METHODS FOR ETHANOL PRODUCTION FROM THE ENZYMATIC HYDROLYSIS AND FERMENTATION OF SUGAR BEET PULP

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Rorick, Rachel Elizabeth, M.S., Department of Agricultural and Biosystems Engineering, College of Engineering and Architecture, North Dakota State University, August 2010. Methods for Ethanol Production from the Enzymatic Hydrolysis and Fermentation of Sugar Beet Pulp. Major Professor: Dr. Scott Pryor.

Sugar beet pulp (SBP), the residue remaining after sucrose extraction, is currently sold as an animal feed. Humans cannot digest the cellulose in the pulp unlike ruminant animals. The pulp is primarily comprised of cellulose, hemicellulose, and pectin which can be hydrolyzed with commercial enzymes into fermentable sugars such as, glucose, arabinose, galacturonic acid, xylose, and galactose. These sugars can be fermented to produce ethanol. This research tested the variation of several enzymes, enzyme loading rates, solids loading rates, and fermenting organisms to increase ethanol yields from sugar beet pulp.

Several commercial enzymes (cellulases, hemicellulases, pectinases, and proteases) were tested to determine impact on SBP hydrolysis. Two commercial enzyme preparations (Viscozyme and Pectinex) were compared. Viscozyme produced the highest sugar yields because of increased cellulose hydrolysis, while Pectinex showed less cellulase activity. All enzyme treatments resulted in similar hemicellulose and pectin hydrolysis. Pretreatment with proteases reduced sugar yields from hydrolysis by 10-30% compared to hydrolysis without pretreatment.

Escherichia coli KO11, a genetically modified organism (GMO), and *Saccharomyces cerevisiae* were used to ferment SBP hydrolyzate to increase ethanol yields (g EtOH/g SBP) and concentrations (g/L). In the "Parallel" fermentation, pectinase was used to solubilize pectin and hemicellulose. After separation, the liquid stream was fermented with *E. coli* KO11 and the high-

cellulose solid fraction was fermented using *S. cerevisiae* and additional cellulase enzymes (Celluclast and Novozyme 188). The "Parallel" method initially produced under 0.15 g EtOH/g SBP but was improved with pH regulation to yield 0.23 g EtOH/g SBP. The separation method limited ethanol production.

The ethanol yields from three additional fermentation methods ("*E. coli* KO11 Only", "Serial", and "Reverse Serial") were compared. The "*E. coli* KO11 Only" method was the baseline fermentation for comparison of the remaining three fermentation methods. SBP was hydrolyzed with pectinase, cellulase, and cellobiase before fermentation with *E. coli* KO11 to yield 0.192 g ethanol/ g SBP. The total hydrolysis of the SBP limited ethanol production. The "Serial" fermentation began by solubilizing pectin and hemicellulose with pectinases. All of the flask contents were fermented with *E. coli* KO11. The remaining cellulose-rich SBP was then hydrolyzed with cellulases and fermented by *S. cerevisiae*. Initial ethanol yields were under 0.15 g EtOH/g SBP but improved to 0.238 g EtOH/g SBP. Acetic acid concentrations limited ethanol production by *S. cerevisiae*. The "Reverse Serial" simultaneous saccharification and fermentation (SSF) started with pectinases, cellulases, cellobiases, and *S. cerevisiae*. Remaining arabinose and galacturonic acid were fermented with *E. coli* KO11 to produce a peak ethanol yield of 0.299 g EtOH/g SBP.

The methods approached and exceeded published results (0.277 g EtOH/g SBP) (Doran and Foster, 2000) to successfully increase ethanol yields. Ethanol concentrations were limited by high SBP moisture content and low solids loading rates.

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GENERAL INTRODUCTION

The demand for liquid transportation fuels continues to increase in the United States. Combining this demand with growing greenhouse gas emissions concerns, the development of biofuels has become a focus for many researchers. The Energy Independence and Security Act of 2007 requires the United States to produce 36 billion gallons of renewable fuel by 2022 (RFA, 2010b). Of the 36 billion gallons of fuel, 16 billion gallons must be produced from cellulosic feedstocks. To reach this goal, many different types of cellulosic feedstocks, such as sugar beet pulp, switchgrass, corn stover, citrus peel, and corn cobs, will need to be used (DOE and USDA, 2005).

North Dakota and Minnesota can benefit from the growing importance of alternative energy. The vast farmland already provides corn for starch-based ethanol and oilseeds for biodiesel. In the Red River Valley, sugar beets could provide a useful cellulosic feedstock. In the past five years, 53% of the national sugar beet harvest came from North Dakota and Minnesota, most of which was grown in the Red River Valley. After the sucrose is extracted for use as table sugar, around 760,000 dry tons of sugar beet pulp remains from locally processed beets (Malmskog, 2010). Currently, the pulp is dried and sold as an animal feed with varying profit margins for producers (Spagnuolo et al., 1997). If energy costs are high and corn and other feed prices are low, the drying costs outweigh potential profit (Doran and Foster, 2000). If this pulp was used to produce ethanol, sugar beet growers and processors could benefit because the variability from drying costs would be eliminated. Still, the profit margin would be affected by the

market value of ethanol. Assuming complete conversion of the different sugars maintained in the pulp structure, 136 million gallons of ethanol could potentially be produced nationally (based on personal communication with David Malmskog) (2010). Before this is possible, some challenges in hydrolyzing sugar beet pulp and fermenting the resulting sugars need to be overcome.

The use of cellulosic feedstocks for ethanol production is currently limited technically and economically by several logistical constraints (Fales et al., 2007). Feedstock harvest, storage, and transportation are all important considerations. In many cases, the mass of the feedstocks requires new harvesting equipment that is expensive for developers and farmers. Storage facilities for the farm and plant are necessary, and the feedstocks may require drying to avoid microbial degradation. Finally, transportation of feedstocks is expensive because of the low bulk density of most biomass feedstocks (Buchanan et al., 2008).

Sugar beet pulp is advantageous as a feedstock because these concerns have already been addressed by the sugar processing industry. Beet harvesting equipment and transportation methods already deliver the product to sucrose processing plants. Once the sucrose is extracted, the pulp can be directly hydrolyzed and the subsequent sugars fermented on-site. Fermentation equipment and ethanol holding tanks would need to be installed, but only minimal other changes would be necessary. One economic challenge could be the small size of individual processing plants. It may be too costly for each plant to make small quantities of ethanol. The five American Crystal Sugar processing plants in North Dakota and Minnesota produce an average of 85,000 dry tons of pulp, which

could produce a maximum of 8.1 million gallons per plant. Individual corn ethanol plants produce between 10 and 100 million gallons of ethanol (M&A, 2010). Multiple plants in close proximity to another could develop a central fermentation plant where pulp could be fermented to approach yields from corn ethanol plants.

Most cellulosic feedstocks are composed of high percentages of cellulose with smaller percentages of hemicellulose and lignin. Biomass recalcitrance, the natural ability of cell walls to resist enzyme and acid degradation, must be overcome before the cellulose and hemicellulose can be accessible to cellulase enzymes. To overcome biomass recalcitrance, pretreatment with acids or bases is necessary and can make up about 30% of total industrial costs (Hendriks and Zeeman, 2009). Once pretreatment is completed, the cellulose can be hydrolyzed to yield glucose, which is easily fermented using conventional yeasts strains. Sugar beet pulp is a unique biomass feedstock because the hemicellulose and pectin contents are high while the cellulose and lignin are relatively low. The low lignin and high pectin content eliminates the need for expensive pretreatment required for conversion of most other biomass feedstocks (Spagnuolo et al., 1997).

Sugar beet pulp is also fairly unique in the composition of sugars resulting from hydrolysis of cellulose, hemicellulose, and pectin. Cellulose content is relatively low in SBP resulting in low glucose concentrations in hydrolyzate. Hemicellulose is primarily a xylose polymer in most forms of biomass, but SBP hemicellulose is composed of arabinose with lower concentrations of xylose and galactose. Most other feedstocks contain negligible pectin contents. SBP contains 15% pectin that can be hydrolyzed to galacturonic acid, another potentially

fermentable sugar (Micard et al., 1996; Spagnuolo et al., 1997). The variety of five- and six-carbon sugars provides a challenge for fermentation to ethanol (Doran et al., 2000). Traditional yeasts used in fermentation metabolize glucose to the exclusion of other sugars. Some yeast strains can co-ferment glucose and xylose, which can be beneficial to other biomass feedstocks. Due to the low xylose concentrations from hydrolyzed sugar beet pulp, the modified yeast strains would not substantially increase the ethanol yields in fermentation (Sedlak et al., 2003). Minimal research concerning the fermentation of arabinose and galacturonic acid has been conducted because most biomass feedstocks have limited pectin content (Ingram et al., 1987; Sedlak and Ho, 2001). To maximize the ethanol production from the sugar beet pulp, a different microorganism that metabolizes the glucose, arabinose, and galacturonic acid is required.

Ethanol production from biomass feedstocks requires two major steps that can be completed separately or in combination: polysaccharide hydrolysis and fermentation of the resulting sugars. The structural polysaccharides in cellulose, hemicellulose, and pectin must be hydrolyzed to their component sugars through the use of acids or enzymes.

After hydrolysis, the resulting sugars must be converted to ethanol. Due to the diversity of sugars yielded from sugar beet pulp, two different microorganisms were considered. The gram negative bacterium *Escherichia coli* KO11 is genetically modified to use glucose, arabinose, and galacturonic acid in fermentation, while the yeast *Saccharomyces cerevisiae* only uses glucose.

Sugar beet pulp is a non-traditional cellulosic feedstock that has potential benefits in North Dakota and Minnesota. Although challenges and costs from harvest, transportation, storage, and pretreatment are limited, the feedstock itself provides new challenges to be considered. The enzymes and microorganisms used in the hydrolysis and fermentation must maximize the yields of soluble sugars and concentration of ethanol in fermentation broth.

An Explanation of the Dissertation Organization

This disquisition has a general literature review discussing the work that has been completed in cellulosic biofuels. The three following chapters contain three papers for publication that discuss the research I have completed. I developed and conducted all of the research with guidance from my advisor, Dr. Scott Pryor, and his lab technician, Nurun Nahar. The data analysis and paper writing was also completed by me. Both Dr. Pryor and Ms. Nahar edited the papers. The first two papers have been published for presentations that I have given at regional and national American Society of Agricultural and Biological Engineering (ASABE) conferences. The third paper will be submitted for publication in the Journal of Biological Engineering following completion of my thesis. General conclusions and complete references complete the document.

LITERATURE REVIEW

ENERGY

Energy is essential in the United States to heat homes, power industry, and fuel cars and trucks. The multiple demand sectors (transportation, industrial, residential, commercial, and electrical) require energy from different supply sources (petroleum, natural gas, coal, renewable, nuclear, and electrical) to satisfy the energy demand (EIA, 2009). The 99.2 quadrillion British thermal units (Btu) of energy consumed in the United States in 2008 is projected to increase 14 percent by 2035 (EIA, 2009; EIA, 2010). To continue to meet the energy demands of Americans, energy production must also continue to increase. The Energy Information Administration (EIA) projection model suggests that the use of renewable fuels for electricity and liquid transportation fuels will see the greatest increase due to favorable legislation, increased funding, and less favorable fossil fuel costs. Although fossil fuels are still projected to constitute most of the United State's energy supply throughout the 25 year projection, the percentage could fall from 84% to 78% (EIA, 2010). In 2008, renewable energy constituted 7% of the total primary energy consumption. This 7% could be broken down into seven renewable sectors: 34% hydroelectric power, 28% wood, 19% biofuels, 7% wind, 6% waste, 5% geothermal, and 1% solar (EIA, 2009).

BIOFUELS

Of the seven renewable energy sectors, biofuels has seen the most dramatic increase in the past 10 years (EIA, 2009). Biofuel production includes both ethanol and biodiesel which can be blended into gasoline or diesel and used

with current vehicles. The increase in liquid fuels that can accommodate current transportation infrastructure is important, because the energy consumption in the transportation sector has increased dramatically over the past 10 years (EIA, 2009). Even though biofuels production continues to increase, petroleum-based fuels constitute approximately 95% of America's liquid fuel supply. There is still oil that can be easily extracted from the ground, but this finite resource will eventually become too expensive for continued extraction or will be used for higher value products than fuel (Prugh, 2006). Experts debate when global oil production will peak and subsequently fall (Aleklett, 2006; Cavaney, 2006; Flavin, 2006; Kaufmann, 2006; Smil, 2006). Between 2000 and 2005 the world oil production still increased by about 7% (Prugh, 2006). Currently, the United States imports 12,440 thousand barrels of oil per day (EIA, 2010). The United States is not in danger of running out of oil in the near future, but the development of biofuels could help lessen oil dependence in coming years.

Biofuel Feedstocks

Biomass feedstocks include all plants and plant-derived materials that can be used for fuel production (DOE and USDA, 2005). The many agricultural plants and forestry residues can be classified in two major categories, first-generation and next-generation feedstocks. First-generation feedstocks include grains and vegetables that are harvested for sugar, starch, and oil content. Feedstocks, such as corn, sugar cane, and soybeans, have conventional processing methods already in place. Next-generation feedstocks are mainly cellulose-based (with significant amounts of hemicellulose and lignin) and are harvested so that the total

biomass can be used for fuel production. Cellulosic feedstocks like crop residues, wood, and perennial grasses are more abundant than first-generation crops, but the technologies for ethanol production have not yet been demonstrated at full commercial scale.

All fuel feedstocks, from petroleum to agricultural crops, are finite. Although biobased feedstocks are renewable on an annual basis, the yields of crops per acre each year limit the total amount of biomass feedstocks available for biofuels production. In order to replace 30% or more of the United State's current petroleum demand, about 1 billion dry tons of biomass feedstock per year would be necessary (DOE and USDA, 2005). To reach this goal, many types of biomass must be harvested, transported, stored, and processed at affordable costs. By making some assumptions about future agricultural capabilities, the Department of Energy (DOE) and United States Department of Agriculture (USDA) (2005) predict that 428 million dry tons of annual crop residues, 377 million dry tons of perennial crops, 87 million dry tons of grains, 106 million dry tons of animal manure, and 368 million dry tons of forest resources (1.366 billion total dry tons) will potentially be available annually without sacrificing food. The most optimistic scenarios in that report assumed that:

- Corn, wheat, and small grain yields will increase by 50%
- Soybean residue-to-grain ration will increase to 2:1
- 75% of annual crop residue will be harvested requiring new technology
- Perennial bioenergy crops will be grown on 55 million acres of cropland, idle cropland, and cropland pasture

- Excess manure not used for fertilizer will be used for biofuels
- Other available residues will be used completely (DOE and USDA, 2005)

This aggressive projection for biomass utilization requires a diversity of feedstocks and techniques to produce liquid fuel and other biobased energy. Currently, the most established ethanol processing method in the United States is for corn. Corn yields and ethanol production methods increase and improve each year with 10.7 billion gallons of ethanol produced in 2009 (RFA, 2010a). Ethanol production from corn and other starch crops uses enzymes to hydrolyze the starch polymers into glucose monomers. Yeasts can metabolize the resulting glucose to produce ethanol. The high ethanol yields and government incentives help to make starch-based fermentation more affordable.

The next-generation feedstocks or cellulosic feedstocks still have many challenges before they can be utilized as effectively as corn for ethanol production. The recalcitrant nature of cellulosic biomass presents challenges for cellulosic ethanol because biomass hydrolysis is difficult and results in large energy consumption and costs (Scheller et al., 2010). Unlike corn, which is mainly composed of starch, cellulosic feedstocks have multiple components (primarily cellulose, hemicellulose, and lignin) that support and protect the plant from microbial attack. The percentage of each component varies based on the specific feedstock and growing conditions but a representative analysis of cellulose, hemicellulose, and pectin percentages is shown in Table 1.

Other challenges facing cellulosic ethanol production include harvest, transportation, and storage. Most harvest equipment is developed for primary food

or forage crops. Dedicated energy crops or plant residues require new or modified harvest equipment. To harvest the plant residue, farmers may have to complete multiple passes, increasing farm costs and soil compaction (DOE and USDA, 2005). The development of new harvest equipment requires time and money for agricultural equipment producers (Fales et al., 2007). Farmers will also have to invest in new, expensive equipment (Buchanan et al., 2008). Once the challenges with harvesting are overcome, transporting the feedstocks becomes another concern. Perennial grasses, plant residues, and forest resources tend to have a low bulk density. Because trucks cannot be filled to capacity on a weight basis, transportation costs per dry ton increase. Also, the distance between farms and processing centers increases the cost for shipping (DOE and USDA, 2005). Finally, the biomass has to be stored at either the farm or processing site (Buchanan et al., 2008). Depending on the type of biomass, large storage areas that can maintain the temperature and moisture content for safe storage are necessary. A favorable biofuels market can lessen the opposition to these changes (DOE and USDA, 2005).

The diversity of biomass that has potential for biofuels production presents many challenges for processors. Different composition with varied feedstocks requires a variety of pretreatments, enzymes, and microorganisms to produce ethanol economically. Also, current infrastructure requires adaptations to achieve the sustainable supply of 1 billion dry tons of biomass to be processed annually in the United States.

Table 1. Representative Compositional Analysis of Cellulosic Feedstocks (DOE, 2010)

	Feedstock Composition (% dry basis)		
Feedstock	**Cellulose**	**Hemicellulose**	**Lignin**
Corn Stover	36.51	22.82	19.25
Sugarcane Bagasse	41.82	25.47	23.89
American Sycamore	37.24	19.57	27.30
Hybrid Poplar	41.47	16.65	25.87
Switchgrass	35.42	26.54	17.12
Big Bluestem	34.35	25.68	17.09

SUGAR BEET PULP

Sugar beet pulp (SBP), a byproduct of the sugar processing industry, is regionally significant in North Dakota and Minnesota. On average, 53% of the nation's sugar beet harvest came from the two states in the past five years (USDA, 2010). After sucrose extraction, the remaining pulp is dried, pelleted, and sold as an animal feed. Up to 30-40% of the energy costs for beet processing can come from drying of the pulp (Doran and Foster, 2000). When the cost of natural gas is high, the drying costs can outweigh the profits from animal feed sales. Also, potential carbon taxation could make drying too expensive for processors. The approximately 30 million tons of sugar beets that are grown and processed annually in the United States result in around 1.425 million dry tons of sugar beet pulp as a co-product (Malmskog, 2010). The use of sugar beet pulp as an ethanol feedstock could be more profitable than processing for animal feed. It may also reduce the carbon footprint of the sugar processing industry which may be essential for industry survival if carbon taxing or cap and trade legislation is

enacted (Pates, 2010). An annual national average of approximately 136 million gallons of ethanol could be produced from SBP. The beet pulp has potential as a biomass feedstock for ethanol production with some atypical advantages and disadvantages.

The proportions of pectin and lignin in sugar beet pulp provide advantages for hydrolysis. Lignin, a binding polymer in plant cell walls, normally ranges from 15-25% of other agricultural residues and cellulosic biomass feedstocks, but only composes 3-4% of sugar beet pulp (DOE, 2010; Spagnuolo et al., 1997). This low lignin content in the SBP helps eliminate the need for expensive pretreatment of the pulp. Pectin is found at minimal concentrations in other forms of cellulosic biomass but contributes 24-32% of beet pulp dry weight (Spagnuolo et al., 1997). The pectin is easily hydrolyzed to yield primarily galacturonic acid, a fermentable sugar (DOE, 2010).

The harvest, storage, and transportation challenges facing most feedstocks are already addressed in sucrose extraction from sugar beets. Harvesting will not be an additional expense, as the beet pulp is a by-product of the sucrose extraction and therefore harvest costs are already internalized for the sugar processing industry. The sugar beets are also already stored by processors after harvest and so no additional storage space or technology would be required. The resulting beet pulp could be fermented directly following sucrose extraction. Many sugar beet facilities are small in size so on-site ethanol fermentation may not be profitable. Transporting the pulp to a centralized fermenting center could increase profits for smaller beet processing facilities.

Sugar beet pulp also presents challenges as a feedstock. Sugar beet pulp has a cellulose content of 22-30%, which is less than that of most other biomass feedstocks which have 30-50% cellulose (DOE, 2010; Spagnuolo et al., 1997). When hydrolyzed, less glucose will be available for fermentation. The hemicellulose content of sugar beet pulp (24-32%) is comparable to the 20-40% found in other cellulosic feedstocks. The cellulose, hemicellulose, and pectin in sugar beet pulp can be hydrolyzed to yield a variety of sugars including glucose, arabinose, and galacturonic acid, respectively, while galactose, xylose, mannose, and rhamnose are produced in lower concentrations (Spagnuolo, 1997). The composition of sugar beet pulp was analyzed by Microbac Laboratories, Inc. (Boulder, CO) and is summarized in Table 2. The diversity of sugars requires a microorganism with a diverse metabolism to maximize ethanol yields. Some of the most promising microorganisms that have the ability to co-ferment multiple sugars at the same time are modified *Saccharomyces cerevisiae*, *Escherichia coli*, and *Pichia stipitis* strains (Agbogbo et al., 2006; Buttke and Ingram, 1980; Sedlak and Ho, 2001).

