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Chemical Pretreatment And Enzymatic Hydrolysis Of Mixed Source-Separated Organic (SSO) And Wood Waste

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CHEMICAL PRETREATMENT AND ENZYMATIC HYDROLYSIS OF MIXED SOURCE-SEPARATED ORGANIC AND (SSO) AND WOOD WASTE

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ABSTRACT

This paper examines the effectiveness of two pretreatments on Source-Separated Organic waste (SSO) mixed with wood wastes: long term lime for SSO mixed with forestry waste (hardwoods), and the cellulose solvent-organic solvent lignocellulose fractionation (COSLIF) method, with SSO and demolition waste (softwoods). For long term lime treatment, the highest overall conversions from cellulose to glucose and xylose were 50.4 %, and 43.5 % respectively. The best temperature found for long term lime pretreatment was 65 °C. The COSLIF pretreatment glucose yield was found to be 93.7 %. The highest enzyme hydrolysis yield found was 93.5 % for a cellulase loading of 30 FPU/ g glucan at 50 °C. The best hydrolysis yield found at lower loading (10 FPU / g glucan), was 83.5 %. At 40 and 50 °C, all peak hydrolysis yields were achieved between 12 and 24 hours. A drop in temperature below 40 °C caused a slowing of the hydrolysis rate.

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GLOSSARY OF TERMS

Arabinan	An arabinan molecule is a polysaccharide of arabinose monomers. Total arabinan in biomass is the total polysaccharide arabinose found in hemicellulose.
β 1-4 Glucosic Bond	1-4 bond refers to the fact that the 1^{st} carbon atom is linked to the 4^{th} carbon atom of the next glucose unit. Beta is the nomenclature used to signify the fact that the first downward facing hydroxide molecule is attached to the second (Beta) carbon in each glucose unit.
Cellulose	An organic compound found in the cell wall of plants. Cellulose is a polysaccharide chain of β(1→4) linked D-glucose units that is crystalline in structure.
Cellulolytic	Cellulose utilizing.
Disaccharide	A carbohydrate made up of two sugars.
Galactan	A galactan molecule is a polysaccharide of galactose monomers. Total galactan in biomass represents the total polysaccharide galactose found in hemicellulose.
Glucan	A glucan molecule is a polysaccharide of glucose monomers. Total glucan in biomass represents the total polysaccharide glucose found in the cellulose and hemicellulose.
Hemicellulose	An organic compound found in the cell wall of plants. Hemicellulose is comprised of any number of polysaccharide sugars, which are in turn comprised mainly of the following monosaccharides: xylose, mannose, arabinose, galactose, and glucose. The polysaccharide chains are much smaller than cellulose, and the structure is amorphous.
Hydrolysis	A chemical reaction by which polymers are broken down and made soluble.
Lignin	Lignin fills the spaces in the cell wall between cellulose, hemicellulose and pectin. It lends structural strength to the cell wall as well as protection from degradation.
Lignocellulosic (Biomass)	Biomass that contains lignin, cellulose, and hemicellulose.
Monosaccharide	A carbohydrate made up of one sugar. Usually water-soluble.
Mannan	A mannan molecule is a polysaccharide of mannose monomers. Total mannan in biomass is the total polysaccharide mannose found in hemicellulose.

Polysaccharide	Polymetic carbohydrates formed of repeating mono and di-saccharides joined by glucosic bonds.
Saccharification	Hydrolysis of polysaccharides to soluble sugars.
Saccharolytic	Sugar utilizing.
Simultaneous Saccharification and Fermentation	A process by which polysaccharides are broken down by enzymes to monosaccharides and fermented in a single tank.
Source Separated Organic Waste (SSO)	Organic waste that is separated from other waste in the household, and then collected separately.
Xylan	A Xylan molecule is a polysaccharide of xylose monomers. Total xylan in biomass represents the total polysaccharide xylose found in hemicellulose.

1 INTRODUCTION

The price of oil has increased over the last decade from $23.17 per barrel in January 2000 up to a peak of $137.11 per barrel in July of 2008 (US Energy Information Administration). Prices at the pump reached as high as $ 4.10 per gallon (Kumar et al., 2009). During the same period, a better understanding of how the consumption of fossil fuels was affecting our climate pushed the public toward a desire for "cleaner" energy. As the price of oil raced higher and this desire for cleaner energy began to take hold, demand for biofuels (fuels from renewable resources) was greatly enhanced. Governments in North America and Europe began implementing gasoline substitution targets in order to encourage biofuel production, to satisfy both fuel security and environmental concerns. Since the conversion of food plants, which contain easily accessed sugars and oils, are more easily processed to fuel than plants containing sugar in the form of cellulose, the major share of biofuel production has been from food crops; corn for the ethanol program in North America ,and rapeseed oil for Biodiesel in Europe. The logic of using crop land for biofuel production in light of worldwide rising food prices has sparked what is known as the, "food versus fuel" debate. The arguments against using food for fuel will be outlined in **Section 3.1** (Literature Review). This paper, and the associated project, offer an alternative to using food crops. The project hopes to show that ethanol can be made from more socially and environmentally acceptable sources, and possibly at greater profit margins.

Lignocellulosic biomass for ethanol fermentation is both environmentally friendly, and socially acceptable, because it does not compete with food for land use. The four main categories that lignocellulosic biomass for ethanol use fall under are: agricultural residue (e.g. corn stover), dedicated energy crops (e.g. switchgrass), forestry residue (e.g. sawmill residue or tree clipping), and municipal waste (paper or organic). Lignocellulosic biomass is plant matter, or material made from plant matter, that contains lignin, cellulose, and hemicellulose. The cellulose has to be broken down to glucose, and

the hemicellulose broken down to xylose and other sugars, which can be fermented by the same microorganisms that ferment ethanol in the corn ethanol process. In this paper, Source-Separated Organic Waste (SSO) was mixed with demolition and forestry waste, and broken down to sugars for ethanol fermentation. The two main differences for fermenting ethanol from lignocellulosic biomass as opposed to food crops (corn or sugar cane) are: 1) the lignin shield that protects the sugar components in the plant wall needs to be opened, and 2) the bonds of the long chains of cellulose and hemicellulose sugars need to be removed. The change in the lignin shield is brought about by chemical pretreatment, and the bonds of the sugar chains are removed by enzymatic hydrolysis.

1.1 Chemical pretreatment

The first step of pretreatment generally involves a mechanical reduction in size of the material to be fermented. This could be accomplished by milling, grinding, or in the case of this project, screw pressing. In most cases, the biomass is then further broken open by chemical pretreatment, to break the bonds of the lignin shield. A number of pretreatment options are available, the most prominent being dilute acid, hot water, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), and lime (Mosier et al., 2005) This paper will explore the efficiency of two kinds of pretreatment: long term lime treatment, and the cellulose-solvent organic-solvent based lignocellulosic fractionation (COSLIF) method.

1.1.1 Long term lime treatment

The lime option holds a number of advantages for the breakdown of mixed SSO and forestry waste. Lime has been assessed in a cost comparison study by Eggman and Elander (2005) to be less expensive than both hot water and ARP. The high lignin content of the wood portion of the waste possibly rules out AFEX technology, because it is not efficient for biomass with high lignin content (Kumar et al., 2009). Most importantly, at pilot plant scale, it could be more promising than dilute acid pretreatment, which is to date the cheapest (Eggman and Elander, 2005), and most comprehensively modeled form of pretreatment (NREL, 2002). The reason it could be more promising is that at pilot scale, the increased costs due to lost economies of scale, wouldn't affect lime pretreatment as much as dilute acid. The pre-treatment reactor costs are significantly lower for lime than dilute acid (Eggman and Elander, 2005), therefore the increase in relative fixed costs for the scaled down pilot plant would be greater for dilute acid. Fixed costs for lime are much lower because the treatment can be applied at ambient pressure and modest temperature over a long period of time, which requires no special steel treatment vessel (Kim, 2005; Ramirez, 2005). A full scale plant could also benefit from long term lime treatment as the capital saved from smaller fixed costs for pretreatment could be reinvested in a larger facility. The increased variable costs would be offset by the negative feedstock costs associated with using SSO.

The objective of the lime treatment portion of the paper will be to judge the effectiveness of lime treatment as on option for mixed SSO and forestry waste. Treatment effectiveness will be judged by the proportion of glucose and xylose contained in the biomass that is released by enzymatic hydrolysis.

1.1.2 COSLIF method

Using the cellulose solvent-organic solvent based lignocellulosic fractionation (COSLIF) method holds a number of advantages for the SSO mixed with demolition waste. The COSLIF method has already been proved effective with corn stover, switchgrass, poplar wood, and douglas fir (Zhang et al., 2007). Those feedstocks would be similar in composition to the components in the SSO/demolition waste. The demolition wood waste used for this study is mainly made up of softwood, particularly douglas fir. The COSLIF method itself has a number of advantages over other pretreatment options: Modest reaction conditions (50°C, atmospheric pressure), low sugar degradation, no inhibitor formation or special reactor needed as in dilute acid treatment, extremely high enzyme digestibility, extremely fast hydrolysis rates, and easy recovery of acid with separation between solid sugars and liquid acid (Zhang et al., 2007). If a demolition wood waste mix was successfully digested by the COSLIF method, it would indicate an extremely high likelihood of success in digesting almost any other mix of biomass with SSO. (The demolition wood is mainly composed of softwood. Softwood has very high lignin content in comparison to other biomass.)

The objective of the COSLIF portion of the paper will be to assess the effectiveness of the COSLIF method to pretreat SSO mixed with demolition waste under a number of different enzyme hydrolysis conditions. Different enzyme loading conditions will be tested to find the optimal loading for fermentation profit maximization. Varying enzyme hydrolysis temperature conditions will also be tested to find the ideal temperature for fermentation. Effectiveness of the pretreatment at the different conditions will be assessed by the amount of glucose released by enzymatic hydrolysis in relation to the total glucose available in the untreated biomass.

1.2 Enzymatic hydrolysis

Enzymes are added after chemical pretreatment to convert the long chain polymeric sugars of cellulose and hemicellulose, into monomeric sugars like glucose, xylose, mannose, galactose and arabinose. The effectiveness of the chemical pretreatment is judged by the effectiveness of the enzymatic hydrolysis. If the proportion of glucose or xylose released by enzymatic hydrolysis is high in comparison to the total glucose or xylose available in the biomass, then the pretreatment is deemed effective. The proportion of glucose or xylose released is judged to be high, if it compares favorably to the proportion of glucose or xylose released by enzyme hydrolysis in other pretreatment studies of similar biomass, at similar enzyme loadings. The release of the other sugars found in hemicellulose such as galactose, arabinose, and mannose, are often ignored because bacteria typically used to ferment the enzyme hydrolysis liquor are not capable of fermenting those sugars at this time. The most common system today is the breakdown of cellulose with enzymes from the fungi *Trichoderma reesei*, followed by a fermentation using the saccharolytic bacteria *Sacchomyces cerevisea* (baker's yeast). Since baker's yeast only ferments glucose, some studies only include glucose conversions.

To assess the effectiveness of enzyme sugar conversion in comparison to other studies, a common dosage/loading of cellulase must be administered. A common language for dosage/loading was developed by the United States National Renewable Energy Laboratory (NREL) from the cellulase studies of Ghose (1987) and Miller (1959). Dosage is reported by the addition of activity units per gram glucan, glucose, or dry biomass. The activity units are called Filter Paper Units (FPU) because the procedure to find the activity level of cellulase used for the experiment, involves solving for the concentration of cellulase that will convert 4 % of a 50 mg strip of Whatman filter paper to 2.0 mg of glucose in 1 hour. The most common loadings tested are 15 and 60 FPU per gram glucan. Glucan is the amount of glucose stored in the cellulose and hemicellulose components of biomass.

1.3 Project objectives

The overall project, of which this thesis is a part, seeks to use Source Separated Organic Waste (SSO) from Toronto's Green Bin Program, as a fuel source for ethanol fermentation. The motivation behind the research is twofold:

- Successful completion would provide an avenue to rescue Toronto's ailing green bin waste diversion strategy, as well as encourage other cities to divert organics away from landfill.
- SSO could be an environmentally friendly, cost-effective fuel source for an ethanol plant. It would be a renewable, cellulosic source, that doesn't compete with the food supply for land use, or have the negative environmental impacts associated with fuel from food. SSO as a feedstock, could also be capable of producing ethanol at less cost than fuel from food alternatives, because of the negative price gained from tipping fees that governments will be willing to pay to divert it from landfill.

1.4 Project stages

The project can be broken into five stages. This study forms a part of the Stage 2.

Stage 1: Characterization of mixed SSO and wood waste as a biomass feedstock in order to examine the potential of utilizing SSO for ethanol production.

Stage 2: Investigation of appropriate chemical and lowest cost biological pretreatments to best break down the mixed SSO/wood feedstock to its component sugars for ethanol fermentation.

Stage 3: Investigation of the feasibility of converting pre-treated SSO to ethanol in two stages of enzymatic hydrolysis and fermentation utilizing the cellulase enzyme and the bacterium *Zymomonas mobilis*.

Stage 4: Production of ethanol and acetate in a continuous-culture fermentor.

Stage 5: Design and operation of a bench-scale ethanol plant.

2 STUDY SCOPE AND OBJECTIVES

The major focus of this study was to prove that Source Separated Organic Waste (SSO), mixed with wood waste, is capable of being broken down to component sugars. The study aimed at contributing to proving that the sugars in SSO and mixed waste could be a more profitable feedstock source than corn for ethanol. The scope of this paper was to address the effectiveness of two different pretreatments, in preparing a mix of Source Separated Organic (SSO) and wood wastes, for enzymatic hydrolysis. The first is a long term lime pretreatment. The conditions of long term lime treatment were: the addition of lime at ambient pressure, moderate (55, 65 and 75 °C) temperatures, oxidative conditions (supply of compressed air), and a relatively long (6 weeks) period of time. The conditions were modeled after the successful long term lime studies of Kim (2005) and Ramirez (2005), with a slightly higher temperature range, and longer duration. Within the lime treatment portion of the paper, the following results were arrived at:

- Effectiveness of long term lime to prepare SSO mixed with hardwood for enzymatic saccharification. The assessment of effectiveness was reported in terms of overall yield (glucose solubilized from initial cellulose composition), and compared to similar studies.
- The ideal temperature and timing of the best overall yield.

The second pretreatment covered in this paper is the COSLIF method. The COSLIF method is comprised of a phosphoric acid hydrolysis, an organic solvent wash, and a water rinse. The ideal timing of the phosphoric hydrolysis portion, composition of the organic solvents, estimated overall yield at high enzyme loading (60 FPU cellulase / g glucan), and best cellulase cocktail (Accellerase 1500), was previously found by Ehsanipour (2010). The goal of this study was to find a more precise answer for the overall glucose yield, to present the hydrolysis profile of the waste digestion, and estimate the best enzyme conditions for cost effective fermentation. The Ehsanipour (2010) study was extended, by

replacing approximations of the following conditions with calculated results based on experimental findings:

- Pretreatment Yield
- Total mass of glucose and non-glucose components, remaining after pretreatment.
- Hydrolysis Yield

Enzyme loading with the Accellerase 1500 cellulase experimented with at a temperature of 50 °C and a pH of 5.0, to determine what loading would return the most profit. The effectiveness of the enzyme hydrolysis was also assessed with a drop in temperature to 40, 35, and 30 °C. The results of the temperature range hydrolysis were used to find the best temperature for future fermentation pairing with the rapidly sacchorolytic bacteria *Zymomonas Mobilis*. The following points summarize the findings that this study accomplished:

- The effect of enzyme loading, through hydrolysis profiles of glucose released versus time, for loadings of 10, 15, 30, and 60 FPU / g glucan.
- The calculation of best loading in terms of profit. (Profit assessed by ethanol revenue in terms of sugar yield at that loading versus cost of cellulase at that loading)
- The effect of temperature on enzyme hydrolysis, through hydrolysis profiles of glucose released versus time, for temperatures of 30, 35, and 40 °C.
- The assessment of optimum temperature for enzyme hydrolysis, if Accellerase 1500 cellulase was to be paired with *Z. Mobilis* for simultaneous saccharification and fermentation of COSLIF treated material.
- The effect of an even smaller enzyme loading at lower temperature, shown by the hydrolysis profile of a 5 FPU loading, at 35 °C.

3 LITERATURE REVIEW

The literature review will first outline the environmental and social advantages of using SSO over food crops. It will address the problems with the Toronto green bin program, and how using green bin waste for ethanol fermentation could not only solve this problem, but generate more profit from ethanol than food crops. The literature review will then cover basic cellulose structure, and the mechanisms by which different pre-treatments break open the material to make it amenable for enzymatic saccharification. An introduction to enzyme action will then be provided for those not familiar with pretreatment technology. The introduction will be followed by a synopsis of studies that include an enzyme hydrolysis profile, and/or hydrolysis at varied temperature, to outline the reasoning behind the conditions of the COSLIF study. A short synopsis of the fermentation capabilities of *Z. Mobilis* will also be presented, to further explain the temperature ranges chosen for the COSLIF study. The section on enzyme hydrolysis will be followed by an overview of lime treatment, COSLIF treatment, and other pretreatments, so as to provide a basis for comparison to the results of this project.

3.1 Food versus Fuel

As more and more crops such as maize, soy, and rapeseed are being consumed for biofuel production, world prices for a basket of food goods have risen dramatically and people have begun to ponder the connection. In an article written for the World Bank, the proportion of food price increases attributed to biofuels was estimated at 65% (Mitchell, 2008). On the other hand, the United States Department of Agriculture (USDA) calculated that only 13.5-18% of food price increases were the result of biofuels (Mitchell, 2008). The main difference between the percentage points quoted is the arguable link between grain stock shortages, export bans, and speculative activity that is attributed to biofuels, as well as the role of dietary changes in China and India with increased wealth. The International Food Policy Research Institute (IFPRI) generated a viewpoint that is about half way between that of the USDA

and Mitchell estimates. The IFRPI impact model assessed the role of biofuels to be 33% of the food price increases, after the mandate of biofuel substitution targets by European and North American governments (Rosegrant, 2008). Using the figures found by the IFPRI model, Oxfam (2008) subsequently noted that of the 100 million people pushed into poverty by increasing food prices, 30 million would have been put there by biofuel policies. Whether research has shown biofuels to be responsible for 30 million in poverty, or whether it's more along the USDA lines of 10 million, everyone researching biofuels can agree that they are playing at least a small role in food price increases. Food based biofuels therefore have to take a share of the responsibility in causing poverty. The push into poverty, no matter to what extent biofuels were responsible, should be justified by some sort of benefit. If there were no benefit to biofuel programs, it would be irresponsible to continue to increase hunger in the third world.

The two main arguments that are espoused in the media for instituting biofuel targets seem to be energy security and environmental benefits. The first is not rational given more attractive options. Yes, if a country produces biofuel it needs to purchase less oil and is therefore more secure, but if it uses less oil it also needs to buy less and is therefore more secure. Dollar for dollar there is nothing more effective than increasing engine efficiency for reducing the demand for gasoline. The costs are in fact negative, which cannot be said for biofuels. In a Swedish study, the cost for increasing engine efficiency was as low as -8000 Kronor, or approximately -1100 USD over the lifespan of the car; meaning that the money saved in gasoline over the lifespan of the car, was greater than the cost of making it more efficient (Koplow, 2007). Therefore, until a country had at least spent enough money on efficiency until extra improvements actually had a cost that was above zero, there is no way that biofuels from food can be touted as a reasonable energy security initiative.

That leaves environmental benefits as the reason why European and North American governments might claim they have justification for biofuel policy. However, that argument only holds true if there actually are environmental gains. Recent articles on land use changes and the nitrogen uptake of food crops from chemical fertilizers have disproved this myth. In 2008, Fargione et al. did a study to show the effect of converting land to produce crops for Biofuel. **Figure 3.1** shows that it can take as much as 423 years to repay the carbon deficit created by converting forest and grassland into cropland.

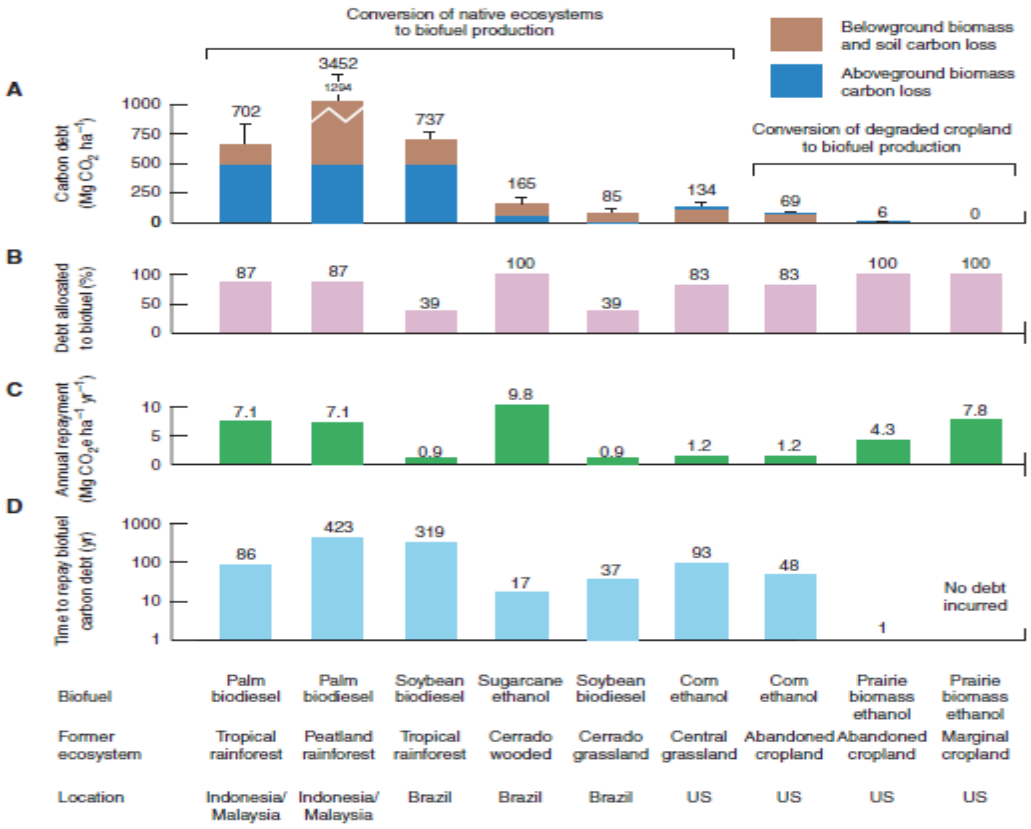


Figure 3.1 Carbon deficit repayment periods for ethanol production (Fargione et al., 2008).

Row A in **Figure 3.1** shows the release of carbon as the biomass on the land is either burnt, or left to decompose. Row B attributes the proportion that biofuel is responsible for (e.g. Corn Ethanol is 83% because only 83% of the corn is used for fuel, some of the by-product (17%) is recovered and sold as

animal feed. Row C represents the benefit of value of the carbon stored in the biofuel plants as they grow over just burning gasoline. Row D is the time in years it would take to repay the carbon released from conversion of the land. The effects of converting peatland rainforest to palm biodiesel production are catastrophic. It would take 423 years to repay the carbon debt making palm biodiesel ludicrous as an environmental initiative. Corn ethanol is not a reasonable option either, with a 93 year payback on grassland, or 48 year payback on abandoned cropland.

Compounding the problem of touting biofuels from food sources as having an environmental benefit is the fact that the gains found in "Row C" of **Figure 3.1** above are widely disputed. The numbers are assumptions that the yearly carbon storage in crops grown for fuel would have a benefit over burning gasoline. A recent study by Crutzen et al. (2008) has shown that even were the fuel to plant/fertilize/harvest/transport the crop zero, the net greenhouse gas benefit would be negative for both corn ethanol and rapeseed biodiesel. Crutzen et al. (2008) have suggested that because of the nitrogen uptake efficiency of these crops from chemical fertilizers, more nitrous oxides are expelled during growth respiration and bacterial denitrification of runoff, then carbon dioxide is saved from gas substitution. Though less nitrous oxides are released then carbon dioxide saved, nitrous oxides are approximately 296 times more potent a greenhouse gas than carbon dioxide. Release of nitrous oxides, combined with the fuel spent planting, fertilizing, harvesting, and transporting the crops, indicate that the paypack periods shown in **Figure 3.1**, are in reality infinite. Since the net carbon benefit is negative compared to burning gasoline, the deficits can never be repaid, and growing of corn and rapeseed crops for biofuel cannot be considered environmentally friendly.

Biofuels from food have been shown to be of little benefit, but that does not mean that all biofuels are environmentally unsound. As shown in the last column of **Figure 3.1,** prairie biomass (grass) grown on

abandoned crop land or land only marginally suited for crops, has either a 1 year or no carbon deficit to pay back. There are abundant sources of biomass whose conversion to ethanol creates no carbon debt. Any kind of organic waste would fall into that category. Agricultural waste like corn stover, forestry waste like wood chips from milling, and municipal waste like the organic fraction of the garbage collection are all prime examples. They all contain sugars in the form of cellulose and hemi-cellulose, which once broken apart can be fermented to ethanol. The product is called cellulosic ethanol because the ethanol is fermented from the sugars contained in the cellulose that plant matter is made of. So if abundant sources for cellulosic ethanol exist why do people continue to use food for biofuel? The answer is that to date, the process used to create cellulosic ethanol has been more expensive than food ethanol or biodiesel.

3.2 SSO as an ethanol feedstock – saving the green bin program

Toronto's decision to collect organic waste was largely motivated by the closure of the Keele Valley Landfill. Keele Valley, located north of Toronto at Major Mackenzie Blvd. and Keele St., was the city's major landfill site from it's opening in 1983 until its closure in 2002. In 1998, in anticipation of the closure of the Keele Valley site, Toronto began to ship its landfill waste to Michigan at an average cost of $63 per tonne (Estey, 2006). The contract with Michigan ends in 2010, and the U.S. Federal Government mandated a closure of the cross border garbage shipments over security concerns. In response to that announcement, in 2000, the City of Toronto assembled a group, Task Force 2010, to come up with a new waste diversion scheme. The report issued by the task force, stated clearly the goals of 30% residential waste diversion by 2003, 60% by 2006, and 100% by 2010 (City of Toronto, 2001). A central part of the waste diversion strategy was the Green Bin Program.

Figure 3.2 Green bin.

Organic kitchen and household waste would be separated by the household and collected separately from the regular garbage in the curbside Green Bin. That material would then either be aerobically digested to produce compost, or anaerobically digested to produce both electricity from methane, and a smaller amount of compost. The program was extremely successful in terms of participation but a less successful public adherence to the proper materials to be placed in the bin, has led to disastrous results. Collection jumped 120% in two years, from 114 681 tonnes in 2005 to 251 368 tonnes in 2007 (Toronto Star, March 1, 2009). The composting facilities Toronto was planning on sending the waste to, did not have the capacity to handle the volume. To compound that problem, some of the material was not passing compost standards. In the March 1st article in the Star, excess waste not processed to compost was reportedly being shipped to New York, and a number of facilities handling the waste were doing so improperly to keep up with demand. Improper handling forced the Ministry of the Environment to shut down facilities for odour complaints. When the material doesn't pass standards, it has to be shipped to a landfill as well, which defeats the purpose of a separate collection. In a conversation with Doug Beatty, who was managing the anaerobic processing section of the Toronto Dufferin Transfer station in 2006, staff would often see items that do not belong in the green bin. They found light bulbs, metal, and hard plastics in the green bin material. When those materials are found even in slight amounts, the compost is inhibited from being used as a soil enhancement for parks, forests, and especially arable farm land. The operators of the Dufferin Transfer station anaerobic digester have even had machinery break downs caused by a hand gun or steel bar in the green bin material. In the Mirzajani (2009) SSO/demolition waste characterization study, completed

as a part of this project, it was found that heavy metal concentrations often inhibited green bin material from being used as category A compost (unrestricted use), and sometimes even category B compost (restricted use).

Fortunately, if the material is processed through anaerobic digestion to create fuel products such as ethanol or methane, the foreign objects and original composition of the green bin waste becomes irrelevant. At the Dufferin Transfer Station, the compost material that comes out of the methane generation process has always passed compost standards (Doug Beatty, personal communication, 2006). In the ethanol fermentation process, the leftover solids would be consumed in the boiler for energy. So if the project can prove that this SSO to ethanol process can be cost competitive with food crop based ethanol, there will be incentive to capitalize on this and build fermentation facilities. The industrial partner to this project, aims at demonstrating that at the pilot scale. Successful results at pilot scale will encourage full scale adaptation, and create a demand for the green bin waste. This demand could allow the City of Toronto to not only process the material properly, but possibly lower the $90 - $127 per tonne (Doug Beatty, personal communication, 2006) that the city is currently paying to process it.

3.3 SSO as an ethanol feedstock - clean and cost competitive

No additional land is required to generate the scraps of food and paper products that are thrown in the garbage. Therefore the carbon deficit created by food crops for biofuel discussed in the **Section 3.1** does not apply to SSO. Similarly, no additional nitrogen oxides will be released from the respiration of crops during chemical fertilizer uptake. Additionally, no fuel will be consumed in the planting/harvesting and transportation of crops. It does take fuel to deliver the SSO to the ethanol plant, but if the plant is located near a city, like the Dufferin Transfer station in Toronto, that fuel will be far less than what is used in alternative transport to a landfill, which is usually a lot further from city centers.

In 2006, according to manager Doug Beatty, the Dufferin Transfer station was being paid a tipping fee of $127 per tonne to process the green bin material. At that time, Toronto was collecting 30% less SSO than they are now (Toronto Star, March 1, 2009). This signifies a potentially feasible opportunity in the event that SSO is proved a viable feedstock for ethanol. In 2002, the NREL in conjunction with the Harris Group, published a comprehensive cost assessment study for lignocellulosic ethanol. **Figure 3.3** shows the NREL assessed the cost of biomass to be 31% of the total costs.

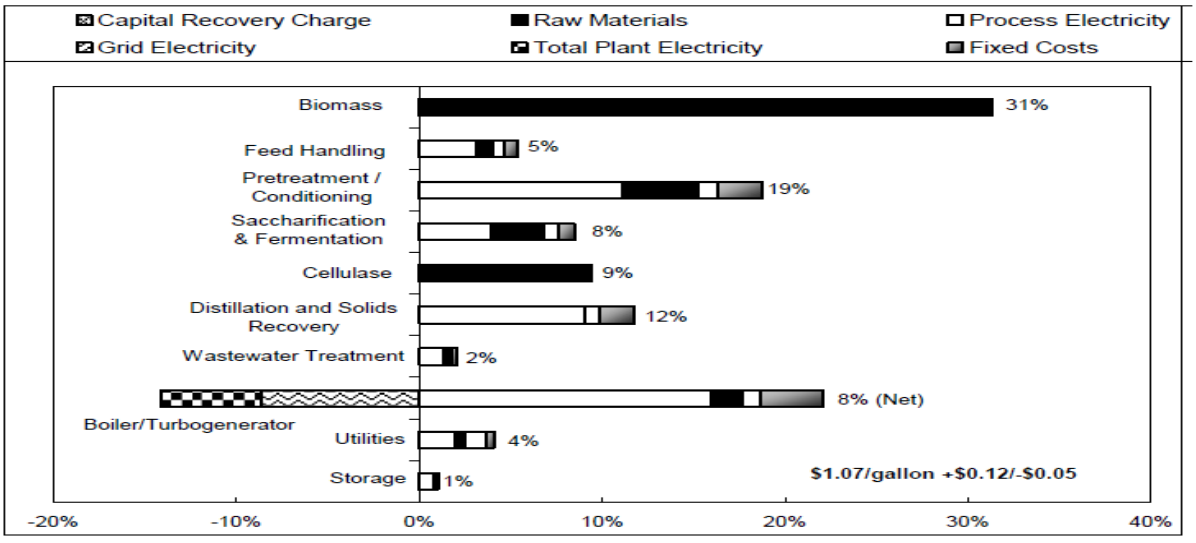


Figure 3.3 Cost contribution of lignocellulose plant details (NREL, 2002).

In the same paper, the delivered cost of the corn stover was stated as $30 per dry tonne. In comparison, the cost of the delivered green bin waste is negative $ 127 per wet tonne. The moisture content of the pure SSO sample received for this study was 81.6% (**Table 4.1**). To make the moisture content more conservative for representing an average sample, a value of 75% is assumed. That would mean the solid content is only 25%, and the delivered cost of a dry tonne of SSO is negative $ 508. **Table 3.1** shows the conversion of that negative biomass cost into a negative cost per gallon of ethanol generated.

Table 3.1 Derivation of per gallon ethanol figure for negative biomass cost.

A	B*	C = A x B	D = 1/C
Liters to U.S. gallons	Liters ethanol in 1kg dry SSO/softwood	Gallons in 1kg dry SSO/softwood mix	kg SSO/softwood needed for 1 gallon
0.26	0.265	0.0689	14.514
E	**F****	**G = E+ 0.2 x F**	**H***
Tipping fee $ / tonne wet SSO	Assumed cost of a tonne of wood waste ($)	Tipping fee per tonne SSO + 20% wood	Maximum Fraction solids in SSO/softwood mix
-127	60	-115	0.5
I = H x 1000 kg	**J = B x I**	**J = C x H**	**K =F/J**
Maximum kg solids in one wet tonne SSO/softwood	Litres ethanol in wet tonne of SSO/softwood	Gallons of ethanol in wet tonne	Cost $ / gallon produced
500	132.5	34.45	-3.34

* Mirzajani, (2009), figures taken from a 6 month study in seasonal fluctuation of mixed SSO and softwood.

** Assume the price of the material blended with the SSO to be twice the price of the NREL estimate for corn stover to be conservative. Even though for this particular supply, the wood waste is free.

Since the market price of ethanol futures is currently under $ 2 per gallon (Barchart.com, May 2010) more money can be made from processing the SSO than in selling the ethanol produced. If the SSO was being processed at an ideal, 2000 metric tonne per day plant, as in the NREL (2002) assessment, this biomass negative cost would be three times the price of the projected total cost of $ 1.07 per gallon. However, the major hurdle to exploiting SSO fermentation is that the technology of SSO to ethanol conversion is unproven, and therefore a pilot scale facility would be more likely until the results encourage scale-up. **Figure 3.4** shows the rise of non-feedstock costs because of loses in economies of scale for smaller facilities.

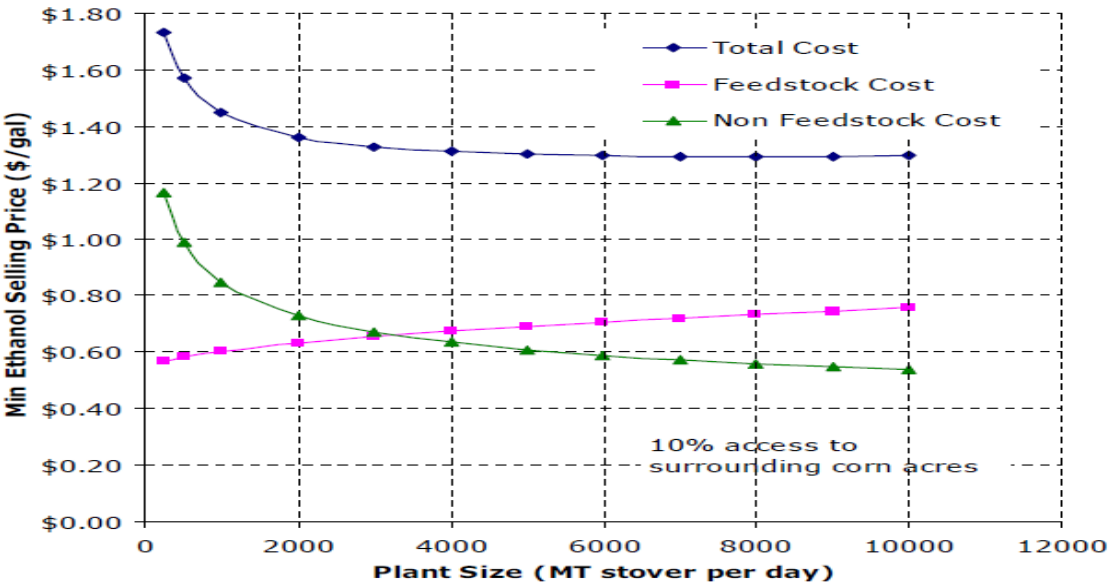


Figure 3.4 Ethanol cost as a function of plant size (NREL, 2002).

The highest point on the graph appears to be about 250 MT / day, which is large for a pilot plant. The capacity of Toronto's Dufferin Transfer station anaerobic digestion plant is only about 70 MT / day. To model what the increase in cost might be for 70MT / day, the trend of increase in non-feedstock costs can be continued along the green line in **Figure 3.4** above. There is an increase in non-feedstock costs per gallon of 10 cents between 2000 MT and 1000MT, then about 15 cents between 1000 and 500, and finally almost 20 cents between 500 and 250. Therefore, the increase from 250 MT to 125MT should be about 25 cents, and the increase from 125MT to 67.5MT about 30 cents. At that pilot scale size, the total non-feedstock costs would then be approximately $1.20 + $ 0.25 + $ 0.30 = $1.75 per gallon ethanol produced. Even in the unlikely event that the distillation and pretreatment costs were doubled for a lower sugar content and a more recalcitrant biomass, the price would only rise to $ 1.75 + $ 0.54 = $ 2.29. (Pretreatment is 19%, and distillation 12% of total cost (**Figure 3.4**). Extra Cost -- 1.75 x 0.31 = 0.54). The negative biomass cost would still pay for the entire process.

In light of these facts, there is an almost desperate need to prove the viability of SSO as an ethanol feedstock. It would not only be good for the environment, but have obvious financial investment benefits. With a negative cost associated with production, larger facilities would be inevitable, enabling cost decreases with economies of scale, as well as additional demand for the SSO. As demand for the SSO increased, the government would be able to lower the tipping fee incentive paid, hopefully to the point that a separate organics collection would be cheaper than landfilling. In fact, if the tipping fee dropped to $ 63 / wet tonne, the price Toronto was paying to ship the material to Michigan in 1998 (Estey, 2006), then the biomass cost would drop to - $1.48. With a minimum selling price of $1.50/ gallon, the price of ethanol futures before the rise in gasoline prices in August 2009 (Barchart.com), the revenue would be $2.98 / gallon produced, enough to overcome even the inflated pilot scale costs of $2.29 / gallon and clear a healthy profit.

3.4 Cellulose structure

Cellulose is a part of the makeup of plant cell walls, and is the most abundant component of plant biomass (Lynd et al., 2002) Cellulose is a polymer of repeated cellobiose molecules joined by 1-4β glucosic bonds. Since cellobiose is made up of two β-D(+) glucose molecules, linked by the 1-4β glucosic bonds, cellulose can also be considered a series of glucose molecules. That chain of cellobiose/glucose molecules can run as long as 25000 glucan residues (Desvaux, 2005). **Figure 3.5** is a good representation of the molecular chain and how those chains fit into a fibril of cellulose.

