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Sara Lynn Walton

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**BIOLOGICAL CONVERSION OF HEMICELLULOSE EXTRACT INTO  
VALUE-ADDED FUELS AND CHEMICALS**

By

Sara Lynn Walton

B.S. University of Maine, 2005

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Chemical Engineering)

The Graduate School

The University of Maine

December, 2009

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December 2009

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# **BIOLOGICAL CONVERSION OF HEMICELLULOSE EXTRACT INTO VALUE-ADDED FUELS AND CHEMICALS**

By Sara Lynn Walton

Thesis Co-Advisors: Dr. Adriaan R.P. van Heiningen and Dr. G. Peter van Walsum

An Abstract of the Thesis Presented  
In Partial Fulfillment of the Requirements for the  
Degree of Doctor of Philosophy  
(in Chemical Engineering)  
December, 2009

The research presented in this thesis describes the fermentation of hemicellulose extracts to produce ethanol and lactic acid. Hardwood hemicellulose extracts made at a variety of conditions using the green liquor and hot water extraction processes were characterized and then fermented by *Escherichia coli* K011 and *Bacillus coagulans* MXL-9. Hemicellulose extracts were found to contain a dilute amount of xylo-oligosaccharides and acetic acid as the major components, and many minor components including other organic acids, lignin-derived phenolics, and sugar degradation products. In order to generate ethanol economically, the fermentation must produce at least a 4% ethanol solution, which requires a starting sugar concentration of at least 8%. Since hemicellulose extracts did not contain high enough sugar titers, methods for concentrating the extracts prior to fermentation were investigated. Evaporation was found to be an ineffective method of concentrating sugars because it also concentrates such chemicals as acetic acid and sodium which inhibit fermentation. Ultrafiltration was then examined as a possible method of concentrating the oligomeric fraction of the

extracts without effecting the concentrations of smaller molecules such as acetic acid and sodium. It does however concentrate the lignin fraction, which causes inhibition of fermentation organisms. Both *E. coli* and *B. coagulans* were found to consume all of the principal monosaccharides found in lignocellulosic biomass, including xylose and arabinose which cannot be utilized by traditional industrial yeast strains. These bacteria had higher tolerance to the inhibitors found in hemicellulose extracts than any other organism tested. The highest product titers achieved were 1% ethanol and 3% lactic acid.

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## CHAPTER 1: INTRODUCTION

The effort to replace petrochemical products with those derived from renewable resources has generated significant interest in the use of lignocellulose to generate fuels and chemicals. Agricultural and forestry wastes provide a readily available, inexpensive source of lignocellulose. Lignocellulose is composed of three polymeric structures: cellulose, hemicelluloses and lignin. Cellulose fibers may be used in the production of pulp, or hydrolyzed into its glucose constituents. Glucose is readily fermented into a variety of products by many organisms. In pulping, hemicelluloses and lignin are generally not converted into higher value products; instead they are burned for their heating values. Hemicelluloses are polymers of various mono-sugars, of which xylose is the most prevalent component in hardwoods and agricultural biomass. Xylose is a five carbon sugar which is not as readily fermented by many organisms. Hemicelluloses have the potential to be removed from wood in a pre-treatment step prior to pulping. They do not have the high heating value of lignin, and the existing pulping industry could benefit from creating a higher value product from the hemicelluloses. Economical production of fuels such as ethanol or chemicals such as lactic acid from lignocellulosic feed-stocks depends on the ability of fermentative organisms to utilize all major sugars. Metabolic engineering has created new strains of organisms which have the ability to consume both five and six carbon sugars, withstand inhibitors released during hydrolysis, and create a single product preferentially. The bacteria *Escherichia coli* K011 was developed for improved ethanol production from xylose substrates. Among the fermentation products, fuel ethanol has generated significant interest due to the rising costs of oil. Continued advancement in hydrolysis of lignocellulose and organism development are necessary to

approach complete conversion of feedstocks into value added chemicals in order to make fermentation of biomass a cost effective process.

## 1.1 Literature Review

### 1.1.1 Lignocellulose Composition

Lignocellulose consists of cellulose, hemicelluloses, and lignin. Cellulose is a homogeneous linear polymer of 1,4 linked  $\beta$ -D-glucopyranose units making up 35-50% of lignocelluloses, with a degree of polymerization (DP) ranging from 10,000 to 15,000 units. Hemicelluloses are amorphous heterogeneous polymers comprising 20-35% of lignocellulosic biomass with a DP of 80-200 units (Saha, 2003). The monosugars comprising hemicelluloses include the pentoses D-xylose and L-arabinose, as well as the hexoses D-glucose, D-galactose, and D-mannose. Sugar acids make up the remainder of the structure of hemicelluloses. Xylans (Figure 1) and glucomannans (Figure 2) are the two predominant types of hemicelluloses; the composition and proportion of these groups varies by species. Hardwoods generally contain 15-30% xylans and only 2-5% glucomannans (Sjöström, 1993). Xylans have a homopolymeric backbone consisting of 1,4-linked  $\beta$ -D-xylopyranose units and may contain side-chains of glucuronic acid or its 4-O-methylether. Hardwood xylans are highly substituted with acetyl groups, containing on average 7 per 10 xylose units (Duff and Murray, 1996). Glucomannans have a heterogeneous backbone of 1,4-linked  $\beta$ -D-glucopyranose and  $\beta$ -D-mannopyranose (Sjöström, 1993).

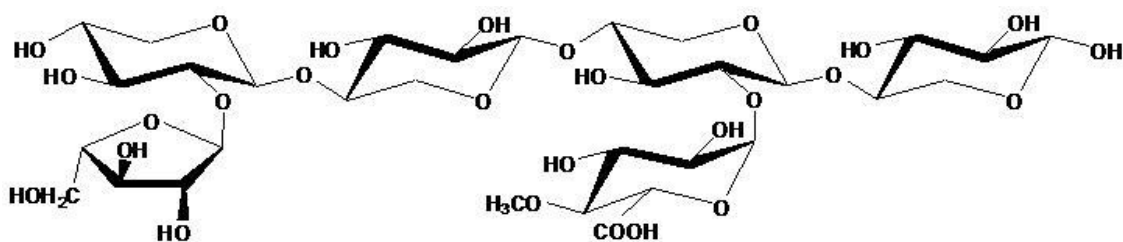


Figure 1. Structural representation of xylan  
 Constituents:  $\beta$ -D-xylopyranose (backbone),  $\alpha$ -L-arabinofuranose (bottom left), 4-O-methyl- $\alpha$ -D-glucopyranosyluronic acid (bottom right) (Sjöström, 1993)

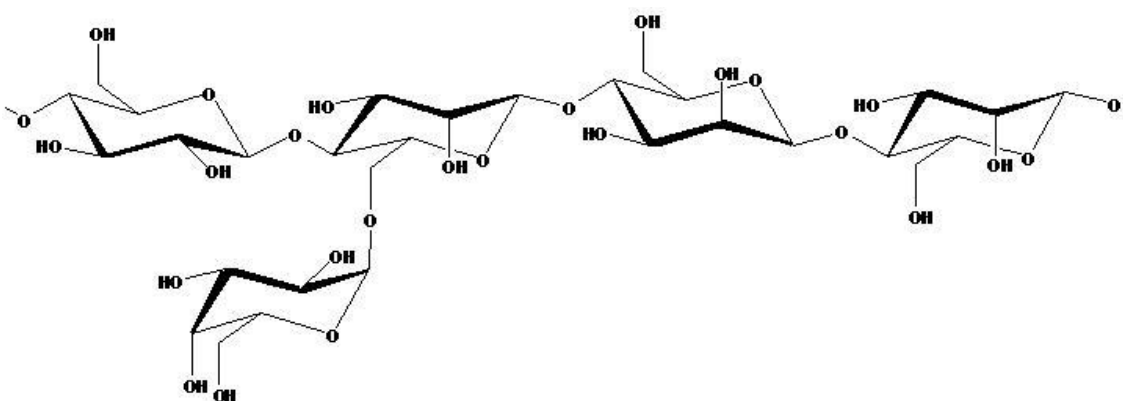


Figure 2. Structural representation of galactoglucomannans (Sjöström, 1993)  
 Constituents:  $\beta$ -D-glucopyranose (left),  $\beta$ -D-mannopyranose (right three),  $\alpha$ -D-galactopyranose (branch)

Lignin is an aromatic polymer of phenylpropanoid precursors which surrounds and strengthens the cellulose-hemicellulose matrix. Softwoods generally contain more lignin than hardwoods. Lignin comprises 20-25% of biomass and is mainly composed of coniferyl alcohol, sinapyl alcohol and hydroxycinnamyl alcohol which are polymerized in a random fashion (Duff & Murray, 1996).

#### 1.1.2 Hemicellulose Extraction

Hemicelluloses, due to their lower degree of polymerization and the presence of branched side groups, can be more readily dissolved into aqueous solution than other

components of lignocellulose. During Kraft pulping, the most widely used chemical pulping method, up to half of the hardwood hemicellulose is removed, accounting for up to 15% of the wood weight (Sjöström, 1993). These hemicelluloses are dissolved into the “black liquor,” which is burned to create energy during the chemical recovery process. Hemicellulose has a heating value of 13.6 MJ/kg which is only half that of lignin (Sjöström, 1993). A better use for hemicellulose would be as a chemical feedstock for the creation of new products. Selective removal of the hemicelluloses prior to pulping can be accomplished without degrading the wood fibers. One proposed method of hemicellulose removal uses green liquor as a pretreatment chemical (van Heiningen, 2006). Green liquor is an alkaline aqueous solution generated in the pulping recovery process which is comprised of sodium hydroxide, sodium carbonate and sodium sulfide. Under alkaline conditions xylan is dissolved in oligomeric form, while glucomannans are degraded by the peeling reaction (Fengel and Wegener, 1984). Alkaline extraction is therefore possible as a treatment of hardwoods, which contain mainly xylan. Extraction using only hot water generates an acidic extract due to release of acetyl groups from wood, which may then result in degradation of cellulose fiber by acid hydrolysis. Extraction using alkaline chemicals results in a final liquor that is near-neutral pH, preserving the pulp yield and pulp production (Mao *et. al.*, 2008).

### 1.1.3 Hydrolysis of Hemicellulose into Monosaccharides

In fermenting hemicelluloses into useful products, it is necessary to hydrolyze the oligosaccharide down to its component monosaccharide constituents. Chemical or enzymatic treatments may be used to accomplish this hydrolysis. Acid hydrolysis

produces degradation products, such as furfural, and hydroxymethyl furfural (HMF), which are harmful to fermenting organisms. Structures of furfural, HMF and their precursors can be seen in Figure 3. Furfural is produced by dehydration of xylose in the presence of acid, and glucose similarly reacts with acid to produce HMF. Severity of chemical hydrolysis is determined by the temperature, time, and acid concentration. The highest amount of hemicellulose monosugars are recovered at intermediate severity. A much higher severity is required to form glucose from cellulose due to the crystalline nature of cellulose.

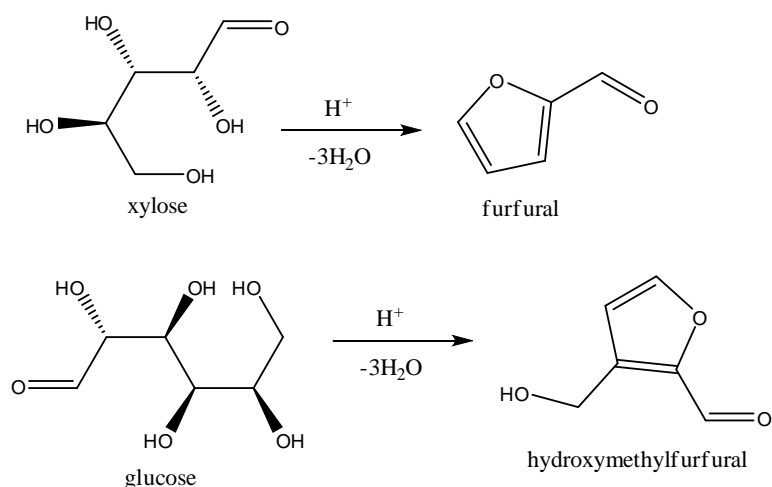


Figure 3. Formation of degradation products furfural and HMF. (Sjöström, 1993)

Chemical hydrolysis was found to produce higher pentose yields than enzymatic methods (Duarte *et al.*, 2004). Enzymatic processes, however, have the advantage of less severe operation, generating a monosugar stream free of sugar and lignin degradation products which would inhibit microbial growth. Dilute acid hydrolysis is dependent on temperature, reaction time, and concentration of the acid catalyst. Enzymatic hydrolysis depends on all of these factors, as well as substrate structure and enzymatic activities.

Optimal performance during enzyme hydrolysis is achieved with a mixture of different enzymes working synergistically. In hemicellulose degradation, the most important enzyme is endo-1,4- $\beta$ -xylanase, which attacks the main polymer chain. Xylo-oligomers are hydrolyzed to xylose by  $\beta$ -xylosidases. Accessory enzymes such as acetyl xylanesterases,  $\alpha$ -arabinofuranosidases and  $\alpha$ -glucuronidases are necessary to liberate substituent sugars from the main chain (Duarte *et al.*, 2004).

#### 1.1.4 Fermentation of Lignocellulose-derived Monosaccharides

Economically viable fermentation of lignocellulosic biomass requires an organism capable of utilizing all of the principal hexose and pentose sugars: glucose, mannose, galactose, xylose and arabinose. While hexose sugars are readily utilized by many organisms, there are relatively few that naturally ferment pentoses. Among these are *Pichia stipitis*, *Pachysolen tannophilus*, and *Candida shehatae*. Traditional brewer's yeast, *Saccharomyces cerevisiae* only utilizes glucose and fructose. Other organisms, such as *Escherichia coli*, can naturally utilize a variety of sugars, but produce a mixture of products, including ethanol, acetic acid, lactic acid, and succinic acid.

##### 1.1.4.1 Metabolic Engineering of Bacteria

Genetic engineering to improve fermentation and product formation is known as metabolic engineering. Metabolic engineering has been used to incorporate the ability to co-ferment five and six carbon sugars, as well as to block metabolic pathways leading to undesired products. Bacteria have proven simpler than yeast to engineer due to their less complex genome. One approach is to incorporate ethanol production into bacteria such as *Escherichia coli* or *Klebsiella oxytoca*, knocking out pathways to competing products

such as lactic acid. Another method transfers pentose utilization capability into natural hexose fermentors such as *Zymomonas mobilis*. These co-fermenting strains tend to preferentially utilize glucose over xylose.

Among the bacteria which have been developed to selectively produce ethanol, the greatest success has been achieved with *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*. *E. coli* and *K. oxytoca* ferment a wide range of sugars, but lack selectivity. *Z. mobilis* shows high ethanol yields but only utilizes glucose and fructose (Dien *et al.*, 2003). The ideal organism for fermenting lignocellulosic sugars must metabolize all major sugars found in wood, while producing minimal side products.

*E. coli* has advantages such as its ability to metabolize many sugars, its lack of requirement for complex growth medium, and its prior acceptance in industrial use. Its major disadvantages include a limitation to a narrow and neutral pH range, making it less hardy than yeast. Ethanol is produced by *E. coli* from pyruvate using pyruvate formate lyase, through a pathway which also leads to acetic and succinic acids (see Figure 4). Yeasts and *Z. mobilis* produce ethanol without side products because their metabolism uses pyruvate decarboxylase (*pdc*). Insertion of the *Z. mobilis pdc* genes as well as its alcohol dehydrogenase (*adh*) genes via plasmid transformation resulted in an *E. coli* strain that produced ethanol almost exclusively (Dien *et al.*, 2003). Transformation with plasmids is less stable than mutations which are incorporated into the organism's chromosome, therefore the *pdc* and *adh* genes were integrated under the native lac promoter, and the resulting construct was termed the PET (production of ethanol) operon. The PET operon was then inserted into the *E. coli* pyruvate formate lyase gene (Dien *et al.*, 2003). An antibiotic resistance to chloramphenicol was associated with the genetic

changes, and only mutants growing in the presence of 600 mg/L chloramphenicol were selected. The terminal fumarate reductase gene of the succinate pathway was later disrupted, and the final strain was named *E. coli* K011.

*K. oxytoca* and *E. chrysanthemi* also have been transformed using the PET operon. While both have shown lower ethanol yields than *E. coli*, *K. oxytoca* has the distinction of being able to grow on cellobiose, which is appealing for cellulose fermentation. Strain M5A1 was shown to ferment faster than *E. coli*.

*Z. mobilis* shows a higher ethanol tolerance than other bacteria and is able to grow in up to 120 g/L ethanol, whereas *E. coli* is generally inhibited above 35 g/L. Other advantages of *Z. mobilis* include its simple nutritional needs and a lower production of biomass which allows more carbon to the products. *Z. mobilis* was engineered to ferment xylose by incorporating four *E. coli* genes coding for xylose isomerase, xylulose kinase, transketolase, and transaldolase into a plasmid. Arabinose utilization was engineered through the incorporation of an additional three *E. coli* genes which code for L-arabinose isomerase, L-ribulose kinase, and L-ribulose-5-phosphate-4-epimerase (Dien *et al.*, 2003). In *Z. mobilis* AX101, these seven genes were incorporated into the chromosomal DNA.

#### 1.1.4.2 *Escherichia coli* K011

Metabolic engineering to enhance one aspect of cell growth often has unintended consequences on other processes. In the case of *E. coli* K011, this was decreased cell growth due to partitioning of pyruvate between NAD<sup>+</sup> regeneration and biosynthesis which resulted in increased need for complex growth media (Underwood *et al.*, 2002). The presence of *Z. mobilis*' *pdc* and *adhb* enzymes redirects pyruvate into ethanol instead of the native lactate and formate, but also restricts flow through 2-ketoglutarate.

Biosynthesis proceeds through the 2-ketoglutarate arm of the tricarboxylic acid cycle and is regulated through the citrate synthase enzyme. The metabolic pathway of *E. coli* K011 for xylose can be seen in Figure 4. Metabolism of xylose produces only one-third of the ATP generated in glucose metabolism, or 0.67 ATP per xylose, because phosphorylation and uptake have separate energy requirements (Underwood *et al.*, 2002). These lower ATP levels contribute to undesired consequences that reduce growth and ethanol productivity. The consequences can be masked in the presence of complex growth media such as Luria Broth, but are apparent in mineral salts medium without complex nutrients. Lower rates of ethanol production and lower yields are caused by depression of native genes for biosynthetic enzymes.

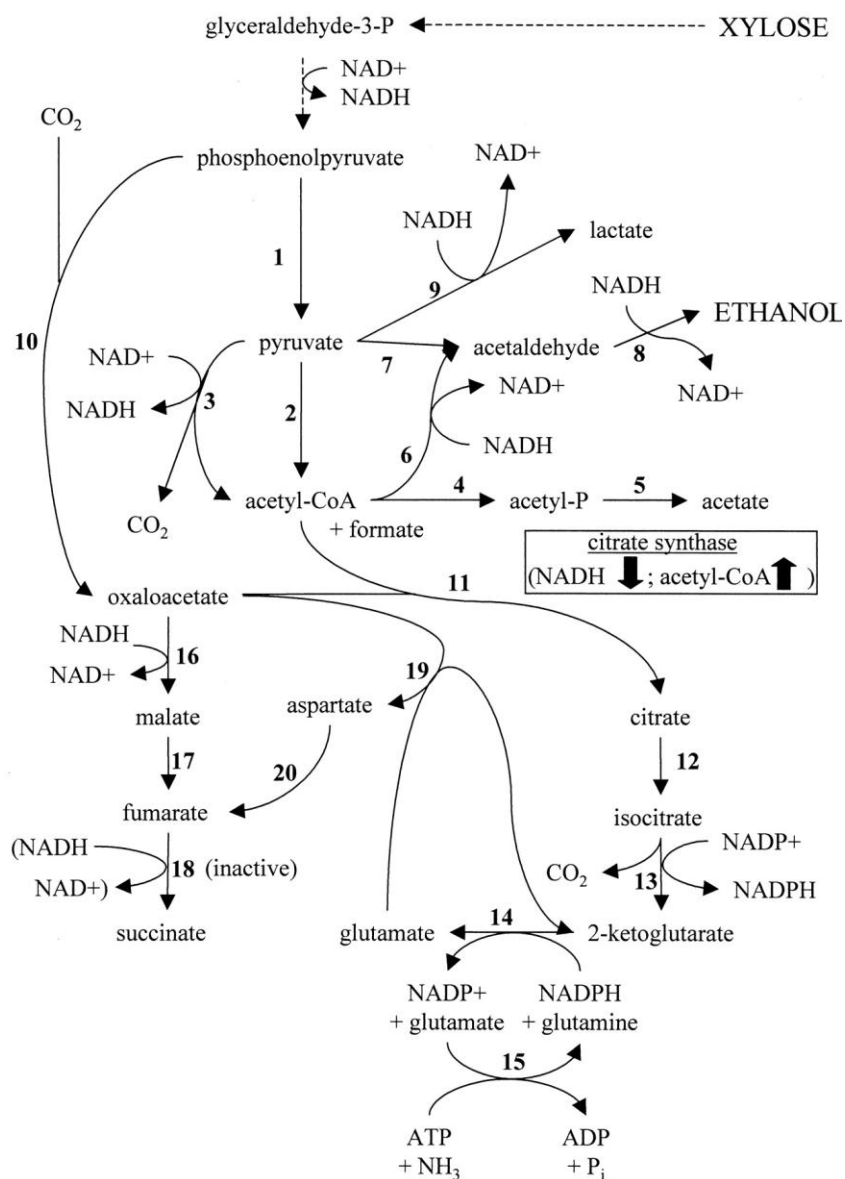


Figure 4. Carbon flow through the central metabolism in *E. coli* K011  
 Enzymes: 1, pyruvate kinase; 2, pyruvate formate-lyase; 3, pyruvate dehydrogenase; 4, phosphotransacetylase; 5, acetate kinase; 6, alcohol/aldehyde dehydrogenase; 7, *Z. mobilis* pyruvate decarboxylase; 8, *Z. mobilis* alcohol dehydrogenase II and *E. coli* alcohol/aldehyde dehydrogenase; 9, lactate dehydrogenase; 10, phosphoenolpyruvate carboxylase; 11, citrate synthase; 12, aconitase; 13, isocitrate dehydrogenase; 14, glutamate synthase; 15, glutamine synthetase; 16, malate dehydrogenase; 17, fumarase; 18, fumarate reductase; 19, aspartate transaminase; 20, aspartase. (Underwood *et al.*, 2002)

#### 1.1.5 Inhibition of Fermentation

Microbial organisms are subject to inhibition not only by the product ethanol, but by several inhibitors released during hydrolysis of lignocellulose into its monomer sugars. During extraction, acetic acid is released from the hemicellulose fraction. Acetic acid in addition to the sugar degradation products furfural and 5-hydroxymethyl furfural, and phenolics derived from lignin degradation, are known inhibitors of fermentation. Salts formed by chemicals used in the extraction process are an additional significant inhibitor to microbial fermentation. Salts and organic compounds with low molecular weight are able to penetrate cell membranes, while higher molecular weight inhibitors influence the expression and activity of sugar and ion transporters within the cell membrane (Klinke *et al.*, 2004). Different organisms experience varying degrees of inhibition in lignocellulosic hydrolysates. *E. coli* is generally more tolerant of furfural and 5-hydroxymethyl furfural than other organisms. Potential for inhibition was shown to be dependent on the chemical structure for aromatic compounds, and for *E. coli* is closely related to the functionality of the aliphatic side-chain (Klinke *et al.*, 2004). In order of increasing inhibition, the side-chains are: alcohols, acids, and then aldehydes. Inhibition was also correlated to the hydrophobicity, where hydrophobic parts of membrane transport systems, proteins and enzymes are potential sites of inhibition. Some compounds, such as furfural, exhibit even higher inhibition potential in the presence of other known inhibitors, indicating a synergistic inhibition mechanism (Klinke *et al.*, 2004).

Acetic acid in its undissociated form is able to penetrate the bacteria cell walls and acidify the cytoplasm. The proton gradient across the cell membrane is disrupted, interfering with cellular processes (Takahashi *et. al.*, 1999.) The effects of varying sodium acetate concentration on ethanol production by *E. coli* K011 were studied in media containing either 80g/L glucose or 50g/L xylose. In glucose fermentation, acetate concentrations up to 12g/L did not significantly affect the ethanol yield or productivity, but at 15g/L acetate there was a sharp decrease in production (Takahashi *et. al.*, 1999). During xylose fermentation, ethanol yield and productivity were not affected up to 10g/L acetate. It was observed that the degree of acetate inhibition was strongly correlated to pH, since the undissociated form of acetic acid is more prevalent at lower pH. The optimum fermentation pH in the presence of acetic acid was found to be 7, where pH 6 is optimum for cultures which aren't growing in acetic acid (Takahashi *et. al.*, 1999.)

There are two approaches to overcoming acetic acid and salt inhibition; the first is to remove the inhibitors before fermentation, and the second is to develop fermenting organisms capable of withstanding higher concentrations of inhibiting chemicals. Removing acetic acid can be done by liquid-liquid extraction. Removing inhibitors requires costly equipment and additional processing steps. An alternative is the development of microorganisms which can withstand higher concentrations of acetic acid, furfural and other inhibitors. Adapted strains may be selected for by exposing the culture to increasingly higher concentrations of the hydrolysate, and selecting for those organisms that exhibit the most rapid growth. Adaptation of *P. stipitis* on hardwood acid prehydrolysate was able to achieve a 1.6-fold increase in ethanol yield and 2.1-fold productivity increase over the parent strain (Nigam, 2001).

#### 1.1.6 Concentration of Hemicellulose Oligomers

In the present study it was found that the oligosaccharide content of hemicellulose extracts was too dilute for fermentation, necessitating the addition of a concentration unit operation. Two potential operations for removal of excess water are evaporation and membrane filtration. In evaporation the vapor from a boiling liquid solution is removed, leaving a more concentrated liquid solution. The liquid feed to an evaporator is typically dilute, with low viscosity, so relatively high heat transfer coefficients can be obtained; as evaporation proceeds, the liquid becomes more viscous and heat transfer coefficients drop (Geankopolis, 2003). Circulation or turbulence is necessary to prevent the heat transfer coefficient from becoming too low. An agitated-film evaporator is one type of evaporator which was available for the present experiments of hemicellulose extract concentration. The apparatus consists of a single, large jacketed tube containing an internal agitator, where liquid enters through the top and is spread out in a turbulent film by the agitator blades (Geankopolis, 2003). The agitated thin film evaporator is well suited to highly viscous or heat sensitive materials; however it has a higher cost and smaller capacity than other types of evaporators (Geankopolis, 2003).

Membrane filtration is a less energy intensive method of concentrating the hemicellulose oligomers, with the added advantage that it does not concentrate small molecules such as salts or acids. Figure 5 shows a comparison between Normal Flow Filtration (NFF) and Tangential Flow Filtration (TFF). In NFF, pressure is applied across the membrane in the same direction the feed flows. Particles which are small enough will pass through the membrane, while those that are too large build up on the membrane, forming a filter cake which impedes flow of additional feed. In TFF, the feed is pumped

tangential to the membrane and the applied pressure, allowing only a portion of the feed to pass through the membrane into the filtrate. The retained feed is swept along so that it does not build up at the surface of the membrane to impede flow.

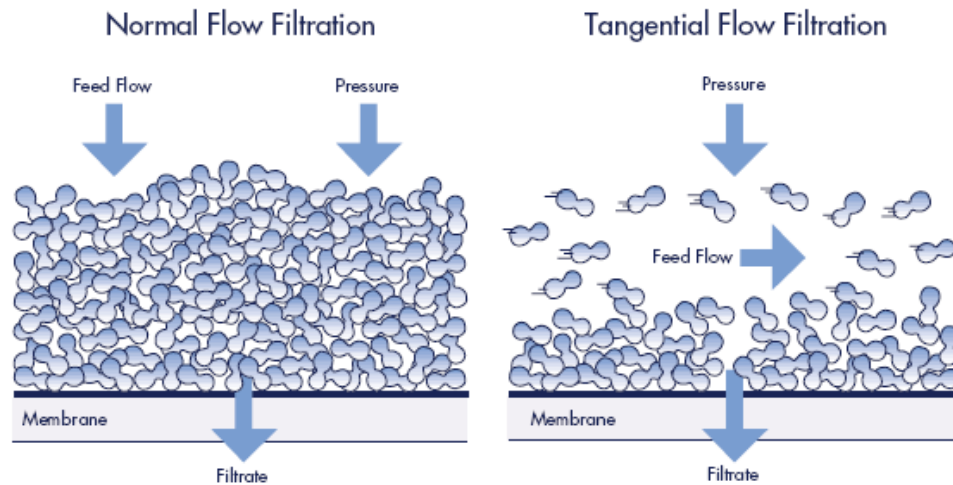


Figure 5. Comparison of flow schemes in Normal and Tangential Flow Filtration (Millipore, 2003)

A TFF system diagram is shown in Figure 6. A pump applies the pressure to drive feed through the channel between two membrane surfaces of the module. A portion of the feed is forced into the filtrate stream, while the retentate stream is recycled back into the feed. Concentration gradients develop between the bulk conditions at the center of the channel and the more concentrated membrane surfaces, and also along the length of the feed channel between inlet and outlet (Millipore, 2003).

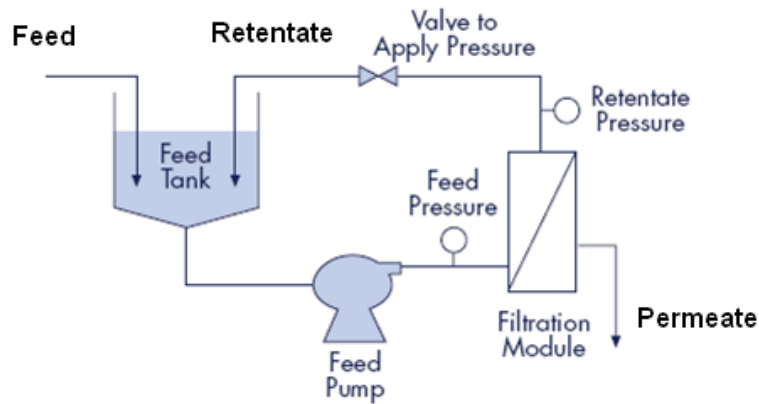


Figure 6. Diagram of Tangential Flow Filtration System (Millipore, 2003)

#### 1.1.7 Kraft Pulping

The Kraft pulping process uses a solution of sodium hydroxide and sodium sulfide, termed white liquor, to cook woodchips. Kraft white liquor has a pH greater than 13 (Smook, 2002). The lignin in the wood undergoes an alkaline hydrolysis reaction which lowers its molecular weight, creating smaller fragments which are soluble in the cooking liquor in the form of sodium salts (Smook, 2002). Spent cooking liquor containing dissolved lignin and hemicellulose is termed black liquor; it is evaporated and then burned in a recovery furnace. The resulting smelt consisting of sodium carbonate and sodium sulfide is dissolved into water to form “green liquor” and then causticized by calcium hydroxide addition to reform sodium hydroxide and calcium carbonate. In Kraft pulping terminology, total alkali refers to all sodium salts and total titratable alkali (TTA) refers to NaOH, Na<sub>2</sub>S and Na<sub>2</sub>CO<sub>3</sub>, where all chemical weights are calculated as sodium equivalents expressed as Na<sub>2</sub>O (Sjöström, 1993). The charge of “active alkali,” or NaOH and Na<sub>2</sub>S typical for hardwood Kraft pulping is 14-18% of the total dry wood (Sjöström, 1993, Ragauskas *et al.*, 2006). Hardwood Kraft pulping typically occurs at 140 - 160°C

with a digester residence time of 60-120 min. and generates pulp at yields of 47-49% on starting dry wood (Ragauskas *et al.*, 2006). Kraft pulp contains approximately 85% of the initial cellulose, 50% of the hemicellulose, and only 10% of the lignin (Gullichsen, 1999). Black liquor contains the remainder of the lignin, cellulose and hemicellulose in the form of carbohydrate degradation products such as aliphatic carboxylic acids, predominantly as hydroxyl monocarboxylic acids (Sjöström, 1993).

## 1.2 Objectives

The principal goal of this research was to determine the fermentability of hemicellulose extracted from woody biomass prior to pulping. Hemicellulose extracts produced under a variety of conditions were characterized to relate extraction severity with concentrations of saccharides, organic acids, inorganic salts and lignin degradation products. It was established that all extraction conditions led to the formation of hemicellulose extracts that were too dilute to economically undergo conventional methods of fermentation and product recovery. The concentration of saccharide components needed to be raised prior to fermentation. Evaporation and ultrafiltration were both evaluated as potential methods. Fermentation of hemicellulose extracts into ethanol by *Escherichia coli* K011 and into lactic acid by *Bacillus coagulans* MXL-9 was carried out to determine product yields which could be achieved and to identify mechanisms of fermentation inhibition.

## 1.3 Outline of This Dissertation

Chapter 2 of this dissertation explains the experimental methods employed in the ensuing research. A summary of the woodchip preparation and chemical make-up is

given. The hemicellulose extraction vessels and operating conditions are described. Methods for extract processing, including concentration, hydrolysis and neutralization, are detailed. The organism culturing techniques and fermentation recipes are included, along with procedures for chemical analysis.

In Chapter 3 the complete compositional determination of hemicellulose extracted from southern hardwood under a variety of conditions is presented. Water, green liquor and sodium carbonate are examined as extraction media at H-Factors of 400, 600, and 800 hrs. Green liquor and sodium carbonate were each tested at concentrations of 2, 4 and 6% on an oven-dry wood basis. The extracted woodchips underwent kraft pulping to determine whether yield and pulp properties were maintained at the same level as an unextracted control.

Chapter 4 describes the concentration of hemicellulose extracts by evaporation and by ultrafiltration of oligomers. Oligomer sizes vary based on the method of extraction performed, where water extraction of hardwood chips had average molecular weights ranging from 0.9 to 2.2 kD and green liquor extraction produced oligomers of 7 to 12 kD. Ultrafiltration membranes with nominal cut-off sizes of 1 kD to 50 kD were examined, as well as microfiltration of 0.22 to 0.45  $\mu\text{m}$ . Membrane performance was evaluated to identify those with high sugar retention and high permeate flux rates.

In Chapter 5 the fermentation of hemicellulose extracts into ethanol is examined, with a focus on the chemicals that will lead to inhibition of microbial growth. *Escherichia coli* K011 was utilized in the production of ethanol from hemicellulose and model glucose-xylose mixtures containing varying levels of inhibiting compounds. Acetic acid and

sodium were identified as the most significant causes of inhibition and completely prevented cell growth at three-fold evaporation levels.

Chapter 6 details the conversion of hemicellulose extracts into lactic acid by *Bacillus coagulans* MXL-9. This bacterium was isolated by the USDA-ARS from dairy manure compost for its ability to utilize pentose sugars. It was found to utilize all of the principal monosaccharides found in lignocellulosic biomass, tolerate inhibitors well, and produce lactic acid at high yield. Under anaerobic growth conditions, *Bacillus coagulans* does not produce carbon dioxide and therefore has a theoretical maximum product yield which is nearly double that of ethanol producing organisms.

Chapter 7 provides supplemental experimental data not otherwise discussed in the previous chapters. Additional commentary on the design considerations necessary to translate hemicellulose extraction into a viable industrial process is provided, as well as experimental results pertaining to processing steps that are not otherwise detailed. This includes a summary of the work on enzyme hydrolysis and SSF in comparison with acid hydrolysis. Inhibitor removal is also discussed by means of over-liming, liquid-liquid extraction and the use of hydrophobic polymeric absorbents.

This dissertation concludes with Chapter 8, which summarizes the findings of the previous chapters.

Chapters 3-6 were prepared as manuscripts for publication. A modified version of Chapter 5 was accepted by the journal of Bioresource Technology. Chapter 3 will be submitted to Industrial and Engineering Chemistry Research and Chapter 6 is being submitted to the Journal of Industrial Microbiology & Biotechnology.

## **CHAPTER 2: EXPERIMENTAL METHODS**

The following chapter details methods and materials used throughout all experimental work, including equipment descriptions and analytical procedures. Some methods such as HPLC analysis are common to all chapters, while others are more specific to a given experiment.

### **2.1 Raw Materials**

#### **2.1.1 Wood chips**

Several sources of woodchips were used for different experiments, the majority of which were conducted on mixed hardwood chips. Mixed southern hardwood chips obtained from International Paper were used in Chapter 3, while mixed northern hardwood chips obtained from the Old Town, ME pulp mill were used in Chapter 5. Extract produced from Siberian Larch at the Helsinki University of Technology appears in Chapter 6, along with both northern and southern hardwoods. Aspen strands produced at the University of Maine Advanced Engineering Wood Composites Center were utilized for some experiments in Chapter 4.

