Exploring Pretreatment Methods and Enzymatic Hydrolysis of Oat Hulls

by

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Abstract

This thesis describes a way to achieve higher conversion rates of sugars from lignocellulosic biomass that can then be used for cellulosic ethanol production. Using oat hulls as the biomass, several chemical and physical pretreatment techniques were explored to overcome the recalcitrance and allow access to cellulose and hemicellulose. Experimentation with enzyme cocktails and dosing was done to obtain the highest conversions of cellulose and xylan to produce sugars. High solids-loading of the substrate, 14-16%, enabled higher conversion rates and would amount to lower cost of production in a commercial facility; however, end-product inhibition by the accumulation of inhibitors is also realized. To remove inhibition, a solid-liquid separation step was implemented which allowed enzymes to operate at a higher efficiency. The best combination of pretreatment and enzymatic hydrolysis led to a glucose of 89% and xylose yield of 84%, for trials conducted in a 20L bioreactor.

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List of Abbreviations

AF100	Alternafuel 100L
ANOVA	analysis of variance
CBM	cellulose binding module
CD	catalytic domain
CI	crystallinity index
DM	dry mass
DP	degrees of polymerization
HPLC	high performance liquid chromatography
MFX	Multifect Xylanase
NaOH	sodium hydroxide
NREL	National Renewable Energy Laboratory
VOC	volatile organic compounds

1. Introduction

Energy is needed to sustain life on earth. There are two sources of energy: renewable such as sunlight, wind, hydrothermal and geothermal and non-renewable sources such as fossil fuels. Today, most of our energy demands are fulfilled by fossil fuels such as petroleum, gas and coal. Many countries face energy insecurity as they rely on foreign countries and import their petroleum supply (Elliott 2000). The creation of renewable energy is a growing global concern as fossil fuel supplies run out and an energy crisis may arise. The need for renewable energy is growing due to an increased focus on climate change and energy security as well as increasing population, oil prices and decreasing availability of water and land. Biofuels are renewable energy resources as they are created from biomass such as plants. Bioethanol and biodiesel are common biofuels used today, with emphasis on bioethanol as the leading gasoline alternative. Biofuels are not a new type of fuel; they have been around for a long time but their usage diminished in the mid-20^{th} century due to an increase in the use of lower cost petroleum products. In the 1970s, a crisis in oil supply led to renewed interest in biofuels (Fischer and Preonas 2010). Bioethanol production from biomass is a promising alternative to gasoline; however, the cost of production is a major deterrent to its commercialization. It is estimated that the global annual production of biomass is 1×10^{11} tons, giving 2×10^{21}J of energy, ten times greater than the 2×10^{20}J from annual petroleum production. Therefore, the resources are available; however, the process optimization and economics need to be improved in order for bioethanol to gain wide acceptance (Binder and Raines 2010).

Bioethanol, C_2H_5OH, is a liquid fuel which is derived from plant material, such as agricultural wastes and plant crops. Bioethanol derived from sugarcane has been used as a transportation fuel since 1975 when the Proalcool program, a national alcohol program, was started in Brazil to overcome the energy crisis. In 1979, Brazil also introduced ethanol-fueled vehicles which made the Proalcool program gain momentum. Through the addition of government sponsorships, tax incentives and subsidies, Brazil has become energy self-sufficient by producing bioethanol (Rosillo-Calle and Cortez 1998). In 2006, Brazil was surpassed by the United States, which produced 18 billion liters, as the largest producer of bioethanol (Hettinga et al. 2008).

There are both advantages and disadvantages in using ethanol over conventional gasoline. The volumetric energy fraction of ethanol is about 25% lower then gasoline, resulting in a one-third reduction in the total energy per volume of ethanol consumed. However, ethanol combustion results in a 15% higher efficiency compared to gasoline. Ethanol is an oxidized fuel, 34.7wt% oxygen, compared to gasoline, which contains no oxygen. The oxygenated ethanol results in a lower stoichiometric air-to-fuel ratio. This means that a larger mass or volume of ethanol is needed to yield the same caloric output from combustion of gasoline. Ethanol also has a higher octane number than gasoline, allowing the fuel to be burned at a higher compression ratio and reduces engine knock (Kar and Deveci 2006). A compression ratio is defined as the ratio of the volume between the piston and cylinder head before and after a compression stroke. Therefore, a higher compression ratio results in higher power output, efficiency and better fuel economy. The improved combustion efficiency means that the overall fuel economy is better than that predicted based on energy content alone. Ethanol also has a higher latent heat of vaporization (1177kJ/kg at 60*C) compared to gasoline (348kJ/kg at 60*C), resulting in engine cooling and a cleaner operation. The use of ethanol as a transportation fuel leads to significant reductions in CO_2(g), NO_x(g) and VOC emissions. The carbon dioxide released during burning of the fuel is equal to the amount of carbon dioxide fixed by the plant during photosynthesis, implying a zero net production of carbon dioxide. Therefore, bioethanol results in a net reduction of carbon dioxide as compared to fossil fuels, such as gasoline (Naik et al. 2010). Alternatively, the emissions of aldehydes such as acetaldehyde and formaldehyde are increased (Otero, Panagiotou and Olsson 2007). Cellulosic ethanol could reduce green house gas emissions by 85%, if it completely replaces gasoline (Bergeron, P., 1996). Due to the many advantages of ethanol over gasoline, it seems promising that ethanol can replace gasoline and has the potential to be more cost effective and efficient.

Bioethanol can be used as a fuel additive, or a complete alternative to petroleum gas. Ethanol can be combined with gasoline at low levels, e.g., 10% or E10, for use in unmodified cars. Flex-fuel vehicles can accommodate mixtures of ethanol higher than E10, including pure ethanol, E100. Ethanol acts as a replacement for the fuel additive methyl t-butyl ether (MTBE). MTBE had been used extensively as an octane enhancer and to reduce carbon monoxide and ozone production from the emission of volatile organic compounds. However, MTBE can make its way into

ground water, contaminating drinking water with severe detrimental effects on health (Green and Lowenbach 2001). In 2000, the US Energy Policy Act issued an ANPR (Advance Notice of Proposed Rulemaking) under the TSCA (Toxic Substance Control Act) to limit the use of MTBE as a fuel additive in gasoline (Yao et al. 2009). The use of MTBE was essentially discontinued in 2006 when the U.S. Government waived oxygenate requirements while refusing to issue a liability waiver to oil companies that included MTBE in their fuel (http://www.epa.gov/oust/fedlaws/publ_109-058.pdf)

Bioethanol produced from agricultural crops such as corn, oats and barley is termed first generation bioethanol. The plant biomass contains starch and the pathway to production is different than that of second generation biofuels. Second generation biofuels such as lignocellulosic ethanol are made from lignocellulosic biomass, which can include agricultural wastes such as oat hulls, corn husks and soy hulls or non-agricultural biomass such as wood chips. Second generation biofuels are more difficult to produce from the biomass than first generation biofuels, due to differences in their structures. However, second generation biomass offers some valuable benefits over its predecessor, such as: the avoidance of competition with human food and animal feed which might otherwise lead to rising food costs and increased land usage, it is relatively inexpensive, it is locally available in many countries and reduces the environmental risks such as soil degradation and water and air pollution associated with first generation biomass (Naik et al. 2010). Average GHG emissions from first-generation ethanol are 62g and 56g CO_2 Eq.MJ^{-1} for wheat-grain and sugarbeet respectively, while second generation ethanol emissions ranged from 24-30g CO_2 Eq.MJ^{-1} for woody crops and wheat straw. Energy requirements for first-generation ethanol are also much higher with wheat and sugarbeet both at 0.7 MJ_{in}:MJ_{fuel} compared with a range of 0.1–0.35 MJ_{in}:MJ_{fuel} for second-generation ethanol (Whitaker et al. 2010).

2. Literature Review

2.1 Lignocellulosic biomass

Lignocellulosic biomass includes agricultural residues, dedicated energy crops, wood, grass, municipal solid waste. Lignocellulose is composed of cellulose, hemicellulose and lignin which are strongly bonded by non-covalent cross linkages. Each of these three components can be degraded by a variety of microorganisms through production of various hydrolytic and oxidative enzymes. The composition of lignocellulosic biomass varies greatly by type of species but contains approximately 30-50% cellulose, 20-30% hemicellulose and 10-25% lignin (Sanchez 2009).

Table 2-1: Lignocellulose composition of agricultural residues, based on dry wt%

	Cellulose	Hemicellulose	Lignin
Corn stover	36.5	28.1	10.4
Oat hulls	48.4	16.1	16.2
Soybean hulls	48.0	17.0	2.0
Cotton seed hulls	49.0	17.0	24.0
Rice husks	31.3	24.3	13.3
Tomato plant vines	25.7	6.0	10.5
Sweet potato vines	19.8	14.6	7.7

Adapted from http://www.ecn.nl/phyllis/single.html

Oat hulls will be used exclusively for this thesis. In Canada, the annual production of oats ranges from 3.5 to 4.5 million tonnes. A further 1.5 million tonnes are produced in the U.S. It is estimated that the hull accounts for 25% of the oat, which would mean 0.9 to 1.1 million tonnes of oat hulls generated in Canada and 0.4 million tonnes of oat hulls in the U.S. (Thompson et al. 2000). Due to the significant amount of waste that is generated from oats, oat hulls are a viable source for bioethanol production. (http://www.agr.gc.ca/pol/mad-dam/index_e.php?s1=pubs&s2=go-co&s3=php&page=go-co_2009-12-15). The estimated potential ethanol production from the total amount of oat hulls in Canada and the U.S is 612 million litres.

2.2 Lignocellulose Chemistry

2.2.1 Cellulose

Polysaccharides are synthesized by plants, animals, and humans to be stored for food, structural support, or metabolized for energy. Cellulose is a polysaccharide made up of glucose monomers, and is the most abundant organic polymer on Earth (Zhu et al. 2006) (Habibi 2010). It is the main structural component of the rigid plant cell wall and offers strength to the plant. Cellulose is strong, crystalline, resistant to hydrolysis and is water insoluble (Habibi 2010). Its structure is defined by a linear chain homopolymer with the formula $(C_6H_{10}O_5)_{n,}$ where n is the degrees of polymerization (DP) of the polymer. Cellulose is a polydisperse product; the DP value gives an indication of the number of anhydroglucose units joined by glycosidic bonds in the cellulose chain; this number can vary dramatically depending on the source. On average, cellulose contains several hundred to over ten thousand β-D-glucopyranose units linked by (1, 4)-glycosidic bonds. The glycosidic bonds are formed between the hemiacetal group of one monomer and the hydroxyl group of another monomer. β-D-glucopyranose is the pyranose ring form of glucose, which exists as the C_1 chair conformation and has the lowest energy conformation for D-glucopyranose. The structure of cellulose is displayed in Figure 2-1, below. Cellobiose is a dimer formed by the hydrolysis of two glucose monomers by a β(1,4)-glycosidic bond (Habibi 2010) (Bidlack et al. 2002) (Sanchez 2009). The acetyl linkage of the glucose monomers is a β(1,4)-glycosidic bond, unlike starch which contains an α(1,4)-glycosidic bond. The difference in acetyl linkages in cellulose results in an inability for humans to digest cellulose, due to a lack of the enzyme that hydrolyzes this glycosidic bond and an inability of this enzyme to penetrate this highly ordered structure (Habibi 2010) (Bidlack et al. 2002).

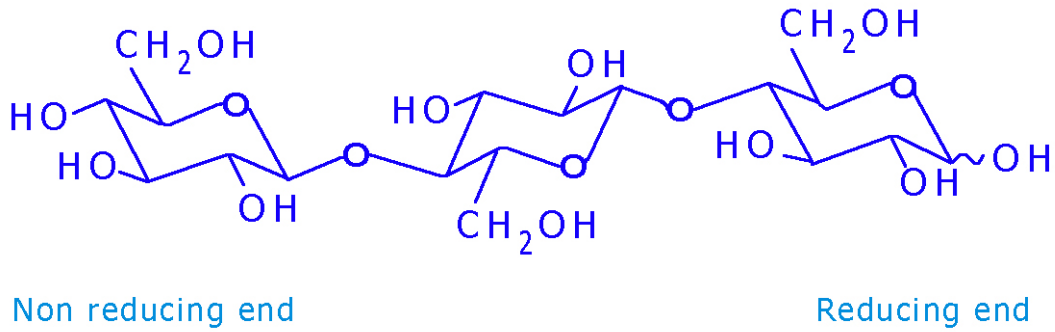

Figure 2-1: Structure of cellulose

Cellulose molecules form intra- and intermolecular hydrogen bonds. The intramolecular bonds hold glucose residues together to form a long cellulose chain. Intermolecular hydrogen bonds form between roughly 40 cellulose chains to create a microfibril which is a packed network of cellulose with a sheet-like formation giving a stiff and rigid structure. These highly ordered regions of cellulose with lateral hydrogen bonding between linear cellulose fibres and are known as crystalline cellulose. The microfibrils prevent cellulases from breaking down this resistant structure and also prevent water molecules from penetrating into the structure. Native cellulose consists of 50-90% enzyme-resistant crystalline cellulose and the rest of the structure is amorphous, which is less ordered. A parameter termed crystallinity index (CI) gives a relative proportion of crystalline and amorphous regions in the particular cellulosic substrate. The amorphous regions are much more easily hydrolyzed than crystalline regions. Amorphous regions are found in a small portion in native cellulose and are also produced from native cellulose by pretreatment methods (Fan et al. 1980). Cellulose morphology consists of a well organized network of fibrillar elements. The primary cell wall contains 8-25% cellulose microfibrils. The secondary cell wall contains 40-80% cellulose microfibrils within three layers known as S_1 and S_2, S_3 lamellae (Krassig 1993) (Klemm, Schaumder and Heinze), (Bidlack et al. 2002) (Habibi 2010).

Cellulose I is the native crystalline form of cellulose found in nature. Cellulose I is composed of a mixture of two polymorphs, cellulose I_α, which is a metastable triclinic unit cell with one cellulose chain, and cellulose I_β, which contains two cellulose chains in its stable monoclinic unit cell (Koyoma et al. 1997). Crystal modifications to this structure result in different forms of

cellulose produced with varying unit cell dimensions. Cellulose II can be prepared by regeneration, dissolving cellulose into an aqueous medium and then recrystallization, or exposing native cellulose to the process of mercerization which will be described later. The conversion of cellulose I into cellulose II is an irreversible process. Cellulose III_I and III_{II} crystal modification can be obtained by treating cellulose I and cellulose II respectively, with liquid ammonia or an organic amine followed by washing with alcohol. Cellulose IV_I and IV_{II} are the final known crystal modification of cellulose, obtained by heating of III_I and III_{II}, respectively (Park et al. 2010 and O'Sullivan 1997).

2.2.2 Hemicellulose

Hemicellulose is a polysaccharide found in the primary cell well (25-50%) and secondary cell wall (10-40%) of lignocellulosic material (Bidlack et al. 2002). Hemicellulose is synthesized by glycosyltransferases, which are present in the Golgi membrane. Hydrogen bonding takes place between hemicellulose and cellulose with the aid of pectin, a structural heteropolysaccharide, to form a network of cross-linked fibres. Unlike cellulose, hemicellulose has a random, branched, amorphous structure and is easily degradable. Hemicellulose chains are much shorter than cellulose, with average DP values less than 200. Hemicellulose is also a much more complex structure than cellulose; it is composed of several different monomers, many of which are five carbon sugars, making it a heteropolymer. The type and quantity of sugar present in the polymer varies widely between plant species. Xylan, mannan and galactan form the backbone of hemicellulose and arabinan, glucuronic acid and galactan form the side chains. Xylan is the largest component of hemicellulose, constituting up to 70% of the structure (Dashtban et al. 2009). The number of sugars in the side chains varies widely, giving a linear or branched structure. The main classes of hemicelluloses are: D-xyloglucans, composed of D-xylopyranose attached to a cellulose chain; D-xylans, composed of D-xylose; D-mannans, composed of D-mannose; and D-galactans, composed of D-galactose. Like cellulose, the monomers are linked by $\beta(1\rightarrow4)$ bonds. The branched structure of hemicellulose is commonly acetylated or methylated. Hemicellulose forms a physical barrier which surrounds cellulose. In the primary cell wall, hemicellulose interacts with other polymers to maintain the physical properties of the wall. In many types of plants, hemicellulose can also act as a seed storage carbohydrate. Hemicellulose

strengthens the secondary cell wall and facilitates transport of water to the plant (Scheller and Ulvskov 2010) (Dashtban et al. 2009).

2.2.3 Lignin

Cellulose and hemicellulose are tightly bound to lignin. Lignin is a major structural component of the secondary cell wall of plants and some algae. Lignin is the second most abundant organic polymer on Earth, after cellulose. Lignin is a complex phenolic polymer which is made up of the monolignols coniferyl, sinapyl and p-coumaryl alcohols. Lignin is hydrophobic in nature and makes the plant cell walls impermeable to water. Due to its complex structure, lignin is the most recalcitrant component of lignocellulose followed by cellulose due to its highly crystalline structure and then hemicellulose. Recalcitrance is defined by difficulty to treat and inability to break down the lignocellulose (Freudenberg 1996). The empirical formula for lignin is $C_9H_{10}O_2(OCH_3)_n$, where n is the ratio of CH_3 to C_9 groups. The values of n for hardwood, softwood and grasses are 1.4, 0.94 and 1.18 respectively. The chemical structure of lignin can vary dramatically, making it difficult to define.

Lignin is biosynthesized in a process known as lignification. This involves a free radical coupling reaction of coniferyl, sinapyl and p-coumaryl alcohols. Enzymes termed peroxidases and laccases initiate the process by breaking the covalent bond between the phenolic oxygen and its hydrogen. The bond is broken and one electron stays with the oxygen and the other goes with the hydrogen, generating a free radical. The initial free radical attempts to stabilize itself by moving the electron throughout the molecule by a process known as resonance stabilization. In doing this, a series of resonance-stabilized free radicals are formed which are in constant equilibrium with one another. When one free radical meets another, they combine to share their electrons, making a new covalent bond. Two monomers would come together to make a dimer; the dimer still has a free phenolic hydroxyl group and an enzyme can remove the hydrogen and electron to make another free radical. The dimer can combine with a monomer or another dimer and this process continues until a polymeric structure results. This reaction produces a polydisperse polymer product with no extended sequences of regularly repeating units; its composition is generally characterized by the relative abundance of *p*-hydroxyphenyl, guaiacyl

and syringyl units and by the distribution of interunit linkages in the polymer, as well as hydroxycinnamyl alcohol end groups. This process is very metabolically costly to the plants; lignin cannot be broken down by the plant, so the process is irreversible and energy consuming (Adler 1977) (Roberts 1996).

2.3 Bioconversion of Lignocellulose to Ethanol

The conversion of lignocellulosic biomass to ethanol involves a series of steps: pretreatment to enhance digestibility of the biomass, enzymatic hydrolysis to break down the cellulose into glucose monomers, fermentation to convert the sugars into ethanol and distillation to remove water from the ethanol (Taherzdeh and Karimi 2008). In order to maximize the yield of the final product, ethanol, each of these steps must be optimized to achieve the maximum conversion rate. This thesis will focus on the first two steps in order to improve the potential yield of glucose for the latter two steps. The pretreatment and enzymatic hydrolysis steps will be discussed in further detail later in this section.

During fermentation, the sugars produced during hydrolysis are converted to ethanol. Fermentation involves the break down of glucose or xylose by micro-organisms. The most common micro-organism used for this process is the yeast *Saccharomyces cerevisiae.* In xylose-fermenting yeasts, such as *Pichia stipitis* and *Candida shehatae,* xylose is reduced to xylitol by xylose reductase, which is then oxidized to xylulose by xylitol dehydrogenase. *Saccharomyces cerevisiae* and most available fermenting microorganisms cannot ferment xylose due to the lack of xylose reductase and xylitol dehydrogenase (Zyl et al., 1989). However, the ethanol production rate from *Saccharomyces cerevisiae* is five-fold higher than fermentation with xylose-fermenting yeast acting on both glucose and xylose (Agbogbo et al. 2006) The first stage of the fermentation of glucose is known as glycolysis, where glucose is converted in a multi-step reaction to pyruvate and energy is consumed in the form of two ATP molecules and two molecules of NAD+ are reduced to NADH. In the second stage of the reaction, pyruvate is then decarboxlyated to form acetaldehyde, which is then reduced to ethanol by alcohol dehydrogenase. During the second stage of the reaction, NADH is reoxidized to NAD+ to

recover the ATP molecules consumed in the first step. For each glucose molecule fermented, two ethanol and two carbon dioxide molecules are produced (Li et al. 2003). The overall reaction is:

$$C_6H_{12}O_6\,(l) + H_2O\,(l) \rightarrow 2C_2H_5OH\,(l) + 2CO_2\,(g) + H_2O\,(l) + \text{heat}$$

The process stream from fermentation is known as beer and usually contains up to 14% ethanol. For bioethanol to be used as a fuel, water must be removed from this beer. In mixtures with gasoline, ethanol must be concentrated to at least 99.5%. Distillation is a method that separates the ethanol and water mixture. The boiling point of ethanol is 78.4°C and the boiling point of water is 100°C (Junqueira et al. 2009) (Udeye et al. 2009). Distillation cannot completely separate ethanol and water, but results in an azeotrope of 96% ethanol and 4% water. The resulting azeotropic mixture obtained by distillation is known as a Rectified Spirit (RS). Dehydration of the Rectified Spirit can further remove water to make the ethanol suitable for as a fuel additive. Dehydration is most often done using molecular sieves; an older method employed azeotropic distillation with the addition of an entrainer, but this is rarely used now as the process is very energy intensive. Dehydration using molecular sieves uses the technology of Pressure Swing Adsorption (PSA). The molecular sieve, made of zeolites, is packed into two columns and operates through a cycle of dehydration/regeneration. Zeolites are microporous substances that have the ability to selectively adsorb chemical species onto their surface; ethanol molecules are too large to adsorb onto zeolite, so there is no competition for adsorption sites. When the RS is introduced to the first column under pressure, dehydration occurs; the zeolites adsorb water in the vapour phase onto their surface. Following saturation of the first column, the anhydrous ethanol vapours are passed to the second column. Desorption under low pressure using a vacuum occurs in the first column during regeneration to remove water from the zeolites and regenerates the molecular sieves for further use; dehydration occurs in column two during this time. The cycle continues with dehydration in column one while regeneration occurs in column two. Anhydrous ethanol is obtained after dehydration is complete (Bastidas et al. 2010) (Pruksathorn and Vitidsant 2009).

2.3.1 Pretreatment

The main goal of pretreatment is to make the biomass more susceptible to enzymatic attack, thereby increasing conversion rates and yields of sugar. Pretreatment breaks apart the bonds connecting lignin, cellulose and hemicellulose. Figure 2-2 depicts the effect of pretreatment on the structure of lignocellulose. Pretreatment methods typically use chemical, physical, mechanical or biological techniques to break down lignocellulose (Hector et al. 2008). Chemical pretreatment methods alter the crystal structure of glucose and hemicellulose. Common chemical pretreatments use sodium hydroxide, ammonia, lime, ammonia fibre explosion, concentrated acid and dilute acid. The treatment with sodium hydroxide, termed mercerization, will be discussed in further detail later in the thesis. Physical pretreatment methods break down the lignocellulose and reduce the particle size, which increases the surface area for enzyme attack. They also decrease the crystallinity of cellulose, which is resistant to enzyme attack, and reduce the degree of polymerization. By breaking down lignocellulose, lignin is removed and access to cellulose and hemicellulose is enhanced. Physical pretreatment methods involve grinding and milling such as ball milling, steam explosion, mechanical refining and szego milling; the latter two will be expanded on further. Biological pretreatment methods involve the use of white or brown rot fungi to degrade lignin and hemicellulose, and are considered environmentally friendly pretreatment methods. Cellulose, hemicellulose and lignin each have different reactivities towards these pretreatment technologies, making the process very difficult to generalize. These cell wall polysaccharides are recalcitrant and much more difficult to break down than storage polysaccharides like starch and therefore, a pretreatment step is necessary before enzymatic hydrolysis can take place. The type of pretreatment method chosen ultimately depends on the type of lignocellulose (Taherzdeh and Karimi 2008) (Mosier et al. 2005) (Kumar et al. 2009).

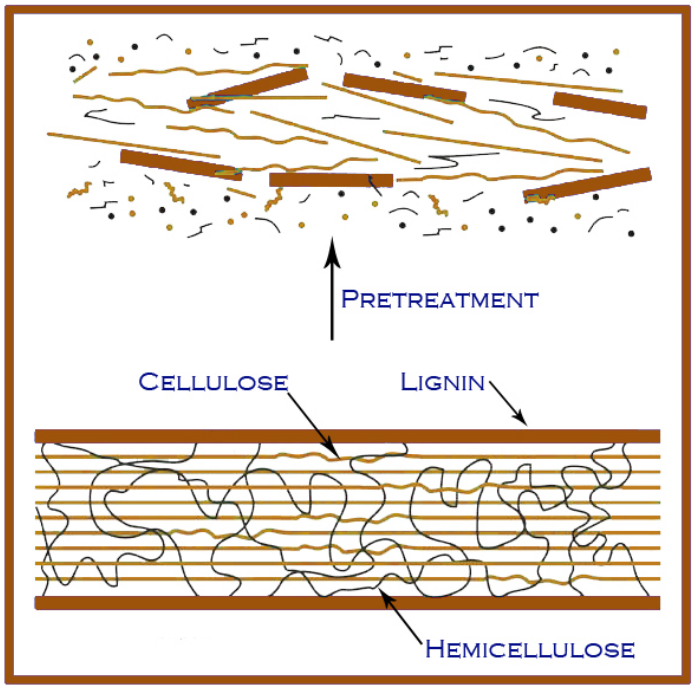

Figure 2-2: Effect of pretreatment on lignocellulose structure (adapted from Hector et al. 2008).

2.3.1.1 Chemical Pretreatment Methods

Mercerization

In 1844, John Mercer was the first to notice that cellulose went through many changes when treated with sodium hydroxide. Treatment of cellulosic materials with sodium hydroxide was then called mercerization and the cellulose subjected to the treatment termed alkali cellulose (Heines 1944). Mercerization is used widely in industry to treat fibre, for example it is used in the manufacturing of viscose, the finishing of textiles and the production of tissues. Mercerization is a process of treating fiber with sodium hydroxide at elevated temperature for a certain amount of time depending on the reaction conditions. Mercerized cellulose is obtained when a concentrated solution of sodium hydroxide is used to treat the cellulose, followed by washing with water. The mercerized cellulose is also referred to as Cellulose II, a cellulose hydrate, due to the modification of the crystal structure of native cellulose, Cellulose I. From a chemical stand-point, the mercerized cellulose is very similar to the native cellulose. Structurally, the mercerized cellulose is very different and results in a change in the position of the chain links and cellulose macromolecules. It is also more hygroscopic, is intensely coloured and is more

readily hydrolyzed by acids. The physiochemical process produces swelling of fibres, an increase in diameter and reduction in length, partial dissolution, and a change from helical fibres to flat round fibres. Swelling also results in the loss of low-molecular weight fractions and decreases the crystallinity of the fibres. The factors that affect the extent of swelling are adsorption of alkali, concentration of alkali, temperature, fiber structure and the medium in which the swelling takes place (Kim et al. 2006) (Borysiak and Doczekalska 2008). The extent of change depends on the severity parameter, which takes temperature and concentration of the caustic used into consideration. Borysiak and Doczekalska (2008) have shown that increasing temperature and concentration of caustic both increase the amount of cellulose II produced.

Using X-ray technology, it has been shown that structural changes in cellulose II result from a rotation of the glucose residues in the unit cell of cellulose and a shift of the chains in a direction perpendicular to the fiber axis. Mercerization loosens the intermicellar structure, resulting in an increase in the volume of intermicellar spaces. Also, a decrease in intramolecular hydrogen bonds and a corresponding formation of intermolecular hydrogen bonds occurs. As a result, the number of hydrogen bonds per anhydroglucose unit increases. Therefore, the free energy of the mercerized cellulose is smaller than that of native cellulose. When alkali cellulose is washed, it becomes decomposed and is termed ‘cellulose hydrate’, which now has the formula $C_6H_{10}O_5.H_2O$. If cellulose hydrate is completely dried, it will have the same chemical composition as native cellulose (Nikitin 1966).

2.3.1.2 Physical Pretreatment Methods

Disc refining

Disc refiners are used extensively in paper manufacturing. They are several variations of disc refiners, including single-disc, double-disc and multi-disc, but this thesis will deal exclusively with double disc refiners. Double-disc refiners are made up of a rotating disc which runs close to a stationary disc with the material to be treated running under pressure between the two separated discs. Another set-up for the double disc refiner requires two discs rotating in opposite directions while the feed runs through the two discs. When the material makes its way to the discs, the

rotating disc provides the power to shear the fibres apart, and the fibres are ejected by pressurized, centrifugal action. The consistency of the biomass, the type of biomass used and the distance between the two discs affects the disc friction and thus determines the extent of the refining. Disc refining can cause external fibrillation of the pretreated fibres. Fibrillation results in a change in the outer layers of the fibre, removing the primary wall and then the secondary wall. The extent of removal of the primary and secondary wall is usually rather small (Whalley and Mitchell, 1997).

Szego milling

A szego mill (Szego Mill SM-320 from General Comminution Inc) is a machine designed for grinding fibrous materials. It has a planetary ring-roller designed for very fast and efficient grinding. It consists of four rollers with helical grooves and ridges, rolling inside a vertical cylinder called the stator, which is the grinding surface. The internal diameter of the stator is 320mm and has four 110mm diameter rollers with a height of 400mm which move radially within the stator. The fibres are subjected to crushing and shearing between the roller ridges and the stator as they move down the stator. The grooves aid transport of material through the mill. The crushing force is created mainly by the radial acceleration of the rollers; shearing action is induced by the high velocity gradients generated in the mill. Hence, the primary forces acting on the particles are the crushing and shearing forces produced by the circumferential motion of the rollers. The speed of rotation around the central shaft can vary between 400 and 1200 rpm**.** With each pass through the rollers, the substrate is reduced in particle size which increases surface area and makes enzyme attack feasible (http://www.general-comminution.com/new_page_1.htm).

2.3.2 Enzymatic Hydrolysis

In order to break down the very recalcitrant structure of cellulose, hydrolysis reactions aided by enzymes are needed. After pretreatment, the biomass should be much more susceptible to enzymatic attack. Enzymes are added to the biomass, acting as catalysts to break down the glycosidic bonds of the polysaccharides. The mechanism involved in the enzymatic degradation of lignocellulose depends on the chemical nature and physical structure of the substrate.

Structural features of the substrate that affect enzymatic hydrolysis are crystallinity, specific surface area, accessibility to enzyme adsorption, degree of polymerization and the unit cell dimensions of the lignocellulose (Taherzdeh and Karimi 2008). Several enzymes are needed to work together synergistically to break down the various polysaccharides. Enzymes are estimated to contribute to 10-20% of the total cost of bioethanol production (Barta et al. 2010). On a commercial scale, the cost of enzymes needs to be lowered in order for ethanol production to be cost effective. This would require enzymes with the best activity in order to minimize the amount needed and keep the cost at a minimum.