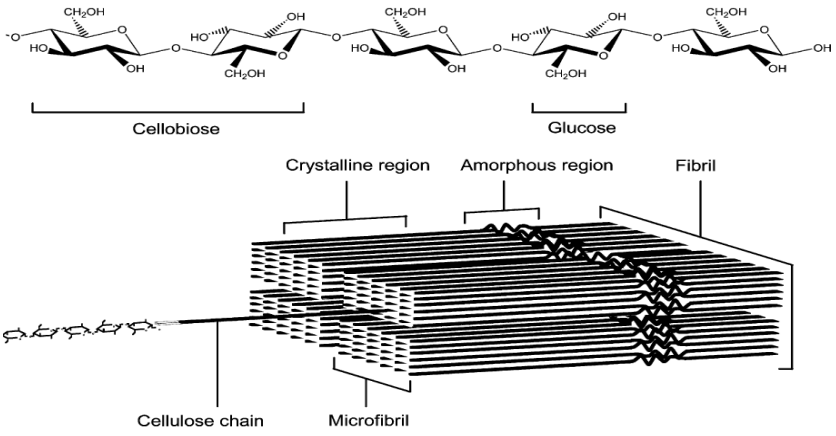


Figure 3.5 Structure of cellulose and cellulose fibril (Desvaux, 2005).

The crystalline region above represents the cellulose chains, and the amorphous region hemicellulose. Hemicellulose is made up of much shorter chains of five and six carbon sugars: xylose and arabinose (five carbon), as well as mannose, galactose, and glucose (six carbon). When lignin fills the spaces between the cellulose and hemicellulose in the cell wall, the substance is also referred to as lignocellulose. If one imagines the cross section of one of the microfibrils above, it would look as if wrapped in a protective lignin coating (**Figure 3.6**).

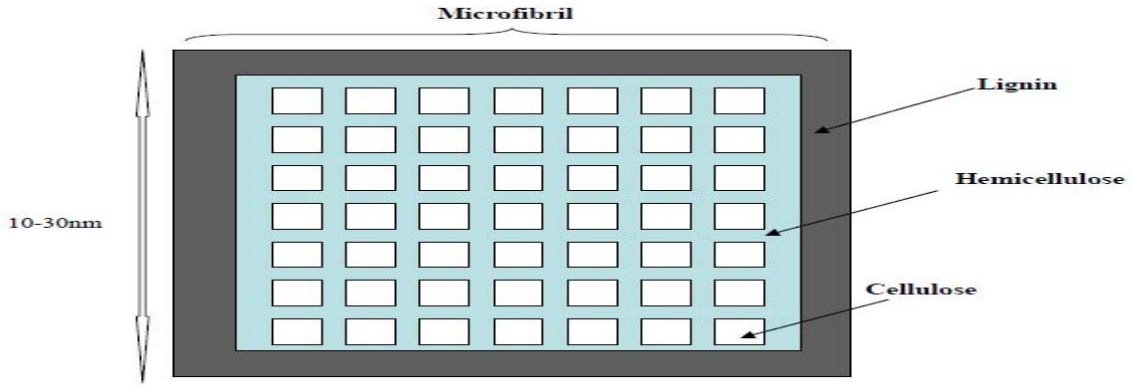


Figure 3.6 Cross section of cellulose microfibril (Gupta, 2008).

Breaking past this lignin barrier, to get access to the sugars found in the cellulose and hemicellulose, is the function of chemical pretreatment.

3.5 Pretreatment

The goal of pretreatment is to hydrolyze the cellulose and hemicellose found in biomass to monomeric sugars. The three processes typically used for ethanol fermentation are dilute acid, concentrated acid, and enzymatic saccharification (Broder et al., 1995); of which enzymatic saccharification is the most common (Kumar et al., 2009). Enzymatic saccharification is common enough that when most papers discuss pretreatment, they are referring to the chemical or physical pretreatment necessary to disrupt the lignocellulose structure and expose the cellulose to enzymatic attack.

> "Pretreatment is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars" (Mosier et al., 2005).

In this paper, pretreatment will be taken to have that meaning as well. Without pretreatment, the protective lignin layer between microbrils renders the β-glucosic bonds of the cellulose inaccessible to enzymes, limiting hydrolysis yields to under 20% of theoretical (Lynd et al., 2002). With pretreatment, the hydrolysis yields of glucose and xylose from cellulose and hemicellulose can reach over 95% of theoretical (Eggman and Elander, 2005). **Figure 3.7** illustrates the purpose of pretreatment in preparing a cellulose microfibril for enzyme saccharification.

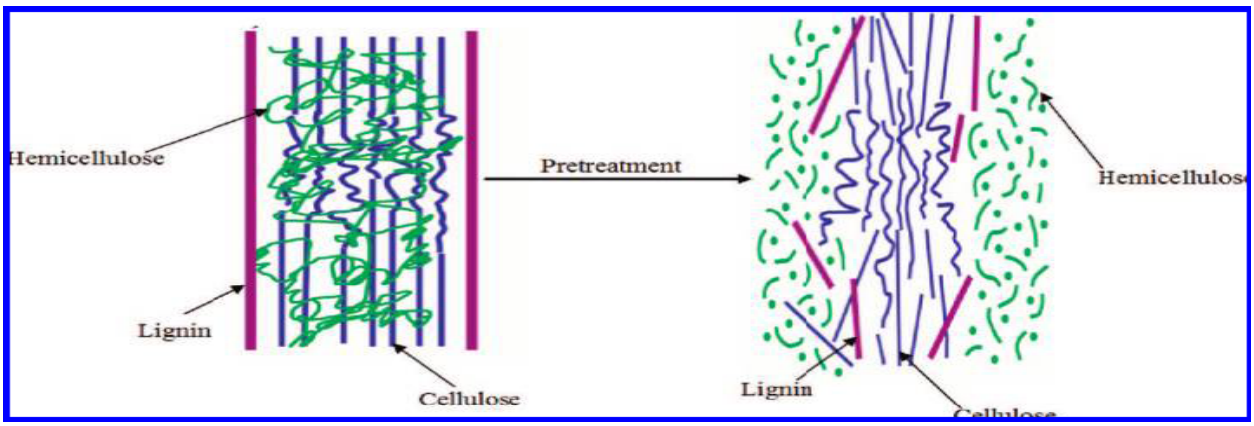


Figure 3.7 Effect of pretreatment on cellulose microfibril (Kumar et al., 2009).

The goal is to break the lignin shield surrounding each microfibril, as well as disrupt the crystalline structure of the cellulose (Mosier et al., 2005).

Physical pretreatments include: dry and wet vibratory ball milling, compression milling, steam explosion, hot water, and hydrothermolysis (Mosier et al., 2005). Chemical pretreatments include: Concentrated mineral acids (H_2SO_4, HCl), ammonia based solvents (NH3, Hydrazine), alkaline H_2O_2, NaOH, $Ca(OH)_2$,ozone, organosolv (Lexis acids, $FeCl_3$, $(Al)_2SO_4$ in aqueous alcohols), glycerol, dioxane, phenol, ethylene glycol, aprotic solvents (DMSO), and metal complexes (ferric sodium tartrate, cadoxen,

and cuoxan) (Mosier et al., 2005). Biological pretreatment is possible as well, the most effective being an application of white-rot fungi, which excrete lignin degrading enzymes (Kumar et al., 2009). Despite the fact that there are numerous pretreatment processes, only steam explosion, hot water treatment, dilute acid, flow through acid, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), and Lime are considered cost effective (Mosier et al., 2005) Each of these technologies works to either remove or alter lignin and/or hemicellulose. By achieving one or the other, or both, accessible surface area is increased, because porosity is increased by a combination of hemicellulose solubilization, lignin solubilization, and lignin redistribution (Lynd et al., 2002). **Table 3.2** summarizes the effects that each of these pretreatments has on the chemical composition and structure of lignocellulose.

Table 3.2 Effect of pretreatment on chemical composition and structure (Adapted from Mosier et al., 2005).

PRETREATMENT	Increases accessible surface area	Decrystallizes cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Uncatalyzed steam explosion					
Liquid hot water		ND			
pH controlled hot water		ND			ND
Flow -through liquid hot water		ND			
Dilute Acid					
Flow-through Acid					
AFEX					
ARP					
Lime		ND			

MAJOR EFFECT	MINOR EFFECT	LITTLE OR NO EFFECT	ND=NOT DETERMINED

It should be noted that at the time of the Mosier et al. (2005) article, the COSLIF process had not yet been experimented with.

3.6 Enzyme hydrolysis

Following pretreatment, the sugars in the treated biomass are hydrolyzed by enzymes excreted from cellulolytic organisms. These organisms excrete extra cellular cellulase and xylanase which break the 1-4 β glycosidic bonds of polysaccharide cellulose and hemicellulose, into monosaccharide and oligosaccharide sugars. The monosaccharide sugars can be consumed by the cellulolytic organism itself, or other saccharolytic organisms. The most common system today is the breakdown of cellulose with enzymes from the fungi Trichoderma reesei, followed by a fermentation using the saccharolytic yeast *Saccharomyces cerevisea* (baker's yeast).

The three major components/activities of cellulase are: endoglucanases, exoglucanases (also called cellodextrinases), and ß-glucosidases.

> "Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri, 97). ß-glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose"
>
> (Lynd et al., 2002)

The activities of the cellulase components are illustrated in **Figure 3.8**.

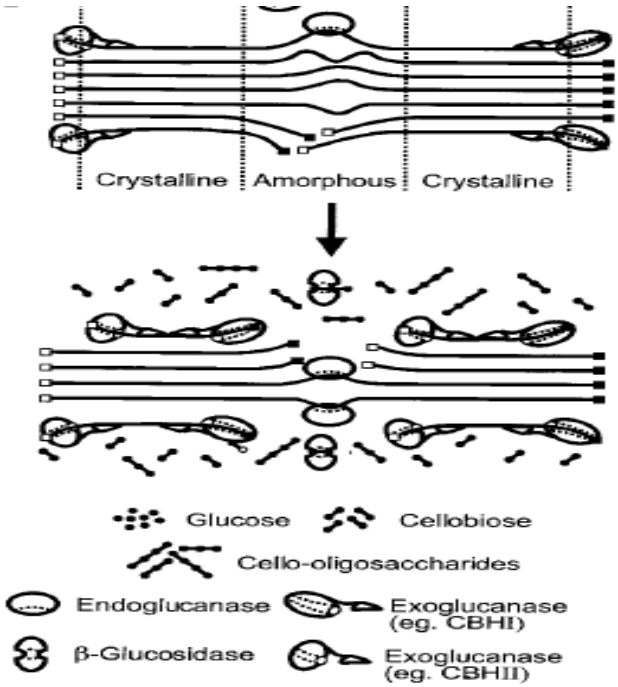


Figure 3.8 Schematic representation of *T. reesei* cellulase action on a cellulose microfibril (Adapted from Lynd et al., 2002).

The enzymes needed for the system shown above can either be generated on-site, or purchased from a supplier. The two largest enzyme suppliers in the world are Genencor International, and Novozymes Biotech (NREL, 2002). They have both dramatically reduced the cost of cellulase through research and development partnerships with the United States Department of Energy (DOE). The Accellerase 1500 enzyme cocktail produced by Genencor was previously found to be more effective on COSLIF treated material than a mixture of Celluclast and Novozyme 188, produced by Novozymes (Ehsanipour, 2010). Genencor has since released an Accellerase Duet enzyme cocktail that is advertised to have 3 times the activity level of Accellerase 1500. For those reasons, the Accellerase 1500 enzyme cocktail was used for the experiments in this study. An attempt to acquire Accellerase Duet was made, but it is only for sale by the tonne or tanker.

To assess the effectiveness of enzymes on biomass, a common language for dosage was developed by the NREL from the cellulase studies of Ghose (1987) and Miller (1959). Dosage is reported by the addition of activity units per gram glucan, glucose, or dry biomass. The activity units are called Filter Paper Units (FPU) because the procedure to find the activity level involves solving for the concentration of cellulase that will convert 4 % of a 50 mg strip of Whatman filter paper to 2.0 mg of glucose in 1 hour. The NREL procedure for measurement of cellulase activity can be found in **APPENDIX 3**.

Since this paper will include enzyme hydrolysis profiles for the COSLIF treatment, at varying enzyme loadings and temperatures, a number of studies were examined to find the conditions which would allow for comparison of the results. The studies were chosen for their prominence in journal article citations, similarity to the mixed SSO hardwood and softwood feedstock, and/or pretreatment process. **Table 3.3** contains a summary of the conditions/finding of those studies.

Table 3.3 Summary of studies that include a hydrolysis profile, and or temperature range.

Study	FPU range	Time Profile (hrs)	Temp. and pH	Graphs generated	Results
Kaar et al., 2000 (Lime on corn stover)	1,3,5,7, 10,15	0,12,24,36,60,80, 110,144,196	40 and 50 4.8 pH	-- sugar yield by time at 5 FPU showing glucose and xylose -- sugar yield by enzyme loading at 40 and 50 °C, points are at 100Hrs -- full profile 40 and 50 °C graphs for sugar yield by time at numerous FPU	-- Not much further conversion after 72H -- 40°C had higher results at longer times (100H) -- significant increases in yield until 10FPU
Teymouri et al., 2005 (AFEX on corn stover)	3,7,15,60	0,24,48,72,168	4.8 pH, 50	- glucan and xylan conversion by Time at different FPU	Only 4 % and 5 % difference between 60 and 15FPU. Increasing to 13% and 21% when only 7FPU
Wyman et al., 2005 (Acid on Stover)	3,7,15,60	None but samples taken at 0, 24, 72	50, 4.8 pH	None	Very little difference between 60 and 15 FPU then 3% drop to 7.
Mosier et al., 2005 (LHW on stover)	7.5, 15, 60	None	50, 4.8pH	Sugar yield by FPU chart	Little difference between 60 and 15 FPU, 15% and 5% drop to 7.5 FPU for glucose and xylose respectively.

Table 3.3 continued					
Stenberg et al., 2000 (SSF of steam treated softwood)	5, 10, 21, 32	None for just enzymes	37, 4.8 pH	Ethanol yields by time	Big difference in loadings. 50% of theoretical conversion at 5FPU up to 80% at 32FPU
Kumar and Wyman (A), 2009 Diff Pre with Poplar	3, 7.5, 15, 60 7.5 and 15 also with addition of xylanase	4,8,24,48,72	50, 4.8pH	Yield by FPU loading bar chart for all the diff technologies. Yield by FPU and FPU + xylanase for diff. technologies.	Big differences in digestibility all the way until 60FPU. Xylanase significantly improved glucose release.
Kumar and Wyman (B), 2009 Diff Pre with Corn Stover	Loading by mg Protein With addition of different mg Protein xylanase	72hrs	50, 4.8 pH	Yield of glucose and xylose by Total Protein for starting 14.5mg protein cellulase per g starting glucan (approximately 7.5FPU) and for starting 29mg protein cellulase per g starting glucan. (15FPU)	Depending on treatment ,it is sometimes better to add xylanase than more cellulase, and vice versa.
Sharma et al., 2002 Steam exploded and NaOH treated sunflower stalks	5, 10, 15, 25	12, 24, 36, 48, 60, 72	50 as well as 40-60 with 72hrs constant and 25FPU 4.8 as well as 4.0-6.0 range with 72hrs, 25FPU constant.	Chart with FPU at top, hours for the rows and yields for values in chart. Yield vs temp Yield vs pH Yield vs. Solids loading	Great difference between 5 and 10 FPU then becomes small. Big differences at different temp. and pH.
Saha et al., 2008 (Lime and Rice Hulls)	0.05ml / g biomass for cellulase, b-glucosidase and hemicellulase	72	From 25 to 65 pH from 4 to 7	Sugar release by pH Sugar release by Temp.	Peaks at 45C and 5.0 pH Between 4.5 and 5.5 ph relatively the same, 4.0 and 6.0 a little less, drop after 6. Between 25 and 50 relatively same, drop after 50.
Zhang et al., 2007 COSLIF on stover, switchgrass, poplar, fir	15	0,1,2,5,9,12,24, 48, 72	50, 4.8 pH	Digestibility by Time where digestibility is percent of pre-treated glucan solubilized not overall %.	As high as 94% conversion in just 12 hours, with 97% plateau reached by 24 hours.

Major Points found in studies shown in **Table 3.3** are:

- There are usually three to four loadings tested. The loadings under 7.5 or 10 FPU consistently deliver too little sugar.
- Most studies find little difference between 15 and 60 FPU, but there are exceptions in Steinberg et al. (2000), as well as Kumar and Wyman (2009). Those studies tested softwood and poplar,

woods similar to the demolition and forestry waste used in this study, therefore larger loadings may be necessary.

- Xylanase can be more effective than additional cellulase but not always.
- Temperature effects have varied; best results between 40 and 50 °C.
- There can be large differences in conversion at different temperatures (Sharma et al., 2002), however there can also be little difference within similar ranges, (Saha, 2009).
- 0, 24, and 72 hours are the most common sampling times.

From this summary, 0, 12, 24 and 72 hours were chosen for the sampling times. Though a 12 hour sample wasn't taken in some studies, the Zhang et al. (2007) study found that most of the sugar had been released by 12 hours. Therefore a 12 hour figure could be needed to establish the plateau for conversion efficiency. Since conversion efficiencies seemed to drop off below 10 FPU / g glucan loadings, 10 FPU was at first chosen as the lowest of the loading range. When it was discovered as the most cost efficient in later testing, 5 FPU / g glucan was added to the 35 °C hydrolysis testing. The addition of xylanase instead of cellulase from lower loading would have been a good idea. However, Genencor's new product line, Accellerase Duet, already includes xylanase supplementation. Therefore as xylanase will be present in the cellulase cocktail in the event of commercialization, there is no need to test for whether it should be added.

There could be a large or small difference in temperature effects, therefore hydrolysis at 30, 35 and 40 °C should be assessed to find the enzymes best pairing temperature for simultaneous saccharification and co-fermentation (SSCF). As discussed in previous papers associated with this project (Mirzajani, 2009; Ehsanipour, 2010), the SSCF process was chosen because it allows for saccharification of the biomass sugars and subsequent fermentation to ethanol in one step. The Co-fermentation aspect refers to the fact that xylose and glucose are both fermented in the same digester, instead of separate

fermentations using different microorganisms. Therefore the fermenting microbe used for the process must be able to utilize both xylose and glucose. *Zymomonas mobilis*, strain 8b, is a rapidly saccharolytic bacteria capable of just that. Since *Z. mobilis* was chosen for the fermentation studies of future research associated with this project, the temperature range studied in this paper, 30, 35 and 40°C, would cover the middle ground between maximum enzyme digestion and ideal bacterial conditions. **Figure 3.9** shows the fermentation profile of *Z. mobilis* at 30 and 37 °C. Even spiked with acetate, the inhibition of the bacteria is little enough that it can utilize the sugars within 24 hrs. Ideally, if the Accellerase 1500 enzyme cocktail can solubilize sugars at 30 °C, as fast as at 50 °C , then the SSCF system would be the fastest possible. Even if the enzymes are capable of solubilizing the sugar in 24 hours at 35 or 40 °C, it would still be an incredible leap forward in comparison to SSCF systems with other pretreatments.

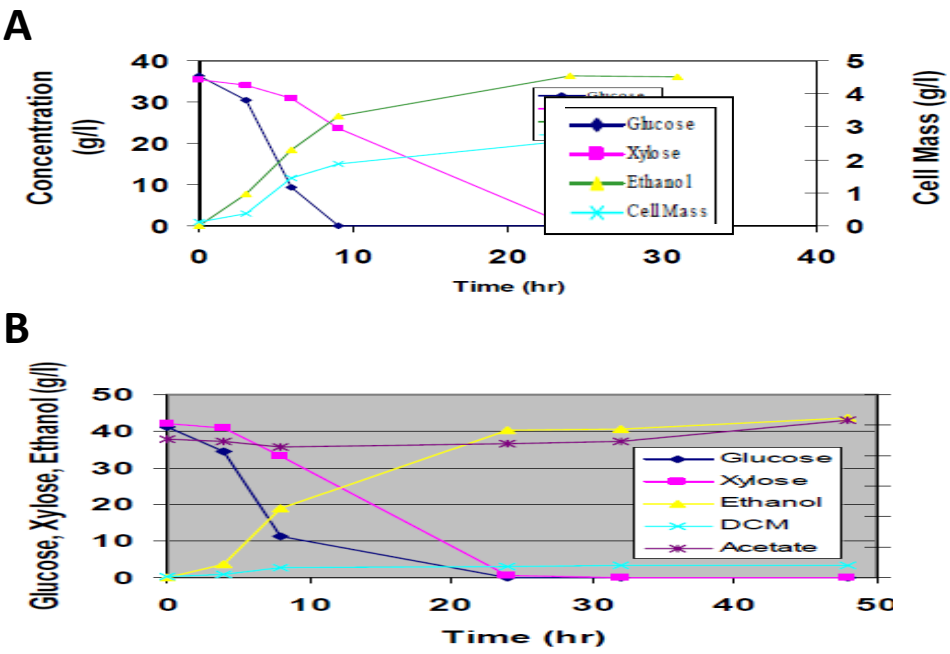


Figure 3.9 Fermentation profile of *Z. mobilis* at 30 °C (**A**) and 37 °C (**B**) (Zhang, 2003).

3.7 Pretreatment effectiveness

Pretreatment effectiveness is judged by hydrolysis yields and/or overall yields. The overall yield is a measure of the combined sugars released during pretreatment and subsequent enzymatic hydrolysis, compared to the total sugar existing in the initial biomass. Hydrolysis yields are a measure of the sugars released during enzyme action, compared to the total sugars available in the post pretreated biomass. **Table 3.4** summarizes some of the pretreatment efficiencies of studies similar to those covered in this paper (long term lime and COSLIF), as well as the other technologies previously mentioned as currently cost effective. The top technologies are generally in the 90 % overall conversion range when it comes to a feedstock like corn stover. However, when both dilute acid and the COSLIF method are used on a more recalcitrant biomass like softwood, the yield is much lower.

Table 3.4 Pretreatment efficiencies of similar studies and other cost effective techniques (Adapted from Wyman et al., 2005; Kim, 2005; Ramirez, 2005; Zhang et al., 2007).

PRETREATMENT	STUDY	FEEDSTOCK	ENZYME LOADING	HYDROLYSIS YIELD % glucose released	OVERALL YIELD % xylose/glucose released
Dilute Acid	Lloyd and Wyman, 2005	Corn Stover	15 FPU/g glucan	NR	92.4/91.5
Flowthrough	Liu and Wyman, 2005	Corn Stover	15 FPU/g glucan	NR	96.6/61.8
Controlled pH	Mosier et al., 2005	Corn Stover	15 FPU/g glucan	NR	87.2/63.0
AFEX	Teymouri et al., 2004	Corn Stover	15 FPU/g glucan	NR	94.4/89.1
ARP	Kim et al., 2005; Kim and Lee, 2005	Corn Stover	15 FPU/g glucan	NR	89.4/71.6
Long term lime	Kim, 2005	Corn Stover	15 FPU/g glucan	NR	91.3/51.8
Long term lime	Ramirez, 2005	Poplar Wood	15 FPU/g glucan	NR	80.7/66.9
COSLIF	Zhang et al., 2007	Corn Stover	15 FPU/g glucan + 60 IU/g glucan β-glucosidase	97	90.1/NR
COSLIF	Zhang et al., 2007	Poplar Wood	15 FPU/g glucan + 60 IU/g glucan β-glucosidase	97	NR
COSLIF	Zhang et al., 2007	Softwood	15 FPU/g glucan + 60 IU/g glucan β-glucosidase	75	NR
Dilute Acid	Soderstrom et al., 2003	Softwood	15 FPU/g dry mass + 23 IU/g dry mass β-glucosidase	NR	77

NR = NOT REPORTED

3.8 Long term lime pretreatment

Long term lime treatment is the application of $Ca(OH)_2$ at ambient pressure and moderate temperature over a relatively long period of time (weeks not hours). Kim (2005) first evaluated the technique on corn stover with temperatures ranging from 25 to 55 °C. In that study, both oxidative and non-oxidative conditions were experimented with by supplying the sample with either compressed air or nitrogen. The oxidative condition was far more effective in solubilizing lignin, which made for increased enzymatic digestion. Four weeks at 55 °C was found to be the optimal time and temperature. Further treatment caused solubilization of the biomass sugars. Since only the solids are passed on to enzyme hydrolysis in this system, solubilization of the sugars in the pretreatment liquor was a negative.

The treatment was repeated by Ramirez (2005) on poplar wood using the same equipment and conditions. In the Ramirez (2005) study a 65 °C temperature was tested as well, and the study ended on the fourth week. Week four at 65 °C was found to be the peak of conversion, though no plateau had been reached. Therefore further treatment and/or a higher temperature could have resulted in higher digestibility. Again, the oxidative condition was far more effective than the non-oxidative.

In light of the optimal and near optimal conditions above, 55, 65, and 75 °C were chosen for the temperature range in this study of SSO mixed with forestry waste (hardwood). Oxygen was supplied to all samples, since it would be redundant to test non-oxidative conditions while expecting lower conversions. The test was initially planned to run 6 weeks in order to attempt to reach a plateau with the hardwood in the samples. However, equipment failure led to the 55 °C only being tested for 3 weeks, and the 75 °C sample for 5 weeks.

Figure 3.10 shows the experimental setup for the Kim (2005), and Ramirez (2005) studies.

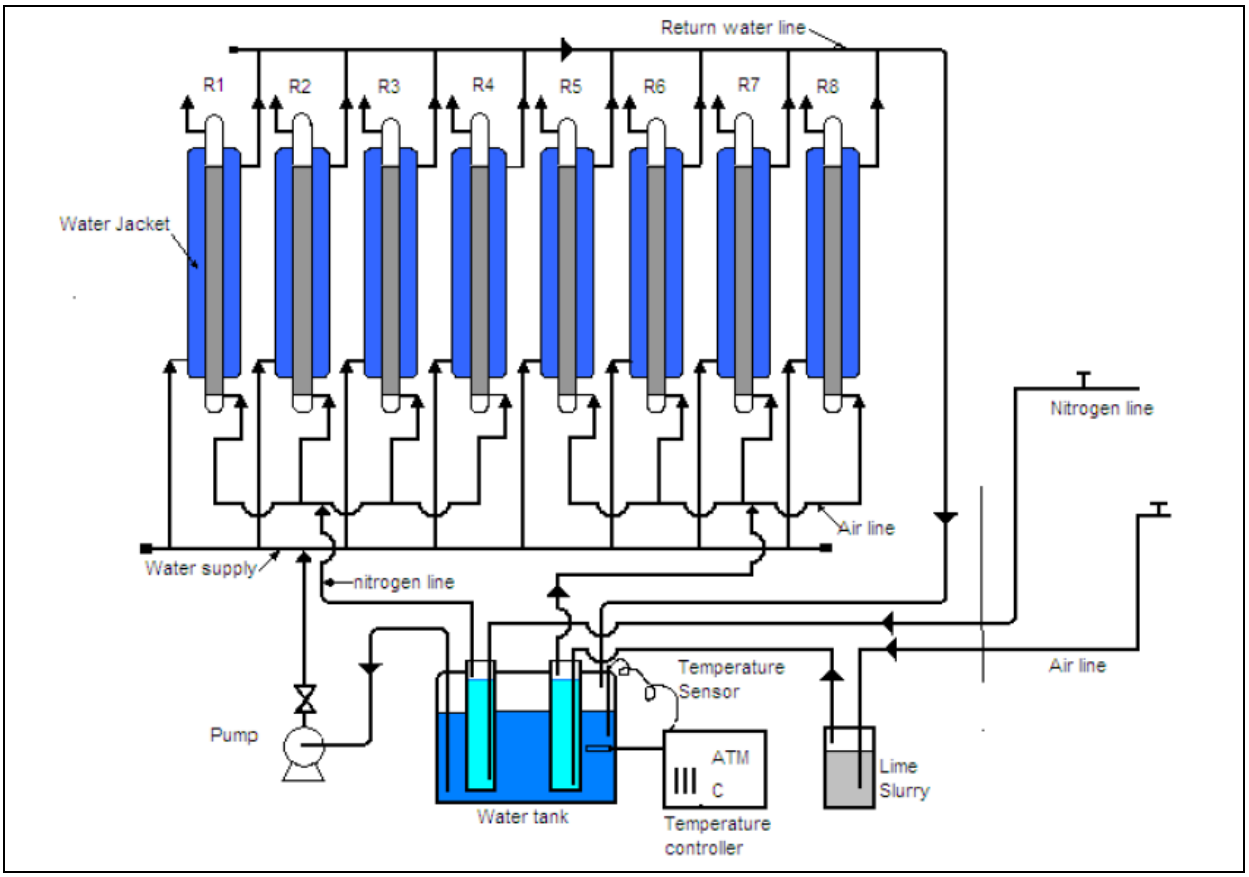


Figure 3.10 Experimental setup for Kim (2005) and Ramirez (2005) long term lime studies.

The compressed air line in **Figure 3.10** shows the air passing through lime slurry to scrub CO_2, as well as the water tank, which is set to the desired sample temperature. The reasoning for that is to prevent the air from changing the temperature in the samples, or reacting with the lime. Those conditions were repeated in this study.

In both studies, the pretreatment yield (mass of components remaining in relation to their initial mass before pretreatment) was found by quantitative saccharification of the pretreated material. The results for temperatures of 55 and 65 °C are shown in **Table 3.5**.

Table 3.5 Pretreatment yields in g / 100 g raw biomass.

Study	Component	Temp. °C	Week						
			0	1	2	3	4	5	6
Kim, 2005. (Corn Stover)	Glucose	55	100	100	96.6	96.6	96.6	91.7	91.7
	Xylose	55	100	76.4	68.1	68.1	68.1	68.1	68.1
	Ash, Extractives, Lignin, Acetyle	55	100	0.62	0.62	0.605	0.59	0.59	0.59
Ramirez, 2006 (Poplar)	Glucose	55	100	100	100	95	90	N/A	N/A
		65	100	100	90	90	90	N/A	N/A
	Xylose	55	100	85.7	85.7	78.6	71.4	N/A	N/A
		65	100	100	85.7	85.7	85.7	N/A	N/A
	Ash, Extractives, Lignin, Acetyle	55	100	68	68	68	68	N/A	N/A
		65	100	68	56	56	56	N/A	N/A

These values were subsequently used to estimate pretreatment yields for this study, for reasons explained in the methods section in chapter 5.

3.9 COSLIF pretreatment

The Cellulose solvent-Organic solvent Lignocellulosic Fractionation method of pretreatment involves using phosphoric acid at ambient pressure and moderate temperature (50 °C) to disrupt the bonds of lignin, dissolve cellulose and hemicellulose fibrils by breaking the hydrogen bonds between chains, weakly hydrolyze cellulose and hemicellulose, and remove acetyle groups from hemicellulose. The material is then washed in acetone or ethanol to precipitate the partially dissolved cellulose and

hemicellulose, and dissolve some of the lignin (Zhang et al., 2007). **Figure 3.11** shows the COSLIF process configuration.

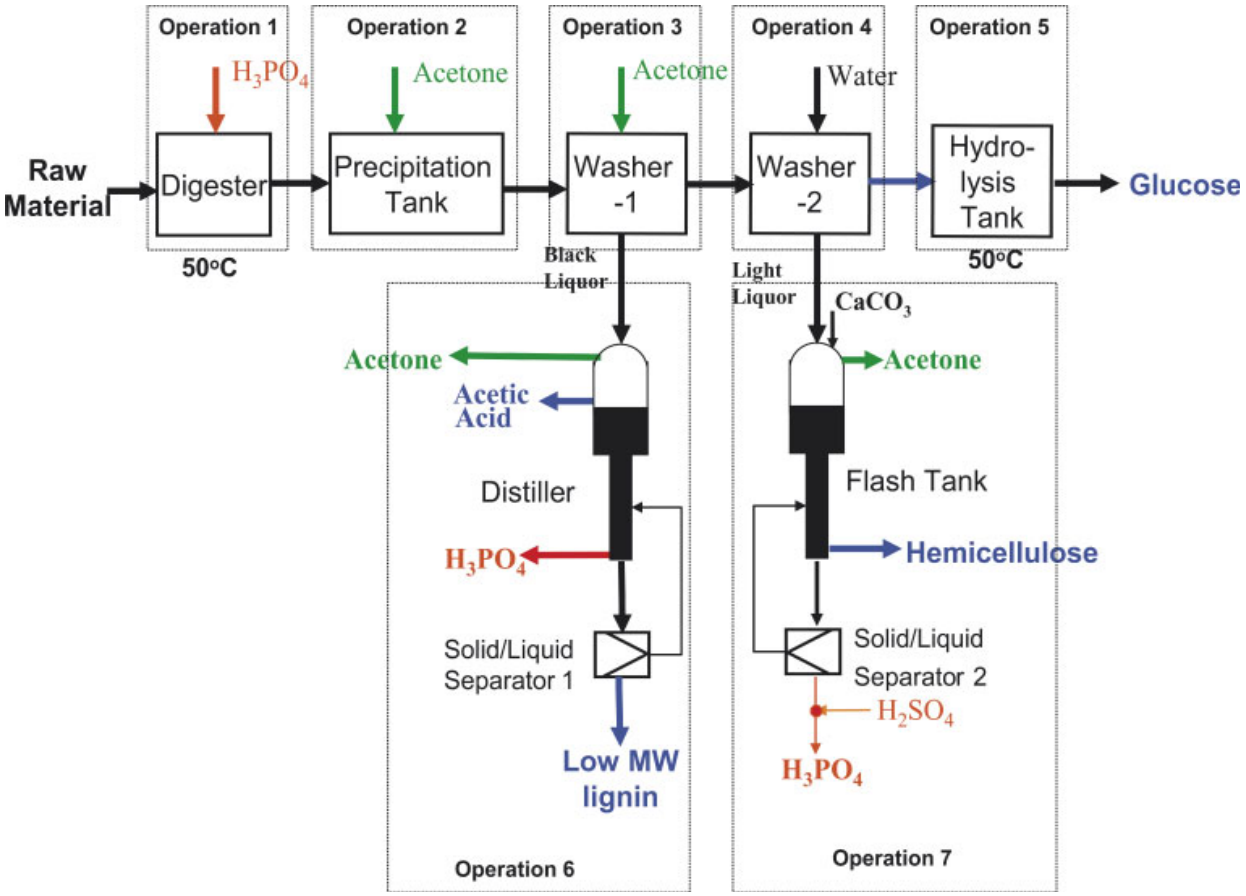


Figure 3.11 COSLIF process configuration (Zhang et al., 2007).

Operation 6 in **Figure 3.11**, shows the separation of acetone and phosphoric acid which are then recycled to be used again. If the recycle efficiencies are high enough that the solvent costs can compete with other pretreatments, the COSLIF process offers a number of advantages:

1. The separation of hemicellulose shown in Operation 7 of **Figure 3.11**, allows for separate processing, which can be worth a lot more than the ethanol revenue from fermentation. Hemicellulose has been used as plant gum for thickeners, adhesives, protective colloids, emulsifiers and stabilizers (Zhang et al., 2007). Consequently, hemicellulose and its derivates

have a 5-20 fold selling price over the value to be gained by selling ethanol from hemicelluloses fermentation (Zhang et al., 2007)

2. There is no need for a special reactor, as there would be for dilute acid treatment. No special reactor is needed because phosphoric acid is less corrosive than sulphuric acid, the reactor vessel operates at ambient pressure, and the temperature is also a very moderate 50 °C. (As shown in Operation 1 of **Figure 3.11**)
3. The hydrolysis yields are not only very high, but extremely fast. According to Yang and Wyman (2008), one of the factors involved in achieving an advanced low cost pretreatment, is that the pretreated cellulose should be able to yield over 90 % conversion in less than 5, and preferably less than 3 days. **Figure 3.12** shows the hydrolysis profile of corn stover, poplar wood, and douglas fir. The conversions are at 94% by 12 hours, and 97% by 24 hours, for corn stover and poplar wood. The conversions are equally fast but not as high at 75% for douglas fir, but that speaks more to the recalcitrant nature of softwoods than a failing of the process.

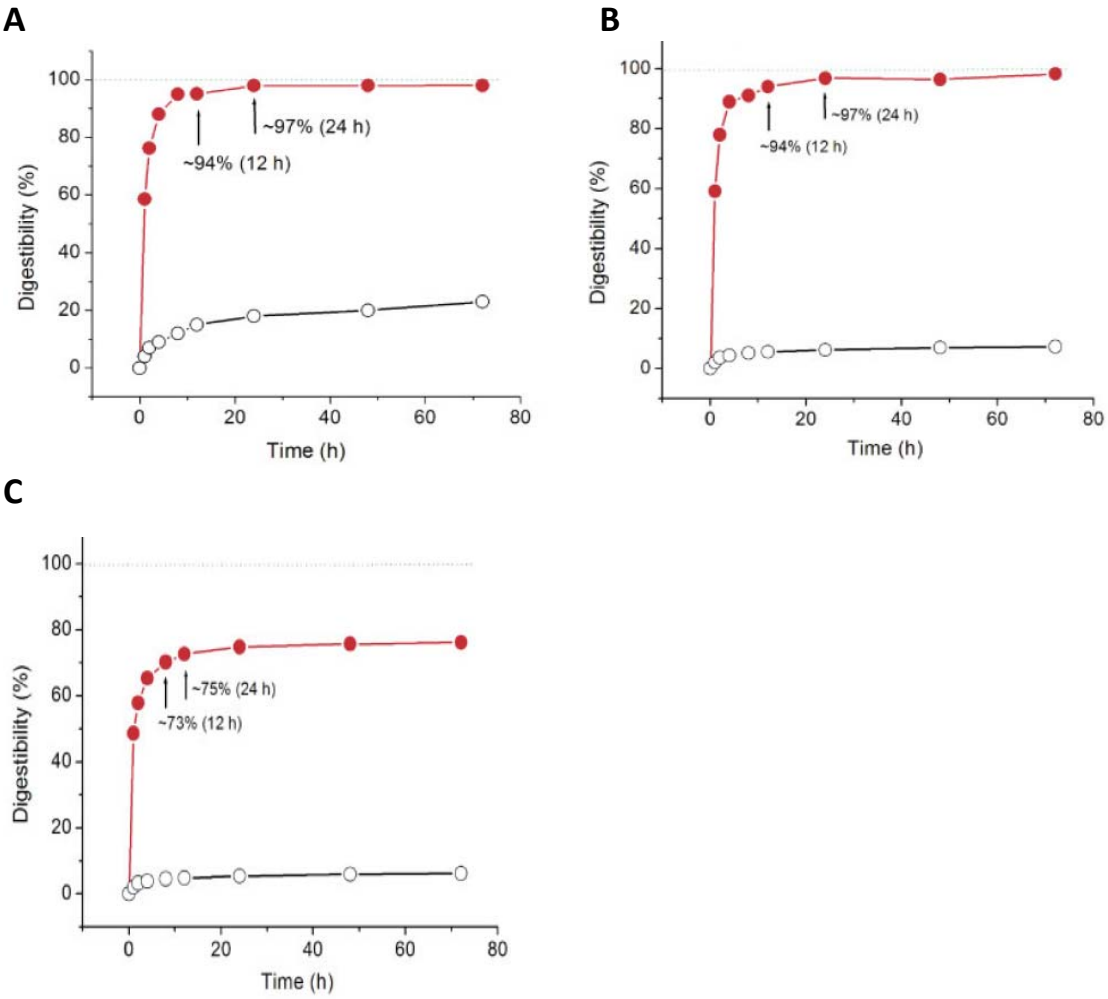


Figure 3.12 COSLIF hydrolysis profiles for corn stover (**A**), poplar wood (**B**), and douglas fir (**C**) (Zhang et al., 2007).

