DEVELOPMENT AND OPTIMIZATION OF GLUCOSE AND ETHANOL PRODUCTION FROM EASTERN REDCEDAR

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DEVELOPMENT AND OPTIMIZATION OF GLUCOSE AND ETHANOL PRODUCTION FROM EASTERN REDCEDAR

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Abstract:
The overall objective of this project was to develop and optimize a process to efficiently extract glucose from cellulose in Eastern redcedar and to demonstrate ethanol production at high efficiencies and titers. To accomplish this goal, the first step was to develop a pretreatment process for efficiently extracting sugars from redcedar wood. A statistical approach was taken for determining the vital few factors affecting redcedar pretreatments. Subsequently, the optimum conditions for achieving maximum overall wood glucan-to-glucose yield (% of theoretical) was determined using response surface methodology. A functional model relating four important pretreatment parameters (pretreatment temperature, hold time, sulfuric acid and sodium bisulfite loading) and wood glucan-to-glucose yield was obtained. The model was validated achieving 87% of theoretical wood glucan-to-glucose yield. Then, the operability of pretreated redcedar at high solids (substrate) loading was tested. Rheological challenges observed at high solids loading were overcome by adding stainless steel balls to shake flask reactors. The highest glucose concentration, 126 g L^{-1}, was obtained using 20% solids loading in the presence of stainless steel balls as a mixing aid, which was subsequently fermented by *S. cerevisiae* D_5A to produce 52 g L^{-1} of ethanol. Such a concentrated stream of products would reduce the capital and operating costs of a commercial process. Afterwards, the effects of two wood zones (sapwood versus heartwood) and two particle sizes (2.5 mm versus 0.5 mm) on wood glucan-to-ethanol yield were investigated. Results demonstrated that particle size had no effect on wood glucan-to-ethanol yield, which was a significant finding because energy costs during milling operations could be reduced. Additionally, it was observed that ethanol yields were 13% lower with heartwood than with sapwood. Finally, the effect of redcedar oil on ethanol fermentations by yeast and enzymatic hydrolysis of pure cellulose was determined. The presence of redcedar oil at 1% (w/w) inhibited enzymatic hydrolysis of cellulose to glucose by 33%, but had only a marginal inhibitory effect on ethanol fermentations during the first 9 h of fermentation. Therefore, it is recommended to remove redcedar oil from the raw material prior to hydrolysis of redcedar by cellulase.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

1.1 References 10

II. OBJECTIVES 15

III. REVIEW OF LITERATURE 17

3.1 Physical and chemical nature of softwood 17

3.2 Wood size reduction 26

3.3 Pretreatments 28

3.3.1 Softwood pretreatments 29

3.3.1.1 Dilute acid pretreatment 30

3.3.1.2 Steam explosion pretreatment assisted with acids 31

3.3.2 Pretreatment severity 37

3.4 Ethanol production strategies 38

3.4.1 Separate hydrolysis and fermentation (SHF) 39

3.4.2 Simultaneous hydrolysis and fermentation (SSF) 39

3.4.3 Non isothermal simultaneous hydrolysis and fermentation (NSSF) 41

3.4.4 Separate hydrolysis and co-fermentation (SHCF) 42

3.4.5 Simultaneous saccharification and co-fermentation (SSCF) 43

Chapter Page

3.4.6 Strategies to improve ethanol titers 44

3.5 References 46

IV. DEVELOPMENT OF AN EFFICIENT PRETREATMENT PROCESS FOR ENZYMATIC SACCHARIFICATION OF EASTERN REDCEDAR 58

4.1 Introduction 60

4.2 Materials and methods 64

4.2.1 Biomass 64

4.2.2 Pretreatments 64

4.2.3 Enzymes and enzymatic hydrolysis of biomass 66

4.2.4 Statistical analysis 67

4.3 Results and Discussion 67

4.3.1 Preliminary screening study on chemical loading 67

4.3.2 Effect of hold time, temperature and bleed-out 72

4.3.3 Effect of chemical loading and surfactants 73

4.3.4 Effect of bisulfite loading, hold time and impregnation 78

4.3.5 Effect of hold time on glucan loss and wood glucan-to-glucose yield 81

4.4 Conclusions 87

4.5 References 88

V. ACID BISULFITE PRETREATMENT OF EASTERN REDCEDAR FOR FERMENTABLE GLUCOSE PRODUCTION: OPTIMIZATION THROUGH RESPONSE SURFACE METHODOLOGY 93

5.1 Introduction 95

Chapter Page

5.2 Materials and methods 97
5.2.1 Biomass 97
5.2.2 Compositional analysis 98
5.2.3 Acid bisulfite pretreatments 99
5.2.4 Enzymes and enzymatic hydrolysis of pretreated redcedar 100
5.2.5 Experimental design 101
5.3 Results and discussion 104
5.3.1 Pretreatment optimization 104
5.3.2 Model validation 116
5.4 Conclusions 120
5.5 References 121

VI. EFFECT OF HIGH SOLIDS LOADING ON ENZYMATIC HYDROLYSIS OF ACID BISULFITE PRETREATED EASTERN REDCEDAR 126
6.1 Introduction 128
6.2 Material and methods 130
6.2.1 Biomass characterization 130
6.2.2 Pretreatments 131
6.2.3 Effect of high solids loading 132
6.2.4 Fermentability of enzymatic hydrolysate at 20% solids loading 134
6.2.5 Effect of enzyme dosage 135
6.2.6 Statistical analysis 135
6.3 Results and discussion 136

Chapter Page

6.3.1 Redcedar pretreatments 136

6.3.2 Effect of high solids loading 138

6.3.3 Fermentability of enzymatic hydrolysate obtained at 20% solids loading 146

6.3.4 Effect of enzyme dosage 149

6.4 Conclusions 153

6.5 References 153

VII. SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF EASTERN REDCEDAR BY *SACCHAROMYCES CEREVISEAE* D_5A: EFFECT OF WOOD ZONES AND PARTICLE SIZE 159

7.1 Introduction 161

7.2 Materials and Methods 164

7.2.1 Biomass characterization 164

7.2.2 Pretreatments 166

7.2.3 Enzymes, yeast strain and inoculum preparation 167

7.2.4 Simultaneous saccharification and fermentation 167

7.2.5 Statistical design of experiment and analysis 168

7.3 Results and discussion 169

7.3.1 Composition of the sapwood and heartwood zones 169

7.3.2 Particle size distribution 171

7.3.3 Pretreatments 173

7.3.4 Simultaneous saccharification and fermentation 178

7.4 Conclusions 189

Chapter Page

7.5 References 190

VIII. INFLUENCE OF EASTERN REDCEDAR OIL ON YEAST FEREMENTATIONS AND ENZYMATIC HYDROLYSIS OF MICROCRYSTALLINE CELLULOSE 195

8.1 Introduction 197

8.2 Materials and methods 199

8.2.1 Effect of redcedar oil loading during fermentations 199

8.2.2 Effect of redcedar oil loading during enzymatic hydrolysis 201

8.2.3 Statistical analysis 202

8.3 Results and discussion 203

8.3.1 Effect of redcedar oil loading on yeast fermentations 203

8.3.2 Effect of redcedar oil loading during enzymatic hydrolysis 207

8.4 Conclusions 210

8.5 References 210

IX. FUTURE WORK 214

APPENDICES 217

LIST OF TABLES

Table Page

Table 3.1 Composition of lignocellulosic biomass 19

Table 3.2 Proportions of different type of linkages in softwood lignin 23

Table 4.1 Effect of chemical loading and surfactant during enzymatic hydrolysis of pretreated redcedar 76

Table 4.2 Weights of wood components and the percentage recovered after acid bisulfite pretreatments at 200 °C, 3.75 g per 100 g of sulfuric acid loading and 20 g per 100 g of sodium bisulfite loading with varying pretreatment time 79

Table 5.1 List of experimental factors and levels for the central composite design 103

Table 5.2 Experimental design matrix and results of the central composite design 107

Table 5.3 ANOVA for response surface reduced quadratic model 109

Table 6.1 Biomass composition before and after acid bisulfite pretreatment expressed as % of dry matter for two different batches of Eastern redcedar 137

Table 6.2 Glucose yield in kg Mg^{-1} of dry wood and % of theoretical achieved at different solids loadings 145

Table Page

Table 7.1 Chemical compositions of sapwood and heartwood zones of Eastern redcedar 170

Table 7.2 Biomass components loss after pretreatment and sugars recovered in pretreated solids and prehydrolyzates 174

Table 7.3 Concentration of fermentation inhibitors in prehydrolysate 179

Table 7.4 Wood glucan-to-ethanol yield expressed as % of theoretical and L Mg^{-1} of dry wood and achieved in different treatments 184

Table 8.1 Estimated redcedar oil content in biomass-prehydrolysate slurry 200

Table 8.2 Effect of redcedar oil on ethanol fermentations by *S. cerevisiae* D_5A 206

LIST OF FIGURES

Figure Page

Fig. 1.1 Conceptual design for biochemical conversion of Eastern redcedar into ethanol and co-products 6

Fig. 3.1 Building blocks of lignin 21

Fig. 3.2 Common linkages in softwood lignin 22

Fig. 3.3 Lignin structure of softwoods 24

Fig. 3.4 Lignin sulfonation and degradation reaction 35

Fig. 3.5 Different strategies for ethanol production 40

Fig. 4.1 Effect of chemical loading on pretreatment of Eastern redcedar at 96 h of enzymatic hydrolysis. 70

Fig. 4.2 Effect of pretreatment temperature, time and bleed-out on pretreatment of Eastern redcedar at 96 h of enzymatic hydrolysis. 74

Fig. 4.3 Effect of sodium bisulfite and hold time on pretreatment of Eastern redcedar at 96 h of enzymatic hydrolysis. 79

Figure Page

Fig. 4.4 Effect of pretreatment time on glucan-to-glucose yield (%) of pretreated redcedar and overall wood-glucan-to-glucose yield (%) based on glucan content of untreated redcedar. 82

Fig. 5.1 Effect of lignin removal on wood glucan-to-glucose yield 105

Fig. 5.2 Quadratic response surface plots showing the interaction between two factors while keeping the other two fixed at certain levels during acid bisulfite pretreatments of redcedar. 114

Fig. 5.3 Process mass balance of acid bisulfite pretreatments and saccharification of Eastern redcedar 118

Fig. 6.1 Glucose and cellobiose profiles during enzymatic hydrolysis of pretreated redcedar at different solid loadings (% w/w, dry basis) and 46 FPU g^{-1} glucan enzyme loading. 139

Fig. 6.2 Effect of solid loading (% w/w, dry basis) on glucan-to-glucose yield of pretreated Eastern redcedar at 96 h for solids loading below 16% and 144 h for solids loading at or above 16% with an enzyme dosage of 46 FPU g^{-1} glucan. 141

Fig. 6.3 Glucose consumption, ethanol production and ethanol yield (% of theoretical) of *S. cerevisiae* D_5A using enzymatic hydrolysate obtained from 20% solid loadings at 37 °C. 147

Fig. 6.4 Glucose concentration and glucan-to-glucose yield during enzymatic hydrolysis of pretreated redcedar at different enzyme loadings (FPU g^{-1} glucan) and 16% solids loading. 151

Figure Page

Fig. 7.1 Particle size distribution of crumbled and ground sections of sapwood and heartwood. 172

Fig. 7.2 Glucose consumption, ethanol production and ethanol yield during SSF of pretreated redcedar with the four treatments combinations at 8 % dry solid loading and 46 FPU g^{-1} glucan enzyme loading. 182

Fig. 8.1 Effect of redcedar oil loading on cell mass concentrations, glucose consumption and ethanol production of *S. cerevisiae* D_5A. 204

Fig. 8.2 Effect of redcedar oil loading on glucan-to-glucose yield and inhibition at 96 h with different glucan loading and enzyme loading. 208

CHAPTER I

INTRODUCTION

There is plentiful energy available below ground in the form of crude oil, coal and natural gas. Our society has been dependent on these energy sources since the start of the industrial revolution. While coal and natural gas is commonly used for electricity generation, crude oil is converted into a multitude of products such as plastics, synthetic fibers and transportation fuels. All three energy sources are non-renewable and large emitters of CO_2. Therefore, our society is on a search for renewable and cleaner energy resources to meet the demands of the increasing population and growing economies.

Microorganisms such as yeast, bacteria and fungi can synthesize fuels and chemicals from renewable biomass, such as food crops, agricultural residues, energy crops and woody biomass, using their unique metabolic pathways. However, biomass will have to be processed to extract sugars that can serve as carbon and energy sources for these microorganisms. Depending on the biomass type, sugars are stored in different forms. They can be present as simple sugars, such as in sugarcane, sweet sorghum and sugar beets, or in the form of starch (polymer of glucose joined by α-1,4 linkages) as in corn or as cellulose (polymer of glucose joined by β-1,4 linkages). Crops like sugarcane and corn are staple foods; hence, farmlands cannot be diverted solely for making fuels. Lignocellulosic biomass can play a big role in the production of transportation fuels and

commodity chemicals. Lignocellulosic biomass is comprised of cellulose, hemicellulose and lignin, of which, cellulose and lignin are the two most abundant, naturally occurring polymers on Earth (Ragauskas et al., 2006). Polysaccharides, namely, cellulose and hemicellulose, comprise 60 to 70% of the plant material and can be potentially converted to fuel ethanol. The US has a potential to produce 1.1 billion tons of lignocellulosic biomass annually without impacting US farm and forest products, which accounts for displacing more than 30% of the current fossil fuel usage by the year 2030 (Perlack, 2005; US Department of Energy, 2011). Over the last decade, there has been tremendous research in the use of lignocellulosic materials for ethanol production. About 28 cellulosic ethanol projects were under development or construction in 2010 (Renewable Fuels Association, 2010) and it is estimated that 1 billion gallons of cellulosic ethanol will be produced in the US in 2013 (Renewable Fuels Association, 2013). Sandia National Laboratories estimated that the US could produce 75 billion gallons per year of lignocellulosic biofuels by 2030 (West et al., 2009). This study assumes that agricultural residues, such as corn stover and wheat straw, energy crops, such as switchgrass and miscanthus, and short rotation woody biomass, such as willow and poplar, would be the major feedstock for biofuel production.

The central plains of the US are dealing with a serious issue with encroachment of Eastern redcedar (*Juniperus virginiana* L.) due to its ability to adapt well to different soils, climatic conditions and topographies (Hiziroglu et al., 2002). Eastern redcedar (hereafter referred as redcedar) is a member of the cypress family (*Cupressaceae*) and is common to the central and eastern US (Iddrisu, 2008). Between 1985 and 2015, a 231% increase in redcedar acreage was estimated in Oklahoma (McKinley, 2012). Recent

studies show that redcedars are spreading at a rate of 57 trees per hectare per year in the prairie lands of Kansas (Price et al., 2010) and at a rate of 121,000 hectares per year in the plains of Oklahoma (McKinley, 2012). According to an estimate made by McKinley (2012), 26% of the overall land base of Oklahoma will be covered with redcedars by 2015. Gold et al. (2003) reported that 53% of the total redcedars available in the US were in Arkansas, Kansas, Tennessee and Missouri. Redcedar has also invaded parts of Alabama, Mississippi, Texas, Florida and Iowa (Semen and Hiziroglu, 2005).

The encroachment of redcedars has brought many ecological concerns to farmers, ranchers and wildlife species (Zhang and Hiziroglu, 2010). First, land availability for grazing is greatly reduced due to the presence of redcedar. Second, a recent study showed that a single redcedar tree could absorb up to 30 gallons of water per day (Truitt, 2011). Their extensive root systems inhibit water recharge in aquifers and their thick foliage captures 25% of rainfall, thereby limiting rain from reaching soil (Truitt, 2011). Third, redcedar leaf litter on the soil was observed to affect soil hydraulic properties, such as water repellency and sorptivity (Wine et al., 2012). Fourth, the presence of essential oils in redcedar wood increases the risk of wildfires in regions where wind and low humidity conditions commonly exist (Zhang and Hiziroglu, 2010). Fifth, redcedar infestations have decreased turkey roost sites, grasslands birds and songbirds that are common to prairie lands (National Resources Conservation Service, 2012). Sixth, forage production is affected due to the encroachment of redcedars. The National Resources Conservation Service (2012) reports that as high as 50% reduction in forage production could be observed with 600 redcedar trees per hectare. Finally, pollens from redcedar have become a common source for allergies. The losses incurred by the state of

Oklahoma due to these ecological effects were estimated to be $447 million (National Resources Conservation Service, 2012).

Common control strategies for the spread of redcedars in Oklahoma are prescribed fires, application of pesticides and mechanical clearing. Mechanical clearing of redcedar, although highly encouraged due to its selectivity, is cost intensive unless a marketable product for these woods can be identified. Alternative utilization of redcedar in the form of particleboard, fence posts, mulch, novelty items, anticancer drugs, such as podophyllotoxin, and cedar oil for perfumes and preservatives has recently gained attention (Dunford et al., 2007; Eller et al., 2010; Gawde et al., 2009; Hiziroglu et al., 2002; Semen and Hiziroglu, 2005). However, wood of good quality is a prerequisite for the lumber industry (Hiziroglu et al., 2002). Additionally, processing units utilizing redcedar wood for oil extraction are finding difficulties with the end use of the wood after oil extraction. Mulch application of oil extracted redcedar wood will be restricted as it will no longer have the ability to deter pests due to the loss of essential oil. Production of biofuels from the polysaccharides of redcedar will be very beneficial to the farmers, ranchers and the state of Oklahoma because all their ecological threats with redcedar will be addressed and renewable fuel can be locally produced. As mentioned earlier, redcedar invasion has been commonly observed in 10 states of the US. With such a wide availability of redcedar across the US, redcedars easily can become a promising source for cellulosic biofuels. Until now, there has been no research conducted to convert redcedar into biofuels. Hence, a broad objective of this dissertation was to develop a process for efficiently extracting fermentable sugars from the polysaccharides of redcedar and demonstrating ethanol production at high efficiencies and titers.

The biochemical platform for ethanol production involves three important processing steps: pretreatment, enzymatic hydrolysis (also commonly referred as saccharification) and fermentation. Pretreatments are conducted to alter biomass structure so that they become amenable for hydrolysis and/or fermentations. During enzymatic hydrolysis, cellulose and hemicellulose are converted into monomeric sugars that subsequently can be converted into ethanol using yeast or bacteria. Fig. 1.1 shows a process for the biochemical conversion of redcedar into ethanol and other co-products. The process begins with harvest operations and separation of the leaf fraction from the wood. As previously mentioned, the needles of redcedar contain podophyllotoxin, a precursor molecule for the manufacture of anticancer drugs (Gawde et al., 2009). The wood fraction can be chipped or ground and subjected to redcedar oil extraction using steam distillation or hydrodistillation (Dunford et al., 2007). Oil free wood can then be pretreated and subjected to simultaneous saccharification and fermentation (SSF) where enzymatic hydrolysis and fermentations occur in a single step for ethanol production. Finally, ethanol will have to be distilled and passed through molecular sieves for getting fuel grade ethanol.

The steps involved in developing a process to convert redcedar into ethanol were broken down into four sub-objectives. First, selection and optimization of redcedar pretreatments was very important because pretreatments impact subsequent steps, such as ethanol yield, capital and operating cost, enzyme utilization, fermentation, distillation and waste disposal (Saville, 2011). Redcedar is a softwood and generally softwood species are more difficult candidates for bioconversion processes to produce biofuels than hardwoods and agricultural residues because of their rigid structure and high lignin

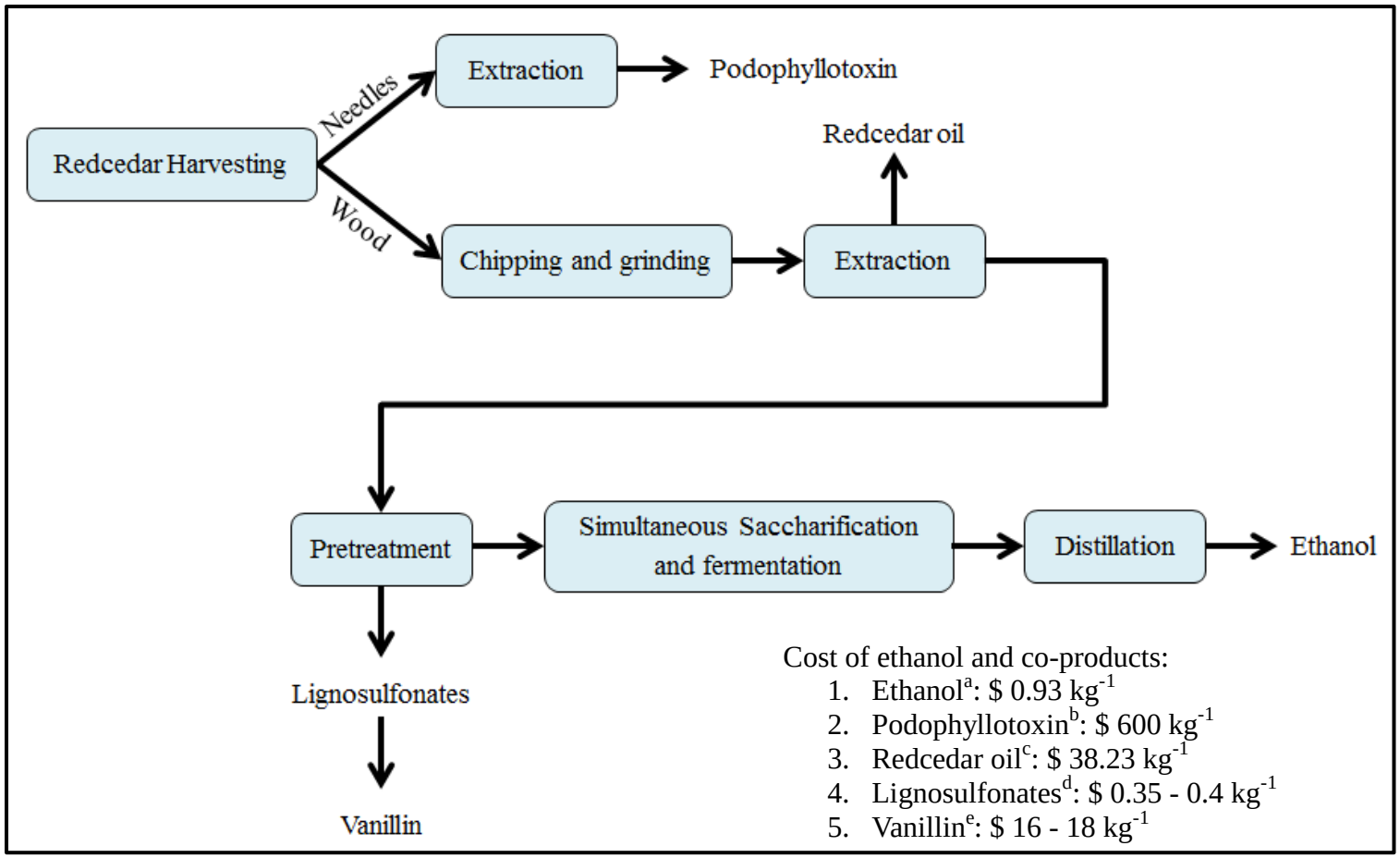

Fig. 1.1 Conceptual design for biochemical conversion of Eastern redcedar into ethanol and co-products.
[a]Ethanol cost from May 2013 issue of Ethanol Producer Magazine; [b]Podophyllotoxin cost from http://www.ebiochem.com/product/podophyllotoxin-20386; [c]Redcedar oil cost from web source http://www.texarome.com/price_list_wholesale.htm; [d]Lignosulfonate cost from web source http://www.alibaba.com/showroom/lignosulfonate.html; [e]Vanillin as ethyl vanillin. Cost from web source http://www.alibaba.com/showroom/ethyl-vanillin.html

content (Ramos et al., 1992). The complex physical and chemical nature of softwoods limits the number of pretreatment options available. Recently, acid bisulfite pretreatment (a variant of sulfite pulping process) had shown success for pretreating softwoods (Lan et al., 2013; Shuai et al., 2010; Zhu et al., 2009) and hence it was selected for the current study. A statistical approachwas undertaken to develop and optimize acid bisulfite pretreatment of redcedar to achieve high wood glucan-to-glucose yield.

The second objective of this research was to determine the ability to conduct enzymatic hydrolysis at high solids loading for achieving high glucose and ethanol concentrations. A bio-refinery utilizing redcedar as a feedstock would operate at high solids (substrate) loading to increase product concentrations and decrease capital and operating costs (Jørgensen et al., 2007; Kristensen et al., 2009). However, challenges such as increased viscosity resulting in mass transfer limitations, mixing difficulties and inhibition from toxic products, such as fermentation inhibitors and lignin, are common to operations at high solids loading (Alvira et al., 2013; Jørgensen et al., 2007; Roche et al., 2009). Thus, a study was conducted to observe the effect of solids loading on enzymatic hydrolysis of pretreated redcedar between 2 and 20% dry solids (w/w) as measured by glucose concentration produced and glucan-to-glucose yield of pretreated redcedar. Mixing difficulties were anticipated at high solids loading and stainless steel balls were used as a mixing aid. Additionally, the fermentability of the enzymatic hydrolysate by yeast was also determined. Enzymes account for 16% of the cost of ethanol (Humbird et al., 2011). Hence, minimizing enzyme dosage during saccharifications of redcedar would be ideal. The effect of lowering enzyme dosage at high solids loading was also studied to facilitate the determination of the

economic trade-off between enzyme dosage, residence time and the desired glucan-to-glucose yields.

The third objective of this study was to observe the effect of biomass particle size and wood zones on ethanol yields (% of theoretical). Size reduction of wood is an energy intensive step utilizing up to 33% of the total electricity that is required for an ethanol production process (Hinman et al., 1992). Forest Concepts, LLC has developed a low energy consuming size reduction process for making precision feedstock particles from woody biomass for biochemical and thermochemical conversion processes (Dooley et al., 2013). A study was conducted to demonstrate the feasibility of applying the biomass crumbles® (crumbles® is a trademark of Forest Concepts, LLC, Auburn, WA) produced by Forest Concepts, LLC during ethanol production process from redcedar. Additionally, the technology developed by Forest Concepts, LLC also allowed the separation of the wood zones into heartwood and sapwood, which are physiologically different from each other. Previous studies on pulping had shown that heartwood was inferior to sapwood for pulping operations (Esteves et al., 2005; Miranda et al., 2007). Since the objective of pulping and pretreatments is to preserve cellulose and hemicellulose, we hypothesized that the two zones of wood would produce different ethanol yields. To test our hypothesis, an experiment was conducted to compare the ethanol yields from the two wood zones during simultaneous saccharification and fermentation of pretreated redcedar.

The final objective of this research was to study the effect of redcedar oil during enzymatic hydrolysis and fermentations. Redcedar oil is an essential oil that has evolved as a natural defense mechanism for the protection of redcedar and it has antibacterial, antiviral, antifungal and insecticidal agents (Clark et al., 1990; Dunford et al., 2007; Eller et al., 2010;

Semen and Hiziroglu, 2005). Essential oils extracted from different plants and trees are cytotoxic to numerous microorganisms (Bakkali et al., 2008) and are inhibitory to α-glucosidases (Basak and Candan, 2013), but no studies have been done to study the effect of redcedar oil on *S. cerevisiae* and enzyme cocktails that are used for saccharification. It was hypothesized that redcedar oil would be inhibitory to yeast and enzyme cocktails. To test our hypothesis, an experiment was conducted to study the effect of three levels of redcedar oil during enzymatic hydrolysis of microcrystalline cellulose and ethanol fermentations by yeast.

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CHAPTER II

OBJECTIVES

The overall objective of this project was to develop and optimize a process for efficiently extracting fermentable sugars from the polysaccharides of redcedar and to demonstrate ethanol production from redcedar at high efficiencies and titers. The specific objectives of this research were as follows:

1. To identify the most important process parameters affecting acid bisulfite pretreatments of Eastern redcedar using factorial treatment designs and to determine near optimal pretreatment conditions that would result in maximum wood glucan-to-glucose yield.
2. To optimize pretreatment temperature, pretreatment time, sulfuric acid loading and sodium bisulfite loading, during acid bisulfite pretreatment of redcedar by response surface methodology for maximizing wood glucan-to-glucose yield.
3. To determine the effect of solids loading on enzymatic hydrolysis of pretreated redcedar between 2 and 20 % dry solids (w/w) as measured by glucose concentration produced and glucan-to-glucose yield of pretreated redcedar.
4. To investigate the effect of two wood zones (sapwood versus heartwood) and two particle sizes (crumbles® versus ground) on wood glucan-to-ethanol yield after

acid bisulfite pretreatment and simultaneous saccharification and fermentation (SSF) of redcedar.

5. To study the effect of Eastern redcedar oil during ethanol fermentations using *Saccharomyces cerevisiae* D_5A and enzymatic hydrolysis of microcrystalline cellulose.

CHAPTER III

REVIEW OF LITERATURE

3.1 Physical and chemical nature of softwood

In nature, woody biomass (softwoods and hardwoods) are built structurally denser and chemically stronger than other biomass types (Zhu and Pan, 2010). Softwoods, in particular, are more refractory than hardwoods and agricultural residues because of their physical (rigid structure) and chemical (high lignin content) properties (Galbe and Zacchi, 2002). This makes softwoods a difficult candidate for the bioconversion processes in comparison to other feedstocks.

Softwoods have longer fiber lengths in comparison to hardwoods and agricultural residues (Mansfield et al., 1999). For example, the fiber length of spruce, pine and Douglas fir (softwoods) was 3.5 mm in comparison to some hardwoods that averaged 1 nm (Shackford, 2003). Additionally, the fiber width of softwoods such as spruce, pine and Douglas fir was about 40 microns, which was two times larger than the fiber width observed with eucalyptus and other hardwoods (Shackford, 2003). Larger fiber dimensions result in greater resistance to pretreatments such as steam explosion because the explosive decompression may not be sufficient for bringing alteration in structure

(Mansfield et al., 1999). Larger fiber size could possibly require harsher pretreatment to increase the digestibility of softwoods. This is one of the prime reasons why steam explosion of softwoods is usually done in the presence of sulfuric acid or sulfur dioxide as a catalyst (Schell et al., 1998).

Porosity of the wood is another physical property of wood that could affect pretreatments by influencing the rate of penetration of chemicals and steam through the wood (Ramos, 2003). The trunk of woody biomass is mainly comprised of two zones: sapwood and heartwood, each serving different physiological roles (Wiedenhoeft and Miller, 2005). Sapwood is comprised of living cells functioning primarily to transport water and nutrients and store food reserves (Ramos, 2003). On the other hand, heartwood is the innermost part of the wood comprised of physiologically inactive cells with the primary function of tree structural support. Heartwood is generally characterized by low moisture content, low permeability, low porosity and high extractives content in comparison to sapwood (Ramos, 2003; Wiedenhoeft and Miller, 2005). Hence, chips from trees that were 6-8 years old could be more easily treated using steam pretreatment than older trees (Ramos, 2003).

Softwoods contain high lignin content, which makes them tougher than any other type of lignocellulosic biomass. Lignin is the cementing material that provides elasticity and mechanical strength to the wood (McGinnis and Shafizadeh, 1980). Table 3.1 shows the composition of different lignocellulosic biomass commonly investigated for ethanol production. Clear differences between the three biomass types can be observed. Softwoods have the highest lignin followed by hardwoods and herbaceous crops.

Table 3.1 Composition of lignocellulosic biomass - Adapted from Zhu and Pan (2010).

Biomass type	Biomass	Glucan, %	Xylan, %	Galactan, %	Arabinan, %	Mannan, %	Lignin, %
Herbaceous crops	Corn stover	30.6	16.0	0.7	1.9	0.5	18.2
	Switchgrass	35.9	19.6	0.5	1.5	0.4	23.1
	Wheat straw	32.6	19.2	0.8	2.4	0.3	16.9
Hardwoods	Salix	41.4	15.0	2.3	1.2	3.2	26.4
	Yellow poplar	42.1	15.1	1.0	0.5	2.4	23.3
	Eucalyptus	48.1	10.4	0.7	0.3	1.3	26.9
Softwood	Spruce	43.2	5.7	2.7	1.4	11.5	28.3
	Douglas fir	44.0	2.8	4.7	2.7	11.0	32.0
	Eastern redcedar*	30.2 – 40.3	5.8 – 8.5	2.0 – 4.6	1.4 – 2.3	6.0 – 8.5	32.2 – 33.8

*Redcedar composition was determined in our laboratories during different studies

Hardwoods have slightly higher glucan than softwoods followed by herbaceous crops, while the xylan content of herbaceous crops is higher than hardwoods and softwoods. In addition to the amount of lignin, the chemical nature of lignin also makes softwood a difficult candidate for the bioconversion process. For example, softwood lignin is primarily (95%) made of coniferyl alcohol (made of guaiacyl units) (Fig. 3.1), while hardwoods share both coniferyl and sinapyl alcohol (made of guaiacyl and syringyl units) (Glasser, 1980; Keshwani, 2010; Ramos, 2003). Hardwood lignin has higher methoxyl content and is less condensed, making it amenable to chemical pretreatments (Ramos, 2003). Guiacyl aromatic rings in softwoods have a single methoxyl group on the C3 carbon that allows it to have a cross linking structure, making them recalcitrant (Glasser, 1980). Thus, the primary nature and distribution of guaiacyl type lignin is believed to make softwoods recalcitrant (Ramos et al., 1992). The lignin monomeric units (Fig. 3.1) are linked together predominantly by ether bridges connecting α and β- carbons on side chains to the phenyl rings of other units (Ingruber, 1985), shown in Fig. 3.2. The distribution of the type of linkages in softwood is shown in Table 3.2 and a model lignin structure of a softwood lignin is shown in Fig. 3.3.

Lignin offers the following challenges during the overall conversion process (Chandra et al., 2007; Larsson et al., 1999; Pan et al., 2005; Ramos, 2003; Saville, 2011):

a) Lignin in biomass can act as a physical barrier and limit the access of cellulose to enzymes. This problem is higher with lignocellulosic biomass with a high lignin content, such as in softwoods. During pretreatments, condensed lignin can re-polymerize on the surface of biomass, thereby limiting the access of underlying cellulose to the enzymes.

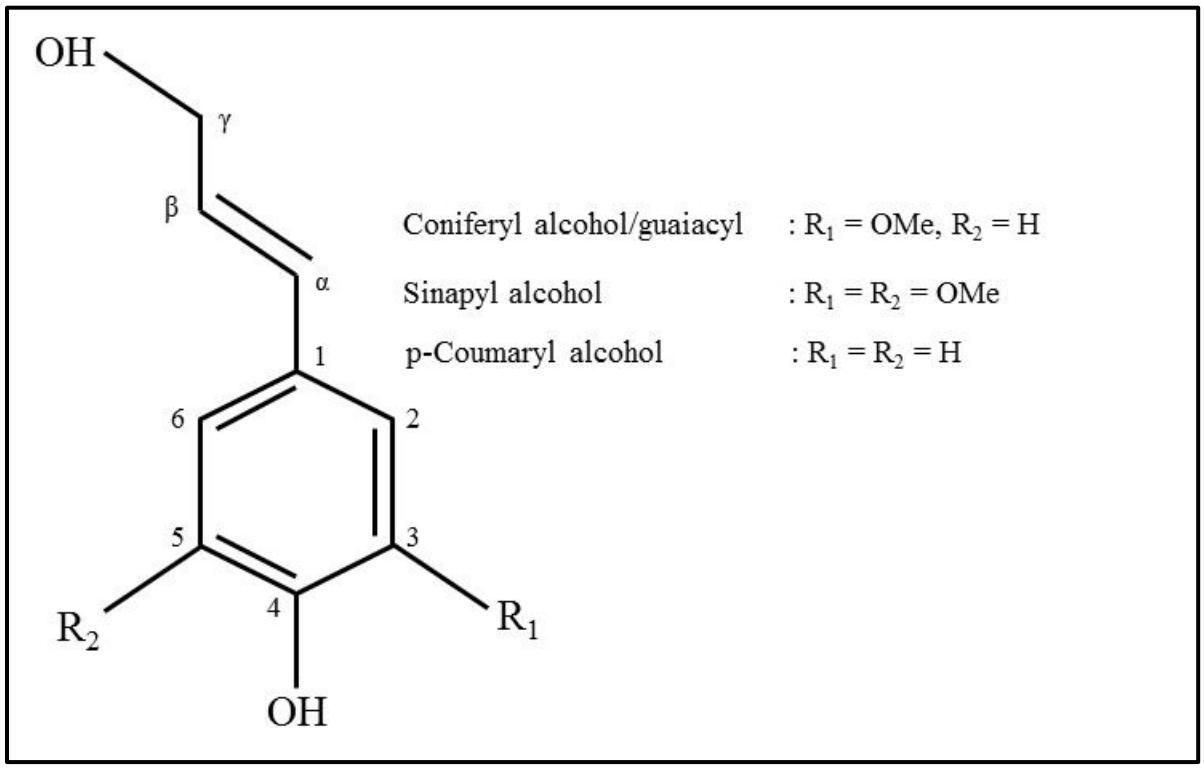

Fig. 3.1 Building blocks of lignin - Adapted from Chakar & Ragauskas (2004).

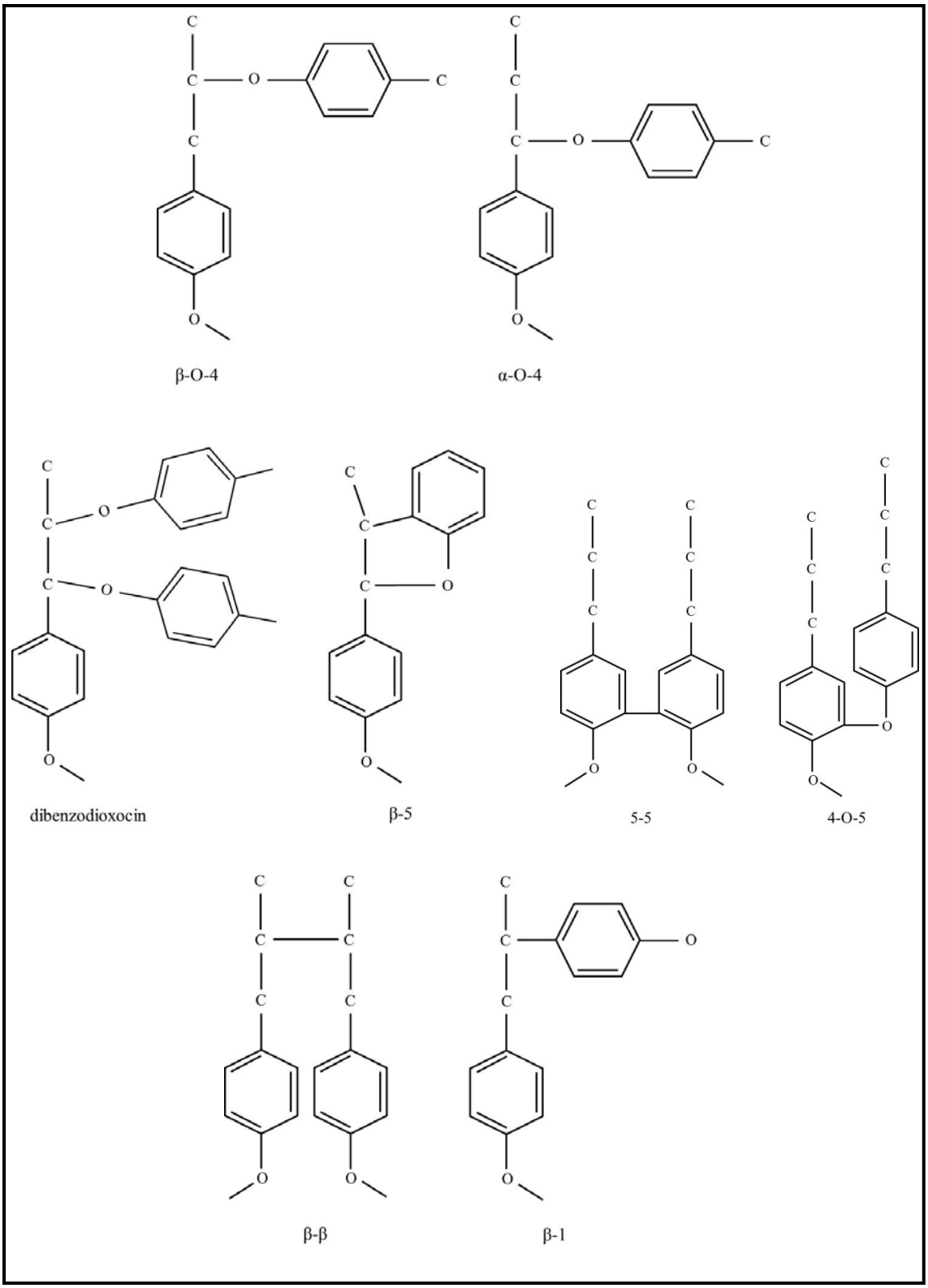

Fig. 3.2 Common linkages in softwood lignin – Adapted from Chakar and Ragauskas (2004).

Refer to Table 3.2 to observe the proportions of these linkages observed in softwoods

Table 3.2 Proportions of different type of linkages in softwood lignin - Adapted from Chakar and Ragauskas (2004).

Linkage type	Dimer structure	Percentage
β-O-4	Phenylpropane β-aryl ether	50
β-5	Phenylcoumaran	9 - 12
5-5	Biphenyl	15 - 25
5-5/ α-O-4	Dibenzodioxicin	10 - 15
4-O-5	Diaryl ether	4
β-1	1,2-Diary propane	7
β- β	β-β-linked structures	2

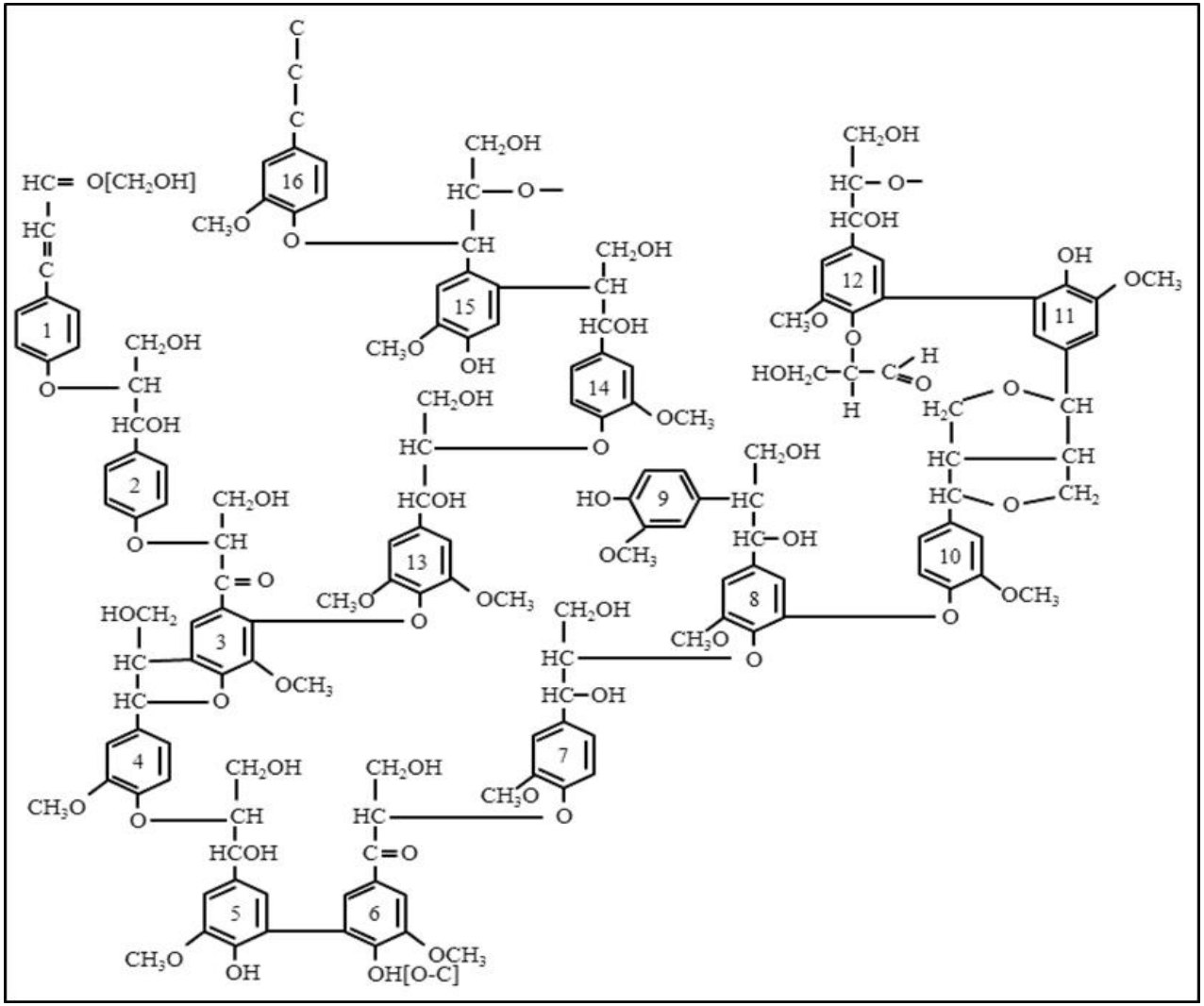

Fig. 3.3 Lignin structure of softwoods – Adapted from Ingruber (1985).

b) Lignin usually bonds with cellulose and hemicellulose molecules using ester, ether or ketal groups. The vicinity of large amounts of lignin to carbohydrates in softwoods could pose a problem because it could result in formation of non-specific bonds between the enzymes and lignin, which inhibits enzyme activity

c) Breakdown of lignin releases phenolic compounds like vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, vannillic acid, hydroquinone, catechol, homovanillic acid and 4-hydroxybenzoic acid that can inhibit enzymes and microorganisms during saccharification and fermentation process.