##### **2.1.1.1 Southern Hardwood Chips**

Mixed southern hardwood chips were used in the extraction and Kraft pulping experiments, consisting of sweet and black gum (35%), oak (35%), maple (15%), poplar and sycamore (12%), and southern magnolia (3%). Raw chips were obtained from a southern Kraft pulp mill following chip screening at the mill. Since the chip sample had been previously screened no additional screening was performed in the laboratory to exclude oversize chips. This procedure was followed to achieve a chip size distribution

representative of industrial Kraft processing. Since the chips were not rescreened, it would be expected that the mill chips had a broader chip size distribution than conventional laboratory screened chips. The chip size distribution in laboratory screened chips often is quite narrow and tightly controlled. As such the mill chips used in the experiments reported here in Chapter 3 would be expected to contain a higher percentage of thick oversized chips, leading to higher rejects during pulping. The chemical composition of southern hardwood chips (% by weight on dry wood basis) is: 43.6 % glucose, 15.5% xylose, 2.2 % mannose, 1% galactose, 0.5% arabinose, 25.8% Klason lignin, 3.2% acid soluble lignin, and 2.8% acetyl groups (Tunc, 2008).

#### 2.1.1.2 Northern Hardwood Chips

Mixed northern hardwood chips comprised primarily of maple (~50%) with lesser amounts of beech, birch and poplar, were obtained from Red Shield Pulp & Chemicals (Old Town, ME, USA). Woodchips were screened to include only chips which passed through 22 mm round hole screens and collected on a screen with 16 mm round holes. After screening the chips were air dried. The woodchips were composed of 42.1% glucan, 18.3% xylan and 24.2% lignin (Um and van Walsum, 2009).

#### 2.1.1.3 Aspen Strands

Aspen strands were produced at the University of Maine Advanced Engineering Wood Composites Center. Three Aspen (*Populus tremuloides*) trees were selected due to expected differences in hemicellulose type and content within and between trees (Fengel and Wegener, 1984). Aspen trees had an average of 12.4 m height and 22.8 cm diameter. Each tree was felled and bucked into logs of 1.5 m length, manually debarked, and

stranded to target dimensions of 10.2 cm in length with a thickness of 0.8 mm using a Carmanah 12/48 ring-strander. A counter knife angle of 70 degrees was used at a ring speed of 638 rpm. Fines and medium strands were removed using an Acrowood Trillium and Diamond Roll combination screen. Juvenal and mature wood were mixed and considered uniform for all treatments.

#### 2.1.1.4 Siberian Larch

Siberian Larch chips utilized at the Helsinki University of Technology were obtained from a mill in the Baikal region of Russia. Chips were screened to exclude oversize chips and fines, then stored frozen prior to experiments. (Hörhammer *et al.*, 2009)

#### 2.1.2 Green liquor and Sodium Carbonate

Green liquor was generally prepared in the laboratory from sodium carbonate, sodium sulfide and sodium hydroxide at the ratios shown in Table 1. Some experiments in Chapters 4 and 5 were performed using industrial green liquor obtained from the Old Town, ME pulp mill, which was found to have 77 g/L total titratable alkali (TTA).

Table 1. Chemical Loading for Extraction

	Water	Green Liquor				Carbonate		
	0%	2%	4%	6%	2%	4%	6%	
Wood chips, o.d. (kg)	7	7	7	7	7	7	7	
Water (kg)	21	21	21	21	21	21	21	
Na <sub>2</sub> CO <sub>3</sub> (g)	0	150	300	450	230	460	690	
Na <sub>2</sub> S (g)	0	75	150	225	0	0	0	
NaOH (g)	0	15	30	45	0	0	0	
Anthraquinone [AQ] (g)	7	7	7	7	7	7	7	

### 2.1.3 Sugars and Other Chemicals

Wherever possible, sugars and other chemicals were obtained through Acros Chemicals or Sigma at the highest purity available. D-glucose (99%), D-xylose (99%), D-mannose (99%), D-galactose (99%) and L-arabinose (99%) were used for standards and model fermentation substrates, though D-arabinose (99%) was inadvertently used in a few early fermentations before it was realized that this is not the naturally occurring stereoisomer. L-fucose (97%) was used as an internal standard in HPLC analysis.

## 2.2 Hemicellulose Extraction

### 2.2.1 Rotating Digester

Woodchips were extracted with 0% (water), 2%, 4% and 6% TTA of either sodium carbonate or green liquor in a custom-built rotating digester at the University of Maine Process Development Center. In each batch, 7 kg of wood (on an oven-dry basis) was added to the digester at a liquor to wood ratio of 4:1. The liquor to wood ratio was defined as the mass of extraction liquor ( $M_L$ ) added to the extraction vessel plus the water in the wood ( $M_w$ ) divided by the dry mass of wood, ( $M_{\text{wood}}$ )

$$r_{L/W} = \left[ \frac{M_L + M_w}{M_{\text{Dry Wood}}} \right]$$

Woodchips contained 48% moisture. All cooks also contained 0.05% anthraquinone (AQ) which has been shown to increase pulp yield and delignification (Upadhyaya and Singh, 1986). As an example of digester loading, for 2% green liquor the required chemical addition was 15 g NaOH (97% purity), 75 g Na<sub>2</sub>S (61.5%), 150 g Na<sub>2</sub>CO<sub>3</sub> (100%) and 6.75 g AQ (51.9%). The extraction was performed at a maximum

temperature of 160°C for target H-factors of 400, 600 and 800 hrs at each chemical loading. The H-factor is a kinetic model applicable to alkaline pulping that expresses cooking time and temperature as a single variable. It was developed to predict the temperature or cooking time needed to obtain a given lignin content in the pulp, when measured in terms of the Kappa number. The H-factor is given by the expression

$$H = \int_{t_o}^t e^{[43.181 - (\frac{16,113}{T(t)})]} dt$$

where time (t) is in hours and the temperature (T) is given in degrees Kelvin (Sixta, 2006). The numerical constants in the expression are related to the universal gas constant and activation energy for removal of lignin during kraft cooking. The extracted woodchips then underwent kraft pulping. In total, 21 different extraction cooking conditions were tested and compared to the un-extracted control kraft cooks. Water extraction at the 800 hr H-Factor was performed in triplicate and the standard deviations for each component are used as the basis for error bars of all other conditions presented in the figures. The rotating digester was used to produce the majority of extracts described in this work, with exceptions noted in the following sections. This includes all of the ceramic membrane ultrafiltrations and all *Bacillus coagulans* fermentations excepting the larch extracts.

### 2.2.2 Rocking Digester

Woodchips were extracted with 3% green liquor in a 20 L rocking digester at 160°C for 110 min. In each batch, 2 kg of oven-dry wood was added to the digester with 8 L of liquor. The green liquor solution contained 3.52 g NaOH, 10.3 g Na<sub>2</sub>S, and 32.64

g Na<sub>2</sub>CO<sub>3</sub> per kilogram of oven dry wood. The 8 L of green liquor solution included the 10% woodchip moisture and any impurities in the green liquor chemicals. The digester heat-up period lasted 50 min. before steady state was reached, and cool down after 110 min. at operating temperature required an additional 50 min. Reactor pressure reached 130 psig during extraction. Extracts produced in the rocking digester were used in the evaporation study, the *E. coli* fermentations of evaporated extracts, and in Millipore ultrafiltration experiments. Additionally they were used in simultaneous saccharification and fermentation testing on the evaporated extracts.

### 2.2.3 Larch Extraction

Larch extracts were received from the Helsinki University of Technology (Finland), where they were prepared by hot water extraction of Siberian larch at 160°C for 60 min. in a 2.5 L rotating autoclave at a liquor to wood ratio of 3.5 L/kg.

## 2.3 Extract Concentration

### 2.3.1 Evaporation

An agitated thin film evaporator (Artisan Industries, Waltham, MA) was used to concentrate approximately 40 liters of 3% green liquor produced from northern hardwood chips in eight batches using the rocking digester. The evaporator was operated in continuous flow with product recycling. Samples of 3 to 5 L were removed from the evaporator at roughly 40 min. intervals. The evaporator was operating at 1050 RPM agitation, 35 psi steam pressure, and a flow rate of 500 mL/min. Samples were then hydrolyzed at pH 1.0 with sulfuric acid at 130°C for 1 hr. The pH was raised to between

5 and 6 with calcium hydroxide and the solution was then filtered through glass microfiber filters to remove the resulting gypsum and precipitated lignin.

### 2.3.2 Ultrafiltration

Three different ultrafiltration systems were constructed and tested. The Millipore Pelicon 2 Mini (Millipore, Billerica, MA) cartridge system operated at the lowest feed pressure (14 psi), using polymeric membranes ranging from 5 kD to 0.22  $\mu\text{m}$  with a surface area of 0.1  $\text{m}^2$ . This system also had the lowest hold-up volume requirements, requiring approximately 100 mL to maintain flow (i.e. 1 L minimum feed to obtain 10 fold concentration). The pressure drop across the membrane was 5 psi. A peristaltic pump was used.

The GE Sepa CF II (GE Osmonics, Minnetonka, MN) high pressure system operated at 500-800 psi with a minimum operating volume of 300 mL. The GE system utilizes a flat sheet polymer filter with a surface area of 140  $\text{cm}^2$ . Membrane sizes included 1 kD, 4 kD, 5 kD, 30 kD and 0.3 $\mu\text{m}$ , as well as reverse osmosis membranes. A diaphragm pump was used which caused higher pressure fluctuations, and a pulse dampener was installed to minimize this fluctuation. The pressure drop across the membrane was more variable, ranging from 25 to 60 psi.

The Novasep Kerasep system (Novasep, Pompey, France) consists of a ceramic membrane with a surface area of 0.08  $\text{m}^2$ , constructed of monolithic  $\text{TiO}_2\text{-Al}_2\text{O}_3$  containing 19 channels. The operating pressure is 50-60 psi, with a pressure drop across the membrane of 2 psi. A centrifugal pump with a maximum flow rate of 13 gal/min was used and the minimum operating volume was 4 L. All systems were operated with continuous withdrawal of permeate and recycle of retentate back to the feed tank.

## 2.4 Extract Hydrolysis

### 2.4.1 Acid hydrolysis

Samples were hydrolyzed at pH 1.0 with sulfuric acid in an autoclave (Hirayama, Japan). Depending upon the final pH and the carbohydrate content of the extract, the hydrolysis time and temperature were varied in accordance with the data of Garrote and co-workers (Garrote *et al.*, 2001). The mildest hydrolysis conditions were used with the extracts produced using hot water extraction since the low pH conditions in the extraction experiments produced carbohydrates that had been partially hydrolyzed. The liquid extracts produced by increasing the alkali application rate using green liquor (2%, 4% and 6%) required higher severity hydrolysis conditions. The optimum hydrolysis condition for the water extracted samples was 120°C for 30 minutes, while alkaline extracts were hydrolyzed at 130°C for 30 to 60 minutes depending upon the green liquor application rate in the extraction step.

### 2.4.2 Enzyme hydrolysis

Enzyme hydrolysis was performed using commercially available xylanase obtained from Fluka and derived from *Trichoderma viride*. Additional enzymes were obtained from Novozymes biomass hydrolysis kit, including NS50012, NS50030, NS2202 and NS50014. Novozymes classifies NS50012 as a multi-enzyme complex, NS50030 and NS50014 as xylanases and NS2202 as hemicellulase. Hydrolysis and SSF were conducted in either 100 mL serum vials with a 50 mL working volume, or in 400 mL Dasgip fermentation vessels having a working volume of 250 mL. Agitation was maintained at 150 RPM. NS50012 contains a wide range of carbohydrases including

arabinase,  $\beta$ -glucanase, cellulase, hemicellulase, pectinase and xylanase. It can break down cell walls and has the ability to liberate bound materials as well as degrading a variety of non-starch polysaccharides. The enzyme activity provided by Novozymes is 100 Fungal  $\beta$ -glucanase units (FBG)/g or approximately 13,700 polygalacturonase units (PGU)/g. NS50030 is a purified endo-xylanase with high specificity toward soluble pentosans which can liberate pentose sugars from hemicellulose fractions. Its activity is given as 500 Farvet Xylan units (FVU)/g. NS2202 contains a mixture of  $\beta$ -glucanase and xylanase enzyme activities, as well as some side cellulase, hemicellulase, and pentosanase activities. It has an activity of 45 FBG/g. NS50014 is an endo-xylanase with activity towards xylans and arabino-xylans. Enzyme optimum conditions fall between pH 4.5-6.0 for NS50012 and NS50030 and pH 5.0-6.5 for NS2202. Temperature ranges for these enzymes are given as 25-55°C for NS50012, 35-55°C for NS50030 and 40-60°C for NS2202. To avoid confusion between the varying enzymatic activity units of measurement, enzyme loadings were recorded as volume added per volume of extract.

## 2.5 Neutralization and Overliming

After hydrolysis, the solutions were filtered through a glass microfiber filter to remove acid-insoluble Klason lignin. The solution pH was then raised to neutral conditions by careful addition of solid calcium hydroxide and filtered through glass microfiber filters to remove the precipitated gypsum. The neutralized extracts were then refrigerated for future use in fermentation experiments. Alternatively for a few experiments where overliming was examined, the pH was raised to 10-11 with calcium hydroxide, then lowered to neutral with 3N HCl. Calcium carbonate and 3N sodium

hydroxide were each tested for pH adjustment, but were not used because of foaming in the case of calcium carbonate and dilution in the case of sodium hydroxide.

## 2.6 Inhibitor Removal

### 2.6.1 Hydrophobic Absorption

Amberlite XAD-4 hydrophobic polymeric absorbent resin was used to pack a Bio-Rad Poly-Prep column. Columns are made of polypropylene, measuring 9 cm in height with a 10 mL reservoir and 2 mL bed volume above a porous 30  $\mu\text{m}$  polyethylene bed support which retains fine particles.

### 2.6.2 Liquid-Liquid Extraction

Liquid-liquid extraction using trioctylphosphine oxide (TOPO) dissolved in undecane as an organic phase will strip acetic acid from hemicellulose extracts. The aqueous hemicellulose phase is mixed with the organic phase (TOPO in undecane) at a ratio of 1:1 v/v. The solution is then heated to 70° C and shaken intermittently for 7 seconds at a time. Next, the solution is centrifuged at a speed of 9000 rpm for 20 minutes to separate the two immiscible phases. The amount of acetic acid in the aqueous phase is determined by HPLC while the organic phase is sent to a distillation system to recover the acetic acid. The concentration of acetic acid in the distillation top recycle is determined by titration using 0.05 M NaOH with phenolphthalein as an indicator.

## 2.7 Kraft Pulping

Control pulping using raw chips was done in the digesters described above at 16% effective alkali (EA) to a target H-factor of 1350 hours. Extracted woodchips were

pulped after draining the extraction liquor and adding cooking liquor such that the total combined H-factor of the extraction step and Kraft pulping step was maintained at 1350 hours. Therefore, the H-factor targets in the Kraft pulping were 950, 750 and 550 hrs and corresponded to H-factors during the extraction step with 400, 600 and 800 hr H-factors, respectively. The target Kappa number for all pulps was 17, which is equivalent to a total lignin content of about 2.55% in the brownstock pulp. Since it is extremely difficult to reach the target Kappa number without performing multiple experiments, pulps with experimental values ranging between 15 and 19 were deemed to have reached the target lignin content. EA was the only parameter which was varied to control the Kappa number of the pulp if the initial Kraft cook did not meet the Kappa target.

## 2.8 Fermentation

### 2.8.1 *Escherichia coli* K011

Small scale fermentations with *Escherichia coli* K011 were performed in 125 mL serum vials at 37 °C and 200 RPM in an incubated shaker (Innova40, New Brunswick Scientific, Edison, NJ). Larger scale controlled fermentations were performed in 3 L BioFlow110 bio-reactors at the same temperature and pressure (New Brunswick Scientific, Edison, NJ). An ARS 400 automated reactor sampling system (Groton BioSystems, Boxborough, MA) was used to provide continuous sampling. Bacterial growth media solution contained 25 g/L LB medium. The antibiotic chloramphenicol was added at 40 mg/L to select for only the *E. coli* K011 strain. Thiamine was added at 1 mg/L and a trace metals solution at 5 mL/L, consisting of per liter: 5 g disodium EDTA, 0.22 mg zinc sulfate heptahydrate, 0.5 g calcium chloride, 0.5 g ferrous sulfate, 0.1 g ammonium molybdate tetrahydrate, 0.16 g cupric chloride, 0.16 g cobalt chloride. The

inoculum represented 5% of the working volume. Model inhibitor solutions contained mixtures of glucose and xylose at varying concentrations. Sodium acetate, sodium sulfate, sodium chloride and sodium carbonate were tested individually over a range of concentrations to determine baseline inhibition. Mixtures of sodium acetate and sodium sulfate were tested for synergistic inhibition, for comparison to hemicellulose extracts of 3.7 wt%, 5.4 wt% and 9.8 wt%. Small scale fermentations were performed in duplicate or triplicate, while 3 L bio-reactors were operated singly but simultaneously with the same inoculum for different substrates being tested.

#### 2.8.2 *Bacillus coagulans* MXL-9

*Bacillus coagulans* MXL-9 was provided by the USDA ARS National Center for Agricultural Utilization Research (Peoria, IL), and stock cultures maintained on media containing 10 g/L tryptone, 5 g/L yeast extract, 2 g/L  $K_2HPO_4$  and 1.5% agar (if applicable). Fermentation was performed in 400 mL DASGIP bio-reactors with a working volume of 250 mL (DASGIP BioTools, Shrewsbury, MA). The pH was maintained at 6.5 by automatic addition of 2N KOH. Vessels were sparged with nitrogen prior to inoculation and maintained negative redox values, indicating anaerobic growth. Temperature was maintained at 50°C and agitation at 250 RPM by magnetic stirring. Vessels containing growth media and hemicellulose extract (if applicable) were autoclaved at 121°C for 20 min. to sterilize prior to aseptic additions. Minimal salts solution containing 26.1 g/L  $K_2HPO_4$ , 11.3 g/L  $KH_2PO_4$ , and 25 g/L  $NH_4NO_3$  was added aseptically to growth media at 20 mL/L. After autoclaving, 1 mL/L of the following sterile stocks were added: 1.05 M Nitrilotriacetic acid, 0.59 M  $MgSO_4 \cdot 7H_2O$ , 0.91 M  $CaCl_2 \cdot 2H_2O$ , and 0.04 M  $FeSO_4 \cdot 7H_2O$ . For fermentation of pure xylose, a 100 g/L

solution was autoclaved separately from growth media. Inoculating cultures were grown on media containing 20 g/L xylose in all experiments. For inhibition experiments, acetic acid was added in the form of ammonium acetate and sodium was added in the form of sodium sulfate. The inoculum represented 5% of the working volume.

### 2.8.3 Additional Fermentation Organisms

*Pichia stipitis* CBS 6054 was obtained from Dr. Thomas Jeffries of the Forest Products Laboratory and its growth media contained 5 g/L YNB, 6.56 g/L peptone, 2.25 g/L urea (added aseptically) and, if applicable, 15 g/L agar and 10 g/L xylose. *Pichia stipitis* was grown in 100 mL Erlenmyer flasks with foam stoppers, or in serum vials with a working volume of 50 mL on a shaking incubator at 37°C and 160 RPM.

*Clostridium phytofermentans* is an obligate anaerobe obtained from the laboratory of Dr. Sue Leschine at the University of Massachusetts, Amherst. GS-2 growth media was used, containing 1.5 g/L  $\text{KH}_2\text{PO}_4$ , 2.9 g/L  $\text{K}_2\text{HPO}_4$ , 2.1 g/L urea, 2 g/L cysteine·HCl, 10 g/L MOPS, 3 g/L sodium citrate·2H<sub>2</sub>O, 6 g/L yeast extract, 1 mL and 0.1% Resazurin. The pH was adjusted to 7 with KOH. After autoclaving, a salt solution is added as 10% of the final volume and contains 10 g/L  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ , 1.5 g/L  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , and 0.0125 g/L  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ .

## 2.9 Chemical Analyses

### 2.9.1 High Performance Liquid Chromatography

Ethanol, lactic acid, formic acid, acetic acid, and furans were analyzed by high performance liquid chromatography (HPLC) equipped with refractive index and UV detection (Shimadzu, Columbia, MD), using the Aminex HPX-87H (H) column (Bio-

Rad, Hercules, CA). The column was operated with a 5 mM sulfuric acid mobile phase at a flow rate of 0.6 mL/min and oven temperature of 60 °C. Samples were filtered through 0.22 µm syringe filters or centrifuged for 10 min. at 14,500 RPM prior to injection. Sugars were measured using an Aminex HPX-87P (P) column with a water mobile phase at a flow rate of 0.6 mL/min and oven temperature of 80°C to separate glucose, xylose, mannose, galactose and arabinose. Internal standards of fucose were used for the H-column and erythritol for the P-column. The H-column can additionally provide analysis of glucose, arabinose and the sum of xylose, mannose and galactose, which co-elute on that column at the approximate distribution of 85% xylose, 12% galactose, and 3% mannose for hardwood hemicellulose extracts.

#### 2.9.2 Lignin determination

Klason lignin content was determined by filtration and gravimetric measurement of the acid hydrolyzed samples, and acid soluble lignin was measured by absorbance at 205 nm using a Thermo-Electron Corp. spectrophotometer following Tappi Useful Method 250.

#### 2.9.3 Other Chemical Analyses

Total organic carbon was measured using a Shimadzu analyzer. Sodium analysis was performed by the University of Maine Soil Testing Laboratory using ICP-AES, method EPA 200.7.

#### 2.9.4 Gel Permeation Chromatography

Molar mass distributions (MMDs) were obtained using a size exclusion chromatography (SEC) system with UV and IV-DP (intrinsic viscosity-differential

pressure) detection, the former detector being set at 295 nm. The solvent system was 0.8% lithium chloride in dimethylacetamide (LiCl/DMAc). Complete dissolution of the freeze dried samples in the solvent system was achieved, making it possible to obtain quantitative solution state analysis of the polymers present. Calibration standards used were pullullan standards (polydextrans) in the molar mass range of 342-22,800 Dalton. The method applied to obtain the MMDs was the universal calibration method, which utilizes the Mark Houwink constants of the polysaccharides in specific solvent systems. The Mark Houwink equation relates intrinsic viscosity,  $\eta$ , to molecular weight,  $M$ :

$$[\eta] = K \cdot M^a$$

The Mark Houwink constants for cellulose in this particular system have been reported in literature and were applied in this study, where  $K$  was 0.00278 and the exponential,  $a$ , was 0.96.

## CHAPTER 3: EXTRACTION OF HEMICELLULOSE PRIOR TO PULPING

### 3.1 Introduction

#### 3.1.1 Why a Biorefinery

Woody biomass is an abundant and renewable material with potential to replace the feed-stocks of many fuels and chemicals currently made from fossil fuels (Perlack *et al.* 2005). The concept of an integrated forest products bio-refinery draws on the technology and infrastructure already present in the pulp and paper industry to generate new products in addition to the existing core business. A bio-refinery would utilize all components of the biomass to make a range of products including fuels, chemicals, heat, and power in proportions that maximize economic returns (Ragauskas *et al.*, 2006).

During the kraft pulping process cellulose fibers remain nearly undegraded, while the majority of the hemicellulose and lignin components are dissolved into the waste pulping liquor to be burned in the chemical recovery process to generate energy (Sjöström, 1993). Cellulose presently has a higher commercial value by manufacture into pulp and paper products than can be derived by hydrolyzing it into glucose for conversion to liquid bio-fuels (van Heiningen, 2006). The combustion of lignin, which has a high heating value, provides the bulk of the energy derived from the recovery furnace (Ragauskas *et al.*, 2006). Hemicellulose has less than half the heating value of lignin and is not currently utilized to its maximum potential (Tunc and van Heiningen, 2008). Many micro-organisms are capable of fermenting the sugars of hemicellulose into products which could achieve higher economic returns than the current combustion process.

### 3.1.2 Undesirable Side Reactions

During Kraft pulping, the hemicelluloses are degraded into low value isosaccharinic acids, but extracting the hemicellulose under milder alkaline conditions prior to pulping would remove the hemicelluloses in higher value oligomeric form (Ragauskas *et al.*, 2006). The most important reactions responsible for degradation of polysaccharides and reduction in chain length are the alkaline peeling and alkaline hydrolysis reactions (Fengel and Wegener, 1984, Smook, 2002). Alkaline peeling begins by an enolization of the end carbonyl group, followed by isomerization to a  $\beta$ -alkoxy-carbonyl which undergoes rapid decomposition, splitting off the end unit and forming a new aldehyde end unit, which in turn can be split again (Rydholm, 1964). Peeling continues until a stopping reaction occurs, usually 50-65 monomers from each starting point, when an alkali-stable conformation is reached (Rydholm, 1964). Possible end products of peeling include gluco-isosaccharinic acid in the case of cellulose or mannan, xylo-isosaccharinic acid in the case of xylan, lactic acid, formic acid, 2-hydroxy-butanoic acid and 2,5-dihydropentanoic acid (Fengel and Wegener, 1984). Hemicelluloses are peeled to a greater extent than cellulose, and glucomannans are peeled more readily than xylans. Molecules which are split off during peeling may be further decomposed through hydrolysis and intramolecular rearrangements to hydroxy acids. Alkaline hydrolysis is responsible for cleaving acetyl groups from the hemicellulose chain (Rydholm, 1964). These reactions which occur during pulping will also impact the alkaline extraction process.

### 3.1.3 Use of Anthraquinone

Anthraquinone is used as a pulping catalyst and stabilizes polysaccharides against alkaline peeling by causing oxidation of aldehyde end groups on the carbohydrates to stable aldonic acids (Fengel and Wegener, 1984). A well known redox mechanism has been proposed where anthraquinone is reduced to anthrahydroquinone by reaction with the aldehyde end groups on the carbohydrates and then reoxidized by reaction with lignin thus accelerating the delignification reactions (Kocurek, 1983)

### 3.1.4 Advantages and Disadvantages of Hemicellulose Extraction

Hemicellulose extraction prior to kraft pulping is anticipated to improve pulp mill operations by reducing kraft cooking times, enhancing cooking liquor penetration into chips, yielding improved pulp properties and increasing production capacity for pulp mills which are limited by the recovery-furnace throughput (van Heiningen, 2006). The recovery boiler and lime kiln may be off-loaded by about 20%, allowing higher throughput in mills where these operations are the bottleneck (Mao *et al.*, 2008). A disadvantage of the near neutral hemicellulose process is that less steam is produced in the recovery boiler because some of the energy content in the wood is contained in the by-products and steam is required in the processing. In addition, considerable sulfuric acid is required for the hydrolysis step in the process and lime is required for neutralization of the extract. This gives rise to gypsum which must be removed and most likely results in a waste disposal problem.

### 3.1.5 Manufacture of Dissolving Pulps

The practice of pre-extracting hemicellulose is already employed in the manufacture of dissolving grades of pulps, which produce high purity cellulose for products such as rayon, cellophane and cellulose acetate (Ragauskas *et al.*, 2006). This modified kraft pulping method relies on water pre-extraction, alternatively termed autohydrolysis, which releases acetyl groups from the hemicellulose in the form of acetic acid. The acetic acid lowers the pH to between 3-4, and subsequently results in solubilization of hemicelluloses. Water pre-extraction is suitable to dissolving pulp production, but the carbohydrate degradation during autohydrolysis leads to a large loss in pulp yield and pulp strength properties when followed by conventional kraft cooking to produce papermaking pulps.

### 3.1.6 Near Neutral Hemicellulose Extraction Process

Alternative pre-extraction methods using alkaline chemicals to maintain the pH of the final extraction liquor have been investigated (Mao *et al.* 2008, Al-dajani and Tschirner, 2008). A schematic diagram of the proposed near-neutral hemicellulose extraction process using green liquor as the solvent is shown in Figure 7. In the process, extracted woodchips serve as the feed to the conventional Kraft pulp mill, while the extracted liquor undergoes concentration by evaporation or ultra-filtration, hydrolysis using sulfuric acid, separation of lignin, acetic acid recovery, neutralization of acids using lime, separation of precipitated calcium sulfate, fermentation into ethanol or other bio-fuels such as butanol, and lastly alcohol recovery by distillation and product purification. In the scheme shown in Figure 7, acetic acid is also recovered as a valuable co-product using liquid/liquid extraction followed by stripping and distillation.

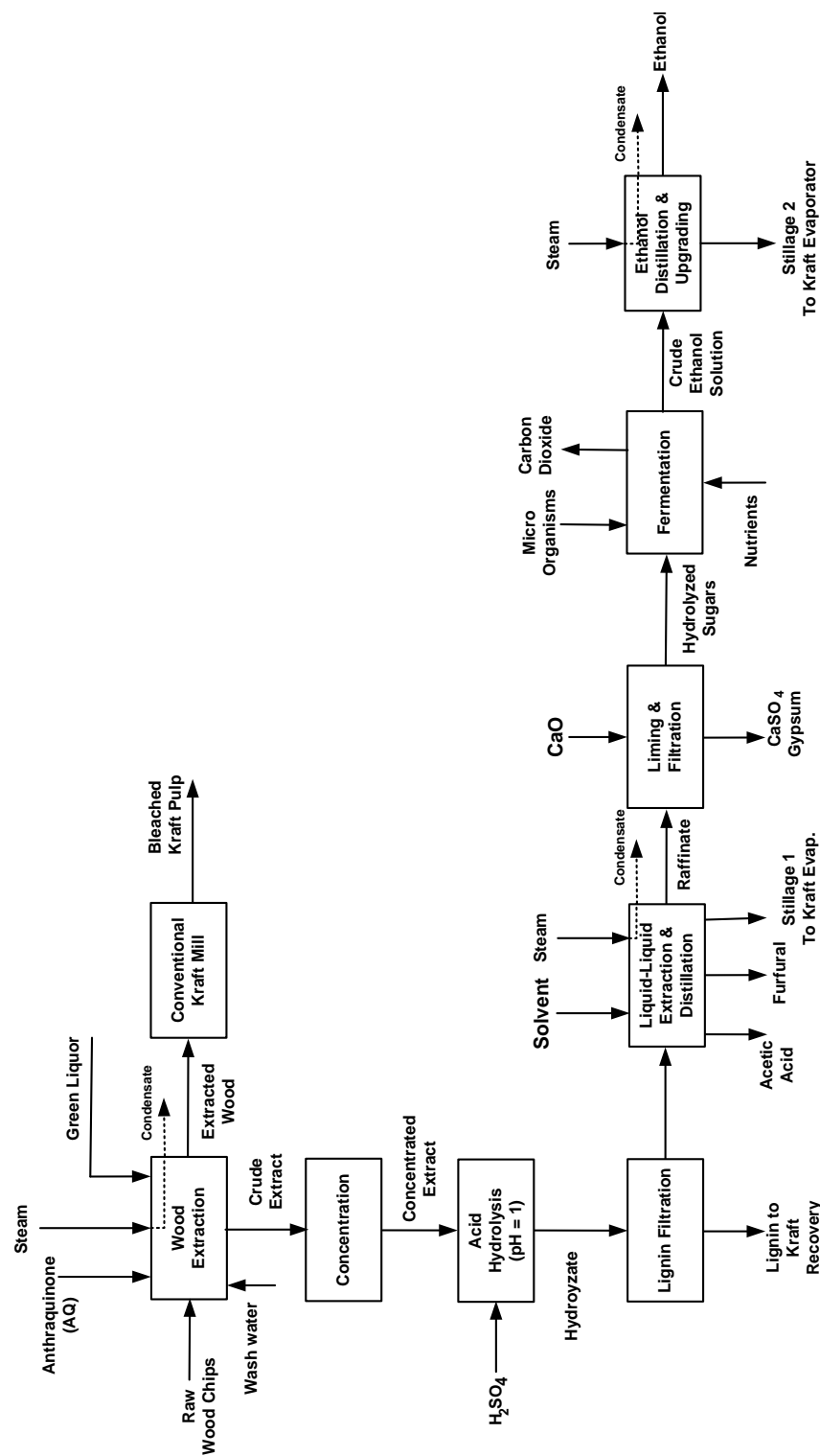


Figure 7. Schematic of near neutral hemicellulose extraction process

### 3.1.7 Objectives and Scope

The objective of this work was to assess the composition of hemicellulose extracts made under a variety of conditions and determine which were best suited to the bio-refinery operation. The hemicellulose extraction conditions tested in this work, 0-6% TTA green liquor, dissolves a portion of the hemicellulose and lignin in a manner similar to black liquor, but maintains the pH at near-neutral conditions so that the degradation of the oligosaccharides and monomeric sugars into hydroxyacids is minimized. This solution was hydrolyzed using sulfuric acid and fermented to determine its effect on micro-organisms which produce ethanol and other chemicals. The extracted woodchips then underwent Kraft cooking to determine if the resulting pulp yield was greater than or equal to a control pulp produced using raw chips. The optimum process will generate an aqueous hemicellulose extract solution containing the highest possible concentration of oligosaccharides while preserving or increasing the pulp yield.

## 3.2 Results and Discussion

### 3.2.1 Hemicellulose Extract Properties

The hemicellulose pre-extraction experiments resulted in liquid solutions containing dilute oligosaccharides, acetic acid liberated from the hemicellulose polymers, lignin, a lesser amount of monomeric sugars, organic acid degradation products, and residual salts from the alkaline chemicals used. The proportions of the various components is determined by the alkalinity (% TTA), and cooking severity (H-factor) in the extraction stage of the process. The two different alkaline chemicals tested – green liquor and sodium carbonate – did not result in significantly different extract solutions or

properties of the resulting pulp. The ensuing figures give representative trends only for green liquor. All extractions were performed at a liquor-to-wood ratio of 4 L/kg?

Figure 8 shows the pH of the extracted liquor as a function of alkalinity and H-factor for the green liquor extraction experiments. For water extractions at 0% TTA the pH of the final extraction liquor drops to 3.5. Addition of 2% green liquor or carbonate at 400 hr H-factor maintained the extract liquor pH near 5.7, but higher H-factors decrease the pH further, indicating more complete consumption of the added alkaline chemicals followed by an increase in acetic acid release. Addition of 4% green liquor or carbonate for H-factors of 400 and 600 hrs maintained the pH slightly above 7 in the final extract liquor, but at the highest H-factor of 800 hrs the pH fell below neutral.

All extracts prepared with 6% green liquor or pure sodium carbonate maintained the pH above neutral conditions, indicating incomplete consumption of the alkali. During subsequent acid hydrolysis any extract with unconsumed alkali, as indicated by a pH above 7, released CO<sub>2</sub> gas upon addition of sulfuric acid, causing the liquid to foam. For processing, the formation of foam required a vessel to have at least 50% headspace to prevent overflow. Foam formation was particularly high in the 6% alkali extracts. For the process to be run efficiently, the residual alkali must be such that excessive foaming does not occur.

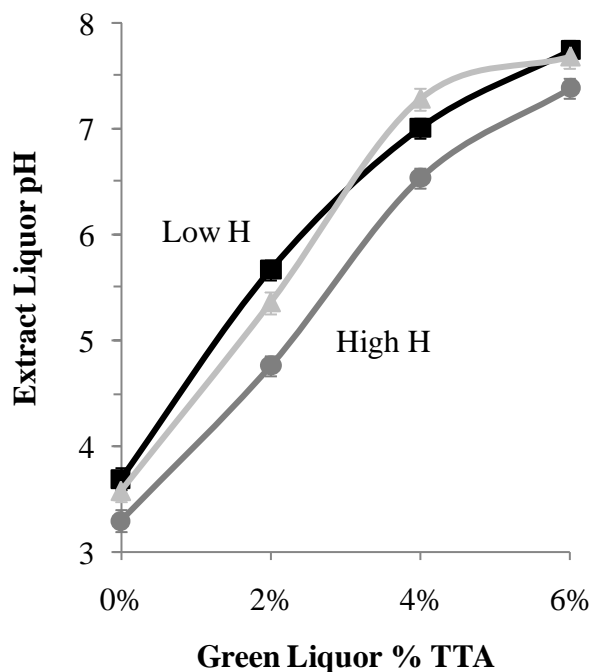


Figure 8. Hemicellulose extract liquor final pH as a function of alkaline charge and H-factor. 400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●). Data shown for green liquor closely matches that of carbonate.

The conductivity of the extract was measured and increased with alkalinity, ranging from 1 mS/cm in water extracts to 18 mS/cm in 6% carbonate extracts or 14 mS/cm in 6% green liquor extracts. Total solids in the extract were lowest in 2% alkaline extracts at low H-factor (1.9% solids). Solids increased with increasing H-factor as more lignocellulosic material was dissolved and went into solution. Water extracts ranged from 2.2% solids at low H-factor to 3.9% solids at high H-factor. The highest solids levels (5.3%) were present in 6% alkaline extracts at high H-factor, though an increasing portion of the solids are inorganic in nature when increasing charges of green liquor are used.

