocellulosic biomass (Bissemaker and Zhang, 2013).

Table 6.1 Quantitative analysis of inter-unit linkages in milled wood lignin (MWL) isolated from wheat straw lignin (Zeng et al., 2013)

Inter-unit linkages	**Observed chemical shift in HSQC (δ_C / δ_H ppm)**	**Quantity (per100 aromatic ring)**
Arylglycerol-β-ether dimer (β-*O*-4)	Cross-peaks: 71.1/4.74 (71.8/ 4.8) and 82.7 / 5.1	29.8
Phenylcoumaran (β -5)	Cross-peaks: 85.9/5.5 and 53.0/3.4	5.4
Resinols (β - β')	Cross-peaks: 84.8/4.7 and 53.5/3.1	1.3
α, β-Diaryl ether	79.6 / 5.5	0.5
Dibenzodioxin (5-5'/4-*O* β')	83.4 / 4.9	0.2
Spirodienone (β – 1)	85.5 / 3.8	0.4

Chapter 7 - Evaluation of lignin-specific sorghum mutants as a potential biomass feedstock for biochemical production

Abstract

Background: Sorghum is a model energy crop because of its high photosynthetic efficiency, abiotic stress tolerance, and wide applications as a food, feed and fuel. The brown midrib (*bmr*) sorghum mutation leads to an alteration of biomass lignin composition, and thereby possesses better forage digestibility for livestock. The *bmr* sorghum mutants could provide a benefit for biofuels production as well. In this study, three sorghum cultivars [Atlas, Early Hegari (EH), and Kansas Collier (KC)] and two *bmr* mutants (*bmr*6 and *bmr*12) of each cultivar were evaluated and compared for grain and biomass yield, biomass composition, and 2,3-butanediol (an important platform chemical) production from biomass. **Results:** The agronomic data showed that the *bmr* mutation led to increased grain yield and decreased biomass yield in EH, whereas the opposite was true for KC. The biomass composition indicated that *bmr* mutants had 10 to 25% and 2 to 9% less lignin and carbohydrate contents, respectively, and 24 to 93% more non-structural sugars than their parents in all sorghum cultivars, except EH *bmr*12. The total fermentable sugars obtained from hydrolysis of pretreated biomass and water extraction of raw biomass prior to pretreatment was 22 to 36% more in *bmr* mutants than in parents for Atlas and KC, but not for EH. The quality of biomass sugars from all sorghum lines was not significantly different than synthetic sugars for 2,3-butanediol production using *Bacillus licheniformis* DSM 8785. **Conclusions:** The *bmr*6 mutation in KC background produced the most promising feedstock, among the six evaluated sorghum *bmr* mutants, for 2,3-butanediol production without significant decrease in grain yield. The *bmr*12 mutation in KC and *bmr*6 and *bmr*12 mutation in Atlas background also led to improvement on feedstock quality for biofuels production, but the *bmr* mutation had an adverse

effect in EH background. This indicated that the genetic background of the parent cultivar as well as type of *bmr* mutation significantly affect the biomass quality as a feedstock for biochemical production. Alkali pretreatment led to release of high quality sugars from all sorghum cultivars for 2,3-butanediol production using *Bacillus licheniformis*, and the sugar quality was unaffected by sorghum cultivar and *bmr* mutation type.

Keywords: *bmr,* biomass composition, pretreatment, fermentation, 2,3-butanediol, platform chemical

Background

In 2013, 23.4 billion gallons of bioethanol was produced globally from maize, sugarcane and other food materials (Guragain et al., 2016). United States (US) alone produced 13.3 billion gallons ethanol, which consumed 30% of US maize (Klein-Marcuschamer & Blanch, 2015). The US Renewable Fuels Standards (RFS2) set a goal of producing 36 billion gallons of transportation fuel per year from renewable resources by 2022 (Kamireddy et al., 2013). In addition, a number of platform and bulk chemicals, including 2,3-butanediol, should be produced via biological routes to minimize dependency on petroleum-derived products. 2,3-butanediol is an important platform chemical for the production of number of high-value products, including foods, pharmaceuticals, fuels, polymers, and chemicals (Celińska & Grajek, 2009; Ge et al., 2011; Qi et al., 2014). The global demand for 2,3-butanediol is estimated around 32 million tons per year (Li et al., 2013). Current approaches to production of bio-based fuels and chemicals are inadequate to replace petroleum products without affecting global food supply. Therefore, abundantly available lignocellulosic biomass must be exploited for bio-based fuels and chemicals production (Solomon et al., 2007).

The major roadblock to the utilization of lignocellulosic biomass is the need for an energy-intensive pretreatment process prior to hydrolysis of carbohydrate polymers because of the presence of a strong outer lignin layer (Guragain et al., 2011; Tomas-Pejo et al., 2008). Lignin is a heterogeneous polyphenolic polymer made up of three types of monomers, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), that are linked by carbon-carbon, ester, or ether linkages. Grass lignin also contains a considerable amount of *p*-coumarate and ferulate monomers (Guragain et al., 2015). In addition to total lignin content, composition of lignin monomers and inter-unit linkages also affect biomass pretreatment efficiency. Higher S/G ratio, ester and ether linkages improve pretreatment efficiency, whereas high carbon-carbon linkages decrease it (Studer et al., 2011).

Sorghum is considered a model energy crop because of its high photosynthetic efficiency, abiotic stress tolerance, and wide applications as a food, feed, and fuel. It can be cultivated on degraded lands or infertile soils that are unfavorable for other crops, including maize (Vanholme et al., 2013). The brown midrib (*bmr*) mutation of sorghum leads to decreased lignin content and altered lignin composition (Vanholme et al., 2013). Phenotypically, the presence of the *bmr* gene(s) is characterized by brown coloration in mid-leaf veins in the sorghum plant (Sattler et al., 2010). Among various known *bmr* mutants, *bmr*6, *bmr*12, and *bmr*18 are agronomically acceptable in sorghum (Kamireddy et al., 2013). Allelic genes *bmr*12 and *bmr*18 decrease caffeic acid *o*-methyl transferase (COMT) activity, and *bmr*6 decreases cinnamyl alcohol dehydrogenase (CAD) activity (Oliver et al., 2005). As shown in **Figure 7.1**, the COMT enzyme is responsible for a methyl group addition to 5-hydroxy-conferylaldehyde; therefore, a decrease in the enzyme activity leads to a decrease in syringyl (S) and accumulation of the 5-hydroxy guaiacyl monomer. The CAD enzyme is responsible for a decrease in each cinnamyl aldehyde and its corresponding

cinnamyl alcohol at the final step of monolignol biosynthesis. Therefore, decreased CAD enzyme activity leads to decreases in all three lignin monomers (H-, G- and S-lignin) (Sattler et al., 2010).

The *bmr* mutation of sorghum possesses better forage digestibility for livestock (Srinivasa et al., 2012); therefore, it can also be a promising feedstock for biofuels and biochemicals production. However, our previous study (Guragain et al., 2014) showed that biomass with lower lignin content does not necessarily have better bioethanol production efficiency. This indicated that the effect of *bmr* mutation to improve biomass susceptibility for digestion could be attributed to the genetic background of the plant, and hence selection of an optimal genotype for the *bmr* mutation is vital to develop a superior feedstock for biofuels and biochemicals production (Feltus & Vandenbrink, 2012). In addition, the altered lignin biosynthesis pathways in engineered plants frequently results in dwarfing and thereby leads to an unacceptable biomass yield penalty (Bonawitz & Chapple, 2013). Studies comparing biofuels and biochemicals production efficiency from *bmr* sorghum mutants and their parent cultivars are limited; to date no work has been done investigating platform chemicals biosynthesis, such as 2,3-butanediol. A number of bacteria, including *Klebsiella*, *Enterobacter*, and *Bacilli* genera, produce 2,3-butanediol from different sugar sources. *Klebsiella* sp. is an efficient bacteria to produce 2,3-butanediol from a broad substrate spectrum, but it is considered a pathogenic (biosafety level 2) microorganism (Jiang et al., 2015). *Bacillus licheniformis* DSM 8785 is another efficient bacteria to produce 2,3-butanediol in high concentration from glucose, and is labelled as nonpathogenic (biosafety level 1) bacterial strain (Jurchescu et al., 2013). In addition, this bacterial strain has not be evaluated so far for 2,3-butanediol production from biomass derived sugars.

In this study, three sorghum cultivars (Atlas, Early Hegari [EH], and Kansas Collier [KC]) and two *bmr* mutants (*bmr*6 and *bmr*12) of each cultivar were evaluated and compared for

agronomic traits, and bioprocessing efficiency to produce 2,3-butanediol from stover using *Bacillus licheniformis* DSM 8785. The objective of this study was to test the hypothesis that the *bmr* mutation significantly changes sorghum biomass composition and leads to better-quality feedstock for second-generation biochemicals production using a robust microbial culture, *Bacilluls licheniformis*. **Figure 7.2** shows the schematic representation of this study from sorghum cultivation to 2,3-butanediol production using sorghum stover as feedstock.

Material and Methods

Sorghum cultivation and field study

The forage/grain sorghum cultivars Atlas, Early Hegari (EH), and Kansas Collier (KC) were introgressed with two *bmr* alleles (*bmr*6 and *bmr*12) at USDA-ARS, Lincoln, Nebraska, USA. The wild types and *bmr* alleles introgressed lines were evaluated in a randomized complete block design with two replications after the rainy season (October) in 2012 at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru, Telangana, India. Each cultivar was planted in two 2-m-long rows using recommended spacing and fertilizer. The total rainfall and average daily temperature during the crop growth period were 762 mm and 26°C, respectively. Various agronomic traits were measured during field study, including days to 50% flowering period, plant height (m), fresh stalk yield (t/ha), stover yield (t/ha), and grain yield (t/ha).

Biomass preparation

A 200-g sample of dried and chopped (3 to 6 mm long) stover for all *bmr* mutants and their wild-type sorghum cultivars were brought from ICRISAT to Bioprocessing and Renewable Energy Laboratory, Kansas State University (KSU), Kansas. The samples were ground using a Thomas-Wiley Laboratory Mill (Model 4) fitted with a 2-mm sieve. The ground biomass samples were

further separated to obtain a specific particle size by sieving in a shaker (W.S. Tyler, Model – RX 29, Serial – 25225) fitted with two sieves of size 20 mesh (841 μm) and 80 mesh (177 μm). The size range of biomass was chosen based on the particle size required for biomass composition analysis without further size separation (Sluiter et al., 2007). Around 2 kg *bmr*12 mutant of forage sorghum (GW8528) stalk was also ground and sieved to get the same biomass size. This sorghum cultivar was grown at the field plot of the Kansas State University Department of Biological and Agricultural Engineering, and the biomass sample was used to optimize the pretreatment processes.

Biomass pretreatment

The pretreatment process was first optimized to maximize sugar release from biomass. Preliminary experiments were carried out to compare acid pretreatment using 1% (v/v) sulfuric acid and 10% solids loading at 140°C for 40 min, and alkali pretreatment using 1% (w/v) sodium hydroxide (NaOH) and 10% solids loading at 121°C for 30 min. The results showed that alkali-pretreated biomass released almost three times more total sugars during enzymatic hydrolysis than acid-pretreated biomass. Then, optimum NaOH concentration for biomass pretreatment was determined by evaluating five different concentrations of NaOH solutions, 0.5, 0.75, 1.0, 1.25 and 1.5% (w/v). A 10-g ground biomass sample was mixed with 100 ml alkali solution for each concentration in a 500-ml Erlenmeyer flask and autoclaved at 121°C for 30 min. The biomass slurry was then filtered using a 200-mesh (74 μm) sieve. Approximately 15 ml filtrate was collected to measure sugars and inhibitors produced during pretreatment, and the solid residue was washed with excess distilled water until the filtrate was clear and neutral to litmus paper. The pretreated samples were then dried overnight at 45°C and hydrolyzed as explained in the following section. Released sugars were measured to determine the optimum alkali concentration for

pretreatment. Finally, the same process was followed for the pretreatment of all sorghum samples using the optimized alkali concentration.

Enzymatic hydrolysis of pretreated biomass

Pretreated biomass (2 g) was mixed with 40 ml citrate buffer (4.8 pH and 0.05 M) in a 125-ml conical flask with a screw cap. Cellic CTec2 and Cellic HTec2 enzymes (Novozymes, Inc., Franklinton, NC, USA) were added at the rate of 5.4 and 0.6% (w/w), respectively, of biomass and incubated in a shaker at 50^{o}C and 150 rpm. 500 µl samples were drawn at different time intervals from each flask to measure released monomer sugars. Hydrolyzates were separated by centrifuging the biomass slurry at 13,000 rpm (maximum g-force 20,400×g) for 15 min.

Fermentation of hydrolyzate

The *Bacillus licheniformis* DMS8785 culture was procured from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The culture was revived in nutrient broth and preserved in 15% glycerol media comprising 0.5 ml for each revived culture and 30% glycerol in 1.5-ml culture tubes. To prepare inoculum, 1 ml stock culture was aseptically added into 80 ml sterilized (121^{o}C for 15 min) nutrient broth in a 1000-ml Erlenmeyer flask and incubated at 30^{o}C and 200 rpm for 15 h. For fermentation, a 2 ml filter-sterilized supplement nutrient was aseptically added into the 16 ml sterilized (121^{o}C for 15 min) biomass hydrolyzate in 125-ml flasks. The supplement nutrient solution was prepared in the concentration so that when 2 ml of concentrated solution was added to make 20 ml total fermentation media, the final concentration would be as follow. Micronutrients (per liter): 5 g yeast extract, 5 g bacto tryptone, 7 g dipotassium phosphate, 5.5 g monopotassium phosphate, 1 g ammonium sulfate, 0.25 g magnesium sulfate heptahydrate, 0.12 g sodium molybdate dihydrate,

0.021 g calcium chloride dihydrate, 0.029 g cobalt nitrate hexahydrate, and 0.039 g ferrous ammonium sulfate hexahydrate. Trace elements (per liter): 0.002 g nicotinic acid, 0.000172 g sodium selenite, 0.000037 g Nickel (II) chloride hexahydrate, 0.005 g manganese chloride tetrahydrate, 0.001 g Boric acid, 0.000172 g aluminum potassium sulfate dodecohydrate, 0.00001 g^1 Copper(II) chloride dihydrate, and 0.00554 g disodium ethylenediaminetetraacetate (Jurchescu et al., 2013). Control flasks were also prepared by using the same volume of synthetic medium containing 25 g/L glucose and 12 g/L xylose instead of biomass hydrolyzate. Freshly prepared 2 ml inoculum was added into each flask and incubated in a shaker at 30ºC and 200 rpm for 20 h. Samples were collected at 0, 12 and 20 h of fermentation to measure products and residual sugars.

Analytical procedures

Biomass moisture was determined using an electric moisture meter (IR35M-00015V1, Denver Instrument GmbH, Goettingen, Germany). Extractives, lignin, glucan, xylan, and arabinan were measured using standard protocols (Sluiter et al., 2005; Sluiter et al., 2007). Sugars (glucose, xylose, sucrose, fructose and arabinose), 2,3-butanediol, glycerol, acetoin, and ethanol were measured using an HPLC instrument (Shimadzu Corporation, Japan) equipped with an LC-20AB pump, an SIL-20 AC auto sampler, an SPD-M 20A photodiode array detector, and a Phenomenex RCM-Monosaccharide Ca^+ column (300 × 7.8 mm). Deionized water was used as mobile phase at a flow rate of 0.6 mL min^{-1}. The column oven and refractive index detector (RID-10A) were maintained at 80°C and 65°C, respectively (Guragain et al., 2013). To measure HMF, furfural, acetic acid, lactic acid and formic acid, an ROA organic acid column (300 × 7.8 mm) and both RID and PDA (Photodiode Array)-UV detectors were used in the same HPLC system.

Total phenolics were determined using a modified Folin-Ciocalteu Reagent (FCR) method (Amendola et al., 2012). In brief, a 0.1 ml sample (neutralized and diluted, if required) was mixed

with 5 ml FCR in a 15-ml centrifuge tube. After mixing for approximately 5 min, 3.5 ml 11.5% (w/v) sodium carbonate solution was added and mixed well. A blank was prepared using 0.1 ml deionized water instead of the sample. The mixture was incubated at 40°C for 1 h, and absorbance was taken at 745 nm. Concentration of total phenolics was determined using a standard curve. The initial experiment showed that the standard curve using only one phenolic (gallic acid) did not work well for samples containing two or more phenolics. Therefore, a standard curve was prepared by taking several concentrations of a mixture of five different phenolics: vanillic acid, catechol, gallic acid, guaiacol and vanillin.

All experiments were carried out in triplicate, and data were statistically analyzed for least significant difference (LSD) at 95% confidence level ($P < 0.05$) using JMP software (SAS Institute Inc., Cary, NC, USA).

Results and discussion

Agronomic data

Table 7.1 shows that days to 50% flowering in *bmr*6 mutants from all backgrounds were longer (61, 84 and 84 days in EH, Atlas and KC mutants respectively) than in wild parents (53, 75 and 73 in EH, Atlas and KC, respectively). The mutants from EH and Atlas backgrounds exhibited small decreases in height (1.2 m and 2.0 m on average), but mutants from the KC background had significantly more plant height (2.2 m). A similar trend was observed in fresh stalk yield and stover yield. Atlas wild-type recorded the highest fresh stalk yield and stover yield (22.1 t/ha and 6 t/ha) followed by Atlas and KC mutants. These results indicate that the effects of the *bmr* mutation on agronomic traits depends on the parent sorghum lines in which the mutation is introduced [15]; therefore, each sorghum cultivar must be evaluated separately to select the promising *bmr* sorghum

lines that lead to increase in stover yield without significant decrease in grain yield and increase in flowering time (Rao et al., 2010).

Composition of raw biomass

The composition of raw biomass samples was determined for total lignin, carbohydrate polymers (glucan, xylan and arabinan) and extractives (sum of water-soluble and alcohol-soluble extractives) contents. **Table 7.2** shows that lignin content of *bmr* mutants was 10 to 25% lower than in parent cultivars, except EH *bmr*12. The highest decrease (25%) was observed in EH *bmr*6 and Atlas *bmr*12, and the lowest decrease (10%) was in Atlas *bmr*6. EH *bmr*12 had 12% more lignin than its parent line, perhaps because of excessive production of the 5-hydroxy guiacyl lignin monomer, which surpassed the decrease in syringyl-lignin monomer with introgression of the *bmr*12 gene in EH; *bmr12* mutation decreases activities of COMT enzymes, leading to a decrease in syringyl-lignin monomer and an elevation of 5-hydroxy guiacyl lignin monomer synthesis in plants (Sattler et al., 2010). Glucan and xylan content decreased in all *bmr* mutants compared with their parents, except EH *bmr*12, but change in arabinan content was not statistically significant at the 95% confidence level. Total carbohydrate polymers decreased by 4% in EH *bmr*6, by 9% in both *bmr* mutants of Atlas, and by 9 and 2% in KC *bmr*6 and KC *bmr*12, respectively, compared with their parent cultivars. But the carbohydrate polymers increased by 10% in EH *bmr*12 compared to its parent cultivar. Total extractives content increased by 6 to 35% in all *bmr* mutants compared with their parents, except EH *bmr*12, in which a decrease of 36% was observed. The highest increase in extractives content (35%) compared with the parent cultivar was observed in Atlas *bmr*6, followed by a 31% increase in Atlas *bmr*12, a 15% increase in KC *bmr*6, a 12% increase in EH *bmr*6, and a 6% increase in KC *bmr*12.

Figure 7.3 shows that water-soluble extractives accounted for almost 90% of total extractives, and the remaining was alcohol-soluble extractives. The water-soluble extractives include non-structural sugars, nitrogenous material and other inorganic materials. Alcohol-soluble extractives include waxes, chlorophylls and other minor components (Sluiter et al., 2005). Sucrose, glucose and fructose were the major non-structural sugars in water-soluble extractives, accounting for more than 50% of total extractives except in EH *bmr*12. The highest amount of non-structural sugars (28% of biomass) was found in KC *bmr*6, and the lowest (3% of biomass) in EH *bmr*12. The *bmr* mutation led to an increase in non-structural sugars by 86 and 93% compared with the parent in Atlas *bmr*6 and Atlas *bmr*12, respectively. Similarly, KC *bmr*12 and KC *bmr*6 had 58 and 66%, respectively, more non-structural sugars than the parent plant. EH *bmr*6 had 24% more non-structural sugars than its parents; however, EH *bmr*12 led to a decrease in non-structural sugars by 82%.

Biomass composition results showed that *bmr* mutation led to significant alterations of biomass composition, and the effect considerably depends on the parent line in which the mutation is introduced as well as type of mutation. In addition, sorghum stover contains a huge amount of non-structural sugars, which is further elevated in *bmr* mutants. Achieving additional benefits from *bmr* mutation requires these non-structural sugars to be extracted with hot water prior to biomass pretreatment because if left in biomass, presence of a number of inhibitory compounds results in the sugars released in pretreatment slurry useless as fermentable sugars for microbes. Alternatively, juice can be extracted from fresh stalks immediately after harvesting crops to recover a maximum proportion of non-structural sugars, and later combined with biomass hydrolyzate for biofuel production.

Optimization of biomass pretreatment

Effectiveness of dilute acid and alkali at same concentration were first compared for the pretreatment of *bmr* sorghum. The results (not shown here) showed that alkali pretreatment led to significantly higher sugar yield than acid pretreatment after enzymatic hydrolysis. As a consequence, the alkali (Sodium hydroxide - NaOH) concentration was optimized for biomass pretreatment at 121°C for 30 min with 10% solid loading. **Figure 7.4** shows that by increasing NaOH concentration for pretreatment from 0.5% to 1.5% (w/v) resulted in a gradual decrease in solid mass recovery after pretreatment from 58% to 39%. On the other hand, increasing NaOH concentration from 0.5% to 1.25% increased sugars release from 38% to 86% of pretreated biomass after enzymatic hydrolysis, but increases beyond 1.25% NaOH concentration conferred no additional benefit. Based on raw biomass weight, the maximum total sugar yield (36 g/g raw biomass) was obtained from 1.25% NaOH pretreated biomass, which was taken as the optimum alkali concentration for the comparative evaluation of various sorghum genotypes.