2.3.2.1 The Cellulase System

Cellulases are the most important enzymes in the enzymatic hydrolysis enzyme cocktail; they are responsible for converting cellulose to the valuable end-product glucose. In order to be effective, cellulases must have the following properties: ability to hydrolyze crystalline cellulose completely, withstand slightly acidic pH (4-5), withstand process stress and should be cost effective (Bajaj et al. 2009) (Tolan and Foody 1999). The ability to engineer cellulases with these desirable properties is key to commercialization of bioethanol. Microorganisms are responsible for 90% of the degradation of cellulosic biomass; of this, fungi account for 80%. Enzymes are produced from the microorganisms through fermentation. The aerobic fungus *Trichoderma reesei* produced the first high-efficiency cellulase used in lab-scale and commercial applications in 1950 (Reese et al. 1950). Today, *Trichoderma reesei* is still the most commonly used fungus since it is well understood and very efficient at hydrolyzing cellulose. A cellulase system, which is a mixture of hydrolytic enzymes, is required to saccharify cellulose into glucose monomers. Cellulose is hydrolyzed by this system of enzymes that acts synergistically and hydrolyses the β-(1,4) glycosidic linkages. An endoglucanase, endo-1,4- β -D-glucanase; randomly cleaves the cellulose to produce free chain ends. An exoglucanase, also known as a cellobiohydrolase, exo-1,4-β-D-glucanase, cleaves cellobiose units from the non-reducing ends. The soluble disaccharide is then hydrolysed by β-D-glucosidase to glucose monomers (Tolan and Foody 1999) (Ting et al. 2009). Cellulases usually have two domains: a catalytic domain (CD) and a cellulose binding module (CBM), which are connected by a linker region. In *Trichoderma reesei*, the CBM is made up of 35 amino acids and the linker region contains many serine and threonine

residues. CBMs increase cellulase activity by increasing the amount of CDs on the biomass. The role of the linker region however, is unknown. At the start of enzymatic hydrolysis, the amorphous non-crystalline regions of the biomass are attacked by cellulase due to their ease of accessibility. As the total amount of non-crystalline regions begins to decrease, the rate of enzymatic hydrolysis begins to drop; this is the rate-limiting factor for enzymatic hydrolysis. This occurs because the decrease in non-crystalline regions means an increase in crystalline regions, which reduces accessibility to glycosidic bonds. Apart from biomass recalcitrance, cellulase efficiency is limited by: end-product inhibition, thermal deactivation, non-specific binding to lignin and irreversible adsorption of the enzymes to the substrate (Ting et al. 2009).

Hydrolysis of cellulose involves a series of steps. First, the CBM facilitates adsorption of cellulase onto the substrate. The rate of adsorption is influenced by the viscosity and agitation of the reactor system. In a well-mixed system, adsorption can be obtained within 5 minutes (Tolan and Foody 1999). Endoglucanase and cellobiohydrolase then locate bonds for hydrolysis and form an enzyme substrate complex. Endoglucanase breaks β-glycosidic bonds at random locations on the cellulose chain via the CD. The chain end of cellulose is inserted in the CD domain and cellobiohydrolase converts the free chain end into cellobiose units (Bansal et al. 2009).The structure of cellobiohydrolase is indicative of its function; the tunnel-like structure of the catalytic domain traps cellulose in place and facilitates the release of cellobiose only. This structure has many substrate binding sites and likes crystalline cellulose as the substrate. Alternatively, the catalytic domain structure of endoglucanase is open and results in arbitrary scission of cellulose, cleaving cellulose, cellobiose and cellotriose. Endoglucanases operate on the amorphous regions of cellulose, and have little activity towards crystalline regions (Mousdale 2008). After the hydrolysis of the β-glycosidic bond takes place within the complex, the enzymes move forward along the cellulose chain. After the chain is broken down almost completely into cellobiose, β-glucosidase breaks the β-glycosidic bond between the glucose dimer, generating glucose monomers. Desorption of the enzymes from the substrate then occurs (Bansal et al. 2009).

The cellulolytic enzymes are inhibited by their products, cellobiose and glucose, disrupting the cellulase system just described. Cellobiose is an inhibitor of endoglucanases and

cellobiohydrolases (Zhao et al. 2004) (Tolan and Foody 1999). Therefore, cellobiose accumulation will impede the release of glucose during enzymatic hydrolysis, since the enzymes predominantly responsible for its release are inhibited. In order to overcome this inhibition, cellobiose needs to be broken down by β-glucosidase. Therefore, it can be stated that the rate of conversion of cellulose to glucose is determined largely in part by the activity of β-glucosidase. In commercial cellulase preparations, the presence of β-glucosidase is usually very low due to its low activity and production yield from *Trichoderma reesei*. The activity and presence of endoglucanases and cellobiohydrolases are both high, leading to the high concentrations of cellobiose found in the hydrolyzate. Adding β-glucosidase from another source, such as the enzyme Novo188 produced from the fungi *Aspergillus niger*, is a way to overcome this limitation and increase the activity of β-glucosidase (Chauve et al. 2010). However, β-glucosidase is also inhibited by glucose. Therefore, adding Novo188 prior to a solid-liquid separation may not have any noticeable effect, since glucose has accumulated and the β-glucosidase present in the cellulase enzyme system is already inhibited. A solid-liquid separation step to remove the accumulated glucose will allow β-glucosidase to act on its substrate without significant interference by inhibitors. At high solids-loadings, the effect of these inhibitors is more pronounced as the concentrations of glucose and cellobiose will be higher than at a low solids-loading (Andric et al. 2010). Therefore, a key focus of this thesis is design of an the appropriate enzyme system and reaction process suitable for high solids loadings, so that the effect of inhibitors can be minimized.

2.3.2.2 Hemicellulases:

Hydrolysis of hemicellulose into its constituent monomers requires the action of several enzymes. β-D-xylanases, β-D-mannases, β-D-galactanases as well as glycosidases β-D-xylosidase α- and β-D-galactosidase and β-D-mannosidase are all needed in this enzyme system. The structure of hemicellulose is complex due to its variable structure; therefore, several enzymes must work together to break down its structure. Hemicellulases contain a CBM and CD, like cellulases. The CD contains glycoside hydrolases which hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. Xylanases hydrolyze the β-1,4 bond in xylan, creating xylooligomers which are further

reduced to xylose by xylosidases. Mannan-based hemicellulose is hydrolyzed by β-mannases to β-1,4-manno-oligomers, which can then be hydrolyzed to mannose by β-mannosidase. α-L-Arabinofuranosidases and α -L-arabinanases hydrolyze arabinofuranosyl-containing hemicelluloses. α -D-Glucuronidases hydrolyze the a-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain of xylans. Hemicellulolytic esterases include acetyl xylan esterases that hydrolyze the acetyl side chains on xylan. Feruloyl esterases hydrolyze the ester bond between the arabinose substitutions and ferulic acid, which is the bond connecting xylan to lignin (Shallom and Shoham 2003).

2.4 Scale-up considerations

One of the main objectives of several experimental studies performed during recent years has been to increase the solids loading during processing of lignocellulosics. This results in reduced water consumption, which greatly reduces the energy demand in distillation and evaporation. Scale up would also increase product concentrations and plant productivity. Although scale up is desirable, the accompanying higher solids loading can decrease product yields from some substrates. The high-solids loading increases the viscosity of the slurry and can result in poor mixing, which contributes to decreased enzyme adsorption. Kristensen et al. 2009 showed an inverse linear relationship between increasing solids content and decreasing cellulase adsorption. The effect of inhibition is greater at high solids loadings and can also reduce conversion rates to the main product, glucose. Inhibition does not always affect the maximum yield of sugars, but affects conversion rate; given enough time, the yields may continue to increase, but this would result in longer operating times and therefore, additional costs (Kristensen et al. 2009). This thesis will experiment with a high solids loading of 16%, while aiming to overcome the negative effect of inhibition on hydrolysis.

3. Thesis Objectives

The main objective of this project is to convert oat hulls into reducing sugars that can potentially be used for bioethanol production. In order to do so, pretreatment and enzymatic hydrolysis of the biomass will be performed. The following points will be the main focus of this research to achieve the main objective.

- Since each biomass substrate responds differently to pretreatment methods, an appropriate pretreatment method will be designed for oat hulls to break down the recalcitrant structure. A variety of pretreatments involving chemical, mechanical and physical methods will be tested for their effectiveness in enzyme accessibility and resulting hydrolysis.

- The effect of particle size on enzymatic hydrolysis will be explored. Physical pretreatment methods should alter the particle size of oat hulls. I hypothesize that smaller particles will improve conversion, and tests will be conducted to identify which range of particle sizes gives the greatest conversion to products during enzymatic hydrolysis.

- Enzyme cocktails will be explored by using various cellulases, xylanases and β-glucosidase. The determination of the appropriate enzyme cocktail will allow for efficient hydrolysis of oat hulls and increased conversion of reducing sugars. The cost of enzymes represents a significant portion of the total commercial cost of producing ethanol (Barta et al. 2010); therefore, the enzyme doses will be minimized in order to reduce cost, while still aiming for high yield of products.

- Strategies to overcome end-product inhibition and increase the functionality of the enzymes involved will be tested. Overcoming inhibition of enzymes will allow for a more efficient hydrolysis and higher yield of products.

Though research in conversion of biomass to ethanol is vast and the process of its production is known, its commercialization is limited by the fact that the process is too costly and not efficient enough. This research intends to show that using oat hulls as a substrate, high yields of glucose are possible using the appropriate pretreatment and enzyme cocktails. If the limitation of poor yields during enzymatic hydrolysis is removed, then this step can be optimized and the final yield of ethanol can be increased. This would result in increased production efficiency of ethanol, which is a key stepping stone to its commercialization. Also, increasing the conversion of the co-product of hydrolysis, xylose, would allow for utilization of this resource and economic benefit.

4. Experimental methods

4.1 Pretreatments

Oat hulls were received from SunOpta Inc. in two forms: one which was pre-processed by grinding to reduce the particle size and the other was raw in its natural state. The pretreatment of raw oat hulls is very important because the starting material is large in size (Figure 4-1). Various pretreatment methods and effects on conversion rates will be explored in this chapter. In order to reduce the particle size to an optimal range (Yu et al. 2002a; Yu et al. 2002b), milling methods were explored. Initial methods involved the use of a coffee grinder to achieve a particle size of 250μm, based on the recommendations of Yu et al. 2002a, b. However, with increasing demand for substrate and reaction scale-up, an alternate method for grinding was needed. Szego milling was selected for this purpose. Mercerization of oat hulls was also explored, as well as disc refining. Finally, the caustic and disc-refining pretreatments were combined (Figure 4-2).

Figure 4-1: Raw oat hulls

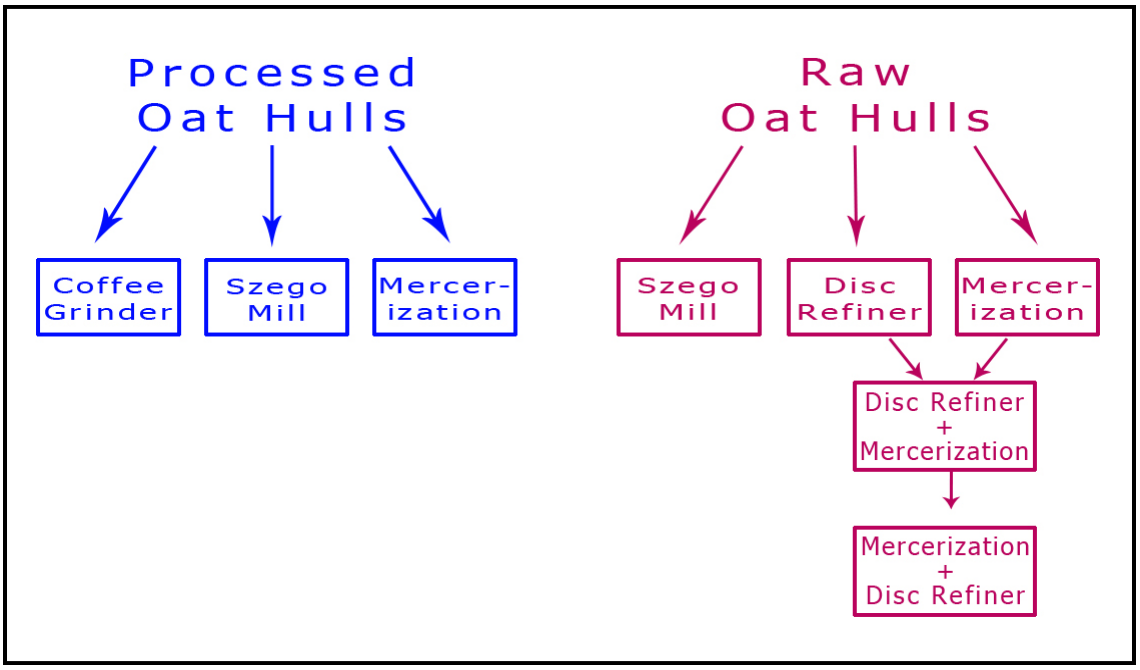

Figure 4-2: Flow chart of pretreatments using processed and raw oat hulls

4.1.1 Coffee grinder

A coffee grinder was used as the first pretreatment method to reduce the particle size of processed oat hulls. This pretreatment involved grinding the processed oat hulls through several passes in a coffee grinder. Once the particle size was visibly reduced, the oat hulls were sieved using a shaking incubator as the sieve shaker, set at 350rpm. A 250µm sieve was used for collection. The sieve was left in the shaking incubator for an extended period of time until the ground oat hulls were separated by size. Raw oat hulls were larger in size and could not be processed using the coffee grinder because the blades could not handle the increased size.

4.1.2 Szego mill

A General Comminution Inc. szego mill was used as a physical pretreatment method to reduce the particle size of processed and raw oat hulls. The oat hulls were fed continuously by gravity into the szego mill and discharged at the bottom of the mill. The oat hulls were subjected to crushing and shearing between the roller ridges and the stator as it moved down the cylinder.

The speed of rotation around the stator was set at 1200 rpm**.** The oat hulls were subjected to four passes through the szego mill to decrease the particle size during each subsequent pass. During milling, superfine particles were collected by attaching a vacuum and a vacuum bag to the bottom of the mill where the particles were ejected. These superfine particles would normally be released from the machine as dust, but were collected. After szego milling, the ground oat hulls were sieved using the shaking incubator to shake the sieve. A 250μm mesh screen sieve was used, and fractions smaller and larger than 250μm were collected separately. The superfine particles were not sieved.

4.1.3 Disc refiner

Raw oat hulls were passed through a Sprout- Waldron disc refiner which had a plate gap of 75/1000 of an inch. The oat hulls were placed in a reservoir and water was added, at which point the oat hulls would float. Oat hulls were pumped into the disc refiner using a Moyno Pump. This process was difficult for the raw oat hulls, which were less dense than water and floated while passing through the disc refiner system. After passing through the plates, the oat hulls were recirculated back through the reservoir and disc refiner until the resulting ground oat hulls obtained in the reservoir were dense and no longer floated. During this process, the temperature was observed at the outlet of the refiner to be sure the temperature remained below 60°C. After disc refining, the slurry was filtered/strained to remove water, leaving wet refined oat hulls.

4.1.4 Mercerization

A more aggressive chemical pretreatment of processed and raw oat hulls was investigated. Sodium hydroxide of varying concentrations (0.125, 0.25, 0.5 and 1.0M) was heated to 95°C in a beaker placed on a hot plate, at which point raw oat hulls were added to the liquid to bring the concentration to 10% w/w oat hulls. Figure 4-3 depicts this experimental set-up. The oat hulls were heated for up to two hours and continuously stirred to allow the oat hulls to maintain contact with the liquid. Initially, the oat hulls would float on top of the caustic, but as pretreatment progressed, the oat hulls sank. After one or two hours, the beaker was removed from the hot plate and cooled. The oat hulls were strained using a strainer and this filtrate was

collected for further analysis. The oat hulls were then washed with a volume of water equal to twice the volume of caustic used for the pretreatment. This washed filtrate was also collected for analysis.

Figure 4-3: Experimental set-up of mercerization of oat hulls

4.1.5 Disc refining and mercerization

The two pretreatment methods were combined, with the disc refining followed by caustic pretreatment. The same method for disc refining described in section 4.1.3 was used. The caustic pretreatment of raw oat hulls was performed using 1M sodium hydroxide and the same method described in section 4.1.4.

4.1.6 Mercerization and disc refining

In these trials, the order of the pretreatments was reversed, this time starting with caustic pretreatment. 5.4kg of raw oat hulls were pretreated with 1M NaOH for two hours at 95°C. 54kg of 1M NaOH total was used to treat the oat hulls, for a concentration of 10% w/w oat hulls. After

the caustic pretreatment, the oat hulls were strained and washed with 108L of water, twice the volume of sodium hydroxide used. The concentrated filtrate and the wash water were collected for analysis. Following the caustic pretreatment, the oat hulls were disc refined. Having done the caustic pretreatment first, the mechanical energy requirement of the disc refiner was lowered because the oat hulls now sank when added to the reservoir with water. One pass through the disc refiner, with a plate gap of 75/1000 of an inch, was sufficient, making it much easier and quicker to process. After disc refining, the oat hulls were filtered to remove the additional water using a Buchner funnel, vacuum pump and a Whatman grade 42 cellulose filter paper, which has a pore size of 2.5μm. The filtrate from this operation was also collected.

4.2 Particle Size Distribution

The effect of particle size on conversion rates was explored. The optimal particle size for oat hulls, 250μm, determined by (Yu et al. 2002) was examined to determine if this applied to processed and szego milled oat hulls. A Mastersizer 2000 laser diffraction particle size analyzer was used, which had a detection range of 0.02μm to 2000μm. The instrument was calibrated with deionized water and samples were run in triplicate. Mastersizer 2000 software was used to analyze the data, and average values for each sample were obtained. Oat hulls that were treated with a coffee grinder and sieved using a 250μm mesh were also analyzed. Raw and processed szego milled oat hulls that were separated by sieving, i.e., less than 250μm, greater than 250μm and superfine were analyzed. Disc refined and caustic pretreated oat hulls had a larger particle size and their particle size distribution could not be analyzed using this method. The particle size distribution results showed that the coffee ground oat hulls had a much more uniform distribution than the szego milled oat hulls. The distribution curves for the coffee ground and szego milled fractions are displayed in Figures 4-3 – 4-6. Coffee ground oat hulls had an average particle size of 190μm. Three particle sizes of szego milled oat hulls analyzed; less than 250μm, greater than 250μm and superfine. It was determined that particles less than 250μm had an average size of 150μm, particles greater than 250μm had an average size of 230μm and superfine particles had an average size of 90μm.

4.3 Characterization of Oat Hulls

Oat hulls were characterized by using the NREL protocol for characterization of biomass (Appendix A2). An acid hydrolysis for determination of sugar content and analysis of lignin content were performed. Each sample was analyzed in duplicate and the average was used for all calculations. The total sugars, lignin, ash and acetate content were determined, as depicted in Tables 4-1 and 4-2.

Table 4-1: Average composition (%) of received oat hulls; raw and processed

	Lignin	Glucan	Xylan	Galactan	Arabinan	Ash	Acetate	Mass closure
Raw	21.9	32.1	29.2	1.5	3.5	6.0	2.2	96.5
Processed	22.6	33.7	27.7	1.8	3.5	5.4	2.2	94.1

Table 4-2: Average composition of pretreated raw oat hulls (%)

Pretreatment Method				
	Disc refining	Mercerization	Disc refining + Mercerization	Mercerization+ disc refining
Lignin	25.4	21.6	20.9	20.0
Glucan	29.0	42.9	47.3	48.7
Xylan	31.2	26.9	25.0	23.8
Galactan	4.3	2.2	1.8	1.7
Arabinan	3.4	3.2	3.2	3.1
Ash	4.1	0.8	0.7	0.6
Acetate	3.0	1.4	0.8	0.4
Mass closure	100.3	98.9	99.7	98.2

From the tables above, it is noted that the composition of oat hulls changes after pretreatment. Mercerization caused swelling of fibres and exposes the lignocellulose structure. Mercerization partially delignifies the fibres and thus alters the glucan and xylan content. The raw oat hulls experience partial conversion of xylan when they are mercerized, leading to a decrease in xylan content; this is also proven by the decrease in the acetate content. Thus, cellulose increases, while lignin, xylan and acetate all decrease. The same trend is seen for the combination of disc refining and mercerization pretreatment from the raw oat hulls. The composition of disc refined oat hulls differs the least from the raw oat hulls. Some of the glucan gets redistributed and the xylan and lignin content therefore increase. The increase in xylan is also accompanied by an increase in acetate content. However, these variations are quite small and could be within normal variability of the characterization method.

4.4 Moisture Content Analysis

The moisture content of the pretreated fibres was determined using a Mettler Toledo MJ33 Infrared Moisture Analyzer. Approximately one gram of the fibre was placed on the balance, which was then heated to 100°C until a constant weight was achieved. The analysis usually took 20 minutes to complete. Three replicates were analyzed for each sample and the average was reported. The moisture content was reported in percent dry mass (DM) as displayed in Table 4-3. The dry mass was needed to determine the solids loading and dry mass of the slurry in the hydrolysis step. Pretreatment using the coffee grinder and szego mill was done using air dried oat hulls; the dry mass of these raw and processed oat hulls was also analyzed

Table 4-3: Average dry mass (%) of oat hulls

Type of oat hull	Dry mass (%)
Disc refined	33.0
Mercerized	18.3
Disc refined and mercerized	22.6
Mercerized and disc refined	20.5
Processed	92.1
Raw	93.4

4.5 Enzymatic Hydrolysis

The oat hulls from all the pretreatment methods and oat hulls that were not pretreated were subject to enzymatic hydrolysis in a shaking incubator. Sodium acetate buffer was prepared at pH 4.8 and a 50mM concentration. 20g of dried oat hulls were added to an Erlenmeyer flask followed by 125ml of buffer to give a solids loading of 14%. The Erlenmeyer flask was covered with aluminum foil to prevent evaporation of the samples. The Erlenmeyer flasks were placed in a Lab-Line shaking incubator pre-heated to 55°C to allow the solid and liquid portions to form a homogeneous slurry. The shaking incubator was set at 150 rpm. Figure 4-4 shows the set-up of the shaking incubator. Once the temperature of the slurry reached 55°C, enzyme was added to each sample; E_1 (see Table 4-4). When enzyme was added, this marked the initiation of the trial, or time zero. At various time points (2, 4, 6, 8, 10, 12, 24, 48 and 72 hours), samples were collected from each flask. A micropipette was used for sampling, using a pipette tip with the end cut off, since the slurry was viscous. 1.5mL samples were collected and placed in a microcentrifuge tube. The samples were immediately placed in the freezer to quench the reaction. The pH of the slurry was measured at each sample collection point, using a bench-top pH meter, Fisher Scientific Accumet pH meter 915. The pH was measured to make sure a consistent pH of 4.8 was maintained, or else sodium hydroxide would need to be added to increase the pH. Each sample was analyzed in triplicate.

Figure 4-4: Enzymatic hydrolysis in a shaking incubator

Enzymes

Enzyme cocktails were explored by varying the types and quantities of enzymes. Cellulases, cellobiases and xylanases were used for the hydrolysis. Initial reactions involved the use of developmental cellulases NS22074 and NS50073 from Novozymes. Novozyme 188 was the only β-glucosidase used. Xylanases Alternafuel 100L (Dyadic) and Multifect Xylanase (Genencor) were also tested for their effectiveness. Determinations as to which cellulase and xylanase are the best for this substrate were made over the course of several reactions, varying enzyme doses, reaction conditions and solids loadings.

The determination of enzyme dose was based on economics as well as conversion rates and yields of sugars. Since a deterrent to the large-scale production of ethanol is enzyme cost (Barta et al. 2010), this was an important consideration when determining enzyme dose. While starting off with larger enzyme doses, a switch to lower enzyme doses was tested to see how low the dose could be without sacrificing significant conversion of sugars. Table 4-4 below gives the varying enzyme doses used to initiate enzymatic hydrolysis; four enzyme doses were experimented with for each type of oat hull.

Table 4-4: Enzyme cocktails (μL) added at initiation of hydrolysis, E_1, and after solid-liquid separation, E_2

Shaking incubator, 20g of dried substrate

	E_1	E_2		E_1	E_2
1a	100 NS22074 50 AF100	100 NS22074 100 AF100 10 NS188	**1b**	100 NS50073 50 AF100	100 NS50073 100 AF100 10 NS188
2a	150 NS22074 100 AF100	150 NS22074 100 AF100 10 NS188	**2b**	150 NS50073 100 AF100	150 NS50073 100 AF100 10 NS188
3a	200 NS22074 150 AF100	200 NS22074 150 AF100 20 Novo188	**3a**	200 NS50073 150 AF100	200 NS50073 150 AF100 20 Novo188
4a	100 NS22074 50 MFX	100 NS22074 100 MFX 10 NS188	**4b**	100 NS50073 50 MFX	100 NS50073 100 MFX 10 NS188
5a	150 NS22074 100 MFX	150 NS22074 100 MFX 10 NS188	**5b**	150 NS50073 100 MFX	150 NS50073 100 MFX 10 NS188
6a	200 NS22074 150 MFX	200 NS22074 150 MFX 20 NS188	**6b**	200 NS50073 150 MFX	200 NS50073 150 MFX 20 Novo188

4.6 Solid-liquid separation

At a residence time of 24 hours, the hydrolysis slurry was filtered through a Whatman cellulose filter paper, grade 42, which has a pore size of 2.5μm. Using a Buchner funnel and a vacuum pump, the slurry was filtered until almost all of the liquid was removed and the solids changed from dark brown to beige. The solids were collected to continue with the batch reaction and the filtrate was removed and frozen. The solids were added to the same Erlenmeyer flask or steel reactor, along with fresh sodium acetate buffer. Again, the slurry was heated to 55°C in the shaking incubator, at which point the second enzyme dose (E_2) was added, (see Table 4-4). The purpose of the separation step was to remove inhibitors that had accumulated over the first 24 hours. The hydrolysis continued for another 48 hours from this point, giving a total hydrolysis of 72 hours. Hydrolysis trials were also run without a solid-liquid separation for comparison; in this case the total initial enzyme dose from time zero is displayed in Table 4-5.

Table 4-5: Enzyme cocktails (μL) added at initiation of hydrolysis, E_1, without solid-liquid separation

Shaking incubator, 20g of dried substrate

	E_1		**E_1**
1a	200 NS22074 100 AF100 10 NS188	**1b**	200 NS50073 100 AF100 10 NS188
2a	300 NS22074 200 AF100 10 NS188	**2b**	300 NS50073 200 AF100 10 NS188
3a	400 NS22074 300 AF100 20 Novo188	**3a**	400 NS50073 300 AF100 20 Novo188
4a	200 NS22074 100 MFX 10 NS188	**4b**	200 NS50073 100 MFX 10 NS188
5a	300 NS22074 200 MFX 10 NS188	**5b**	300 NS50073 200 MFX 10 NS188
6a	400 NS22074 300 MFX 20 NS188	**6b**	400 NS50073 300 MFX 20 Novo188

A wide range of enzyme doses were explored using the "_coffee ground_" and szego milled substrate. These enzyme doses are not displayed in Tables 4-4, but cover a range of 50-750μL for NS22074 and NS50073, 25-500μL for Alternafuel 100L and Multifect Xylanase and 5-250μL for Novo188. These results were omitted for simplicity and ease of comparison to other pretreatment methods. A larger enzyme dose was tested for all of these enzymes, and eventually these values were reduced to see how much they could be reduced while still maintaining sufficient yields of glucose and xylose. These preliminary enzyme doses were used to refine the range of enzyme doses used as the basis for all further trials with pretreated substrates. Also, after the extensive trials with varying enzyme doses for "_coffee ground_" and szego milled substrates, it was determined that Novo188 was not needed in the initial enzyme cocktail when solid-liquid separation took place; this will be discussed later in further detail. Also, the use of NS50073 and Multifect Xylanase was discontinued due to the fact that trends showed that NS22074 and Alternafuel 100L were the more effective cellulase and xylanase respectively, which will also be discussed in further detail later.

Enzyme doses were reported in weight (wt) % as well as on a volumetric basis. The wt % will allow for ease of comparison when enzyme doses are scaled up. The weight of the enzyme was determined by multiplying a density of 1.1g/mL by the volume of the enzyme (mL). The wt% was then calculated by taking the weight of the enzyme dose and diving by the weight of the substrate on a dry mass basis.

4.7 Calculations

To compare the results from hydrolysis trials following different pretreatment methods, a single factor analysis of variance (ANOVA) was used to determine statistical differences, based on the mean glucose and xylose yields. A difference that is statistically significant has a confidence level of over 95%. A difference that is not statistically significant will have a confidence level of less than 85%. Final glucose and xylose yields for a particular set of enzyme doses or pretreatment methods were compared using standard deviations of the mean. All errors bars represent the standard error of the mean.

5. Hydrolysis Results and Discussion

The glucose and xylose concentrations from the various hydrolysis trials were tracked as a function of hydrolysis trials. The total concentration of each sugar at time points after the solid-liquid separation step was added to the concentration of that sugar obtained in the filtrate during filtration. From these resulting concentrations, the yields of glucose and xylose were calculated based on the maximum possible glucose and xylose concentrations that can be achieved based on the glucan and xylan content shown in Tables 4-1 and 4-2. These calculations were made using equations presented in Appendix A3. These equations account for addition of a hydroxyl group upon hydrolysis of cellulose and hemicellulose to yield glucose and xylose monomers. The total glucan and xylan present in the oat hulls was determined through characterization of each type of oat hull; (see Tables 4.1 and 4.2). These characterizations were made based on dried oat hulls;

therefore, the DM values in Table 4.3 were used to calculate the total glucan and xylan available for a particular sample. It is acknowledged that glucose and xylose yields on their own do not fully represent glucan and xylan conversions, since oligosaccharides are omitted. To evaluate different pretreatment methods, the average glucose and xylose yields were compared, based on three replicates run for each enzyme dose.

5.1 No Pretreatment

To prove that pretreatment is a necessary precursor to enzymatic hydrolysis, hydrolysis of raw and processed oat hulls without pretreatment was performed. From these trials 21% glucose and 15% xylose yields were obtained for raw oat hulls. Processed oat hulls led to yields of 22 and 17%. These results were obtained using enzyme dose 1a from Table 4-4. A wide range of enzyme doses was used for these initial trials, and it was determined that this dose gave the highest yields. NS22074 and Alternafuel100L were the preferred cellulase and xylanase that led to the highest yields of glucose and xylose respectively. Without solid-liquid separation, the yield for the raw oat hulls decreased to 15% glucose and 8% xylose, and to 17% glucose and 9% xylose for processed oat hulls; see Figures 5-1 and 5-2. These results were obtained using enzyme dose 1a from Table 4-5; 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation. The solid-liquid separation significantly increased the yield of glucose and xylose and was a valuable step to reduce the presence of inhibitors that would otherwise lower yields. The results of hydrolysis without pretreatment will be used as a baseline to establish if a particular pretreatment method was effective in increasing product yields. Since the yields from hydrolysis trials without pretreatment were consistently low, irrespective of the enzymes used or dose, exploration of the enzymes and doses will be omitted here. Subsequent trials involving pretreatment will show the full range of enzyme and doses used, which significantly increase the yield of both glucose and xylose.