Cellulosic Ethanol Production

Pretreatment

Lignocellulosic biomass has strong interactions between the cellulose, hemicellulose, and lignin. In this network of interactions, lignin binds the plant mass and provides a barrier to enzyme access (Wang et al., 2009). Pretreatment is required to improve enzymatic hydrolysis. Hydrolysis without pretreatment yields about 20% of the possible five- and six-carbon sugars in biomass, but

pretreatment can remove lignin and/or hemicellulose to increase sugar yields to approximately 90% of the theoretical original sugar (Sun and Cheng, 2002). The desirable improvement in hydrolysis comes with high production costs as pretreatment has been estimated to account for up to 30% of the total processing costs (Lynd et al., 1996). A considerable amount of research has been completed to determine methods that effectively increase digestibility of biomass and reduce the sale price of the ethanol end product (Hendriks and Zeeman, 2009; Sendich et al., 2008). Multiple physical and chemical pretreatment methods have been considered to decrease processing costs while increasing hydrolysis yields (Mosier et al., 2005; Sun and Cheng, 2002).

Table 2. Compositional Analysis of Sugar Beet Pulp

Sugar Beet Pulp Composition (% dry basis)	
Component	**Percentage**
Glucose	28%
Arabinose	20%
Galacturonic Acid	15%
Galactose	7%
Xylose	3%
Protein	11%
Ash	4%
Other	12%

The simplest physical pretreatment is milling, where the biomass particle size is reduced by grinding. This decreases the degree of polymerization and increases the surface area and the shearing (Sun and Cheng, 2002). These

changes in the biomass increase hydrolysis yields by 5-25% and reduce hydrolysis time necessary by 23-59%. Physical pretreatment processes alone are generally considered energy intensive and do not yield sufficient sugars compared with most thermochemical pretreatment methods (Hendriks and Zeeman, 2009; Mosier et al., 2005; Sun and Cheng, 2002).

Most thermochemical pretreatments utilize either acids or bases at elevated temperatures. Acid pretreatments function primarily by solubilizing and removing hemicellulose (Mosier et al., 2005). Dilute or strong acids can be used, but strong acids tend to produce more inhibitory sugar degradation products, such as furfural and hydroxymethyl furfural. Once the hemicellulose is removed, cellulose access for enzymes is greatly increased. Alkaline pretreatments with lime, sodium hydroxide or ammonia function primarily by modifying or solubilizing lignin. Biomass also tends to swell during alkaline pretreatment, and this opens the structure for increased enzyme accessibility (Hendriks and Zeeman, 2009; Mosier et al., 2005).

An increase in temperature to 150-180°C can influence the solubility of both hemicellulose and lignin. In steam pretreatment or steam explosion, chipped biomass in a pressurized container is treated with saturated steam at a temperature of 160-240°C for a few seconds to minutes (Sun and Cheng, 2002). The pressure is released to cool the biomass. Steam explosion releases the pressure much quicker compared to standard steam pretreatment to explode the particles and causes lignin transformation and hemicellulose degradation (Hendriks and Zeeman, 2009). Liquid hot water (LHW) also increases biomass

temperature in batch or steady flow conditions. The degradation of hemicellulose allows for greater access to cellulose fibers in hydrolysis. During these thermal pretreatments, the harsh conditions can further degrade the desirable sugar monomers creating enzyme inhibitors. Limitation of inhibitor formation is essential to the success of enzyme hydrolysis (Hendriks and Zeeman, 2009; Mosier et al., 2005; Sun and Cheng, 2002).

Addition of acids or bases to thermal pretreatment has advantages for lignin and hemicellulose removal. Ammonia fiber explosion (AFEX) exposes lignocellulosic biomass to liquid ammonia at high pressures and temperatures (Sun and Cheng, 2002). This process degrades lignin with little to no affect on hemicellulose. Biomass with lignin contents less than about 15% benefitted from the treatment, but higher lignin content may be better pretreated with other methods (Sun and Cheng, 2002). CO_2 pretreatments also treat biomass at high temperature (approximately 200°C) and pressure and remove hemicellulose in biomass. The increase in hydrolysis is mainly due to increased biomass pore size from hemicellulose removal (Hendriks and Zeeman, 2009).

Biological pretreatments degrade lignin and hemicellulose to increase access to the cellulose portion of biomass. Brown-rot, white-rot, and soft-rot fungi have all been considered, but tend to degrade the desirable cellulose along with the lignin and hemicellulose (Sun and Cheng, 2002). Although energy requirement are low for this biological pretreatment, most hydrolysis yields that follow are low and lignin and hemicellulose are unavailable for other uses (Mosier et al., 2005; Sun and Cheng, 2002).

Sugar Beet Pulp Pretreatment

Sugar beet pulp does not require pretreatment for successful saccharification because of low lignin content. Although this is true, researchers tested different pretreatments on sugar beet pulp to determine if enzymatic hydrolysis could be improved (Chamy et al., 1994; Foster et al., 2001; Micard et al., 1997)

The effects of drying, grinding, and storage of sugar beet pulp were tested to determine their effect on cellulose hydrolysis (Micard et al., 1997). The comparison treatments hydrolyzed the SBP with pectinases only, and then washed the pulp afterward. Some of the depectinised pulp was dried, ground, and stored for 0.5 or 3 months, and hydrolyzed with cellulases. The remaining pulp was hydrolyzed with cellulases right away. The half month storage time proved that storage of the pulp had the greatest effect on cellulose degradation. The ground and dried pulp was stored for 15 days which allowed the pulp to be degraded by cellulase enzymes. Compared to the wet pulp and the 3 month stored pulp, the 15 day storage produced the highest sugar yields (Micard et al., 1997).

Chamy et al. (1994) attempted to use acid pretreatment to selectively hydrolyze hemicellulose and increase the hydrolyzed hemicellulose to hydrolyzed cellulose ratio (H/C). This goal differed from that of typical pretreatment, because a cellulose-rich fraction was kept intact, instead of improving the complete hydrolysis of the biomass feedstock. H_2SO_4 pretreatment increased both hemicellulose and cellulose hydrolysis, but produced a higher and more favorable H/C ratio than HCl. When 75 g/L of sugar beet pulp was treated with 0.72N

H_2SO_4, 70% of the hemicellulose was hydrolyzed, while the cellulose hydrolysis was limited to 4.4% (Chamy et al., 1994).

Another pretreatment method considered for sugar beet pulp was ammonia pressurization depressurization (APD), also known as Ammonia Fiber Explosion or Expansion (AFEX). Foster et al. (2001) tested the effectiveness of two APD pretreatments followed by enzymatic hydrolysis on sugar beet pulp to determine if sugar yields would increase with pretreatment. Ammonia was loaded at 0.5:1, 0.75:1, and 1:1 ammonia to pulp ratios. Sugar beet pulp was treated with two different combination of commercial cellulase, cellobiase, hemicellulase, and pectinase enzymes for 48 hr following pretreatment. When only cellulases and cellobiases were used, APD appeared to increase glucose yields. As cellulose constitutes less than one-third of sugar beet pulp, hemicellulases and pectinases were added to hydrolyze the remaining pulp. When all enzymes were used, the APD with 0.75:1 and 1:1 ammonia to beet pup produced lower glucose, arabinose, and galacturonic acid sugar yields than the untreated pulp. APD does not alter the cellulose content of sugar beet pulp, because glucose yields were similar at all ammonia loading rates. The hemicellulose and pectin portions of the pulp may be altered, because arabinose and galacturonic acid yields decreased with increased ammonia loading rates (Foster et al., 2001).

Pretreatment of sugar beet pulp was found to both help and hinder hydrolysis. The cellulose fraction of SBP left behind after pectinase treatment was better hydrolyzed when dried, ground, and stored for 15 days (Micard et al., 1997). H_2SO_4 was found to degrade 70% of the hemicellulose and only 5% of the

cellulose to separate the five-carbon sugars from the six-carbon sugars for future fermentation applications (Chamy et al., 1994). APD did not improve overall hydrolysis yields because hemicellulose and pectin may have been altered. Less arabinose and galacturonic acid were yielded in hydrolysis (Foster et al., 2001). Overall, the cost and time of pretreatment seemed to outweigh the slight benefits from pretreatment of sugar beet pulp.

HYDROLYSIS

ACID HYDROLYSIS

Acids can be used to hydrolyze the cellulose, hemicellulose, and pectin in sugar beet pulp. Although effective, the cost of stainless steel tanks, pumps, valves, and other system components and the risks associated with strong acids have made acid hydrolysis less popular than enzymatic hydrolysis. The acid must also be recovered after hydrolysis to make the process economical (Sun and Cheng, 2002). The hydrolysis of more recalcitrant biomass feedstocks could benefit from acids. The Blue Fire Ethanol Corporation (Irvine, CA), one of the few commercial cellulosic biomass ethanol plants, treats hardy biomass feedstocks with concentrated acids for hydrolysis (BFH, 2010). As discussed above, Chamy et al. (1994) used acids as a pretreatment before complete sugar beet pulp hydrolysis.

Acid hydrolysis is often used for composition analysis of biomass feedstocks. Spagnuolo et al. (1997) used acid hydrolysis as a reference comparison for the soluble sugar concentrations produced from enzymatic hydrolysis of sugar beet pulp. SBP was soaked in 12 M sulfuric acid for 3 hours at

35°C. Water was added to dilute the sulfuric acid concentration to 1 M, and saccharification took place for 4 hours at 100°C. The complete acid hydrolysis released 666 mg of monosaccharies per gram of pulp (dry basis), with glucose, arabinose, and galacturonic acid making up 88% of total sugars. The sugar components were identified by HPLC analysis as 24.3% glucose, 19% arabinose, 15.3% galacturonic acid 4% galactose, 1.4% xylose, 1.4% mannose, and 1.2% rhamnose of the theoretical (Spagnuolo et al., 1997).

Enzymatic Hydrolysis

Enzymatic hydrolysis uses enzymes to degrade biomass feedstocks. Because most enzymes cannot survive in extreme acidic or alkaline conditions, enzymatic hydrolysis requires milder reaction conditions and reduces industrial costs compared to acid hydrolysis (Spagnuolo et al., 1997). To maximize degradation of different biomass feedstocks the enzyme activity, temperature, and pH must be optimized for the enzyme activities in use. Also, many enzymes are inhibited by end-products of hydrolysis (Sun and Cheng, 2002). The recalcitrant nature of lignocellulosic biomass also requires high enzyme loading rates to achieve substantial degradation (Banerjee et al., 2010). Many enzymes are thermodynamically unstable and have limited temperature ranges. The optimization of enzymes and reaction conditions that maximize hydrolysis has been studied for many lignocellulosic feedstocks (Banerjee et al., 2010; Gao et al., 2010; Gil et al., 2010; Kuhad et al., 2010; Ray et al., 2010).

The greatest financial challenge with enzymatic hydrolysis is the production costs of enzymes (Banerjee et al., 2010). Enzymes used for hydrolysis are

naturally produced by bacteria and fungi (Sun and Cheng, 2002). Increased enzyme production from living organisms at lower costs is a focus of major distribution companies, such as Novozymes, Genencor, DSM Innovation Center, and Verenium (Banerjee et al., 2010). As the cost of enzymes decrease and the yields of hydrolysis increase, enzymatic hydrolysis has promise for many biomass feedstocks.

Enzymatic Hydrolysis of Sugar Beet Pulp and Similar Biomass

Multiple types of enzymes (cellulases, hemicellulases, cellobiases, and pectinases) have been considered for the degradation of biomass feedstocks into soluble sugars. Most feedstocks do not require pectinases because of the limited pectin content in the substrate. Pectin-rich biomass provides different challenges because pectinases and cellulases must be used to maximize the soluble sugars in the hydrolyzate. As the more plentiful biomasses have limited pectin content, more research focuses on cellulase use compared to pectinase use. Research to improve hydrolysis of both sugar beet pulp and grapefruit waste peels provide important information about the use of pectinases, cellulases, and cellobiases individually and in combination (Micard et al., 1996; Spagnuolo et al., 1997; Spagnuolo et al., 1999; Wilkins et al., 2007b).

Dried sugar beet pulp (10% moisture content) was tested for hydrolysis with three cellulases, one hemicellulase, and three pectinases (Spagnuolo et al., 1997). The pectinases produced the most effective overall hydrolysis ranging between 24.7 and 44.6% total sugars. More arabinose and galacturonic acid were released by pectinases compared to the other enzymes, but low cellulase activity produced

glucose yields between 2 and 30%. Hemicellulases alone caused very little degradation of the SBP, and only one cellulase showed significant hydrolysis with 20% of the theoretical sugar yields (Spagnuolo et al., 1997). Wilkins et al. (2007b) also found that hydrolysis with pectinase increased yields of total sugars and dissolved dry matter of grapefruit waste peels compared to those without. Grapefruit waste peels are comparable to sugar beet pulp because of similar pectin content. Greater pectinase loading rates (5 and 10 mg protein/g peel dry matter) were required to improve all the total sugar yields. Lower cellulase loadings (1 mg protein/g peel dry matter) increased hydrolysis yields of glucose, xylose, galactose, total sugars, and dissolved dry matter, but galacturonic acid and arabinose yields were not improved (Wilkins et al., 2007b). Both pectin-rich materials were degraded with pectinases and cellulases individually, but total sugar yields were not maximized.

To increase the overall sugar yields from hydrolysis of sugar beet pulp and grapefruit peel wastes, pectinases were tested in combination with cellulases, hemicellulases, and cellobiases. Spagnuolo et al. (1997) found the best results came from pairing cellulases with hemicellulases or pectinases. Overall, the synergistic effect increased hydrolysis to 69.2% of total acidic saccharification. The best indicator of increased hydrolysis was seen in the glucose yields which increased between 16.1-77.6% when a combination of cellulases and pectinases were used (Spagnuolo et al., 1997). The use of pectinase and beta-glucosidase enzymes produced greater sugar and dissolved solids yields from grapefruit waste peels compared to treatment with cellulase and beta-glucosidase enzymes

(Wilkins et al., 2007b). This showed that pectinases contain some cellulase activity, where as cellulases did not have any noticible pectinase activity. Although pectinase had some cellulase activity, addition of some cellulases was recommended to increase glucose and galactose concentrations for future fermentations (Wilkins et al., 2007b). Combinations of cellulases, hemicellulases, and pectinases have also been considered, because the combination of two enzymes used by Spagnuolo et al. (1997) successfully increased the sugar yields. Three of the five combinations tested increased sugar yields to 76.6-86.5% of the acid hydrolysis sugar yield. When the enzyme combination that included cellulase, hemicellulase, and pectinase was doubled saccharification was increased from 84.5-100% (Spagnuolo et al., 1997). Research with pectin-rich material recommended the use of pectinases and cellulases to increase hydrolysis yields.

The degradation of individual components (cellulose, hemicellulose, and pectin) in sugar beet pulp was also studied (Micard et al., 1996; Spagnuolo et al., 1999). The research goal of Micard et al. (1996) was to release arabinose, rhamnose, and galacturonic acid while keeping the cellulose intact through the use of commercially prepared enzymes in one unique set. Two pectinases produced the highest yields of galacturonic acid, arabinose, rhamnose, and galactose between 80-98% of theoretical yields. The cellulase activity varied but only about 10% of theoretical glucose was hydrolyzed (Micard et al., 1996). Spagnuolo et al. (1997) showed a similar difference between two other pectinases (Viscozyme and Pectinx), was 15% more glucose was released from Viscozyme. Semicontinuous hydrolysis of sugar beet pulp was attempted with ultrafiltration to hydrolyze the

hemicellulose of sugar beet pulp while the pectin and cellulose remained intact (Spagnuolo et al., 1999). A 1000 Da cut-off membrane separated hydrolyzed soluble sugars from enzymes and deproteinated beet pulp (DBP) and reduced substrate feedback inhibition. After seven cycles where DBP was added to the filtration cell, there was no decrease in the enzyme efficiency with an average of 97.6% of the arabinose hydrolyzed. Only 94.2% of arabinose was released in enzymatic hydrolysis without ultrafiltration. The membrane used lower enzyme loading (3.5 times less) compared with conventional enzyme hydrolysis (Spagnuolo et al., 1999). Single enzyme preparations and ultrafiltration improved hydrolysis of individual components of sugar beet pulp.

Sugar beet pulp can be successfully hydrolyzed with enzymes. Combinations of pectinases and cellulases increased total degradation significantly, but hydrolysis of one component of sugar beet pulp is also possible. Depending on the goal after hydrolysis, each method has applications.

MICROORGANISMS

Saccharomyces cerevisiae is traditionally used to ferment glucose to ethanol, but the diversity of sugars from hydrolyzed hemicellulose requires microorganisms with diverse metabolisms (Dumsday et al., 1999). One of the major roadblocks affecting ethanol production from lignocellulosic biomass is the lack of a microorganism that can ferment the diversity of sugars encountered from different feedstocks (Dien et al., 2003). Many researchers are now developing engineered microorganisms that have the potential to ferment a variety of sugars

hydrolyzed from biomass, such as glucose, xylose, arabinose, cellobiose, and galacturonic acid, with high ethanol yields (Chandrakant and Bisaria, 1998).

SACCHAROMYCES CEREVISIAE

Saccharomyces cerevisiae, a fermentative yeast species, has been the traditional glucose-fermenting microorganism used in industrial production of ethanol for liquid fuel (Sedlak and Ho, 2001). *S. cerevisiae* has an optimum pH range between 4 and 5 and an optimum temperature range between 30-35°C (Bollok et al., 2000). Glucose, a hexose, is the primary carbon source for energy and growth. Ethanol is the main by-product produced in an anaerobic environment (Figure 1) (Entian and Barnett, 1992). The glucose metabolism works well for corn ethanol production because of the starch-based biomass feedstock, but other lignocellulosic feedstocks present challenges because of composition diversity.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Figure 1. *S. cerevisiae* Metabolism

Like most microorganisms, *S. cerevisiae* can be inhibited by chemicals and conditions during fermentation. Acetic acid and lactic acid have both decreased the productivity of yeasts (Narendranath et al., 2001). Concentrations as low as 0.05-0.1% for acetic acid and 0.2-0.8% for lactic acid have decreased cell growth and ethanol production (Narendranath et al., 2001). The minimum inhibitory concentration (MIC) was 100 mM (0.6% w/v) and 278 mM (2.5%) w/v for acetic and lactic acids, respectively (Narendranath et al., 2001). When acetic and lactic

acid are present in solution together, a synergistic effect occurs. Ethanol production and final ethanol titers were decreased in a corn mash hydrolzate at 30, 34, and 37°C (Graves et al., 2007). Undissociated acetic acid, found in solution at a low pH, is antimicrobial and inhibits *S. cerevisiae* by dissipating across the plasma membrane of the yeast cell (Arneborg et al., 1997). Inside the yeast cell where there is a higher intracellular pH, the acetic acid dissociates and acidifies the cell. Homeostasis attempts to regulate the cell pH by pumping protons out of the cell membrane (Arneborg et al., 2000). The proton pumping to maintain appropriate cellular conditions interrupts the production of energy for cell growth (Arneborg et al., 2000; Arneborg et al., 1997).

Ethanol concentrations have been found to influence yeast cell growth (Arneborg et al., 1997; Krisch and Szajani, 1997). Too much ethanol can inhibit cell growth and function of *S. cerevisiae* by altering the membrane permeability and structure. One study showed that ethanol concentration greater than 16% (v/v) were toxic to free yeast cells, and no yeast growth occurred above 20% (v/v) (Krisch and Szajani, 1997). On the other hand, production and addition of ethanol to fermentation media increased yeast cells resistance to acetic acid (Arneborg et al., 1997).

As the importance of lignocellulosic feedstocks for biofuels production grows, so does the need for a recombinant microorganism that can produce ethanol from multiple sugars. *S. cerevisiae* already has many desirable traits that make industry-scale fermentation possible; it is user-friendly, safe, and effective (Sedlak et al., 2003). Genetically altering *S. cerevisiae* has created new strains

that can ferment glucose, xylose, and arabinose to increase ethanol yields (Sedlak and Ho, 2001), but no efforts have been made to incorporate galacturonic acid metabolism.

More work remains to be done for commercial utilization of modified co-fermenting yeast strains. Sedlak et al. (2003) compared the ethanol yields and continuous fermentation stability of two new strains of *Saccharomyces* yeasts, LNH33 and LNH-ST. Both strains were modified to co-ferment glucose and xylose by introduction of three xylose metabolizing genes. Although LNH33 could not sustain successive culture generations, LNH-ST successfully co-fermented glucose and xylose in continuous and batch fermentations with both stock solutions and corn hydrolzate (Toon et al., 1997). To further improve the *S. cerevisiae* for use in lignocellulosic hydrolysates, DNA microarray analysis was used to understand enzyme encoding and gene expressions for alcohol fermentation. Although the recombinant *S. cerevisiae* co-ferments glucose and xylose, glucose is still consumed much quicker. The increase and decrease of certain metabolic genes was found to rely on the glucose concentrations in solution. The results will help improve co-fermentation (Sedlak et al., 2003). Another improvement to *S. cerevisiae* is the incorporation of the *E. coli* araBAD operon into *S. cerevisiae* (Sedlak and Ho, 2001). This operon allowed the glucose-xylose co-fermenting strain of *S. cerevisiae* to ferment L-arabinose also. Although more arabinose was consumed in fermentations where *S. cerevisiae* contained the operon, no increase in ethanol concentration occurred (Sedlak and

Ho, 2001). As modification of *S. cerevisiae* continues to improve co-fermentation abilities, more applications with lignocellulosic biomass will become possible.