To illustrate how fast these rates are, **Figure 3.13** shows the hydrolysis profile for Lime and AFEX treated biomass. To compare fairly, the top line in the Lime study is at an enzyme loading of 15 FPU / g glucan, as are the COSLIF studies above. A 600 mg glucose release / g dry biomass would be a 60% conversion. The pink curve (second down) is also 15 FPU / g glucan.

A) **B)**

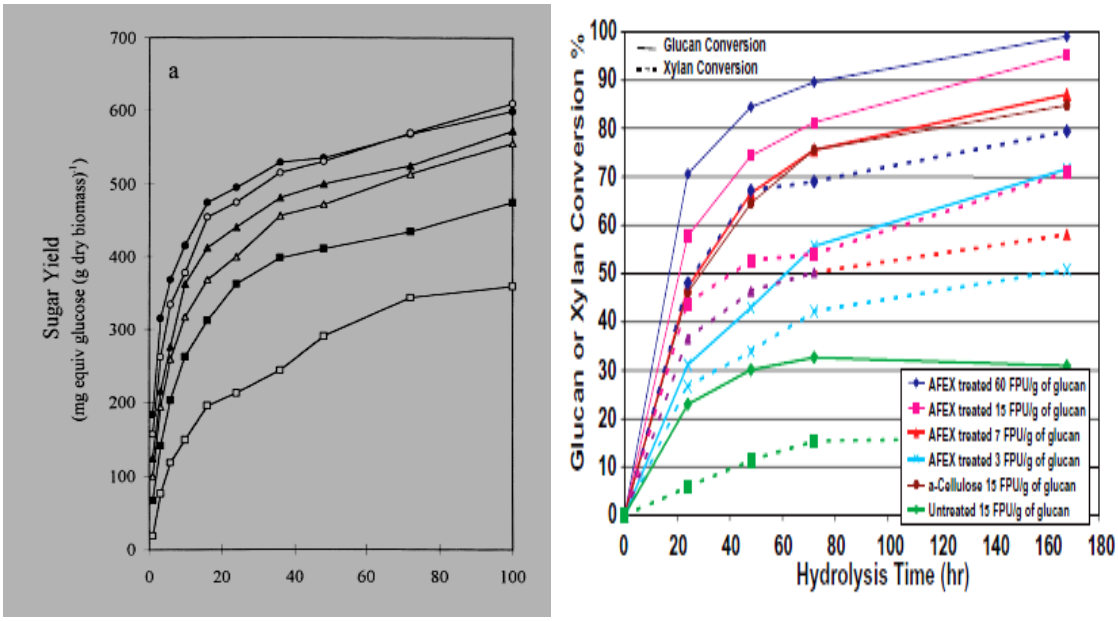


Figure 3.13 Enzyme hydolysis profile for lime (A) and AFEX (B) studies (Kaar et al., 2000; Teymouri et al., 2005).

The COSLIF method was previously studied as a part of this overall project, by Ehsanipour (2010), who found the ideal timing of the phosphoric acid hydrolysis (2hr), estimated overall yield at a high enzyme loading of 60 FPU cellulase / g glucan (89.5%), and solved the question of which commercial enzyme cocktail worked best (Accellerase 1500). In addition, she found that ethanol was a more effective organic solvent for the light liquor washing procedure. This study built on the results of Ehsanipour (2010), utilizing the same treatment method, to seek a more precise answer for the overall glucose yield, present the hydrolysis profile of the waste digestion, and estimate the best enzyme conditions for cost effective fermentation.

4 MATERIALS AND PREPARATION METHODS

Two types of feedstock were investigated for their amenability to long term lime and COSLIF pretreatment: Source-Separated Organic Waste (SSO) mixed with softwood, and SSO mixed with hardwood. The softwood and hardwood used in the blend are from other lignocellulosic waste streams available in Toronto. The softwood chips were taken from ground demolition waste, and the hardwood chips from live and dead tree clippings. The tree clippings were from parks and residential areas (municipal forestry waste) in the city of Toronto. The term softwood is used to represent the demolition waste because of the higher proportion of softwoods used in building construction, especially older structures. Douglas Fir and Cedar are two examples. Our feedstock supplier confirmed that the demolition wood was mainly softwood, and that the forestry waste from the city of Toronto was mainly hardwood, like maple, birch, and poplar (Mike Crupi, Vartek Industrial; personal communication).

There are three main reasons why adding woodchips is an advantage for a mixed SSO feedstock:

- Source separated organic waste composition is variable, as people's diets and waste patterns can vary over the seasons. Highly variable sugar levels can cause a modeling problem when estimating many of the parameters of the system. Higher sugar levels require the addition of more enzyme, and longer residence times. Therefore adding woodchips would add a steadying amount of cellulose/hemicellulose to the feedstock stream, and provide a buffer against this variability.
- Organic waste has an odour, which can be a problem for feedstock storage; as one can imagine from the smell of a green bin in Toronto. However, when processed at high heat and pressure in the thermal screw, 150 °C and 50 Bar (Mirzajani, 2009), most of the bacteria in the material is destroyed, halting or slowing its decomposition; thereby removing the cause of the odour. In

order to accomplish this, a dry material has to be added to the wet green bin waste to increase the mixed materials frictional resistance within the screw.

- The wood chips in this case arise from other waste sources, thereby providing an environmentally friendly mix, with little or no extra cost.

Testing the SSO mixed with softwood in the COSLIF process has another advantage. Since the high lignin composition of softwood makes it extremely hard to break down, a high sugar yield of treated SSO/softwood would be an indication that the SSO could be mixed with almost anything organic, and also achieve high yields in that process.

4.1 Feedstock preparation

All material was processed by a thermal screw belonging to Vartek Industrial. The screw administrates high pressure and temperature from the application of friction forces as the material is pressed and screwed through the chamber. The product that leaves the screw is pulverized to the extent that even tiny hair-like projections from the mulched, more homogenized material can be seen. The processed material is relatively odourless. **Figure 4.1** shows the before and after look of the material.

Figure 4.1 SSO material before and after thermal screw.

After the sample has been processed by the screw, it was mixed in a pile and a portion sent to the laboratory for testing. To increase the homogeneity of the sample, as well as enable comparison with similar studies, the standard NREL procedure "Preparation of Samples for Compositional Analysis, method B" was followed as closely as possible (Hames et al., 2005) The NREL procedure is so widely used it could be called standard protocol (Zhang et al., 1996; Teymouri et al, 2005; Wyman et al., 2005; Kim, 2005; Ramirez,2005).

A synopsis of the procedure used in these experiments is as follows:

1. Samples not being dried immediately are to be stored in a refrigerator freezer (0-4°C).
2. Samples to be dried are spread thinly, (< 1") on trays previously dried at 44°C and weighed.
3. Samples are dried at < 45°C for 48-96 hours, and then re-weighed until the weight decrease on an hourly basis is less than 1.0%, and the moisture content below 10.0%.
4. Samples are then ground/milled further before being sieved. The material that passes a No.20 (0.841mm) ASTM-E11 screen, but is retained on the No.80 (0.180mm) sieve are kept to be used for the remainder of the experimentation. (The milling was accomplished by simple Hamilton Beach and Cuisinart coffee grinders.)
5. Samples once again are kept in a refrigerator freezer (0-4°C) until use.

In addition, samples are well mixed in bag before being withdrawn for experiments.

Figure 4.2 is a visual representation of the material through the various steps of this process.

Figure 4.2 Visual representation of sample preparation. (**A**) post-thermal screw, (**B**) oven dried, (**C**) after mill (Adapted from Ehsanipour, 2009).

One of the problems in working with a heterogeneous feedstock is that a careful balance has to be struck in the milling process, between chopping the wood into small enough pieces, and not over

grinding the SSO to ash (< No. 80 sieve). The SSO/wood mixes were ground in an attempt to achieve about a 50% usable ratio (50% of the total particles between the No. 20 and 80 sieves). Using Hamilton Beach or Cuisinart coffee grinders, this meant holding and compressing the grind button for approximately two seconds, three times repeatedly. **Figure 4.3** shows the homogenization proportions.

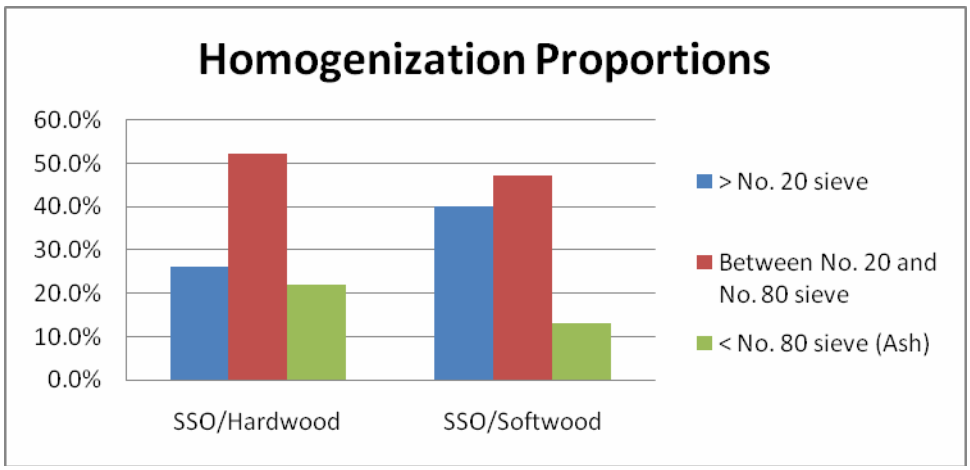


Figure 4.3 Homogenization proportions of SSO and wood waste.

Pure SSO as well as the SSO/wood mixtures were analyzed for their as-received moisture contents. The results are shown in **Table 4.1**.

Table 4.1 As-received moisture contents.

Sample Type	Mass As Received (g)	Mass at 45°C (g)	Average % solids of 45°C dried material at 105°C	As Received Moisture Content
SSO	2395.84	445.12	0.962	82.1 %
SSO /20% Hardwood (Forestry Waste)	1853.36	562.41	0.951	70.4 %
SSO / 20% Softwood (Demolition waste)	2215.43	772.53	0.957	66.6%

4.2 Compositional analysis

The sugar, lignin, and ash composition of the mixed biomass was determined by quantitative saccharification using the NREL standard procedure, "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008). Analysis was carried out by High Performance Liquid Chromatography (HPLC), using an Aminex HPX-97P column with Refractive Index (RI) detector. The full procedure undertaken can be found in **Appendix 1.** The process consists of adding 300 mg of material to concentrated acid for an hour, then diluting the acid and raising the temperature to 121°C for a further hour. The sugars contained in the cellulose and hemicellulose are solubilized, while lignin and ash remain. The liquid is adjusted closer to neutral and tested by HPLC to quantify the sugars. The remaining material once filtered, is weighed after spending 24 hours at 105 °C, and again after time spent in a 575 °C muffle furnace, to determine the amount of lignin.

An attempt was made to adapt a procedure slightly different to the NREL standard procedure in order to lower the variation between samples. All of the standard procedures were followed in saccharification of the samples, but instead of removing 20ml of liquid to neutralize and test by HPLC, 10ml from each of three samples were taken instead, and combined to form a single HPLC tested sample. For each run, 30 samples were combined to make 10 for testing, in effect increasing the sampling size from 0.3 g to 0.9 g, in an attempt to lower variation. It should be noted that a scale sensitive to 1 mg was used, not 0.1 mg as stipulated by the standard procedure. Therefore the percentage figures of the components are reported only to the first decimal place. The compositional proportions for the sugar and lignin results of both substrates are shown in **Table 4.2** and **Table 4.3**. The mannan and arabinan components were grouped together, because even in the analysis of the pure sugar standards, the mannose and arabinose peaks were indistinguishable.

Table 4.2 Sugar and lignin proportions of mixed SSO and hardwood feedstock**.**

	Components (g / 100 g Biomass)				
	GLUCAN	XYLAN	GALACTAN	MANNAN AND ARABINAN	LIGNIN
SAMPLE					
1	34.0	8.2	1.9	4.9	25.4
2	39.2	9.0	2.1	5.0	25.4
3	37.3	9.3	2.1	4.9	25.5
4	38.2	10.2	2.1	5.2	25.8
5	37.9	10.2	2.1	4.9	25.1
6	35.5	10.0	1.9	4.7	25.2
7	32.8	9.1	2.1	4.7	24.8
8	26.3	7.3	1.9	3.7	24.6
9	29.5	8.3	1.9	4.1	24.0
10	29.2	7.9	1.9	3.3	24.9
AVERAGE	**34.0**	**9.0**	**2.0**	**4.5**	**25.1**
STANDARD DEV	4.4	1.0	0.1	0.6	0.5
95% C.I.	**+/- 2.8**	**+/- 0.6**	**+/- 0.1**	**+/- 0.4**	**+/- 0.3**

Table 4.3 Sugar and lignin proportions of mixed SSO and softwood feedstock.

	Components (g / 100 g Biomass)				
	GLUCAN	XYLAN	GALACTAN	MANNAN AND ARABINAN	LIGNIN
SAMPLE					
1	26.0	3.3	1.3	6.2	25.8
2	32.6	5.6	3.1	8.4	25.3
3	33.5	3.5	1.9	6.9	24.4
4	32.3	5.5	3.1	8.7	27.0
5	33.1	5.1	1.8	7.0	28.6
6	32.2	4.7	1.8	6.6	25.6
7	21.6	3.5	1.5	4.5	24.9
8	28.7	4.7	1.7	5.9	25.3
9	24.1	4.1	1.7	5.2	25.7
AVERAGE	**29.3**	**4.4**	**2.0**	**6.6**	**25.9**
STANDARD DEV	4.5	0.9	0.6	1.4	1.2
95% C.I.	**+/- 2.9**	**+/- 0.6**	**+/- 0.4**	**+/- 0.9**	**+/- 0.7**

The compositional analysis of the SSO mixed with softwood falls within range of typical values for this material, established by an extensive six month study of seasonal fluctuation, performed in association with this project. **Table 4.4** shows the variability of glucan and lignin proportions in the seasonal fluctuation of SSO mixed with softwood, over a six month period.

Table 4.4 Seasonal fluctuation of glucan and lignin proportions in SSO/softwood over a six month period (Adapted from Mirzajani, 2009).

Component	Sept. 2008	Nov. 2008	March 2009	April 2009	May 2009	June 2009
Average Glucan	25.83 %	38.19 %	30.61 %	27.30 %	34.80 %	27.10 %
95 % CI	± 2.0 %	± 2.0 %	± 0.7 %	± 2.6 %	± 3.6 %	± 2.0 %
Average Lignin	25.86 %	21.51 %	18.96 %	29.78 %	21.41 %	22.00 %
95 % CI	± 2.6 %	± 1.5 %	± 1.1 %	± 0.5 %	± 1.6 %	± 0.4 %

Each average value in **Table 4.4** refers to the average of ten tests, each of which had 10 samples. Therefore, the figures for each month are an average of 100 samples. The confidence interval for the average value refers to a confidence interval generated from the standard deviation between the averages of the ten sets of samples tested each month.

A separate analysis of the mixed SSO and softwood used for this experiment was performed by MBI International. The results on average were 26.8 % glucan, 5.4 % xylan, 1.2 % arabinan, 5.7 % mannan, and 1.2 % galactan (Ehsanipour, 2010). These values lie within the range found in this compositional analysis, with a slight difference in xylan and galactan. The larger galactan and smaller xylan numbers could be a result of the proximity of those peaks in HPLC analysis. In fact, the galactan peak, while visually present on the Chromatogram, was not detected in the analysis program. Therefore the peak was determined by manual correction. The manual correction may have been over-corrected. The average result of 29.3 % glucan was used for subsequent COSLIF calculations for pretreatment yield as well as overall yield. Since 29.3 % glucan is high, in light of the MBI International findings for the same

material, the pretreatment and overall yields found in the results section may be on the conservative side.

NOTE FOR THOSE NOT FAMILIAR WITH BIOMASS NOMENCLATURE:

Initial sugar proportions are traditionally reported in the polymetric form. Glucan is the total glucose found in cellulose and hemicellulose. Since it has yet to be hydrolyzed, which will add mass from water bonds, there are anhydrous corrections to find the glucose available. The anhydrous correction from glucose to glucan is 0.9, therefore every 0.9 grams of glucan yields 1.0 gram of glucose in hydrolysis. The xylose to xylan correction is 0.88.

5 STUDY METHODOLOGY

The NREL standard procedure, "Determination of Structural Carbohydrates and Lignin in Biomass" as well as a slightly modified version (larger sample size) of the NREL, "Enzymatic Saccharification of Biomass" procedure, were used to determine pretreatment yields, hydrolysis yields, and overall yields of xylose and glucose, as well as the change in lignin content. The first procedure will furthermore be referred to as quantitative saccharification, and the second procedure as enzymatic saccharification. The full explanation of the steps involved in the quantitative and enzymatic saccharifications done for this project, as well as the equations for calculations, can be found in **Appendix 1**, and **Appendix 2**. A brief description of quantitative saccharification can be found in the **Section 4.2**, and the outline of the enzymatic saccharifications performed is as follows:

1. A known amount of dry biomass was added to a known amount of buffer solution (pH 4.8).
2. Enzymes of a known activity level were added to the mixture and incubated on a shaker at 50°C, and at a speed great enough to suspend the solids.
3. A sample was taken to be tested by HPLC, at different time intervals up to 72 hours.
4. The hydrolysis yield was found by **Equation 5.3** and **Equation 5.4**. (Total liquid multiplied by the sugars determined in mg/ml by HPLC, divided by the available sugar in the dry biomass)

The activity level of the enzymes in Filter Paper Units (FPU) was determined by the NREL standard procedure, "Measurement of Cellulase Activities". The procedure followed, as well as the activity results for both the lime treatment and COSLIF treatment, can be found in **Appendix 3.**

Figure 5.1 shows a schematic diagram for the overall, pretreatment, and hydrolysis yields. **Figure 5.1** indicates the positions in the process that measurements and saccharifications were performed as well.

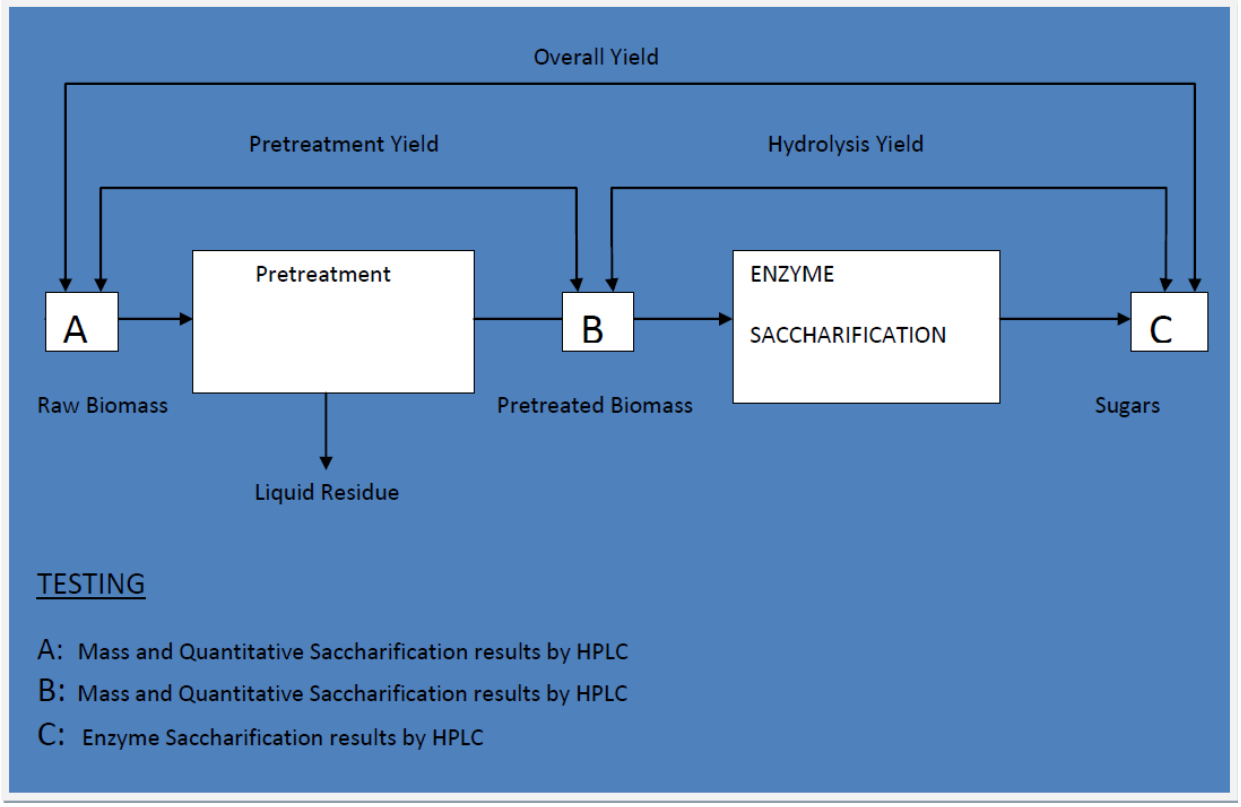


Figure 5.1 Schematic diagram of yields and testing.

Dry mass was determined by moisture tests at point **A,** and a quantitative saccharification was performed to find the compositional proportions of the material. Dry mass and saccharification procedures were repeated at point **B**, and the difference in values determined the pretreatment yield. After the enzyme saccharification, the sugars found in mg/ml by HPLC analysis were compared to the quantitative saccharification results of **B**, and the hydrolysis yield determined. The pretreatment yield, multiplied by the enzyme saccharification yield, is the overall yield. **Equations 5.1** through **5.5**, illustrate how these values are derived.

$$\text{Glucose Pretreatment Yield} = \frac{QSGB \times MB}{QSGA \times MA} \qquad \textbf{[EQUATION 5.1]}$$

QSGB – Quantitative Saccharification Proportion Results of Glucose at B.

QSGA – Quantitative Saccharification Proportion Results of Glucose at A.
(Glucose at A = Glucan/0.9)
MB – Dry Mass at B
MA – Dry Mass at A

$$\text{Xylose Pretreatment Yield} = \frac{QSXB \times MB}{QSXA \times MA} \qquad \textbf{[EQUATION 5.2]}$$

QSGB – Quantitative Saccharification Proportion Results of Xylose at B
QSGA – Quantitative Saccharification Proportion Results of Xylose at B
(Xylose at A = Xylan/0.88)
MB – Dry Mass at B
MA – Dry Mass at A

$$\text{Glucose Hydolysis Yield} = \frac{GHPLC \times VES}{QSGB \times MB} \qquad \textbf{[EQUATION 5.3]}$$

GHPLC – Glucose content in mg/ml found by HPLC analysis of enzymatic saccharification
VES – Total volume of enzymatic saccharification
MB – Dry Mass at B

$$\text{Xylose Hydrolysis Yield} = \frac{XHPLC \times VES}{QSXB \times MB} \qquad \textbf{[EQUATION 5.4]}$$

XHPLC – Xylose content in mg/ml found by HPLC analysis of enzymatic saccharification
VES – Total volume of enzymatic saccharification
MB – Dry Mass at B

Overall Yield = Pretreatment Yield × Hydrolysis Yield **[EQUATION 5.5]**

For the COSLIF method, the xylose was mainly removed during pretreatment, and could be quantified in the liquid residue by HPLC analysis. Since the purpose of the COSLIF testing was to understand the enzyme loadings needed for the pretreated solid material, the xylose yields and quantities in the liquid residue were ignored.

Unfortunately, for lime pretreatment, this procedure could not be used to find the various yields. A calculation using the initial quantitative saccharification, a modified version of the NREL enzymatic saccharification, and pretreatment yields from similar experiments were used. The reason for the modified calculation is that the material evidenced behavior different from that of other types of biomass, and could not be tested by quantitative saccharification. At point **B** in **Figure 5.1**, a quantitative saccharification needs to be performed to find the sugar content and change in lignin. When the lignin results from the first week were compiled, the compositional proportion was not possible. The mass of the lignin remaining after the quantitative saccharification had increased beyond even what might be possible if no lignin had been solubilized. In some cases the lignin content showing after the saccharification was as high as 68%. This meant that the saccharification results could not be valid as some of the sugars had not been solubilized. The enzyme saccharification results were incorrect as well, and it wasn't until they were redone that the answer to the mystery presented itself. In one instance, only half of the pretreated material to be tested was added to the citrate buffer solution. Since a simultaneous enzyme saccharification was intended, those samples were placed in the refrigerator overnight. In the morning, the samples were checked again for pH before addition of enzyme. It was found that the pH had risen in the buffered solution from 4.8 to upwards of 7.0. This meant that there must have been some residual lime in the sample, not previously neutralized by pH adjustment following the washing procedure. The unusual amount of washing needed to stabilize pH (dozens of washes), that hadn't been reported in similar studies (Kim, 2005; Ramirez,2006), led to the suspicion that the lime had formed some sort of bond with the mixed SSO material. Repeated washing and pH adjustment were not enough to overcome this bond. Steps were taken to counteract this for both the enzyme saccharification and quantitative saccharification needed at point **B** in **Figure 5.1.** The samples for the first two weeks were adjusted to 4.8 for the enzyme saccharification, and then left in the refrigerator overnight. The samples were readjusted to 4.8 again and then kept overnight and

checked before addition of enzyme. The pH was then checked at the end of 72 hrs. In the majority of samples the pH was found to be above 4.8, but not above 6.0, where the enzymes would be deactivated. In the next round of samples (weeks 3 and 4), the pH was adjusted to 4.8 using 1M citric acid while still in the DDW wash, checked repeatedly, adjusted back to 4.8 when needed, and then dried after the solution was stable. After adding the dried pre-treated material to the citrate buffer, the samples were kept in the refrigerator overnight, and in most cases required pH readjustment. After 24 hours, the solution was checked for pH. In all cases, the samples again needed pH readjustment, and the addition of more enzymes because of pH deactivation. In samples from week 3 on, this adjustment and addition of enzymes took place twice, and the solution in some cases still rose to a pH of almost 7.0. When calculating the sugar conversions from the HPLC analysis, the extra ml of citric acid and enzyme were added to the total ml of the solution.

Since the lime was forming an unidentified bond with the mixed SSO material, great enough to overcome release at a pH of 4.8 in the final rinsing solution, the quantitative saccharification results for point **B** in **Figure 5.1** were unlikely to be accurate. To ensure that this was true, week six of the SSO/hardwood mix was tested. The result was that only 15% of the material was glucose. A 15% composition figure was impossible, given that the material started at 34%, and should have been rising. Therefore the quantitative saccharification at point **B** in **Figure 5.1** was found to be unreliable. The pretreatment and hydrolysis yields, and by extension the overall yields, were not possible to find through normal methods. Consequently, an approximation to find the overall yields had to be found to link the enzyme saccharification results to the initial quantitative saccharification. To solve this problem, the pretreatment composition ratios of long term lime studies at similar conditions were used to approximate the losses in sugars and other components throughout the pretreatment. **Table 5.1** shows pretreatment yields of sugar and non-sugar components found from the literature review of other long term lime studies, as well as what was used to approximate those values for this study.

Table 5.1 Pretreatment yields in g / 100 g raw biomass.

Study	Component	Temp. °C	Week						
			0	1	2	3	4	5	6
Kim, 2005 (Corn Stover)	Glucose	55	100	100	96.6	96.6	96.6	91.7	91.7
	Xylose	55	100	76.4	68.1	68.1	68.1	68.1	68.1
	Ash, Extractives, Lignin, Acet.	55	100	62	62	60.5	59	59	59
Ramirez, 2005 (Poplar)	Glucose	55	100	100	100	95	90	N/A	N/A
		65	100	100	90	90	90	N/A	N/A
	Xylose	55	100	85.7	85.7	78.6	71.4	N/A	N/A
		65	100	100	85.7	85.7	85.7	N/A	N/A
	Ash, Extractives, Lignin, Acetyl	55	100	68	68	68	68	N/A	N/A
		65	100	68	56	56	56	N/A	N/A
Approximate yields used for this study	Glucose	55	100	100	96.6	95	90	N/A	N/A
		65	100	90	90	90	90	90	90
		75	100	90	90	90	90	90	N/A
	Xylose and Other Sugars	55	100	76.4	68.1	68.1	68.1	N/A	N/A
		65	100	76.4	68.1	68.1	68.1	68.1	68.1
		75	100	76.4	68.1	68.1	68.1	68.1	N/A
	Ash, Extractives, Lignin, Acetyle	55	100	62	62	60.5	59	N/A	N/A
		65	100	62	50	48.5	47	47	47
		75	100	62	50	48.5	47	47	N/A

The smaller the pretreatment yield, the lower the conversion for overall sugars will be. Therefore to be conservative, the values showing the most reduction from either study were used. At 65 °C, since there

was not a consistent loss of more sugar with higher heat in the Ramirez (2005) study, the values from the 55 °C Kim (2005) study were used and not adjusted lower because of additional heat. For the ash, extractives, lignin, and acetyle components however, the Ramirez (2005) study showed a 12 % drop in weeks 2 and 4 from the additional heat. Therefore, the lower Kim (2005) numbers were used, but adjusted down 12 %; the difference between the Ramirez (2005) and Kim (2005) studies. There was no data for 75 °C, therefore the conversions found at 65 °C were used. Since the overall conversion efficiencies calculated for the SSO/hardwood mix at 75 °C were not commercially viable, or greater than conversion at 65 °C, extrapolation of conversion values from 55 and 65 °C was deemed unnecessary.

5.1 Adapted method to calculate overall lime treatment yields

In a normal situation, the enzyme yield results from the HPLC, divided by the multiplication of the dry mass by the post-pretreatment quantitative saccharification proportions would solve the hydrolysis yield (**Equations 5.3, 5.4)**. The hydrolysis yield would then be multiplied by the pretreatment yield to find the overall yield. In this case, the enzyme yield results from the HPLC, were divided by the multiplication of the dry mass to an estimated conversion from the original quantitative saccharification (**Equation 5.6**), which includes the pretreatment yield, to solve for the overall yield. The conversion equations are found on the following page.

$$\text{Modified Glucose Overall Yield} = \frac{GHPLC \times VES}{GCon \times MB} \qquad \textbf{[EQUATION 5.6]}$$

GHPLC – Glucose content in mg/ml found by HPLC analysis of enzymatic saccharification
VES – Total volume of enzymatic saccharification
MB – Dry Mass at B
GCon – Glucose Conversion (Proportion from quantitative saccharification)

Glucose conversion equation **[EQUATION 5.7]**

$$= \frac{QSG}{(QSG \times Tbl.5.1) + (QSX \times Tbl.5.1) + (QSOS \times Tbl.5.1) + (AELA \times Tbl.5.1)}$$

QSG – Initial Quantitative Saccharification proportion results for Glucose

QSX – Initial Quantitative Saccharification proportion results for Xylose
QSOS – Initial Quantitative Saccharification proportion results for Other Sugars
AELA – Ash, Extractives, Lignin, Acetyle = 1 – (QSG + QSX + QSOS)
Tbl.5.1 – Estimated pretreatment yield (g /100 g raw biomass), by temperature category and week, divided by 100, from **Table 5.1.**

Modified Xylose Overall Yield = $\frac{XHPLC \times VES}{XCon \times MB}$ **[EQUATION 5.8]**

XHPLC – Xylose content in mg/ml found by HPLC analysis of enzymatic saccharification
VES – Total volume of enzymatic saccharification
MB – Dry Mass at B
XCon – Xylose Conversion

Xylose conversion (XCon) estimate **[EQUATION 5.9]**

$$= \frac{QSX}{(QSG \times Tbl.5.1) + (QSX \times Tbl.5.1) + (QSOS \times Tbl.5.1) + (AELA \times Tbl.5.1)}$$

QSG – Initial Quantitative Saccharification proportion results for Glucose

QSX – Initial Quantitative Saccharification proportion results for Xylose
QSOS – Initial Quantitative Saccharification proportion results for Other Sugars
AELA – Ash, Extractives, Lignin, Acetyle = 1 – (QSG + QSX + QSOS)
Tbl.5.1 – Estimated pretreatment yield (g /100 g raw biomass), by temperature category and week, divided by 100, from **Table 5.1.**

Example of the adapted method

Conventional Procedure:

Initial Proportions (From initial quantitative saccharification):

- 30 g glucose
- 10 g xylose
- 10 g other sugars
- 50 g ash, extractives, lignin and acetyle (AELA)

  Total = 100 g

After 1 week of treatment at 55 °C, the quantitative saccharification of the dried out material shows that all sugars are reduced by 10%, and the AELA is reduced by 50%. Pretreatment yields are then 90% for sugars and 50% for AELA. Therefore there would be 27 g glucose, 9 g xylose, 9 g other sugars, and 25 g AELA.

The post pre-treatment quantitative saccharification result would show glucose remaining divided by the total remaining. This would be 27 / (27 + 9 + 9 + 25) $\cong$ 38.571 %

For this example, let the post enzyme HPLC analysis be 15 mg / ml glucose, the dry amount of material added 1000 mg, and the total volume 20 ml.

Enzyme saccharification results would then be -- 15 $\times$ 20 / 1000 $\times$ 38.571 % $\cong$ 77.78%

Overall yield for glucose after one week would be 77.78 % $\times$ 90 % = 70.0 %.
(Enzyme saccharification yield $\times$ pretreatment yield).

Adjusted Method

There are no quantitative saccharification results after the pretreatment so an approximation is used for the pretreatment yields. In this case, **Table 5.1** would contain the values of 90, 90, 90, and 50 for glucose, xylose, other sugars and AELA respectively, in the week one column of the 55 °C row)

Initial proportions would be the same and the enzyme saccharification results would be the same.

Using **Equation 5.7** solve for glucose conversion**,**

$$= \frac{QSG}{(QSG \times Tbl.5.1) + (QSX \times Tbl.5.1) + (QSOS \times Tbl.5.1) + (AELA \times Tbl.5.1}$$

= 30 / [(30 x 0.9) + (10 x 0.9) + (10 x 0.9) + (50 x 0.5)

≈ 0.42857

Therefore by **Equation 5.6**, the overall yield is 15 (GHPLC) × 20 (VES) / 1000 (MB) × 0.42857 (GCON)

= 70 %.

5.2 Lime pretreatment

The experimental conditions were set to best replicate, with the equipment available, those that existed in the long term lime studies on corn stover and poplar wood by Kim (2005) and Ramirez (2005), previously mentioned in **Section 4**. The main highlights of those conditions are:

1. A supply of CO_2 scrubbed air to deliver oxygen.
2. 10 % total solids.
3. 0.5 g / g dry biomass lime loading (overliming).

5.2.1 Apparatus, material, and reagents

The materials, reagents and equipment listed below were used in the long term lime testing:

1. All equipment/reagents needed to perform the NREL laboratory analytical procedures for cellulase assay, quantitative saccharification, and enzymatic saccharification as listed in **Appendix 1**, **Appendix 2**, and **Appendix 3**.
2. Biomass prepared by the technique outlined in **Section 4.1**.
3. Water bath capable of maintaining 55, 65, and 75 C.
4. 5/16” OD Vinyl tubing.
5. 7/16” OD Vinyl tubing.
6. 1.2 L square tuperware containers with malleable lids.
7. Plastic manifolds (8 spigot).
8. 1 large, 10 spigot manifold.
9. Plastic bottles with screw caps capable of withstanding drilled holes in lid.
10. Insulated Styrofoam.
11. 5 L container with input and output ports, coupled with a heating blanket/sensor.
12. 10 L air compressor.
13. Air regulator.
14. $Ca(OH)_2$
15. HCl (5N)
16. Citric Acid Monohydrate

5.2.2 Experimental setup procedure

1. For each sample, a Ziplock tuperware container with soft plastic top (malleable), was drilled with nine, 4/16" holes around the inside edges and in the center, as shown in **Figure 5.2**.

Figure 5.2 Lime sample container.

Nine 5/16" outside diameter clear vinyl tubes CT series "K0010" were pushed into the holes, until the resting position was at approximately 1 cm from the bottom of the container. These lines were attached to an 8 spigot plastic manifold. The extra line was used as a vent.

2. 80 g of dried biomass was added to the container, along with 40 g of lime, and 800 ml of DDW.

3. The sample was stirred until well mixed, and the pH measured to ensure it was approximately 12.

4. Samples were placed in water baths set to 55, 65, and 75 °C. The plastic manifolds were attached to bottles with hard plastic screw caps, containing a slurry of lime and DDW (pH ≅ 12). The connecting tubing was 7/16" OD and inserted through 6/16" holes drilled in the screw caps. (**Figure 5.3**)

5. The lines leaving the lime slurry bottles passed through holes drilled in insullated styrofoam that raised the level of the water bath lid. (**Figure 5.3**)

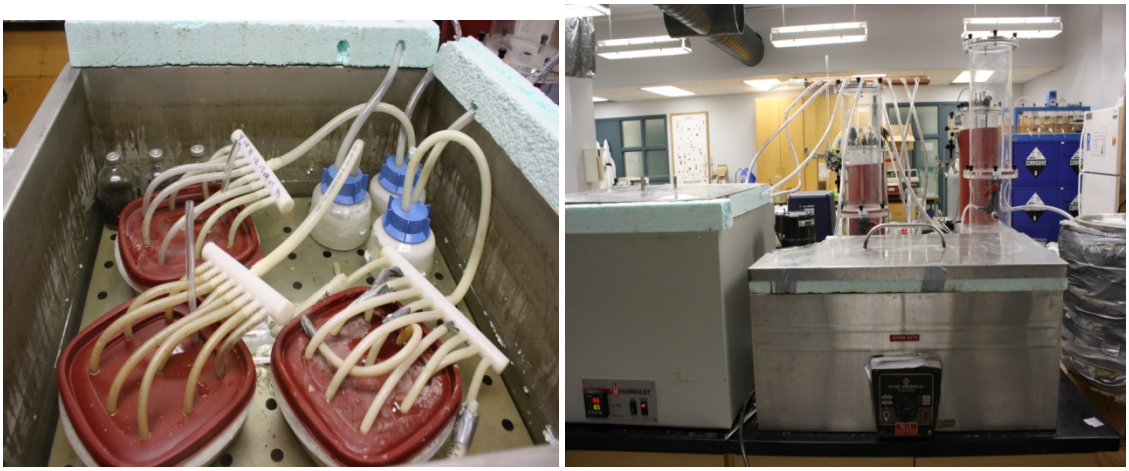


Figure 5.3 Lime sample water bath configuration.

6. The lines leaving the water baths were connected to a central manifold which was connected to a 5 L lime slurry housed in an Armstrong Anaerobic Digester (for CO_2 scrubbing), equipped with a heating blanked and set to 50 °C as shown in **Figure 5.4**.

Figure 5.4 Lime sample compressed air manifold and CO_2 scrubber.

The purpose of preheating and scrubbing the CO_2 from the compressed air through the heated lime slurries, was to ensure the temperature of the pretreatment remained at the desired

temperature set by the water bath, and to prevent the CO_2 from reacting with, and consuming, the lime in the samples.

7. The CO_2 scrubber was attached to a Rigid, 10 L air compressor with regulator, as shown in **Figure 5.5.**

Figure 5.5 Rigid air compressor for lime pretreatment.