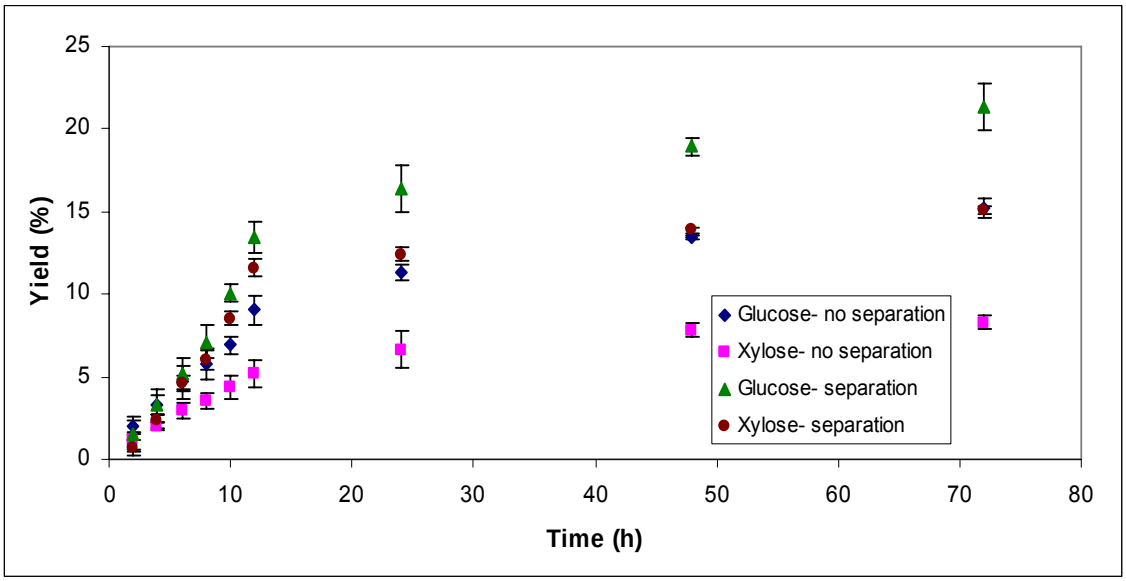

Figure 5-1: Hydrolysis yields of raw oat hulls with and without solid-liquid separation
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

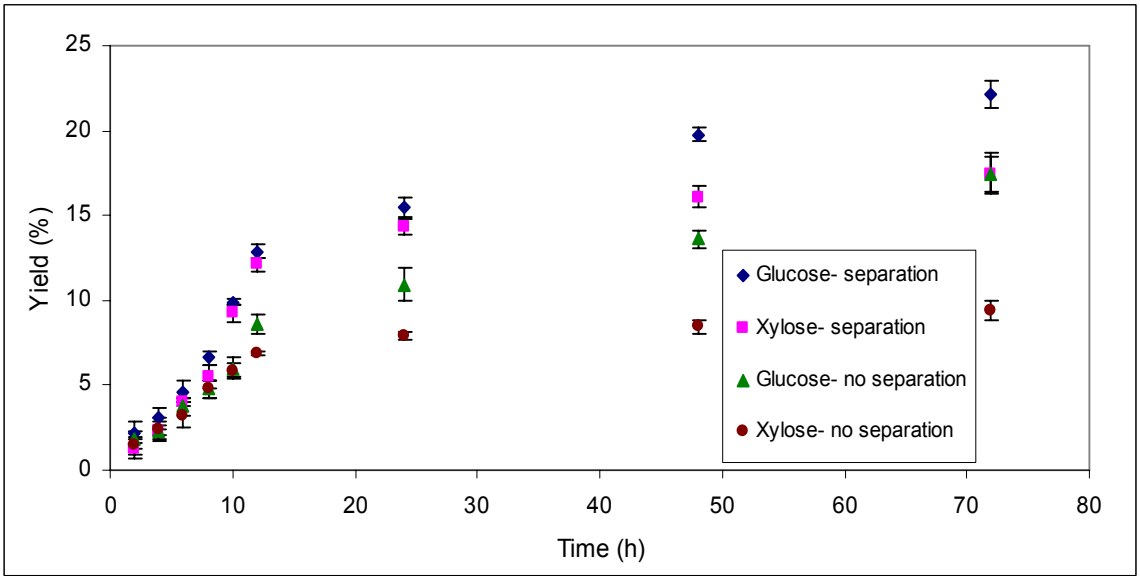

Figure 5-2: Hydrolysis yields of processed oat hulls with and without solid-liquid separation
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.2 "_Coffee ground_" oat hulls

5.2.1 Comparison of NS22074 and NS50073

These two cellulases were examined for their effectiveness in converting the substrate to glucose and xylose, using the shaking incubator. A range of enzyme doses (Tables 4-4) were evaluated, and the trend showed that NS22074 was more effective than NS50073. When the cellulase doses were varied, the other enzymes remained constant for comparison. Figure 5-3 shows compares the two cellulases based on production of glucose from "coffee ground" oat hulls. Each pair of bars corresponds to a particular enzyme dose, shown in Table 4-4. For each pair, the doses of the other enzymes in the cocktail (i.e., xylanase and β-glucosidase) were constant. The hydrolysis results for the "coffee ground" oat hulls show a preference for NS22074 as the cellulase over the entire range of doses considered; the enzyme doses 1-6 correspond to those presented in Table 4-4, with a) using NS22074 and b) NS50073. It was also determined from these results that NS22074 dose 1a) works the best since it gave the highest yield of glucose; since NS22074 doses 1b and 1c) are larger but not statistically different. This enzyme cocktail is comprised 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation.. This enzyme dose gave a yield of 71% glucose, which is not statistically different from the 72% yield of glucose obtained from enzyme dose 2a) and 73% from enzyme dose 3a). Therefore, the lower enzyme dose 1a) was most beneficial. Figure 5-3 also highlights another important point; synergism of the enzyme cocktails. The enzyme dose 1a) contained 100μL of NS20074 while enzyme dose 4a) contained the same amount of this cellulase, but Multifect Xylanase was used in place of Alternafuel 100. The fact that the glucose yield was statistically higher for 1a) is due to synergism; where NS22074 and Alternafuel 100L have worked together to produce a higher yield of glucose. It will be shown that the higher yield of xylose produced by Alternafuel 100L, as opposed to Multifect Xylanasae, makes access to glucan easier and therefore facilitates its conversion. The yield of glucose increases when Alternafuel 100L is added to NS22074 alone, indicating that these two enzymes work synergistically together (see Appendix B3).

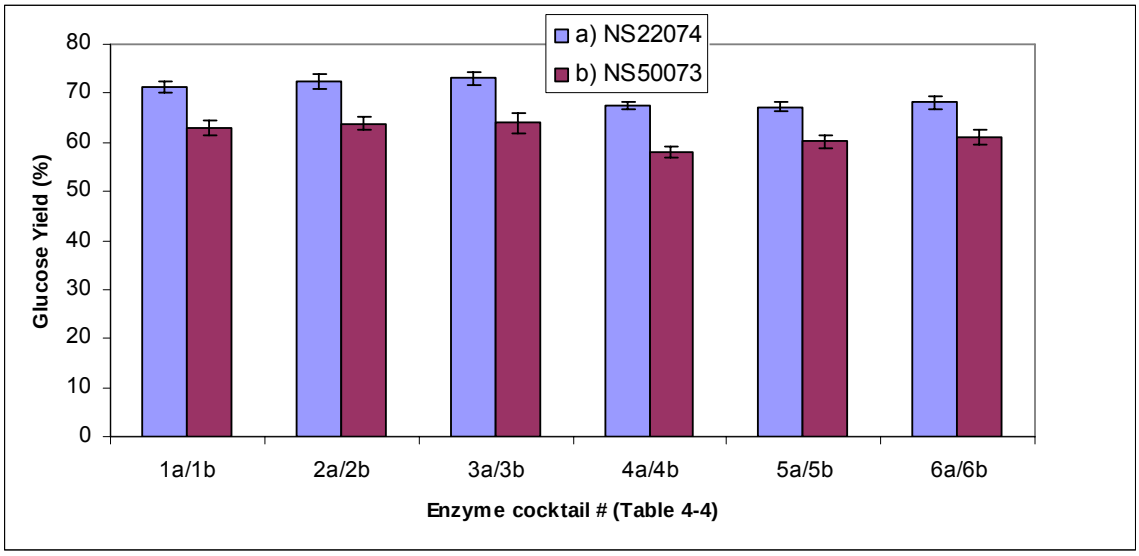

Figure 5-3: Final glucose yields (%,) obtained at 72 hours, comparing NS22074 and NS50073 for oat hulls pretreated with a coffee grinder

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.2.2 Comparison of Alternafuel 100L to Multifect Xylanase

Two xylanases, Alternafuel 100L and Multifect Xylanase, were explored for their effectiveness to convert xylan into xylose. However, due to potential synergy between cellulase and xylanase, it is also useful to examine the effect of xylanase on glucose yields. Figure 5-4 below depict the results, which led to the decision to choose Alternafuel 100 as the best xylanase for oat hulls; since it consistently provided a higher xylose yield. To facilitate comparison, the quantities of Alternafuel 100 and Multifect Xylanase were kept constant in each set of side-by-side trials. As well, the concentration of the cellulase and cellobiase, Novo188, were the same in all side-by-side trials. Over the range of doses considered for both xylanases, it was apparent that Alternafuel 100L had the greatest efficiency for hydrolysis trials in a small scale shaking incubator. Alternafuel 100L dose 3a) from Table 4-5 gave the greatest yield of xylose, 69%. However, this yield was not statistically different from that obtained using Alternafuel 100L dose 2a), 67%, or using 1a), 66% glucose. Therefore dose 1a) was preferred, since it was the lowest enzyme dose that gave statistically favourable results. This enzyme cocktail (1a) is comprised of 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation. From Figure 5-4 it is also apparent that the cellulase also affects the yield of xylose. 1a/1b, 2a/2b and 3a/3b all have

the same doses of Alternafuel 100, but the b) trials use NS50073 instead of NS22074 used in a) trials. Since 1a, 2a and 3a) give higher xylose yields this is most likely due to the prevalence of NS22074, which in previous trials was shown to be the most effective cellulase. These Alternafuel 100L and NS22074 work synergistically; the increased glucan conversion by NS22074 opens up the lignocellulose structure and makes access to xylan more accessible. When NS22074 is added to Alternafuel 100L alone, the yield of xylose increases; therefore, these two enzymes work synergistically together to increase the yield of glucose and xylose (see Appendix B3).

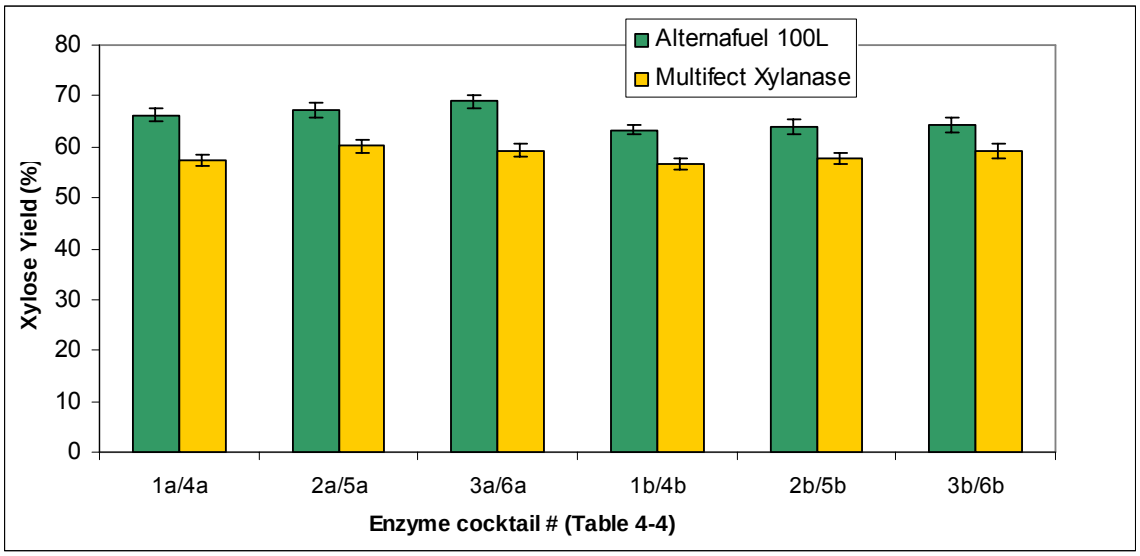

Figure 5-4: Final xylose yields (%) obtained at 72 hours, comparing Alternafuel 100L and Multifect Xylanase using oat hulls pretreated by coffee grinder
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.2.3 Effect of addition of Novo 188

Novozyme 188 is a β-glucosidase, and the dosage and presence of this enzyme at various points during hydrolysis was investigated. Cellobiose is an inhibitor which impedes the action of endoglucanases and cellobiohydrolases. A β-glucosidase, such as Novozyme 188, is needed in order to break down the accumulated cellobiose. However, β-glucosidase is inhibited by accumulation of glucose (Chauve et al. 2010). Novozyme 188 was added before the solid-liquid separation, and also after this step. Adding Novozyme 188 after the separation was shown to

effectively decrease the prevalence of cellobiose, compared to omitting this enzyme from the cocktail. After the solid-liquid separation, the rate of hydrolysis slows and the accumulation of glucose does not affect the action of Novozyme 188 to a great degree. Therefore, Novozyme 188 is permitted to act upon cellobiose, turning it into glucose monomers, and the action of the endoglucanases and cellobiohydrolases is no longer inhibited so as to release further glucose monomers. When Novozyyme188 was added before the solid-liquid separation, there was essentially no added benefit; no statistical difference in the amount of glucose released was observed. This is because the high concentrations of glucose, plateauing at roughly 24 hours, counteract Novozyme 188. Also, the solid-liquid separation step removes inhibitors such as cellobiose; therefore, the addition of this enzyme is not needed until after the separation, where cellobiose can again accumulate. Figure 5-5 compares the results when Novozyme 188 is added to the initial enzyme cocktail and after solid-liquid separation, versus its addition only after the separation; these doses are 1a) from Table 4-4 and 1a) modified with addition of 10µL to E_1. These data indicate very little difference in glucose and xylose yields between these trials, i.e., adding Novozyme 188 after solid-liquid separation worked just as well as splitting the dose before and after separation.

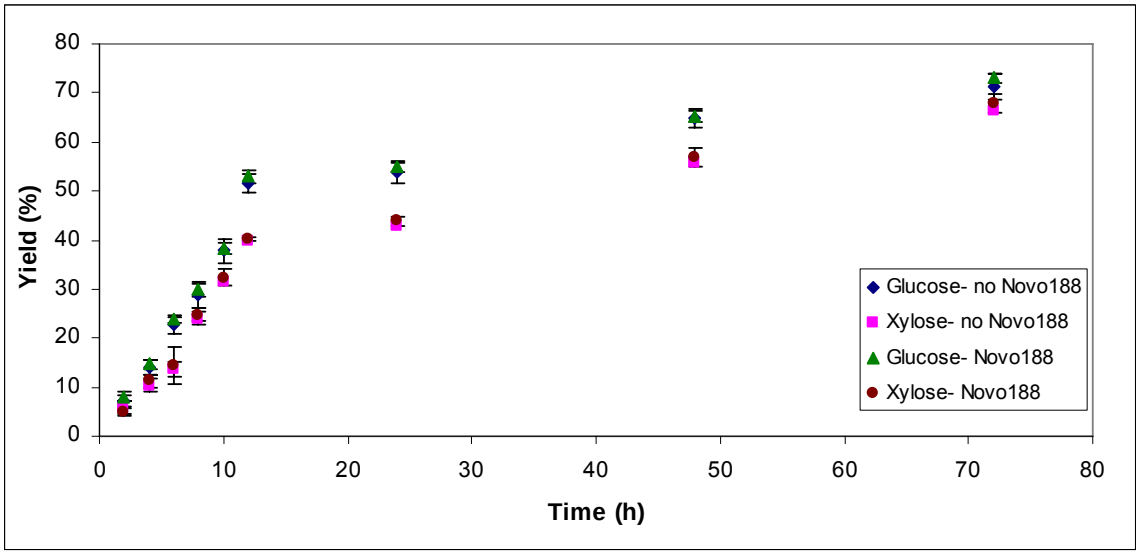

Figure 5-5: Yield of glucose and xylose (%) for addition of Novo188 before and after solid-liquid separation

*no Novo188= enzyme dose 1a), Novo188 refers to enzyme dose 1a) with 10µL Novo188 added to E_1

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

Similar outcomes were observed with other enzyme cocktails and with different Novo188 doses; no statistical significance of adding Novo188 to E_1.

5.2.4 Effect of solid-liquid separation

Without solid-liquid separation, there was a clear trend towards decreased yields of glucose and xylose; this was true for all enzyme doses. For simplicity, the results of hydrolysis trials without solid-liquid separation are shown below for enzyme dose 1a in Table 4-4. For this enzyme dose, the yields dropped significantly from 71% glucose and 66% xylose to 55% glucose and 40% xylose (Figure 5-6).

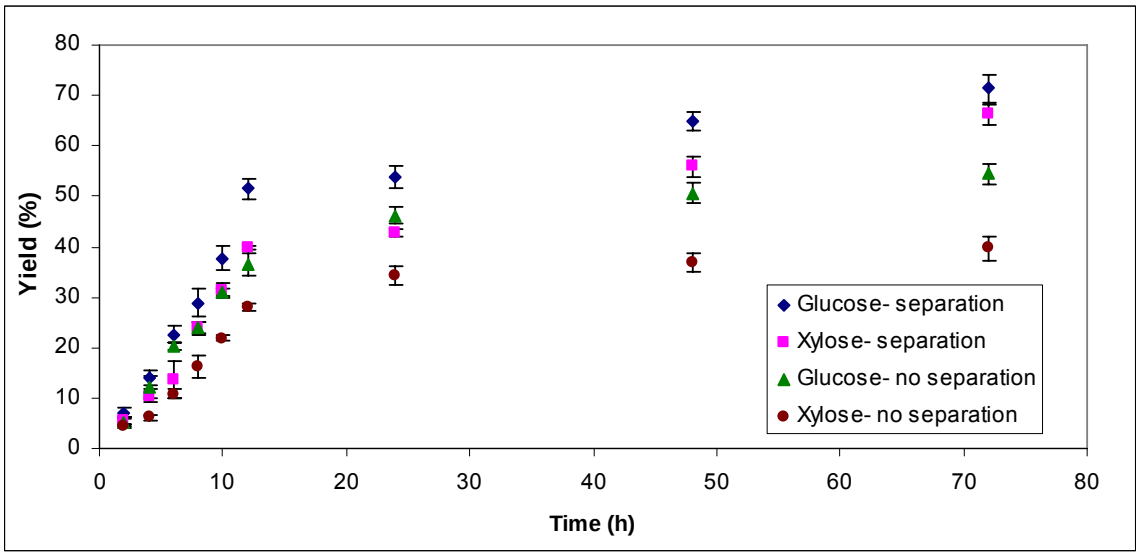

Figure 5- 6: Yields of glucose and xylose for "_coffee ground_" oat hulls, with and without solid-liquid separation
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.3 Szego milled oat hulls

5.3.1 Comparison of cellulases

The two cellulases, NS22074 and NS50073, were again compared for their effectiveness for production of sugars, this time using szego milled raw and processed oat hulls of varying sized. Figures 5-7 to 5-12 display the results of these trials. The szego milled oat hulls were more efficiently hydrolyzed by NS22074 than NS50073 for each particle size. The results for raw and processed oat hulls were not statistically different, across the entire range of cellulase doses and particle size; it can be concluded that these two substrates are essentially the same, once they have been processed through the szego mill.

The raw and processed szego milled oat hulls, less than 250μm, gave the greatest yields of glucose when NS22074 was used; this was true across the entire range of cellulase doses, Figure 5-7 and 5-8. Using enzyme dose 3a) gave the greatest yields of glucose for both raw and processed oat hulls of this size, but this was not statistically different from the lowered enzyme doses 1a and 2a). NS22074 dose 1a) gave glucose yields of 74% for raw oat hulls and 75% for processed oat hulls. NS22074 dose 1a) is the most beneficial, due to the decreased enzyme loading and increased yield of glucose. 1a) enzyme cocktail is comprised of 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation.

It was expected that a smaller particle size would lead to increasing yields of products, due to increased surface area and enzyme accessibility. However, this was not the case for both superfine raw and processed szego milled oat hulls, Figures 5-9 and 5-10. Superfine particles released less glucose than the particle size “less than 250μm.” Superfine particles led to yields of 68% for raw szego milled oat hulls and 69% for processed szego milled oat hulls. These yields were obtained from NS22074 dose 1a), (0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation) which produced the greatest glucose yields. Again, NS22074 was statistically more effective than NS50073, for all enzyme cocktails. As expected, the largest

particle size gave the worst yield of glucose, Figures 5-11 and 5-12. This is most likely due to the decreased surface area and inaccessibility to glucan. Enzyme dose 1a) (0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation) was the lowest enzyme dose that still maintained efficient yield of glucose for this particle size. 62% and 63% glucose were released from raw and processed szego milled oat hulls respectively; there was no statistical difference between the two substrates.

In all these trials, NS22074 was clearly superior to NS50073 as the cellulase. Therefore, it was decided that further studies would be only NS22074 only. Evidence has proved that irrespective of the particle size or dose, NS22074 always resulted in higher yields. For further pretreatment studies, it can be reasonably stated that the same trend in favour of NS22074 will probably be seen.

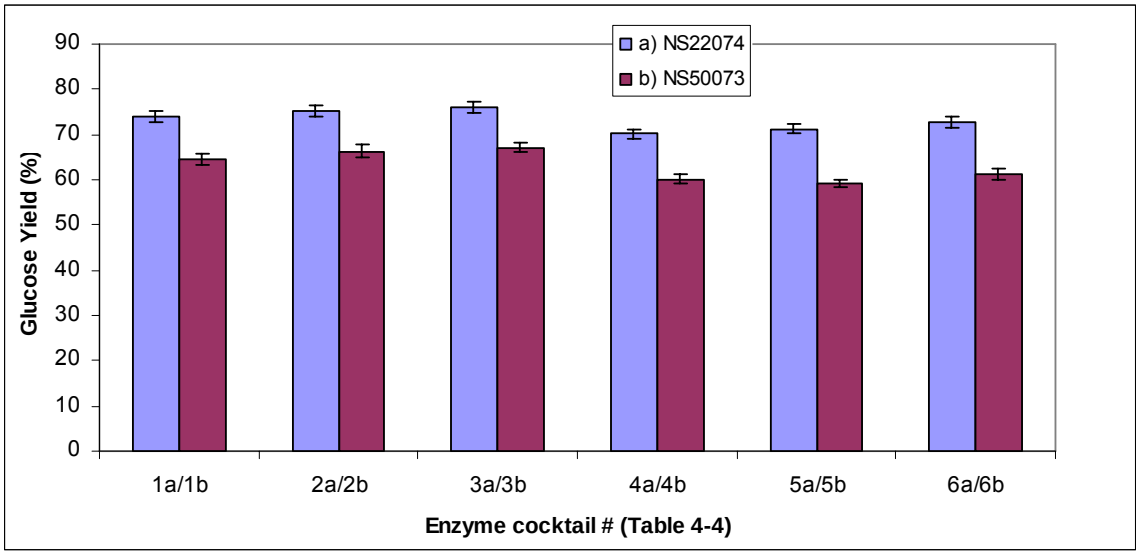

Figure 5-7: Final glucose yields (%) obtained at 72 hours, comparing NS22074 and NS50073 for szego milled raw oat hulls, less than 250µm.
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

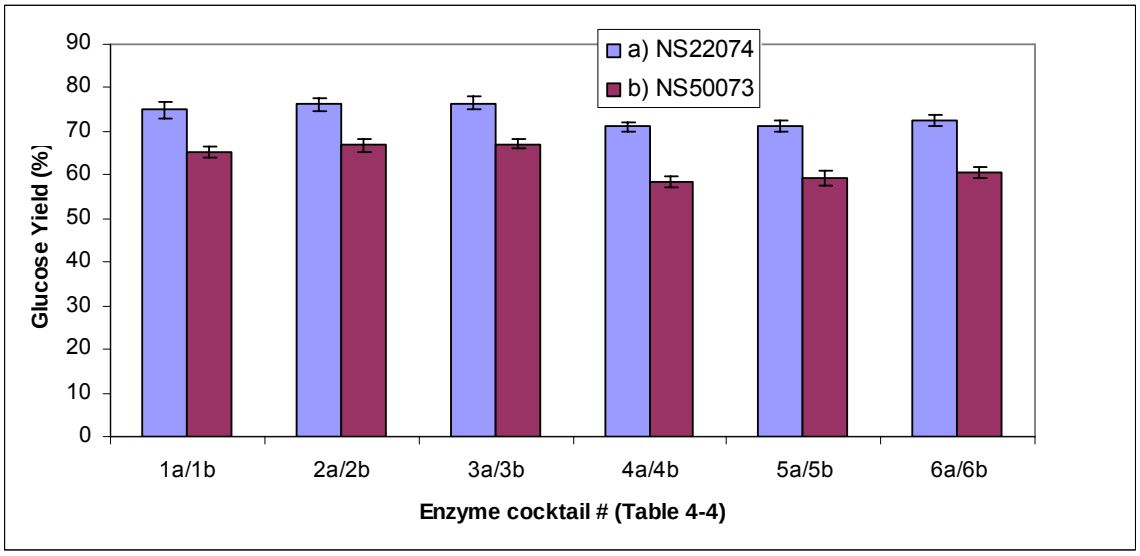

Figure 5-8: Final glucose yields (%,) obtained at 72 hours, comparing NS22074 and NS50073 for szego milled processed oat hulls, less than 250µm.

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

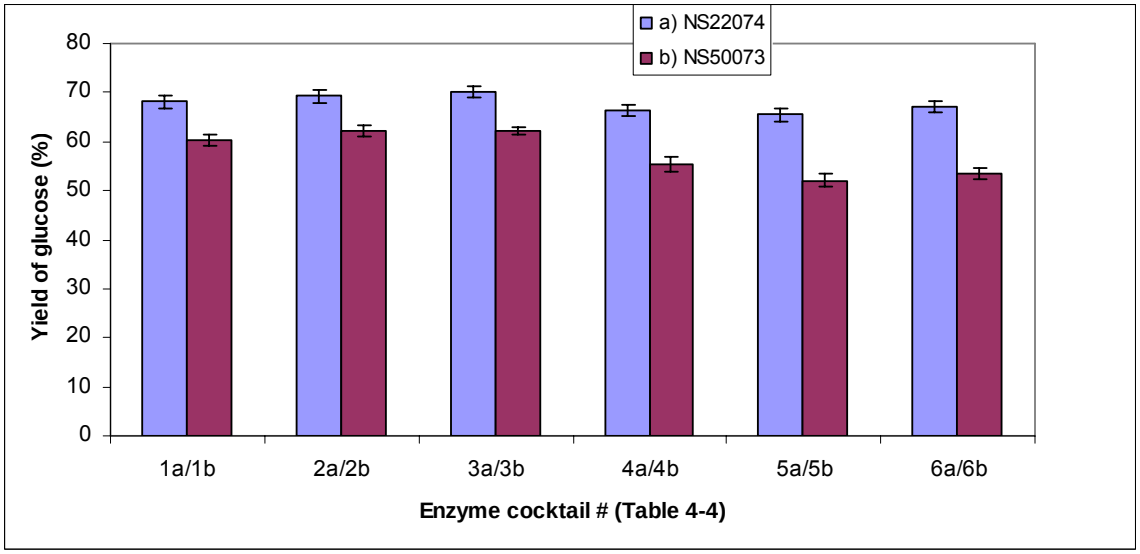

Figure 5-9: Final glucose yields (%,) obtained at 72 hours, comparing NS22074 and NS50073 for szego milled raw oat hulls, superfine

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

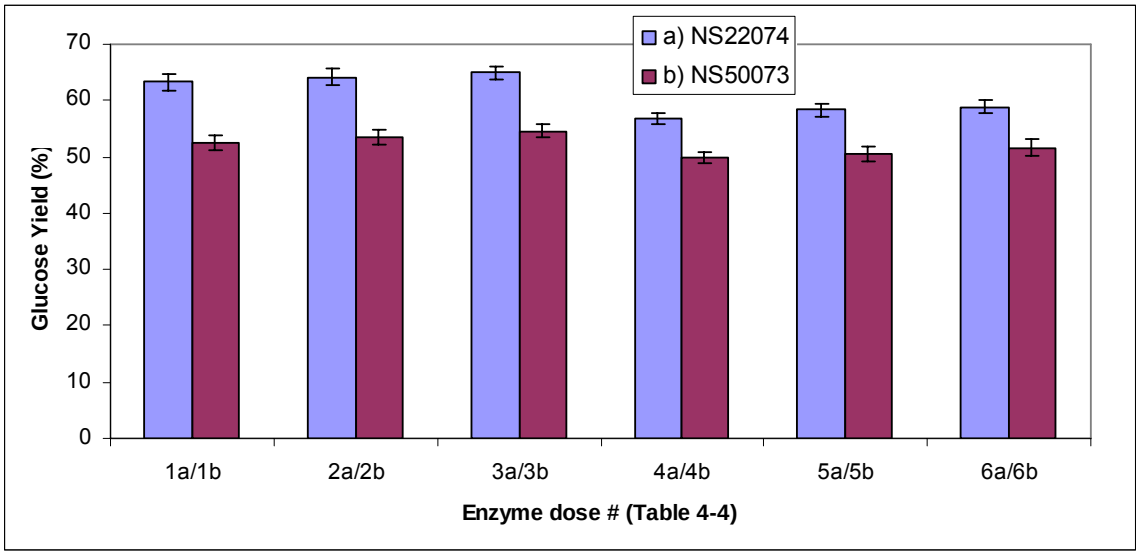

Figure 5-10: Final glucose yields (%,) obtained at 72 hours, comparing NS22074 and NS50073 for szego milled processed oat hulls, superfine

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

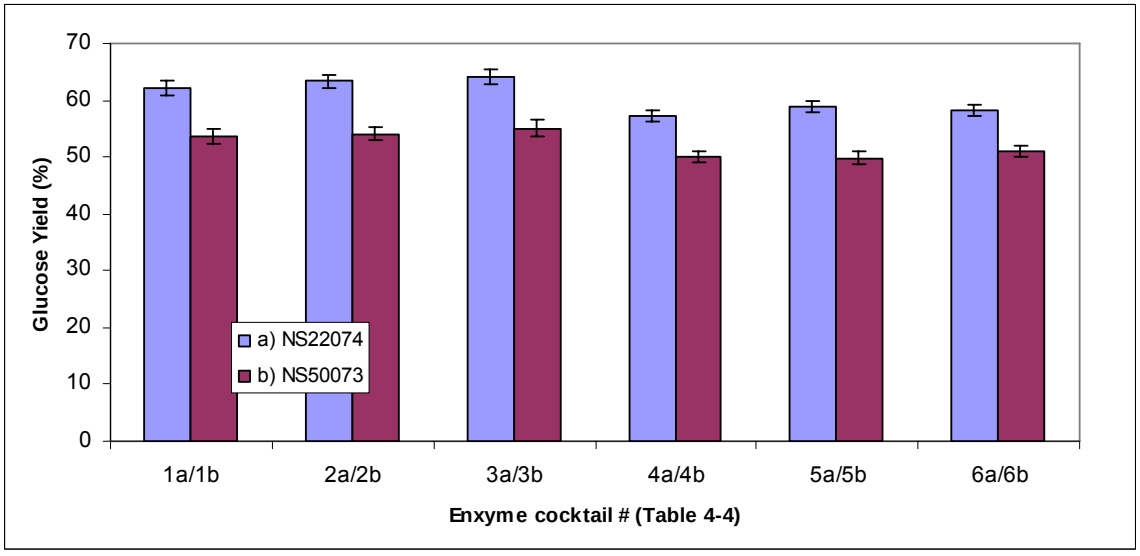

Figure 5-11: Final glucose yields (%,) obtained at 72 hours, comparing NS22074 and NS50073 for szego milled raw oat hulls, greater than 250µm

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

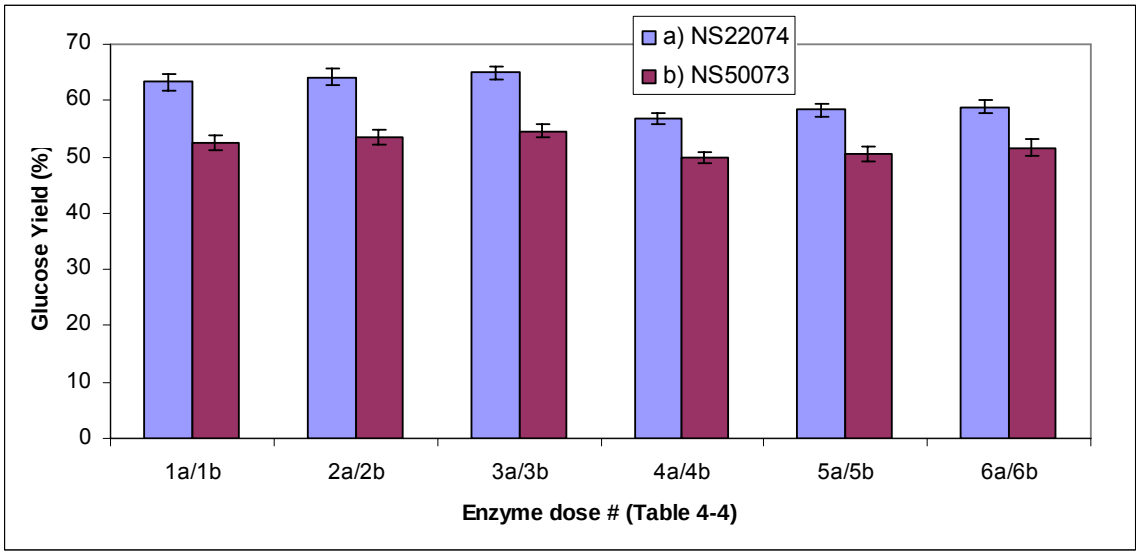

Figure 5-12: Final glucose yields (%,) obtained at 72 hours, comparing NS22074 and NS50073 for szego milled processed oat hulls, greater than 250µm
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.3.2 Comparison of Alternafuel 100L to Multifect Xylanase

Figures 5-13 to 5-15, below, depict the decision to choose Alternafuel 100 as the best xylanase for oat hulls, since it produced a higher xylose yield for all particle sizes and enzyme doses. To facilitate comparison, the quantities of Alternafuel 100 and Multifect Xylanase were kept constant in each set of side-by-side trials. As well, the concentration of the cellulase (NS22074 or NS50073) and cellobiase (Novo188) were the same in all side-by-side trials trials. Over the range of doses considered, for both xylanases, it was apparent that Alternafuel 100L had the greatest efficiency. It was concluded in cellulase trials that raw and processed oat hulls released essentially the same amount of glucose; the same is true for xylanase trials. For simplicity, only data from trials with processed szego milled oat hulls will be displayed in the following graphs; the szego milled raw oat hulls follow similar trends and are not statistically different from trials using processed oat hulls.