Cellulose and hemicellulose are polysaccharides that serve as substrates for ethanol production. Cellulose is the major structural polymer of a plant cell wall and usually exists as long thread-like fibers called microfibrils. It is a linear polysaccharide containing monomeric units of anhydro-D-glucose units with a β-(1→4)-linkage. This bonding allows the microfibril structure to develop strong inter-molecular and intra-molecular hydrogen bonding (Keshwani, 2010). Microfibrils are usually embedded on a matrix that contains hemicellulose and lignin. The microfibril structure has a high degree of polymerization (high number of glucosyl residues per cellulose chain) and crystallinity. These fibers are usually broken down during the pretreatment process, which decreases the degree of polymerization of cellulose and creates less crystalline structure. However, excessive loss of cellulose fibers must be prevented because it can decrease the overall wood glucan-to-ethanol yield.

Hemicellulose of softwoods contains partially acetylated galacto-glucomannans and substantial amounts of arabino-(4-o-methylglucurono) xylan (McGinnis and Shafizadeh, 1980). Xylans of softwood have anhydro-β-D-xylopyranose units as

backbone and α-linked branch points for L-arabino furanose units and 4-o-methyl-D-glucuronic acid (McGinnis and Shafizadeh, 1980). Unlike cellulose, they do not form microfibrils, but they can form hydrogen bonds with the cellulose and/or lignin and are referred to as "cross linking glucans" (Ramos, 2003). Cellulose and hemicellulose are usually linked to lignin through benzyl ester, benzyl ether and/or glycosidic linkages forming the lignin-carbohydrate-complex (Lawoko et al., 2006).

3.2 Wood size reduction

Biomass processing begins with a size reduction step and is a prerequisite for converting wood polysaccharides into ethanol. Conventional size reduction techniques include rotary hammer mills, knife mills, shredders, chippers and disk (or attrition) mills (Zhu, 2011). While hammer and knife mills are not well suited for wet biomass, disk mills can easily handle wet biomass (Schell and Harwood, 1994). Hammer milling and disk milling are the two techniques that are well suited for large scale production (Zhu, 2011). Hammer milling has been commonly used for the production of composites and pellets, while disk milling has been used for fiberization in pulping industries.

Size reduction of wood is an energy intensive step. Preliminary estimates showed that size reduction consumes 33% of the total electricity that is required for an ethanol production process (Hinman et al., 1992). Schell and Harwood (1994) compared the energy usage by a pilot-scale hammer mill and disk mill for reducing the size of freshly chipped wood with 60 % moisture content and observed that hammer mill used less energy (288 to 367 MJ Mg^{-1} of dry wood) than disk mills (439 to 1984 MJ Mg^{-1} of dry wood). The particle size distribution for hammer mills varied between 0.3 to 4.8 mm,

while disk mills resulted in 0.4 to 2.3 mm size distribution. Thus, the hammer mill consumed less energy but ended up with a larger-size distribution. Other reports have shown that 468 MJ Mg^{-1} of dry wood was required to reduce hardwood chips to 1.6 mm particle size using hammer mills and knife mills (Cadoche and López, 1989). The same study also concluded that energy used during size reductions would go below 108 MJ Mg^{-1} of dry biomass when the desired particle size was in the range of 3 to 6 mm. Datta (1981) observed that 72 to 144 MJ Mg^{-1} of dry wood was required to achieve 0.6 to 2 mm particles from hardwood chips and the energy usage increased by an order of 2.5 to 10 folds (360 to 720 MJ Mg^{-1} of dry wood) for obtaining finer particles (0.15 to 0.3 mm).

Post-chemical pretreatment size reduction was recently proposed and validated to reduce the energy usage during ethanol production (Zhu, 2011; Zhu and Pan, 2010; Zhu et al., 2010a). During this process, size reduction of woody biomass occurs in two steps. First, woody biomass was reduced to chip size prior to pretreatments. During pretreatments, the wood structure is softened and chemical components including hemicellulose and lignin are removed from the biomass. Pretreated wood chips were then reduced to finer particles using disk milling. Zhu et al. (2010a) observed a threefold reduction in energy consumption by using post-chemical pretreatments when compared to conventional pre-chemical pretreatment size reduction methods. Besides reduction in energy usage, post-chemical pretreatment size reduction avoids the challenges with solid/liquid separation step following pretreatments and allows operations at a low liquid-to-solid ratio, which reduces the water demand during biomass pretreatments (Zhu, 2011).

Recently, Forest Concepts, LLC developed a low energy consuming size reduction process for making precision feedstock particles from high moisture content woody biomass (Lanning et al., 2012). Logs of biomass are first passed through a rotary veneer lathe to peal the surface of the wood and then the peeled surface passes through a rotary shear configurable crumbler (crumbler® is a registered trademark of Forest Concepts, LLC, Auburn, WA) to give a desired particle size (Lanning et al., 2012). Biomass crumbles® (crumbles® is a registered trademark of Forest Concepts, LLC, Auburn, WA) with widths of 1.5 mm to 6 mm screen size could be obtained by adjusting the cutter wheel shafts (Lanning et al., 2012). The energy consumed to obtain a final dimension of 1.6 × 2 × 2 mm (length × width × thickness) from logs of hybrid poplar with about 50% moisture content was 150 MJ Mg^{-1} of dry wood (Lanning et al., 2012). In a different study, Dooley et al. (2013) observed that processing high moisture Douglas fir into 2.5 to 4.2 mm particles consumed 20% of the energy that was required by hammer mills for achieving similar particle size. At the same time, crumbling produced a more uniform shape and size (Dooley et al., 2013).

3.3 Pretreatments

Pretreatment of lignocellulosic biomass is the first step in the biochemical production of ethanol. The main objective of pretreatment is to make the biomass amenable for enzymatic hydrolysis and fermentation (Alvira et al., 2010). The selection of a pretreatment process is very critical due to the differences in the physical and chemical modes of action during different pretreatment technologies (Alvira et al., 2010).

The following are the desirable features for a pretreatment process (Mosier et al., 2005; Saville, 2011):

a) High recovery of all fermentable carbohydrates, especially glucose and xylose.
b) Minimal amounts of sugar degradation products, namely furfural, 5-hydoxy-methyl-furfural (HMF), formic acid and levulinic acid. This prevents inhibitory effects on fermentation and removes the requirement of detoxification.
c) Low capital and operating costs.
d) Pretreated solids are highly digestible during enzymatic hydrolysis.
e) Minimal requirement of size reduction, thereby reducing the cost associated with milling.
f) Reduced downstream operation cost by being operational at high solid loading, which avoids dilution of sugars and ethanol.
g) Catalysts with a low cost or inexpensive catalyst recycle systems.
h) Co-product formation, such as lignin or lignosulfonates, which could be easily recovered in pure form.
i) Applicability to a wide variety of feedstocks.

3.3.1 Softwood pretreatments

The complex physical and chemical nature of softwoods limits the number of pretreatment options available. For example, pretreatment methods based on ammonia such as ammonia recycle percolation (ARP), soaking in aqueous ammonia (SAA) and ammonia fiber expansion (AFEX), and hot water pretreatment have not shown success

with softwoods (Chandra et al., 2007). This is because ammonia based pretreatments and hot water pretreatments use acetic acid released from the biomass as a chemical catalyst and softwoods have low acetyl content (Kumar et al., 2009). Acidic pretreatments such as dilute acid pretreatments (Nguyen et al., 1998), steam explosion pretreatments assisted with acids (Kumar et al., 2010; Monavari et al., 2009; Schell et al., 1998) and sulfite pretreatment to overcome the recalcitrance of lignocellulose (SPORL) (Shuai et al., 2010; Zhu et al., 2009; Zhu et al., 2010a; Zhu et al., 2010b) have been relatively more successful with softwoods.

3.3.1.1 Dilute acid pretreatment

Dilute acid pretreatments commonly use sulfuric acid as a catalyst at concentrations below 4 wt% (Alvira et al., 2010). At temperatures around 160 to 200 °C, hemicellulose is dissolved in the liquid fraction called prehydrolysate, leaving cellulose and lignin with the biomass (Kumar et al., 2009). Due to low pH and high temperatures, fermentation inhibitors such as furfural, 5-hydoxy-methyl-furfural (HMF), formic acid and levulinic acid are produced. The biggest drawback of this process is the increased capital cost due to special material design requirements (Kumar et al., 2009). However, it offers high reaction rates and increased cellulose hydrolysis (Kumar et al., 2009).

Dilute acid hydrolysis of softwoods is less studied compared to steam explosion catalyzed by acids and SPORL pretreatments because extensive loss of glucan and formation of fermentation inhibitors are observed. In a study by Nguyen et al. (1998), Douglas fir and pine were mixed in 0.4% sulfuric acid solution at 200 to 230 °C for 1 to 5 min. Pretreatments resulted in 90 to 95% removal of hemicellulose from biomass and

20% loss of cellulose. Fermentations with 10% solid loading resulted in 80 to 85% of theoretical ethanol yields (Nguyen et al., 1998).

3.3.1.2 Steam explosion pretreatment assisted with acids

Steam explosion assisted with acids is the most widely studied technology for pretreatment of softwoods (Galbe and Zacchi, 2002). Steam explosion uses high pressure saturated steam to heat the biomass quickly, followed by a violent discharge of pressure into a collecting tank that creates explosive decompression of fibers (Kumar et al., 2009; Mosier et al., 2005). This process of sudden discharge is called explosion (Ramos, 2003). Alternatively, when the pressure inside the vessel is discharged by bleeding the steam pressure through a needle valve, it is not considered as explosion (Ramos, 2003). Bleed-outs are generally done to separate volatile compounds that could be inhibitory to subsequent processing steps (Brownell et al., 1986).

Steam explosion removes hemicellulose from the biomass and brings modification of lignin structure (Kumar et al., 2009). During steam pretreatments, condensation of high-pressure steam causes "wetting" of the biomass (Carvalheiro et al., 2008). When the pressure inside the reactor is released suddenly, moisture evaporates causing breakage of inter- and intra-molecular linkages of biomass (Carvalheiro et al., 2008). Lignin structure is disrupted due to homolytic cleavage of β-o-4 ether and other acid labile linkages, releasing lignin monomers and phenolic compounds (Ramos, 2003). Higher release of lignin occurs when pretreatment severity is increased (Ramos, 2003). Pretreatment severity or the reaction ordinate factor (R_0) is a model that relates pretreatment temperature and hold time to describe the impact of temperature and hold time on lignocellulosic components during steam explosions (See section section 3.3.2

for more details) (Overend et al., 1987). However, at very high severities, lignin condenses and deposits over the surface of the biomass (Pan et al., 2005; Ramos, 2003; Shevchenko et al., 1999).

Softwoods require addition of acid catalysts such as H_2SO_4 or SO_2 during steam explosion for the production of digestible substrates (Ballesteros et al., 2000; Monavari et al., 2009; Schell et al., 1998). Single step or two-step acid catalyzed steam explosions have been compared. It was observed that single step acid catalyzed steam explosions have lower ethanol yield and require larger enzyme loadings than two-step acid catalyzed steam explosions (Nguyen et al., 2000). When a two-step acid catalyzed steam explosion of softwoods was done where the first step was at a relatively lower severity in comparison to the second step, hemicellulose recovery improved, overall sugar yield increased by 10% and the required enzyme loading decreased by 50% (Nguyen et al., 2000). Similarly, Söderström et al. (2003) reported improvements in wood glucan-to-ethanol yield with a two-step sulfuric acid catalyzed steam explosion for softwoods. In a later study by Söderström et al. (2005), H_2SO_4 and SO_2 pretreatments were compared using softwood. It was observed that SO_2 based two-step steam explosion process resulted in more reactive material with fewer inhibitory compounds and higher ethanol yields than a H_2SO_4 based, two-step steam explosion process. Generally, the advantages of a two-step pretreatment with SO_2 are outweighed by increased energy and operational cost in comparison to a one-stage pretreatments (Galbe and Zacchi, 2002).

SO_2 catalyzed steam explosions were relatively more successful in comparison to H_2SO_4. Advantages that SO_2 over H_2SO_4 are as follows (Galbe and Zacchi, 2007; Monavari et al., 2009; Ramos et al., 1992; Wayman et al., 1984):

a. SO_2 addition during pretreatments could be performed before steam addition or with steam addition.
b. SO_2 gets distributed over the biomass faster than H_2SO_4 and is much cheaper in comparison to H_2SO_4.
c. SO_2 is incorporated in the biomass as lignosulfonates that can be recovered as a co-product.
d. The quality of lignin is poor when treated in the presence H_2SO_4, while SO_2 leaves a lignin as lignosulfonates with better marketable quality.
e. SO_2 pretreatment alters the hydrophobic nature of lignin into hydrophilic nature. Hence, the fermentability of SO_2 pretreated substrates was better than H_2SO_4 pretreated substrates over a wide range of severity factors.
f. Finally, SO_2 addition was shown to reduce time and temperature requirements during steam treatments with enhanced fractionation and recovery of sugars.

A problem with SO_2 catalyzed steam explosions is that the mild nature of SO_2 does not dissolve large quantities of lignin (Shuai et al., 2010) and the toxicity of the gas raises many health issues.

3.3.1.3 SPORL pretreatments

Sulfite pretreatment to overcome the recalcitrance of lignocellulose (SPORL) is a variant of sulfite pulping technology that was used to produce paper and pulp. Bisulfite salt (made of sodium, ammonium, magnesium, potassium or calcium) and sulfuric acid are the two chemicals required for this process. Bisulfite salts are involved with lignin sulfonation reactions (Bryce, 1980). Detailed chemistry of the delignification reaction is as follows:

Delignification has been thoroughly studied for sulfite pulping (Bryce, 1980). Since, the chemicals for sulfite pulping and SPORL are the same, knowledge from sulfite pulping can be applied to understand the chemical reactions occurring during SPORL. The chemistry of sulfonation is shown in Fig. 3.4. SO_2 based pretreatments and SPORL work alike by forming bisulfite (HSO_3^-) ions, crucial for sulfonation. At acidic pH (1.5) and room temperature, sodium bisulfite solution contains 40% bisulfite and 60% SO_2 gas (Bryce, 1980). The equilibrium reactions can be represented in Eqs. 3.1 and 3.2.

$$NaHSO_3 \leftrightarrow Na^+ + HSO_3^- \quad (3.1)$$

$$HSO_3^- + H^+ \leftrightarrow H_2SO_3 \leftrightarrow H_2O + SO_2 \quad (3.2)$$

The first step of the sulfonation reaction is the attack of an acid group on the C-α position that is referred to as hydrolysis (Bryce, 1980). As shown in Table 3.2, the proportion of monomeric lignin that is linked at the α-carbon is only 10 to 15% in comparison to other lignin linkages. Thus, most of the α-carbon on phenolic monomers will be exposed to hydrolytic attack creating a relatively unstable carbonium ion (C+) called the quinone-methide intermediate. This intermediate molecule immediately reacts with the negatively charged bisulfite (HSO_3^-) ions to form lignosulfonates (Glasser, 1980). The β-carbon position that is frequently engaged with ether linkages is then sulfonated by a sulfitolysis reaction. The sulfitolysis reaction breaks the monomeric lignin from the polymeric form (Ingruber, 1985). Thus, a lignin unit is obtained with both α and β carbons sulfonated in the form of lignosulfonates. This reaction continues and results in the delignification of biomass. The lignosulfonates released from biomass get dissolved into the liquid fraction (prehydrolysates or cooking liquor) when the pH of the pretreatment liquor is below 7 without recondensing (Glasser, 1980). The residual

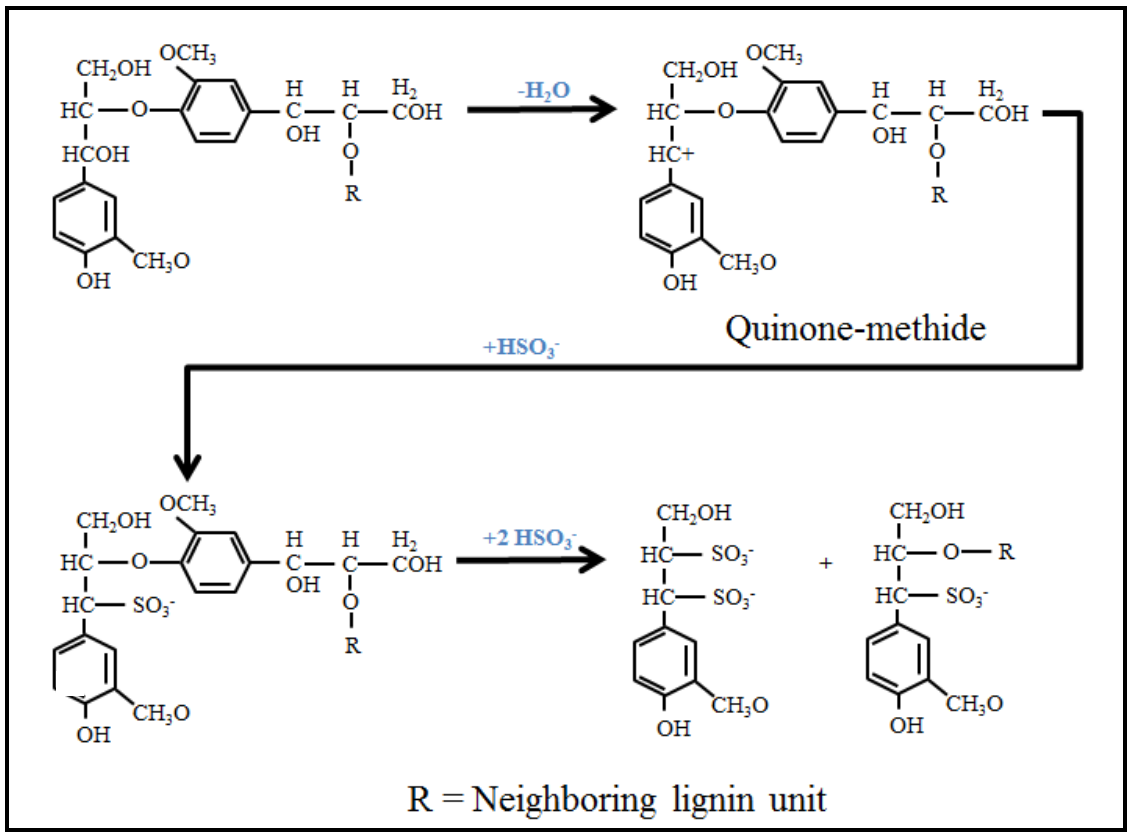

Fig. 3.4 Lignin sulfonation and degradation reaction – Adapted from Ingruber (1985).

lignin in biomass is also in a sulfonated form. Furthermore, lignin condensation can occur when lignin in the biomass has been sulfonated and bisulfite ions do not reach the carbonium ions. The unstable carbonium ions have a tendency to condense with another carbonium ions nearby, resulting in a condensation reaction (Ingruber, 1985). Additionally, the extractives (phenolic constituents) present in wood, such as flavotannins, pinosylvins and resorcinol, also favor condensation reactions, thereby blocking the active site of sulfonation and decreasing conversion yields (Bryce, 1980). For this reason, the bark of the wood had to be removed before conducting acid bisulfite pulping (Bryce, 1980). In general, delignification reactions are faster with acid bisulfite pulping (pH 1 to 2) in comparison to bisulfite pulping conducted at pH 4.

Besides playing an important role in delignification (sulfonation) reactions, sulfuric acid also plays a critical role in the removal of hemicellulose from the wood. Hemicellulose is prone to acid hydrolysis because of the low pH, higher temperatures and lower degree of polymerization of hemicellulose in comparison to cellulose (Ingruber, 1985). Xylans are almost completely hydrolyzed to xylose, which is evident from its predominance in the spent liquor (or prehydrolysate fraction) (Bryce, 1980; Ramachandriya et al., 2013; Zhu et al., 2009).

Sufficient time for delignification and hemicellulose removal is provided through impregnation and residence time (Bryce, 1980). Lignin removal increases the porosity (both pore width and volume) of the biomass, which favors an increased rate of enzymatic hydrolysis (Casey, 1980; Grethlein, 1985; Stone and Scallan, 1968). Additionally, the degree of polymerization of xylan and cellulose is reduced. Sulfonation of lignin also increases the hydrophilicity of lignin, which benefits subsequent enzymatic

hydrolysis process by decreasing non-productive binding of enzymes to lignin (Zhu et al., 2009). Fractionation of hemicellulose and lignin increases the surface area of cellulose available to the enzymes resulting in faster rates of hydrolysis (Ramos et al., 1992). SPORL has demonstrated success with softwoods (Lan et al., 2013; Shuai et al., 2010; Tian et al., 2010; Zhu and Pan, 2010; Zhu et al., 2009; Zhu et al., 2010a; Zhu et al., 2010b; Zhu et al., 2011).

3.3.2 Pretreatment severity

Temperature and time are the most important factors that affect pretreatments. Overend et al. (1987) came up with a reaction coordinate expression (R_0 or log R_0), also referred to as the severity parameter, that related the two variables. The severity factor was similar to the H factor (a model that relates time and temperature to determine the rate of delignification during pulping process) and P factor (a model that relates time and temperature to determine hemicellulose removal during pulping process), which were based on theories developed for paper and pulp making (Ramos, 2003). This expression can be given as:

$$R_0 = \boldsymbol{t}.\boldsymbol{e}^{[(\boldsymbol{T}-\mathbf{100})/\mathbf{14.75}]} \qquad (3.3)$$

where, T = Pretreatment temperature in °C;

t = Pretreatment hold time in min

100˚C is the reference temperature and 14.75 is value of activation energy where process kinetics obey first order law and are of first order kinetics.

The R_0 factor has been successfully used to predict process parameters such as sugar recovery yield, sugar yield from enzymatic hydrolysis, delignification and hemicellulose removal, but it has not worked well for pretreatments that involve mineral

acids (Ramos, 2003). For this reason, a combined severity factor was introduced by Chum et al. (1990) to account for the amount of acid catalysis that is shown in Eq. (3.4).

$$\text{Combined severity, } CS = \log R_0 - pH \qquad (3.4)$$

These models help to compare the results between various pretreatment conditions and processes. Generally, higher severity factors result in increased removal of hemicellulose, but also in increased degradation of sugar, thereby decreasing the wood glucan-to-ethanol yield (Alvira et al., 2010). Thus, a balance occurs between temperature, time and acid concentration must be determined for reduced economic cost with low sugar losses (Alvira et al., 2010). An acceptable combined severity factor depends on the type of feedstock. In the literature, sometimes severity factor was used to measure the digestibility of the material and at other times they are used to measure recovery of biomass components. As an example, Alfani et al. (2000) recommended a severity factor of 3.94 for achieving high glucose yield from steam explosion of wheat straw. While Heitz et al. (1991) observed that highest pentosan recovery of 65% was achieved when severity factor for pretreating *Populus tremuloides* was 3.8.

3.4 Ethanol production strategies

Pretreatment is followed by enzymatic hydrolysis where fermentable sugars are extracted from pretreated biomass and subsequently converted into ethanol. Four common strategies used for ethanol production from pretreated biomass are separate (or sequential) hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), separate hydrolysis and co-fermentation (SHCF) and simultaneous

saccharification and co-fermentation (SSCF). A diagrammatic representation of these strategies is shown Fig. 3.5.

3.4.1 Separate hydrolysis and fermentation (SHF)

During SHF, the pretreated biomass is hydrolyzed to monomeric sugars and subsequently fermented to ethanol. A major advantage of this process is that saccharification and fermentation can occur at their optimal conditions. The optimum temperature for saccharification lies between 45 and 50 °C (depending on the type of microorganism used for enzyme production), while the optimum for fermentation is between 30 to 37 °C (depending on the type of fermenting microorganism), respectively (Taherzadeh and Karimi, 2007). Inhibition of enzymes by glucose, cellobiose and hemicellulose sugars is the major drawback of this process (Xiao et al., 2004). It was demonstrated that the presence of 6 g L^{-1} of cellobiose can reduce cellulase activity by 60% (Philippidis and Smith, 1995; Philippidis et al., 1993). Similarly, about 50% inhibition of β-glucosidase was observed when glucose was supplemented between 20 and 100 g L^{-1} during cellobiose hydrolysis (Xiao et al., 2004). Likewise, cellulases were inhibited by 35% and between 10 to 15% in the presence of 20 g L^{-1} of glucose and 10 g L^{-1} of hemicellulose sugars (galactose, mannose and xylose), respectively, during cellulose hydrolysis (Xiao et al., 2004). Enzyme inhibition due to sugars becomes predominant when saccharification is conducted at high solids loading (Alvira et al., 2013; Jørgensen et al., 2007). Microbial contamination of prehydrolysate rich in monomeric sugars is also a concern of the SHF scheme (Taherzadeh and Karimi, 2007).

3.4.2 Simultaneous hydrolysis and fermentation (SSF)

SSF is the most common method of ethanol production where enzymatic

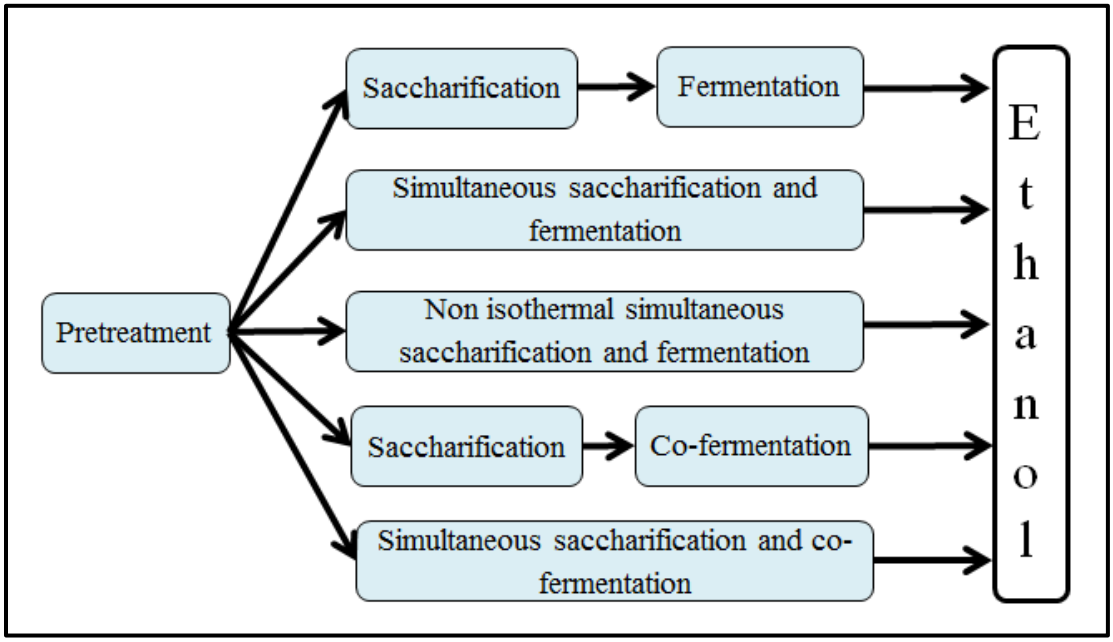

Fig. 3.5 Different strategies for ethanol production.

hydrolysis and fermentation occur simultaneously inside a reactor. The fermenting microorganism consumes glucose as soon as it is produced by enzymatic hydrolysis eliminating the issues with glucose inhibition of cellulases and the risk for contamination. A major disadvantage of SSF is that the enzymes work less efficiently as they are operated at sub-optimal temperatures. Other issues include inhibition of cellulases by ethanol (Wu and Lee, 1997). It was reported that 30 g L^{-1} of ethanol reduces cellulase activity by 25% (Wyman, 1996). Despite its drawbacks, SSF was 13% better in comparison to SHF with respect to ethanol yield over glucose (Drissen et al., 2009).

3.4.3 Non isothermal simultaneous hydrolysis and fermentation (NSSF)

Non-isothermal simultaneous saccharification and fermentation (NSSF) has been vaguely defined in the literature. In NSSF, saccharification and fermentation occurs simultaneously but in two separate reactors at the optimum temperatures of enzymatic hydrolysis and fermentation (Taherzadeh and Karimi, 2007). The sugar rich enzymatic hydrolysate can be continuously filtered and then pumped to the fermenter for ethanol production. This mode increases plant productivity and efficiency because the two steps are occurring concurrently and at optimum temperatures for saccharification and fermentations. A major drawback with SSF is the inability to conduct fermentations for long periods of time because the cells cannot be recycled as they are bound with biomass. Thus, Wu and Lee (1998) showed that ethanol yield attainable in 96 h with SSF was achieved in 40 h with NSSF mode with dilute acid pretreated switchgrass. Recently, Ishola et al. (2013) showed continuous operations using NSSF with enzymatic hydrolysis of SO_2 pretreated spruce conducted at 14.4% (w/w) solids loading. Hydrolysis was conducted in 2.5 L reactors with 10 and 12% solids loading. A microfiltration unit made

of polyethylene with a polypropylene housing with a filter module with a pore size of 0.2 µm, effective filtration area of 0.025 m^2 and a free flow area of 0.24 cm^2 was used to filter enzymatic hydrolysate. The filtrate was pumped to a 1.5 L fermentation tank for ethanol production. Results showed that 85% of theoretical ethanol yield could be obtained and the system was continuously operated for 4 weeks.

3.4.4 Separate hydrolysis and co-fermentation (SHCF)

Certain biomass types such as hardwoods and agricultural residues contain 5 to 20% xylan and arabinan (Hahn-Hägerdal et al., 2006), but the most common ethanol producing microorganisms, like *S. cerevisiae*, cannot utilize xylose and arabinose as a carbon source. The inability to utilize five-carbon sugars tremendously decreases the potential to produce high ethanol yields from hardwoods and agricultural residues. To overcome this issue, SHCF was introduced where the five-carbon and six-carbon sugars released by enzymatic hydrolysis can be fermented to ethanol using a microorganism that can concomitantly utilize five-carbon and six-carbon sugars. A major challenge of this scheme is the inability of microorganisms to effectively produce ethanol from glucose and xylose released from pretreated biomass. *Pichia stipitis* is a natural xylose fermenting microorganism that can ferment xylose and glucose to ethanol at reasonable yield and productivity, but experiences severe inhibitions with compounds generated from pretreatments (Hahn-Hägerdal et al., 1994). Microbial adaptation of yeast cultures to prehydrolysates have been carried out to avoid sugar losses that are commonly observed during detoxification methods (Tian et al., 2010). A SHCF study conducted by Tian et al. (2010) showed that *S. cerevisiae* Y5 strain adapted to prehydrolysate yielded 270 L of ethanol Mg^{-1} of lodgepole pine. However, this study utilized glucan and

mannan present in prehydrolysate to produce ethanol because of low xylan content (5.5%) of the raw material.

The fractionation of hemicellulose occurs differently with different pretreatment methods. For instance, alkaline pretreatments have low to moderate hemicellulose removal with pretreated biomass (Alvira et al., 2010) while acidic pretreatments removes a large portion of hemicellulose from the biomass (Alvira et al., 2010; Shuai et al., 2010; Zhu et al., 2009). If hemicellulose sugars are predominantly available in prehydrolysate, neutralization and/or detoxification may be required before the prehydrolysate could be utilized by the microorganisms (Sánchez and Montoya, 2013). Despite the challenges SHCF offers an advantage of lower capital costs since no additional vessel is required for pentose fermentation. It also allows hydrolysis and fermentation to be operated at their optimum temperatures and the option for developing continuous reactor schemes with cell recycling option.

3.4.5 Simultaneous saccharification and co-fermentation (SSCF)

During SSCF, enzymatic hydrolysis of pretreated biomass and fermentation of five-carbon and six-carbon sugars to ethanol occurs using one microorganism without additional enzymes. A key factor in SSCF is the ability of the microorganism to produce ethanol at high yields without being inhibited by any pretreatment and/or enzymatic hydrolysis products. Genetically modified strains of *S. cerevisiae* (Jin et al., 2010), *Z. mobilis* (McMillan et al., 1999) and *E. coli* (Kang et al., 2010) have been developed and validated in SSCF for ethanol production. Co-cultures of *P. stipitis* and *Brettanonymes clausennii* have also been employed for SSCF, yielding as high as 369 L of ethanol Mg^{-1} of aspen (Parekh et al., 1988). A complete process design and economic evaluation for

ethanol production using SSCF was demonstrated by Humbird et al. (2011). This process applied dilute acid pretreatment of corn stover followed by SSCF of glucose and xylose to ethanol using a genetically modified strain of *Z. mobilis*.

3.4.6 Strategies to improve ethanol titers

The cost associated with distillations can be drastically reduced by producing more than 4% (w/w) ethanol concentrations (Öhgren et al., 2006). Higher titers of ethanol can be achieved by operating enzymatic hydrolysis and/or fermentations at high solids (substrate) loading. Prehydrolysis and fed-batch operation of SSFs are the common modes of operation to enable ethanol production of high titers (Hoyer et al., 2013; Pessani et al., 2011). Prehydrolysis is carried out by liquefying lignocellulosic biomass at the optimum temperature for enzymatic hydrolysis for a defined time followed by addition of yeast or bacteria. On the other hand, fed-batch operation involves the addition of fresh pretreated substrate after the viscosity of lignocellulosic biomass decreases. Hoyer et al. (2013) showed that prehydrolysis of SO_2 impregnated steam exploded pretreated spruce at 48 ºC for 22 h prior to yeast fermentation resulted in an overall wood glucan-to-ethanol yield of 72% (final ethanol concentration 48 g L^{-1}), which was twelve folds higher than SSF that was conducted without prehydrolysis. A different study by Öhgren et al. (2007) showed a 21% improvement of ethanol productivity with 16 h prehydrolysis of steam pretreated corn stover when compared to traditional SSF. However, the final ethanol concentrations achieved in this study remained the same at 34 g L^{-1} with and without prehydrolysis.

Generally, a buildup of fermentation inhibitors such as furans, weak acids and phenolics are commonly observed as the solids loading increases (Larsson et al., 1999),

but yeast have an in-built mechanism to detoxify these compounds (Hahn-Hägerdal et al., 2006). For instance, furfural present during fermentations can be converted to furfuryl alcohol, which is less inhibitory to yeast (Larsson et al., 1999). This phenomenon has been successfully exploited by adjusting hydrolysate feed rate to match the inhibitor conversion capacity of yeast. With yeast having to deal with a lesser degree of detoxification, ethanol productivity can be increased (Rudolf et al., 2005). Pessani et al. (2011) reported that fed-batch operations lowered enzyme demand by 33% for hydrolyzing 12% dry solids hot water pretreated switchgrass.

Novel bioreactor designs have been employed to overcome technical barriers of mass transfer limitation and mixing difficulties for using lignocellulosic biomass at high solids loading. Bioreactor designs such as laboratory-scale roller bottle reactors (RBRs) (Roche et al., 2009), bench scale helical stirring bioreactors (Zhang et al., 2010), horizontal five chambered liquefaction reactors (Jørgensen et al., 2007), and laboratory scale peg mixers (Zhang et al., 2009) have been developed and validated. Roche et al. (2009) developed RBRs that were continuously rolled and provided sufficient mixing when tested up to 30% dry solids loading. RBRs resulted in 89% higher glucose concentrations than conventional shake flask reactors for hydrolyzing pretreated corn stover at 30% dry solids loading producing 170 g L^{-1} of glucose in 7 days of operation. Zhang et al. (2009) showed the feasibility of using a peg mixer, which is typically used in pulping operations, for high solids enzymatic hydrolysis of extensively delignified pretreated hardwoods. The use of a peg mixer achieved 210 g L^{-1} of glucose from organosolv pretreated poplar during enzymatic hydrolysis at 30% solids loading. Additionally, the fermentability of the enzymatic hydrolysate obtained at 20% solids

loading was successfully demonstrated by achieving 63 g L^{-1} of ethanol (83% of theoretical) in 96 h. A different study conducted by Jørgensen et al. (2007) showed that hydrolyzing pretreated wheat straw in a five chambered liquefying reactor resulted in 76 and 86 g of glucose kg^{-1} of enzymatic hydrolysate at 20% and 40% dry solids loading, respectively. However, only 60% and 35% glucan-to-glucose yield was achieved while operating at 20% and 40% solids loading, respectively, indicating strong product inhibitions as the solids loading was increased. The same study also conducted prehydrolysis for 8 h at 50 °C followed by a SSF and reported 48 g of ethanol per kg of enzymatic hydrolysate of concentrations at 40% dry solids loading. Another study compared the performance of a helical impeller and common Rushton impeller during SSF of steam exploded corn stover and observed 51 g L^{-1} and 44 g L^{-1} of ethanol with the two impellers, respectively (Zhang et al., 2010). The same study also observed a thirty fold reduction in power consumption with helical impellers compared to Rushton impellers.

3.5 References

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CHAPTER IV

DEVELOPMENT OF AN EFFICIENT PRETREATMENT PROCESS FOR ENZYMATIC SACCHARIFICATION OF EASTERN REDCEDAR

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"Ramachandriya, K.D., Wilkins, M.R., Hiziroglu, S., Dunford, N.T., Atiyeh, H.K. 2013. Development of an efficient pretreatment process for enzymatic saccharification of Eastern redcedar. *Bioresource Technology*, 136, 131-139."

Abstract

This study investigates the potential for extracting sugars from the polysaccharides of Eastern redcedar. Pretreatment temperature, time, sulfuric acid loading, sodium bisulfite loading and impregnation time were varied using factorial treatment design experiments for identifying near optimal overall wood glucan-to-glucose yields during acid bisulfite pretreatments. The highest overall wood glucan-to-glucose yield of 87% was achieved when redcedar was impregnated with pretreatment liquor containing 3.75 g of sulfuric acid per 100 g of dry wood and 20 g of sodium bisulfite per 100 g of dry wood at 90 °C for 3 h followed by increasing the temperature to 200 °C with a hold time of 10 min. Hemicellulose and lignin removal during pretreatments made the substrate amenable to enzymatic hydrolysis using 0.5 mL of Accelerase® 1500 g^{-1} of glucan at 2% (w/w) solid loading. Preliminary mass balances showed 97% glucan recovery at pretreatment condition with 87% overall wood glucan-to-glucose yield and 59% delignification.

Keywords: Sodium bisulfite pretreatment, Enzymatic saccharification, Softwood, Bioenergy.

4.1 Introduction

Eastern redcedar (*Juniperus viriginiana* L.) is a member of the cypress family (*Cupressaceae*) and is one of the most widely distributed conifers in the US. It is commonly found in central and eastern US. Although generally referred to as cedar, it is actually one of 13 juniper species in the US (Hiziroglu et al., 2002). Eastern redcedar (hereafter referred to as redcedar) is considered a very invasive species as it adapts well to different soils, climactic conditions and topographies (Hiziroglu et al., 2002). Redcedar's encroachment in the Great Plains of the US is a very serious problem. Between 1985 and 2015, a 231% increase in redcedar acreage is estimated in Oklahoma (McKinley, 2012). Recent studies show that redcedars are spreading at a rate of 57 trees per hectare per year in the prairie lands of Kansas (Price et al., 2010) and at a rate of 121,000 hectares per year in the plains of Oklahoma (McKinley, 2012). According to an estimate made by McKinley (2012), 26% of the overall land base of Oklahoma will be covered with redcedars by 2015. The encroachment of redcedars has brought many ecological concerns to farmers, ranchers and wildlife species, reduced ground water yields and an increased risk of wildfires which resulted Oklahoma a massive estimated loss of $447 million in 2012 in Oklahoma (Natural Resources Conservation Service. 2013).

Common control strategies for the spread of redcedars in Oklahoma are prescribed fires, application of pesticides and mechanical clearing. Mechanical clearing of redcedar, although highly encouraged due to its selectivity, is cost intensive unless a valuable use for the wood can be identified that can use low quality wood not suitable for lumber. Processing units utilizing redcedar wood for oil extraction are available, but they

have difficulties using the wood after oil extraction. Mulch application of oil extracted redcedar wood will be restricted as it will no longer have the ability to deter pests due to the loss of aromatic oil. A viable option that has not been focused on is the conversion of polysaccharides in redcedar into transportation fuels such as ethanol and butanol. Such a conversion process will provide the flexibility to use woody biomass of any quality and can make use of redcedar wood after oil extraction.

Redcedar encroachment has resulted in the availability of enormous amounts of redcedar wood across the Great Plains. A recently published report estimated the availability of 11.5 million dry metric tons of above ground redcedar biomass in just 17 counties in Northwest Oklahoma (Starks et al., 2011). Assuming, 80% of the above ground biomass is wood and 75% of the glucan in redcedar can be converted into ethanol (McKinley, 2012), 2 billion L (530 million gallons) of ethanol can be produced from existing redcedar in this small region. When the geographical distribution of redcedar across the US is taken into account, redcedars can easily become a promising source for cellulosic biofuels.

Redcedar is a softwood and generally softwood species are more difficult candidates for bioconversion processes to produce biofuels than hardwoods and agricultural residues because of their rigid structure and high lignin content (Ramos, 2003). Redcedar contains on a dry basis 40.3 ± 1.5 % glucan, 8.5 ± 0.0 % xylan, 2.0 ± 0.6 % galactan, 1.4 ± 1.0 % arabinan, 6.0 ± 1.2 % mannan and 33.7 ± 0.6 % lignin (mean ± 1 standard deviation) (Pasangulapati et al., 2012). The lignin content of redcedar is 5 to 25% higher than other softwoods investigated for ethanol production such as spruce, Douglas fir and pine (Zhu and Pan, 2010).

Pretreatment of lignocellulosic biomass is the first step in the biochemical production of ethanol where the biomass is converted to a form amenable to enzymatic hydrolysis and fermentation. The complex physical and chemical nature of softwoods limits the number of pretreatment options available. Therefore, the selection of a pretreatment process is critical due to the differences in the physical and chemical modes of action during different pretreatment technologies. Hot water pretreatment and alkaline pretreatment methods based on ammonia, such as ammonia recycle percolation (ARP), soaking in aqueous ammonia (SAA) and ammonia fiber expansion (AFEX), have not shown success in achieving high glucose yields after enzymatic hydrolysis of pretreated wood (Ramos, 2003). Acidic pretreatments such as dilute acid pretreatments (Nguyen et al., 1998), steam explosion pretreatments assisted with acids (Kumar et al., 2010; Monavari et al., 2009) and sulfite pretreatment to overcome the recalcitrance of lignocellulose (SPORL) (Shuai et al., 2010; Zhu and Pan, 2010) have been relatively more successful than hot water and ammonia pretreatments in achieving high glucose yields.

