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Sodium hydroxide pretreatment of corn stover and subsequent enzymatic hydrolysis: An investigation of yields, kinetic modeling and glucose recovery

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SODIUM HYDROXIDE PRETREATMENT OF CORN STOVER AND
SUBSEQUENT ENZYMATIC HYDROLYSIS:
AN INVESTIGATION OF YIELDS, KINETIC MODELING
AND GLUCOSE RECOVERY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Engineering
at the University of Kentucky

By
Alicia Abadie Modenbach

Lexington, Kentucky

Director: Dr. Sue E. Nokes, Professor of Biosystems and Agricultural Engineering

Lexington, Kentucky

2013

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ABSTRACT OF DISSERTATION

SODIUM HYDROXIDE PRETREATMENT OF CORN STOVER AND SUBSEQUENT ENZYMATIC HYDROLYSIS: AN INVESTIGATION OF YIELDS, KINETIC MODELING AND GLUCOSE RECOVERY

Many aspects associated with conversion of lignocellulose to biofuels and other valuable products have been investigated to develop the most effective processes for biorefineries. The goal of this research was to improve the efficiency and effectiveness of the lignocellulose conversion process by achieving a more basic understanding of pretreatment and enzymatic hydrolysis at high solids, including kinetic modeling and separation and recovery of glucose.

Effects of NaOH pretreatment conditions on saccharide yields from enzymatic hydrolysis were characterized in low- and high-solids systems. Factors associated with pretreatment and hydrolysis were investigated, including duration of pretreatment at different temperatures and NaOH loadings, as well as different solids and enzyme loadings. Under relatively mild pretreatment conditions, corn stover composition was essentially equivalent for all time and temperature combinations; however, components were likely affected by pretreatment, as differences in subsequent cellulose conversions were observed. Flushing the hydrolyzate and reusing the substrate was also studied as a method for inhibitor mitigation while increasing overall glucose yields. Flushing the PCS throughout the hydrolysis reaction eliminated the need to wash the pretreated biomass prior to enzymatic hydrolysis when supplementing with low doses of enzyme, thus reducing the amount of process water required.

The robustness of an established kinetic model was examined for heterogeneous hydrolysis reactions in high-solids systems. Michaelis-Menten kinetics is the traditional approach to modeling enzymatic hydrolysis; however, high-solids reactions violate the main underlying assumption of the equation: that the reaction is homogeneous in nature. The ability to accurately predict product yields from enzymatic hydrolysis in high-solids systems will aid in optimizing the conversion process.

Molecularly-imprinted materials were studied for use in both bulk adsorption and in column chromatography separations. Glucose-imprinted materials selectively adsorbed glucose compared xylose by nearly 4:1. Non-imprinted materials were neither selective in the type of sugar adsorbed, nor were they capable of adsorbing sugar at as high a capacity as the glucose-imprinted materials. Liquid chromatography with imprinted materials was not a suitable means for separating glucose from solution under the conditions investigated; however, many factors impact the effectiveness of such a separation process and warrant further investigation.

KEYWORDS: high-solids loadings; enzymatic hydrolysis; pretreatment; heterogeneous reactions; separation

Alicia A. Modenbach
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July 30, 2013
Date

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July 30, 2013

To my family and friends,
I could not have done this without you.

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CHAPTER 1: INTRODUCTION

In early 2012, the global population surpassed seven billion people which is increasing the demands for food and energy worldwide. Currently, first-generation starch and sugar crops (like corn and sugarcane) are being used to produce the liquid transportation fuel ethanol as a substitute for gasoline. Nearly 40% of the corn produced in the United States is converted to fuel ethanol (United States Department of Agriculture 2013). Sugar and starch are easily fermented into ethanol, and the technology is mature. However using these crops as a renewable feedstock for ethanol pits the fuel supply in direct competition with the food supply. One proposed alternative is the use of lignocellulose as a renewable feedstock to supply energy demands. Lignocellulose is the most abundant and renewable source of carbon on the planet, being the main structural component of plants. Harnessing the energy stored in lignocellulose has been tapped as one solution for meeting the growing energy demands without decreasing the food supply.