ESCHERICHIA COLI KO11

Escherichia coli was one of the first microorganisms modified with fermentative plasmids from *Zymomonas mobilis* (Ingram et al., 1987). *E. coli* already had the ability to metabolize a variety of five- and six-carbon sugars, such as glucose, xylose, arabinose, and lactose, but the major end products were succinate, lactate, and acetate. Low ethanol concentrations were modified by the addition of pyruvate decarboxylase and alcohol dehydrogenase II genes via the pLOI295 plasmid. Ethanol concentrations then reached over a third of the total fermentation product concentrations (Ingram et al., 1987). This modified strain, *Escherichia coli* KO11 has been used in research for many different lignocellulosic feedstocks including sugar beet pulp, brewery wastewater, cotton gin residues, and corn cob hydrolysates (Agblevor et al., 2003; Doran and Foster, 2000; Doran et al., 2000; Grohmann et al., 1994; Lima et al., 2002; Rao et al., 2007; Takahashi et al., 2000)

E. coli KO11 is also a hardy bacterium that is resistant to many processing challenges that could occur in an industrial setting (Moniruzzaman et al., 1998). *E. coli* KO11 has an optimum pH range of 6-8 (Dien et al., 2003). Ethanol production in extreme pH changes (3 and 10) was shown to be detrimental to the bacteria's ethanol production. Once the pH was readjusted to 6, ethanol production resumed but took much longer to complete. A similar effect was noted for temperature.

When the temperature (32-37°C) was also altered to 5°C and 50°C and returned to optimum, *E. coli* KO11 resumed ethanol production.

Challenges caused by feedstock variability and industrial sanitation conditions were also considered. Both microbial contamination and biomass particle size did not restrict ethanol production, although process time did increase (Moniruzzaman et al., 1998). *E. coli* KO11 can survive and continue to function in unfavorable circumstances.

An advantage that *E. coli* KO11 provides for fermentations is the atypical ability to ferment galacturonic acid. Grohmann et al. (1994) proved the fermentation path of galacturonic acid by growth of *E. coli* KO11 in a 2% galacturonic acid solution. The ethanol, acetic acid, and carbon dioxide yields of 80, 78, and 80% showed that ethanol was produced, but at lower yields 0.237 g ethanol/g galacturonic acid compared to the standard 0.51 g ethanol/g glucose associated with typical yeasts (Grohmann et al., 1994). The fermentation of galacturonic acid is helpful for feedstocks rich in pectin, such as sugar beet pulp, citrus peel waste, and apple pomace (Doran et al., 2000).

The acetic acid by-products of galacturonic acid fermentation could be used as a valuable end product, but the lower pH in the fermentation media could limit *E. coli* KO11 cell growth (Grohmann et al., 1994; Ingram et al., 1987). The decrease in pH to below 5, resulted in lower cell density for both wild and recombinant strains of *E. coli* (Ingram et al., 1987).

The ethanol tolerance of *E. coli* KO11 is much less than that of traditional yeasts which presents some challenges for profitable ethanol production. The

concentrations of sugars in typical biomass hydrolyzates would only produce about 50 g/L of ethanol maximum due to solids loading restrictions (Moniruzzaman et al., 1998). In multiple strains of *E. coli*, increased ethanol concentrations ranging from 1-4% ethanol were found to selectively inhibit the production of saturated fatty acids. At 6% ethanol, unsaturated fatty acid production was also limited (Buttke and Ingram, 1980). The associated damage in the cell membrane can decrease the ethanol tolerance of *E. coli*. Yomano et al. (1998) isolated *E. coli* KO11 mutants that produced over 60 g/L of ethanol from 140 g/L xylose or 85% of the maximum theoretical yield (0.51 g ethanol/g glucose or xylose). The parent *E. coli* KO11 strain yielded 59.5 g/L of ethanol from xylose or 80% of the maximum yield in 120 hr. Glucose solutions yielded 52.7 g/L of ethanol or 74% of the maximum yield in 96 hr. Although other microorganisms with greater ethanol tolerance have been modified to metabolize xylose, *E. coli* KO11 produced the highest ethanol yields (Yomano et al., 1998).

Other Microorganisms

Pichia stipitis, a type of yeast, has potential as a fermenting organism for cellulosic biomass feedstocks that are high in glucose and xylose because of the ability to produce ethanol yields over 50 g/L (Agbogbo et al., 2006; Slininger et al., 1990). Operating temperature and pH ranges of 28-34°C and 3-8, respectively, make *P. stipitis* a desirable microorganism because of stability in dynamic environments (Slininger et al., 1990). *P. stipitis* can naturally ferment xylose (Agbogbo et al., 2006). *P. stipitis* is also respiro-fermentative, meaning that both respiration and fermentation can occur simultaneously at moderate oxygen

concentrations. Moderate aeration increased ethanol production and decreased fermentation time. The rates of biomass production and xylose transport were also increased. Too much aeration decreased ethanol yields. (Agbogbo and Wenger, 2007).

Although *P. stipitis* fermented both glucose and xylose, glucose concentration had to reach less than 2% before xylose was consumed (Agbogbo et al., 2006). The lack of co-fermentation by *P. stipitis* could increase fermentation time and make *P. stipitis* less favorable for industrial application. As seen in experimentation, glucose consumption occurred at a faster rate than xylose consumption, with fermentations completing two days faster (Agbogbo et al., 2006). *P. stipitis* is limited in its application for SBP because it only ferments glucose and xylose, which leaves other five- and six- carbon sugars, such as arabinose and galacturonic acid, that comprise a majority of the sugars present in the hydrolyzate.

P. stipitis does have one advantage over many other yeasts and bacteria. Acetic acid can be consumed, which increases fermentation pH (Agbogbo and Wenger, 2007). Although the end products from the metabolism process are not known, acetic acid normally decreases pH and inhibits microorganisms (Agbogbo and Wenger, 2007).

Fermentation Applications

Separate Hydrolysis and Fermentation (SHF)

The development and knowledge of different capabilities of various microorganisms has been applied in many types of fermentation. The different

temperature and pH requirements of enzymes and microorganisms may make separate hydrolysis and fermentation (SHF) desirable. An SHF allows for optimization for the growth conditions of both the enzymes and microorganisms (Olofsson et al., 2008). Both options have been thoroughly researched for use with many different lignocellulosic feedstocks.

Early fermentation of citrus peel waste separated hydrolysis and fermentation to limit the inhibition of microorganisms caused by limonene (Grohmann et al., 1994). In an SHF with *E. coli* KO11, the glucose, galactose, fructose, and galacturonic acid were completely consumed within the first 24 hours. *E. coli* KO11 fermentations increased ethanol yields by 25-35% compared with yeast fermentations of similar hydrolyzates, because more sugars were metabolized in the fermentation (Grohmann et al., 1994).

Simultaneous Saccharification and Fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) has become an industry standard for fermentations. SSF processes hydrolyze biomass with enzymes and ferment resulting sugars with microorganisms at the same time (Wilkins et al., 2007a). This process eliminates end-product inhibitors for enzymes, so that the sugar monomers that compose the biomass are continually released and consumed and thus sugar concentrations remain relatively low. Capital costs can also be reduced by SSF because hydrolysis and fermentation occur in the same vessel, eliminating costs for multiple vessels and hydrolyzate separation (Olofsson et al., 2008).

A successful example of decreased inhibition is the simultaneous saccharification and fermentation (SSF) of citrus peel waste decreased the enzyme loading requirements by 6% for pectinase and 92% for cellulase. The *S. cerevisiae* in the fermentation vessel consumed glucose quickly once hydrolysis occurred, thus eliminating the inhibition feedback responses of the enzymes in solution (Wilkins et al., 2007a).

Although an SSF with *S. cerevisiae* decreased the necessary enzyme loading for citrus peel waste, the opposite occurred when sugar beet pulp was fermented in an SSF with *E. coli* K011 (Doran and Foster, 2000). The study compared fermentations of wet, pressed, and pelletized sugar beet pulp at 10 and 12% solids loading rates. The greatest ethanol yield and concentration occurred in the fermentation with the greatest solids and enzyme loadings. *E. coli* K011 produced 33 g/L of ethanol for a yield of 0.277 g ethanol/g SBP (Doran and Foster, 2000).

Problem Statement

Ethanol is an important liquid fuel that functions with the current transportation infrastructure in the United States. As transportation needs continue to grow, petroleum cannot be the only energy input. Starch-based ethanol is helpful but limited because humans can consume starch as food. Lignocellulosic biomass uses woody plants and crop residues that humans cannot digest. Sugar beets are a regionally significant cash crop in North Dakota and Minnesota that have the challenges of harvest, storage, and transportation addressed in the current sucrose extraction process. The sugar beet pulp produced annually can be

easily hydrolyzed with enzymes because of the low lignin content. The atypical low cellulose and high hemicellulose and pectin fractions of sugar beet pulp requires careful consideration of enzymes and microorganisms for successful ethanol production.

Research has shown that the use of pectinases and cellulases together increases the total hydrolysis of sugar beet pulp (Spagnuolo et al., 1997). Components of sugar beet pulp, like hemicellulose, can be selectively hydrolyzed, with the other components remaining mostly intact (Micard et al., 1996; Spagnuolo et al., 1999). Once the sugars contained in sugar beet pulp are released, microorganisms that can ferment all sugars are needed to increase ethanol yields. *E. coli* KO11 and *S. cerevisiae* both have desirable characteristics to produce high ethanol titers yields from the pulp. *E. coli* KO11 can ferment the glucose, arabinose, and galacturonic acid that comprise the majority of SBP hydrolyzate (Dien et al., 2003). Although the commercial use of *E. coli* KO11 is currently limited, the gene modifications hold promise for successful commercial fermentation. *S. cerevisiae*, while limited to metabolism of glucose, can withstand high ethanol concentrations and is already an industrial standard yeast (Sedlak and Ho, 2001).

To maximize the potential ethanol production from sugar beet pulp, pectinase, cellulase, and cellobiase enzymes will hydrolyze different components (cellulose, hemicellulose, and pectin) strategically to take advantage of each organism's favorable characteristics. *E. coli* KO11 will be used to ferment the glucose, arabinose, and galacturonic acid and produce lower ethanol

concentration, while *S. cerevisiae* will ferment remaining glucose to increase ethanol concentrations.

The final goal of increased ethanol yields and titers was achieved by progressive experimental steps. The protease and pectinase (Viscozyme and Pectinex) enzymes were tested to determine the effect on hydrolysis of sugar beet pulp. The pectinase enzymes that best maximize pectin and hemicellulose hydrolysis but limit cellulose hydrolysis were used before fermentation with *E. coli* KO11 to maximize ethanol production from arabinose and galacturonic acid. Cellulases produced glucose that can be fermented to high ethanol yields with *S. cerevisiae*. After preliminary investigations to determine the success of these fermentations, four methods that combine the ethanol produced by both *E. coli* KO11 and *S. cerevisiae* were tested and compared.

This thesis researched pectinase enzymes that would maximize hemicellulose and pectin hydrolysis, while minimizing cellulose hydrolysis. Combining that pectinase with cellulases, cellobiases, and microorganisms to test two microorganisms that can ferment soluble sugars hydrolyzed from sugar beet pulp. Increased solids loading rates improved hydrolyzate sugar concentrations to increase ethanol concentrations/titers. *S. cerevisiae* will ferment glucose, while *E. coli* KO11 will ferment the remaining sugars (arabinose, galacturonic acid, and galactose) to improve ethanol yields.

PAPER 1: ENZYMATIC HYDROLYSIS OF PECTIN AND HEMICELLULOSE IN SUGAR BEET PULP

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ABSTRACT

Sugar beet pulp (wet), a byproduct from the table sugar industry, was used in enzymatic treatment to determine the effect of solids and enzyme loading rates on hydrolysis of pectin, hemicellulose, and cellulose for ethanol production. Long-term research goals include hydrolyzing and fermenting glucose separately from other five and six carbon sugars. Two commercial enzymes, Viscozyme and Pectinex, were used to hydrolyze pectin and hemicellulose prior to cellulose hydrolysis. These enzymes were tested separately and in combination with maximum total enzyme loading rates of 100 μL/dry g. A protease, Flavourzyme, was also tested as a pretreatment to Pectinex treatments to determine if hemicellulose and pectin hydrolysis could be increased. Optimal loading rates resulted in high hemicellulose and pectin hydrolysis with low cellulose hydrolysis (low glucose yields). Separate hydrolysis and fermentation of the sugar mixtures is desired to increase final solids loading rates and ethanol titers. Viscozyme treatments resulted in the highest hemicellulose and pectin hydrolysis while limiting cellulose hydrolysis.

Keywords. Sugar beet pulp, hydrolysis, hemicellulose, pectin, cellulose, ethanol

INTRODUCTION

The conversion of cellulosic material into ethanol has been gaining considerably more attention recently. With concerns over increasing oil prices, net carbon dioxide emissions, and the use of foodstuffs for fuel production, a wide variety of cellulosic biomass sources are being investigated for ethanol production (Ruth, 2008). Sugar beet pulp, a by-product of the table sugar industry, shows promise as a potential biofuel feedstock (Micard et al., 1996). Due primarily to its limited geographic importance, it has received much less attention than other biomass feedstocks such as corn stover, wheat straw, perennial grasses, or hybrid poplar. Beet pulp could have economic benefits in states such as North Dakota and Minnesota, where 14.7 million tons of sugar beets (56% of the nation's total harvest) were produced in 2007 (USDA, 2008). Currently sugar beet pulp is dried, pelleted, and sold as a relatively low-value animal feed. The high cost and energy for drying, pelleting, and transporting the pulp limits profits for this product. Producers and processors will benefit from developing a higher value use of beet pulp that does not require drying.

Sugar beet pulp has characteristics that make it advantageous for ethanol production. The primary compositional advantages of sugar beets are the low lignin content (3-4%) and high levels of readily digestible pectin (24-32%). This allows beet pulp to be hydrolyzed relatively easily without the use of harsh and expensive thermochemical pretreatments required for most other biomass feedstocks (Spagnuolo et al., 1997). An ammonia pressurization depressurization pretreatment was determined to substantially increase cellulose hydrolysis but

showed no improvements when used with hemicellulases and pectinases (Foster et al., 2001). The logistics and technology for feedstock harvest, transportation, and storage may be a limiting factor for most conventional biomass sources, as well. Because the beet pulp is generated on site, these issues will generally not be a limiting factor if the pulp is to be fermented on site at the sugar processing facility.

Compared to other biomass feedstocks, sugar beet pulp has relatively low levels of cellulose (22-30%) and moderate levels of hemicellulose (24-32%) (Spagnuolo et al., 1997). Because of the low cellulose content, the yield of readily fermentable glucose as a percentage of total sugars is low. The hemicellulose of most biomass streams is typically comprised of xylose as the largest sugar component. Sugar beet pulp, however, has low levels of xylose (1.7%) with arabinose making up the largest portion of the hemicellulose (Doran and Foster, 2000). The higher levels of arabinose (20.9%) and pectin-derived galacturonic acid (21.1%) are less readily fermented and require genetically modified organisms to ferment efficiently in the presence of glucose (Doran and Foster, 2000).

Spagnuolo et al. (1997) tested different combinations of hemicellulolytic, cellulolytic, and pectinolytic enzymes to determine which combination yielded the maximum total sugars. Pectinase treatments resulted in the greatest hydrolysis levels, while the cellulase treatments alone resulted in much lower sugar yields. Combinations of cellulolytic and pectinolytic enzymes acted synergistically, while hemicellulase addition had no such effect. These results suggest that the pectin found in sugar beet pulp creates a barrier that must first be broken before complete

hydrolysis of the pulp can occur. Micard et al. (1997) also studied the complete enzymatic degradation of sugar beet pulp. They tested both sequential and combined treatments with pectinase and cellulase enzymes with different pretreatments of grinding and washing. Cellulose was found to be more completely hydrolyzed when pectinase was added prior to cellulase treatment (Micard et al., 1997).

Ethanol fermentation using genetically modified bacteria requires high loading rates of enzymes in order to produce a desirable ethanol yield in a simultaneous saccharification and fermentation process. Doran and Foster (2000) analyzed three recombinant ethanologenic bacterial strains, *Escherichia coli* KO11, *Klebsiella oxytocaa* P2, and *Erwinia chrysanthemi* EC 16, to determine which produced the highest levels of ethanol from sugar beet pulp hydrolyzate. When fermenting arabinose and galacturonic acid, *E. coli* KO11 yielded the highest ethanol concentration of 25.5 g/L followed by *K. oxytocaa* P2, and *E. chrysanthemi* EC 16 at 18.3 g/L and 17.3 g/L, respectively. The loading rates of the enzymes were possibly too high for industrial scale processes for the relatively low ethanol titers that resulted in the experiment. Another challenge in using the genetically modified bacteria is the inability of these organisms to survive at high ethanol concentrations (Doran and Foster, 2000). Separating hemicellulose and pectin sugar streams from cellulose could allow for increased ethanol titers as the glucose could be fermented by conventional yeast strains that can survive much higher ethanol concentrations.

This paper aims to explore the possibility of using enzymes that have hemicellulase, pectinase, or protease activity to separate cellulose from the hemicellulose and pectin. Enzyme loading rates were studied to maximize hydrolysis of hemicelluloses and pectin while minimizing the cellulose hydrolysis.

MATERIALS AND METHODS

SUBSTRATE

Sugar beet pulp was obtained from American Crystal Sugar Company in Moorhead, MN. The wet pulp had a moisture content of 88% (wet basis) and was stored at -20°C until thawed for experiments. Thawing had no effect on the pulp.

ENZYMES

All enzymes used in this study were trademark products of the Novozymes Corporation (Bagsvaerd, Denmark) and purchased from Sigma-Aldrich (St. Louis, MO). All enzymes were stored at 4°C when not in use.

This experiment used two commercial preparations of arabinases and pectinases, Viscozyme and Pectinex, to hydrolyze pectin and hemicellulose within the sugar beet pulp. Viscozyme L is a multi-enzyme complex with hemicellulase, cellulase, arabinase, β-glucanase, and xylanase activities. Pectinex Ultra SPL is a highly pectinolytic enzyme complex containing pectintranseliminase, polygalacturonase, and pectinesterase activities with lower levels of hemicellulase and cellulose activities. Both Viscozyme and Pectinex have an optimum pH and temperature of 5 and 40°C, respectively. The enzymes were tested both independently and in combination to quantify interaction. The benefit of these enzymes was the limited cellulase activity that minimized the cellulose hydrolysis. By first releasing the galacturonic acid, arabinose, and galactose more efficient ethanol fermentation can occur in later steps using genetically altered bacteria. The cellulose can be hydrolyzed to glucose fermented separately to increase total ethanol yields.

Flavourzyme is a fungal protease/peptidase complex that contains both endoprotease and exopeptidase activities. Flavourzyme has an optimum pH and temperature of 6 and 50°C, respectively.

Hydrolysis of Sugar Beet Pulp

Sugar beet pulp (1.235 dry g) was loaded in 125-mL Erlenmeyer flasks at a loading rate of 5% weight per volume of buffer and then autoclaved at 121°C for 20 minutes to prevent unwanted microbial growth. After autoclaving, the sterile buffer (50 mM citric acid buffer, pH 5 for hemicellulase/pectinase or 100 mM phosphate buffer, pH 6 for protease) and corresponding enzymes were then added according to the solids and enzyme loadings rates used in each experiment. Flasks were then incubated in a water bath at 100 rpm and 40°C or 50°C for hemicellulase/pectinase and protease treatments, respectively. Samples (2 ml) were taken 1-2 times per day for up to 48 hours. All treatments were conducted in triplicate (n=3).

Pectinex and Viscozyme were tested individually and in combinations according to Table 3. Pectinex loading rates of 100, 150, 200, and 250 μL/dry g were also tested against Viscozyme at a loading rate of 100 μL/dry g to determine if similar yields for arabinose, galactose, xylose, and galacturonic acid could be attained while minimizing glucose yields.

Flavourzyme was recommended as a protease for sugar beet pulp by the manufacturer. For experiments using a protease pretreatment, Flavourzyme was added at a loading rate of 8.1 μL/dry g in a 100 mM phosphate buffer solution at pH 6. Samples were taken for HPLC analysis after 24 hr. The remaining solids

were vacuum filtered using a Buchner Funnel and Whatman No. 41 (pore size of 20-25 μm) filter paper in a sterile hood and returned to their original flasks for further hydrolysis of pectin and hemicellulose. Citric acid buffer (50 mM, pH 5) was added to filtered biomass for a solids loading rate of 25 ml/original dry g. Flasks were then heat treated in an autoclave at 90°C for 20 min to deactivate any remaining protease prior to addition of subsequent enzymes. After the samples cooled, Pectinex was added at a loading rate of 100 μL/original dry g. Flasks were then incubated in a water bath at 40°C and 100 rpm; samples were taken for HPLC analysis at 0, 6, 19, and 24 hr.

Table 3. Viscozyme and Pectinex Loading Combinations

Viscozyme Loading μL/dry g	Pectinex Loading μL/dry g
100	0
75	25
50	50
25	75
0	100

Analysis of Soluble Sugars

Samples were centrifuged and filtered through a 0.2-μm nylon filter (Pall Corporation, West Chester, PA) prior to HPLC analysis. Cellobiose, glucose, arabinose, galactose, and fructose were quantified using a Waters (Milford, MA) HPLC and refractive index detector. Sugars were separated using a Bio-Rad (Hercules, CA) Aminex HPX-87P column with a mobile phase of water at a flow of

0.6 mL/min; the column and detector temperatures were 50°C and 85°C, respectively. Galacturonic acid was quantified and separated using a Waters HPLC. Separation was done using a Bio-Rad Aminex 87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min at 60°C. Detection was carried out using a photodiode array detector (Waters Corporation) at 210 nm wavelength. All sugars were quantified using a 3-point external standard curve for each component with the limits of quantification in Table 4.