SPORL is a recently studied technology for softwood pretreatments (Zhu et al., 2009). It is a variant of sulfite pulping that was used to produce pulp and paper from woody biomass. Bisulfite salt (made of Na^{+}, NH^{4+}, Mg^{2+}, K^{+} or Ca^{2+}) and sulfuric acid are the two chemicals required for this process. These chemicals play an important role in achieving delignification using sulfonation reactions resulting in a lignosulfonate rich prehydrolysate (Bryce, 1980). The presence of sulfuric acid also results in a significant removal of hemicellulose (Bryce, 1980; Zhu et al., 2009). Numerous studies have shown

the effectiveness of SPORL with softwoods (Shuai et al., 2010; Zhu and Pan, 2010; Zhu et al., 2009).

The current study reports on a modified SPORL process for redcedar. Unlike previous studies using SPORL, finely ground biomass screened with a 2 mm screen was used in the present work as mechanical size reduction enhances biomass digestibility (Sun and Cheng, 2002). Pretreatment affects subsequent processes and hence its optimization is the first and most important step. Optimization experiments are generally sequential in nature and begin with screening experiments that aim to identify the more important factors affecting a process, while eliminating the less important ones (Myers and Montgomery, 1995). These screening studies are often referred as phase zero of optimization experiments (Myers and Montgomery, 1995). The next phase (phase I) of process optimization is referred to as the path of steepest ascent. During this phase, levels of factors are adjusted such that near optimum responses are obtained (Myers and Montgomery, 1995). The objective of this study was to determine the near optimal pretreatment conditions for the maximum wood glucan-to-glucose yield from redcedar. Factors were identified that affect the acid bisulfite process, which were pretreatment time, pretreatment temperature, sulfuric acid loading (g per100 g of dry wood), sodium bisulfite loading (g per100 g of dry wood) and impregnation time. These factors were varied sequentially using factorial treatment designs to identify the factor levels that result in the greatest yield of glucose from enzymatic hydrolysis of pretreated redcedar.

4.2 Materials and methods

4.2.1 Biomass

Eastern redcedar (*Juniperus virginiana* L.) chips were acquired from a local manufacturer in Oklahoma. The chips contained both heartwood and softwood fractions of the trunk from redcedar trees that were 20 to 25 years. The biomass was ground using a Thomas-Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, USA) equipped with a 2 mm screen. After grinding, the moisture content of the biomass was determined by a convection oven method (Sluiter et al., 2008a). Biomass was stored in zip-lock bags at room temperature prior to pretreatments and/or compositional analysis. The standard procedure developed by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008c) was used for compositional analysis of redcedar and is detailed in Pasangulapati et al. (2012).

4.2.2 Pretreatments

Acid bisulfite pretreatments were done in a 1-L bench top pressure reactor (Parr series 4250, Parr Instrument Company, Moline, IL, USA) equipped with an agitator, a heater and a control unit. The reactor was initially loaded with 100 g of dry biomass and then filled with a mass of pretreatment liquor to achieve a liquid-to-solid mass ratio of 5:1 resulting in a total mass of 600 g in the reactor. The pretreatment liquor was composed of deionized water, sulfuric acid and/or sodium bisulfite. The concentrations of these chemicals were varied for different factorial design experiments. The range of sulfuric acid loadings and sodium bisulfite loadings varied between 0.00 and 5.00 g per 100 g of dry wood and 0 and 20 g per 100 g of dry wood, respectively. The reactor was agitated at 150 rpm and biomass was soaked at 90 °C for 3 h for all studies except the

preliminary screening study on chemical loading. This soaking process is commonly referred as impregnation, which is commonly used in the pulping process (Bryce, 1980). Impregnation allows sufficient time for the diffusion of chemicals to different parts of the wood for delignification (Bryce, 1980). At the end of 3 h, the reactor temperature was increased to a desired set point and held at that temperature for a desired time. In this study, different time-temperature combinations were investigated. Temperature was varied between 180 °C and 220 °C and hold time was varied between 5 min and 40 min. At the end of pretreatment hold time, the reactor was cooled in an ice bath until the temperature was less than 55 °C. For the study on hold time, temperature and bleed-out (section 4.3.2), steam and other vapors were bled-out through a check valve to reduce reactor pressure and then the reactor was cooled in an ice bath. After cooling the reactor, the solid and liquid fractions were separated using vacuum filtration through a Whatman #5 filter paper. About 5 to 6 g of sample were taken before washing the solids and dried in an oven at 105 °C to determine the moisture content of dry solids after pretreatment (Sluiter et al., 2008a). The wet solids were then rinsed with 500 mL of deionized water at 60 °C four times to remove soluble sugars and fermentation inhibitors. The moisture content of washed pretreated solids was also determined using a standard NREL procedure (Sluiter et al., 2008a). The pretreated solids were stored in plastic zip lock bags at 4 °C until use for enzymatic hydrolysis.

The composition of pretreated solids was determined using the standard NREL procedure without extraction (Sluiter et al., 2008c). Extraction of pretreated solids was not required because the non-structural components of biomass were extracted during pretreatment (Sluiter et al., 2008c). The composition of degradation products in

prehydrolysate was also determined using the standard NREL procedure (Sluiter et al., 2008b) using a HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID) with 0.01 N sulfuric acid as eluent at 0.6 mL min^{-1} with a 50 min run time. Composition of sugars in the prehydrolysate was determined after acid hydrolysis of prehydrolysate to break down the polysaccharides released after pretreatments using a protocol developed by NREL (Sluiter et al., 2008b) and HPLC analysis as described above.

4.2.3 Enzymes and enzymatic hydrolysis of biomass

Accelerase 1500 kindly provided by Genencor Inc. (Palo Alto, CA, USA) was the enzyme cocktail used for this study. Enzymatic hydrolysis of pretreated biomass was done to determine the efficacy of pretreatments. Enzyme loading of 0.5 mL g^{-1} of glucan (50 FPU g^{-1} glucan) was used for these studies. This loading was recommended by the manufacturer as a starting point for optimization. A low solid loading of 2% (w/w) was used to determine the efficacy of pretreatments. Such a low solid loading provides unbiased determination of efficacy of pretreatments as the inhibitory effect of glucose is minimal. Enzymatic hydrolysis was carried at pH 5.0 using 0.05 M sodium citrate buffer and 50 °C in an incubator shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA) at 200 rpm. One and a half milliliters of sample were withdrawn at 0, 6, 12, 18, 24, 48, 72 and 96 h to determine the amount of sugar released during enzymatic hydrolysis. The samples were then centrifuged at 13,000 rpm for 10 min using a benchtop microcentrifuge (Fisher Scientific, Pittsburgh, PA). The supernatant was collected, filtered through 0.45 µm nylon syringe filters (VWR International, West Chester, PA,

USA) and frozen until analyzed. Enzymatic hydrolysis was stopped at 96 h as a very small amount of glucose was produced after 72 h of hydrolysis.

Enzyme activity was measured using the standard protocol developed by NREL (Adney and Baker, 2008). Analytical grade chemicals required for the assay were purchased from Sigma Aldrich (St. Louis, MO).

4.2.4 Statistical analysis

Analysis of variance was conducted using the generalized linear model (GLM) procedure in SAS release 9.2 (SAS, Cary, NC, USA). P values were calculated for each analysis and are shown in the text. The correlation between glucan-to-glucose yields of pretreated redcedar and delignification was conducted using the correlation (CORR) procedure in SAS.

4.3 Results and Discussion

The approach that was taken during screening experiments and studies conducted to find the near optimum responses using a series of factorial design experiments are discussed in the following sections.

4.3.1 Preliminary screening study on chemical loading

Sulfuric acid and sodium bisulfite are the two chemicals that play an important role in delignification of biomass in acid bisulfite pretreatment; thus, their loadings play an important role in improving biomass digestibility. Zhu et al. (2009) found that sulfuric acid loadings of 1.80 to 3.75 g per 100 g of dry wood and sodium bisulfite loadings of 8 to 10 g per 100 g of dry wood at 180 °C and 30 min resulted in more than 90%

conversion of glucan-to-glucose during the enzymatic hydrolysis of SPORL pretreated spruce and red pine. Another study carried out by Shuai et al. (2010) also resulted in 91% glucan conversion from SPORL pretreated spruce using a sulfuric acid loading of 5 g per 100 g of dry wood and sodium bisulfite loading of 9 g per 100 g of dry wood. The temperature and time used by Zhu et al. (2009) and Shuai et al. (2010) for pretreatment of softwoods was 180 °C and 30 min, respectively. Thus, it was hypothesized that under these conditions of pretreatment temperature and time, pretreated redcedar could produce similar glucose yields.

A factorial design experiment was conducted with two factors: sulfuric acid loading (g per100 g of dry wood) and sodium bisulfite loading (g per 100 g of dry wood). The levels of sulfuric acid loading were 0.00, 1.25 and 2.50 and sodium bisulfite tested was 0, 5 and 10. Nine treatment combinations were tested without replication on pretreatments at 180 °C and 30 min to observe the effect of chemical loading on the yield of glucan-to-glucose in pretreated redcedar during enzymatic hydrolysis. Though the pretreatments were not replicated, two subsamples were taken from each pretreatment for enzymatic hydrolysis. Replications of pretreatments were not carried out due to limited availability of biomass and the large experimental units used. This experimental design allowed for screening through different types of pretreatment methods. For example, the 0% sulfuric acid and 0% sodium bisulfite condition represents a hot water pretreatment. Other conditions with no sodium bisulfite represent dilute acid pretreatments. Similarly, conditions with sodium bisulfite as the only chemical represent bisulfite pretreatments. Acid bisulfite pretreatment was represented when both sulfuric acid and sodium bisulfite were present. These experiments were conducted in the absence of impregnation because

a study by Zhu et al. (2009) showed that impregnation did not significantly affect acid sulfite pretreatment of softwood. After pretreatments, biomass was washed and enzymatic hydrolysis was performed.

The glucan-to-glucose conversion yields of pretreated redcedar at various chemical loadings are shown in Fig. 4.1. Glucan-to-glucose yield (Eq. 4.1) for the enzymatic hydrolysis step is a measure of digestibility of the biomass after pretreatments Eq. 4.1 is based on the glucan content of the biomass after pretreatments and not the content of glucan in the untreated wood; hence, it should not be confused with overall wood glucan-to-glucose yield (%).

$$\text{Glucan-to-glucose yield (\%)} = \frac{\text{Glucose(t)-Glucose(0)}}{\text{SL} \times \text{f(Pretreated biomass)} \times 1.11} \times 100 \qquad (4.1)$$

Where, Glucose(t) and Glucose(0) are the glucose concentrations in % (w/v) at time t and 0 hours, respectively, SL is the dry solid loading used for enzymatic hydrolysis which was 2% (w/w), and f(Pretreated biomass) represents the fraction of glucan in pretreated biomass. 1.11 is the conversion factor for glucan to glucose.

Hot water pretreatment had the lowest glucan-to-glucose yield (Fig. 4.1) indicating that redcedar pretreatments require chemical catalysts to break down their complex structure. Untreated redcedar was also hydrolyzed enzymatically under the same solid and enzyme loadings produced 2.9% glucan-to-glucose yield, which was much lower than spruce that resulted in 20% glucose-to-glucan yield (Shuai et al., 2010). Dilute acid pretreatment did not show much improvement in glucan conversion in comparison to hot water pretreatment. Statistical analysis showed that the main effects for sulfuric acid ($p = 0.203$) and sodium bisulfite ($p = 0.080$) were not significant. However, the condition of a sulfuric acid loading of 2.50 g per 100 g of dry wood and

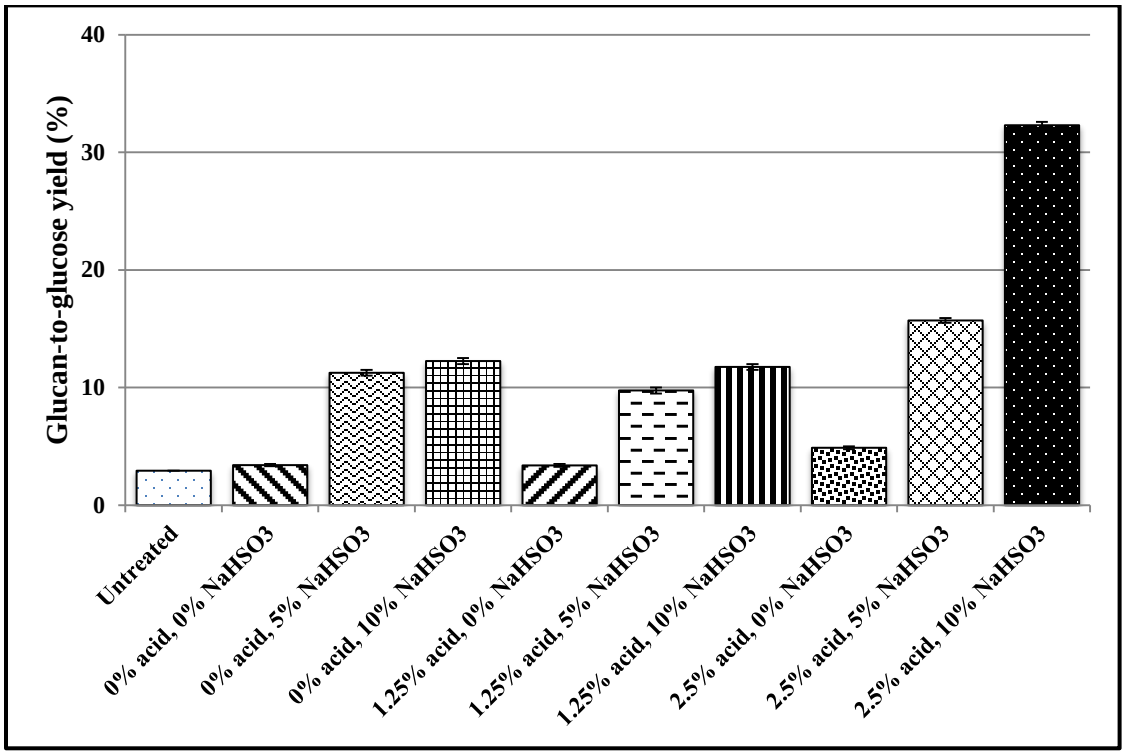

Fig. 4.1 Effect of chemical loading on pretreatment of Eastern redcedar at 96 h of enzymatic hydrolysis.

Acid and $NaHSO_3$ represents sulfuric acid and sodium bisulfite, respectively. The number provided in x-axis labels represents the loading of chemicals in g per 100 g of dry wood. Glucan-to-glucose yield of pretreated redcedar is defined in Equation 4.1. Error bars shows the standard error of the subsamples within each treatment combination.

sodium bisulfite loading of 10 g per 100 g of dry wood resulted in the highest glucan-to-glucose yield of 32%, which was 105% to 900% greater than the yields at other chemical loadings. This is probably due to an interaction between the sulfuric acid and bisulfite main effects that were confounded with the error term due to lack of replication. The results obtained in this study are much lower than studies conducted on other softwoods using the SPORL process at similar conditions (Shuai et al., 2010; Zhu et al., 2009). Low glucan-to-glucose yield could be attributed to the lignin content of redcedar. Lignin accounted for 33.7% of redcedar dry matter while other softwoods such as ponderosa pine, spruce, red pine and Douglas fir had 26.9, 28.3, 29 and 32% lignin, respectively (Zhu and Pan, 2010). Mass balances before and after pretreatment showed hemicellulose loss, but no lignin loss. Lignin loss during pretreatments (also commonly referred as delignification) is defined in Eq. 4.2 and is obtained by performing a component balance before and after pretreatment:

$$\text{Lignin loss, w/w (\%)} = \frac{\text{Lignin}_{bp}\text{-Lignin}_{ap}}{\text{Lignin}_{bp}} \times 100 \qquad (4.2)$$

where, Lignin_{bp} is the mass of lignin in redcedar before pretreatment (in g) and lignin_{ap} is the mass of lignin in pretreated redcedar (in g). It was hypothesized that higher pretreatment severity was required to increase the enzymatic digestibility of redcedar. Sulfuric acid loading of 2.50 g per 100 g dry wood and sodium bisulfite loading of 10 g per 100 g dry wood was applied for the next study, which was intended to determine the effect of higher pretreatment termperatures and hold times.

4.3.2 Effect of hold time, temperature and bleed-out

The hypothesis for this experiment was that by increasing pretreatment temperature and including a 3 h impregnation at 90 °C, the chemicals added would obtain sufficient time to delignify biomass and improve glucan-to-glucose yields from the pretreated redcedar. Additionally, it was also hypothesized that a sudden reduction of the pretreatment reactor pressure by opening a needle valve, similar to a bleed-out process (but not explosion) may have some degree of explosive decompression of the cell wall components and could improve the digestibility of the material. The terms 'bleed-out' and 'explosion' have been used interchangeably in the literature. An explosion is generally referred to as a sudden violent discharge of pressure into a collecting tank that creates explosive decompression of fibers (Ramos, 2003). Bleed-out generally refers to the process of pressure release that involves reducing the pressure through a needle valve (Ramos, 2003). With the current set up of the pretreatment reactor-needle valve configuration, explosion experiments were difficult to perform and hence "bleed-outs" were incorporated.

For the present study, sulfuric acid and sodium bisulfite loadings were held constant at 2.50 g per 100 g dry wood and 10g per 100 g dry wood, respectively. Factors varied were temperature, hold time and bleed-out. The three levels of temperature selected were 180 °C, 200 °C and 220 °C and the two levels of pretreatment time selected were 10 min and 20 min. Pretreatments were conducted with and without bleed-out. A total of 12 factorial combinations were evaluated without replication of pretreatments. The response variable for these experiments was glucan-to-glucose yield (%) of

pretreated redcedar (Eq. 4.1). Though the pretreatments were not replicated, two subsamples were taken from each pretreatment for enzymatic hydrolysis.

Fig. 4.2 shows the glucan-to-glucose yield (%) obtained during the different pretreatment combinations. An interesting trend observed in this study was the effect of bleed-out that significantly lowered the conversion yields at all levels of temperature and time (Fig. 4.2). The reduction in glucan-to-glucose yields with bleed-out when compared to the treatment conditions without bleed-out varied between 34% and 56%. Similar observations have been reported in the literature with steam explosion experiments (Pan et al., 2005). The reduction of yields was possibly due to lignin condensation on biomass when pressure was suddenly released (Pan et al., 2005). The severity of pretreatment and lignin condensation could be responsible for lowering yields. This shows that bleed-out during pretreatment was not favorable at the conditions tested.

In this study, pretreatments at 200 °C for 20 min and 220 °C for 10 min resulted in comparable glucan-to-glucose yields of about 31%, which was similar to the yield (32%) achieved previously (Fig. 4.1). A maximum lignin loss of only 1% was observed. The lack of lignin loss probably was responsible for lower glucan yields of pretreated redcedar. The pretreatment condition at 200 °C and 20 min was selected for subsequent studies because it resulted in 206% lower amounts of total fermentation inhibitors such as formic acid, acetic acid, levulinic acid, 5-hydoxy-methyl-furfural (HMF) and furfural than the pretreatment at 220°C for 10 min (data not shown).

4.3.3 Effect of chemical loading and surfactants

The existing literature on softwood pretreatments with SPORL process have used sulfuric acid loadings up to 5 g per 100 g dry wood (Shuai et al., 2010; Zhu et al., 2009)

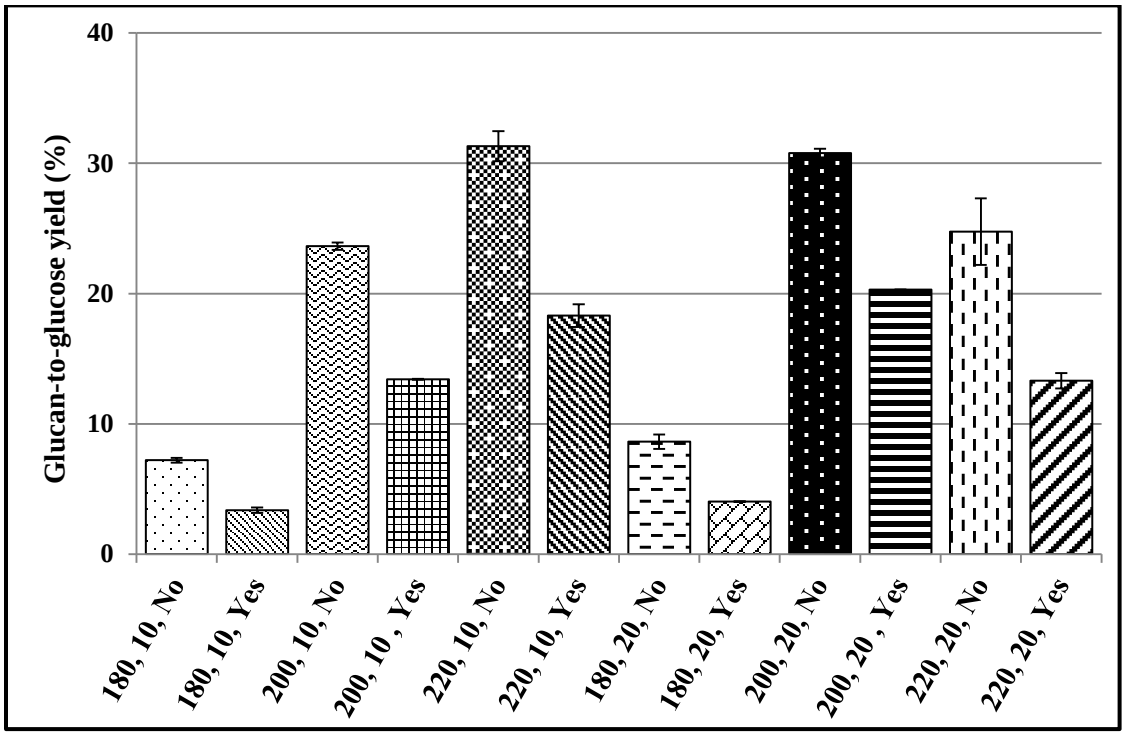

Fig. 4.2 Effect of pretreatment temperature, time and bleed-out on pretreatment of Eastern redcedar at 96 h of enzymatic hydrolysis.

The first number represents pretreatment temperature in °C and the second represents hold time in min. Pretreatments with bleed-out are designated as 'Yes' and without bleed-out are represented by 'No'. Sulfuric acid and sodium bisulfite loading were 2.5 and 10 g per100g of dry wood. Glucan-to-glucose yield of pretreated redcedar is defined in Equation 4.1. Error bars shows the standard error of the subsamples within each treatment combination.

while sodium bisulfite loadings were not above 10 g per 100 g dry wood (Zhu et al., 2009) to produce highly digestible (>90% glucan-to-glucose yield) pretreated biomass. However, acid bisulfite pulping processes for pulp manufacture have used sodium bisulfite loadings as high as 25 g per 100 g dry wood (Bryce, 1980). Thus in the present work, the hypothesis was that increased sulfuric acid and sodium bisulfite loadings would result in greater glucan-to-glucose yields of pretreated redcedar. Sulfuric acid was tested at two levels (g per 100 g dry wood): 3.75 and 5.00, and sodium bisulfite was tested at two levels (g per 100 g dry wood): 10 and 15. Thus, four combinations of pretreatments were performed without replication. Impregnation time and temperature were 3 h and 90 °C and the pretreatment time and temperature were kept constant at 20 min and 200 °C for all pretreatments.

Table 4.1 shows the glucan-to-glucose yields (%) of pretreated redcedar at the four different conditions. A large improvement in glucan-to-glucose yields was achieved in this study in comparison to the two previous experiments that employed lower chemical loadings. Around 62% glucan-to-glucose yield was obtained with pretreatment conditions containing sulfuric acid and sodium bisulfite loadings of 3.75 g per 100 g and 15 g per 100 g dry wood, respectively. A decrease in glucan-to-glucose yields between 51.0% and 53.9% was observed with an increase in sulfuric acid loading from 3.75 to 5 g per 100 g of dry wood, showing that 3.75 g per 100 g dry wood loading was sufficient for the pretreatment process ($p = 0.0225$). A significant increase in glucan-to-glucose yield from 44.6% to 60.3% was obtained when sodium bisulfite loading was increased from 10 to 15 g per 100 g dry wood ($p = 0.0041$). Sodium bisulfite is an important chemical used during sulfonation and delignification and, hence, its higher loading was important.

Table 4.1 Effect of chemical loading and surfactant during enzymatic hydrolysis of pretreated redcedar.

Sulfuric acid loading (g per100 g biomass)	Sodium bisulfite loading (g per100 g biomass)	Delignification (%)	Yield (%) without PEG-8000	Yield (%) with PEG-8000
3.75	10	2.41	45.93 ± 0.82	56.44 ± 0.55
3.75	15	12.01	61.81 ± 0.65	68.79 ± 0.72
5	10	4.13	44.49 ± 1.50	50.45 ± 0.20
5	15	6.59	58.87 ± 0.41	64.22 ± 0.54

Yield listed are averages ± standard deviation for two subsamples at 96 h of enzymatic hydrolysis.

Yield (%) represents the glucan-to-glucose yield of pretreated redcedar defined in Eq. 1.

PEG-8000 is a surfactant and stands for poly-ethylene glycol-8000.

Since redcedar had more lignin content in comparison to other softwoods, more sodium bisulfite was required for pretreating redcedar. From the mass balances around the pretreatment reactor, it was observed that a sulfuric acid and sodium bisulfite loading of 3.75 and 15 g per 100 g of dry wood, respectively, resulted in 12% lignin loss (Table 4.1).

Lignin in biomass can act as a physical barrier that limits enzyme access to cellulose. This problem will be pronounced with softwoods that have higher lignin contents than hardwoods and agricultural residues. Lignin binds with cellulose andhemicellulose molecules using ester, ether or ketal bonds (Ramos, 2003). The vicinity of large amounts of lignin to carbohydrates in softwoods poses a problem because it results in formation of non-specific bonds between enzymes and lignin, which inhibits enzyme activity. Because only 12% lignin loss was achieved during the best pretreatment condition obtained, it was hypothesized that the large amounts of residual lignin significantly affected enzymes through non-specific bonds. To test this hypothesis, poly-ethylene glycol-8000 (PEG-8000) at 0.05 g g^{-1} glucan was added to pretreated redcedar with the four treatment combinations. The selection of PEG was based on previous reports that suggested that PEG binds with lignin, thereby preventing enzyme-lignin interaction (Börjesson et al., 2007).

An increase in glucan-to-glucose yield between 8 and 20% was observed with addition of PEG-8000 (Table 4.1). Our results are similar to other studies that have shown an increase in enzymatic hydrolysis yields with addition of surfactants (Börjesson et al., 2007). Our hypothesis on non-specific bonds between enzymes and lignin in

biomass was supported by this data and increased lignin removal from redcedar was determined to be essential to achieve greater glucan-to-glucose yields.

4.3.4 Effect of bisulfite loading, hold time and impregnation

The previous experiment showed that an increase of sodium bisulfite loading from 10 to 15 g per 100 g dry wood improved the glucan-to-glucose yield of pretreated redcedar by 35%; thus, it was hypothesized that increased delignification and digestibility of pretreated biomass could be achieved by increasing the loading of sodium bisulfite from 15 to 20 g per 100 g dry wood. Another hypothesis was that the hold time of 20 min could be insufficient for delignification reactions to occur. Thus, a factorial experiment was designed to validate the hypothesis. The two factors selected were bisulfite loading and pretreatment time. Two levels of bisulfite loading (g per 100 g dry wood): 15 and 20, and three levels of pretreatment time: 20, 30 and 40 min, were selected. An experiment was conducted with six factorial combinations without replication and the enzymatic hydrolysis of pretreated, washed solids was done as mentioned previously with two subsamples per treatment combination.

Fig. 4.3 shows the glucan-to-glucose yields (%) of pretreated redcedar obtained with different factorial combinations. Pretreatment time ($p < 0.0002$) and bisulfite loading ($p < 0.0026$) significantly affected glucan-to-glucose yields. Increased pretreatment time from 20 min to 40 min resulted in a decrease in glucan-to-glucose yields. Irrespective of pretreatment time, pretreatments carried at 20% sodium bisulfite averaged 89% glucan-to-glucose yield while 15% sodium bisulfite loadings averaged only 48%. Digestibility of pretreated solids was enhanced due to the removal of lignin. A significant correlation ($p = 0.0002$) between lignin loss and glucan-to-glucose yields

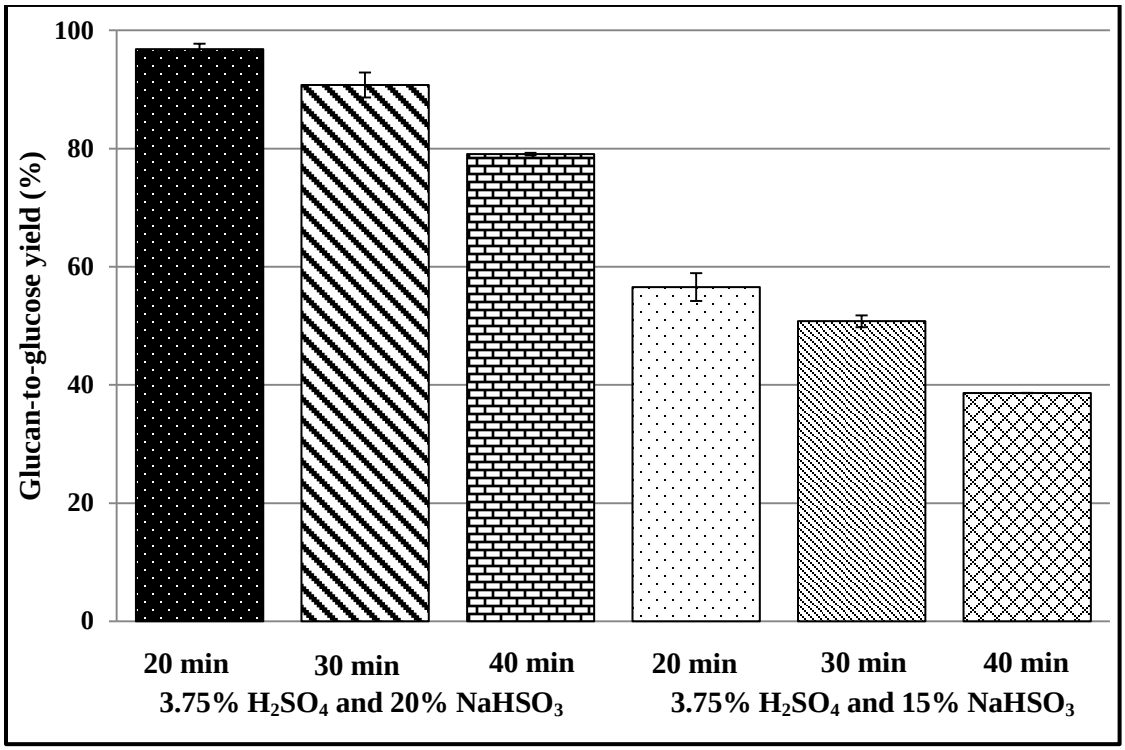

Fig. 4.3 Effect of sodium bisulfite and hold time on pretreatment of Eastern redcedar at 96 h of enzymatic hydrolysis.

The loading of chemicals shown in % is based on g per100g of dry wood. Glucan-to-glucose yield of pretreated redcedar is defined in Equation 4.1. Error bars shows the standard error of the subsamples within each treatment combination.

was observed. The pretreatment condition with 3.75 g of sulfuric acid per 100 g of dry wood and 20 g of sodium bisulfite per 100 g of dry wood resulted in 54% lignin loss. These observations were consistent with other experiments conducted with SPORL technology, although the lignin loss in other studies did not exceed 32% (Shuai et al., 2010; Zhu et al., 2009). The lignin removed from the biomass gets collected as lignosulfonates in the pretreatment liquor, which have market potential as dispersants and/or could be upgraded to vanillin (Fatehi and Ni, 2011; Glasser, 1980; Yu et al., 2012).

Another experiment was done to observe the effect of impregnation time on pretreatments. Pretreatment was done for 20 min at 200 °C with 3.75 g per 100 g dry wood and 20 g per 100 g dry wood sulfuric acid and sodium bisulfite loading, respectively. The wood was either impregnated with the pretreatment solution for 3 h at 90 °C or not impregnated at all before pretreatment. Our observations indicate that the experimental condition with no impregnation achieved only 55% glucan-to-glucose yield of pretreated redcedar (data not shown) while the control provided 89% glucan-to-glucose yield of pretreated redcedar. This indicates that impregnation time plays an important role in improving the digestibility of the redcedar. Impregnation allows the chemicals to penetrate the wood chips and bring uniform delignification (Bryce, 1980). Our results are contradictory to the observations of Zhu et al. (2009), which reported that 1 to 3 h of impregnation time at 90 °C did not improve the digestibility of softwoods after SPORL pretreatment. This could be due to the higher lignin content and the nature of lignin in redcedar compared to other softwoods.

Our hypothesis on achieving higher delignification with increasing sodium bisulfite loading was supported; however, 47% of glucan present in the original wood was lost during pretreatment with 20 g per 100 g dry wood sodium bisulfite loading, 3.75 g per 100 g sulfuric acid loading at 200 °C and 20 min. Glucan loss was calculated by mass balances around the pretreatment reactor using Eq. 4.3.

$$\text{Glucan loss, w/w (\%)} = \frac{\text{Glucan}_{bp} - \text{Glucan}_{ap}}{\text{Glucan}_{bp}} \times 100 \quad (4.3)$$

where, glucan_{bp} is the mass of glucan in redcedar before pretreatment (in g) and glucan_{ap} is the mass of glucan in pretreated redcedar (in g). Pretreatment time of 20 min was long enough to produce sufficient delignification, but at the expense of high glucan loss. This could be due to the higher severity of pretreatments at longer hold times. Such a significant loss of glucan is not desirable because it decreases the overall glucose yield of the process.

4.3.5 Effect of hold time on glucan loss and wood glucan-to-glucose yield

The research hypothesis for this part of the study was that pretreatment times in the previous experiment were too long and resulted in high glucan loss; thus, the pretreatment time was decreased to 5 min and 10 min and compared to 20 min. There were no replications on pretreatments but two subsamples of pretreated redcedar were used for enzymatic hydrolysis.

Fig. 4.4 shows the effect of pretreatment time on glucan-to-glucose yields (%) of pretreated redcedar. Pretreatments times of 10 and 20 min resulted in over 80% yield. Fig. 4.4 also shows the effect of pretreatment time on wood glucan-to-glucose yields as defined in Eq. 4.4.

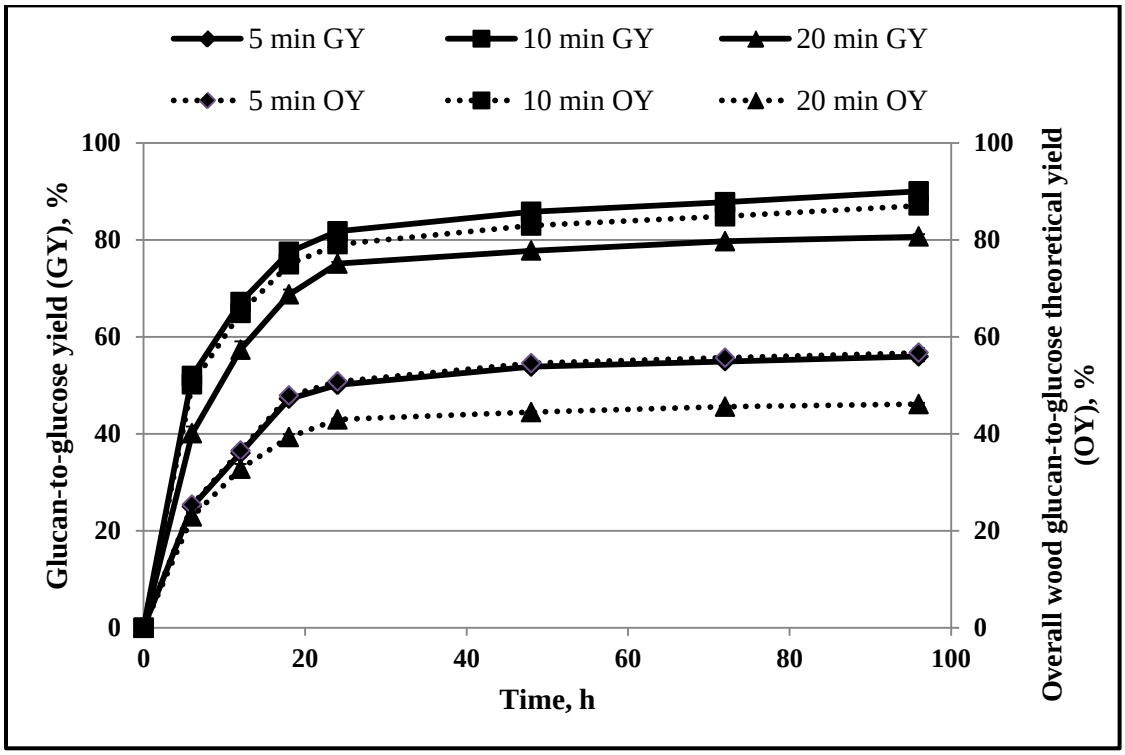

Fig. 4.4 Effect of pretreatment time on glucan-to-glucose yield (%) of pretreated redcedar and overall wood-glucan-to-glucose yield (%) based on glucan content of untreated redcedar.

Solid lines represent the glucan-to-glucose yield (%) and dashed line represent wood-glucan-to-glucose yield (%) for different pretreatment time. Pretreatment condition: 3 h of impregnation at 90 °C, 3.75 g per 100 g of sulfuric acid loading, 20 g per 100g of sodium bisulfite loading, pretreatment temperature of 200 °C. Each data point is the average of two of subsamples ± standard error.

$$\text{Wood glucan-to-glucose yield (\%)} = \frac{[\text{Glucose(t)-Glucose(0)}]+\text{Glucose(pre)}}{\text{SL} \times \text{f(Untreated biomass)} \times 1.11} \times 100 \qquad (4.4)$$

Where, Glucose(t) and Glucose(0) are the glucose concentration in % (w/v) obtained from enzymatic hydrolysis of pretreated biomass at time t and 0 hours, respectively. Glucose (pre), SL is the dry solid loading used for enzymatic hydrolysis which was 2% (w/w), and f(Untreated biomass) represents glucose in the prehydrolysate fraction and fraction of glucan in pretreated biomass, respectively. 1.11 is the conversion factor for glucan to glucose.

The overall wood-glucan-to-glucose yield accounts for the glucan lost during the pretreatment process. It is calculated from the amounts of glucose released from pretreated biomass during enzymatic hydrolysis and glucose available in prehydrolyzate fraction. Table 4.2 shows the component balance of biomass before and after pretreatment with % recovery of each component. It can be observed that a pretreatment time of 5 min did not result in any glucan loss, but yielded a pretreated redcedar that cannot be enzymatically digested completely. This shows recalcitrance of native cellulose in redcedar to enzymatic hydrolysis. With no glucan lost during pretreatments at 5 min, the cellulose still has a high degree of polymerization (high number of glucose residues per chain). Other studies have observed that cellulose hydrolysis becomes limited with cellulose beyond a definite molecular weight range (Mansfield et al., 1999). When the biomass is not properly pretreated, the amorphous regions are attacked initially by the enzymatic systems leaving the crystalline regions intact (Mansfield et al., 1999), which doesn't allow access for the enzymes to reach fibers (Krassig, 1993). Although 52% of lignin was removed during pretreatment for 5 min, it was insufficient to make biomass amenable to enzymatic hydrolysis without lowering the degree of

Table 4.2 Weights of wood components and the percentage recovered after acid bisulfite pretreatments at 200 °C, 3.75 g per 100 g of sulfuric acid loading and 20 g per 100g of sodium bisulfite loading with varying pretreatment time.

Component/ Fermentation inhibitors	Untreated redcedar	Acid bisulfite pretreated redcedar			Prehydrolyzate[b]		
		5 min	10 min	20 min	5 min	10 min	20 min
Glucan[a], g	40.32	41.23/102.26	37.93/94.07	22.53/55.88	0.56/1.39	1.06/2.63	0.53/1.31
Xylan[a], g	8.45	2.49/29.47	0.65/7.69	0.40/4.73	0.01/0.12	0.44/5.20	0.42/4.97
Galactan[a], g	1.98	0/0	0/0	0/0	0.06/3.03	0.39/19.69	0.17/8.58
Arabinan[a], g	1.40	0/0	0/0	0/0	0/0	0/0	0/0
Mannan[a], g	6.00	1.64/27.33	0.96/16	0.42/7	0/0	0.79/13.17	0.09/1.5
Lignin, g	33.65	17.15	13.70	8.41	16.50[c]	19.95[c]	25.24[c]
Acetic acid, gL^{-1}	NA	NA	NA	NA	4.11	3.47	4.27
HMF, gL^{-1}	NA	NA	NA	NA	2.40	1.76	2.72
Furfural, gL^{-1}	NA	NA	NA	NA	0.71	1.32	0.91

[a]The first number represents the mass of component observed in pretreated redcedar or prehydrolyzate and the second number represents the recovery of the components based on initial mass of each component.
[b]Mass of different components in the prehydrolyzate fraction was obtained as monomers and was converted into polymeric sugars using a multiplication factor of 0.90 for glucan, galactan and mannan and 0.88 for xylan and arabinan.
[c]Based on balance of lignin.
NA – Not applicable

polymerization of cellulose.

Wood glucan-to-glucose yield is an important metric that helps calculate the amount of sugars in the original feedstock that will be available to make products such as ethanol, butanol and/or other chemicals. The highest overall wood-glucan-to-glucose yield of 87% was obtained with a 10 min hold time. Only 6% of glucan was lost during pretreatment with a 10 min hold time. At 5 min, glucan loss was negligible, but digestibility was poor resulting in an overall yield of 57%. With 20 min of pretreatment time, the overall yield was only 47% because of excessive glucan loss during pretreatments. The highest overall yield obtained in this study was comparable with other studies in the literature. For example, Shuai et al. (2010), Zhu et al. (2010b) and Zhu et al. (2009) obtained an overall wood glucan-to-glucose yield of 80%, 84% and 86% with SPORL pretreatment of spruce, lodgepole pine and red pine, respectively. Other studies on softwood have reported overall glucan yields based on initial glucan in biomass between 50% and 75% (Ewanick et al., 2007; Monavari et al., 2009a).

Hemicellulose and lignin removal played an important role in increasing digestibility of redcedar. Sulfuric acid plays a crucial role in the removal of hemicellulose from wood. Hemicellulose is prone to acid hydrolysis because of the low pH, high temperatures and lower degree of polymerization of hemicellulose in comparison to cellulose (Ingruber, 1985). Xylans are almost completely hydrolyzed to xylose during pulping operations (Bryce, 1980; Zhu et al., 2009). From our mass balance (Table 4.2), complete removal of galactan and arabinan was observed during pretreatment of redcedar. There was also a removal of large fractions of xylan and mannan from the wood. The recoveries of xylan and mannan with the pretreatment

carried for 10 min were 13% and 29%, respectively, which are less than SPORL experiments conducted with other softwoods (Zhu et al., 2009). This could be due to the higher loading of chemicals used to overcome the recalcitrance of redcedar. However, a glucan recovery of 94% was achieved with pretreatments carried at 10 min, which is consistent with other literature (Shuai et al., 2010; Zhu et al., 2009).