5.2.3 Lime experiment procedure

1. Set up instructions were followed.
2. Water baths were filled with water and set to desired heat.
3. Heat was set on CO_2 scrubber to 50 °C.
4. The compressor was turned on with regulator closed, and slowly adjusted upward until all samples were bubbling rapidly (too fast to count).
5. Lime slurry levels in water baths were checked every 24 hours and replaced when necessary.
6. pH of CO_2 scrubber was checked weekly to ensure pH remained approximately 12.
7. Samples were removed weekly, and mixed well before drawing off 150 ml for testing.
8. The 150 ml removed were poured into a settling column, and rinsed with DDW.
9. The solids were allowed to settle, and the supernatant poured off.
10. The slurry was adjusted to a pH of 7.0, using 5N HCl.
11. The solids were rinsed again in DDW repeatedly. Each time the solids were allowed to settle, and then the supernatant poured off.
12. The pH was checked and readjusted to 7.0. Steps 11 and 12 were repeated until the slurry was clear. **Figure 5.6** shows the progression from cloudy to clear.

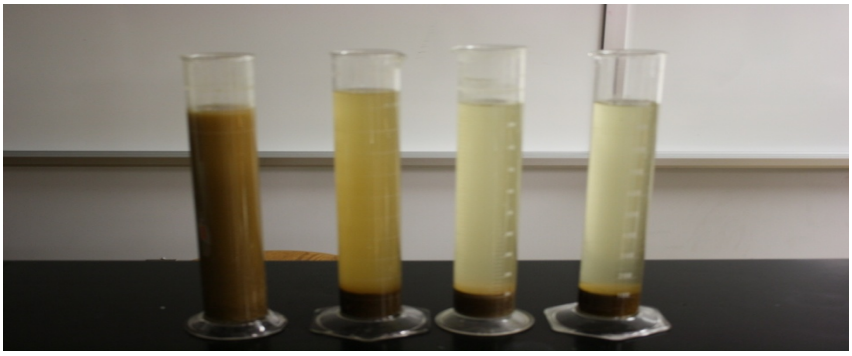


Figure 5.6 Progression of sample from cloudy to clear.

13. From Week 3 on, pH was adjusted to 4.8 with 1M citric acid.
14. The slurry was mixed and the pH measured repeatedly until no change had occurred over a 12 hour period.
15. The sample was filtered through 0.45 µm Whatman filter paper and set on a tray to dry, as shown in **Figure 5.7**. The tray was placed in an oven at 44 °C or less, and left for 48 hours.

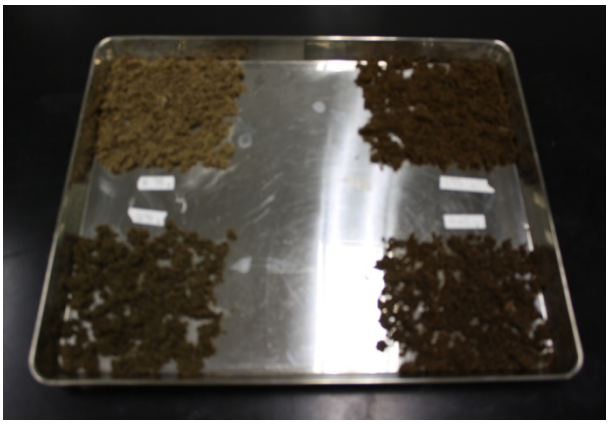


Figure 5.7 Lime treated samples after drying at 44 °C.

16. Dried samples were bagged in a marked Ziplock bag, and placed inside a larger Ziplock bag marked with the weeks the samples were taken. Samples were stored in the freezer until needed for enzyme/quantitative saccharification.
17. A quantitative saccharification (**Appendix 1**) was performed on all samples to ascertain the post-pretreatment glucose and xylose proportions. (Though the tests were not accurate)
18. Total solids content was found by triplicate samples (Ratio of current mass to mass after 24 hours oven drying at 105 °C)
19. Enzymatic saccharification of material (**Appendix 2**) was performed to find the hydrolysis yield.
20. The alternative method as explained in **Section 5.1**, was used to estimate overall yield.

5.3 COSLIF pretreatment

The method used for this paper was adapted from a study, Ehsanipour (2010), which confirmed the effectiveness of the COSLIF method on SSO mixed with softwood. The Ehsanipour (2010) study was in itself adapted from Zhang et al. (2007), with changes made to some of the washing solvents (acetone to ethanol), and varied acid hydrolysis times. In the Ehsanipour (2010) study, a two hour optimum time was found for the phosphoric acid portion of the treatment, and the overall conversion rate from cellulose to glucose was found to be approximately 90 % at 60 FPU / g glucan cellulase loading.

The method used for this study aimed to add precision to the overall yield figures, by finding both the pretreatment yield and hydrolysis yield. FPU loadings per gram glucan were varied to assess the optimum loading for commercialization.

5.3.1 Apparatus, materials, and reagants

1. All equipment/reagents needed to perform the NREL laboratory analytical procedures for cellulase assay, quantitative saccharification, and enzymatic saccharification as listed in **Appendix 1**, **Appendix 2**, and **Appendix 3.**
2. Biomass prepared by the technique outlined in **Section 4.1.**
3. 250 ml plastic screw top bottles.
4. A centrifuge capable of housing those bottles, as well as generating a speed of 7000 rpm.
5. Phosphoric Acid (85% assay).
6. Acetone (99.5% assay)
7. Ethanol (95% assay).

5.3.2 COSLIF experimental procedure

1. The mass of the bottles was recorded at room temperature. (A) 4.000 g of SSO mixture was added to each weighed bottle.
2. 32ml of phosphoric acid (85%) was added to bottle.
3. Bottles were incubated in rotary shaker at 50 °C, 150 rpm, for 2 hours.

4. The phosphoric acid reaction was stopped by adding cool acetone (20 ml/g = 80 ml) and mixed well by shaking.
5. Bottles were centrifuged at 7000 rpm for 10 min at 24 °C.
6. The supernatant was collected.
7. The pellet of solids was washed in approximately 160 ml of 95% pure ethanol and then centrifuged at the same rate/time.
8. The supernatant was collected.
9. Steps 7 and 8 were repeated.
10. The pellet was then washed in 160 ml of DDW, centrifuged, and the supernatant collected, three times.
11. The bottles were then dried at 45 °C for 48 hours, or until the change in mass was less than 0.05% in the space of 1 hour.
12. The mass was recorded at room temperature. (B)
13. The dry material from bottle 1 was ground and mixed well using a pestle until there were no clumps larger than 1 mm. Three, 300 mg samples were taken from the ground material and placed in dry 125 ml serum bottles. The bottles were labeled sample 1.
14. Step 13 was repeated for bottles 2 through 10.
15. A quantitative saccharification was performed on the material in the serum bottles(**Appendix 1**).
16. A portion of the remaining material was split into ten, 1 g samples and dried at 105 °C for 24 hours. Total solids were calculated for the 45 °C dried material. (TS) The average total remaining material was found --> (B-A)*(TS).
17. The average moisture content, glucose concentrations from the quantitative saccharification, and losses from pre-treatment were used to calculate the amount of water needed to bring the glucose concentration of samples to 20 g/L.
    - Example - 4.000 g of 100% dried material is found to be reduced to 2.000 g on average. The average glucose content is 90.0% of the remaining material. Therefore, if the container and dried 4.000 g of sample weighed 124 g before pretreatment, and 134 g after the rinses and centrifugation, there would be 10g of water. Since there is 1.800 grams of glucose (0.900 * 2.000), 80 ml of DDW would need to be added to bring the glucose content to 20 g/L. (1.8 g / 0.09 L = 20 g/L)
18. Pretreatment yield was calculated from the ratio of glucose remaining in the bottle, to glucose in original sample.

19. <u>FOR SUBSEQUENT SAMPLES</u>
    Steps 1-11 were repeated.
20. An enzymatic hydrolysis was performed on the material as per the method described in **Appendix 2**.
21. Hydrolysis yield was calculated as per the method described in **Appendix 2**.

An overview of the process is shown in **Figure 5.8**.

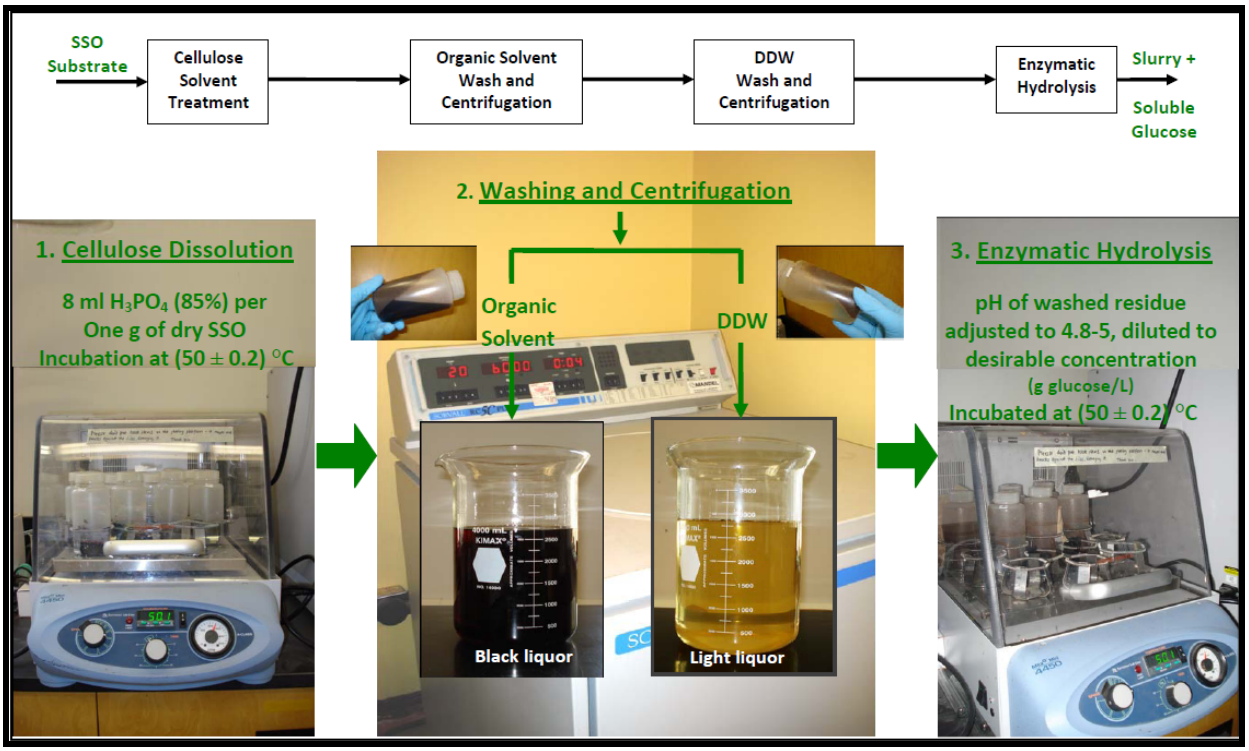


Figure 5.8 COSLIF process overview (Ehsanipour, 2010).

All sugar concentrations for the enzymatic and quantitative saccharifications were analyzed by HPLC with the Refractive Index (RI) detector shown in **Figure 5.9**. The column used for detection was a Biorad Aminex HPX – 87P.

Figure 5.9 Perkin Elmer LC autosampler, 200 series.

6 RESULTS AND DISCUSSION

6.1 Long term lime pretreatment results

The results of the long term lime study are divided into three subsections. The first subsection deals with the effect the lime bond had in preventing quantitative saccharification testing, as well as inhibition of enzyme hydrolysis. The remaining sections present the overall yields achieved and a summary of the results in comparison to similar long term lime studies.

6.1.1 Long term lime general findings

As discussed previously in the methods section, the behavior exhibited by the treated material during the washing procedure, post treatment quantitative saccharification, and enzyme hydrolysis, resulted in low conversion yields. The lime formed an unknown bond with the material/chemicals in the highly heterogeneous mixed SSO and hardwood substrate. This phenomenon was previously observed in homogeneous corn stover (Kim, 2005) and poplar (Ramirez, 2005) feedstock studies, but not nearly to so drastic an extent. In those studies, the slurry was neutralized, centrifuged, and rinsed repeatedly until the solution was clear. Ramirez (2006) warned that, "It has been observed that no matter how carefully the neutralization has been done, the pH increases again and further neutralization may be required." In this study, a centrifuge of the size required was not available, therefore the solids were allowed to settle in 1000 ml beakers before being tested for pH and readjusted. The slurry had to be readjusted dozens of times before it finally remained stable at a pH of 7.0. There is no mention of this in the previous studies, which would be unusual, given that it would take days/weeks just to allow for the slurry to stabilize. In addition, there is no mention in either the Ramirez (2006) or Kim (2005) study of a necessary divergence from the NREL enzymatic or quantitative saccharification procedures that follow. In this study, the quantitative saccharification appeared not to work even after the slurry had remained at constant pH for over 24 hours. The enzyme saccharification buffer solution required repeated

readjustment back to a pH of 4.8. It is possible that the lime formed a unique bond with the mixed SSO and hardwood substrate that prevented proper hydrolysis. **Table 6.1** and **Table 6.2** illustrate how the lime interfered with proper quantitative saccharification.

Table 6.1 Post acid hydrolysis lignin test results, week 1, 55 °C.

Sample	Mass of filter (g)	Mass at 105°C = Acid Insoluable Residue and filter (g)	105°C Residue (g)	Mass at 575°C of ash and filter paper (g)	Ash (g)	Klasson Lignin (g)	% Klasson Lignin (g)
1	0.114	0.308	0.194	0.123	0.009	0.631	63.1
2	0.114	0.206	0.092	0.123	0.009	0.283	28.3
3	0.114	0.207	0.093	0.123	0.009	0.286	28.6
4	0.114	0.207	0.093	0.124	0.010	0.283	28.3
5	0.114	0.308	0.194	0.146	0.032	0.553	55.3
6	0.114	0.207	0.093	0.122	0.008	0.290	29.0
7	0.114	0.207	0.093	0.122	0.008	0.290	29.0
8	0.114	0.206	0.092	0.123	0.009	0.283	28.3
9	0.114	0.207	0.093	0.125	0.011	0.280	28.0
10	0.114	0.294	0.18	0.131	0.017	0.556	55.6
						AVERAGE	37.3 %

Since the lignin proportion of the original material was 25.1 %, and a large portion of the loss of mass in the pretreated material should be from lignin, an increase in lignin content to 37 % is impossible. Some of the samples had 50-60% lignin, as well as larger ash contents than the other samples, which could be the higher amount of salts created by the lime and hydrochloric acid. The results indicate that an incomplete hydrolysis occurred. The NREL, "Determination of Structural Carbohydrates and Lignin in Biomass," stipulates in point 5.6 of the Interferences Section, "This procedure is not suitable for samples containing added acid, base, or catalyst" (Sluiter et al., 2008). Since the lime attachment to the biomass appeared to increase with time, week 6 should have shown the most interference. **Table 6.2** shows the

quantitative saccharification results of week 6 of the 65 °C sample to outline the extent of the failed acid hydrolysis.

Table 6.2 Quantitative saccharification results, week 6, 65 °C.

Sample	glucan
1	12.1%
2	13.5%
3	14.1%
4	15.3%
5	14.1%
6	13.0%
7	16.2%
8	15.0%
9	15.3%
10	15.0%
AVERAGE	**14.4%**

Since the glucan proportion started at 34 %, and should be getting larger as the treatment progresses, 14.4% shows that the quantitative saccharification results are that of an incomplete hydrolysis. Since quantitative saccharification is required to find both pretreatment and enzymatic hydrolysis yields, neither of those yields could be calculated. However, as explained in the methods section, an overall yield was determined from the enzymatic results, and a pretreatment yield estimate, based on the corn stover and poplar wood long term lime studies.

As with the quantitative saccharification, behavior not reported in the other long term lime studies occurred with the enzyme hydrolysis. The enzyme hydrolysis of weeks one and two showed very little sugar conversion. In retrospect, the low conversion should not have been surprising, since the standard method followed likely resulted in a rise in pH that inhibited the enzymes. After the pretreatment slurry was neutralized to a pH of 7.0, the solids were dried at 45 °C, and later added to a citrate buffer at pH 4.8. Since the enzyme hydrolysis does not conventionally call for a pH check of the buffer solution,

the probable rise was not detected. Weeks one and two were re-tested and the pH rise discovered by accident when half of the samples were left in the fridge overnight, to be simultaneously saccharified with samples to be prepared the next day. The pH was checked before addition of enzymes and the rise from 4.8 to above 7 was discovered. The samples were then adjusted back to 4.8, and repeatedly tested until no noticeable change occurred by the end of a 12 hour period. For samples from week 3-6, the post treatment DDW/substate slurry was adjusted and re-adjusted by 1M citric acid until the slurry remained steady for 12 hours at to a pH of 4.8. The material was then filtered and dried before being added to the 4.8 pH buffer solution. All of the samples required additional adjustment after being added to the buffer. The fact that this was necessary, highlights the unique bond the lime has formed with the mixed SSO and hardwood.

Compounding the need to repeatedly adjust the pre-hydrolysis liquor is the fact that the enzyme action released more lime into solution, even after the buffered material was stable at a pH of 4.8. The pH was checked after 24 hours, and it was occasionally found even higher than 7. Since the optimum pH range of Accellerase 1500 enzyme is 4.6 - 5.0, and complete inactivation occurs at pH greater than 7, (Genencor,http://www.genencor.com/wps/wcm/connect/09a976004fa2d4ceaecbbe4895e3224e/ACCELLERASE+1500+product+information+sheet.pdf?MOD=AJPERES&CACHEID=09a976004fa2d4ceaecbbe4895e3224e) the release of lime into the solution prevented proper enzyme hydrolysis. In weeks 1 and 2, the pH was increased but remained below 6.0, and the material was not readjusted. However, in weeks 3-4, one adjustment back to a pH of 4.8 was necessary after 24 hours, accompanied by a further addition of 15 FPU cellulase, as the pH was in some cases higher than 7. In weeks 5 and 6, an adjustment and additional enzyme were added to the samples at 24 and 48 hours, and the pH nevertheless rose to levels that could inactivate the Accellerase 1500 enzyme. At that point, no additional enzyme was added. Any pretreatment that required multiple enzyme addition to generate sugars would not be cost effective.

6.1.2 Long term lime overall yields

Figure 6.1, **Figure 6.2**, and **Figure 6.3**, show the overall conversions for glucose and xylose at the different temperatures tested.

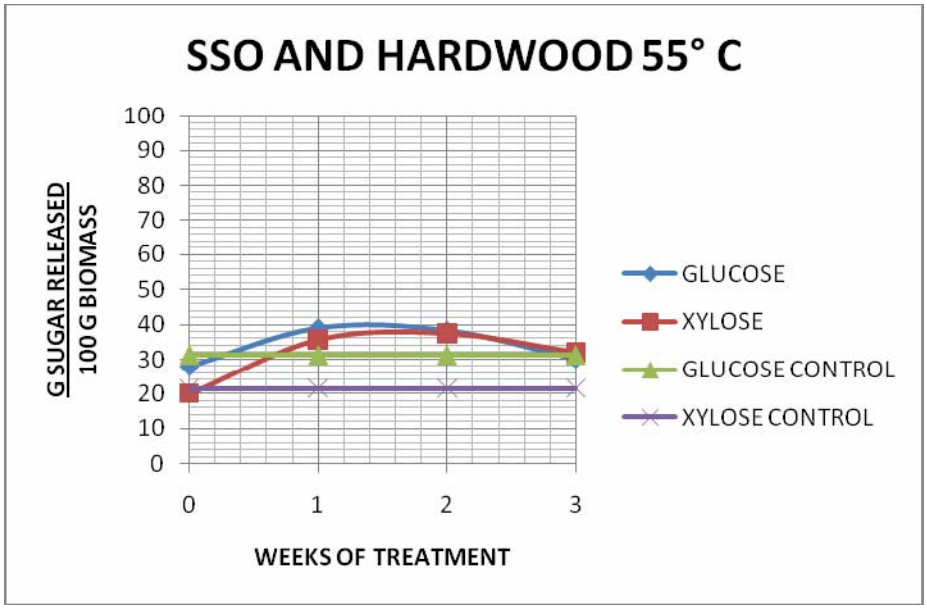


Figure 6.1 Overall lime treatment yield of glucose and xylose at 55 °C.

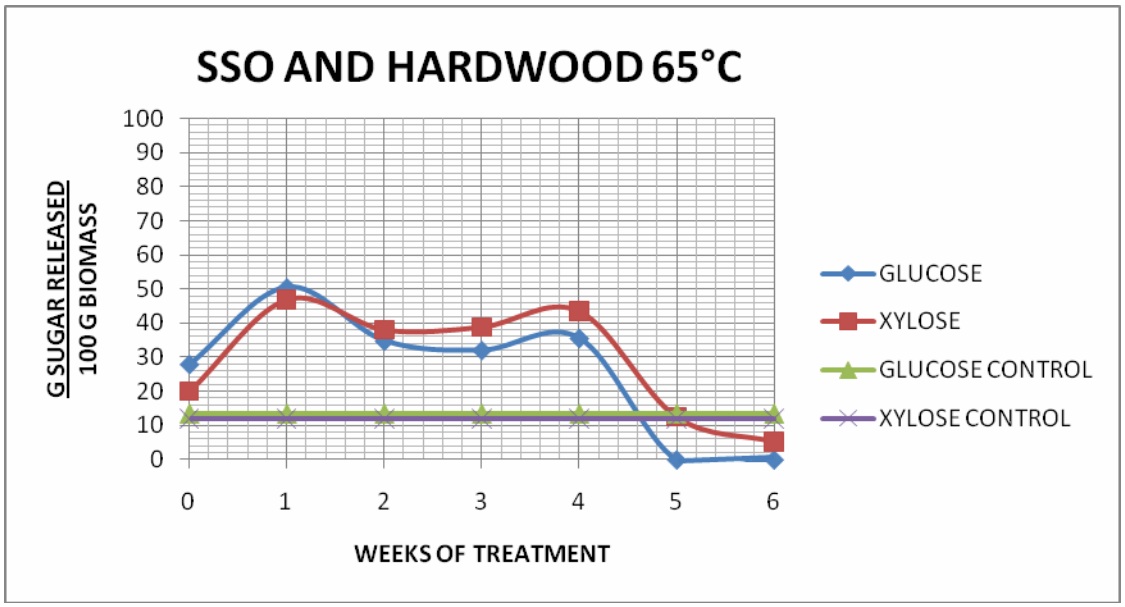


Figure 6.2 Overall lime treatment yield of glucose and xylose at 65 °C.

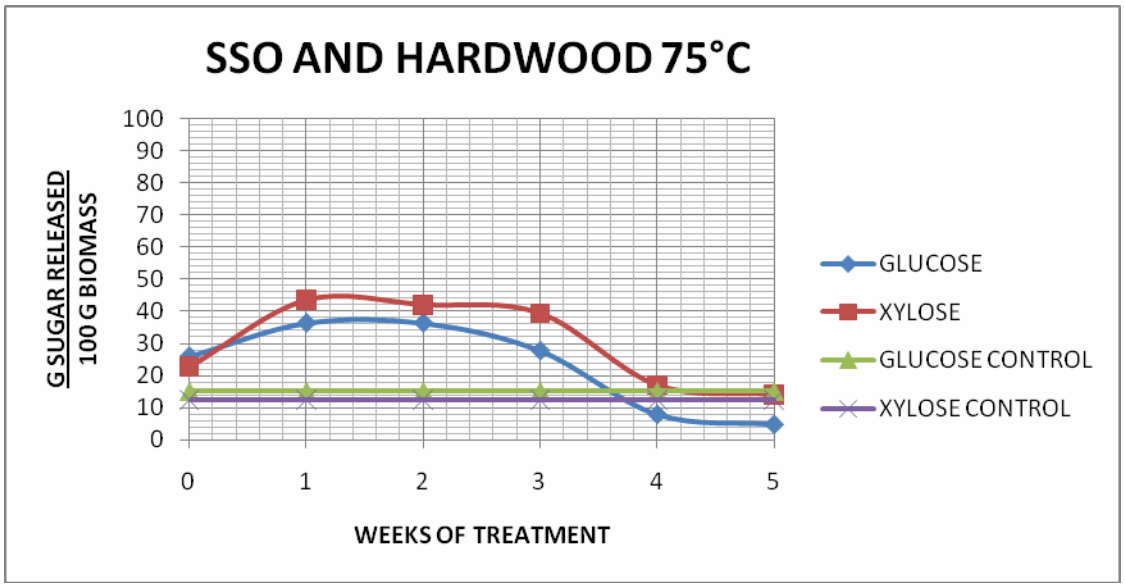


Figure 6.3 Overall lime yield of glucose and xylose at 75 °C.

The results are unlike similar studies, but consistent. There was an initial rise in the glucose/xylose release, then a leveling before plummeting to near zero glucose and very little xylose at week 4 or 5. In the corn stover and poplar wood studies, the conversion peaks were not achieved until week 4 (Kim,

2005; Ramirez, 2005). The unknown lime bond allowed for release of lime into buffered and adjusted enzyme hydrolysis liquor. The pH increase to levels unsuited for enzymes is likely the cause of the low conversion rates. The effect that the lime had in bonding with the material seemed to happen faster at 75 °C, as it needed pH adjustment in week 2, while the 55 and 65 °C samples did not. It is possible that that is the reason the conversion rates at 75 ° C were even lower than at 65 °C. At 55 °C, the test was only conducted until week three, because the water bath broke down in the first week, and the sample was then compromised.

Given the lime release of the material during enzymatic hydrolysis, even if the conversion rates had been above 80%, lime treatment of this material would not be commercially viable. If more than one dose of cellulase is needed at a loading rate as high as 15 FPU / g dry biomass, the process cannot be commercially competitive with other pretreatment techniques. In the following COSLIF pretreatment section, a loading of 30 FPU / g glucan is shown to be commercially unacceptable at an overall conversion rate of 89 %. Since the lime treated biomass is about 50 % glucan, just one dose at 15 FPU / g dry biomass is too expensive.

6.1.3 Long term lime pretreatment results summary

The highest overall conversions from cellulose to glucose were found in the first week. The amount of glucose released, compared to the initial quantity in the biomass, was 38.5 %, 50.4 %, and 48.8 %, for conditions of 55, 65, and 75 °C, respectively. The highest xylose conversions took place in the first week of treatment as well, for 55 and 75 °C, but for the 65 °C sample, the best conversion was in the fourth week. The percentage released in comparison to the initial quantity in the biomass was 38.5 %, 43.5 %, and 44.1 %, for 55, 65 and 75 °C, respectively. The Kim (2005) study found that 91.3 % and 51.8 % overall glucose and xylose conversions were possible for corn stover at 55 °C, and that the peak overall conversions occurred after 4 weeks. The Ramirez (2005) study on poplar wood was only four weeks

long, but the best overall conversions were 80.7 % and 66.9% for glucose and xylose, in the fourth week, at 65 °C. **Figure 6.4** shows the best results from this study compared to the corn stover and poplar wood studies on long term lime treatment.

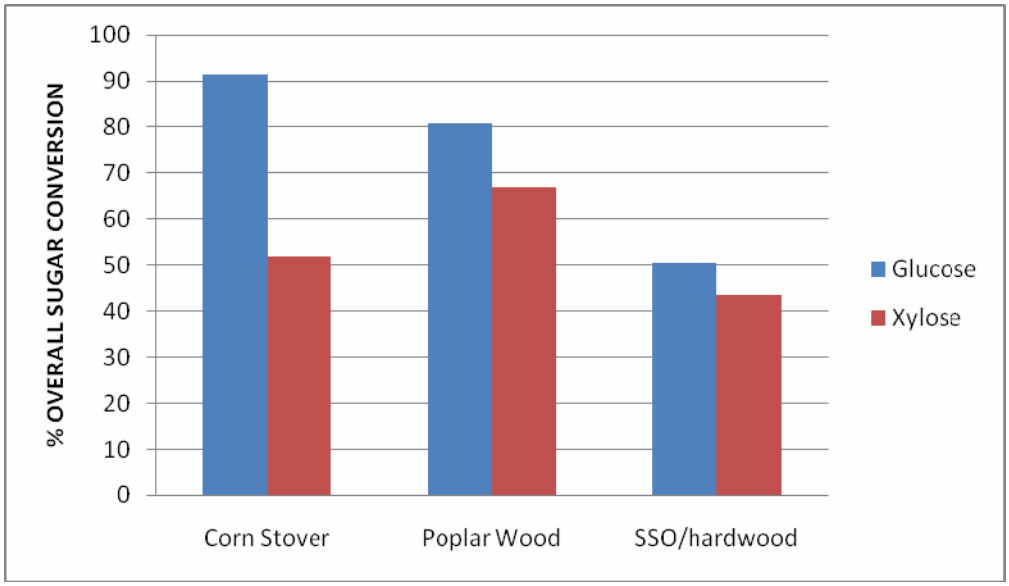


Figure 6.4 Sugar conversion efficiencies for this and other long term lime studies corn stover (Kim, 2005) poplar wood (Ramirez, 2005).

The peak conversions found for this study on long term lime treatment of SSO mixed with forestry waste (hardwood) were much lower than that found for similar biomass. The peak conversions were found in the first week in comparison to the fourth week for both the corn stover and poplar wood studies. The conversions might have been higher in this study in subsequent weeks, if the material had not released lime into solution during enzyme hydrolysis. This deficiency required repeated addition of enzymes and pH adjustment. The effect of pH adjustments was negated after the addition of more enzyme, because it is likely that the enzyme action caused a subsequent further rise in pH. Since the conversion efficiencies were low in the first week, before multiple enzyme loadings would be necessary, long term lime is not a viable pretreatment for SSO mixed with forestry waste.

6.2 COSLIF pretreatment results

6.2.1 COSLIF pretreatment yield

To find the pretreatment yield, ten bottles with 4.000 g (3.837 dry) of SSO/softwood were treated by the COSLIF method, dried, weighed, and quantitatively saccharified to be analyzed by HPLC, in order to ascertain the total mass and total glucose remaining. The complete set of measurements and HPLC absorbance readings can be found in **Appendix 1**, while the summary of findings are shown in **Table 6.3** and **Table 6.4**.

Table 6.3 Post-COSLIF treated total mass and glucose.

BOTTLE	INITIAL DRY MASS (g)	POST PRETREATMENT DRY MASS (g)	TOTAL MASS REMAINING	HPLC ANALYSIS (g / L)	PROPORTION GLUCOSE	GLUCOSE IN BOTTLE (g)
1	3.837	2.815	73.4%	1.469	0.448	1.261
2	3.837	2.494	65.0%	1.634	0.499	1.243
3	3.837	2.473	64.4%	1.563	0.477	1.179
4	3.837	2.106	54.9%	1.470	0.448	0.944
5	3.837	2.566	66.9%	1.588	0.484	1.243
6	3.837	2.943	76.7%	--	--	--
7	3.837	2.898	75.5%	--	--	--
8	3.837	2.207	57.5%	1.563	0.477	1.052
9	3.837	2.490	64.9%	1.647	0.502	1.251
10	3.837	2.442	63.7%	1.623	0.495	1.209
AVERAGE		**2.543**	**66.3%**	**1.570**	**0.479**	**1.173**
Standard Deviation		0.276	7.2%	0.069	0.021	0.115

The 66.3 % total mass remaining is larger in comparison to the Zhang et al. (2007) study, which found that only 49.9 % of the corn stover survived the process. Therefore, more lignin and hemicellulose are carried through to hydrolysis. The advantages of increased lignin and hemicellulose would be that chemical lignin recovery would be less intensive, and a co-fermentation of the xylose with the glucose

would yield more ethanol. The disadvantage would be that lignin would increase the enzyme dosage necessary. Xylose was small enough in proportion to elute in the same peak as glucose on the HPLC, so an estimate was made to subtract the portion beyond 13.5-6 minutes in the chromatogram results. (13.5 minutes is the approximate time for xylose peak elusion, shown on the sample HPLC chromatogram). The subsequent hydrolysis analysis of this estimated xylose showed an average peak of 2.1 mg / ml in the 60 FPU / g glucan cellulase treated samples. Since the total volume was 58.7 ml, and the total dry biomass 2.543 g, the pretreated sample xylose composition is at least 4.8 % xylose (4.3 % xylan). This is approximately the same composition ratio as the biomass before pretreatment, therefore about 66.3% of the xylose was left intact during pretreatment. The Zhang et al. (2007) study on corn stover showed that 21% of the xylose was passed on to the hydrolysis stage. Therefore, if operating a simple facility that was not selling the pretreatment solubilized hemicellulose separately, the revenue from xylose fermentation would increase. If the HPLC correction figures were too conservative, the glucose conversion rates were even higher. Bottle 6 and 7 are missing data in **Table 6.3**, because there was an injection malfunction of all three of the triplicate quantitative saccharifacation samples in both bottles.

Table 6.4 shows the pretreatment yields from each bottle. In some cases the yield was 100 % or above, which is impossible. The reason there are 100 % yields is that the initial glucose levels are taken from the average initial quantitative saccharification. With 95% confidence, the samples should be 29.3 % $\pm$ 2.9% glucan. Therefore some of the samples would have had more glucose to start, and some would have had less.

Table 6.4 COSLIF pretreatment yield.

BOTTLE	TOTAL INITIAL MASS OF GLUCOSE (g) ***	POST – TREATMENT GLUCOSE PROPORTION	MASS OF DRY MATERIAL (g)	TOTAL MASS OF GLUCOSE POST TREATMENT (g)	PRETREATMENT YIELD
1	1.249	0.448	2.817	1.261	100.8
2	1.249	0.498	2.496	1.243	99.4
3	1.249	0.476	2.475	1.179	94.2
4	1.249	0.448	2.107	0.944	75.5
5	1.249	0.484	2.568	1.243	99.4
8	1.249	0.476	2.209	1.052	84.1
9	1.249	0.502	2.492	1.251	100.0
10	1.249	0.495	2.444	1.209	96.6
				AVERAGE	93.7
				Standard Deviation	9.2

*** Estimate based on original quantitative saccharification average for the material (29.3% glucan = 32.5 % glucose x 4.000 g added to bottle X 95.9 % total solids)

The average yield, shown in **Table 6.4**, is comparable with the Zhang et al. (2007) study done on corn stover, which had a 92.9 % yield. Since less material was solubilized than in the Zhang et al. study, it follows that less glucose would be solubilized as well.

6.2.2 COSLIF enzyme hydrolysis to determine optimal loading

An enzyme hydrolysis at 50 °C was performed under different cellulase loadings to ascertain the most cost effective result. That result was then experimented with at temperatures more suitable to simultaneous fermentation with *Z. Mobilis*, strain 8b. As mentioned in the **Methods Section**, the samples were at first adjusted by approximation to 20 g/L for hydrolysis, because HPLC machine failure delayed the quantification of pretreatment composition results. The loadings were therefore also an approximation of 10, 15, 30, and 60 FPU per g glucan. The actual loadings after back calculation (**Table A2-7, Appendix 2**) were 9.74, 14.61, 29.22, and 58.44 FPU per g glucan. Those loadings will henceforth

be referred to as 10, 15, 30, and 60 FPU. The loading 9.74 was carried over to the temperature range section as well, but also referred to as 10 FPU.

Figure 6.5 shows the glucose digestibility (% conversion from pretreated sample total glucose) for the 50 °C samples at different cellulase loadings.

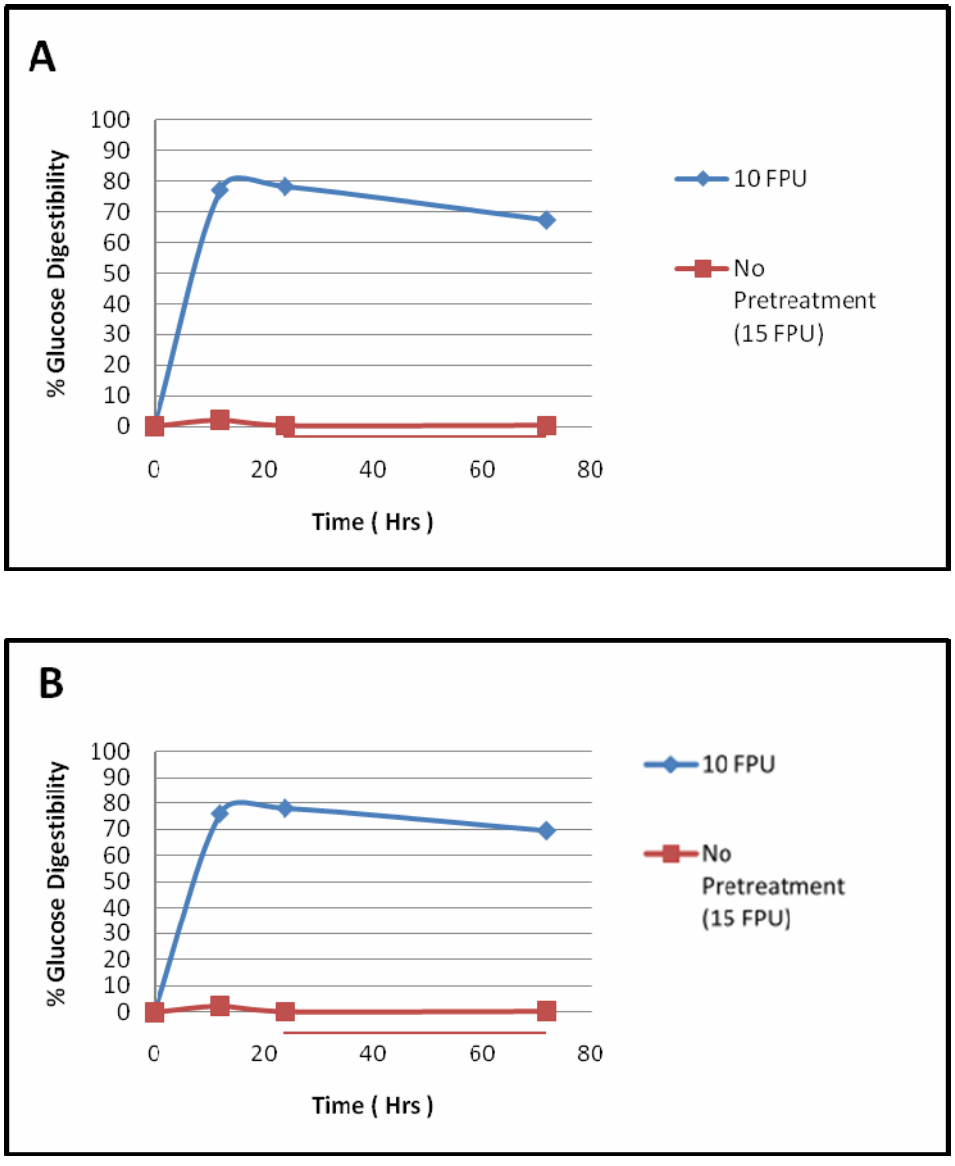


Figure 6.5 COSLIF 50 °C enzyme hydrolysis for **A**) 10 FPU, **B**) 15 FPU, **C**) 30 FPU, and **D**) 60 FPU.