From the graphs below, the same trend that was evident in cellulase trials is seen here. The particle size has a significant effect on the yield of xylose. The oat hulls “less than 250µm” gave

the greatest yield of xylose, for all xylanase doses and types. The superfine particles gave unanticipated results, with yields lower than the previous particle size (compare Figure 5-13 and 5-14). The largest particle sized gave the lowest yields of xylose across the entire range of doses and for both xylanases. For all particle sizes, Alternafuel 100L dose 1a) was the most beneficial because it was not statistically different from dose 1b and 1c) and therefore gave the highest yield of glucose. Overall, the best result was obtained from oat hulls "less than 250µm" using Alternafuel 100L dose 1a) which contains 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation.; giving a 73% yield of xylose. Since Alternafuel 100L consistently worked best on oat hulls, regardless of their size, all further trials used this xylanase.

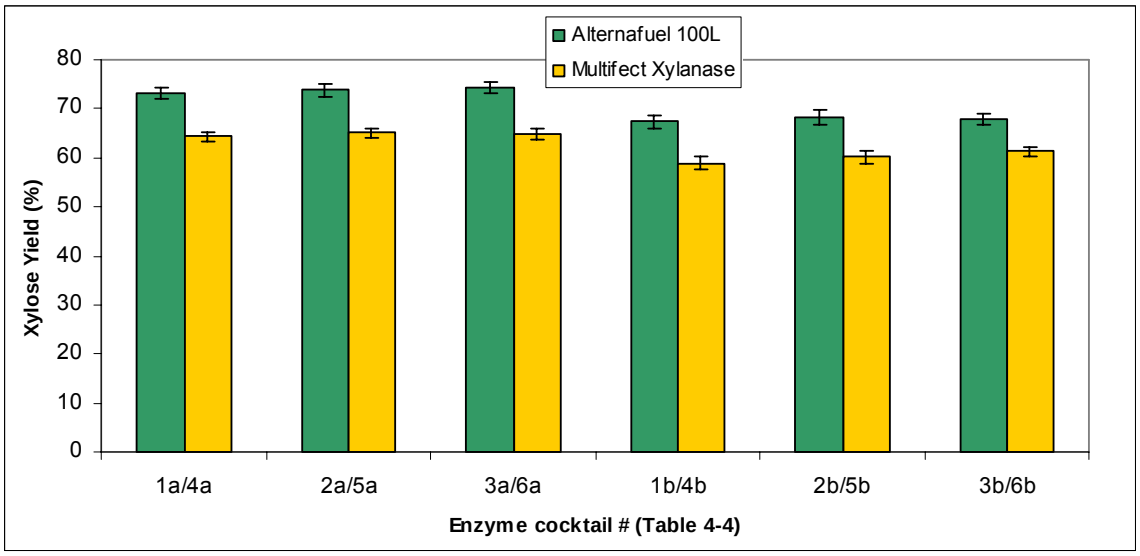

Figure 5-13: Final xylose yields, %, obtained at 72 hours comparing Alternafuel 100L and Multifect Xylanase for szego milled processed oat hulls, less than 250µm.
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

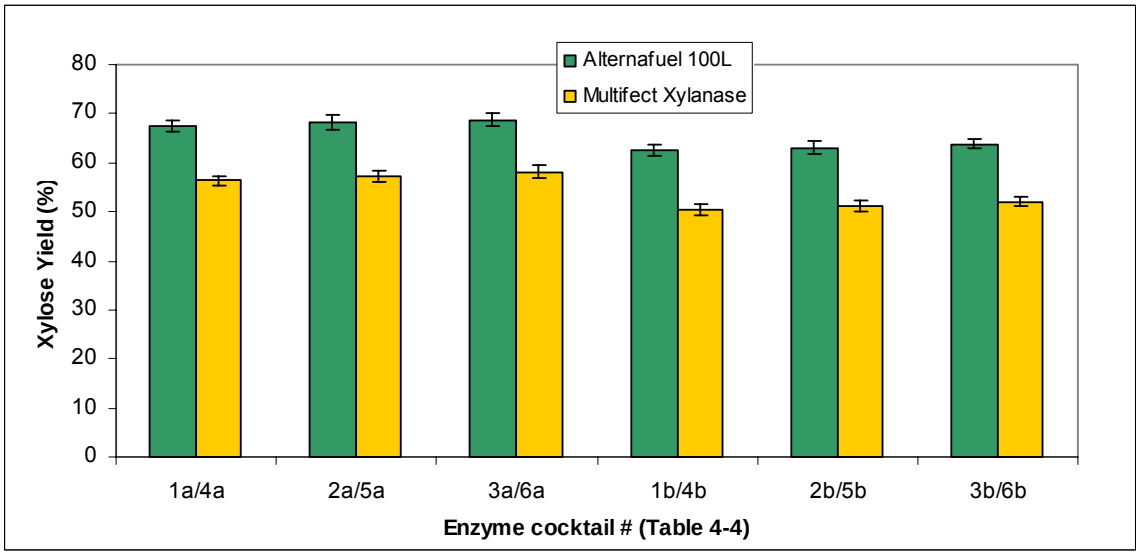

Figure 5-14: Final xylose yields, %, obtained at 72 hours comparing Alternafuel 100L and Multifect Xylanase for szego milled processed oat hulls, superfine.
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

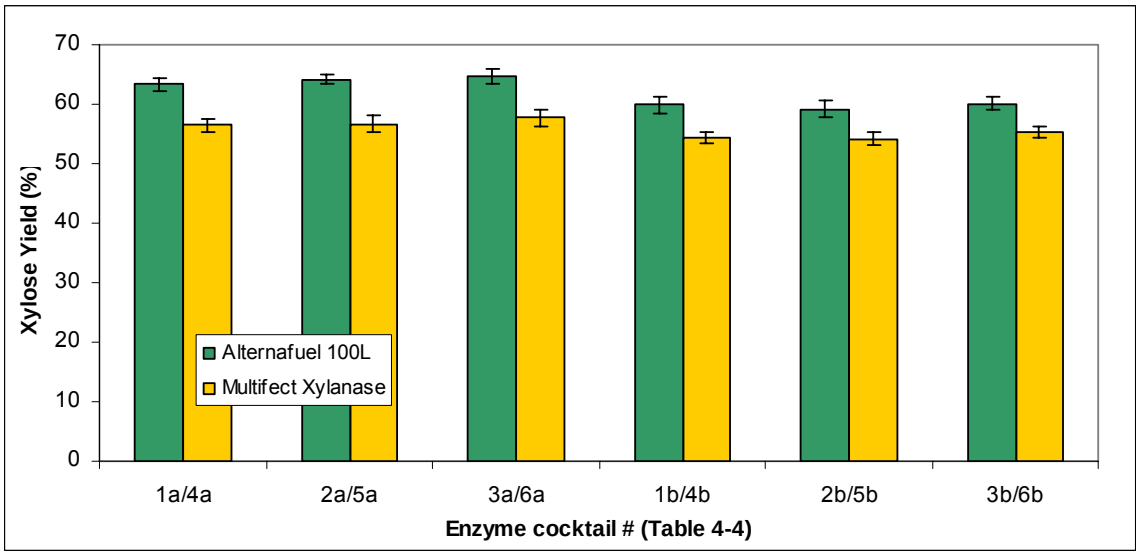

Figure 5-15: Final Xylose yields, %, obtained at 72 hours based comparing Alternafuel 100L and Multifect Xylanase for szego milled processed oat hulls, greater than 250µm.
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.3.3 Effect of particle size

The best yields were obtained from enzyme dose 1a from Table 4-4 and a particle size less than 250µm; szego milled processed oat hulls gave yields of 75% glucose and 73% xylose (Figure 5-

17). Szego milled raw oat hulls gave yields of 74% glucose and 72% xylose (Figure 5-16). There was no statistical difference in terms of yields between the raw and processed substrates. Across the range of enzyme doses, the superfine particle gave a lowered yield of both glucose and xylose compared to the particles less than 250µm. The largest particle size, greater than 250µm, responded as anticipated and produced the lowest yields of glucose and xylose across the entire range of enzyme doses.

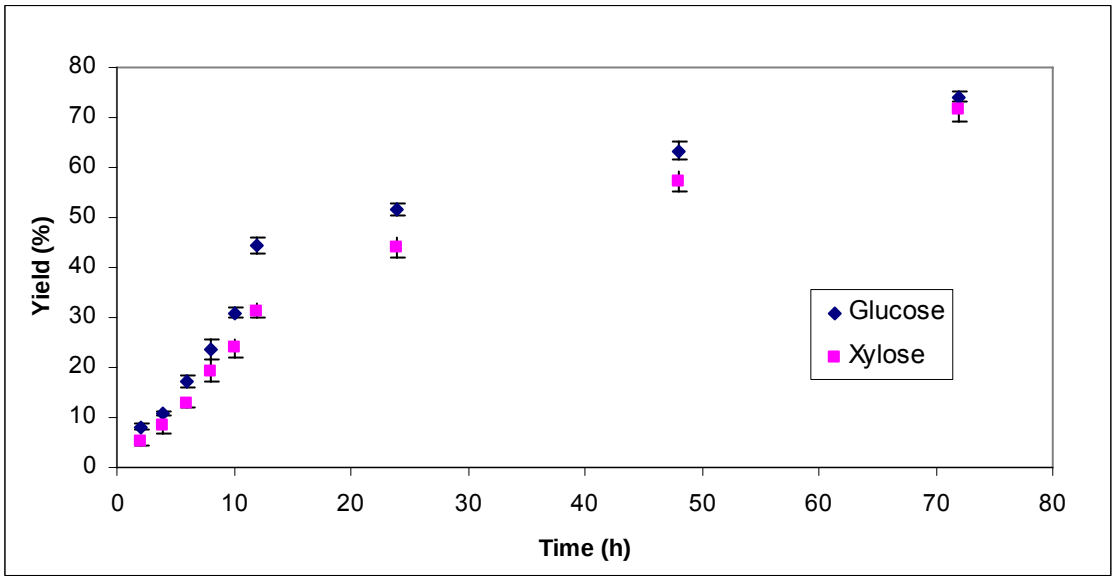

Figure 5-16: Yield of glucose and xylose for szego milled raw oat hulls, less than 250µm
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

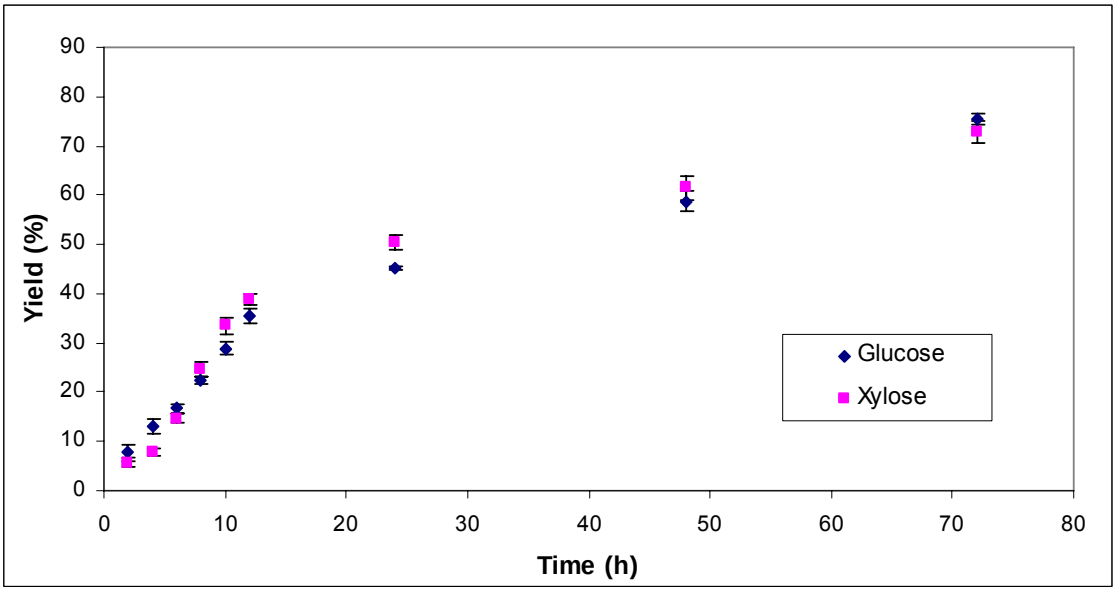

Figure 5-17: Yield of glucose and xylose for szego milled processed oat hulls, less than 250µm
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

Figure 5-18 below depicts the synergistic action of the cellulase and xylanase mixture. The yield of glucose is lowered when NS22074 is used as the cellulase alone; when Alternafuel 100L is added with NS22074, the yield of glucose increases. Conversely, when Alternafuel 100L is used as the xylanase alone, the yield of xylose is lower than depicted in Figure 5-18; adding NS22074 increases the yield of xylose. When NS22074 and Alternafuel 100L are used in conjunction, the yields of glucose and xylose both increase compared to trials in which NS22074 was combined with Multifect Xylanase. Figure 5-18 below shows this observation for processed szego milled oat hulls, "less than 250µm" using enzyme dose 1a from Table 4-4. Both of the xylanases do contain some cellulase activity, which accounts for the higher glucose conversion in the Alternafuel 100L trial, as the cellulases were the same for both trials. The enhanced xylan conversion produced by using Alternafuel 100L opens up the lignocellulose structure and facilitates access to cellulose. The cellulase and xylanase operate synergistically; by breaking down the hemicellulose, the xylanase allows the cellulase to work more effectively on the cellulose. The same can be said about the cellulase; increased conversion of glucan allows access to xylan by xylanases. Both cellulases and xylanases operate more effectively in conjunction than on their own. The higher concentration of both glucose and xylose produced by Alternafuel 100L led to the decision to use Alternafuel 100L as the xylanase in all subsequent trials.

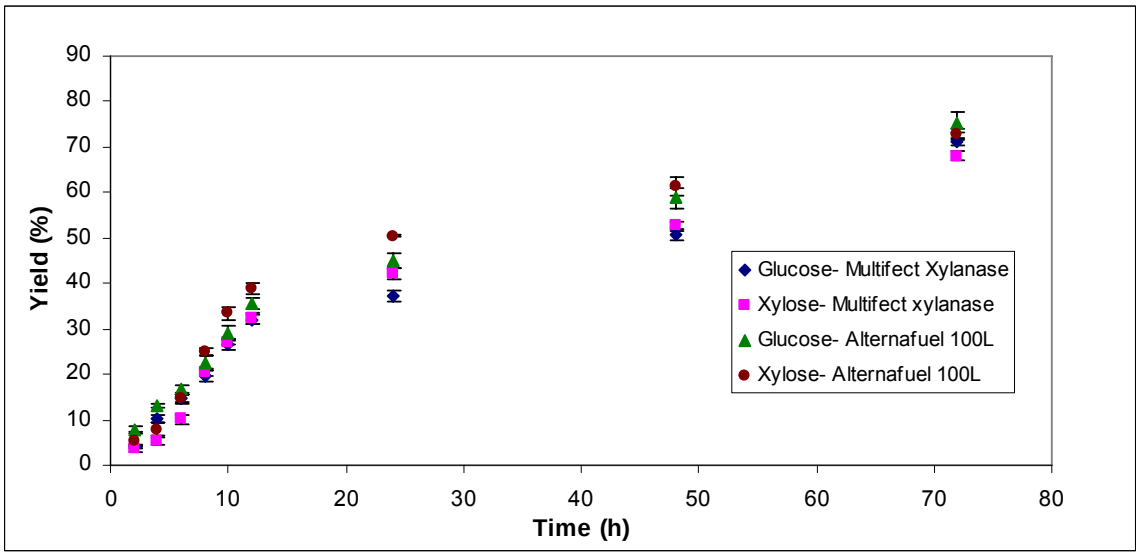

Figure 5-18: Hydrolysis yields from synergistic action of NS22074 with Alternafuel 100L and Multifect Xylanase

***The xylanase used is displayed next to the Glucose or Xylose marker in order to simplify which xylanase was added to NS22074 in the enzyme cocktail

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.3.4 Effect of Novo188 addition

For simplicity the effect of Novo188 addition will only be shown for szego milled processed oat hulls, "less than 250µm"; this substrate has consistently given the greatest yields and will be a good basis for comparison. The effect of adding a Novo188 was explored by varying its addition before and after the solid-liquid separation. 10 µL of Novo188 was added to E_1 of 1a and compared to enzyme dose 1a from Table 4-4. It was expected that the addition before the separation would have little effect since Novo188 is inhibited by the high concentration of glucose at that point. This is what was observed during trials; adding Novo188 before the separation had no statistically significant effect on the yield of glucose (Figure 5-19). Adding Novo188 after the separation helped to convert cellobiose dimers into glucose monomers and helped delay the onset on inhibition by cellobiose. An additional source of β-glucosidase was needed at this point in order to break down cellobiose effectively and prevent it from inhibiting both endo-glucanase and cellobiase again. Since the rate of glucose production decreases after the solid-liquid separation, the inhibition of β-glucosidase by glucose is minimized.

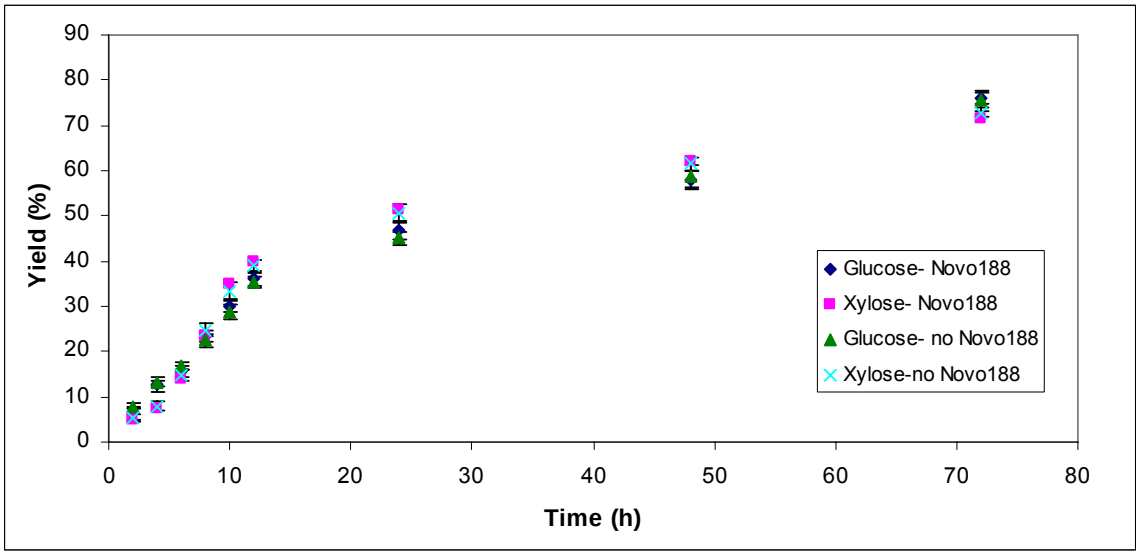

Figure 5-19: Yield of glucose and xylose (%) for addition of Novo188 before and after solid-liquid separation

*no Novo188 refers to enzyme dose 1a. Novo188 refers to 10µL of Novo188 added to E_1 of 1a

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.3.5 Effect of solid-liquid separation

There was an overall trend in favour of hydrolysis with solid-liquid separation versus hydrolysis without the separation. This trend appears across both processed and raw oat hulls for all enzyme doses and particle sizes. For simplicity, only results of hydrolysis trials without the separation for processed oat hulls less than 250µm and using enzyme cocktail 1a from Table 4-4 are shown below (Figures 5-20). The yields of hydrolysis without solid-liquid separation dropped to 55% glucose and 48% from 75% glucose and 73% xylose with separation.

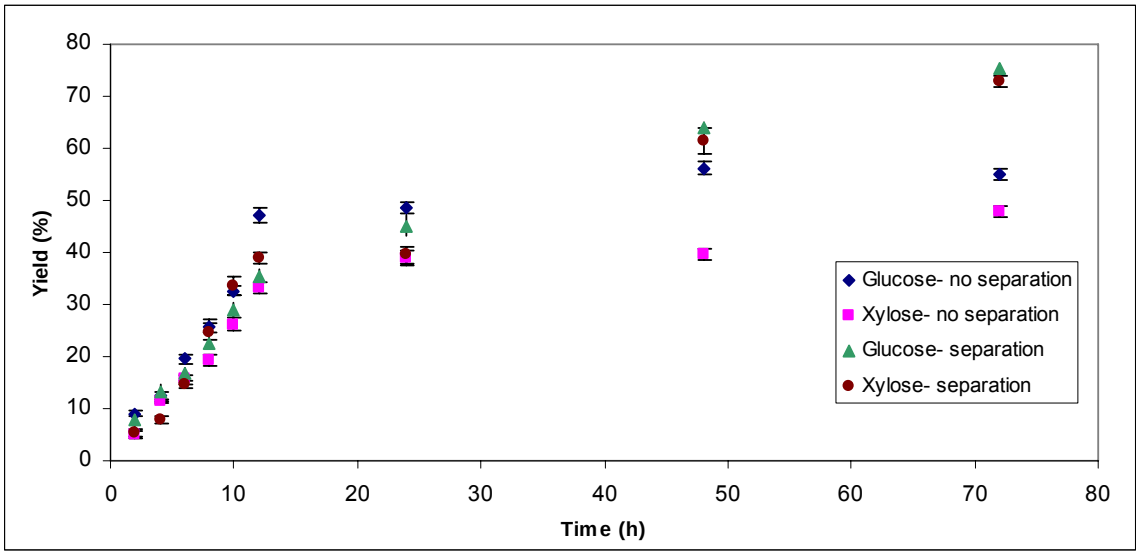

Figure 5-20: Yields of hydrolysis with and without solid-liquid separation for processed szego milled oat hulls, less than 250µm, using dose 1a from Table 4-4
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.4 Disc refined oat hulls

By this time, it had been determined that the cellulase NS227074 and the xylanase Alternafuel 100L were the most effective at converting the oat hulls to glucose and xylose, respectively. Over a range of enzyme doses for NS50073 and Multifect Xylanase there was a trend for increased yields of hydrolysis from NS22074 and Alternafuel 100L, when both "_coffee ground_" and szego milled oat hulls were analyzed. Therefore, all future studies used NS50074 and Alternafuel 100L. It is expected that even though the pretreatment method will be altered, the same trend in favour of NS22074 and Multifect Xylanase would still be seen. The effect of enzyme dose was studied extensively with previous pretreatment methods, and thus, the dosage of NS22074, Alternafuel 100L and Novo188 that gave the best conversions in the "coffee grinder" and szego mill trials were also used in these trials with disc-refined oat hulls. Based on prior results, the use of Novo188 before solid-liquid separation will be eliminated from here on; since it did not lead to increased yields. Previous trials also demonstrated the benefits of a solid-liquid separation step. For simplicity, only results from trials that include a solid-liquid separation step are presented here. Even so, unless otherwise stated, all the trials were conducted

with and without a solid-liquid separation. Results from trials without solid-liquid separation are shown in Appendix B2.

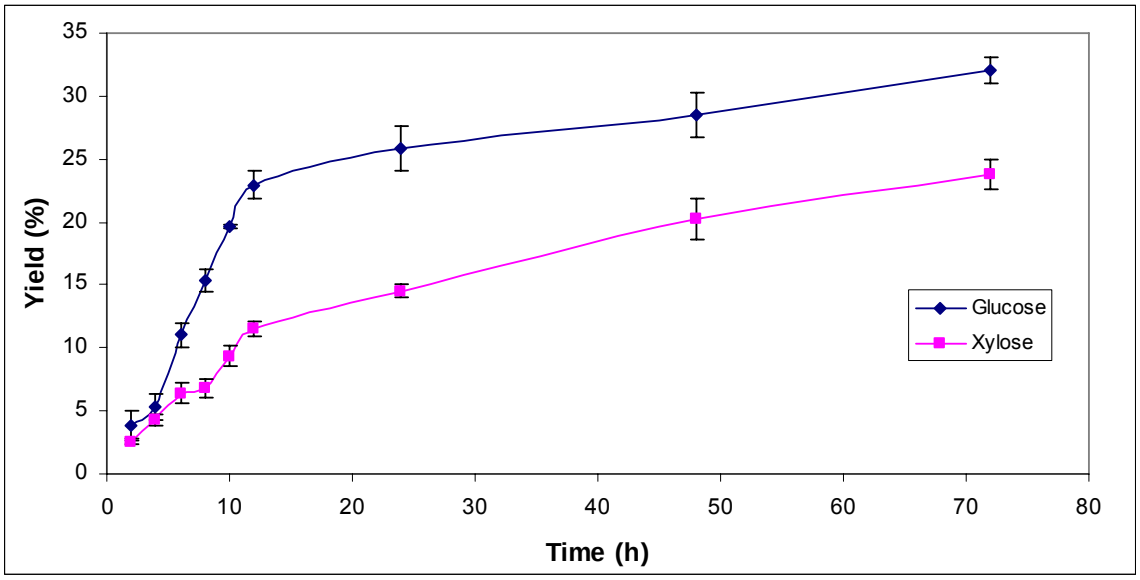

Figure 5-21: Yield of reducing sugars following enzymatic hydrolysis of raw disc refined oat hulls

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

Enzyme dose 1a, using NS22074 and Alternafuel 100L, from Table 4-4 gave the greatest yield of hydrolysis for disc refined oat hulls. This enzyme cocktail is comprised of 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation. From Figure 5-22 above, it is evident that disc refining alone did not produce a reasonable glucan conversion (32%), or xylan conversion (24%). Disc refining alone did not impact the structure of lignocellulose in a way that increased enzyme accessibility and conversion efficiency. After the oat hulls were disc refined, the size of the fibres appeared similar to that before pretreatment, which implies that the surface area did not increase significantly and enzyme adsorption was not significantly impacted. Mechanical pretreatments serve to break the physical structure of the fibres, but the disc refiner alone was not beneficial for this particular type of fibre. The mechanical forces between the two plates of the refiner did not cause sufficient disruption of the physical structure or an alteration of the bonds that hold the lignocellulose together.

5.5 Mercerization

The effect of mercerization on enzymatic hydrolysis was explored using various concentrations of sodium hydroxide and by varying the time of reaction during the pretreatment. Various enzyme doses were explored, Table 4-4, and it was determined that dose 2a gave the greatest yields statistically. This enzyme cocktail is made of 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation. Since this pretreated substrate contains more glucan, 42.9%, as opposed to the starting raw oat hulls with 32.1%, the increased enzyme dose compared to previous pretreatment methods, accounts for increase in glucan characterization.

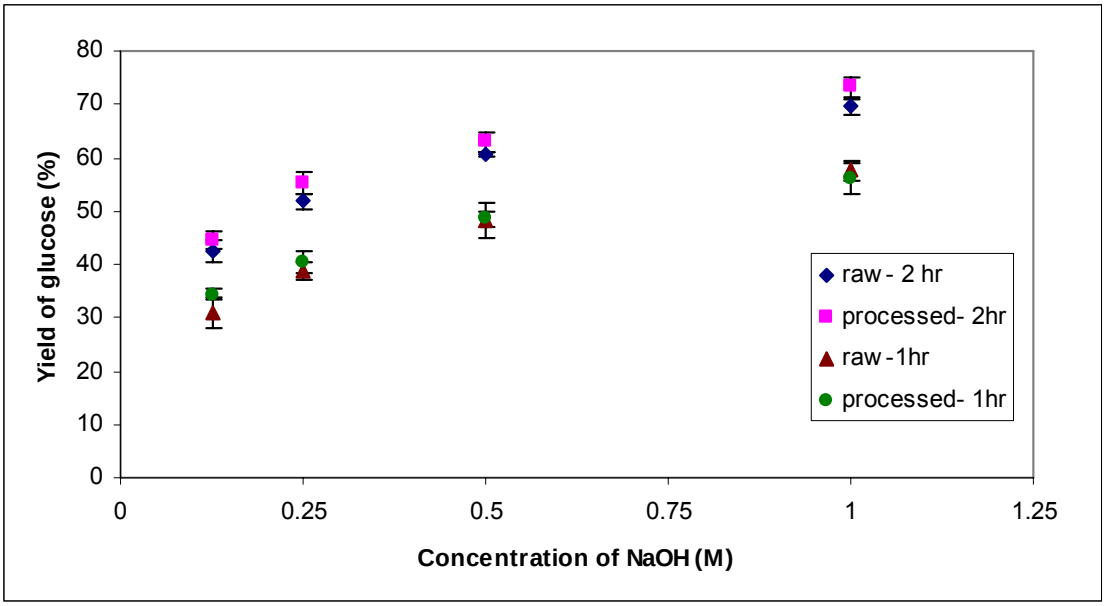

Figure 5-22: Final yield of glucose (%) obtained after 72 hours, from hydrolysis of mercerized raw and processed oat hulls.
mercerization using varying concentrations of caustic for 1 and 2 hours
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

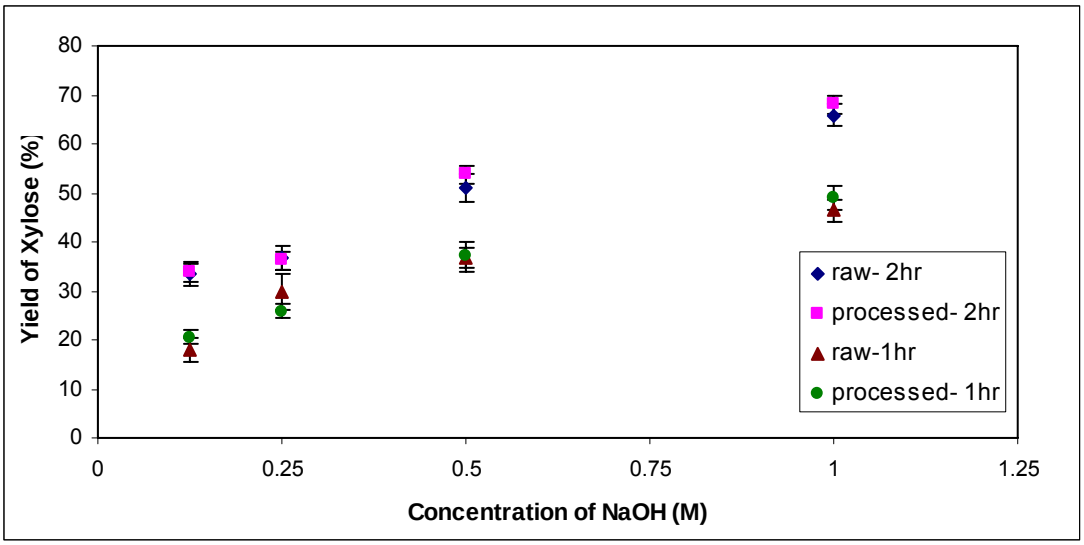

Figure 5-23: Final yield of xylose (%) obtained after 72 hours, from hydrolysis of mercerized raw and processed oat hulls
mercerization using varying concentrations of caustic for 1 and 2 hours
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

Comparing glucose and xylose yield data of mercerized raw oat hulls versus mercerized processed oat hulls revealed there was no significant difference between the two types of oat hulls, for each concentration of caustic used, and for both 1 hour and 2 hours of mercerization. Figures 5-22 and 5-23 above clearly show that the conversion of raw and processed oat hulls to glucose and to xylose follow very similar trend lines; and although there appears to be a slight benefit with processed oat hulls, the difference is not statistically significant. Both types of oat hulls are large in size, though the raw oat hulls are larger, and undergo swelling, a reduction in length and increase in diameter, which increases enzyme accessibility. It is also evident from these graphs that the best pretreatment method involves mercerization using 1M NaOH for 2 hours. From ANOVA tests, it was found that there is a statistical difference between trials run at 1 hour and trials run at 2 hours, considering both glucose and xylose yields. There is also a statistical difference between each increasing concentration of NaOH used. Therefore, 1M NaOH works best for oat hulls with a reaction time of 2 hours for mercerization, giving the highest glucan conversion (73%) and xylan conversion (68%).