Table 4: HPLC Quantification Standards

	Concentrations (g/L)		
Sugars	**Low**	**Medium**	**High**
Cellobiose	0.2	1	2
Glucose	1	5	10
Xylose	0.2	1	2
Galactose	0.2	1	2
Arabinose	0.8	4	8
Fructose	1	5	10
Galacturonic Acid	1.6	4	8

To determine which enzyme treatments were the best at limiting cellulose hydrolysis while maximizing hemicellulose and pectin hydrolysis, the saccharification yields were calculated in a weight percentage based on the composition data acquired from Spagnuolo et al. (1997).

Cellulose hydrolysis was quantified by combining free glucose concentrations with cellobiose-glucose concentrations because both cellobiose and glucose result from cellulose hydrolysis. The cellulose-derived glucose

component was calculated by subtracting the fructose concentration from the measured glucose concentration. Preliminary results showed the glucose yields were unexpectedly high and the presence of fructose indicated that residual sucrose (glucose, fructose disaccharide) was responsible for these high glucose concentrations. The concentration of glucose derived from sucrose was assumed to be equal to the concentration of fructose in the hydrolyzate. Cellobiose-glucose was found using a conversion factor of 1.053 g glucose/g cellobiose.

RESULTS AND DISCUSSION

Figure 2 shows the results of hydrolysis with combinations of Viscozyme and Pectinex. The 100% Viscozyme treatment produced the greatest hydrolysis of hemicellulose, pectin, and cellulose at total enzyme loading rates of 100 µL/dry g. The 100% Pectinex treatment yielded the lowest concentrations of all sugars. The three combinations of enzymes yielded galacturonic acid percentages comparable to that of both the 100% Pectinex and 100% Viscozyme treatments, showing little difference in the pectin hydrolysis. Galacturonic acid yields were greater than 100% (Figure 2), which was probably due to inconsistency in sugar beet pulp composition between the feedstock used here and the reference composition. Arabinose, galactose, and glucose yields for samples containing any percent of Pectinex were 10-20% lower than the Viscozyme treatment. Addition of Pectinex at any level had a negative impact on yields of sugars from both hemicellulose and cellulose. Although Viscozyme hydrolyzed larger amounts of the hemicellulose and pectin, larger amounts of the cellulose were also hydrolyzed to free glucose.

Since equivalent volumetric loading rates of Viscozyme and Pectinex did not produce the same yields of various sugars, Pectinex loading rates were increased in increments of 50 µL/dry g to determine if higher Pectinex loading rates could achieve the same sugar yields as Viscozyme. Figure 3 shows the results of the comparison of Viscozyme treatments with increased Pectinex loading rates. Again, the galacturonic acid yields were greater than 100% due to differences in the composition of sugar beet pulp used in the experiment compared to the theoretical values from Spagnuolo et al. (1997). The Pectinex loading rates of 200

and 250 µL/dry g produced similar sugar yields as the 100 µL/dry g of Viscozyme (Figure 3). The aim of this experiment was to determine which enzyme and corresponding loading rate would maximize the hemicellulose and pectin hydrolysis, while minimizing the cellulose hydrolysis. Table 5 shows the hydrolyzate sugar concentrations and a ratio of glucose concentration (including the glucose from cellobiose hydrolysis) to total other sugars (arabinose, galactose, and galacturonic acid).

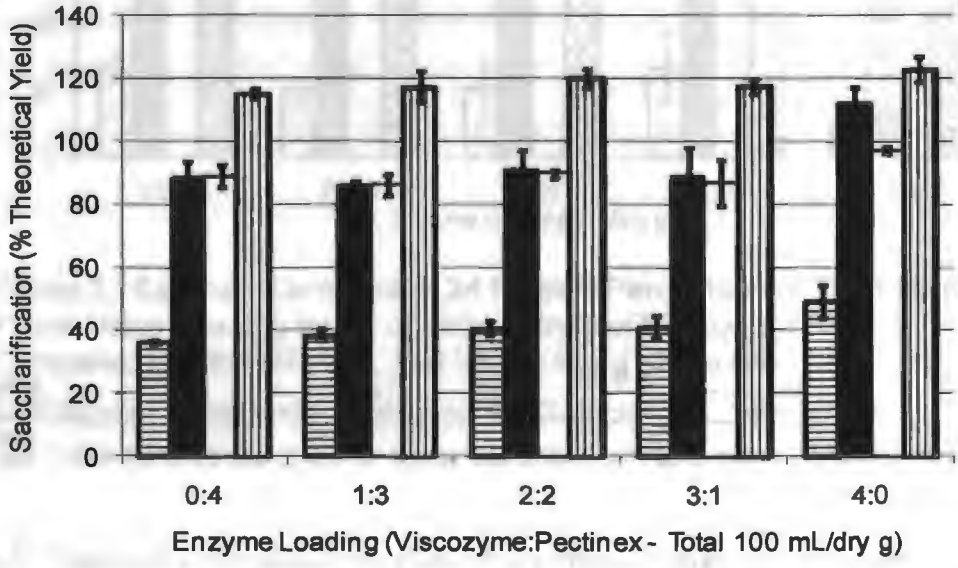

Figure 2. Saccharification (after 48 hr) as Percentage of Theoretical Yields Using Combinations of Viscozyme and Pectinex ■Glucose (Free and Cellobiose), ■ Galactose, □ Arabinose, ▤ Galacturonic Acid, Error bars represent sample standard deviation

The presence of cellobiose in the hydrolyzate is very different for Viscozyme and Pectinex. Viscozyme yields much lower levels of cellobiose but higher glucose concentrations, meaning that Pectinex had lower β-glucanase activity.

Although Pectinex had lower glucose yields, the higher concentrations of glucose from cellobiose result in similar total possible glucose concentrations for all the loading rates.

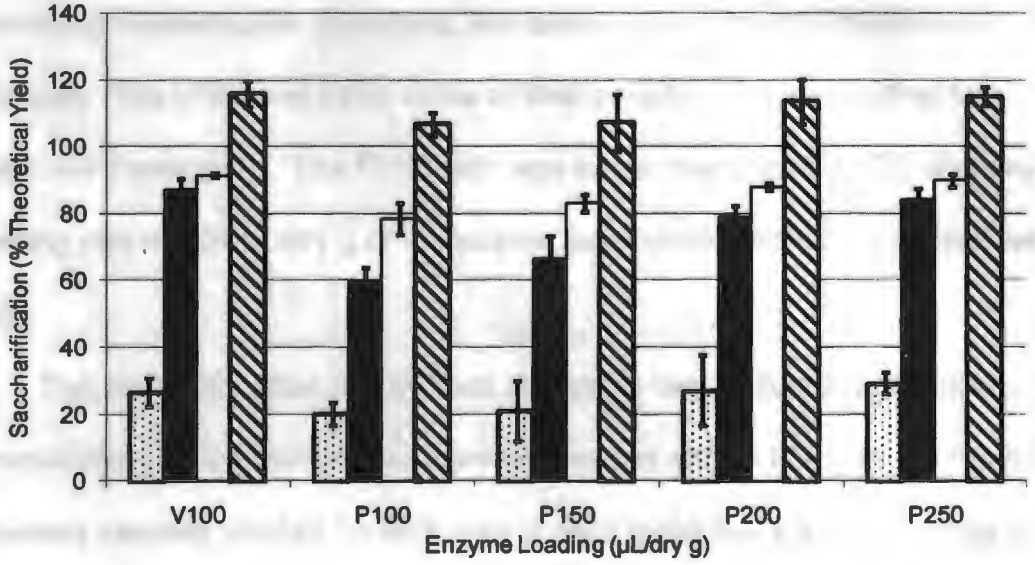

Figure 3. Saccharification (after 24 hr) as a Percentage of Theoretical Yields Using Loading Rates of 100 µL/dry g of Viscozyme (V) Compared to 100, 150, 200, and 250 µL/dry g of Pectinex (P) Glucose (Free and Cellobiose), Galactose, Arabinose, Galacturonic Acid, Error bars represent sample standard deviation

Table 5. Ratio of Total Glucose (Cellulose Hydrolysis) to Total Other Sugars

Treatment	Glucose from Cellulose (g/L)	Glucose from Cellobiose (g/L)	Total Possible Glucose (g/L)	Other Total Sugars (g/L)	Glucose : Other Sugars
V100	2.410	0.282	2.692	13.936	0.193
P100	0.042	1.985	2.027	12.161	0.167
P150	0.355	1.776	2.131	12.589	0.169
P200	1.007	1.740	2.747	13.457	0.204
P250	1.304	1.663	2.966	13.745	0.216

The most important goal of this experiment was to maximize the hemicellulose and pectin hydrolysis. The Viscozyme treatment (V100) achieved the best hydrolysis by producing the highest concentration of total other sugars at 13.94 g/L. The closest Pectinex treatment (P250) yield was 13.75 g/L. After maximizing hemicellulose hydrolysis, the second goal was to minimize cellulose hydrolysis. The V100 and P250 ratios of total possible glucose to other total sugars were compared. The P250 ratio was larger than that of V100, showing that a loading rate of 100 µL/dry g of Viscozyme best hydrolyzed the hemicellulose and pectin.

The saccharification of individual sugars by hemicellulase/pectinase enzymes with and without protease pretreatment is shown in Figure 4. Protease-pretreated samples yielded 10-30% less of each individual sugar compared with the samples that were not pretreated. Destruction of hemicellulases and pectinases by residual proteases should have been minimized or eliminated through filtration and inactivation by 90°C heat treatment of solids prior to hemicellulase/pectinase addition. In addition, Flavourzyme and Pectinex have different optimum temperatures and pHs. Further explanation of the cause for inhibition of further hydrolysis was not explored. Flavourzyme pretreatment failed to achieve the goal of increasing hemicellulose and pectin hydrolysis and will not be used in the future.

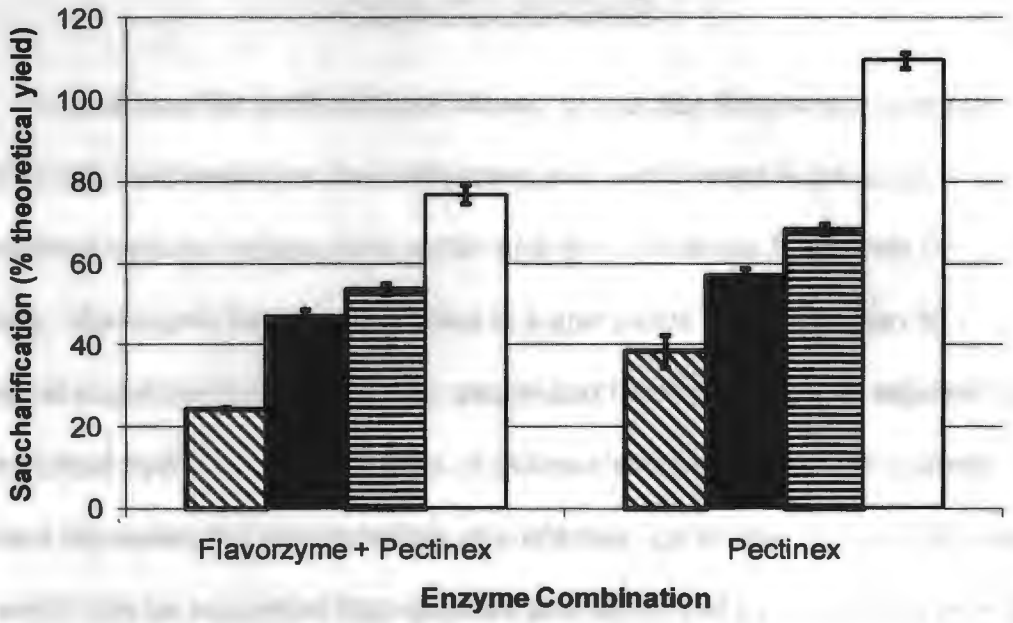

Figure 4. Saccharification (after 48 hr) as a Percentage of Theoretical Yields Using Pectinex with or without a Flavourzyme Pretreatment of Sugar Beet Pulp
■ Glucose (Free and Cellobiose), ■ Galactose, ■ Arabinose, □ Galacturonic Acid, Error bars represent sample standard deviation

CONCLUSIONS

To maximize the profitability of ethanol production from sugar beet pulp, the enzymes that best hydrolyze hemicelluloses and pectin need to be used. Protease pretreatment reduced subsequent pectin and hemicelluloses hydrolysis by Pectinex. Viscozyme treatment resulted in sugar yields of greater than 85% of theoretical sugar beet composition for pectin and hemicellulose with approximately 30% cellulose hydrolysis (lower yields of glucose and cellobiose). Viscozyme treatment increased the concentration of arabinose, galactose and galacturonic acid, which can be separated from glucose and fermented by genetically modified organisms such as *E. coli K011*. Future research will focus on separate sugar streams for subsequent fermentation to ethanol or other products.

ACKNOWLEDGMENTS

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To write the first paper in my thesis, I researched literature, designed experiments, collected data, and analyzed results with guidance from my advisor, Dr. Scott Pryor, and his lab technician, Ms. Nurun Nahar. The paper is my original work that was edited by both Dr. Pryor and Ms. Nahar. This paper was published for the 2008 North/Central American Society of Agricultural and Biological Engineering (ASABE) conference. I presented the results at the conference.

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PAPER 2: ENZYMATIC HYDROLYSIS AND FERMENTATION OF SUGAR BEET PULP

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ABSTRACT

Pressed sugar beet pulp, a byproduct of the table sugar industry, has potential as a biofuel feedstock that will benefit producers and processors in North Dakota and Minnesota. The goal of this research is to maximize ethanol titers and yields through enzymatic hydrolysis of sugar beet pulp and fermentation of the five- and six- carbon sugars. Hemicellulose and pectin were hydrolyzed and fermented separately from cellulose in order to increase ethanol titers and yields. A commercial pectinase was used to hydrolyze hemicellulose and pectin in the pulp. The resulting solid and liquid streams were either processed sequentially in a serial fermentation or separated and fermented in parallel. The first hydrolyzate stream, containing high concentrations of glucose, arabinose, and galacturonic acid, was fermented using *Escherichia coli* KO11. The remaining solids had a high cellulose content and were processed via simultaneous saccharification and fermentation using commercial cellulases and *Saccharomyces cerevisiae*.

Keywords. Sugar beet pulp, hydrolysis, fermentation, *E. coli* KO11, *S. cerevisiae*, ethanol, hemicellulose, pectin

INTRODUCTION

Biofuels produced from renewable resources have the potential to decrease US petroleum consumption. Currently, most biofuels are produced using starch-based feedstocks. Cellulosic biomass such as switchgrass, corn stover, orange peels, and sugar beet pulp can also be used for ethanol production. Such feedstocks are abundant and have less direct impacts on food production compared to starch-based feedstocks. Sugar beet pulp, a by-product of the table sugar industry, is a unique biofuel feedstock that could be regionally significant in North Dakota and Minnesota. Beet production in the Red River Valley of these states was 14.9 million tons of sugar beets (56% of the nation's total harvest) in 2008 (USDA, 2008). Once sucrose is extracted, the beet pulp is dried, pelleted, and sold as a relatively low-value animal feed. Profits from the animal feed depend greatly on the economics of the energy and feed industries but tend to be moderate because of high energy requirements for drying, pelleting, and transporting the pulp. Use of beet pulp in a process that does not require drying will benefit producers and processors.

The composition of sugar beet pulp is different from traditional biomass feedstocks, because the lignin and hemicellulose content is lower, while the cellulose and pectin content is higher (Table 6).

Because of the low lignin and high pectin content in sugar beet pulp, expensive thermochemical pretreatment is not needed for effective hydrolysis. The pectin is readily hydrolyzed into galacturonic acid and removal of pectin improves cellulose hydrolysis (Spagnuolo et al., 1997). The relatively low cellulose

and high hemicellulose content are disadvantages that lead to lower concentrations of readily fermentable glucose and higher concentrations of arabinose (Spagnuolo et al., 1999).

Table 6. Sugar Beet Pulp and Other Biomass Feedstocks Composition (% dw basis)

	Sugar Beet Pulp (Foster et al., 2001)	Other Biomass Feedstocks (Lee et al., 2007)
Cellulose	20-24%	30-50%
Hemicellulose	25-36%	20-40%
Pectin	20-25%	0%
Lignin	1-2%	15-25%

Enzymatic hydrolysis of sugar beet pulp was studied to determine loading rates that would maximize sugar concentrations. Micard et al. (1997) compared a two-step hydrolysis of sugar beet pulp with a single-step hydrolysis to determine which cellulase yielded the highest glucose concentration. In the two-step process commercial pectinases were applied in the first step followed by commercial cellulases in the second step, while the single-step process applied both pectinases and cellulases concurrently. Cellulose hydrolysis was found to be more complete when pectinase was added separately (Micard et al., 1997). Spagnuolo et al. (1997) compared different commercial enzyme preparations separately and in combination to determine which enzymes most completely hydrolyzed sugar beet pulp. The use of Pectinex resulted in low glucose yields with high yields of arabinose and galacturonic acid (Spagnuolo et al., 1997).

Escherichia coli KO11 is a recombinant ethanologenic bacterium that has the ability to ferment glucose, xylose, arabinose, and galacturonic acid (Dien et al., 2003). *E. coli* KO11 has the disadvantage, however, of a lower ethanol tolerance (40-60 g/L) compared to yeasts (Doran and Foster, 2000). Doran and Foster (2000) studied the fermentation of sugar beet pulp hydrolyzate with three recombinant ethanologenic bacterial strains, *Escherichia coli* KO11, *Klebsiella oxytocaa*, and *Erwinia chrysanthemi* EC 16. All three were analyzed to determine which produced the highest concentrations of ethanol from sugar beet pulp hydrolyzate. *E. coli* KO11 was most efficient at fermenting arabinose and galacturonic acid and yielded the highest ethanol concentration of 25.5 g/L followed by *K. oxytocaa*, and *E. chrysanthemi* EC 16 at 18.3 g/L and 17.3 g/L, respectively. *E. coli* KO11 fermented the glucose, arabinose, and galacturonic acid in solution. At a 12% solids loading rate with 0.57% (v/v) cellulase and 1% (v/v) pectinase loadings, an ethanol concentration of 40 g/L was produced in an simultaneous saccharification and fermentation (SSF) with *E. coli* KO11 (Doran and Foster, 2000).

The yeast *Saccharomyces cerevisiae* is traditionally used to produce ethanol because the yeast can convert glucose to ethanol with high yields and can withstand ethanol concentrations greater than 130 g/L. Its disadvantage when used with beet pulp is that conventional strains cannot metabolize the other sugars present in the hydrolyzate. To ferment the diversity of sugars hydrolyzed from sugar beet pulp, *S. cerevisiae* needs to be used in combination with another microorganism that can ferment a variety of five- and six-carbon sugars.

This paper explores two paths for the hydrolysis and fermentation of sugar beet pulp using both *E. coli* KO11 and *S. cerevisiae*. The first method (parallel fermentation) included fermentation after separation of the liquid and solid streams following initial hydrolysis with pectinases. The liquid stream was fermented by *E. coli* KO11, while the solids were treated with a simultaneous saccharification and fermentation (SSF) using commercial cellulase enzymes in combination with *S. cerevisiae*. The second method (serial fermentation) included a sequential hydrolysis and fermentation (SHF) process where a pectin and hemicellulose hydrolysis was followed by fermentation with *E. coli* KO11. This was followed by an SSF with cellulases and *S. cerevisiae*.

MATERIALS AND METHODS

SUBSTRATE

Pressed sugar beet pulp was provided by American Crystal Sugar Company (Moorhead, MN) from their Moorhead facility. Moisture content was determined to be of 71.2% (wet basis). Pulp was stored at -20°C until used.

ENZYMES

Pectinex, Celluclast, and Novozyme 188 were purchased from Sigma-Aldrich (St. Louis, MO). Pectinex Ultra SPL (Novozymes, Inc.; Bagsvaerd, Denmark), a commercial preparation with arabinase pectinase, and some cellulase activity, was used to hydrolyze pectin and hemicelluloses within the sugar beet pulp. Pectinex has an optimum pH of 5 and temperature of 40°C. Celluclast (Novozymes, Inc.), a commercial preparation of cellulase, has limited cellobiase activity and was used in combination with Novozyme 188 (Novozymes, Inc.), a commercial preparation of cellobiases, to completely hydrolyze the cellulose in the sugar beet pulp. Enzyme activity of Celluclast and Novozyme 188 were determined to be 60.7 FPU/mL and 661 CBU/mL, respectively. Both have an optimum pH of 4.8 and temperature of 50°C. All enzymes were stored at 2°C when not in use.

MICROORGANISMS

A slant of *E. coli* KO11 (ATCC 55124) was obtained from American Crystal Sugar Company. Inoculum was grown on a solution of 50 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, 5/L g NaCl, and 40 mg/L chloramphenicol. The culture was incubated at 37°C and 100 rpm for 24 hr. The culture was mixed with

80% sterile glycerol to produce a 40% glycerol solution. Aliquots (1 mL) were dispensed into cryovials and stored at -20°C until use. One cryovial was added to 200 mL of pulp hydrolyzate as an inoculum for all *E. coli* KO11 fermentations.

S. cerevisiae (Type II – YSC2) was obtained from Sigma-Aldrich (St. Louis, MO). Lyophilized yeast (1 g) was combined with 2 g glucose in 100 mL of water. The inoculum was incubated in a water bath at 30°C and 25 rpm for 24 hr and added to each flask at 10% (v/v).