### 3.2.2 Sugar Content of Hemicellulose Extract

The concentration of dissolved component sugars that are present in the extraction liquor following hydrolysis decreases with increasing green liquor addition. This is illustrated in Figure 9 for glucose and Figure 10 for xylose. Glucose is released at low levels for all extraction conditions, ranging from 0.2 to 0.6 g/L in alkaline extractions, and 1.1 to 1.9 g/L in water extractions depending upon the extraction severity. In hardwoods, glucose is a constituent of glucomannan which makes up less than 5% of the total wood and likely contributes a small amount of the extracted glucose (Gullichsen, 1999). Additional glucose would first come from the amorphous low molecular weight cellulose, which is easily solubilized and would have gone into solution as well in the black liquor during pulping. In hardwoods such as birch, cellulose makes up 40% of the initial wood weight but only 34% based on the original mass of the wood is found in the Kraft pulp. Therefore 6% of the mass of the cellulose based on original wood is dissolved during pulping (Gullichsen, 1999). Any glucose present in the hydrolyzed extract originating from autohydrolysis of crystalline or higher molecular weight cellulose would be detrimental to the pulp yield. Figure 9 indicates that addition of alkali protects the cellulose fibers, while water extraction begins to impact them negatively as far as yield loss in the pulp. The glucose degradation product 5-hydroxymethylfurfural (HMF) is not generated in measurable quantities for any alkaline extraction, and the greatest amount measured in a water extraction was only 0.09 g/L.

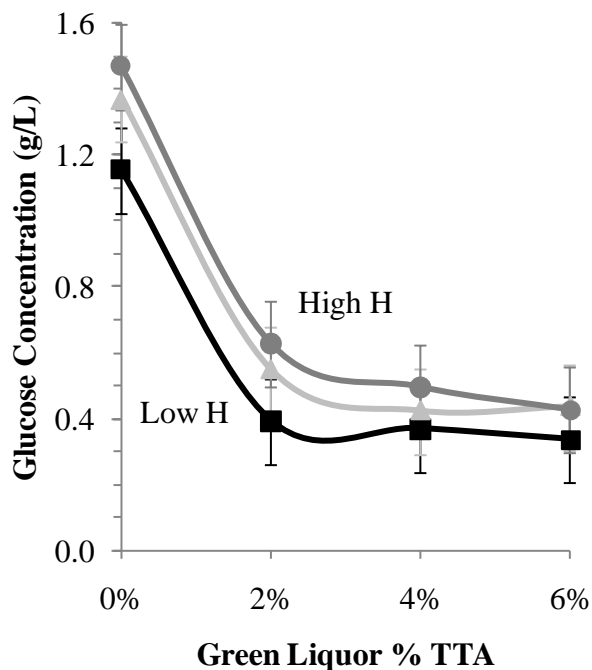


Figure 9. Glucose concentration in hemicellulose extraction liquor as a function of green liquor charge and H-factor.

400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●)

Arabinose is a minor constituent of hardwood hemicellulose which is dissolved into the extract solution. Arabinose removal was found to have a maximum of 1.3 g/L which was achieved in water extracts at all H-factors tested. It was inferred from these results that all of the available arabinose most likely had been removed at the lowest H-factor. Alkali extractions produced even lower concentrations of arabinose in the hydrolyzed extract because arabinose is easily degraded by alkaline peeling reactions.

The principal monosaccharide in the hydrolyzed extracts from hardwood was xylose. The xylose concentration, seen in Figure 10, was significantly higher in water extracts than with any amount of alkali. In water extracts, xylose concentration showed a strong correlation to H-factor, but the alkaline extracts did not show as strong a correlation. Liquid extracts produced using 2% carbonate or 2% green liquor at higher

H-factors begin to exhibit higher xylose concentrations, where there is a corresponding drop in pH. The greatest concentration of xylose, 25 g/L, was generated in water extracts at 800 hrs H-Factor which did not have AQ added (Figure 4). AQ addition to water extractions lowered the xylose concentration by 2-3 g/L compared to the same H-Factor extraction experiment performed without AQ. The highest xylose concentration reached in any of the alkaline extractions was 8 g/L at 2% TTA and occurred at an H-Factor of 800 hrs. The xylose degradation product furfural was not generated in significant quantities for any hemicellulose extracts. Before acid hydrolysis, only water extracts contained measurable quantities of furfural generated during the extraction itself – up to 0.9 g/L at the highest H-factor. Furfural levels in the hydrolyzed extract can be minimalized if the hydrolysis severity is tailored to the individual feedstock. Alkaline extracts did not generate more than 0.2 g/L of furfural in any case tested at the optimum acid hydrolysis condition.

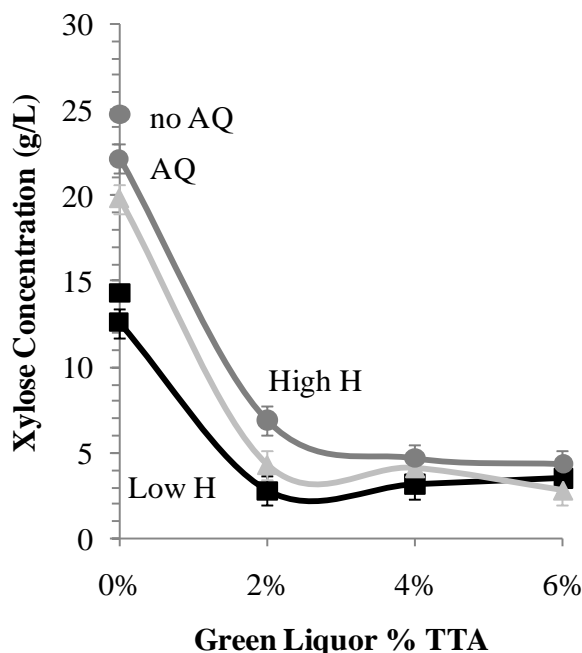


Figure 10. Xylose (XMG) concentration in hydrolyzed extraction liquor as a function of green liquor charge and H-factor.  
400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●)

### 3.2.3 Accumulation Trends of Other Components in Hemicellulose Extract

When evaluating optimum extraction conditions there are several important considerations. Impact on pulp yield is critical, but effect on downstream fermentation operations is also of great importance, as many by-products of the extraction process have inhibitory effects on growth of microorganisms (Palmqvist and Hahn-Hagerdal, 2000). Compounds with the potential to accumulate to inhibitory concentrations include sodium, furfural, lignin, formic acid and acetic acid. The alkali charge determines how much sodium will remain in the final liquor. The measured concentration of sodium in the extract liquor varied from 3 g/L to 9 g/L depending upon the TTA application rate; 2-6% TTA based on wood. To minimize sodium inhibition the extraction should be done with the minimal amount of alkali needed to protect the pulp quality.

#### 3.2.3.1 Acetic Acid

Acetic acid is a principal by-product of the hemicellulose extraction process. Acetyl groups are liberated from the hemicellulose in the form of acetate when the pH is above the pKa of acetic acid (4.8). At lower pH the acetyl groups lead to the formation of acetic acid. In the extract, the amount of acetyl groups removed from the hemicelluloses and the split between the acetate and the acid form will depend upon pH of the extraction liquor. Figure 11 shows the concentration of acetic acid in hydrolyzed green liquor extracts as a function of alkaline charge and H-factor. Acetic acid content increases with both increasing H-factor and increasing alkalinity and reaches a maximum of 10.5 g/L at the highest alkaline charge and H-factor (Figure 11). However, once the maximum is reached no further increase in acetyl groups is observed and this suggests that no additional acetyl groups are removed from hemicellulose polymers in the secondary wall of the fiber. In alkaline extracts, the acetyl groups are cleaved entirely from the hemicellulose chain during the extraction process and subsequent acid hydrolysis does not result in an increase in acetic acid. However, in water extracts more than half of the acetyl groups remain bound to the hemicellulose prior to acid hydrolysis. Concentration of acetic acid in low H-factor water extracts increases from 1.3 g/L before hydrolysis to 3.5 g/L after hydrolysis, 2 g/L to 5 g/L at medium H-factor and 3 g/L to 6 g/L at the highest H-factor. Acetic acid is both a valuable co-product and a potential inhibitor to downstream fermentation. Acetic acid can be removed by liquid-liquid extraction before fermentation, as described in Figure 7 and purified by distillation and sold as a by-product.

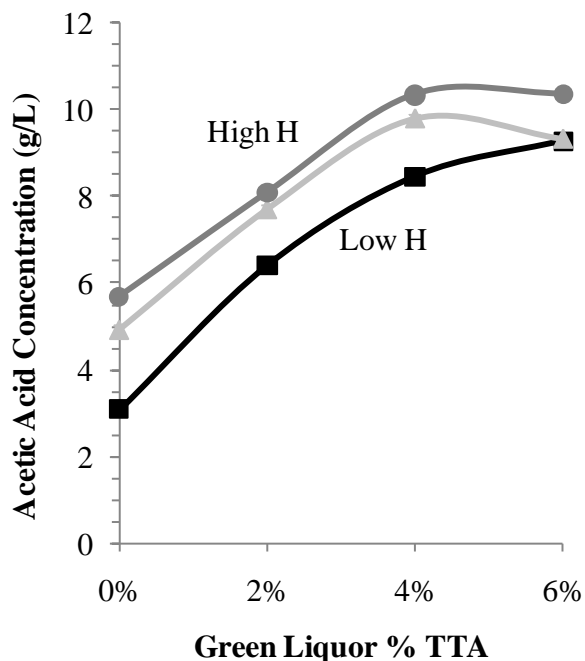


Figure 11. Acetic acid concentration in hydrolyzed extraction liquor as a function of green liquor charge and H-factor.  
 400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●)

The total acetyl group content in the original mixed southern hardwoods was measured to be 2.8% on wood, which corresponds to 4% as acetic acid. At the 4:1 liquor to wood ratio, 10 g/L acetic acid in the extract also corresponds to 4% acetic acid on wood, and indicates that all acetyl groups were removed from wood at the highest severity green liquor extractions. At the lowest severity of water extraction only 30% of acetyl groups were removed from wood.

### 3.2.3.2 Lactic Acid and Formic Acid

The alkaline peeling reactions lead to formation of lactic acid and formic acid through degradation of the extracted carbohydrates. The concentration of lactic acid in the hydrolyzed extracts is shown in Figure 12. Lactic acid is not formed at all during

water extractions, indicating it is only formed through alkaline degradation. Formic acid, seen in Figure 13, is formed during water extraction to a small extent, indicating that acid hydrolysis caused by the cleaved acetyl groups may also be a formation mechanism. Both the lactic and formic acid concentrations increase steadily with increasing alkalinity and also to a lesser extent with increasing H-Factor. The maximum lactic acid concentration was 2 g/L and the maximum formic acid concentration was 3.6 g/L; both were reached at 6% TTA green liquor and an H-Factor of 800 hrs.

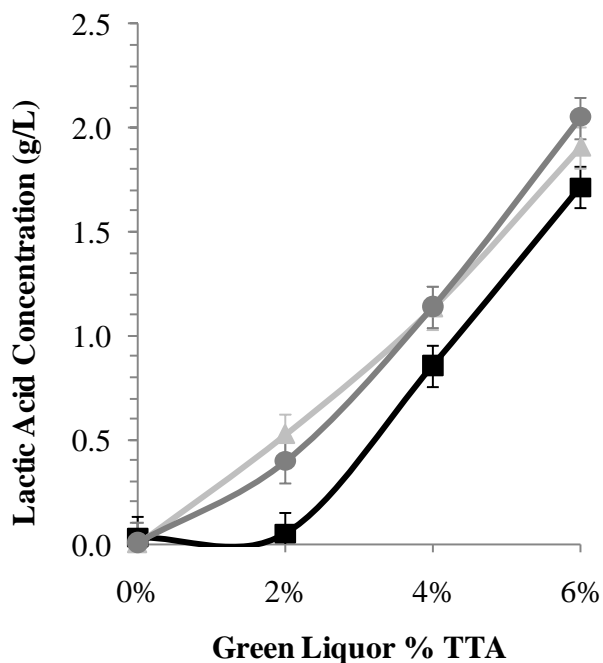


Figure 12. Lactic acid concentration in hydrolyzed extraction liquor as a function of green liquor charge and H-Factor.  
400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●)

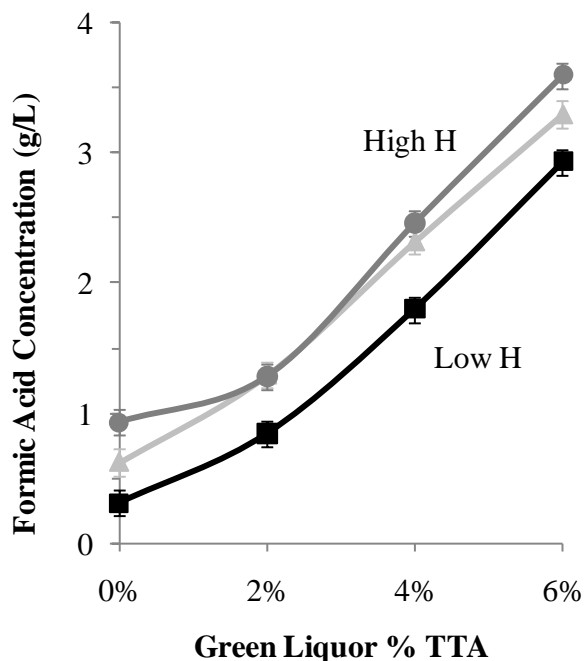


Figure 13. Formic acid concentration in hydrolyzed extraction liquor as a function of green liquor charge and H-Factor.

400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●)

### 3.2.3.3 Lignin Degradation Products

Lignin degraded phenolics are potential inhibitors to the downstream fermentation operation. Insoluble lignin can be removed by filtration prior to fermentation, but acid soluble lignin remains. Acid soluble lignin concentrations were slightly higher in green liquor extracts than carbonate extracts because sulfide aids in delignification. The highest concentration of acid soluble lignin, 9 g/L, was achieved at an H-factor of 800 hrs and 6% green liquor, while 6% carbonate at the same severity only produced 6 g/L of acid soluble lignin. Figure 14 shows the concentrations of soluble lignin in green liquor extractions following hydrolysis. All of the curves shown in Figure 14 resulted from experiments in which 0.05% anthraquinone (AQ) was added to the extraction step to aid in pulping the partially macerated chips following extraction. The water extraction

experiments included in Figure 14 were also conducted with AQ for consistency. Water extraction experiments performed without AQ had much lower concentrations of acid soluble lignin, which are indicated with dotted lines. AQ is added during pulping to increase delignification, so this result was anticipated. Water extracts without AQ contained approximately 3 g/L acid soluble lignin, compared to 7 g/L in water extracts that did contain 0.05% AQ. Acid soluble lignin can be removed by ion exchange methods if it would inhibit fermentation but would of course add to the complexity and capital and operating cost of the process.

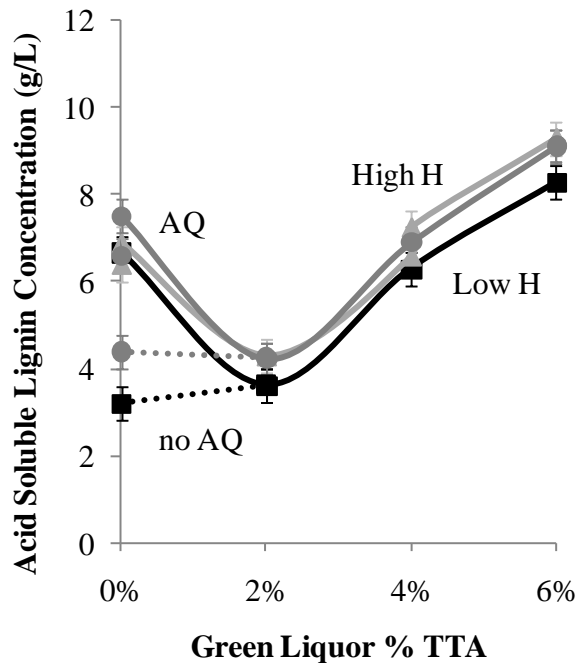


Figure 14. Acid soluble lignin concentration in hydrolyzed extraction liquor as a function of green liquor charge and H-factor.

400 hr H-Factor (■); 600 hr H-Factor (▲); 800 hr H-Factor (●)

### 3.2.4 Kraft Pulping of Hemicellulose Extracted Woodchips

Typical wood composition for birch wood before and after Kraft pulping is illustrated in Table 2. Approximately half of the xylan and mannan polymers are lost during normal Kraft pulping. It is the objective here to recover these polymers as component sugars while maintaining the composition of the pulp similar to that obtained during conventional Kraft pulping with equal pulp yield and physical properties.

Table 2. Typical wood chemical distribution for hardwood (birch) before and after Kraft pulping (Gullichsen, 1999).

Component	Wood (as a % of original wood)	Kraft Pulp
Cellulose	40-41	34
Xylan	25-30	16
Glucomannan	2-5	1
Other carbohydrates	0-4	0
Lignin	20-22	1.5-3
Extraneous compounds	2-4	<0.5

Kraft pulping of extracted wood chips is affected by many variables. Consequently it is difficult to compare pulp qualities and yields on a uniform basis for the combined processes of pre-extraction and Kraft cooking. The H-factor and white liquor charge of Kraft pulping for extracted woodchips were adjusted to correspond to the pre-extraction H-factor and alkali charge so that the combined operations would represent uniform cooking severity. For an accurate direct comparison of pulp yields between varying pre-extraction conditions, all pulps should have had a uniform Kappa number of 17. The final Kappa number of the unbleached pulp varied between 15 and 19 because it proved difficult to hit the target kappa number 17 exactly.

### 3.2.5 Effective Pulp Yield

Rejects in a Kraft pulp mill are often re-pulped to recover additional wood pulp. Rejects result from oversized chips as well as knots and reaction wood that have high lignin content. Because screened mill chips were used that had a greater proportion of oversized chips compared to laboratory chips, the pulp rejects were high in some experiments (see Table 3). In a mill situation, screened rejects would be re-pulped by recycling them back to the digester. Because mill chips were used in the current experiments the effective pulp yield was used as the indicator for pulp obtainable from the process. The effective pulp yield ( $Y_{Eff}$ ) was estimated as the mass of screened pulp ( $M_S$ ) plus 60% of the mass of the screened rejects ( $M_R$ ) divided by the initial mass of dry wood sent to the extraction vessel ( $M_{Dry\ Wood}$ ).

$$Y_{Eff} = \left[ \frac{M_S + 0.6 * M_R}{M_{Dry\ Wood}} \right]$$

The factor of 60% is the industrially observed efficiency for re-pulping screened rejects. Figure 15 shows the effective pulp yield achieved at each pre-extraction condition, in comparison to the un-extracted control.

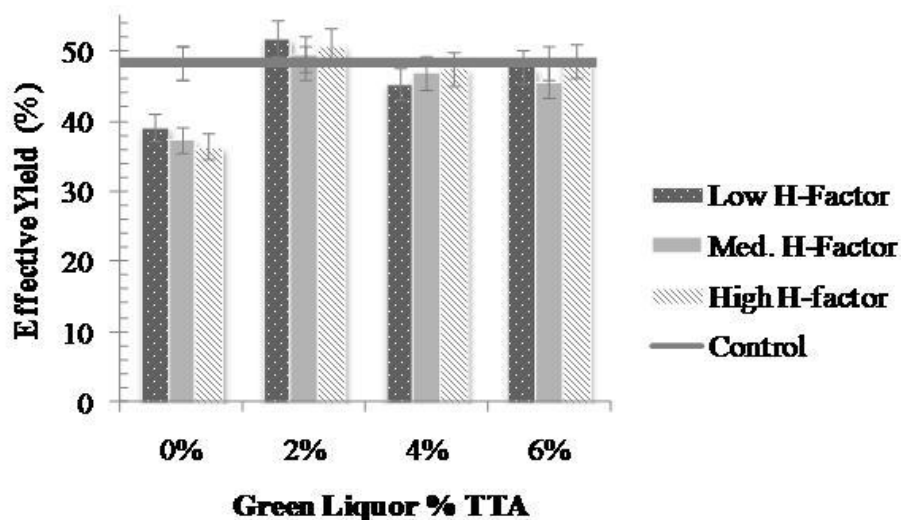


Figure 15. Effective pulp yield for green liquor extracted woodchips

For green liquor extraction, 2% alkali charge may achieve as high as 50% effective pulp yield, an increase of 3% over the control. Water extraction however shows a clear drop in pulp yield, averaging as much as 12 percentage points lower than the control for the experiments in which AQ was added as a pulping additive. Green liquor addition at 4% and 6% were slightly below or on equal to the control pulp yield within the accuracy of the experiments.

### 3.2.6 Pulp Physical Properties

Measurement of pulp yield is a strong indicator of whether hemicellulose extraction has impacted the cellulose fiber, but it is not the only consideration when considering pulp quality. For a successful extraction process the physical properties of the resulting pulp must not be negatively impacted. Extensive testing of pulp properties at all hemicellulose extraction conditions was performed. The results are summarized in Table 3. Nearly all extracted pulps experienced an increase in pulp viscosity, which is an

indication of the degree of polymerization of cellulose in the pulp (give reference). For hot water extraction the viscosity in all cases was more than doubled compared to the control kraft pulp without pre-extraction. Green liquor extracted pulps all had higher viscosity than carbonate extraction at the corresponding charge and H-factor. The least impact on viscosity was observed at low H-factor and 2% or 4% alkaline charge. Measures of physical strength such as the fiber strength factor and the wet zero-span breaking length both show that the un-extracted control is roughly the same as hot water extraction at low H-factor, but medium and high H-factor extractions have lower strength. Conversely all alkaline charges at all H-factors tested have higher values of fiber strength and breaking length than the control. Pulp drainage as measured by Canadian Standard Freeness was highest in hot water extraction. Alkaline extracted pulps tended to be near or slightly higher than the control.

Table 3. Effect of hemicellulose extraction on pulp quality and yield

Extract H-Factor (hrs)	Extract % TTA	Total Yield on OD Wood (%)	Screened Yield on OD Wood (%)	Rejects on OD Wood (%)	Kraft EA (%)	Residual EA (g/L)	Black Liquor Solids (g/L)	Kappa # (mL)	Viscosity (cps)
Control		49.5	46.4	3.1	15.6	4.2	167.1	17.4	36.6
400	0	42.7	33.8	8.9	12.0	0.3	130.2	17.3	74.2
400	2	53.5	49.4	4.1	14.2	5.1	151.3	16.5	36.2
400	4	46.4	43.8	2.5	13.9	7.3	146.8	15.9	37.7
400	6	49.4	45.2	4.2	10.5	3.0	126.5	18.8	55.8
600	0	42.2	30.2	12.1	12.0	0.1	122.7	17.8	87.2
600	2	51.3	47.2	4.1	15.3	8.5	160.7	17.4	41.1
600	4	48.3	44.6	3.7	13.9	10.4	159.3	16.1	38.7
600	6	46.8	43.7	3.1	11.9	7.2	135.7	17.9	49.0
800	0	40.3	30.5	9.8	12.0	0.5	130.2	17.6	77.2
800	2	56.0	42.9	13.0	14.8	1.4	126.1	17.7	91.2
800	4	49.5	44.8	4.7	13.3	7.3	144.0	18.8	53.9
800	6	51.4	44.7	6.6	11.3	6.0	126.6	18.6	62.4

### 3.3 Conclusions

Extraction of woodchips prior to Kraft pulping has the potential to remove hemicellulose selectively without degrading the cellulose fiber. Addition of 2% green liquor maintains the pH of the final extract at near neutral conditions and prevents acetic acid from causing excessive hydrolysis of the hemicellulose remaining in the extracted wood. Water extraction releases the highest amount of oligosaccharides, but does so at the expense of pulp yield. The highest concentration of sugars was 30 g/L in water extracts at high H-factor, but the corresponding pulp yield was only 35%. Control Kraft pulping experiments conducted with mill chips showed an effective pulp yield value of 47%, while pulping green liquor extracted woodchips led to similar pulp yields. For the 2% green liquor extraction experiment effective pulp yields of about 50% were obtained. Carbonate and green liquor extraction resulted in similar aqueous extract composition and pulp yields. The hemicellulose extraction process can be tailored to optimize the economic trade-off between oligosaccharide concentration and pulp yield based on the data presented in this work. Recovery of acetic acid is possible and would represent a valuable by-product.

## CHAPTER 4: CONCENTRATION OF HEMICELLULOSE EXTRACTS

### 4.1 Introduction

Economically viable conversion of hemicellulose extracts into value-added fuels and chemicals through fermentation requires a high initial concentration of saccharides. If ethanol is the fermentation product, the minimum concentration is 4-5% w/w ethanol in the feed to the distillation column for economic recovery (Galbe *et al.* 2007). Due to the stoichiometry of ethanol fermentation, this requires at least 80-100 g/L of monosaccharides in the feed to fermentation. The hemicellulose extract solutions contain only 5-30 g/L of sugar depending on severity, and must therefore undergo concentration prior to fermentation to increase the oligosaccharide content approximately ten-fold.

Evaporation was examined as the subsequent method for removing excess water, but the concomitant increase of sodium, acetic acid and lignin resulted in a solution that completely inhibited cell growth at only three-fold concentration for 3% green liquor extracts (Chapter 5). It was therefore proposed to examine membrane ultrafiltration as a means of concentrating hemicellulose oligomers. Ultrafiltration is a pressure-driven membrane separation process currently used in many industries, including separation of oil-water emulsions, processing blood and plasma, fractionation of proteins, recovery of whey proteins in cheese manufacturing, removal of bacteria following wine production, and clarification of fruit juices (Geankopolis, 2003).

A variety of ultrafiltration membrane sizes are available; commonly tested sizes include 1, 5, 15 and 50 kD molecular cut-off weights. A schematic of the ultrafiltration system used is shown in Figure 16. Hemicellulose oligomers larger than the membrane cut-off will remain in the retentate and become more concentrated as permeate is removed. The permeate will contain some saccharide losses including smaller oligomers and any monosaccharides that may have been present after extraction. The smaller molecules such as monosaccharides, organic acids and inorganic salts will be in equilibrium on each side of the membrane such that the concentration is the same in both the permeate and retentate fractions. Acetyl groups bound to hemicellulose oligomers can be retained and result in an increase in acetic acid concentration after subsequent hydrolysis. Lignin exists as a polymer and in some cases bound to carbohydrates, which increases its likelihood of being retained by the membrane. Lignin is also the attributed cause of membrane fouling which reduces the permeate flow rate.

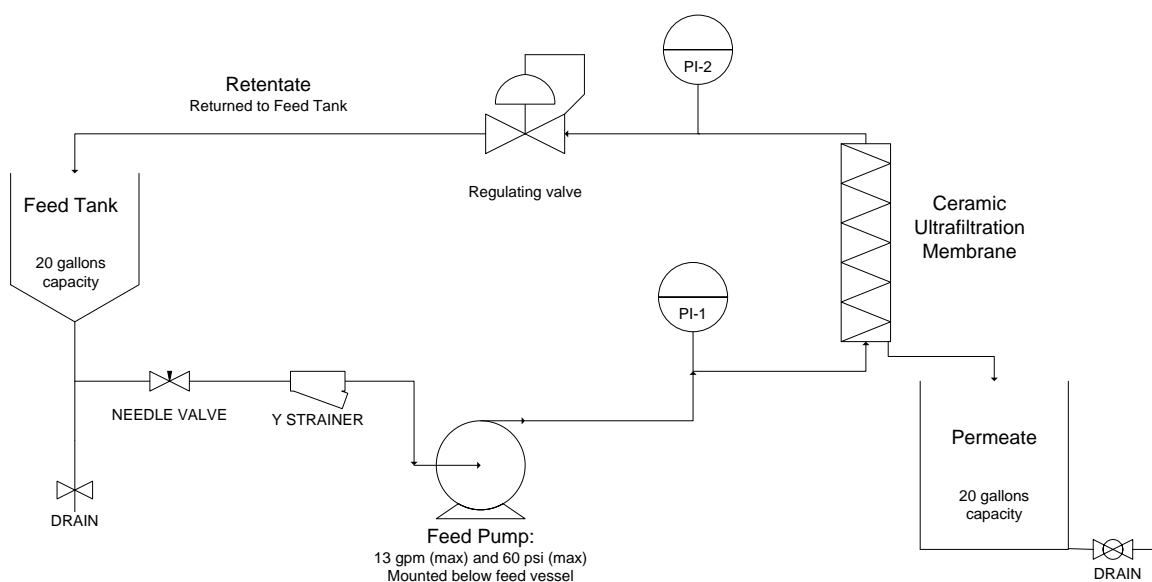


Figure 16. Ultrafiltration system schematic

Xylose based oligosachharides derived from hemicelluloses have potential for novel applications in food and pharmaceutical use, as low calorie sweeteners, dietary fiber supplements, and building block materials for biodegradable water soluble films, capsules and tablets (Nabarlatz *et al.*, 2007). Recent studies have shown ultrafiltration to be an effective process for purifying xylo-oligosaccharides derived from autohydrolysis of corn cobs, (Yuan *et al.*, 2004) almond shells, (Nabarlatz *et al.*, 2007) and rice husks (Vegas *et al.*, 2007). Ultrafiltration of hardwood autohydrolysis extracts for production of bio-based fuels and chemicals has also been described (Wood *et al.*, 2008).

Ultrafiltration has additionally been applied to kraft black liquor streams for recovery of lignin and hemicellulose (Wallberg *et al.*, 2006). Recovery of lignin from pulping black liquors is being investigated for mills with surplus energy to sell as a fossil fuel replacement in heat production (Wallberg *et al.*, 2003). Kraft black liquor has similar properties to the hemicellulose extracts produced prior to kraft pulping and is therefore of interest in comparing membrane performance data. A study by Wallberg *et al.* (2003) found that ultrafiltration of 16 wt% solids kraft black liquor using a 15 kD ceramic membrane effectively separated high molecular weight lignin from low molecular weight cooking chemicals, which is a necessary separation to for an economically viable process. It was observed that monovalent ions, such as sodium and sulfur which are important cooking chemicals, had virtually no retention by the membrane, while multivalent ions were retained to a high degree due to their association with the organic material. Multivalent ions include Fe, Ca, Mg, and Mn which are present in the wood, but at such low concentrations that their increased concentration should not be detrimental to downstream fermentation operations. Additionally this study found that membrane

fouling was heavily dependent on the process temperature, where at 60°C the permeate flux leveled off at a limiting value of 120 L/m<sup>2</sup>·h, but at 75°C and 90°C no limiting flux was reached in the pressure range studied (up to 200 kPa).

Design of a membrane filtration system must take into consideration a variety of parameters which effect filtration performance, including membrane surface area and pore size, operating pressure, temperature, feed flow rate and feedstock composition. The greatest trade-offs will be between membrane cut-off size and permeate flow rate, where a smaller pore size allows greater retention of saccharides but also requires longer processing time, thereby necessitating either larger membrane area or larger holding tanks. Successful ultrafiltration of hemicellulose oligomers would achieve both high volume concentration and high xylan concentration at a reasonably fast permeate flow rate. The objective of this research was to determine if ultrafiltration was a viable processing option for concentrating hemicellulose extracts prior to fermentation. The following work describes ultrafiltration of hardwood hemicellulose extracts made under a variety of conditions. Both polymeric and ceramic membranes were studied. Ultrafiltration pore sizes ranging from 1 kD to 50 kD were examined, as well as addition of either a preliminary microfiltration step at 0.22 to 0.45 µm or centrifugation to reduce fouling of the subsequent ultrafiltration. Permeate fluxes, xylo-oligosaccharide retention factors, and fermentation of the resulting concentrates are compared for the differing pore sizes and membrane materials.

## 4.2 Results and Discussion

### 4.2.1 Evaporation

Hemicellulose extracts made using 3% green liquor contain approximately 11.4% of the original wood mass, mainly as de-acetylated oligosaccharides, where the acetyl groups are present as sodium acetate. (Um and van Walsum, 2009) The initial aqueous extracts had a solids content of 3.7 wt%, a pH of 5.6 and following acid hydrolysis contained 4.5 g/L of monosaccharides, which was significantly less than the target 80 g/L needed to economically produce ethanol. The extracts also contained acetic acid at 12 g/L, sodium at 5 g/L and lesser amounts of formic acid, lactic acid and acid soluble lignin. Furfural and HMF were present at low concentrations because they are not released under near-neutral and alkaline extraction conditions and the acid hydrolysis did not cause significant sugar degradation. The initial extracts were too dilute, so an evaporator was used to remove water and increase oligosaccharide concentration. Evaporation also increased the concentration of components which inhibit fermentation such as acetic acid, sodium from the green liquor, and lignin derived phenolics. Three samples were taken during the evaporation process, ranging from the initial 3.7 wt% solids to the final product of 9.8 wt% solids. Each of these samples was acid hydrolyzed then neutralized with calcium hydroxide, and the resulting chemical analysis is presented in Table 4. From the original extraction liquor containing 4.5 g/L total monosaccharides, evaporation was used to produce solutions containing 6.9 g/L and 11.7 g/L monosaccharides. The evaporated sample at 9.8 wt% solids contained 25 g/L acetic acid and 18 g/L sodium, potentially high enough to have significant inhibitory effects on the fermentation. The 11.7 g/L monosaccharide concentration achieved through this extent of evaporation is

still insufficient to produce ethanol at the 40 – 50 g/L level needed for economic distillation. The resulting evaporated solution at 9.8 wt% solids was highly viscous, which creates handling problems and made significant further concentration by evaporation unfeasible. Fermentation of these three extract solutions into ethanol is discussed in Ch. 5.2.2. It can be seen that all components increase proportionally with the solids content as expected, except for the UV lignin which increased more strongly. This most likely due to the increased concentration of carbohydrate degradation products such as furfural, which also strongly absorb UV light at 280nm used for UV lignin detection.

Table 4. Concentrations of aqueous hemicellulose extracts after acid hydrolysis for varying evaporation levels

% Solids	Concentration (g/L)		
	3.7%	5.4%	9.8%
Total Organics	11.3	21.6	35.0
Glucose	0.79	1.21	2.03
Xylose (XMG) <sup>a</sup>	3.20	5.12	8.66
Arabinose	0.46	0.62	1.02
Acetic Acid	10.79	14.97	25.00
Lactic Acid	0.72	1.32	1.94
Formic Acid	1.84	2.70	3.88
HMF <sup>b</sup>	0.008	0.075	0.171
Furfural	0.268	0.303	0.436
Sodium	5.6	11.4	18.3
Sulfate	1.15	2.45	3.34
Klason Lignin <sup>c</sup>	2.3	4.6	4.1
Acid Soluble Lignin	2.6	5.4	12.3

- a. Xylose, mannose and galactose co-elute, approx. 85% xylose, 12% galactose, 3% mannose
- b. 5-Hydroxy-methylfurfural
- c. Klason lignin removed by filtration prior to fermentation

The concentration of sugars even at 3-fold evaporation was too low to be a commercially viable process, but the high viscosity reached and the high inhibitor levels made further evaporation undesirable.

#### 4.2.2 Ultrafiltration

##### 4.2.2.1 Extract Molecular Weight Distributions

Analysis of the molecular weight distribution of hemicellulose extracts by GPC revealed that hot water extracts at high H-factor have the lowest degree of polymerization (DP), while green liquor extracted hemicellulose oligomers are significantly larger. The GPC spectra also provide qualitative information on how the lignin and carbohydrates are bound in various extracts. Comparison of the peak elution times on the UV detector, which measures lignin, and the RI detector, which measures carbohydrates, revealed that significant portions of the hemicellulose fraction were bound to lignin through lignin-carbohydrate complexes (LCC). Figure 17 shows that hot water extracts have three distinct fractions – a free carbohydrate peak elutes first, followed by lignin-bound carbohydrates which are indicated by UV and RI peaks with the same elution time, and finally a free lignin fraction elutes. Green liquor extracts, shown in Figure 18, have only a single carbohydrate peak, which elutes at the same time as a UV peak, and is likely an indication that all of the carbohydrates in green liquor extracts are bound to lignin, whereas the acidic conditions in hot water extraction result in cleavage of the LCC bonds. The neutralization effect of green liquor preserves a greater number of LCC bonds.