5.6 Disc refining and Mercerization

Using the range of enzyme doses in Table 4-4 it was determined that dose 2a gave the greatest yields; 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation. Disc refining was performed on raw oat hulls, which were then subjected to mercerization using 1M NaOH for 2 hours, based on the results obtained in the previous section. An increase in yield of glucose and xylose was obtained compared to mercerization alone, which gave 73% glucose and 68% xylose yields. The average glucose yield from trials with combined pretreatments was 81%, and the average xylose yield was 76% (Figure 5-24).

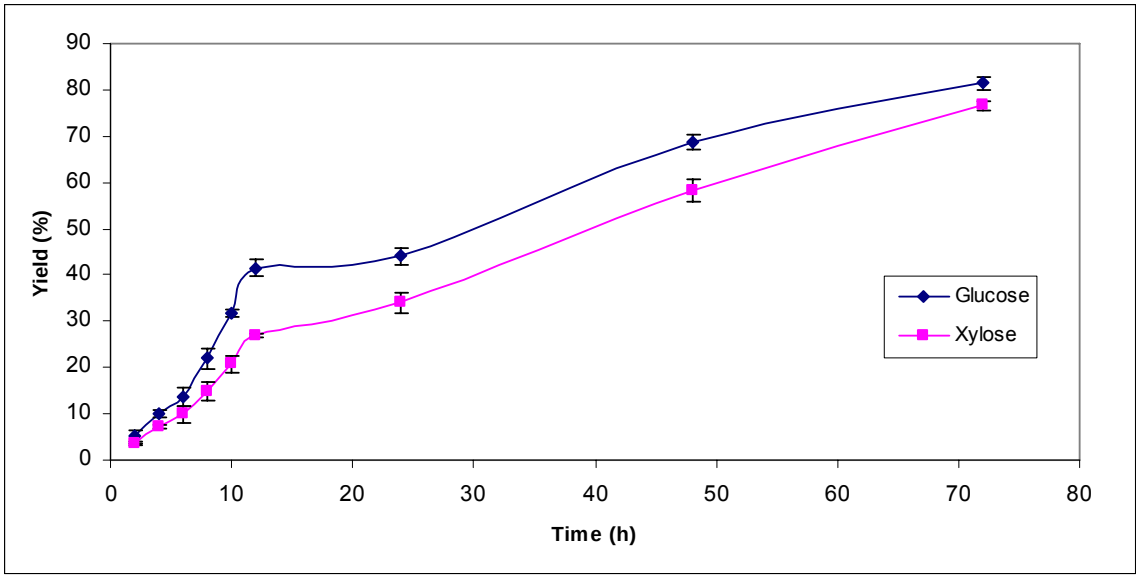

Figure 5-24: Yield of reducing sugars following enzymatic hydrolysis of disc refined and mercerized raw oat hulls
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.7 Mercerization and disc-refining

Across the range of enzyme doses in Table 4-4, it was determined that 2a gave the greatest yields; 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation. The mercerized and disc

refined oat hulls had an average glucose yield of 82% and a xylose yield of 75%; (Figure 5-25). In comparing the order of mercerization and disc-refining, there was no statistical significance in yield, irrespective of which pretreatment is performed first. Although there is no difference in yield of sugars, the mechanical energy benefit of performing mercerization first is significant. If disc refining precedes mercerization, the process of refining is very difficult to perform, the mechanical energy use is larger and the length of the process increases because one pass through the refiner is not sufficient. Furthermore, the combined pretreatment of mercerization and disc refining was more beneficial compared to mercerization alone. The conversion of glucose and xylose both increased upon addition of disc refining to the mercerization step.

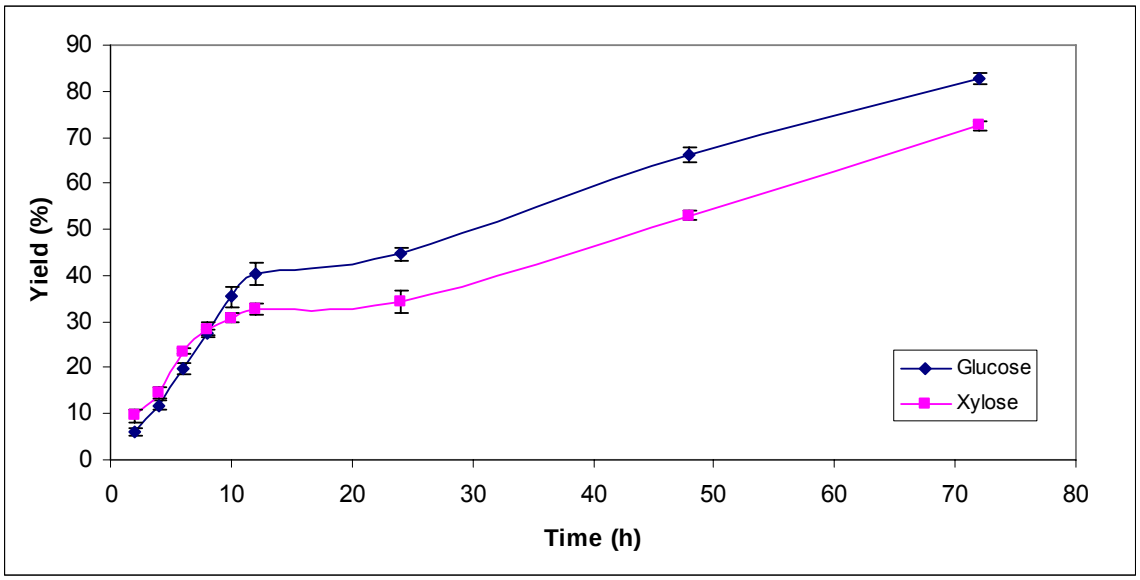

Figure 5-25: Yield of reducing sugars following enzymatic hydrolysis of mercerized and disc refined raw oat hulls
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

5.8 Discussion

Without pretreatment, raw and processed oat hulls gave a very low conversion to reducing sugars. Raw oat hulls had a glucose yield of 21% and a xylose yield of 15%, while processed oat hulls had a glucose yield of 22% and a xylose yield of 17% without pretreatment. There is no statistical difference between the raw and processed oat hulls without pretreatment. The low

conversion clearly demonstrates that pretreatment is necessary to enhance the conversion of lignocellulose to reducing sugars. Pretreatment is necessary to disrupt the structure of lignocellulose and increase enzyme accessibility to cellulose and hemicellulose while reducing barriers imposed by lignin. Without pretreatment, an excess of enzyme would need to be added. That would increase costs for ethanol production on a commercial scale. Pretreatment techniques can be relatively inexpensive and can overcome recalcitrance of the biomass when used correctly. Substrates are very selective for the type of pretreatment that works best.

Experiments using a coffee grinder and szego mill as the pretreatment method showed promising conversion of cellulose and hemicellulose to glucose and xylose. The optimal enzyme dose, 1a from Tables 4-4, gave a yield of 71% glucose and 66% xylose with the coffee grinder; 74% glucose and 72% xylose yields were obtained for szego milled raw oat hulls and 75% glucose and 73% xylose yields were obtained for szego milled processed oat hulls. Collectively, these values are not statistically different. The pretreatment using the szego mill gave a statistically higher yield of the products than "_coffee ground_" oat hulls; using the most optimal enzyme dose of 0.6wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06 wt% Novo188 after separation. The particle size distribution of the oat hulls pretreated using both techniques offers insight into the difference between yields. Overall, a good yield of glucose and xylose was achieved due to the fact the oat hulls were pretreated before hydrolysis. The physical pretreatment presumably increased accessibility of the enzymes to cellulose and hemicellulose, likely by breaking the intermolecular bonds that hold lignin, cellulose and hemicellulose together. Physical pretreatment methods such as these typically decrease the crystalline regions of cellulose and increase the proportion of random amorphous regions that are much more susceptible to enzymatic attack (Taherzdeh and Karimi 2008) (Mosier et al. 2005) (Kumar et al. 2009).

The results from the trials with szego milled oat hulls show that a smaller particle size generally enhances hydrolysis, likely due to increased surface area and accessibility of the enzymes to the substrate. The results from hydrolysis trials with superfine particles are counterintuitive, however, since a smaller particle size is equated with increased surface area and enzyme accessibility. Disruption of the physical structure of the lignocellulose is a reasonable

explanation for the decreased conversion rate of superfine oat hulls compared to the 250μm particles. This change in structure may be due to hornification. Hornification is a term used to describe structural changes in fibre due to drying or recycling. Upon drying, physical and chemical changes can occur in the fibres resulting in shrinkage and formation of intramolecular hydrogen bonds. Some of the effects of hornification on the fibres are irreversible. When water is removed, the cell wall structure becomes much less flexible and less swollen than before drying. The intramolecular hydrogen bonds that form between cellulose fibrils and the tight packing of these fibrils cause a decreased accessible surface area. The removal of water is the main cause of hornification and results in the loss of pores in the cell wall matrix of the fibre. Loss of water results in an increase in crystalline regions and a decrease in amorphous regions of cellulose which are most susceptible to enzyme attack. Upon re-wetting, only partial opening of these pores occurs. The high temperature of the szego mill may have induced drying and thus, removal of water from the fibres and removal of pores on the cell wall. The decreased pores make access to enzymes very difficult. Weise 1998 noted that hornification of fibre occurs mostly in the fines fraction. When the fines are subjected to enzymatic hydrolysis and water is re-introduced, the fines show a decreased ability to re-swell (Weise 1998). The largest particle size did have the worst conversion rates and yields, as expected. These particles were not broken down sufficiently, implying less surface area and/or a lower fraction of amorphous regions which are more susceptible to enzymatic attack; this means that enzyme accessibility still remained an issue.

Two cellulases and two xylanases were tested on oat hulls pretreated using a coffee grinder and szego mill. The trend of increased conversion rates by NS22074 can be explained by the selectivity of the substrate. Each substrate has different structural characteristics and amounts of glucan, xylan and lignin. The cellulase system of NS22074 is better suited to hydrolysis of oat hulls than NS50073. There was also a trend for increased conversion to xylose, and a slight increase in glucose when Alternafuel 100L was used in place of Multifect Xylanase. The preference for Alternafuel 100L over Multifect Xylanase implies different activities or substrate selectivity for the enzymes binding sites. NS22074 and Alternafuel 100L were selected as the best cellulase and xylanase, and were used in all subsequent trials. It is assumed that irrespective

of the pretreatment method, these two enzymes would continue to produce greater conversions of glucose and xylose compared to the other enzyme cocktails explored.

When hydrolyzates from the oat hulls pretreated by the coffee grinder and szego mill were analyzed by HPLC for sugar content, an unknown peak was found in the chromatogram. Though its identity was undetermined, its presence offers insight into the presence of inhibitors and intermediates over the course of the reaction. The peak was present at a retention time of roughly seven minutes. This unknown is believed to be an oligosaccharide. From the chromatograms, its intensity initially increases during the course of the reaction. At the same time, the concentration of glucose seems to plateau at approximately 12 hours, which correlates with the increased intensity of the unknown. By removing the filtrate during the solid-liquid separation, the unknown is removed from the system. After the solid-liquid separation, it is evident from HPLC analysis that it is present in the filtrate and does not reappear in the hydrolyzate until after approximately 48 hours into the reaction. Once this unknown is removed, the enzymes have the ability to operate effectively on the substrate again, and the concentration of glucose increases rapidly.

The solid-liquid separation step enhanced the hydrolysis of oat hulls subjected to all pretreatment methods. Solid-liquid separation removed cellobiose, monomers and other soluble oligosaccharides in the filtrate. The accumulation of cellobiose inhibits the action of cellulase, specifically endo-glucanases and cellobiohydrolases, which inhibits the production of further cellobiose and other cello-oligosaccharides. Also, the concentration of glucose plateaus at roughly 12 hours. The glucose concentration obtained up to 24 hours is high enough to inhibit the native β-glucosidase activity, which leads to cellobiose accumulation. Adding Novo188 after the solid-liquid separation step helped to avoid inhibition by cellobiose. After adding Novo188, the cellobiose was broken down to glucose monomers and accumulation of cellobiose was not observed until approximately 72 hours into the reaction. Without this solid-liquid separation, the cellobiose concentration increases and further glucose production is inhibited after approximately 12 hours. The addition of Novo188 seems to lower the prevalence of cellobiose after the solid-liquid separation as it is converted to glucose. By comparison, adding Novo188 before the solid-liquid separation has no effect on overall conversion of cellobiose to glucose

because glucose conversion rate is high initially, and the glucose concentration would inhibit the Novo188. This was seen when Novo188 was added in the initial enzyme dose for hydrolysis of "coffee grinder" and szego mill pretreated oat hulls. After the solid-liquid separation, the glucose is removed in the filtrate and the Novo188 can act upon the substrate without inhibition by glucose. The glucose production rate after the solid-liquid separation is not as high as it was initially; therefore Novo188 has a chance to work effectively on cellobiose to produce glucose. There is no statistical difference between addition of Novo188 before or after the solid-liquid separation step, in terms of glucan conversions. All trials were run with and without solid-liquid separation. In every instance, there was a significant difference between trials with and without the separation. The solid-liquid separation improved conversion rates and yields of products dramatically. For ease of presentation, only the results from the hydrolysis trials performed with solid-liquid separation are presented here; data from trials without the solid-liquid separation step may be found in Appendix B.

The disc refining trials showed that this pretreatment method was not sufficient on its own. Disc refining of raw oat hulls gave a glucan conversion of 32% and a xylan conversion of 24% during hydrolysis. The mechanical pretreatment method therefore did not appreciably increase enzyme accessibility, although the results were better than those for hydrolysis of raw oat hulls without refining. Mechanical pretreatment method likely did not sufficiently decrease the size of the oat hulls to a level needed to materially enhance enzyme accessibility and hydrolysis. Also, the change in crystallinity and extent of intermolecular hydrogen bonds was presumably very low. The resulting characterization profile of disc refined oat hulls did not change significantly from the starting material, implying that the chemical composition was not altered, i.e., there was no delignification or hemicellulose conversion.

Mercerization was a very useful pretreatment technique. Following pretreatment with 1M NaOH for 2 hours, the subsequent hydrolysis led to a 73% glucose yield and a 68% xylose yield from processed oat hulls, compared to a glucose yield of 70% and a xylose yield of 66% from raw oat hulls. There was no statistical difference between the glucose and xylose yields of mercerized raw and mercerized processed oat hulls. Increasing the severity of mercerization from 1 to 2 hours and from 0.125M to 1M NaOH increased the hydrolysis yields of glucose and xylose.

Mercerization results in swelling of the fibres (Kim et al. 2006) (Borysiak and Doczekalska 2008) and, based on these results, significantly increases accessibility of the enzymes to cellulose and hemicellulose, enhancing hydrolysis. From the characterizations, (see Tables 4.1 and 4.2) it was seen that some of the lignin was removed, which led to a redistribution of glucan and xylan. Since the glucan content increased during mercerization, and xylan and lignin both decreased, glucan is more accessible to the enzymes. This validates the findings of improved hydrolysis of glucan following mercerization, since the ability of the enzyme to access the substrate is crucial. The increased accessibility to glucan is due to decreasing connections to lignin and xylan and a more porous structure. This allows the cellulase to find its way to glucan and also decreases the non-specific binding to lignin.

It was shown in this study that oat hulls benefit from a combination of mercerization and disc refining pretreatment methods. Adding disc refining to mercerization increased the yield of sugars compared to mercerization alone. On its own, disc refining was not very effective, but when added to mercerization, the benefits were apparent, with a statistically significant improvement in glucose and xylose yields. Since there is no statistical difference between which pretreatment is performed first, the mechanical energy should be considered. Performing mercerization followed by disc refining would lower the mechanical energy input, because mercerization alters the fibre structure and density, making mercerized oat hulls easier to process through the disk refiner than raw oat hulls. This combination of pretreatments gave the highest conversion of glucan (83%) and xylan (73%) compared to the other pretreatments studied. This was done using 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation. Trials to validate these results during scale up are described in section 6.

6. Scale-up reactions

Alterations and modifications to process conditions usually require an economic consideration. When evaluating the economics of scaling up the reaction, the following areas need to be

considered: enzyme cost, reactor cost, production costs and biomass costs. These costs can be considerable and can result in decreased profits for a company if not properly taken into account. At this level, the effect of inhibitors and cost both increase. Once inhibitors increase, it can be difficult to obtain the same results that were seen during smaller scale trials. The bench scale reactions carried out in a shaking incubator were scaled up to a 2L steel reactor and a 20L ChemGlass Bioreactor. Only the enzyme doses corresponding to the best yields of glucose and xylose were used for each large scale hydrolysis. The purpose of the scale-up reactions was to test the effect of inhibition that occurs on a large scale, while aiming to maintain at least the same yields of hydrolysis that were seen during small scale trials.

6.1 Enzymatic hydrolysis in a steel reactor

Following the shaking incubator trials, hydrolysis trials were scaled up to a 2L jacketed steel reactor. 1L of 50mM sodium acetate buffer, pH4.8, was added to the reactor, followed by the appropriate amount of solids to give a solids loading of 14%. The reactor was connected to a water bath filled with deionized water preheated to 55°C. The two components were mixed with an overhead stirrer at 150rpm until a slurry formed and the temperature of the slurry reached 55°C. Figure 6-1 depicts the set-up of these components for hydrolysis. Enzyme was added at this point, E_1, and sampling was done in the same manner as in the shaking incubator trials. Each sample was analyzed in triplicate. Solid-liquid separation by filtration was performed at a residence time of 24 hours. After the separation, the solids were added to fresh buffer and the second enzyme dose was added at this time, E_2; see Table 6-1. Each sample was also run without a solid-liquid separation and the total enzyme dose was added at E_1; Table 6-2. The enzyme doses added were based upon those added in the shaking incubator trial that gave the highest yield of products. Only the trials that gave the best results during the smaller scale trials were scaled-up.

Figure 6-1: Set-up of enzymatic hydrolysis in a steel reactor

Table 6-1: Enzyme doses added at certain time points during hydrolysis, where solid-liquid separation took place
1L steel reactor, 160g substrate on dry basis

Type of oat hull	E_1	E_2
Processed	800µL NS22074 400µL Alternafuel 100L	800µL NS22074 800µL Alternafuel 100L 80µL NS188
Raw	800µL NS22074 400µL Alternafuel 100L	800µL NS22074 800µL Alternafuel 100L 80µL NS188
"_Coffee ground_"	800µL NS22074 400µL Alternafuel 100L	800µL NS22074 800µL Alternafuel 100L 80µL NS188
Szego milled	800µL NS22074 400µL Alternafuel 100L	800µL NS22074 800µL Alternafuel 100L 80µL NS188
Disc refined	800µL NS22074 400µL Alternafuel 100L	800µL NS22074 800µL Alternafuel 100L 80µL NS188
Mercerized	1200µL NS22074	1200µL NS22074

	800µL Alternafuel 100L	800µL Alternafuel 100L 80µL NS188
Disc refined + mercerized	1200µL NS22074 800µL Alternafuel 100L	1200µL NS22074 800µL Alternafuel 100L 80µL NS188
Mercerized + disc refined	1200µL NS22074 800µL Alternafuel 100L	1200µL NS22074 800µL Alternafuel 100L 80µL NS188

Table 6-2: Enzyme doses added at the start of hydrolysis, where no solid-liquid separation took place
1L steel reactor, 160g substrate on dry basis

Type of oat hull	**E_1**
Processed	1600µL NS22074 1200µL Alternafuel 100L 80µL NS188
Raw	1600µL NS22074 1200µL Alternafuel 100L 80µL NS188
"_Coffee ground_"	1600µL NS22074 1200µL Alternafuel 100L 80µL NS188
Szego milled	1600µL NS22074 1200µL Alternafuel 100L 80µL NS188
Disc refined	1600µL NS22074 1200µL Alternafuel 100L 80µL NS188
Mercerized	2400µL NS22074 1600µL Alternafuel 100L 80µL NS188

Disc refined + mercerized	2400µL NS22074 1600µL Alternafuel 100L 80µL NS188
Mercerized + disc refined	2400µL NS22074 1600µL Alternafuel 100L 80µL NS188

6.2 Enzymatic hydrolysis in a ChemGlass reactor

Mercerized and disc refined oat hulls were hydrolyzed at a larger scale using the jacketed ChemGlass Bioreactor. The ChemGlass Bioreactor was connected to a water bath filled with deionized water and heated to 55°C. The reactor was equipped with a motor to rotate the agitator and a monitor to view and control the speed of agitation. Figure 6-2 below displays the ChemGlass reactor set-up. 7.8 kg of pretreated oat hulls, DM=20.5%, were added to 3.8 kg of sodium acetate buffer, pH 4.8, to give a DM of 16% and a final volume of 10L. Since the pretreated oat hulls were caustic, 20ml of acetic acid was added to the slurry to bring the pH down to 4.8. The slurry was allowed to mix at 250 rpm until a temperature of 55°C was reached. The enzyme doses used are displayed in Table 6-3. The enzyme dose used for this trial was based on the enzyme dose that gave the best yield of sugars during the shaking incubator trials using the same substrate. The enzyme cocktail is made of 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation. This trial was run in duplicate; only one trial was run without a solid-liquid separation, due to availability of substrate. The enzyme doses added to the hydrolysis without solid-liquid separation are displayed in Table 6-4.

Figure 6-2: Experimental setup in the ChemGlass reactor

Table 6-3: Enzyme doses used in ChemGlass reactor at various time points, where solid-liquid separation was performed

10L ChemGlass reactor, 1.6 kg dry substrate

t_0	t_{24}
12mL NS22074	12mL NS22074
8mL Alternafuel 100L	8mL Alternafuel 100L
	0.8mL Novo188

Table 6-4: Enzyme doses used in ChemGlass reactor at the start of hydrolysis, where no solid-liquid separation took place

10L ChemGlass reactor, 1.6kg dry substrate

t_0
24mL NS22074
16mL Alternafuel 100L
0.8mL Novo188

6.3 Results

The results were tracked the same way as previously mentioned at the start of Section 5. The yield of glucose and xylose were the main focus, while oligosaccharides were omitted. The yield calculations are available in Appendix A3. Table 4-1 and 4-2 were used to determine the total available glucan and xylan, and Table 4-3 was used to determine the availability based on dry mass. Pretreatment methods were compared based on the average glucose and xylose yields from three replicates run for each enzyme dose. This was done for trials with and without solid-liquid separation; for simplicity trials without solid-liquid separation can be found in Appendix B2. For the steel reactor trials, the same calculations were performed as with the shaking incubator trials. To compare the results from hydrolysis trials following different pretreatment methods, the standard deviations of the mean at each time point were compared. A single factor analysis of variance (ANOVA) was used to determine statistical differences, based on the mean glucose and xylose yields between enzyme doses within a given pretreatment method. A difference that is statistically significant has a confidence level of over 95%. A difference that is not statistically significant will have a confidence level of less than 85%. All errors bars represent the standard error of the mean. When the ChemGlass reactor was used, the amount of substrate was limited and therefore the average of two replicate trials with solid-liquid separation was obtained. Only one trial using the ChemGlass reactor was run without solid-liquid separation.

6.3.1 Steel reactor trials

The scale-up of enzymatic hydrolysis trials from the shaking incubator led to overall increased yields of glucose and xylose for the various pretreatment methods, the significance of these yields will be explored.

6.3.1.1 No pretreatment

To validate the efficiency of pretreatment methods on yield of products, hydrolysis trials were run without pretreatment of oat hulls (Figure 6-3 and 6-4). Hydrolysis of raw oat hulls with a solid-liquid separation step gave yields of 25% glucose and 19% xylose; without separation, 16% glucose and 10% xylose were obtained. Hydrolysis of processed oat hulls with a solid-liquid separation gave yields of 28% glucose and 21% xylose; without separation, 18% glucose and 12% xylose were obtained. It was determined through student's t-tests that there is no statistical difference between yields for raw and processed oat hulls. Also, when compared to hydrolysis in the shaking incubator, a significant increase in yield was obtained. Since these yields are very low, it can be stated that pretreatment is necessary to obtain higher yields of products during enzymatic hydrolysis. It is expected that all of the pretreatments to follow will increase the yields of hydrolysis compared to from those results obtained without pretreatment.

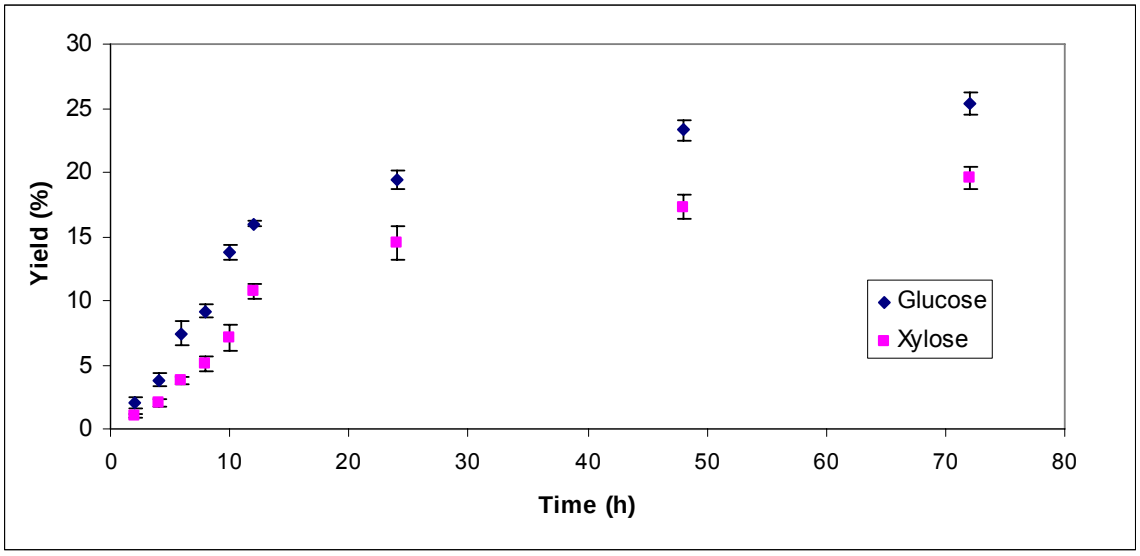

Figure 6-3: Hydrolysis of raw oat hulls in a steel reactor
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

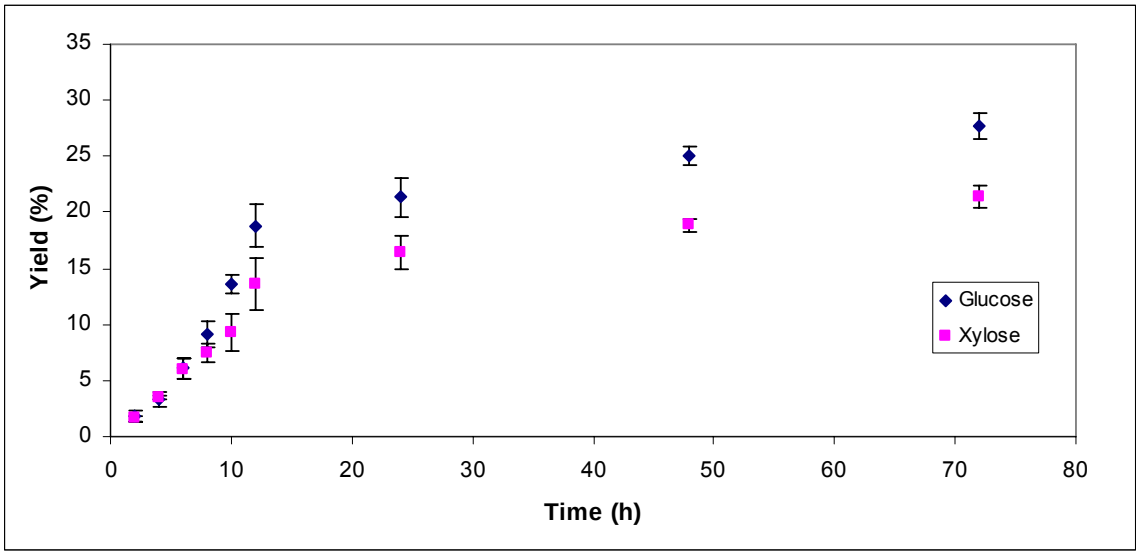

Figure 6-4: Hydrolysis of processed oat hulls in a steel reactor
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

6.3.1.2 Coffee grinder

With solid-liquid separation, a yield of 73% glucose and 68% xylose was obtained (Figure 6-5). Figure 6-6 depicts hydrolysis of processed oat hulls pretreated with a coffee grinder without solid-liquid separation; a glucose yield of 53% and a xylose yield of 42% were obtained. There was a definite statistical significance of adding the separation step, leading to increased yields of both products. Compared to shaking incubator trials using the same enzyme dose and having the separation step, no statistical difference was obtained from the scale-up to the 2L steel reactor, where 71% glucose and 66% xylose yields were obtained.