EXPERIMENTAL DESIGN

Hydrolysis and fermentation of the pectin and hemicellulose fractions of beet pulp were carried out separately from cellulose hydrolysis and fermentation. Pectinex was applied first to yield galacturonic acid, arabinose, and other hemicellulose sugars; this fraction was subsequently fermented with *E. coli* KO11. The remaining cellulose fraction was either: 1) separated following initial hydrolysis and treated in as a separate stream (parallel fermentation) or 2) hydrolyzed and fermented with *S. cerevisiae* in the same flask following the initial *E. coli* fermentation (serial fermentation). Schematics of these systems are shown in Figures 5 and 6, respectively. Results were produced in triplication (n=3).

Pressed pulp (10 dry grams) was loaded into 500-mL Erlenmeyer flasks and autoclaved at 121°C for 20 minutes. Citrate buffer (50 mM, pH 5) was added to make a 5% w/v solids loading rate. Pectinex was loaded at 200 µL/dry g. Total volume of the flask was 200 mL. The flasks were incubated in a water bath at 40°C and 100 rpm for 24 hours. Samples were collected at 24 hours for HPLC analysis.

For the parallel fermentation system (Figure 5), the initial hydrolyzate and remaining solids were separated by centrifugation at 5,000 rpm for 30 min. The liquid (approximately 200 mL) and solids fractions were decanted to separate 500-mL flask. The hydrolyzate pH was adjusted to 6.5 by 6N NaOH for fermentation with *E. coli*. The fermentation was inoculated by addition of a 1-mL cryovial of *E. coli* KO11. Chloremphenicol (40 mg/L) was added to prevent contamination. The flasks were sealed with a vented rubber stopper and then incubated at 37°C and 100 rpm for 120 hr. The solids fraction was resuspended in 176 mL of 50 mM citrate buffer (pH 5) and chlorenphenical was added at 40 mg/L. Simultaneous saccharification and fermentation (SSF) of the cellulose fraction was carried out by adding Celluclast (25 FPU/g original cellulose), Novozyme 188 (60 CBU/g cellulose), and 20 mL of *S. cerevisiae* inoculum. The flasks were incubated at 40°C and 100 rpm for 120 hr. Samples were taken for HPLC analysis at 0, 6, 16, 24, 32, 42, 49, 61, 74, 86, 98, 112, and 120 hr.

For the serial fermentation system (Figure 6), after initial hydrolysis with Pectinex, the pH was adjusted to 6.5 using 6N NaOH for fermentation with *E. coli* KO11 as described for the parallel fermentation. The vented flasks were incubated in a water bath at 37°C and 100 rpm for 120 hr. Samples were collected at 0, 8, 18, 24, 30, 44, 48, 58, 72, 96, and 120 hr for HPLC analysis. After 120 hr of the *E. coli* KO11 fermentation, the pH was adjusted to 5 with 3N HCl, and Celluclast (25 FPU/g original cellulose), Novozyme 188 (60 CBU/g cellulose), and 20 mL of the *S. cerevisiae* inoculum were added to each flask before incubation in a water bath

at 40°C and 100 rpm for 120 hr. Samples were taken at 0, 6, 16, 24, 32, 42, 49, 61, 74, 86, 98, 112, and 120 hr for HPLC analysis.

Analysis of Soluble Sugars

Samples were centrifuged and filtered through a 0.2-μm nylon filter (Pall Corporation, West Chester, PA) prior to HPLC analysis. Cellobiose, glucose, arabinose, galactose, fructose, and xylose were separated using a Bio-Rad (Hercules, CA) Aminex HPX-87P column with a mobile phase of water at a flow of 0.6 mL/min; the column and detector temperatures were 85°C and 50°C, respectively.

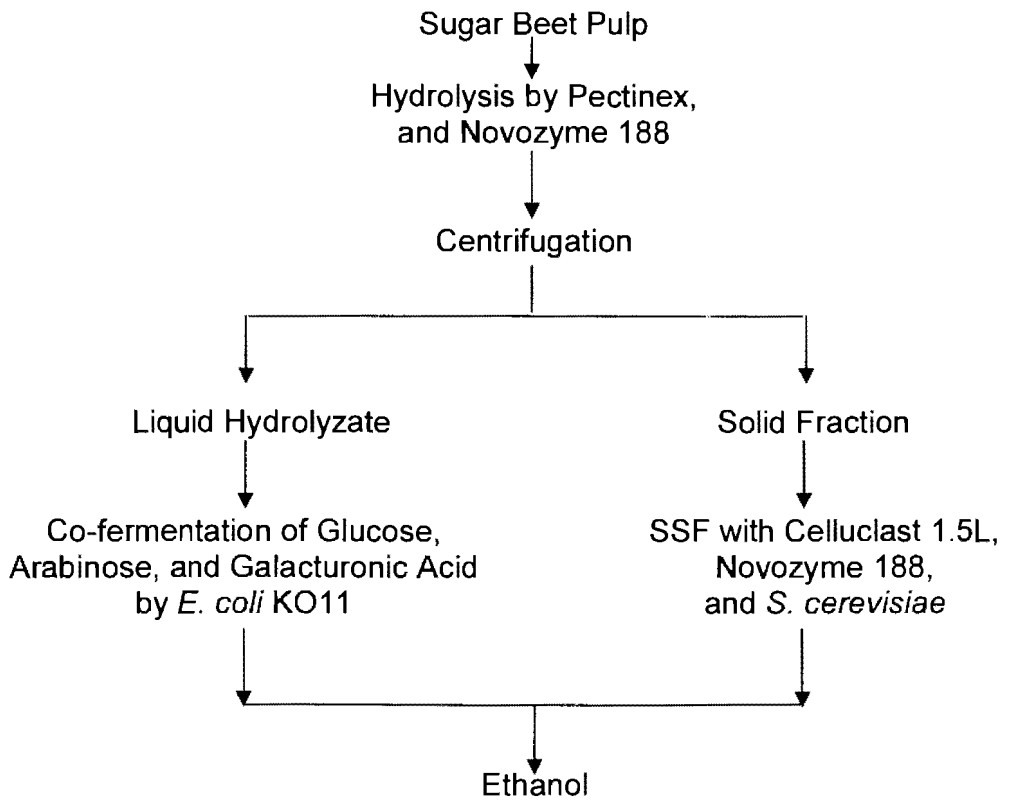

Figure 5. Parallel Fermentation Flow Chart

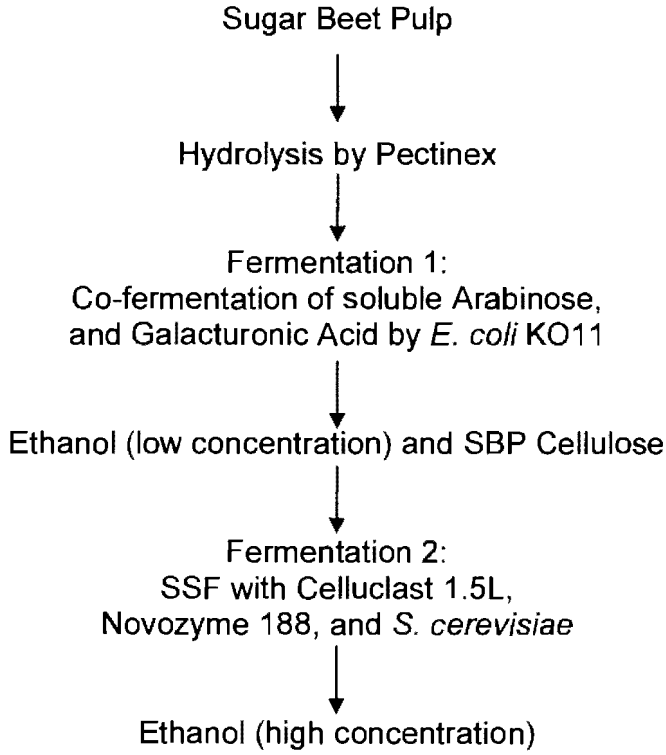

Figure 6. Serial Fermentation Flow Chart

Galacturonic acid was quantified and separated using a Waters HPLC using a Bio-Rad Aminex 87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min. A photodiode array detector (Waters Corporation) at 210 nm wavelength was used for detection. Ethanol was separated using a Bio-Rad (Hercules, CA) Aminex HPX-87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min at 60°C. All sugars were quantified using a 3-point external standard curve for each component. Standard concentrations are shown in Table 7.

Table 7. HPLC Quantification Standards

	Concentrations (g/L)		
Sugars	**Low**	**Medium**	**High**
Cellobiose	0.2	1	2
Glucose	1	5	10
Xylose	0.2	1	2
Galactose	0.2	1	2
Arabinose	0.8	4	8
Fructose	1	5	10
Galacturonic Acid	1.6	4	8

RESULTS

The parallel fermentation produced two separate ethanol streams. The *E. coli* KO11 fermentation (Figure 7) consumed glucose, arabinose, and galacturonic acid to produce ethanol. Glucose was consumed the most rapidly and reached low concentrations of 1 g/L after 24 hours. The arabinose and galacturonic acid were consumed more slowly than the glucose; concentrations stabilized at less than 2 g/L by approximately 96 hr. Once the sugar concentrations reached about 1 g/L, activity slowed. Galactose and xylose were present at under 1 g/L and not consumed by the *E. coli*. The maximum ethanol concentration was approximately 7 g/L, with a yield of 0.39 g ethanol/g sugar utilized. The actual yield was comparable to the theoretical yield of 0.42 g ethanol/g sugar utilized, which assumed complete fermentation of the sugars.

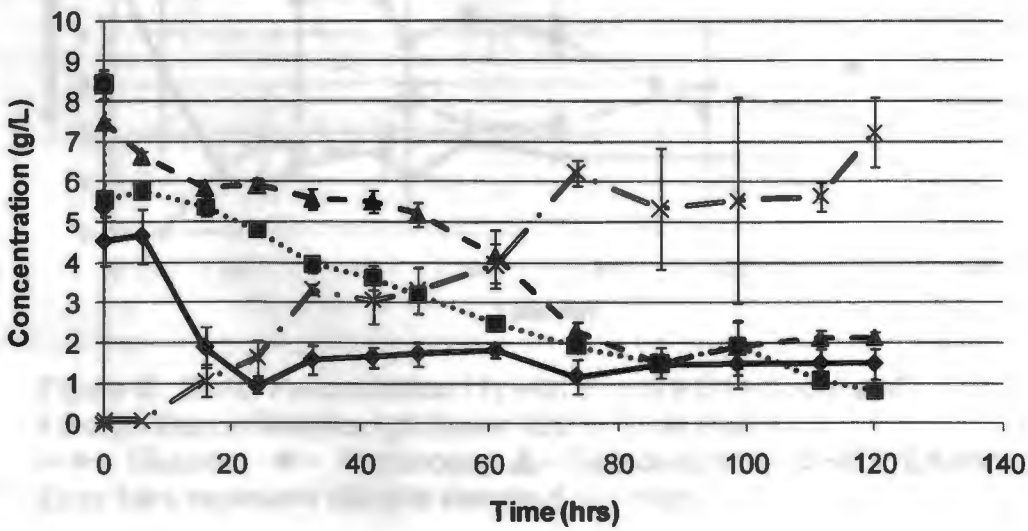

Figure 7. Parallel *E. coli* KO11 Fermentation Sugar and Ethanol Concentrations (g/L)
—◆— Glucose,···■··· Arabinose,–▲– Galacturonic Acid,–✕– Ethanol, Error bars represent sample standard deviation

Figure 8 shows changes in primary sugar and ethanol concentrations during the *E. coli* KO11 segment of the serial fermentation. Glucose was consumed within 24 hr and stabilized at approximately 1.5 g/L. Utilization of galacturonic acid and arabinose were much less than seen in the parallel fermentation system with concentrations decreasing to 5 and 3 g/L, respectively. Again galactose and xylose were present at under 1 g/L. Final ethanol concentrations reached 2 g/L, giving an approximate yield of 0.22 g ethanol/g sugar utilized. The theoretical yield was calculated to be 0.47 g ethanol/g sugar utilized, based on complete fermentation of the sugars.

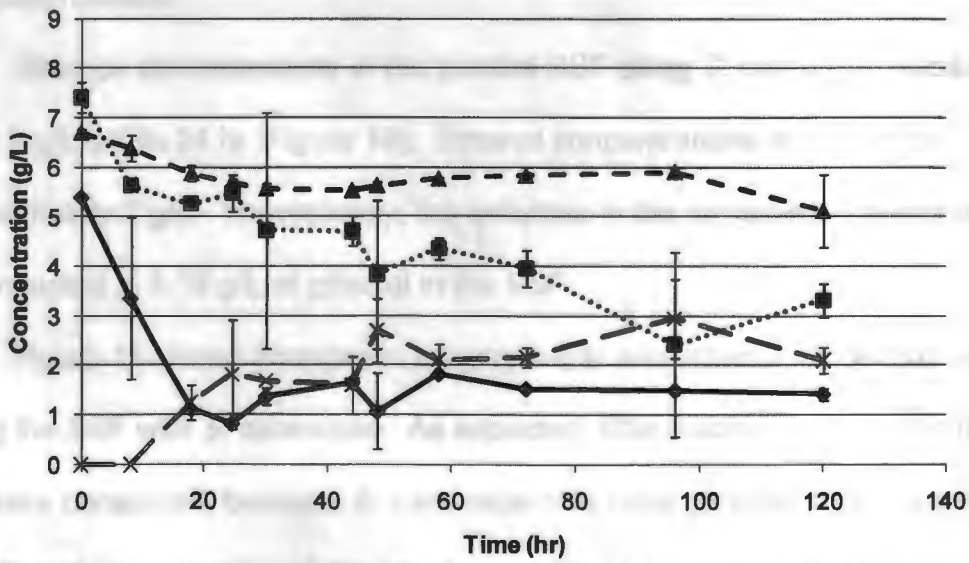

Figure 8. Serial Fermentation (1) with *E. coli* KO11 Sugar and Ethanol Concentrations (g/L) over 120 hr Time Period
—◆— Glucose;···■··· Arabinose,–▲– Galacturonic Acid;–×– Ethanol, Error bars represent sample standard deviation

Comparison of the parallel and serial *E. coli* KO11 fermentations (Figure 9) showed that the galacturonic acid concentrations and pH impacted fermentation performance. In the parallel and serial fermentations, the pH (Figure 9A) and

galacturonic acid concentrations (Figure 9B) decreased for the first 24 hr. At 24 hr, the parallel and serial pH reached approximately 5.9 and 5.6, respectively. Both 24 hr pH readings were below the optimal growth range (pH 6-8) of *E. coli* KO11. For the next 24 hr, the galacturonic acid utilization in the parallel fermentation slowed and pH increased very slowly until it was within the optimum growth range. At that point, the galacturonic acid concentrations quickly dropped and pH began to rise more quickly. Ethanol concentrations increased accordingly (data not shown). Although the serial fermentation followed a similar trend for the first 24 hr, the pH never recovered and the galacturonic acid utilization and ethanol production ceased.

Glucose concentrations in the parallel SSF using *S. cerevisiae* decreased to under 1 g/L within 24 hr (Figure 10). Ethanol concentrations increased to approximately 2 g/L. Theoretically, the cellulose in the remaining solids should have resulted in 5.16 g/L of ethanol in the SSF.

Figure 11 shows changes in primary sugar and ethanol concentrations during the SSF with *S. cerevisiae*. As expected, little arabinose and galacturonic acid were consumed, because *S. cerevisiae* only uses glucose to produce ethanol. The ethanol concentration at the conclusion of the serial fermentations (both *E. coli* KO11 and *S. cerevisiae*) was 6.11 g/L. Only 2.57 g/L of ethanol were produced during the *S. cerevisiae* fermentation. Based on the cellulose that remained intact throughout the stage 1 *E. coli* KO11 fermentation, a theoretical yield of 5.12 g/L of ethanol could have been produced from the stage 2 *S. cerevisiae* fermentation.

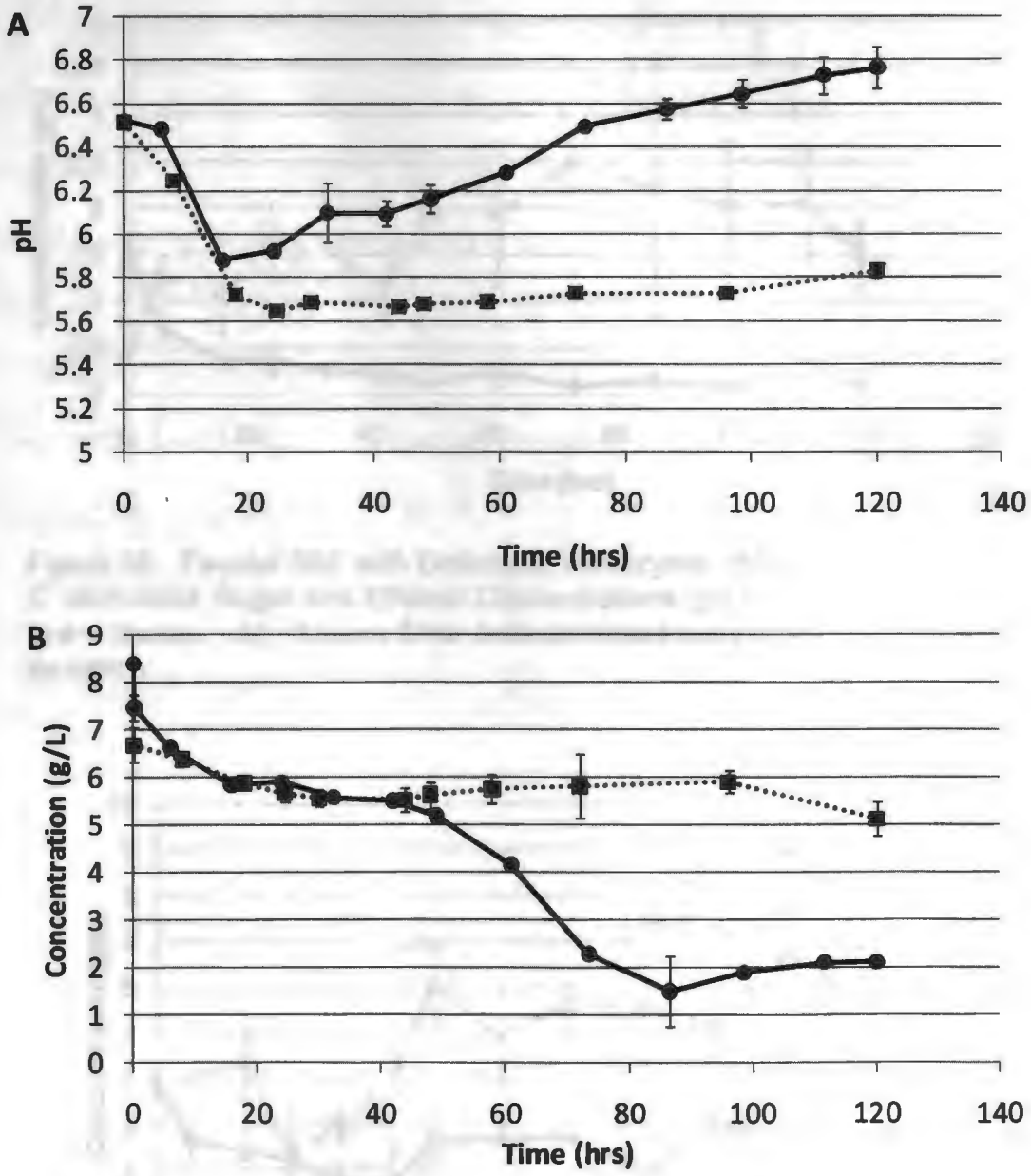

Figure 9. (A) The Change in pH for Parallel and Serial Fermentations over Time (B) The Change in Galacturonic Acid Concentrations for Parallel and Serial Fermentations over Time

——— Parallel,••••• Serial, Error bars represent sample standard deviation

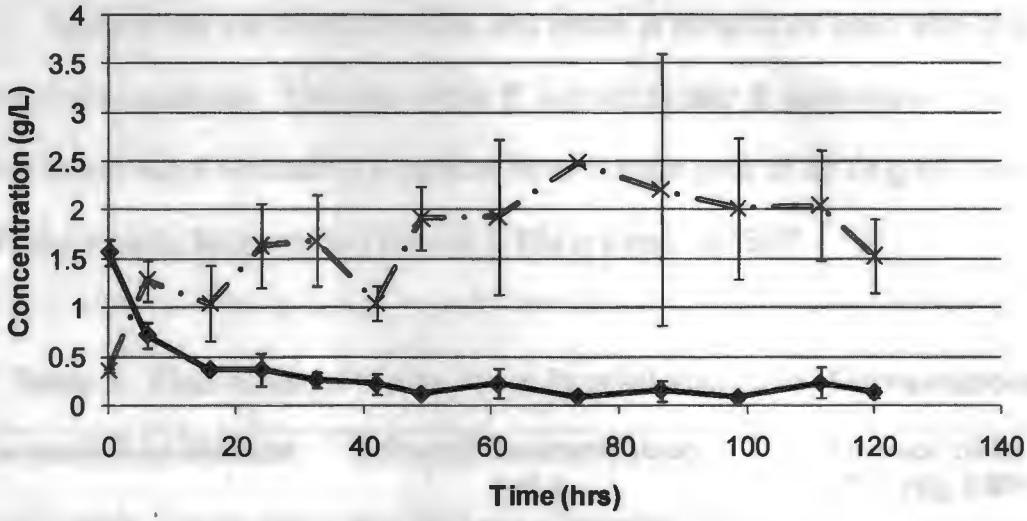

Figure 10. Parallel SSF with Celluclast, Novozyme 188, and *S. cerevisiae:* Sugar and Ethanol Concentrations (g/L) —◆— Glucose, —×— Ethanol, Error bars represent sample standard deviation

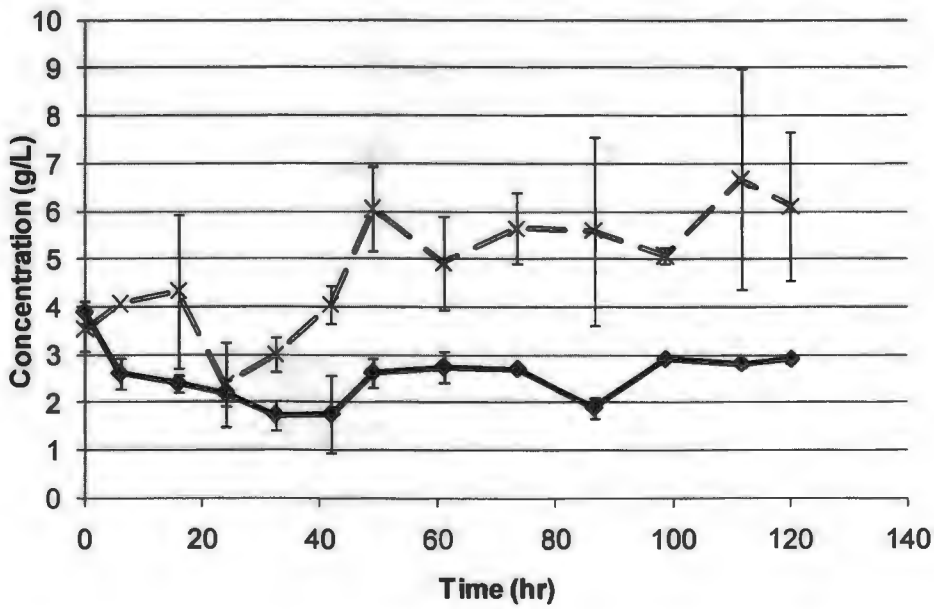

Figure 11. Serial Fermentation (2) with *S. cerevisiae* Sugar and Ethanol Concentations (g/L) over 120 hr —◆— Glucose, —×— Ethanol, Error bars represent sample standard deviation

Table 8 lists the concentrations and yields of ethanol for each step of both fermentation methods. The sum of the *E. coli* KO11 and *S. cerevisiae* fermentation steps resulted in a parallel fermentation yield of 0.178 g ethanol/g SBP and a serial fermentation yield of 0.134 g ethanol/g SBP.