Developing second generation feedstocks, (for example agricultural residues like corn stover and wheat straw, or dedicated energy crops, like switchgrass and miscanthus), as an energy supply has many advantages over continued use of fossil resources including improving sustainability and potentially slowing climate change. For instance, use of lignocellulose as an energy source has the potential to reduce greenhouse gas emissions because the plant cycle is a net zero carbon dioxide emitter (Chang 2007). Essentially, carbon released upon combusting fuel derived from lignocellulose is used during the production of lignocellulose, resulting in a carbon cycle on the order of a year in contrast to the carbon cycle of fossil fuels that required millions of years to form. As of 2011, the United States were the largest consumers of energy in the world, consuming 95 quadrillion BTUs. Nearly 30% of all the energy used was imported from other countries (United States Energy Information Administration 2013). Concurrently, fossil reserves are in limited supply and tend to be located in volatile regions of the world, so lignocellulose could potentially provide a more politically sustainable (domestic) source of energy. A localized, domestic energy source could also stimulate rural economic development (Brown 2003).

The major issues associated with the use of lignocellulose as an energy source are the recalcitrant nature of the material (Jorgensen et al. 2007a; Zhao et al. 2012) and the ability to replace the wide range of products manufactured cheaply from fossil resources (Kadam et al. 2008; Menon and Rao 2012). Lignocellulose structure, by design, is difficult to depolymerize, so as to provide the plant protection against attack by microorganisms and other pests. Much research has gone into developing technologies capable of breaking down lignocellulose into its major components for economical use in downstream conversion processes. As more and more petroleum is replaced by renewable energy sources, many commodity chemicals will need to be manufactured from other sources because fossil fuel serves as a basic building block for the commercial chemicals industry. One proposed solution to this problem, and the economical production of biofuel, is the concept of the biorefinery. In the biorefinery concept, every component of the material is exploited, much like in the traditional petroleum refinery. The suite of products manufactured including liquid transportation fuels, commodity chemicals and precursory chemical building blocks would be dictated by the market and selected to extract the greatest value possible out of lignocellulose.

1.1 CONVERSION OF LIGNOCELLULOSE

Several unit operations are required in the conversion of lignocellulose to valuable products, including pretreatment, hydrolysis, fermentation and product recovery (Figure 1.1). Multiple options are available for each of these unit operations, and each has its own advantages and challenges associated with it.

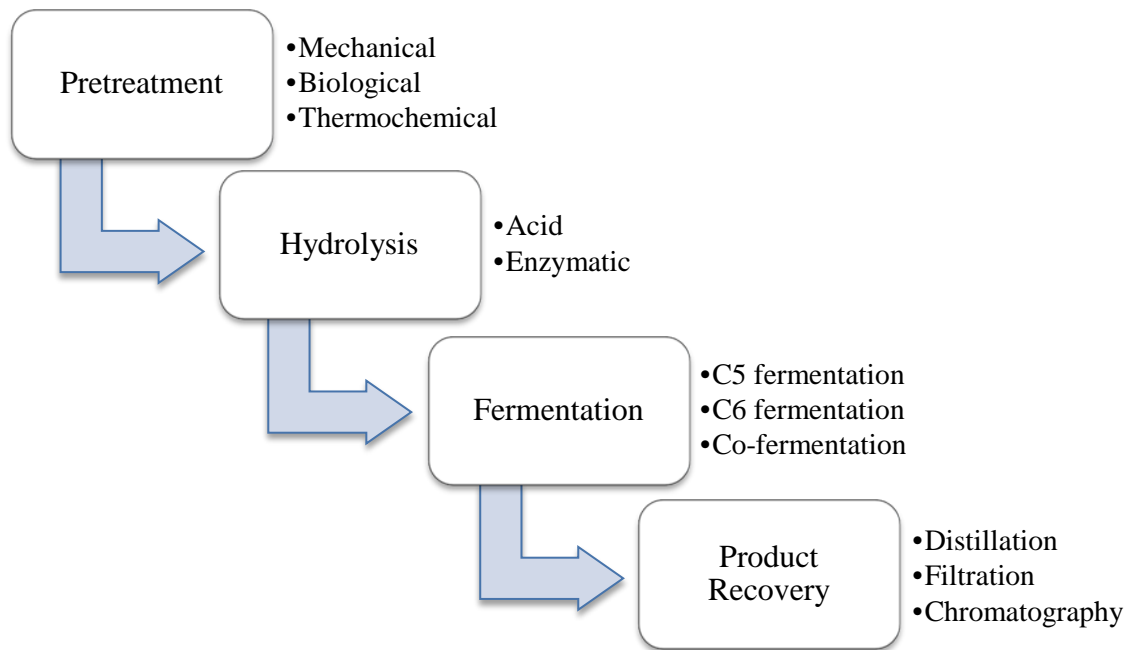


Figure 1.1. Unit operations typical of the lignocellulosic conversion process.