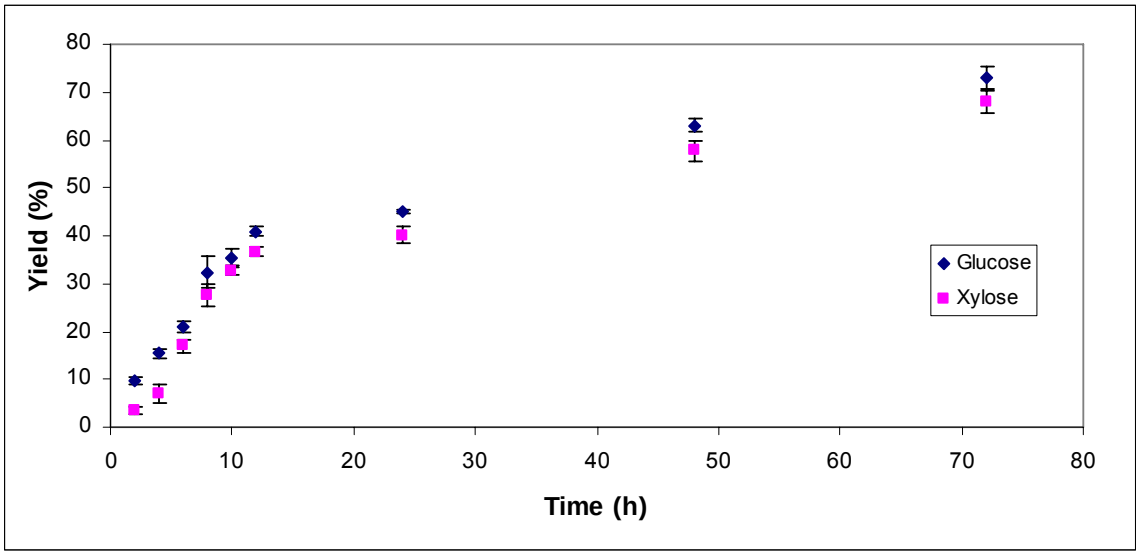

Figure 6-5: Yield of glucose and xylose (%) during hydrolysis of "_coffee ground_" oat hulls in a steel reactor

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

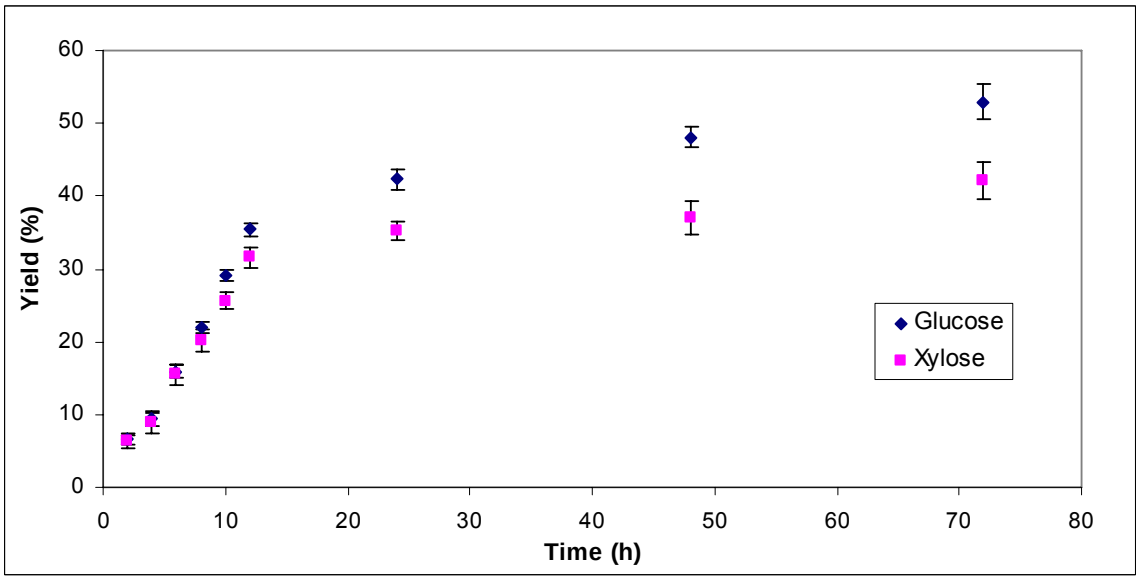

Figure 6-6: Yield of glucose and xylose (%) during hydrolysis of "_coffee ground_" oat hulls in a steel reactor, without solid-liquid separation

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

6.3.1.3 Szego mill

The hydrolysis of szego milled raw oat hulls gave a yield of 76% glucose and 69% xylose, Figure 6-7. The hydrolysis of szego milled processed oat hulls gave a yield of 78% glucose and 74% xylose, Figure 6-8. It was determined from these results that there is no statistical difference between the hydrolysis of raw and processed substrates that are szego milled. This compares to the shaking incubator trials, where a lack of statistical difference was also found, see Figures 5-16 and 5- 17. There is also no statistical difference between yields in the shaking incubator and steel reactor for both raw and processed oat hulls. This may be due to the fact that the smaller particle size produced by this pretreatment method helps to improve mixing and reduces viscosity. Regardless of the reaction vessel, the mixing is efficient and allows enzyme accessibility and adsorption onto the oat hulls. Without solid-liquid separation, szego milled raw oat hulls gave yields of 57% glucose and 46% xylose, whereas processed oat hulls gave yields of 55% glucose and 48% xylose. The results without the separation are statistically different from those with separation for both raw and processed oat hulls.

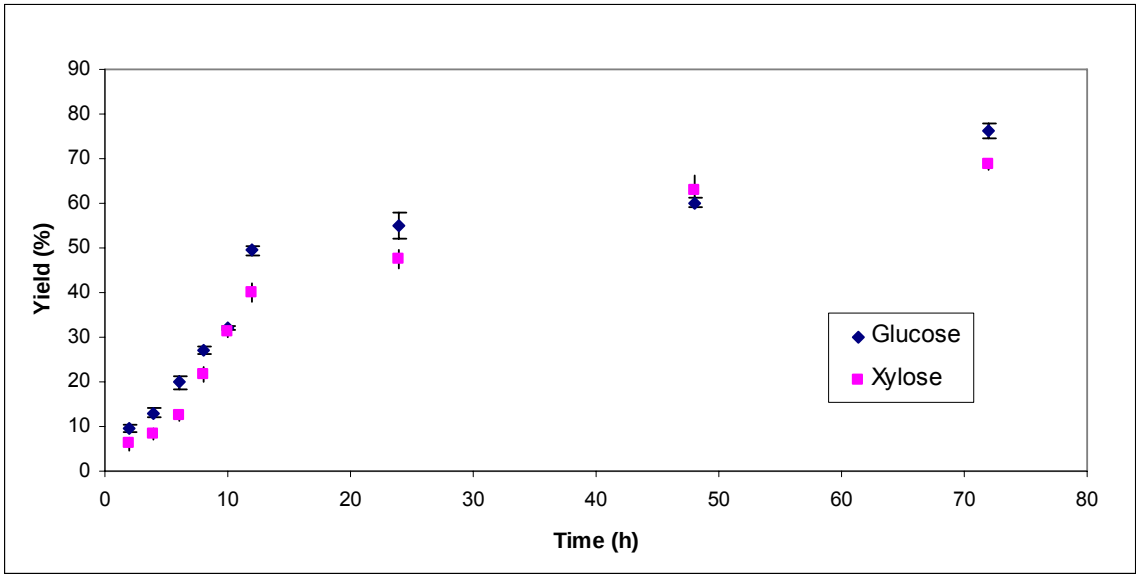

Figure 6-7: Yield of glucose and xylose (%) during hydrolysis of szego milled raw oat hulls in a steel reactor, with solid-liquid separation

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

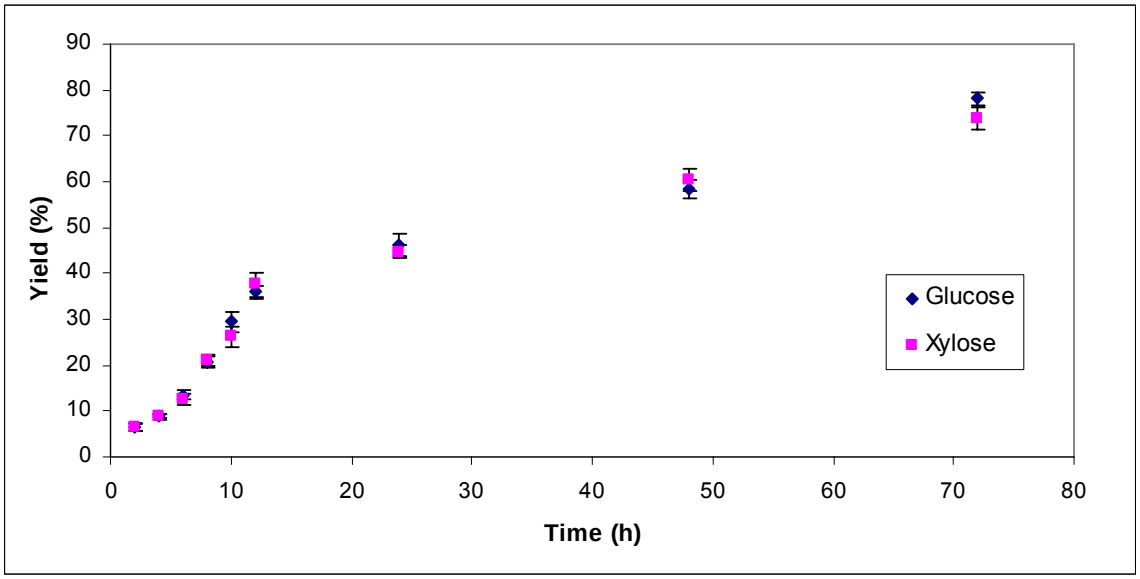

Figure 6-8: Yield of glucose and xylose (%) during hydrolysis of szego milled processed oat hulls in a steel reactor, with solid-liquid separation

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

6.3.1.4 Disc refined

Disc refined raw oat hulls gave yields of 37% glucose and 29% xylose (Figure 6-9). Although these yields increased compared to yields without pretreatment (25% glucose and 18% xylose) the yields were less than anticipated. As shown in the small scale trials, disc refining on its own is not an efficient pretreatment method and thus, it was combined with mercerization to see if any added benefit is achieved. Without solid-liquid separation, the yields were statistically lower; 28% glucose and 21% xylose yields.

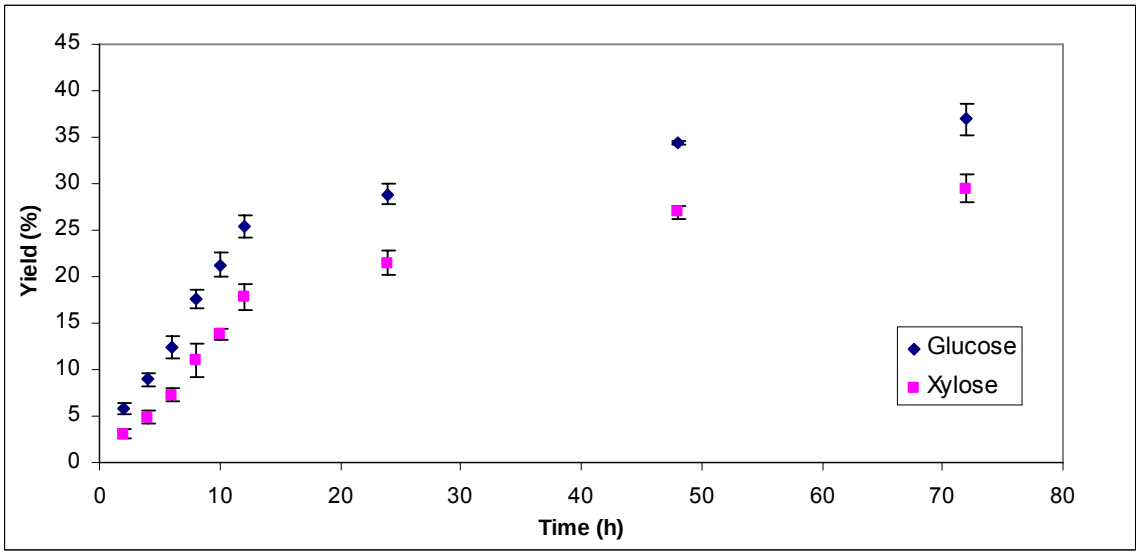

Figure 6-9: Yield of glucose and xylose (%) during hydrolysis of disc refined oat hulls in a steel reactor, with solid-liquid separation

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

6.3.1.5 Mercerization

Scale-up of mercerization trials used the optimal conditions obtained in shaking incubator trials; 95°C for two hours on a hot plate (see section 5.4). Hydrolysis of raw mercerized oat hulls led to a yield of 75% glucose and 73% xylose (Figure 6-10). Hydrolysis of processed mercerized oat hulls led to a yield of 77% glucose and 72% xylose (Figure 6-11). There was no statistical difference between hydrolysis of raw and processed mercerized oat hulls. There was however, a statistical difference obtained from scale up of shaking incubator trials for both raw and processed substrates. Shaking incubator trials gave yields of 70% glucose and 66% xylose for raw oat hulls and 73% glucose and 68% xylose for processed oat hulls. The mixing in the shaking incubator may be the cause of the decreased yields; the oat hulls, being large in size, would settle on the bottom of the flask and led to increased viscosity of the slurry.

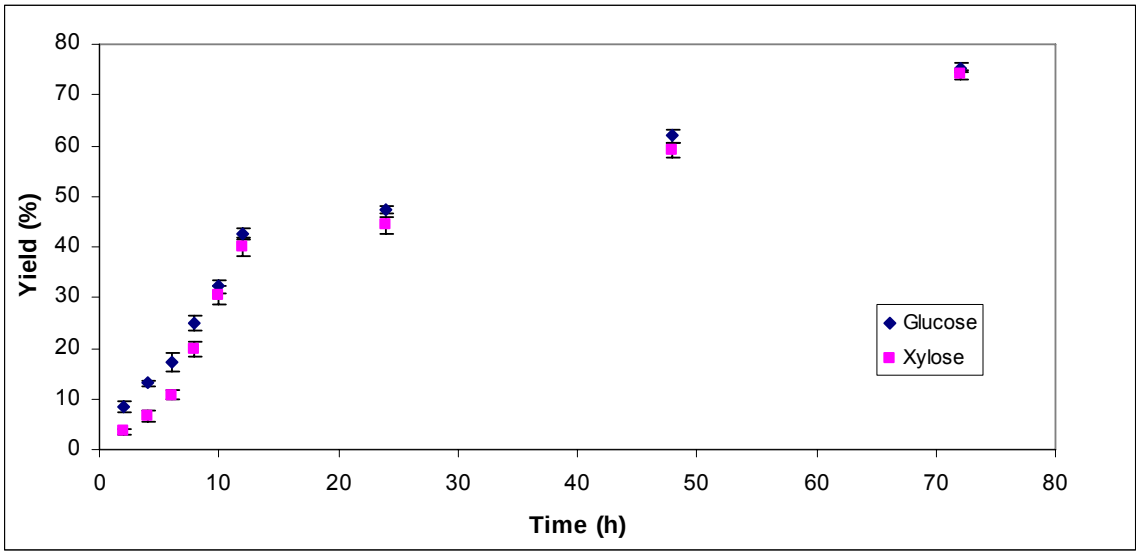

Figure 6-10: Yield of glucose and xylose (%) during hydrolysis of mercerized raw oat hulls in a steel reactor, with solid-liquid separation

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

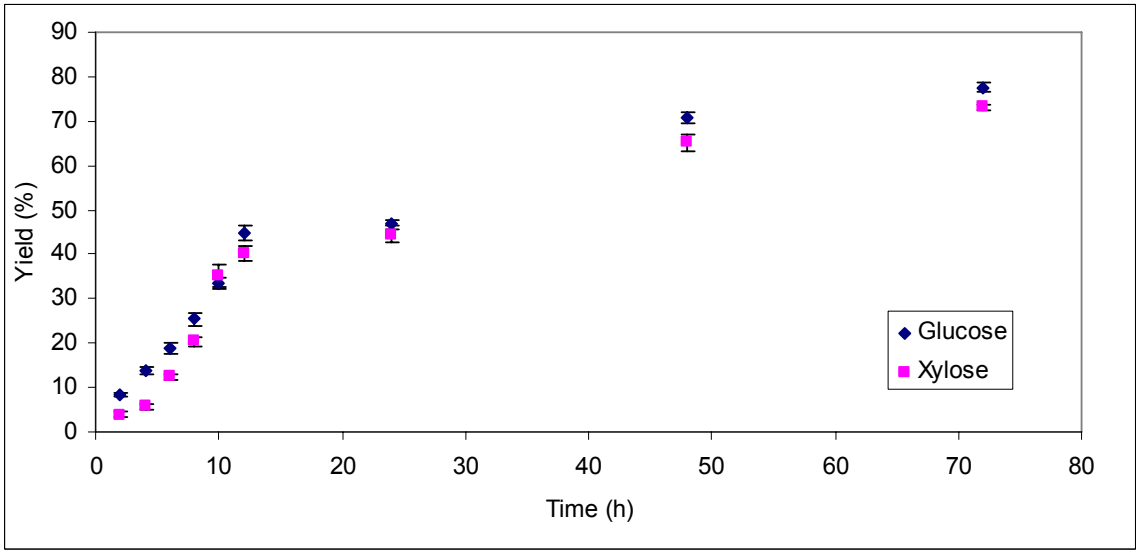

Figure 6-11: Yield of glucose and xylose (%) during hydrolysis of mercerized processed oat hulls in a steel reactor, with solid-liquid separation

Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

6.3.1.6 Disc refined and mercerized

Disc refined and mercerized oat hulls led to a hydrolysis yield of 85% glucose and 80% xylose (Figure 6-12). These results are statistically different from those obtained during the shaking incubator trials; a yield of 81% glucose and 76% xylose was obtained. The results obtained for the combined pretreatment are statistically better than those obtained for mercerization or disc refining alone; these two pretreatments work together to enhance the efficiency of hydrolysis.

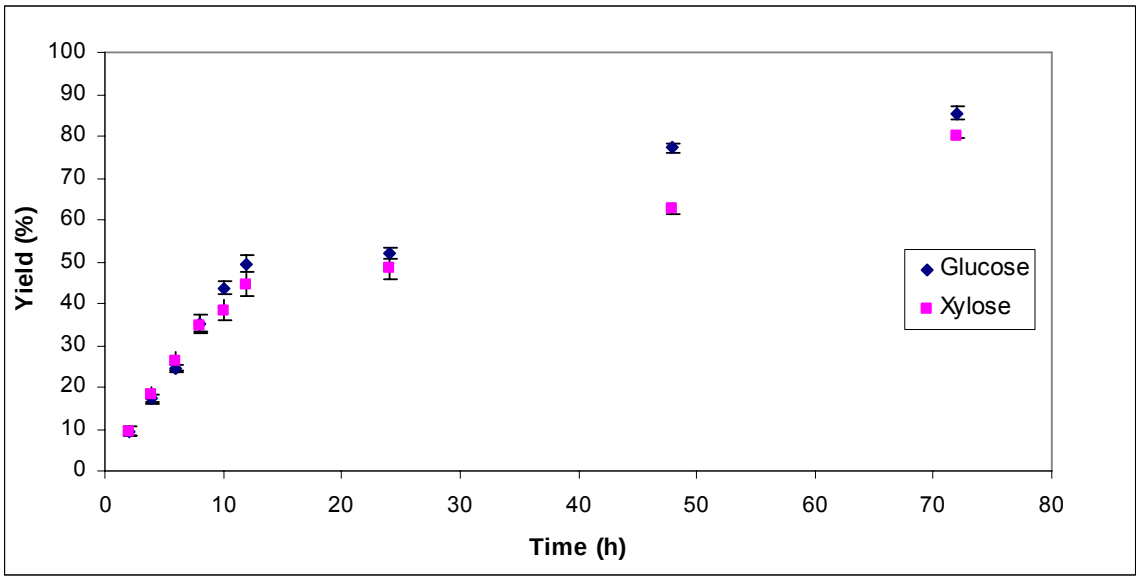

Figure 6-12: Yield of glucose and xylose (%) during hydrolysis of disc refined and mercerized oat hulls in a steel reactor, with solid-liquid separation
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

6.3.1.7 Mercerized and disc refined

Hydrolysis of mercerized and disc refined oat hulls led to a yield of 86% glucose and 81% xylose (Figure 6-13). These results are statistically different from those obtained during shaking incubator trials under the same conditions; 82% glucose and 75% xylose yield. However, these results are not statistically different from the results of hydrolysis of disc refined and mercerized oat hulls in a steel reactor. Even with the shaking incubator trials, there was no statistical difference between the order of these two pretreatments. For the combination of caustic and disc refining, there was a statistical increase in hydrolysis yields in the shaking incubator. This is

most likely due to the increased efficiency of mixing in the steel reactor as well as the size of the oat hulls, which increase viscosity.

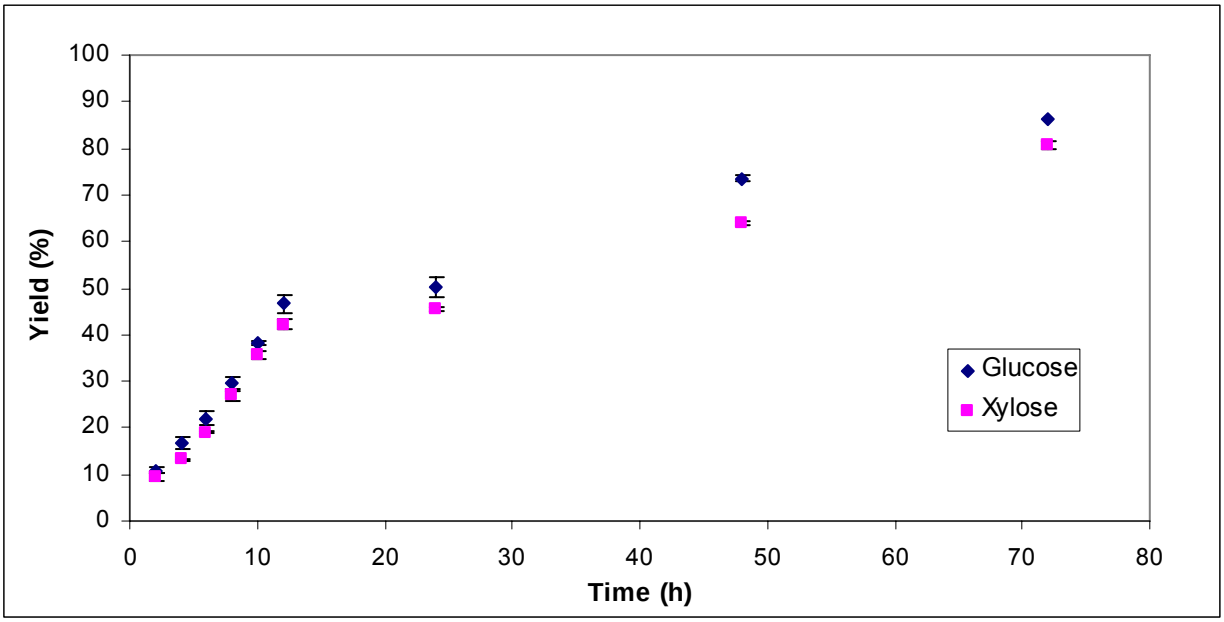

Figure 6-13: Yield of glucose and xylose (%) during hydrolysis of mercerized and disc refined oat hulls in a steel reactor, with solid-liquid separation
Data points represent the mean of triplicate values, and error bars represent the standard error of the mean

A summary of all the pretreatment methods performed on oat hulls and using the steel reactor for hydrolysis is presented in Figure 6-14. The enzyme cocktails used for the comparisons reflect the enzyme doses that gave the best yields of glucose and xylose, as previously mentioned. The graph shows that the best yields were obtained using mercerized + disc refined oat hulls. Results with no pretreatment gave the poorest yields.

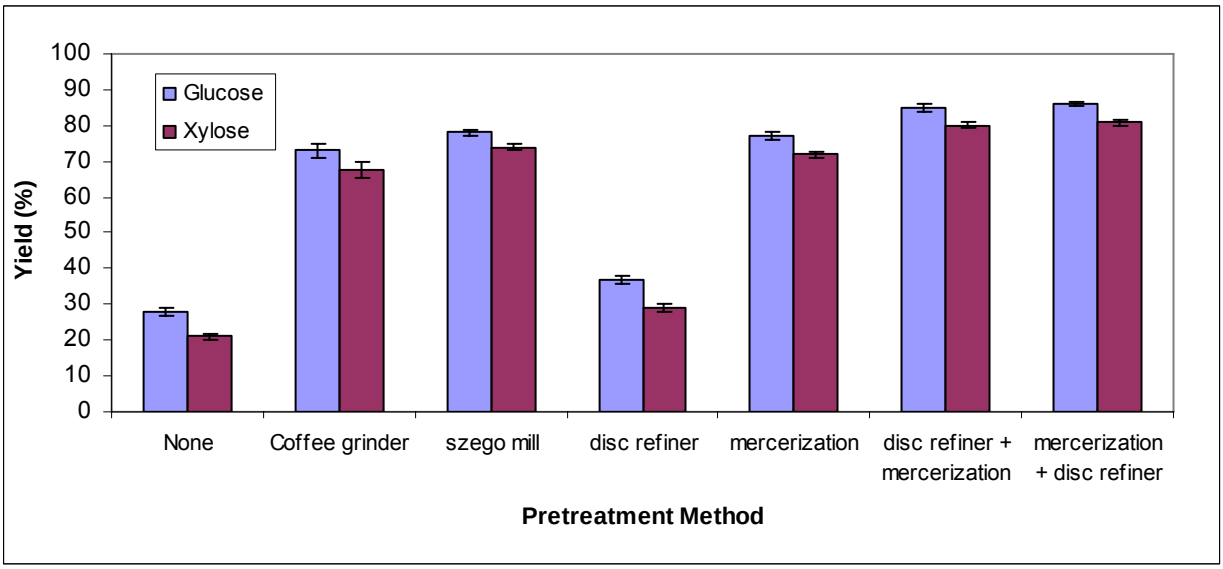

Figure 6-14: Comparison of pretreatment methods on yields of glucose and xylose (%) during steel reactor hydrolysis trials

6.3.2 ChemGlass reactor trials

The mercerized and disc refined oat hulls in the ChemGlass reactor gave an average glucose yield of 89% and an average xylose yield of 84% after 72 hours of hydrolysis. One hydrolysis was run without solid liquid separation and gave a glucose yield of 60% and a xylose yield of 59%; results are displayed in Figures 6-15 and 6-16 below.

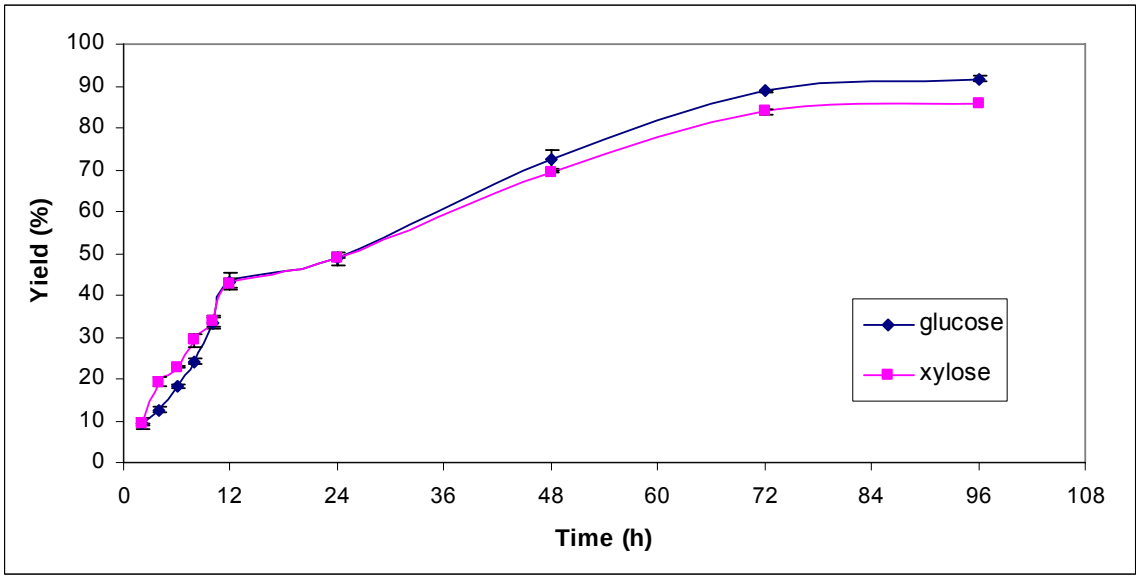

Figure 6-15: Yield of glucose and xylose (%) during hydrolysis of mercerized and disc refined oat hulls, with solid-liquid separation

Data points represent the mean of duplicate values, and error bars represent the standard error of the mean

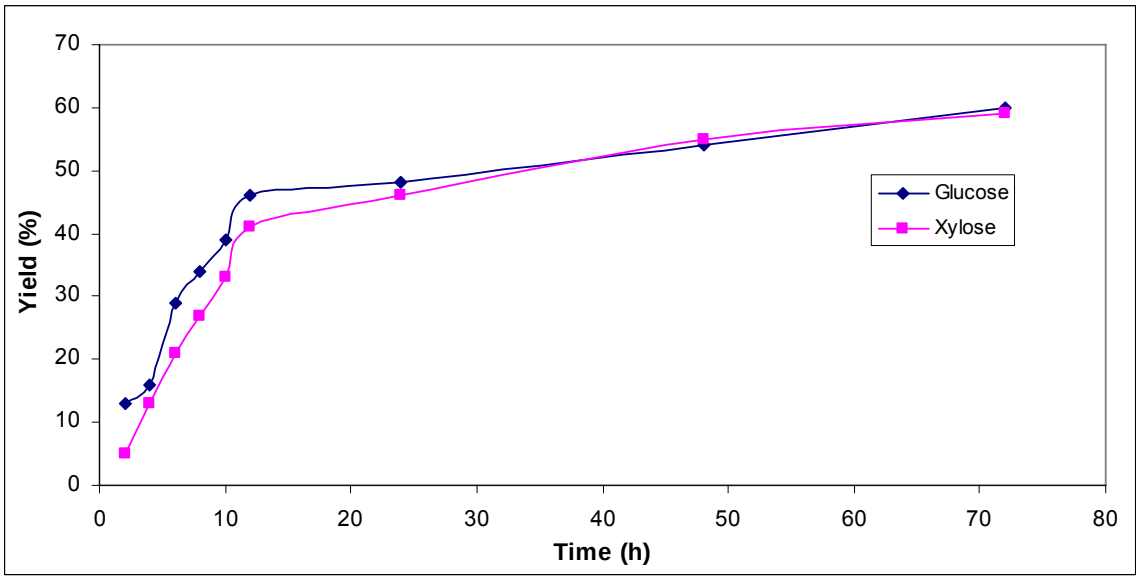

Figure 6-16: Yield of glucose and xylose (%) during hydrolysis of mercerized and disc refined oat hulls, without solid-liquid separation

Data points represent the mean of duplicate values, and error bars represent the standard error of the mean

6.4 Discussion

Steel reactor trials produced increased yields overall compared to the shaking incubator trials. However, not all of these results were statistically significant. The hydrolysis of "_coffee ground_" and szego milled oat hulls in the shaking incubator and steel reactor were not statistically different. The reason for this may be explained by particle size; these two pretreatments gave oat hulls with a particle size less than 250μm. This small particle size has the ability to form a homogeneous slurry. This leads to increased efficiency of mixing. Therefore, irrespective of the reaction vessel, the enzymes can easily access the substrate due to efficient mixing and are able to adsorb onto it. With the disc refined, mercerized and combination pretreatments, the particle size was not sufficiently reduced to aid slurry formation and increase mixing. This was not a problem in the steel reactor, where the agitator compensated for the larger particle size and higher viscosity. However, the shaking incubator was not able to increase the efficiency of mixing. Therefore, oat hulls settled on the bottom of the flask, which means decreased enzyme accessibility and less adsorption of enzyme on the substrate. For the disc refining, mercerization and combination trials, a statistical difference was reported when the reaction was scaled up. The increase in yields of glucose and xylose, for the most part, increased only after the solid-liquid separation. This may be an indication that the effect of inhibitors in the large reaction vessel was greater and thus irrespective of mixing efficiency, the yields did not increase from shaking incubator trials. However, after the solid-liquid separation, the yields of glucose and xylose were greater than in shaking incubator trials. Once the inhibitors were removed, the increased enzyme accessibility due to mixing efficiency was realized.

For all of the hydrolysis trials in the steel reactor, the solid-liquid separation produced increased yields of hydrolysis; which in all cases were statistically significant. The solid- liquid separation removed inhibitors such as cellobiose and glucose and allowed for efficient action of the cellulase system. Overall, the hydrolysis of mercerized and disc refined oat hulls gave the best yields of hydrolysis; though the order of these pretreatments is irrelevant. During 72 hours of hydrolysis in a steel reactor, where solid-liquid separation takes place, these pretreatments led to yields of 86% glucose and 81% xylose. 0.8 wt% NS22074 before and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06wt% Novo188 after separation were the

enzyme doses used to obtain these yields. These yields were the highest obtained as of yet and due to these favourable results, provide a good basis for research in commercialization of this process.