Mass recovery after pretreatment and composition of pretreated biomass

Table 7.3 shows that the solids mass recovery during alkali pretreatment varied from 39% (KC *bmr*6) to 55% (EH *bmr*12). The KC *bmr* mutants had significantly lower mass recovery than their parent, but it was statistically equal to Atlas *bmr* mutants and their parents. Glucan, xylan and arabinan content in pretreated biomass (**Table 7.3**) were almost double that in the raw biomass (**Table 7.2**) for all sorghum lines because of removal of a large proportion of extractives and lignin during pretreatment. However, the percentage carbohydrate increase due to pretreatment was not equal in these sorghum lines, which indicates that loss of biomass components during pretreatment varied significantly among sorghum lines. For example, EH *bmr*12 had 10% more total carbohydrate than its parent in raw biomass but 3% less than its parent in pretreated biomass;

opposite results were observed for Atlas *bmr*6 and its parent line. Decreases in lignin content in pretreated biomass compared with raw biomass seemed very low or even negative in some samples because most of the biomass samples contained more than 30% extractives, which were almost completely removed during pretreatment. This led to increased lignin content in some pretreated biomass despite partial delignification. For example, lignin content in EH *bmr*6 was 9.3% and 12.9% in raw and pretreated samples, respectively, even though 37% of raw biomass lignin was removed during pretreatment. Similarly, lignin content in both raw and pretreated KC *bmr*6 was 9.2%, whereas 62% of raw biomass lignin was removed during pretreatment. Maximum delignification (around 70% of raw biomass lignin) was observed in EH, Atlas and Atlas *bmr*6. Pretreated biomass had almost five times less total extractives content than raw biomass in all sorghum lines, indicating that 80 to 90% of extractives were removed during alkali pretreatment.

Sugar loss and inhibitory compounds produced during pretreatment

During biomass pretreatment, hemicellulose is partially hydrolyzed to monomer sugars, and a number of toxic compounds, including phenolics, acetic acid, formic acid, hydroxymethylfurfural (HMF) and furfural, are produced as a result of depolymerization of lignin and degradation of released sugars. These compounds are considered toxins because they inhibit sugar-fermenting microbes (Feldman et al., 2015). **Table 7.4** shows that total sugar release during pretreatment was 2.5 to 17.3% of raw biomass, which is close to the non-structural sugars content (**Figure 7.3**). This result indicates that hemicellulose was not hydrolyzed significantly during pretreatment. Higher acetic acid production (3.1 to 4.5% of raw biomass) compared with phenolics (2.6 to 3.6% of raw biomass) and minimum hydrolysis of hemicellulose indicate that sorghum lignin is extensively acylated; biomass lignin is partially acylated at γ-carbon of lignin monomer (Guragain et al., 2015; Martínez et al., 2008). Formic acid was produced at 0.4 to 0.7% of raw

biomass. Degradation of xylose and arabinose produces furfural, and further degradation of furfural produces formic acid (Jönsson et al., 2013). HMF and furfural were also measured in all biomass samples, but their values were very low (less than 0.01% of raw biomass), and hence are not reported here. These low values show that 1.25% NaOH pretreatment did not significantly degrade sugars to HMF, and the small amount of furfural produced during this process almost completely further degraded to formic acid.

Hydrolysis of pretreated biomass

Total sugar yield based on raw biomass was 19, 16 and 14% more in *bmr*12 of EH, Atlas and KC, respectively, than in their parents, whereas the *bmr*6 mutants did not yield a significantly higher amount of total sugar than their parents (**Figure 7.5**). Based on total carbohydrate content in raw biomass, total sugar yield was 8 to 27% more in *bmr* mutants than in their parents. In EH background, total sugar yield in *bmr*6 based on total carbohydrate in raw biomass was significantly more than *bmr*12 at 95% confidence level, but opposite was true based on total raw biomass weight, which was due to higher carbohydrate loss in *bmr*6 during pretreatment process. In Atlas background, *bmr*12 had significantly higher total sugars yield than *bmr*6 based on both raw biomass weight as well as total carbohydrate content. In KC background, both *bmr* mutants had statistically equal total sugar yield. These results indicated that *bmr* mutation led to an increase in the hydrolysis efficiency of biomass; however, the effects varied significantly among biomass types as well as *bmr* types.

Fermentation of biomass hydrolyzate

Figure 7.6 shows that 2,3-butanediol yield per gram of sugars consumed during fermentation was not significantly different among biomass hydrolyzates and the control at a 95%

confidence level. This result indicates that quality of released sugars does not vary among sorghum cultivars and their *bmr* mutants, and are comparable with the quality of synthetic sugars for 2,3-butanediol production using *Bacillus licheniformis*. Average 2,3-butanediol yield was very low (around 0.3 g per g sugars consumed), however, in all samples because of the production of a significant amount of byproducts, including acetic acid, glycerol, lactic acid, and ethanol (data not reported); theoretical maximum yield is 0.50 g 2,3-butanediol per g glucose (Jurchescu et al., 2013). Fermentation parameters, including pH, aeration and agitation, must be optimized to minimize byproduct formation to funnel maximum carbon from sugars to 2,3-butanediol (Celińska & Grajek, 2009).

Overall mass balance from sorghum stover to fermentable sugars

Overall mass balances from raw biomass to total fermentable sugars (sum of total sugars released by hydrolysis of pretreated biomass and non-structural sugars obtained from water extraction of raw biomass) are shown in **Figure 7.7**. The EH *bmr*12 mutant yielded the highest total sugars (0.41 g/g raw biomass) from hydrolysis; however, it had the lowest total fermentable sugars because of a very low amount of non-structural sugars (0.03 g/g raw biomass). Total fermentable sugars yield in the EH *bmr*6 mutant is 12% more than in its parent, but it was 12% less than parent cultivar for the EH *bmr*12 mutant. In addition, the stover yield in both EH *bmr* mutants was almost four times less than their parent (**Table 7.1**), indicating that *bmr* mutation of EH sorghum is not a good approach to produce feedstock for biofuels production. On the other hand, total fermentable sugars in Atlas *bmr*12 and KC *bmr*12 mutants were 36 and 30%, respectively, more than their parents; Atlas *bmr*6 and KC *bmr*6 mutants yielded 22 and 27%, respectively, more than the parents. Stover yield in KC *bmr*6 and KC *bmr*12 mutants had 33 and 58%, respectively, more than their parent cultivar (**Table 7.1**). In addition, the grain yield in KC *bmr*6 was not significantly different than

its parent cultivar, but KC *bmr*12 had lower grain yield than its parent. Atlas *bmr* mutants had lower stover yield than their parents; however, their yields were much better than EH *bmr* mutants. Overall, introgression of *bmr*6 gene into KC cultivar led to the most promising feedstock among the tested sorghum lines for second-generation biofuels and biochemical production without significant decrease in grain yield. Introgression of *bmr*12 gene in KC and *bmr*6 and *bmr*12 genes in Atlas also led to improved feedstock quality for biofuels and chemicals production, but the *bmr* mutation in EH had an adverse effect.

Conclusions

The *bmr* mutation in sorghum cultivars significantly affected their flowering time, grain and stover yields as well as composition of biomass. These effects led to improvement on quality of biomass for platform chemicals, like 2,3-butanediol production in some cultivar and *bmr* types, while an adverse effect was observed in others. Introgression of *bmr*6 gene into KC cultivar led to the most promising feedstock among the tested sorghum lines for second-generation biofuels and biochemicals production without significant decrease in grain yield. Introgression of *bmr*12 gene into KC, and *bmr*6 and *bmr*12 genes into Atlas also led to improvement on feedstock quality, but it has an adverse effect in EH cultivar. These results indicated a significant interaction effect between the *bmr* gene and the genetic background of the sorghum lines into which the *bmr* gene is introduced. Therefore, each sorghum line must be evaluated separately to select the promising sorghum cultivars for biofuels and biochemicals production. In addition, the quality of released sugars from alkali pretreated biomass is unaffected by cultivar and *bmr* mutation types, and the sugars quality was as good as synthetic sugars for 2,3-butanediol production using a robust microbial culture, like *Bacillus licheniformis.*

Table 7.1 Agronomic data of different sorghum genotypes

Genotypes	50% flowering time (day)	Plant height (m)	Fresh stalk yield (t/ha)	Stover yield (t/ha)	Grain yield (t/ha)
Early Hegari (EH)	53 ± 1.4^{d}	1.2 ± 0.0^{d}	9.8 ± 0.6^{d}	4.0 ± 0.3^{de}	1.3 ± 0.1^{c}
EH *bmr*6	61 ± 4.2^{c}	1.2 ± 0.2^{d}	5.0 ± 0.6^{e}	1.1 ± 0.1^{f}	3.1 ± 0.6^{b}
EH *bmr*12	59 ± 0.7^{c}	1.3 ± 0.1^{d}	5.4 ± 0.6^{e}	1.1 ± 0.1^{f}	1.5 ± 0.0^{c}
Atlas (AT)	75 ± 0.7^{b}	2.3 ± 0.1^{a}	22.1 ± 2.8^{a}	6.0 ± 0.6^{b}	4.8 ± 0.8^{a}
AT *bmr*6	84 ± 0.0^{a}	2.0 ± 0.1^{abc}	18.9 ± 0.8^{b}	4.8 ± 0.1^{c}	4.1 ± 0.4^{a}
AT *bmr*12	75 ± 0.7^{b}	1.9 ± 0.0^{bc}	16.4 ± 0.6^{c}	3.9 ± 0.3^{e}	1.7 ± 0.0^{c}
Kansas Collier (KC)	73 ± 0.0^{b}	1.8 ± 0.1^{c}	10.6 ± 0.2^{d}	4.5 ± 0.1^{cd}	1.7 ± 0.4^{c}
KC *bmr*6	84 ± 0.7^{a}	2.2 ± 0.1^{ab}	18.9 ± 0.1^{b}	6.0 ± 0.1^{b}	1.6 ± 0.1^{c}
KC *bmr*12	85 ± 1.4^{a}	2.0 ± 0.4^{abc}	16.1 ± 0.3^{c}	7.1 ± 0.1^{a}	0.3 ± 0.1^{d}

bmr = brown midrib sorghum mutant. Data are average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each other at the P<0.05 level.

Table 7.2 Raw biomass composition of different sorghum genotypes

Genotypes	Composition (%, g / g biomass)				
	Glucan	Xylan	Arabinan	Lignin	Extractives
Early Hegari (EH)	25.4 ± 0.5^{cd}	15.4 ± 0.4^{b}	2.0 ± 0.6^{a}	12.5 ± 0.7^{cd}	35.4 ± 1.1^{b}
EH *bmr*6	25.3 ± 1.1^{cd}	13.8 ± 1.3^{d}	1.9 ± 0.8^{a}	9.3 ± 0.5^{g}	39.7 ± 0.3^{a}
EH *bmr*12	27.1 ± 1.3^{ab}	18.0 ± 0.9^{a}	2.0 ± 0.1^{a}	13.9 ± 0.1^{ab}	22.8 ± 0.9^{d}
Atlas (AT)	28.5 ± 0.4^{a}	15.8 ± 0.7^{b}	2.0 ± 0.7^{a}	14.3 ± 1.3^{a}	26.8 ± 2.8^{c}
AT *bmr*6	26.2 ± 0.6^{bc}	13.7 ± 0.5^{d}	2.2 ± 0.3^{a}	12.9 ± 0.9^{bc}	36.1 ± 1.5^{b}
AT *bmr*12	25.0 ± 0.8^{cd}	15.2 ± 0.6^{bc}	2.1 ± 0.6^{a}	10.8 ± 0.1^{ef}	35.1 ± 2.4^{b}
Kansas Collier (KC)	24.5 ± 0.9^{de}	15.2 ± 0.4^{bc}	1.5 ± 0.4^{a}	11.5 ± 0.4^{de}	33.8 ± 0.2^{b}
KC *bmr*6	21.8 ± 1.6^{f}	14.1 ± 0.5^{cd}	1.4 ± 0.2^{a}	9.2 ± 0.4^{g}	38.9 ± 1.1^{a}
KC *bmr*12	23.2 ± 0.6^{ef}	15.8 ± 1.0^{b}	1.5 ± 0.1^{a}	9.6 ± 0.7^{fg}	35.9 ± 0.1^{b}

bmr = brown midrib sorghum mutant. Data are average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each other at the P<0.05 level.

Table 7.3 Mass recovery after pretreatment and composition of pretreated biomass

Sorghum genotype	Mass recovery (%, g/g)	Biomass composition (%, g/g biomass)				
		Glucan	Xylan	Arabinan	Lignin	Extractive
Early Hegari (EH)	44.6 ± 2.0^{b}	48.8 ± 2.2^{abc}	29.8 ± 1.2^{a}	2.7 ± 0.2^{abcd}	8.0 ± 0.2^{g}	6.2 ± 0.6^{e}
EH *bmr*6	45.2 ± 1.0^{b}	45.1 ± 2.0^{d}	25.9 ± 0.3^{c}	2.1 ± 0.1^{d}	12.9 ± 0.3^{a}	7.7 ± 0.1^{cd}
EH *bmr*12	55.3 ± 0.6^{a}	46.9 ± 1.2^{bcd}	29.3 ± 1.9^{ab}	2.4 ± 0.1^{abcd}	8.5 ± 0.3^{fg}	7.5 ± 0.7^{d}
Atlas (AT)	44.7 ± 2.4^{b}	49.4 ± 1.9^{ab}	27.1 ± 0.6^{bc}	2.8 ± 0.6^{abc}	9.7 ± 0.4^{cd}	7.1 ± 0.5^{d}
AT *bmr*6	42.8 ± 0.8^{bc}	51.2 ± 0.9^{a}	27.3 ± 1.1^{bc}	3.0 ± 0.8^{a}	8.5 ± 0.3^{fg}	7.4 ± 0.4^{d}
AT *bmr*12	45.0 ± 2.4^{b}	46.4 ± 1.0^{cd}	28.3 ± 1.7^{abc}	2.5 ± 0.5^{abcd}	11.7 ± 0.7^{b}	8.5 ± 0.6^{bc}
Kansas Collier (KC)	44.4 ± 1.2^{b}	47.1 ± 1.0^{bcd}	28.8 ± 1.3^{ab}	2.9 ± 0.4^{ab}	10.2 ± 0.5^{c}	9.2 ± 0.4^{b}
KC *bmr*6	38.7 ± 1.8^{d}	46.2 ± 0.4^{d}	27.9 ± 2.7^{abc}	2.3 ± 0.1^{bcd}	9.2 ± 0.6^{de}	11.0 ± 0.4^{a}
KC *bmr*12	41.5 ± 1.3^{c}	46.8 ± 2.1^{bcd}	28.7 ± 0.9^{ab}	2.2 ± 0.1^{cd}	8.8 ± 0.2^{ef}	11.1 ± 0.8^{a}

bmr = brown midrib sorghum mutant. Data are average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each other at the P<0.05 level.

Table 7.4 Sugars and inhibitory compounds released during pretreatment

Phenotypes	Released compounds (%, g/g biomass)			
	Total sugars	Phenolics	Acetic acid	Formic acid
Early Hegari (EH)	16.6 ± 0.8^{a}	3.6 ± 0.3^{a}	4.5 ± 0.3^{a}	0.68 ± 0.03^{ab}
EH *bmr*6	14.7 ± 2.0^{a}	3.3 ± 0.4^{ab}	3.2 ± 0.4^{b}	0.51 ± 0.04^{cd}
EH *bmr*12	2.5 ± 0.1^{c}	3.6 ± 0.1^{a}	4.1 ± 0.0^{ab}	0.42 ± 0.01^{d}
Atlas (AT)	6.6 ± 0.8^{b}	3.3 ± 0.2^{ab}	3.1 ± 0.4^{b}	0.58 ± 0.06^{bc}
AT *bmr*6	14.4 ± 2.7^{a}	3.2 ± 0.4^{ab}	3.5 ± 0.6^{b}	0.68 ± 0.10^{ab}
AT *bmr*12	10.3 ± 4.1^{b}	3.0 ± 0.3^{bc}	3.3 ± 1.4^{b}	0.67 ± 0.22^{b}
Kansas Collier (KC)	8.3 ± 0.6^{b}	3.0 ± 0.1^{bc}	4.6 ± 0.4^{a}	0.85 ± 0.07^{a}
KC *bmr*6	17.3 ± 3.1^{a}	2.6 ± 0.2^{c}	3.4 ± 0.6^{b}	0.69 ± 0.11^{ab}
KC *bmr*12	15.6 ± 1.5^{a}	2.6 ± 0.2^{c}	3.9 ± 0.3^{ab}	0.71 ± 0.02^{ab}

bmr = brown midrib sorghum mutant. Data are average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different from each other at the P<0.05 level.

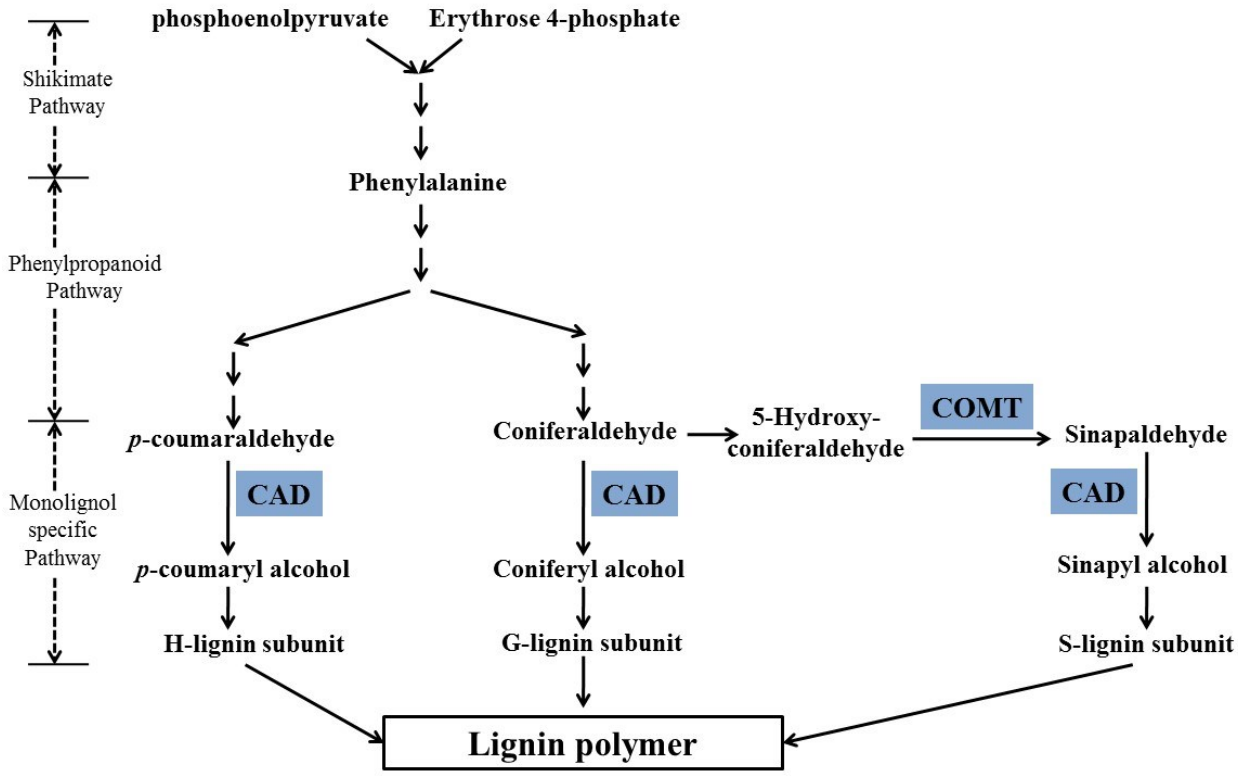


Figure 7.1 Monolignols biosynthetic pathways in plants. COMT= Caffeic acid O-methyltransferase, CAD= Cinnamyl alcohol dehydrogenase (Sattler et al., 2010)**.**

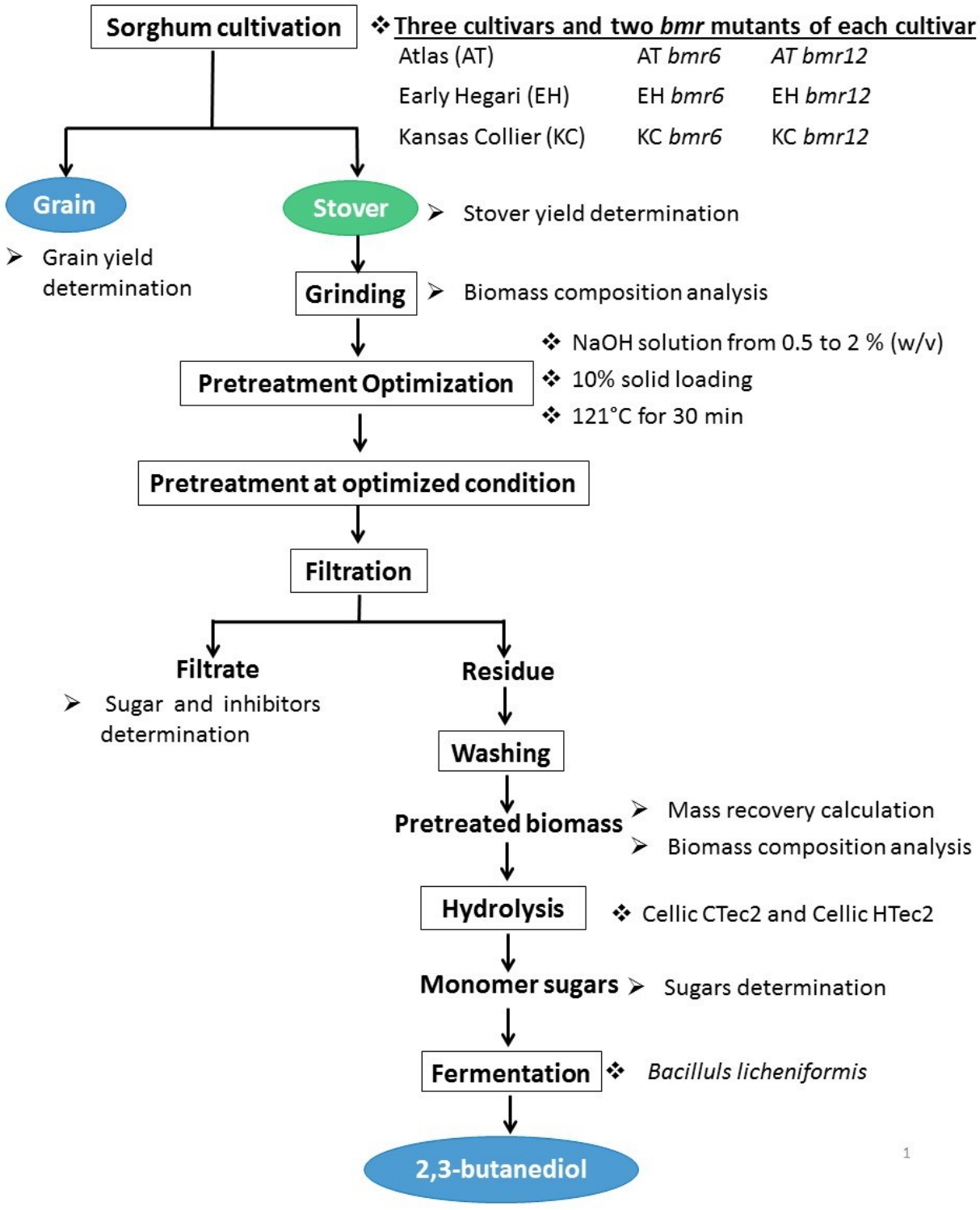


Figure 7.2 Schematic representation of 2,3-butanediol production from sorghum stover