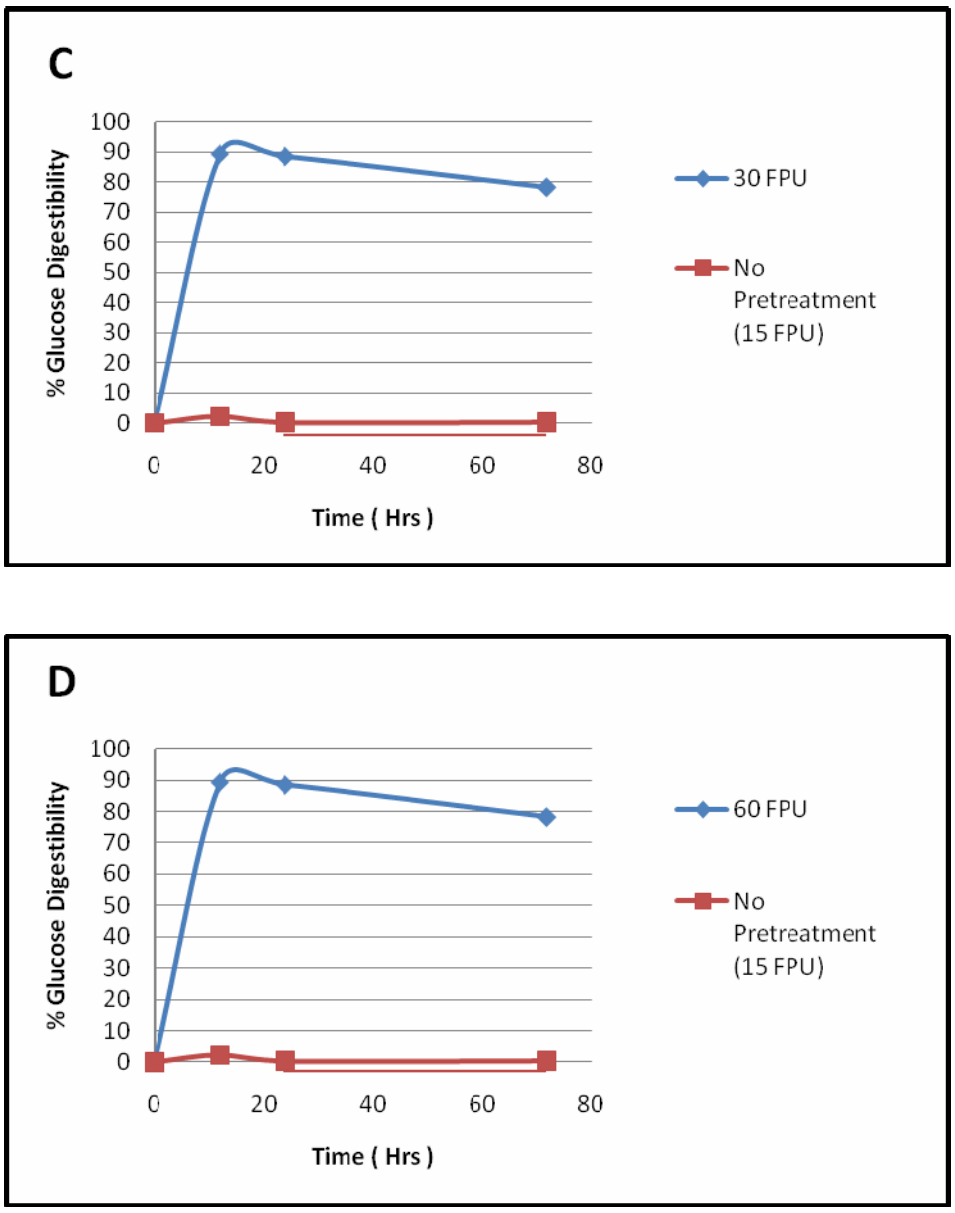


Figure 6.5 COSLIF 50 °C enzyme hydrolysis for **A**) 10 FPU, **B**) 15 FPU, **C**) 30 FPU, and **D**) 60 FPU.

The lower loadings achieved over 90 % digestibility. The near identical results between 10 and 15 FPU could be a result of lower initial glucose in the 15 FPU samples, but if this is true all three samples were equally low at 18.896, 18.691, and 18.535 g of glucose released. The lowest reading is within 2% of the highest. More likely, there was little difference between the effects of the two loadings on this material. Kaar et al. (2002) had similar findings with lime treated corn stover, as the 10 and 15 FPU loading

conversions were virtually the same at 50 °C, and the 10 FPU loading conversion at 40 °C was actually higher. There are a number of studies that show very little (<5%) difference between 15 and 60 FPU loadings at peak conversion (Teymouri et al., 2005; Wyman et al., 2005; Mosier et al., 2005), which could explain the even results for 30 and 60 FPU loadings.

As mentioned in **Section 6.2.1**, a rough estimate for xylose was made from correction of the glucose chromatograms, the results of which can be found in **APPENDIX 2**. The results showed that xylose ranged from approximately 1.7 to 2.0 g/L in the 49.98 ml hydrolysate (at 24 hours). The xylose concentrations indicate that about 2.2 % and 2.6 % of the total 5.0 % original xylose survived the process and were hydrolyzed by the enzymes. In comparison, the Zhang et al. (2007) COSLIF study found that only 1.9 % of the original 20% xylose survived to be hydrolyzed. Therefore, while the softwood lignin structure inhibits greater conversion of glucose, it does help to prevent dissolution of the hemicellulose, which would increase the overall ethanol fermentation. Again, as mentioned in **Section 6.2.1**, if the glucose was corrected too conservatively, then the glucose yield rates are slightly higher, and the xylose levels slightly lower.

The most remarkable result found by the Zhang et al. (2007) COSLIF study, and confirmed in these experiments on a mixed SSO/softwood substrate, is the rate at which the sugars are digested. The peaks in this study were arrived at between 12 and 24 hours. The Zhang et al. (2007) COSLIF study found similar results in that all substrates had peaks achieved by 24 hours, and the 12 hour conversion rates were at least 97 % of the peak. If the enzymes could perform at the same rate in a temperature range acceptable to a partnered bacteria in a simultaneous saccharification system, the speed of the fermentation would be limited only by the speed of the saccharolytic bacteria. This presents a distinct advantage over other pretreatments. If the majority of conversion is achieved in only 12 hours, it could mean at least a halving of the simultaneous fermentation time, allowing for double the output with the

same size tank. (The literature review of numerous papers did not uncover a treatment that allowed 97 % of hydrolysis conversion within 24 hours). With a set amount of investment capital, the smaller requirements for the fermentation tank would allow more money to be spent elsewhere, such as pre-treatment and distillation capacity. Larger capacity would result in more SSO processed and fermented.

One disparity from the Zhang et al. (2007) COSLIF study that should be noted is the decline in glucose after 12-24 hours in all of the samples. A possible conclusion for this phenomenon is that there was microbial contamination despite precautions taken to prevent it. The samples were not treated with sodium azide to stop all microbial growth, as would be done in the NREL procedure for enzyme hydrolysis, because the original intent was to use the hydrolysate for further fermentation studies. Fortunately, the results for the best cost effective loading were not put in jeopardy by the assumption of even the most disparate effects of microbes between samples. The proof of the previous statement will be shown in **Table 6.6**. **Table 6.5** through **Table 6.7** show how the optimal cellulase loading was arrived at given the price of ethanol, conversion efficiencies, and price of cellulase. **Table 6.5** shows the profit difference between cost and revenue at different enzyme loadings. The revenue is earned from ethanol generated from the fermented hydrolysis sugars at a specific loading. The cost is the cost of enzyme at that specific loading. Both revenue and cost are expressed on a per kg glucan basis. For every kilogram of glucan added to a hydrolysis digester, that amount of profit would be made from the hydrolysis. **Table 6.5** is shown in excel format, with a letter heading above each item. If an equation is needed to generate an item in the table, the equation is shown in the letter heading.

Example → Q = N/L, Q is the L of cellulase / kg glucan, N is the cellulase loading in FPU / g glucan, and L is the activity of the cellulase in FPU / ml. Therefore the L of cellulase needed per kg glucan added to the hydrolysis is the cellulase loading divided by the cellulase activity.

The following points provide an explanation for some of the items in **Table 6.5**:

- *Z. mobilis* is the fermentation organism to be used for subsequent studies associated with this project. *Z. mobilis* is capable of fermenting 1.0 grams of glucose to 0.5 g of ethanol (Zhang, 2003)
- Since ethanol has a density of 0.789 kg / L, *Z. mobilis* can ferment 1.0 kg of glucose to 0.634 L of ethanol, which is 0.167 gallons.
- The anhydrous correction for glucose to glucan is 0.9. Therefore *Z. mobilis* can ferment 1.0 kg of glucan to 0.186 gallons of ethanol (0.167/0.9 = 0.186)
- Pretreatment yield is taken from **Section 6.2.1**
- The $ 2.00 delivered cost of Accellerase Duet cellulase enzyme was quoted by Aaron Kelly, Senior Manager- Business Development, Genencor, May 4th, 2010.
- This study used Accellerase 1500 for testing because Accellerase Duet is only available by tonne or tanker. Genencor claims that Accellerase Duet has an activity level that is 3 times that of Accellerase1500(Genencor,http://www.genencor.com/wps/wcm/connect/genencor/genencor/products_and_services/business_development/biorefineries/accellerase/accellerase_product_line/accellerase_product_line_en.htm). Since activity is measured in units / ml, the volume of Accellerase Duet enzyme needed to accomplish the Accellerase 1500 lab results would be one third that of the Accellerase 1500 volume used.
- The mass of 10 ml of enzyme was measured to be 10.220 g. Therefore, the density is 1.022 kg / L.
- The price of ethanol was assumed to be $ 2.00, a price well under what ethanol futures are trading for until past 2012 (Barchart.com). This figure is used
- Enzyme hydrolysis yields can be seen in **Figure 6.5**, or taken from **Appendix 2**.
- The overall yield is the pretreatment yield multiplied by the hydrolysis yield.
- The revenue from the addition of 1.0 kg of glucan to the enzyme digester is the overall yield of glucose, multiplied by *Z. mobilis* conversion of that kg of glucan to ethanol, multiplied by the price of ethanol.
- The cost associated with converting that added kg of glucan is the price of a kg of enzyme, multipled by the estimated litres of enzyme added, multiplied by the density of the enzyme.

Table 6.5 shows the profit regimes for different loadings of the 50 °C enzyme hydrolysis research results.

Since the 10 and 15 FPU loadings had the same conversion rate, the cheaper option is to use 10 FPU.

The jump to achieve higher conversion at 30 FPU is not rewarded by better profit, since the cost of the enzyme per kg glucan converted outweighs the additional ethanol generated.

Table 6.5 Profit regimes per kg glucan converted from 50 °C COSIF enzyme saccharification.

AA	B = AB	AC = B/A	AD	AE = C/D	AF	AG =E*F
Density Ethanol kg / L	Z. Mobilis ethanol conversion kg / kg glucose (Zhang, 2003)	Z. Mobilis conversion L / kg glucose	Liters / Gallon	Z. Mobilis conversion in gallons of 1 kg glucose	kg glucan / kg glucose	Z mobilis conversion in gallons of 1 kg of glucan
0.789	0.500	0.634	3.785	0.167	0.900	0.186

AH	AI	AJ	AK	AL	AM
PRETREATMENT YIELD	Delivered cost of enzyme $ / kg (Genencor)	Measured Density enzyme kg / L	Claimed Activity Ratio of Accelerase Duet to Accellerase 1500	Activity of Accellerase 1500 FPU / ml	Price of a gallon of ethanol ($)
0.937	2.00	1.022	3	71	2.00

AN	AO	P = O*H	Q = N/L	AR = Q/K	AS = P*G*M	AT = I*R*J T	AU = S-T
FPU Loading / g glucan	**ENZYME HYDROLYSIS YIELD**	**OVERALL yield**	L of Accelerase 1500 per kg of glucan	Estimated L of Accellerase Duet per kg glucan	Revenue in terms of loading / kg glucan ($)	Enzyme cost in terms of loading / kg glucan ($)	**Enzyme Profit difference per kg glucan ($)**
10	**0.818**	**0.767**	0.141	0.047	0.285	0.096	**0.189**
15	**0.817**	**0.766**	0.211	0.070	0.285	0.144	**0.141**
30	**0.935**	**0.876**	0.423	0.141	0.326	0.288	**0.038**
60	**0.933**	**0.874**	0.845	0.282	0.325	0.576	**-0.251**

6.2.3 COSLIF enzyme loading maximization sensitivity analysis

If the argument is made that microbial contamination could have disproportionally affected the 15 FPU loading over the 10 FPU loading, even though the total drop in sugars appears larger in the 10 FPU samples, the conversion rates can be altered to be more conservative toward the conclusion. **Table 6.6** on the following page shows the final conversion results needed to make the 15 FPU loading equal to 10 FPU, and what the price difference would be if 30 and 60 FPU loadings had 100 % hydrolysis, possibly taken away by the microbes (All other components held constant).

Table 6.6 Profit regime given larger conversion assumptions for 15, 30, and 60 FPU loadings.

FPU Loading ml / g glucan	**ENZYME HYDROLYSIS YIELD**	**OVERALL yield**	L of Accelerase 1500 per kg of glucan	Estimated L of Accellerase Duet per kg glucan	Revenue in terms of loading ($)	Enzyme cost in terms of loading ($)	**Enzyme Profit difference per kg glucan ($)**
10	**0.818**	**0.767**	0.141	0.047	0.285	0.096	**0.189**
15	**0.956**	**0.896**	0.211	0.070	0.333	0.144	**0.190**
30	**1.000**	**0.937**	0.423	0.141	0.349	0.288	**0.061**
60	**1.000**	**0.937**	0.845	0.282	0.349	0.576	**-0.227**

The loadings of 30 and 60 FPU remain far more expensive, while 15 FPU would require a 95.6 % hydrolysis yield to be more cost effective than 10 FPU; a yield far above that recorded for softwood in the Zhang et al. study at the same enzyme loading. **Table 6.7** shows the effects of adding the real price of current ethanol futures through to 2011 (Barchart.com), or the results obtained if Accellerase Duet performs at an activity level less than advertised (Holding all other components constant). The profit margin between 10 FPU and all of the other loadings, widens dramatically.

Table 6.7 Profit regime given current (lower) ethanol prices (**A**), less than advertised performance in Accellerase Duet (**B**).

A - Ethanol = $ 1.70							
FPU Loading ml / g glucan	**ENZYME HYDROLYSIS YIELD**	**OVERALL yield**	L of Accelerase 1500 per kg of glucan	Estimated L of Accellerase Duet per kg glucan	Revenue in terms of loading ($)	Enzyme cost in terms of loading ($)	**Enzyme Profit difference per kg glucan ($)**
10	0.818	0.767	0.141	0.047	0.243	0.096	**0.147**
15	0.817	0.766	0.211	0.070	0.242	0.144	**0.098**
30	0.935	0.876	0.423	0.141	0.277	0.288	**-0.011**
60	0.933	0.874	0.845	0.282	0.276	0.576	**-0.299**
B – Accellerase Duet = 2 x activity of Accellerase 1500							
FPU Loading ml / g glucan	**ENZYME HYDROLYSIS YIELD**	**OVERALL yield**	L of Accelerase 1500 per kg of glucan	Estimated L of Accellerase Duet per kg glucan	Revenue in terms of loading ($)	Enzyme cost in terms of loading ($)	**Enzyme Profit difference per kg glucan ($)**
10	**0.818**	**0.767**	0.141	0.047	0.285	0.144	**0.141**
15	**0.817**	**0.766**	0.211	0.070	0.285	0.216	**0.069**
30	**0.935**	**0.876**	0.423	0.141	0.326	0.432	**-0.106**
60	**0.933**	**0.874**	0.845	0.282	0.325	0.864	**-0.538**

Figure 6.6 summarizes the difference in profit between a 10 FPU cellulase loading and 15, 30 and 60 FPU loadings in the varied scenarios. The first scenario is that of **Table 6.6**, which assumes greater than the maximum likely pretreatment yields, to compensate for possible microbial contamination. The second scenario is the difference in profit of the varied loadings from current ethanol prices (lower than that used for maximum price up to 2012). The third scenario is the analysis of profit difference given that Accellerase Duet performs at an activity level less than what is advertised by Genencor. To be clear, profit is taken to be the profit on a per gallon ethanol produced basis, from the inputs and outputs of the fermentation/saccharification tank.

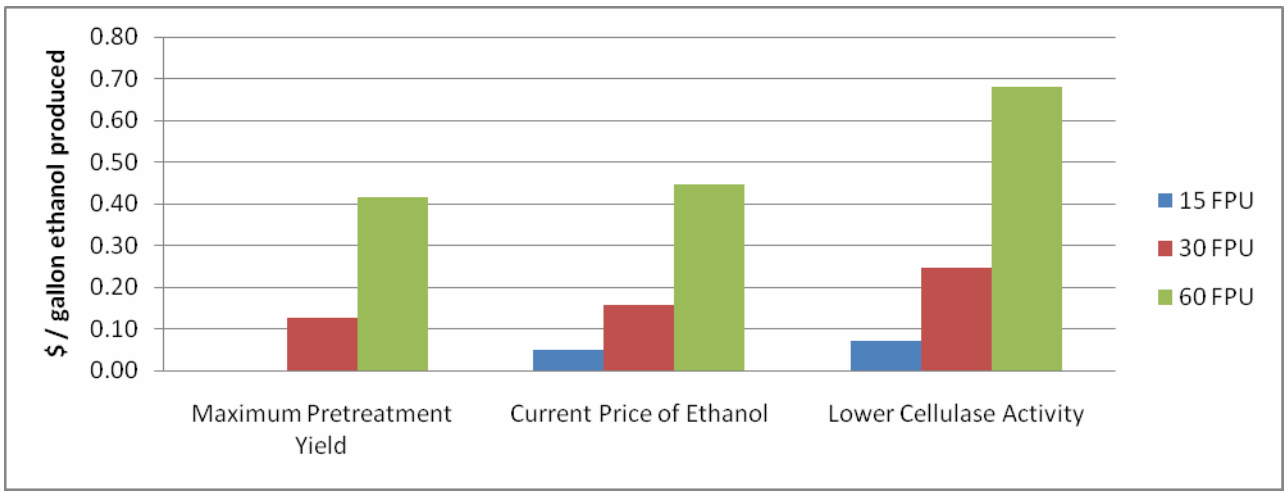


Figure 6.6 Sensitivity analysis summary - increased profit from 10 FPU loading over 15, 30, and 60 FPU.

6.2.4 COSLIF enzyme hydrolysis to determine optimum temperature

Enzyme action was tested at 30, 35, and 40 °C to ascertain the fall in activity, in order to assess the proper temperature to pair the enzyme with *Zymomonas mobilis*, a rapidly saccharolytic bacteria capable of fermenting glucose as well as xylose. If a simultaneous saccharification and co- fermentation (SSCF) is to be effective, a temperature balance between the organism's ideal temperature and the enzyme action has to be struck. Cazetta et al. (2007) found that *Z. mobilis* is more effective at 30 °C than 35 or 40 °C. Zhang et al. (2003) found *Z. mobilis* capable of fermenting 37 g / L glucose and 37 g / L xylose to completion in 12 hours and 24 hours respectively at 30 °C. When the bug was inhibited by acetate and a higher temperature of 37 °C, *Z. mobilis* strain 8b was still capable of fermenting to completion in 24 hours.

Since the majority of sugar in a COSLIF treated material is glucose, if the enzyme action was near completion in only 12 hours at 30 °C, like it was at 50 °C, then the SSCF process would be extraordinarily quick. Unfortunately, that was not the case for the results in this study. **Figure 6.7** shows the enzyme hydrolysis profile at 30 °C.

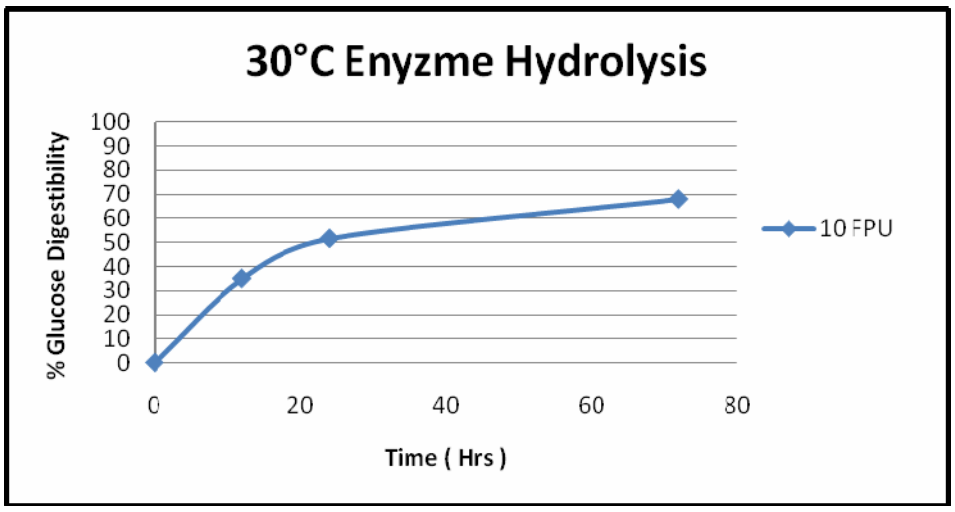


Figure 6.7 COSLIF enzyme hydrolysis profile at 30 °C.

The total glucose achieved by 72 hours was less than that of the 50 °C samples, and the rate of hydrolysis far slower. In fact, it is likely that peak had not yet been reached, as no plateau is evident from the results.

At 40 °C however, the enzyme action appears to be only slightly slowed, with the peak conversion achieved between 12 and 24 hours, and the conversion at 12 hours 91 % of that found in the 50 °C study. **Figure 6.8** shows the 40 °C hydrolysis profile.

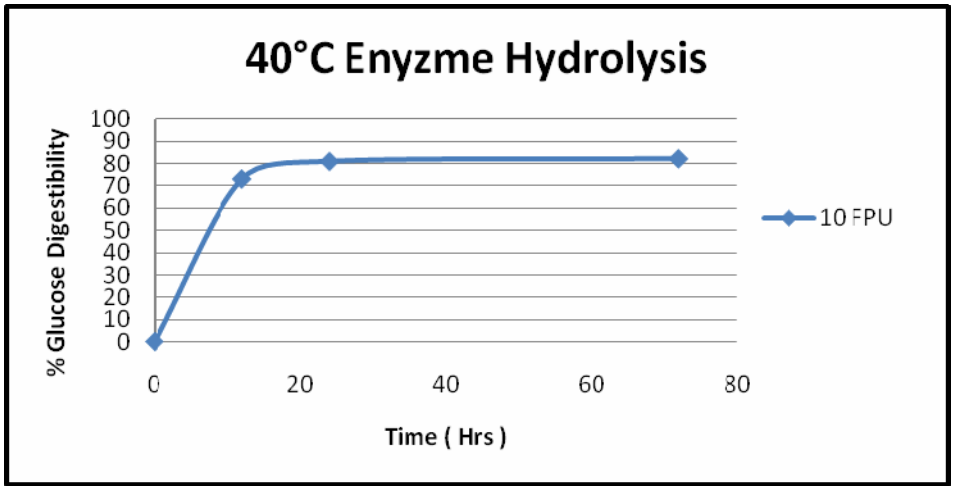


Figure 6.8 COSLIF enzyme hydrolysis profile at 40 °C.

Consequently, the 35 °C samples were expected to be a possible mid-ground between the near perfect enzyme action at 40 °C, and the optimum bacterial condition of 30 °C. For all of the temperature range bottles, a 48 hour sample was taken to be tested if needed, to better determine the hydrolysis plateau. These 48 hour samples were analyzed for the 35 °C range, as the 24 hour figures were not near the peak. Since the lowest FPU loading was the best in the economic optimization tests, a lower loading of 5 FPU was added to the 35 °C testing as well. **Figure 6.9** shows the 35 °C hydrolysis profile.

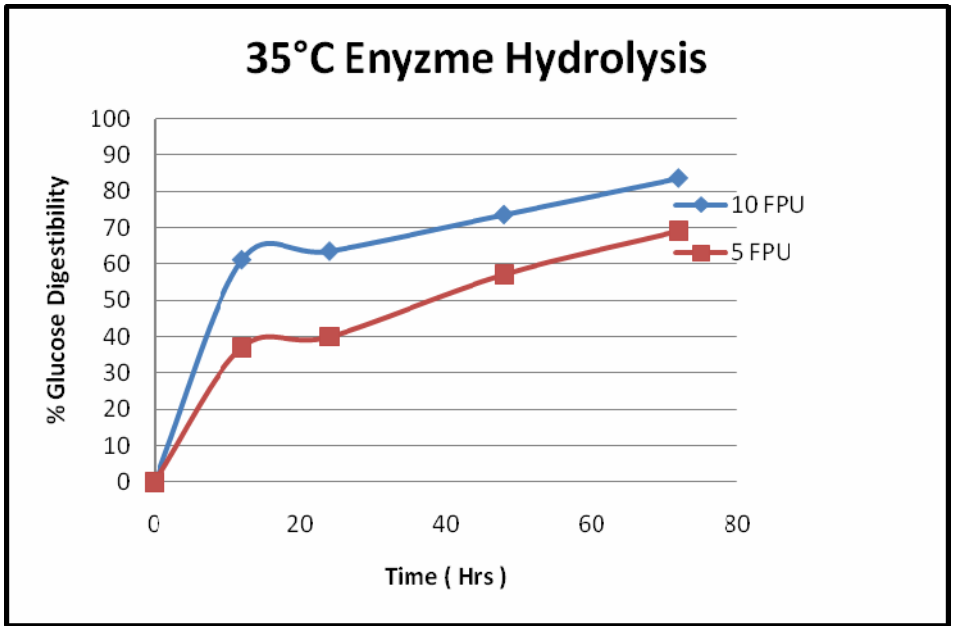


Figure 6.9 COSLIF enzyme hydrolysis profile at 35 °C.

The 5 FPU loading resulted in a 24 % drop in digestibility by 24 hours. With the high level of lignin in the SSO/softwood substrate, it was no surprise that enzyme loadings could only be dropped so far. Sharma et al. (2002) found as much as a 2 fold increase in sugar release from 5 to 10 FPU loadings when treating steam exploded sunflower stalks. From the 35 °C profile, it was also obvious that this temperature condition is much less effective than 40 °C at 24 hours. In looking at the HPLC chromatograms for 35 °C, a trend can be seen that does not exist in the 40 °C samples. On the 12 and 24 hour profiles as shown

in **Figure 6.9** and **Figure 6.10**, there is a disproportionate amount of cellobiose (10.6 min peak), to that of the 48 and 72 hour images (**Figure 6.11** and **Figure 6.12**), as well as in comparison to the 12 hour 40 °C chromatogram (**Figure 6.13**). It is possible that the lower temperature is disproportionately slowing the β-glucosidase enzyme in the Accellerase 1500 enzyme cocktail, which would slow the conversion of cellobiose to glucose.

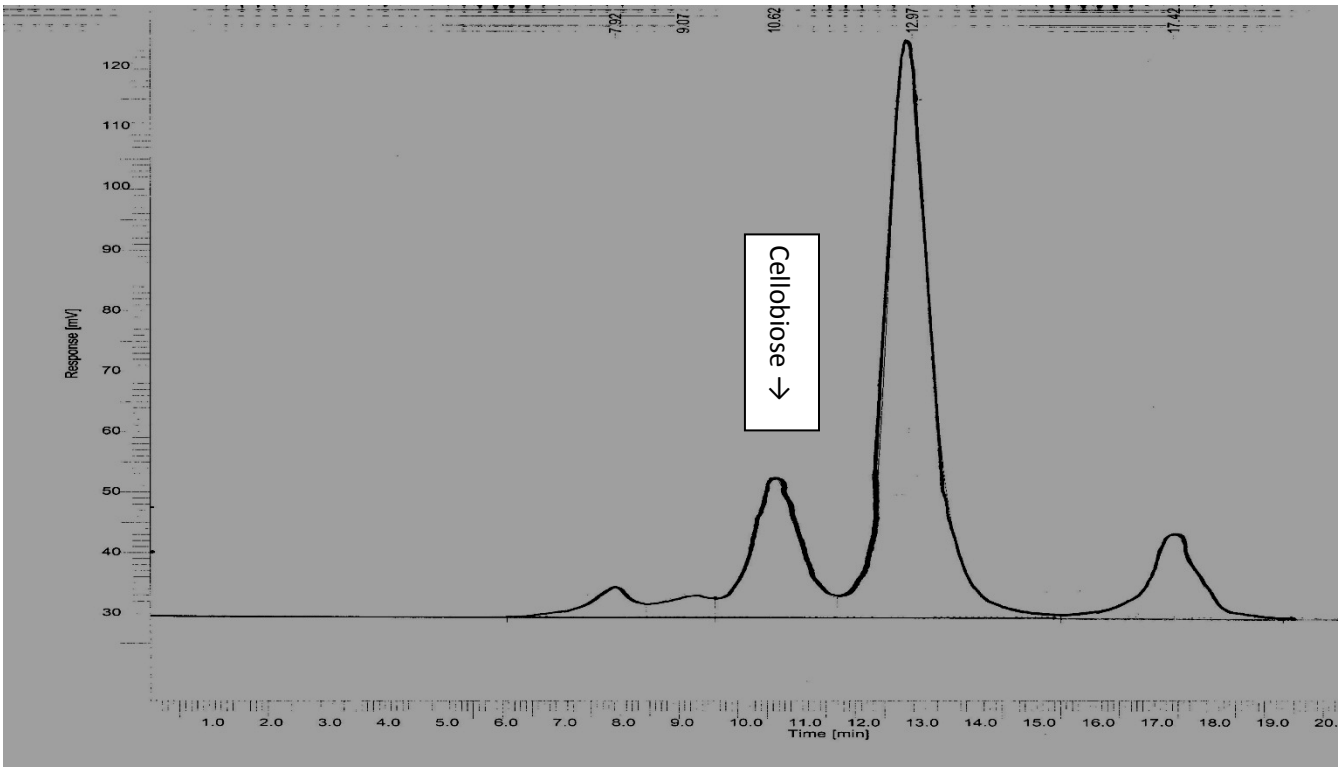


Figure 6.9 COSLIF 12 hour chromatogram for 5 FPU loading at 35 °C.

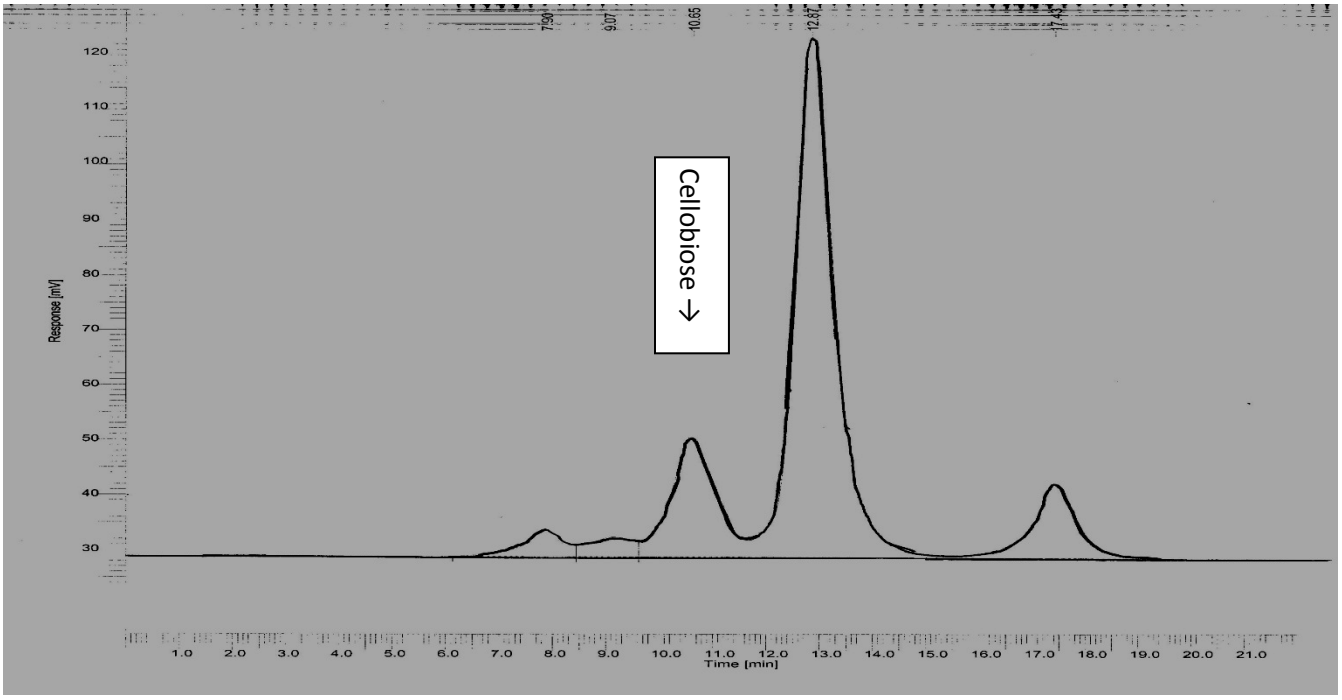


Figure 6.10 COSLIF 24 hour chromatogram for 5 FPU loading at 35 °C.

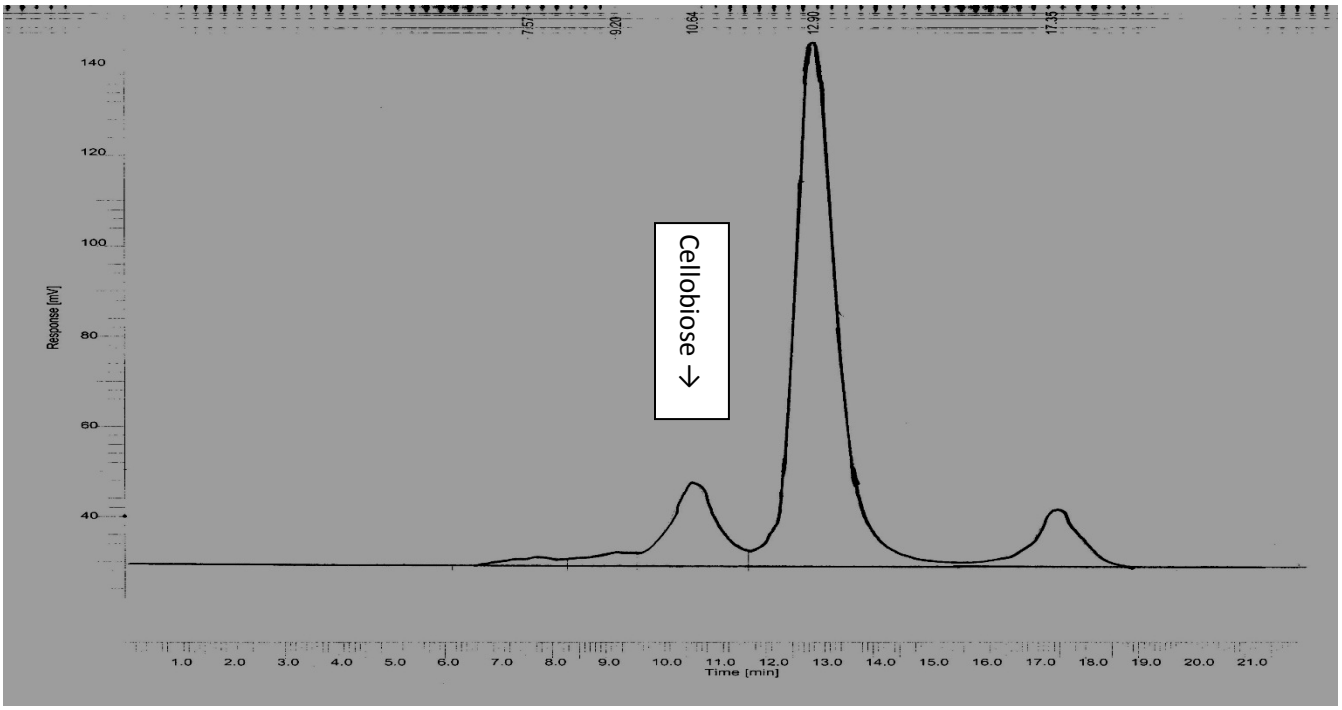


Figure 6.11 COSLIF 48 hour chromatogram for 5 FPU loading at 35 °C.

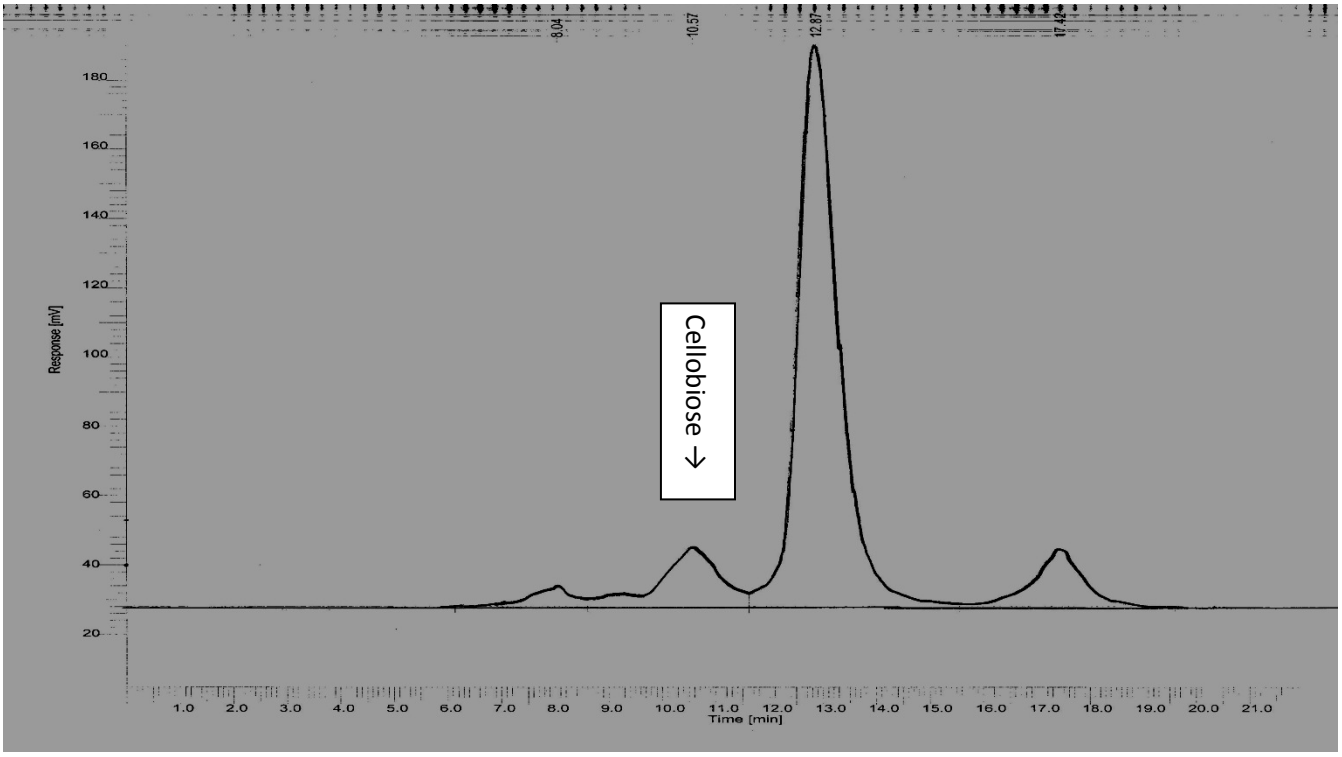


Figure 6.12 COSLIF 72 hour chromatogram for 5 FPU loading at 35 °C.