A mass balance of redcedar before and after pretreatment shows that acid bisulfite pretreatment removed from 49% to 75% lignin when acid bisulfite pretreatments were carried out at 200 °C, 3.75 g per 100 g of sulfuric acid loading and 20 g per 100 g of sodium bisulfite loading with varying pretreatment time (Table 3). Delignification is accomplished by a sulfonation reaction that involves both sulfuric acid and sodium bisulfite. The first step of sulfonation is the attack of an acid group on the C-α position that is referred to as hydrolysis resulting in an unstable carbonium ion (C+) called the quinone-methide intermediate (Bryce, 1980). This intermediate molecule immediately reacts with the negatively charged bisulfite (HSO_3^+) ions to form lignosulfonates (Glasser, 1980). The β-carbon position that is frequently engaged with ether linkages is then sulfonated by a sulfitolysis reaction, which eventually breaks the monomeric lignin from the polymeric form (Ingruber, 1985). Thus, a lignin unit is obtained with both α and β carbons sulfonated in the form of lignosulfonates. This reaction continues and results in the delignification of biomass. The lignosulfonates released from biomass are dissolved into the liquid fraction (prehydrolyzates or cooking liquor) when the pH of the pretreatment liquor is below 7 (Glasser, 1980). Lignin removal increases the porosity (both pore width and volume) of the biomass, which favors an increased rate of enzymatic hydrolysis (Casey, 1980; Stone and Scallan, 1968). Additionally, the degree

of polymerization of xylan and cellulose is reduced. Sulfonation of lignin also increases the hydrophilicity of lignin, which benefits subsequent enzymatic hydrolysis by decreasing non-productive binding of enzymes to lignin (Zhu et al., 2009). Fractionation of hemicellulose and lignin from the biomass increases the surface area of cellulose available to the enzymes resulting in faster rates of hydrolysis (Ramos et al., 1992). The pretreatment condition that resulted in an overall wood glucan-to-glucose yield of 87% had 59% lignin loss. These observations were consistent with other experiments conducted with SPORL technology, although the lignin loss in other studies did not exceed 32% (Shuai et al., 2010; Zhu et al., 2009). Lignosulfonates obtained after pretreatments can be sold as dispersants and/or be upgraded to vanillin (Bryce, 1980; Glasser, 1980) as a co-product of the biorefining process.

Fermentation inhibitors produced during biomass pretreatments must be kept low to prevent their inhibitory effects during subsequent fermentation. The concentrations of fermentation inhibitors monitored during this study are listed in Table 3. Our results were much lower than dilute acid pretreatments of softwood (Shuai et al., 2010) and comparable with previous studies carried by Shuai et al. (2010) and Tian et al. (2010) that reported 2.7 to 5.3 g L^{-1} of acetic acid, 2.0 to 2.7 g L^{-1} of HMF and 1.3 to 2.2 g L^{-1} of furfural with SPORL pretreatment of spruce carried at 180°C for 30 min with 5 g per 100 g of sulfuric acid loading and 9 g per 100 g of sodium sulfite loading.

4.4 Conclusions

Acid bisulfite pretreatment of redcedar successfully made the biomass amenable to enzymatic hydrolysis. As high as 87% overall wood glucan-to-glucose yield was

achieved when pretreatments were conducted with impregnation of 3 h at 90 °C with a pretreatment liquor consisting of sulfuric acid at 3.75 g per 100 g of dry wood and sodium bisulfite at 20 g per 100 g of dry wood loading, pretreatment temperature of 200 °C and hold time of 10 min. Mass balances indicated removal of large parts of hemicellulose and lignin. Delignification was important to attain high glucan-to-glucose yield of pretreated redcedar and was achieved by increasing sodium bisulfite loading.

Acknowledgements

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CHAPTER V

ACID BISULFITE PRETREATMENT OF EASTERN REDCEDAR FOR FERMENTABLE GLUCOSE PRODUCTION: OPTIMIZATION THROUGH RESPONSE SURFACE METHODOLOGY

Abstract

The primary goal of this study was to determine the optimal pretreatment conditions to efficiently obtain fermentable glucose from Eastern redcedar. Response surface methodology (RSM) based on a uniform precision rotatable central composite design (CCD) was used to design the experiments and analyze the influence of four pretreatment process variables: pretreatment temperature, hold time, sulfuric acid loading and sodium bisulfite loading on wood glucan-to-glucose yields. The highest wood glucan-to-glucose yield of 91% was predicted at the optimum conditions of 200 ºC, 7.5 min of hold time, 3.75 g of sulfuric acid loading per 100 g of dry wood and 22.5 g of sodium bisulfite per 100 g of dry wood. The predicted model was validated by conducting experiments at the optimized conditions, resulting in 87 ± 2% of theoretical wood glucan-to-glucose yield. Mass balances showed that 70% delignification and 89 to 100% loss of hemicellulose polymers during pretreatments.

Keywords: Acid bisulfite pretreatment, Eastern redcedar, Response surface methodology, softwood, enzymatic saccharification.

5.1 Introduction

Eastern redcedar (*Juniperus virginiana* L.) (hereafter referred to as redcedar) is a softwood commonly available in the central plains of the United States. Redcedar is considered a weed in the state of Oklahoma because of its invasive nature. It is spreading at a rate of 762 acres per day and it is estimated that 26% of the overall land base of Oklahoma will be covered with redcedars by 2015 if their spread remains uncontrolled (McKinley, 2012). The encroachment of redcedars has brought many ecological concerns to farmers, ranchers and wildlife species (Zhang and Hiziroglu, 2010). First, land availability for grazing is greatly reduced due to the presence of redcedar. Second, a recent study showed that a single redcedar tree could absorb up to 30 gallons of water per day (Truitt, 2011). Their extensive root systems inhibit water recharge in aquifers and their thick foliage captures 25% of rainfall, thereby limiting rain from reaching soil (Truitt, 2011). Third, redcedar leaf litter on the soil was observed to affect soil hydraulic properties such as water repellency and sorptivity (Wine et al., 2012). Fourth, the presence of volatile acids in redcedar wood increases the risk of wildfires in regions where wind and low humidity conditions commonly exist (Zhang and Hiziroglu, 2010). Fifth, redcedar infestations have decreased turkey roost sites, grasslands birds and songbirds that are common to prairie lands (National Resources Conservation Service, 2012). Sixth, forage production is affected due to the encroachment of redcedars. The National Resources Conservation Service (2012) reports that as high as 50% reduction in forage production could be observed with 250 redcedar trees per acre. Finally, pollens from redcedar have become a common source for allergies. The losses incurred by the state of Oklahoma due to these ecological effects were estimated as $ 447 million

(National Resources Conservation Service, 2012). Production of biofuels from the polysaccharides of redcedar will be very beneficial to the farmers, ranchers and the state of Oklahoma because all their ecological threats with redcedar will be addressed and renewable fuel can be locally produced. Besides Oklahoma, redcedar encroachment is common to the states of Arkansas, Alabama, Kansas, Florida, Iowa, Missouri, Mississippi, Tennessee, Texas (Gold et al., 2003; Semen and Hiziroglu, 2005). With such a wide availability of redcedar across the US, they can easily become a promising source for cellulosic biofuels.

There is a tremendous potential for producing ethanol from the polysaccharides of redcedar. Approximately, 2 billion L (530 million gallons) of ethanol could be produced from redcedar present in just 17 counties in Northwest Oklahoma (Ramachandriya et al., 2013). For developing an efficient ethanol production process from redcedar, optimization of pretreatment is the most important step because it affects subsequent processes of the bioconversion, such as ethanol yield, capital and operating cost, enzyme utilization, fermentation, distillation and waste disposal (Saville, 2011). Statistical and mathematical techniques, such as response surface methodology, are generally followed to develop, improve and optimize processes (Myers and Montgomery, 1995). This approach reduces the number of experiments thereby reducing the cost and time with research and development, allows to sensitively interpret statistical differences and indicates interaction between two variables that usually go unnoticed during the traditional “one-factor at a time” approach (Anderson and Whitcomb, 2005).

During a previous study, acid bisulfite pretreatment was chosen for pretreating redcedar and screening experiments were performed to identify the most important

factors affecting redcedar pretreatments, which were pretreatment temperature, pretreatment time, sulfuric acid loading and sodium bisulfite loading (Ramachandriya et al., 2013). Preliminary screening was followed by a path of steepest ascent where levels of factors were adjusted to achieve near optimum response (Ramachandriya et al., 2013), but the levels of independent variables affecting pretreatments were not optimized. Hence, the aim of the present study was to optimize pretreatment temperature, pretreatment time, sulfuric acid and sodium bisulfite loadings, during acid bisulfite pretreatment by response surface methodology for maximizing the response variable which was wood glucan-to-glucose yield. Another objective of this study was to obtain a functional relationship between the four "vital few" controllable factors and the response variable.

5.2 Materials and methods

5.2.1 Biomass

Eastern redcedar (*Juniperus virginana* L.) chips were acquired from the Oklahoma State Forestry Services (Idabel, McCurtain County, OK, USA). The chips contained both heartwood and sapwood fractions of the trunk from redcedar trees. The biomass was ground using a Thomas-Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, USA) equipped with a 2 mm screen. After grinding, the moisture content of the biomass was determined by a convection oven method (Sluiter et al., 2008a). Biomass was stored in resealable bags at room temperature prior to pretreatments and/or compositional analysis.

5.2.2 Compositional analysis

For compositional analysis of the raw material, biomass was sieved through Tyler number +9/+60 sieve plates and the fraction of biomass that was retained on the +60 sieve plate were used for compositional analysis. About 80% of the ground biomass was retained on the +60 sieve plate and the remaining portion was fines. Sieving of biomass was important because the NREL protocols for compositional analysis were developed for particle size between 180 µm and 850 µm (Hames et al., 2008). Ethanol extraction of sieved redcedar was then carried out using an accelerated solvent extractor (ASE) (Model 300, Dionex Corporation, Sunnyvale, CA, USA) to remove non-structural material using National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008d). The amount of extractives (on a percent dry weight basis) was calculated directly by evaporating ethanol at room temperature in a fume hood and measuring the residual mass (Sluiter et al., 2008d).

Following extraction, the biomass was air dried at 35 °C in a vacuum incubator (Model 285A, Fisher Scientific, Pittsburgh, PA, USA) and was analyzed for structural carbohydrates and lignin using a two-step acid hydrolysis procedure developed by NREL (Sluiter et al., 2008c). Structural carbohydrates were analyzed using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 mL min^{-1} and the column temperature was maintained at 85 °C. The HPLC with Chemstation software (Agilent Technologies) was calibrated at five levels using known concentrations of compounds before being used to quantitate the concentration of compounds. Acid soluble lignin (ASL) content of

biomass was determined using a UV–Vis spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA, USA) at a wavelength of 205 nm and an extinction coefficient of 110 L g^{-1} cm^{-1}. Acid insoluble lignin (AIL) was determined gravimetrically (Sluiter et al., 2008c).

5.2.3 Acid bisulfite pretreatments

Pretreatments were performed in a 1 L bench top pressure reactor (Parr series 4250, Parr Instrument Company, Moline, IL, USA) equipped with an agitator, a heater and a control unit. The reactor was filled with 100 g of dry redcedar and 500 g of pretreatment liquor to achieve a liquid-to-solid mass ratio of 5:1. The pretreatment liquor comprised of deionized water, sulfuric acid and sodium bisulfite. The concentration of sulfuric acid and sodium bisulfite varied from one experimental run to other based on the experimental design. In this study, Sulfuric acid loading varied between 3.25 and 4.25 g per 100 g of dry wood while sodium bisulfite loading varied between and 15 g and 25 g per 100 g of dry wood. The reactor was agitated at 150 rpm and biomass was soaked in the pretreatment liquor at 90 °C for 3 h for all pretreatments. In a previous study, we showed soaking was important to obtain redcedar amenable for enzymatic hydrolysis (Ramachandriya et al., 2013). At the end of 3 h, the reactor temperature was increased to a desired set point that varied between 180 °C and 220 °C and was held at the temperature for a desired time that varied between 5 min and 15 min based on the experimental design. At the end of the pretreatment hold time, the reactor was cooled in an ice bath until the reactor temperature was 55°C. After cooling the reactor, the slurry was filtered using vacuum filtration through a Whatman #4 filter paper for solid and

liquid recovery. Moisture content of the solid fraction was measured using a standard NREL procedure (Sluiter et al., 2008a). The wet solids were then rinsed with 500 mL of 60 °C deionized water four times to remove soluble sugars and fermentation inhibitors and the moisture content of washed solids was determined (Sluiter et al., 2008a). The wet washed solids were then stored in plastic resealable bags at 4 °C until use for enzymatic hydrolysis and compositional analysis. The chemical composition of pretreated solids was determined using NREL protocols (Sluiter et al., 2008c). The concentrations of sugars was determined using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA) with column conditions explained in section 5.2.2 and protocols outlined by NREL (Sluiter et al., 2008b). The concentrations of fermentations inhibitors in the prehydrolysate were analyzed using a HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA) and a refractive index detector (RID). The eluent was 0.01 N sulfuric acid at a flow rate of 0.6 mL min^{-1} with a 30 min run time (Sluiter et al., 2008b).

5.2.4 Enzymes and enzymatic hydrolysis of pretreated redcedar

Accelerase® 1500 kindly provided by Genencor Inc. (Palo Alto, CA, USA) was the enzyme cocktail used for this study. Enzymatic hydrolysis of pretreated redcedar was done at 2% (w/w) dry solids loading to determine the efficacy of pretreatments. Enzyme loading of 0.5 mL g^{-1} of glucan (50 FPU g^{-1} glucan) was used for these studies. This loading was recommended by the manufacturer as a starting point for optimization (Genencor, 2012). The combination of low solids loading and high enzyme loading used in this study provides unbiased determination of efficacy of pretreatments as the

inhibitory effect of glucose is minimal. The enzymatic hydrolysis were carried out in 250 mL baffled flasks containing a total mass of 100 g incubated at 250 rpm and 50 °C with 0.05 M sodium citrate buffer at pH 5. The cellulase activity of Accelerase® 1500 was determined using standard protocol developed by NREL (Adney and Baker, 2008). Analytical grade chemicals required for the enzyme assay were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2.5 Experimental design

For the current study, four significant independent variables: sulfuric acid loading (g per 100 g of dry wood), sodium bisulfite loading (g per 100 g of dry wood), pretreatment temperature (°C) and pretreatment hold time (min) were considered for optimization using a rotatable uniform precision central composite design (CCD) of response surface methodology. These were determined to be the four "vital few" factors affecting redcedar pretreatments in a previous study using a series of factorial design and "one-factor at a time" experiments (Ramachandriya et al., 2013). CCD's are built from two-level factorial designs with center points and axial points (Anderson and Whitcomb, 2005). Center points help to estimate the pure error for the design while the axial points makes the design rotatable, which helps to achieve equally precise predictions from the center point of the design (Anderson and Whitcomb, 2005). A total of 32 experimental runs were employed for optimization comprising of 16 factorial (2^k), 8 axial points (2k) and 8 center points (where k is the number of controllable factors which is 4 in this study). Our previous study indicated near optimal wood glucan-to-glucose yield when redcedar was pretreated at 200 °C for 10 min with sulfuric acid and sodium bisulfite loading of 3.75 g per 100 g of dry wood and 20 g per 100 g of dry wood, respectively

(Ramachandriya et al., 2013). So, these levels were selected as center level for current study and a narrow region of interest was selected to optimize pretreatments. The range of the coded and actual factor levels selected for this study is given in Table 5.1. The functional quadratic equation relating the four "vital few" factors and the response variable can be expressed as in Eq. (5.1):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (5.1)$$

Where Y is the predicted response variable; β_0 is the constant; $\beta_1, \beta_2, \beta_3$ and β_4 are quadratic coefficients; $\beta_{11}, \beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}$ and β_{34} are interaction coefficients; A, B, C and D are factors representing pretreatment temperature, pretreatment hold time, sulfuric acid loading and sodium bisulfite loading, respectively.

Statistical analysis to obtain the predicted responses and optimal levels of the variables for maximizing wood glucan-to-glucose yield was performed using the ADX interface in SAS release 9.3 (SAS, Cary, NC, USA). Coefficients of the full model were analyzed and the insignificant ones ($P \geq 0.10$) were eliminated from the model. Influential diagnostics to determine leverage points and normality of the data was conducted using the regression (REG) procedure in SAS. Additionally, the correlation between wood glucan-to-glucose yields and delignification was conducted using the correlation (CORR) procedure in SAS.

Table 5.1 List of experimental factors and levels for the central composite design (CCD).

Factor symbol	Experimental factor	Coded and experimental levels[a]				
		-α = -2	**-1**	**0**	**1**	**α = 2**
Temp	Pretreatment temperature, ºC	180	190	200	210	220
Time	Pretreatment hold time, min	5	7.5	10	12.5	15
Acid	Sulfuric acid loading, g per 100 g of dry wood	3.25	3.50	3.75	4.00	4.25
Bisulf	Sodium bisulfite loading, g per 100 g of dry wood	15	17.5	20	22.5	25

[a]"0" level corresponds to center point conditions and ±α corresponds to axis points. The experimental level for center point was selected from a previous study (Ramachandriya et al., 2013)

5.3 Results and discussion

5.3.1 Pretreatment optimization

Redcedar used for this study contained 34.2 ± 0.3 % glucan, 7.9 ± 0.1 % xylan, 3.7 ± 0.0 % galactan, 1.2 ± 0.1 % arabinan, 8.5 ± 0.1 % mannan, 32.1 ± 0.3 % lignin and 3.2 ± 0.0 % extractives (mean ± 1 standard error). Lignin, glucan and mannan content of redcedar was 5% lower, 15% lower and 42% higher, respectively, when compared to a previous study (Ramachandriya et al., 2013); whereas, the composition of other hemicellulose polysaccharides were similar between the two studies. The variation in raw material composition between Ramachandriya et al. (2013) and the current study was due to differences in the age of the trees and location of harvest. Despite compositional differences mass balances around the pretreatment reactor showed 25 to 72% lignin loss (also referred as delignification), 0.2 to 32% glucan loss and 80 to 100% hemicellulose polysaccharides loss during acid bisulfite pretreatments, which were comparable with the results obtained by Ramachandriya et al. (2013). The extent of delignification and recovery of monomeric sugars during pretreatments differed with respect to the levels of controllable factors.

Fig. 5.1 shows a scatterplot with lignin loss observed during the 32 experimental runs versus its corresponding wood glucan-to-glucose yield. A significant correlation between delignification and wood glucan-to-glucose yields was observed during this study ($p < 0.0001$) with a pearson correlation coefficient (r) of 0.8177, showing a strong linear relationship between the two variables. A cause and effect relationship between lignin loss and increase in wood glucan-to-glucose yield can be established because delignification increases the porosity of the biomass, which subsequently increases the

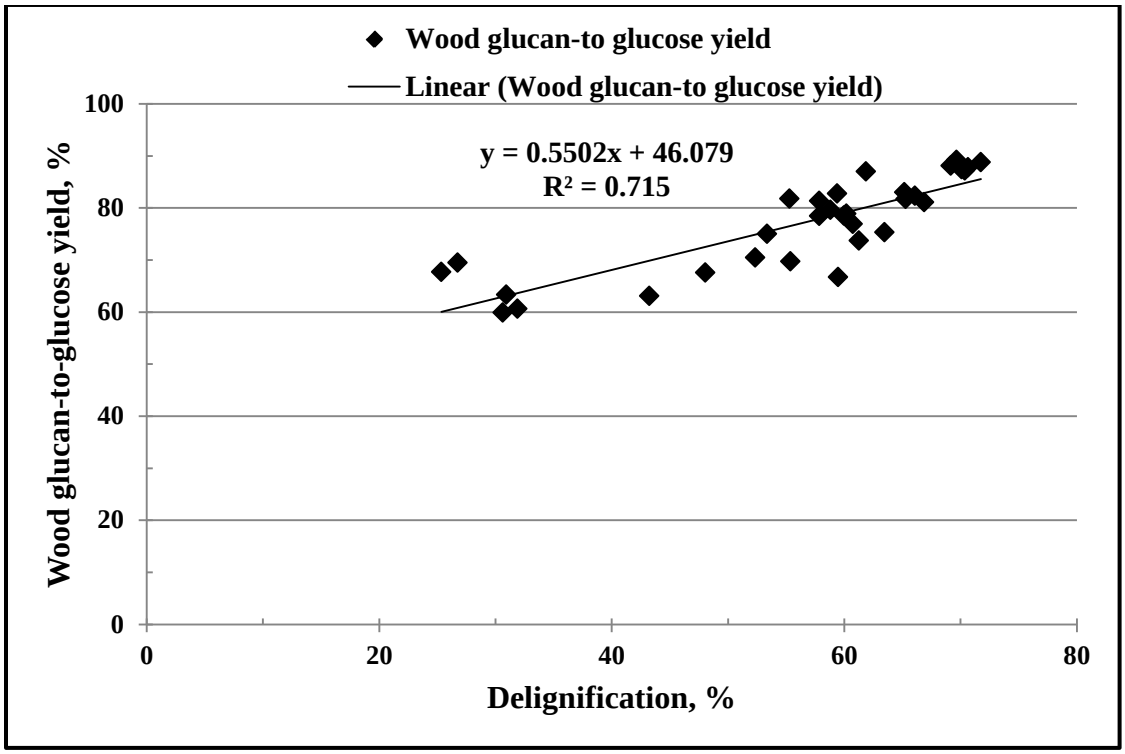

Fig. 5.1 Effect of lignin removal on wood glucan-to-glucose yield.

rate of enzymatic hydrolysis (Stone and Scallan, 1968). Additionally, acid bisulfite pretreatment increases the hydrophilicity of lignin, which decreases the unproductive binding of lignin with enzymes (Zhu et al., 2009). Our results are consistent with previous literature that showed such a linear relationship between delignification and glucose yields obtained from pretreated biomass during acid bisulfite pretreatment of redcedar and switchgrass (Ramachandriya et al., 2013; Zhang et al., 2013).

Glucose was the only monomeric sugar that was produced at high concentrations during enzymatic hydrolysis. Therefore, wood glucan-to-glucose yield was the most important response variable for evaluating the efficacy of pretreatments as it accounts for glucan lost during pretreatments and delignification is related to wood glucan-to-glucose yield as previously discussed. Wood glucan-to-glucose yield was calculated from the amount of glucose that was produced using different pretreatments with respect to the theoretical maximum amount of glucose that could be produced based on the glucan present in the untreated wood as shown in Eq. (5.2):

$$\text{Wood glucan-to-glucose yield (\%)} = \frac{(\text{Glucose}_{96\text{ h}} - \text{Glucose}_{0\text{ h}}) + \text{Glucose}_{\text{pre}}}{\text{SL} \times \text{f(glucan)}_{\text{untreated}} \times 1.11} \times 100 \qquad (5.2)$$

where, $\text{Glucose}_{96\text{ h}}$, $\text{Glucose}_{0\text{ h}}$ and $\text{Glucose}_{\text{pre}}$ are the concentration of glucose (% w/v) obtained during enzymatic hydrolysis at 96 h, 0 h and in the prehydrolysate, respectively. SL is the dry solid loading used for enzymatic hydrolysis which was 2% (w/w), $\text{f(glucan)}_{\text{untreated}}$ represents the glucan fraction in raw material, and 1.11 is the mass conversion factor of glucan to glucose (g g^{-1}).

Table 5.2 shows the experimental design matrix and the wood glucan-to-glucose yield (% of theoretical) for each experimental run. The extent of wood glucan-to-glucose yields achieved during pretreatments differed with respect to the levels of controllable

Table 5.2 Experimental design matrix and results of the central composite design.

No.	Point type	Temp, °C	Time, min	Acid loading, %[a]	Bisulfite loading, %[a]	Experimental yield, %[b]	Predicted yield, %[c]
1	Factorial	190	7.5	3.50	17.5	69.5	73.1
2	Factorial	190	7.5	3.50	22.5	82.7	83.3
3	Factorial	190	7.5	4.00	17.5	60.2	62.3
4	Factorial	190	7.5	4.00	22.5	83.0	81.3
5	Factorial	190	12.5	3.50	17.5	70.4	72.2
6	Factorial	190	12.5	3.50	22.5	69.7	70.0
7	Factorial	190	12.5	4.00	17.5	77.7	77.1
8	Factorial	190	12.5	4.00	22.5	82.3	83.6
9	Factorial	210	7.5	3.50	17.5	78.5	76.4
10	Factorial	210	7.5	3.50	22.5	81.3	86.5
11	Factorial	210	7.5	4.00	17.5	59.2	65.6
12	Factorial	210	7.5	4.00	22.5	81.1	84.6
13	Factorial	210	12.5	3.50	17.5	63.1	67.3
14	Factorial	210	12.5	3.50	22.5	66.0	65.1
15	Factorial	210	12.5	4.00	17.5	69.5	72.2
16	Factorial	210	12.5	4.00	22.5	73.7	78.7
17	Axial	180	10.0	3.75	20.0	63.4	63.6
18	Axial	220	10.0	3.75	20.0	70.1	62.0
19	Axial	200	5.0	3.75	20.0	88.1	85.5
20	Axial	200	15.0	3.75	20.0	79.6	78.8
21	Axial	200	10.0	3.25	20.0	73.7	71.3
22	Axial	200	10.0	4.25	20.0	79.7	74.2
23	Axial	200	10.0	3.75	15.0	75.0	73.8
24	Axial	200	10.0	3.75	25.0	89.2	90.5
25	Center	200	10.0	3.75	20.0	87.0	82.1
26	Center	200	10.0	3.75	20.0	87.2	82.1
27	Center	200	10.0	3.75	20.0	76.9	82.1

28	Center	200	10.0	3.75	20.0	78.9	82.1
29	Center	200	10.0	3.75	20.0	81.3	82.1
30	Center	200	10.0	3.75	20.0	87.8	82.1
31	Center	200	10.0	3.75	20.0	88.8	82.1
32	Center	200	10.0	3.75	20.0	81.3	82.1

[a]Acid loading and bisulfite represents sulfuric acid and sodium bisulfite and their loadings are expressed as g of chemical per 100 g of dry redcedar.
[b]Yield corresponds to wood glucan-to-glucose yield expressed as % of theoretical was calculated using Eq. (5.2).
[c]Predicted yield for experimental runs were calculated using the response calculator in SAS 9.3 ADX interface.

Table 5.3 ANOVA for response surface reduced quadratic model.

Source	DF[a]	Sums of square	Mean square	F-value	Pr > F
Model	10	1864.36	186.44	8.92	<.0001
Temp (A)	1	3.94	3.94	0.19	0.6686
Time (B)	1	67.00	67.00	3.21	0.0877
Acid (C)	1	12.70	12.70	0.61	0.4441
Bisulfite (D)	1	418.50	481.50	20.04	0.0002
Temp*Temp (A^2)	1	697.25	697.25	33.39	<.0001
Temp*Time (A.B)	1	66.59	66.59	3.19	0.0886
Time*Acid (B.C)	1	244.45	244.45	11.71	0.0026
Time*Bisulfite (B.D)	1	148.60	148.60	7.24	0.0127
Acid*Acid (C^2)	1	155.13	155.13	7.43	0.0109
Acid*Bisulfite (C.D)	1	77.35	77.35	3.70	0.0679
Error	21	438.52	20.88		
Lack of fit	14	291.83	20.85	0.99	0.5325
Pure Error	7	146.68	20.95		
Total	31	2302.87			

[a]DF represents degrees of freedom; Temp (factor A), time (factor B), acid (factor C) and bisulfite (factor D) refers to pretreatment temperature (°C), hold time (min), sulfuric acid loading (g per 100 g of dry wood) and sodium bisulfite loading (g per 100 g of dry wood).

factors. The ANOVA evaluations of the data are shown in Table 5.3. The curvature in the responses occurs due to the squared effects for temperature ($p < 0.0001$) and sulfuric acid loading ($p = 0.0109$). Significant two factor interactions were time*acid ($p = 0.0026$), time*bisulfite ($p = 0.0127$), temp*time ($p = 0.0886$) and acid*bisulfite ($p = 0.0679$). The p values of temp*time and acid*bisulfite showed marginal significance, each resulting in 91 and 93% confidence that the interactions were significant, respectively. Despite their marginal significance, there have been numerous reports in the literature that observed the interaction between pretreatment temperature and hold time during acidic pretreatments (Alvira et al., 2010; Chum et al., 1990), and between sulfuric acid and sodium bisulfite loadings during acid bisulfite pretreatments (Ingruber, 1985; Ramachandriya et al., 2013; Zhu et al., 2009). Significant time*acid and time*bisulfite interactions highlight that hold time plays an important role in allowing sulfuric acid and sodium bisulfite to perform sufficient delignification to produce high wood glucan-to-glucose yields. Other two-factor interactions and squared factors, namely temp*acid, temp*bisulfite, squared effect of time and bisulfite were removed from the model because their p values were 0.400, 0.674, 0.444 and 0.212, respectively. The quadratic regression model was significant ($p < 0.0001$) and the lack of fit to the quadratic model was not significant ($p = 0.5325$); thus, it can be concluded that the second-order model was an adequate approximation of the true response surface.

The coded and actual predictive regression models that were obtained from wood glucan to glucan yield are shown in Eqs. (5.3) and Eq. (5.4), respectively.

$$\text{Coded Yield} = 82.12 - 0.40\cdot\text{Temp} - 1.67\cdot\text{Time} + 0.73\cdot\text{Acid} + 4.16\cdot\text{Bisulfite} - 4.83\cdot\text{Temp}^2 - 2.33\cdot\text{Acid}^2 - 2.04\cdot\text{Temp}\cdot\text{Time} + 3.91\cdot\text{Time}\cdot\text{Acid} - 3.11\cdot\text{Time}\cdot\text{Bisulfite}$$

$$+ 2.20 \cdot Acid \cdot Bisulfite \quad (5.3)$$

$$\text{Actual Yield} = -2169.75 + 20.10229 \cdot Temp + 2.163167 \cdot Time + 150.1136 \cdot Acid - 6.540167 \cdot Bisulf - 0.048317 \cdot Temp^2 - 37.34714 \cdot Acid^2 - 0.0816 \cdot Temp \cdot Time + 6.254 \cdot Time \cdot Acid - 0.4982 \cdot Time \cdot Bisulfite + 3.518 \cdot Acid \cdot Bisulfite \quad (5.4)$$

Where, Yield represents wood glucan-to-glucose yield (% of theoretical), Temp represents pretreatment temperature (°C), Time represents hold time (min), and Acid and Bisulfite represents sulfuric acid and sodium bisulfite loading in g per 100 g of dry wood, respectively. The coefficients of actual model were not rounded because they are subject to more serious rounding error than the coded model (Anderson and Whitcomb, 2005).

The coded model facilitates knowledge of the process and works only if the factors are converted into their coding scale of -1 to 1 for the low versus high end (Anderson and Whitcomb, 2005), respectively, shown in Table 5.1 . The intercept of 82.12 obtained in the coded model represents wood glucan-to-glucose yield at the center point of the design. Additionally, a positive sign in front of terms in the coded model reveals a synergistic effect while a negative sign reveals an antagonistic effect of independent variables relative to the center point of the design (Anderson and Whitcomb, 2005). On the other hand, the coefficients in the actual model shown in Eq. (5.4) can be used to plug in values based on the actual units of measure of the independent variables and find out the predictive wood glucan-to-glucose yield (Anderson and Whitcomb, 2005). However, care must be taken that extrapolation of the data are not made because the precision of estimating responses decreases rapidly beyond the coded and actual levels of -1 to 1 (Anderson and Whitcomb, 2005). The actual model could be used for

performing economic analysis of the process by changing the levels of independent variables within the coded and actual levels of -1 and 1.

The distribution of residuals (deviation between predicted and actual values) was analyzed to evaluate influential points and normality of the data (Appendix B.1). Influential points (also referred as leverage) are responses that are unusual and hence can control certain model properties like the coefficient of determination (R^2) and standard errors of regression coefficients (Myers and Montgomery, 1995). Our analysis showed that the residuals followed a normal distribution and there were no leverage points (Appendix B.1). However, experimental run number 3, 11, 14 and 17 were identified as outliers (Table 5.2). Despite replicating these four experiments, there were no differences observed in the responses showing that our observations were not an experimental error. A closer examination of the responses revealed that low wood glucan-to-glucose yields with these experimental runs (Table 5.2) compared to other runs would have made them outliers (Table 5.2). The experimental runs numbered 3 (190 ºC, 7.5 min, 3.5 g sulfuric acid per 100 g of dry wood and 17.5 g sodium bisulfite per 100 g of dry wood) and 11 (210 ºC, 7.5 min, 4 g sulfuric acid per 100 g of dry wood and 17.5 g sodium bisulfite per 100 g of dry wood) resulted in 31% delignification due to the low level of sodium bisulfite loading (17.5 g per 100 g of dry wood), while experimental run number 17 (180 ºC, 10 min, 3.75 g sulfuric acid per 100 g of dry wood and 20 g sodium bisulfite per 100 g of dry wood) also resulted in 31% delignification due to the low temperature (180 °C) that may have been insufficient to drive the delignification reaction. Unlike experimental runs 3, 11 and 17, the experimental run 14 (210 ºC, 12.5 min, 3.5 g sulfuric acid per 100 g of dry wood and 22.5 g sodium bisulfite per 100 g of dry wood)

had 60% delignification but achieved only 66% of the theoretical wood glucan to glucose yield because of high glucan loss (22%) from redcedar during pretreatment and 84% digestibility of pretreated redcedar. These observations highlight the importance of selecting the right levels of process parameters and how deviations in processing conditions from the near optimal conditions would lower wood glucan-to-glucose yields. The four runs that were identified as outliers were included in the model. The coefficient of determination (R^2) for the predictive model was calculated as 0.8096, indicating that the model could explain 81% of the variability in the response variable and adjusted R^2 (R^2_{Adj}) was determined as 0.7189. Adjusted R^2 provides a more accurate goodness-of-fit measure than raw R^2 because it counteracts the tendency of over fitting the data (Anderson and Whitcomb, 2005). Hence, the predictive model obtained in this study could efficiently explain 72% of the variability in the wood glucan-to-glucose yield.

Response surface plots indicate the sensitivity of the response variable to each of the factors and the extent by which the factors interplay and affect the response variable (Kuehl, 2000). The two-factor interactions between the independent variables based on the model equation were demonstrated by plotting surface plots in Fig. 5.2a to 5.2f. As there were 4 independent variables in this study, the surface plots were plotted between two independent variables by keeping the remaining two independent variables constant. The range of the independent variables selected in the axis represents the entire region of interest for this study. Surface plots obtained were typical responses like rising ridge, simple maximum and inverse saddle. For example, Figs. 5.2a and 5.2b demonstrate a classic example of a rising ridge. In Fig. 5.2a, it can be observed that for any hold time the wood glucan-to-glucose yield remained lower at low (180 °C) and high level (220 °C)

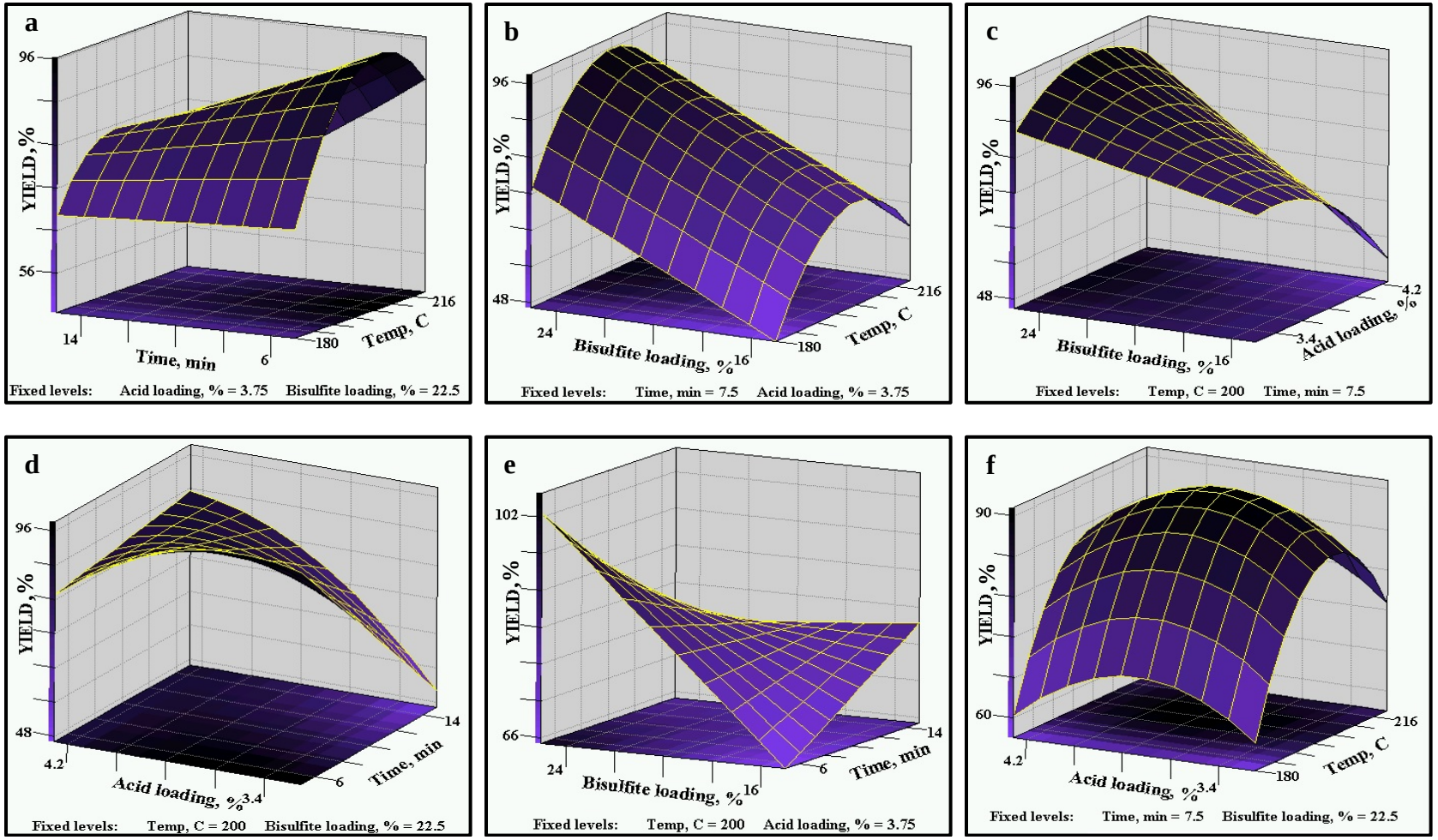

Fig. 5.2 Quadratic response surface plots showing the interaction between two factors while keeping the other two fixed at certain levels during acid bisulfite pretreatments of redcedar.

Yield represents wood glucan-to-glucose yield, % of theoretical defined in Eq (5.2). Acid and bisulfite represents sulfuric acid and sodium bisulfite and the loadings are expressed as g per 100 g of dry redcedar.

of temperature when sulfuric acid and sodium bisulfite loadings were kept constant at 3.75 and 22.5 g per 100 g of dry wood, respectively. This was because low temperatures resulted in insufficient delignification and high temperatures resulted in excessive glucan loss. But, when the temperature was near the mid-level (200 °C), the predicted yield increased linearly as pretreatment hold time was reduced. Similarly, linear increase in wood glucan-to-glucose yield was predicted when sodium bisulfite levels increased from 16 to 25 g per 100 g of dry wood at mid-levels of temperature (200 °C) when sulfuric acid loading was held constant at 3.75 g per 100 g of dry wood and pretreatment time was held constant at 7.5 min (Fig. 5.2b), which reiterates the importance of sodium bisulfite during acid bisulfite pretreatments. Fig. 5.2c also shows a rising ridge, although the surface plot it is not as distinct as Figs. 5.2a or 5.2b. Since sulfuric acid and sodium bisulfite work in tandem to achieve delignification of biomass, getting the right dosage of these chemicals is important to optimize the process. Fig. 5.2c shows that at low and high levels of sulfuric acid loading, the yield would be low for any given sodium bisulfite loading when redcedar was pretreated at 200 °C for 7.5 min. This was because reducing sulfuric acid loading will not allow the delignification reactions to move forward as sulfuric acid is required for the creation of a carbonium ion (C^+) on α-carbon on lignin monomers, which is the first step of delignification during acid bisulfite reactions (Bryce, 1980). Excessive sulfuric acid results in high glucan loss because cellulose fibrils are prone to hydrolytic cleavage.

Fig. 5.2d exhibits an inverse saddle (also referred to as “mini-max”) interaction between pretreatment hold time and sulfuric acid loading. Saddle shaped response plots contain two peaks and in such cases it is easy to get stuck in the saddle or push up the

lesser peak in the region of interest (Anderson and Whitcomb, 2005). In Fig. 5.2d, the two peaks were at high levels of hold time (15 min) and sulfuric acid loading (4.25 g per 100 g of dry wood) and low levels of hold time (5 min) and sulfuric acid loading (3.25 g per 100 g of dry wood) with a maximum predicted yield of 88% and 96%, respectively.

The surface plots between hold time and sodium bisulfite loading (Fig. 5.2e) indicated that wood glucan-to-glucose yield could be increased to 100% by increasing the sodium bisulfite to 25 g per 100 g of dry wood at 5 min of hold time, 200 °C and sulfuric acid loading of 3.75 g per 100 g of dry wood. However, this was not the recommended optimized pretreatment conditions because the level of sodium bisulfite loading and hold time where 100% yield was predicted was the axial points. During RSM experiments, it is recommended not to extrapolate beyond coded levels of -1 and 1 because the precision of estimating responses decreases rapidly at the axial points (Anderson and Whitcomb, 2005).

Fig. 5.2f shows a classic example of a simple maximum which demonstrates that 91% of theoretical wood glucan-to-glucose yield could be achieved at 3.75 g of sulfuric acid per 100 g of dry wood and 200 °C when hold time and sodium bisulfite loading were kept constant at 7.5 min and 22.5 g per 100 g of dry wood, respectively. The numerical optimization function of SAS software showed that the levels of controllable factors listed in the previous sentence were the optimized conditions for pretreating redcedar to achieve maximum wood glucan-to-glucose yields.

5.3.2 Model validation

Validation experiments were conducted at pretreatment temperature of 200 °C, pretreatment hold time of 7.5 min with 3.75 g per of sulfuric acid per 100 g of dry wood

and 22.5 g of sodium bisulfite per 100 g of dry wood to observe if the predicted response of 91% could be achieved experimentally. Validating RSM models is important due to the uncertainty of predictions that are commonly shown using prediction intervals (Anderson and Whitcomb, 2005). For this study, the prediction interval for 95% confidence was 86.7 and 95.5 around the expected outcome of 91% wood glucan-to-glucose yield. The results of enzymatic hydrolysis and mass balances around the pretreatment reactor for the validation experiments showed a wood glucan-to-glucose yield of 87 ± 2% (average ± standard error of 4 replications). Although the validation wood glucan-to-glucose yield was 4% lower than the predicted yield, the outcome was within the prediction interval. Hence, the predictive model was validated.

Fig. 5.3 shows the preliminary mass balance of acid bisulfite pretreatments of redcedar at the optimized pretreatment conditions. On average 9% glucan was lost during pretreatments and was either converted into fermentation inhibitors or was available in monomeric and polymeric form. Minimizing glucan loss is important during pretreatments because glucan is the main substrate for glucose production during enzymatic hydrolysis. Our data is comparable with previous studies that have shown between 6 to 14% glucan loss during acid bisulfite pretreatments of softwoods (Ramachandriya et al., 2013; Shuai et al., 2010; Tian et al., 2010; Zhu et al., 2009; Zhu et al., 2010). The overall wood glucan-to-glucose yield obtained at the optimized condition was 87%, which was comparable with some previous reports that have achieved between 70% and 93% of theoretical wood glucan-to-glucose yields using spruce and red pine using acid bisulfite pretreatments and SO_2 assisted steam explosion (Monavari et al., 2009; Shuai et al., 2010; Zhu et al., 2009). Although, Zhu et al. (2009) and Shuai et al.