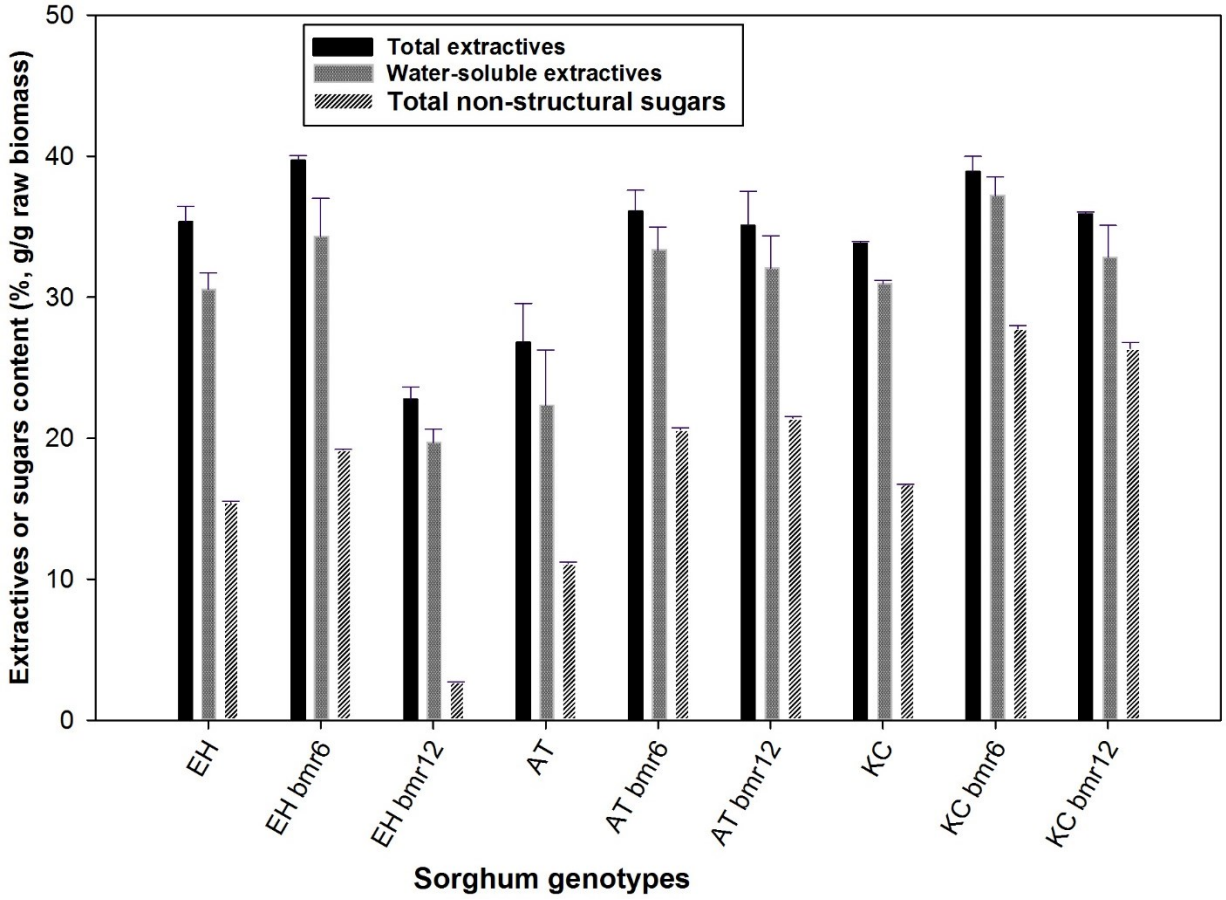


Figure 7.3 Biomass extractives. EH = Early Hegari, AT = Atlas, KC = Kansas Collier, *bmr* = brown midrib sorghum mutant. Total extractives is sum of water-soluble extractives and alcohol (95% ethanol)-soluble extractives. Non-structural sugars are the sugars extracted from biomass in water. All the extractions were done using Soxhlet extraction set. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

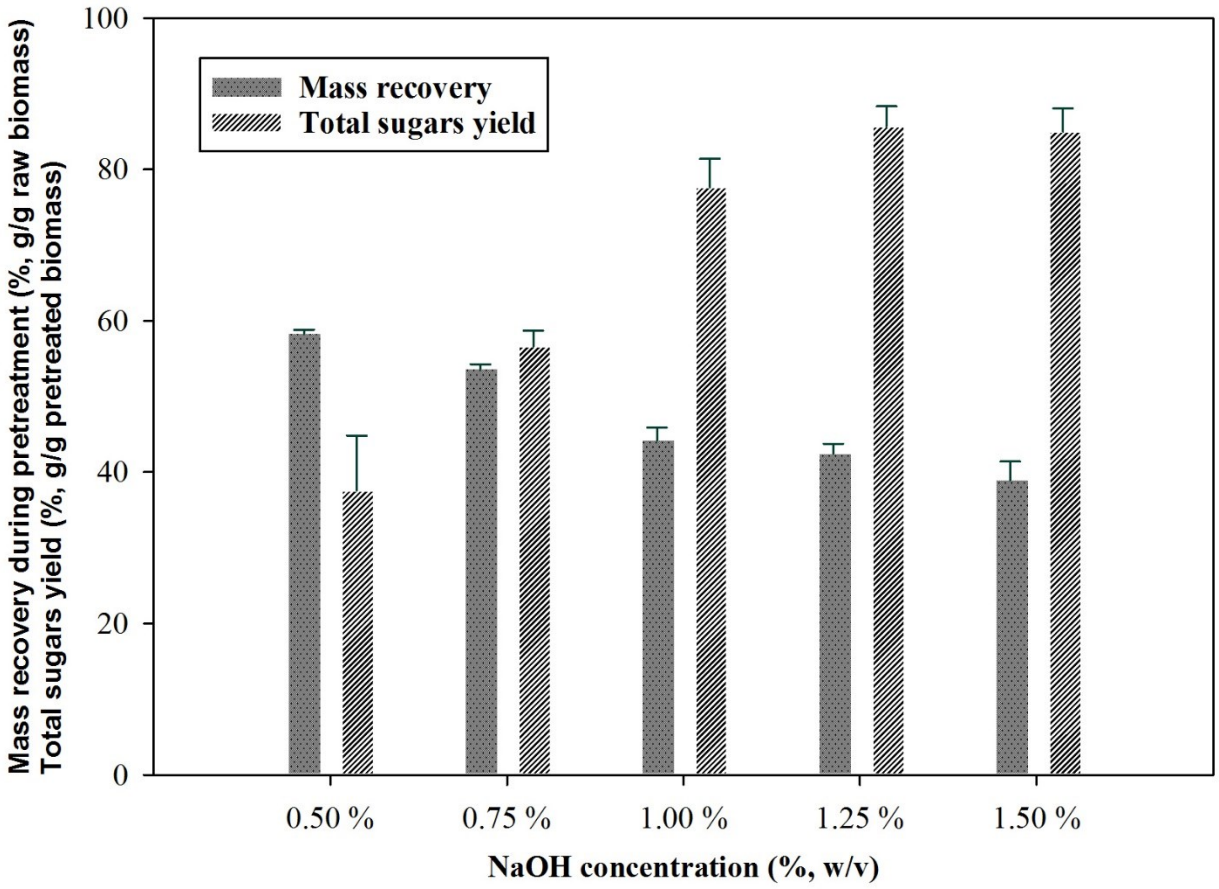


Figure 7.4 Optimization of sodium hydroxide (NaOH) concentration for *bmr* sorghum pretreatment. Pretreatment was carried out at 121°C for 30 min using 0.5 to 1.5% (w/v) NaOH with 10% (w/v) solid loading, followed by hydrolysis at 50°C for 48 h with 5% (w/v) solid loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of solid. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

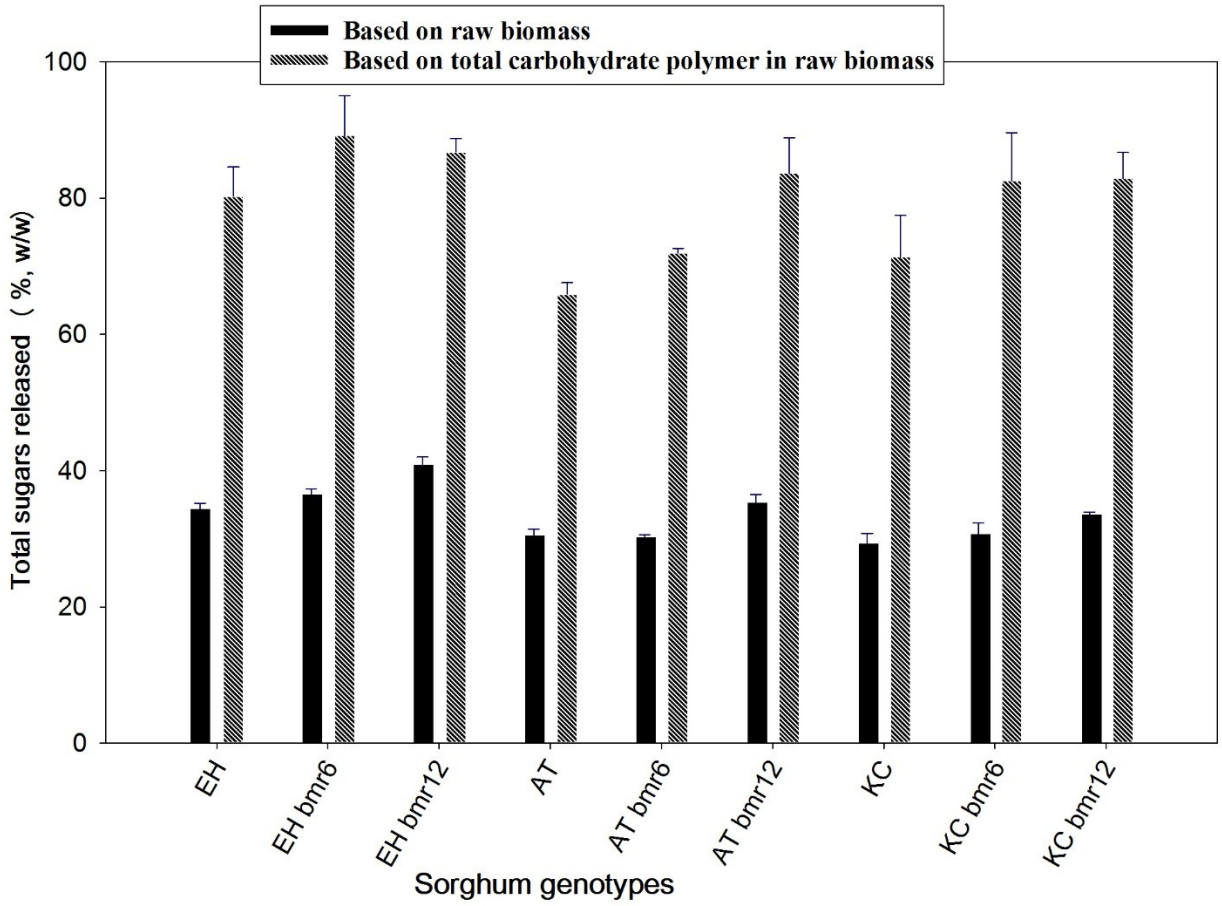


Figure 7.5 Total sugars released during hydrolysis of pretreated biomass. EH = Early Hegari, AT = Atlas, KC = Kansas Collier, *bmr* = brown midrib sorghum mutant. Hydrolysis was carried out at 50°C for 48 h with 5% (w/v) biomass loading in citrate buffer (4.8 pH and 0.05 M) using enzyme loading of 6% (w/w) of biomass. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

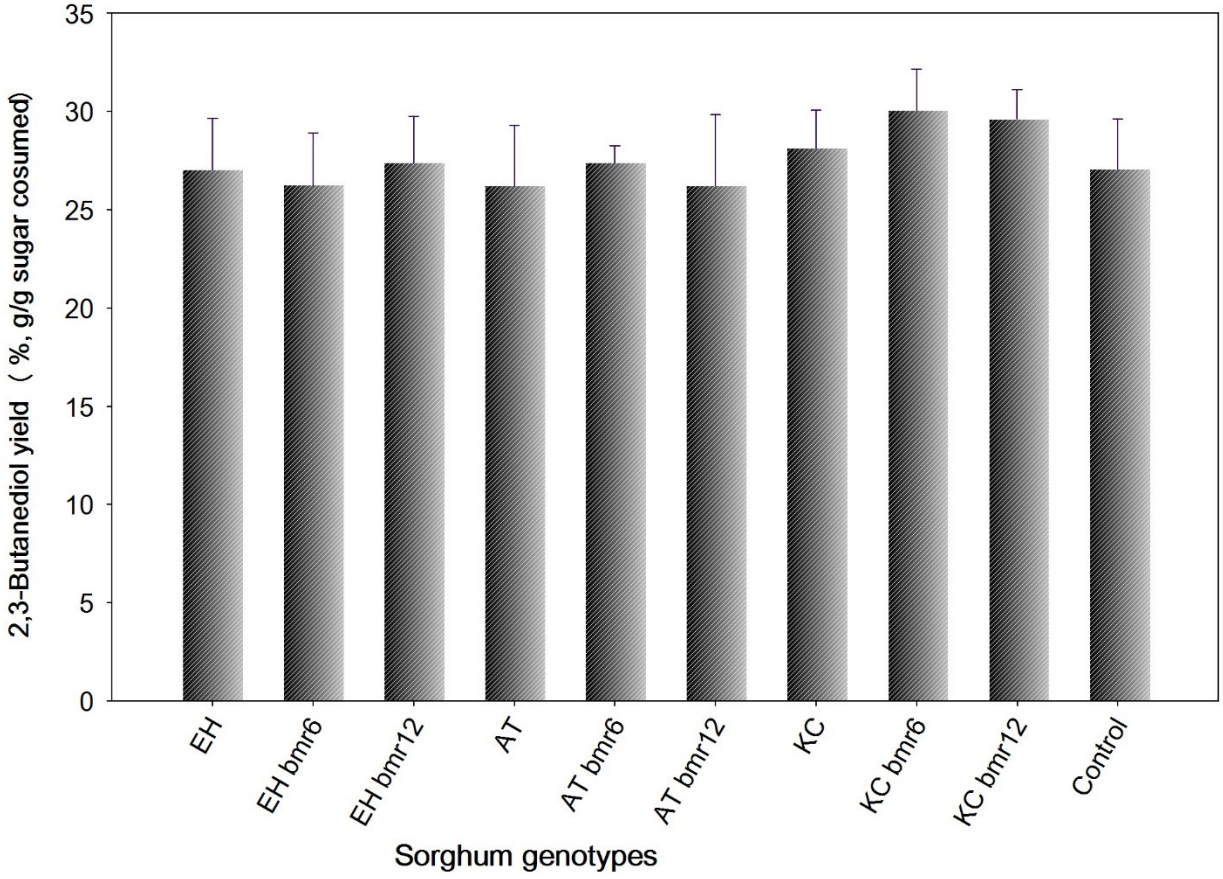
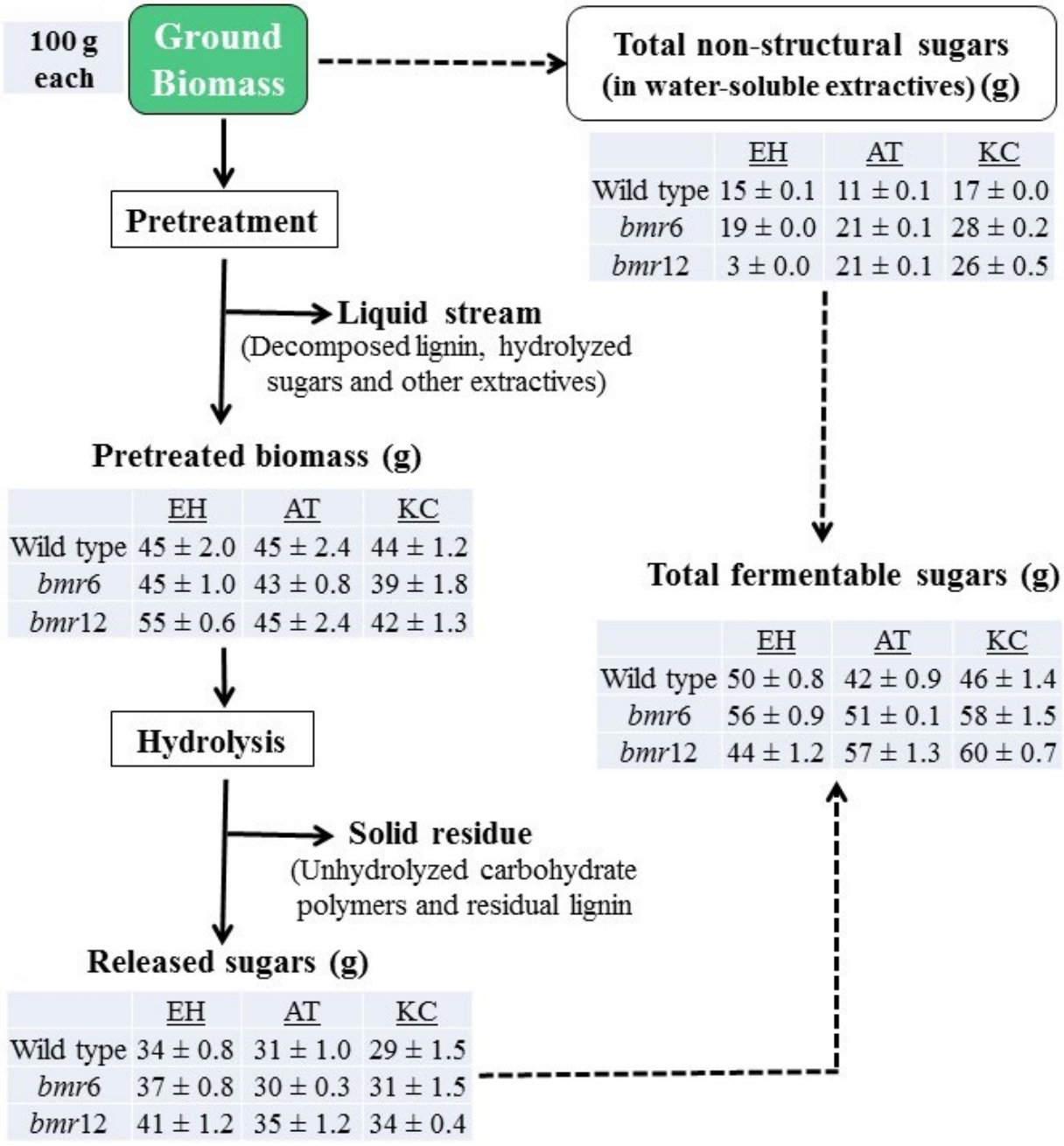


Figure 7.6 2,3-Butanediol fermentation from biomass hydrolyzate. EH = Early Hegari, AT = Atlas, KC = Kansas Collier, *bmr* = brown midrib sorghum mutant, control = synthetic sugars solution with similar concentration of hydrolyzate. Fermentation was carried out at 30°C and 200 rpm for 12 h using *Bacillus licheniformis* DSM 8785. Data are average values of triplicate experiments, and error bars represent sample standard deviation.



	EH	AT	KC
Wild type	15 ± 0.1	11 ± 0.1	17 ± 0.0
*bmr*6	19 ± 0.0	21 ± 0.1	28 ± 0.2
*bmr*12	3 ± 0.0	21 ± 0.1	26 ± 0.5

	EH	AT	KC
Wild type	45 ± 2.0	45 ± 2.4	44 ± 1.2
*bmr*6	45 ± 1.0	43 ± 0.8	39 ± 1.8
*bmr*12	55 ± 0.6	45 ± 2.4	42 ± 1.3

	EH	AT	KC
Wild type	50 ± 0.8	42 ± 0.9	46 ± 1.4
*bmr*6	56 ± 0.9	51 ± 0.1	58 ± 1.5
*bmr*12	44 ± 1.2	57 ± 1.3	60 ± 0.7

	EH	AT	KC
Wild type	34 ± 0.8	31 ± 1.0	29 ± 1.5
*bmr*6	37 ± 0.8	30 ± 0.3	31 ± 1.5
*bmr*12	41 ± 1.2	35 ± 1.2	34 ± 0.4

Figure 7.7 Overall mass balance from sorghum stover to total fermentable sugars. EH = Early Hegari, AT = Atlas, KC = Kansas Collier, *bmr* = brown midrib sorghum mutant. Data are average values of triplicate experiments ± sample standard deviation.