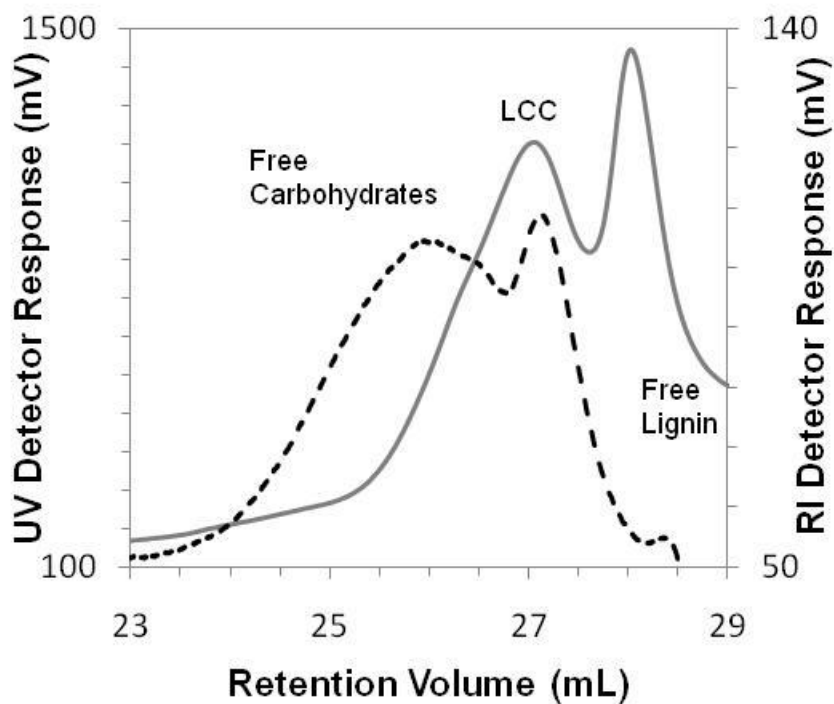


Figure 17. Overlay of UV and RI detector response for GPC analysis of water extract Water extracted southern hardwood chips, 800 hr H-Factor. RI detector response (- - -) and UV detector response (—)

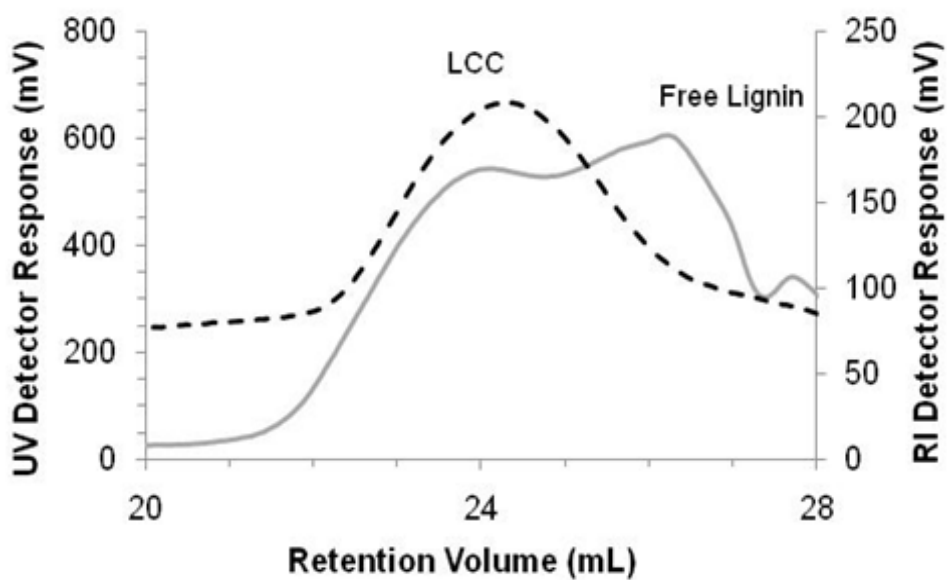


Figure 18. Overlay of UV and RI GPC data for 2% green liquor extract 2% green liquor extracted southern hardwood chips, 800 hr H-Factor. RI detector response (- - -) and UV detector response (—)

Differences in the carbohydrate fractions can be observed in Figure 19, a comparison of the RI spectra for extracts made at varying green liquor charge. Water extraction clearly results in the lowest molecular weight fraction, and is the only condition where two distinct peaks can be observed, differentiating between free carbohydrates and those bound to lignin. Table 5 shows the average molecular weight distributions and degree of polymerization for selected extraction conditions. Oligomers in 2% green liquor extract are significantly larger than those of hot water, and 4% green liquor leads to significant further size increase, but the trend of increasing alkali do not continue for 6% green liquor, where the DP shows a slight decrease from 4%. At a given alkali charge, oligomer size also decreases with increasing H-Factor as the alkaline peeling reaction reduces chain length over time.

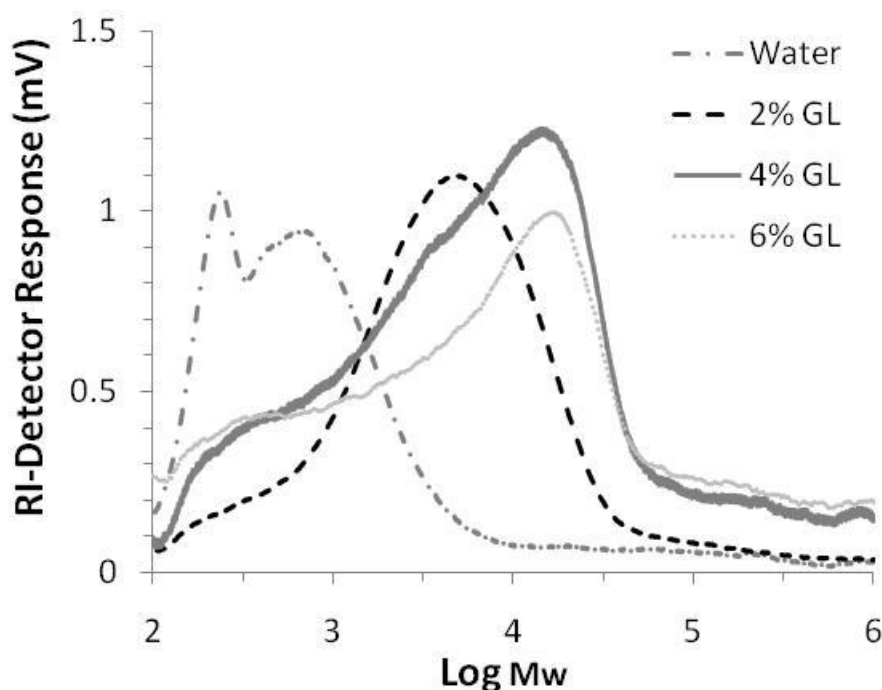


Figure 19. GPC spectra at varying green liquor charge

The oligomer sizes in an extract solution encompass a broad range of molecular weights, where the values given in Table 5 are average values estimated at the peak maximum. The broad size distribution combined with the prevalence of large lignin-carbohydrate complexes enables the concentration of sugars by ultrafiltration. The smallest ultrafiltration membranes commonly available have a nominal molecular weight cut-off of 1 kD, which is small enough to retain all green liquor extracts and water extracts of 600 hr or lower H-Factor based on the average molecular weights presented in Table 5. Larger filters of 5 or 10 kD pore size were expected to be appropriate for the green liquor extracts.

Table 5. Hemicellulose oligomer molecular weight distributions and DP analysis

Extraction media	H-Factor	Molecular weight (Da)	Degree of Polymerization (based on xylose)
Water	400	2238	17
Water	600	1231	9
Water	800	859	6
2% Green Liquor	400	11205	85
2% Green Liquor	600	8946	68
2% Green Liquor	800	7100	54
4% Green Liquor	800	12100	91
6% Green Liquor	800	10900	83

#### 4.2.2.2 Ultrafiltration of Hemicellulose Oligomers

Though ultrafiltration is commonly practiced in a variety of industrial processes such as protein purification for biopharmaceutical applications, its' use on woody biomass derived hemicellulose at an industrial scale may prove cost prohibitive due to the highly fouling nature of the extract solution. Membrane technology is capital intensive and may require frequent replacement of membranes resulting in large operating costs as well. Ceramic membranes offer the most likely industrial option as they can withstand

harsher operating conditions of time, temperature and pH. Bench scale testing for hemicellulose extract filtration utilized both ceramic and polymeric membranes. In high pressure systems the foaming of extracts posed a potential processing concern, as tanks had to be oversized to prevent foam overflow. Foam originates as CO<sub>2</sub> from decomposition of uronic acids and NaHCO<sub>3</sub> in combination with sodium soaps formed from the extractives and Na<sub>2</sub>CO<sub>3</sub>.

To assess membrane performance, the permeate flux rates, volume reduction and effectiveness of membrane retention were compared. The volume reduction, VR, is given by:

$$VR = \frac{V_p}{V_o}$$

where V<sub>p</sub> is the permeate volume and V<sub>o</sub> the initial feed volume. The target VR value was 0.9 for a ten-fold concentration, though this proved difficult to reach in practice. The fraction of a component that is found in the retentate is given by:

$$Retention = \frac{V_o \cdot (1 - VR) \cdot C_r}{V_o C_o} = \frac{(1 - VR) \cdot C_r}{C_o}$$

where C<sub>r</sub> is the concentration of the retentate and C<sub>o</sub> the concentration of the initial feed. Because xylan is the principal component of hardwood hemicellulose, the retention of xylose is used as an indicator of how well a membrane performed. Xylose is measured in the feed, permeate and retentate fractions following acid hydrolysis of each. Ideally retention would be 100% if all xylose was in oligomeric form and too large to pass through the membrane pores.

#### 4.2.2.3 Polymeric Membranes

Initial testing on ultrafiltration of hemicellulose extracts was conducted using a small scale Millipore Pellicon II system utilizing membrane cartridges constructed of polyethersulfone or PVDF. A range of membrane sizes were tested, with the idea that pre-filtration would be performed through a large pore size such as 0.22  $\mu\text{m}$  or 0.65  $\mu\text{m}$ , which would remove large impurities without affecting the concentration of oligomers. Oligomers were thought to range from 0.8 to 12 kD in size, necessitating the use of the smallest available pore sizes - 5 or 10 kD ultrafiltration membranes for this system. A summary of the observed ultrafiltration performance for the Millipore system is shown in Table 6. The largest membrane tested was 0.65 $\mu\text{m}$ , with 2 L of a hot water extract concentrated 10-fold on a volume basis to 200 mL of retentate. A slight increase in xylose concentration of 1.2-fold indicated that this membrane was actually able to retain 12% of the oligomeric xylan and/or xylose which is part of lignin-carbohydrate complexes. The 800 H-factor water extract tested using this 0.65 $\mu\text{m}$  membrane had a measured molecular weight of only 0.8 kD, so no retention would have been expected, and in fact the permeate concentration was not depleted of monomeric xylose.

When green liquor extracts with an approximate average molecular weight of 12 kD were pre-filtered through a 0.22  $\mu\text{m}$  membrane to a 20-fold volume reduction, an unexpected 4.8-fold concentration in xylose was observed. In this case the permeate displayed a decrease in xylose from 4.3 g/L to 3.1 g/L. The retention of 23% of xylose was not anticipated through such a large pore size, and indicates that lignin-carbohydrate complexes result in higher molecular weight compounds that can be retained more easily. Alternatively the same feedstock when filtered through a 5 kD polymeric membrane

without prefiltration resulted in 76-81% xylose retention. Fermentation of the 4% green liquor 0.22  $\mu\text{m}$  and 10 kD retentate samples by *E. coli* K011 is described in Ch. 5.2.3.

Table 6. Ultrafiltration performance summary of Millipore Pellicon system

Filter Cut-off Size	Extrxn Media	TTA (%)	H-Factor	Feed Volume (mL)	Retentate Volume (mL)	Volume Concentration Factor	Feed Xylose Conc. (g/L)	Retentate Xylose Conc. (g/L)	Xylose Conc. Factor	Volume Reduction	Retention (%)	Flux ( $\text{L}/\text{m}^2/\text{hr}$ )
0.65 $\mu\text{m}$	H <sub>2</sub> O	0	800	2000	200	10.0	10.7	13.2	1.2	0.90	12.4	18
0.22 $\mu\text{m}^{\text{a}}$	GL	4	800	2500	120	20.8	4.3	20.5	4.8	0.95	23.1	10
50kd	GL	4	800	1000	100	10.0	1.9	10.9	5.8	0.90	58.2	12
10kD <sup>b</sup>	GL	4	800	2100	500	4.2	3.1	6.1	2.0	0.76	46.6	3
10kD	GL	2	600	500	150	3.3	3.6	9.3	2.6	0.70	78.2	4
5kD	GL	4	800	1700	1000	1.7	4.3	6.0	1.4	0.41	80.8	2
5kd	GL	4	800	1000	125	8.0	1.9	11.5	6.1	0.88	76.1	2

All experiments performed with Northern hardwood chips as raw material

- a. Feedstock evaporated from 3.7 wt% to 4 wt% total solids prior to filtration
- b. Feedstock ultra-filtered through 0.22 $\mu\text{m}$  filter

The GE SEPA CF system was also examined for ultrafiltration of hemicellulose extracts, and a performance summary is provided in Table 7. The flat sheet polymeric membranes composed of PVDF, PS, PES or TF had the lowest surface area of the three systems tested and were generally plagued by fouling problems. Pre-filtration performed through a 0.35  $\mu\text{m}$  membrane was only able to reach a volume reduction of 0.12 before the filter was too clogged to operate. The filter proved to have a high xylose retention of 91%, but was not feasible because of the fouling. Many of the experiments performed on this system did not yield significant xylose concentration in the product, either because fouling prevented a sufficient volume reduction or because pre-filtered feedstocks were unknowingly depleted in xylose. Only filtration experiments achieving a final xylose concentration of over 20 g/L were included in Table 7. This includes the 2 kD filtration

of pre-filtered water extracts which showed a xylose retention of 92%, and of 2% green liquor extracts with a 77% xylose retention.

Table 7. Ultrafiltration performance summary for GE SEPA CF system

Filter Cut-off Size	Extrxn Media	TTA (%)	H-Factor	Feed Volume (mL)	Retentate (mL)	Volume Concentration Factor	Feed Xylose Conc. (g/L)	Retentate Xylose Conc. (g/L)	Xylose Conc. Factor	Volume Reduction	Retention (%)	Flux (L/m <sup>2</sup> /hr)
0.35um	H2O	0	800	18548	16378	1.13	22.4	23.2	1.0	0.12	91.4	42
2kD <sup>a</sup>	H2O	0	800	1450	605	2.40	12.1	26.7	2.2	0.58	92.3	60
2kD	GL	2	800	7310	1815	4.03	7.2	22.5	3.1	0.75	77.3	71

a. Feedstock pre-filtered through 0.65 µm Millipore filter.

#### 4.2.2.4 Ceramic Membrane Filtration

Extracts made using 2% green liquor at an H-Factor of 600 hrs had an average molecular weight of 8.9 kD. Filtration of these extracts was performed using the ceramic membrane at nominal cut-off sizes of 0.45 µm, 50 kD, and 1 kD. The 0.45 µm filter retained 62% of xylose, while the 50 kD filter was able to retain 90% of the xylose. Both of these filters were applied directly to hemicellulose extracts without any pretreatment such as centrifugation or coarse filtration to reduce the rate of membrane fouling. The feed to the 1 kD filter did undergo a preliminary filtration through the 0.45 µm filter to reduce fouling, but this lowered the retention, as only 71% of xylose that was able to permeate 0.45 µm was retained by the 1 kD filter. Extensive processing may also result in oligomer degradation due to shear forces breaking up the agglomerated LCCs and due to exposure to slightly elevated temperatures. Permeate flow rates through each of these filters are shown in Figure 20, which illustrates that larger pore sizes have faster throughput.

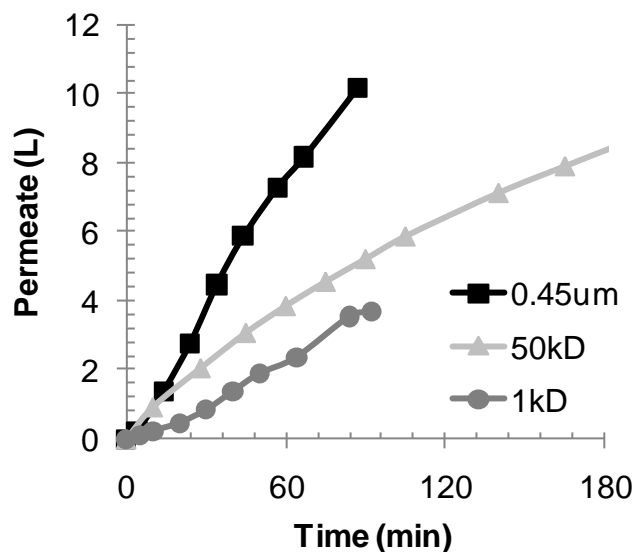


Figure 20. Effect of filter pore size on permeation rate of hemicellulose extracts Molecular weight cut-offs of 1 kD (●), 50 kD (▲), and 0.45  $\mu$ m (■).

The compositional analysis of feed, retentate and permeate fractions for the filter sizes 0.45  $\mu$ m and 50 kD is shown in Table 8. The 0.45  $\mu$ m filter was intended only as a pre-filtration step to remove the largest particulates which cause membrane fouling and reduced flow rates. Unexpectedly, the 0.45  $\mu$ m filter retained so much of the hemicellulose that the permeate contained only 0.8 g/L xylose from the initial 3.9 g/L. This is beneficial because if a larger filter can be used to concentrate extracts effectively, much faster flow rates can be achieved and capital costs will be lower. However, because extracts must be hydrolyzed before the composition is known, the permeate from this filter was used as feed to the 1 kD filtration before it was found to contain such low xylose concentration. The 1 kD filter was an effective concentrator, as the retentate contained nearly all of the input xylose and none was measurable in the final permeate, but it was not run long enough to obtain a significantly concentrated product.

Table 8. Composition of 0.45µm and 50kD filter streams for 2% green liquor hemicellulose extracts, 600 hrs H-Factor

Sample	Concentration (g/L)				
	Glucose	Xylose (XMG)	Arabinose	Acetic Acid	Klason Lignin
0.45µm Filter					
Feed	1.2	3.9	0.4	6.7	2.9
3x Retentate	1.7	6.8	0.5	6.3	3.6
3x Permeate	1.2	0.8	0.2	6.2	1.2
50kD Filter					
Feed	3.3	3.9	0.3	9.2	3.5
7x Retentate	4.2	27.1	1.0	10.4	16.4
7x Permeate	1.9	2.0	0.3	8.6	1.8

It was observed that larger pore size filters were able to concentrate xylan more effectively than the estimated oligomers sizes would predict possible. This is due in part to the broad molecular weight distributions which provide only an average oligomer size and do not indicate the size of the largest oligomers. The pore sizes provided for a given filter are nominal estimations, which do not necessarily reflect the actual effective pore sizes. The geometry of the pores will also influence how effectively oligomers are retained. The flow patterns will also influence efficiency, as oligomers are linear, branched molecules which will be more easily retained if they approach the membrane pore in a perpendicular orientation. The tendency for lignin to stick to membrane surfaces also will have an effect on the geometry of the pores, as a coating may build-up over time and decrease the pore size, impeding the flow of material through the membrane.

Hot water extract produced from aspen strands had an average molecular weight of 7.8 kD and was filtered using ceramic membranes of 1 kD and 5 kD without any prior centrifugation or coarse filtration. Two trials were performed at the 5 kD size – the first

with 30.5 L of feed concentrated 4.7 fold over 15 hrs and the second with 132 L of feed concentrated 2.5 fold over 25 hrs. The former retained xylose at 91% efficiency while the latter operated at 88% retention. With increased time of filtration the xylose concentration in the permeate rises due to oligomer degradation, causing the retention to decrease. Hot water extracted hemicelluloses are not completely de-acetylated, which leads to an increased acetic acid concentration in the retentate because acetyl groups bound to the hemicellulose are unable to permeate the membrane. The retention of acetic acid through the 5 kD filter was 65% for the former trial and 70% for the latter. Retention efficiencies for the 1 kD filter were 97% of xylose and 77% of acetic acid.

Table 9. Autohydrolysis of aspen strands: composition of aqueous extract before and after ultrafiltration with 1kD and 5kD ceramic membranes

Sample	Concentration (g/L)				
	Glucose	Xylose (XMG)	Arabinose	Acetic Acid	Klason Lignin
5kD Filter - 360 hr H-Factor					
Feed	3.2	26.4	0.0	6.8	-
4.7x Retentate	6.7	106.5	0.0	20.1	-
4.7x Permeate	2.4	8.1	0.0	3.6	-
5kD Filter - Mixed 360 & 220 hr H-Factor					
Feed	4.9	19.2	0.4	5.9	2.9
2.4x Retentate	6.2	41.0	0.8	9.2	14.9
2.4x Permeate	2.9	4.7	0.5	2.5	1.6
1kD Filter - 135 hr H-Factor					
Feed	1.86	5.75	0.90	2.37	1.71
2x Retentate	2.64	9.91	1.32	3.27	15.75
2x Permeate	0.67	0.36	0.33	1.30	0.73

Optimal hydrolysis conditions for hot water extracts are less severe than those needed to hydrolyze green liquor extracts. The 4.7-fold retentate fraction was hydrolyzed at pH 1 and 120°C for 3 different reaction times: 60, 30 and 10 minutes. For consistency with the feed and permeate which were only done at 60 min., that is the condition presented in

Table 9. However, Table 10 presents the data obtained for all 3 hydrolysis times, showing a dramatic increase in xylose and corresponding decrease in furfural at lower hydrolysis time. A maximum of 149 g/L fermentable sugar was obtained with a 10 min hydrolysis, though the corresponding 1.3 g/L of furfural indicates that an even lower hydrolysis severity could possibly achieve higher conversion.

Table 10. Composition of hot water extracted aspen strands with varying acid hydrolysis times

Hydrolysis Time (min)	Glucose	Xylose (XMG)	Formic Acid	Acetic Acid	HMF	Furfural
60	6.6	106.5	0.9	20.4	0.2	3.6
30	8.4	112.5	0.8	22.5	0.2	2.8
10	9.3	139.4	0.9	26.2	0.3	1.3

The performance summary for all ultrafiltrations performed using the ceramic membrane system is given in

Table 11. The overall flux of permeate through the ceramic membranes is significantly higher for this system when compared to the lower pressure Millipore polymeric membrane system. The high pressure GE system had generally higher flux rates, and was more comparable to ceramic membranes. Ceramic membranes were however the easiest to clean when fouling occurred to raise flux rates, whereas the flat sheet membranes utilized in the GE system were often too degraded by the high pressure to be reused. Retentate from the 50 kD filtration of green liquor extracts was later fermented by both *E. coli* K011 and *Bacillus coagulans* MXL-9, which is described in Ch. 6.2.

Table 11. Ultrafiltration performance summary of Novasep ceramic membranes

Filter Cut-off Size	Feedstock	Extrxn Media	TTA (%)	H-Factor	Feed Volume (L)	Retentate Vol. (L)	Vol. Conc. Factor	Feed Xylose Conc. (g/L)	Retentate Xylose Conc. (g/L)	Xylose Conc. Factor	Volume Reduction	Retention (%)	Flux (L/m <sup>2</sup> /hr)
0.45um	SHW	GL	2	600	16	6	2.8	3.9	6.8	1.8	0.65	62.3	71
1kD <sup>a</sup>	SHW	GL	2	600	10	7	1.6	0.8	0.9	1.1	0.36	71.1	23
50kD	NHW	GL	2	600	35	5	7.6	3.9	27.2	6.9	0.87	90.2	23
1kD	Aspen	H2O	0	135	8	5	1.8	5.8	9.9	1.7	0.44	96.9	9
5kD	Aspen	H2O	0	360	30	7	4.7	26.4	112.5	4.3	0.79	90.9	20
5kD	Aspen	H2O	0	Mix	132	54	2.4	19.3	41.0	2.1	0.59	87.6	38

a. Feedstock filtered through 0.45  $\mu$ m membrane prior to 1 kD filtration.

#### 4.3 Conclusions

Characterization of the molecular weight distribution of extracts revealed that green liquor extraction produces significantly larger oligomers than autohydrolysis, and that nearly all carbohydrates are bound to lignin as lignin-carbohydrate complexes. Water extracts have three distinct polymeric fractions comprised of free carbohydrates, free lignin, and LCCs. The average molecular weight for water extracts is approximately 0.7 kD, indicating a DP of 6 xylose units. Green liquor extraction at 4% resulted in the largest oligomer sizes of 12 kD, and DP of 90 xylose units. The binding of lignin and carbohydrates results in larger molecular weight agglomerations, which allow hemicellulose sugars to be concentrated using ultrafiltration.

Evaporation and ultrafiltration were each evaluated as means of concentrating the sugars in hemicellulose extracts. The targeted 8-10% sugar concentration proved difficult to reach by either method. Evaporation by 3-fold led to a highly viscous solution with toxic levels of acetic acid and sodium at only 1.1% sugar. Ultrafiltration was shown

to be a more promising method of concentrating green liquor extracted hemicellulose oligomers than evaporation because there was no concurrent concentration of the inhibiting organic acids and inorganic salts. Ultrafiltration of green liquor extracts through a 50 kD membrane produced a retentate with 3.2% sugar available for fermentation without increasing acetic acid or salt concentrations. The main drawbacks to ultrafiltration are the slow permeation rates and tendency for lignin to foul the membranes, as well as the increased concentration of lignin in the retentate which will lead to lower fermentation yields. Hot water extracts contain acetyl groups that remain bound to the oligosaccharide fraction, and unlike green liquor extract, this leads to increased acetic acid content following ultrafiltration. Ultrafiltration of hot water extracts at 5kD was able to achieve sugar concentrations of 150 g/L, higher than the target, but will require removal of inhibitors by ion exchange prior to fermentation.

## CHAPTER 5: FERMENTATION OF HEMICELLULOSE EXTRACTS INTO ETHANOL BY *ESCHERICHIA COLI* K011

### 5.1 Introduction

Hemicelluloses are removed from wood in oligomeric form and must be hydrolyzed by either acid or enzymes into their monosaccharide constituents before they can be fermented into ethanol. The principal monosaccharide in aqueous hemicellulose extracts is xylose, which requires a metabolically engineered organism capable of converting both pentose and hexose sugars into ethanol at high yield, such as *Escherichia coli* K011 (Bothast *et al.* 1999, Dien *et al.* 2003). The ethanol solution from fermentation should contain 4-5% w/w ethanol in the feed to the distillation column for economic recovery (Galbe *et al.* 2007). Due to the stoichiometry of ethanol fermentation, this requires at least 80-100 g/L of monosaccharides in the feed to fermentation.

Fermentation of lignocellulosic derived substrates is subject to inhibition from several components. In hemicellulose extraction liquor, acetic acid and sodium salts are likely to have significant impacts on micro-organism viability and ethanol production (Klinke *et al.* 2004, Nigam 2001). Lignin-derived phenolics and sugar degradation products such as furfural and hydroxymethylfurfural (HMF) may also contribute to microbial inhibition (Palmqvist and Hahn-Hagerdal 2000). Levulinic acid and formic acid can be formed as products of HMF breakdown, or formic acid may also form from furfural under acid conditions at elevated temperatures during acid hydrolysis (Almeida, 2007). Salts and organic compounds with low molecular weight are able to penetrate cell membranes, while higher molecular weight inhibitors influence the expression and activity of sugar and ion transporters within the cell membrane (Klinke *et al.* 2004).

Acetic acid in its undissociated form is able to penetrate the bacteria cell walls and acidify the cytoplasm, disrupting the proton gradient across the cell membrane and interfering with cellular processes (Takahashi *et al.* 1999). The effects of varying sodium acetate concentration on ethanol production by *E. coli* K011 were previously studied in media containing either 80 g/L glucose or 50 g/L xylose (Takahashi *et al.* 1999). In glucose fermentation, acetate concentrations up to 12 g/L did not significantly affect the ethanol yield or productivity, but at 15 g/L acetate there was a sharp decrease in production (Takahashi *et al.* 1999). During xylose fermentation, ethanol yield and productivity were not affected up to 10 g/L acetate (Takahashi *et al.* 1999). It was observed that the degree of acetate inhibition was strongly correlated to pH, since the undissociated form of acetic acid is more prevalent at lower pH. The optimum fermentation pH in the presence of acetic acid was found to be 7, where pH 6 is optimum for cultures which aren't growing in acetic acid (Takahashi *et al.* 1999). Different organisms experience varying degrees of inhibition in lignocellulosic hydrolysates. *E. coli* is generally more tolerant of furfural and 5-hydroxymethyl furfural than other organisms (Klinke *et al.*, 2004). Potential for inhibition was shown to be dependent on the chemical structure for aromatic compounds. For *E. coli* the aromatic inhibition is closely related to the functionality of the aliphatic side-chain (Klinke *et al.*, 2004). Alcohols are the side-chain with the lowest inhibition, followed by acids, and then aldehydes. Inhibition was also correlated to the hydrophobicity, where hydrophobic parts of membrane transport systems, proteins and enzymes are potential sites of inhibition (Klinke *et al.* 2004, Zaldivar *et al.* 1999). Some compounds, such as furfural, exhibit even higher inhibition potential in the presence of other known inhibitors, indicating a

synergistic inhibition mechanism (Klinke *et al.* 2004, Zaldivar *et al.* 1999). Previous work by Zaldivar *et al.* showed *E. coli* was completely inhibited by 25 g/L acetic acid, 17.5 g/L formic acid, 3.5 g/L furfural or 4 g/L of 5-HMF. The purpose of this work was to compare the inhibition effects on model hemicellulose extracts with varying sodium and acetate concentrations to the inhibition observed during fermentation of actual hardwood hemicellulose extracts. The feasibility of evaporation as the method for concentrating dilute hemicellulose extracts was assessed.

## 5.2 Results and Discussion

Initial experiments were conducted to establish a baseline performance yield of *E. coli* K011 on pure sugars and explore the optimal fermentation conditions. It was known that this organism could consume the five major biomass sugars (glucose, xylose, mannose, galactose and arabinose), but the performance on hemicellulose extracts had not been investigated. Most previous studies of *E. coli* K011 focused on only one sugar individually, while a few examined binary glucose/xylose mixtures, but relatively little was published about the simultaneous consumption of all sugars. Much of the data presented in literature was for small scale shake flask or test tube fermentation without control of pH, which is important to obtaining high yields in the presence of acetic acid. This work began with an examination of pure sugar fermentation in well-controlled 3 L bioreactors, progressed into mixtures of five sugars and then model extract systems containing inhibitors, and culminated in the fermentation of concentrated hemicellulose extracts.

### 5.2.1 Fermentation of Pure Sugars

It is essential to the production of ethanol at high yields from hemicellulose extracts that all of the biomass derived sugars can be utilized. This is particularly important for hardwood hemicellulose extracts where xylose is the principal component, because in comparison to glucose relatively few organisms consume xylose.

#### 5.2.1.1 Glucose Fermentation

It is expected that the most efficient performance for *E. coli* fermentation should be achieved in fermentation of pure glucose with no inhibitors present, as the cells would have no stresses or substrate competition. At the conditions studied in well controlled 3 L bioreactors and summarized in Table 12, the highest ethanol yield achieved was 79% of the theoretical maximum at a rate of 1g/L/hr.

Table 12. Fermentation of glucose by *E. coli* K011 in a 3 L bioreactor

Temp. (°C)	Avg. pH	Gas Flow (LPM)	Initial Glucose (g/L)	Final Glucose (g/L)	Maximum Ethanol (g/L)	Time for Max EtOH (hr)	Production Rate at Max. EtOH (g/L/hr)	Overall Ethanol Yield
30	6.3	.5 Air	59.2	0.0	22.7	33.0	0.69	75.2%
30	6.1	.5 Air	60.1	0.0	22.9	23.3	0.98	74.7%
37	6.8	.5 Air	53.9	0.0	21.6	23.6	0.92	78.6%
37	6.1	.07 Air	74.5	11.7	21.4	43.7	0.49	66.8%

#### 5.2.1.2 Xylose Fermentation

*E. coli* K011 was engineered to consume xylose with greater speed and efficiency than the native strain possessed. In comparison to pure glucose fermentation, the cells utilize xylose at a slower rate, ranging from 0.4 to 0.8 g/L/hr at the conditions tested in Table 13. Most of the fermentations did achieve higher yields than the glucose experiments shown in Table 12, with the maximum yield being 98.5% of theoretical.

The cultures for inoculating were however all grown on xylose, which may have contributed to the decreased efficiency of glucose metabolism.

Table 13. Fermentation of xylose by *E. coli* K011 in a 3 L bioreactor

Temp. (°C)	Avg. pH	Gas Flow (LPM)	Initial Xylose (g/L)	Final Xylose (g/L)	Maximum Ethanol (g/L)	Time for Max EtOH (hr)	Production Rate at Max. EtOH (g/L/hr)	Overall Ethanol Yield
30	6.1	0.5 Air	33.5	0.0	14.2	33.4	0.43	83.1%
30	6.2	0.5 Air	37.9	0.5	13.5	38.5	0.35	70.8%
37	6.1	None	76.8	14.5	30.4	52.0	0.58	95.7%
37	6.0	0.5 Air	52.2	0.0	24.3	29.4	0.83	91.1%
37	6.4	0.5 Air	64.6	0.0	23.1	42.7	0.54	70.1%
37	6.8	0.5 Air	81.4	0.0	40.9	55.2	0.74	98.5%

#### 5.2.1.3 Temperature Effects

Several different *E. coli* fermentation temperatures are commonly used in literature, generally ranging from 30°C to 37°C (Burchhardt and Ingram, 1992, Underwood *et al.*, 2002). A comparison of the end points of this range was conducted for pure xylose in well controlled 3 L bioreactors. Xylose consumption and ethanol production were both marginally faster at 37°C, as seen in Figure 21 A and B. From 61.5 g/L of xylose, *E. coli* K011 produced 19 g/L ethanol in 23 hrs at 37°C compared to 17.7 g/L ethanol in 38 hrs. For all subsequent experiments 37°C was used as the fermentation temperature.

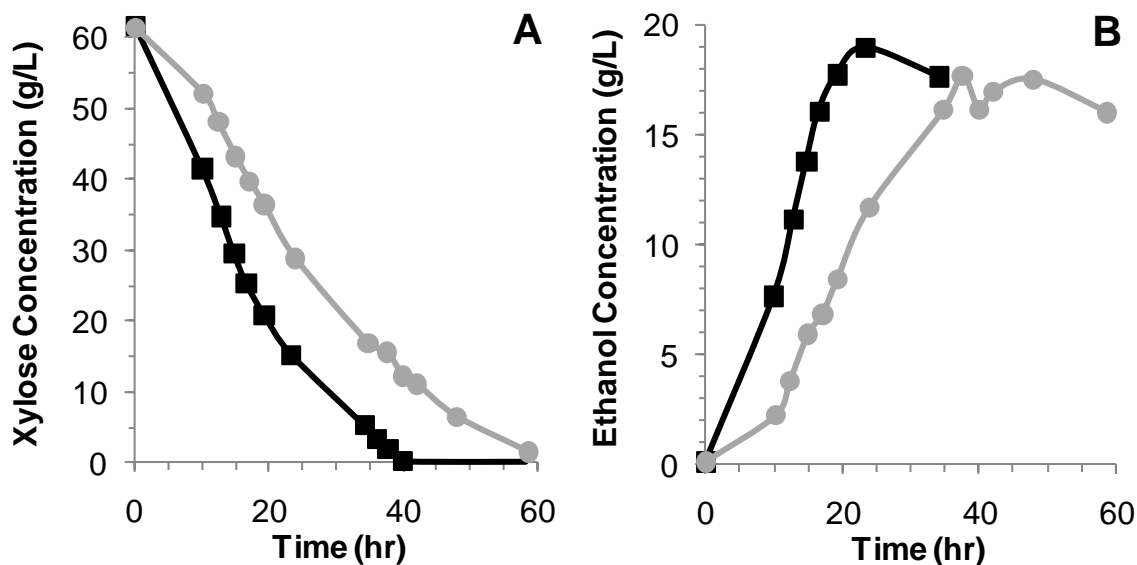


Figure 21. Effect of temperature on *E. coli* fermentation  
Fermentation performed at temperatures of 37°C (■) and 30°C (●)

#### 5.2.1.4 Aeration Effects

As a facultative aerobe, *E. coli* K011 can grow under aerobic or anaerobic conditions. It is known to produce more cells under aerobic conditions and produce more ethanol under anaerobic conditions. Some fermentation experiments are conducted in two phases, with an initial aerobic phase to build up cell mass followed by an anaerobic production phase. The 3 L bioreactor systems used in this work could be sparged with air, nitrogen or oxygen. A comparison between the various aeration schemes was conducted simultaneously in the 4 reactors, where one vessel was sparged with nitrogen through the entire fermentation, one with oxygen, another with air until 8 hours followed by nitrogen for the remainder, and the fourth had no sparging. For each of these gas additions, Figure 22 shows the concentration profiles of substrates and products. The feedstock was a mixture of 60g/L each of pure glucose and xylose. Anaerobic conditions

maintained by nitrogen sparging resulted in the fastest utilization of sugars and production of ethanol. However, the reactor with no gas flow achieved the highest ethanol concentration, which is likely because the lack of gas flow prevented ethanol vapor from being stripped into the exhaust gas stream. While an exhaust gas condenser is employed to condense ethanol vapor, it was probably not completely effective. Once the organisms begin growing they will consume any oxygen that might have been present in the reactor and thus create anaerobic conditions, so sparging with nitrogen is not necessary to the growth of facultative aerobes. The two phase growth experiment results were similar to anaerobic and no flow, with slightly lower ethanol production – also likely due to vapor losses in the exhaust gas stream. Aerobic conditions clearly showed the worst fermentation performance, with incomplete glucose utilization, nearly no xylose consumption and only 4.4 g/L ethanol produced compared to 34.7 g/L in the maximum no-flow experiment. The lack of dependence on gas sparging to generate optimum fermentation conditions is beneficial because it avoids the cost of compressing and handling gas. In hemicellulose extracts it is also beneficial to avoid sparging because it causes excessive foaming. A lack of gas flow out of the reactor does however require an alternate method of measuring exhaust gases, since many detectors, including the CO<sub>2</sub> detector used in these experiments, requires gas flow.