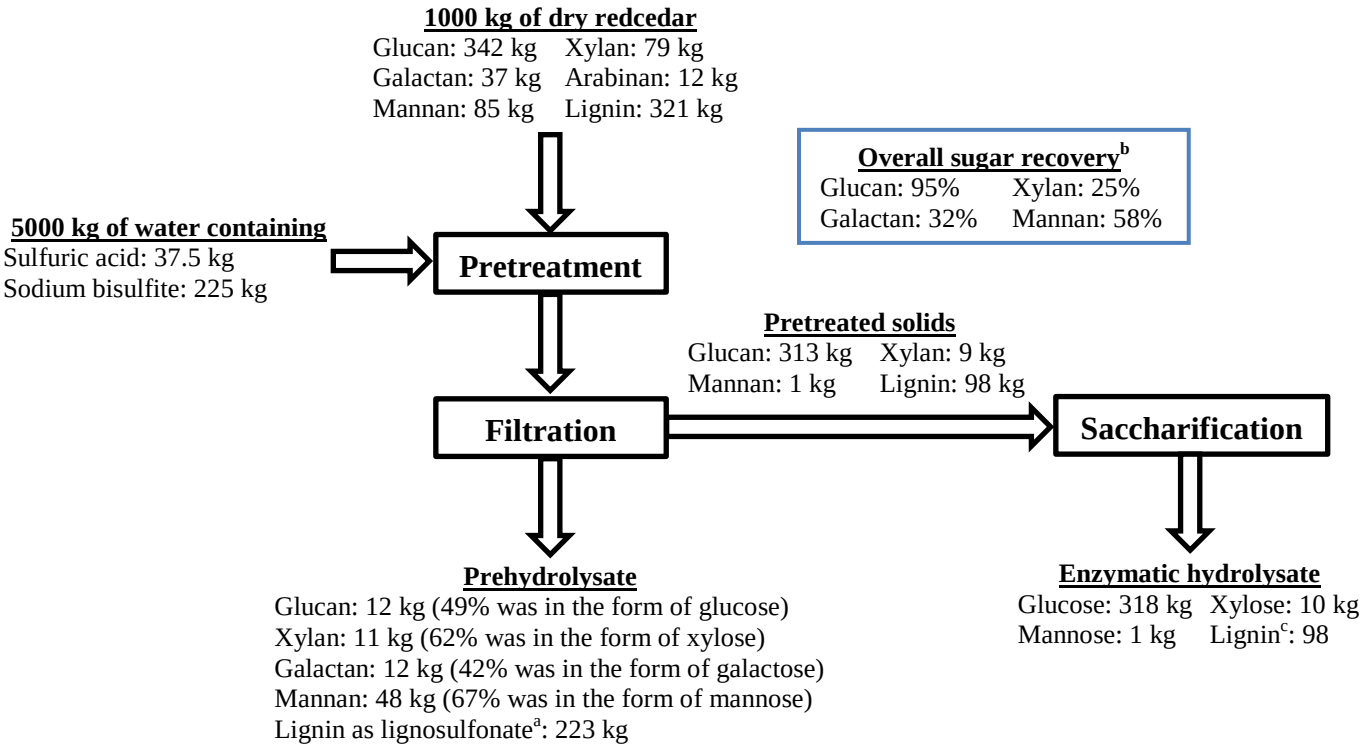

Fig. 5.3 Process mass balance of acid bisulfite pretreatments and saccharification of Eastern redcedar.
[a]Lignin as lignosulfonate was determined by differences between lignin content of raw material and lignin content of pretreated redcedar.
[b]Overall sugar recovery of biomass components was determined by the recovery of components after pretreatments based on initial mass of each component. Pretreatment conditions: 3 h of impregnation at 90 ºC, pretreatment temperature of 200 ºC, pretreatment hold time of 7.5 min, sulfuric acid and sodium bisulfite loading of 3.75 and 22.5 g per 100 g of dry wood, respectively.
[c]Lignin in enzymatic hydrolysis was carried from lignin in pretreated solids.

(2010) had 92% and 93% overall wood glucan-to-glucose yield, they observed 14% and 6% of glucan recovered in the prehydrolysate, respectively. Retaining a larger fraction of glucan in the pretreated biomass is important because co-fermentation of pretreated redcedar and neutralized prehydrolysates during subsequent processing steps would not be necessary in trying to utilize all the glucan in raw material. Fig. 5.3 also shows that 318 kg of glucose can be produced from a ton of dry redcedar. However, Zhu et al. (2009) and Shuai et al. (2010) achieved 372 kg and 403 kg of glucose per ton of dry spruce, respectively. This was because spruce had 42 to 43% glucan in the raw material while redcedar contained only 34% glucan in the current study. During a previous study, Ramachandriya et al. (2013) had observed that redcedar was comprised of 40% glucan. The variations in the composition of redcedar could be due to differences in the age of the trees and location of harvest.

In the validation study, loss of hemicellulose polymers varied between 89 and 100%, which was also comparable with previous literature (Ramachandriya et al., 2013; Shuai et al., 2010; Zhu et al., 2009; Zhu et al., 2010). Loss of hemicellulose sugars has been commonly detected during acid bisulfite pretreatments because they are susceptible to hydrolytic cleavage in the presence of acids. Additionally, 70% delignification of pretreated redcedar was also observed when pretreatments were conducted at the optimized condition. The amount of delignification achieved in this study was 19% higher than Ramachandriya et al. (2013), which used redcedar and conditions that were the same as the center point of the current study, and 119% higher than acid bisulfite pretreatments of spruce reported by Zhu et al. (2009) and Shuai et al. (2010). The optimized conditions used 22.5 g of sodium bisulfite per 100 g of dry wood, while the

study conducted by Ramachandriya et al. (2013), Zhu et al. (2009) and Shuai et al. (2010) employed 20 g, 9 g and 9 g of sodium bisulfite per 100 g of dry softwood, respectively. The higher loading of sodium bisulfite used in the current study resulted in higher lignin loss. Higher delignification would remove the unproductive binding of lignin to the enzymes and increase the hydrophilicity of lignin in pretreated biomass increasing glucose yields and rates of reaction (Zhu et al., 2009). Additionally, lignosulfonates collected in the prehydrolysates can be potential co-products like dispersants and/or starting material for the production of vanillin (Bryce, 1980; Glasser, 1980).

Hemicellulose polymers that dissolve in the prehydrolysate are generally converted into fermentation inhibitors. During pretreatments, the acetyl groups on hemicellulases breakdown to form acetic acid while hexoses and pentoses degrade into 5-hydroxymethylfurfural (HMF) and furfural, respectively (Shuai et al., 2010). Successive decomposition of HMF produces formic acid and levulinic acid (Shuai et al., 2010). The concentrations of acetic acid, formic acid, levulinic acid, HMF and furfural at the optimized conditions were 4.3 ± 0.1 gL^{-1}, 0.8 ± 0.0 g L^{-1}, 0.3 ± 0.0 gL^{-1}, 1.4 ± 01 g L^{-1} and 1.0 ± 0.0 g L^{-1}, respectively. These compositions were in agreement with previous studies on acid bisulfite pretreatments of softwoods (Ramachandriya et al., 2013; Shuai et al., 2010; Tian et al., 2010).

5.4 Conclusions

Acid bisulfite pretreatment of Eastern redcedar was optimized for achieving maximum wood glucan-to-glucose yields. Delignification was important to make biomass amenable for enzymatic hydrolysis. The highest wood glucan-to-glucose yield

of 87% was achieved at the optimum conditions of 200 ºC, 7.5 min, 3.75 g of sulfuric acid per 100 g of dry wood and 22.5 g of sodium bisulfite per 100 g of dry wood. Using RSM, a functional model relating the four "vital few" controllable factors and the response variable was obtained and validated.

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CHAPTER VI

EFFECT OF HIGH DRY SOLIDS LOADING ON ENZYMATIC HYDROLYSIS OF ACID BISULFITE PRETREATED EASTERN REDCEDAR

Abstract

This study investigates the effectiveness of extracting glucose from Eastern redcedar at high solids loading. Enzymatic hydrolysis of pretreated redcedar was performed employing 0.5 mL of Accelerase® 1500 g^{-1} of glucan (46 FPU g^{-1} glucan) using solids loading ranging from 2 to 20% dry solids (w/w). Rheological challenges observed at high solids loading were overcome by adding stainless steel balls to shake flask reactors. The highest glucose concentration, 126 g L^{-1}, was obtained using 20% solids loading in the presence of stainless steel balls as a mixing aid. This enzymatic hydrolysate was easily fermented into ethanol using *S. cerevisiae* D_5A to produce 52 g L^{-1} of ethanol. Reducing enzyme dosage at 16% solids loading from 46 FPU g^{-1} glucan to 11.5 FPU g^{-1} glucan reduced glucose concentrations and glucan-to-glucose yields. This study has demonstrated the possibility of extracting sugars from the invasive species of Eastern redcedar with high solid loadings and their conversion into ethanol.

Keywords: Acid bisulfite pretreatment, Softwood, Fermentation, Cellulosic ethanol, high solids loading

6.1 Introduction

Eastern redcedar (*Juniperus virginiana* L.) (hereafter referred to as redcedar) is an invasive softwood species spreading at an alarming rate in the central US plains. Approximately 2 billion L (530 million gallons) of ethanol could be produced from redcedar wood from just 17 counties in Northwest Oklahoma (Ramachandriya et al., 2013). Recently, the Enid Regional Development Alliance (ERDA) of Oklahoma has identified a bio-refinery location in Enid, Oklahoma and have plans for processing 2,000 dry Mg of redcedar per day into fuels and chemicals (Enid Regional Development Alliance, 2012). A previous study identified near optimal pretreatment conditions for pretreating redcedar using sulfuric acid and sodium bisulfite and achieved 87% overall wood glucan-to-glucose yield (Ramachandriya et al., 2013); however, the enzymatic hydrolysis of pretreated redcedar was carried out at low (2% w/w dry basis) solids loading. Ideally, a bio-refinery utilizing redcedar as a feedstock would operate at high solids (substrate) loading to increase product concentrations and decrease capital and operating costs (Jørgensen et al., 2007; Kristensen et al., 2009). However, challenges such as increased viscosity resulting in mass transfer limitations and mixing difficulties and inhibition from toxic products such as fermentation inhibitors and lignin are common to operations at high solids loading (Alvira et al., 2013; Jørgensen et al., 2007; Roche et al., 2009).

In order to overcome the technical barriers for using lignocellulosic biomass at high solids loading, new bioreactor designs and strategies have been employed. Novel bioreactor designs such as laboratory-scale roller bottle reactors (RBRs) (Roche et al., 2009), bench scale helical stirring bioreactors (Zhang et al., 2010), horizontal five

chambered liquefaction reactors (Jørgensen et al., 2007) and laboratory scale peg mixers (Zhang et al., 2009) have been developed and validated; however, shake flask studies are still the most common method of evaluating digestion of biomass (Roche et al., 2009). Furthermore, novel strategies such as prehydrolysis and fed-batch operation of simultaneous saccharification and fermentations (SSFs) have also been demonstrated (Hoyer et al., 2013; Lan et al., 2013; Pessani et al., 2011). Prehydrolysis is carried out by liquefying lignocellulosic biomass at the optimum temperature for enzymatic hydrolysis for a defined time followed by addition of yeast or bacteria. In a fed-batch operation, fresh substrate is added after the viscosity of lignocellulosic biomass decreases. Most studies conducted with high solids loading are carried out in conjunction with fermentations (Hoyer et al., 2013; Zhang et al., 2010; Zhu et al., 2011) to avoid end-product inhibition of enzymes due to glucose and cellobiose (Xiao et al., 2004). However, a major drawback of SSF from a commercial standpoint is the inability to recirculate fermenting microorganisms for continuous operation since the organisms are mixed with biomass (Ishola et al., 2013; Olofsson et al., 2008). Recently, Ishola et al. (2013) showed continuous operation of a simultaneous saccharification, filtration and fermentation (SSFF) layout with 14.4% (w/w) suspended pretreated spruce for 4 weeks with 85% of theoretical ethanol yield. The development of continuous operating schemes such as SSFF will require enzymatic hydrolysis to be performed at high solids loading.

The objective of the present study was to determine the effect of solids loading on enzymatic hydrolysis of pretreated redcedar between 2 and 20% dry solids (herein all solids loading represented as % refers to dry solids on a w/w basis) as measured by glucose concentration produced and glucan-to-glucose yield of pretreated redcedar.

Since technical challenges with mixing are common to hydrolysis of pretreated biomass above 15% dry solids (Alvira et al., 2013), enzymatic hydrolysis of pretreated redcedar above 15% were conducted both in the presence and absence of stainless steel balls that were used as a mixing aid. The effect of lowering enzyme dosage at high solids loading was also studied. Additionally, the fermentability of enzymatic hydrolysate that was obtained at 20% dry solids loading was tested.

6.2 Material and methods

6.2.1 Biomass characterization

Eastern redcedar (*Juniperus virginana* L.) chips were acquired from the Oklahoma State Forestry Services (Idabel, McCurtain County, OK, USA). The chips contained both heartwood and sapwood fractions of the trunk from redcedar trees. The biomass was ground using a Thomas-Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, USA) equipped with a 2 mm screen. After grinding, the moisture content of the biomass was determined by a convection oven method (Sluiter et al., 2008a). Biomass was stored in resealable bags at room temperature prior to pretreatments and/or compositional analysis.

For compositional analysis, biomass was sieved through Tyler number +9/+60 sieve plates. The samples that were collected on the +60 sieve plate were used for compositional analysis. About 80% of the ground biomass was retained on the +60 sieve plate and the remaining portion was fines. Sieving of biomass was important because the NREL protocols for compositional analysis were developed for particle size between 180 µm and 850 µm (Hames et al., 2008). Ethanol extraction of sieved redcedar was then

carried out using an accelerated solvent extractor (ASE) (Model 300, Dionex Corporation, Sunnyvale, CA, USA) to remove non-structural material using National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008d). The amount of extractives (on a percent dry weight basis) was calculated directly by evaporating ethanol at room temperature in a fume hood and measuring the residual mass.

Following extraction, the biomass was air dried and was analyzed for structural carbohydrates and lignin using a two-step acid hydrolysis procedure developed by NREL (Sluiter et al., 2008c). Structural carbohydrates were analyzed using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 mL min^{-1} and the column temperature was maintained at 85 °C. The total run time for each sample was 30 min. The HPLC with Chemstation software (Agilent Technologies) was calibrated at five levels using known concentrations of compounds before being used to quantitate the concentration of compounds. Acid soluble lignin (ASL) content of biomass was determined using a UV–Vis spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA, USA) at a wavelength of 205 nm and an extinction coefficient of 110 L g^{-1} cm^{-1}. Acid insoluble lignin (AIL) was determined gravimetrically (Sluiter et al., 2008c).

6.2.2 Pretreatments

Acid bisulfite pretreatments were done in a 1-L bench top pressure reactor (Parr series 4250, Parr Instrument Company, Moline, IL, USA) equipped with an agitator, a heater and a control unit. The reactor was initially loaded with 100 g of dry biomass and then filled with a mass of pretreatment liquor to achieve a liquid-to-solid mass ratio of

5:1. The pretreatment liquor was composed of deionized water, sulfuric acid and sodium bisulfite. Sulfuric acid and sodium bisulfite loadings were 3.75 g per100 g of dry wood and 20 g per 100 g of dry wood, respectively. The reactor was agitated at 150 rpm and biomass was soaked in the pretreatment liquor at 90°C for 3 h. At the end of 3 h, the reactor temperature was increased to 200 °C and held for 10 min. These pretreatment conditions were identified as near-optimal in a previous study (Ramachandriya et al., 2013). At the end of the pretreatment hold time, the reactor was cooled in an ice bath until the temperature was less than 55 °C. After cooling the reactor, the solid and liquid fractions were separated using vacuum filtration through a Whatman #4 filter paper. About 5 to 6 g of sample were taken after filtration and dried in an oven at 105 °C to determine the moisture content of wet solids after pretreatment (Sluiter et al., 2008a). The remaining wet solids were then rinsed with 500 mL of deionized water at 60 °C four times to remove soluble sugars and fermentation inhibitors. The moisture content of washed pretreated solids was also determined using a standard NREL procedure (Sluiter et al., 2008a). Pretreated solids were then stored in plastic resealable bags at 4 °C until use for enzymatic hydrolysis. The compositions of pretreated solids and prehydrolysate were determined using protocols developed by NREL (Sluiter et al., 2008b; Sluiter et al., 2008c).

6.2.3 Effect of high solids loading

The first batch of redcedar with composition shown in Table 6.1 was used for this study. Accelerase® 1500 was generously provided by Genencor Inc. (Palo Alto, CA, USA) and it was the enzyme cocktail used for this study. An enzyme loading of 0.5 mL g^{-1} of glucan was used for these studies. This loading was recommended by the

manufacturer as a starting point for optimization. Enzymatic hydrolysis was done at pH 5 using 0.05 M sodium citrate buffer and 50°C in an incubator shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA) at 250 rpm. The experiments were carried out in 250 mL baffled flasks containing a total mass of 100 g. The cellulase activity of Accelerase® 1500 was determined as 92 FPU mL^{-1} of enzyme using a standard protocol developed by NREL (Adney and Baker, 2008). Analytical grade chemicals required for the enzyme assay were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The different dry solids loading levels were 2%, 4%, 8%, 12%, 16% and 20%. Additionally, the effect of mixing aid was also determined at 16% and 20% dry solids loading. Twenty stainless steel metal balls (302 SS, 6.35 mm diameter, Grade 100) were added to hydrolysis reactors with 16% and 20% dry solids loading to aid mixing. All experiments were conducted in duplicate.

One and a half mL of sample were withdrawn at 0, 6, 12, 18, 24, 48, 72 and 96 h to determine the amount of sugar released during enzymatic hydrolysis. For 16% dry solids loading, the initial sample could not be taken due to the high viscosity of the material. The first sample was taken at 12 h after liquefaction was observed. Thereafter, samples were taken at 18, 24, 30, 36, 48, 60, 72, 84, 96, 108, 120 and 144 h. Similarly, liquefaction at 20% dry solids loading was observed at 36 h; hence, samples were taken at 36, 42, 48, 54, 60, 72, 84, 96, 108, 120, 132 and 144 h. Samples were centrifuged at 13,000 rpm for 10 min using a benchtop microcentrifuge (Fisher Scientific, Pittsburgh, PA, USA). The supernatant was collected, filtered through 0.45 µm nylon syringe filters (VWR International, West Chester, PA, USA) and frozen until analyzed. Composition of

sugars released during enzymatic hydrolysis was determined using HPLC as explained earlier in section 2.1.

6.2.4 Fermentability of enzymatic hydrolysate at 20% solid loading

Saccharomyces cerevisiae D_5A was used to test the fermentability of enzymatic hydrolysate at 20% dry solids loading. The yeast was maintained at 4 °C on YPD agar slants containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar. Prior to fermentations, a loopful of culture was aseptically transferred into 100 mL of YPD media containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 50 g L^{-1} glucose in a 250 mL baffled flask reactor and was incubated at 37 °C for 17 h at 250 rpm on an orbital shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA). Yeast cells were concentrated to obtain an initial OD of 0.5 in each fermentation by centrifuging the cells at 3,500 rpm for 10 min in a bench-top centrifuge (Sorvall, Legend RT, Asheville, NC) and washing the cells with 0.89% (w/v) sterile sodium chloride solution.

The enzymatic hydrolysates obtained from hydrolyses with 20% dry solids loading with the treatment that contained metal balls were mixed together to form one hydrolysate for fermentations. The glucose concentration of this solution was 126 g L^{-1}. Fermentations were carried out in 250 mL baffled flasks containing 50 g of hydrolysate. Care was taken that the metal balls were not transferred to the fermentation flasks. Yeast extract and peptone were added into the reactor at 10 g L^{-1}, 20 g L^{-1}, respectively. The flasks were inoculated with 0.5 OD (~0.14 g dry cells L^{-1}) of *S. cerevisiae* D_5A and were incubated at 37 °C at 250 rpm on an orbital shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA). This experiment was performed in triplicate.

Ethanol, acetic acid, glycerol, glucose and xylose were monitored using HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was 0.01 N sulfuric acid at a flow rate of 0.6 mL min^{-1} with a 30 min run time.

6.2.5 Effect of enzyme dosage

The second batch of redcedar with composition shown in Table 6.1 was used for this study. Four levels of enzyme dosage tested were 11.5 FPU g^{-1} glucan (0.125 mL g^{-1} of glucan), 23 FPU g^{-1} glucan (0.25 mL g^{-1} of glucan), 34.5 FPU g^{-1} glucan (0.375 mL g^{-1} glucan) and 46 FPU g^{-1} glucan (0.5 mL g-^{1} of glucan). A dry solids loading of 16% was chosen for this experiment because it resulted in glucan-to-glucose yields comparable to all lower dry solids loadings tested. The total mass in the reactors was 100 g and experiments were performed in triplicate. Enzymatic hydrolysis was carried out at pH 5 using 0.05 M sodium citrate buffer and 50 °C in an incubator shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA). Samples were taken at 12, 18, 24, 30, 36, 48, 60, 72, 84, 96, 108, 120 and 144 h and sugar concentrations were monitored using the procedure detailed in section 2.3.

6.2.6 Statistical analysis

Analysis of variance was conducted using the generalized linear model (GLM) procedure in SAS release 9.3 (SAS, Cary, NC, USA). P values were calculated for each analysis and are shown in the text. Differences among various treatments in Table 6.2 and Fig. 6.2 were determined using Tukey's honest significant difference test at a 95% confidence interval.

6.3 Results and discussion

6.3.1 Redcedar pretreatments

Two different batches of redcedar were procured from the supplier. The compositions of redcedar of the two batches (shown in Table 6.1) were quite different from each other and from our earlier study which used a different batch of redcedar (Ramachandriya et al., 2013). The first batch was used for the study on effect of high solids loading and the second was used to study the effect of enzyme loading. Acid bisulfite pretreatment makes biomass amenable for enzymatic hydrolysis by reducing the degree of polymerization of cellulose and extensively removing hemicellulose and lignin fractions (Zhu et al., 2009). Like a previous study with redcedar, 61.3% of lignin and 90 to 96% of the hemicellulose polymers were removed during pretreatments (on a dry mass basis) (Ramachandriya et al., 2013). Moreover, an average glucan loss of 10% was observed in the present study, which is comparable to the glucan loss observed in Ramachandriya et al. (2013). Other studies conducted on different softwoods using acid bisulfite pretreatment have reported similar levels (9.6 to 12%) of glucan loss during pretreatments (Zhu et al., 2009; Zhu et al., 2010).

Glucan, xylan and mannan recoveries in the pretreated redcedar averaged 91.2 ± 1.5 %, 20.1 ± 1.8 % and 32.0 ± 2.3 %, respectively (values in this section are reported as a mean ± standard error of 8 pretreatments) and were comparable with our previous study (Ramachandriya et al., 2013). Some of the dissolved solids that accumulated in the prehydrolysate were converted into formic acid, acetic acid, levulinic acid, 5-hydroxy-methyl-furfural (HMF) and furfural with concentrations of 0.3 ± 0.1 g L^{-1}, 3.7 ± 0.1 g L^{-1}, 0.1 ± 0.0 g L^{-1}, 1.6 ± 0.1 g L^{-1} and 1.2 ± 0.1 g L^{-1}, respectively, which were comparable

Table 6.1 Biomass composition before and after acid bisulfite pretreatment expressed as % of dry matter for two different batches of Eastern redcedar.

Biomass component	Batch 1[a]		Batch 2[b]	
	Untreated redcedar (%)[c]	Pretreated redcedar (%)[c]	Untreated redcedar (%)[c]	Pretreated redcedar (%)[c]
Glucan	34.2 ± 0.3	67.9 ± 2.4	30.2 ± 0.2	53.7 ± 0.0
Xylan	7.9 ± 0.1	1.5 ± 0.1	5.8 ± 1.0	1.7 ± 0.1
Galactan	3.7 ± 0.0	0.0 ± 0.0	4.6 ± 0.4	1.5 ± 0.2
Arabinan	1.2 ± 0.1	0.0 ± 0.0	2.3 ± 0.2	0.0 ± 0.0
Mannan	8.5 ± 0.1	1.5 ± 0.0	5.6 ± 0.1	0.6 ± 0.0
Lignin[d]	32.2 ± 0.3	26.3 ± 0.1	33.8 ± 1.8	31.3 ± 0.7
Ash	4.3 ± 0.1	ND	9.8 ± 0.1	ND
Extractives	3.2 ± 0.0	ND	6.7 ± 0.0	ND

[a]Used to study the effect of high solids loading
[b]Used to study the effect of enzyme dosage
[c]Values listed above are averages ± standard deviation; n=2
[d]Acid soluble lignin and acid insoluble lignin is included
ND – Not determined

with other studies (Lan et al., 2013; Ramachandriya et al., 2013; Shuai et al., 2010).

6.3.2 Effect of high solids loading

Figs. 6.1a and 6.1b shows the time course of glucose and cellobiose concentrations obtained during enzymatic hydrolysis as a function of increasing solids loading between 2% and 20%. Since liquefaction was observed after 12 h and 36 h at 16% and 20% dry solids loading, respectively, samples were not taken prior to this time. The first 6 h of enzymatic hydrolysis resulted in a rapid production of glucose and cellobiose for dry solids loadings between 2 and 12%. However, the rapid production of glucose and cellobiose occurred between 12 h and 18 h for 16% and between 36 h and 48 h for 20% dry solids loading. Thereafter, the rate of glucose production decreased and the concentration of cellobiose was reduced due to the conversion of cellobiose into glucose owing to the β-glucosidase activity of the enzyme cocktail.

Increasing solids loading resulted in a linear increase in glucose concentrations, which demonstrated that there were no issues with end-product inhibition. At every dry solids loading tested, glucose concentrations followed a typical batch hydrolysis pattern (Cara et al., 2007; Xiao et al., 2004). When solids loadings were below 16%, there was free moisture in the reactors and liquefaction occurred faster than the treatments above 16% because the suspensions were well mixed. However, at a dry solids loading of 16% or higher, there was no free moisture present in the reactors, which made the structure of the material degrade more slowly producing a thick paste. The transformation into a thick paste occurred at 12 h and 36 h with 16% and 20% dry solids loading, respectively. More than 100 g L^{-1} of glucose could be obtained at dry solids loadings above 16%. Operating at these high dry solids loadings is important because of

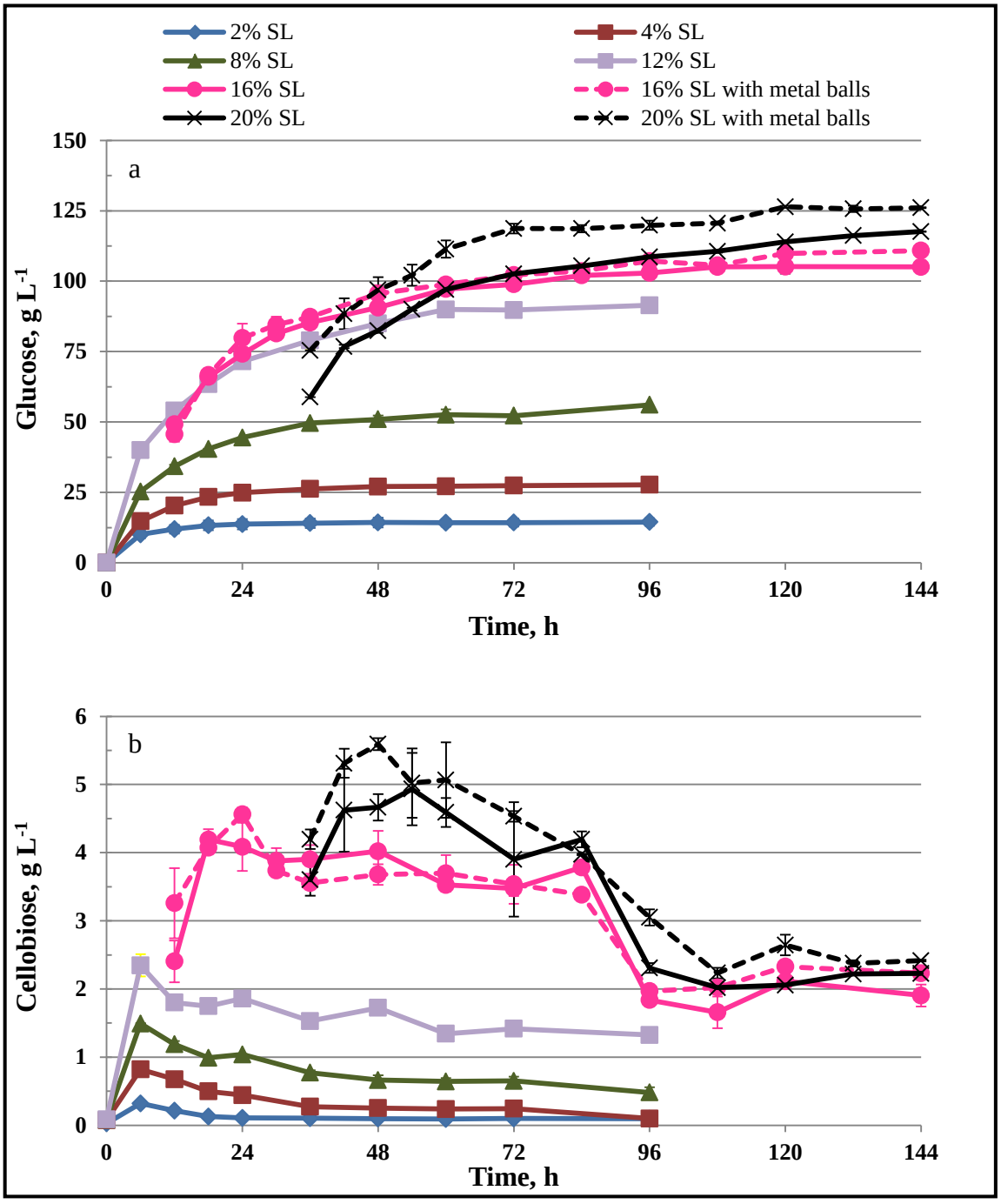

Fig. 6.1 Glucose (6.1a) and cellobiose (6.1b) profiles during enzymatic hydrolysis of pretreated redcedar at different solid loadings (SL) (% w/w, dry basis) and 46 FPU g^{-1} glucan enzyme loading.
Each data point is an average of two replicates and error bars represent one standard error.

the high ethanol concentrations that can be produced during fermentations and lower energy consumption during distillation (Kristensen et al., 2009; Öhgren et al., 2006).

It is also essential to produce glucose efficiently in addition to achieving high glucose concentrations during enzymatic hydrolysate. Glucan-to-glucose yield (Eq. 6.1) of pretreated redcedar is a metric to determine the efficacy of the hydrolysis process and is shown in Fig. 6.2.

$$\text{Glucan-to-glucose yield (\%)} = \frac{\text{Glucose(t)-Glucose(0)}}{\text{SL} \times \text{f(Glucan in pretreated biomass)} \times 1.11} \times 100\ \% \qquad (6.1)$$

Where, Glucose(t) and Glucose(0) are glucose concentrations at time t and 0 h, respectively, SL represents the dry solids loading used for enzymatic hydrolysis, f(Glucan in pretreated biomass) represents the fraction of glucan in pretreated biomass and 1.11 is the conversion factor for glucan to glucose.

The glucan-to-glucose yields for dry solids loadings between 2 and 16% at the end of saccharification experiment (96 h for flasks below 16% and 144 h for flasks at or above 16%) were similar and above 85% ($p = 0.0001$). There was a decrease in glucan-to-glucose yield of pretreated redcedar from 87% at 16% dry solids loading to 77% at 20% dry solids loading. The reduction in glucan-to-glucose yield is likely due to the rheological challenges at high solids loading of 20% that has been commonly observed in other studies (Cara et al., 2007; Jørgensen et al., 2007; Roche et al., 2009; Tengborg et al., 2001). To test if problems with mixing that are common to high solids saccharification lowered glucan-to-glucose yields, experiments were performed at 16% and 20% with 20 stainless steel balls added to shake flasks. Results were compared with the control flasks with similar dry solids loadings without the metal balls. It was observed that the addition of metal balls to the shake flasks improved glucan-to-glucose

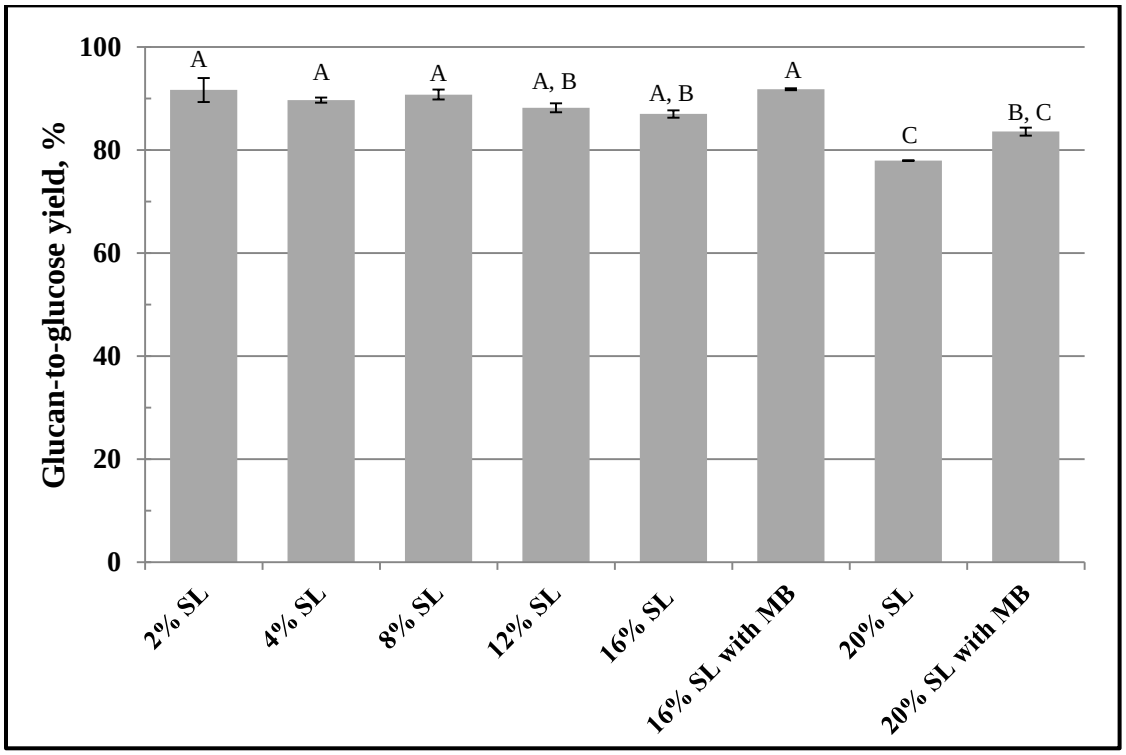

Fig. 6.2 Effect of solid loading (SL) (% w/w, dry basis) on glucan-to-glucose yield of pretreated Eastern redcedar at 96 h for solids loading below 16% and 144 h for solids loading at or above 16% with an enzyme dosage of 46 FPU/g glucan.
Error bars shows the standard error of two replicates. Bars with the same letter are not statistically different at 95% confidence level. MB represents the treatments which had metal balls as mixing aid.

yield at both 16% and 20% ($p = 0.0006$), resulting in a maximum glucose concentration of 126 g L^{-1} with 84% glucan-to-glucose yield at 20%. This clearly shows that mixing the slurry at high solids loading above 16% caused the reduction in glucan-to-glucose yield.

These results were comparable to other studies conducted at high solids loading with different type of pretreatments and lignocellulosic biomass. Agricultural residues and hardwoods are commonly studied biomass types and have performed well with novel approaches and reactor designs for high solids hydrolysis. Recently, Roche et al. (2009) showed that a lab scale roller bottle reactors (RBRs) produced 16 % higher glucose concentrations than conventional shake flask reactors for hydrolyzing pretreated corn stover at 20% dry solids loading and achieved 125 g L^{-1} of glucose (Roche et al., 2009). Zhang et al. (2009) showed the feasibility of using a peg mixer, which is typically used in pulping operations, for high solids enzymatic hydrolysis of extensively delignified pretreated hardwoods. The use of peg mixer achieved 158 g L^{-1} of glucose from organosolv pretreated poplar with an 85% glucan-to-glucose yield during enzymatic hydrolysis at 20% solids loading. Jørgensen et al. (2007) showed that hydrolyzing pretreated wheat straw in a five chambered liquefying reactor resulted in 76 and 86 g of glucose per kg of enzymatic hydrolysate at 20% and 40% dry solids loading, respectively. Another study showed only 71 g L^{-1} of glucose could be obtained when pretreated poplar was hydrolyzed at 20% dry solids loading using a combination of enzyme cocktails (Di Risio et al., 2011). Cara et al. (2007) conducted enzymatic hydrolysis on pretreated olive tree pruning biomass and achieved a maximum glucose concentration of 64.5 g L^{-1} at 20% dry solids loading. To the best of the authors' knowledge, 126 g L^{-1} of glucose

achieved by hydrolyzing pretreated redcedar in the current study is the highest reported glucose concentration observed during enzymatic hydrolysis of any softwood species.

Several previous studies have shown a 22 to 30% decrease in glucan-to-glucose yield of pretreated lignocellulosic biomass as the dry solids loadings were increased from 2 to 20% during enzymatic saccharification (Cara et al., 2007; Jørgensen et al., 2007; Kristensen et al., 2009; Tengborg et al., 2001). However, only a 15% decrease in glucan-to-glucose yields was observed in this study as dry solids loading was increased from 2 to 20%. Also, the glucan-to-glucose yield at 20% dry solids loading in the presence of metal balls was only 9% lower than the yield obtained with 2% dry solids loading. It is possible that removal of 61% of lignin from redcedar could have aided saccharification by decreasing the number of non-specific lignin-enzyme binding sites. This is supported by the promising results shown by Zhang et al. (2009) on pretreated hardwoods containing 1.5 to 2.5% lignin. Additionally, acid bisulfite pretreatments alter the nature of the lignin by sulfonating them and making them hydrophilic, which could have also helped the saccharification process by decreasing non-productive binding of enzymes to lignin (Zhu et al., 2009). The concentrations of xylose and mannose varied between 0.5 to 3.2 g L^{-1} and 0.3 to 1.3 g L^{-1}, respectively between the different levels of dry solids loading tested in the present study. Lower concentrations of xylose and mannose in the enzymatic hydrolysate could have reduced the inhibitory effect of hemicellulose monomers on cellulases (Xiao et al., 2004).

Glucose yield in kg per dry Mg of raw material is an important metric that aids in comparing the glucose production potential of redcedar with other softwoods. Table 6.2

shows glucose yield and % of theoretical glucose yield at the different solids loading, which were calculated using Eqs. 6.2 and 6.3, respectively:

$$\text{Glucose yield (kg per dry Mg)} = [f(\text{Glucan}) \times 1000] \times 1.11 \times [(100\text{-Glucan loss}_{pret})/100] \times \eta_{hyd} \quad (6.2)$$

$$\text{Glucose yield (\% of theoretical)} = \frac{[f(\text{Glucan}) * 1000] \times 1.11 \times [(100\text{-Glucan loss}_{pret})/100] \times \eta_{hyd}}{[f(\text{Glucan}) \text{ X } 1000] \times 1.11} \times 100 \quad (6.3)$$

Where, f(Glucan) denotes the glucan fraction of redcedar (on as received basis) and [f(Glucan) × 1000] represents the kg of glucan in redcedar in one Mg of dry wood. The conversion factor for glucan-to-glucose is 1.11. Glucan loss_{pret} is the percent of glucan lost during pretreatment (on mass basis), which was calculated as 10% from the glucan mass balance before and after pretreatment, and η_{hyd} is the glucan-to-glucose yield of enzymatic hydrolysis at different dry solids loadings shown in Fig. 6.2.

The amount of glucose per dry Mg of redcedar that could be produced at any dry solids loading between 2% and 16% were statistically similar ($p = 0.1209$). A maximum glucose yield of 314 kg per dry Mg of redcedar was achieved at 2% solids loading and at 16% solids loading with metal balls. This was only 15% lower than the yield Zhu et al. (2009) observed with enzymatic hydrolysis on acid bisulfite pretreated spruce. The lower glucose yield of redcedar was due to redcedar containing 21% lower glucan content than spruce. Glucose yield (% of theoretical) is the ratio between glucose yield that is achieved during hydrolysis to the maximum possible glucose yield. The results obtained in the present study are consistent with previous reports that have achieved between 75% and 86% of theoretical glucose yields using spruce and pine (Monavari et al., 2009; Shuai et al., 2010; Zhu et al., 2009).

Table 6.2 Glucose yield in kg Mg^{-1} and % of theoretical achieved at different solids loadings.

Solids loading, % w/w dry basis	Glucose yield, kg Mg^{-1}	Glucose yield, % of theoretical
2[a]	313.1 ± 8.0^{A}	82.4 ± 2.1^{A}
4[a]	306.4 ± 1.7^{A}	80.7 ± 0.5^{A}
8[a]	310.1 ± 3.4^{A}	81.6 ± 0.9^{A}
12[a]	$301.4 \pm 3.0^{A,B}$	$79.3 \pm 0.8^{A,B}$
16[b]	$297.2 \pm 2.4^{A,B}$	$78.2 \pm 0.6^{A,B}$
16 with metal balls[b]	313.6 ± 0.7^{A}	82.5 ± 0.2^{A}
20[b]	266.3 ± 0.3^{C}	70.1 ± 0.1^{C}
20 with metal balls[b]	$285.5 \pm 2.7^{B,C}$	$75.2 \pm 0.7^{B,C}$

Values listed above are averages ± standard deviation; n=2.

[a]Experiments were conducted for 96 h.

[b]Experiments were conducted for 144 h.

[A,B]Values listed in the same column with the same letter are not statistically different at 95% confidence intervals.

6.3.3 Fermentability of enzymatic hydrolysate obtained at 20% solids loading

Acid bisulfite pretreatment of biomass causes extensive sulfonation of lignin moieties (Zhu et al., 2009). It is possible that some of the sulfite species present in pretreated redcedar would be released into the enzymatic hydrolysate. Schimz (1980) showed that low levels of sulfites resulted in death of *S. cerevisiae* because they inhibited ATP production and caused mutagenesis. Although Lan et al. (2013) and Zhu et al. (2011) demonstrated high titers of ethanol using sulfite pretreated biomass without any inhibition of *S. cerevisiae* strains, these studies were conducted at 8 g per 100 g of sodium bisulfite loading. The levels of sodium bisulfite loading used in the current study were 2 to 2.5 fold higher than previous studies (Lan et al., 2013; Shuai et al., 2010; Zhu et al., 2009). Thus, there was a necessity to explore if the higher levels of sodium bisulfite used in this study would inhibit *S. cerevisiae* fermentations.

Fig. 6.3 shows glucose consumption, ethanol production and ethanol yield (% of theoretical) of *S. cerevisiae* D_5A using the enzymatic hydrolysate obtained by saccharifying pretreated redcedar at 20% solids loading (values in this section are based on % w/w dry solids) using metal balls as a mixing aid. Although the glucose concentration in the enzymatic hydrolysate was 126 g L^{-1}, the addition of yeast fermentation medium diluted the glucose concentration to 113 g L^{-1} at the start of fermentations. Nearly all the glucose was metabolized in 42 h of fermentation. Ethanol concentrations as high as 52 g L^{-1} (~ 6.6% by volume) were obtained with a 90% of theoretical glucose-to-ethanol yield. Achieving ethanol concentrations above 4% (w/w) is important because it drastically reduces distillation costs (Öhgren et al., 2006).