Chapter 8 - Optimization of 2,3-butanediol fermentation using *Klebsiella oxytoca* ATCC 8724 from biomass-derived sugars

Abstract

2,3-butanediol (BD) is a valuable platform chemical with a number of industrial applications. Biomass-derived sugars is a sustainable feedstock for BD production. High BD titer is critical to reduce downstream processing cost. In this study, we evaluated both synthetic sugars and biomass-derived sugars for efficient BD fermentation in batch and fed-batch fermentation processes at shake-flask (50 ml working volume) and 7-L bioreactor (5 L working volume) using *Klebsiella oxytoca* ATCC 8724. The results showed that the culture can utilize both glucose and xylose, with BD productivity using the glucose medium was almost twice as well compared to the xylose medium. The mixed sugars medium (with glucose and xylose in 2:1 ratio) was less efficient compared to the xylose medium because of the catabolic repression from glucose, leading to reduced substrate uptake rate. Biomass-derived sugars were as efficient as synthetic sugars at lower total sugar concentrations (2.5%, w/v), but not at higher total sugar concentrations (9%, w/v) unless the biomass hydrolyzates were detoxified to remove acetic acid and other inhibitory compounds. The concentration of these toxins was not enough to adversely affect the xylose utilization in biomass hydrolyzate with lower sugar concentration. One vvm aeration without pH control at 37°C and 200 rpm represented the optimum conditions for BD fermentation with 7% (w/v) initial glucose concentration in a 7-L bioreactor. A BD titer of 91 g/L was obtained using fed-batch process in glucose medium. BD productivity gradually decreased upon each subsequent feeding due to the reduced substrate uptake rate; therefore, two feedings was optimum resulting in a final BD titer, yield and productivity of 80 g/L , 0.47 g/g glucose, and 0.79 $gL^{-1}h^{-1}$, respectively. Fed-batch fermentation using the xylose medium was also beneficial to increase BD titer.

However, mixed sugars medium was not appropriate for fed-batch fermentation; feeding additional sugars before complete utilization of xylose resulted in utilization of only glucose, leaving xylose untouched, whereas sugar feeding after complete utilization of xylose resulted in very limited glucose and no additional xylose utilization. These results showed that the development of appropriate biomass processing technology to obtain separate cellulose and hemicellulose hydrolyzates is beneficial for efficient BD fermentation.

Keywords: 2,3-butanediol, *Klebsiella oxytoca*, fed-batch fermentation, biomass hydrolyzate

Introduction

2,3-butanediol (BD) is a valuable platform chemical with considerable potential for the chemical industry. The worldwide demand for BD is estimated around 32 million tons per year (Li et al., 2013). The production of 2,3-butanediol (BD) via microbial bioprocessing from resources such as plant-based biomass holds much promise for renewable BD. Though, one of the challenges with utilizing biomass feedstocks is the efficient utilization of hemicellulose-derived monosaccharides including xylose. Development of improved microbial bioprocessing technologies to produce BD from biomass holds opportunity to improve the feasibility of BD as a platform chemical for food, fuel, chemical, pharmaceutical, and polymer markets as shown in **Figure 8.1** (Celińska & Grajek, 2009; Ji et al., 2011; Li et al., 2013; Qi et al., 2014).

BD is a four-carbon alcohol that exists in three stereoisomers: L-(+)-BD (S,S – dextrorotatory form), D-(-)-BD (R,R – Levorotatory form), and meso-BD (optically inactive form). Microbial production of BD has more than 100 years of history. Industrial BD production started in 1933. The huge demand for 1.3-butadiene during World War II intensified research on

BD production to use as feedstock for the production of 1,3-butadiene. However, the availability of a cheaper petroleum-derived alternative replaced BD for 1,3-butandiene production after the War. The energy crisis in the 1970s resulted in revival of BD production using microbial bioprocessing (Ji et al., 2011). A number of microbial species, including *Klebsiella, Enterobacter, Bacillus* and *Serratia* genera can produce BD from different feedstocks. Among them, *K. oxytoca, K. pneumoniae, E. aerogenes, B. licheniformis,* and *S. marcescents* are the most promising microorganisms for efficient BD fermentation (Ji et al., 2011; Jurchescu et al., 2013). These microorganisms produce BD in a mixed acid fermentation process with several intermediate compounds; a number of other end-products are also synthesized depending on the type of microorganisms and fermentation conditions, as shown in **Figure 8.2** (Celińska & Grajek, 2009; Han et al., 2013; Jansen et al., 1984). For example, most of the BD producing microorganisms are facultative anaerobes, which obtain energy from both respiration and fermentation pathways. The acetate production pathway is predominant in oxygen-saturated, aerobic environments. Under anaerobic conditions, lactate will be the major product while intermediate levels of oxygen favor BD, ethanol and acetoin production. A number of other operating parameters, including pH, temperature, and substrate concentration also significantly affect the formation of metabolites (Celińska & Grajek, 2009). In addition, the type of microorganism determines the production of different stereoisomers of BD; for example, *K. oxytoca* produces a racemic mixture of L-(+)-BD and meso-BD. Similarly, *B. lechiniformis* produces a racemic mixture of D-(-)-BD and meso-BD, and *S. marcescens* produces optically pure meso-BD (Ji et al., 2011).

For the sustainable production of bio-based fuels and chemicals, abundantly available lignocellulosic biomass feedstocks must be used. Fermentable monosaccharides derived from hydrolyzed biomass polysaccharides (hemicellulose and cellulose) include a mixture of glucose

and xylose; therefore, the capability of BD producing organisms to utilize both sugars is critical. *K. oxytoca* is a highly efficient BD fermenting microorganism capable of using a wide range of biomass feedstocks, including glucose and xylose (Cheng et al., 2010). In addition, high BD titer is desirable in fermentation broth to reduce downstream processing. However, batch fermentation is limited due to substrate inhibition from the high initial sugar concentration needed to achieve high BD titer. Instead, fed-batch fermentation is more appropriate to achieve the desired high titer without substrate inhibition. Thus, the objective of this study was to optimize the fermentation conditions for *Klebsiella oxytoca* ATCC 8724 to produce BD from both synthetic and biomass-derived sugars using fed-batch fermentation to produce high BD titer and productivity.

Materials and methods

Microorganisms

Klebsiella oxytoca ATCC 8724 obtained from American Type Culture Collection (Manassas, Virginia, USA) was used in this experiment. The culture was revived in nutrient broth medium, and several stock culture tubes were prepared comprising 0.5 ml for each revived culture and 30% (v/v) sterilized glycerol. The stock culture tubes were stored at -80°C until used for inoculum preparation.

Preparation of biomass hydrolyzate

Switchgrass samples obtained from the Kansas State University Agronomy Farm in Manhattan, Kansas were ground to less than 1 mm size using a Thomas-Wiley Laboratory Mill (Model 4). Sorghum stalks was obtained from Texas A&M University, College Station, Texas, and ground by Mesa Reduction Engineering & Processing Inc., Auburn, New York. The ground biomass samples were mixed with 1.25% (w/v) sodium hydroxide (NaOH) solution with 1:10 ratio (40 g biomass in 400 ml alkali solution) in 500-ml flask, and autoclaved at 121°C for 30 min. The

biomass slurry was filtered and washed with excess water, and dried overnight at 45°C. The pretreated biomass was mixed with citrate buffer (4.8 pH and 0.05M) at two different solids loading: 12% (24 g biomass in 200 ml buffer) and 4% (8 g biomass in 200 ml buffer) in 500-ml flask with a screw cap. Cellulases (Cellic CTec2) and hemicellulases (Cellic HTec2) enzymes provided by Novozymes, Inc., Franklinton, North Carolina were used for biomass hydrolysis at the rate of 6% (w/w) of biomass with 10:1 ratio of CTec2:HTec2. The biomass slurry was incubated in a temperature-controlled shaker (Innova 4300, New Brunswick Scientific, New Jersey, USA) at 50°C and 125 rpm for 48 h. The liquid hydrolyzate was finally separated by centrifuging the biomass slurry at 13,000 rpm (maximum g-force 20,400×g) for 15 min and stored at -20°C until used for BD fermentation.

Concentrated wood (poplar) hydrolysates were obtained from Technology Holding LLC (Salt Lake City, Utah, USA), which contained 415 g/L of glucose and 132 g/L of xylose. The hydrolysates were diluted to a final total sugars concentration of 8% to 9% (w/v); similar to the total sugar concentration of sorghum stalk hydrolysates prepared using a 12% (w/w) solids loading during hydrolysis. .

Inoculum preparation

Inoculum preparation was performed by adding 0.3 ml stock culture to 100 ml sterilized (at 121°C for 15 min) nutrient broth medium in 1000-ml flask and incubated in a temperature-controlled shaker (Innova 4300, New Brunswick Scientific, NJ, USA) at 37°C and 200 rpm. The inoculum was removed after 7 h of incubation (corresponding to the exponential growth or log phase) and aseptically added to the fermentation medium at the rate of 5% (v/v) of total fermentation medium volume.

Fermentation

Shake-flask

Shake-flask fermentations were carried out in 500-ml flasks with a 50 ml working volume. The medium for shake-flask fermentations was prepared using different concentrations of synthetic sugars (glucose, xylose or mixed sugars) or biomass hydrolyzate supplemented with recommended nutrients: 2 gL^{-1} monopotassium phosphate, 10.5 gL^{-1} dipotassium phosphate, 3.3 gL^{-1} diammonium phosphate, 6.6 gL^{-1} ammonium sulfate, 0.25 gL^{-1} magnesium sulfate heptahydrate, 0.05 gL^{-1} ferrous sulfate heptahydrate, 0.001 gL^{-1} zinc sulfate heptahydrate, 0.001 gL^{-1} manganese (II) sulfate monohydrate, 0.01 gL^{-1} calcium chloride dehydrate, 0.05 g L^{-1} ethylenediaminetetraacetic acid (Jansen et al., 1984). The sugar solutions and biomass hydrolyzates were autoclaved at 121°C for 15 min in the 500-ml flasks. Once cooled, a 10x concentrated nutrient solution was filter sterilized (0.22 μm, Millipore) and added aseptically (10%, v/v) to each flask. The freshly prepared inoculum was added to each flask and incubated in temperature-controlled shaker at 37°C and 200 rpm. 0.5 ml sub-samples were obtained over the course of fermentation to measure the products and residual sugars. Filter sterilized concentrated sugar solutions were used as feeding medium for fed-batch fermentation.

Bioreactor scale-up

The optimal fermentation medium determined from the batch-flask study was scaled up to a 7-L bioreactor (Bioflo 110, New Brunswick Scientific Inc., Enfield, Connecticut, USA) with a 5-L working volume. The fermenter with 3,750 ml sugar solution containing sugars in the amount sufficient to make the desired sugars concentration in final 5 L fermentation medium were autoclaved at 121°C for 30 min. Three autoclavable sample bottles were fitted with the fermenter before autoclaving: one bottle contained 10% (w/v) sodium hydroxide solution, another contained

antifoam and a third was used to pump sterilized nutrient solution and inoculum. One liter solution with required nutrients in the amount sufficient for 5 L fermentation medium was filter sterilized (Millipore, 0.22 μm) and pumped into the sterilized fermenter. The fermentation process was optimized for pH and aeration when operating parameter were set at 200 rpm agitation and 37°C temperature to maximize BD concentration, yield per gram sugars, and productivity. Around 10 ml samples were drawn periodically to measure biomass growth, products formation and sugars consumption. Filter sterilized concentrated sugar solutions were added at appropriate time interval to carry out the fed-batch fermentation process.

Analytical methods

Monosaccharides (glucose, and xylose), and fermentation products/byproducts including, BD, acetoin, and glycerol were measured using High Performance Liquid Chromatography (HPLC). The HPLC instrument (Shimadzu Corporation, Japan) was equipped with an LC-20AB pump, an SIL-20 AC auto sampler, an SPD-M 20A photodiode array detector, and a Phenomenex RCM-Monosaccharide Ca^{+} column (300 × 7.8 mm). Flow rate of mobile phase (deionized water) was 0.6 ml min^{-1}. The column oven and refractive index detector (RID-10A) were maintained at 80°C and 65°C, respectively. Other fermentation byproducts, such as acetic acid, lactic acid, formic acid, and ethanol, were measure using ROA organic acid column (150 × 7.8 mm); both RID and PDA (Photodiode Array)-UV detectors were used in the same HPLC system. A 0.005 N sulfuric acid in deionized water was used as mobile phase with a flow rate of 0.6 ml min^{-1}.

Statistical methods

All shake-flask fermentations were performed in triplicate and fermentations performed in the bioreactor were performed in duplicate. The data were statistically analyzed for the least

significant difference (LSD) test at 95% confidence level ($P < 0.05$) using JMP software (SAS Institute Inc., Cary, North Carolina, United States).

Results and discussion

Optimization of sugar concentration

Glucose, xylose and mixed sugars media were evaluated separately to determine the optimum sugar concentration for BD fermentation. Four different concentrations of sugar solutions were taken in these experiments for each type of medium: 3%, 5%, 7%, and 9% (w/v) for glucose and xylose media, and 6%, 9%, 12%, and 15% (w/v) for mixed sugars medium. **Figure 8.3(a)** shows that the final BD concentration increased with an increase in the initial glucose concentration of the fermentation medium from 3% to 9%. BD yield and productivity also increased with an increase in initial glucose concentration in fermentation medium from 3% to 7%; however, 7% and 9% did not differ significantly. BD yield at 9% initial glucose concentration was 0.43 g/g glucose, and productivity (at 36 h) was 1.10 $gL^{-1}h^{-1}$. This result showed that 9% initial glucose concentration is optimum for batch fermentation based on the highest BD concentration in final fermentation broth. In case of fed-batch process, 7% initial glucose concentration can be taken as the optimum because of initial slower glucose uptake rate until first 12 h of fermentation with 9% glucose medium compared to 7% glucose medium.

Similar to glucose media, BD yield and productivity increased as the initial xylose concentration increased from 3% to 7% in xylose medium as shown in **Figure 8.3(b)**, but the productivity significantly decreased at 9% initial xylose concentration due to a long lag phase (12h). BD yield and productivity (at 36 h) at 7% xylose medium were 0.36 g/g xylose consumed and 0.64 $gL^{-1}h^{-1}$, respectively. Based on these data, 7% xylose concentration was optimum for BD fermentation using xylose medium.

Above results show that *K. oxytoca* ATCC 8724 can consume both glucose and xylose to produce BD; however, substrate uptake rate and, therefore, BD formation rate was almost double in glucose medium vs xylose medium. Because of slower BD formation rate in xylose medium, a higher proportion of sugar was utilized for cell maintenance, thereby reducing BD yield per gram of substrate compared to glucose media: 0.43 g/g in 7% glucose medium and 0.36 g/g in xylose medium. The similarity between glucose and xylose was the increase in BD yield with an increase in sugar concentration until the optimum sugar concentration was reached. BD yields at 3%, 5% and 7% sugar concentration were 0.33 g/g, 0.37 g/g, and 0.43 g/g, respectively, in glucose medium, and 0.27 g/g, 0.33 g/g, and 0.36 g/g, respectively, in xylose medium. This was because a higher proportion of sugar was used for cell maintenance, and therefore a lower proportion of sugar was used for product formation at suboptimal sugar concentrations compared to optimum sugar concentration.

After separate optimization of glucose and xylose media, a mixed sugar medium containing mixture of glucose and xylose was evaluated for BD production. Our previous studies (Guragain et al., 2014; Guragain et al., 2013) showed that biomass hydrolysates comprised of approximately a 2:1 ratio of glucose and xylose. Therefore, mixed sugar media containing a 2:1 ratio of glucose and xylose were prepared at different total sugar concentrations and tested for the optimal mixed sugar concentration. **Figure 8.3(c)** shows that *K. oxytoca* ATCC 8724 rapidly consumed glucose at first, and the xylose was consumed only after glucose was depleted in the medium at total sugars concentrations of 6% and 9% (w/v). This was not true for higher total sugars concentration (12% and 15%), in which the glucose consumption was very slow, and no xylose was consumed until 91 h of fermentation. The BD yield at 6% and 9% total sugars were equal (0.35 g/g), but the productivity was slightly higher at 9% total sugars (0.50 $gL^{-1}h^{-1}$ at 60 h fermentation) than 6% total

sugars (0.47 $gL^{-1}h^{-1}$ at 48 h fermentation). Additionally, the final BD concentration was also significantly higher at 9% total sugars; therefore, 9% was the optimum total sugars concentration for BD fermentation using mixed sugar medium.

The BD productivity at optimum sugar concentration (7% for glucose and xylose, and 9% for mixed sugars) was 2.3 and 1.3 times higher in glucose and xylose medium, respectively than for mixed sugars medium. The lowest productivity in mixed sugar media was due to a slower substrate uptake rate as well as diauxic growth. This is most likely due to the glucose catabolite repression resulting in hindered xylose uptake (Ji et al., 2011). The substrate utilization in optimum mixed sugars medium (9% total sugars) can be divided into two phases: a first phase from 0 to 24 h in which glucose was utilized, and a second phase from 24 to 60 h characterized by xylose consumption. The BD productivity during the first phase was 0.79 $gL^{-1}h^{-1}$, 28% lower than BD productivity using only glucose with a similar total sugar concentration (9% glucose). This may indicate that xylose also inhibits the rate of glucose uptake reducing BD productivity in the mixed sugar fermentation. The BD productivity during the second phase of the mixed sugar fermentation (24 to 60 h) was 0.30 $gL^{-1}h^{-1}$, which was not significantly different than the BD productivity at 3% xylose media; however, it was significantly less than the productivity at the optimum initial xylose concentration (0.55 $gL^{-1}h^{-1}$ at 7% initial xylose concentration).

Above results indicate that mixed sugar fermentation is significantly less efficient compared to using a single sugar source (glucose or xylose) for the production of BD using *K. oxytoca* ATCC 8724. The inability of *K. oxytoca* to simultaneously utilize glucose and xylose was the major problem for the reduced BD productivity in mixed sugar media. To overcome this, *K. oxytoca* was grown for multiple generations to evoke nutrient adaptation by sub-culturing five times in 7% xylose containing media. The inoculum was prepared after the fifth sub-culture and

used for a mixed sugars fermentation with total sugars 9% and 12%. Unfortunately, no significant change in substrate uptake and product formation rate was observed using this technique (data not reported).

Comparison of biomass hydrolyzate with synthetic sugar for BD fermentation

The total sugar concentration in biomass hydrolyzates prepared with 4% and 12% (w/v) solids loading during hydrolysis of alkali pretreated biomass (sorghum stalks and switchgrass) was approximately 2.5% and 9% (w/v), respectively. The concentrated wood (poplar) hydrolysates were diluted to achieve a total sugar concentration of 9%. The pH of all hydrolyzates was adjusted to 6.9±0.1 using concentrated NaOH solution. Two sugar solutions with a total sugar concentration of 2.5% and 9% (w/v) of glucose and xylose (approximately 2:1 ratio) were taken as control media.

Figure 8.4(a) shows that glucose and xylose utilization, and BD formation rates were not significantly different between the controls and biomass hydrolyzates at the lower sugar concentration (2.5 % total sugar); both glucose and xylose was completely utilized in all media. Similar results were achieved for the 9% total sugars control and wood hydrolyzate, but not for sorghum hydrolyzate, as shown in **Figure 8.4(b).** For the sorghum hydrolyzate containing higher total sugars, an initial lag phase of ~6 h was observed prior to glucose utilization; xylose was not consumed at all even after depletion of glucose in the fermentation medium. One possibility for this observation is the presence of acetic acid that was detected at a concentration of 3 g/L in the sorghum hydrolysates. Acetic acid is a known microbial inhibitor produced during hydrolysis of hemicellulose polymer (Hu & Ragauskas, 2012). The acetic acid was produced at a low concentration (less than 0.5 g/L) in hydrolyzate containing lower total sugars (2.5%, w/v), which did not significantly affect xylose uptake. Acetic acid was present in low concentrations (less than 0.5 g/L) in wood hydrolyzate because the supplier (Technology Holding LLC) claimed that a

detoxification step was used to remove potential inhibitory compounds. These results show that detoxification of inhibitory compounds, including acetic acid, is essential for *K. oxytoca* ATCC 8724 BD fermentation using optimum total sugar concentration (9%) in biomass hydrolyzate.

Fed-batch fermentation in shake flask

The *K. oxytoca* ATCC 8724 was capable of utilizing both glucose and xylose sugars, but BD production was more efficient in glucose medium. The final BD titer at the optimum initial sugar concentration was only 28.2 g/L and 23.1 g/L for glucose and xylose, respectively; higher BD titer is desired to reduce downstream processing cost. Fed-batch fermentation in shake flasks was carried out for both glucose and xylose media by feeding sterilized concentrated sugar solution over the fermentation process time. **Figure 8.5a** shows that BD concentration gradually increased up to the fourth glucose feeding. BD yield per gram of sugar consumed was fairly constant, but the productivity gradually decreased as the fed-batch proceeded because of reduced substrate uptake rate, indicating a trade-off between BD titer and productivity. No significant amount of glucose was consumed after 148 h in the fourth feeding and, hence, data is not shown here. The final BD titer reached 91.6 gL^{-1} in 148 h of fermentation, for which productivity was 0.62 $gL^{-1}h^{-1}$. Based on these results, two feedings with 71 h of fermentation time is considered optimum, which generated the highest BD yield (0.42 g/g glucose consumed, 84% of theoretical maximum) and a good productivity (0.91 $gL^{-1}h^{-1}$).

The fed-batch results using xylose medium, **Figure 8.5b**, was less effective compared to the glucose medium. A lower BD titer was achieved because xylose consumption halted after the second feeding. Therefore, only one feeding was chosen as the optimum for xylose media, in which final BD titer was 32.8 g/L, with a BD yield and productivity of 0.36 g/g and 0.46 $gL^{-1}h^{-1}$, respectively.