The results from the larger scale trials with caustic and disc refined pretreated oat hulls in the ChemGlass reactor were surprising due to the high conversion rate and yield. The glucose and xylose yields were 89 and 84%, respectively, over 72 hours of hydrolysis. Again, 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation were the enzyme doses used. At a larger scale, one would expect the effects of inhibition to be greater and thus lower overall conversion and yields. However, the results were opposite; the larger scale trial gave a better yield than the small scale trials in the shaking incubator. The results from the scale up trials were statistically better than those in the shaking incubator. This may be due in part to the increased efficiency of mixing in ChemGlass reactor. The better mixing could increase adsorption of the enzymes onto the oat hulls. In the shaking incubator trials, some inefficient mixing of mercerized and disc refined raw oat hulls was observed, likely due to their relatively large particle size. The orbital action of the incubator could not prevent some settling of particulate pretreated oat hulls at the bottom of the Erlenmeyer flasks. However, mixing in the ChemGlass reactor was efficient and no settling occurred. Also, the speed of rotation of the agitator in the ChemGlass reactor was easier to monitor and much more accurate than in the shaking incubator. The speed was very high initially; otherwise the slurry would not mix at all. The ability to control the mixing and having it set at a higher speed was crucial to the process, and likely enhanced adsorption of the enzymes to their substrate. These two factors would greatly contribute to the increased yields that were observed. Without solid-liquid separation, 60% glucose and 59% xylose yields were obtained. These results prove that inhibitors play a significant role in hydrolysis, and once they are removed, the yield of the products will increase. Since this is a large scale, the effect of inhibitors is intensified and the solid-liquid separation step is essential to significantly increase the amount of products obtained from hydrolysis.

7. Conclusions

7.1 Summary

This thesis has investigated the use of pretreatments and enzyme cocktails to achieve high conversion rates and yields of reducing sugars. In evaluating their effectiveness towards the substrate, oat hulls, several observations were made. The structure of the biomass is recalcitrant and pretreatment methods are needed to break down the physical structure and facilitate enzyme access. Without pretreatment, enzymatic hydrolysis resulted in a glucose yield of 21% and a xylose yield of 15% for processed oat hulls versus an 18% glucose yield and 12% xylose yield for raw oat hulls. These values are not statistically different.

Szego milling is an effective means to pretreat oat hulls, leading to a glucose yield of 75% and a xylose yield of 71% on a small scale, using processed oat hulls. When raw oat hulls were used in the szego mill, a glucose yield of 72% and a xylose yield of 67% were obtained. These yields were obtained using an enzyme cocktail made up of 0.6 wt% NS22074 before and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06 wt% Novo188 after separation. When a 2L steel reactor was use to hydrolyze the szego milled oat hulls, the same trend was seen in favour of the processed oat hulls as the substrate. Processed oat hulls gave a glucose yield of 79% and a xylose yield of 76% while raw oat hulls gave a glucose yield of 74% and xylose yield of 68%; the same enzyme doses used as small scale trials. While the hydrolysis of szego milled processed hulls suggests a slightly better yield than hydrolysis of raw oat hulls at both scales with the same conditions, they yields with szego milled raw and processed oat hulls were statistically equivalent. Though the starting material of processed oat hulls is smaller than the raw oat hulls, they each get broken down sufficiently when szego milled. There was generally no significant difference between trials run in the shaking incubator and the 2L steel reactor. The trials in the shaking incubator were expected to have decreased yields due to inefficiency of mixing compared to the steel reactor. However, since the particle size was so small and a homogeneous slurry easily formed, the mixing was easily facilitated by the shaking

incubator. The steel reactor also had great mixing due to the viscosity reduction by enzymes in the slurry and the agitator that aided mixing.

Milling of raw oat hulls using the coffee grinder also gave promising yields in shaking incubator trials; 71% glucose and 66% xylose yields. In steel reactor trials, a glucose yield of 73% and a xylose yield of 70% were obtained. 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06 wt% Novo188 after separation were the enzyme doses used. Again, there was no statistical difference between trials run at small and large scales. There is also no statistical difference between using a coffee grinder or szego mill on processed oat hulls, both at a small and large scale. While the milling/grinding methods worked on a lab scale, they would be very inefficient when large amounts of the substrate are needed for hydrolysis. Obtaining enough substrate for the steel reactor trials was very difficult and time consuming; only a small amount can be processed through the coffee grinder at once.

Disc refining alone was not efficient in converting oat hulls to reducing sugars; a yield of 32% glucose and 24% xylose was obtained in shaking incubator trials. In trials using the 2L steel reactor, a 37% yield of glucose and a 29% yield of xylose were obtained. The enzyme dose used for both scales was 0.6 wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06wt% Novo188 after separation. The scale-up produced a statistically significant increase in the yield of products. Disc refined oat hulls are still relatively large, and in the shaking incubator, a well-defined slurry does not form, making mixing of this mixture very difficult. The large particles settled easily in the shaking incubator, while in the steel reactor, the overhead agitator prevented settling of pretreated oat hulls. The lower yield of both glucose and xylose in the incubator trials implies decreased enzyme accessibility, likely due to poorer mixing. Although disk refining reduced the particle size somewhat, the composition of the disc refined oat hulls is similar to that of the starting raw oat hulls (Tables 4-1 and 4-2). Disc refining did not alter the physical structure of the oat hulls enough to change their susceptibility to enzymatic attack. The swelling of the fibres and the decrease in size of the oat hulls were not sufficient to allow increased access to cellulose and hemicellulose. The disc refined oat hulls maintain much of their recalcitrant structure and

enzyme accessibility is limited; implying that, on its own, this pretreatment method is ineffective.

Mercerization on its own was a viable option for pretreatment, with a 73% glucose yield and a 68% xylose yield during shaking incubator trials of processed oat hulls pretreated for 2 hours with 1M NaOH. Trials in the 2L steel reactor had statistically greater yields: a 76% glucose yield and a 72% xylose yield, using the same pretreatment conditions. The enzyme doses used were: 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation. 1M NaOH and 2 hours for pretreatment were found to be the best combination for subsequent hydrolysis. Increasing the severity parameter increased the yield of products. Mercerization changed the composition of the raw and processed oat hulls, (Tables 4-1 and 4-2), causing partial delignification and redistribution of xylan increased the glucan content. This resulted in increased access to cellulose and therefore improved cellulase efficiency; which explains the increased yields of glucose and xylose obtained compared to hydrolysis without mercerization.

Disc refining and mercerization pretreatments gave a yield of 81% glucose and 78% xylose during shaking incubator trials. In 2L steel reactor trials, the yields were 84% glucose and 80% xylose, which are statistically better than the yields from the shaking incubator trials. The combination of mercerization and disc refining gave the best hydrolysis yields of glucose and xylose on a small scale; 83% and 73%, respectively. 0.8 wt% NS22074 before separation and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation were the enzyme doses used. The two pretreatment methods were analyzed and it was concluded the order of the pretreatments did not lead to statistically different hydrolysis yields in shaking incubator and steel reactor trials. The results from the larger scale trials using the 20L ChemGlass reactor were statistically better than those from the shaking incubator trials, with an 89% glucose yield and an 84% xylose yield over 72 hours. The 2L steel reactor trials produced a yield of 86% glucose and 81% xylose; these results were also statistically better than those from the shaking incubator trials, but not statistically different from the ChemGlass reactor trials. These findings indicate that the mixing is a very important feature of the hydrolysis of mercerized fibres, because enhances enzyme accessibility and adsorption onto the substrate. The

mixing in the 2L steel reactor is superior to that in the shaking incubator, because settling of the mercerized oat hulls is reduced and reduced more uniform slurry is generated.

The effect of particle size was also explored. When the pretreatment involved physical means to reduce particle size, e.g., a coffee grinder or szego mill, the particle size distribution was analyzed. It was evident from the hydrolysis results of that a smaller particle size was beneficial. A smaller particle size generally increased the conversion of glucan and xylan. The smaller particle size after physical pretreatments also increases the surface area for enzymatic attack, which should enhance adsorption of enzymes onto the substrate. The amount of substrate binding to the enzyme through the enzyme's binding domain would increase, improving the enzyme's ability to operate on the substrate and the yield of products. A larger particle size implies less surface area and a decreased ability for the enzyme to adsorb to the oat hulls, explaining the lower conversion upon hydrolysis of these larger particles. However, a surprising finding was obtained from hydrolysis trials with very small particle sizes obtained by collecting superfine particles from szego milling. The superfine particles did not lead to increased production of reducing sugars; hornification may be the cause of this. Hornification would lead to a decrease in swelling and prevalence of amorphous regions, which leads to decreased enzyme accessibility.

The exploration of enzyme cocktails and doses was critical to this research. The type of enzyme selected is highly dependent on the substrate. In this thesis, two cellulases and two xylanases were investigated for their efficiency in converting glucan and xylan respectively, to reducing sugars glucose and xylose. A clear trend identifying the preferred cellulase and xylanase was noted, in this case indicating that a combination of Novozymes developmental enzyme NS22074 and Alternafuel 100L were optimal. Also, the quantity of enzyme added was very important, and represents an economic decision important in scale up considerations. On a commercial basis, the cost of ethanol production is affected by enzyme cost; therefore, limiting the amount of enzyme used is necessary. This brings a trade-off between cost of enzyme and yield of sugars – factors that are also influenced by the type and effectiveness of the pretreatment step. In this research, the enzyme dose was lowered to an acceptable range, where the yield of glucose and xylose wasn't significantly affected. The way the enzymes work together synergistically is very important. Finding the right balance between the enzymes that make up the cocktail and the

quantity of each component is essential. While one enzyme alone may operate on the substrate effectively, when other enzymes are introduced into the system, they can affect the outcome.

It was also clear that inhibitors severely affected the rate of conversion and yield. When inhibitors accumulated, the rate of conversion slowed dramatically and consequently affected the overall yield of reducing sugars. Glucose concentrations plateaued at approximately 12 hours, at which point the rate of hydrolysis of glucan slowed. When glucose accumulated, the inhibition of β-glucosidase occurred. With inhibition of β-glucosidase, cellobiose accumulated, which led to further inhibition of endo- and exo-glucanases. To overcome the inhibition produced by the products of hydrolysis, a solid-liquid separation step was introduced. This step worked very well to remove the inhibitors and accumulated products and allow the enzymes to work without significant inhibition. The solid-liquid separation step was one of the most valuable outcomes from this research, demonstrating increased overall conversion of sugars. For instance, when mercerized and disc refined oat hulls were hydrolyzed, it led to a glucose yield of 83% xylose yield of 73% compared to a 61% glucose yield and a 43% xylose yield if the solid-liquid separation step was omitted.

7.2 Implications

Overall, the combination of chemical and mechanical pretreatment provided the best hydrolysis yields for oat hulls, both on a small and large scale. The highest yields of products were obtained during scale-up. Szego milling gave glucose and xylose yields of 79% and 76 %, respectively, while the combined chemical and mechanical pretreatment led to glucose and xylose yields of 86% and 81%, in steel reactor trials. It is believed that on a commercial level, where tonnes of oat hulls would need to be processed, szego milling would be a very efficient way to process the large amount of material. Sieving the oat hulls represents a significant energy requirement as this process can be very lengthy. However, on a commercial scale, sieving would most likely be eliminated due to time and cost constraints in additional to the energy requirement. If sieving was performed, a portion of the oat hulls would be unused due to increased particle size which would result in significant yield losses. Without sieving, a larger particle size would be used and

the yield of products would decrease accordingly. However, the szego milling would be a very fast process if sieving was eliminated and the overall production time would thus decrease; leading to higher throughput and cost savings. The coffee grinder worked well for bench scale trials in a shaking incubator but this method would be inappropriate for large scale trials. The energy requirement to grind the oat hulls is large, as only a small amount can be processed at a time. Using 1M NaOH at 95°C is not a very severe process. When the disc refining is performed after the caustic pretreatment, the mechanical energy requirement is lowered. Thus, only one pass through the refiner is needed and mechanical energy use is minimized. The issue with this combined pretreatment is the washing step that occurs after mercerization. A large amount of water would be needed on a commercial scale, which would increase the cost of production. Also, neutralization with acid is needed in order to decrease the pH before hydrolysis, which is an additional cost consideration. The total time for pretreatment using the disc refiner and mercerization would be much longer compared to szego milling increasing costs of production. However, the mercerization/disk refining process is able to use all of the substrate, a significant economic benefit considering that this method also generates the highest yields. Ultimately, a more detailed economic assessment is required, taking into account equipment and process costs, chemical costs and feedstock costs in a comprehensive process design.

The enzymes used for hydrolysis of oat hulls should include NS22074 and Alternafuel 100L. The β-glucosidase activity of the cellulase system should be enhanced by supplementing with Novo188. NS50073 and Multifect Xylanase were also explored, but resulted in lower conversion of glucan and xylan. Therefore, it is important to note that each substrate responds differently to certain enzymes and it is important to tailor the enzyme cocktail to fit the characteristics of the substrate. The enzyme dose is an important consideration; on a commercial scale the cost of enzymes is considerable. The enzyme dose selected needs to consider both economics yields of products. For raw, processed and disc refined oat hulls, the best choice of enzyme doses are 0.6wt% NS22074 before separation and after separation, 0.3 wt% before and 0.6wt% after separation for Alternafuel 100L and 0.06 wt% Novo188 after separation .When the content of glucan increased significantly following pretreatment using mercerization and a combination of mercerization and disc refining, the best enzyme doses were 0.8wt% NS22074 before separation

and after separation, 0.6 wt% Alternafuel 100L before and after separation and 0.06 wt% Novo188 after separation.

The solid-liquid separation is a necessary step to overcome inhibition and increase conversion of the products. Overall, solid-liquid separations increased the yields of glucose and xylose by 20-25%. The timing of the solid-liquid separation at 12 hours coincides with the time when glucose and xylose concentrations plateau, and the rate of conversion decreases. Performing the solid-liquid separation before 24 hours would decrease the time required to reach the optimal conversion of the products. At a commercial scale for production of ethanol, this means decreased time for production, faster product formation, decreased energy input, and most importantly, economic savings.

In this thesis, it has been shown that a smaller particle size generally leads to better enzymatic hydrolysis. A switch to a larger particle size, e.g., due to fewer passes through the szego mill, reduces conversion rates and yields. The particle size directly affects the efficiency of enzyme hydrolysis, likely due to decreased surface area and adsorption of the enzymes onto the oat hulls. As the particle size decreases, the surface area increases and so does the ability of the enzymes to bind through the catalytic domain to the substrate. There is, however, a minimum particle size which works best, below which a further decrease in particle size is detrimental. The superfine particles obtained during szego milling likely went through hornification and did not produce the greatest yield of products. Overall, physical pretreatment methods increase hydrolysis results by increasing surface area and breaking intermolecular bonds within lignocellulose to allow greater access to glucan and xylan. The increased accessibility allows the enzymes to act efficiently. The enzymes can then work synergistically to produce a greater yield of products.

7.3 Recommendations

- To fully assess the benefits of szego milling and mercerization + disk refining, a cost-benefit analysis should be performed based on a large scale process design.

- Performing the solid-liquid separation at 12 hours instead of 24 hours would be beneficial. This would likely decrease the time for the hydrolysis overall and would lead to cost and energy savings at a commercial level of ethanol production.

- Identification of unknown would allow for complete mass balance, and facilitate a more targeted strategy for inhibitor removal

- Perform multivariate analysis of the enzymatic hydrolysis to account for the various enzymes used and the properties of the reaction system

- Improve upon the disc refining pretreatment by exploring variations in the disc refiner plates and operating conditions

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APPENDICES

Appendix A: Experimental Methods

A1. Biomass Characterization Protocol

This protocol was adapted from NREL`s "Determination of Structural Carbohydrates and Lignin in Biomass" protocol.

Preparing the samples for analysis and hydrolysis:

- Biomass to be characterized should be dried at 45°C in a convection drying oven for a minimum of 24 hours. The DM of the samples should then be above 90% before proceeding.
- The dried samples are then processed through a knife mill. Resulting particles are placed in a sieve shaking with sieve
- After sieving the samples are ready for analysis and the DM of each sample should be taken, e.g., using a Mettler Toledo MJ33 Infrared Moisture Analyzer.
- Place a filtering crucible for each sample in a 575°C muffle furnace for a minimum of 4 hours.
- Place the filtering crucibles in a desiccator and wait at least 15 minutes before closing the lid. Let the crucibles remaining in the desiccator for at least one hour, record this time. Weight the crucibles to the nearest 0.1mg and record weight.
- Place the crucibles back in the muffle furnace and ash to constant weight. Constant weight is defined as less than a 0.3mg change in the weight within one hour of reheating the crucible.
- Weigh 300.0mg +/- 10.0mg of each sample and place in a pressure tube. Each sample should be analyzed in duplicate.
- Add 3.00mL +/- 0.01mL of 72% sulphuric acid to each pressure tube. Using a Teflon stir rod, mix each sample for at least one minute.
- Place the pressure tubes in a water bath at 30°C for 60+/- 5 minutes. Stir the samples every five to ten minutes during the acid hydrolysis.
- After the hydrolysis, remove samples from the water bath and add 84mL +/-0.04mL deionized water to each pressure tube. Tightly cap the pressure tubes and invert to mix the samples.
- A sugar recovery standard (SRS) should be prepared which contains known concentrations of D-(+)glucose, D-(+)xylose, D-(+)galactose, -L(+)arabinose, and D-(+)mannose. Weigh out the required amounts of each sugar, to the nearest 0.1 mg, and add 10.0 mL deionized water. Add 348 μL of 72% sulfuric acid. Transfer the SRS to a pressure tube and cap tightly.
- Place the tubes in an autoclave safe rack, and place the rack in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121°C. After completion of the autoclave cycle, allow the hydrolyzate to slowly cool to near room temperature before removing the caps

Analysis of acid insoluble lignin:

- Vacuum filter the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles, using a vacuum pump. Capture the filtrate in a filtering flask.
- Transfer an aliquot, approximately 50 mL, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl groups if necessary.

Acid soluble lignin determination must be done within six hours of hydrolysis. If the hydrolysis liquor must be stored, it should be stored in a refrigerator for a maximum of two weeks. It is important to collect the liquor aliquot before proceeding to step

- Use deionized water to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible. Rinse the solids with a minimum of 50 mL fresh deionized water. Hot deionized water may be used in place of room temperature water to decrease the filtration time.
- Dry the crucible and acid insoluble residue at 105 +/- 3 °C until a constant weight is achieved, usually a minimum of four hours.
- Remove the samples from the oven and cool in a desiccator. Record the weight of the crucible and dry residue to the nearest 0.1 mg.
- Place the crucibles and residue in the muffle furnace at 575 +/- 25 °C for 24 +/- 6 hours.
- Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight. Place the crucibles back in the furnace and ash to a constant weight.

Analysis of acid soluble lignin:

- On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid.
- Use the hydrolysis liquor aliquot obtained after filtration to measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer; use 240nm for oat hulls. Dilute the sample with deionized water as necessary to bring the absorbance into the range of 0.7 – 1.0, recording the dilution. Record the absorbance to three decimal places. Reproducibility should be + 0.05 absorbance units. Analyze each sample in duplicate, at minimum. (This step must be done within six hours of hydrolysis.)

Analysis of structural carbohydrates:

- Prepare a series of calibration standards containing the compounds that are to be quantified, using D-(+)glucose, D-(+)xylose, D-(+)galactose, -L(+)arabinose, and D-(+)mannose standards. Use a four point calibration in a range of 0.1 to 4.0mg/mL.
- Prepare an independent calibration verification standard (CVS) for each set of calibration standards. Use reagents from a source or lot other than that used in preparing the calibration standards. Prepare the CVS at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.
- Filter the standards through 0.2µm filters into autosampler vials, sealed and labelled.
- Using the hydrolysis liquor obtained after filtration, transfer an approximately 20mL aliquot of liquor to a 50 mL Erlenmeyer flask.
- 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.)

- 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. Prepare each sample in duplicate, reserving one of the duplicates for analysis later if necessary. 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.
- Analyze the standards and samples by HPLC using a Biorad Aminex HPX-87P column equipped with the appropriate guard column.
  HPLC conditions:
  Injection volume: 20μL
  Mobile phase: HPLC grade water, 0.2 μm filtered and degassed
  Flow rate: 0.6 mL / minute
  Column temperature: 80 - 85°C
  Detector temperature: as close to column temperature as possible
  Detector: refractive index
  Run time: 35 minutes
- 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.
- 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 degradation 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.

Analysis of acetate content:

- Prepare 0.005 M (0.01 N) sulfuric acid for use as a HPLC mobile phase. In a 2L volumetric flask, add 2.00 mL of standardized 10N sulfuric acid and bring to volume with HPLC grade water. Filter through a 0.2 μm filter and degas before use.
- Prepare a series of calibration standards containing the compounds that are to be quantified; acetic acid and formic acid. A range of 0.02 to 0.5 mg/mL is suggested. An evenly spaced four point calibration is suggested. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.
- Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using components obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each compound contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.
- Prepare the sample for HPLC analysis by passing a small aliquot of the liquor collected in filtration through a 0.2 μm filter into an autosampler vial. Seal and label the vial. If it is suspected that the sample concentrations may exceed the calibration range, dilute the samples as needed, recording the dilution. The concentrations should be corrected for dilution after running.

- Analyze the calibration standards, CVS, and samples by HPLC using a Biorad Aminex HPX-87H column equipped with the appropriate guard column.

  HPLC conditions:
  Sample volume: 50 µL
  Mobile phase: 0.005 M sulfuric acid, 0.2 µm filtered and degassed
  Flow rate: 0.6 mL / minute
  Column temperature: 55 -65°C
  Detector temperature: as close to column temperature as possible
  Detector: refractive index
  Run time: 50 minutes

Calculations:

- Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content

$$ODW = \frac{Total\ solids\ \%\ x\ Weight_{air\ dry\ sample}}{100}$$

- Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL)

$$\%\ AIR = \frac{Weight_{crucible\ plus\ AIR} - Weight_{crucible}}{ODW_{sample}} \times 100$$

$$\%\ AIL = \frac{(Weight_{crucible\ plus\ AIR} - Weight_{crucible}) + (Weight_{crucible\ plus\ ash} - Weight_{crucible})}{ODW_{sample}} \times 100$$

- Calculate the amount of acid soluble lignin present using calculation below

$$\%\ ASL = \frac{UVabs \times Volume_{filtrate} \times Dilution}{\varepsilon \times ODW_{sample}} \times 100$$

  UVabs = average UV-Vis absorbance for the sample at 240nm
  $Volume_{hydrolysis\ liquor}$ = volume of filtrate, 87 mL
  Dilution = ($Volume_{sample}$ + Volume $_{diluting\ solvent}$) $Volume_{sample}$
  ε = Absorptivity of biomass at specific wavelength, 240nm

- Calculate the total amount of lignin

  $\%\ Lignin = \%ASL + \%AIL$

- 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.

- For the sugar recovery standards (SRS), calculate the amount of each component sugar recovered after dilute acid hydrolysis, accounting for any dilution made prior to HPLC analysis. Average any replicate ($\%R_{sugar}$) values obtained for each individual sugar and report

$$R\ \%_{\ sugar} = \frac{\text{concentration detected by HPLC, mg/mL}}{\text{known concentration of sugar before hydrolysis, mg/mL}} \times 100$$

- Use the percent hydrolyzed sugar recovery values calculated in step 11.8 to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$), accounting for any dilution made prior to HPLC analysis.

$$C_x = \frac{C_{HPLC} \times \text{factor dilution}}{\%R_{\ ave\ sugar x}/100}$$

Where: C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL.
$\%\ R_{ave.\ sugar}$ = average recovery of a specific SRS component.
$C_x = C_{cor.\ sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis

- Calculate the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 for C-5 sugars (xylose and arabinose) and a correction of 0.90 for C-6 sugars (glucose, galactose, and mannose)

$$C_{anhydro} = C_{corr} \times \text{Anhydro correction}$$

- Calculate the percentage of each sugar

$$\%\ Sugar = \frac{C_{anhydro} \times V_{filtrate} \times 1g/1000\ mg}{ODW} \times 100$$

Where: $V_{filtrate}$ = volume of filtrate, 87.00 mL.

- Calculate the acetate percentage

$$\%\ Acetate = \frac{C_{AA,HPLC} \times \text{Volume}_{\ filtrate} \times \text{Conversion factor}}{ODW_{sample}} \times 100$$

Where: $C_{AA,HPLC}$ = concentation in mg/mL of acetic acid as determined by HPLC
$\text{Volume}_{hydrolysis\ liquor}$ = volume of filtrate, 87 mL
Conversion factor = 0.683, the conversion from acetic acid to acetate in biomass

A2. HPLC Analysis

HPLC setup

A Waters HPLC system equipped with a Waters 410 Differential Refractometer, a Waters 717plus Autosampler, a Waters 510 HPLC Pump and a BioRad Column heater was used for all sample analysis. The flow rate was set at 0.6ml/min and the temperature at 85°C. A BioRad Aminex 87-P column was used with a guard column as well. HPLC grade water was used as the mobile phase. Each sample was run for 20 minutes using an injection volume of 0.2mL. The Waters Millenium[32] software was used to analyze the data.

Sample Preparation

The frozen samples collected at various time points during hydrolysis as well as the filtrate were allowed to thaw after remaining in the freezer overnight. The samples were then centrifuged using a Hettich Universal/K2S centrifuge at 3000U/min for 15 minutes. The supernatant was collected and the pH was increased to 5 using calcium carbonate and pH paper to test. The pH adjusted supernatant was then filtered through a 0.2μm syringe filter into an HPLC vial. At least 500μL of the hydrolyzate was needed for analysis.

Standard Preparation

Standards were prepared for quantification of sugars. Sugars obtained from Sigma were used: D-(+)-Glucose, minimum 99.5%, D-(+)-Xylose, minimum 99% and D-(+)-Cellobiose, minimum 98%. The calibration curves for glucose were prepared in a range of 5mg/mL to 80mg/mL. The xylose calibration curves were prepared in a range of 1-20mg/mL and cellobiose 1-5mg/mL. The retention times for the three sugars are as follows: cellobiose~10 minutes, glucose ~12minutes and xylose ~13minutes. These known retention times were used to identify the peaks in a sample chromatograph. A calibration curve was used to correlate the peak height of the unknown sample concentration with the known concentration corresponding to that same peak height. A fresh set of glucose, xylose and cellobiose standards were run with each sample set. Figure A1- A3 are examples of calibration curves generated from HPLC analysis of standards.

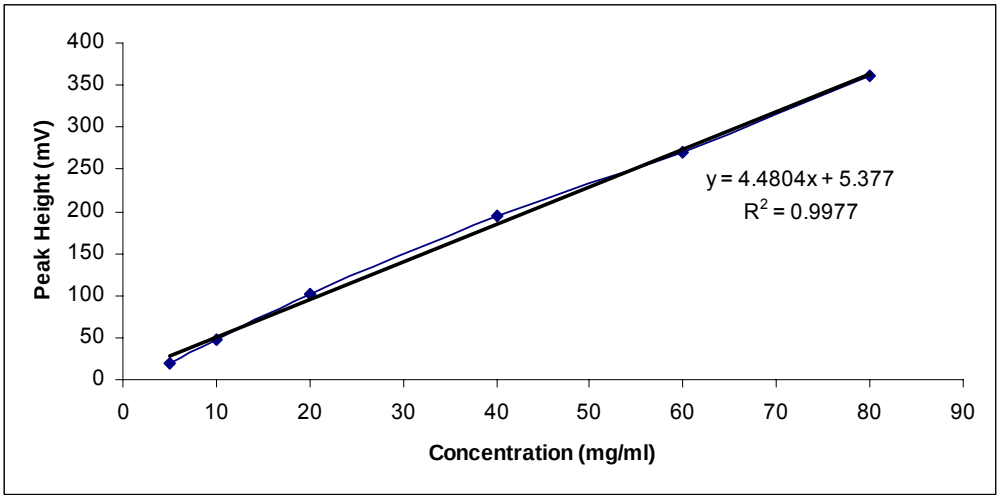


Figure A1: Glucose standard curve

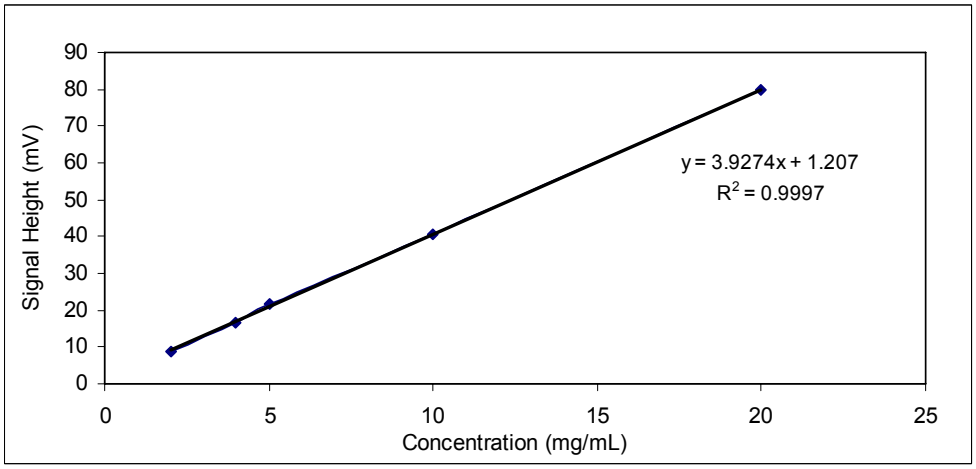


Figure A2: Xylose standard curve

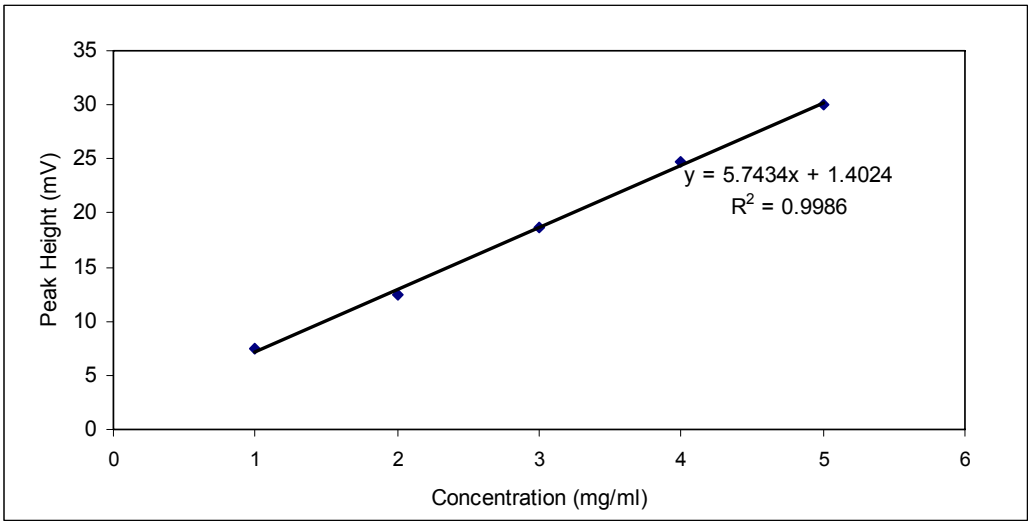


Figure A3: Cellobiose standard curve

A3. Percent Yield Calculations

Using NREL formulas to calculate the concentration of glucose:

$$\% \text{ yield of glucose} = \frac{[\text{glucose}] * \text{Volume}}{1.11 * [\text{glucan}]}$$

Where the constant 1.1 accounts for the loss of water during hydrolysis of cellulose.

$$\% \text{ yield of xylose} = \frac{[\text{xylose}] * \text{Volume}}{1.13 * [\text{xylan}]}$$

Where the constant 1.13 accounts for water loss during hydrolysis of xylan to xylose monomers.