Our results are comparable with the fermentation profiles of an industrially

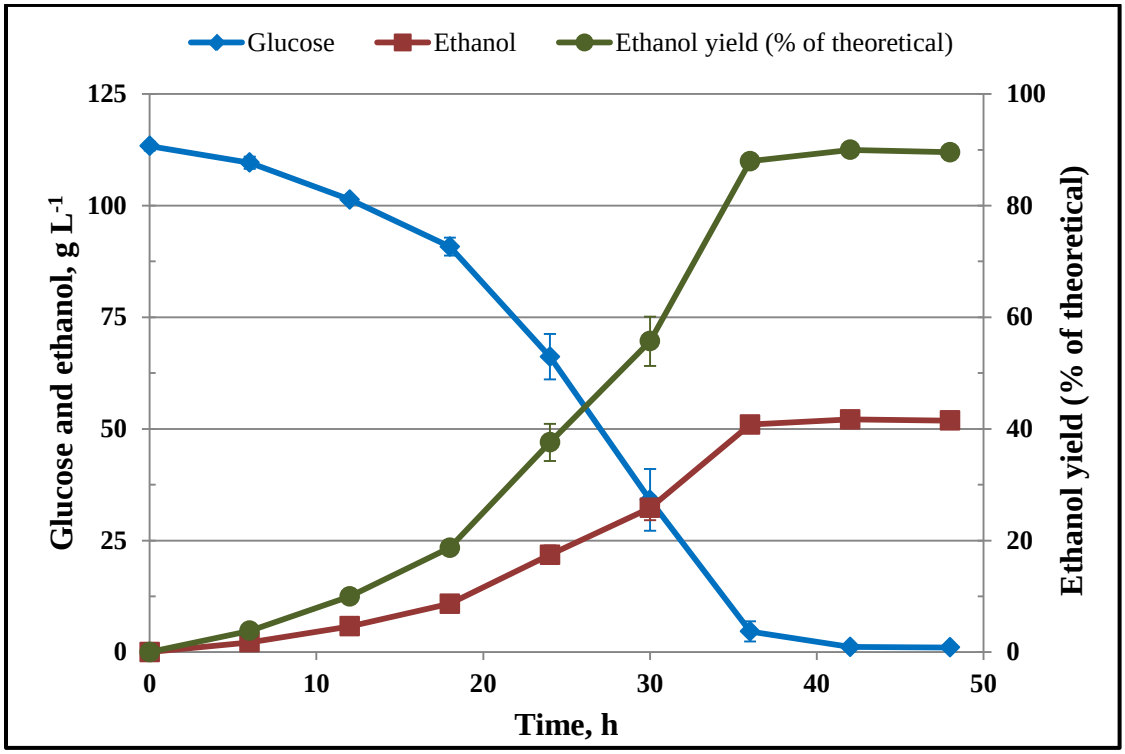

Fig. 6.3 Glucose consumption, ethanol production and ethanol yield (% of theoretical) of *S. cerevisiae* D_5A using enzymatic hydrolysate obtained from 20% solid loadings at 37 °C.

Each data point is an average of three replicates and error bars represent one standard error.

adapted *S.cerevisiae* T2 strain utilizing unbleached hardwood pulp enzymatic hydrolysate containing 110 g L^{-1} of glucose (Zhang et al., 2009). Glycerol was the only co-product produced in the current study with the highest concentration of 1.9 g L^{-1} in 48 h. Negligible concentrations of acetic acid (0.02 g L^{-1}) was produced in this separate hydrolysis and fermentation study, which is different from the previous literature that showed 0.5 g L^{-1} acetic acid production when SSF was performed on pretreated switchgrass using *S. cerevisiae* D_5A (Faga et al., 2010; Pessani et al., 2011). The present study demonstrated the fermentability of enzymatic hydrolysate obtained at 20% solids loading from redcedar without any inhibition of *S. cerevisiae*.

Ethanol yield per Mg of raw material is an important metric to help compare the ethanol production potential of redcedar with other softwoods. Ethanol yield was calculated from experimental data using Eq. 6.4:

$$\text{Ethanol yield (L per dry Mg)} = \frac{[f(\text{Glucan}) \times 1000] \times 1.11 \times 0.51 \times [(100\text{-Glucan loss}_{pret})/100] \times \eta_{hyd} \times \eta_{ferm}}{0.789} \quad (6.4)$$

Where, f(Glucan) denotes the glucan fraction of redcedar (on as received basis) and [f(Glucan) × 1000] represents the kg of glucan in redcedar in one Mg of dry wood. The conversion factor for glucan-to-glucose and glucose-to-ethanol are 1.11 and 0.51, respectively. Glucan $loss_{pret}$ is the percent of glucan lost during pretreatments, which was calculated as 10% from glucan mass balance before and after pretreatments. η_{hyd} is the efficiency of enzymatic hydrolysis at 20% solids loading with metal balls (glucan-to-glucose yield in Eq. 1) that was calculated as 84%, η_{ferm} is the ethanol yield (% of theoretical) that was found to be 90% (Fig. 3) and 0.789 is the density of ethanol in kg L^{-1}.

Ethanol yield of 166.4 L per dry Mg (~44 gallon per dry Mg) of redcedar was achieved during the current study when operated in a separate hydrolysis and fermentation (SHF) mode conducted at 20% solids loading and 46 FPU g^{-1} glucan (0.5 mL g^{-1} glucan) in the presence of metal balls as mixing aid. Ethanol yield from this study was 20% lower than lodgepole pine (Zhu et al., 2010) and the same as acid bisulfite pretreated aspen (Zhu et al., 2011). The ethanol yield as compared to the theoretical level (ratio between ethanol yield that was achieved in the current study to the theoretical ethanol that could be produced from raw material) was only 67.6% from redcedar in the current study, which corresponded well with other studies that reported 68% of theoretical ethanol yield with acid bisulfite pretreated lodgepole pine (Zhu et al., 2010) and 64.5% of theoretical ethanol yield from aspen (Zhu et al., 2011). However, ethanol yield as compared to the theoretical level was much lower than SO_2-based pretreatments conducted on spruce that reported 85% of theoretical ethanol yield when operated in a SSFF mode (Ishola et al., 2013) and 95% of theoretical ethanol yield from acid bisulfite pretreated lodgepole pine using simultaneous saccharification and combined fermentation (SSCF) mode (Lan et al., 2013), where pretreated pine was fermented with non-detoxified and concentrated prehydrolysate fraction. One way to improve ethanol yield from redcedar is by minimizing glucan loss during pretreatments and utilizing the xylan fraction.

6.3.4 Effect of enzyme dosage

The cost of enzymes and the slow rate of enzymatic hydrolysis are the two most important obstacles for ethanol production from lignocellulosic biomass (Newman et al., 2013). Although the cost of cellulases has been lowered significantly, a techno-economic

analysis by Humbird et al. (2011) showed that 16% of the overall ethanol cost was still accounted for by the cost of enzymes. Experiments in the present study were performed using 46 FPU of Accelerase® 1500 g^{-1} glucan (0.5 mL g^{-1} glucan). To test the effect of enzyme dosage on glucose yield, various enzyme loadings were used to hydrolyze pretreated redcedar at 16% dry solids loading. 16% dry solids loading was selected because it produced more than 100 g L^{-1} of fermentable glucose without the need of additional stainless ball as a mixing aid. Thus, the effect of reducing enzyme dosage was observed at four different levels: 46 FPU g^{-1} glucan (0.5 mL g^{-1} of glucan), 34.5 FPU g^{-1} glucan (0.375 mL g^{-1} glucan), 23 FPU g^{-1} glucan (0.25 mL g^{-1} of glucan) and 11.5 FPU g^{-1} glucan (0.125 mL g^{-1} of glucan).

Fig. 6.4 shows the effect of enzyme loading on glucose titer and yield at 16% dry solids loading. At any given sampling time, it was apparent that decreasing enzyme dosage decreased the glucose concentration, which consequently lowered the glucan-to-glucose yields (Fig. 6.4). Reducing the enzyme dosage lowered enzyme binding to cellulose, thereby decreasing glucan-to-glucose yields. However, it was observed that glucose production rates increased linearly between of 0.015 g L^{-1} h^{-1} and 0.019 g L^{-1} h^{-1} after 144 h for enzyme loadings above 34.5 FPUg^{-1} glucan and 46 FPU g^{-1} glucan, respectively but the glucose production rate was approximately two folds higher (0.033 g L^{-1} h^{-1}) with 11.5 FPU g^{-1} glucan of enzyme loading after 144 h. This correlated with a 32% increase in glucan-to-glucose yield of pretreated redcedar between 144 h and 672 h with 11.5 FPU g^{-1} glucan of enzyme loading. Higher glucose productivity after 144 h with enzyme dosage of 11.5 FPU g^{-1} glucan could be due to the fact that around 40% of

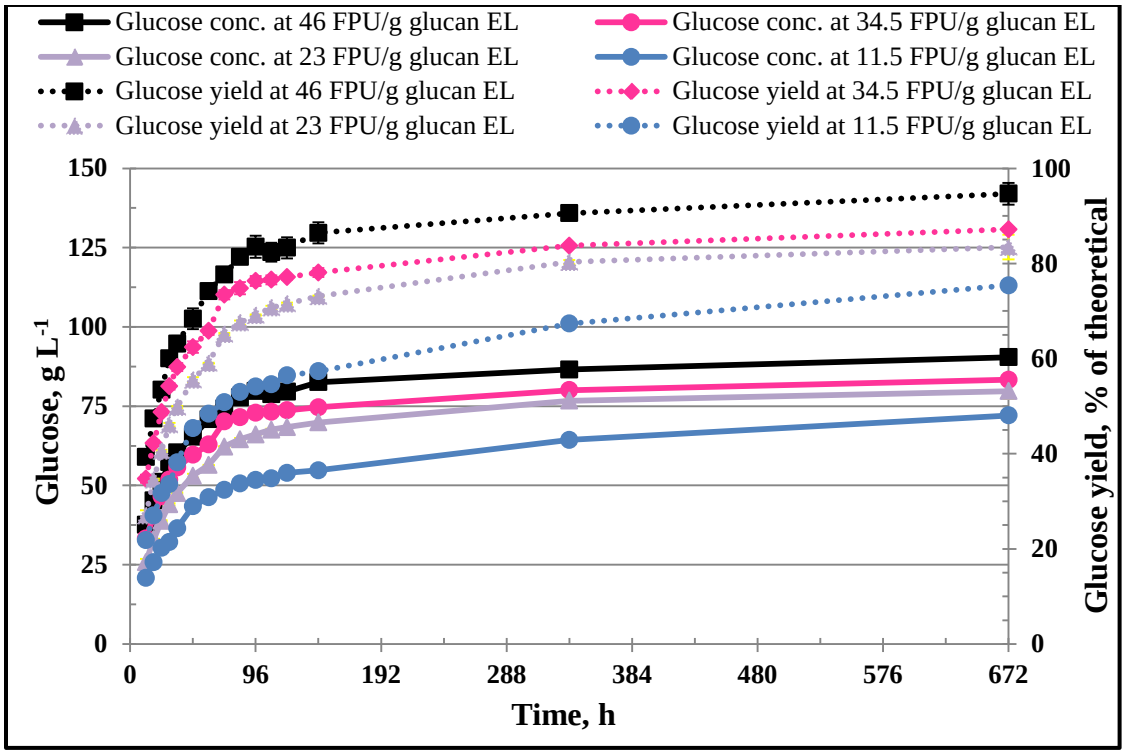

Fig. 6.4 Glucose concentration and glucan-to-glucose yield during enzymatic hydrolysis of pretreated redcedar at different enzyme loadings (FPU g^{-1} glucan) and 16% solids loading.

Each data point is an average of three replicates and error bars represent one standard error.

glucan was still available for enzymatic hydrolysis. Moreover, steady glucose production between 144 h and 672 h at all enzyme dosages shows that the enzymes neither lost their activity nor experienced end-product inhibition. The results obtained in this study corroborates the observations of Wang et al. (2011) that slower product formation was due to lowered binding capacity of enzymes to cellulose and slower three dimensional diffusion of enzymes in the solution.

The highest glucose concentration achieved when pretreated redcedar at 16% solids loading was digested at 46 FPU g^{-1} glucan enzyme loading was 82.6 g L^{-1}. At same levels of dry solids and enzyme loading, 27% higher glucose concentration was achieved in the study on effect of solids loading. This was due to lower glucan content in the batch of redcedar used for the effect of enzyme dosage study as compared to the batch of redcedar used for the effect of solids loading study (Table 6.1). Despite the differences in glucan content of the two batches, both studies resulted in 87% glucan-to-glucose yield in 144 h of hydrolysis.

A 50% reduction in enzyme dosage from 46 to 23 FPU g^{-1} glucan decreased glucose concentrations by 15% while a 75% reduction in enzyme to 11 FPU g^{-1} glucan resulted in decreased glucose concentration by 34% at 144 h of saccharification. Although the enzyme loading of 46 FPU g^{-1} glucan resulted in the highest glucan-to-glucose yield at 144 h, lower enzyme loadings will decrease the operational cost of the process. An economic trade-off will have to be made between enzyme dosage, residence time and the desired glucan-to-glucose yields.

6.4 Conclusions

Acid bisulfite pretreatments of redcedar made the biomass amenable for enzymatic saccharification at high dry solids loading. A linear increase in glucose concentration without significant decrease in glucan-to-glucose yield was achieved as dry solids loading increased. Stainless steel balls used during shake flask hydrolysis helped to overcome rheological challenges at dry solids loadings above 16%. Fermentation of the enzymatic hydrolysate obtained at 20% dry solids produced high titers of ethanol of 52 g L^{-1} without any signs of inhibition of the yeast used. Lowering enzyme dosage below 46 FPU g^{-1} glucan lowered glucose concentration and productivity.

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CHAPTER VII

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF EASTERN REDCEDAR BY *SACCHAROMYCES CEREVISEAE* D_5A: EFFECT OF WOOD ZONES AND PARTICLE SIZE

Abstract

This study investigated the effect of two wood zones (sapwood versus heartwood) and two particle sizes (crumbles® versus ground) on wood glucan-to-ethanol yield after acid bisulfite pretreatment and simultaneous saccharification and fermentation (SSF) of Eastern redcedar (*Juniperus virginiana* L.). SSFs were conducted at 8% solids loading (w/w dry basis) using Accelerase® 1500 at 46 FPU g^{-1} glucan enzyme loading and *Saccharomyces cerevisiae* D_5A. Results demonstrated that the particle size had no effect on wood glucan-to-ethanol yield. However, heartwood glucan-to-ethanol yields were significantly lower than sapwood yields. The highest wood glucan-to-ethanol yield of 187 L dry Mg^{-1} (95% of theoretical) was achieved with sapwood crumbles in 240 h. Ground sapwood, crumbled heartwood and ground heartwood achieved ethanol yields of 89%, 81% and 80% of theoretical in 240 h, respectively. Preliminary mass balances showed 100% glucan recovery with crumbled sapwood and extensive (72%) delignification.

Keywords: Eastern redcedar, SSF, Acid bisulfite pretreatment, Sapwood, Heartwood.

7.1 Introduction

The trunk of woody biomass is mainly comprised of two zones: sapwood and heartwood, each serving different physiological roles (Wiedenhoeft and Miller, 2005). Sapwood is comprised of living cells functioning primarily to transport water and nutrients and store food reserves (Ramos, 2003). On the other hand, heartwood is the innermost part of the wood comprised of physiologically inactive cells with a primary function of offering structural support to the tree. Heartwood is generally characterized by low moisture content, low permeability, low porosity and high extractives content in comparison to sapwood (Ramos, 2003; Wiedenhoeft and Miller, 2005). These physiological differences have significant impacts during pulping operations and there may be substantial differences in the sugar yields from the two zones for ethanol production using the biochemical platform. For instance, heartwood pulp yields of Eucalyptus and maritime pine were 8 and 20% lower compared to their respective sapwood pulp yields (Esteves et al., 2005; Miranda et al., 2007). Pulp yield is a measure of the amount of fibers (cellulose and hemicellulose) that are retained after pulping processes (Casey, 1980). Since, cellulose and hemicellulose are the substrates for pulp, sugars as well as ethanol production, increased pulp yield can translate into higher sugar and ethanol yields. Wood porosity is the only physical property that was studied thoroughly for ethanol production (Ramos, 2003) and no report was found comparing the sapwood and heartwood zones of woody biomass. Wood porosity affects the rate of penetration of chemicals and steam through the wood during pretreatments (Ramos, 2003). Since younger trees have more sapwood compared to older ones, they were easily pretreated using steam explosion (Ramos, 2003). Higher porosity of hardwoods is one of

the reasons that makes them relatively better feedstock than the softwoods for the biochemical conversion process to fuels (Galbe and Zacchi, 2002; Wiedenhoeft and Miller, 2005).

The chemical composition of sapwood and heartwood zones can be considerably different. Ritter and Fleck (1923) showed that cellulose and lignin content in the sapwood fraction of many American softwoods were higher than in the heartwood fraction. Another study conducted by Bertaud and Holmbom (2004) on spruce showed that heartwood lignin and cellulose content was 5% higher and 4% lower than sapwood lignin and cellulose, respectively. Heartwood is considered inferior to sapwood during pulping operations as heartwood decreases pulping yields (Esteves et al., 2005; Miranda et al., 2007). Hence, understanding the chemical heterogeneity of wood zones is important for selecting process conditions that will help to effectively utilize both sapwood and heartwood zones for ethanol production.

To the best of the authors' knowledge, there have been no reports comparing ethanol production from heartwood and sapwood. This could be due to the fact that conventional size reduction techniques such as rotary hammer mills, chippers and grinders are unable to fractionate wood into different zones. Additionally, these size reduction methods also produce excessive fine materials like dust, have a random particle size distribution and are very energy intensive (Dooley et al., 2013). Forest Concepts, LLC have developed a low energy consuming size reduction process for making precision feedstock particles from woody biomass for biochemical and thermochemical conversion processes (Dooley et al., 2013). Logs of biomass are first passed through a rotary veneer lathe to peal the surface of the wood and then the peeled surface passes

through a rotary shear configurable crumbler® (crumbler® is a registered trademark of Forest Concepts, LLC, Auburn, WA) to give a desired particle size (Dooley et al., 2013). Fractionation of the wood zones is possible because of the veneering process. Biomass crumbles® (crumbles® is a registered trademark of Forest Concepts, LLC, Auburn, WA) with widths of 1.5 mm to 6 mm screen size could be obtained by adjusting the cutter wheel shafts (Lanning et al., 2012). The energy consumed to reduce the size of hybrid poplar to 2 mm screen size using Forest Concepts, LLC method was 150 MJ Mg^{-1} of dry wood (Lanning et al., 2012), while typical size reduction techniques such as hammer mill and disk mill used from 470 to 2,160 MJ Mg^{-1} of dry wood of energy for milling lignocellulosic biomass to 2 mm screen size (Schell and Harwood, 1994; Sun and Cheng, 2002).

Eastern redcedar (*Juniperus virginiana* L.) (hereafter referred to as redcedar) was chosen for this study because it is a predominant softwood species available in the central plains of the United States with an approximate ethanol production potential of 2 billion L (530 million gallons) from just 17 counties in Northwest Oklahoma (Ramachandriya et al., 2013). The objective of this study was to determine the effect of wood zones (sapwood versus heartwood) and particle size (2.5 mm size crumbles versus 0.5 mm size ground particles) on wood glucan-to-ethanol yield from redcedar using acid bisulfite pretreatment and simultaneous saccharification and fermentation (SSF).

7.2 Materials and Methods

7.2.1 Biomass characterization

Eastern redcedar (*Juniperus virginana* L.) heartwood and sapwood crumbles were provided by Forest Concepts, LLC (Auburn, WA, USA). Some of the heartwood and sapwood crumbles were ground using a Thomas-Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, USA) for compositional analysis and for studying the effect of particle size on ethanol production. Particle size distribution of the crumbled and ground biomass were determined following ASABE standard method (ASABE Standards, 2006) using a sieve shaker (Dura Tap^{TM} shakers, CSC Scientific, Fairfax, VA) consisting seven sieves that were American Society of Testing and Material (ASTM) specified, a lid and a pan. For determining the size of crumbles, screen size ranged from 4.750 mm to 0.355 mm. On the other hand, screen size ranged from 2 mm to 0.125 mm for determining the size of ground biomass. 50 g of samples were added to the top plate and the samples were screened for 10 min. After sieving, the mass of samples collected on each sieve was measured. Duplicate samples were used to determine the particle size distribution and the geometric mean length (by mass) of the crumbled sapwood, crumbled heartwood, ground sapwood and ground heartwood using ASABE standard protocols (ASABE Standards, 2006).

The moisture content of crumbles and ground redcedar was determined by a convection oven method (Sluiter et al., 2008a). Biomass was stored in resealable bags at room temperature prior to pretreatments and/or compositional analysis. For compositional analysis, ground fractions of heartwood and sapwood were sieved through Tyler number +9/+60 sieve plate and the fractions retained on +60 sieve plate were used

for compositional analysis. About 80% of the ground fractions of heartwood and sapwood were collected on the +60 sieve plate and the remaining portions were fines. Sieving was important because the NREL protocols for compositional analysis were developed and optimized for samples with particle size between 180 µm and 850 µm (Hames et al., 2008). Ethanol extraction of redcedar collected on +60 sieve plate was then carried out using an accelerated solvent extractor (ASE) (Model 300, Dionex Corporation, Sunnyvale, CA, USA) to measure non-structural material using National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008d). The extractives were collected in a beaker and were allowed to air-dry in a fume hood. The amount of extractives (on a percent dry weight basis) was calculated directly by measuring the residual mass of the beaker (Sluiter et al., 2008d).

Following extraction, the biomass was air dried and analyzed for structural carbohydrates and lignin using a two-step acid hydrolysis procedure developed by NREL (Sluiter et al., 2008c). Structural carbohydrates were analyzed using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale. CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 mL min^{-1} and a column temperature of 85 °C. The total run time for each sample was 30 min. Acid soluble lignin (ASL) content of biomass was determined using a UV–Vis spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA, USA) at a wavelength of 205 nm and an extinction coefficient of 110 L g^{-1} cm^{-1}. Acid insoluble lignin (AIL) was determined gravimetrically (Sluiter et al., 2008c).

7.2.2 Pretreatments

Crumbled and ground biomass were pretreated using acid bisulfite pretreatments were performed in a bench top pressure reactor (Parr series 4250, Parr Instrument Company, Moline, IL, USA). The total volume of the reactor was 1 L and it was equipped with an agitator, a heater and a control unit. The amounts of dry redcedar and pretreatment liquor loaded into the reactor were 100 g and 500 g, respectively, to achieve a liquid-to-solid mass ratio of 5:1. The pretreatment liquor contained sulfuric acid and sodium bisulfite at loadings of 0.0375 g g^{-1} of dry wood and 0.2 g g^{-1} of dry wood, respectively. The reactor was agitated at 150 rpm and biomass was soaked in the pretreatment liquor at 90 °C for 3 h. Then, the reactor temperature was increased to 200 °C and held for 10 min. These pretreatment conditions were identified as near-optimal for preparing redcedar for cellulase hydrolysis in a previous study (Ramachandriya et al., 2013). At the end of the pretreatment hold time of 10 min, the reactor was cooled in an ice bath until the reactor temperature was 55 °C. After cooling the reactor, the slurry was filtered using vacuum filtration through a Whatman #4 filter paper for solid and liquid recovery. Moisture content of the unwashed solid fraction was measured using a standard NREL procedure (Sluiter et al., 2008a). The wet solids were then rinsed with 500 mL of deionized water for four times at 60 °C to remove soluble sugars and fermentation inhibitors and the moisture content of washed solids was determined (Sluiter et al., 2008a). The wet washed solids were then stored in plastic resealable bags at 4 °C until use for enzymatic hydrolysis and compositional analysis. The compositions of pretreated solids and prehydrolyzate were determined using NREL protocols (Sluiter et al., 2008b; Sluiter et al., 2008c).

7.2.3 Enzymes, yeast strain and inoculum preparation

Accelerase® 1500 was generously provided by Genencor Inc. (Palo Alto, CA, USA) and it was the enzyme cocktail used in this study. The cellulase activity of Accelerase® 1500 was determined as 92 FPU mL^{-1} of enzyme using a standard protocol developed by NREL (Adney and Baker, 2008). An enzyme loading of 0.5 mL g^{-1} glucan (46 FPU g^{-1} glucan) was used for these studies. This volume loading was recommended by the manufacturer as a starting point for process optimization.

Saccharomyces cerevisiae D_5A yeast was used for SSFs. This yeast ferments hexoses, i.e. glucose and mannose, but not pentoses. The yeast was maintained at 4 °C on YPD agar slants containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar. Prior to fermentations, 100 mL preculture was prepared using YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 50 g L^{-1} glucose in a 250 mL baffled flask reactor. This was incubated at 37 °C for 16.5 h at 250 rpm on an orbital shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA). Yeast cells were concentrated to obtain an initial optical density (OD) of 0.5 by centrifuging the cells at 3,500 rpm for 10 min in a bench-top centrifuge (Sorvall, Legend RT, Asheville, NC, USA) and washing with 0.89% (w/v) sterile sodium chloride solution.

7.2.4 Simultaneous saccharification and fermentation

The SSF experiments were performed in 250 mL shake flask reactors with a working volume of 100 mL containing 10 mL of 10X yeast fermentation medium, 5 mL of 1 M sodium citrate buffer at pH 5.5, 8% dry solids loading (w/w), 0.5 mL g^{-1} of glucan enzyme loading, and 1 mL of concentrated yeast cells. The flasks were sealed with a rubber stopper fitted with a one way valve (Check valve, Fisher Scientific, Pittsburgh,

PA, USA) to exhaust CO_2 produced during SSFs and to maintain anaerobic conditions. A solid loading of 8% was chosen because operating at solids loadings above 10% causes many technical problems due to increased viscosity and end-product inhibition (Jørgensen et al., 2007). Concentrated yeast fermentation medium (10X) supplied a nitrogen source for yeast cells and was prepared by mixing 100 g L^{-1} and 200 g L^{-1} of yeast extract and peptone, respectively, in deionized water and sterilizing it at 121 °C for 30 min. The flask reactors were incubated at 37 °C on an incubating shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA) at 200 rpm. Samples (1.5 mL) were collected at 0, 6, 12, 18, 24, 36, 48, 60, 72, 96, 120, 144, 168, 192 and 240 h. The samples were centrifuged at 13,500 rpm for 10 min and the supernatant was filter sterilized using 0.22 µm nylon filters (VWR International, West Chester, PA, USA) prior to product analysis. Ethanol, acetic acid, glycerol, succinic acid, glucose and xylose concentrations were measured using HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was 0.01 N sulfuric acid at a flow rate of 0.6 mL min^{-1} with a 30 min run time. The HPLC with Chemstation software (Agilent Technologies) was calibrated at five levels using known concentrations of compounds before being used to quantitate the concentration of compounds.

7.2.5 Statistical design of experiment and analysis

A completely randomized factorial design was constructed to test two factors at two levels. The independent variables tested include two zones of redcedar wood: sapwood and heartwood, and two particle sizes: crumbles (2.5 mm particle size) and ground (0.5 mm particle size). Four treatment combinations obtained were: sapwood

ground, sapwood crumbles, heartwood ground and heartwood crumbles. Two replications were performed per pretreatments resulting in a total of 8 pretreatments. From each of the 8 pretreatments, two subsamples were taken to perform SSFs. The main response variable for comparing the different treatment combinations was the wood glucan-to-ethanol yield. However, other response variables such as biomass component losses, glucan recovery in pretreated solids and prehydrolyzate and concentrations of fermentation inhibitors were also compared between the four treatment combinations.

Analysis of variance (ANOVA) was conducted using the generalized linear model (GLM) procedure in SAS release 9.3 (SAS, Cary, NC). P values were calculated and are shown in the text. Post-hoc analysis for determining the differences between various treatments shown in Tables 7.1 to 7.4 were determined using Tukey's honestly significant difference test (Abdi and Williams, 2010) at a 95% confidence interval.

7.3 Results and discussion

7.3.1 Composition of the sapwood and heartwood zones

Table 7.1 shows the composition of sapwood and heartwood zones of redcedar. The extractives content of heartwood was 25% higher than sapwood. The extractives are generally produced by the parenchyma cells at the heartwood-sapwood boundary (Wiedenhoeft and Miller, 2005) and are transported to the heartwood section, thereby increasing the extractives content of heartwood. Sapwood and heartwood had similar glucan and lignin contents expressed as weight percentage of dry wood (Table 7.1). These results are different from the observations of Ritter and Fleck (1923) who found that the cellulose content in the sapwood fraction of bald cypress, white pine, yellow

Table 7.1 Chemical compositions of sapwood and heartwood zones of Eastern redcedar[a].

Component	Sapwood (%)	Heartwood (%)
Glucan	34.7 ± 0.5	34.6 ± 0.0
Xylan	8.9 ± 0.2	8.5 ± 0.0
Galactan	2.6 ± 0.0	3.0 ± 0.1
Arabinan	1.0 ± 0.0	0.7 ± 0.0
Mannan	6.7 ± 0.4	7.4 ± 0.0
Lignin[b]	33.7 ± 0.4	34.3 ± 0.1
Extractives	4.0 ± 0.0	4.9 ± 0.2
Ash	0.5 ± 0.0	0.1 ± 0.0

[a]Values listed above are means ± standard error of two subsamples.
[b]Acid soluble lignin and acid insoluble lignin are included in lignin content.

cedar and incense cedar were 4 to 10% higher than their respective cellulose content of heartwood on extractives free basis. On the other hand, the present study showed that the lignin content of heartwood was only 2% higher than in sapwood, which is within values obtained in previous studies that showed 1 to 9% higher lignin in heartwood compared to sapwood (Bertaud and Holmbom, 2004; Ritter and Fleck, 1923). Similarly, the ash content of sapwood was five folds higher than heartwood which was consistent with the report by Ritter and Fleck (1923). The hemicellulose contents in the sapwood and heartwood were 19.2 ± 0.7 % and 19.6 ± 0.1 %, respectively. Similar chemical composition of sapwood and heartwood zones is advantageous for the biochemical conversion process to produce ethanol because similar pretreatment and fermentation conditions can be used.

7.3.2 Particle size distribution

Figs. 7.1a and 7.1b shows the particle size distribution of the crumbles and ground biomass of the two wood zones, respectively. Sapwood crumbles and heartwood crumbles had 83% and 86% of the particles retained on sieves with 2.36 mm and 2.00 mm sieve openings. In contrast, sapwood ground and heartwood ground had 70% and 79% of the particles retained on sieves with 0.60 mm and 0.35 mm sieve openings. Despite of a slight difference in the particle size distribution between the sapwood and heartwood crumbles, their geometric mean particle length was 2.5 ± 0.3 mm and 2.5 ± 0.3 mm, respectively. On the other hand, the geometric mean particle length of sapwood

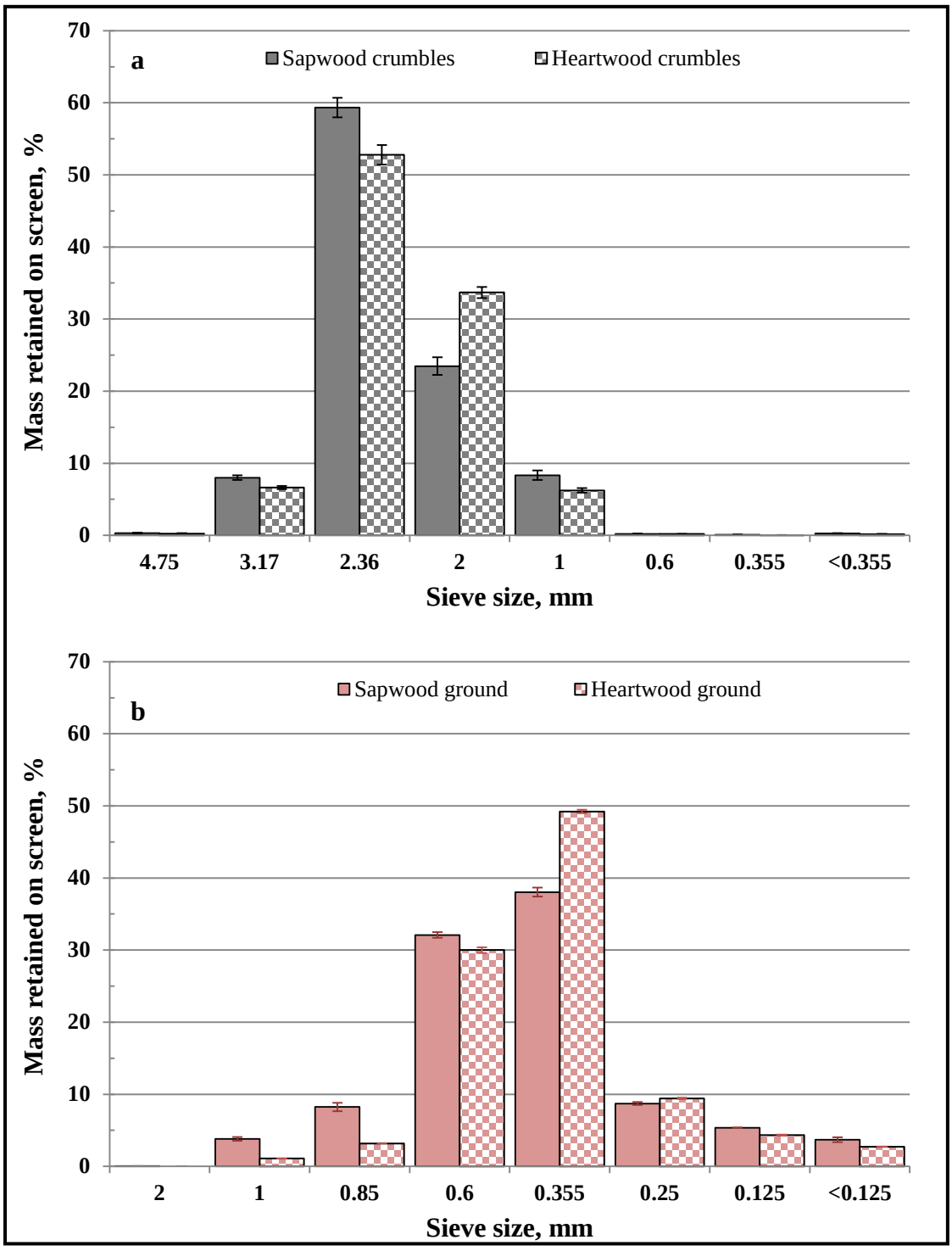

Fig. 7.1 Particle size distribution of crumbled® (a) and ground (b) sections of sapwood and heartwood. Each data point is an average of two replications and error bars represent one standard error.

ground and heartwood ground was 0.5 ± 0.1 mm and 0.5 ± 0.1 mm, respectively. Thus, on an average the ground particles were five folds smaller that the crumbled particles. The size of the crumbled particles was comparable with a previous study by Dooley et al. (2013) that reported 2.3 mm mean particle length when the same crumbler developed by Forest Concepts, LLC was used to study the effect of material orientation using Douglas fir.

7.3.3 Pretreatments

During acid bisulfite pretreatments, sulfuric acid and sodium bisulfite work in tandem to remove large fractions of hemicellulose and lignin, thereby making lignocellulosic biomass amenable for enzymatic hydrolysis and fermentation (Ramachandriya et al., 2013; Zhu and Pan, 2010; Zhu et al., 2009; Zhu et al., 2010). Table 7.2 shows the loss in biomass components after pretreatment with four different treatment combinations, which were calculated by mass balances around the pretreatment reactor using Eq. (7.1):

$$\text{Glucan or lignin or hemicell loss, \%} = \frac{(\text{Glucan or lignin or hemicell})_{bp} - (\text{Glucan or lignin or hemicell})_{ap}}{(\text{Glucan or lignin or hemicell})_{bp}} \times 100 \qquad (7.1)$$

where $(\text{Glucan or lignin or hemicell})_{bp}$ is the mass of the component in 100 g of dry wood before pretreatments and $(\text{Glucan or Lignin or hemicell})_{ap}$ is the mass of the component in 100 g of dry wood after pretreatment. Hemicellulose component is the sum of xylan, galactan, arabinan and mannan fractions.

Hemicellulose removal of 91 to 95% was observed for all treatment combinations after acid bisulfite pretreatments (Table 7.2). Sulfuric acid plays an important role in the removal of hemicellulose polysaccharides as they are prone to acid hydrolysis due to low

Table 7.2 Biomass components loss after pretreatment and sugars recovered in pretreated solids and prehydrolyzates for the four combinations of wood treatments[a].

Treatment	Loss, %[b]			Recovery in pretreated solids, %[c]			Recovery in prehydrolyzate, %[d]		
	Glucan	Lignin	Hemi[e]	Glucan	Xylan	Mannan	Glucan	Xylan	Mannan
Sapwood ground	7.5 ± 4.0^{A}	$47.6 \pm 8.8^{A,B}$	95.1 ± 2.2^{A}	92.5 ± 4.0^{A}	5.1 ± 2.0^{B}	$3.8 \pm 3.7^{A,B}$	3.5 ± 0.4^{A}	7.2 ± 1.6^{B}	15.5 ± 7.4^{B}
Sapwood crumbles®	4.0 ± 1.2^{A}	71.6 ± 0.5^{A}	94.0 ± 0.2^{A}	96.0 ± 1.2^{A}	10.1 ± 0.3^{A}	0.1 ± 0.0^{B}	4.4 ± 0.2^{A}	18.0 ± 2.0^{A}	36.8 ± 1.4^{A}
Heartwood ground	6.5 ± 0.2^{A}	$24.3 \pm 2.0^{B,C}$	91.2 ± 0.5^{A}	93.5 ± 0.2^{A}	$6.6 \pm 0.5^{A,B}$	12.2 ± 0.7^{A}	4.2 ± 0.3^{A}	$12.1 \pm 0.2^{A,B}$	18.9 ± 0.9^{B}
Heartwood crumbles®	5.7 ± 1.9^{A}	20.8 ± 0.9^{C}	91.0 ± 0.3^{A}	94.3 ± 1.9^{A}	$8.7 \pm 0.1^{A,B}$	$10.2 \pm 0.6^{A,B}$	4.1 ± 0.2^{A}	15.9 ± 1.6^{A}	$27.4 \pm 2.5^{A,B}$

[a]Values listed in this table are means ± standard error; n=2

[b]Component losses were calculated using Eq. (7.1).

[c,d]Glucan, xylan and mannan recovered in pretreated solids and prehydrolysate were calculated using Eq. (7.2) and Eq. (7.3), respectively.

[e]Hemi: Hemicellulose fraction is represented by the sum of xylan, galactan, arabinan and mannan fractions.

[A,B,C]Values listed in the same column with the same letter are not statistically different at 95% confidence intervals.

pH of the pretreatment liquor, high pretreatment temperatures and low degree of polymerization (Ingruber, 1985). The loss in hemicellulose measured in the present study was comparable with previous reports that have shown significant removal of hemicelluloses with acid bisulfite pretreatments (Ramachandriya et al., 2013; Zhu et al., 2009). Lignin loss (commonly referred to as delignification) during acid bisulfite pretreatments is crucial to improve the digestibility of pretreated biomass (Ramachandriya et al., 2013). The highest lignin loss observed in this study was with sapwood crumbles (72%) followed by sapwood ground (48%), heartwood ground (24%) and heartwood crumbles (21%). In a previous study, 59% of lignin was removed after pretreating ground redcedar containing both the sapwood and heartwood fractions of redcedar at the same conditions used in the present study (Ramachandriya et al., 2013). Despite the similarity in lignin content of sapwood and heartwood, heartwood treatments showed 69% and 53% less delignification than sapwood crumbles and sapwood ground, respectively. Although sapwood crumbles had higher delignification than sapwood ground, they were statistically similar due to a large standard error between the two replicates (Table 7.2). The differences in delignification between the two wood zones could be due to differences in physiological properties like porosity between the two wood zones. Although the porosities of sapwood and heartwood zones of redcedar were not determined in this study, heartwood structure is generally less porous than sapwood (Ramos, 2003; Wiedenhoeft and Miller, 2005). Wood porosity affects the rate of penetration of chemicals and steam through the wood (Ramos, 2003). During acid bisulfite pretreatments, biomass delignification happens in two steps. First, the α-carbon on phenolic monomers is prone to hydrolytic attack creating a relatively unstable

carbonium ion (C+) called the quinone-methide intermediate (Bryce, 1980). Then, bisulfite (HSO_3^+) ions available in the pretreatment liquor attack the carbonium ion and the β-carbon on lignin resulting in cleavage of the lignin monomer and the production of lignosulfonates (Bryce, 1980). In order to achieve significant delignification of biomass, sulfuric acid and sodium bisulfite must reach to the lignin monomers in the middle lamella and secondary cell wall. It is likely that lower porosity of the heartwood zone reduced the amount of delignification.

Glucan loss in all four treatment combinations were similar ($p = 0.7579$) and varied between 4 and 8%. Minimizing glucan loss during pretreatments benefits the overall process because glucan is the substrate for hydrolysis and subsequent ethanol production. The results in the present study are comparable with the glucan loss (6 to 14%) reported on softwood pretreatment using acid bisulfite pretreatment Ramachandriya et al., 2013; Shuai et al., 2010; Tian et al., 2010; Zhu et al., 2009; Zhu et al., 2010).

High recovery of polysaccharides in pretreated solids is a desirable feature for a pretreatment process (Saville, 2011). Softwoods are rich in glucan and mannan with lower amounts of xylan relative to hardwoods and agricultural residues. Hence, glucan and mannan are the main substrates for yeast and bacteria to make ethanol. Therefore, it is desirable to retain as much as glucan and mannan as possible in the pretreated biomass. Table 7.2 shows the percentage recovery of glucan, xylan and mannan fractions in pretreated redcedar and the prehydrolyzate fraction for the four treatments, which was calculated using Eq. (7.2) and Eq. (7.3):

$$\text{Gluc or xyl or mann recovery in pretreated biomass (\%)} = \frac{(\text{Glun or xyl or mann})_{\text{pretreted biomass}}}{(\text{Gluc or xyl or mann})_{\text{untreated biomass}}} \times 100 \qquad (7.2)$$

$$\text{Gluc or xyl or mann recovery in prehydrolyzate (\%)} = \frac{(\text{Gluc or xyl or mann})_{\text{prehydrolyzate}}}{(\text{Gluc or xyl or mann})_{\text{untreated biomass}}} \times 100 \quad (7.3)$$

where $(\text{Gluc or xyl or mann})_{\text{pretreted biomass}}$, $(\text{Gluc or xyl or mann})_{\text{untreated biomass}}$ and $(\text{Gluc or xyl or mann})_{\text{prehydrolyzate}}$ are masses of glucan,. xylan and mannan in pretreated biomass, untreated biomass and prehydrolyzate, respectively. Sum of Eq. (7.2) and Eq. (7.3) will give overall recovery of biomass components.

Glucan recovered in pretreated biomass was between 92 and 96% for all treatments. Such a high recovery of glucan in pretreated biomass is required to achieve high ethanol yields. The overall recovery of biomass components can be obtained by adding component recovered in pretreated biomass and prehydrolyzate. In the present study, overall glucan recovered varied between 96 and 100% and was consistent with a previous study (Ramachandriya et al., 2013).

Overall, xylan and mannan recovered varied between 9 and 30% and 8 and 41%, respectively. The results in the present study are comparable with a previous report that achieved 13% and 29% xylan and mannan recoveries, respectively, using ground redcedar containing both sapwood and heartwood fractions at the same pretreatment conditions (Ramachandriya et al., 2013). Acid bisulfite pretreatment removes lignin and hemicellulose (discussed earlier in section 7.3.2) which explains the low recoveries of xylan and mannan in pretreated solids. Zhu et al. (2009) also observed only 7% xylan and 2% mannan recovery in pretreated spruce during acid bisulfite pretreatments at 180 °C for 30 min with a sulfuric acid and sodium bisulfite loading of 0.37 g g^{-1} of dry wood and 0.9 g g^{-1} of dry wood, respectively. Large parts of xylan and mannan in spruce were collected in the prehydrolyzate fraction resulting in an overall xylan and mannan recovery of 76 and 88% (Zhu et al., 2009). Such a high recovery may have been due to

lower pretreatment temperature and sodium bisulfite loading than those used in the present study.

Hemicellulose sugars dissolved in the prehydrolyzate are generally converted into fermentation inhibitors. The concentrations of fermentation inhibitors observed in the four different treatment combinations are listed in Table 7.3. During pretreatments, the acetyl groups on hemicelluloses breakdown to form acetic acid while hexoses and pentoses are degraded into 5-hydroxymethylfurfural (HMF) and furfural, respectively (Shuai et al., 2010). Successive decomposition of HMF produces formic acid and levulinic acid (Shuai et al., 2010). Except acetic acid, the concentrations of other fermentation inhibitors were similar for all the treatment combinations used in this study. The acetic acid concentration in the prehydrolzate was significantly higher with sapwood compared to heartwood ($p = 0.0022$). This is because the acetyl content of sapwood is generally higher than heartwood in softwoods (Ritter and Fleck, 1923). The concentrations of fermentation inhibitors obtained in the present study were comparable to previous reports with acid bisulfite pretreatments used for softwood (Lan et al., 2013; Ramachandriya et al., 2013; Shuai et al., 2010; Tian et al., 2010).