The fed-batch fermentation in shake-flasks was performed for wood hydrolyzate with 9% (w/v) total sugars and control, but not for sorghum hydrolyzate because xylose was not utilized in this medium. As shown in **Figure 8.6**, feeding a concentrated sugars solution or wood hydrolyzate was performed after 36 h. Glucose was almost completely consumed in both the control and wood hydrolyzate in 24 h of fermentation, but only a limited amount of xylose was consumed prior to feeding; xylose was not consumed at all after feeding. Final BD titer was significantly higher in wood hydrolyzate compared to the control because of the higher concentration of initial glucose in wood hydrolyzate; however, BD yield per gram of sugar consumed were not significantly different between wood hydrolyzate and control media. Results showed that the culture does not consume xylose if sugars are fed prior to the complete consumption of initial sugars in the medium. The fed-batch experiments were repeated by feeding concentrated sugar solution (or wood hydrolyzate) after complete utilization of both glucose and xylose (at 72 h of fermentation). In such experiments, neither glucose nor xylose was consumed at all after feeding, and, hence, the final BD titer was not increased (data not shown here). These results indicate that fed-batch fermentation using *K. oxytoca* ATCC 8724 is beneficial to achieve higher BD titer only for the fermentation media containing a single sugar, but not with mixed sugars; glucose is more efficient than xylose in the single sugar medium. Therefore, appropriate bioprocessing strategies must be developed for lignocellulosic biomass to obtained separate streams of glucose and xylose, that is, cellulose must be separated from hemicellulose (or hemicellulose hydrolyzate) prior to its hydrolysis. This can be achieved using acid pretreatment, in which hemicellulose is hydrolyzed during pretreatment, and residual cellulose is separated from hemicellulose hydrolyzate, and then hydrolyzed using cellulase enzymes (Lloyd & Wyman, 2005).

Scale-up of 2,3-butanediol fermentation process

The shake-flask fermentations showed that 7 to 9% glucose media lead to maximum BD production using *K. oxytoca* ATCC8724. The fermentation process was scaled-up to a 5-L working volume bioreactor to allow better control over pH, aeration, agitation, dissolved oxygen level, and temperature to further improve BD production. Since pH and aeration are the major factors to be considered for efficient BD fermentation (Ji et al., 2009; Wong et al., 2014), multiple fermentations were performed to optimize pH and aeration, while maintaining a constant agitation of 200 rpm and temperature of 37°C.

The first fermentation was carried out by controlling the pH at 6.8 ± 0.2 using sterilized 10% sodium hydroxide (NaOH) and aeration with sterile air at 1 vvm. **Figure 8.7** shows that the fermentation at controlled pH led to a low production of BD even after 91 h and two fed-batch steps; final BD titer was 29.1 g/L, and yield was 0.17 g/g glucose consumed. However, glucose was efficiently utilized to produce other products (lactic acid, acetic acid, and ethanol) during this fermentation process; a total of 110.6 g/L products were produced with a total product yield of 0.63 g/g glucose consumed. Lactic acid (73.6 g/L) made up more than two-thirds of the total product, followed by BD (29.1 g/L), acetic acid (5.4 g/L), and ethanol (2.5 g/L). Interestingly, the lactic acid was of very high optical purity: 99.98% D-lactic acid. This shows that *K. oxytoca* ATCC 8724 is a promising lactic acid producing organism under a specific fermentation regime. Optically pure lactic acid is another important platform chemical used to produce a number of high-value products, including poly-lactic acid (Zhang & Vadlani, 2013). Further research is required to optimize the fermentation process to maximize lactic acid yield and productivity using this culture, which was outside the scope of this research.

A second fermentation was carried out without pH control and aeration, which resulted in very low glucose uptake and cell biomass growth. A small amount of BD (less than 20 g/L), lactic acid, and ethanol were produced (data not shown here). A third fermentation was carried out without pH control and with the two-stage aeration scheme which supplied 1vvm aeration for first 8 h of fermentation (until optical density of the fermentation broth reached >2.0) to facilitate maximum cell biomass yield, followed by 0.33 vvm aeration to facilitate BD production (Ji et al., 2009). **Figure 8.8** shows that the initial pH of the fermentation medium gradually decreased from 7.0 to 5.3 due to production of organic acids, such as lactic acid and acetic acid, and, interestingly, remained almost constant at 5.3 throughout the fermentation. During the first stage, a majority of glucose was utilized for cell growth and organic acid production; BD production started after 6 h of fermentation characterized by rapid glucose utilization. BD yield per gram glucose consumed remained constant over the entire fermentation; even after feeding. However, productivity significantly decreased due to slow glucose utilization, and thereby slow BD formation after glucose feeding was started. Additionally, BD and total product formation showed similar trends after the first feeding indicated that BD was the major product being formed during the later stages of fermentation. Cell biomass production (multiplication of bacterial cells) remained constant around 0.02 g/g until the final feeding in which cell biomass yield per gram glucose decreased to 0.016 g/g. A third feeding was performed at 121 h of fermentation, but little to no glucose uptake was observed (data not shown). Maximum BD titer achieved from this fermentation was 65.2 g/L.

A fourth fermentation was carried out with constant aeration and without pH control. **Figure 8.9** shows that BD yield significantly increased reaching 0.47 g/g glucose consumed; 94% of the theoretical maximum yield (Jansen et al., 1984). The higher BD yield was due to a lower formation of byproducts, and thereby leading to smaller difference between BD yield and total

product yield compared to the previous experiment (**Figure 8.8**). The cellular growth was rapid during the first 22 h of fermentation, and the total cell mass concentration remained constant thereafter. This lead to a significant decrease in biomass yield per gram glucose consumed in subsequent stages of fermentation. In addition, BD productivity significantly decreased in each subsequent feeding process because of the reduced substrate uptake rate. The BD productivity and yield decreased by 23% and 9%, respectively, from the second to the third feeding, during which BD titer increased by only 4%, indicating that the third feeding was not beneficial. Therefore, two feedings was optimum for this fermentation; two feedings produced a final BD titer of 79.4 g/L, yield of 0.47 g/g glucose consumed, and productivity of 0.79 $gL^{-1}h^{-1}$. The final BD titer and productivity for two feedings in 7-L bioreactor (**Figure 8.9**) was similar to the third feeding in the shake-flask fermentation (**Figure 8.5a**); however, BD yield in 7-L fermenter was significantly higher than the shake-flask fermentation.

Comparison of glucose and xylose media for BD fermentation in 7-L bioreactor

Previous fermentations performed using the 7-L bioreactor (mentioned earlier) determined that 1 vvm aeration without pH control at 37°C and 200 rpm were the optimal conditions for BD fermentation using a 7% (w/v) initial sugar concentration. Glucose and xylose media were separately evaluated and compared for BD fermentation in 7-L bioreactor at the optimized condition. **Figure 8.10** shows that both BD yield and productivity were significantly greater in glucose medium compared to xylose medium, but total product yields were not significantly different indicating that a higher amount of byproduct formation occurred using the xylose media. In xylose medium, acetoin was the major byproduct (5.1 g/L^{1}), followed by acetic acid (3.1 g/L) and a small amount of lactic acid and ethanol; whereas, lactic acid (4.5 g/L) was the major byproduct with small amount of acetoin, acetic acid, and ethanol in the glucose medium. Cell

biomass yield per gram of sugar consumed was significantly higher using the xylose medium compared to the glucose medium due to a longer fermentation time and a slower but continuous biomass growth. However, maximum specific growth rate (μ_{max}) was significantly greater using the glucose medium compared to the xylose medium; μ_{max} for glucose and xylose medium were 2.08 $h^{-1}$and 1.0 h^{-1}, respectively.

Use of 7-L bioreactor with a 5 L working volume resulted in higher BD yield, but lower productivity in both glucose and xylose medium at 7% initial sugar concentration as compared to shake-flask with 50 ml working volume. In glucose medium, BD yields were 0.47 g/g and 0.43 g/g glucose consumed, and productivities were 0.91 $gL^{-1}h^{-1}$ and 1.03 $gL^{-1}h^{-1}$ in bioreactor and shake-flask fermentation, respectively. Similarly, in xylose medium, BD yields were 0.43 g/g and 0.36 g/g xylose consumed, and productivities were 0.55 $gL^{-1}h^{-1}$ and 0.64 $gL^{-1}h^{-1}$ in bioreactor and shake-flask fermentation, respectively. This indicated that there is an opportunity to improve the fermentation process to achieve a higher BD productivity and yield at bioreactor level.

Conclusions

Glucose was the best medium for 2,3-butanediol (BD) fermentation using *Klebsiella oxytoca* ATCC 8724, followed by xylose, and the mixed sugar was the least efficient medium in terms of BD concentration, yield, and productivity. Biomass-derived sugars are as efficient as synthetic sugars at low (2.5%, w/v) total sugars concentration; however, higher total sugar concentration in biomass hydrolyzate resulted in a simultaneous increase in the concentration of inhibitory compounds, including acetic acid, and thereby hindering xylose utilization. The xylose utilization rate using detoxified biomass hydrolyzate with high total sugars (8%, w/v) was similar to the synthetic sugars solution, indicating that detoxification of biomass hydrolyzate is vital for BD fermentation. At the optimized fermentation conditions, high BD titer (91g/l) was obtained in

a fed-batch process using glucose medium; however, fed-batch with mixed sugars was not beneficial. Development of an appropriate biomass processing technology capable of separating the different sugar streams would be highly valuable for efficient BD fermentation.

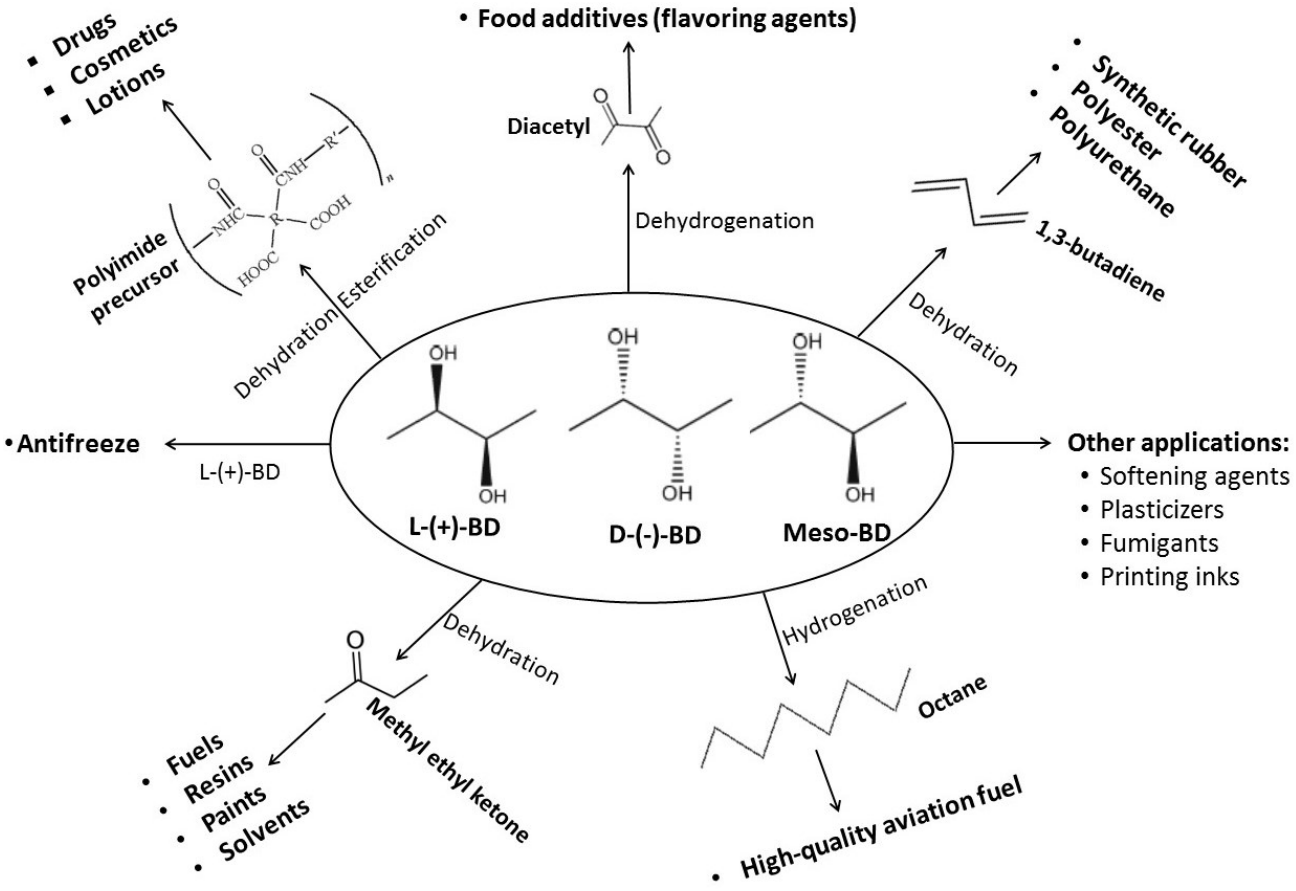


Figure 8.1 Some important derivatives of 2,3-butanediol, and their potential applications (Qi et al., 2014; Li et al., 2013; Ji et al., 2011; Celinska and Grajek, et al., 2009).

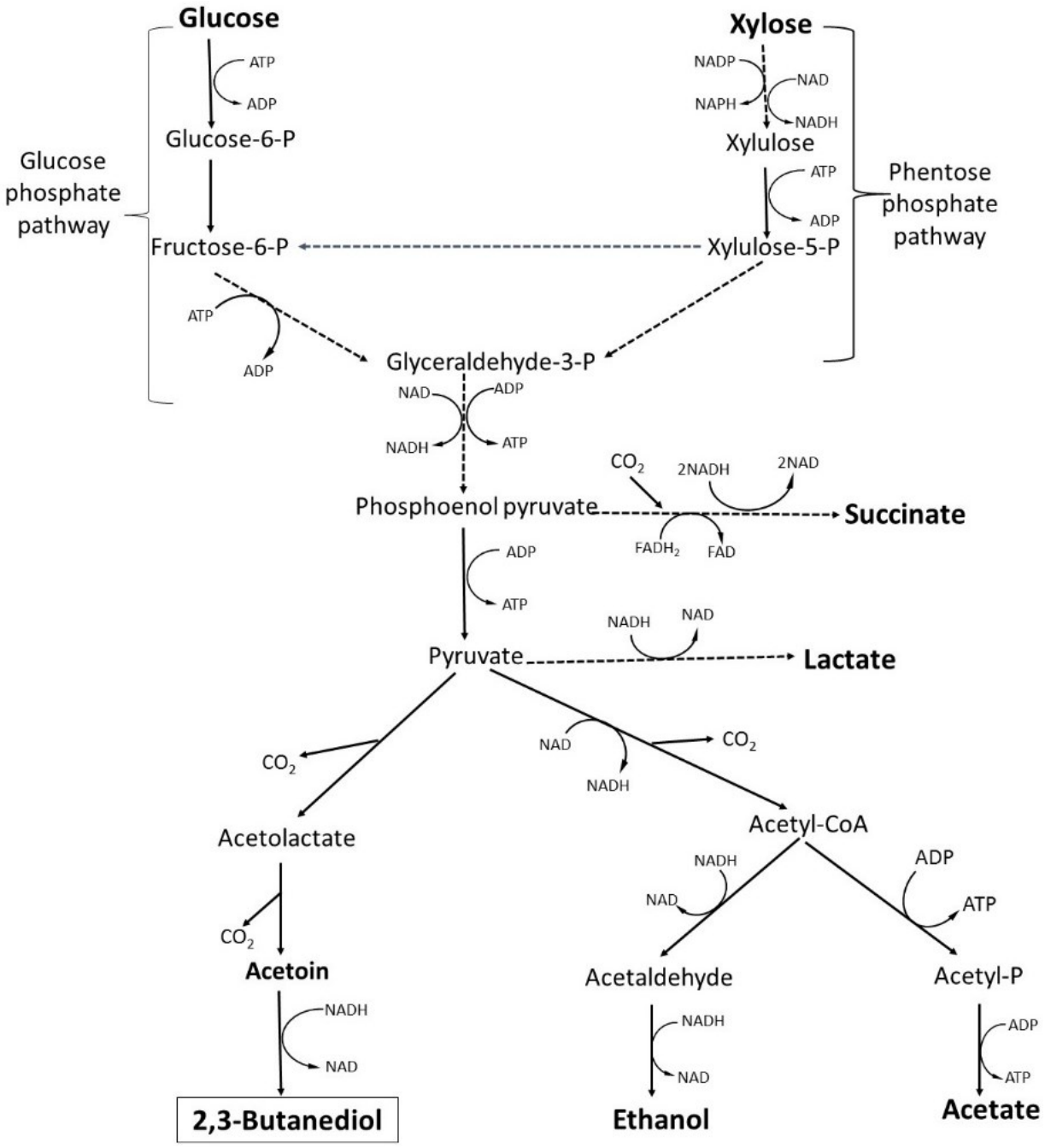


Figure 8.2 Different metabolic routes of glucose and xylose metabolism during 2,3-buanediol fermentation (Han et al., 2012; Celinska and Grajek et al., 2009; Jansen et al., 1984).

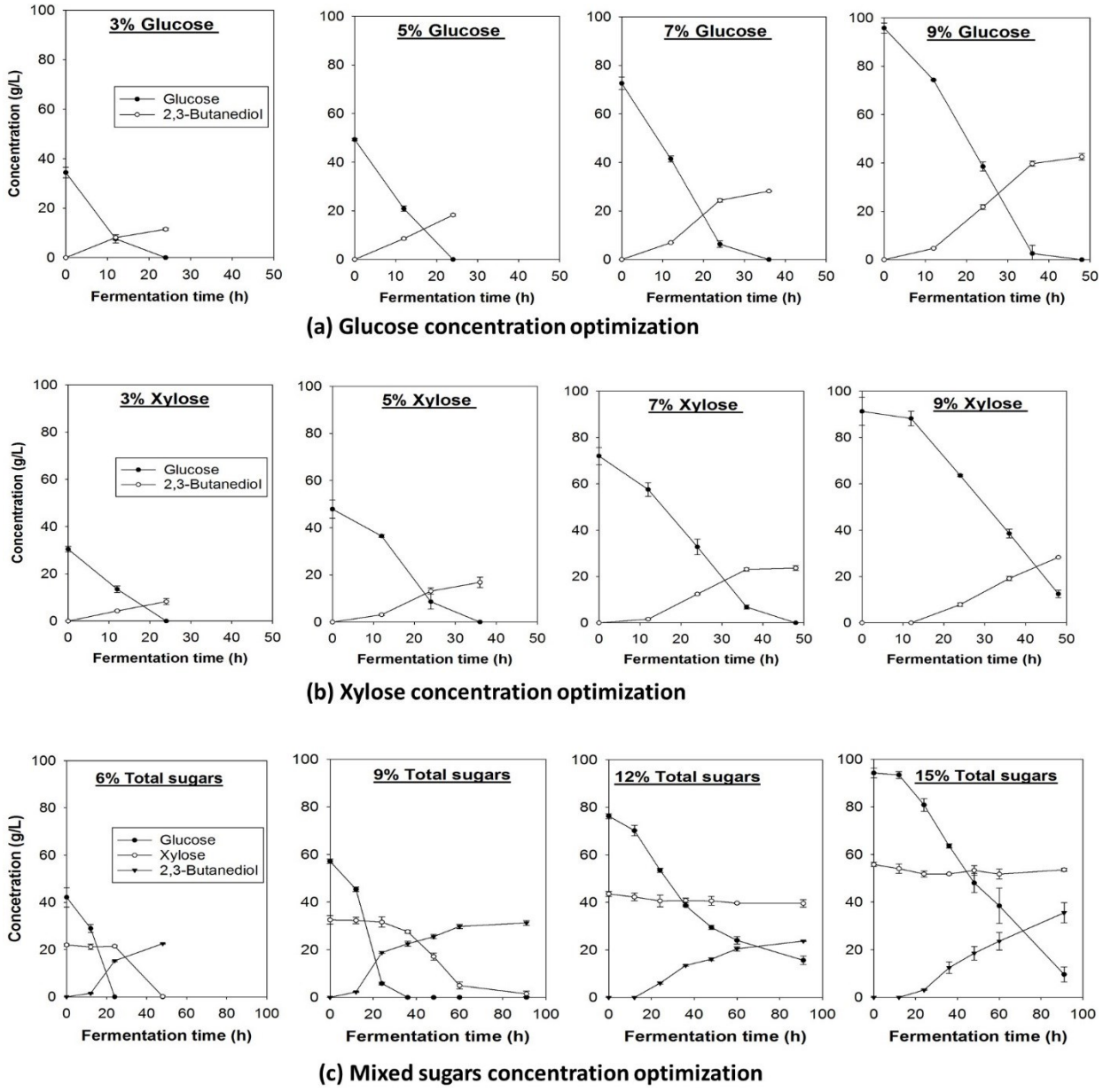


Figure 8.3 2,3-butanediol fermentation in shake-flasks with *Klebsiella oxytoca* ATCC 8724 using glucose, xylose and mixed sugars media with different initial sugars concentrations. Fermentation was carried out in 500-ml flask with 50 ml working volume at 37°C and 200 rpm. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