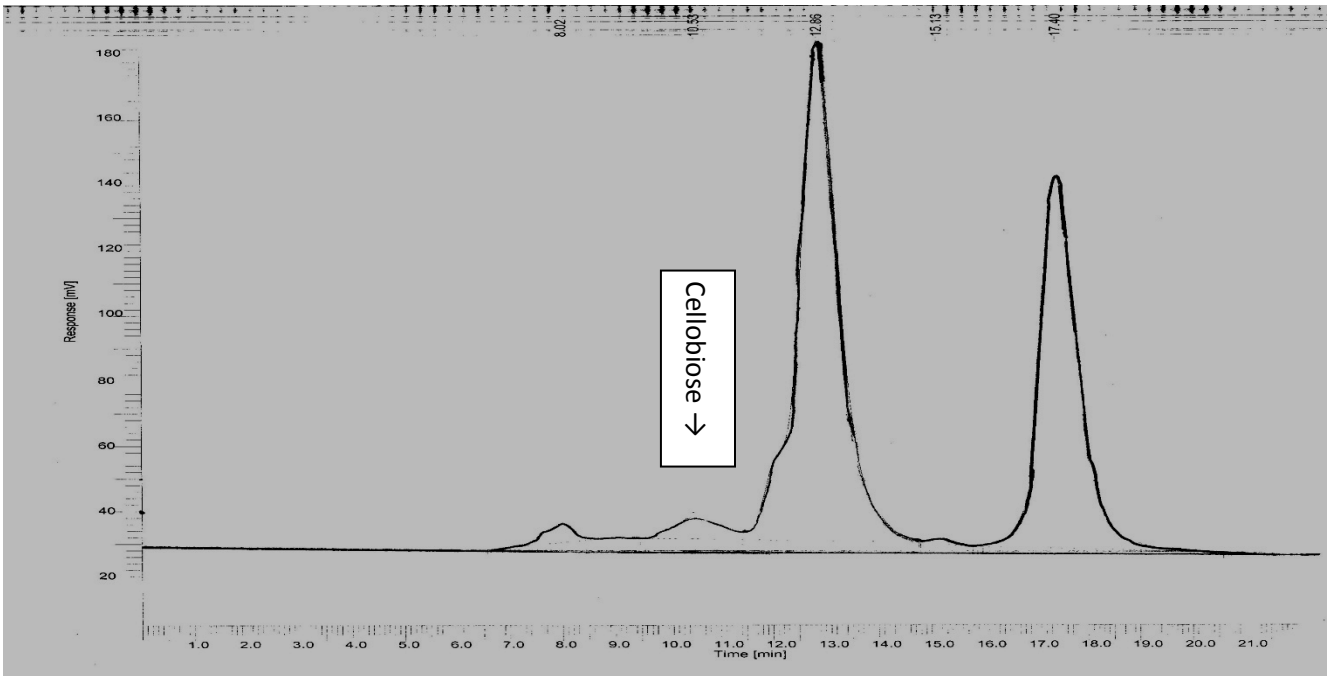


Figure 6.13 COSLIF 12 hour chromatogram for 40 °C.

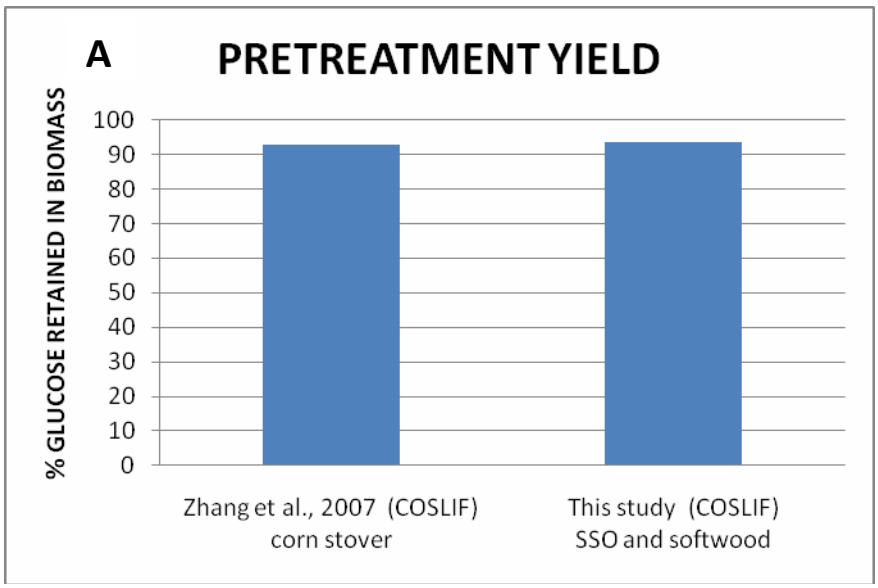
Regardless of the reasoning behind the slower cellobiose/glucose conversion at 35 °C, since *Z. mobilis* functions to ferment within 24 hours at 37 °C, the ideal temperature for an SSCF system will be between 37 and 40 °C. The ideal loading for that system will be approximately 10 FPU / g glucan.

The 10 FPU / g glucan loading was checked against the claim Genencor has made that, "Accellerase DUET puts you face-to-face with a startling economic threshold: 50 cents enzyme cost per gallon of ethanol."(Genencor,http://www.genencor.com/wps/wcm/connect/genencor/genencor/products_and_services/business_development/biorefineries/accellerase/accellerase_product_line/accellerase_product_line_en.htm) From the values calculated in **Table 6.5**, *Z. mobilis* can ferment 1 kg of glucan to approximately 0.186 gallons of ethanol. If the overall conversion of a 10 FPU loading at 40 °C is considered, then 0.151 gallons would be fermented from 1 kg glucan added to the fermentor (0.186 x 0.81) **Table A2-3** (**Appendix 2**) shows there is a little more than 0.1 g xylose / g glucose released. This would increase the total ethanol to approximately 0.151 + 0.015 = 0.166 gallons, per kg glucan added, since xylose utilization is approximately the same as glucose (Zhang, 2003). Therefore it would require 1/0.661 = 6.024 kg of glucan to generate a gallon of ethanol. The enzyme cost per kg of glucan at 10 FPU is $ 0.096 (**Table 6.3**). The actual loading was in fact 9.74 FPU (**Table A2-7, Appendix 2**), which would make the actual cost approximately $0.093 / kg glucan. Therefore, the total cost in enzyme to convert a 6.024 kg of glucan to 1 gallon of ethanol would be approximately $ 0.56, which is indeed very close to the Genencor claim.

6.2.5 COSLIF pretreatment results summary

The pretreatment glucose yield was found to be 93.7 %. The highest enzyme hydrolysis yield found was 93.5 % for a loading of 30 FPU/ g glucan at 50 °C. These yields combined represent an overall yield of 87.6 %. The best hydrolysis yield found at lower loading (10 FPU / g glucan), was 83.5 %. The overall yield was 78.2 %. This is comparable to the 77 % yield found in the Soderstom et al. study (2003), on

dilute acid treatment of softwood, which had a much higher loading of 15 FPU cellulase / g dry biomass + 23 IU β-glucosidase / g dry biomass. Both the pretreatment yield and the hydrolysis yield are similar to those found in the COSLIF study done by Zhang et al. (2007), using a loading of 15 FPU cellulase / g glucan + 60 IU/g glucan β-glucosidase. The Zhang et al. (2007) study found pretreatment and hydrolysis yields of 92.9 % and 97.0% for corn stover. The hydrolysis yield was 75.0 % for softwood. **Figure 6.14**, summarizes the comparisons to those studies with the results of this experiment.



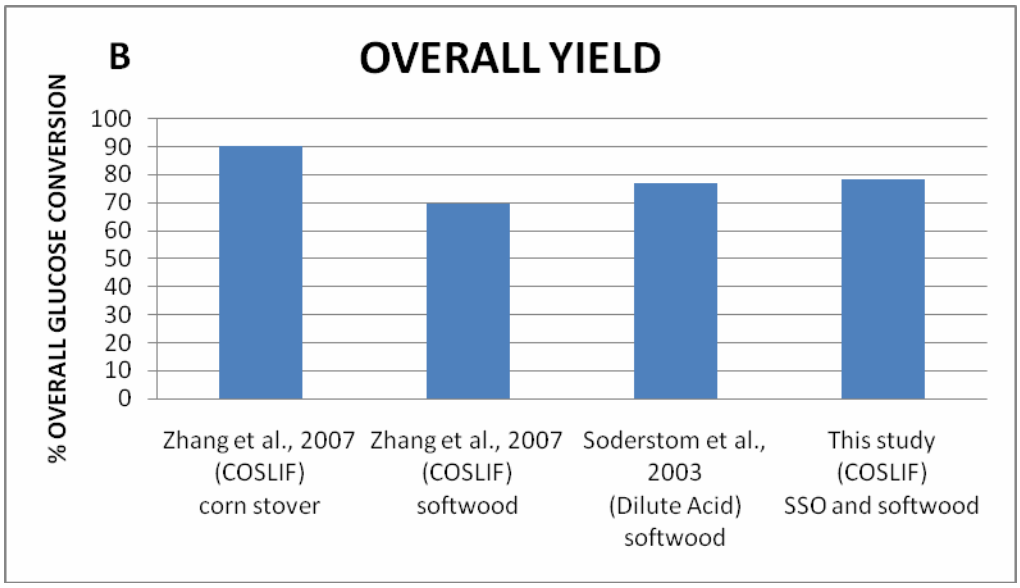


Figure 6.14 COSLIF pretreatment results comparison (**A**),
COSLIF and dilute acid results comparison for overall yield (**B**).

The higher pretreatment yield for mixed SSO and softwood, to that reported for corn stover, is as would be expected with a higher lignin content to protect the material from degradation. Consequently, higher lignin content is a reason to expect a less effective hydrolysis in comparison to corn stover, as was found in both this study and the Zhang et al. (2007) figures for softwood.

At a higher loading of 30 FPU / g glucan, the 87.6 % overall yield is very close to the approximate yield of 89.5% found for a loading of 60 FPU / g glucan in the previous COSLIF study, completed as a part of this project (Ehsanipour, 2010).

In terms of enzyme cost maximization, the lowest (10 FPU) loading tested was found to be the most cost effective, based on current ethanol and cellulase pricing. A drop in temperature slowed the rate of enzyme action but did not affect the overall conversion, except for the 30 °C samples. In fact, the peak conversion rate was slightly higher for 35 °C than for 40 °C, though it took longer to reach the peak. The same phenomenon of higher conversion rates after time for lower temperature, was found in a study on

lime treated corn stover by Kaar et al. (2000) when studying the difference between 40 and 50 °C on enzyme action.

The 40 °C enzyme hydrolysis peaked between 12 and 24 hours, while the 35 °C hydrolysis peaked at 72 hours. Since the bacteria of interest for this project, *Zymomonas mobilis 8b*, has been shown to ferment large quantities of sugar to ethanol within 24 hours at 37 °C, (Zhang, 2003), the best temperature for a simultaneous saccharification and fermentation would likely be between 37 °C and 40 °C.

7 CONCLUSIONS AND RECOMMENDATIONS

This study assessed two pre-treatments for their effectiveness to prepare SSO mixed with wood waste for enzymatic hydrolysis, and compared them to other pretreatment techniques. SSO and forestry waste (hardwood) was treated by the long term lime method, and SSO and demolition waste (softwood) by the COSLIF method.

7.1 Conclusions

Conclusions about the effectiveness of both lime and COSLIF treatments for the mixed SSO wood waste are as follows:

1. Long term lime treatment is not an effective pretreatment for SSO and forestry waste. The need for multiple additions of cellulase, that are made inactive by lime release even after post treatment stabilization, indicates that the cost of the treatment would be prohibitive.
2. COSLIF is an effective treatment for SSO and demolition waste (softwood). Even at a lower loading (10 FPU), than a similar study on softwood (15 FPU + cellobiase, Zhang et al., 2007), the glucose release was higher.
3. Since the conversion rates for COSLIF treated SSO and softwood were high, there is a high degree of certainty that SSO and a mixture of almost any organic material would achieve high conversion rates as well. Softwood has extremely high lignin content and has been found previously by Zhang et al. (2007) to be far less amenable to COSLIF treatment than corn stover, poplar wood, or switchgrass.
4. COSLIF is a good pretreatment for SSO mixed with demolition waste in terms of the rate of enzyme hydrolysis. The rapid rate found in other studies was confirmed in this study on SSO/softwood. The ramifications of rapid hydrolysis are that a simultaneous saccharification

and fermentation system could operate much more quickly than systems employing other pretreatments. COSLIF treated SSO and softwood were fully hydrolyzed in approximately 12 hours, which means almost any mixed SSO biomass would be hydrolyzed in the same period. All other conditions being equal, this represents a doubling of capacity in the same size digester for pretreatments showing a 24 hour time to reach near peak figures for enzyme hydrolysis. Using COSLIF instead of pretreatments requiring a 72 hour hydrolysis, 6 times more material could be processed in the same size digester.

5. Lowering temperature slows the rate of hydrolysis for COSLIF treated SSO/softwood, but not necessarily the overall yield. The slower rate was barely noticeable at 40 °C, but pronounced at 35 °C.
6. Since the peak conversion was reached between 12 and 24 hours at 40 °C, but the largest conversion was not found until 72 hours at 35 °C, the ideal temperature for a simultaneous saccharification and fermentation with *Z. mobilis 8b*, will be between 37 °C and 40 °C. *Z. mobilis*, strain 8b, can ferment to completion at 37 °C within 24 hours (Zhang et al., 2003).
7. The ideal loading for most profit generated per kg glucan converted to ethanol, out of a range of 10, 15, 30 and 60 FPU, was 10 FPU. The best loading was found using the price of ethanol futures, as well as current costs of Genencor Accellerase brand enzymes. The activity level ratio of Accellerase Duet, to that of the Accellerase 1500 used in this study, is advertised as 3 to 1. If the price of ethanol used is more conservative at current prices, or the Accellerase Duet enzyme is less active than advertised, the 10 FPU loading becomes even more profitable in relation to the other loadings.
8. There was a significant (25%) drop in glucose conversion between 5 and 10 FPU loadings at 35°C by the 12th and 24th hour marks. Therefore, the ideal loading in the simultaneous

saccharification and fermentation at 37 to 40 °C, will be somewhere between 5 and 10 FPU, likely closer to 10 FPU.

7.2 Recommendations for future study

The research results of this paper have shown that the COSLIF method can be effectively employed to break down SSO mixed with demolition waste. The enzyme hydrolysis was accomplished at a cellulase loading of only 10 FPU per gram glucan in the treated biomass. The hydrolysis rapidly reached a peak between 12 and 24 hours, at 40 °C. However, in light of the commercialization goals of this project, more information is needed to confirm the efficacy of the COSLIF process in comparison to the cheapest, and most effective pre-treatment to date: dilute acid. Recommendations for testing that would allow comparison of the COSLIF method to dilute acid pretreatment for commercialization are as follows:

1. A fermentation study of *Z. mobilis* utilizing the post COSLIF hydrolysis hydrolysate with addition of 1% v/v corn steep liquor. Corn steep liquor is the cheapest nutrient additive, and has already been tested effective for *Z. mobilis* fermentation (Lawford and Rouseau, 1997). Included in that study would be fermentation of a broth of pure sugars, mirroring the concentrations found in the COSLIF hydrolysate. The pure sugar fermentation would allow for conclusions to be drawn about the existence of possible fermentation inhibitors, caused by the COSLIF process.
2. A fermentation study of *Saccharomyces cerevisiae* (baker's yeast), on the same hydrolysate if *Z. mobilis* was found to be inhibited by the broth.
3. A simultaneous saccharification and fermentation study of the COSLIF pretreated material with *Z. mobilis*, or yeast in the event that *Z. mobilis* is inhibited. The fermentation would be a continuous study started at a temperature of 40 °C, and kept there until output was stable. Since results for the ideal temperature between 37 and 40 °C have not yet been found, the

temperature would be decreased by a degree at a time until 37 °C, establishing output stability between drops, and recording the overall sugar and ethanol levels. Once the ideal temperature was reached, the cellulase loading could be lowered slowly until the decrease in ethanol, at a conservative price, equaled the decreased cost in enzyme dosage. The experimental conditions could be repeated at different digester residence times, varying upwards from 12 hours until peak ethanol conversions could be reached. The greatest expected profit regime for the fermentation/saccharification tank could be found from the above conditions given the cost (size) of the tank, cost of cellulase, and revenue from ethanol. These costs/revenues would be quantified by combinations of residence time, cellulase loading, and ethanol yield.

4. Most importantly, the recycle efficiencies of the COSLIF process would have to be tested. Whereas dilute acid treatment costs are well known and modeled, COSLIF has not been employed at the commercial scale. The commercialization of the COSLIF method relies on the ease of recycle for the comparably far more expensive solvents. The losses for each solvent during a pass through the process would have to be calculated, as well as the saturation point of the phosphoric acid, acetone, and ethanol. Each time the solvents pass through the process, some portion of the sugar, lignin, and other components in the biomass will be solubilized. At some point, these dissolved components will start to interfere with the efficiency of the dissolution and/or precipitation of the biomass components. After the limits of recycling efficiency are found, cost comparisons of the two processes can begin to be made.

Although the long term lime results were not encouraging, the process could be very well suited to a pilot plant facility. Consequently, further research into separating lime from mixed SSO long term lime treated biomass could be valuable. A washing process that removed the bonds between lime and mixed SSO, without removing or degrading the fermentable sugars, could still prove long term lime an ideal treatment process.

REFERENCES

Adney, B., & Baker J. (2008) *Measurement of Cellulase Activities*. Laboratory Analytical Procedure (LAP), Technical Report, NREL/TP-510-42628. National Renewable Energy Laboratories (NREL).

Broder, J. D.; Barrier, J. W.; Lee, K. P.; Bulls, M. M. Biofuels system economics. (1995) *World Resource Re*view. Volume *7* (4), p. 560–569.

Beatty, D. (Manager of the Dufferin Transfer station in 2006) Personal communication from site visit, 2006.

Cazetta, M.L., Celliogoi, M.A.P.C., Buzato, J.B., Scarmino, I.S. (2007) Fementation of molasses by Zymomonas mobilis: Effects of temperature and sugar concentration on ethanol production. *Bioresource Technology*, (98), p.2824-2828.

City of Toronto. *Waste Diversion 2010 Task Force Report*. Toronto: June, 2001. Electronic resource, accessed November 20, 2006 at http://www.toronto.ca/taskforce2010/report

Crutzen, P.J., Mosier, A.R., Smith, K.A., Winiwarter, W. (2008) N2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmospheric Chemistry and Physics*, (8), p.389-395.

Crupi, Mike. (Owner of Vartek Ind.) Personal communications from site visit and ongoing association as project material supplier.

Desvaux, M. (2005) Clostridium cellulolyticum: model organism of mesophilic cellulolytic clostridia. *FEMS Microbiology Reviews*, (29), 741-764.

Estey, M. November 17, 2006. *Out of mind, but no longer out of sight*. The Ubyssey. Accessed December 13, 2006 at www.ubyssey.bc.ca

Eggman, T., & Elander, R.T. (2005) Process and economic analysis of pretreatment technologies. *Bioresource Technology*, (96), p.2019-2025.

Ehsanipour, M. (2009) *Acid pretreatment and fractionation of source separated organic waste for lignocellulosic saccharification*. Master's Thesis, Environmental Applied Science and Management. Ryerson University, Toronto.

Fargione, J., Hill, J., Tilman, D., Polasky, S., Hawthorne, P. (2008) Land Clearing and the Biofuel Carbon Debt. *Science,* (319), p.1235-1237.

Genencor, (2010) (online product information sheet) Accellerase 1500 Cellulase Enzyme Complex for Lignocellulosic Biomass Hydrolysis. Accessed on April 10, 2010, at http://www.genencor.com/wps/wcm/connect/09a976004fa2d4ceaecbbe4895e3224e/ACCELLERASE+1500+product+information+sheet.pdf?MOD=AJPERES&CACHEID=09a976004fa2d4ceaecbbe4895e3224e

Genencor (webpage) Accellerase Product line. Accessed April 10, 2010 at http://www.genencor.com/wps/wcm/connect/genencor/genencor/products_and_services/business_development/biorefineries/accellerase/accellerase_product_line/accellerase_product_line_en.htm

Ghose, T.K. (1987) Measurement of Cellulase Activities. *Pure and Applied Chemistry*, (59), p.257-268.

Gupta, R. (2008) *Alkaline pretreatment of biomass for ethanol production and understanding the factors influencing the cellulose hydrolysis.* PhD Dissertation. Aubern University, Alabama.

Hamelinck, C.N., Van Hooijonk, G., Faaij, A.P.C. (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy*, (28), p.384-410.

Hames, B., Ruiz, R., Scarlata, C., Sluiter, A., Sluiter, J., Templeton, D. (2008) *Preparation of Samples for Compositional Analysis*. Laboratory Analytical Procedure (LAP), Technical Report NREL/TP-510-42620. National Renewable Energy Laboratories (NREL).

Kaar, W. E., & Holtzapple, M. T. (2000) Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover, *Biomass and Bioenergy*, (18), p.189-199.

Kim, Se Hoon. (2005) *Lime pretreatment and enzymatic hydrolysis of Corn Stover*. PhD Dissertation, Chemical Engineering. Texas A & M Universtiy.

Kim, T. H., and Lee Yoon.Y. (2003) Pretreatment of corn stover by soaking in aqueous ammonia. *Applied Biochemistry and Biotechnology*, (121-124), p.1119-1132

Kim, T.H., Lee, Y.Y., Kim, J.S. (2005) Pretreatment of corn stover by low-liquid ammonia percolation process. *Bioresource Technology*, (96), p.2007-2013.

Koplow, D. (2007) *Biofuels – at what cost ? Government support for ethanol and biodiesel in the United States : 2007 Update.* The Global Subsidies Initiative (GSI) of the International Institute for Sustainable Development (IISD). Geneva, Switzerland. Accessed on Feb 14, 2009 at http://www.iisd.org/pdf/2007/biofuels_subsidies_us_update.pdf

Kumar, P., Barret, D.M., Delwiche, M.J., Stroeve, P. (2009) Methods fro Pretreatment of Lignocelllosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial and Engineering Chemistry Research*, (48), p.3713-3729.

Kumar, R., & Wyman, C. (2009,A) Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. *Bioresource Technology*, (100), p.4203-4213.

Kumar, R., & Wyman, C. (2009,B) Effects of cellulase and Xylanase Enzymes on the Deconstruction of Solids from Pretreatment of Poplar by Leading Technologies. *Biotechnology Progress*, (25), 2, p.302-314.

Lawford, H.G., & Rouseau, J.D. (1997) Corn Step Liquor as a Cost=Effectie Nutrition Adjunct in High-Performance Zymomonas Ethanol Fermentations. *Applied Biochemistry and Biotechnology*, (63-65), p.287-304.

Liu, C., &Wyman, C. (2005) Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. *Bioresource Technology*, (96), 18, p. 1978-1985.

Lloyd, T., &Wyman, C. (2005) Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresource Technology*, (96), 18, p.1967-1977.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, (66), 506-577.

Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M. (2005) Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology*, (16), p. 577-583.

Miller, G.L. (1959) Use of Dinitosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, (31), p.426-428.

Mirzajani, M. (2009) The amenability of pre-treated source separated organic (SSO) waste for ethanol production. Masters Thesis, Department of Civil Engineering, Ryerson University.

Mitchell, D. (2008) A note on Rising Food Prices. Policy research working paper 4682. The World Bank Development Prospects Group. Accessed February 2009, at http://econ.worldbank.org/external/default/main?pagePK=64165259&piPK=64165421&theSitePK=469372&menuPK=64216926&entityID=000020439_20080728103002.

Mosier, N. S., Wyman, C., Dale, B., Elander, R., Lee, Y. Y.,Holtzapple, M., Ladisch, M. R. (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology,* (96), p. 673–686.

National Renewable Energy Laboritories (NREL), 2002. *Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover.* Technical Report. NREL/TP-510-32438

Oxfam. (2008) *Another inconvenient truth: How biofuel policies are deepening poverty and accelerating climate change.* Briefing Paper. Accessed on Feb.2, 2009 at www.oxfam.org/policy/another-inconvenient-truth

Ramirez, R.S. (2005) Long-term lime treatment of poplar wood. Master's thesis, Chemical Engineering, Texas A & M University.

Rosegrant, M. (2008) *Biofuels and Grain Prices: Impacts and Policy Responses.* International Food Policy Research Institute (IMPRY). Accessed on Feb 2, 2009 at http://www.ifpri.org/pubs/testimony/rosegrant20080507.asp

Saha, B.C., &Cotta, M.A. (2008) Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. *Biomass and Bioenergy*, (32), p.971-977.

Selig, M., Weiss, N., Ji, Y. (2008) *Enzymatic Saccharification of Lignocellulosic Biomass.* Laboratory Analytical Procedure (LAP), Technical Report NREL/TP-510-42629. National Renewable Energy Laboratories (NREL).

Sharma, S.K., Kalra, K.L., Grewal, H.S. (2002) Enzymatic saccharification of pretreated sunflower stalks. *Biomass and Bioenergy*, (23), p.237-243.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. (2008) Determination of Stuctural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure (LAP), Technical Report NREL/TP-510-42618. National Renewable Energy Laboratories (NREL)

Stenberg, K., Bollok, M., Reczey, K., Galbe, M., Zacchi, G. (2000) Effect of Substrate and Cellulase Concentration on the Simultaneous Saccharification and Fermentation of Steam-Pretreated Softwood for Ethanol Production. *Biotechnology and Bioengineering*, (68), 2, p.204-210.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B.E. (2005) Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresource Technology*, (96), 18, p.2014-2018.

Toronto Star, March 1, 2009. *Green bin waste trucked to N.Y. Ontario municipalities 'scrambling' to cope with surge in kitchen refuse and plant closings.* Moira Welsh, Environmental Reporter. Page A1 and A6.

Thoenes, P. (2007) Biofuels and Commodity Markets – Palm Oil Focus. FAO, Commodities and Trade Division. Accessed on March 22, 2009 at http://www.rlc.fao.org/es/prioridades/bioenergia/pdf/commodity.pdf
USDA and DOE. (2008) *Responses to questions for Senator Bingaman.* Accessed on Feb 2, 2009 at http://www.energy.gov/media/Secretaries_Bodman_and_Schafer_Ltr_to_Sen_Bingaman.pdf

U.S. Energy Information Administration. Online petroleum statistics. Accessed September 18, 2008 at: http://tonto.eia.doe.gov/dnav/pet/hist/wtotworldw.htm.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M.A., Ladisch, M.R., Lee, Y.Y. (2005) Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, (96), 18, p.1959-1966.

Yang, B., & Wyman, C.E. (2007) Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioproducts, Biorefineries, (2), p.26-40

Zhang, M. (2003) *Zymomonas mobilis: Special Topics Session Microbial Pentose Metabolism.* 25th Symposium on Biotechnology for Fuels and Chemicals. National Bioenergy Center, National Renewable Energy Laboratory. Accessed July, 2009 at http://www1.eere.energy.gov/biomass/pdfs/34264.pdf

Zhang, P. Y-H., & Lynd, L. (2004) Toward an Aggregated Understanding of Enzymatic Hydrolysis ofCellulose: Noncomplexed Cellulase Systems. *Biotechnology and Bioengineering*, Vol. 88, No.7, p. 797-824.

Zhang, P.Y-H., Ding, S-Y., Mielenz, J.R., Cui, J-B., Elander, R.T., Laser, M., Himmel, M.E., McMillan, J.R., Lynd, L.R. (2007) Fractionating Recalcitrant Lignocellulose at Modest Reaction Conditions. *Biotechnology and Bioengineering,* (97), 2, p.214-223.

APPENDIX 1 QUANTITATIVE SACCHARIFICATION

A1.1 Procedure for determination of structural carbohydrates and lignin in biomass

The procedure is taken for the most part directly from the NREL, "Determination of Structural Carbohydrates and Lignin in Biomass" Laboratory Analytical Procedure (LAP), Revised April 2008, Technical Report # NREL/TP-510-42618. (Sluiter et al., 2008)

Terminology

1. *Oven dry weight (ODW)*- the weight of biomass mathematically corrected for the amount of moisture present in the sample at the time of weighing.
2. *Prepared biomass*- biomass prepared according to LAP "Preparation of Samples for Biomass Compositional Analysis".
3. *Extractives free biomass* - Biomass after exhaustive water and ethanol extraction (refer to LAP "Determination of Extractives in Biomass").
4. *Acid insoluble lignin*- the residue remaining on an ashless 0.2µm filter paper
5. *Structural carbohydrates*-Polymeric carbohydrates, namely cellulose and hemicellulose.
6. *Non-structural components*- Non-chemically bound components of biomass that include but are not limited to sucrose, nitrate/nitrites, protein, ash, chlorophyll, and waxes.

Apparatus

1. Analytical balance, accurate to 1 mg.
2. Drying oven, with temperature control of 105 ± 3oC
3. Muffle furnace, equipped with a thermostat, set to 575 ± 25 °C or equipped with optional ramping program
4. Pot and hot plate, controled at 30 ± 3 °C
5. Autoclave, suitable for autoclaving liquids, set to 121 ± 3 °C
6. Filtration setup, equipped with a vacuum source
7. Desiccator containing desiccant
8. HPLC system equipped with refractive index detector and the following columns:
   Shodex sugar SP0810 or Biorad Aminex HPX-87P column (or equivalent) with ionic form H+/CO3- deashing guard column
   Biorad Aminex HPX-87H column (or equivalent) equipped with an appropriate guard column

Reagants

1. Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20oC)
2. Calcium carbonate
3. Water, purified, 0.2 µm filtered
4. Sugar standards : D(+)glucose, D(+)xylose, D(+)galactose, L(+)arabinose, and D(+)mannose

Materials

1. Glass serum bottles, 125 ml capacity, with septum and clamp seal.
2. Plastic stir rods sized to fit in serum bottle and extent at least 3 cm longer.
3. Ashless, 0.2 µm Whatman filters.
4. Filtration flasks, 250 mL
5. Adjustable pipettor, 1.00 ml.
6. pH paper, range 1-11.
7. pH meter.
8. Disposable syringes, 3 mL, fitted with 0.2 syringe filters.
9. Autosampler vials.

Procedure

1. Weigh 300 mg of the sample prepared by the NREL Laboratory Analytical Procedure (LAP), "Preparation of samples of Biomass Compositional Analysis" and add to serum bottle.
2. Before, after or during the test, add ten 1.000 g samples to some sort of heat resistant dish, and find the moisture content by taking the difference in weight of the material after exposure to 105 ± 3 °C for 24 hrs, or until the change in weight is less than 0.1% over the space of one hour. A minimum total solids for this procedure is 85%.
3. Add 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72% sulfuric acid to serum bottle. Use a Plastic (acid resistant) stir rod to mix for one minute, or until the sample is thoroughly mixed.
4. Place the bottle in a water bath set at 30 ± 3 °C or equivalent system (pot and hot plate) and incubate the sample for 60 ± 5 minutes. Using the stir rod, stir the sample every five to ten minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.
5. Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath. Dilute the acid to a 4% concentration by adding 84.00 ± 0.04 mL deionized water using an automatic burette or scale. Dilution can also be done by adding 84.00 ± 0.04 g of purified water using a balance accurate to 0.01 g. Stopper the caps securely. Mix the sample by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

Analysis of Lignin

1. Vacuum filter the autoclaved hydrolysis solution through one of the previously weighed ashless filters. Capture the filtrate in a filtering flask.
2. Use deionized water to wash all remaining solids out of the serum bottle into the filtering flask. Rinse the solids with a minimum of 50 mL fresh deionized water
3. Dry the filter and acid insoluble residue at 105 ± 3 °C until a constant weight is achieved, usually a minimum of four hours.
4. Remove the samples from the oven and cool in a desiccator. Record the weight of the filter and dry residue to the nearest 1 mg.
5. Place the crucibles and residue in the muffle furnace at 575 ± 25 °C for 24 ± 6 hours.
6. A furnace with temperature ramping may also be used Furnace Temperature Ramp Program:
   - Ramp from room temperature to 105 °C
   - Hold at 105°C for 12 minutes
   - Ramp to 250 °C at 10°C / minute
   - Hold at 250 °C for 30 minutes

- Ramp to 575 °C at 20 °C / minute
- Hold at 575 °C for 180 minutes
- Allow temperature to drop to 105 °C
- Hold at 105 °C until samples are removed

7. Carefully remove the crucible from the furnace directly into a desiccator and cool. Weigh the filters and ash to the nearest 1 mg and record the weight.

Analyze the sample for structural carbohydrates

1. Prepare a series of calibration standards containing the compounds that are to be quantified. Suggested range is 0.1 to 4.0 mg / ml. Use a four point calibration. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.
2. A fresh set of standards is not required for every analysis. A large batch of standards may be produced, filtered through 0.2 µm filters into autosampler vials, sealed and labeled. The standards may be stored in a freezer and removed when needed. Thaw and vortex frozen standards prior to use. During every use, standards should be observed for unusual concentration behavior. Unusual concentrations may mean that the samples are compromised or volatile components have been lost. Assuming sufficient volume, standards and CVS samples should not have more than 12 injections drawn from a single vial. In a chilled autosampler chamber, the lifetime of standards is approximately three to four days.
3. Using the hydrolysis liquor obtained in step 2 of the Lignin analysis procedure, transfer an approximately 20 mL aliquot of each liquor to a 50 mL Erlenmeyer flask.
4. Use calcium carbonate to neutralize each sample to pH 5 – 6. Avoid neutralizing to a pH greater that 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5 – 6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant. The pH of the liquid after settling will be approximately 7. (Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.) After reaching a pH of 4, the sample can alternatively be swirled around a set pH meter probe and adjusted slowly.
5. Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 µm filter into an autosampler vial. Seal and label the vial. If necessary, neutralized samples may be stored in the refrigerator for three or four days. After this time, the samples should be considered compromised due to potential microbial growth. After cold storage, check the samples for the presence of a precipitate. Samples containing a precipitate should be refiltered, while still cold, through a 0.2 µm filters.
6. Analyze the calibration standards and samples by HPLC using a Shodex sugar SP0810 or Biorad Aminex HPX-87P column equipped with the appropriate guard column.
7. HPLC conditions:
8. Injection volume: 10 – 50 µL, dependent on concentration and detector limits
9. Mobile phase: HPLC grade water, 0.2 µm filtered and degassed
10. Flow rate: 0.6 mL / minute
11. Column temperature: 80 - 85°C
12. Detector temperature: as close to column temperature as possible
13. Detector: refractive index
14. Run time: 22 minutes
15. Note: The de-ashing guard column should be placed outside of the heating unit and kept at ambient temperature. This will prevent artifact peaks in the chromatogram.
16. Check test sample chromatograms for presence of cellobiose and oligomeric sugars. Levels of cellobiose greater than 3 mg/mL indicate incomplete hydrolysis. Fresh samples should be hydrolyzed and analyzed.
17. Check test sample chromatograms for the presence of peaks eluting before cellobiose (retention time of 4-5 minutes using recommended conditions). These peaks may indicate high levels of sugar degradations products in the previous sample, which is indicative of over hydrolysis. All samples from batches showing evidence of over-hydrolysis should have fresh samples hydrolyzed and analyzed.

Calculations

1. Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content as determined by the LAP "Standard Method for the Determination of Total Solids in Biomass".

$$\text{ODW} = \frac{Weight\ (< 45\ ^{\circ}C\ dried\ sample) \times \%\ total\ solids}{100}$$

2. Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis.

$$\%\ \text{AIR} = \frac{Weight\ of\ filter\ plus\ AIR - Weight\ filter}{ODW\ sample} \times 100$$

$$\%\ \text{AIL} = \frac{(Weight\ of\ filter\ plus\ AIR - Weight\ filter) - (Weight\ filter + ash - Weight\ filter)}{ODW\ sample} \times 100$$

3. Create a calibration curve for each analyte to be quantified using linear regression. From these curves, determine the concentration in mg/mL of each component present in the samples analyzed by HPLC, correcting for dilution if required.