7.3.4 Simultaneous saccharification and fermentation

It was hypothesized that the two wood zones may result in significantly different wood glucan-to-ethanol yield (defined in Eq. 7.4 and Eq. 7.5), which is the measure of the efficiency of the entire ethanol production process. This hypothesis was based on studies conducted by Esteves et al. (2005) and Miranda et al. (2007) that showed heartwood pulp yields were 8% and 20% lower than sapwood pulping yields during Kraft's pulping of softwoods. Although pulping and acid bisulfite pretreatments have

Table 7.3 Concentration of fermentation inhibitors in prehydrolysate[a].

Treatment	Fermentation inhibitors, gL^{-1}				
	Acetic acid	**Formic acid**	**Levulinic acid**	**HMF**	**Furfural**
Sapwood ground	6.29 ± 0.43^{A}	0.48 ± 0.00^{A}	0.44 ± 0.08^{A}	2.96 ± 0.18^{A}	2.91 ± 0.21^{A}
Sapwood crumbles®	$5.47 \pm 0.02^{A,B}$	0.48 ± 0.05^{A}	0.30 ± 0.01^{A}	2.68 ± 0.31^{A}	2.59 ± 0.29^{A}
Heartwood ground	4.29 ± 0.09^{B}	0.42 ± 0.01^{A}	0.43 ± 0.02^{A}	3.13 ± 0.18^{A}	2.59 ± 0.03^{A}
Heartwood crumbles®	4.40 ± 0.00^{B}	0.37 ± 0.07^{A}	0.35 ± 0.00^{A}	2.76 ± 0.17^{A}	2.28 ± 0.06^{A}

[a]Values listed in this table are means ± standard error of two replicates on pretreatments

[A,B]Values listed in the same column with the same letter are not statistically different at 95% confidence intervals.

different goals, they work in a similar manner by removing lignin and preserving as much as cellulose and hemicellulose fractions as possible. Another hypothesis of this study was that ground biomass (0.5 mm particle size) would result in higher wood glucan-to-ethanol yield than 2.5 mm particle size crumbles. Higher glucan-to glucose yield and/or glucan-to-ethanol yield at reduced pretreatment severity has been observed due to particle size reduction as milling increases the specific surface area, reduces the degree of polymerization (DP) and shears the biomass (Hendriks and Zeeman, 2009).

The overall wood glucan-to-ethanol yield (% of theoretical) defined in Eq. (7.4) gives a measure of the efficiency of the overall ethanol production process and is calculated from the amount of ethanol that was produced in different treatments with respect to the theoretical maximum ethanol that could be produced based on the glucan present in the untreated wood. Wood glucan-to-ethanol yield per dry Mg of raw material is an important metric used to compare ethanol production potential of redcedar with other softwoods and was calculated using Eq. (7.5):

$$\text{Wood glucan-to-ethanol yield (\%)} = \frac{[f(\text{glucan})_{\text{pretreated}} \times 1000] \times 1.11 \times 0.51 \times \text{ETOH yield}}{[f(\text{glucan})_{\text{untreated}} \times 1000] \times 1.11 \times 0.51} \times 100 \qquad (7.4)$$

$$\text{Wood glucan-to-ethanol yield (L dry Mg}^{-1}) = \frac{[f(\text{glucan})_{\text{pretreated}} \times 1000] \times 1.11 \times 0.51 \times \text{ETOH yield}}{0.79} \qquad (7.5)$$

where, $f(\text{glucan})_{\text{pretreated}}$ and $f(\text{glucan})_{\text{untreated}}$ represents the fraction of glucan in pretreated and untreated redcedar and $[f(\text{glucan})_{\text{pretreated}} \times 1000]$ and $[f(\text{glucan})_{\text{untreated}} \times 1000]$ represents the mass of glucan in one ton of pretreated and untreated redcedar, respectively. Theoretical mass conversion factor of glucan to glucose and glucose to ethanol were 1.11 (g g^{-1}) and 0.51 (g g^{-1}), respectively, and 0.79 is ethanol density in kg L^{-1} and ETOH yield is a measure of efficiency of the SSF process, for each treatment was measured using Eq. (7.6).

$$\text{Ethanol yield}_{SSF}\ (\%) = \frac{ETOH_{240\ h} - ETOH_{0\ h}}{SL \times f(glucan)_{pretreated} \times 1.11 \times 0.51} \times 100 \quad (7.6)$$

where, $ETOH_{240\ h}$ and $ETOH_{0\ h}$ are ethanol concentrations (% w/v) obtained during SSF at 240 h and 0 h, respectively. SL is the dry solid loading used for SSF which was 8% (w/w), $f(glucan)_{pretreated}$ represents the fraction of glucan in pretreated redcedar, 1.11 is the mass conversion factor of glucan to glucose (g g^{-1}) and 0.51 is the mass conversion factor of glucose to ethanol (g g^{-1}).

Fig. 7.2 shows ethanol production, ethanol $yield_{SSF}$ (% of theoretical) and glucose consumption of *S. cerevisiae* D_5A with the four different treatment combinations. Glucose concentrations increased for the first 6 h for sapwood crumbles, 12 h for sapwood ground and 18 h for heartwood ground and crumbles indicating that the rate of enzymatic hydrolysis occurred faster than the glucose consumption rate by yeast during these times in the respective treatments (Fig.7.2a). During the initial period of SSF, yeast cells may have experienced an initial lag phase before starting to ferment glucose released by enzymatic hydrolysis. Thereafter, a rapid decrease in glucose concentrations was observed with concomitant increase in ethanol concentrations demonstrating that fermentations were occurring faster than enzymatic hydrolysis (Fig. 7.2b). Glucose concentration decreased to below 2 g L^{-1} after 36 h indicating rapid conversion of glucose to ethanol. Glucose concentrations during SSF of sapwood ground were inconsistent between the two replicates until 18 h resulting in a huge error bar in Fig. 7.2a, but the deviation between samples decreased as SSF progressed. Similar deviation was also observed with ethanol concentrations and ethanol $yield_{SSF}$ in Fig. 7.2b. Acetic acid, glycerol and succinic acid concentrations obtained as co-products of yeast fermentations

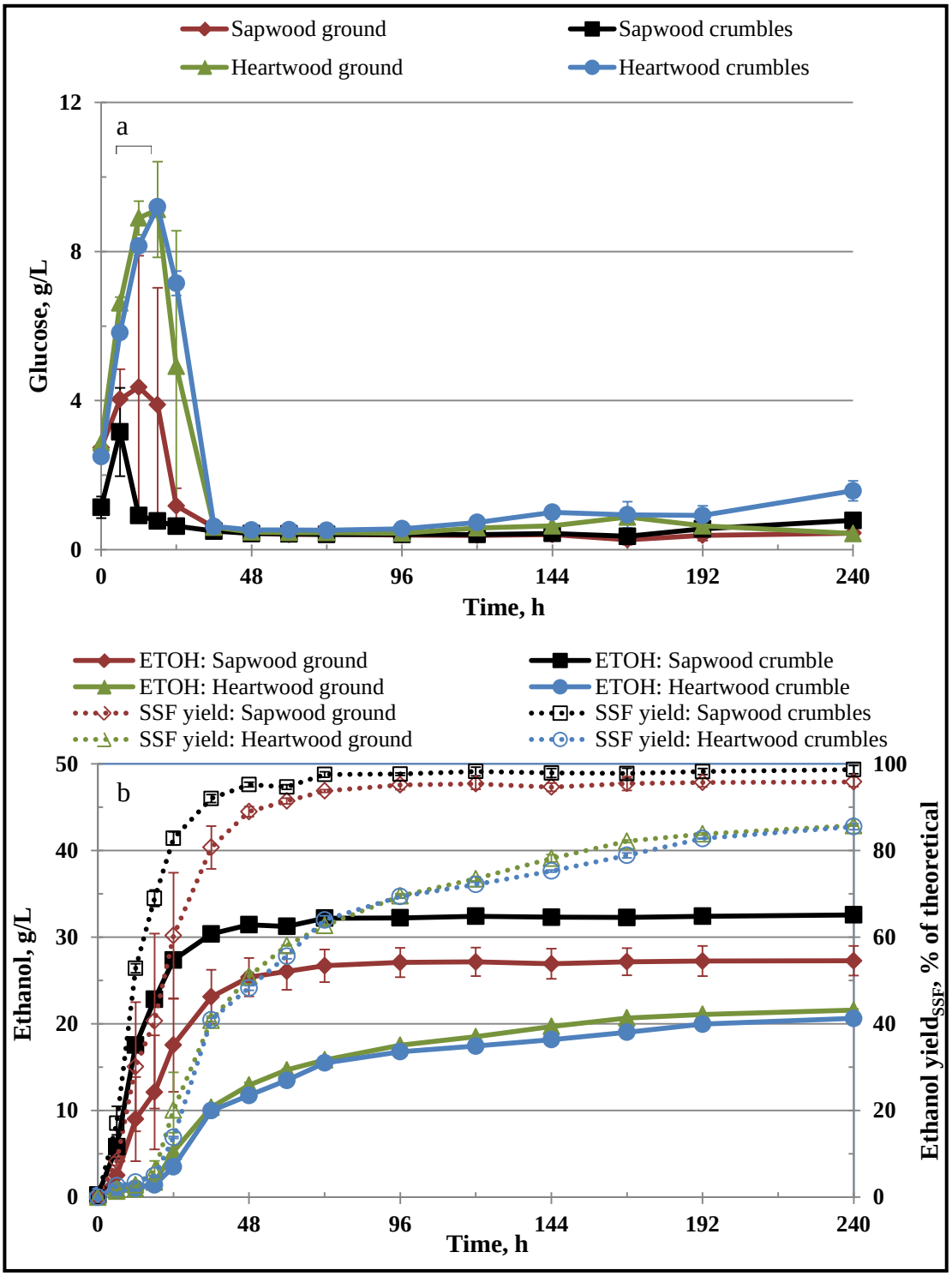

Fig. 7.2 Glucose consumption (1a), ethanol production (1b) and ethanol yield (1b) during SSF of pretreated redcedar with the four treatments combinations at 8 % dry solid loading and 46 FPU g^{-1} glucan enzyme loading.

Each data point is an average of three subsamples and error bars represent one standard error.

during SSFs varied between 0.3 to 2.2 g L^{-1}, 1.3 to 1.6 g L^{-1} and 0.8 to 2.3 g L^{-1}, respectively, for the four different treatments. Their trends were not shown because of the low product concentrations.

Initial ethanol production rates were highest with sapwood crumbles followed by sapwood ground and the heartwood treatments. The maximum ethanol concentration obtained with sapwood crumbles (33 g L^{-1}) was 19% greater than sapwood ground (27 g L^{-1}), while heartwood ground and heartwood crumbles produced 21 g L^{-1} of ethanolin 240 h. The difference in ethanol concentration between sapwood crumbles and sapwood ground was because pretreated sapwood crumbles contained more glucan (72%) than sapwood ground (62 %). However, ethanol $yield_{SSF}$ from sapwood were similar (99% with sapwood crumbles versus 96% with sapwood ground), showing that acid bisulfite pretreatments of both sapwood ground and sapwood crumbles resulted in a very digestible and fermentable material.

Ethanol $yields_{SSF}$ of the two heartwood treatments were both 86% at 240 h, which was 12% and 15% lower than the ethanol $yield_{SSF}$ of sapwood ground and sapwood crumbles, respectively (Fig.7.2b). Although ethanol yield provides information on effectiveness of the SSF process, it does not account for the glucan lost during pretreatments. Wood glucan-to-ethanol yields obtained with different treatments are listed in Table 7.4. Statistical analysis indicated no two-way interaction between wood zone and particle size ($p = 0.2485$) and no difference between the two particle sizes tested ($p = 0.1943$). However, a significant main effect for wood zone was observed ($p = 0.0057$). Pretreatments and SSF of sapwood crumbles produced 95% wood glucan-to-ethanol yield while sapwood ground resulted in 89% wood glucan-to-ethanol yield. On

Table 7.4 Wood glucan-to-ethanol yield expressed as % of theoretical and L Mg^{-1} of dry wood and achieved in different treatments.

Treatments	Wood glucan-to-ethanol yield[a], % of theoretical	Wood glucan-to-ethanol yield[a], L Mg^{-1}
Sapwood ground	88.7 ± 1.7^{A}	171.7 ± 1.6^{B}
Sapwood crumbles®	94.8 ± 0.5^{A}	186.8 ± 1.1^{A}
Heartwood ground	80.2 ± 2.0^{B}	158.1 ± 4.0^{C}
Heartwood crumbles®	80.6 ± 2.7^{B}	158.2 ± 5.2^{C}

Values listed above are averages ± standard error; n=2 for pretreatments and 3 subsamples for SSF of pretreated redcedar.

[a]Based on ethanol produced from glucan content of raw material defined in Eq (7.4) and Eq (7.5) calculated at 240 h.

[A,B]Values listed in the same column with the same letter are not statistically different at 95% confidence intervals.

the other hand, heartwood ground and heartwood crumbles produced 80 and 81% wood glucan-to-ethanol yield, respectively. Insignificant difference between the particle sizes could translate into enormous cost savings with respect to size reduction. These results were contrary to the hypothesis that ground redcedar would perform better than crumbles. Although determining the cost of energy reduction was not the scope of this study, previous work by Forest Concepts, LLC has shown that only 150 MJ Mg^{-1} of dry wood was consumed to reduce the size of hybrid poplar to 2 mm screen size (Lanning et al., 2012). This is three to fourteen folds lower than typical size reduction techniques such as a hammer mill and disk mill, which use 470 to 2160 MJ Mg^{-1} of oven dry wood of energy for milling lignocellulosic biomass to 2 mm screen size (Schell and Harwood, 1994; Sun and Cheng, 2002).

The results in the present study demonstrate that no size reduction of pretreated crumbles was required after acid bisulfite pretreatments. Previous studies with acid bisulfite pretreatments of softwoods with larger particle size (6 to 38 mm in two dimensions with 1 to 5 mm thickness) wood chips required size reduction before enzymatic hydrolysis or SSF to obtain high hydrolysis or ethanol yields (Tian et al., 2010; Zhu and Pan, 2010; Zhu et al., 2009; Zhu et al., 2011). Size reduction after acid bisulfite pretreatments resulted in 15% to 78% energy savings (Zhu, 2011), but still consumed at least 550 MJ Mg^{-1} of dry wood. The present study has demonstrated the feasibility of using 2.5 mm size crumbles for ethanol production using redcedar using a low energy size reduction technique.

Another significant observation in the present study was the difference between sapwood and heartwood glucan-to-ethanol yields (% of theoretical) shown in Table 7.4

despite the fact that the glucan and lignin contents of these zones were similar (Table 7.1). To the best of authors' knowledge, this is the first time a detailed study was conducted to examine the effect of wood zones with respect to ethanol production. Sapwood treatments averaged 14.1% higher wood glucan-to-ethanol yield (% of theoretical) than heartwood treatments that subsequently resulted in higher wood glucan-to-ethanol yield per dry Mg of raw material for sapwood (179.2 L Mg^{-1}) treatments compared to heartwood (158.2 L Mg^{-1}). This difference in yield could significantly affect a bio-refining process that handles thousands of dry Mg of raw material on a daily basis. These results were similar to the observations of Esteves et al. (2005) and Miranda et al. (2007) who achieved 8 and 20% lower pulping yield with heartwood in comparison to sapwood with Eucalyptus and maritime pine, respectively. Although pulps are not used for ethanol production, pulp yields are a measure of the amount of fibers (cellulose and hemicellulose) that are retained after pulping processes and is defined as the amount of oven dry fibers that could be recovered after pulping (Casey, 1980).

A higher ethanol yield with sapwood was due to higher delignification of sapwood in comparison to heartwood. On average, sapwood pretreatments achieved 60% delignification while heartwood pretreatments reached only 23% delignification (Table 7.2). Higher porosity of heartwood when compared to sapwood could have reduced the rate of penetration of sulfuric acid and sodium bisulfite reducing delignification efficiency with heartwood treatments subsequently decreasing the digestibility of pretreated biomass. Ramachandriya et al. (2013) showed that digestibility of pretreated redcedar could be increased by increasing sodium bisulfite loading as it removed higher levels of lignin from the biomass. Thus, higher sodium bisulfite loading for heartwood

would be recommended for achieving wood glucan-to-ethanol yields similar to sapwood treatments.

It is also likely that heartwood treatments may have experienced some kind of inhibition during enzymatic hydrolysis that lowered ethanol yields in comparison to sapwood treatments. The evidence of yeast inhibition can be observed in Fig. 7.2a, which showed that yeast cells experienced a lag phase for the first 12 h of SSF using heartwood because the rate of glucose production was higher than the rate of fermentation, resulting in accumulation of 9 g L^{-1} of glucose in the reactors. It is likely that the yeast cells experienced this lag phase due to the presence of redcedar oils in heartwood. A previous study by Dunford et al. (2007) have shown that heartwood redcedar oil content (on weight basis) could be about two folds higher than the sapwood. To examine the effect of redcedar oil on glucose fermentations by *S. cerevisiae* D_5A, a preliminary study was conducted in the presence of 0.5% (v/v) redcedar oil. Cell growth and ethanol production were inhibited by 46% and 50%, respectively at 9 h of fermentation. However, the yeast cells acclimated to redcedar oil and produced the same cell mass and ethanol concentration as yeast cells not in the presence of redcedar oil at 24 h of fermentation (data not shown). It may be possible that the pretreated solids retained a smaller fraction of redcedar oil even after extensive washing that resulted in slower rates of SSF and lower wood glucan-to-ethanol yield. Testing this was beyond the scope of this study.

SSF of sapwood crumbles achieved the highest wood glucan-to-ethanol yield of 95% followed by sapwood ground (89%), heartwood crumbles (81%) and heartwood ground (80%). A high wood glucan-to -ethanol yield with sapwood crumbles was a

result of low glucan loss during pretreatments (4%) and high ethanol yield (99%). Wood glucan-to-ethanol yield as compared to the theoretical was comparable with a study conducted by Lan et al. (2013) who obtained 95% of theoretical ethanol yield from acid bisulfite pretreated lodgepole pine using simultaneous saccharification and combined fermentation (SSCF) where pretreated pine was fermented with a non-detoxified and concentrated prehydrolyzate fraction. In a different study by Tian et al. (2010), 90% of theoretical wood glucan-to-ethanol yield was obtained when enzymatic hydrolysate produced at 10% dry solid loading of sulfite pretreated lodgepole pine was fermented with detoxified prehydrolyzate using *S. cerevisiae* Y5 adapted to grow on prehydrolyzates. The maximum wood glucan-to-ethanol yield (% of theoretical) from the present study was 12% to 47% higher than other softwoods to ethanol studies (Ishola et al., 2013; Zhu et al., 2010; Zhu et al., 2011).

The highest wood glucan-to-ethanol yield per dry Mg of raw material was 186.8 L Mg^{-1} (49.4 gallon Mg^{-1}) and was obtained with sapwood crumbles. This yield was 8% higher than the yield from bisulfite pretreated aspen (Zhu et al., 2011) and 14% and 34% lower than yields from acid bisulfite pretreated lodgepole pine conducted by Zhu et al. (2010) and Tian et al. (2010). Lower wood glucan-to-ethanol yield per dry Mg of redcedar was due to lower glucan content of the redcedar compared to lodgepole pine. The redcedar used in the present study contained only 35% glucan while a previous study had 40% of glucan (Ramachandriya et al., 2013) and lodgepole pine has 42 to 43% glucan (Tian et al., 2010; Zhu et al., 2010). Variation in redcedar composition could be due to differences in location and age of the trees.

Our results have demonstrated that ethanol can be produced from both sapwood and heartwood zones with 13% higher yield with sapwood. Thus, this property of the wood should be considered as a quality variable while processing redcedar for ethanol production using the biochemical platform.

7.4 Conclusions

Eastern redcedar crumbles (2.5 mm sieve size) and ground (0.5 mm sieve size) produced similar wood glucan-to-ethanol yields showing that the low energy consuming size reduction process developed by Forest Concepts, LLC was effective and no further size reduction of crumbles was required before or after acid bisulfite pretreatments. Our results demonstrate that ethanol can be produced from both the wood zones, but with 13 % lower ethanol yield with heartwood in comparison to sapwood.

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CHAPTER VIII

INFLUENCE OF REDCEDAR OIL ON YEAST FERMENTATIONS AND ENZYMATIC HYDROLYSIS OF MICROCRYSTALLINE CELLULOSE

Abstract

Essential oil are known to be inhibitory to yeast and starch hydrolyzing enzymes. The presence of redcedar oil in Eastern redcedar (*Juniperus virginiana* L.) could negatively affect the bioprocessing of redcedar for the production of ethanol and hence its effect during enzymatic hydrolysis and yeast fermentations needs to be explored. This study investigates the effect of redcedar oil during ethanol fermentations using *Saccharomyces cerevisiae* D_5A and enzymatic hydrolysis of microcrystalline cellulose. Results show that the presence of 0.5% and 1% (w/w) redcedar oil only had a marginal inhibitory effect on ethanol fermentations by yeast during the first 9 h of fermentation. As high as 22 g L^{-1} of ethanol (92% of theoretical yield) was produced with all treatments within 18 h of fermentation. Enzymatic hydrolysis was conducted with two different cellulose loadings: 2% and 4% (w/w), two different enzyme loadings (Accelerase® 1500): 25 FPU g^{-1} cellulose and 50 FPU g^{-1} cellulose and three different redcedar oil loadings: 0%, 0.5% and 1% (w/w). Results showed that the presence of 0.5% and 1% (w/w) redcedar oil inhibited cellulose-to-glucose yields by 26 and 33%, respectively. Appropriate steps for removing redcedar oil from the raw material such as oil extractions should be taken during ethanol production from redcedar using hydrolysis-fermentation route.

Keywords: Essential oil, Redcedar oil, Easter redcedar, Enzyme inhibition, Cellulose hydrolysis.

8.1 Introduction

Essential oils are volatile mixtures containing secondary metabolites of plant material and are characterized for diverse compositions and activities (Bakkali et al., 2008). They have evolved as a natural defense mechanism for the protection of plants as antibacterial, antiviral, antifungal and insecticidal agents (Bakkali et al., 2008). Additionally, they also offer protection against herbivores by reducing their appetite for such plants (Bakkali et al., 2008). Their lipophilic nature allows them to pass through and permeabilize the microbial cell wall, causing ion loss, reduction in membrane potential, collapse of proton pumps and depletion of the ATP pool (Bakkali et al., 2008; Di Pasqua et al., 2006; Helander et al., 1998; Richter and Schlegel, 1993). The cytotoxic property of essential oils has been exploited by the livestock industry to cut down greenhouse gas emissions by killing or reducing the activity of the microbial flora of rumens (Calsamiglia et al., 2007; Lin et al., 2013; Patra and Yu, 2012). Patra and Yu (2012) observed 18 to 87% inhibition of methane production with essential oils from clove, eucalyptus, garlic, origanum and peppermint. Other studies have also reported adverse effects on fiber digestion by inhibition of cellulolytic bacteria (Calsamiglia et al., 2007; Macheboeuf et al., 2008; Patra and Yu, 2012). Although these studies do not show the effect of essential oils on glucosidases (enzymes that catalyze the breakdown of glycosidic linkages found in starch, cellulose or glycogen into monomeric glucose), a study conducted by Basak and Candan (2013) showed that 1,8-cineole, a major component of laurel essential oil, inhibited starch hydrolyzing enzymes competitively, while other components such as 1-(S)-α-pinene and R-(+)-limolene were uncompetitive inhibitors to α-glucosidase.

The wood of Eastern redcedar (*Juniperus virginiana* L.) contains 2.4 g of essential oils per 100 g of dry redcedar, commonly referred to as redcedar oil, comprising of 39 g of cedrol+viddrol per 100 g redcedar oil, 4.7 g of thujopsene per 100 g of oil and 4.2 g of cedrene per 100 g of oil as its main components (Dunford et al., 2007). Redcedar oil has long been used for its odor in soap and perfume applications, adulteration with sandalwood oil, and as insecticides due to its antibacterial, antifungal and antitermitic activities (Adams et al., 1988; Clark et al., 1990; Huddle, 1936). The bioconversion of softwoods such as Eastern redcedar into ethanol employs enzyme cocktails for saccharification of pretreated biomass and yeast or bacteria for the production of biofuels (Ramachandriya et al., 2013). Although there are reports that demonstrate the inhibitory effect of essential oils on α-glucosidases (Basak and Candan, 2013), cellulase producing microorganisms (Calsamiglia et al., 2007; Lin et al., 2013) and numerous bacteria and yeasts (Bakkali et al., 2008), no studies have been performed to assess the influence of redcedar oil during enzymatic hydrolysis and yeast fermentations. The presence of redcedar oil will be of primary concern when the raw material is subjected to pretreatments without redcedar oil extraction followed by combined prehydrolysate and biomass fermentations, where both the prehydrolysate and pretreated biomass are fermented together to improve overall ethanol yields. Hence, to develop a biofuel production process using redcedars, it is important to understand the impact of redcedar oil during enzymatic hydrolysis and yeast fermentations. Therefore, a study was conducted to observe the effect of enzyme oil loading during glucose fermentation by *Saccharomyces cerevisiae* D_5A strain and enzymatic hydrolysis of microcrystalline cellulose.

8.2 Materials and methods

8.2.1 Effect of redcedar oil loading during fermentations

Saccharomyces cerevisiae D_5A yeast was used for fermentations. The yeast was maintained at 4 °C on YPD agar slants containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar. Prior to fermentations, 100 mL preculture was made using YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 45 g L^{-1} glucose in a 250 mL baffled flask reactor. This was incubated at 37 °C for 15 h at 250 rpm on an orbital shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA) under aerobic conditions to produce high cell mass concentrations. Yeast cells were transferred to fermentation stage by transferring 5% of inoculum of actively growing cells. The fermentation stage were performed in 250 mL shake flask reactors with a working volume of 100 mL containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 45 g L^{-1} glucose at pH 5.5. The flasks were sealed with a rubber stopper fitted with a one way valve (Check valve, Fisher Scientific, Pittsburgh, PA, USA) to exhaust CO_2 produced during fermentations and to maintain anaerobic conditions.

To observe the effect of redcedar oil during yeast fermentations, three oil loadings were used: 0% (control), 0.5% and 1% (w/w). Redcedar oil loadings of 0.5 and 1% (w/w) were selected based on an estimation that 0.4% to 1.2% (w/w) of redcedar oil would be present in biomass-prehydrolysate slurry (Table 8.1). Redcedar oil was acquired from Aromatic Cedar Products (Asheville, NC, USA). The oil was produced by steam distillation to produce 100% pure oil. The density of redcedar oil was 0.96 g mL^{-1}. Cell densities, pH and fermentation products (ethanol, acetic acid, succinic acid and

Table 8.1 Estimated redcedar oil content in biomass-prehydrolysate slurry.

LSR[a]	5:1	4:1	3:1	2:1	1:1
Mass of dry wood, g	100	120	150	200	300
Mass of pretreatment liquor, g	500	480	450	400	300
Oil percentage, %	0.4	0.5	0.6	0.8	1.20

[a]LSR represents the ratio between pretreatment liquor to dry redcedar.

Calculations were made assuming the redcedar oil content of redcedar was 2.43% that was based on the average value of redcedar oil from Dunford et al. (2007).

glycerol) were monitored at 0, 3, 6, 9, 12, 18 and 24 h. Cell mass concentrations were measured in optical density (OD) units at 600 nm using a UV-visible spectrophotometer (Model No. 2100, UNICO spectrophotometer, Dayton, NJ, USA). Fermentation broth pH was measured using a pH meter (Thermo Orion, Beverly, MA, USA). The samples were centrifuged at 13,500 rpm for 10 min and the supernatant was filter sterilized using 0.22 µm nylon filters (VWR International, West Chester, PA) prior to product analysis. Ethanol, acetic acid, glycerol, succinic acid and glucose were monitored using HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA) and a refractive index detector (RID). The eluent was 0.01 N sulfuric acid at a flow rate of 0.6 mL min^{-1} with a 30 min run time.

8.2.2 Effect of redcedar oil loading during enzymatic hydrolysis

Enzymatic hydrolyses were carried out in 250 mL baffled flasks containing a total mass of 100 g incubated at 250 rpm and 50 °C with 0.05 M sodium citrate buffer at pH 5. To observe the effect of redcedar oil during enzymatic hydrolysis, microcrystalline cellulose (Catalog No. 435236, Sigma Aldrich) was chosen as the substrate. Pretreated biomass was not chosen as the substrate because the redcedar oil content of pretreated biomass was not determined and it was hypothesized that the pretreated biomass could possibly contain some redcedar oil that will interfere with the objective of this study.

For this study, three parameters were selected: cellulose loading, enzyme loading and red cedar oil loading. The levels of cellulose loading were 2% and 4% (w/v), the levels of enzyme loading were 0.125 mL g^{-1} cellulose and 0.25 mL g^{-1} cellulose and the levels of oil loading was 0% (control), 0.5% and 1% (w/w). Thus, 12 factorial combinations were obtained.

Samples (1.5 mL) were collected at 0, 3, 6, 9, 12, 18, 24, 36, 48, 60, 72 and 96 h. The samples were centrifuged at 13,500 rpm for 10 min and the supernatant was filter sterilized using 0.22 µm nylon filters (VWR International, West Chester, PA) prior to product analysis. The response variable was glucose concentration, which was analyzed using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 mL min^{-1} and the column temperature was maintained at 85 °C. The HPLC with Chemstation software (Agilent Technologies) was calibrated at five levels using known concentrations of compounds before being used to quantitate the concentration of compounds.

Accelerase® 1500, generously provided by Genencor Inc. (Palo Alto, CA, USA), was the enzyme cocktail used for this study. This enzyme cocktail was produced from a genetically modified strain of *Trichoderma reesei* and contained multiple enzyme activities; mainly exoglucanase, endoglucanase, hemicellulase and β-glucosidase (Genencor, 2012). Enzyme activity was measured using the standard protocol developed by NREL (Adney and Baker, 2008) and was determined to be 100 FPU mL^{-1} of enzyme. Analytical grade chemicals required for the assay was purchased from Sigma Aldrich (St. Louis, MO, USA).

8.2.3 Statistical analysis

For the experiment conducted to observe the effect of redcedar oil on yeast fermentations, the cell mass concentration, ethanol production and glucose consumption trends were compared between each other using repeated measures design in SAS 9.3. P-

values were calculated and are shown in text. Additionally, post hoc analysis was also performed using Tukey's honestly significant difference test (Abdi and Williams, 2010) to compare sample means of cell mass concentration, ethanol and glucose concentration shown in Table 8.2. Experiments were performed in quadruplicate and all statistical comparisons were made at 95% confidence interval.

The experiment to observe the effect of oil loading during enzymatic hydrolysis was conducted using a randomized complete block factorial design with 3 blocks. Analysis of variance (ANOVA) was conducted using generalized linear model (GLM) procedure in SAS 9.3 (SAS, Cary, NC, USA). P-values were calculated for the whole model. The insignificant interaction effects ($p > 0.05$) were eliminated and the program was re-run to obtain the p-values that are shown in the text.

8.3 Results and discussion

8.3.1 Effect of redcedar oil loading on yeast fermentations

Fig. 8.1 shows cell mass concentrations, glucose consumption and ethanol production during batch fermentation of *S. cerevisiae* D_5A using glucose as substrate. Fermentations occurred rapidly and all the glucose was consumed within 12 h of fermentation. Maximum cell mass concentrations of 5.2 g L^{-1} and ethanol concentrations of 22 g L^{-1} were achieved both in the presence and absence of redcedar oil. Insignificant differences between the overall trends of cell mass concentration ($p = 0.4943$), glucose consumption ($p = 0.3539$) and ethanol production ($p = 0.2246$) were observed.

A closer analysis of the data showed a slight inhibition to growth, glucose consumption and ethanol formation before 12 h of fermentation. The growth rate,

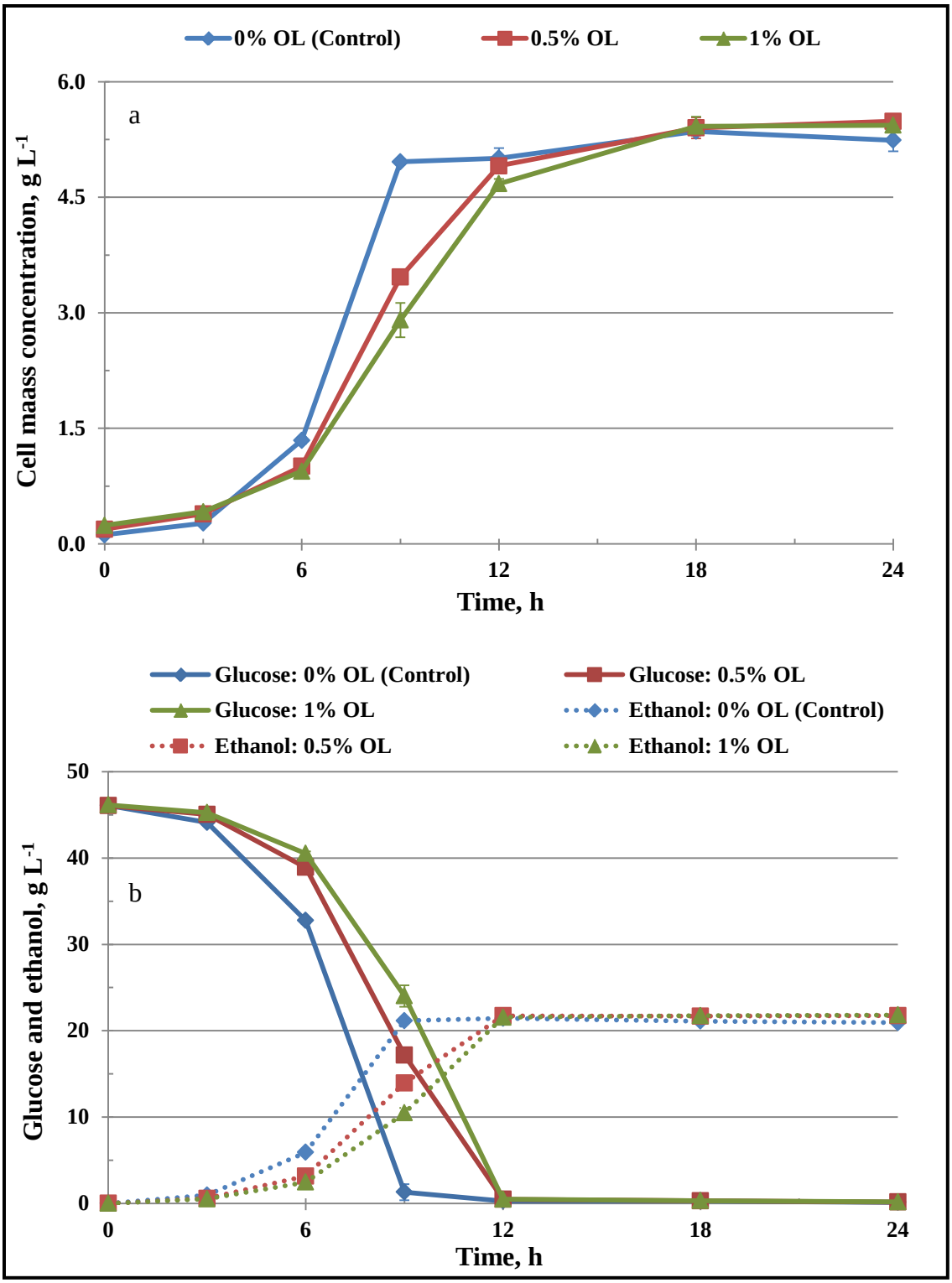

Fig. 8.1 Effect of redcedar oil loading (OL) on cell mass concentrations (a), glucose consumption (b) and ethanol production (b) of *S. cerevisiae* D_5A.
Each data point is an average of four replicates and error bars represent one standard error.

glucose uptake rate, ethanol formation rate and ethanol yield (% of theoretical) were calculated (Table 8.2). The presence of redcedar oil decreased the growth rate of yeast cells, glucose uptake rate and ethanol production rate by 69%, 30.5% and 30.5%, respectively. Despite lower growth rates, glucose uptake rates and ethanol production rates between 3 h and 9 h of fermentation, same concentrations of cell mass, residual glucose and ethanol after 12 h resulting in comparable ethanol yields (Table 8.2). This shows that the initial delay in glucose consumption and ethanol production were due to the reason that the yeast cells were getting adapted to the new environment.

Our observations were contrary to Veljković et al. (1990), which showed a 4 fold slower growth of *S. cerevisiae* in the presence of 0.5% juniper berry oil and a 5 fold increased time to achieve 30 g L^{-1} of ethanol. Other reports have also shown a substantial delay in ethanol production with *S. cerevisiae* in the presence of 0.05% (w/v) of cinnamon, clove, garlic, tomato, oregano and thyme essential oils (Conner et al., 1984). Likewise, inhibitions to growth and ethanol production were observed in with *S. cerevisiae* in presence of 0.05 to 0.2% orange peel oil (Wilkins et al., 2007). Additionally, 50% lethality of *S. cerevisiae* was reported with 0.05 % (v/v) of *Origanum compactum* essential oil, 0.16% (v/v) of *Coriandrum sativum* essential oil and 0.8% (v/v) of *Cinnamomum camphora, Artemisia herba-alba* and *Helichrysum italicum* essential oils (Bakkali et al., 2005). These differences could be due to the differences between the compositions of essential oils obtained from different plant materials. Fermentation co-products were acetic acid and glycerol, whose concentrations varied between 0.9 to 1.2 g L^{-1} and 1.0 to 1.1 g L^{-1}, respectively, between the three treatments, The results obtained

Table 8.2 Effect of redcedar oil on ethanol fermentations by *S. cerevisiae* D_5A.

Oil loading (OL), % (v/v)	Growth rate, h^{-1}	Glucose uptake rate, $g\ L^{-1}\ h^{-1}$	Ethanol production rate, $g\ L^{-1}\ h^{-1}$	Ethanol yield, % of theoretical[a]
0% OL[b]	0.44 ± 0.01^{A}	7.13 ± 0.20^{A}	3.37 ± 0.06^{A}	90.91 ± 0.06^{B}
0.5% OL[c]	0.26 ± 0.01^{B}	4.95 ± 0.03^{B}	2.35 ± 0.00^{B}	92.16 ± 0.03^{A}
1% OL[c]	0.27 ± 0.01^{B}	4.97 ± 0.03^{B}	2.34 ± 0.00^{B}	91.45 ± 0.29^{B}

[a]Ethanol yield was determined at 12 h.

[b]Glucose uptake rate and ethanol production rate was calculated between 3 h and 9 h.

[c]Glucose uptake rate and ethanol production rate was calculated between 3 h and 12 h.

[A,B]Values in the same column with the same letter are not statistically different at 95% confidence interval.

in this study clearly indicate that redcedar oil was not inhibitory to yeast cells and ethanol production under the conditions tested.

8.3.2 Effect of redcedar oil loading during enzymatic hydrolysis

Fig. 8.2 shows the cellulose-to-glucose yields for each factorial combination. Cellulose-to-glucose yield is the measure of the amount of glucose that was produced compared to the theoretical and was calculated using Eq. (8.1).

$$\text{Cellulose-to-glucose yield (\%)} = \frac{\text{Glucose}_{96\text{ h}} - \text{Glucose}_{0\text{ h}}}{\text{Cellulose loading} \times 1.11} \times 100\ \% \qquad (8.1)$$

Where, $\text{Glucose}_{96\text{ h}}$ and $\text{Glucose}_{0\text{ h}}$ are glucose concentrations in % (w/v) at 96 h and 0 h, respectively, cellulose loading in % (w/w) and 1.11 is the conversion factor for cellulose to glucose.

Statistical analysis indicated that a 3-way interaction between cellulose loading, enzyme loading and redcedar oil loading was insignificant for cellulose-to-glucose yields ($p = 0.1109$). Additionally, there was no significant block effect ($p = 0.1211$) and all two-way interactions among treatments were insignificant for cellulose-to-glucose yields (cellulose loading*enzyme loading, $p = 0.5845$; cellulose loading*oil loading, $p = 0.6444$; enzyme loading*oil loading, $p = 0.3231$). Among the three main effects, cellulose loading was insignificant ($p = 0.8156$), while enzyme loading and oil loading were observed to be significant (both p values were less than 0.0001). The main effect of cellulose loading was insignificant because increase in cellulose loading did not increase cellulose-to-glucose yields. On the other hand, increase in enzyme loading improved cellulose-to-glucose yields and increasing oil loading lowered cellulose-to-glucose yields (Fig. 8.2).

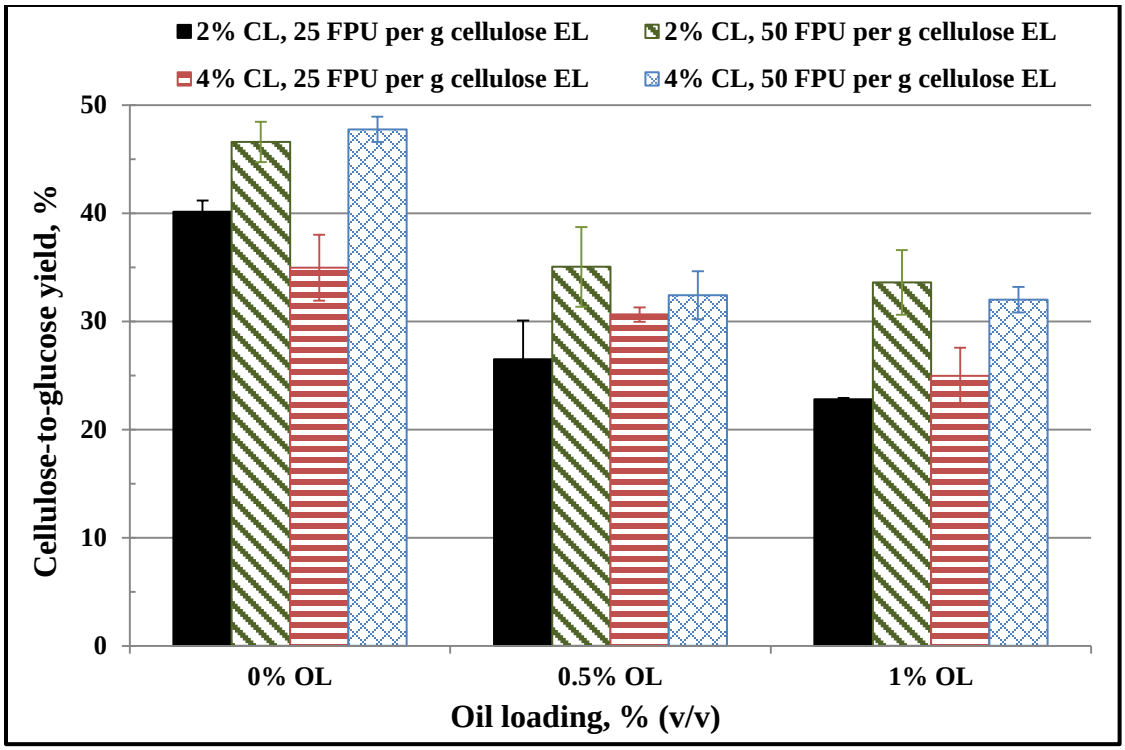

Fig. 8.2 Effect of redcedar oil loading (OL) on cellulose-to-glucose yield (A) and inhibition (B) at 96 h with different cellulose loading (CL) and enzyme loading (EL). Error bar shows the standard error of three samples

The highest cellulose-to-glucose yield achieved in this study was 48 %. Although it would be expected to achieve higher glucose yields from crystalline cellulose, but in reality native cellulose are so tightly packed that even water molecules cannot penetrate these regions (Krassig, 1993). Our observations were consistent with other literature that obtained 41% cellulose-to-glucose yield with a cellulose loading of 5% and enzyme (Celluclast 1.5 L) loading of 15 FPU g^{-1} of microcrystalline cellulose (Ouyang et al., 2010).