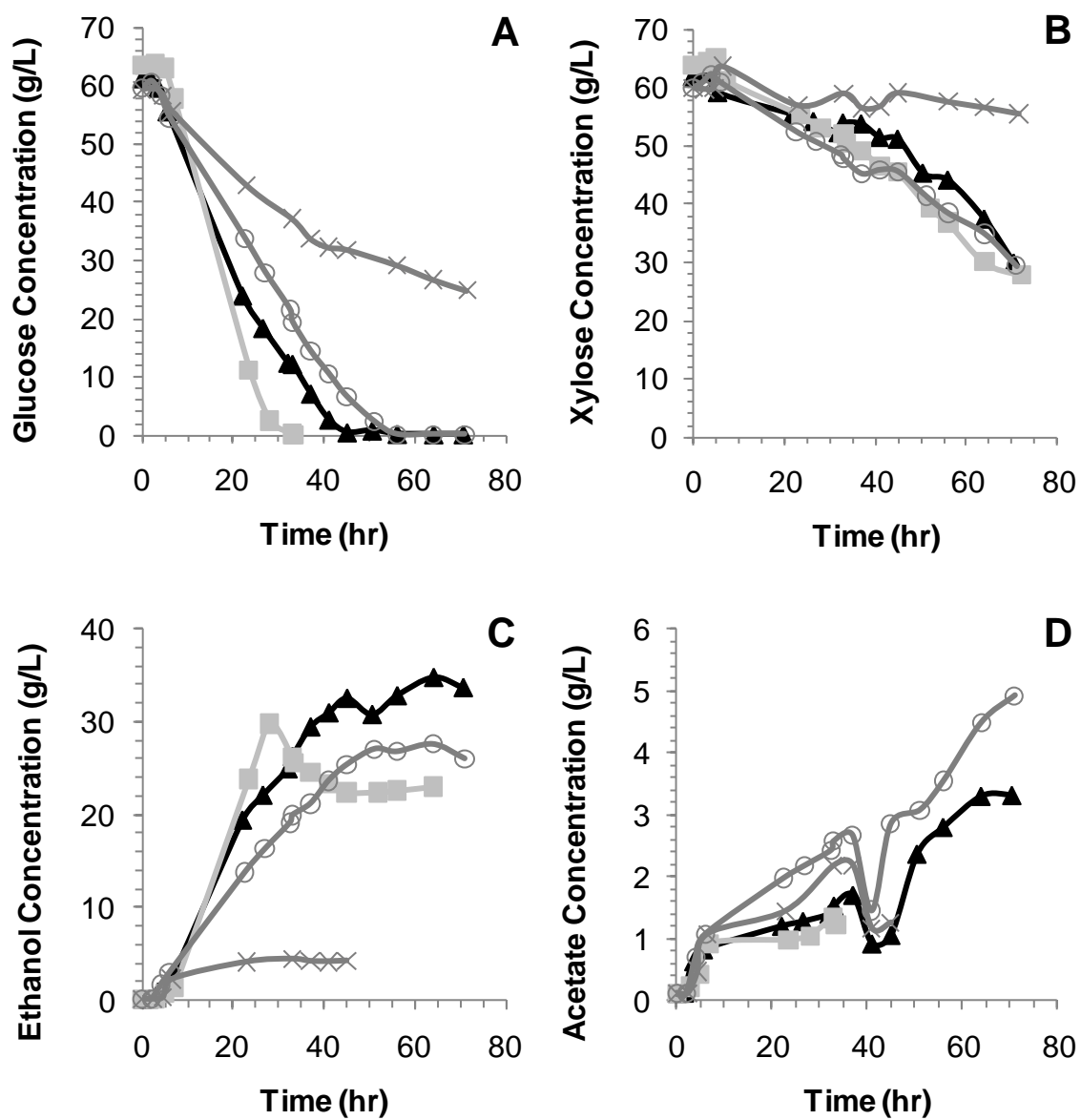


Figure 22. Effect of aeration scheme on fermentation of *E. coli*  
 No gas flow(▲), Anaerobic conditions maintained with 1 LPM nitrogen flow(■), Two phase gas flow with 8 hrs of 1 LPM air and 1 LPM nitrogen for remaining time (○), Aerobic conditions maintained with 1 LPM oxygen flow (x).

#### 5.2.1.5 Mannose and Galactose

Utilization of all the biomass derived sugars is vital to the successful conversion of hemicellulose extracts. While glucose is easily consumed by all organisms, the ability to consume the other hexose sugars mannose and galactose is also essential. Figure 23 shows the successful conversion of mannose and galactose by *E. coli* K011 at high yields. The sugars were fermented individually in separate small scale flask experiments. From an initial 63 g/L of mannose, 30 g/L of ethanol were formed with a yield of 95%. Mannose followed an almost identical trend, where 63 g/L were converted into 24.7 g/L ethanol with a 77% yield. The ethanol production rates from mannose and galactose were 0.52 and 0.43 g/L/hr respectively.

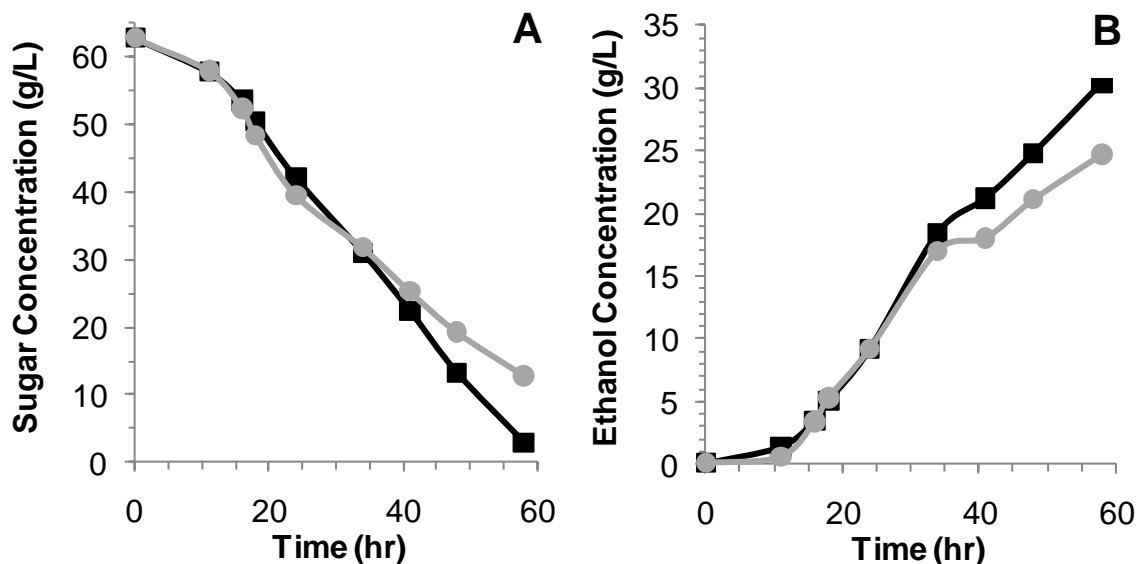


Figure 23. Fermentation of Mannose and Galactose by *E. coli*  
Mannose (■) and galactose (●) fermentations by *Escherichia coli* K011 in shake flasks.

#### 5.2.1.6 Five Sugar Mixture Fermentation

A five sugar mixture was prepared with concentrations proportional to those found in concentrated green liquor extracts and fermented without any inhibitors present to establish a baseline performance of *E. coli* K011 for simultaneous consumption of glucose, mannose, galactose, xylose and arabinose. Metabolism of all sugars and production of ethanol is shown in Figure 24. Glucose was rapidly consumed in 10 hours at the rate of 0.9 g/L/hr. Mannose was entirely consumed in 24 hours at the significantly slower rate of 0.27 g/L/hr – the same rate was observed for xylose consumption, which required 96 hours because xylose had the highest initial concentration. Galactose and arabinose showed similar consumption trends, both with a rate of 0.14 g/L/hr. From the initial 64 g/L total monosugars, 20g/L of ethanol were produced at a yield of only 62% of theoretical.

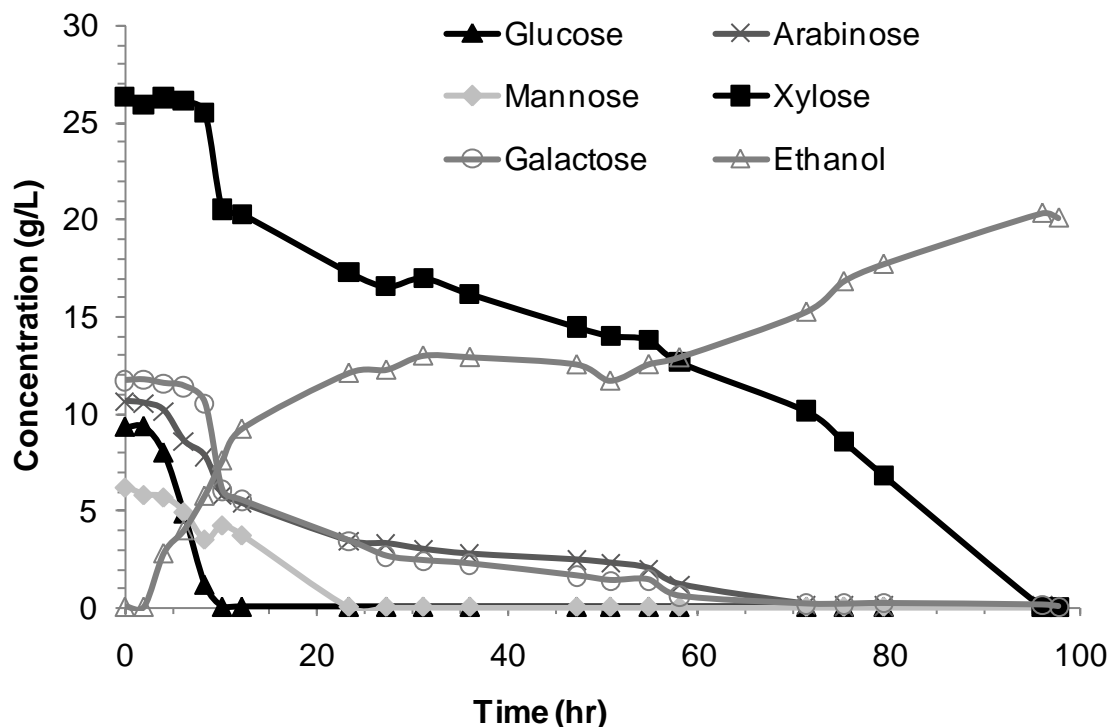


Figure 24. Fermentation of five sugar mixture by *E. coli* K011 in 3 L bio-reactor. The pH was maintained at 6 by 2 N KOH addition. Temperature was 37°C. Agitation was 250 RPM. Air flow of 0.1 LPM maintained by thermal mass flow controller and exhaust gas was measured for CO<sub>2</sub> content. Online optical density probe provided continuous cell mass monitoring.

On-line monitoring of cell mass and pH shown in Figure 25 provided additional insight into the fermentation process for the five sugar mixture. Following complete consumption of glucose at 10 hours there was a decrease in cell mass and at the same time an increase in pH associated with cell lysis, where higher pH intracellular material was released. Glucose metabolizing cells that could not adjust to the consumption of other sugars likely died at that point. Cell mass began increasing again after 60 hours when only xylose remained. After 96 hours when all of the sugars were completely consumed the cell mass began decreasing again and was accompanied by an additional rise in pH when cell lysis of the xylose metabolizing cells occurred.

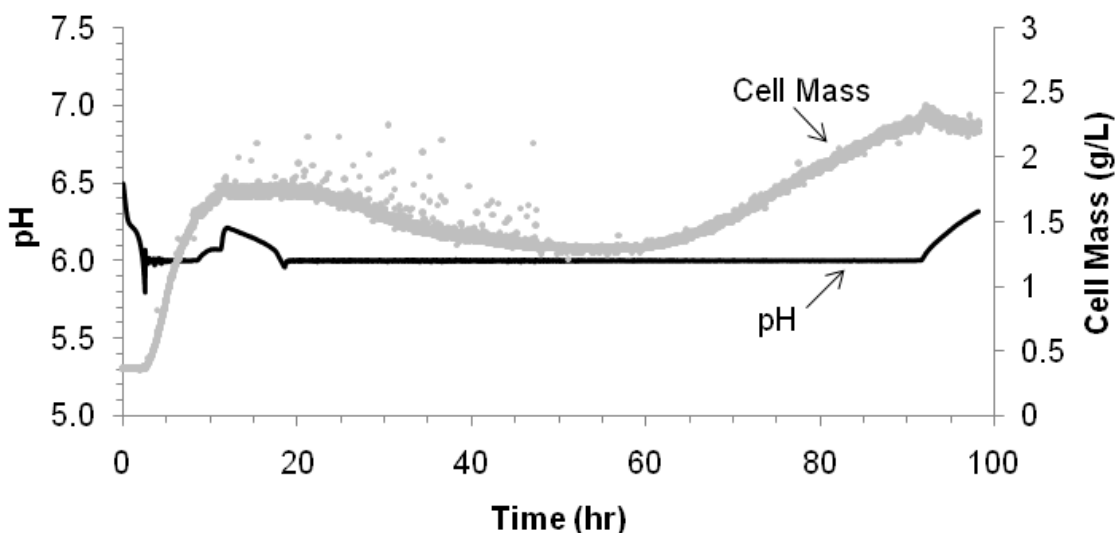


Figure 25. Cell growth and pH trends during fermentation of five sugar mixture

#### 5.2.1.7 Carbon balance

Fermentation of the five sugar mixture was done with complete on-line analysis of cell mass and carbon dioxide, as well as off-line HPLC analysis of all substrates and products. This allowed a complete carbon balance over the course of the fermentation to be calculated. The mole balance for carbon is seen in Figure 26. At each data point where HPLC samples were taken, the contributions associated with ethanol, acetic acid, lactic acid, and carbon dioxide are shown in comparison to the total moles of carbon in consumed sugars. The sum of all products closely agreed with that of the sugar substrates consumed. Ethanol and carbon dioxide were the most significant products, formed in approximately equal molar quantities. Some of the carbon which is unaccounted for in the mole balance was incorporated into production of cell mass.

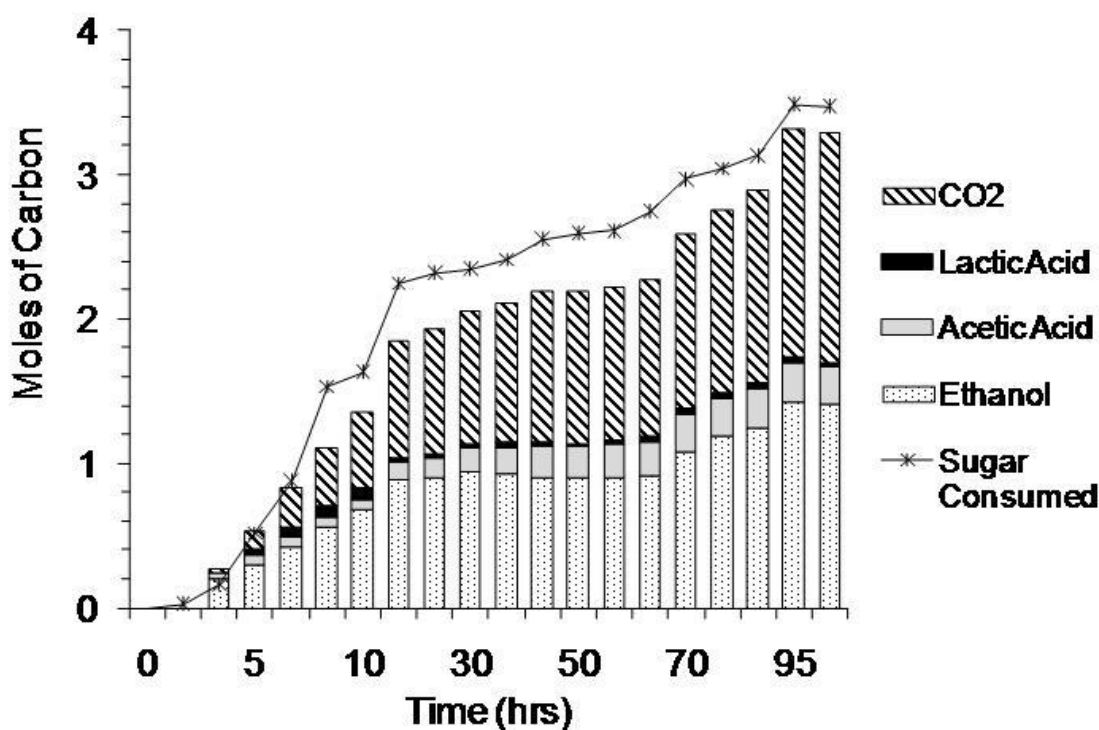


Figure 26. Carbon mole balance for five sugar fermentation

#### 5.2.1.8 Acetic acid inhibition

Before fermenting the hemicellulose extracts, a baseline of expected inhibition was established using model systems. Fermentation of a mixture of pure monosaccharides containing 20 g/L glucose and 30 g/L xylose was done with *E. coli* K011 in the presence of varying acetic acid levels (added as sodium acetate) at pH 7 to determine the expected level of acetate inhibition. Figure 27 shows that this organism had a good tolerance for acetic acid, where the ethanol production at 12.5 g/L of acetic acid even exceeded that of the control. As concentrations increased up to 25 g/L of acetic acid the ethanol production showed a much greater lag phase, but did not significantly affect the ultimate concentration of ethanol obtained. The culture was able to adapt to the presence of acetic acid, probably because acetate is a naturally produced intermediate in

*E. coli* metabolism pathways. Fermentation in the presence of acetic acid was done at pH 7, which is higher than the optimum pH of 6 for *E. coli* when not in the presence of acetic acid. Maintaining fermentation pH at 7 allows the acetic acid to remain in a more dissociated form which does not penetrate into the cytoplasm. Previous work by Zaldivar *et al.* showed 25 g/L acetic acid completely inhibited growth of *E. coli* in 4 mL culture tubes which were not pH controlled, but growth was achieved at that concentration in pH controlled 3 L bioreactors, as seen in Figure 27.

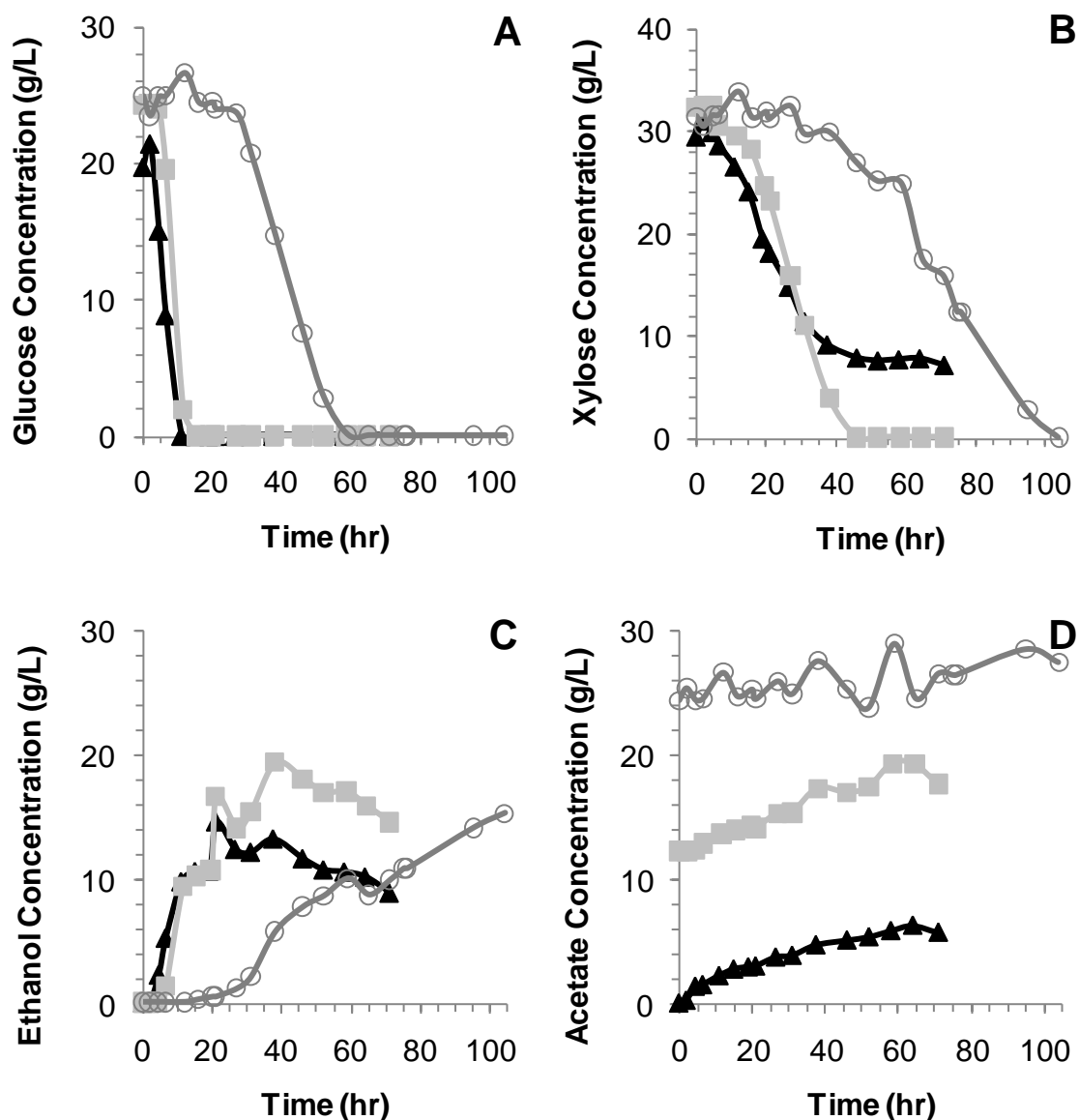


Figure 27. *E. coli* K011 fermentation in the presence of varying acetic acid levels. Fermentation was conducted in LB medium containing 20 g/L glucose and 30 g/L xylose. Conditions were maintained at 37°C and controlled at pH 7 in a 3 L bioreactor. 0 g/L acetic acid (▲); 12.5 g/L acetic acid (■); 25 g/L acetic acid (○).

#### 5.2.1.9 Sodium inhibition

Additional baseline performance testing was done to explore the inhibitory effects of sodium. A variety of different counter-ions were tested including sulfate, carbonate, chloride, and acetate, but all results are presented in terms of the sodium ion

concentration. In the hemicellulose extracts, sodium is present as a mixture that includes bicarbonate, carbonate, sulfate and acetate. The first salt examined was sodium sulfate in fermentation of a solution containing 40 g/L glucose and 40 g/L xylose. Figure 28 shows the ethanol production as a function of sodium ion concentration, added as sodium sulfate. In the presence of sodium sulfate *E. coli* showed a more classical inhibition, where increased sodium levels had decreased ethanol production. Glucose was preferentially consumed before xylose, so most of the ethanol production could be attributed to glucose consumption, while the yields on xylose were much lower.

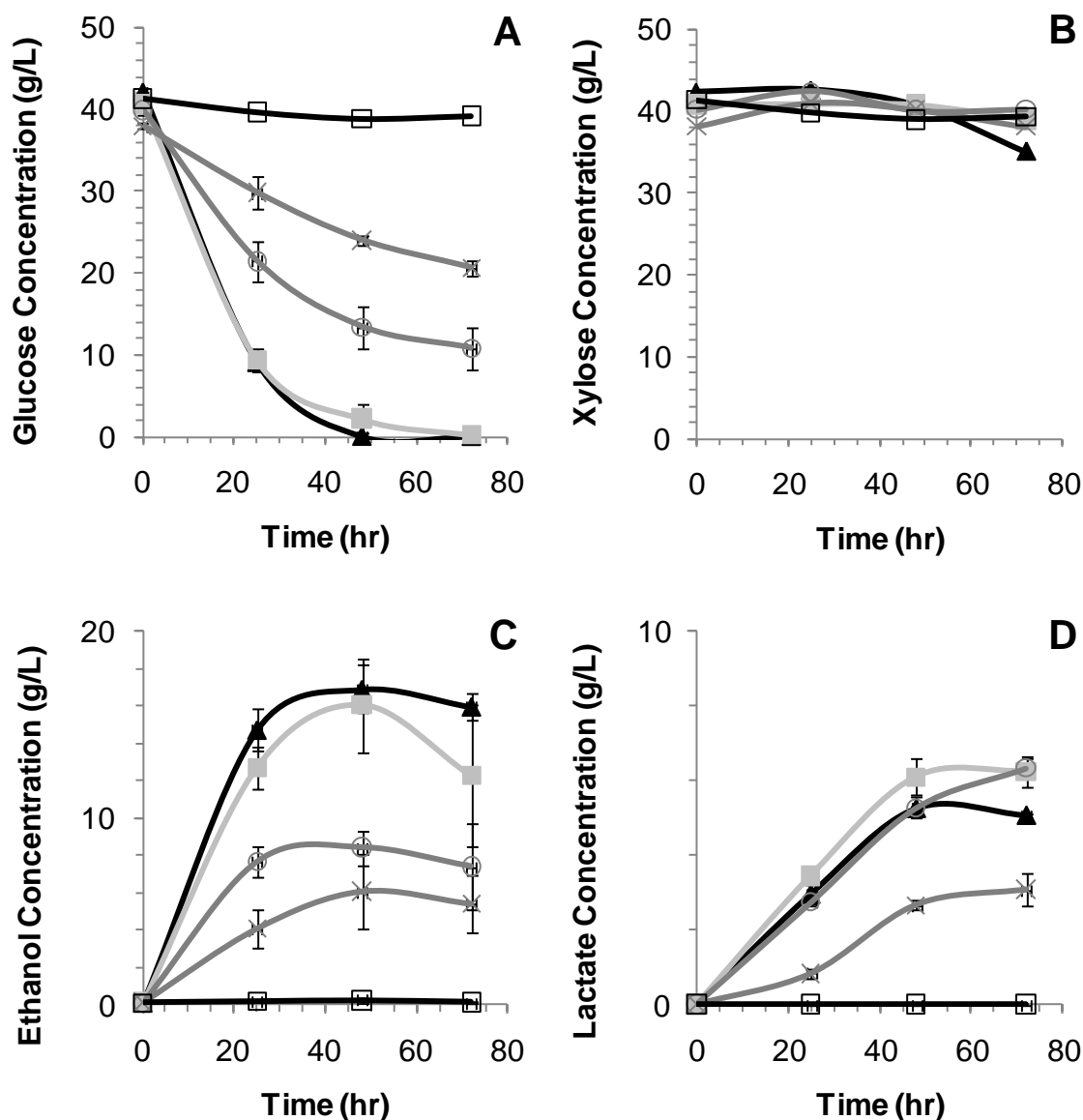


Figure 28. *E. coli* K011 fermentation in the presence of varying sodium levels. Sodium added as sodium sulfate. Fermentation was conducted in shake flasks by *E. coli* K011 in LB medium containing 40 g/L glucose and 40 g/L xylose. Temperature was maintained at 37°C and the initial pH was 7. 0 g/L sodium (▲); 4 g/L sodium (■); 12.5 g/L sodium (○); 25 g/L sodium (x); 41 g/L sodium (□).

The experimental data are summarized in Table 14, which shows that for 4 g/L sodium ions 95% of glucose and only 0.1% of xylose were consumed after 70 hrs.

Table 14. Summary of fermentation results in response to Sodium concentrations

Sample ID <sup>a</sup>	Glucose		Xylose		Ethanol Produced <sup>d</sup> g/L	Acetic Acid Produced <sup>e</sup> (g/L)	Yield % of max. theory <sup>f</sup>	Percent of Control <sup>g</sup>
	g/L <sup>b</sup>	% initial <sup>c</sup>	g/L	% initial				
Control	42.0	100.0	7.0	3.9	16.9	1.2	75.0	100.0
4 g/L Na (Sulfate)	38.0	95.0	1.5	0.1	16.1	0.8	78.2	95.3
12 g/L Na (Sulfate)	27.0	66.0	0.0	0.0	8.4	0.5	57.0	49.7
25 g/L Na (Sulfate)	14.0	37.0	0.0	0.0	6.1	0.3	99.0	36.1
41 g/L Na (Sulfate)	2.0	6.0	0.0	0.0	0.2	0.0	4.0	1.2
Control	4.9	99.5	19.2	99.7	11.4	0.5	92.3	100.0
5 g/L Na (Carbonate)	5.0	99.5	16.4	85.6	8.8	2.7	81.2	77.7
12 g/L Na (Carbonate)	4.8	99.6	17.9	95.3	9.0	2.9	77.7	79.1
20 g/L Na (Carbonate)	4.7	99.4	13.4	73.5	6.4	3.0	69.8	56.7
30 g/L Na (Carbonate)	4.4	96.7	4.1	25.2	2.3	1.8	53.5	20.5
Control	4.9	99.5	19.2	99.7	11.4	0.5	92.3	100.0
5 g/L Na (Chloride)	4.8	99.5	7.7	39.0	5.5	0.1	85.5	48.1
12 g/L Na (Chloride)	4.8	99.5	2.6	14.0	3.7	0.2	98.4	32.8
20 g/L Na (Chloride)	5.6	100.0	1.6	8.5	3.3	0.4	88.8	29.0
30 g/L Na (Chloride)	0.7	12.1	1.1	5.8	0.1	0.0	1.3	0.7
Control	19.7	99.5	22.4	75.8	14.7	5.7	92.7	100.0
5 g/L Na (Acetate)	24.2	99.6	32.3	99.7	19.4	5.4	67.2	132.1
10 g/L Na (Acetate)	24.9	99.6	31.4	99.7	15.4	3.1	53.6	104.8
Control	4.9	99.5	19.2	99.7	11.4	0.5	92.3	100.0
Model Hemi. Extract	5.2	100.0	10.6	51.7	7.3	-0.6	91.0	64.6
Model 2x Hemi. Extract	5.1	100.0	4.4	23.1	3.8	-0.9	77.0	33.7
Model 3x Hemi. Extract	6.8	62.6	1.5	7.8	1.8	-1.0	41.7	15.7
Hemi. Extract	0.8	54.0	2.6	92.0	1.2	0.3	71.1	NA
2x Conc. Hemi. Extract	0.8	60.0	3.8	92.0	2.1	0.2	90.6	NA
3x Conc. Hemi. Extract	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA

- Concentrations compared in terms of g/L sodium ion content for various compounds
- Initial glucose concentration minus final glucose concentration
- Glucose consumed divided by initial glucose
- Maximum ethanol concentration reached minus initial ethanol from inoculums
- Acetic acid at final time minus initial acetic acid, negative if acetate was consumed
- Based on maximum conversion of 0.51g ethanol per gram of glucose or xylose, calculation based on sugar consumed not on total available sugar
- Ethanol concentration divided by that of the control sample

The initial work performed with equal or nearly equal glucose and xylose concentrations was a reflection of the methods described in the literature on binary inhibition testing, but proved to be an inaccurate reflection of hardwood hemicellulose extract composition. Further experiments were conducted with reduced glucose levels of only 5 g/L to allow observation of the change in metabolism that must occur when glucose is depleted, but to make xylose at 20 g/L the more significant substrate, which better reflects the ratio of glucose to xylose that would be found in actual hemicellulose extracts.

Sodium inhibition for all counter-ions is detailed in Table 14. The toxicity of the salt was observed to vary for different counter-ions, where sodium acetate was the least inhibitory and sodium chloride was the most inhibitory. Presence of 30 g/L sodium ions in the sodium chloride form completely inhibited ethanol production compared to 40 g/L in the form of sodium sulfate. Sodium acetate was not tested at a concentration high enough to completely prevent all growth. It was observed that sodium carbonate generated the highest proportion of acetate as a metabolic byproduct, where all concentrations of sodium carbonate had higher acetate production than the control. For all other anions the net acetate production was highest for the control. Regardless of which counter-ion was present, the 18 g/L sodium found in concentrated hemicellulose extracts was not enough to cause complete inhibition by itself.

#### 5.2.1.10 Fermentation of Model Extract Systems

Following individual tests of the inhibitors, sodium and acetic acid were combined at concentrations modeling hemicellulose extracts at the various evaporation levels to assess the synergistic effects of the two inhibitory compounds. Sodium acetate was added for the acetic acid, and the additional sodium needed came from sodium

sulfate. Figure 29 shows the ethanol production for the model extracts that contained 5 g/L glucose and 20 g/L xylose. The model indicated that significant inhibition would be expected for the 9.8 wt% solids extracts, where the ethanol production achieved was only 15% that of a non-inhibited control, compared to 65% of the non-inhibited control for the initial unevaporated extracts. The results of model experiments outlined in Figure 29 suggest that the 3.7 wt%, 5.4 wt% and 9.8 wt% solids hemicellulose extracts contain acetic acid and sodium at concentrations that are expected to cause significantly inhibited growth, but still produce some ethanol.

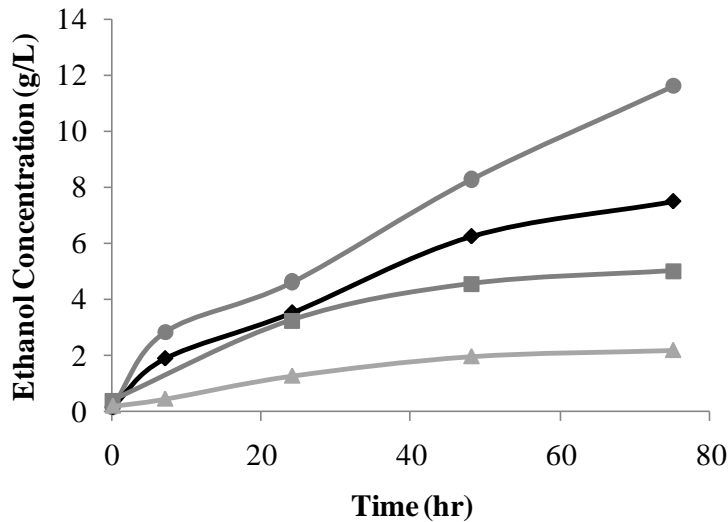


Figure 29. Ethanol production in model hemicellulose extracts by *E. coli* K011. Fermentation was conducted in serum vials with LB medium containing 5 g/L glucose and 20 g/L xylose. Temperature was maintained at 37°C and the initial pH was 7. The control containing no inhibitors (—●—); 3.7 wt% solids extract model with 5 g/L sodium and 10 g/L acetic acid (—◆—); 5.4 wt% solids extract model with 12 g/L sodium and 15 g/L acetic acid (—■—); 9.8 wt% solids extract model with 20 g/L sodium and 25 g/L acetic acid (—▲—).

### 5.2.2 Fermentation of Evaporated Extracts

Figure 30 shows the results of fermenting hemicellulose extract at these three consistencies. Hemicellulose extracts at 3.7 wt% and 5.4 wt% solids were both readily fermentable, producing 1.2 g/L and 2.1 g/L of ethanol, respectively. These concentrations of ethanol are too dilute for economic product recovery which targets 40-50 g/L, but do represent approximately 85% of the theoretical yields based on sugar consumption. The hemicellulose extracts at 9.8 wt% solids were not initially fermentable, despite predictions that the acetic acid and sodium were not concentrated enough to entirely prevent growth. The extracts also contain lignin degraded phenolics, which together with the sodium and acetate appear to be enough to prevent *E. coli* growth.