4. Calculate the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or162/180) for C-6 sugars (glucose, galactose, and mannose)

   $C_{anhydro} = C_{HPLC}$ x Anhydro Correction

5. $$\%\ \text{Sugar} = \frac{Canhydro \times Volume\ filtrate}{ODW} \times 100$$

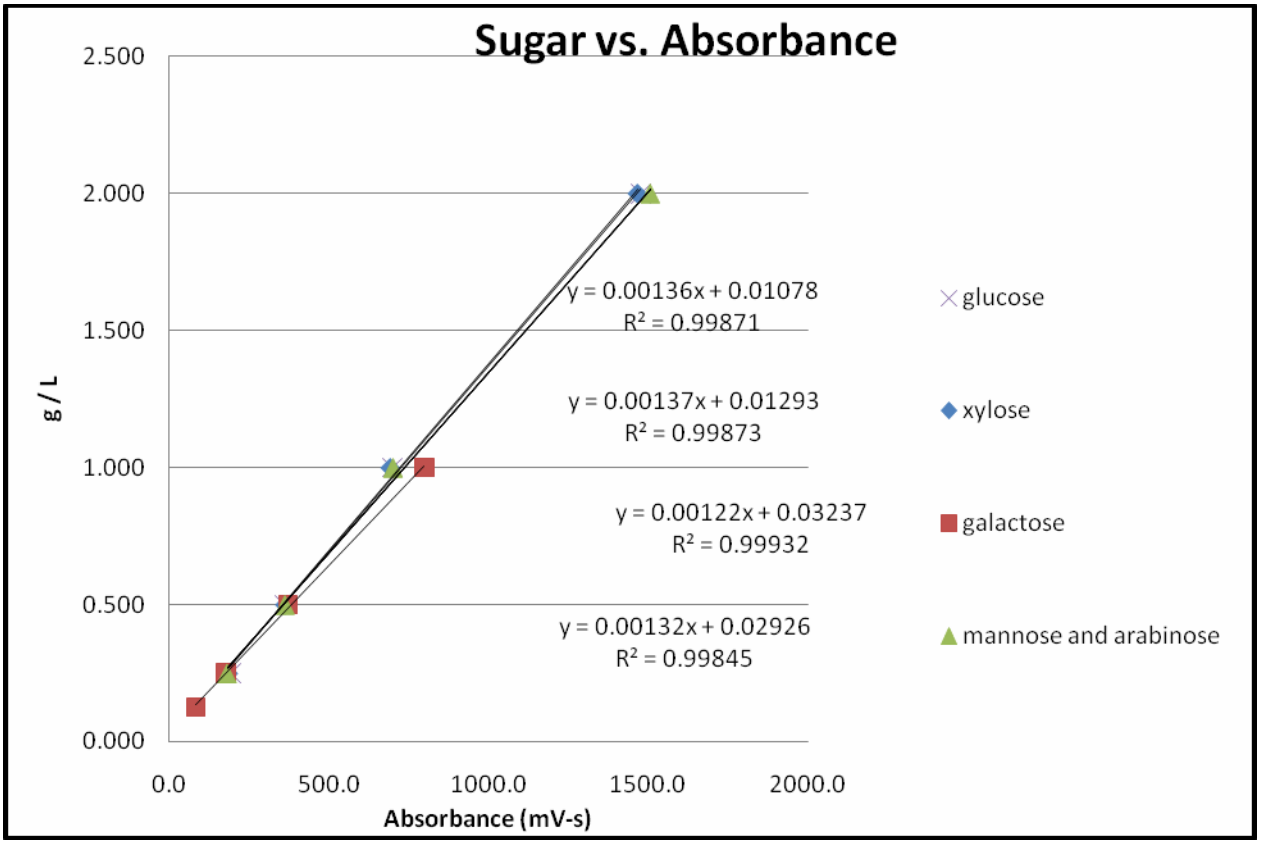
   Volume filtrate = 87 ml

A1.2 Quantitative saccharification for untreated biomass

Table A1-1 Sugar standards and their HPLC absorbance readings for initial composition

Sugar Standards (mg/ml)						Absorbance (mV s)			
Standard	glucose	xylose	galactose	mannose and arabinose		glucose	xylose	galactose	mannose and arabinose
1	2.000	2.000	1.000	2.000		1475.1	1465.9	798.7	1505.9
2	1.000	1.000	0.500	1.000		699.1	692.6	372.7	701.4
3	0.500	0.500	0.250	0.500		360.2	363.2	177.2	363.4
4	0.250	0.250	0.125	0.250		193.3	186.1	83.8	182.3

Figure A1-1 Standard Curves for initial composition



Moisture Content

Table A1-2 Initial Moisture of 45°C dried SSO and hardwood

Sample	Mass Foil (g)	Mass of filter + waste at 45°C (g)	Mass at 105°C (g)	Mass of 45C dried sample (g)	TS	MOISTURE
1	1.299	2.299	2.248	1.000	0.949	0.051
2	1.311	2.311	2.256	1.000	0.945	0.055
3	1.305	2.305	2.259	1.000	0.954	0.046
4	1.304	2.304	2.267	1.000	0.963	0.037
5	1.309	2.309	2.253	1.000	0.944	0.056
6	1.310	2.310	2.254	1.000	0.944	0.056
7	1.291	2.291	2.248	1.000	0.957	0.043
8	1.305	2.305	2.251	1.000	0.946	0.054
9	1.309	2.309	2.255	1.000	0.946	0.054
10	1.289	2.289	2.252	1.000	0.963	0.037
AVERAGE					0.951	0.049
STD DEV					0.008	0.008

Table A1-3 Initial Moisture of 45°C dried SSO and Softwood

Sample	Mass Foil (g)	Mass of filter + waste at 45°C (g)	Mass at 105°C (g)	Mass of 45C dried sample (g)	TS	MOISTURE
1	1.310	2.310	2.264	1.000	0.954	0.046
2	1.291	2.291	2.247	1.000	0.956	0.044
3	1.310	2.310	2.269	1.000	0.959	0.041
4	1.305	2.305	2.260	1.000	0.955	0.045
5	1.309	2.309	2.266	1.000	0.957	0.043
6	1.304	2.304	2.257	1.000	0.953	0.047
7	1.311	2.311	2.267	1.000	0.956	0.044
8	1.303	2.303	2.259	1.000	0.956	0.044
9	1.290	2.290	2.252	1.000	0.962	0.038
10	1.303	2.303	2.262	1.000	0.959	0.041
AVERAGE					0.957	0.043
STD DEV					0.003	0.003

HPLC and lignin results

Table A1-4 Absorbance results and corresponding HPLC mg/ml found for SSO and hardwood

Absorbance (mV-s)					HPLC sugar concentration mg/ml			
Sample	glucose	xylose	galactose	mannose and arabinose	glucose	xylose	galactose	mannose and arabinose
1	877	213	24	101	1.238	0.305	0.062	0.162
2	912	207	30	103	1.287	0.296	0.069	0.165
3	866	213	30	99	1.223	0.304	0.069	0.160
4	887	235	30	107	1.253	0.335	0.069	0.170
5	880	235	30	100	1.243	0.335	0.069	0.161
6	824	229	24	95	1.164	0.327	0.062	0.155
7	761	208	30	94	1.076	0.298	0.069	0.153
8	608	166	24	69	0.861	0.241	0.062	0.121
9	683	190	24	79	0.967	0.273	0.062	0.134
10	677	179	24	59	0.959	0.259	0.062	0.107
Anhydrous Correction	0.9	0.88	0.9	0.9	Oven Dry Weight (mg) 285.3	Total volume (ml) 87.0		

As mentioned in the materials section, mannose and arabinose were eluting in the same peak and are therefore reported together.

Table A1-5 Absorbance results and corresponding HPLC mg/ml found for SSO and softwood

Absorbance (mV-s)					HPLC sugar concentration mg/ml			
Sample	glucose	xylose	galactose	mannose and arabinose	glucose	xylose	galactose	mannose and arabinose
1	672	81	12	152	0.951	0.124	0.047	0.229
2	847	143	66	213	1.196	0.209	0.113	0.310
3	869	86	30	172	1.227	0.131	0.069	0.256
4	838	140	66	221	1.184	0.205	0.113	0.321
5	860	129	27	174	1.215	0.190	0.065	0.259
6	836	119	27	163	1.181	0.176	0.065	0.245
7	557	86	18	106	0.791	0.131	0.054	0.169
8	744	119	24	142	1.053	0.176	0.062	0.217
9	623	103	24	125	0.883	0.153	0.062	0.194
10	672	81	12	152	0.951	0.124	0.047	0.229
Anhydrous Correction	0.9	0.88	0.9	0.9	Oven Dry Weight (mg) 287.0	Total volume (ml) 87.0		

Table A1-6 Lignin results for SSO and hardwood

Sample	Mass of filter (g)	Mass at 105°C = Acid Insoluable Residue and filter (g)	105°C Residue (g)	Mass at 575°C of ash and filter paper (g)	Ash (g)	Klasson Lignin (g)	% Klasson Lignin (g)
1	0.114	0.361	0.247	0.144	0.03	0.254	25.4
2	0.114	0.363	0.249	0.146	0.032	0.254	25.4
3	0.114	0.367	0.253	0.149	0.035	0.255	25.5
4	0.114	0.368	0.254	0.147	0.033	0.258	25.8
5	0.114	0.36	0.246	0.145	0.031	0.251	25.1
6	0.114	0.36	0.246	0.144	0.03	0.252	25.2
7	0.114	0.366	0.252	0.154	0.04	0.248	24.8
8	0.114	0.355	0.241	0.144	0.03	0.246	24.6
9*	0.114	0.273	0.159	0.136	0.022	0.240	24.0
10*	0.114	0.279	0.165	0.137	0.023	0.249	24.9
						AVERAGE	25.1%

* Double sample poured through filter together, therefore calculations would use double the ODW.
The others are triplicate samples, therefore a triple ODW is used.

Example – Sample 1 – Klasson Lignin

$$= \frac{(\text{Mass at } 105^{\circ}C \text{ with ash and filter}) - (\text{Mass of filter + ash at } 575^{\circ}C)}{3 \times ODW} = \frac{0.361 - 0.144}{3 \times 285.3} \cong 25.4\%$$

Table A1-7 Lignin results for SSO and softwood

Sample	Mass of filter (g)	Mass at 105°C = Acid Insoluable Residue and filter (g)	105°C Residue (g)	Mass at 575°C of ash and filter paper (g)	Ash (g)	Klasson Lignin (g)	% Klasson Lignin (g)
1	0.114	0.293	0.179	0.145	0.031	0.258	25.8
2	0.114	0.303	0.189	0.158	0.044	0.253	25.3
3	0.114	0.284	0.170	0.144	0.030	0.244	24.4
4	0.114	0.322	0.208	0.167	0.053	0.270	27.0
5	0.114	0.319	0.205	0.155	0.041	0.286	28.6
6	0.114	0.297	0.183	0.150	0.036	0.256	25.6
7	0.114	0.303	0.189	0.160	0.046	0.249	24.9
8*	0.114	0.396	0.282	0.178	0.064	0.253	25.3
9*	0.114	0.400	0.286	0.179	0.065	0.257	25.7
10*	0.114	0.397	0.283	0.173	0.059	0.260	26.0
						AVERAGE	25.9 %

* Samples are in triplicate, therefore triple ODW, others are in duplicate, therefore double ODW.

Sugar and lignin proportions for initial quantitative saccharification

Table A1-8 Composition of SSO and hardwood

	Components (g / 100 g Biomass)				
	GLUCAN	XYLAN	GALACTAN	MANNAN AND ARABINAN	LIGNIN
SAMPLE					
1	34.0	8.2	1.9	4.9	25.4
2	39.2	9.0	2.1	5.0	25.4
3	37.3	9.3	2.1	4.9	25.5
4	38.2	10.2	2.1	5.2	25.8
5	37.9	10.2	2.1	4.9	25.1
6	35.5	10.0	1.9	4.7	25.2
7	32.8	9.1	2.1	4.7	24.8
8	26.3	7.3	1.9	3.7	24.6
9	29.5	8.3	1.9	4.1	24.0
10	29.2	7.9	1.9	3.3	24.9
AVERAGE	**34.0**	**9.0**	**2.0**	**4.5**	**25.1**
STANDARD DEV	4.4	1.0	0.1	0.6	0.5
95% C.I.	**+/- 2.8**	**+/- 0.6**	**+/- 0.1**	**+/- 0.4**	**+/- 0.3**

Example calculation: Sample 1 – Glucan = (HPLC (mg/ml) x Total ml / ODW) x anhydrous correction

ODW = 300 mg X TS = 300 x 0.951 = 285.3 mg.
HPLC = 1.238 mg/ml
Total ml is always 87.
Anhydrous Correction glucose = 0.9
Sample 1 glucose = (1.238 x 87 / 285.3) x 0.9 = 34.0 (Rounded to the nearest tenth of a percent)

Table A1-9 Composition of SSO and softwood

	Components (g / 100 g Biomass)				
	GLUCAN	XYLAN	GALACTAN	MANNAN AND ARABINAN	LIGNIN
SAMPLE					
1	25.8	3.3	1.3	6.2	25.8
2	32.4	5.5	3.1	8.4	25.3
3	33.3	3.5	1.9	7.0	24.4
4	32.1	5.4	3.1	8.7	27.0
5	32.9	5.0	1.8	7.0	28.6
6	32.0	4.7	1.8	6.6	25.6
7	21.4	3.5	1.5	4.6	24.9
8	28.6	4.7	1.7	5.9	25.3
9	24.0	4.1	1.7	5.3	25.7
10	0.0	0.0	0.0	0.0	26.0
AVERAGE	**29.2**	**4.4**	**2.0**	**6.6**	**25.9**
STANDARD DEV	4.4	0.9	0.6	1.4	1.2
95% C.I.	**+/- 2.9**	**+/- 0.6**	**+/- 0.4**	**+/- 0.9**	**+/- 0.7**

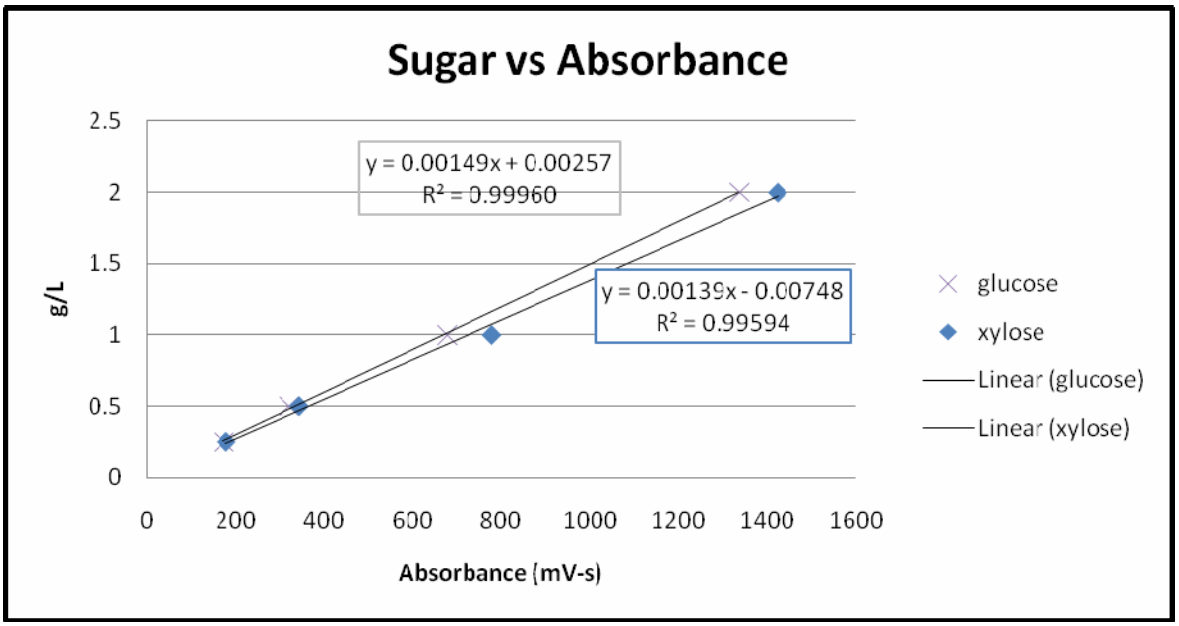
A1.3 (Failed) Quantitative saccharification post lime pretreated sample

Standards

Table A1-10 Sugar standards and their HPLC absorbance readings for lime treated composition.

Sugar Standards (mg/ml)				Absorbance (mV s)	
Standard	glucose	xylose		glucose	xylose
1	2.000	2.000		1338	1424
2	1.000	1.000		678	778
3	0.500	0.500		320	343
4	0.250	0.250		173	178

Figure A1-2 Standard Curves for lime treated composition



Moisture Content

Table A1-10 Initial Moisture of 45°C dried SSO and hardwood

Sample	Mass Foil (g)	Mass of filter + waste at 45°C (g)	Mass at 105°C (g)	Mass of 45C dried sample (g)	TS	MOISTURE
1	1.305	1.555	1.542	0.948	0.052	1.305
2	1.319	1.569	1.559	0.960	0.040	1.319
3	1.310	1.560	1.550	0.960	0.040	1.310
AVERAGE					0.956	0.044
STD DEV					0.007	0.007

HPLC results

Table A1-11 Absorbance results and corresponding HPLC mg/ml found for SSO and hardwood treated at 65°Cfor 6 weeks.

Absorbance (mV-s)				HPLC sugar concentration mg/ml	
Sample	glucose	xylose		glucose	xylose
1	295	72		0.442	0.107
2	298	72		0.447	0.107
3	311	72		0.466	0.107
4	337	72		0.505	0.107
5	310	72		0.464	0.107
6	286	72		0.429	0.107
7	356	76		0.533	0.113
8	331	72		0.496	0.107
9	336	72		0.503	0.107
10	331	72		0.496	0.107
Anhydrous Correction	0.9	0.88		Oven Dry Weight (mg)	Total volume (ml)
				285.3	87.0

Sugar proportions for six weeks of lime treatment at 65 °C.

Table A1-12 Composition of SSO and hardwood after 6 weeks at 65°C.

	Components (g / 100 g Biomass)	
	GLUCAN	XYLAN
SAMPLE		
1	12.1	2.9
2	13.5	3.3
3	14.1	3.3
4	15.3	3.3
5	14.1	3.3
6	13.0	3.3
7	16.2	3.4
8	15.0	3.3
9	15.3	3.3
10	15.0	3.3
AVERAGE	14.4	3.2
STANDARD DEV	1.2	0.1

A1.4 Quantitative saccharification for COSLIF pretreated sample

Moisture Content of 45 °C dried material before and after treatment

Table A1-12 Moisture content of 45°C dried SSO and softwood before COSLIF treatment

Sample	Mass Foil (g)	Mass of filter + waste at 45°C (g)	Mass at 105°C (g)	Mass of 45C dried sample (g)	TS	MOISTURE
1	1.310	2.310	2.269	1.000	0.959	0.041
2	1.291	2.291	2.248	1.000	0.957	0.043
3	1.310	2.310	2.269	1.000	0.959	0.041
4	1.305	2.305	2.268	1.000	0.963	0.037
5	1.309	2.309	2.266	1.000	0.957	0.043
6	1.304	2.304	2.267	1.000	0.963	0.037
7	1.311	2.311	2.271	1.000	0.960	0.040
8	1.303	2.303	2.262	1.000	0.959	0.041
9	1.290	2.290	2.252	1.000	0.962	0.038
10	1.303	2.303	2.256	1.000	0.953	0.047
AVERAGE					0.959	0.041
STD DEV					0.003	0.003

Table A1-13 Moisture of 45°C dried COSLIF pretreated material

Sample	Mass Foil (g)	Mass of filter + waste at 45°C (g)	Mass at 105°C (g)	Mass of 45C dried sample (g)	TS	MOISTURE
1	1.281	1.581	1.565	0.300	0.947	0.053
2	1.291	1.591	1.576	0.300	0.950	0.050
3	1.285	1.585	1.570	0.300	0.950	0.050
4	1.289	1.589	1.575	0.300	0.953	0.047
5	1.288	1.588	1.573	0.300	0.950	0.050
6	1.282	1.582	1.568	0.300	0.953	0.047
7	1.285	1.585	1.571	0.300	0.953	0.047
8	1.285	1.585	1.570	0.300	0.950	0.050
9	1.290	1.590	1.574	0.300	0.947	0.053
10	1.286	1.586	1.572	0.300	0.953	0.047
AVERAGE					0.951	0.049
STD DEV					0.003	0.003

Total mass retained per post pretreated bottle

Table A1-14 Total mass retained per post pretreated bottle

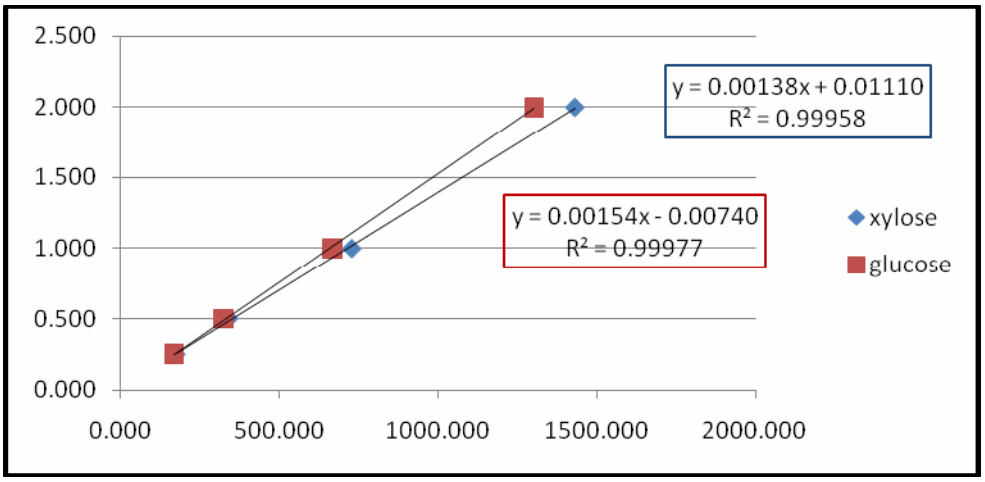
Bottle	Mass Container (g)	SSO/softwood (g)	Dry Mass (g)	Total Mass BEFORE Pretreatment	45°C Dried Mass Remaining After Pretreat	45°C dried SSO/softwood remaining	Dry SSO/softwood remaining	TOTAL % MASS REMAINING
1	37.145	4.000	3.837	40.982	40.106	2.961	2.815	73.4%
2	37.397	4.000	3.837	41.234	40.020	2.623	2.494	65.0%
3	37.041	4.000	3.837	40.878	39.642	2.601	2.473	64.4%
4	36.308	4.000	3.837	40.145	38.523	2.215	2.106	54.9%
5	37.916	4.000	3.837	41.753	40.615	2.699	2.566	66.9%
6	36.841	4.000	3.837	40.678	39.937	3.096	2.943	76.7%
7	36.807	4.000	3.837	40.644	39.855	3.048	2.898	75.5%
8	36.925	4.000	3.837	40.762	39.247	2.322	2.207	57.5%
9	36.494	4.000	3.837	40.331	39.113	2.619	2.490	64.9%
10	37.691	4.000	3.837	41.528	40.260	2.569	2.442	63.7%
						AVERAGE	2.543	66.3%
						std dev	0.276	7.2%

Sugar standardS for COSLIF quantitative saccharification

Table A1-15 COSLIF quantitative saccharification sugar standards and HPLC absorbance readings.

Sugar Standards (mg/ml)				Absorbance (mV s)	
Standard	glucose	xylose		glucose	xylose
1	2.000	2.000		1338	1424
2	1.000	1.000		678	778
3	0.500	0.500		320	343
4	0.250	0.250		173	178

Figure A1-3 Standard Curves for COSLIF



HPLC results for COSLIF

Table A1-16 Absorbance results and corresponding HPLC mg/ml found for COSLIF pretreated samples.

Absorbance (mV-s)		HPLC sugar concentration mg/ml		Average HPLC sugar concentration (mg/ml) per bottle	
Bottle	glucose	glucose		glucose	
	965	1.479			
	902	1.382			
1	1009	1.546		1.469	
	1077	1.651			
	1069	1.639			
2	1052	1.613		1.634	
	1034	1.585			
	1005	1.540			
3	BI*	--		1.563	
	932	1.428			
	987	1.513			
4		--		1.470	
	1036	1.588			
	BI	--			
5	BI	--		1.588	
	BI	--			
	BI	--		--	
6	BB**	--			
	BI	--			
	BI	--		--	
7	BI	--			
	1022	1.566			
	1017	1.559			
8	BI	--		1.563	
	1088	1.668			
	1097	1.682			
9	1038	1.591		1.647	
	1023	1.568			
	1094	1.677			
10	BB	--		1.623	
				Oven Dry Weight (mg)	Total volume (ml)
Anhydrous Correction		0.9		285.2	87.0

* Bad Injection (HPLC Machine Failure)

** Broken Bottle during acid hydrolysis

Table A1-17 Pretreatment efficiency

Bottle	Average HPLC glucose (mg/ml)	Glucose ratio	Glucan ratio of treated material	Dry SSO/softwood remaining (g)	Glucose available per bottle (g)	Ratio original glucan	Pretreatment Yield	Yield %
1	1.469	0.448	0.403	2.815	1.261	0.293	1.008	100.8
2	1.634	0.499	0.449	2.494	1.243	0.293	0.994	99.4
3	1.563	0.477	0.429	2.473	1.179	0.293	0.942	94.2
4	1.470	0.448	0.404	2.106	0.944	0.293	0.755	75.5
5	1.588	0.484	0.436	2.566	1.243	0.293	0.994	99.4
8	1.563	0.477	0.429	2.207	1.052	0.293	0.841	84.1
9	1.647	0.502	0.452	2.490	1.251	0.293	1.000	100.0
10	1.623	0.495	0.446	2.442	1.209	0.293	0.966	96.6
AVERAGE					1.173		0.937	93.7
Std Dev					0.115		0.092	9.2

Example – Bottle 1

- HPLC (mg/ml) – Table A1-17 above.
- Ratio glucose = HPLC value x Total ml / 300 mg x TS
  = 1.469 x 87 / 300 x 0.951
  ≈ 0.448
- Glucan Ratio = Glucose ratio x 0.9 (Anhydrous Correction)
  ≈ 0.403
- Dry Softwood remaining – Table A1-17 Above
- Glucose Available per bottle = Dry weight remaining x glucose ratio
  = 2.815 x 0.403
  ≈ 1.261
- Ratio of original glucan = from original quantitative saccharification (Table A1-17)
- Pretreatment yield = (glucan ratio x dry material remaining)/(original glucan ratio x dry material added to bottle)
  = (0.403 x 2.815) / (0.293 x 3.837)
  ≈ 1.008

APPENDIX 2 Enzymatic saccharification

The procedure used was adapted from the NREL, "Enzymatic Saccharification of Lignocellulosic Biomass" Laboratory Analytical Procedure (LAP), Revised March 2008, Technical Report # NREL/TP-510-42629. (Selig et al., 2008)

Terminology

1. *Pretreated biomass:* biomass that has been chemically or thermally altered, changing the structural composition.
2. *Cellulase enzyme:* an enzyme preparation exhibiting all three synergistic cellulolytic activities: endo-1,4-β-D-glucanase, exo-1,4-β-glucosidase, and β-D-glucosidase activities, which are present to different extents in different cellulose preparations.
3. *TS* - % Total Solids

Apparatus

1. A suitable shaking or static incubator set at 50 °C ± 1 °C
2. Any fixed speed rotator that can hold scintillation vials and operate in a static incubator.
3. pH meter.
4. Analytical Balance accurate to 1 mg.
5. HPLC equipped with a Refractive Index Detector
6. Biorad Aminex HPX-87P column
7. 100 µL and a 1000 µL Eppendorf Pipetman pipet with tips.
8. HPLC testing vials, 2 ml centrifuge vials, and 20 ml glass scintillation vials.

Reagants

6. Sodium Azide (20 mg/ml in DDW)
7. Sodium Citrate Buffer (0.05 M, pH 4.8)
8. 1M citric acid solution.
9. Cellulase of known activity, FPU/ml (**APPENDIX 3**)
10. 1N Ammonium Hydroxide (COSLIF ONLY)

A2.1 Procedure for lime pretreated material

1. Weigh out 0.750 g of dried, pretreated material and add to each vial. Four vials for each point to be tested, one vial to be used as a blank.
2. Add 150 µL of 2% sodium azide reagent, as well as 14.7 ml of DDW, less the total ml cellulase enzyme to be added to achieve 15 FPU loading.
3. Check that the pH remains 4.8 for at least 12 consecutive hours. Adjust any changes back to pH 4.8 with 1M citric acid.
4. Record total ml of citric acid used to adjust.
5. Bring each vial to 50 C by warming in the incubator.
6. Prepare an equal volume enzyme blank with buffer solution and bring to 50 C as well.
7. Innoculate all samples with 15 FPU / g dry biomass.
   (For this experiment the activity = 41 FPU/ml. Therefore 15/41*0.750 = 0.274 ml)
8. Close vials tightly and place on rotary shaker at 180 rpm (enough to keep solids in suspension)
9. Check vials for pH at 24 and 48 hrs. Adjust if necessary and record adjustment + any additional enzyme added.
10. At desired intervals or completion (In this case 72 hrs) remove samples from incubator and draw 1.5 ml into a 3 ml syringe. Transfer contents of syringe to 2 ml centrifuge vial and store in freezer for testing.
11. Before HPLC testing, thaw vials at room temperature and centrifuge at 15000 rpm for 10 min.
12. Draw liquid portion of vial into syringe and attach a 0.2 µm filter tip. Plunge contents through filter to an HPLC sampling vial.

Calculations

1. Calculate the glucose/xylose concentration in mg/ml from the sample supernatant by comparison to prepared standards. Subtract glucose concentrations, if any, from the substrate and enzyme blanks.

2. Calculate % digestion.

$$\% \text{ digestion} = \frac{(HPLC\ reading\ mg/ml - mg/ml\ of\ blanks) \times Total\ ml\ (15)}{Total\ glucose\ added}$$

   Total glucose added = 0.750 g x Total Solids x % original glucose

A2.2 Procedure for COSLIF treated material

A2.2.1 Normal procedure which was used in the temperature effects testing

1. Weight bottle after **Step 11 in COSLIF Procedure**. Calculate DDW in bottle (Mass bottle after pretreatment – mass bottle – average mass remaining from previous dried samples). Adjust pretreated material in bottle to 20 g/L glucose using average value for glucose remaining in the bottle from the quantitative saccharification of the pretreated material. (**APPENDIX 1**) Add 400 µL of 2% sodium azide and nearly but not all of the DDW. Bring bottles to desired temperature.
2. Adjust to pH 5.0 with 1N ammonium hydroxide.
3. Add remaining DDW less the addition of desired ml of cellulase enzyme.
4. Add enzyme, seal bottles tightly and place on rotary shaker at 180 rpm (enough to keep solids in suspension).
5. Prepare enzyme blanks for each loading in bottles at same volume as samples. Add a substrate blank bottle with equal volume and no enzyme.
6. Take, store, and prepare samples in the same manner as described for the lime pretreated material.

Calculations

1. Calculate the glucose/xylose concentration in mg/ml from the sample supernatant by comparison to prepared standards. Subtract glucose concentrations, if any, from the substrate and enzyme blanks.

2. Calculate % digestion.

$$\% \text{ digestion} = \frac{\left(HPLC\ reading\ mg\ \square\ ml\ -\ mg\ \square\ ml\ of\ blanks\right)}{20}$$

A2.2.2 Procedure used for 50 C samples because HPLC was malfunctioning

At the time of the 50 °C testing, the quantitative saccharification results could not be found due to equipment malfunction, and the glucose was approximated to 20 g/L by using the MBI International original glucan content results, and assuming 100 % pretreatment yield. This method was previously employed by fellow group member Mandana Ehsanipour in her initial testing of the COSLIF. The results were later corrected when the HPLC was functioning again. The procedure is the same, replacing the pretreatment average glucose with the estimate. The calculations are as follows.

Calculations

1. Actual glucose in mg/ml =

$$\frac{\text{Average glucose remaining in bottle from quantitative saccharification}}{\text{Total estimated DDW + enzyme added}}$$

2. Calculate the glucose/xylose concentration in mg/ml from the sample supernatant by comparison to prepared standards. Subtract glucose concentrations, if any, from the substrate and enzyme blanks.

3. Calculate % digestion.

$$\text{\% digestion} = \frac{\left(\text{HPLC reading mg}\frac{\square}{\square}\text{ ml} - \text{mg}\frac{\square}{\square}\text{ml of blanks}\right)}{\text{Actual glucose in}\frac{\text{mg}}{\text{ml}}}$$

A2.3 COSLIF Enzyme Hydrolysis

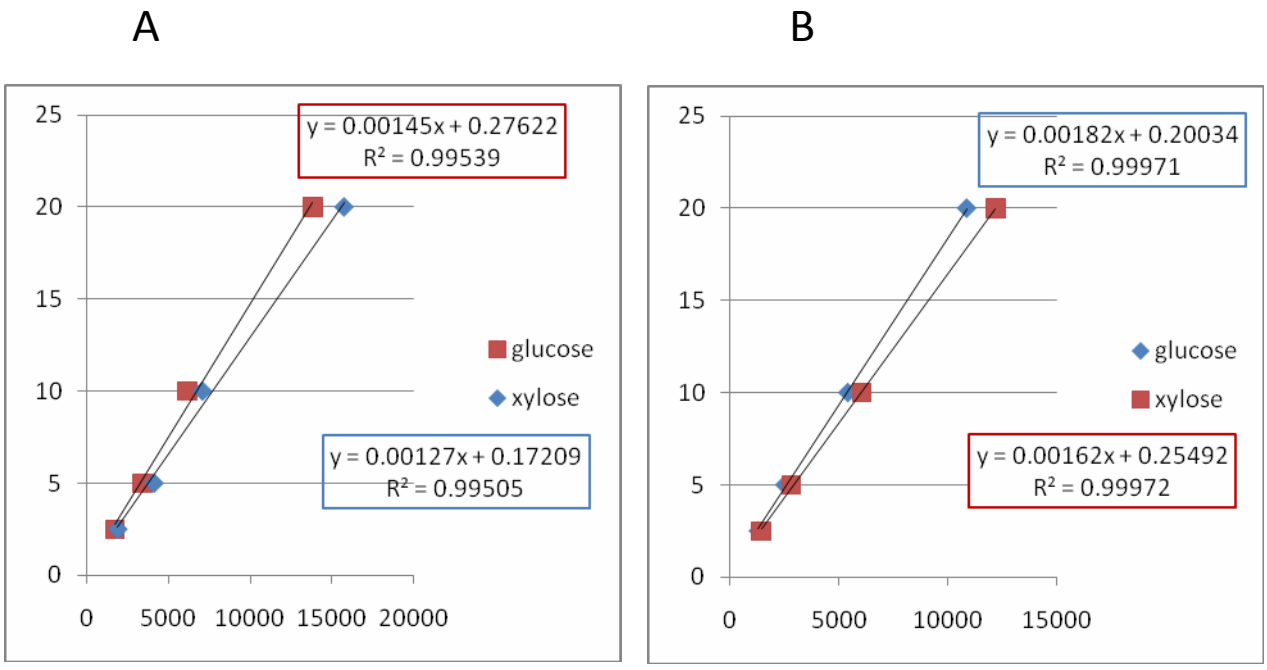
COSLIF sugar standard curves

Table A2-1 COSLIF sugar standards and their HPLC absorbance readings.

Sugar Standards (mg/ml)				Absorbance for 50 °C (mV s)		Absorbance for 30, 35, and 40 °C samples	
Standard	glucose	xylose		glucose	xylose	glucose	Xylose
1	2.5	2.5		1743	1928	1325	1458
2	5	5		3429	4141	2534	2819
3	10	10		6181	7101	5419	6057
4	20	20		13819	15760	10859	12184

The discrepancy between the two sets of standards was caused by the dismantling and reassembly of the faulty Refractive Index Detector.

Figure A2-1 COSLIF standard curves A) 50 C and B) 30, 35, and 40 C



COSLIF absorbance readings and consequent sugar concentration

*****ALL COSLIF ENZYME BLANKS AND SUBSTRATE BLANKS AT ALL TIMES WERE 0 - excepting a single substrate blank at 12 h which was 47 mV-s ≈ 0. The samples were all zero sugar at time 0 as well.**

Table A2-2 12 hour absorbance results and corresponding HPLC mg/ml found for COSLIF pretreated samples.

Absorbance (mV-s)				HPLC sugar concentration mg/ml		Average HPLC sugar concentration (mg/ml) per loading	
Temp	FPU	glucose	xylose	glucose	xylose	glucose	xylose
50	10	12707	600	18.701	0.934	18.954	1.506
		13056	1500	19.207	2.077		
50	15	12841	1380	18.896	1.925	18.707	1.874
		12700	1320	18.691	1.848		
		12592	1320	18.535	1.848		
50	30	14050	840	20.649	1.239	21.979	1.798
		15931	1620	23.376	2.229		
		14921	1380	21.912	1.925		
50	60	15428	1620	22.647	2.229	21.925	2.102
		14881	1500	21.854	2.077		
		14481	1440	21.274	2.001		
40	10	7913	600	14.602	1.227	14.629	1.405
		8491	750	15.654	1.470		
		7380	780	13.632	1.519		
35	10	6919	540	12.793	1.130	12.228	1.211
		6072	540	11.251	1.130		
		6834	690	12.638	1.373		
35	5	4047	900	7.566	1.713	7.426	1.664
		3893	840	7.286	1.616		
		BI	BI	--	--		
30	10	3643	840	6.831	1.616	7.058	1.616
		3622	840	6.792	1.616		
		3900	1230	7.298	2.248		
50 NP**	15	120	0	0.419	0.255	0.444	0.388
		426	120	0.976	0.449		
		537	126	1.178	0.459		
	0*	117	0	0.413	0		

** NP = No Pretreatment, * No pretreatment substrate blank

BI = Bad Injection

Table A2-3 24 hour absorbance results and corresponding HPLC mg/ml found for COSLIF pretreated samples.

Absorbance (mV-s)				HPLC sugar concentration mg/ml		Average HPLC sugar concentration (mg/ml) per loading	
Temp	FPU	glucose	xylose	glucose	xylose	glucose	xylose
50	10	12994	1200	19.118	1.696	19.236	1.772
		13158	1320	19.355	1.848		
50	15	12670	1140	18.648	1.620	19.204	1.810
		13437	1440	19.760	2.001		
		BI	BI	--	--		
50	30	13571	1260	19.954	1.772	21.773	1.950
		15905	1440	23.338	2.001		
		15000	1500	22.026	2.077		
50	60	14353	1500	21.088	2.077		
		14236	1500	20.918	2.077		
		13996	1380	20.570	1.925	20.859	2.026
40	10	8792	870	16.202	1.664	16.208	1.632
		8650	870	15.943	1.664		
		8945	810	16.480	1.567		
35	10	7022	600	12.980	1.227	12.699	1.324
		6713	720	12.418	1.421		
		BI	BI	--	--		
35	5	4120	840	7.699	1.616	8.000	1.826
		4312	1050	8.048	1.956		
		4424	1020	8.252	1.907		
30	10	6091	660	11.286	1.324	9.195	1.729
		5881	570	10.904	1.178		
		4675	930	8.709	1.762		
50 NP**	15	189	210.6	0.544	0.596		
		BI	BI	--	--		
		263	126	0.679	0.459		
	0*	123	0	0.424	0	0.187	0.528

** NP = No Pretreatment, * No pretreatment substrate blank

BI = Bad Injection

Table A2-4 48 hour absorbance results and corresponding HPLC mg/ml found for COSLIF pretreated samples.

Absorbance (mV-s)				HPLC sugar concentration mg/ml		Average HPLC sugar concentration (mg/ml) per loading	
Temp	FPU	glucose	xylose	glucose	xylose	glucose	xylose
35	10	7571	720	13.980	1.421	14.705	1.519
		BI	BI	--	--		
		8368	840	15.430	1.616		
35	5	5749	690	10.664	1.373	11.430	1.389
		6089	660	11.282	1.324		
		6672	750	12.343	1.470		

BI = Bad Injection

Table A2-5 72 hour absorbance results and corresponding HPLC mg/ml found for COSLIF pretreated samples.

Absorbance (mV-s)				HPLC sugar concentration mg/ml		Average HPLC sugar concentration (mg/ml) per loading	
Temp	FPU	glucose	xylose	glucose	xylose	glucose	xylose
50	10	9243	960	17.023	1.810	16.554	1.567
		8728	660	16.085	1.324		
50	15	8974	840	16.533	1.616	17.115	1.648
		9892	900	18.204	1.713		
		9015	840	16.608	1.616		
50	30	9435	720	17.372	1.421	19.253	1.778
		11141	1080	20.477	2.005		
		10830	1020	19.911	1.907		
50	60	9603	900	17.678	1.713		
		9729	1140	17.907	2.102		
		9369	720	17.252	1.421	17.612	1.745
40	10	8436	900	15.554	1.713		
		9330	840	17.181	1.616		
		9017	840	16.611	1.616	16.449	1.648
35	10	9113	870	16.786	1.664		
		BI	BI	--	--		
		9028	810	16.631	1.567	16.708	1.583
35	5	7834	780	14.458	1.519		
		7384	780	13.639	1.519		
		7236	750	13.370	1.470	13.822	1.502
30	10	7305	840	13.495	1.616		
		7314	900	13.512	1.713		
		7398	1020	13.665	1.907	13.502	1.535
50 NP**	15	110	211	0.401	0.597		
		0	233	0.000	0.632		
		0	204	0.000	0.585		
	0*	0	0	0.000	0	0.134	0.605

** NP = No Pretreatment, * No pretreatment substrate blank

BI = Bad Injection

Table A2-6 Calculations to determine amount DDW to add to achieve 20 g/L glucose for 50 C sample

	A	B	C	D	E = B*C*D	F = E*1000/20	G	H =G-A-E	I	J	K = I*E/J	L = F-H-K
Bottle	Weight of container (g)	Weight of 45c dried material (g)	Solids Content	Glucan Content (MBI Int)	Total glucan (g)	ml DDW to make 20 g/L glucose	Total mass post pretreatment (g)	Estimated ml of DDW in pretreated container	FPU loading per g glucose	FPU/ml	ml cellulase	DDW TO ADD (ml)
1	36.60	4.000	0.959	0.268	1.028	51.40	72.35	34.72	10	71	0.145	16.54
2	36.91	4.000	0.959	0.268	1.028	51.40	68.27	30.33	10	71	0.145	20.93
3	37.97	4.000	0.959	0.268	1.028	51.40	73.53	34.53	10	71	0.145	16.73
4	35.87	4.000	0.959	0.268	1.028	51.40	68.91	32.01	15	71	0.217	19.17
5	37.91	4.000	0.959	0.268	1.028	51.40	77.62	38.68	15	71	0.217	12.50
6	37.26	4.000	0.959	0.268	1.028	51.40	66.62	28.33	15	71	0.217	22.85
7	37.22	4.000	0.959	0.268	1.028	51.40	68.44	30.19	30	71	0.434	20.78
8	37.44	4.000	0.959	0.268	1.028	51.40	73.80	35.33	30	71	0.434	15.64
9	36.45	4.000	0.959	0.268	1.028	51.40	69.83	32.35	30	71	0.434	18.62
10	37.65	4.000	0.959	0.268	1.028	51.40	71.85	33.17	60	71	0.869	17.36
11	41.31	4.000	0.959	0.268	1.028	51.40	71.38	29.04	60	71	0.869	21.49
12	37.40	4.000	0.959	0.268	1.028	51.40	63.78	25.35	60	71	0.869	25.18

** I should note that this estimation contained an error in procedure. 51.4 ml of DDW would make 20 g/L glucan, not glucose. It would actually be 22.222 g/L glucose. The actual glucose added in g/L had to be adjusted anyway when the real value was determined from quantitative saccharification so the error was caught before subsequent analysis. **Table ?** on the following page shows the calculations for the actual glucose in each sample, and the adjusted cellulase loadings.