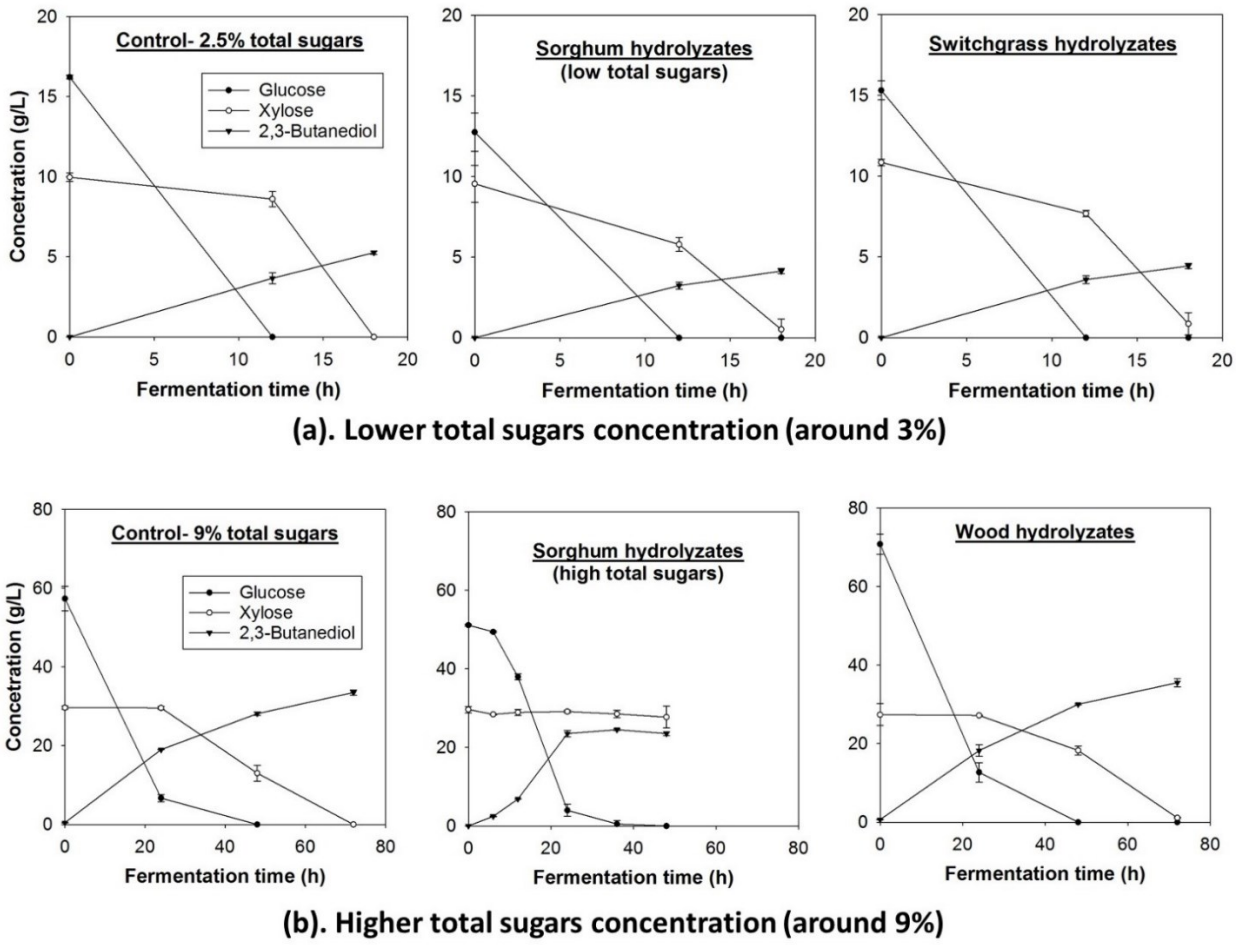
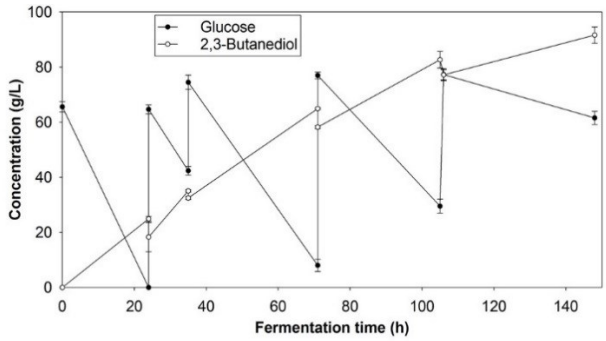
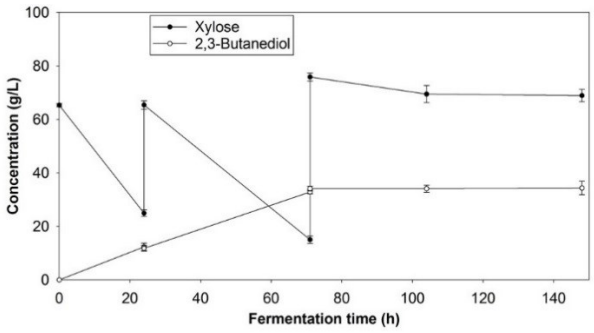


Figure 8.4 2,3-butanediol fermentation in shake-flasks with *Klebsiella oxytoca* ATCC 8724 using various media and different initial sugars concentrations. Control = Synthetic sugars solution containing glucose and xylose. Fermentation was carried out in 500-ml flask with 50 ml working volume at 37°C and 200 rpm. Data are average values of triplicate experiments, and error bars represent sample standard deviation.



Fermentation stage	Fermentation time (h)	Yield (g/g glucose consumed)	Productivity ($gl^{-1}h^{-1}$)
No fed-batch	24	0.38 ± 0.01	1.04 ± 0.04
1st fed-batch	35	0.40 ± 0.02	1.00 ± 0.01
2nd fed-batch	71	0.42 ± 0.01	0.91 ± 0.01
3rd fed-batch	105	0.41 ± 0.02	0.79 ± 0.03
4th fed-batch	148	0.41 ± 0.02	0.62 ± 0.02

(a). Glucose medium



Fermentation stage	Fermentation time (h)	Yield (g/g xylose consumed)	Productivity ($gL^{-1}h^{-1}$)
No fed-batch	24	0.31 ± 0.03	0.51 ± 0.06
1st fed-batch	71	0.36 ± 0.01	0.46 ± 0.01
2nd fed-batch	104	0.33 ± 0.02	0.31 ± 0.01

(b). Xylose medium

Figure 8.5 Fed-batch process for 2,3-butanediol fermentation in shake flask using *Klebsiella oxytoca* ATCC 8724 in glucose and xylose media. Fermentation was carried out in 500-ml flask with 50 ml working volume at 37°C and 200 rpm. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

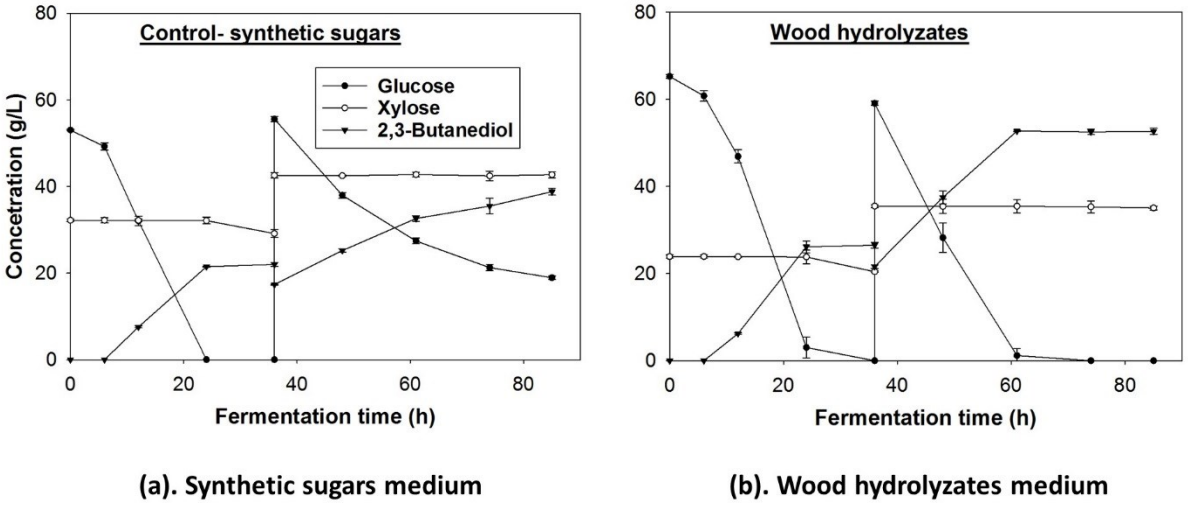


Figure 8.6 Fed-batch process for 2,3-butanediol fermentation in shake flask using *Klebsiella oxytoca* ATCC 8724 in biomass hydrolyzate and control media. Control = Synthetic sugars solution containing glucose and xylose. Fermentation was carried out in 500-ml flask with 50 ml working volume at 37°C and 200 rpm. Data are average values of triplicate experiments, and error bars represent sample standard deviation.

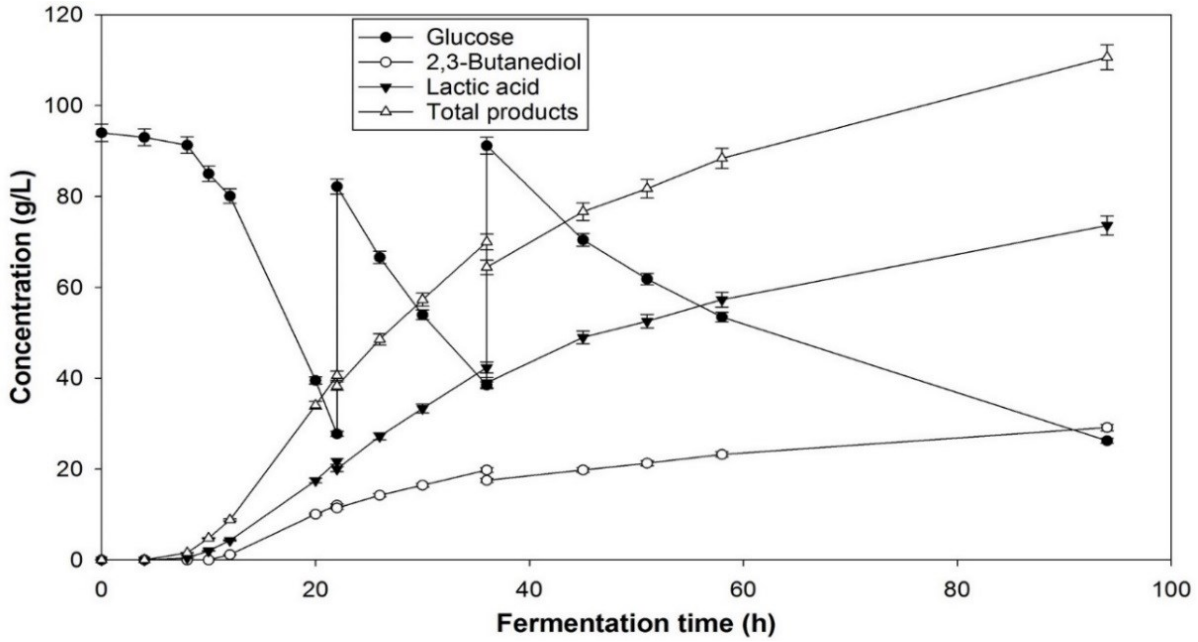
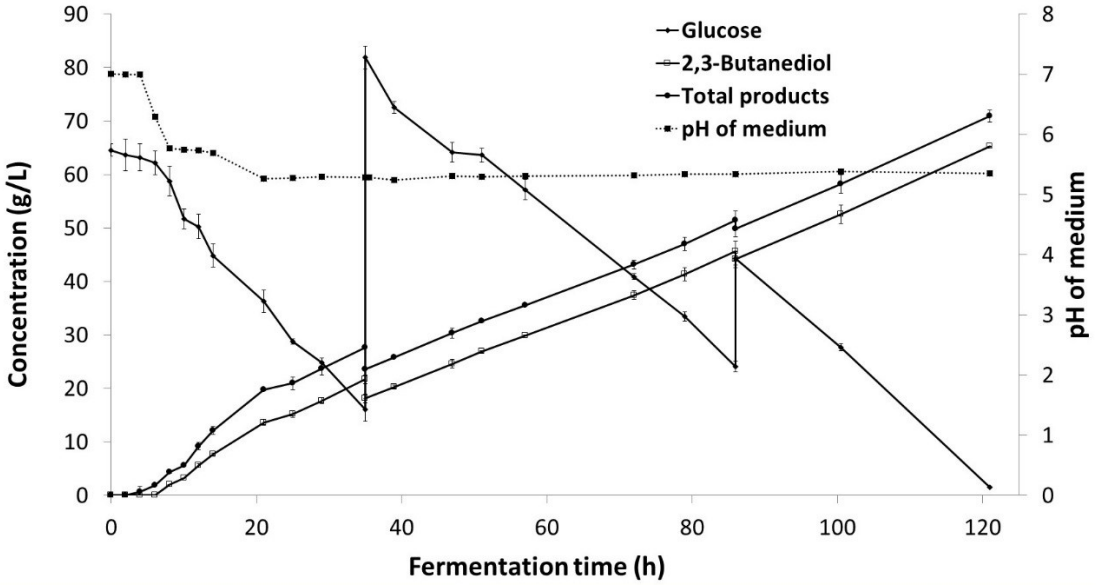
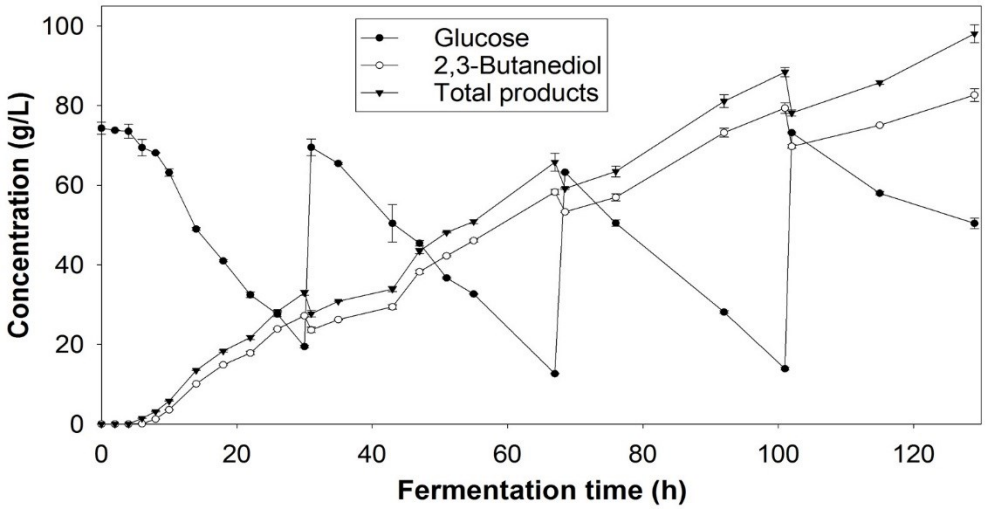


Figure 8.7 Fed-batch process for 2,3-butanediol fermentation using *Klebsiella oxytoca* ATCC 8724 in glucose medium with pH control at 6.8±0.2, and 1vvm aeration. Fermentation was carried out in 7-L bioreactor with 5 L working volume at 37°C and 200 rpm. Data are average values of duplicate experiments, and error bars represent sample standard deviation.



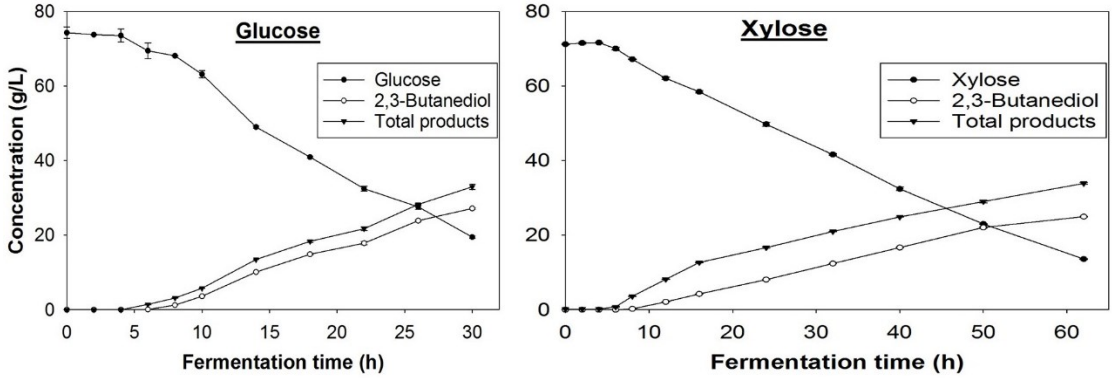
Fermentation stage	2,3-Butanediol yield (g/g sugar consumed)	Total product yield (g/g sugar consumed)	Biomass yield (g/g sugar consumed)	Productivity ($gL^{-1}h^{-1}$)
No fed-batch	0.43 ± 0.01	0.62 ± 0.03	0.020 ± 0.001	0.62 ± 0.02
1st fed-batch	0.42 ± 0.02	0.50 ± 0.02	0.021 ± 0.002	0.53 ± 0.02
2nd fed-batch	0.43 ± 0.03	0.49 ± 0.03	0.016 ± 0.001	0.54 ± 0.01

Figure 8.8 Fed-batch process for 2,3-butanediol fermentation using *Klebsiella oxytoca* ATCC 8724 in glucose medium without pH control, and 1vvm aeration for first 8 h, followed by 0.33 vvm for rest of fermentation process. Fermentation was carried out in 7-L bioreactor with 5 L working volume at 37°C and 200 rpm. Data are average values of duplicate experiments, and error bars represent sample standard deviation.



Fermentation stage	2,3-Butanediol yield (g/g sugar consumed)	Total product yield (g/g sugar consumed)	Biomass yield (g/g sugar consumed)	Productivity ($gL^{-1}h^{-1}$)
No fed-batch	0.47 ± 0.02	0.57 ± 0.03	0.026 ± 0.001	0.91 ± 0.01
1st fed-batch	0.49 ± 0.01	0.55 ± 0.02	0.011 ± 0.000	0.87 ± 0.01
2nd fed-batch	0.47 ± 0.00	0.52 ± 0.01	0.008 ± 0.000	0.79 ± 0.01
3rd bed-batch	0.43 ± 0.02	0.51 ± 0.03	0.007 ± 0.001	0.64 ± 0.01

Figure 8.9 Fed-batch process for 2,3-butanediol fermentation using *Klebsiella oxytoca* ATCC 8724 in glucose medium without pH control, and 1vvm aeration. Fermentation was carried out in 7-L bioreactor with 5 L working volume at 37°C and 200 rpm. Data are average values of duplicate experiments, and error bars represent sample standard deviation.



Yield / Productivity	Glucose media	Xylose media
2,3-Butanediol yield (g/g sugar consumed)	0.47 ± 0.02	0.43 ± 0.01
Total product yield (g/g sugar consumed)	0.57 ± 0.03	0.59 ± 0.01
Biomass yield (g/g sugar consumed)	0.026 ± 0.001	0.031 ± 0.001
2,3-Butanediol productivity ($gL^{-1}h^{-1}$)	0.91 ± 0.01	0.55 ± 0.01

Figure 8.10 2,3-butanediol fermentation using *Klebsiella oxytoca* ATCC 8724 in glucose and xylose media. Fermentation was carried out in 7-L bioreactor with 5 L working volume, 1 vvm aeration, 200 rpm, without pH control, and at 37°C. Data are average values of duplicate experiments, and error bars represent sample standard deviation.

Chapter 9 - Efficient production of 2,3-butaneidol from multiple sugars sources using *Bacillus licheniformis* DSM 8785

Abstract

2,3-butanediol (BD) is an advanced biofuel and promising platform chemical with enormous potential for conversion to industrial chemicals. Microbial production of BD has recently attracted global attention; however, most of the promising BD producing microbes reported so far are pathogenic (biosafety level 2) including *Klebsiella oxytoca,* which is not desirable for commercial bulk chemical production. In this study, a non-pathogenic (biosafety level 1) organism, *Bacillus licheniformis* DSM 8785, was evaluated for BD production from both synthetic and biomass-derived sugars. The fermentation conditions optimized in our earlier study for *Klebsiella oxytoca* ATCC 8724 were used for *B. licheniformis* as well for the comparative evaluation of this culture in different sugar media. The results showed that *B. licheniformis* utilized both glucose and xylose from synthetic sugars medium; however, poor fermentation performance resulted from using a medium containing a mixture of glucose and xylose leading to a reduced BD productivity. Glucose medium generated a significantly higher BD productivity (2.23 $gL^{-1}h^{-1}$) compared to the xylose medium (1.58 $gL^{-1}h^{-1}$). The mixed sugars medium with glucose and xylose in a 2:1 ratio had the lowest productivity (0.91 $gL^{-1}h^{-1}$). BD production using the sorghum stalk hydrolyzate with 4% total sugar was as efficient as synthetic sugars; limited BD production was achieved using hydrolysates containing higher sugar concentrations (8%, w/v, total sugars). Sorghum stalk hydrolyzates containing a higher total sugar content were challenging to utilize due to poor xylose consumption. The culture was unable to utilize xylose in detoxified wood hydrolyzate with an 8% (w/v) total sugar concentration as well, which indicated that the biomass hydrolyzate with higher total sugars is not an appropriate medium for efficient BD fermentation

using *B. licheniformis*, unless an appropriate biomass processing technology is developed to obtain separate cellulose and hemicellulose streams. The highest BD titer obtained from fed-batch fermentation using glucose medium in 7-L bioreactor was 11.5% (w/v) after four feedings, but the productivity suffered (0.48 $gL^{-1}h^{-1}$); two glucose feedings was the optimum fed-batch process in which final BD titer, yield and productivity were 8% (w/v), 0.49 g/g glucose, and 0.94 $gL^{-1}h^{-1}$, respectively. As compared to *K. oxytoca*, the *B. licheniformis* culture had 2.2 and 2.5 times greater BD productivity using glucose and xylose medium, respectively, but was less efficient in biomass hydrolyzate containing 8% or total sugars. This study determined that efficient BD production is possible using a non-pathogenic organism from single sugar medium; a discovery for potential future BD development.