On average, the presence of 0.5% and 1% (w/w) redcedar oil inhibited the cellulose-to-glucose yields by 26 and 33%, respectively. To the best of the authors' knowledge, there have been no studies conducted to understand the mechanism of redcedar oil inhibition on cellulolytic enzymes. Other studies have demonstrated that essential oils can be inhibitory to glucosidases. For example, a study conducted by Basak and Candan (2013) showed that 1,8-cineole, a major component of laurel (*Laurus nobilis* L.) essential oil inhibited α-glucosidase (enzymes that convert starch into glucose) competitively (where binding of inhibitor to the active site prevents the binding of the substrate), while other components such as 1-(S)-α-pinene and R-(+)-limolene were uncompetitive inhibitors (where inhibitor binds to the enzyme-substrate complex) to α-glucosidase. Another study showed 90% inhibition of β-glucosidase when essential oil extracted from lemongrass extracted was used for an in-vitro anti-diabetic test (Mirghani et al., 2012). From the current study, it was clearly observed that redcedar oil at concentrations at or above 0.5% inhibited Accelerase® 1500.

8.4 Conclusions

The presence of redcedar oil marginally inhibited growth of *S. cerevisiae* D_5A, glucose consumption and ethanol production early in the fermentations. However, similar concentrations of cell mass and ethanol was observed after 12 h of fermentation. Additionally, the inhibitory effect of redcedar oil on enzymatic hydrolysis of microcrystalline cellulose was clearly demonstrated. The intensity of inhibition increased as the concentration of redcedar oil was increased. Appropriate steps for removing redcedar oil from the raw material such as oil extractions should be taken during from redcedar using hydrolysis-fermentation route.

Acknowledgements

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CHAPTER IX

FUTURE WORK

This research has developed and optimized a process for efficiently extracting fermentable sugars from the polysaccharides of redcedar and has demonstrated ethanol production from redcedar at high efficiencies and titers. However, further reduction in operating cost, water load and capital cost would be ideal for converting Eastern redcedar into biofuels. The following studies are considered essential.

1. A techno-economic analysis of the overall process is required to determine the selling price of ethanol from the process that has been developed. It would be interesting to compare the price of ethanol from redcedar with the price of gasoline and the ethanol produced from other feedstocks. Economic analysis will also provide an idea of return on investment to stakeholders who wish to start a biorefinery based on this research. The current study has developed a model relating the wood glucan-to-glucose yield with four important pretreatment parameters. This model could be used for performing economic analysis of the process by changing the levels of independent variables. Additionally, a study conducted on lowering enzyme dosage was conducted at 20 % (w/w) solids.

loading will facilitate the determination of the help making economic trade-off between enzyme dosage, residence time and the desired glucan-to-glucose yields However, while doing such economic evaluations, it is important not to forget about the co-product potential. Redcedar can be used to produce anti-cancer drugs, artificial vanilla, lignosulfonates and redcedar oil that have industrial applications in medicine, food, perfumery and aromatherapy. Hence, an integrated bio-refinery economic model using redcedar as feedstock is necessary.

2. Reducing the water load of the pretreatment process is important from an industrial stand-point to reduce costs associated with water usage and create concentrated prehydrolysate streams. All pretreatment studies were conducted with pretreatment liquor to solid ratio (LSR) of 5:1. The reduction of LSR to 3:1 and 2:1 will decrease the water load of the process by 10 and 20%, respectively, which would translate into huge water savings at a commercial scale. However, challenges with mixing during pretreatments may be observed and will have to be addressed.
3. Redcedar oil was found to be inhibitory to cellulolytic activity of Accelerase® 1500, but finding the mechanism of inhibition of redcedar oil on cellulases was beyond the scope of the study. Finding the mechanism of inhibition of redcedar oil on cellulases will be important from a basic science stand-point.
4. Redcedar oil extraction prior to pretreatments would be necessary because the market cost of redcedar oil is thirty fold higher than ethanol cost. However, having two unit operations for redcedar oil extraction and pretreatments would increase the capital cost and operational cost of the process. Since redcedar

pretreatments use 3 h of impregnation at 90 °C and 200 °C of pretreatment temperature, it is highly likely that redcedar oil will be extracted from the biomass during pretreatment. The right temperature for conducting bleed-outs to recover oil will have to be identified. The composition of redcedar oil that is achieved from these studies will have to be compared with the composition of redcedar oil obtained from hydro-distillation to evaluate if the presence of sulfuric acid and sodium bisulfite during pretreatments affected the composition of redcedar oil.

APPENDICES

A.1 SAS codes and outputs for chapter 4

As there were many SAS programs run for statistical analysis, only one example per type is shown here

```
*Preliminary screening study on effect of chemical loading;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA Expt1;
INPUT sub acid base gluconv;
CARDS;
1     0     0     3.5
2     0     0     3.3
1     0     5     11.1
2     0     5     11.6
1     0     10     12.0
2     0     10     12.6
1     1.25     0     3.5
2     1.25     0     3.3
1     1.25     5     9.5
2     1.25     5     10.0
1     1.25     10     11.5
2     1.25     10     12.1
1     2.5     0     5.0
2     2.5     0     4.8
1     2.5     5     15.5
2     2.5     5     15.9
1     2.5     10     32.0
2     2.5     10     32.6
;
*ANOVA for completely randomized factorial design;
*Factorial treatment combination, 3 acid levels, 3 base levels during enzymatic hydrolysis- Subsampling case;
PROC GLM;
CLASS sub acid base;
```

```
MODEL gluconv = acid | base sub(acid*base);
TEST H= acid | base E=acid*base;
MEANS acid | base;
RUN;
```

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
sub	2	1 2
acid	3	0 1.25 2.5
base	3	0 5 10

Number of Observations Read	18
Number of Observations Used	18

The GLM Procedure

Dependent Variable: gluconv

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1258.244444	74.014379	.	.
Error	0	0.000000	.		
Corrected Total	17	1258.244444			

R-Square	Coeff Var	Root MSE	gluconv Mean
1.000000	.	.	11.65556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
acid	2	323.0744444	161.5372222	.	.
base	2	669.3911111	334.6955556	.	.
acid*base	4	264.8488889	66.2122222	.	.
sub(acid*base)	9	0.9300000	0.1033333	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F

Source	DF	Type III SS	Mean Square	F Value	Pr > F
acid	2	323.0744444	161.5372222	.	.
base	2	669.3911111	334.6955556	.	.
acid*base	4	264.8488889	66.2122222	.	.
sub(acid*base)	9	0.9300000	0.1033333	.	.

Tests of Hypotheses Using the Type III MS for acid*base as an Error Term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
acid	2	323.0744444	161.5372222	2.44	0.2029
base	2	669.3911111	334.6955556	5.05	0.0804
acid*base	4	264.8488889	66.2122222	1.00	0.5000

The GLM Procedure

Level of acid	N	gluconv	
		Mean	Std Dev
0	**6**	9.0166667	4.3787746
1.25	**6**	8.3166667	3.9255148
2.5	**6**	17.6333333	12.3470914

Level of base	N	gluconv	
		Mean	Std Dev
0	**6**	3.9000000	0.7823043
5	**6**	12.2666667	2.7659839
10	**6**	18.8000000	10.4646070

Level of acid	Level of base	N	gluconv	
			Mean	Std Dev
0	**0**	**2**	3.4000000	0.14142136
0	**5**	**2**	11.3500000	0.35355339
0	**10**	**2**	12.3000000	0.42426407
1.25	**0**	**2**	3.4000000	0.14142136

Level of acid	Level of base	N	gluconv	
			Mean	Std Dev
1.25	**5**	**2**	9.7500000	0.35355339
1.25	**10**	**2**	11.8000000	0.42426407
2.5	**0**	**2**	4.9000000	0.14142136
2.5	**5**	**2**	15.7000000	0.28284271
2.5	**10**	**2**	32.3000000	0.42426407

```
*ANOVA for finding the effect of pretreatment time;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA Expt5;
INPUT sub time gluconv;
CARDS;
1       20      44.83
2       20      45.36
1       10      83.41
2       10      86.01
1       5       56.43
2       5       58.41
;
*ANOVA for completely randomized design;
*3 time levels during enzymatic hydrolysis- Subsampling case;
PROC GLM;
CLASS sub time;
MODEL gluconv = time sub(time);
TEST H= time E=sub(time);
RUN;
```

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
sub	2	1 2
time	3	5 10 20

Number of Observations Read 6

Number of Observations Used 6

The GLM Procedure

Dependent Variable: gluconv

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1649.479283	329.895857	.	.
Error	0	0.000000	.		
Corrected Total	5	1649.479283			

R-Square	Coeff Var	Root MSE	gluconv Mean
1.000000	.	.	62.40833

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	2	1643.998633	821.999317	.	.
sub(time)	3	5.480650	1.826883	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	2	1643.998633	821.999317	.	.
sub(time)	3	5.480650	1.826883	.	.

Tests of Hypotheses Using the Type III MS for sub(time) as an Error Term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	2	1643.998633	821.999317	449.95	0.0002

The GLM Procedure
Least Squares Means

time	gluconv LSMEAN
5	57.4200000
10	84.7100000
20	45.0950000

B.1 SAS codes and outputs for chapter 5

This study used the ADX interface for optimizations that does not require any SAS programming. Hence, they are not shown. Codes for correlation procedure and influential diagnostics are shown below:

```
*ANOVA for correlation between delignification and wood glucan-to-ethanol yield;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA correlation;
INPUT lignin yield;
CARDS;
48.04   69.46
59.38   82.74
31.88   60.21
65.15   82.97
52.33   70.44
55.35   69.7
59.95   77.72
66.07   82.32
55.28   78.45
57.84   81.33
30.61   59.23
66.86   81.1
43.22   63.08
59.46   65.95
26.74   69.48
63.44   73.74
30.92   63.35
25.34   70.08
69.14   88.11
58.79   79.6
61.24   73.74
70.08   79.67
53.34   74.98
69.64   89.21
61.87   87
70.37   87.18
60.72   76.9
60.17   78.87
57.84   81.33
70.65   87.8
71.74   88.75
```

```
65.26   81.32
;
PROC CORR DATA= correlation;
VAR lignin yield;
RUN;
```

SAS Output

The CORR Procedure

2 Variables: lignin yield

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
lignin	32	56.20969	13.58784	1799	25.34000	71.74000
yield	32	76.74406	8.61628	2456	59.23000	89.21000

Pearson Correlation Coefficients, N = 32
Prob > |r| under H0: Rho=0

	lignin	yield
lignin	1.00000	0.81768
		<.0001
yield	0.81768	1.00000
	<.0001	

```
/* Multiple linear regression of redcedar data_RSM experiment*/
/* Pretreatment time, pretreatment temperature, acid and bisulfite loading were our
predictor variables. */
/* Wood glucan-to-glucose yield was the response variable.*/
DATA RSMexpt;
    INPUT temp time acid bisulf yield;
CARDS;
190   7.5   3.5   17.5  69.46
190   7.5   3.5   22.5  82.74
190   7.5   4     17.5  60.21
190   7.5   4     22.5  82.97
190   12.5  3.5   17.5  70.44
190   12.5  3.5   22.5  69.7
190   12.5  4     17.5  77.22
```

```
190   12.5  4     22.5  82.32
210   7.5   3.5   17.5  78.45
210   7.5   3.5   22.5  81.33
210   7.5   4     17.5  59.23
210   7.5   4     22.5  81.1
210   12.5  3.5   17.5  63.08
210   12.5  3.5   22.5  65.95
210   12.5  4     17.5  69.48
210   12.5  4     22.5  73.74
180   10    3.75  20    63.35
220   10    3.75  20    70.08
200   5     3.75  20    88.11
200   15    3.75  20    79.6
200   10    3.25  20    73.74
200   10    4.25  20    79.67
200   10    3.75  15    74.98
200   10    3.75  25    89.21
200   10    3.75  20    87
200   10    3.75  20    87.18
200   10    3.75  20    76.9
200   10    3.75  20    78.87
200   10    3.75  20    81.33
200   10    3.75  20    87.8
200   10    3.75  20    88.75
200   10    3.75  20    81.32
;
RUN;
ODS GRAPHICS ON;
PROC REG DATA=RSMexpt
     plots (label)=(CooksD RStudentByLeverage DFFITS DFBETAS);
     ID temp time acid bisulf;
MODEL yield = temp time acid bisulf/ INFLUENCE;
/*INFLUENCE option, which produces several influence statistics such as RSTUDENT,
DFFITS, DFBETAS*/
RUN;
QUIT;
```

SAS Output

The REG Procedure
Model: MODEL1
Dependent Variable: yield

Number of Observations Read	32
Number of Observations Used	32

Analysis of Variance

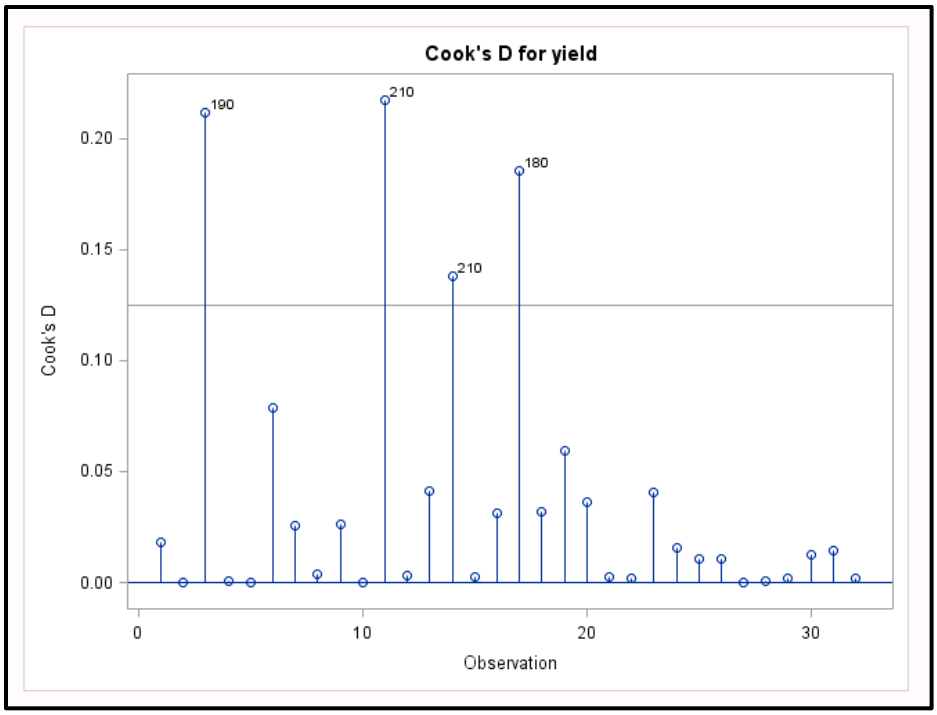
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	507.04092	126.76023	1.91	0.1380
Error	27	1793.67411	66.43237		
Corrected Total	31	2300.71502			

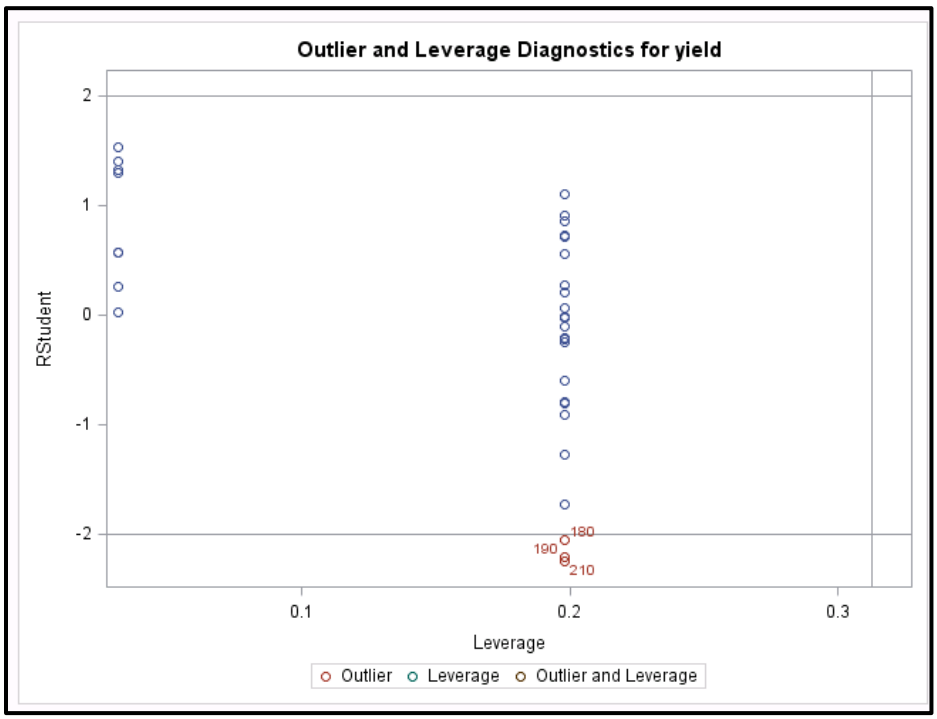
Root MSE	8.15061	**R-Square**	0.2204
Dependent Mean	76.72844	**Adj R-Sq**	0.1049
Coeff Var	10.62267		

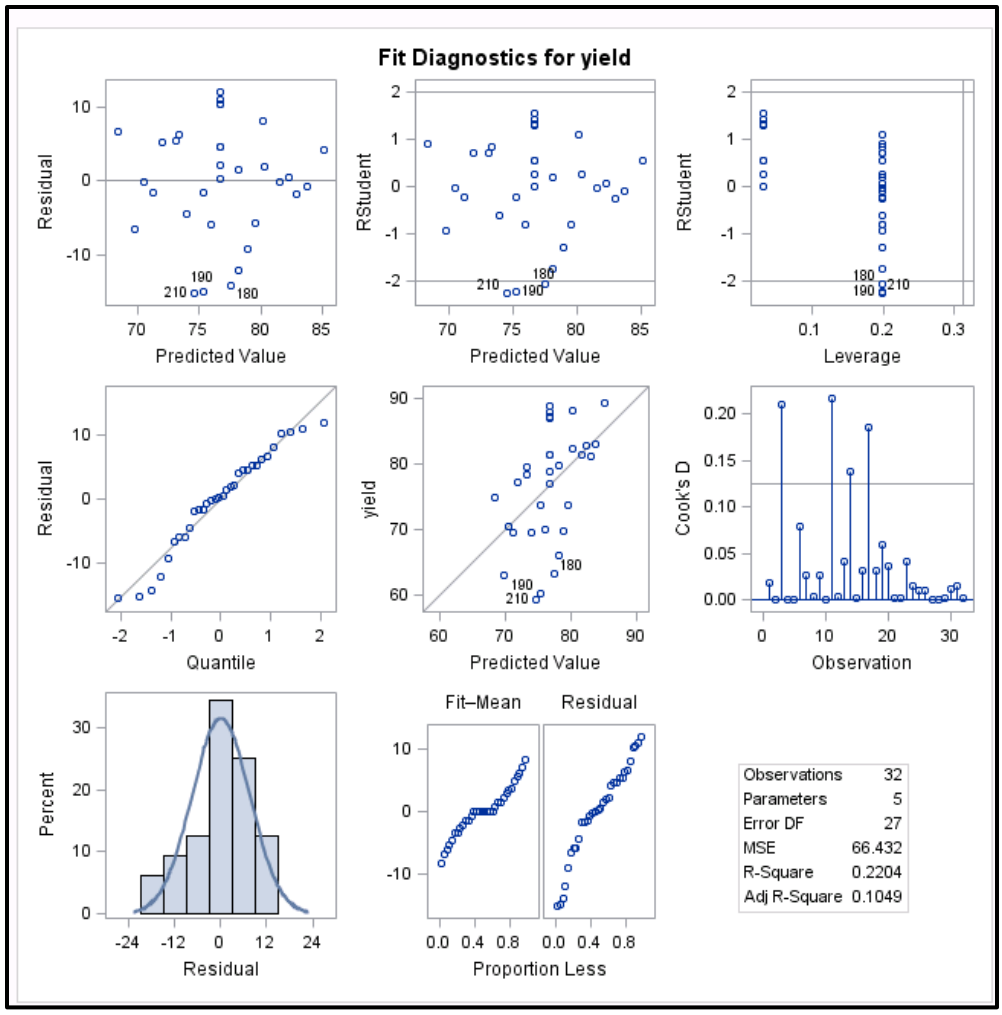
Parameter Estimates

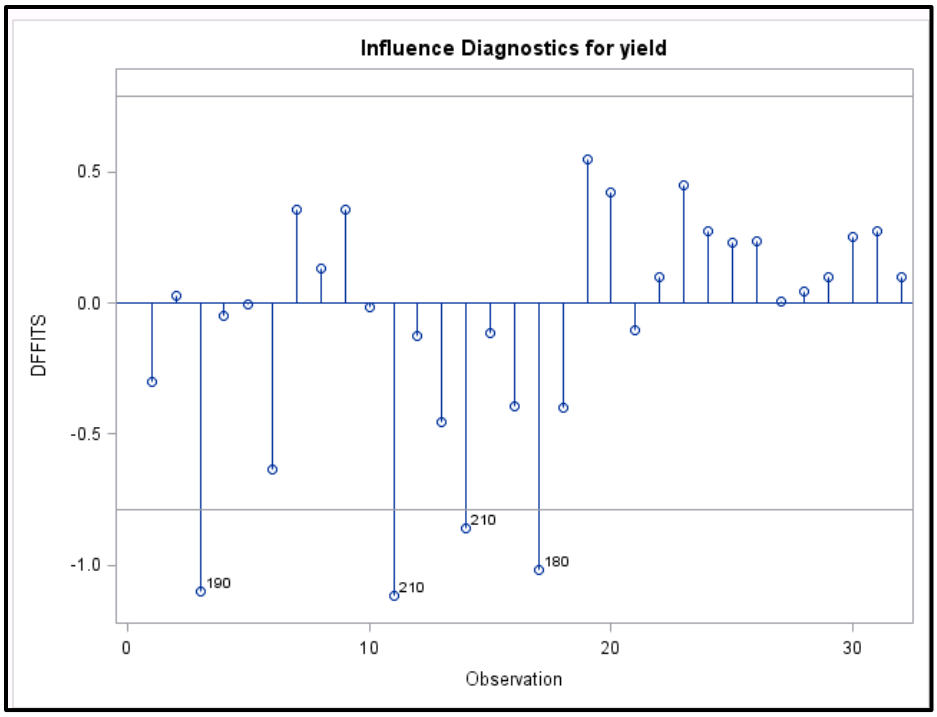
Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	**1**	46.99927	44.19872	1.06	0.2970
temp	**1**	-0.03850	0.16637	-0.23	0.8187
time	**1**	-0.67633	0.66549	-1.02	0.3185
acid	**1**	2.83000	6.65494	0.43	0.6740
bisulf	**1**	1.67900	0.66549	2.52	0.0178

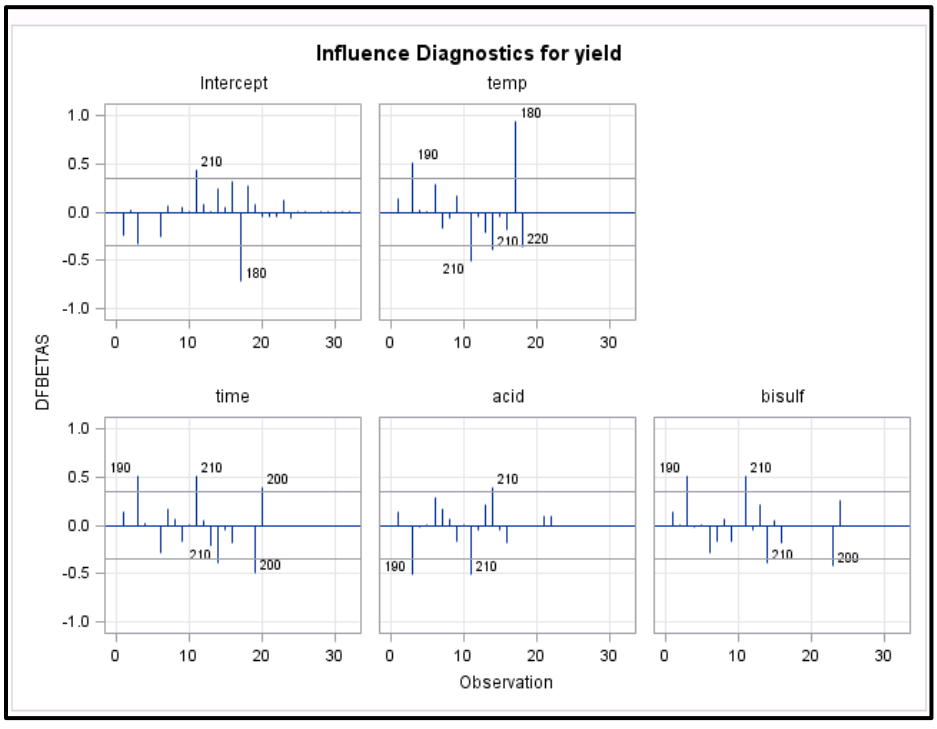
Sum of Residuals	0
Sum of Squared Residuals	1793.67411
Predicted Residual SS (PRESS)	2529.62259

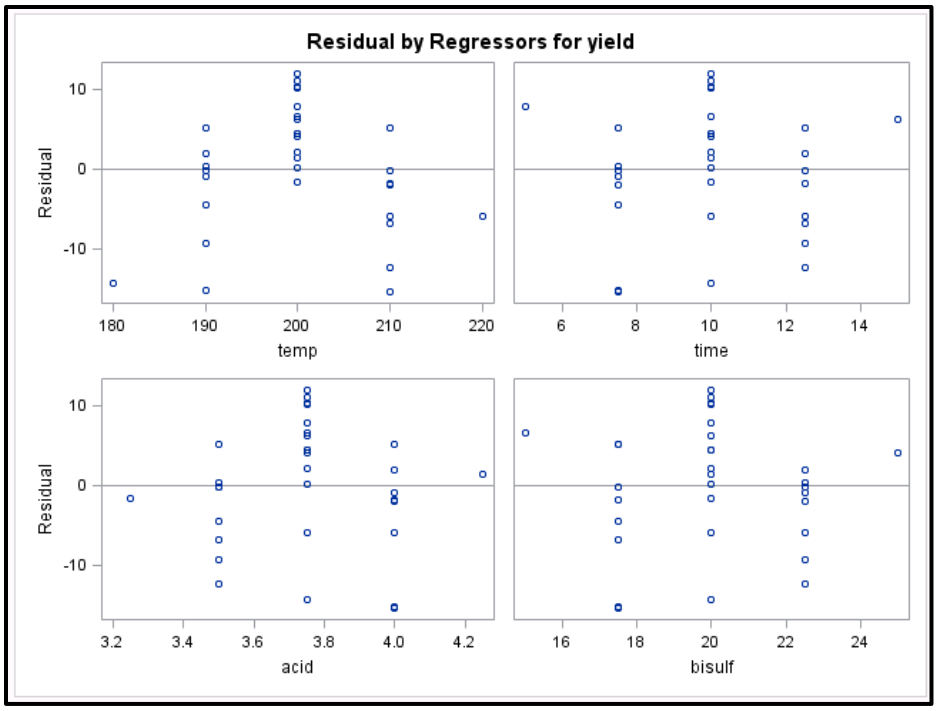

Cook's D for yield
Cook's D
0.20
0.15
0.10
0.05
0.00
190
210
180
210
0
10
20
30
Observation


Outlier and Leverage Diagnostics for yield
RStudent
2
1
0
-1
-2
180
190
210
0.1
0.2
0.3
Leverage
Outlier
Leverage
Outlier and Leverage


Fit Diagnostics for yield
Residual
Predicted Value
RStudent
Predicted Value
RStudent
Leverage
Residual
Quantile
yield
Predicted Value
Cook's D
Observation
Percent
Residual
Fit–Mean
Residual
Proportion Less
Observations 32
Parameters 5
Error DF 27
MSE 66.432
R-Square 0.2204
Adj R-Square 0.1049


Influence Diagnostics for yield
DFFITS
0.5
0.0
-0.5
-1.0
190
210
210
180
0
10
20
30
Observation


Influence Diagnostics for yield
Intercept
temp
time
acid
bisulf
DFBETAS
1.0
0.5
0.0
-0.5
-1.0
210
180
190
180
210
210
220
190
210
200
210
200
210
190
210
190
210
200
0
10
20
30
Observation



C.1 SAS codes and outputs for chapter 6

As there were many SAS programs run for statistical analysis, only one example per type is shown here

```
*ANOVA for effect of high solids loading - Chapter 6;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA mixing;
INPUT rep SL mix $ digestibility;
CARDS;
1       16      N       104.19
2       16      N       105.90
1       16      Y       110.60
2       16      Y       111.09
1       20      N       117.50
2       20      N       117.71
1       20      Y       124.91
2       20      Y       127.26
;
*ANOVA for completely randomized factorial design;
*SL =  Solid loading; N and Y refers to absence and presence of mixing aid, respectively;
PROC GLM;
CLASS rep SL mix;
```

MODEL digestibility = SL mix SL*mix;
RUN;

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
rep	2	1 2
SL	2	16 20
mix	2	N Y

Number of Observations Read	8
Number of Observations Used	8

The GLM Procedure

Dependent Variable: digest

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	491.9704000	163.9901333	150.26	0.0001
Error	4	4.3654000	1.0913500		
Corrected Total	7	496.3358000			

R-Square	Coeff Var	Root MSE	digest Mean
0.991205	0.909245	1.044677	114.8950

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SL	1	386.4200000	386.4200000	354.08	<.0001
mix	1	101.9592000	101.9592000	93.42	0.0006
SL*mix	1	3.5912000	3.5912000	3.29	0.1439

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SL	1	386.4200000	386.4200000	354.08	<.0001
mix	1	101.9592000	101.9592000	93.42	0.0006
SL*mix	1	3.5912000	3.5912000	3.29	0.1439

```
*ANOVA for effect of high of solids loading- Chapter 6;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA mixing;
INPUT rep cond $ digestibility;
CARDS;
1       2       89.30
2       2       93.98
1       4       90.19
2       4       89.17
1       8       91.74
2       8       89.77
1       12      87.34
2       12      89.07
1       16      86.26
2       16      87.68
1       16M     91.57
2       16M     91.98
1       20      77.87
2       20      78.01
1       20M     82.78
2       20M     84.34
;
*ANOVA for completely randomized design ;
*16M and 20M are 16% and 20% SL with metal balls;
PROC GLM;
CLASS rep cond;
MODEL digestibility= cond;
MEANS cond/ Tukey;
RUN;
```

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
rep	2	1 2
cond	8	12 16 16M 2 20 20M 4 8

Number of Observations Read	16
Number of Observations Used	16

The GLM Procedure

Dependent Variable: digest

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	316.8466437	45.2638062	21.02	0.0001
Error	8	17.2271500	2.1533938		
Corrected Total	15	334.0737937			

R-Square	Coeff Var	Root MSE	digest Mean
0.948433	1.675823	1.467445	87.56563

Source	DF	Type I SS	Mean Square	F Value	Pr > F
cond	7	316.8466437	45.2638062	21.02	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
cond	7	316.8466437	45.2638062	21.02	0.0001

The GLM Procedure

Tukey's Studentized Range (HSD) Test for digest

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	2.153394
Critical Value of Studentized Range	5.59618
Minimum Significant Difference	5.8068

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	cond
A	91.775	2	16M
A			
A	91.640	2	2

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	cond
	A			
	A	90.755	2	8
	A			
	A	89.680	2	4
	A			
B	A	88.205	2	12
B	A			
B	A	86.970	2	16
B				
B	C	83.560	2	20M
	C			
	C	77.940	2	20

D.1 SAS codes and outputs for chapter 7

As there were many SAS programs run for statistical analysis, only one example per type is shown here

```
*ANOVA for wood glucan-to-ethanol yield with subsamples;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA yield;
INPUT zone $ size $ rep sub yield;
CARDS;
Sap   Ground   1   1   90.87
Sap   Ground   1   2   92.13
Sap   Ground   2   1   86.25
Sap   Ground   2   2   85.45
Sap   Crum  1   1   94.25
Sap   Crum  1   2   95.88
Sap   Crum  2   1   93.53
Sap   Crum  2   2   95.46
Heart Ground  1   1   82.57
```

```
Heart   Ground      1       2       77.29
Heart   Ground      2       1       84.47
Heart   Ground      2       2       76.38
Heart   Crum   1    1       80.90
Heart   Crum   1    2       74.16
Heart   Crum   2    1       87.15
Heart   Crum   2    2       80.25
;
*ANOVA for completed randomized factorial design;
*Factorial treatment combination, 2 categorical levels for wood zone and 2 levels for
particle size during SSF and 2 subs for each treatment;
PROC GLM;
CLASS rep zone size;
MODEL yield = zone | size  rep(zone*size);
TEST H=zone | size E= rep(zone*size);
MEANS zone size;
RUN;
```

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
rep	2	1 2
zone	2	Heart Sap
size	2	Crum Ground

Number of Observations Read	16
Number of Observations Used	16

The GLM Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	659.0750937	94.1535848	7.73	0.0049
Error	8	97.4867500	12.1858437		
Corrected Total	15	756.5618437			

R-Square	Coeff Var	Root MSE	yield Mean
0.871145	4.056178	3.490823	86.06188

Source	DF	Type I SS	Mean Square	F Value	Pr > F
zone	1	513.5889063	513.5889063	42.15	0.0002
size	1	42.8043063	42.8043063	3.51	0.0978
zone*size	1	32.1205562	32.1205562	2.64	0.1431
rep(zone*size)	4	70.5613250	17.6403313	1.45	0.3035

Source	DF	Type III SS	Mean Square	F Value	Pr > F
zone	1	513.5889063	513.5889063	42.15	0.0002
size	1	42.8043063	42.8043063	3.51	0.0978
zone*size	1	32.1205562	32.1205562	2.64	0.1431
rep(zone*size)	4	70.5613250	17.6403313	1.45	0.3035

Tests of Hypotheses Using the Type III MS for rep(zone*size) as an Error Term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
zone	1	513.5889063	513.5889063	29.11	0.0057
size	1	42.8043063	42.8043063	2.43	0.1943
zone*size	1	32.1205562	32.1205562	1.82	0.2485

The GLM Procedure

Level of zone	N	yield	
		Mean	Std Dev
Heart	**8**	80.3962500	4.34048693
Sap	**8**	91.7275000	3.98379127

Level of size	N	yield	
		Mean	Std Dev
Crum	**8**	87.6975000	8.36145878
Ground	**8**	84.4262500	5.66139291

```
*ANOVA for lignin loss during pretreatments;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA yield;
INPUT cond $ rep ligloss;
CARDS;
SG      1       56.38
SG      2       38.86
SC      1       71.05
SC      2       72.09
HG      1       26.30
HG      2       22.30
HC      1       21.73
HC      2       19.88
;
*ANOVA for completely randomized design;
*Factorial treatment combination, 2 levels for wood zone and 2 levels for particle size during SSF;
PROC GLM;
CLASS rep cond;
MODEL ligloss = cond;
MEANS cond/tukey;
RUN;
```

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
rep	2	1 2
cond	4	HC HG SC SG

Number of Observations Read	8
Number of Observations Used	8

The GLM Procedure

Dependent Variable: ligloss

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3330.111138	1110.037046	27.12	0.0041
Error	4	163.727250	40.931813		
Corrected Total	7	3493.838388			

R-Square	Coeff Var	Root MSE	ligloss Mean
0.953138	15.57637	6.397797	41.07375

Source	DF	Type I SS	Mean Square	F Value	Pr > F
cond	3	3330.111138	1110.037046	27.12	0.0041

Source	DF	Type III SS	Mean Square	F Value	Pr > F
cond	3	3330.111137	1110.037046	27.12	0.0041

The GLM Procedure

Tukey's Studentized Range (HSD) Test for ligloss

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	40.93181
Critical Value of Studentized Range	5.75704
Minimum Significant Difference	26.044

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	cond
	A	71.570	2	SC
	A			
B	A	47.620	2	SG
B				
B	C	24.300	2	HG
	C			
	C	20.805	2	HC

E.1 SAS codes and outputs for chapter 8

As there were many SAS programs run for statistical analysis, only one example per type is shown here

```
*ANOVA for repeated measures for cell mass concentration- Chapter 8;
DM 'LOG; CLEAR; OUTPUT; CLEAR;';
OPTIONS PAGENO=1;
DATA cells;
INPUT trt rep time cmc;
CARDS;
0    1    0    0.12
0    1    3    0.27
0    1    6    1.30
0    1    9    5.03
0    1    12   5.20
0    1    18   5.36
0    1    24   5.49
0    2    0    0.11
0    2    3    0.26
0    2    6    1.27
0    2    9    4.96
0    2    12   4.68
0    2    18   5.29
0    2    24   5.22
0    3    0    0.12
0    3    3    0.28
0    3    6    1.40
0    3    9    4.87
0    3    12   5.10
0    3    18   5.35
0    3    24   4.90
0    4    0    0.12
0    4    3    0.27
0    4    6    1.41
0    4    9    4.98
0    4    12   5.06
0    4    18   5.42
0    4    24   5.35
0.5  1    0    0.19
0.5  1    3    0.29
0.5  1    6    0.98
0.5  1    9    3.64
0.5  1    12   5.15
0.5  1    18   5.06
0.5  1    24   5.45
0.5  2    0    0.20
```

0.5	2	3	0.35
0.5	2	6	0.86
0.5	2	9	3.45
0.5	2	12	4.81
0.5	2	18	5.49
0.5	2	24	5.51
0.5	3	0	0.17
0.5	3	3	0.31
0.5	3	6	1.03
0.5	3	9	3.47
0.5	3	12	4.82
0.5	3	18	5.61
0.5	3	24	5.62
0.5	4	0	0.20
0.5	4	3	0.60
0.5	4	6	1.16
0.5	4	9	3.30
0.5	4	12	4.85
0.5	4	18	5.45
0.5	4	24	5.37
1	1	0	0.25
1	1	3	0.32
1	1	6	0.95
1	1	9	2.27
1	1	12	4.58
1	1	18	5.38
1	1	24	5.48
1	2	0	0.25
1	2	3	0.36
1	2	6	1.12
1	2	9	3.25
1	2	12	4.59
1	2	18	5.76
1	2	24	5.58
1	3	0	0.25
1	3	3	0.38
1	3	6	0.81
1	3	9	3.18
1	3	12	4.68
1	3	18	5.38
1	3	24	5.39
1	4	0	0.21
1	4	3	0.61
1	4	6	0.90
1	4	9	2.92
1	4	12	4.86

```
1       4       18      5.17
1       4       24      5.30
;
PROC PRINT DATA=cells; RUN;
*Expt design - CRD;
*Treatments are different levesls of redcedar oil loading;
*Time would be a related variable, ie cell mass concentrations (CMC) measurements
from the same trts each time;
TITLE2 'UNSTRUCTURED';
PROC MIXED DATA=cells; CLASS trt rep;
MODEL cmc=trt time trt*time/HTYPE=1;
REPEATED/SUBJECT=time TYPE=cs;
RUN;
```

SAS Output

The Mixed Procedure

Model Information

Data Set	WORK.CELLS
Dependent Variable	cmc
Covariance Structure	Compound Symmetry
Subject Effect	time
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
trt	3	0 0.5 1
rep	4	1 2 3 4

Dimensions

Covariance Parameters	2
Columns in X	8
Columns in Z	0

Dimensions

Subjects	84
Max Obs Per Subject	1

Number of Observations

Number of Observations Read	84
Number of Observations Used	84
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	257.32020417	
1	1	257.32020417	0.00000000

Convergence criteria met but final hessian is not positive definite.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	**time**	0.5359
Residual		0.5116

Fit Statistics

-2 Res Log Likelihood	257.3
AIC (smaller is better)	261.3
AICC (smaller is better)	261.5
BIC (smaller is better)	266.2

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	0.00	1.0000

Type 1 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F

Type 1 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
trt	2	78	0.71	0.4943
time	1	78	321.54	<.0001
time*trt	2	78	0.08	0.9273

```
*ANOVA for glucan-to-glucose yield- Chapter 8;
dm 'log; clear; output; clear;';
OPTIONS pageno=1;
DATA yield;
INPUT GL EL OL block yield;
CARDS;
2 0.125 0.00 1 41.5
2 0.125 0.50 1 28.6
2 0.125 1.00 1 22.9
2 0.250 0.00 1 43.5
2 0.250 0.50 1 29.4
2 0.250 1.00 1 29.5
4 0.125 0.00 1 30.4
4 0.125 0.50 1 29.7
4 0.125 1.00 1 22.3
4 0.250 0.00 1 48.3
4 0.250 0.50 1 29.6
4 0.250 1.00 1 30.2
2 0.125 0.00 2 40.75
2 0.125 0.50 2 31.41
2 0.125 1.00 2 22.51
2 0.250 0.00 2 46.33
2 0.250 0.50 2 33.80
2 0.250 1.00 2 31.80
4 0.125 0.00 2 33.80
4 0.125 0.50 2 31.94
4 0.125 1.00 2 30.13
4 0.250 0.00 2 45.51
4 0.250 0.50 2 30.84
4 0.250 1.00 2 31.51
2 0.125 0.00 3 38.09
2 0.125 0.50 3 19.48
2 0.125 1.00 3 22.95
2 0.250 0.00 3 49.93
2 0.250 0.50 3 41.92
2 0.250 1.00 3 39.43
```

```
4    0.125  0.00  3   40.71
4    0.125  0.50  3   30.19
4    0.125  1.00  3   22.49
4    0.250  0.00  3   49.43
4    0.250  0.50  3   36.79
4    0.250  1.00  3   34.26
;
*ANOVA for randomized complete block design;
*Factorial treatment combination, 2 levels for glucan loading (GL), 2 levels of enzyme
loading (EL) and 3 oil loading (OL)with3 blocks;
PROC GLM;
CLASS GL EL OL block;
MODEL yield = GL EL OL block GL*EL GL*OL EL*OL GL*EL*OL;
RANDOM block;
RUN;
```

SAS Output

The GLM Procedure

Class Level Information

Class	Levels	Values
GL	2	2 4
EL	2	0.125 0.25
OL	3	0 0.5 1
block	3	1 2 3

Number of Observations Read	36
Number of Observations Used	36

The GLM Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	2072.575650	159.428896	11.07	<.0001
Error	22	316.931850	14.405993		
Corrected Total	35	2389.507500			

R-Square	Coeff Var	Root MSE	yield Mean
0.867365	11.18249	3.795523	33.94167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GL	1	0.902500	0.902500	0.06	0.8047
EL	1	561.690000	561.690000	38.99	<.0001
OL	2	1321.155150	660.577575	45.85	<.0001
block	2	67.049817	33.524908	2.33	0.1211
GL*EL	1	4.438044	4.438044	0.31	0.5845
GL*OL	2	12.917917	6.458958	0.45	0.6444
EL*OL	2	34.278317	17.139158	1.19	0.3231
GL*EL*OL	2	70.143906	35.071953	2.43	0.1109

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GL	1	0.902500	0.902500	0.06	0.8047
EL	1	561.690000	561.690000	38.99	<.0001
OL	2	1321.155150	660.577575	45.85	<.0001
block	2	67.049817	33.524908	2.33	0.1211
GL*EL	1	4.438044	4.438044	0.31	0.5845
GL*OL	2	12.917917	6.458958	0.45	0.6444
EL*OL	2	34.278317	17.139158	1.19	0.3231
GL*EL*OL	2	70.143906	35.071953	2.43	0.1109

VITA

Karthikeyan D. Ramachandriya

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Doctor of Philosophy

Thesis: DEVELOPMENT AND OPTIMIZATION OF GLUCOSE AND ETHANOL PRODUCTION FROM EASTERN REDCEDAR

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Experience:
Graduate Research Associate, Oklahoma State University, Stillwater, Oklahoma, January 2010 to July 2013.

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