Table 8. Final Ethanol Yields From Parallel and Serial Fermentations

Fermentation Method	Ethanol Concentration (g/L)	Ethanol Yield (g EtOH/g SBP)
Parallel *E. coli* KO11	7.22	0.144
Serial *E. coli* KO11	2.11	0.042
Parallel *S. cerevisiae*	1.53	0.034
Serial *S. cerevisiae*	6.11	0.092

DISCUSSION

The *E. coli* KO11 fermentations confirmed previous research showing that it can use glucose, arabinose, and galacturonic acid (Grohmann et al., 1994). Growth was limited once sugar concentrations were less than 1 g/L. Higher solids loading rates will lead to higher sugar concentrations and thus increased ethanol titers.

When the pH was within the optimum range of *E. coli* KO11 in the parallel fermentation, the actual ethanol yield of 0.39 g ethanol/g sugars utilized was very close to the theoretical yield of 0.42 g ethanol/g sugars utilized. The pH of the serial *E. coli* KO11 fermentation never recovered and less glucose, arabinose, and galacturonic acid were consumed. The serial fermentation only yielded 0.22 g ethanol/ g sugars utilized.

Galacturonic acid and pH data for the serial *E. coli* fermentation suggested that ethanol production decreased after 48 hr because the pH was below the growth range. After the pH decreased from 6.5 to 5.6, ethanol production slowed. The initial hydrolysis reaction was buffered at a pH of 5, so there was a natural tendency for the system to return to that pH even after adjusting to 6.5 prior to fermentation. Active control of pH during the fermentation should allow for more complete and rapid sugar utilization for ethanol production.

Both *S. cerevisiae* fermentations in the parallel and serial fermentation systems resulted in lower ethanol concentrations compared to the predicted theoretical conversion. Although most of cellulose hydrolysis was limited until the SSF, the fermentation yields were lower than predicted. Glucose concentrations

were under 1 g/L for most of the parallel fermentation, but relatively low glucose concentrations are expected in an SSF process. In the serial fermentation, the soluble glucose was not completely consumed by the yeast, as shown by the 3 g/L glucose concentration at 120 hr. Low yields were likely exacerbated by low solid loading rates used.

The parallel and serial fermentation yields of 0.178 g ethanol/g beet pulp and 0.134 g ethanol/g beet pulp, respectively, were less than the 0.245 g ethanol/g beet pulp recorded by Doran and Foster (2000). Although the yields were less than those previously recorded, both parallel and serial fermentation methods are possible to produce ethanol. The yields for each individual step showed that the parallel *E. coli* KO11 fermentation had the highest productivity. Future experiments will actively control pH and increase solids loadings rates to increase ethanol concentrations and yields.

CONCLUSION

The parallel fermentation method separated hydrolyzate and solids after hydrolysis of pectin and hemicellulose. The separate streams were treated with *E. coli* KO11 and *S. cerevisiae*, respectively and both produced ethanol. The serial fermentation method applied the *E. coli* KO11 and *S. cerevisiae* fermentations in sequential order. The change in pH was responsible for the difference in ethanol yields for the parallel and serial fermentations. Future experiments will monitor and adjust the pH regularly throughout fermentations to ensure that the *E. coli* KO11 bacteria are grown in the optimum pH range. Ethanol yields from *S. cerevisiae* fermentations were lower than expected and this was likely exacerbated by low solid loading rates. Even though the ethanol yields were low, both parallel and serial fermentation methods were shown to be possible and work will continue to maximize ethanol yields and titers.

ACKNOWLEDGMENTS

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To write the second paper in my thesis, I researched literature, designed experiments, collected data, and analyzed results with guidance from my advisor, Dr. Scott Pryor, and his lab technician, Ms. Nurun Nahar. Chad Siestsema, an undergraduate lab assistant, helped with media preparation and sampling during experiments. The paper is my original work that was edited by both Dr. Pryor and Ms. Nahar. This paper was published for the 2009 Annual International Meeting of the American Society of Agricultural and Biological Engineering (ASABE). I presented the results at the conference to professionals in my field.

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PAPER 3: ETHANOL PRODUCTION FROM SUGAR BEET PULP USING *ESCHERICHIA COLI* KO11 AND *SACCHAROMYCES CEREVISIAE*

Paper 3 will be submitted to the American Society of Agricultural and Biological Engineering (ASABE) publication, *Journal of Biological Engineering*, following completion of my thesis.

ABSTRACT

The effectiveness of sugar beet pulp as a feedstock for ethanol production was tested in four different fermentation systems using *Escherichia coli* KO11 alone or in combination with *Saccharomyces cerevisiae*. The baseline system used *E. coli* KO11 as the only fermenting organism following hydrolysis with pectinase, hemicellulase, and cellulase enzymes. Ethanol was produced with a peak yield of 0.26 g ethanol/g sugar. A "Serial" fermentation consisted of pectinase treatment followed by fermentation with *E. coli* KO11 and then cellulose hydrolysis and fermentation with *S. cerevisiae* to produce a total peak ethanol yield of 0.322 g ethanol/g sugar. A "Reverse Serial" method also used both microorganisms. The first step was a simultaneous saccharification and fermentation (SSF) using pectinases, cellulases, cellobiases, and *S. cerevisiae*, while the second step was fermentation with *E. coli* KO11. This fermentation system produced a maximum of 0.405 g ethanol/g sugar. A "Parallel" method split the liquid and solid streams following hydrolysis with pectinase. *E. coli* KO11 was used to ferment the liquid stream while cellulases and *S. cerevisiae* were used for hydrolysis and fermentation of the solid fraction. The combined ethanol yield from both fermentations was 0.282 g ethanol/g sugar. Based on yield data, the "Reverse Serial" method has potential to improve ethanol production from sugar beet pulp.

INTRODUCTION

Sugar beet pulp has potential as an alternative energy feedstock. This byproduct of sugar beet sucrose extraction is currently dried and pelleted to be sold as an animal feed. When energy prices are high, drying of the pulp becomes expensive and less profitable for processors. Ethanol production has the potential to increase revenue for processors in a favorable ethanol market, especially in North Dakota and Minnesota where an average of 53% (2003-2008) of US sugar beets are grown (USDA, 2008).

As an ethanol feedstock, sugar beet pulp presents some atypical advantages and disadvantages. Most cellulosic feedstocks have a lignin content of 15-25% and limited pectin, while sugar beet pulp has approximately 2% lignin and 24-32% pectin (DOE, 2010; Spagnuolo et al., 1997). The combination of high, easily hydrolyzed pectin and low lignin content eliminates the need for expensive thermochemical pretreatment (Hendriks and Zeeman, 2009). Logistically, the harvest, transport, and storage challenges that exist for many cellulosic ethanol feedstocks are not a hindrance for beet pulp as these processes are already accounted for with sucrose production. The pulp could be fermented directly following sucrose extraction if the quantity of pulp production at the plant was large enough to take advantage of economies of scale. Smaller plants could consolidate ethanol production at a centralized fermentation plant to limit transportation costs but improve economics based on economy of scale.

Despite the logistical and processing advantages of using sugar beet pulp as an ethanol feedstock, some technical challenges exist as well. The

hemicellulose portion of sugar beet pulp is comparable as a portion of total weight to that of most biomass at around 25-36%, but yields more arabinose than xylose when hydrolyzed (Spagnuolo et al., 1997). Limited research has focused on arabinose fermentation for ethanol, because it is a minor component of most other biomass hydrolyzates (Sedlak and Ho, 2001). The high pectin content will hydrolyze readily into galacturonic acid, but few organisms are able to utilize this for ethanol production. Another disadvantage of sugar beet pulp is the relatively lower cellulose content (20-24%) as compared to other agricultural residues or dedicated energy crops (30-50%) (DOE, 2010; Spagnuolo et al., 1997).

To maximize the sugar yields from enzymatic hydrolysis, acid and enzyme treatments have been considered for sugarbeet pulp and similar high-pectin feedstocks (Foster et al., 2001; Micard et al., 1996; Micard et al., 1997; Spagnuolo et al., 1997; Spagnuolo et al., 1999; Wilkins et al., 2007). Pretreatments such as grinding and drying, ammonia pressurization depressurization (APD), and deproteination did not cause large increases in sugar yields during subsequent hydrolysis (Foster et al., 2001; Micard et al., 1997). Spagnuolo et al. (1997) found pectinases paired with cellulases or hemicellulases produced the highest sugar yields (Spagnuolo et al., 1997). Total sugar yields from hydrolysis of grapefruit waste peel, a similar high-pectin feedstock, has increased during hydrolysis with pectinases and cellulases (Wilkins et al., 2007). Cellulase in combination with β-glucosidase degraded sugar beet pulp most effectively when the pectin was hydrolyzed first prior to cellulose hydrolysis. In this sequential method, 90% total pulp degradation was achieved (Micard et al., 1997).

Conversion of the variety of sugars present in beet pulp hydrolyzate requires an ethanologenic microorganism with a diverse metabolism. The standard fermentation yeast, *Saccharomyces cerevisiae*, metabolizes glucose and is an effective and safe yeast used in industrial ethanol production (Sedlak et al., 2003). Yeasts are hardy microorganisms that have an optimum range of temperature (30-35°C) and pH (4-5) and ethanol tolerance over 120 g/L (Bollok et al., 2000). Although work has modified *S. cerevisiae* to ferment xylose, the commercial strains currently only ferment glucose (Sedlak et al., 2003; Sedlak and Ho, 2001). A genetically modified *Escherichia coli* incorporated two genes from *Zymomonas mobilis* that encoded for alcohol dehydrogenase and pyruvate decarboxylase, allowing ethanol production from glucose, xylose, arabinose, and galacturonic acid (Ingram et al., 1987). Although *E. coli* KO11 can ferment a variety of sugars, it has a limited ethanol tolerance (40-60 g/L) and a higher optimum pH range (6-8) (Dien et al., 2003). In addition to ethanol and carbon dioxide, *E. coli* KO11 produces acetic acid during fermentation of galacturonic acid. A 2% galacturonic acid solution was fermented with *E. coli* KO11, and ethanol, acetic acid, and carbon dioxide were produced at 80, 78, and 80% of the theoretical yields (Grohmann et al., 1994). Due to a diverse metabolism, *E. coli* KO11 has fermented the variety of sugars in hydrolyzates from multiple feedstocks, such as corn cob, grapefruit peels, brewery wastewater, and sugar cane bagasse (Grohmann et al., 1994; Lima et al., 2002). To combine the strengths of *S. cerevisiae* and *E. coli* KO11 and increase total ethanol yields,

methods for sequential and separated fermentations with two microorganisms were developed.

Four distinct methods were developed to use *E. coli* KO11 alone or in conjunction with *S. cerevisiae* to improve ethanol concentrations and yields. The difference in enzyme loadings allowed for the direct comparison of only three of the four methods. Although the peak ethanol yield values were improved, the ethanol concentrations were difficult to increase because of the high moisture content of the sugar beet pulp. The experiments also addressed the possible use of multiple microorganisms to optimally use soluble sugars in ethanol production.

MATERIALS AND METHODS

SUBSTRATE

Pressed sugar beet pulp with a moisture content of 75% (w.b.) was obtained from the Moorhead, MN American Crystal Sugar processing plant (Moorhead, MN). Pulp was stored at -20°C until used in experiments. Compositional analysis of sugar beet pulp was conducted by Microbac Laboratories, Inc. (Boulder, CO) and found to be: glucan (25.21%), arabinan (18.08%), galacturonic acid (15%), galactan (6.63%), and xylan (2.32%).

ENZYMES

Pectinex Ultra SPL, Celluclast 1.5L, and Novozyme 188 were purchased from Sigma-Aldrich (St. Louis, MO). Pectinex Ultra SPL (Novozymes, Inc.; Bagsvaerd, Denmark) has an optimum pH and temperature of 5 and 40°C, respectively, and is a commercial preparation of pectinases and hemicellulases with limited cellulase activity. Celluclast 1.5L (Novozymes, Inc.) is a commercial preparation of cellulase with limited cellobiase activity. Novozyme 188 (Novozymes, Inc.) is a commercial cellobiase preparation. Both have an optimum pH and temperature of 4.8 and 50°C, respectively. The Celluclast 1.5L activity used in the "*E. coli* KO11 Only", "Serial", and "Reverse Serial" methods was determined to be 72.6 Filter Paper Units/mL (FPU/mL) and 6.7 Cellobiose Unit/mL (CBU/mL), while the Celluclast 1.5L activity from the "Parallel" method was 78.72 FPU and 5.32 CBU. The Novozyme 188 activity used in all experiments was 500.5 CBU/mL (Ghose, 1987). In the hydrolysis of sugar beet pulp, Pectinex Ultra SPL was used to hydrolyze hemicellulose and pectin. Cellulcast 1.5L and Novozyme

188 were used together to hydrolyze cellulose. All enzymes were stored at 2°C when not in use.

MICROORGANISMS

E. coli KO11 (ATCC 55124) was provided by American Crystal Sugar Company. The inoculation seed was prepared in a solution of 50 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, 5/L g NaCl, and 40 mg/L chloramphenicol at 37°C and 100 rpm for 24 hr. The resulting cell culture was mixed with sterile 80% glycerol to produce a 40% glycerol solution. Aliquots (1 mL) were dispensed into sterile cryovials and stored until use at -20°C. For each experiment, one cryovial was added to a 100 mL of inoculum media containing 50 g/L glucose, 10 g/L tryptone, and 5 g/L yeast extract. The seed culture was incubated at 37°C and 100 rpm for 18 hr. Sugar beet pulp hydrolyzate was inoculated with 1% v/v inoculum for all fermentations with *E. coli* KO11.

S. cerevisiae was obtained from Dr. Bill Gibbons at South Dakota State University. Cultures were maintained on PDA plates (5 g/L agar and 6 g/L potato dextrose broth) at 30°C. The liquid inoculum was prepared from a 2-3 d old culture plate in media composed of 5 g/L yeast extract and 30 g/L glucose in DI water. The pH was adjusted to pH 4 with 0.3 N HCl. After inoculation, the media was incubated in a water bath at 30°C and 150 rpm for 24 hr and added to fermentation flasks at 1% v/v.

EXPERIMENTAL DESIGN

The "*E. coli* KO11 Only", "Serial", and "Reverse Serial" methods were designed for comparison. Sugar beet pulp (24 g d.b.) was added into 500-mL

Erlenmeyer flasks and autoclaved for 20 min at 121°C. Hydrolysis was carried out at a total solids loading rate of 12% (w/v) in 5 mM citrate buffer (pH 5). Pectinex Ultra SPL was added at 200 µL/dry g for all experiments. Celluclast 1.5L was added at 16.7 FPU/g glucan with additional Novozyme 188 to achieve a total cellobiase loading of 33.3 CBU/g glucan, respectively. All sample flasks were incubated in a water bath at 100 rpm and 40°C (Pectinex) or 37°C (*E. coli* KO11 or SSF with Celluclast, Novozyme 188, and *S. cerevisiae*). Flasks were sealed with rubber stoppers pierced with syringes to release carbon dioxide. The pH of the *E. coli* KO11 and *S. cerevisiae* fermentations were adjusted to 6.5 and 5, respectively, with 6N NaOH every 8 to 16 hr. Samples were taken every 24 hr, filtered, stored at -20°C and analyzed after completion of the experiment. All treatments were carried out in triplicate.

"*E. coli* KO11 Only" Method

Pulp was hydrolyzed with Pectinex, Celluclast, and Novozyme 188 for 48 hr. Chloramphenicol (39 mg/L) and *E. coli* KO11 (1% v/v) were added to each flask. Fermentation was carried out for 7 days (Figure 12). Samples were taken daily and analyzed by HPLC for sugar and ethanol concentrations.

"Serial" Method

Pectinex was used to hydrolyze the pectin and hemicellulose portions of SBP with minimal cellulose hydrolysis. *E. coli* KO11 was used to ferment the hydrolyzate for 7 days. The pH of each flask was readjusted to 5 using 3N HCl before Celluclast 1.5L, Novozyme 188, and *S. cerevisiae* were added to each flask. The simultaneous saccharification and fermentation (SSF) ran for 7 days

(Figure 13). Samples were collected daily and were analyzed by HPLC for sugar and ethanol concentrations.

"Reverse Serial" Method

Pectinex, Celluclast, and Novozyme 188 were used to fully hydrolyze SBP in an SSF with *S. cerevisiae* for conversion of the liberated glucose. Due to an error in experiment set-up, the Celluclast 1.5L was loaded at 16.66 FPU/g glucan with additional Novozyme 188 to reach a total cellobiase loading of 17.1 CBU/g glucan instead of the 16.7 FPU/g glucan and 33.3 CBU/g glucan used in the "*E. coli* KO11 Only" and "Serial" methods. Once the SSF was completed, the pH was increased to 6.5, and the remaining soluble sugars in the hydrolyzate were fermented with *E. coli* KO11 (Figure 14). Samples were collected daily and were analyzed by HPLC for sugar and ethanol concentrations.

"Parallel" Method

The "Parallel" method (Figure 15) separated the *E. coli* KO11 and *S. cerevisiae* fermentations in two different flasks to determine the effect on ethanol yields and concentrations. Pectinex (200 µL/dry g) and Novozyme 188 (33.3 CBU/g glucan) was used to hydrolyze the pectin and hemicellulose portions of SBP with minimal cellulose hydrolysis. The cellobiase was added to further degrade the soluble cellobiose that resulted from the small cellulase activity in the Pectinex. The contents of each flask were then centrifuged at 1,500 rpm for 30 min to separate the liquids and solids. The supernatant was transferred to a new flask, while the remaining solids (mostly cellulose) were returned to the original

flasks. Some residual hydrolyzate remained bound in the solids fraction because of incomplete separation.

The pH of the supernatant flasks was adjusted to 6.5, chloramphenicol (39 mg/L) was added, and the remaining sugars were fermented with *E. coli* KO11 (1% v/v). Samples were collected for 7 days and analyzed by HPLC for sugar and ethanol concentrations.

The moisture content of the solid fraction of hydrolyzed sugar beet pulp was determined to be 85% (w.b.). The solids were resuspended in 39 mL of citrate buffer to achieve a new solids loading rate of 12% w/v (d.b.). Yeast extract (1% w/v) was added as a nitrogen source. The flasks were autoclaved for 15 min at 121°C. Celluclast, Novozyme 188, and *S. cerevisiae* were added to each flask to achieve celluase and cellobiase loadings of 15.8 FPU/g glucan and 12.5 CBU/g glucan to degrade the cellulose-rich pulp. Solids were assumed to have the same glucan content as the original beet pulp. The flasks incubated in a water bath for 7 days. Samples were collected every 24 hours and were analyzed by HPLC for sugar and ethanol concentrations.