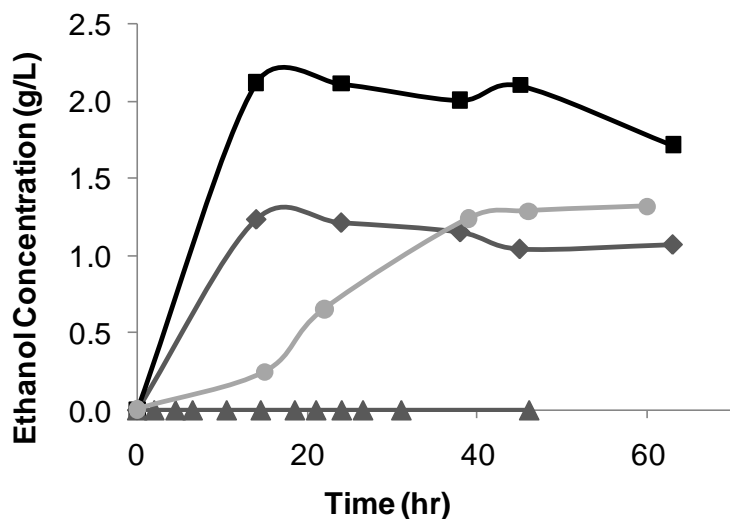


Figure 30. Ethanol production at varying extract evaporation levels. Fermentation conducted by *E. coli* K011 with LB medium at 37°C and controlled at pH 7 in a 3 L bioreactor. Initial 3.7 wt% solids extract (◆); 5.4 wt% solids extract (■); 9.8 wt% solids extract, no strain adaptation (▲), 9.8 wt% solids extract, adapted with large inoculum from 5.4 wt% in log growth (●).

Initial attempts to condition the culture to this high level of inhibitors have shown some success, where one culture was able to produce an amount of ethanol approaching that of the 3.7 wt% solids extract. Conditioning was achieved by using a large inoculum from the 5.4 wt% solids extract in log growth phase, which caused substantial dilution, lowering the acetic acid from 30 g/L to 20 g/L. Additional development of inhibitor-tolerant mutants could possibly increase the allowable extent of evaporation, but the need to increase monosaccharides from 12 g/L to the target 80 g/L will further concentrate acetic acid and sodium, possibly resulting in inhibition that could not be overcome by strain adaptation alone. Several alternatives to the evaporation of the hemicellulose extract feedstock can now be considered. Using ultrafiltration instead of evaporation to concentrate the hemicellulose oligomers does not result in corresponding acetic acid or sodium concentration increases, and requires less energy. Alternative separations technologies such as membrane pervaporation are being developed in an attempt to recover ethanol from dilute sources such as lignocellulosic fermentation so that a lower ethanol concentration can be used than required for economic distillation (Vane, 2005). If evaporation is to be used for concentrating the monosaccharides, it should be done in conjunction with inhibitor removal through such methods as liquid-liquid extraction or ion exchange.

Fermentation experiments performed at larger scale in the fully controlled 3 L bio-reactor systems have a better performance than those done at small scale in 125 mL serum vials on the incubated shaker. Ethanol production for acid hydrolyzed hemicellulose extracts was 30% higher in the bioreactor than at small scale for the 3.7% solids extract, and 20% higher in the bioreactor for 5.4% solids extract. The bioreactor

has automated pH control, which is likely the most important parameter to control in the presence of acetic acid. Small scale experiments have the advantage of allowing many conditions to be tested in a shorter period of time, and do not require as much media or hemicellulose extract. Of the experiments presented in Table 14, only the actual hemicellulose extracts and the sodium acetate inhibition tests were performed in the larger scale controlled bioreactors.

### 5.2.3 Fermentation of Ultrafiltered Extracts

Hemicellulose extracts which had undergone concentration by ultrafiltration were fermented by *E. coli* K011. The first ultrafiltered extracts to be tested were produced from 4% green liquor extracts using the Millipore tangential flow filtration system with membranes of 0.22  $\mu\text{m}$  and 10 kD, shown in Figure 31. In the 0.22  $\mu\text{m}$  retentate a 20-fold volumetric concentration resulted in 5-fold xylose concentration, but also in 3-fold lignin concentration. Acetic acid did not increase and sodium, though not measured, was not expected to increase. A long lag phase of over 3 days was observed in which neither glucose nor xylose were utilized and no product produced. This slow cell growth is attributed to the increased concentration of lignin degraded phenolic substances severely inhibiting the organisms. Only after 7 days were all of the sugars consumed, and from the 18.4 g/L sugars a concentration of 10g/L ethanol was produced. This corresponds to 105% of theoretical yield, which could be a result of the organism consuming dimers that aren't quantified in the feedstock, or a measurement inaccuracy.

Retentate from the 10 kD ultrafiltration was concentrated 4-fold volumetrically but only 2-fold in xylose and 1.5-fold in lignin. Because the feed to this experiment was permeate from the 0.22  $\mu\text{m}$  filtration and was depleted to only 3 g/L of xylose, the total

fermentable sugars were only marginally higher than in the original extract. From the measured 6.4 g/L of total monosugars, 4 g/L of ethanol were produced. As in the 0.22  $\mu\text{m}$  fermentation, this corresponds to a product yield of over 100%.

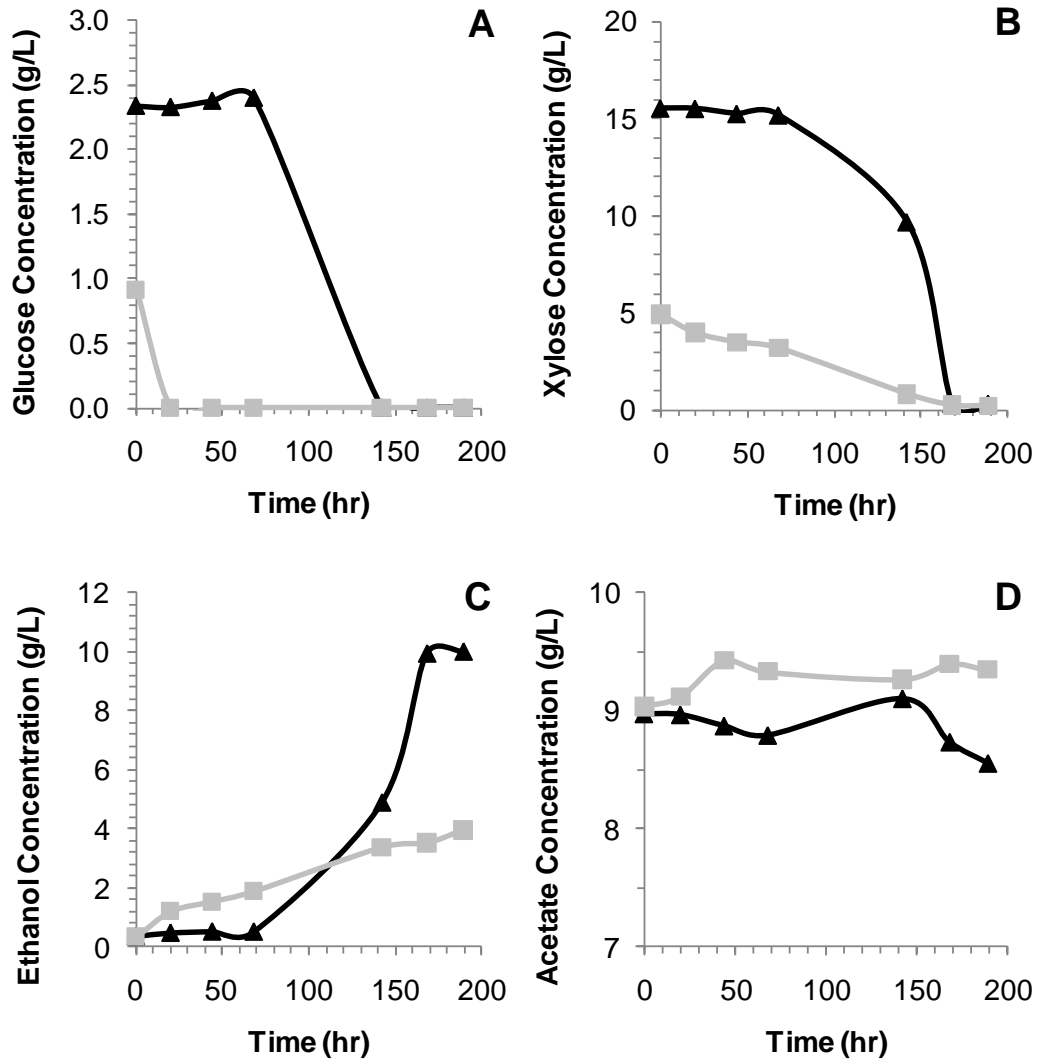


Figure 31. Fermentation by *E. coli* K011 of ultra-filtered extracts  
Feedstocks for fermentation produced from 4% green liquor extracts, 800 H-Factor, by filtration using the Millipore Pellicon II system as described in Ch. 4.2.2.3  
Retentate from 0.22  $\mu\text{m}$  filtration, concentrated 20-fold ( $\blacktriangle$ ), Retentate from 10 kD ultrafiltration, concentrated 4-fold ( $\blacksquare$ )

### 5.3 Conclusions

*Escherichia coli* K011 is an ethanol producing bacteria capable of consuming all of the major lignocellulosic derived monosugars. The organism can withstand some concentrations of acetic acid, sodium and lignin degradation products - the main inhibitors found in hemicellulose extracts. The greatest challenge to overcome is the level of oligosaccharide dilution, which requires removal of excess water prior to hydrolysis and fermentation. Using evaporation to concentrate the hemicellulose extracts presents a substantial inhibition problem. The inhibitors were shown to cause complete inhibition of *E. coli* K011 at 3-fold evaporation before an economically feasible monosaccharide concentration could be achieved. Ultrafiltration concentrated green liquor extracts were able to reach higher ethanol titers because they were not inhibited by increased acetic acid or sodium, though the increase in lignin did lead to longer lag phases.

## CHAPTER 6: FERMENTATION OF HEMICELLULOSE EXTRACTS INTO LACTIC ACID BY *BACILLUS COAGULANS* MXL-9

### 6.1 Introduction

#### 6.1.1 Lactic Acid

Lactic acid has a variety of uses in the food and pharmaceutical industries, and was identified by the USDOE as one of the top 30 potential building block chemicals from biomass (Gruber *et al.*, 2006, Werpy and Petersen, 2004). Lactic acid has the potential to replace chemicals currently derived from petrochemical routes, such as acrylic acid, or the ability to form novel bio-products such as polylactic acid (Werpy and Petersen, 2004). Lactic acid may be produced by synthetic or fermentation routes (Iyer *et al.*, 2000). Synthetic production uses lactonitrile as a starting material and produces a racemic mixture (Gruber *et al.*, 2006). Fermentation processes have become more common because they produce either D- or L-lactic acid at chiral purity near 100% (Gruber *et al.*, 2006). Both isomers can be polymerized but the properties of the polymer vary with the stereo-purity. Optically pure lactic acid is important to the formation of polymers with desirable mechanical properties (Hofvendahl and Hahn-Hägerdal, 2000).

#### 6.1.2 *Bacillus coagulans*

*Bacillus coagulans* is a spore-forming thermophilic lactic acid bacteria first isolated from spoiled milk and food products (Becker and Pederson, 1950). Strain MXL-9 was isolated by the USDA ARS from dairy manure compost for its ability to consume pentose sugars. It is a moderate thermophile, growing at 50-55°C and producing mainly L-lactic acid (Bischoff, 2009). The organism is a facultative anaerobe and produces little

or no CO<sub>2</sub> under anaerobic conditions but large amounts aerobically (Becker and Pederson, 1950). A study conducted by Gordon and Smith in 1949 examined 73 strains of *Bacillus coagulans* and determined that all strains ferment glucose and mannose, while 79% were capable of utilizing xylose and 59% consumed arabinose. A separate study of 23 strains by Becker and Pederson in 1950 revealed that all of the strains tested could consume galactose. The organism can survive between pH 4 and 8, where fermentation optimum lies between 5 and 7 (Becker and Pederson, 1950). The temperature and pH optima for cell growth are closely compatible with those of hydrolytic enzymes, making it well suited for simultaneous saccharification and fermentation (Rhee *et al.*, 2007). Thermophilic organisms are also better suited to industrial processes because they are less easily subject to contamination (Gruber *et al.*, 2006). *Bacillus coagulans* is naturally recalcitrant to genetic manipulation, but recent developments report successful design of a plasmid vector uptake method which could prove useful in optimizing this organism for higher yield lactic acid production (Rhee *et al.*, 2007). The ability of *Bacillus coagulans* to utilize a wide range of sugars under thermophilic conditions makes it well suited to the conversion of lignocellulosic biomass.

### 6.1.3 Hemicellulose Extracts

One promising development in conversion of lignocellulosic biomass to renewable fuels and chemicals is the process of pre-pulping hemicellulose extraction. Extracting hemicellulose prior to pulping creates a new feedstock within the existing pulp and paper industry while preserving cellulose for production of the more valuable pulp (van Heiningen, 2006). In present-day kraft pulp mills, hemicellulose is burned during chemical recovery along with lignin to generate power and steam (Smook, 2002).

Because hemicellulose does not have a high heating value, conversion by biological fermentation processes offers a potential way to increase the value derived from lignocellulosic feedstocks within an integrated bio-refinery. Hemicellulose extraction can be achieved by autohydrolysis in the presence of water prior to the manufacture of dissolving pulp grades, or alternatively in the presence of alkaline chemicals which are necessary to maintain pulp yields in the manufacture of kraft pulp (Ragauskas *et al.*, 2006). Extraction of hardwood species generates an extract rich in xylan oligosaccharides and acetic acid. Softwood extracts have lower acetic acid and are higher in galactose, mannose and arabinose (Sjöström, 1993). All extracted solutions contain low concentrations of glucose derived from dissolution of the amorphous low molecular weight cellulose and from glucomannans. The major portion of cellulose is preserved for pulp production because it achieves greater value as fiber than as a feedstock for commodity fuels and chemicals (van Heiningen, 2006). For an economically viable process it is important that all of the sugars derived from woody biomass, glucose, mannose, galactose, xylose and arabinose, be utilized by the fermentation organism with high conversion yields to the desired product. The concentration of product achieved in the fermentation broth must be high to overcome the costs of recovery and purification.

The following work details a study on the fermentation of hemicellulose derived through extraction of both hardwood and softwood prior to pulping. *Bacillus coagulans* MXL-9 was tested on pure substrates to determine its ability to consume the pentose sugars xylose and arabinose. It was also tested in the presence of varying levels of acetic acid and sodium which are potential inhibitors to bacterial growth at the concentrations contained in hemicellulose extracts.

## 6.2 Results and Discussion

### 6.2.1 Inhibition Effects on Model Systems

An assessment of the suitability of *Bacillus coagulans* MXL-9 for conversion of hemicellulose extracts to lactic acid began with testing its ability to withstand the inhibitory chemicals that have previously been shown to be detrimental to cell growth of *E. coli* (Chapter 5). Acetic acid and sodium inhibition were each evaluated at concentrations ranging from 0 to 30 g/L in bio-reactors controlled at a pH of 6.5. On average, an uninhibited control containing 20 g/L of xylose produced lactic acid at 90% conversion, or 18 g/L of lactic acid. Minor additional side products included acetic acid, formic acid, and ethanol. Figure 32A shows the effect of increased acetic acid concentration on xylose consumption. The control was able to completely consume xylose within 14 hours, while higher acetic acid levels required longer. Fermentations at concentrations of 10 and 20 g/L acetic acid were complete in under a day, but at 30 g/L the fermentation required two days. Despite the slower growth rates, Figure 32B shows that the overall product yields for lactic acid remained high and were all similar to that of the control. Green liquor hemicellulose extracts may contain as much as 10 g/L acetic acid, which could be further concentrated if evaporation methods were employed to increase the monosaccharide concentration prior to fermentation. Acetic acid can be removed before fermentation by liquid-liquid extraction if necessary, though the concentration is not high enough to warrant removal if oligosaccharides are concentrated by ultrafiltration. Hemicellulose extracts produced by water extraction do not contain acetic acid at high enough concentrations to inhibit fermentation significantly.

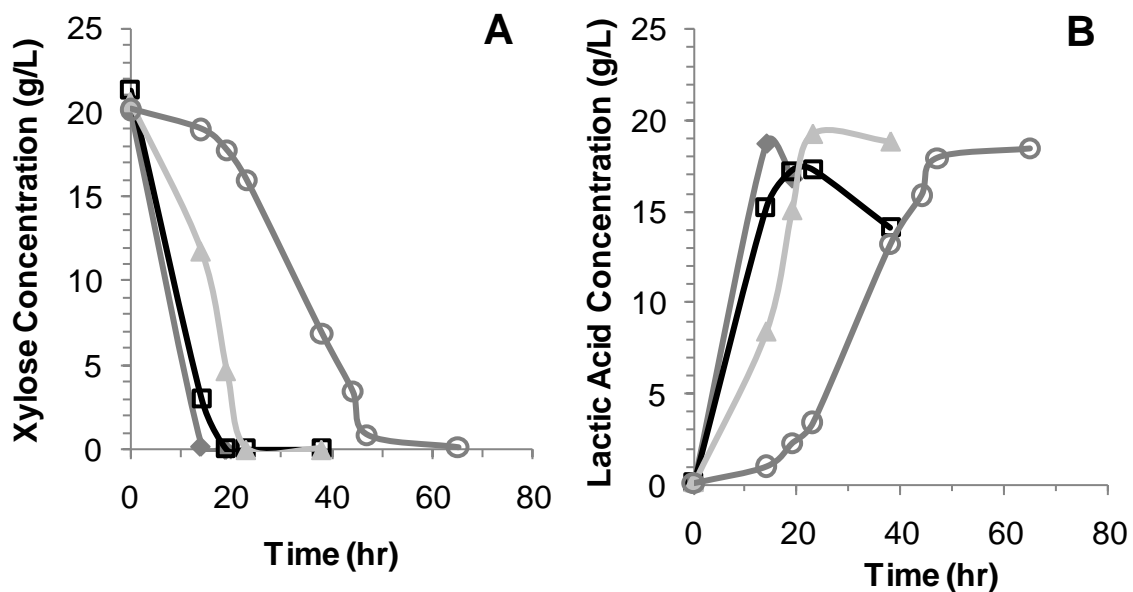


Figure 32. Effect of acetic acid concentration on xylose consumption (A) and lactic acid production (B) by *Bacillus coagulans* MXL-9. Fermentation performed in the presence of: 0 g/L acetic acid (◆), 10 g/L acetic acid (□), 20 g/L acetic acid (▲), 30 g/L acetic acid (○).

When alkaline chemicals such as green liquor are used to perform hemicellulose extraction the residual sodium concentration can also impact the level of microbial inhibition. For an extract made with 2% green liquor the sodium concentration is 3 g/L. If evaporation methods were used to concentrate the dilute monosaccharides 10-fold to 30 g/L sodium, the data in Figure 33A suggests that no xylose consumption could be expected. For sodium concentrations up to 20 g/L the organism required a longer lag phase, seen in Figure 33B, but was able to adjust and completely consume xylose with yields comparable to that of the control. However, at 30 g/L sodium no cell growth occurred over the course of 6 days. Table 15 summarizes the product yields obtained at varying concentrations of acetic acid and sodium. The tolerance of *Bacillus coagulans* MXL-9 to these inhibitors was found to be sufficiently high to predict that it could grow in hemicellulose extracts.

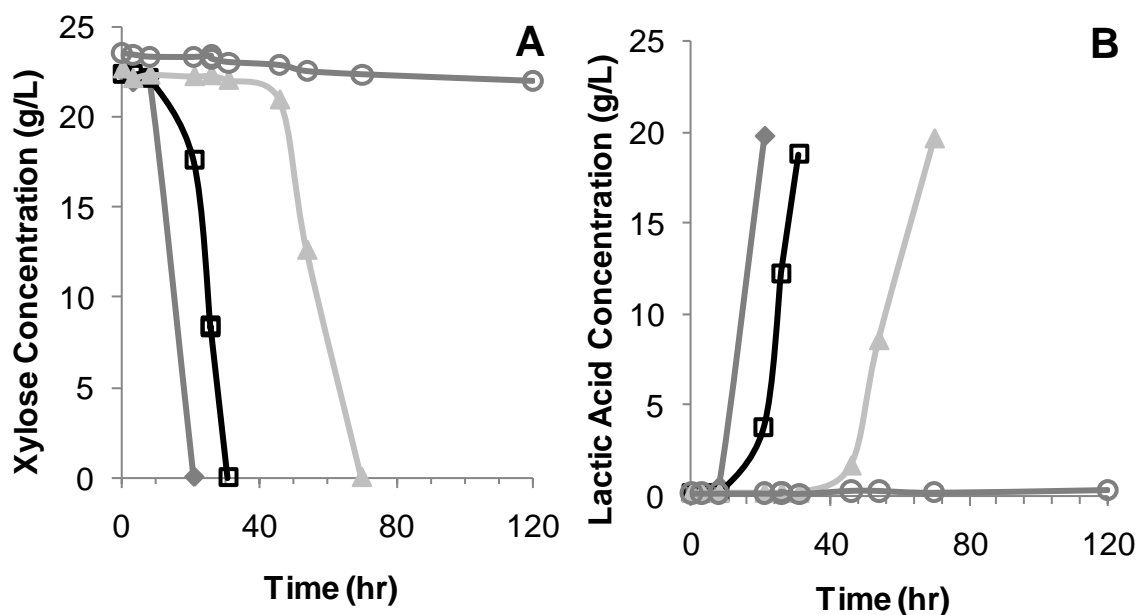


Figure 33. Effect of sodium concentration on xylose consumption (A) and lactic acid production (B) by *Bacillus coagulans* MXL-9. Sodium added in the form of sodium sulfate. Fermentation performed in the presence of: 0 g/L sodium (◆), 10 g/L sodium (□), 20 g/L sodium (▲), 30 g/L sodium (○).

Table 15. Product yields achieved by *Bacillus coagulans* MXL-9 in the presence of varying inhibitor concentrations.

Inhibitor Concentration	Xylose Consumed (g/L)	Lactic Acid Produced (g/L)	Formic Acid Produced (g/L)	Acetic Acid Produced (g/L)	Ethanol Produced (g/L)	Lactic Acid Yield (%)	Total Products Yield (%)
0 g/L Acetic Acid	20.1	18.8	1.4	0.7	1.2	93.2	109.5
10 g/L Acetic Acid	21.4	17.3	1.1	0.0	1.0	81.0	90.7
20 g/L Acetic Acid	20.6	19.3	0.5	0.0	0.8	93.6	99.8
30 g/L Acetic Acid	20.2	18.5	0.4	0.0	0.4	91.4	95.3
0 g/L Sodium	22.5	19.8	1.7	0.9	0.1	87.3	99.3
10 g/L Sodium	22.4	18.8	1.3	0.8	0.0	83.1	92.4
20 g/L Sodium	22.7	19.6	1.3	0.7	0.0	85.9	94.5
30 g/L Sodium	0.8	0.0	0.0	0.0	0.0	2.6	2.6

### 6.2.2 Fermentation of Hemicellulose Extracts

The hemicellulose extracts that were tested for fermentation by *Bacillus coagulans* MXL-9 include hot water extracted Siberian larch (softwood), hot water

extracted mixed hardwoods, and ultra filtered 2% and 4% green liquor extracted hardwood. Extracts were hydrolyzed by sulfuric acid prior to fermentation. Compositional analysis of the hemicellulose extracts tested is given in Table 16. Water extracted larch contained the highest concentration of monosaccharides, which were mainly derived from arabinogalactans. Galactose comprised 55.1% of the available sugar, while arabinose represented 15.6%. Xylose (12.5%), mannose (11.7%) and glucose (5.1%) were also present, resulting in a total of 45 g/L monosugars available for fermentation. Extracts produced from the mixed hardwood chips contained xylose as the principal sugar. Hydrolyzed hot water extracts contained 21.4 g/L of sugar, comprised of 70% xylose, and 7-8% each of galactose, mannose, glucose and arabinose. Hot water extracts do not have the issue of sodium inhibition, and acetic acid is present at lower concentrations than in alkaline extracts, at 1.9 g/L in larch extracts and 5.8 g/L in hardwood extracts. Furfural concentration is slightly higher in the hot water extracts compared to alkaline, as is acid soluble lignin, both of which are potential inhibitors of cell growth.

Table 16. Composition of hemicellulose extracts before and after fermentation by *Bacillus coagulans* MXL-9

	Concentration (g/L)					
	Hot Water Extracted		Conc. Green Liquor		Hot Water Extracted	
	Hardwood		Extracted Hardwood		Siberian Larch	
	0 hrs	28 hrs	0 hrs	22 hrs	0 hrs	73 hrs
Glucose	1.6	0.0	1.0	0.0	2.3	0.0
Mannose	1.6	0.2	0.2	0.0	5.4	0.0
Galactose	1.7	0.5	3.7	0.7	25.3	0.0
Xylose	14.9	0.0	8.9	0.0	5.7	0.0
Arabinose	1.5	0.0	1.8	0.0	7.1	0.0
Lactic Acid	0.7	20.9	1.6	14.5	0.1	33.5
Formic Acid	0.9	1.3	2.4	2.3	0.5	2.5
Acetic Acid	5.8	5.8	8.9	8.8	1.9	3.3
Ethanol	0.0	0.0	0.0	0.0	0.0	0.0
Furfural	0.4	0.0	0.0	0.0	0.3	0.0
Total sugar	21.4	0.7	15.6	0.7	45.9	0.0

The *Bacillus coagulans* MXL-9 is capable of consuming all five of the monosaccharides found in lignocellulose. The organism has a marked preference for glucose and mannose, where Figure 34 shows that both of these hexose sugars in a model sugar system representative of larch extracts were consumed rapidly in the first 10 hours of fermentation. After depletion of glucose and mannose there was an extended 12 hour lag phase where the metabolism shifted, after which consumption of arabinose, xylose and galactose began. After 50 hours the organism had produced 40.5 g/L of lactic acid by consuming 45.8 g/L of monosaccharides, for a yield of 88.6%.

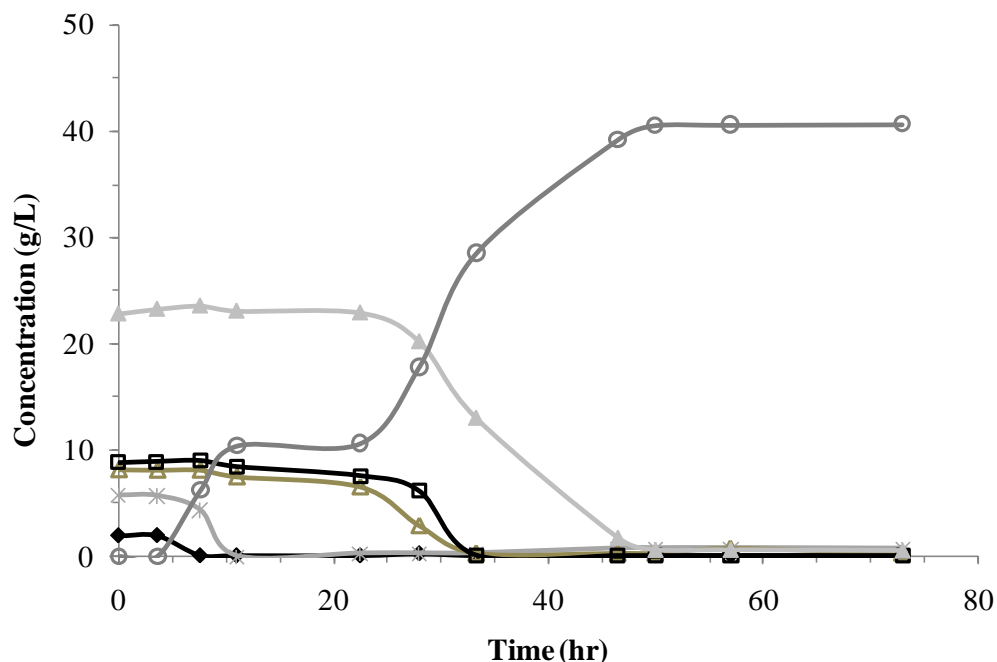


Figure 34. Fermentation of five sugar model representative of larch extract  
Lactic acid (○), glucose (◆), Mannose (✱), Arabinose (△), Xylose (□), Galactose (▲).

The actual larch extract contained several inhibitory substances such as acetic acid, furfural, HMF and lignin degraded phenolics which resulted in a longer initial lag phase and lower yield. Again glucose and mannose were consumed first, followed by a 12 hour lag before xylose, arabinose and galactose consumption. Figure 35 shows that fermentation was complete after 58 hours, where 33 g/L lactic acid was produced from 44 g/L sugar, at a yield of 75%. Furfural (0.25 g/L) was entirely metabolized by the organism within 20 hours, before metabolism of xylose, arabinose and galactose commenced. A variety of other bacteria and yeasts have been shown to possess enzymes for transforming furfural into either furfuryl alcohol or furoic acid which are less toxic to the cells (Gutiérrez *et al.*, 2006). Organisms with the ability to metabolize furfural have been investigated as potential biological detoxification agents in the treatment of lignocellulosic biomass (Lopez *et al.*, 2004). *Bacillus coagulans* MXL-9 is a promising

organism for fermentation of lignocellulose feedstocks due to its ability to simultaneously detoxify the inhibitory furans while converting all of the sugars into lactic acid at high yields.

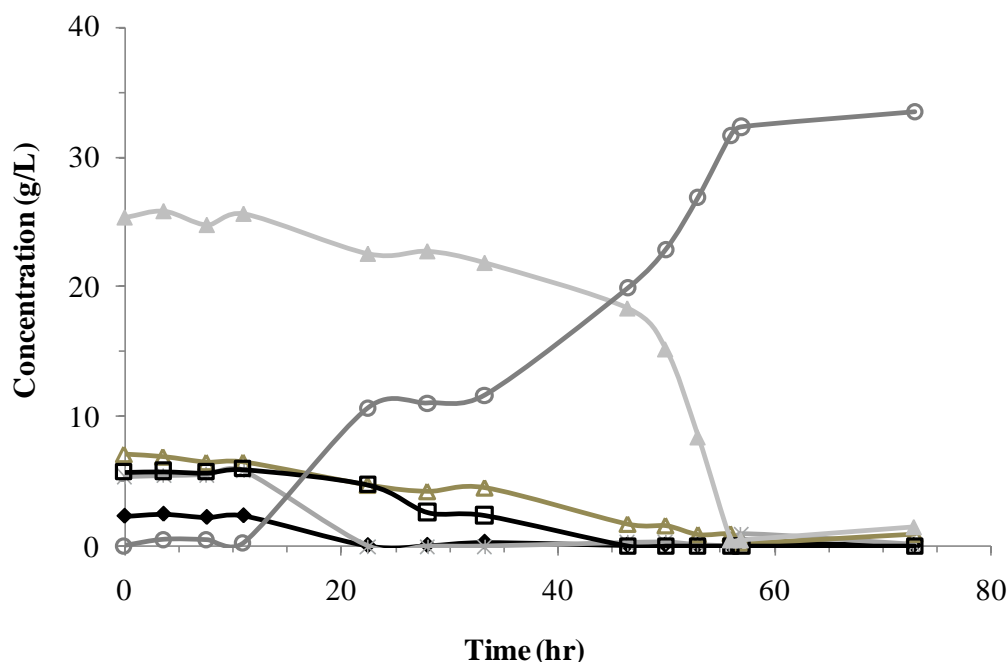


Figure 35. Fermentation of hot water extracted larch  
Lactic acid (○), glucose (◆), Mannose (⊠), Arabinose (△), Xylose (□), Galactose (▲).

The 4% green liquor extract shown in Figure 36 had been concentrated three-fold by ultrafiltration at 15 kilodaltons. The total monosaccharide concentration before fermentation was 15.6 g/L and contained mainly xylose as shown in Table 16. Fermentation was complete after 22 hours. For hardwood hemicellulose extracts an intermediate lag phase was not required for metabolism to shift, likely due to the much lower concentrations of furfural, galactose and arabinose. Green liquor extracts initially contained 1.5 g/L of lactic acid formed from sugar degradation through the alkaline peeling reactions, and an additional 13 g/L of lactic acid were produced by the organism during fermentation. The yield on lactic acid produced through fermentation was 83%.

The green liquor extracts contain the highest acetic acid concentration, 8.9 g/L. While acetic acid is sometimes a byproduct of *Bacillus coagulans*, no additional acetic acid was formed, nor were any other byproducts.

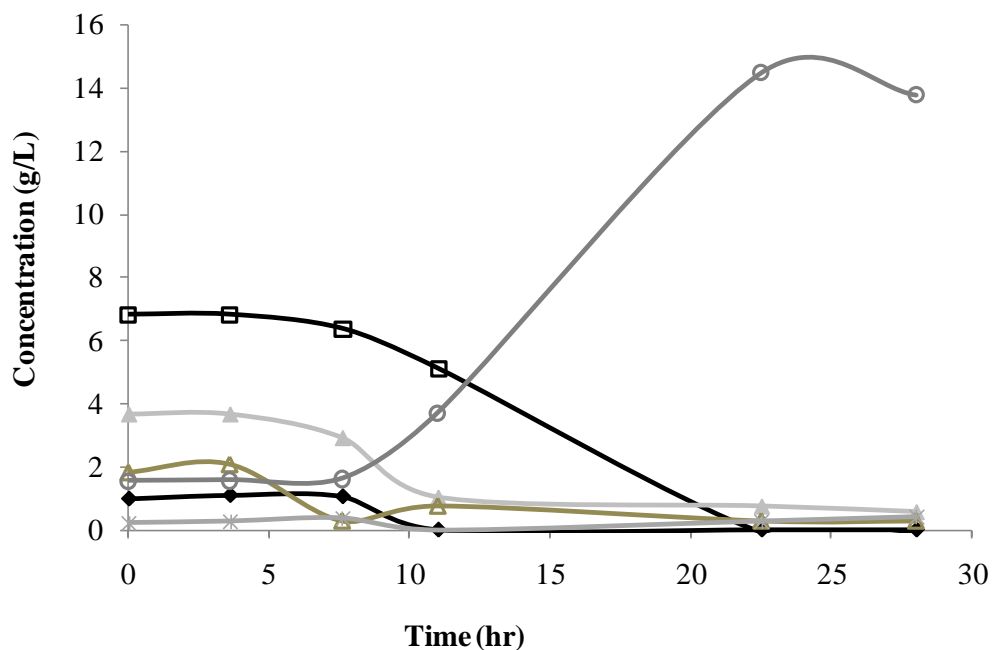


Figure 36. Fermentation of ultrafiltered green liquor extract  
Lactic acid (○), glucose (◆), Mannose (✱), Arabinose (△), Xylose (□), Galactose (▲).

Fermentation of hot water extracted mixed southern hardwoods is shown in Figure 37. The extracts contained 21.4 g/L of monosaccharides, primarily xylose, and 5.8 g/L acetic acid. Following an initial lag phase of 10 hours, all of the sugars were consumed within 24 hours without an intermediate lag phase. Production of 20.8 g/L lactic acid represented a 94% yield based on the initially available monosaccharides, excluding the lactic acid already present in the extracts. Table 16 shows that no byproducts of acetic acid or ethanol were formed and formic acid showed only a minor increase. Furfural (0.4 g/L) was completely metabolized by the organism.

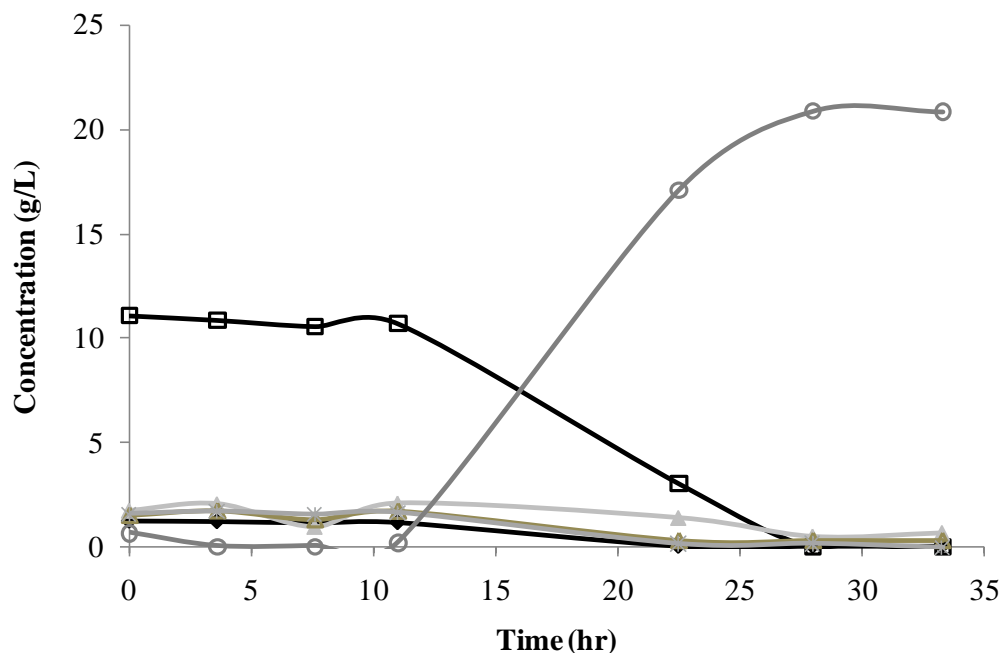


Figure 37. Fermentation of hot water extracted southern hardwood  
Lactic acid (○), glucose (◆), Mannose (※), Arabinose (△), Xylose (□), Galactose (▲).