Keywords: 2,3-butanediol, *Bacillus licheniformis*, fed-batch fermentation, biomass hydrolyzate

Introduction

2,3-butanediol (BD) is an important platform chemical with wide industrial applications as chemical feedstocks and liquid fuels, as shown in **Figure 8.1** (Celińska & Grajek, 2009; Ji et al., 2011; Li et al., 2013; Qi et al., 2014). The microbial BD production started more than a century ago; however, most of the robust BD producing organisms reported so far, including *Klebsiella oxytoca, Klebsiella pneumoniae, Enterobacter aerogenes,* and *Serratia marcescents*, belong to biosafety level 2 (pathogenic) (Jurchescu et al., 2013). A number of nonpathogenic strains, including *Paenibacillus polymyxa*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, also produce BD; however, their BD fermentation efficiency is very low compared to biosafety level 2 organisms (Ji et al., 2011). Recently, some strains of *Bacillus licheniformis* were reported as promising bacterial strains for BD production (Jurchescu et al.,

2013; Li et al., 2014). In addition, most of the research on microbial production of BD so far has used food-based materials, including glucose and sucrose, as feedstocks, which possesses a threat to food security (Ji et al., 2011). Lignocellulosic biomass is a sustainable feedstock for biofuels and biochemicals production using conversion routes as shown in **Figure 1.3.** Studies on BD fermentation from biomass derived sugars are limited. In addition, high BD titer is desirable in fermentation broth to reduce downstream processing cost but batch fermentation is limited due to substrate inhibition at the high initial sugar concentrations needed to achieve high BD titer. Fed-batch fermentation is an appropriate approach to meet high titer without the limitations of substrate inhibition. Thus, the objective of this study was to evaluate the use of *Bacillus licheniformis* DSM 8785, a biosafety level 1 microorganism, to produce BD using different substrates, including synthetic sugars and biomass-derived sugars over various concentrations, and use fed-batch fermentation to achieve high BD titer. BD fermentation efficiencies of this culture was compared with that of *Klebsiella oxytoca* ATCC 8724.

Materials and methods

Microorganisms

The *Bacillus licheniformis* DMS8785 culture procured from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, was used in this experiment. The culture was revived in nutrient broth medium and several stock culture tubes were prepared comprising 0.5 ml each revived culture and 30% (v/v) sterilized glycerol. The stock culture were stored at -80°C until used for inoculum preparation.

Preparation of biomass hydrolyzate

Sorghum stalk hydrolyzate and wood (poplar) hydrolyzate were prepared using the protocol explained in Chapter 8 under “Preparation of biomass hydrolyzate” sub-section.

Inoculum preparation

A study on the growth curve of the bacteria, *Bacillus licheniformis*, was first performed. A 0.3 ml stock culture was added to 100 ml sterilized (at 121°C for 15 min) nutrient broth medium in 1000-ml flask and incubated in temperature-controlled shaker (Innova 4300, New Brunswick Scientific, NJ, USA) at 30°C and 200 rpm. Around 0.3 ml sample were taken every 2 h to measure optical density at 600 nm until 30 h. The measured optical density was plotted against fermentation time to find the optimum inoculum preparation time; that is, half way between beginning and end of the exponential growth phase. The results showed (see Result and discussion section) that 6 to 7 h was the optimum incubation time for inoculum preparation in nutrient broth medium. Therefore, inoculum was prepared by inoculating 0.3 ml stock culture in 100 ml sterilized nutrient broth medium in 1000-ml flask, and incubated at 30°C and 200 rpm for 7 h. The inoculum were directly added in fermentation medium at the rate of 5% (v/v) of total fermentation medium volume.

Fermentation

Shake-flask

Shake-flask fermentations were carried out in 500-ml flasks with a 50 ml working volume. The media for shake-flask fermentation were prepared using specific concentrations of synthetic sugars (glucose, xylose or mixed sugars) or biomass hydrolysates with supplemented nutrients. The supplementary nutrients for *Bacillus licheniformis* (per liter): 5 g yeast extract, 5 g bacto tryptone, 7 g dipotassium phosphate, 5.5 g monopotassium phosphate, 1 g ammonium sulfate, 0.25 g magnesium sulfate heptahydrate, 0.12 g sodium molybdate dihydrate, 0.021 g calcium chloride dihydrate, 0.029 g cobalt nitrate hexahydrate, and 0.039 g ferrous ammonium sulfate hexahydrate. Trace elements (per liter): 0.002 g nicotinic acid, 0.172 mg sodium selenite, 0.037 mg Nickel (II)

chloride hexahydrate, 0.005 g manganese chloride tetrahydrate, 0.001 g Boric acid, 0.172 mg aluminum potassium sulfate dodecohydrate, 0.01 mg Copper(II) chloride dihydrate, and 0.00554 g disodium ethylenediaminetetraacetate (Jurchescu et al., 2013). The sugar solutions and biomass hydrolyzates were autoclaved at 121°C for 15 min in the 500-ml flasks. Once cool, a 10x concentrated nutrient solution was filter sterilized (0.22 μm, Millipore) and added aseptically (10%, v/v) to each flask. The freshly prepared inoculum were added to each flask and incubated in temperature-controlled shaker at 30°C and 200 rpm. 0.5 ml sub-samples were obtained over the fermentation time to measure the products and residual sugars. Filter sterilized concentrated sugar solutions were used as feeding medium for fed-batch fermentation.

Bioreactor scale-up

The fermentation medium evaluated in the batch-flask study were scaled up to a 7-L bioreactor (Bioflo 110, New Brunswick Scientific Inc., Enfield, Connecticut, USA) with a 5 L working volume. The fermenter with 3,750 ml sugar solution containing sugars in the amount sufficient to make the desired sugars concentration in final 5 L fermentation medium were autoclaved at 121°C for 30 min. Three autoclavable sample bottles were fitted with the fermenter before autoclave: one bottle contained 10% (w/v) sodium hydroxide solution, other contained antifoam and third was used to pump sterilized nutrient solution and inoculum. One liter nutrient solution with required nutrients in the amount sufficient for 5 L fermentation medium was filter sterilized (Millipore, 0.22 μm) and pumped into the sterilized fermenter. The fermentation process was optimized for pH and aeration using 200 rpm agitation and 30°C temperature to maximize BD concentration, yield per gram sugar and productivity. Around 10 ml samples were drawn periodically to measure biomass growth, products formation and sugar consumption. Filter

sterilized concentrated sugars solution were added at desired time to carry out the fed-batch fermentation process.

Analytical and statistical methods

The analytical methods to analyze monosaccharides (glucose, xylose and arabinose), and fermentation products/byproducts used in "Analytical methods" sub-section in Chapter 8 were followed in this study. Similarly, statistical methods used in Chapter 8 was followed in this study.

Results and discussion

Growth curve study for the optimization of inoculum preparation time

Figure 9.1 shows that the *Bacillus licheniformis* DSM 8785 in 7% (w/v) glucose medium had no growth period (lag phase) for first 4 h, slow growth period (initiation of log phase) from 4 to 6 h, exponential growth period (log phase) from 6 to 14 h, second slow growth period (initiation of stationary phase) from 14 to 16 h, and finally no growth period after 16 hour (stationary phase). We observed a similar growth curve in nutrient broth medium, but log phase started at 4 h (data not shown here). These results indicate that the optimum inoculum preparation time is 6 to 7 h for nutrient broth medium, and 8 to 10 h for 7% (w/v) glucose medium.

Optimization of initial pH of fermentation medium

Our earlier study using *Klebsiella oxytoca* ATCC 8724 culture (Chapter 8) showed that the initial pH of fermentation medium dropped from around 7 to 5.3, and remained fairly constant throughout the fed-batch experiment; 2,3-butanediol (BD) production initiated only after the pH dropped below 6 (**Figure 8.8**). Different researchers reported different optimum pH for BD fermentation depending upon the microorganisms and substrate used; however, the majority of them reported that a pH 5 to 6 is the optimum for efficient BD production (Celińska & Grajek, 2009). On the other hand, Jurchescu et al. (2013) reported that an initial neutral pH and no pH

control during fermentation resulted in an efficient BD fermentation using *Bacillus licheniformis* DSM 8785, which was consistent with our earlier work using *Klebsiella oxytoca* ATCC 8724 (Chapter 8). We, therefore, evaluated the glucose medium with two different initial pH values (5.5 and 7.0), and with no pH adjustment during the fermentation process.

Figure 9.2 shows that the medium with an initial pH of 7.0 resulted in a significantly higher glucose uptake rate leading to a higher titer and productivity compared to an initial pH of 5.5. Faster cell growth and organic acids (acetic acid and lactic acid) formation was observed during the first 6 h of fermentation at a pH of 7.0 compared to a pH of 5.0. This led to lower BD yield and higher total products (BD, acetic acid, lactic acids, acetoin, and ethanol) yield per gram sugar consumed in the medium with an initial pH of 7.0. These results indicate that an initial pH of 7.0 results in greater cell growth and subsequently in improved BD production.

2,3-Butanediol fermentation in shake-flask using different sugar sources

The sugar concentration optimized for *Klebsiella oxytoca* ATCC 8724 in our earlier study (Chapter 8) was used for *Bacillus licheniformis* DSM 8785 as well; the optimum sugar concentrations were 7% (w/v) for the single sugar medium and 9% for the mixed sugar medium. Therefore, a 7-8 % single sugar (glucose/xylose) medium, and a 9-10% for mixed sugars medium comprising glucose and xylose in a 2:1 ratio were used in this study. In addition, two types of sorghum stalk hydrolyzates with 4% and 8% total sugar concentrations were evaluated in this study.

Figure 9.3 shows that the glucose uptake rate was almost 50% greater compared to the xylose uptake rate leading to significantly higher BD productivity in glucose medium compared to xylose. Cell mass growth and byproduct (acetic acid, lactic acid, ethanol, and acetoin) formation were also significantly higher in the glucose medium compared to xylose. This resulted in a lower

final BD titer and BD yield per gram sugar consumed in glucose medium; the opposite was observed for total products (including BD) yield. The mixed sugar medium was significantly less efficient for BD production compared to the single sugar media in terms of both yield and productivity. The major problem in mixed sugar medium was the lack of simultaneous utilization of glucose and xylose because of the carbon catabolic repression of glucose for xylose utilization (Ji et al., 2011). These results indicated that the mixed sugar medium is not appropriate for efficient BD fermentation using *B. licheniformis*. Using the biomass hydrolyzate with 4% total sugars, both glucose and xylose sugars were utilized for BD fermentation, but at reduced rate compared to the pure sugar mixtures; however, the xylose was not utilized significantly in biomass hydrolyzate with a higher total sugar concentration of 8% (w/v). One possible explanation for the different behavior in sugar utilization between these two biomass hydrolyzates could be the higher concentration of acetic acid in the hydrolysates with 8% total sugar (around 3 g/L) compared to hydrolysates with 4% total sugar (less than 0.5 g/L). Acetic acid is produced during hydrolysis of biomass hemicellulose, and it is an inhibitory compound for microbial fermentation (Hu & Ragauskas, 2012). Detoxification of biomass hydrolyzate is essential to remove these inhibitory compounds. For the hydrolysates containing 8% total sugar, BD productivity was calculated over 24 h of fermentation because almost all glucose was consumed in 24 h of fermentation, but xylose was not significantly consumed in this medium. On the other hand, BD productivity was calculated over 18 h of fermentation in the hydrolysates containing 4% total sugars hydrolyzate, during which both glucose and xylose were completely utilized. This led to a significantly lower BD productivity in hydrolysates containing 4% total sugars because of the slow xylose utilization.

2,3-Butanediol fermentation in a 7-L bioreactor using different sugar sources

The fermentation process performed for shake-flask fermentation with 50 ml working volume was scaled up to a 7-L bioreactor with a 5 L working volume. In this study, two types of biomass hydrolyzates and single sugar media containing either synthetic glucose or xylose were evaluated and compared. Sorghum biomass hydrolyzate were prepared from alkali pretreated sorghum stalks using 12% (w/v) solid loading to achieve a total sugar concentration of 8% (w/v). Concentrated poplar wood biomass hydrolyzate were obtained from Technology Holding LLC (Salt Lake City, Utah, USA) and prepared by diluting to a total sugar concentration of 8% (w/v).

In this study, fermentation conditions optimized for *K. oxytoca* ATCC 8724 were used for *B. licheniformis* DSM 8724. **Figure 9.4** shows that the glucose uptake rate is significantly higher than the xylose uptake rate; this resulted in a BD productivity in glucose medium that was almost double the productivity using the xylose medium. Surprisingly, product yield per gram of sugar consumed in glucose medium was less than for xylose medium because of higher cell biomass formation. The single sugar BD production efficiencies were similar to results from the 7-L bioreactor and shake-flask fermentations; however, final BD concentration, productivity and yield in 7-L bioreactor were significantly lower than the shake-flask fermentations for both glucose and xylose media. These results indicate that BD fermentation conditions, including pH, aeration, and substrate concentration, for *B. licheniformis* DSM 8785 can be further improved to increase BD concentration, productivity and yield in 7-L bioreactor, at least equal to the shake-flask production efficiency.

For biomass hydrolyzate media, xylose utilization was minimal in both the sorghum and wood hydrolyzate. The 7–L bioreactor results for the sorghum hydrolyzates were similar to the shake-flask fermentations. The inability of the culture to utilize xylose in detoxified wood

hydrolyzate medium showed that our assumptions for sorghum hydrolyzate medium based on shake-flask fermentation results were not completely valid. Shown in **Figure 9.3**, xylose was completely utilized in the sorghum hydrolysates containing 4% total sugars with a low acetic acid content (less than 0.5 g/L); this was not true for the 8% total sugars hydrolysate with a high acetic acid content (around 3 g/l). Therefore, we assumed that acetic acid was the major inhibitory compound for inability of the culture to utilize xylose. For the wood hydrolysates, the opposite was observed; the culture did not utilize xylose from hydrolyzate containing low acetic acid content (less than 0.5 g/L). The supplier claimed that they detoxified the wood hydrolyzate to remove potential inhibitory compounds. Due to this, other inhibitory compounds may be present including but not limited to hydroxymethylfurfural (HMF), furfural, acetic acid and phenolics. Alternatively, *B. licheniformis* DSM 8785 utilized xylose from biomass hydrolyzate if glucose was depleted from the medium before the bacteria reached stationary phase of its growth cycle (14 h or less), but did not utilize xylose if the glucose was depleted from the medium after the bacterial reached its stationary phase (after 16 h); this was not true for the medium containing synthetic sugars. These results indicate that biomass hydrolysates containing a mixture of glucose and xylose in high concentrations is not an appropriate medium for efficient BD fermentation using *B. licheniformis* DSM 8785, despite the high production efficiency observed from BD fermentation with single sugar media. Therefore, appropriate biomass processing technology should be developed to separate cellulose and hemicellulose from biomass to generate separate streams of glucose and xylose. Further research is required to evaluate the BD fermentation efficiencies of this culture in separate cellulose and hemicellulose streams from biomass sources.

Fed-batch fermentation in 7-L bioreactor using glucose medium

Fed-batch fermentation process was carried out in 7-L bioreactor with a 5 L working volume using glucose medium for *B. licheniformis* DSM 8785 culture to produce a high titer of BD for efficient downstream processing. **Figure 9.5** shows that the rate of glucose uptake was highest during the first phase of the fermentation process (before glucose feeding), and gradually decreased in subsequent feedings leading to a gradual decrease in BD productivity. The final BD titer achieved was 11.5% (w/v) after the fourth feeding with a BD yield of 0.46 g/g glucose consumed; productivity was reduced from 1.83 $gL^{-1}h^{-1}$ to 0.48 $gL^{-1}h^{-1}$. Here, we need to tradeoff between BD titer, yield and productivity to find the optimum fermentation approach. Based on these data, BD fed-batch fermentation with two feedings is promising since it generates a BD titer of 8% (w/v), BD yield 0.49 (98% of theoretical maximum value), and BD productivity 0.94 $gL^{-1}h^{-1}$. There is still opportunity for further optimization of the fermentation parameters, including the initial glucose concentration, pH, and aeration, for more efficient BD fermentation, at least equal to that of shake flask fermentation for this culture. This indicates that *B. licheniformis* DSM 8785 is an efficient bacteria for BD production from single sugar medium; further research is needed to improve the fermentation performance using mixed and biomass-derived sugars.

Comparison of B. licheniformis DSM 8785 and K. oxytoca ATCC 8724

K. oxytoca ATCC 8724 is one of the most robust BD fermenting bacteria but is classified as a biosafety level 2 microorganism, while *B. licheniformis* DSM 8785 is a nonpathogenic BD fermenting bacteria. The two bacterial cultures were evaluated using different fermentation media with the same initial sugar concentrations and agitation in shake-flask fermentation. In addition, these cultures were compared using fed-batch fermentation in glucose medium at same

fermentation conditions, including, pH, aeration, agitation and initial glucose concentration in 7-L bioreactor with a 5 L working volume.

Table 9.1 shows that *B. licheniformis* had significantly higher substrate uptake rate and thereby higher BD productivity in all media compared to *K. oxytoca*, except sorghum hydrolyzate with 8% (w/v) total sugars. No specific trend was observed in BD yield per gram sugar consumed between these two cultures. That is, a different media was preferred by each type of culture; hence, different BD fermentation performance was observed. Despite their differences in BD fermentation efficiency, some similarities were observed between these two cultures: (1) glucose was the best medium for efficient BD fermentation followed by xylose and mixed sugars; (2) lack of simultaneous utilization of glucose and xylose in the mixed sugars medium due to the carbon catabolic repression of glucose for xylose utilization was the main obstacle for reduced efficiency in the mixed sugar fermentation; and (3) xylose from non-detoxified sorghum stalk hydrolyzate was utilized if the total sugar concentration was 4% (w/v) or less; this was not true for sorghum stalk hydrolyzate with a total sugar concentration 8% (w/v) or more. One significant difference between these two cultures was utilization of xylose in detoxified wood hydrolyzate containing 8% total sugars. These results show that removal of acetic acid and other inhibitory compounds from biomass hydrolyzate resulted in improved fermentation performance comparable to that of synthetic sugars for *K. oxytoca*, but this was not true using *B. licheniformis*. Therefore, development of appropriate biomass technology to fractionate cellulose and hemicellulose into different sugar-rich streams is necessary for efficient BD fermentation using *B. licheniformis*; such approach would also improve BD fermentation efficiency while using *K. oxytoca* culture.

The fed-batch BD fermentation in glucose medium was more efficient using *B. licheniformis* culture (**Figure 9.5**) compared to *K. oxytoca* culture (**Figure 8.9**). Final BD titer was

11.5% and 8.2% (w/v) in *B. licheniformis* and *K. oxytoca*, respectively. Two feedings was optimum for both cultures based on BD concentration, yield and productivity, in which BD titer was equal (around 8%, w/v) in both cultures, but BD productivities and yields for *B. licheniformis* and *K. oxytoca* were 0.94 $gL^{-1}h^{-1}$ and 0.79 $gL^{-1}h^{-1}$, and 0.49 g/g and 0.47 g/g sugar consumed, respectively. The fed-batch fermentation results also showed that *B. licheniformis* is a more efficient producer of BD compared to *K. oxytoca* for BD fermentation while using single sugar media.

Conclusions

2,3-butanediol (BD) productivity using *Bacillus licheniformis* DSM 8785 in glucose, xylose and mixed sugars media were, 2.23 $gL^{-1}h^{-1}$, 1.58 $gL^{-1}h^{-1}$, and 0.91 $gL^{-1}h^{-1}$, respectively. The culture can utilize both glucose and xylose in biomass-derived sugars with a total sugar concentration of 4% (w/v); poor performance was observed with hydrolysates containing a total sugar concentration of 8% (w/v) or higher. The highest BD titer obtained from fed-batch fermentation using glucose medium in 7-L bioreactor was 11.5% (w/v); however, substrate uptake rate and BD productivity reduced with each subsequent feeding cycle. The BD productivity using *B. licheniformis* was 2.2 and 2.5 times greater than *K. oxytoca* in the glucose and xylose medium, respectively. Results from this study show that efficient BD production using a non-pathogenic organism and a single sugar medium is promising; however, an appropriate biomass processing technology is needed to separate cellulose and hemicellulose streams for efficient BD fermentation.