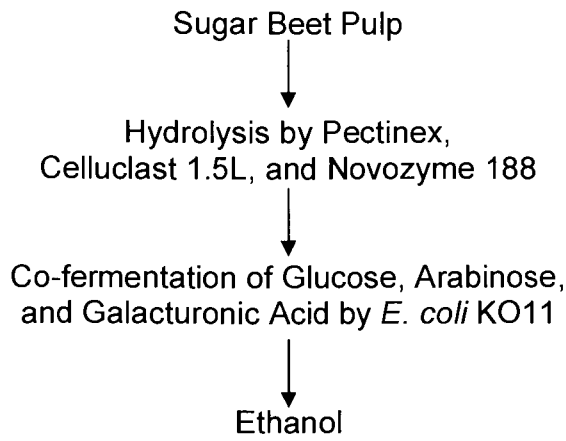

Figure 12. "*E. coli* KO11 Only" Fermentation Flow Chart

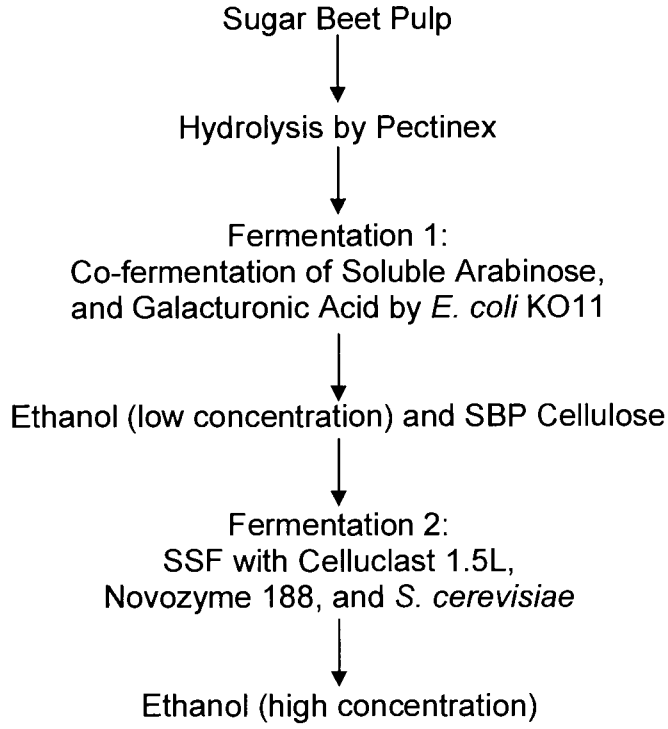

Figure 13. "Serial" Fermentation Method Flow Chart

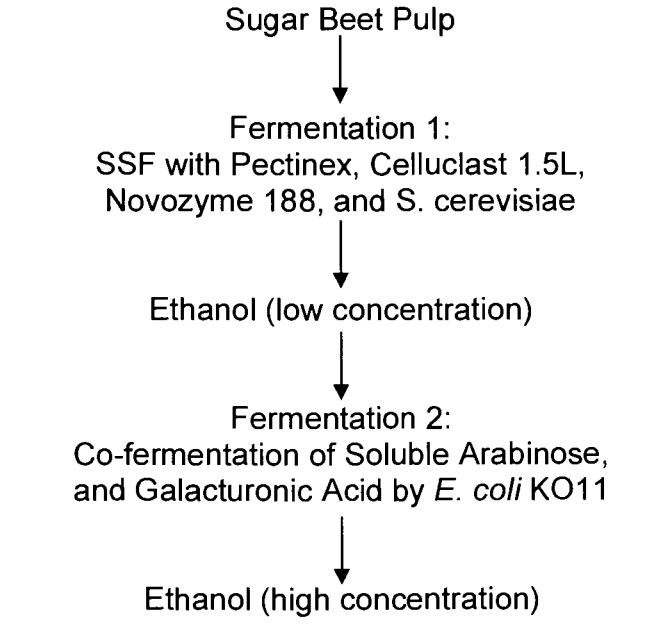

Figure 14. "Reverse Serial" Fermentation Flow Chart

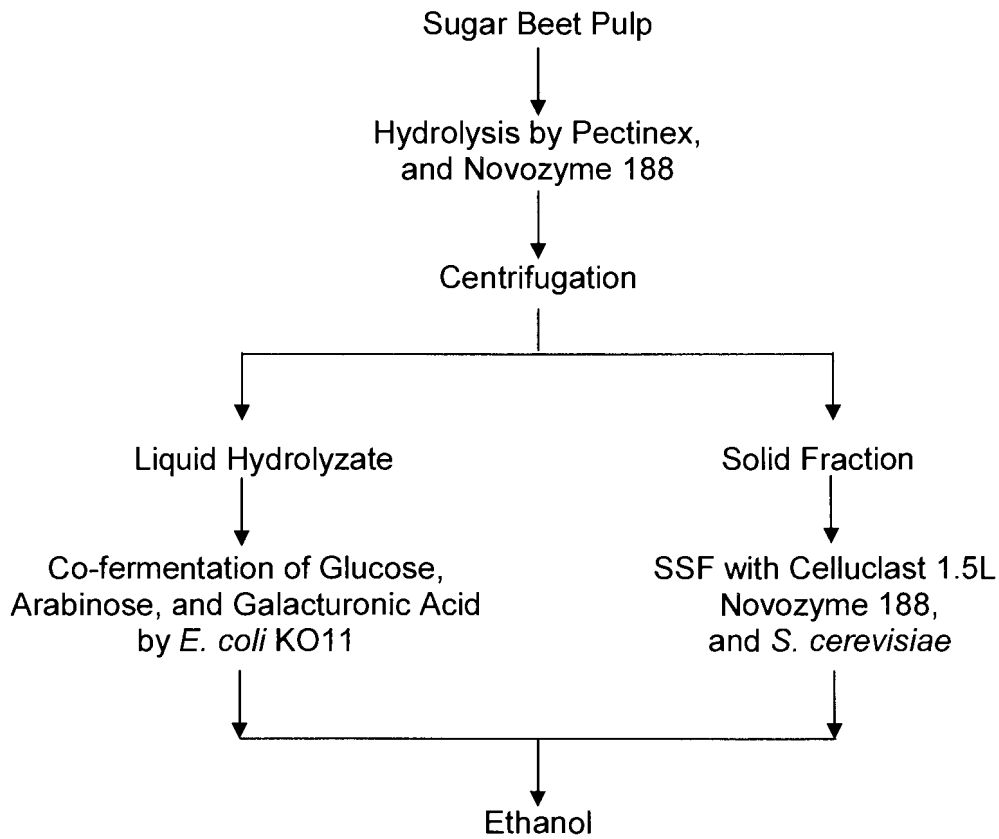

Figure 15. "Parallel" Fermentation Flow Chart

Analysis of Soluble Sugars and Ethanol

Samples (2 mL) were centrifuged and filtered through a 0.2-μm nylon filter (Pall Corporation, West Chester, PA) prior to HPLC analysis. All components were separated with a Waters (Milford, MA) HPLC. Cellobiose, glucose, arabinose, galactose, and fructose were quantified using a refractive index detector (Waters Corporation). Sugars were separated using a Bio-Rad (Hercules, CA) Aminex HPX-87P column with a mobile phase of water at a flow of 0.6 mL/min; the column and detector temperatures were 50°C and 85°C, respectively. Galacturonic acid and acetic acid were separated with a Bio-Rad Aminex 87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min at 60°C. Detection

was carried out using a photodiode array detector (Waters Corporation) at 210 nm wavelength. Ethanol was separated using a Bio-Rad Aminex HPX-87H column with a mobile phase of 5 mM sulfuric acid at a constant flow of 0.6 mL/min at 60°C. All components were quantified using a 3-point external standard curve shown in Table 9. Quantification of neutral sugars used 3 of the 4 concentrations listed depending on the expected concentration range.

Table 9. HPLC Quantification Standards

	Concentrations (g/L)			
Sugars	**Low**	**Medium**	**Medium High**	**High**
Cellobiose	1	3.33	5	10
Glucose	2.5	8.33	12.5	25
Xylose	0.2	0.67	1	2
Galactose	1	3.33	5	10
Arabinose	2	6.67	10	20
Fructose	0.5	1.67	2.5	5
Galacturonic Acid	2.5	12.5		25
Acetic Acid	1	5		10
Ethanol	2.5	25		50

STATISTICAL ANALYSIS

All treatments were reproduced in triplicate. Tukey's test ($p < 0.05$) was used to determine statistical difference in the peak ethanol yields.

RESULTS

The "*E. coli* KO11 Only" fermentation (Figure 16) consumed glucose, arabinose, and galacturonic acid to produce ethanol. Glucose was consumed rapidly with no notable lag but concentrations stabilized at approximately 2 g/L after 48 hr. Arabinose was also consumed with little or no lag, but at a slower rate than glucose. Concentrations decreased steadily until 72 hr with no residual concentrations remaining. Galacturonic acid was consumed more slowly than other sugars during for the first 48 hr. Galacturonic acid utilization rate increased from 0.06 g/L/hr after glucose utilization ceased. Approximately 6 g/L of galactose was also consumed during the fermentation (data not shown). Under 2 g/L of xylose was present in solution and decreased to 1 g/L. After 96 hr, most of the soluble sugars were consumed. The peak ethanol concentration reached approximately 22 g/L, with a yield of 0.26 g ethanol/g sugars.

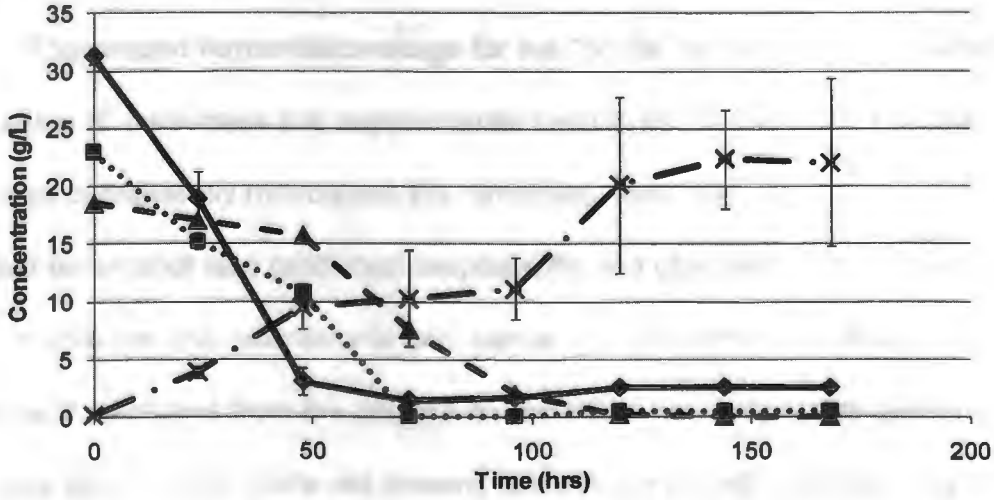

Figure 16. "*E. coli* KO11 Only" Fermentation with Pectinex, Celluclast, Novozyme 188, and *E. coli* KO11 Sugar and Ethanol Concentration (g/L) —◆— Glucose,···■··· Arabinose,–▲– Galacturonic Acid,–✕– Ethanol, Error bars represent sample standard deviation

The "Serial" fermentation results are displayed in Figures 17 and 18. The first fermentation stage, using *E. coli* KO11 produced over 25 g/L of ethanol, with most of the glucose, arabinose, and galacturonic acid consumed (Figure 17). The glucose was metabolized the quickest, nearing complete consumption in 48 hr. Arabinose and galacturonic acid were consumed in an almost identical trend, nearing completion in 96 hr. This differed from the "*E. coli* KO11 Only" fermentation where the galacturonic acid was used more slowly until glucose depletion. Utilization rates for both galacturonic acid and arabinose consistently decreased until under concentration of 2 g/L. This also differed from the "*E. coli* KO11 Only" fermentation because the consumption of glucose did not alter the rate of consumption for other soluble sugars. Again, most of the soluble sugars were consumed by 96 hr. About 4 g/L of galactose was also consumed during that time period (data not shown). The peak ethanol concentration was 28 g/L with a yield of 0.322 g ethanol/g sugar.

The second fermentation stage for the "Serial" fermentation consisted of an SSF using *S. cerevisiae* and supplemental cellulases. In the first 24 hr, the enzymes successfully hydrolyzed the remaining cellulosic sugar beet solids (Figure 18), but no ethanol was produced despite sufficient glucose in the medium (10 g/L). Arabinose and galacturonic acid concentrations remained below 2 g/L. The acetic acid produced from the stage 1 *E. coli* KO11 fermentation of galacturonic acid was about 13 g/L (data not shown), twice the reported minimum inhibition concentration for *S. cerevisiae* (Narendranath et al., 2001). The limited ethanol production by *S. cerevisiae* resulted in a total peak ethanol yield for the entire

method of 0.322 g ethanol/g sugar. If the unfermented 13 g/L of glucose was metabolized an additional 0.075 g ethanol/g sugar could be yielded.

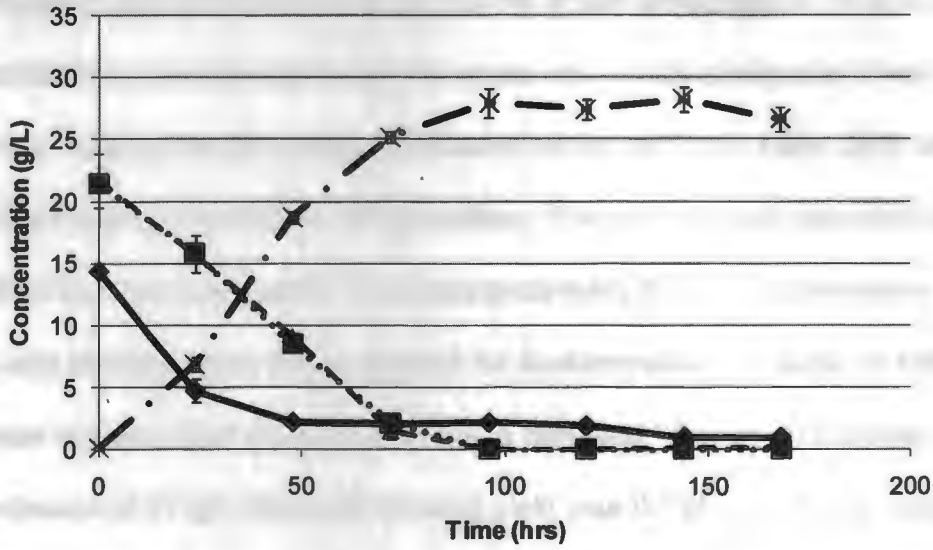

Figure 17. "Serial" Fermentation Method with Pectinex and *E. coli* KO11 Sugar and Ethanol Concentrations (g/L)
—◆— Glucose,···■··· Arabinose,–▲– Galacturonic Acid,
—×— Ethanol, Error bars represent sample standard deviation

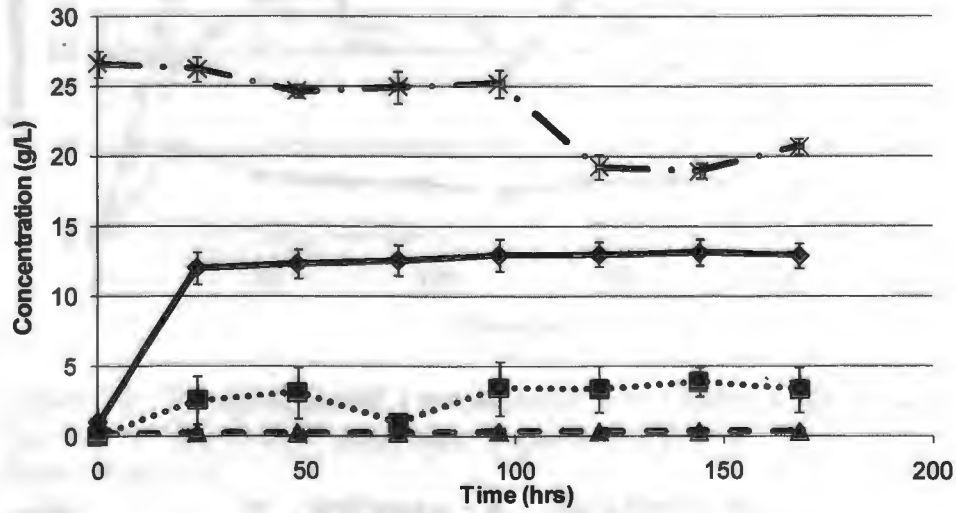

Figure 18. "Serial" Fermentation with Celluclast, Novozyme 188, and *S. cerevisiae* Sugar and Ethanol Concentrations
—◆— Glucose,···■··· Arabinose,–▲– Galacturonic Acid,
—×— Ethanol, Error bars represent sample standard deviation

The first stage of the "Reverse Serial" fermentation was comprised of an SSF using *S. cerevisiae* with pectinases and cellulases (Figure 19). The glucose concentration stayed between 2-5 g/L for the entire fermentation. This low concentration is expected because the yeast consumed glucose as it was produced. Arabinose and galacturonic acid increased in the initial 24 hr and was not utilized during this stage of fermentation. The ethanol concentration increased until 96 hr but then decreased. This was potentially due to contamination. Aerobic acetic acid bacteria were not suspected for contamination because no sharp increases in acetic acid concentration were detected. Based on the peak ethanol concentration of 17 g/L, the peak ethanol yield was 0.194 g ethanol/g sugar.

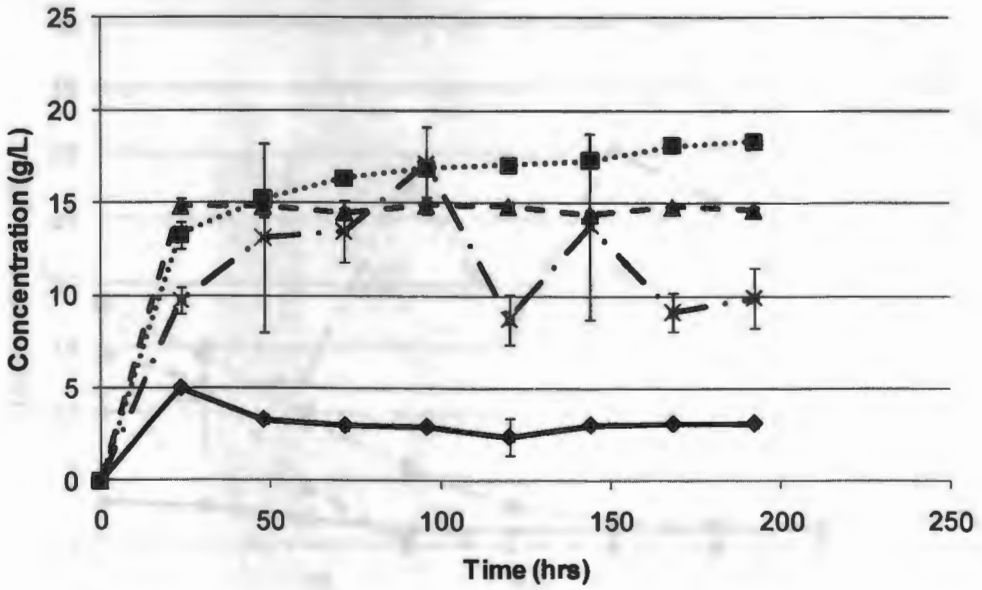

Figure 19. "Reverse Serial" Fermentation with Pectinex, Celluclast, Novozyme 188, and *S. cerevisiae* Sugar and Ethanol Concentrations
—◆— Glucose, ···■··· Arabinose, – ▲ – Galacturonic Acid,
—✕— Ethanol, Error bars represent sample standard deviation

After the stage 1 "Reverse Serial" fermentation, *E. coli* KO11 was used to ferment the remaining glucose, arabinose, and galacturonic acid (Figure 20). The small amount of glucose decreased to under 1 g/L. In the absence of significant glucose concentrations, galacturonic acid was consumed more quickly than the arabinose. This supports the conclusion that galacturonic acid and glucose metabolism are linked while arabinose metabolism occurs independently of the presence of glucose. The ethanol concentration increased to as high as 30 g/L, but ethanol concentration data varied significantly. The stage 2 peak ethanol yield was 0.211 g ethanol/g sugar. The ethanol yield for the combined stage 1 and stage 2 fermentations was 0.405 g ethanol/g sugar.

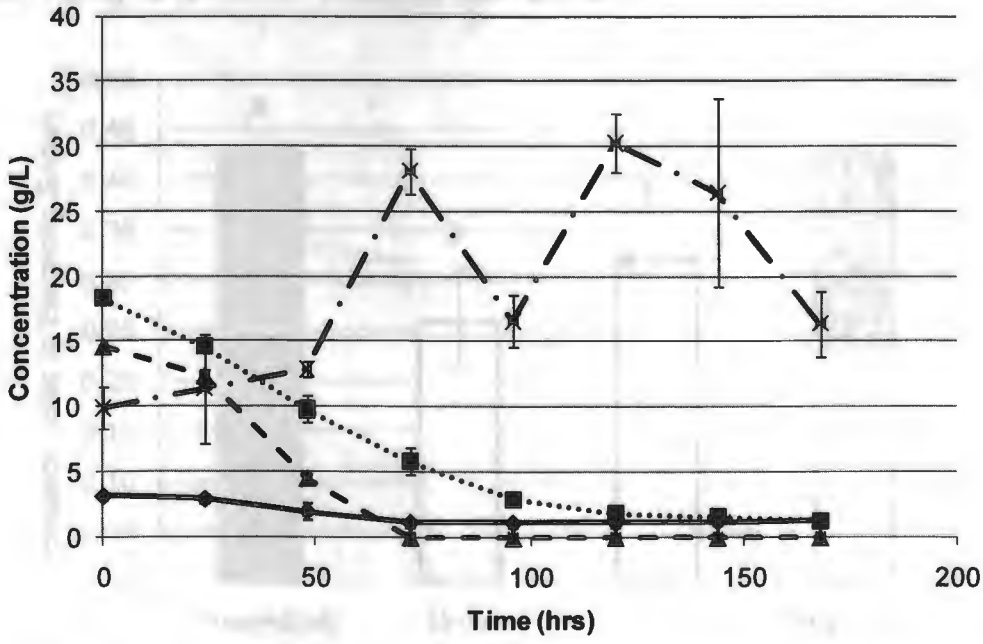

Figure 20. "Reverse Serial" Fermentation with *E. coli* KO11 Sugar and Ethanol Concentrations
—◆— Glucose;···■··· Arabinose,–▲– Galacturonic Acid,
–×– Ethanol, Error bars represent sample standard deviation

The total ethanol yields from the three fermentation methods ("*E. coli* KO11 Only", "Serial", and "Reverse Serial") were compared to each other and the maximum theoretical yield (Figure 21). The "Reverse Serial" fermentation had lower enzyme loadings for Celluclast and Novozyme 188, but the low cellobiose concentrations (under 0.1g/L) showed that the loading change did not have a significant impact on the results. The theoretical ethanol yield was calculated assuming that sugar was completely metabolized to ethanol. The "Reverse Serial" method closely approached the theoretical ethanol yield. The "Serial" and "*E. coli* KO11 Only" methods produced 60-70% of the theoretical yield. Statistical analysis showed that the "Reverse Serial" and Theoretical yields were similar, while the "*E. coli* KO11 Only" and "Serial" methods were similar.