*Bacillus coagulans* is well suited to simultaneous saccharification and fermentation (SSF) because of its compatibility with the temperature and pH optima of enzymes. In addition some strains of this organism have been found to have inherent hemicellulose degrading enzymes, such as strain BL69 which was reported to contain xylanase activity (Heck *et al.*, 2005). Fermentation of unhydrolyzed hemicellulose extracts directly into lactic acid without the need for additional enzyme or chemical hydrolyzing agents would be the ideal performance. Experiments with strain MXL-9 showed that unhydrolyzed hot water extracts of hardwood contained 5 g/L of monosugars and an estimated 19 g/L of oligomeric sugars at the start of fermentation. After a week of fermentation, the culture produced 5 g/L of lactic acid, which indicates that there was not a significant breakdown of oligomeric sugars, but all sugar present in monomeric form was utilized.

### 6.2.3 Comparison of Ethanol and Lactic Acid Fermentations

Fermentation of hemicellulose extracts into lactic acid has an advantage over the fermentative production of fuel ethanol because the metabolic pathway does not result in production of carbon dioxide. Production of ethanol has a maximum yield of only 0.51 grams of ethanol per gram of product due to CO<sub>2</sub> formation, whereas lactic acid producing organisms do not cycle as much carbon into waste products and can therefore achieve higher product yield. This yield increase is particularly beneficial in a process such as converting hemicellulose extracts, which are relatively low in sugar content and high in inhibitor content. Increasing the sugar concentration prior to fermentation is necessary to achieve a high enough product titer for an economically viable process. Methods for increasing sugar concentration also increase the levels of inhibitors such as lignin, and may increase organic acid and salt concentrations. If the fermentation yield can be doubled by avoiding CO<sub>2</sub> generation then only half as much effort is expended to concentrate the feedstock and inhibitors only accumulate by half. Lactic acid is therefore easier than fuel ethanol to produce in an economically feasible manner. The market price for lactic acid is approximately twice as high as that of fuel grade ethanol, estimated at \$1000/MT for lactic acid compared to \$500/MT for ethanol (Horhammer et al., 2009).

A direct comparison of ethanol and lactic acid fermentation is shown in Figure 38. The feedstock was a 2% green liquor extract of hardwood chips which had been concentrated 7-fold by ultrafiltration at 50 kD. The initial monosaccharide concentration was 35 g/L and consisted predominantly of xylose with 4-7% each of glucose, arabinose, mannose and galactose. Also present initially were 12 g/L of acetic acid, 1.8 g/L of formic acid and 0.7 g/L of lactic acid. Fermentation into ethanol by *E. coli* K011 was

slightly faster but far less efficient than the fermentation into lactic acid by *B. coagulans* MXL-9. The highest ethanol titer of 12 g/L was obtained after just 28 hrs but represents a yield of only 32% based on the initial sugar concentration. Alternately the highest lactic acid titer was 26 g/L obtained at 68 hrs, and neglecting the lactic acid initially present in the extract itself, this represents at 74% production yield. Thus lactic acid was shown to be produced at more than twice the efficiency of ethanol from the concentrated green liquor hemicellulose extracts.

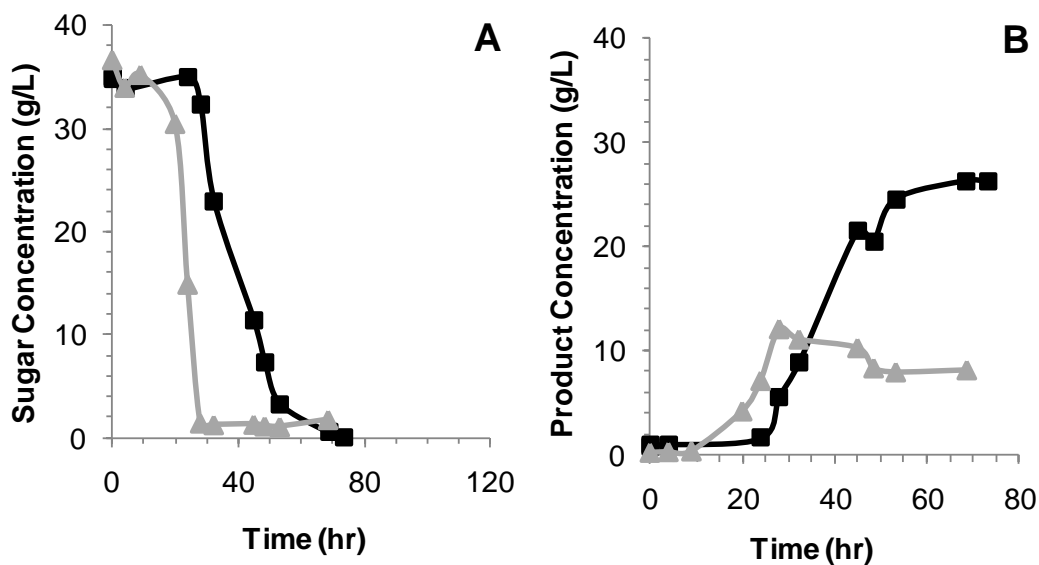


Figure 38. Comparison of lactic acid and ethanol fermentation on ultrafiltered green liquor extracts. 2% green liquor extracts, 600 hrs H-Factor were concentrated 7-fold using a 50 kD ceramic membrane. *Bacillus coagulans* MXL-9 lactic acid fermentation (■), *Escherichia coli* K011 ethanol fermentation (▲)

### 6.3 Conclusions

*Bacillus coagulans* MXL-9 is capable of consuming all of the principal monosaccharides found in hemicellulose extract and producing lactic acid at high yields. In softwood extracts glucose and mannose are consumed preferentially, followed by an

intermediate lag phase during which metabolism shifts to xylose, arabinose and galactose consumption. In hardwood extracts the same preference for glucose and mannose was observed but an intermediate lag phase was not required. This organism has relatively high tolerance for inhibitors found in hemicellulose extract including acetic acid and sodium, and has the ability to detoxify furfural. The ability to consume a wide range of sugars, grow at 50°C and pH 5-7 makes this bacteria well suited to SSF operations for lignocellulosic feedstocks. Hemicellulose extracts containing 45 g/L of monosaccharides were converted into 33 g/L lactic acid, which represents a 14% decrease in yield and 8 hours increased fermentation time compared to an uninhibited control containing the same amounts of each sugar.

## CHAPTER 7: SUPPORTING EXPERIMENTS AND RESULTS

The following chapter details experiments performed on hemicellulose extracts which provide further insight into their characteristics and processing but were not presented in journal publications. Additional details on the process development are provided. Detoxification methods for improving fermentation yields were considered which include use of hydrophobic polymeric absorbents and liquid-liquid extraction. Enzymatic hydrolysis was examined as an alternative to sulfuric acid, and was also tested simultaneously with fermentation. Some additional fermentation microbes were tested on hemicellulose extracts and found to perform poorly due to inhibition.

### 7.1 Introduction

#### 7.1.1 Hemicellulose Extracts Supplemental

Hemicellulose extraction has been investigated by other researchers in this group and results published in studies such as that of Tunc and van Heiningen (2008). Initial work by the group was concerned only with quantifying the amount of material removed from the wood and the subsequent effects on pulping, with no consideration given to the issues of fermentation. This work looks specifically at the feasibility of using hemicellulose extracts as a feedstock for fermentation, and as such the primary concern was with quantifying the sugars and inhibiting compounds in the extract at each stage of processing. The focus therefore shifts from defining mass removed in terms of g/g of original wood to defining concentration in g/L of the extract itself. If processing steps such as hydrolysis result in dilution of the extract solution, the dilution is not corrected for because it cannot be removed without the addition of energy-requiring further steps. There are many unit operations that are necessary to process the raw hemicellulose

extraction liquor into a fermentable solution, and each of these steps required some trial and error in developing the methods employed. Further optimization is still needed. The following sections detail some of the considerations that have already been evaluated in development of concentration, hydrolysis, neutralization, detoxification and fermentation methods.

#### 7.1.1.1 Hemicellulose Extract Concentration

When the idea of fermenting hemicellulose extracts was first proposed, it was not yet known that the sugar content was much too dilute. Distillation of the final product is an energy intensive operation that requires a minimum ethanol feed of 4-5% to be accomplished economically, and to meet this requirement the feed to fermentation must contain at least 8-10% monosugars. The 3% green liquor extracts which had been selected for study on the basis of favorable pulp yield needed to be concentrated by 20-fold to reach this sugar target. Chapter 4 details this initial work where evaporation was used to remove water. Evaporation was not an ideal solution to the ultimate goal of reducing energy input, because any operation where steam must be generated is highly energy intensive.

#### 7.1.1.2 Hydrolysis Methods

A techno-economic study by So and Brown (1999) estimated the relative costs of dilute acid hydrolysis compared to enzyme hydrolysis. For two plants utilizing wood, (not pre-pulping) each with uniform annual ethanol production of 25 million gallons, the SSF production cost was \$1.28/gal while acid hydrolysis was marginally higher at \$1.35/gal. Acid hydrolysis is more efficient, with an ethanol yield of 95 gal/ton compared

to 93 gal/ton for SSF. The capital costs and operating costs for both processes were similar. The process considered in this study converts both cellulose and hemicellulose into ethanol, and the initial sugar concentration of 100 g/L is significantly higher than that of hemicellulose extracts which do not utilize cellulose (So and Brown, 1999). An assessment of whether acid or enzymatic hydrolysis leads to lower production costs for the specific case of the hemicellulose extraction process would depend on the relative yields, reaction times, and reactor costs for each process. Preliminary yield and reaction time data was obtained for each method in this work.

#### 7.1.1.3 Neutralization vs. Overliming of Acid Hydrolyzate

Following acid hydrolysis, the pH of the extract must be raised from 1 to the optimal fermentation pH of the organism being used, between 5 and 7. This may be done directly through neutralization with a base such as calcium hydroxide, or through the process of overliming. Overliming is a commonly practiced method of detoxification for lignocellulosic hydrolysates in which the pH is raised from 1 to 10 by addition of calcium hydroxide, and then lowered back to the neutral fermentation pH by addition of an acid such as HCl or H<sub>2</sub>SO<sub>4</sub> (Mohagheghi *et al.*, 2006). It is practiced especially prior to the fermentation of acid pre-treated biomass because it has been shown to reduce furfural and phenolic compounds. A study by Martinez *et al.* found that optimum overliming of bagasse hemicellulose hydrolysates reduced furans by 51±9%, phenolics by 41±6% and sugar by 8.7±4.5%, where hydrolysates initially contained 95±7 g/L total sugar, 1.3±0.3 g/L furans and 2.9±0.3 g/L phenolics (Martinez *et al.*, 2001). These hemicellulose hydrolysates produced via dilute acid pretreatment allow for more degradation of cellulosic material than the autohydrolysis or alkaline pretreatment generated

hemicellulose extracts considered in this work, and therefore have substantially higher sugar concentrations as well as higher inhibitory furan concentrations. Overliming was initially considered for pre-pulping hemicellulose extracts, but was ultimately deemed an unnecessary step which would increase cost of production without justifiable benefit. Overliming increases cost because of extra calcium hydroxide needed to reach the higher pH, the addition of chemical costs for the acid that is then needed to neutralize, and the added disposal cost associated with increased production of gypsum. The degradation of sugars observed during overliming is less significant in the acid pretreatment methods than in the alkaline pre-pulping extraction where sugar concentrations are already too low to allow for losses.

#### 7.1.2 Enzyme Hydrolysis and SSF

Enzymatic hydrolysis should be considered for the conversion of hemicellulose oligomers into monosaccharides because the biological method is a far more environmentally benign option. Enzymes are significantly slower than acid hydrolysis and may have lower yields, but also do not produce as many degradation products. Reactors for enzyme hydrolysis need to be larger to generate the same amount of sugar in the same time span as acid hydrolysis, but the materials of construction can be less costly because they do not need to withstand low pH or high temperature. Simultaneous enzyme saccharification and fermentation consolidates two steps into one reactor, which can lower capital costs. SSF may require the fermentation organisms and the enzymes to work at conditions which are a compromise between their respective optima.

### 7.1.3 Additional Fermentation Organisms

There are vast numbers of organism which can be found in nature or engineered to consume lignocellulosic biomass. For economic conversion of hemicellulose extracts there are a number of criteria that an organism must meet. The organism must be able to consume all of the pentose and hexose sugars derived from biomass, and withstand inhibition by the acids, salts and lignin derived phenolics present in the extract. While it is possible to remove these compounds prior to fermentation, it is economically more favorable if the microbe can tolerate them so that additional processing steps are not needed. An ideal organism for the conversion of hemicellulose extracts into valuable fuels and chemicals would actually be able to consume oligomers such as xylan directly without requiring hydrolysis. *Clostridium phytofermentans* is an organism that has been shown to degrade xylan into ethanol and hydrogen (Warnick *et al.*, 2002). It is an obligate anaerobe isolated from forest soil which also consumes a variety of other substrates including cellulose and all of the major monosaccharides associated with woody biomass.

### 7.1.4 Inhibitor Removal

In Chapter 5 it was shown that hemicellulose extracts require removal of water, but that performing evaporation concentrates the inhibitory components too quickly. Chapter 4 details the concentration of hemicellulose extracts by ultrafiltration and also shows a substantial rise in inhibitory lignin concentrations. Some work on removal of inhibitors prior to fermentation was conducted, utilizing either hydrophobic polymeric absorption methods or liquid-liquid extraction. Hydrophobic absorption was mainly targeted to the removal of phenolic compounds including lignin degradation products and

furfural. Hydrophobic polymeric absorbents such as XAD-4 resin have been shown to remove furans and phenolic compounds from lignocellulosic feedstocks (Weil *et al.*, 2002).

## 7.2 Results and Discussion

### 7.2.1 Hemicellulose Extraction Supplemental

#### 7.2.1.1 Reactor Operating Temperature and Pressure

Hemicellulose extracts were characterized by the H-Factor of cooking, a kinetic parameter which incorporates the reactor heating and cooling into the severity. The actual temperature profile over the course of extraction in the 20 L rocking digester can be seen in Figure 39. In addition, the reactor pressure is shown for both water extracts and 3% green liquor extracts, where it can be observed that green liquor causes the vessel to reach a higher pressure. During water extraction, the maximum reactor pressure reached was 76 psig. At the extraction temperature of 160°C, this is close to the pressure of saturated steam which is 74.9 psig. However at 3% green liquor the pressure in the vessel reached a maximum of 90 psig due to the evolution of CO<sub>2</sub> from sodium carbonate.

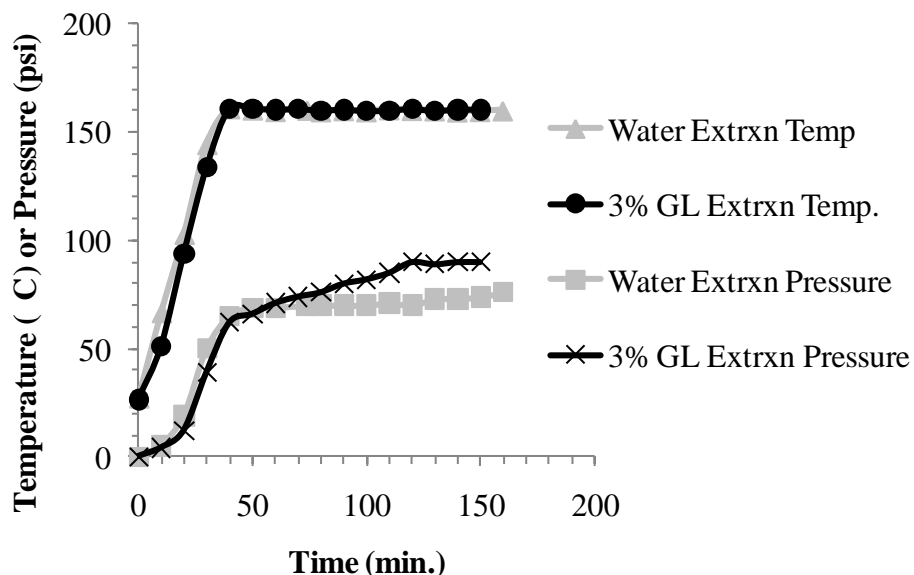


Figure 39. Temperature and pressure profiles of rocking digester  
Extracts produced from northern hardwood chips in the 20 L rocking digester at 160°C for 110 min.

#### 7.2.1.2 Acid Hydrolysis of Hemicellulose Extracts

Initial hemicellulose extract hydrolysis experiments were performed using the standard hydrolysis conditions cited in literature of 4% w/w sulfuric acid (Sluiter *et al.*, 2008). When the xylose concentrations being measured in green liquor extracts were lower than expected, it was realized that the extracts have considerable buffering capacity which was keeping the pH higher than 4% acid would indicate. For example, when 3% green liquor extracts were adjusted to 4% sulfuric acid, the pH only dropped from 5.8 to 1.7 instead of 1.0. Figure 40 shows the titration of extracts of various green liquor concentrations from 0% to 6%, and also of 4% sodium carbonate extracts. It can clearly be seen that higher concentrations of green liquor require more sulfuric acid addition to reach a pH of 1.0, which is the target hydrolysis pH. Water extracts have less buffering capabilities and pH 1 was reached with only 40 mL of 0.5N H<sub>2</sub>SO<sub>4</sub> added to 50 mL of

extract, whereas 6% green liquor extract required 130 mL to reach the same pH. This finding led us to perform all further acid hydrolysis experiments by adding acid as required to reach pH 1.0 for each individual sample, rather than at a uniform concentration of 4% sulfuric acid. It can also be observed from Figure 40 that 4% green liquor and 4% sodium carbonate extracts follow the same titration curve, indicating the importance of carbonate to buffering the pH.

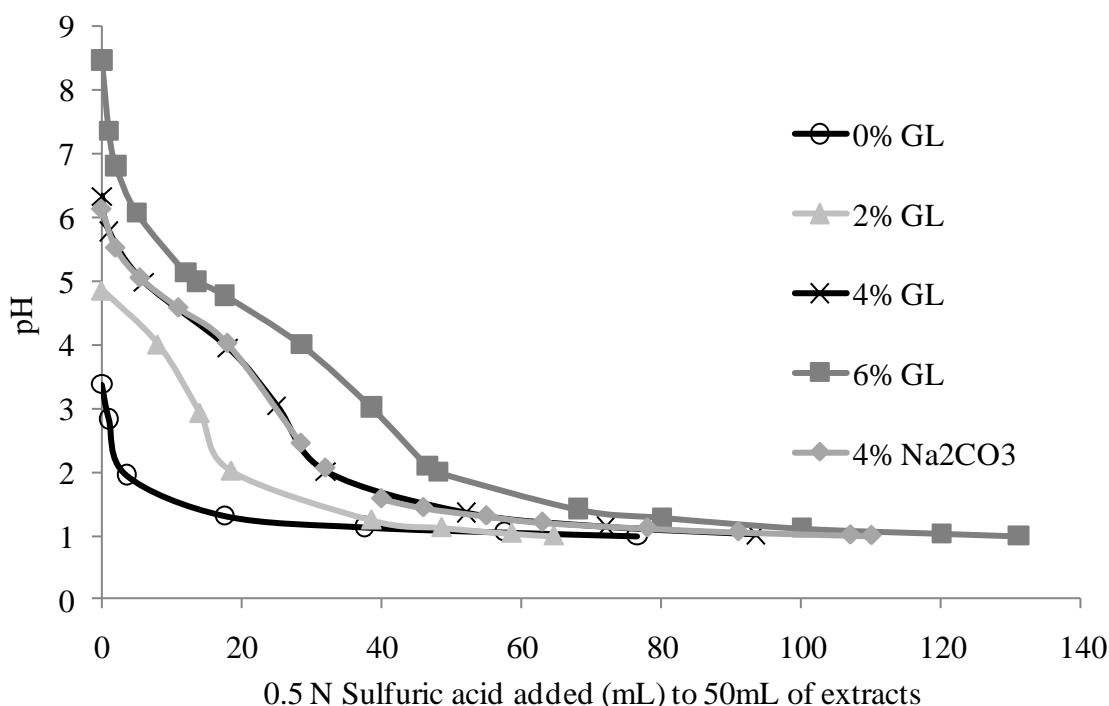


Figure 40. Titration of hemicellulose extracts to determine buffering capacity

#### 7.2.1.3 Neutralization and Overliming of Acid Hydrolyzate

The initial process flow sheet for converting pre-pulping hemicellulose extracts into ethanol was based on the NREL design report by Wooley *et al.*, which included overliming as a detoxification step prior to fermentation. Overliming the hydrolyzate to pH 10 was shown to reduce the concentration of inhibitors such as furfural and lignin

degraded phenolics, but it also caused a minor loss of fermentable sugars (Mohagheghi *et al.*, 2006). In the dilute acid pretreated material which NREL utilized, this loss was deemed acceptable because the benefits of inhibitor removal outweighed the loss, and there was significantly more sugar available. Green liquor hemicellulose extracts which have too little sugar to begin with cannot afford to undergo a process which degrades them. Because green liquor extracts are produced under alkaline conditions they do not contain furfural or HMF in measurable quantities, and if acid hydrolysis is carried out at optimum conditions they will not generate significant furans, therefore detoxification by overliming can be avoided. The extract pH does need to be raised after acid hydrolysis, but it does not need to be taken above neutral, thus saving costs of additional calcium hydroxide and acid to lower the pH from 10 back to neutral. Figure 41 shows five samples of a 3% green liquor extract that were acid hydrolyzed and then raised to different pHs. Samples adjusted to pH 6-8 all contained about 3.5 g/L xylose, while a sample that was overlimed to pH 11 contained only 3.2 g/L. While this is a small difference in concentration, it does represent an 8.5% loss which was consistent with degradation results seen in literature (Martinez *et al.*, 2001).

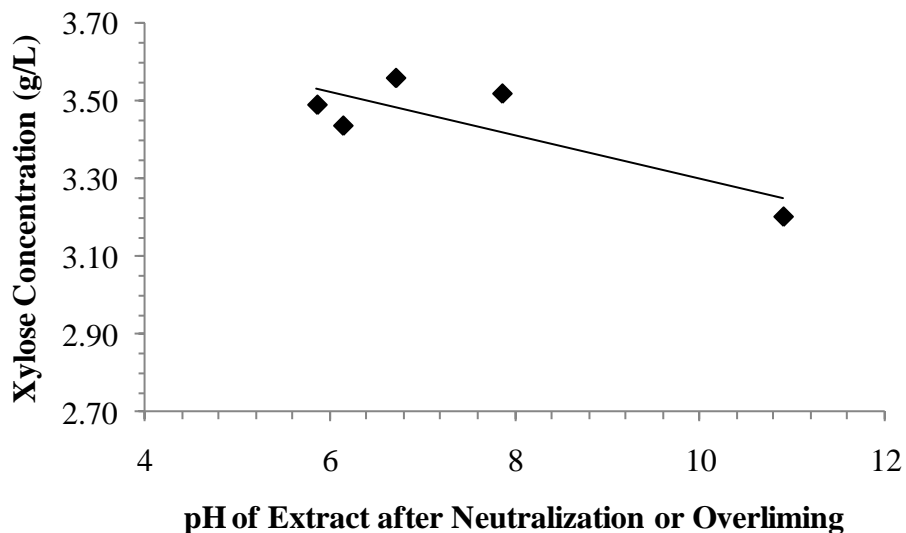


Figure 41. Effect of pH adjustment on extract sugar concentration  
 Extracts were made with 3% green liquor at an H-Factor of 800 hrs. Following acid hydrolysis at pH 1.0 and 120°C for 60 min the pH was adjusted by addition of  $\text{Ca}(\text{OH})_2$ . Four samples were neutralized while one was overlimed.

Extracts which had undergone evaporation to increase the sugar concentration were also observed to experience sugar degradation as the pH was increased. Figure 42 shows the effect of pH adjustment on the xylose content of an evaporated 3% green liquor extract. The extract had been hydrolyzed after centrifugation at 4000RPM for 0, 60 or 120 min. It was later found that centrifugation prior to hydrolysis results in additional sugar loss because the carbohydrates are bound to the lignin. Considering only the 60 minute centrifuged samples however, there was a  $15 \pm 6\%$  drop in xylose between the samples adjusted to pH 5 and pH 8. Changes in lignin concentration were not quantified, but furans can be seen in Figure 43 to decrease with increasing pH. Because the generation of furfural can be minimized by optimizing hydrolysis for each type of extract, overliming is not likely to benefit the overall fermentation yields. It is therefore

not recommended in the preparation of green liquor or water hemicellulose extracts for fermentation.

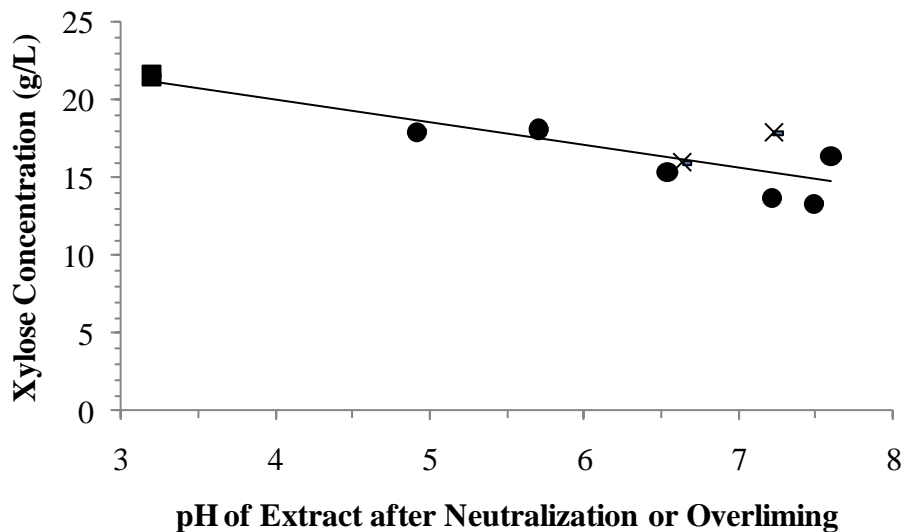


Figure 42. Effect of pH adjustment on evaporated extract sugar concentration  
3% green liquor extracts were evaporated to 30% solids and nine samples were acid hydrolyzed with varying centrifugation times prior to hydrolysis of 0 min (■), 60 min (●) or 120 min (x) at 4000 RPM.

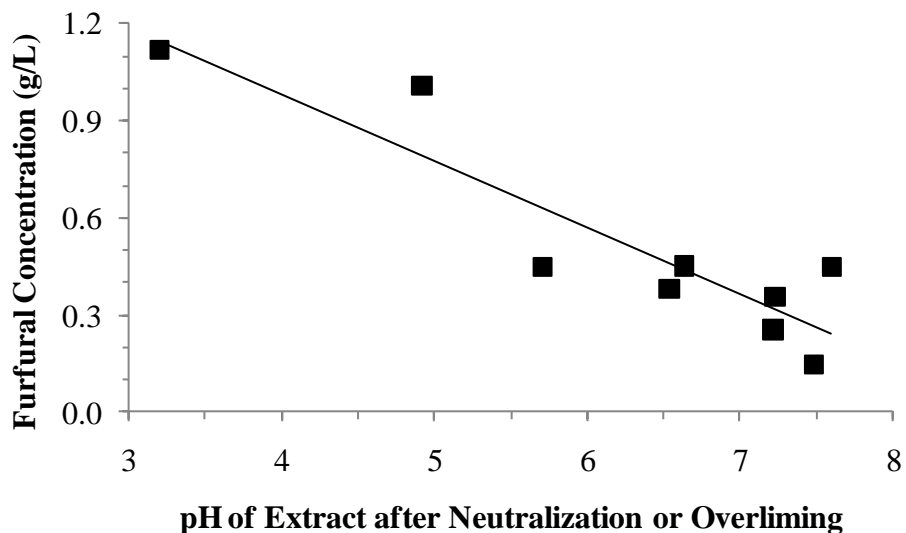


Figure 43. Effect of pH adjustment on evaporated extract furfural concentration

#### 7.2.1.4 Compositional Changes during Extract Processing

Throughout the process of preparing hemicellulose extracts for fermentation there are many steps which can lead to significant sugar losses. Figure 44 shows the compositional changes that occur in a hot water extract between its initial unhydrolyzed form and the final sterilization prior to fermentation. While 25 g/L xylose were present in the hydrolyzed extract, this dropped to 22 g/L after neutralization with calcium hydroxide and even further losses occurred during sterilization of the bioreactor at 121°C for 15 min. Comparing the acetic acid composition over these same processing steps it is clear that dilution is not the cause. Xylose is also not degrading into furfural, because a decrease in furfural is observed during sterilization. Lactic acid content is negligible in water extracts, and formic acid shows a decrease from 0.5 to 0.4 g/L during sterilization, indicating that neither peeling nor hydrolysis reactions occurred. A possible explanation for the drastic xylose reduction may be that condensation reactions are occurring. To avoid loss of the monosugars it is recommended to carry out fermentation without sterilizing the extracts where possible. Both *E. coli*, due to its chloramphenicol antibiotic resistance, and *Bacillus coagulans*, due to its thermophilic growth, could be cultured without sterilization of the bioreactors, though it is unknown how this compares to sterilized fermentation.

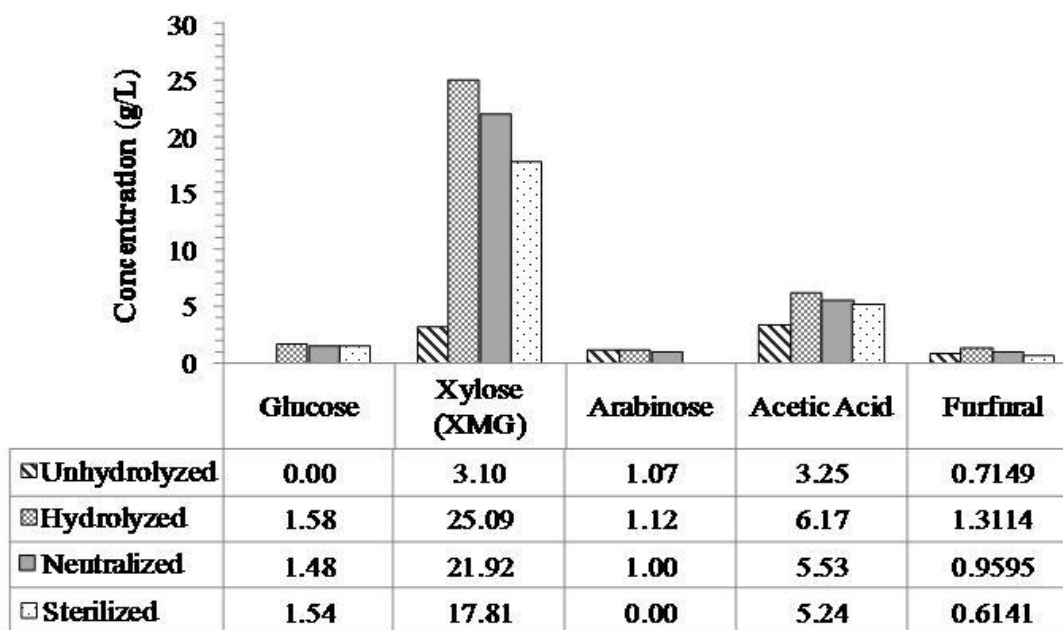


Figure 44. Composition of 800 hr H-Factor water extracts throughout processing

Similarly for 2% green liquor extracts the compositional changes throughout processing are shown in Figure 45. Green liquor extracts have comparatively lower proportions of monosugars present before hydrolysis, and acetic acid only increases by 3% during hydrolysis compared to the 90% increase observed for water extracts. Xylose concentrations dropped dramatically from 6.8 g/L after hydrolysis to only 3.3 g/L after sterilization, a loss of 49%. By observation of the other components it can be seen that dilution could only account for minor losses, and hydrolysis to furfural is clearly not occurring during sterilization. Slight increases in formic acid and lactic acid of 0.5 g/L and 0.7 g/L respectively were noted during sterilization, which could be caused by alkaline peeling reactions. Condensation reactions may further contribute to the loss of xylose. These losses must be avoided for an economically viable fermentation process.

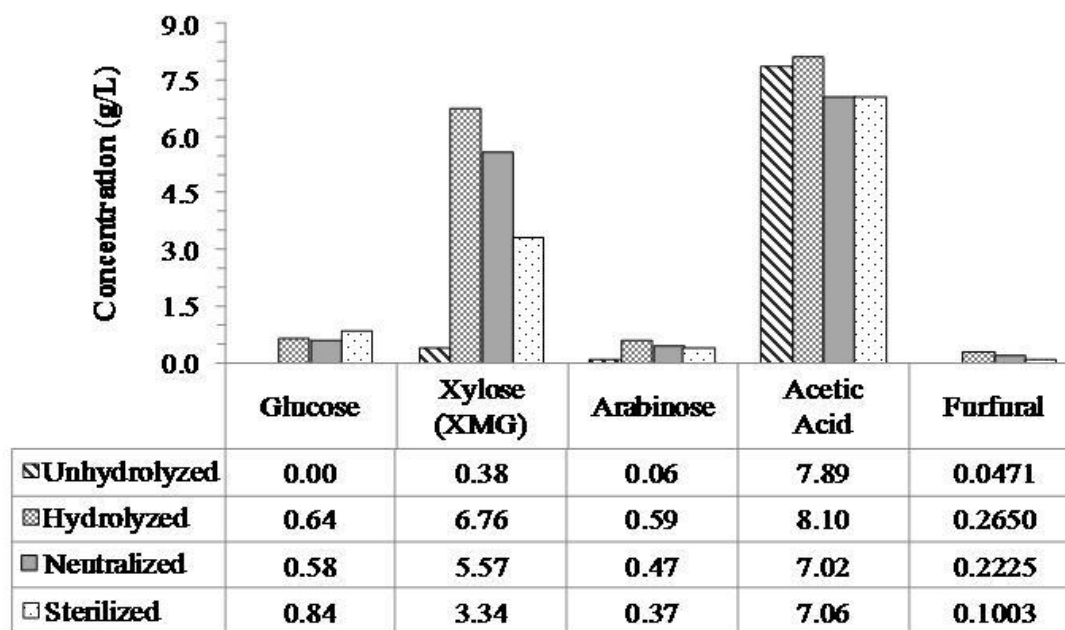


Figure 45. Composition of 800 hr H-Factor 2% green liquor extracts throughout processing

### 7.2.2 Enzyme Hydrolysis and SSF

Enzymes which degrade biomass generally have maximum activity in the pH range of 5-6 and temperature range of 40-60°C. For simultaneous saccharification and fermentation to be successful, the fermentation organism and the enzymes must work at compatible conditions. In the case of *E. coli* K011, the organisms' optimum temperature is 37°C and its optimum pH is 7 because acetic acid is present. A compromise or tradeoff must be decided upon if the organism and enzyme do not have the same optimum conditions. Generally the enzymes appear to be more flexible to adjustment of operating conditions. Figure 46 shows that enzymatic hydrolysis of hemicellulose extracts was slightly better at pH 5, the enzyme optimum than at pH 7, the organism optimum. Nevertheless, SSF cannot be conducted for *E. coli* at pH below 7 because the organisms will not grow.

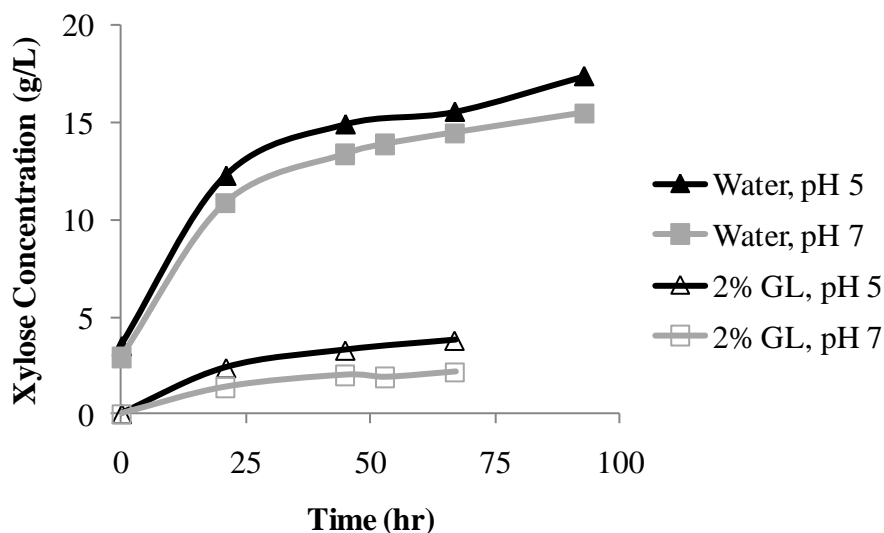


Figure 46. Enzymatic hydrolysis of hemicellulose extracts at enzyme optimum (pH 5) and organism optimum (pH 7) for water extracts and 2% green liquor extracts

Water extracted southern hardwood with an H-Factor of 800 hrs contained 22 g/L of xylose when acid hydrolyzed. Enzyme hydrolysis by Fluka xylanase released 17.4 g/L xylose at pH 5(79% yield) and 15.5 g/L at pH 7 (70% yield). 2% green liquor extract with an H-Factor of 800 hrs contained 6.9 g/L xylose when acid hydrolyzed. Enzymes released 3.8 g/L xylose at pH 5 (55% yield) and 2.2 g/L xylose at pH 7 (32% yield).