Table A2-7 Calculations to determine actual glucose added to 50 °C samples, and actual FPU/ g glucan loadings

	A	B	C	D	E = D – B – C -A	F	G	H	I	J = E+H+I	K = B*1000/J	L = G*H/B	M=L/0.9
Bottle	Mass of bottle (g)	Total glucose in bottle from quant sac (g)	Mass of non-glucose components from quant sac (g)	Total mass post pretreat. (g)	DDW in pretreat. container (ml)	estimate loading FPU/ g glucose	FPU/ ml	ml cellulase added	DDW added	Total ml	g glucose / L	Actual loading FPU/g glucose	Actual Loading FPU/ g glucan
1	36.6	1.173	1.370	72.35	33.21	10	71	0.145	16.54	49.89	23.510	8.77	9.74
2	36.91	1.173	1.370	68.27	28.82	10	71	0.145	20.93	49.89	23.510	8.77	9.74
3	37.97	1.173	1.370	73.53	33.02	10	71	0.145	16.73	49.89	23.510	8.77	9.74
4	35.87	1.173	1.370	68.91	30.50	15	71	0.217	19.17	49.89	23.510	13.15	14.61
5	37.91	1.173	1.370	77.62	37.17	15	71	0.217	12.50	49.89	23.510	13.15	14.61
6	37.26	1.173	1.370	66.62	26.82	15	71	0.217	22.85	49.89	23.510	13.15	14.61
7	37.22	1.173	1.370	68.44	28.68	30	71	0.434	20.78	49.89	23.510	26.30	29.22
8	37.44	1.173	1.370	73.8	33.82	30	71	0.434	15.64	49.89	23.510	26.30	29.22
9	36.45	1.173	1.370	69.83	30.84	30	71	0.434	18.62	49.89	23.510	26.30	29.22
10	37.65	1.173	1.370	71.85	31.66	60	71	0.869	17.36	49.89	23.510	52.59	58.44
11	41.31	1.173	1.370	71.38	27.53	60	71	0.869	21.49	49.89	23.510	52.59	58.44
12	37.4	1.173	1.370	63.78	23.84	60	71	0.869	25.18	49.89	23.510	52.59	58.44

Table A2-8 Calculations to determine amount DDW to add to achieve 20 g/L glucose for 50 C sample

	A	B	C	D	E = D-A-B-C	F = B*1000/20	G	H	I = B*0.9	J = G*I/H	K = I*E/J
Bottle	Weight of container (g)	Total glucose in bottle from quant sac (g)	Mass of non-glucose components from quant sac (g)	Total mass post pretreat. (g)	DDW in pretreat. container (ml)	ml DDW to make 20 g/L glucose	FPU loading / g glucan	FPU/ml	Total glucan (g)	cellulase / g glucan (ml)	DDW TO ADD (ml)
1	41.59	1.173	1.372	83.44	39.42	58.65	9.74	71	1.056	0.145	19.08
2	36.9	1.173	1.372	73.72	34.39	58.65	9.74	71	1.056	0.145	24.11
3	37.12	1.173	1.372	74.72	35.17	58.65	9.74	71	1.056	0.145	23.33
4	35.84	1.173	1.372	81.12	42.85	58.65	9.74	71	1.056	0.145	15.65
5	37.54	1.173	1.372	75.41	35.44	58.65	9.74	71	1.056	0.145	23.06
6	38.53	1.173	1.372	77.27	36.31	58.65	9.74	71	1.056	0.145	22.19
7	37.25	1.173	1.372	82.52	42.84	58.65	9.74	71	1.056	0.145	15.66
8	37.03	1.173	1.372	82.29	42.83	58.65	9.74	71	1.056	0.145	15.67
9	37.67	1.173	1.372	90.30	50.20	58.65	9.74	71	1.056	0.145	8.30
10	36.94	1.173	1.372	83.11	43.74	58.65	0	71	1.056	0.000	14.91
11	36.88	1.173	1.372	75.01	35.70	58.65	5	71	1.056	0.074	22.87
12	37.46	1.173	1.372	77.74	37.85	58.65	5	71	1.056	0.074	20.72
16	37.93	1.173	1.372	78.58	38.22	58.65	5	71	1.056	0.074	20.35

COSLIF hydrolysis yields

Table A2-9 COSLIF hydrolysis yields

		A		B					C = B/A			
				Average HPLC values					Hydolysis yield (g / g glucan added)			
				Time (hrs)					Time (hrs)			
Temp.	FPU	Glucose adjustment (g/L)		12	24	48	72		12	24	48	72
50	10	23.510		18.954	19.236		16.554		0.806	0.818		0.704
50	15	23.510		18.707	19.204		17.115		0.796	0.817		0.728
50	30	23.510		21.979	21.773		19.253		0.935	0.926		0.819
50	60	23.510		21.925	20.859		17.612		0.933	0.887		0.749
40	10	20.000		14.629	16.208		16.449		0.731	0.810		0.822
35	10	20.000		12.228	12.699	14.705	16.709		0.611	0.635	0.735	0.835
35	5	20.000		7.426	8.000	11.430	13.822		0.371	0.400	0.571	0.691
30	10	20.000		7.058	9.195		13.502		0.353	0.460		0.675
50 NP*	15	20.000		0.444	0.187		0.134		0.022	0.009		0.007

*NP = No Pretreatment

A2.4 Lime hydrolysis/overall yields

As previously explained in the **Methods Section,** the hydrolysis yields were converted to overall yields by estimating the change in compostion (% glucose and % xylose) of the pretreated material by comparison to previous long term lime studies. **Table A2-7** below shows the summary of those composition assumptions.

Table A2-10 (**Table 5.1** amended) Approximate pretreatment yields used for lime pretreatment

Component	Temp. °C	Week						
		0	1	2	3	4	5	6
Glucose	55	100	100	96.6	95	90	N/A	N/A
	65	100	90	90	90	90	90	90
	75	100	90	90	90	90	90	N/A
Xylose and Other Sugars	55	100	76.4	68.1	68.1	68.1	N/A	N/A
	65	100	76.4	68.1	68.1	68.1	68.1	68.1
	75	100	76.4	68.1	68.1	68.1	68.1	N/A
Ash, Extractives, Lignin, Acetyle	55	100	62	62	60.5	59	N/A	N/A
	65	100	62	50	48.5	47	47	47
	75	100	62	50	48.5	47	47	N/A

Below is a reiteration of the technique used to solve for overall yields. The accuracy, given that the changes in composition above are true, is proved in the **Methods Section**.

Glucose Overall Yield $= \frac{GHPLC \times VES}{GCon \times MB}$ **[EQUATION 6.6]**

GHPLC – Glucose content in mg/ml found by HPLC analysis of enzymatic saccharification
VES – Total volume of enzymatic saccharification
MB – Dry Mass at B –(*mass of 45 °C dried, treated sample*)
GCon – Glucose Conversion

Glucose conversion (GCon) equation **[EQUATION 6.7]**

$$= \frac{QSG}{(QSG \times Tbl.6.1) + (QSX \times Tbl.6.1) + (QSOS \times Tbl.6.1) + (AELA \times Tbl.6.1)}$$

QSG – Initial Quantitative Saccharification proportion results for Glucose
QSX – Initial Quantitative Saccharification proportion results for Xylose
QSOS – Initial Quantitative Saccharification proportion results for Other Sugars
AELA – Ash, Extractives, Lignin, Acetyle = 1 – (QSG + QSX + QSOS)
Tbl.6.1 – Estimated pretreatment yield (g /100 g raw biomass), by temperature category and week, divided by 100, from **Table 6.1.**

Xylose Overall Yield = $\frac{XHPLC \times VES}{XCon \times MB}$ **[EQUATION 6.8]**

XHPLC – Xylose content in mg/ml found by HPLC analysis of enzymatic saccharification
VES – Total volume of enzymatic saccharification
MB – Dry Mass at B –(*mass of 45 °C dried, treated sample*)
XCon – Xylose Conversion

Xylose conversion (XCon) estimate **[EQUATION 6.9]**

$$= \frac{QSX}{(QSG \times Tbl.6.1) + (QSX \times Tbl.6.1) + (QSOS \times Tbl.6.1) + (AELA \times Tbl.6.1)}$$

QSG – Initial Quantitative Saccharification proportion results for Glucose
QSX – Initial Quantitative Saccharification proportion results for Xylose
QSOS – Initial Quantitative Saccharification proportion results for Other Sugars
AELA – Ash, Extractives, Lignin, Acetyle = 1 – (QSG + QSX + QSOS)
Tbl.6.1 – Estimated pretreatment yield (g /100 g raw biomass),by temperature category and week, divided by 100, from **Table 6.1.**

Table A2-11 on the following page shows the estimated post pretreatment proportions of glucose and xylose found using **EQUATION 6.7** AND **EQUATION 6.9**, **Table A2-10,** and the quantitative saccharification values found for SSO/hardwood, found in **APPENDIX 2**.

Table A2-11 Estimated post pretreatment glucose and xylose proportions

Component	Temp. °C	Week						
		0	1	2	3	4	5	6
Glucose (GCon)	55	0.378	0.459	0.474	0.482	N/A	N/A	N/A
	65	0.378	0.458	0.531	0.537	0.543	90	90
	75	0.378	0.480	0.531	0.537	0.543	0.543	0.543
Xylose (GCon)	55	0.100	0.124	0.128	0.130	N/A	N/A	N/A
	65	0.100	0.123	0.143	0.145	0.146	0.146	0.146
	75	0.100	0.129	0.143	0.145	0.146	0.146	N/A

Example –Week 1, 55 C ⟶ (glucose) - From **Table 6.1 –** Glucose is completely intact
Xylose is reduced to 76.4 % of its original mass
Others sugars reduced to 76.4 % of their original mass
Ash, extract, ect. reduced to 62% of original mass

The original proportion results from quantitative saccharification are: glucan =0.34
Xylan = 0.090
Other sugars = 0.065

Therefore with anhydrous corrections : Glucose = 0.378
Xylose = 0.100
Other sugars = 0.072

Original lignin, ash, extractives and acetyle are 1 – (glucan, xylan, other sugars)
= 0.505

New glucose proportion (GCon) = 0.378 /[(0.378*1.00) + (0.100*0.764) + (0.072*0.764) + (0.505*0.62)]

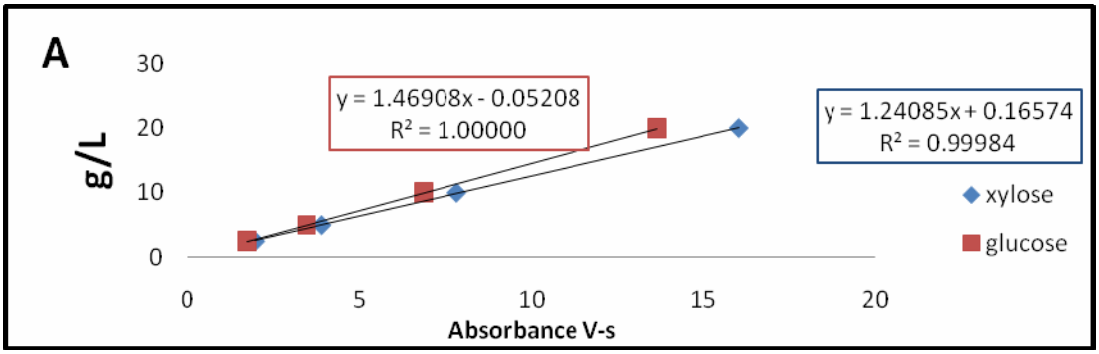
≈ 0.459

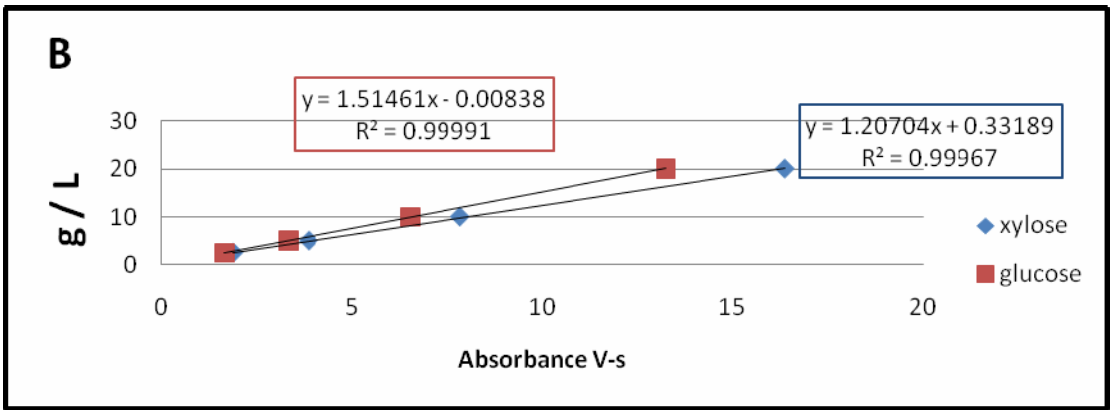
Sugar standard curves

Table A2-12 Lime sugar standards and their HPLC absorbance readings.

Sugar Standards (mg/ml)				Absorbance (V -s)					
				Week 0,1,2		Week 3,4		Week 5,6, control	
Std	glucose	xylose		glucose	xylose	glucose	xylose	glucose	Xylose
1	2.5	2.5		1.724	1.947	1.671	1.895	1.681	1.921
2	5	5		3.45	3.895	3.338	3.864	3.339	3.750
3	10	10		6.849	7.814	6.536	7.847	6.668	7.450
4	20	20		13.645	16.031	13.236	16.362	13.660	15.399

Figure A2-4 Lime Hydrolysis Standard Curves. **A)** Week 0-2, **B)** Week 3, 4, **C)** Week 5, 6, and Control





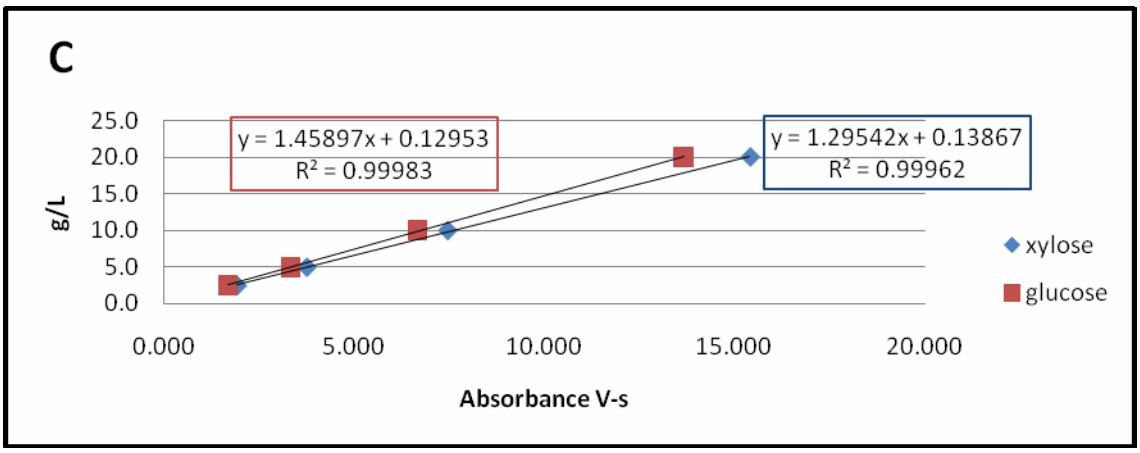


Table A2-13 Average total solids of 45 °C dried material used for hydrolysis

	SAMPLE		
WEEK	55 C	65 C	75 C
0	0.951	0.951	0.951
1	0.977	0.976	0.971
2	0.967	0.967	0.969
3	0.960	0.961	0.969
4	--	0.955	0.983
5	--	0.972	0.964
6	--	0.956	--

CONTROL	0.969	0.960	0.976

Table A2-14 Week 0 Lime treatment HPLC results and corresponding overall conversion figures

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and added cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
55	15	2.871	0.405	4.166	0.668	0	15	723		
		2.653	0.351	3.845	0.601	0	15	723		
		4.849	1.188	7.072	1.640	0	15	723		
	EB	0.0	0.0			0	15	723		
	SB	0.0	0.0			0	15	723	0.280	0.200
65	15	2.871	0.405	4.166	0.668	0	15	723		
		2.653	0.351	3.845	0.601	0	15	723		
		4.849	1.188	7.072	1.640	0	15	723		
	EB	0.0	0.0			0	15	723		
	SB	0.0	0.0			0	15	723	0.280	0.200
75	30	2.871	0.405	4.166	0.668	0	15	723		
		2.653	0.351	3.845	0.601	0	15	723		
		4.849	1.188	7.072	1.640	0	15	723		
	EB	0.0	0.0			0	15	723		
	SB	0.0	0.0			0	15	723	0.280	0.200

* FPU is loading is per g dry biomass. EB – Enzyme Blank, SB - Substrate Blank

An example of the overall conversion calculation can be found after the next table.

Table A2-15 Week 1 Lime treatment HPLC results and corresponding overall conversion figures

.

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and extra cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
55	15	6.171	1.679	9.014	2.249	0	15	733		
		5.758	1.512	8.407	2.042	0	15	733		
						0	15	733		
	EB	0.0	0.0			0	15	733		
	SB	0.0	0.0			0	15	733	0.389	0.355
65	15	6.757	1.736	9.875	2.320	0	15	732		
		7.423	1.994	10.853	2.640	0	15	732		
		8.804	2.643	12.882	3.445	0	15	732		
	EB	0.0	0.0			0	15	732		
	SB	0.0	0.0			0	15	732	0.505	0.469
75	30	6.124	1.979	8.945	2.621	0	15	728		
		4.846	1.970	7.067	2.610	0	15	728		
		6.404	2.256	9.356	2.965	0	15	728		
	EB	1.2	0.2			0	15	728		
	SB	0.0	0.0			0	15	728	0.363	0.435

* FPU is loading is per g dry biomass. EB – Enzyme Blank, SB – Substrate Blank

Example – 55 C: Dry Mass = 0.750 mg x Total Solids from **Table ?** above. (750 x 0.977 = 733)

Overall glucose yield = (Average HPLC mg/ml x Total Volume) / (GCon @ 55 C week 1 x Dry Mass)
(Table ?)

= [(9.014 + 8.407)/2 x (15)] / (0.459 x 733)

= 0.389

Table A2-16 Week 2 Lime treatment HPLC results and corresponding overall conversion figures

.

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and extra cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
55	15	6.171	1.679	8.765	2.393	0	15	725		
		5.758	1.512	8.729	2.218	0	15	725		
						0	15	725		
	EB	0.0	0.0			0	15	725		
	SB	0.0	0.0			0	15	725	0.382	0.374
65	15	6.361	1.959	9.293	2.596	0	15	725		
		6.330	2.094	9.247	2.764	0	15	725		
		5.681	1.905	8.294	2.529	0	15	725		
	EB	0.000	0.000			0	15	725		
	SB	0.000	0.000			0	15	725	0.349	0.380
75	30	5.624	2.040	8.510	2.794	0.374	15.374	727		
		6.503	2.088	9.841	2.852	0.374	15.374	727		
		5.924	2.119	8.964	2.890	0.374	15.374	727		
	EB	0.000	0.000			0.374	15.374	727		
	SB	0.000	0.000			0.374	15.374	727	0.363	0.421

* FPU is loading is per g dry biomass. EB – Enzyme Blank, SB – Substrate Blank

Table A2-17 Week 3 Lime treatment HPLC results and corresponding overall conversion figures

.

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and extra cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
55	15	4.908	1.378	6.682	1.817	1.674	16.674	720		
		4.405	1.354	5.920	1.788	1.674	16.674	720		
		4.014	1.348	5.328	1.780	1.674	16.674	720		
	SB	0.300	0.100			1.674	16.674	720		
	EB	0.200	0.000			1.674	16.674	720	0.301	0.320
65	15	5.535	1.989	7.490	2.681	1.674	16.674	721		
		6.058	2.008	8.283	2.704	1.674	16.674	721		
		4.331	1.341	5.667	1.899	1.674	16.674	721		
	SB	0.384	0.043			1.674	16.674	721		
	EB	0.200	0.000			1.674	16.674	721	0.308	0.388
75	30	4.289	1.786	5.402	2.344	1.471	16.471	727		
		4.660	1.832	5.964	2.399	1.471	16.471	727		
		6.208	2.160	8.308	2.795	1.471	16.471	727		
	SB	0.717	0.119			1.471	16.471	727		
	EB	0.000	0.000			1.471	16.471	727	0.277	0.394

* FPU is loading is per g dry biomass. EB – Enzyme Blank, SB – Substrate Blank

Table A2-18 Week 4 Lime treatment HPLC results and corresponding overall conversion figures

.

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and extra cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
65	15	6.162	2.073	8.919	2.834	1.674	16.674	716		
		5.838	1.984	8.428	2.727	1.674	16.674	716		
		5.264	1.905	7.559	2.631	1.674	16.674	716		
	SB	0.079	0.000			1.674	16.674	716		
	EB	0.189	0.000			1.674	16.674	716		0.308
75	30	1.277	0.804	1.717	1.180	1.474	16.474	737		
		1.056	0.559	1.394	0.863	1.474	16.474	737		
		1.941	0.894	2.686	1.297	1.474	16.474	737		
	SB	0.0	0.0			1.474	16.474	737		
	EB	0.2	0.0			1.474	16.474	737	0.080	0.170

* FPU is loading is per g dry biomass. EB – Enzyme Blank, SB – Substrate Blank

Table A2-19 Week 5 Lime treatment HPLC results and corresponding overall conversion figures

.

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and extra cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
65	15	3.633	1.483	5.154	2.060	1.648	16.648	729		
		0.432	0.471	0.484	0.749	1.648	16.648	729		
		0.124	0.512	0.035	0.802	1.648	16.648	729		
	SB	0.000	0.000			1.648	16.648	729		
	EB	0.189	0.000			1.648	16.648	729	0.079	0.188
75	30	1.339	0.371	1.807	0.619	1.648	16.648	723		
		0.292	0.487	0.280	0.770	1.648	16.648	723		
		1.022	0.894	1.345	1.297	1.648	16.648	723		
	SB	0.0	0.0			1.648	16.648	723		0.141
	EB	0.2	0.0			1.648	16.648	723	0.049	

* FPU is loading is per g dry biomass. EB – Enzyme Blank, SB – Substrate Blank

Table A2-20 Week 6 Lime treatment HPLC results and corresponding overall conversion figures

.

Absorbance (V-s)				HPLC sugar concentration mg/ml (from standard curves)		pH adj. and extra cellulase (ml)	Total Vol. (ml)	Dry mass (mg)	Overall Conversion	
Temp	FPU *	glucose	xylose	glucose	xylose				glucose	xylose
65	15	0.000	0.124	0.000	0.299	1.648	16.648	717		
		0.000	0.095	0.000	0.262	1.648	16.648	717		
		0.000	0.148	0.000	0.330	1.648	16.648	717		
	SB	0.0	0.0			1.648	16.648	717		
	EB	0.2	0.0			1.648	16.648	717	0.000	0.047
55C*	15	4.815	0.675	6.879	1.013	0	15	727		
		3.546	0.515	5.027	0.806	0	15	727		
		3.815	0.330	5.420	0.566	0	15	727		
	SB	0.0	0.0			0	15	727		
	EB	0.2	0.0			0	15	727	0.312	0.216
65C	30	2.293	0.324	3.199	0.558	0	15	720		
		1.399	0.189	1.895	0.384	0	15	720		
		1.633	0.189	2.236	0.384	0	15	720		
	SB	0.0	0.0			0	15	720		
	EB	0.2	0.0			0	15	720	0.135	0.119
75C	30	2.012	0.270	2.789	0.488	0	15	732		
		1.988	0.270	2.754	0.488	0	15	732		
		1.470	0.189	1.998	0.384	0	15	732		
	SB	0.0	0.0			0	15	732		
	EB	0.2	0.0			0	15	732	0.150	0.124

* C = CONTROL

APPENDIX 3

A3.1 Procedure for the measurement of cellulase activity

The procedure is taken almost exactly from the NREL, "Measurement of Cellulase Activity", Laboratory Analytical Procedure (LAP), Revised January 2008, Technical Report # NREL/TP-510-42628. (Adney and Baker, 2008)

Introduction

1. The following method describes a procedure for measurement of cellulase activity using International Union of Pure and Applied Chemistry (IUPAC) guidelines (1). The procedure has been designed to measure cellulase activity in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. For quantitative results the enzyme preparations must be compared on the basis of significant and equal conversion. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC.

2. It is extremely important to keep in mind that the FPU is defined only at this extent of conversion. Reducing sugar yield is not a linear function of the quantity of enzyme in the assay mixture; as discussed by Ghose (1987), twice the amount of enzyme would not be expected to yield twice the reducing sugar in equal time. The assay procedure therefore involves finding a dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4% conversion in 60 minutes (or, in practical terms, finding two dilutions that bracket the 4%-conversion point so closely that the required dilution can be obtained, with reasonable accuracy, by interpolation) and then calculating the activity (in FPU/mL) of the original stock from the dilution required. Further comments on the required calculations, and their significance, are to be found in the Appendix.

3. Assay mixtures may in some cases contain reducing sugars unrelated to hydrolysis of substrate glycosidic bonds by the enzyme. Culture filtrates to be assayed for cellulase may contain nutrient sugars, and the reducing ends of the cellulose polymers of the substrate may sometimes be measurable as glucose equivalents before any enzyme attack. For this reason, controls consisting of (a) enzyme without substrate and b) substrate without enzyme are included with all enzyme assays and sample values are corrected for any blank values.

Apparatus

1. Water bath capable of maintaining 50 °C ± 1°C. (Or alternatively as was used in this study, a pot with hot plate, and rack for holding 15 ml centrifuge tubes.)
2. Spectrophotometer suitable for measuring absorbance at 540nm.

Reagants and Materials

1. **DNS Reagent**

   Mix: 141.6 ml DDW
   3,5 Dinitrosalicylic acid 1.06 g
   Sodium hydroxide 1.98 g

   Dissolve above, then add: Phenol (melt at 50oC) 0.76 mL
   Sodium metabisulfite 0.83 g
   Rochelle Salts 30.6 (Added for COSLIF only)

2. **Citrate Buffer**: For *Trichoderma reesei*, cellulase assays are carried out in 0.05 M citrate buffer pH 4.8. For other cellulase enzymes, the pH and the assay temperature may be different. The assay conditions must be defined when reporting results.

   Citric acid monohydrate 210 g
   DI water 750 mL
   NaOH - add until pH equals 4.3 50 to 60 g

   Dilute to 1 L and check pH. If necessary add NaOH until the pH is 4.5. When the 1 M stock citrate buffer stock is diluted with water to 50 mM the pH should be 4.8. After diluting the citrate buffer check and adjust the pH if necessary to pH 4.8.

Procedure

1. The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standards), prepared as detailed below. The substrate is a 50 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm).
2. Enzyme assay tubes:
3. Place a rolled filter paper strip into each 13 x 100 test tube.
4. Add 1.0 mL 0.05 M Na-citrate, pH 4.8 to the tube; the buffer should saturate the filter paper strip.
5. Equilibrate tubes with buffer and substrate to 50oC.
6. Add 0.5 mL enzyme diluted appropriately in citrate buffer. At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Target 2.1 and 1.9 mg glucose, respectively, for these two dilutions. Depending on the enzyme these targets may be hard to achieve and additional dilutions must be run.
7. Incubate at 50 C for exactly 60 min.
8. At the end of the incubation period, remove each assay tube from the 50 C bath and stop the enzyme reaction by immediately adding 3.0 mL DNS reagent and mixing.

9. Blank and controls:

   Reagent blank: 1.5 mL citrate buffer.

Enzyme control: 1.0 mL citrate buffer + 0.5 mL enzyme dilution (prepare a separate control for each dilution tested).
Substrate control: 1.5 mL citrate buffer + filter-paper strip.

10. Glucose standards:
    A working stock solution of anhydrous glucose (10 mg/mL) should be made up. Aliquots of this working stock should be tightly sealed and stored frozen. The standard should be vortexed after thawing to ensure adequate mixing.
11. Dilutions are made from the working stock in the following manner:
    1.0 mL + 0.5 mL buffer = 1:1.5 = 6.7 mg/mL (3.35 mg/0.5 mL).
    1.0 mL + 1.0 mL buffer = 1:2 = 5 mg/mL (2.5 mg/0.5 mL).
    1.0 mL + 2.0 mL buffer = 1:3 = 3.3 mg/mL (1.65 mg/0.5 mL).
    1.0 mL + 4.0 mL buffer = 1:5 = 2 mg/mL (1.0 mg/0.5 mL).
12. Glucose standard tubes should be prepared by adding 0.5 mL of each of the above glucose dilutions to 1.0 mL of citrate buffer in a 13 x 100 mm test tube.
13. Blanks, controls and glucose standards should be incubated at 50oC along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 mL of DNS reagent.
14. Color development (Miller, 1959):
    a. Boil all tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath.
    b. Let the tubes sit until all the pulp has settled, or centrifuge briefly. Dilute all tubes (assays, blanks, standards and controls) in water (0.200 mL of color-developed reaction mixture plus 2.5 mL of water in a spectrophotometer cuvette works well, use the pipettor to mix by drawing the mixture into the pipettor tip repeatedly). Determine color formation by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A.

Calculations

1. Construct a linear glucose standard curve using the absolute amounts of glucose (mg/0.5 mL) plotted against absorbance at 540nm. The data for the standard curve should closely fit a calculated straight line, with the correlation coefficient for this straight line fit being very near to one. Verify the standard curve by running a calibration verification standard, an independently prepared solution of containing a known amount of glucose which falls about midpoint on the standard curve.
2. Using this standard curve determine the amount of glucose released for each sample tube after subtraction of enzyme blank.
3. Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by means of a plot of glucose liberated against the logarithm of enzyme concentration To find the required enzyme concentration take two data points that are very close to 2.0 mg and draw a straight line between them, use this line to interpolate between the two points to find the enzyme dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar.

4. Calculate FPU:

$$\text{FPU} = \frac{0.37}{(\text{enzyme concentration})\ \text{releasing}\ 2.0\text{mg glucose}}\ \text{units/ml}$$

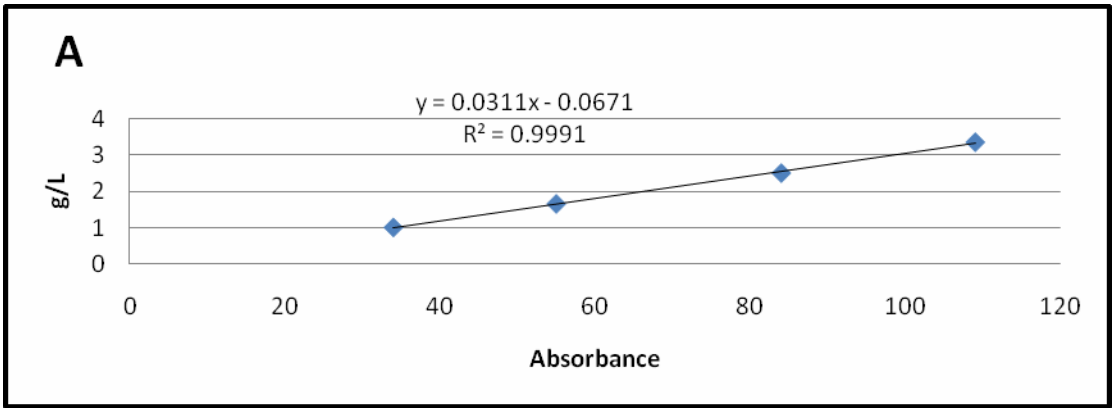
5. Where (enzyme concentration) represents the proportion of original enzyme solution present in the directly tested enzyme dilution (that dilution of which 0.5 mL is added to the assay mixture).

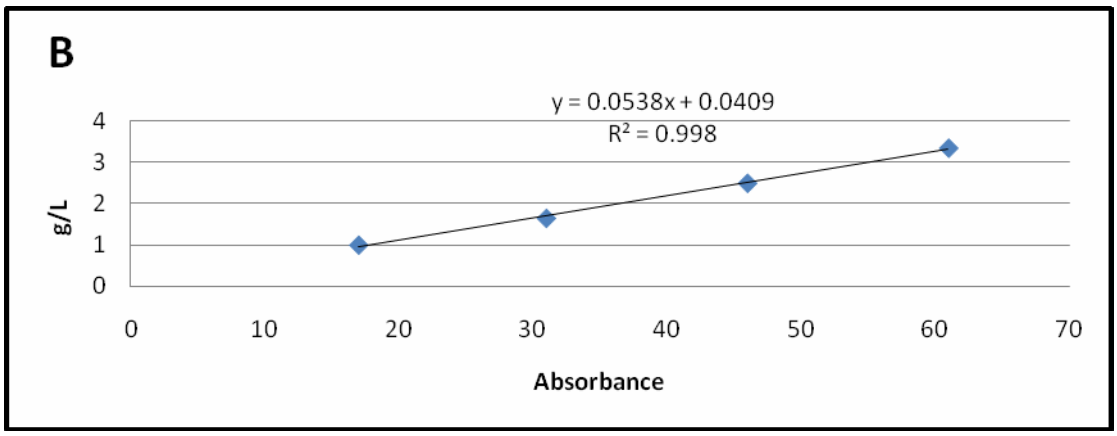
A3.1 Cellulase activity sugar standards

Table A3-1 COSLIF sugar standards and their HPLC absorbance readings.

Sugar Standards (mg/ml)			Absorbance pre-lime testing (540nm)	Absorbance pre-COSLIF (540nm)
Standard	glucose		glucose	glucose
1	1		34	17
2	1.65		55	31
3	2.5		84	46
4	3.35		109	61

Figure A3-1 Cellulase activity standard curves. **A**) Pre-Lime, **B**) Pre-COSLIF



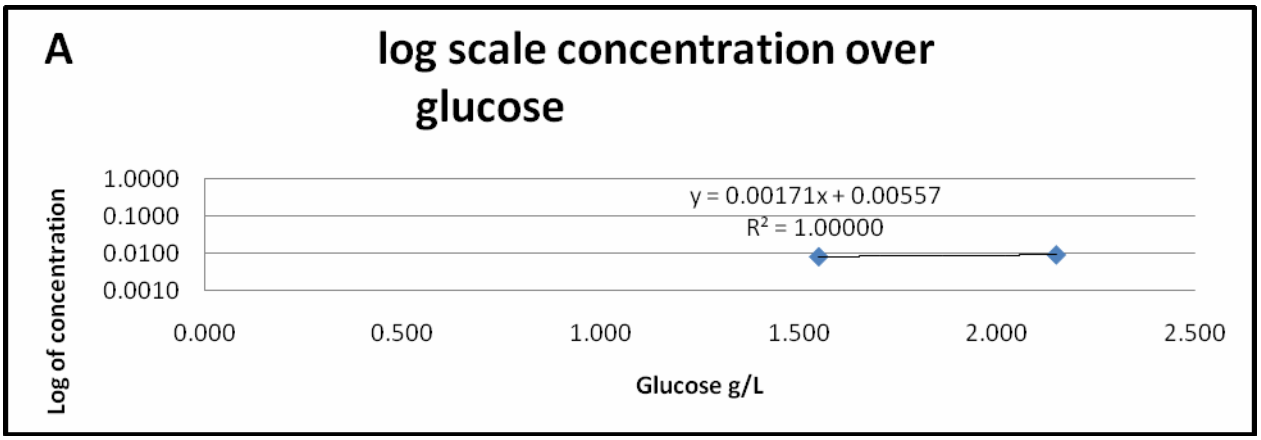


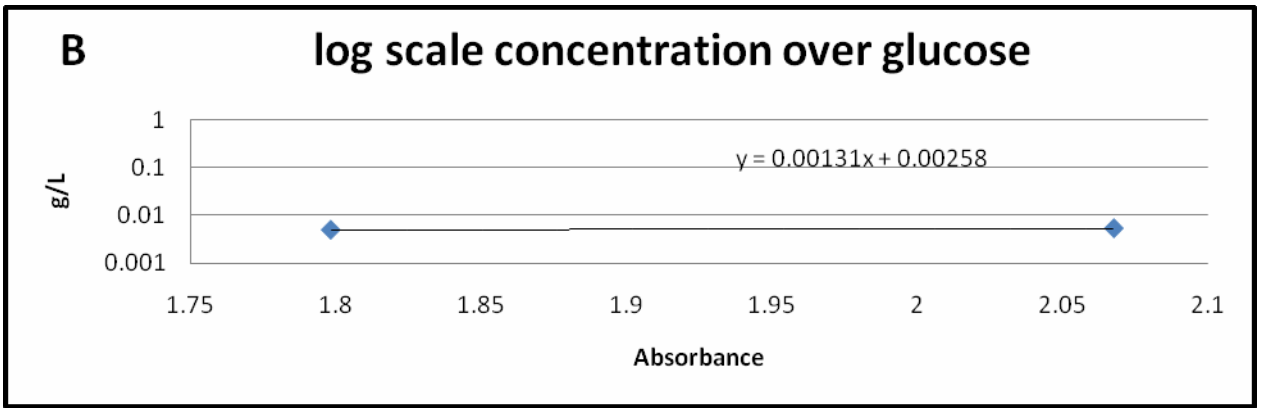
A3.2 Cellulase activity results

Table A3-2 Cellulase activity absorbance and corresponding glucose released

LIME						COSLIF				
Loading FPU	Abs	Average Abs.	glucose	Conc.		Loading FPU	Abs.	Average Abs.	glucose	Conc.
30	85	84	2.669	0.0123		65	39	39.7	2.174	0.0057
	85						40			
	81						40			
35	80	76	2.431	0.0106		70	38	37.7	2.067	0.0053
	72						37			
	76						38			
40	68	67	2.151	0.0093		75	31	32.7	1.798	0.0049
	62						32			
	71						35			
45	55	48	1.550	0.0082		80	21	22.7	1.260	0.0046
	46						24			
	42						23			

Figure A3-2 Cellulase activity concentration curves. **A**) Lime **B**) COSLIF





Lime pretreatment- Concentration to release 2.0 g of glucose ≅ 0.00898

0.00899 ≅ 41.2 FPU (0.37/0.00898)

COSLIF pretreatment- Concentration to release 2.0 g of glucose ≅ 0.00519

0.00520 ≅ 71.2 FPU (0.37/0.00898)