Appendix B: Results

B1. Hydrolysis without solid-liquid separation

Coffee grinder

Shaking incubator trials

Replicate #1

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield (%)
2	3.6	2.8	125	450.0	350	6.0	4.6
4	7.2	5.2	125	900.0	650	12.0	8.5
6	12.3	8.4	125	1537.5	650	20.6	8.5
8	16.6	13.7	125	2075.0	1712.5	27.7	22.5
10	22.9	17.8	125	2862.5	2225	38.3	29.2
12	29.5	23.9	125	3687.5	2987.5	49.3	39.2
24	30.3	26.2	125	3787.5	3275	50.6	43.0
48	37.5	32.5	125	4687.5	4062.5	62.7	53.3
72	42.9	38.4	125	5362.5	4800	71.7	63.0

Replicate #2

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield (%)
2	4.2	3.5	125	525	437.5	7.0	5.7
4	8.9	6.8	125	1112.5	850	14.9	11.2
6	14.6	9.8	125	1825	1225	24.4	16.1
8	19.5	14.6	125	2437.5	1825	32.6	24.0
10	24.3	19.3	125	3037.5	2412.5	40.6	31.7
12	32.5	24.5	125	4062.5	3062.5	54.3	40.2
24	33.5	25.4	125	4187.5	3175	56.0	41.7
48	38.6	33.9	125	4825	4237.5	64.5	55.6
72	40.7	41.1	125	5087.5	5137.5	68.0	67.5

Replicate #3

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield(%)
2	5.1	3.9	125	637.5	487.5	8.5	6.4
4	9	7	125	1125	875	15.0	11.5
6	13.6	10.1	125	1700	1262.5	22.7	16.6
8	15.7	15.5	125	1962.5	1937.5	26.2	25.4
10	20.6	20.1	125	2575	2512.5	34.4	33.0
12	30.5	24.3	125	3812.5	3037.5	51.0	39.9
24	32.8	26.5	125	4100	3312.5	54.8	43.5
48	40.2	35.8	125	5025	4475	67.2	58.8
72	44.5	42	125	5562.5	5250	74.4	68.9

Szego mill

Shaking incubator trials

Raw Oat Hulls

Replicate #1

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield (%)
2	4.3	2.1	125	537.5	262.5	7.5	4.0
4	6.3	3.4	125	787.5	425	11.1	6.4
6	8.9	6.3	125	1112.5	787.5	15.6	11.9
8	12.1	8.9	125	1512.5	1112.5	21.2	16.9
10	16.8	11.4	125	2100	1425	29.5	21.6
12	24.7	16.7	125	3087.5	2087.5	43.3	31.6
24	28.4	22.7	125	3550	2837.5	49.8	43.0
48	34.6	31.4	125	4325	3925	60.7	59.5
72	43.1	39.3	125	5387.5	4912.5	75.6	74.4

Replicate #2

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield (%)
2	4.6	3.4	125	575	425	8.1	6.4
4	5.9	5.1	125	737.5	637.5	10.3	9.7
6	10.1	6.8	125	1262.5	850	17.7	12.9
8	13.5	10.1	125	1687.5	1262.5	23.7	19.1
10	17.8	12.8	125	2225	1600	31.2	24.2
12	24.5	15.6	125	3062.5	1950	43.0	29.5
24	29.5	22.4	125	3687.5	2800	51.7	42.4
48	36.6	28.9	125	4575	3612.5	64.2	54.7
72	41.6	36.4	125	5200	4550	73.0	68.9

Replicate #3

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose yield(%)
2	5.1	2.9	125	637.5	362.5	8.9	5.5
4	6.3	4.8	125	787.5	600	11.1	9.1
6	10.3	7.3	125	1287.5	912.5	18.1	13.8
8	14.9	11.7	125	1862.5	1462.5	26.1	22.2
10	18.3	13.7	125	2287.5	1712.5	32.1	26.0
12	26.7	17.4	125	3337.5	2175	46.8	33.0
24	30.2	24.8	125	3775	3100	53.0	47.0
48	36.9	30.2	125	4612.5	3775	64.7	57.2
72	42.1	37.7	125	5262.5	4712.5	73.8	71.4

Processed oat hulls

Replicate #1

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose yield(%)
2	5.8	2.3	125	725	287.5	9.7	4.6
4	6.7	3.5	125	837.5	437.5	11.2	7.0
6	9.3	7.9	125	1162.5	987.5	15.5	15.8
8	12.8	12.4	125	1600	1550	21.4	24.8
10	16.3	15.7	125	2037.5	1962.5	27.2	31.3
12	20.1	19.2	125	2512.5	2400	33.6	38.3
24	26.9	24.3	125	3362.5	3037.5	44.9	48.5
48	33.5	30.8	125	4187.5	3850	56.0	61.5
72	45.1	36.3	125	5637.5	4537.5	75.4	72.5

Replicate #2

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield (%)
2	4.3	3.1	125	537.5	387.5	7.2	6.2
4	7.9	4.2	125	987.5	525	13.2	8.4
6	10.2	6.9	125	1275	862.5	17.0	13.8
8	13.7	11.5	125	1712.5	1437.5	22.9	23.0
10	17.3	16.8	125	2162.5	2100	28.9	33.5
12	22.2	20.3	125	2775	2537.5	37.1	40.5
24	27.3	26.1	125	3412.5	3262.5	45.6	52.1
48	36.3	32.3	125	4537.5	4037.5	60.7	64.5
72	46.1	37.2	125	5762.5	4650	77.0	74.3

Replicate #3

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose Yield (%)
2	4.1	2.8	125	512.5	350	6.9	5.6
4	8.9	4.1	125	1112.5	512.5	14.9	8.2
6	10.6	7.3	125	1325	912.5	17.7	14.6
8	13.8	13.4	125	1725	1675	23.1	26.8
10	18.3	17.8	125	2287.5	2225	30.6	35.5
12	21.4	18.9	125	2675	2362.5	35.8	37.7
24	26.7	25.5	125	3337.5	3187.5	44.6	50.9
48	35.7	29.3	125	4462.5	3662.5	59.6	58.5
72	44.3	35.9	125	5537.5	4487.5	74.0	71.7

Disc refiner

Shaking incubator trials

Replicate #1

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose yield (%)
2	1.32	1.44	125	165.7	179.7	2.6	2.7
4	2.26	2.02	125	282.4	252.3	4.4	3.8
6	5.67	2.99	125	708.6	373.5	11.0	5.6
8	8.35	3.19	125	1044.1	398.3	16.2	6.0
10	10.21	4.43	125	1276.8	554.1	19.9	8.3
12	12.43	5.88	125	1553.1	734.5	24.1	11.0
24	14.57	7.36	125	1820.9	919.6	28.3	13.8
48	15.99	11.99	125	1998.2	1498.8	31.1	22.5
72	17.24	13.55	125	2154.4	1693.6	33.5	25.4

Replicate #2

Time (h)	Concentration of glucose (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose yield (%)
2	1.9	1.4	125	234.1	179.7	2.7	2.7
4	2.5	2.2	125	307.5	276.3	4.1	4.1
6	5.1	3.2	125	637.5	403.8	6.0	6.1
8	7.2	3.5	125	904.4	436.9	6.5	6.5
10	9.9	5.0	125	1248.5	625.3	9.4	9.4
12	12.0	6.0	125	1499.7	751.6	11.3	11.3
24	12.3	8.0	125	1543.2	1001.5	15.0	15.0
48	13.8	10.0	125	1729.7	1248.5	18.7	18.7
72	16.0	11.9	125	1998.5	1497.2	22.4	22.4

Replicate #3

Time (h)	Concentration (mg/ml)	Concentration of xylose (mg/ml)	volume (ml)	total glucose (mg)	total xylose (mg)	Glucose Yield (%)	Xylose yield (%)
2	2.7	1.2	125	345.3	156.1	5.4	2.3
4	3.5	2.6	125	437.3	322.3	6.88	4.8
6	6.2	3.9	125	779.9	498.7	12.1	7.5
8	8.1	4.1	125	1018.6	516.2	15.8	7.7
10	10.1	5.5	125	1263.2	693.5	19.6	10.4
12	11.0	6.6	125	1375.5	822.9	21.4	12.3
24	12.9	7.8	125	1624.0	979.3	25.2	14.7
48	14.2	10.3	125	1776.6	1292.8	27.6	19.4
72	16.2	12.5	125	2029.0	1567.4	31.5	23.5

Mercerization

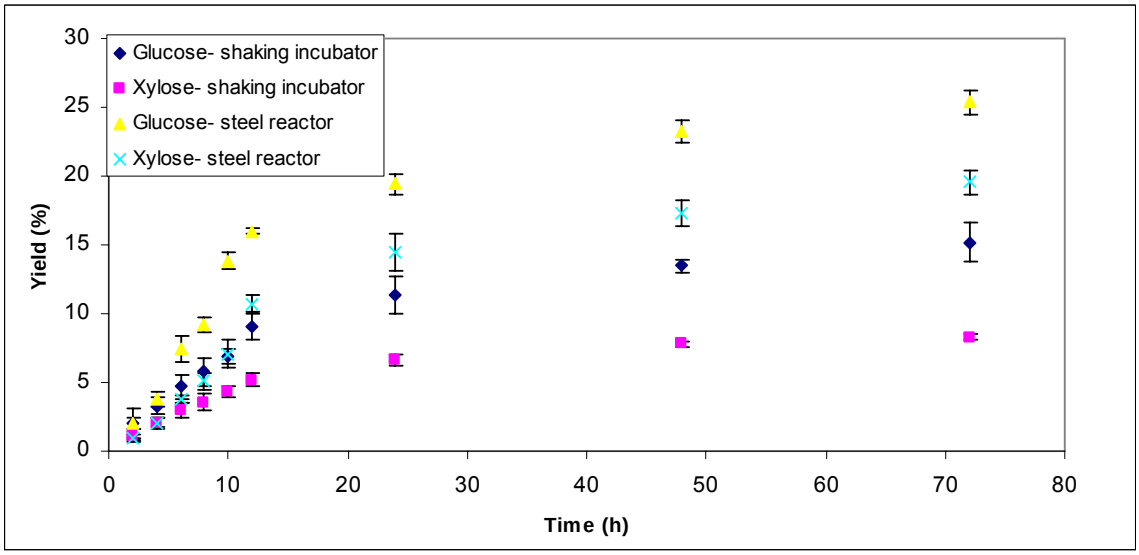
shaking incubator trials

2 hours	Sodium hydroxide (M)	Glucose yield (%)	Xylose yield (%)	**1 hour**	Sodium hydroxide (M)	Glucose yield (%)	Xylose yield (%)
Raw	0.125	42.2 40.1 44.9 **42.4** ***1.970***	33.9 30.2 35.7 **33.3** ***2.27***	**Raw**	0.125	34.2 31.4 27.0 **30.9** ***2.98***	15.5 17.5 21.2 **18.1** ***2.37***
	0.25	50.1 53.9 51.4 **51.8** ***1.56***	36.2 39.8 34.2 **36.8** ***2.32***		0.25	36.4 39.5 40.0 **38.6** ***1.61***	25.9 30.3 33.2 **29.8** ***3.66***
	0.5	61.2 60.4 60.0 **60.5** ***0.52***	48.2 50.2 55.0 **51.2** ***2.84***		0.5	45.1 52.6 46.9 **48.2** ***3.17***	34.7 39.5 35.9 **36.7** ***2.03***
	1	71.1 70.1 67.3 **69.5** ***1.59***	64.2 64.3 69.1 **65.9** ***2.28***		1	55.5 57.5 60.0 **57.7** ***1.82***	43.4 48.3 47.7 **46.5** ***2.21***
Processed	0.125	45.3 46.1 42.1 **44.5** ***1.71***	31.2 34.6 36.0 **33.9** ***1.99***	**Processed**	0.125	32.8 34.8 35.4 **34.3** ***1.12***	20.2 18.9 22.7 **20.6** ***1.58***
	0.25	55.6 52.8 57.4 **55.3** ***1.92***	36.8 33.6 38.1 **36.2** ***1.87***		0.25	40.7 42.7 37.9 **40.4** ***1.96***	25.4 24.5 27.7 **25.8** ***1.34***
	0.5	63.7 64.8 60.3 **62.9** ***1.88***	54.7 51.2 55.4 **53.8** ***1.81***		0.5	46.7 48.8 50.3 **48.6** ***1.47***	33.9 36.2 41.2 **37.1** ***3.07***
	1	72.9 75.8 71.2 **73.3** ***1.89***	68.0 70.4 65.9 **68.1** ***1.86***		1	55.4 59.8 52.8 **56.0** ***2.91***	50.3 51.1 45.3 **48.9** ***2.57***

Mean ***standard deviation***

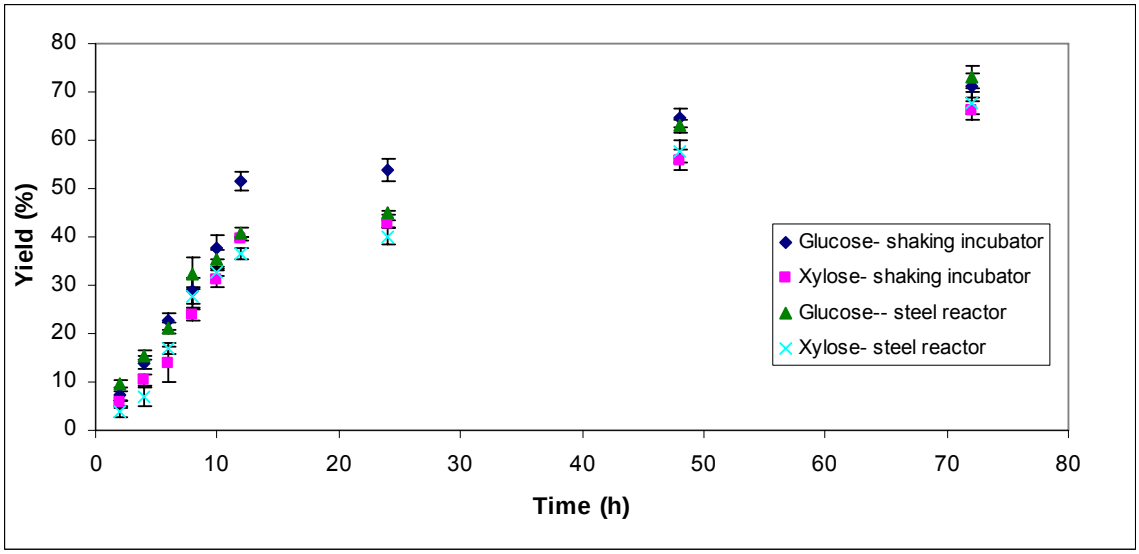
B2: Comparison of trials

No pretreatments- raw oat hulls, shaking incubator vs. steel reactor



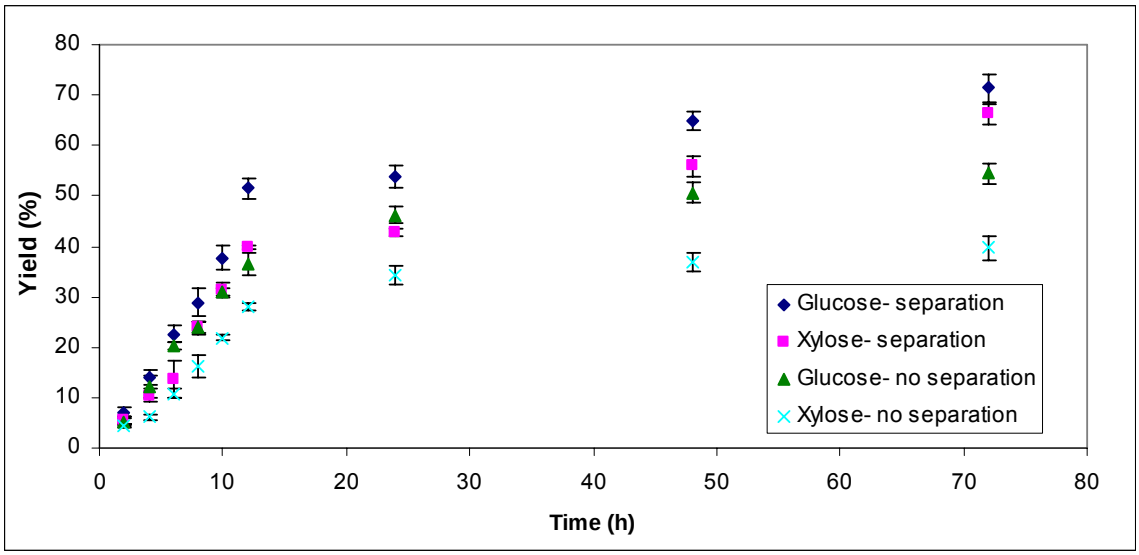
There was a statistical significance observed for hydrolysis of raw and processed oat hulls without pretreatment, when the trials were scaled up. Both glucose and xylose increased significantly during steel reactor trials. This may be a result of increased efficiency of mixing in the steel reactor.

Coffee grinder: shaking incubator vs. steel reactor



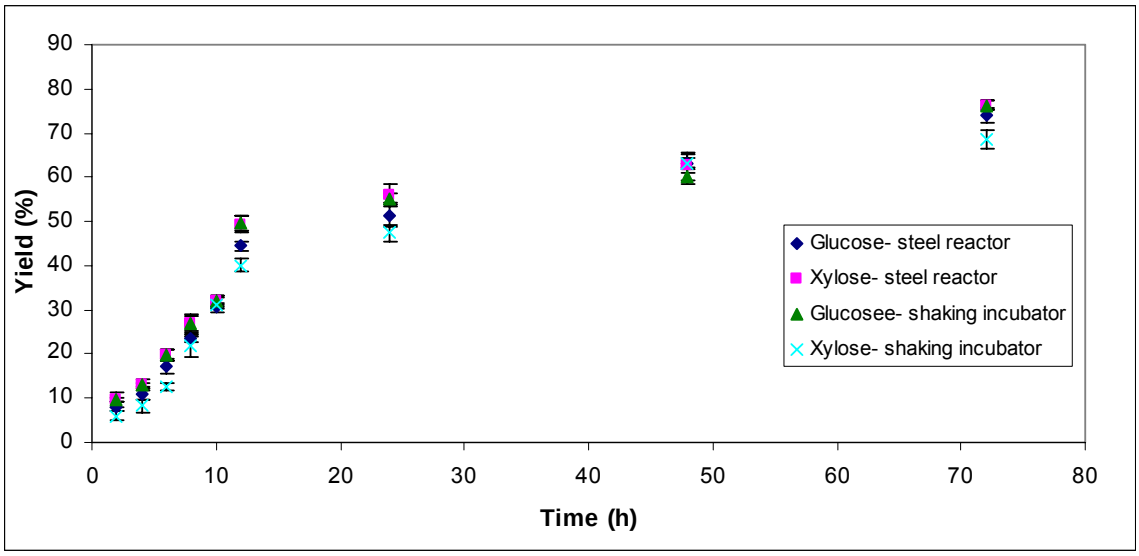
The results of hydrolysis using the coffee grinder were not statistically different for the shaking incubator and steel reactor trials. For both scales, glucose and xylose follow similar trends. The difference at 12 and 24 hours may be due to the increased accumulation of inhibitors in the steel reactor, which led to lowered yields of glucose. However, by 72 hours the yields for both glucose and xylose are comparable.

Coffee grinder: shaking incubator; solid-liquid separation vs. no separation



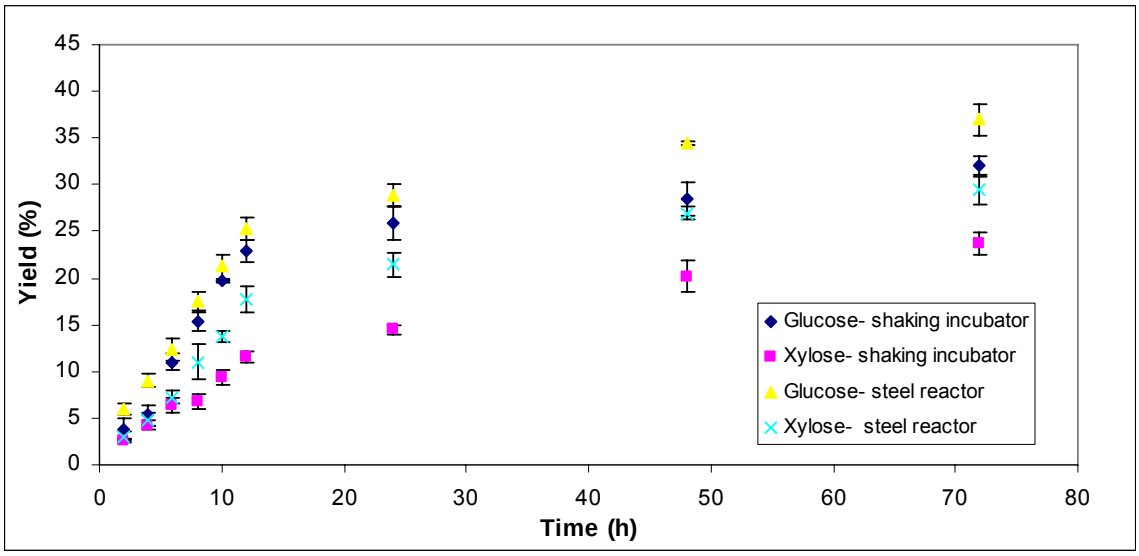
There is a definite statistical significance of having the solid-liquid separation. Both glucose and xylose yields increase when the separation is performed.

Szego milled: raw. shaking incubator vs. steel reactor



There is no statistical difference between the small and large scale trials, in terms of glucose and xylose yields. There is a difference in glucose and xylose yields at 12 and 24 hours. The decreased yield of glucose at these time points may be due to increasing accumulation of cellobiose and other oligosaccharide inhibitors which decrease the effectiveness of the cellulase system. The slight increase in xylose at these points is opposite to the decreased glucose at this point and may be due to increased accessibility of the xylanase at these points.

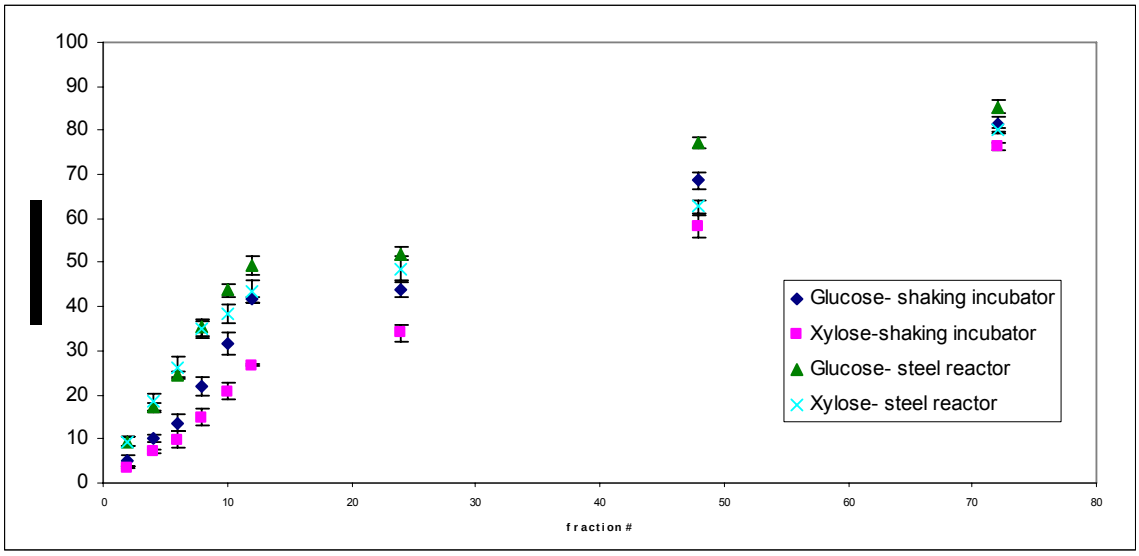
Disc refined oat hulls: shaking incubator vs. steel reactor



There is a difference between both scales, with the steel reactor giving higher yields of conversion overall. After the solid-liquid separation there is clear differences in the yields of glucose and xylose between the steel reactor and shaking incubator. However, before the separation, the difference is still seen but is less apparent. Since the overall trend favours the steel reactor, it is likely that the steel reactor gives higher yields of hydrolysis than the shaking incubator.

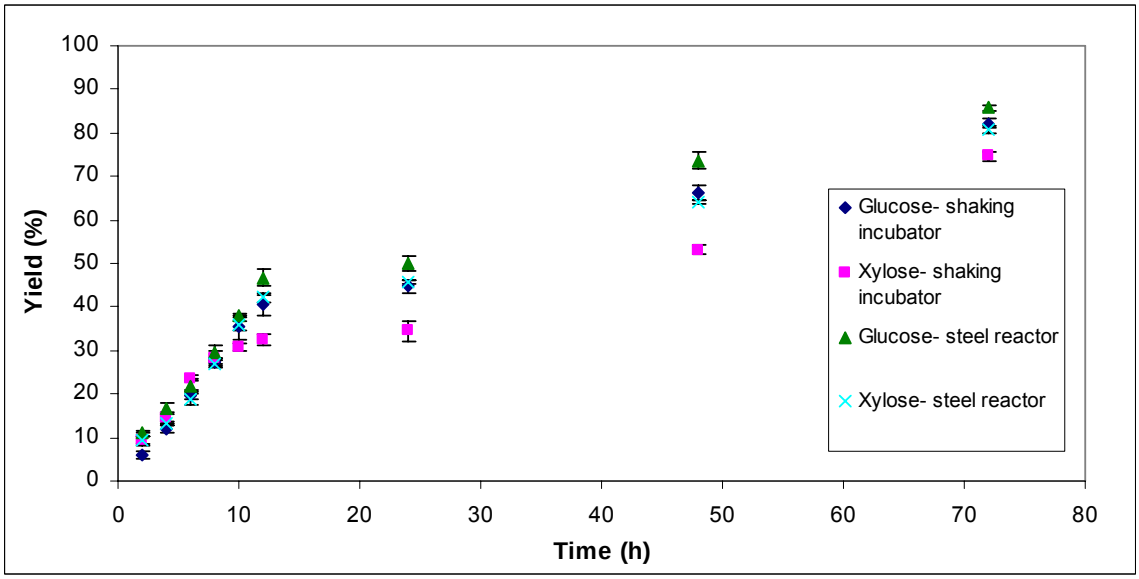
Disc refined and mercerized: steel reactor vs. shaking incubator

From this graph it is evident that there is a statistical difference between trials run in the shaking incubator and steel reactor. While those differences do not appear until after the solid-liquid separation, at which point the inhibitors are removed and glucose and xylose concentrations increase. The steel reactor may have produced increased yields after the separation because inhibitors were removed and efficient mixing led to increased enzyme accessibility.



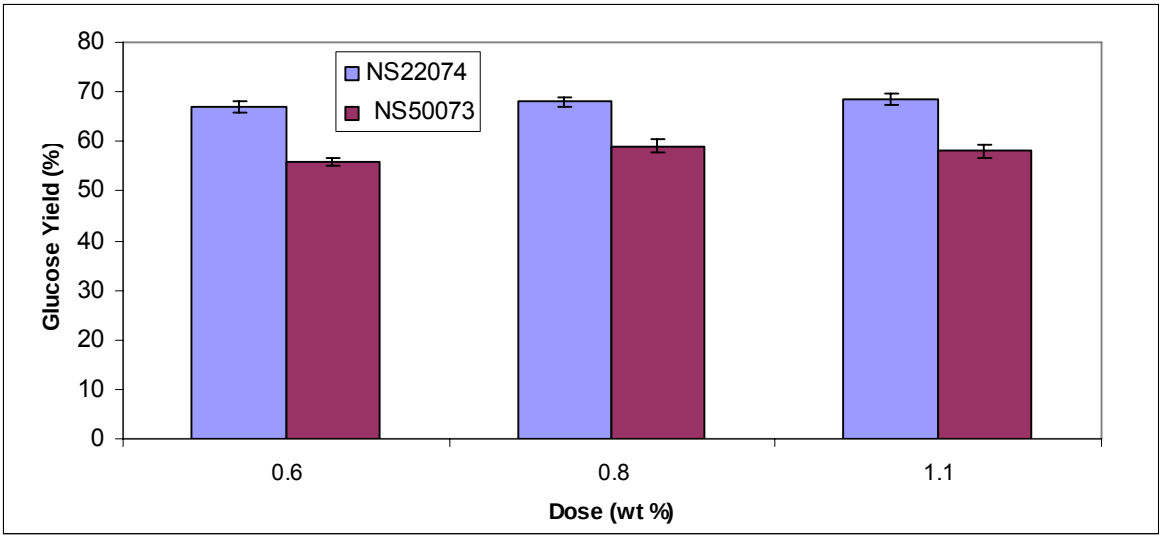
Mercerized and disc refined: shaking incubator vs. steel reactor

With these trials they is also a trend in favour of the steel reactor for hydrolysis, while these observations can be made after the solid-liquid separation only. Before the separation, there is no statistical difference. However, after the separation the yields of glucose and xylose both increase in the steel reactor. Because this is a scale-up the effect of inhibition increase, and when the separation is performed the inhibitors are removed and the increased mixing in the steel reactor allows for efficient enzyme accessibility.



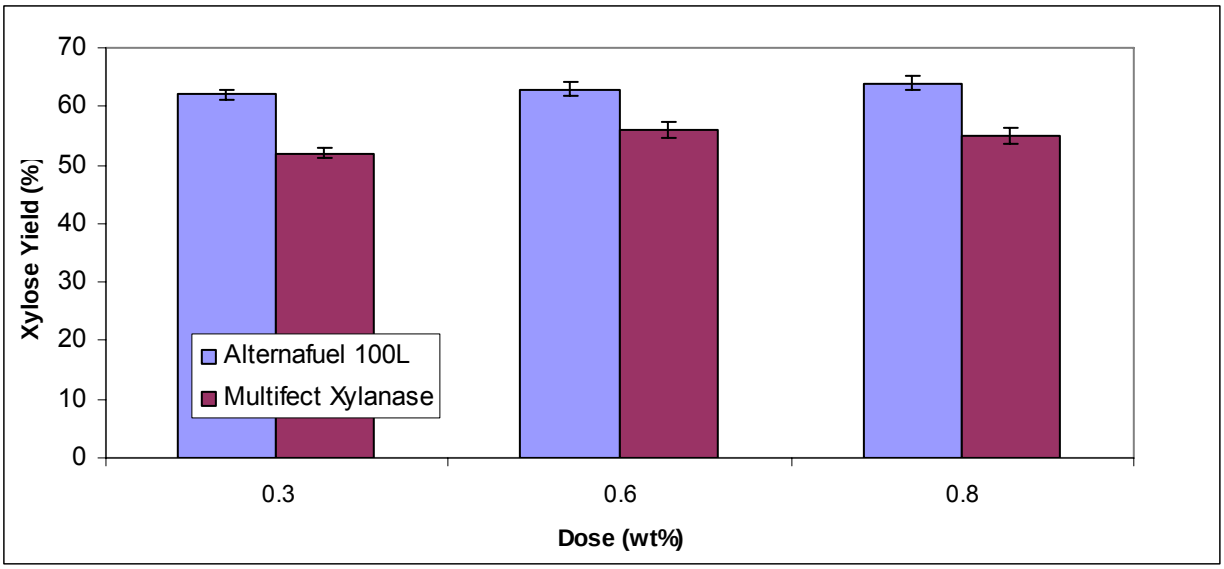
B3: Single enzyme hydrolysis

Raw coffee ground oat hulls were hydrolyzed in the shaking incubator. Hydrolysis was run with NS22074 alone and NS50073 alone, without the addition of xylanases or cellobiases. The graph below shows that across the cellulase doses, NS22074 gave the highest yields of glucose. Since NS22074 gave a higher yield of glucose than NS50073; NS22074 is therefore, the better cellulase. Since previous results showed that the yield of glucose increases when Alternafuel 100L is added to NS2074, it can be stated that these two enzymes work together synergistically.



Final glucose yields (72 hours) obtained from comparison of cellulases across varying enzyme doses

Hydrolysis was also run using Alternafuel 100L and Multifect Xylanase alone, without the addition of cellulases or cellobiases. Alternafuel 100L gave a higher yield of xylose than Multifect Xylanase; therefore, Alternafuel 100L is the best xylanase of the two.



Final xylose yields (72 hours) obtained from comparison of xylanases across varying enzyme doses