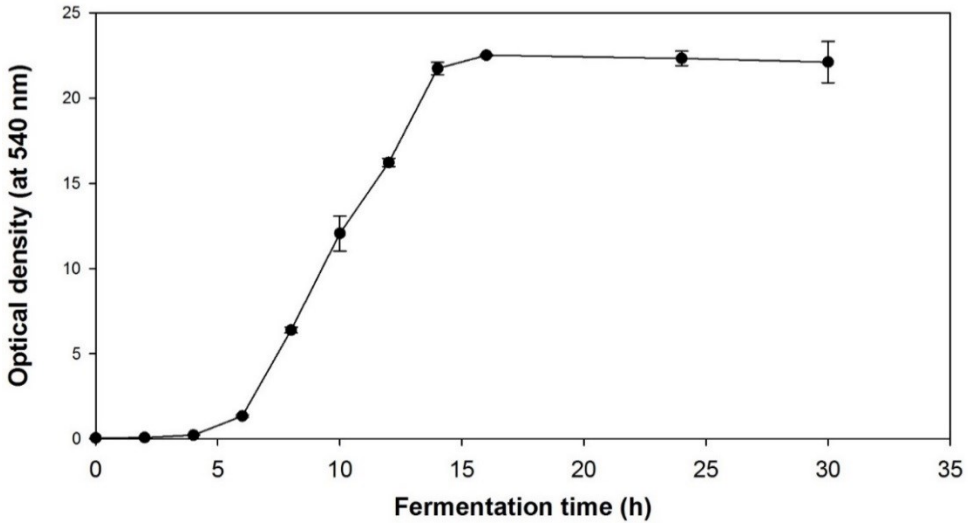
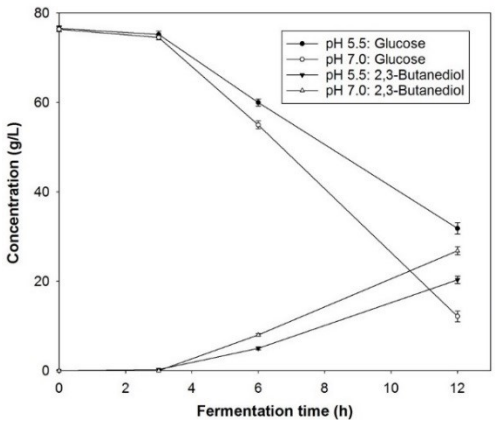
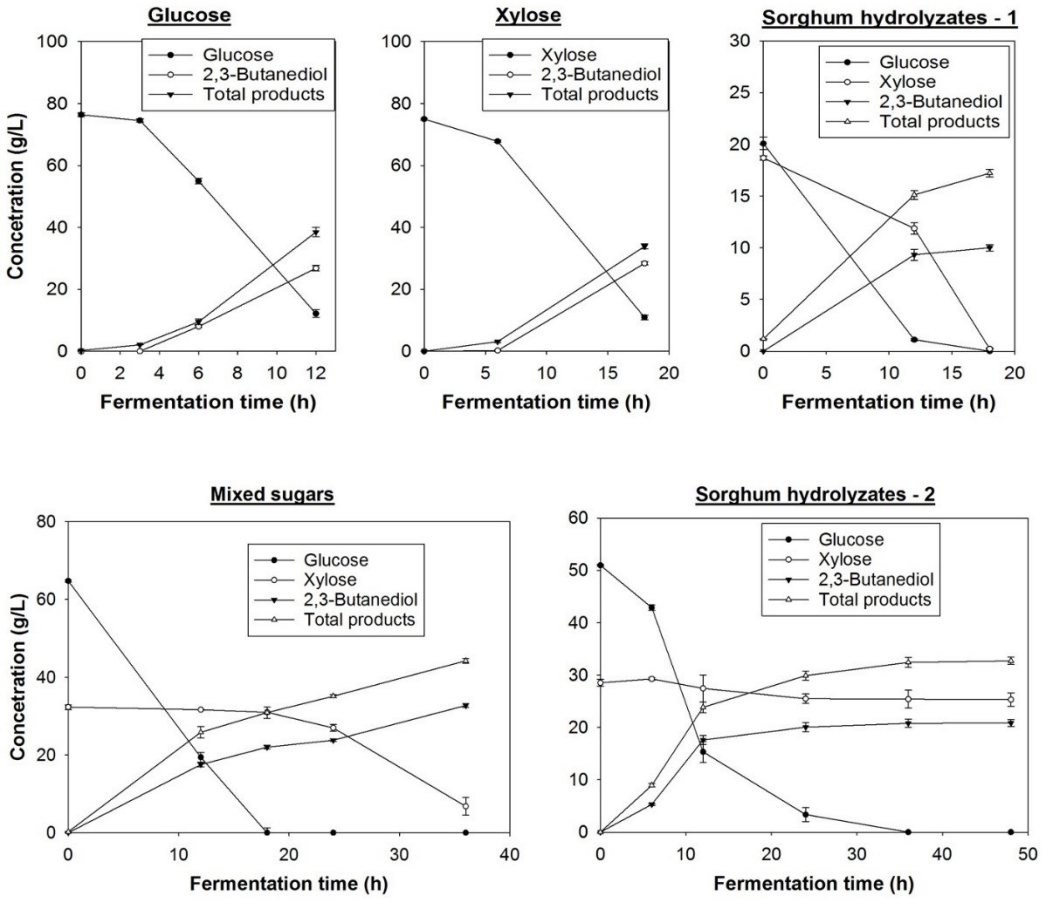


Figure 9.1 Growth curve of *Bacillus licheniformis* DSM 8785 in 70 g/L initial glucose medium with supplement nutrients in shake-flask (50 ml working volume in 500-ml flask) at 30°C and 200 rpm. Data are average values of triplicate experiments, and error bars represent sample standard deviation.



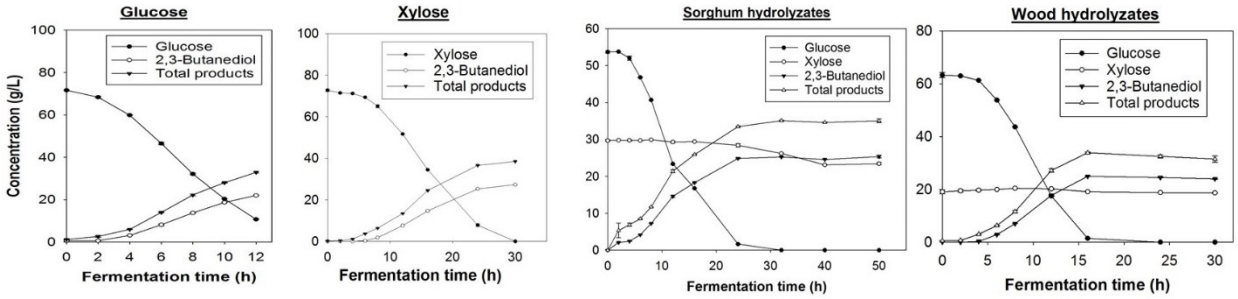
Product yield	Initial pH of medium	
	5.5	7.0
Final 2,3-butanediol concertation (g/L)	20.3 ± 0.3	26.8 ± 0.3
Final total product concertation (g/L)	25.3 ± 0.4	38.5 ± 0.3
2,3-butanediol yield (g/g glucose consumed)	0.45 ± 0.01	0.42 ± 0.1
Total product yield (g/g glucose consumed)	0.56 ± 0.01	0.60 ± 0.02
Productivity ($gL^{-1}h^{-1}$)	1.7 ± 0.03	2.2 ± 0.03

Figure 9.2 2,3-butanediol fermentation using *Bacillus licheniformis* DSM 8785 in glucose medium with different initial pH. Fermentation was carried out in shake flask (50 ml working volume in 1000-ml flask) at 30°C and 200 rpm. Data are average values of triplicate experiments, and error bars represent sample standard deviation.



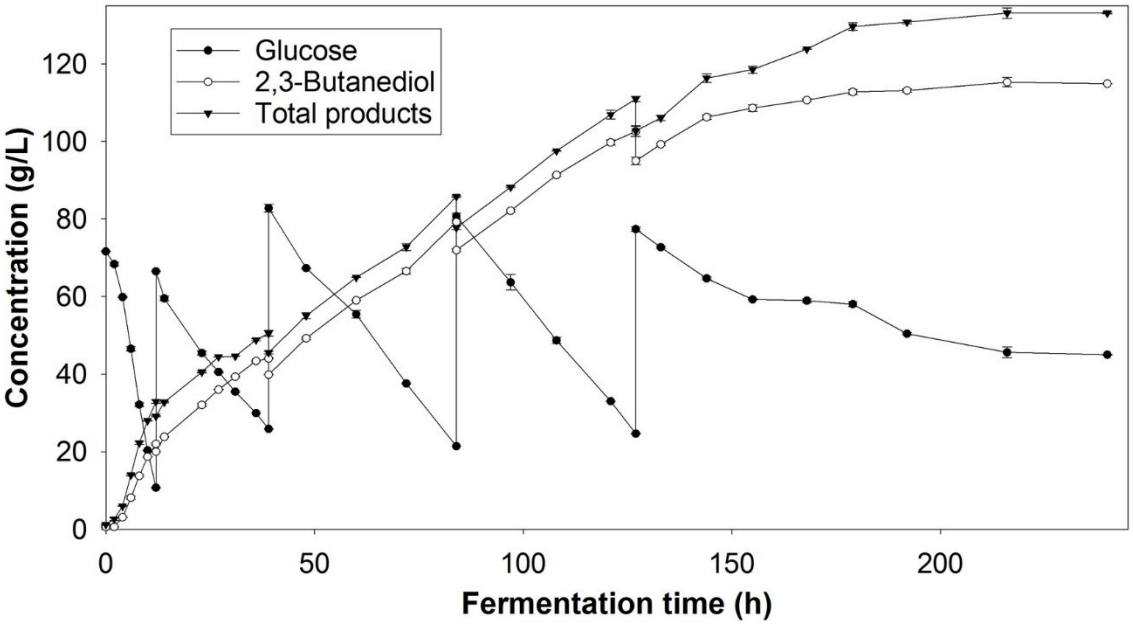
Media	2,3-Butanediol yield (g/g sugar consumed)	Total product yield (g/g glucose consumed)	2,3-Butanediol productivity ($gL^{-1}h^{-1}$)
Glucose	0.42 ± 0.00	0.60 ± 0.00	2.23 ± 0.03
Xylose	0.44 ± 0.01	0.53 ± 0.01	1.58 ± 0.03
Mixed sugar	0.32 ± 0.01	0.41 ± 0.02	0.91 ± 0.01
Sorghum -1	0.26 ± 0.00	0.42 ± 0.00	0.56 ± 0.02
Sorghum -2	0.41 ± 0.01	0.54 ± 0.01	0.82 ± 0.04

Figure 9.3 2,3-butanediol fermentation in shake-flasks using *Bacillus licheniformis* DSM 8785 in various media with different initial sugars concentration. Fermentation was carried out at 30°C and 200 rpm with 50 ml working volume in 1000-ml flask. Data are average values of triplicate experiments, and error bars represent sample standard deviation.



Sugar media	2,3-Butanediol yield (g/g sugar consumed)	Total product yield (g/g sugar consumed)	Biomass yield (g/g sugar consumed)	Productivity (gL-1h-1)
Glucose	0.36 ± 0.00	0.54 ± 0.01	0.075 ± 0.001	1.83 ± 0.02
Xylose	0.39 ± 0.00	0.56 ± 0.01	0.072 ± 0.001	1.05 ± 0.01
Sorghum hydrolyzates	0.45 ± 0.00	0.61 ± 0.01	0.071 ± 0.002	1.04 ± 0.01
Wood hydrolyzates	0.40 ± 0.01	0.55 ± 0.02	0.057 ± 0.001	1.56 ± 0.03

Figure 9.4 2,3-butanediol fermentation in batch bioreactor using *Bacillus licheniformis* DSM 8785 in various media with different initial sugars concentration. Fermentation was carried out in 7-L bioreactor with 5 L working volume, 1vvm aeration, 200 rpm, without pH control, and at 30°C. Data are average values of duplicate experiments, and error bars represent sample standard deviation.



Fermentation stage	2,3-Butanediol yield (g/g sugar consumed)	Total product yield (g/g sugar consumed)	Biomass yield (g/g sugar consumed)	2,3-Butadeiol productivity ($gL^{-1}h^{-1}$)
No fed-batch	0.36 ± 0.00	0.54 ± 0.01	0.075 ± 0.001	1.83 ± 0.02
1st fed-batch	0.43 ± 0.00	0.50 ± 0.01	0.040 ± 0.000	1.13 ± 0.00
2nd fed-batch	0.49 ± 0.00	0.053 ± 0.00	0.022 ± 0.002	0.94 ± 0.00
3rd fed-batch	0.47 ± 0.01	0.51 ± 0.00	0.014 ± 0.001	0.81 ± 0.01
4th fed-batch	0.46 ± 0.00	0.53 ± 0.00	0.012 ± 0.000	0.48 ± 0.00

Figure 9.5 Fed-batch process for 2,3-butanediol fermentation in fermenter using *Bacillus licheniformis* DSM 8785 in glucose medium. Fermentation was carried out in 7-L bioreactor with 5 L working volume, 1vvm aeration, 200 rpm, without pH control, and at 30°C. Data are average values of duplicate experiments, and error bars represent sample standard deviation.

Table 9.1 Comparison of two bacteria cultures for 2,3-butanediol production using different fermentation media.

Fermentation medium	Culture	Conc. (g/L)	Yield (g/g sugar consumed)	Productivity ($gL^{-1}h^{-1}$)	Remarks
Glucose (7 - 8%, w/v)	*B.l.*	26.8 ± 0.3^{e}	0.42 ± 0.00^{b}	2.23 ± 0.03^{a}	• Fermentation time: 12 h for *B.l.* and 24 h for *K.o.*
	K.o.	28.2 ± 0.1^{d}	0.43 ± 0.01^{b}	1.03 ± 0.03^{c}	
Xylose (7 - 8%, w/v)	*B.l.*	28.4 ± 0.5^{d}	0.44 ± 0.01^{a}	1.58 ± 0.03^{b}	• Fermentation time: 18 h for *B.l.* and 36 h for *K.o.*
	K.o.	23.1 ± 0.7^{g}	0.36 ± 0.02^{d}	0.64 ± 0.02^{f}	
Mixed sugars (9 - 10%, w/v)	*B.l.*	32.7 ± 0.5^{b}	0.32 ± 0.01^{e}	0.91 ± 0.01^{d}	• Fermentation time: 36 h for *B.l.* and 60 h for *K.o.*
	K.o.	29.8 ± 0.9^{c}	0.35 ± 0.01^{d}	0.50 ± 0.01^{h}	
Sorghum hydrolyzate (3 - 4%, w/v)	*B.l.*	10.0 ± 0.3^{j}	0.26 ± 0.00^{f}	0.56 ± 0.02^{g}	• Fermentation time: 18 h for both cultures • Biomass hydrolyzate used for *K.o.* had lower total than that used for *B.l.* than
	K.o.	4.1 ± 0.2^{k}	0.19 ± 0.00^{g}	0.23 ± 0.01^{i}	
Sorghum hydrolyzate (8%, w/v)	*B.l.*	19.7 ± 0.9^{i}	0.41 ± 0.01^{bc}	0.82 ± 0.04^{e}	• Fermentation time: 24 h for both cultures • Both cultures did not utilize xylose
	K.o.	21.3 ± 0.8^{h}	0.45 ± 0.01^{a}	0.89 ± 0.03^{d}	
Detoxified wood hydrolyzate (8%, w/v)	*B.l.*	25.0 ± 0.0^{f}	0.40 ± 0.01^{c}	1.56 ± 0.03^{b}	• *B.l.* utilized all glucose in 16h, and did not utilized xylose at all • *K.o.* utilized all glucose in around 30, then slowly all utilized all xylose in 72 h
	K.o.	34.9 ± 1.0^{a}	0.36 ± 0.02^{d}	0.48 ± 0.01^{h}	

Conc. = concertation, *B.l.* = *Bacillus licheniformis* DSM 8785, *K.l.* = *Klebsiella oxytoca* ATCC 8724. Data are average values of triplicate experiments ± sample standard deviation. Values with the same letters, in superscripts, within the same column are not significantly different form each other at $p < 0.05$.

Chapter 10 - Conclusion and future research

Conclusion

Sustainable production of biofuels and biochemicals requires an integrated approach including plant breeding, harvesting and handling, and conversion of diverse biomass feedstocks to fuels, chemicals and power. Various steps of biomass processing were evaluated, and the following conclusions were drawn from this doctoral research.

1. Pelleting of biomass to better streamline handling and logistic issue results in beneficial effect to improve biomass pretreatment and hydrolysis efficiencies.
2. Alkali pretreatment is more effective than acid pretreatment at same processing conditions for grasses and hardwood biomass resources
3. Alkaline ethanol-isopropanol mixture and glycerol are promising solvent systems for the pretreatment of corn stover (grass) and poplar (hardwood), respectively.
4. Pretreatment conditions used in this study were not effective for Douglas fir (softwood); appropriate processing conditions, better solvent and catalyst are required for this biomass.
5. Each potential sorghum cultivar should be separately evaluated for each type of the brown midrib (*bmr*) mutation to develop the best sorghum line as a promising feedstock for biofuels and biochemicals production, with concomitant promising grain yield.
6. Biomass hydrolyzate with separate glucose and xylose streams is beneficial for efficient 2,3-butanediol (BD) fermentation using a non-pathogenic (biosafety level 1) bacterial strain, *Bacillus licheniformis* DSM 8785. This culture is significantly more efficient for BD fermentation in single sugar media than *Klebsiella oxytoca* ATCC 8724; the *K. oxytoca* is a better culture reported so far for BD fermentation from diverse sugars media, but they are pathogenic (biosafety level 2) bacteria.

Future research

1. Biomass lignin characterization

Our study showed that reduced total lignin content in lignocellulosic biomass feedstocks does not necessarily improve pretreatment efficiency. In addition, the efficient biomass pretreatment solvents varied from biomass to biomass. These results indicated a significant effect of lignin structure, and inter-unit lignin linkages on biomass pretreatment and hydrolysis efficiencies. Therefore, characterization of biomass lignin structure, and study of change to lignin structure and composition during various biomass pretreatment methods is critical to better understand the underlying mechanism of above variations, and develop the best pretreatment method for each type of biomass feedstock, including grass, hardwood and softwood biomass. The lignin characterization also helps evaluate the quality of extracted lignin from the alkaline organosolv pretreatment process we performed in this study for high value applications. The solution-state two dimensional heteronuclear single quantum coherence (2D ^{13}C-^{1}H HSQC) NMR is a promising technique for biomass lignin characterization (Kim and Ralph, 2010).

2. Bioprocessing to obtained separate cellulose and hemicellulose hydrolyzates

Our study determined a promising opportunity for efficient 2,3-butaneidol (BD) fermentation using a non-pathogenic (biosafety level 1) bacterial strain, *Bacillus licheniformis* DSM 8785 with very high BD productivity, which is more efficient compared to *Klebsiella oxytoca* ATCC 8724 from single sugar medium; the *K. oxytoca* is the a better culture reported so far for BD fermentation from diverse sugars media, but they are pathogenic (biosafety level 2) bacteria. However, the *B. licheniformis* is inefficient for BD fermentation from biomass hydrolyzates with total sugars (glucose + xylose) 8% or more. Therefore, a separate glucose and

xylose stream is required for efficient BD fermentation using *B. licheniformis*. We proposed a three-step pretreatment method to generate different streams for extractives, lignin, hemicellulose hydrolyzate and cellulose hydrolyzate as shown in **Figure 10.1**. The processing conditions can be varied depending on the biomass types. For example, the proposed condition in **Figure 10.1** can be good for grass biomass with high extractives and non-structural sugar content, such as *bmr* sorghum. However, biomass with very low amount of water-soluble extractives, like poplar biomass, does not need the first pretreatment step, but requires more severe processing conditions for second and third pretreatment steps; for example, addition of dilute acid for second pretreatment step and higher temperature and/or higher NaOH concentration for third pretreatment step.

3. **Detoxification of biomass hydrolyzates**

Depending on the pretreatment method and the severity of the process, a number degradation products are generated during pretreatment, including sugar degradation products, such as hydroxymethylfurfural (HFM), furfural, levulinic acid, and formic acid, and lignin degradation products, such as phenolics and acetic acid. In addition, acetic acid is released during hydrolysis of hemicellulose. These compounds are considered toxins because they inhibit sugar-fermenting microbes. Ideally, appropriate pretreatment method can be developed to avoid or minimize these toxin formation; however, some of the toxins, like acetic acid from hemicellulose hydrolysis, are unavoidable in biomass hydrolysis. Therefore, development of appropriate detoxification methods to remove these toxins from biomass hydrolysis is another important area for future research.

4. **Strain development for *Bacillus licheniformis* DSM 8785**

Our study showed that the commercial culture of *Bacillus licheniformis* DMS 8785 is a promising bacteria for BD fermentation using single sugar media. However, BD productivity severely decreased in mixed sugars medium because the culture could not simultaneously utilize both glucose and xylose sugars due to the carbon catabolic repression (CCR) of glucose for xylose utilization. Therefore, a bacterial strain need to be engineered to express the required gene to eliminate the CRC mechanism or modify the xylose metabolic pathway to avoid the effect of CCR. The research carried out by (Ji et al., 2011) for *Klebsiella oxytoca* bacteria could be followed for this proposed research.

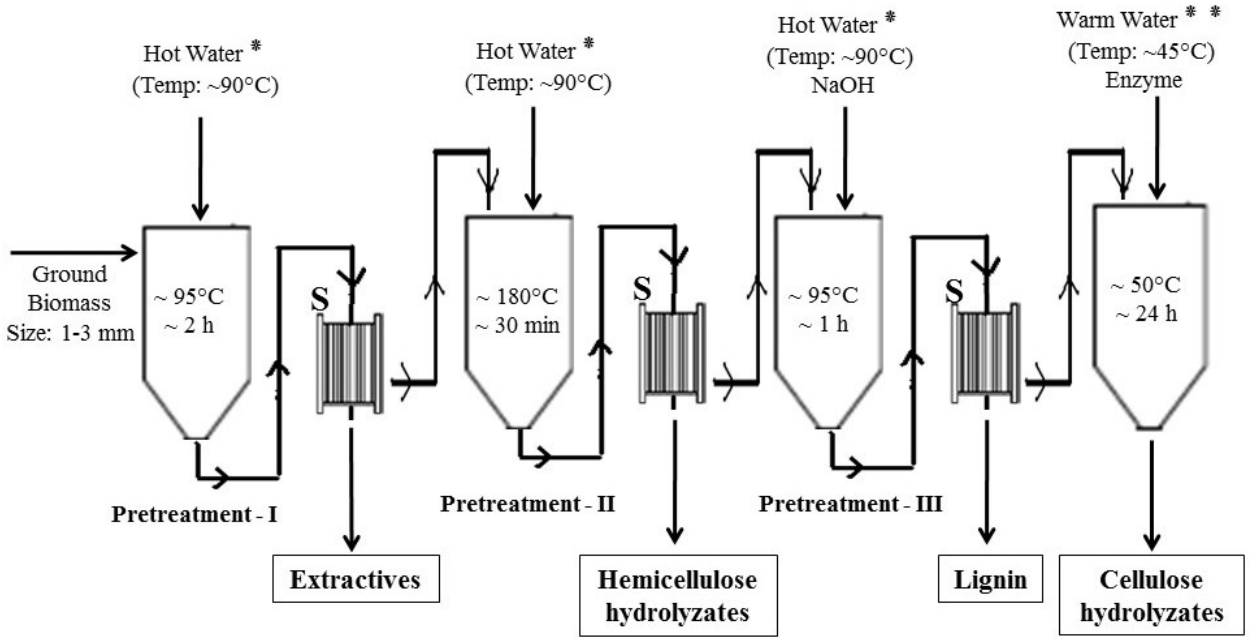


Figure 10.1 Proposed three-step pretreatment and hydrolysis to get separate stream of fermentable sugars.

*= Hot water around 90°C is obtained by cooling biomass slurry after liquid-hot-water pretreatment at around 180°C to around 95°C.

**= Warm water around 45°C is obtained by cooling biomass slurry after alkali pretreatment at 95°C to around 50°C.

S = Separator containing 0.5 mm sieve size to separate liquid and solid fraction of biomass slurry without vacuum pump.

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