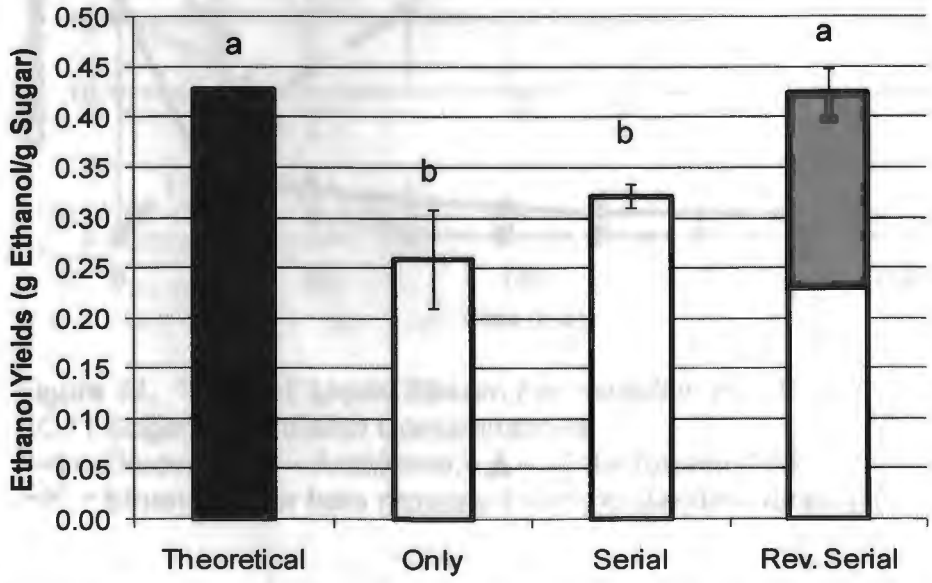

Figure 21. Comparison of Ethanol Yields
■ Theoretical yield (Ethanol based on SBP compositional analysis), □ *E. coli* KO11 Fermentation yield, ■ *S. cerevisiae* Fermentation yield, Error bars represent sample standard deviation

The "Parallel" fermentation separated liquid and solid streams following hydrolysis with Pectinex. The liquid stream was fermented by *E. coli* KO11 and results are shown in Figure 22. Most glucose was consumed in the first 24 hr of the fermentation. Both the arabinose and galacturonic acid were depleted by 72 hr. The ethanol concentration continued to increase through 124 hr indicating utilization of other sugars or continued hydrolysis of solubilized oligomers. The peak ethanol concentration of 28 g/L resulted in an ethanol yield of 0.179 g ethanol/g sugar.

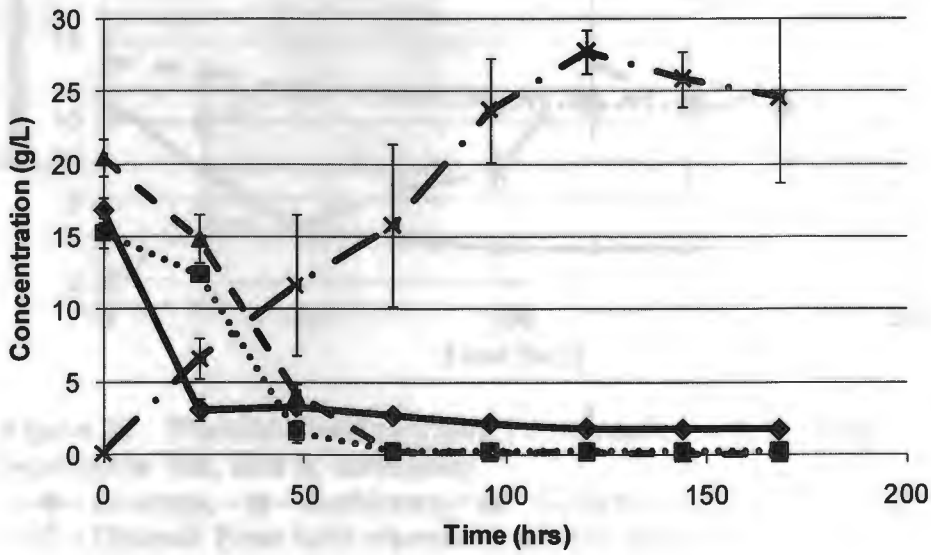

Figure 22. "Parallel" Liquid Stream Fermentation with *E. coli* KO11 Sugar and Ethanol Concentrations
—◆— Glucose,···■··· Arabinose,–▲– Galacturonic Acid,
–×– Ethanol, Error bars represent sample standard deviation

The solid fraction separated after hydrolysis with Pectinex was used for an SSF with *S. cerevisiae* and cellulases (Figure 23). Significant percentages of total arabinose and galacturonic acid (27% and 47%, respectively) were present in the solids fraction fermentation because of incomplete separation of liquids and solids

after initial hydrolysis. Like previous fermentations with *S. cerevisiae*, only the glucose was metabolized. The ethanol increased throughout the 168-hr fermentation with a peak concentration of 13.7 g/L and a peak ethanol yield of 0.103 g ethanol/ g sugar. Combining yields from the liquid and solid stream fermentations, the total ethanol yield was 0.282 g ethanol/g sugar.

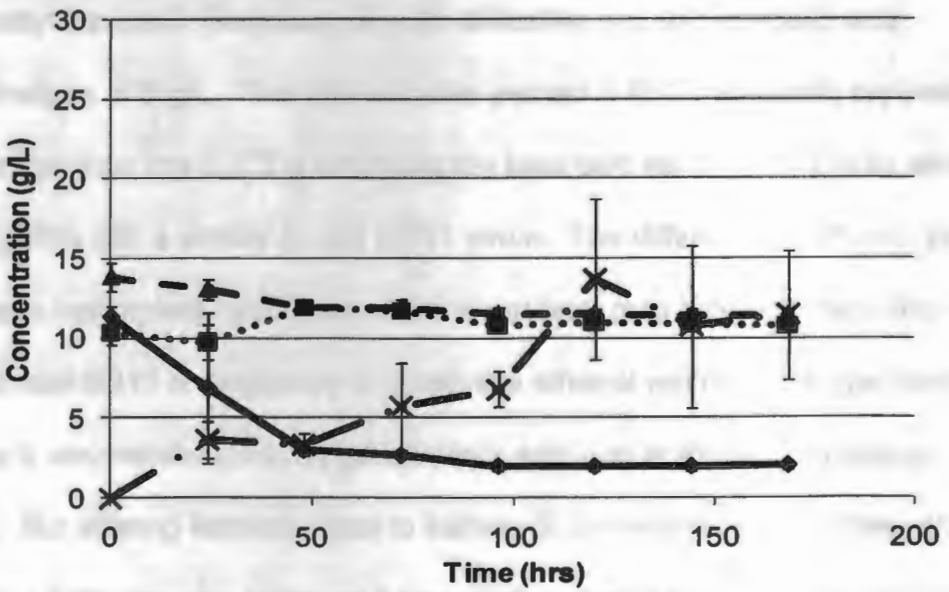

Figure 23. "Parallel" Solids Stream Fermentation with Celluclast, Novozyme 188, and *S. cerevisiae*
—◆— Glucose, ····■···· Arabinose, – ▲ – Galacturonic Acid,
—×— – Ethanol, Error bars represent sample standard deviation

DISCUSSION

The "*E. coli* KO11 Only" method consumed glucose, arabinose, and galacturonic acid to under 1 g/L. The sugars were metabolized simultaneously, showing that *E. coli* KO11 successfully co-fermented sugar beet pulp hydrolyzate. Galacturonic acid was consumed more slowly than the other sugars until glucose was mostly depleted. Galacturonic acid utilization resulted in acetic acid concentrations of 8 g/L. This fermentation yielded 0.192 g ethanol/g dry beet pulp which is less than the 0.277 g ethanol/g dry beet pulp reported by Doran and Foster (2000) with a similar *E. coli* KO11 strain. The difference in ethanol yield could be to incomplete hydrolysis of the sugar beet pulp before fermentation.

E. coli KO11 is necessary to maximize ethanol yields from sugar beet pulp, because it can metabolize both galacturonic acid and arabinose to produce ethanol. But altering fermentations to include *S. cerevisiae* can increase ethanol production from glucose, because it can produce between 0.375 and 0.475 g ethanol/g glucose in pure glucose media fermentations (Arneborg et al., 1997; Narendranath et al., 2001). *E. coli* KO11 yielded 0.376 g ethanol/g glucose pure glucose fermentations (Yomano et al., 1998).

With the greater potential ethanol concentrations, the "Serial" and "Reverse Serial" methods were compared to the "*E. coli* KO11 Only" baseline case. The *E. coli* KO11 fermentation in the "Serial" method began with lower glucose concentrations compared to the "*E. coli* KO11 Only" method. The limited cellulase activity of Pectinex produced about 15 g/L of glucose (45% of theoretical glucose yields) in stage 1, and cellulases yielded an additional 13 g/L in the stage 2 *S.*

cerevisiae fermentation. The lower amount of glucose in the stage 1 fermentation allowed the arabinose and galacturonic acid to be metabolized faster. Along with the ethanol concentrations of 28 g/L, approximately 13 g/L of acetic acid was produced as a byproduct of the fermentation. The higher concentration of acetic acid in the "Serial" method occurred due to the higher galacturonic acid concentrations (about 22 g/L).

When Celluclast, Novozyme 188, and *S. cerevisiae* were added for the second stage in the "Serial" fermentation, the cellulose that remained intact was hydrolyzed to glucose (13 g/L). It was not consumed by the *S. cerevisiae*, however, because the acetic acid produced in stage 1 inhibited growth of the yeast. Acetic acid can inhibit *S. cerevisiae* growth at concentrations as low 0.6% w/v or 6 g/L (Narendranath et al., 2001). Stock yeast inoculums with 0, 6, and 12 g/L of acetic acid were tested and little to no yeast cell growth was seen in both the 6 and 12 g/L flasks (data not shown). In order to make this fermentation scheme function, acetic acid removal or an acetic acid-tolerant ethanogen would be needed, such as *P. stipitis* (Agbogbo and Wenger, 2007). If that glucose was metabolized, an additional 6.63 g/L ethanol could theoretically be produced. The total experimental ethanol yield of 0.238 g ethanol/g sugar beet pulp could increase to 0.294 g ethanol/ sugar beet pulp. This would surpass Doran and Foster's 0.277 g ethanol/g SBP yield (2000). Without the increased ethanol produced in the second stage, the "Serial" method produced a statistically similar ethanol yield to the "*E. coli* KO11 Only" fermentation, but could be comparable to the theoretical and "Reverse Serial" if the remaining glucose had been consumed.

The "Serial" method could be improved by acetic acid removal or use of acetic acid resistant microorganisms, like *P. stipitis*.

The "Reverse Serial" method produced a total peak ethanol yield of 0.405 g ethanol/g sugar that was statistically similar to the theoretical yield. Although the enzyme loadings were lower than that of both the "*E. coli* KO11 Only" and "Serial", the low residual cellobiose showed that the glucose in the sugar beet pulp was consumed. Thus comparisons to the "*E. coli* KO11 Only" and "Serial" methods were still valid. The ethanol concentrations were erratic over time in both the *S. cerevisiae* and *E. coli* KO11 fermentations. There was no statistical difference between ethanol concentrations in the *S. cerevisiae* fermentation, but the three high ethanol concentrations in the *E. coli* KO11 fermentation were statistically different than the other ethanol concentrations. The variability could have been caused by contamination from other microorganisms in the media. Although sterile sampling was practiced, the rubber stoppers were removed every 24 hr and could have allowed contamination. The variability did cause lower final ethanol concentrations, but industrial fermentations would be monitored and stopped once the soluble sugars were consumed and ethanol concentrations peaked.

Compared to published results (0.227 g ethanol/g sugar beet pulp), the peak ethanol concentrations of 0.299 g ethanol/g sugar beet pulp surpassed previous findings. To fully understand the ethanol production potential, the ethanol concentrations need to be more consistent during both fermentations in the "Reverse Serial" method. Improved sterile sampling methods could reduce potential contamination.

Unlike the "Reverse Serial" method, the differences in cellulase loadings separated the "Parallel" method. The cellulose loading was different from the other methods, because cellulases were added to both the liquid and solid streams at the full cellulase loading rate. The "Parallel" method produced a total peak ethanol yield of 0.282 g ethanol/g sugar. Both the liquid and solid stream fermentations followed expected fermentation trends for the two microorganisms. The disadvantage to the "Parallel" method was the residual arabinose and galacturonic acid that remained bound in the solid fraction after solids separation. The centrifugation separation method did not remove all of the hydrolyzate from the pulp, and the quantification of residual liquid and sugar concentrations is difficult. About 12 g/L of both arabinose (27% of total arabinose) and galacturonic acid (47% of total galacturonic acid) were not consumed because *S. cerevisiae* only ferments glucose. To improve the ethanol yield, the separation process must be improved. Vacuum filtration of the solids would decrease arabinose and galacturonic acid remaining in the 45% of the hydrolyzate that remained in the solids fraction after centrifugation. With improved separation, the peak total ethanol yield of 0.27 g ethanol/g sugar beet pulp would likely exceed the 0.277 g ethanol/g sugar beet pulp reported in the literature (Doran and Foster, 2000). Although improvements in the separation process of the "Parallel" method could increase ethanol yields, the cost and time to achieve separation would likely outweigh the yield increase of 0.055 g ethanol/g sugar.

CONCLUSION

Four fermentation methods were used to improve ethanol titers and yields by combining the advantages of both *E. coli* KO11 and *S. cerevisiae*. Although ethanol yields in the "Parallel" method could be potentially increased through improved separation, the difficulty and cost of liquid and solid separation would likely outweigh the potential benefits. Comparing the "*E. coli* KO11 Only", "Serial", and "Reverse Serial" fermentation methods, the "Reverse Serial" produced the greatest peak ethanol yields. Elimination of variability in ethanol concentrations is necessary to confirm the results with this method. The "Serial" method can be improved by removal of acetic acid that inhibits the *S. cerevisiae* in the stage 2 fermentation, or by using an acetic acid tolerant ethanogen, such as *Pichia stipitis*. Finally, the "*E. coli* KO11 Only" fermentation could increase ethanol production with improved bacteria metabolism of the soluble sugars.

The "Serial" and "Reverse Serial" fermentations produced ethanol yields equal to or greater than those previously recorded with a feedstock of sugar beet pulp. Ethanol concentrations were restricted by the moisture content of sugar beet pulp that limited the solids loading rate to only 12% w/v.

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To write the third paper in my thesis, I researched literature, designed experiments, collected data, and analyzed results with guidance from my advisor, Dr. Scott Pryor, and his lab technician, Ms. Nurun Nahar. The paper is my original work that was edited by both Dr. Pryor and Ms. Nahar. This paper will be submitted to the peer-reviewed American Society of Agricultural and Biological Engineering (ASABE) publication, *Journal of Biological Engineering*, for publication. Like previous publications from my thesis research, Dr. Pryor and Ms. Nahar will be the coauthors.

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GENERAL CONCLUSIONS

Ethanol has potential to decrease US dependence on foreign oil. To meet government regulations of 16 billion gallons of ethanol by 2022, many new lignocellulosic feedstocks are under consideration. Sugar beet pulp is regionally significant for North Dakota and Minnesota and has a well-developed infrastructure for harvest, storage, and transportation.

This research tested different pectinases (Viscozyme and Pectinex) and a protease to determine which best hydrolyzed sugar beet pulp to produce high levels of soluble five- and six-carbon sugars. Protease decreased pectinase activities and was not considered further. The hydrolysis of hemicellulose and pectin was comparable for both Viscozyme and Pectinex, but more cellulose was degraded with Viscozyme when the same volumes of enzymes were loaded. When Pectinex volume was increased, the total theoretical glucose yield, which included both glucose and cellobiose, increased. Viscozyme was initially favored because the total soluble glucose concentration was the least of all enzyme loadings. After further consideration, the greater theoretical glucose concentration consisted of more cellobiose that cannot be fermented until cellobiases further hydrolyze it into glucose. Still the soluble cellobiose would be removed in separation of hydrolyzate from pulp. Fermentation with *S. cerevisiae* would benefit increased glucose concentrations later in the process.

Preliminary fermentation schemes showed that pH had a dramatic effect on the yields from both bacteria and yeast. The "Serial" and "Parallel" methods tested in the fermentation were successful, although there were low ethanol yields. The

5% w/v solids loading rate limited sugar concentrations in the hydrolyzate and final ethanol concentrations. These problems were improved for the final fermentation experiments by regular pH adjustments and higher solids loading rate (12% w/v).

Three different methods to optimize the advantageous characteristics of *E. coli* KO11 and *S. cerevisiae* were compared. The "Reverse Serial" produced peak ethanol yields (0.299 g ethanol/g sugar beet pulp) greater than the 0.277 g ethanol/g sugar beet pulp reported by Doran and Foster (2000). The "Serial" method was not statistically different from the "*E. coli* KO11 Only method", but could be improved with acetic acid removal or use of an acetic acid tolerant ethanogen. Glucose (13 g/L) was not consumed because of acetic acid inhibition. The improvement of both the "Serial" and "Reverse Serial" methods has great potential to increase ethanol yields.

Separation of cellulose-rich solids following pectin and hemicellulose hydrolysis in the "Parallel" method was difficult to maximize ethanol concentrations and yields. The residual hydrolyzate that remained in this fraction after separation constituted 27% and 47% of the total arabinose and galacturonic acid, respectively. The time and difficulty associated with improved separation of these soluble sugars from the solids would likely not be outweighed by the additional ethanol *E. coli* KO11 could produce.

Aside from the greater ethanol tolerance of *S. cerevisiae* (>120 g/L ethanol), greater reported actual ethanol yields from glucose substrates (0.375 and 0.475 g ethanol/g glucose) compared to *E. coli* KO11 (0.376 g ethanol/g glucose) (Arneborg et al., 1997; Narendranath et al., 2001; Yomano et al., 1998). The

improvement of both the "Serial" and "Reverse Serial" methods could take advantage of the higher ethanol yields from *S. cerevisiae*.

In the "Serial" method, other microorganisms, like *P. stipitis* could improve fermentation yields because of greater acetic acid tolerance. Also, the removal or precipitation of acetic acid after the *E. coli* KO11 fermentation step could allow more productivity from *S. cerevisiae*. The "Reverse Serial" method could benefit from stopping fermentation when ethanol concentration peak.

Improvements to ethanol yields did occur through this research, but ethanol concentrations did not reach the goal of 40 g/L that Doran and Foster (2000) achieved. To improve ethanol concentrations, increased solids loading rates are recommended. Doran et al. (2000) added sugar beet pulp to fermentations in stages to increase ethanol concentrations to 40 g/L. This solids loading could be adapted to work in the "Serial" and "Reverse Serial" methods. Additional pulp could be added after 96-120 hr in the "Serial" *E. coli* KO11 fermentation and the "Reverse Serial" *S. cerevisiae* fermentation. The "Serial" method would be altered to fluctuate pH levels as pectinases and *E. coli* KO11 have significantly different pH optima. If acetic acid removal was conducted in the second stage *S. cerevisiae* fermentation, more residual glucose could be fermented. The "Reverse Serial" method could benefit the most from addition of more pulp because the first stage SSF already degrades pulp and ferments hydrolyzate simultaneously. As long as final concentrations do not increase past *E. coli* KO11 tolerance in stage 2, ethanol concentrations can be increased. A limitation of this method would be the

increasing acetic acid concentrations that are seen during initial hydrolysis that would limit *S. cerevisiae* activity at higher solids loading rates.

Sugar beet pulp has potential as an economical lignocellulosic feedstock for ethanol production. Improvements in the economies of scale by processors will be an important step in producing a profit from the sale of ethanol. The growing importance of alternative fuel feedstocks and carbon emission regulations makes ethanol production from sugar beet pulp important to the Red River Valley. This research showed that genetically modified organisms (*E. coli* KO11) can be partnered with standard fermentation yeasts (*S. cerevisiae*) to improve ethanol yields from sugar beet pulp. Future improvements including acetic acid removal, real-time fermentation productivity monitoring, and increased solids loading rates can continue to improve the productivity of the methods explored here.

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