To demonstrate SSF at conditions more favorable to the enzymes, the 3% green liquor extracts described in chapters 4 and 5 were tested at pH 6. Seen in Figure 47A, the enzymes were able to release 56% of the xylose from oligomeric form, but *E. coli* K011 was unable to grow so no ethanol was produced. Because the extracts contain 10 g/L acetic acid which is more toxic to the cells at lower pH, the SSF only worked at pH 7, shown in Figure 47B.

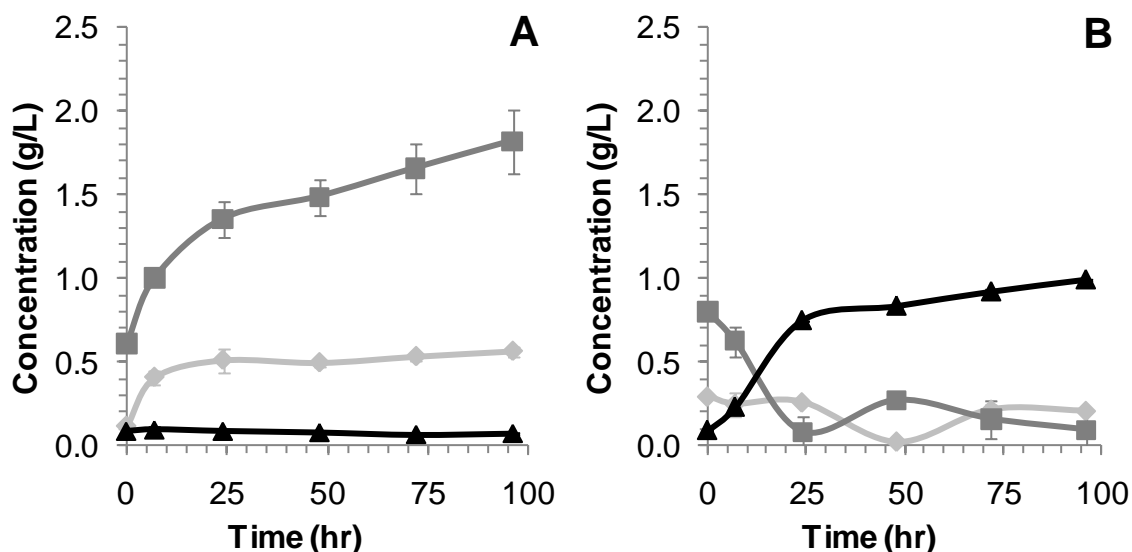


Figure 47. SSF of 3% green liquor extracts at pH 6 (A) and pH 7 (B)  
Glucose (♦), xylose (■) and ethanol (▲)

An alternative to SSF is to perform sequential hydrolysis and fermentation, where the enzymes are initially added to extracts at 50°C, pH 5 and then after hydrolysis has completed the temperature is lowered to 37°C and pH raised to 7 before *E. coli* K011 is added. SSF is more compatible with the *Bacillus coagulans* MXL-9 or other thermophilic fermentation organisms which have optimum conditions similar to enzymes.

Enzymatic hydrolysis and SSF in the presence of Sigma Antifoam O-30 in Dasgip bioreactors was entirely unsuccessful. Antifoam was added because hemicellulose extracts tend to have foaming problems when sparged with nitrogen to obtain anaerobic fermentation conditions. In the experimental set-up where enzyme hydrolysis and SSF performance were being compared side by side for both green liquor and hot water extracts, all reactors were sparged to generate uniform conditions – though sparging would not have been necessary for enzyme hydrolysis itself - and antifoam was added to prevent foam from overflowing. Enzymes showed no activity in the presence of the

antifoam, and no monomers were released from the hemicellulose. In the SSF reactors, ethanol was produced from the initially present monomers, leading to formation of 1.2 g/L ethanol in 3% green liquor extracts and 2.2 g/L ethanol in hot water extracts. The fermentation organisms were not affected by antifoam addition, but failure of the enzymes limited their available substrate. Enzymatic inhibition can likely be attributed to surface interactions between the surfactant molecules and enzyme binding sites. Further experiments in the Dasgip reactors were performed without antifoam to prevent enzyme inactivation, and therefore required that reactors could not be sparged with nitrogen. This led to two phase batch fermentation, wherein any oxygen initially present was consumed, favoring cell growth, and then anaerobic conditions were reached, favoring ethanol production.

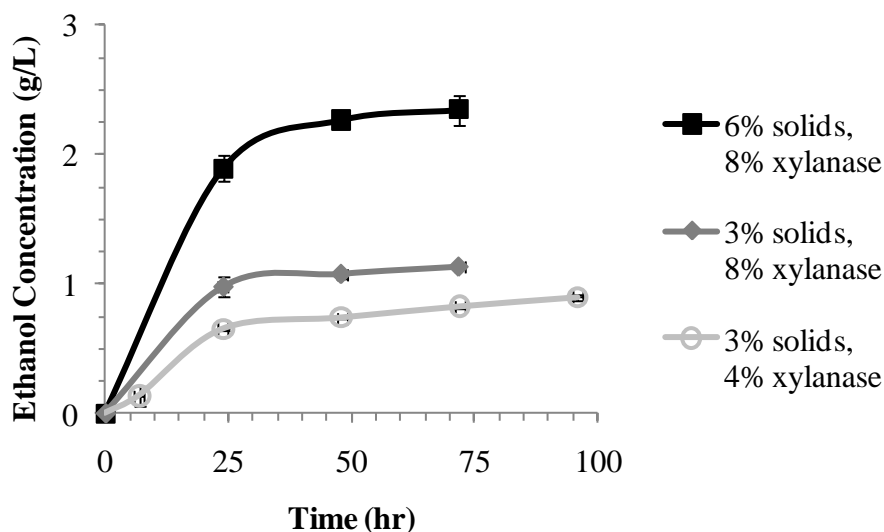


Figure 48. SSF of green liquor extracts at varying Fluka xylanase enzyme loadings

Enzymatic hydrolysis of hot water extracts made at an H-Factor of 800 hrs was tested for a variety of enzyme loading levels using the Fluka xylanase complex and enzymes from the Novozymes biomass hydrolysis kit. After the Fluka enzyme alone was

shown to be 70-80% effective compared to acid hydrolysis, this experiment was conducted to determine if the addition of accessory enzymes could improve the hydrolysis yield. From the Novozymes biomass hydrolysis kit, four enzymes were chosen: NS50012 complex, NS50030 xylanase, NS2202 hemicellulase, and NS50014 xylanase. Each of the four enzymes was added at an equal volume to the enzyme cocktail. Table 17 shows that a loading of 1 mL/L of each Novozymes enzyme released only 46% of the available xylose. As the concentration of the Novozymes enzymes increased the amount of xylose also increased, reaching a yield of 73% at 4 mL/L enzyme loading – equivalent to the yield obtained with just Fluka enzymes. Combining both the Fluka and xylanase enzymes led to further yield increases, where a maximum yield of 97% was obtained at an enzyme loading of 1 g/L Fluka xylanase plus 3 mL/L of each Novozymes enzyme.

Table 17. Enzymatic hydrolysis of hot water extracts at varying enzyme loading

Enzyme Loading	Glucose <sup>a</sup> (g/L)	Xylose (g/L)	Arabinose (g/L)	Ethanol <sup>b</sup> (g/L)	Yield on Xylose (%)	Fermentation Yield <sup>c</sup> (%)
1g/L Fluka	2.8	17.5	0.0	6.5	73.2	63.2
1g/L Fluka + 1mL/L Novozymes <sup>d</sup>	3.7	21.1	0.1	7.0	88.2	55.2
1g/L Fluka + 1mL/L Novozymes	2.8	20.3	0.0	7.7	84.8	65.5
1g/L Fluka + 2mL/L Novozymes	4.0	23.0	0.0	6.7	96.5	48.7
1g/L Fluka + 2mL/L Novozymes	3.2	22.6	0.0	7.9	94.6	60.2
1g/L Fluka + 3mL/L Novozymes	4.0	23.2	1.2	8.3	97.2	57.4
1mL/L Novozymes	3.0	11.1	0.1	4.6	46.4	63.4
2mL/L Novozymes	3.6	12.6	3.8	5.3	52.8	51.5
2mL/L Novozymes	3.2	13.4	3.3	5.8	56.3	56.4
3mL/L Novozymes	3.6	15.5	4.3	7.1	65.0	59.3
4mL/L Novozymes	4.0	17.4	6.1	8.2	73.0	58.0
Acid Hydrolysis at 120°C, 60 min <sup>e</sup>	1.6	23.9	1.1	4.7	100.0	34.4

a. Glucose, xylose and arabinose measured after 7 days of growth at pH 5, 37°C

b. Ethanol measured on day 9; 48 hrs after inoculation

c. Fermentation yield is ethanol divided by 51% of total available sugars

d. Novozymes NS50012 complex, NS50030 xylanase, NS2202 hemicellulase, and NS50014 xylanase each added at the specified dosage

e. Fermentation of acid hydrolyzed extracts conducted in Dasgip reactors, while enzymatic hydrolysis and fermentation was done in 100 mL serum vials.

The enzymatic hydrolysis was carried out over 7 days at optimal conditions for enzymatic hydrolysis, with a pH of 5 and temperature of 50°C. On the 7<sup>th</sup> day the conditions were changed to fermentation optima, 37°C and pH 7, and the *E. coli* K011 was added. Fermentation was carried out for an additional 2 days, and the resulting ethanol production is listed in Table 17. For comparison, the acid hydrolyzed extracts were also fermented for 2 days and produced 4.7 g/L of ethanol. All of the enzyme hydrolyzed extracts reached equivalent or higher titers of ethanol, with a maximum production of 8.3 g/L achieved at the highest enzyme loading of 1 g/L Fluka plus 3 mL/L Novozymes. The fermentation yields on the basis of available sugar consumed were significantly higher for enzymatic hydrolysis than acid hydrolysis of hot water extracts. This is likely a result of the higher furfural concentration in hydrolyzed extracts, and may

be compounded by lignin degradation products generated during acid hydrolysis. Figure 49 shows a comparison of the fermentation by *E. coli* of acid hydrolyzed and enzyme hydrolyzed extracts. In acid hydrolyzed extracts (A), the organism required 24 hrs to detoxify furfural before either glucose or xylose consumption could commence. Ethanol was produced inefficiently, at a yield of only 34%. Enzymatic hydrolysis (B) had slightly less sugar, but with lower concentrations of the inhibitory furans and phenolics, was able to produce 75% more ethanol than acid hydrolysis. The fermentation yields for enzymatic hydrolysis ranged from 49-66% of theoretical, and all were significantly higher than that of acid hydrolysis. The fermentations were conducted at different times which may cause a change in the inoculating strain. The fermentation of acid hydrolyzed extracts was observed to have higher production of byproducts, with a 3 g/L increase in the acetic acid concentration, whereas no byproducts were created in the fermentation of enzymatically hydrolyzed extracts.

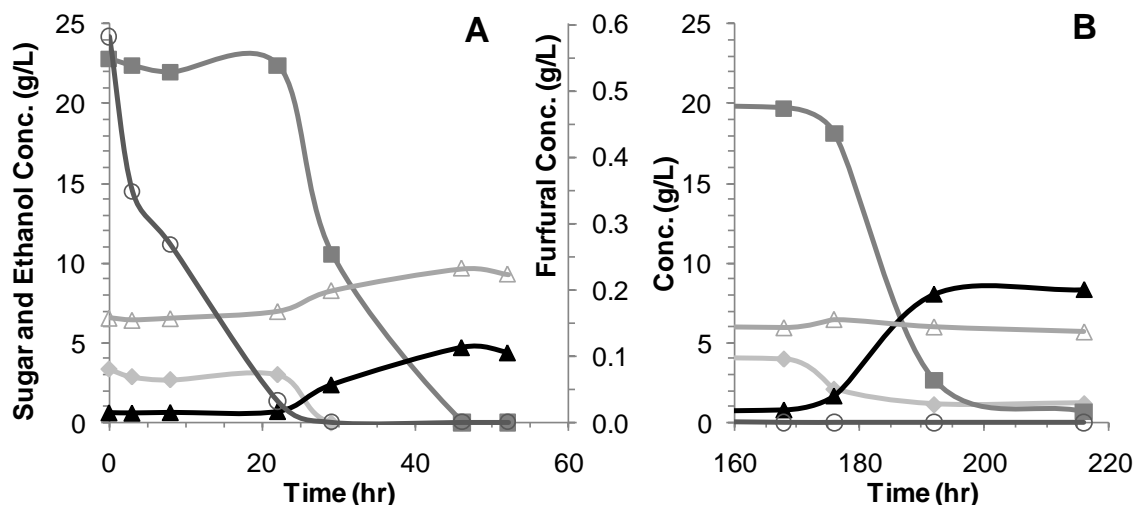


Figure 49. Fermentation by *E. coli* K011 of acid hydrolyzed (A) and enzyme hydrolyzed (B) extracts  
 Acid hydrolysis conducted at pH 1, 120°C for 60 min, enzymatic hydrolysis utilized 1 g/L Fluka xylanase and 3 mL/L Novozymes enzymes. Xylose (■), glucose (◆), Ethanol (▲), Furfural (○)

### 7.2.3 Additional Fermentation Organisms

While fermentation of hemicellulose extracts by *Escherichia coli* K011 and *Bacillus coagulans* MXL-9 are the only organisms described in detail, they are not the only organisms that have been tested. Preliminary results indicate that no other organisms tested are able to tolerate the inhibitory components of green liquor extracts. These organisms include *Pichia stipitis* CBS-6054, *Clostridium phytofermentans*, *T. thermosaccharoluticum* HG-8, and *Moorella thermoacetica*. These strains were all selected based on their ability to metabolize pentose sugars. Table 18 summarizes the characteristics of organisms which have been tested for fermentation of green liquor extracts and indicates what concentration each has been demonstrated to survive. *E. coli* K011 has the highest demonstrated tolerance, though growth on 3x green liquor extract required strain adaptation. *B. coagulans* MXL-9 has been demonstrated at only 1x strength, but inhibitor tests indicate it should also be capable of growth at higher

concentrations. The remaining organisms were not able to tolerate full strength green liquor extract, but were demonstrated at 0.5x concentration.

Table 18. Summary of fermentation organisms - characteristics and green liquor extract tolerance

Organism	Class	Temp.	Strain develop	Mono/ oligo sugar usage	Anaerobe?	Product	Demon- strated product yield (% theory)	Demon- strated GL tolerance
<i>E. coli</i> KO11	Bacteria	meso	GMO	mono	facultative	EtOH	80-90%	3x
<i>Pichia stipitis</i> CBS-6054	Yeast	meso	isolated	mono	micro aerobic	EtOH	40%	0.5X
<i>Clostridium phytofermentans</i>	Bacteria	thermo	isolated	oligo	obligate	EtOH	---	0.5X
<i>T. thermosaccharolyticum</i> HG-8	Bacteria	thermo	isolated	oligo	obligate	EtOH	---	0.5X
<i>Moorella thermoacetica</i>	Bacteria	thermo	isolated	oligo	obligate	HAc	---	0.5X
<i>Bacillus coagulans</i> MXL-9	Bacteria	thermo	isolated	mono	facultative	HLa	72-95%	1X

## 7.2.4 Inhibitor Removal

### 7.2.4.1 Hydrophobic Absorption Chromatography

Treatment with hydrophobic polymeric absorbants (sometimes referred to as ion exchange resins) was tested for removal of inhibitory components from hemicellulose extract prior to fermentation. Acid hydrolyzed hot water extracted southern hardwood prepared at an H-factor of 600 was passed through a chromatography column containing Amberlite XAD-4 hydrophobic absorption resin. While absorption was highly effective at removing phenolic lignin, furfural and acetic acid, it also resulted in significant loss of monosaccharides. Figure 50 shows a comparison of the hemicellulose extract composition before and after undergoing hydrophobic absorption. After hydrophobic

absorption only 53% of the initial monosaccharides were present in the extract. A subsequent water wash of the resin was able to recover the monosaccharides, but then required an additional evaporation step, adding further energy demands and the expense of additional unit operations. Hemicellulose extracts are dark brown in color due to the presence of lignin components, but all color was removed during hydrophobic absorption, resulting in a clear solution. Water washing the column to recover bound sugars did not return color to the treated extract, indicating that lignin remained bound to the hydrophobic resin. Acetic acid is also stripped from the extracts during this process, and is only partially recovered during water washing. Hot water extracted hemicellulose contained 3.92 g/L acetic acid prior to absorption and only 0.86 g/L following. However, water washing of the resin followed by evaporation to remove the added water resulted in a solution containing 2.68 g/L acetic acid, recovering 68% of the initial acetic acid present in the extracts.

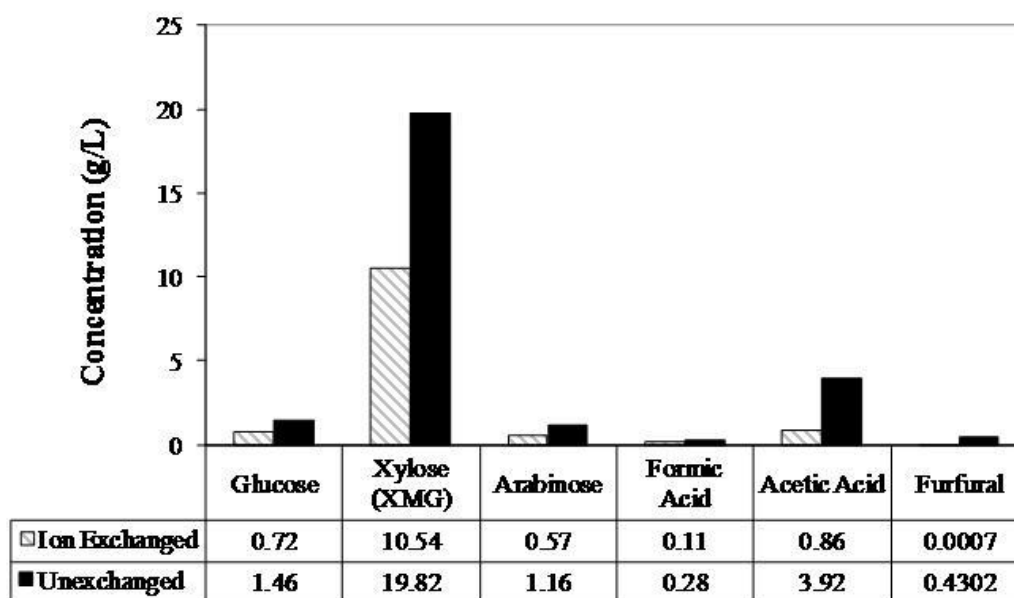


Figure 50. Composition of hemicellulose extracts before and after ion exchange Hot water extracted southern hardwood, 600 H-Factor, treated with Amberlite XAD-4 ion exchange resin.

Fermentation of the hydrophobic absorption treated extracts was performed by *E. coli* K011 and *B. coagulans* MXL-9 in 100 mL serum vials with a working volume of 50 mL. Untreated water extracted hemicellulose is more inhibited when pH is not controlled, and often cells do not grow at all. This can be attributed to the higher concentrations of lignin degraded phenolics and furfural because water extracts do not contain salts and have much lower acetic acid than green liquor extracts. Resin treated extracts were not inhibited in serum vial fermentation and were consumed far more rapidly, producing higher product yields. Figure 51 shows the fermentation performance of *E. coli* K011 in both the untreated extract and the detoxified extract which has been re-concentrated by evaporation after water washing the resin. Prior to autoclave sterilization, both substrates contained 20 g/L xylose. Following sterilization the xylose concentration had decreased to 13 g/L. The untreated water extracts did not

support any cell growth and showed no ethanol production while the detoxified extracts were readily consumed within 18 hours and produced ethanol at 92% of theoretical yield.

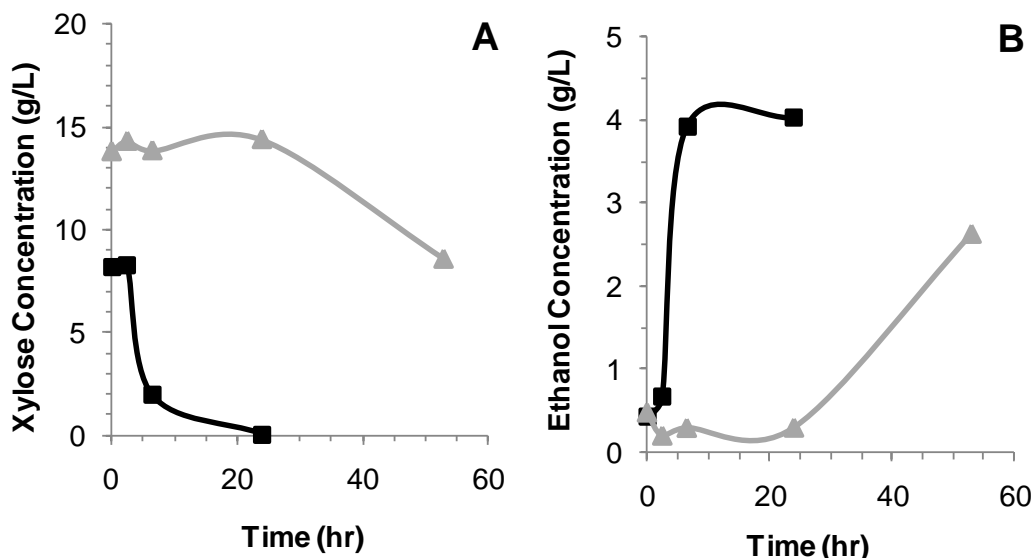


Figure 51. Fermentation of ion exchanged and untreated hemicellulose extracts by *E. coli* K011

Untreated original extracts (▲), Extracts treated with ion exchange resin (■)

Similarly, Figure 52 shows the fermentation of hydrophobic absorption treated and untreated extracts by *Bacillus coagulans* MXL-9. In this case the resin had not been water washed to recover lost sugars, so the starting concentration of xylose in the ion exchanged extracts was significantly lower than that of the untreated extract. The untreated extract however did not support cell growth and no lactic acid was formed. Extracts that had undergone absorption did readily support lactic acid production. Because the pH was not controlled in the serum vial fermentations, cell viability ceased before all of the xylose could be consumed. The yield of lactic acid production from consumed sugars was 92%.

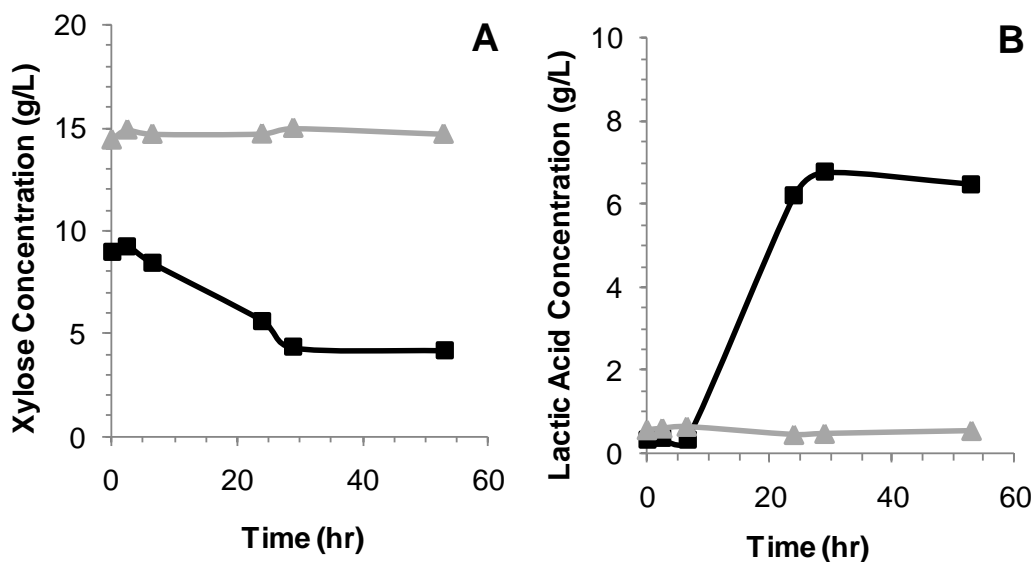


Figure 52. Fermentation of ion exchanged and untreated hemicellulose extracts by *B. coagulans* MXL-9  
 Untreated original extracts (▲), Extracts treated with ion exchange resin (■)

#### 7.2.4.2 Liquid-liquid Extraction

Liquid-liquid extraction using trioctylphosphine oxide (TOPO) dissolved in undecane as the organic phase is a proposed method of removing acetic acid from hemicellulose extracts prior to fermentation (van Heiningen et al., 2009). For this to be a viable process option, TOPO and undecane must be fully recycled within the process due to the high cost of chemicals. A 1% green liquor extract was prepared from mixed northern hardwood chips at a liquor to wood ratio of 4 L/kg, temperature of 160°C and H-Factor of 800 hrs. The extract which contained 13 g/L total sugar and 6 g/L acetic acid underwent liquid-liquid extraction and was then fermented by *E. coli* K011 to determine if residual organic solvents would have a negative effect on fermentation. Liquid-liquid extraction was able to remove 62% of the acetic acid, reducing the concentration to 2.2 g/L. Sugar lost during this step represented 6% of the total available monosugars, reducing the concentration to 12.3 g/L. Figure 53 shows that the TOPO treated extracts

undergo fermentation much faster than untreated extracts, demonstrating that there is no toxicity associated with residual TOPO or undecane. However, the product yield was not higher for the TOPO treated extracts, likely because 6 g/L of acetic acid is not an inhibitory concentration.

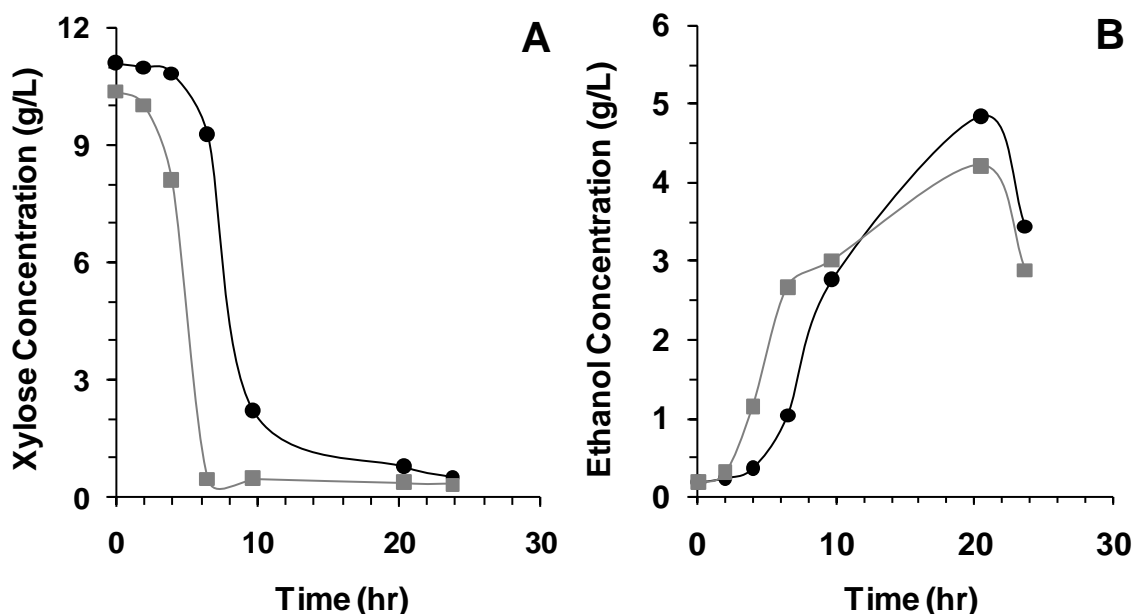


Figure 53. Fermentation of TOPO treated 1% green liquor extract compared to untreated extract

TOPO treated 1% GL extract(■), untreated 1% GL extract (●)

### 7.3 Conclusions

The preceding section provides information intended to be of use in further research to optimize the fermentation of hemicellulose extracts. It highlights some of the processing mistakes to avoid, and gives preliminary results on experiments such as ion exchange and enzymatic hydrolysis that were not fully examined. It was discovered in this work that the optimum acid hydrolysis severity varies based on hemicellulose extract composition, where hot water extracts with low DP require shorter times or lower temperatures than the higher DP green liquor extracts. Some techniques such as

overliming that apply to other types of biomass fermentation processes do not make sense in the context of hemicellulose extract processing. Sugar losses can occur at many points in the processing, and must be minimized. Sterilization of extracts prior to fermentation results in significant reduction of xylose and may not be necessary because the extracts will be sterile after their high temperature generation.

Enzymatic hydrolysis has been shown successful on both green liquor and hot water extracts. For ethanol production, higher yields are achieved through separate hydrolysis and fermentation stages because the enzymes and organisms do not share common pH or temperature optima. Lactic acid production with *bacillus coagulans* would be better suited to the SSF process because the organism is thermophilic. Fermentation of enzyme hydrolyzed green liquor extracts achieved comparable ethanol titers and yields to that of acid hydrolyzed extract fermentation. Hot water extracts which had been enzyme hydrolyzed produced 1.7 times more ethanol than the corresponding acid hydrolyzate, and had significantly higher yields.

Removal of inhibitors by ion exchange leads to greatly improved fermentation rates and yields. Treatment with anion exchange resin XAD-4 showed high affinity for phenols and furans as well as moderate removal of organic acids. Significant binding of fermentable sugar components to the resin did occur during ion exchange, requiring a water washing step to recover sugars followed by an evaporation step to restore a high concentration of sugars prior to fermentation.

## CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

Extracting hemicellulose from wood prior to Kraft pulping is a proposed method of creating new feed-stocks in an integrated forest products bio-refinery. If alkaline chemicals are used to maintain the final extract liquor at near-neutral pH conditions then hemicellulose can be extracted without causing significant yield loss to the more valuable pulp product, or in some cases even improving the pulp yield. Acetyl groups are more prone to be liberated from the hemicellulose polymer during near-neutral extraction than during (acidic) autohydrolysis, and represent a valuable co-product. The aqueous extract contains mainly xylo-oligosaccharides which can be hydrolyzed and utilized by pentose fermenting organisms to produce ethanol, lactic acid or other value-added products.

### 8.1 Hemicellulose Extraction

The effect of green liquor charge and extraction time on hemicellulose extract composition and properties was determined. Water extraction produced the highest concentration of sugars (25 g/L), but also caused an 8% loss in pulp yield. The hemicellulose oligomers were smaller in water extracts, measuring only 0.7 kD, and comprised of both free carbohydrate and LCC fractions. Water extracts have the lowest concentration of acetic acid, ranging from 3 g/L at the lowest severity extraction to 6 g/L at the highest tested severity. Nearly half of the removed acetyl groups remain bound to the hemicellulose oligomers following water extraction, whereas all acetyl groups are free in solution for green liquor extracts. 2% green liquor extraction resulted in the best preservation of pulp yield and had higher sugar concentration than 4% or 6% green liquor. Green liquor extracts had larger average molecular weights, ranging from 7 to 12 kD for the highest severity, and nearly all of the carbohydrate fraction was associated

with lignin by LCC bonds. The green liquor charge should be minimized to avoid inhibitory concentrations of inorganic salts and provide the highest possible concentration of fermentable sugars without negatively impacting pulp yield. The greatest challenge identified in fermenting hemicellulose from wood is overcoming the dilute sugar concentration. Evaporation and fermentation were investigated as potential methods of increasing sugar concentration.

## 8.2 Fermentation by *E. coli* K011

*E. coli* K011 was shown to be capable of growing in hemicellulose extracts, consuming all of the monosugars and producing ethanol at approximately 70% of the theoretical yield in 14 hours. An extract evaporated 2-fold was also fermentable, while a 3-fold evaporated extract was not initially fermentable. Strain conditioning was later found to enable some consumption at this level of concentration. Inhibitor removal prior to fermentation is necessary to produce ethanol economically from evaporated extracts. Removal of inhibitory components including acetic acid, lignin derived phenolics, and furfural, increased the speed of fermentation, and in some cases was able to improve product yields. Testing of *E. coli* on model inhibitor systems revealed that the organism could withstand 25 g/L of acetic acid or 25 g/L of sodium, but would grow slowly and with poor yield. Using evaporation to concentrate the extracts, these levels of inhibitors were reached far before the target 80 g/L sugar concentration was reached. Ultrafiltration generated an extract that increased sugar without increasing acetic acid or lignin, and therefore was more readily fermentable. The highest concentration of ethanol produced from hemicellulose extracts was 12 g/L, obtained in a 7-fold ultrafiltration concentrated extract which was converted at an overall yield of 32%.

### 8.3 *Bacillus coagulans* MXL-9

*Bacillus coagulans* MXL-9 was found capable of growing on pre-pulping hemicellulose extracts, utilizing all of the principal monosugars found in woody biomass. This organism is a moderate thermophile isolated from compost for its pentose utilizing capabilities. It was found to have high tolerance for inhibitors such as acetic acid and sodium which are present in pre-pulping hemicellulose extracts. Fermentation of 20 g/L xylose in the presence of 30 g/L acetic acid required a longer lag phase but overall lactic acid yield was not diminished. Similarly fermentation of xylose in the presence of 20 g/L sodium increased the lag time but did not affect overall product yield, though 30 g/L sodium proved completely inhibitory. Fermentation of hot water extracted Siberian larch containing 45 g/L total monosaccharides, mainly galactose and arabinose, produced 33 g/L lactic acid in 60 hrs and completely consumed all sugars. Small amounts of co-products were formed, including acetic acid, formic acid and ethanol. Hemicellulose extract formed during autohydrolysis of mixed hardwoods contained mainly xylose and was converted into lactic acid with a 94% yield. Green liquor extracted hardwood hemicellulose containing 10 g/L acetic acid and 6 g/L sodium was also completely converted into lactic acid at a 72% yield. The *Bacillus coagulans* MXL-9 strain was found to be well suited to production of lactic acid from lignocellulosic biomass due to its compatibility with conditions favorable to industrial enzymes and its ability to withstand inhibitors while rapidly consuming all pentose and hexose sugars of interest at high product yields.

## 8.4 Recommendations for Future Work

This work demonstrates that the most significant challenge to overcome in creating a commercially viable process is the increase of sugar concentration prior to fermentation. The target values were not achieved, and further work is needed to reach this goal. It is recommended that the green liquor charge be kept below 2%, preferably at 1%, to obtain the highest possible starting sugar concentration without negatively impacting pulp yield. It is also recommended that the Forest Products Biorefinery focus on higher value chemicals, rather than commodities. The scale of the forest products industry is relatively small in comparison to the market for fuels, which makes it harder to compete with other production methods that do not face the challenges of low sugar concentrations or high inhibitory chemical concentrations.

### 8.4.1 Ultrafiltration

It has been shown that ultrafiltration is a promising method for increasing the concentration of sugar in hemicellulose extracts. The ultrafiltration systems tested in this study did not have sufficient capacity to reach the target sugar concentrations because membrane fouling occurred too rapidly. A larger membrane surface area or the ability to operate at high temperature is needed to increase the permeate flux. Operating the filter at process temperatures as close to 160°C as possible, so that the extract does not cool, would avoid precipitation of lignin and reduce fouling. The pH of the extract during filtration is another potential important variable which needs to be studied.

#### 8.4.2 Detoxification

The use of hydrophobic absorbents such as XAD resin was shown to be effective at improving fermentation performance, but requires more in depth study. Detailed cost estimation is needed to determine if this is a viable treatment option. Further testing is also needed to optimize the treatment and minimize sugar loss. It is expected that varying the operating parameters could recover nearly all of the sugar without significant dilution, as the sugar was easily removed by water washing the column.

#### 8.4.3 Product Separation

Some investigation should be undertaken to determine the efficiency of product recovery from the complex mixture of chemicals which make up hemicellulose extracts. For lactic acid, it is unknown how the presence of other organic acids might effect the creation and purification of lactide. Similarly for the distillation of ethanol, it needs to be understood how other components will partition in the separation processes.

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