1.1.1 Pretreatment

The goal of pretreatment is to increase the accessibility of the polysaccharides within the lignocellulose to make them more susceptible to hydrolysis. Generally speaking, pretreatments work by separating the lignocellulose into its structural components: cellulose, hemicellulose and lignin. Without pretreatment, the sugar yields from enzymatic hydrolysis are less than 20% of the theoretical yields; however, with pretreatment, the sugar yields are reportedly $\geq 90\%$ of the theoretical sugar yields (Zhang and Lynd 2004). There are several key characteristics necessary for an effective pretreatment (Alvira et al. 2010; Galbe and Zacchi 2007; Jorgensen et al. 2007a). Pretreatments should:

- have a low capital and operating cost compared to the product of interest;
- not be energy intensive;
- work on a wide variety of feedstocks;
- not result in significant monosaccharide degradation or inhibitory compounds;
- not use chemicals toxic to fermentation organisms;

- maximize digestibility of lignocellulose material;
- maximize recovery of valuable by-products like lignin; and
- be scalable to industrial size.

Many different pretreatment methods have been developed, but most processes can fall into one of three categories: mechanical, biological or thermochemical. Some pretreatment methods are briefly discussed here but those included are by no means an exhaustive list. Many reviews are available that provide detailed overviews of the types of pretreatments available (Carvalho et al. 2008; Galbe and Zacchi 2007; Hendriks and Zeeman 2009; Mosier et al. 2005).

Initially, mechanical means, like chipping, milling and grinding, may be used to reduce the size of the particles, essentially increasing the surface area of the lignocellulose (Galbe and Zacchi 2007). The crystallinity of the cellulose may also be affected during these processes, resulting in the reduction of the degree of polymerization (DP) of the cellulose. Hammer mills and ball mills used to reduce particle size have been shown to enhance the digestibility of the lignocellulose in enzymatic hydrolysis (Alvira et al. 2010). However, the energy requirements for these mechanical processes are exceedingly high and may not be economically feasible at larger scales (Hendriks and Zeeman 2009).

Biological pretreatments with lignin-degrading fungi have been receiving renewed interest recently as an environmentally friendly option. White-rot, brown-rot and soft-rot organisms that produce ligninases have been used to remove lignin, exposing the cellulose and hemicellulose fractions of lignocellulose (Alvira et al. 2010; Galbe and Zacchi 2007). This option is not very energy intensive because it can be performed at low temperatures and does not require any toxic chemicals that may be problematic in the other downstream processes. However, the slow rate of this reaction renders this process ineffective at larger scales. The organisms have been shown to consume some of the sugars during the delignification process, resulting in lower sugar yields (Balat et al. 2008; Galbe and Zacchi 2007) than other non-biological pretreatments.

Thermochemical pretreatment methods are numerous, and many have been studied intensively. Some of the more common methods are dilute acid, liquid hot water (LHW), steam explosion, ammonia fiber explosion (AFEX) and alkaline pretreatment.

Dilute acid pretreatment is typically performed by soaking lignocellulose in acid (sulfuric or phosphoric acid) at concentrations usually below 4 wt % at high temperature (140-200°C) for up to 1 hour (Galbe and Zacchi 2007). The acid mainly removes lignin, which then condenses and precipitates, and hydrolyzes the hemicellulose fraction into its respective monosaccharides. (Balat et al. 2008; Hendriks and Zeeman 2009). Even though dilute acid pretreatment separates the hemicellulose from lignocellulose (one of the objectives of this current work), the solubilized sugars cannot always be recovered in a useable form. While this pretreatment is highly effective in making cellulose susceptible to hydrolysis, it is possible to produce compounds inhibitory to fermentative organisms. For instance, xylose can be further degraded into furfural and 5-hydroxymethyl furfural (HMF) (Vertes et al. 2010). The nature of the acids used in this pretreatment require specialized equipment resistant to corrosion, as well as substrate neutralization prior to other downstream processes (Hendriks and Zeeman 2009; Mosier et al. 2005).

Liquid hot water pretreatments also require specialized equipment because elevated pressures (2.4-2.8 MPa) are used to keep the water in a liquid phase at high temperatures (180-230°C). Water under these conditions is acidic (pH 4-7), resulting in the hydrolysis of hemicellulose, and removal of a significant portion of the lignin, much like the dilute acid pretreatment (Allen et al. 2001; Mosier et al. 2005). Neutralization is not required following pretreatment and fewer inhibitors and degradation products form with LHW than dilute acid pretreatment because the pH is not as acidic for the former option. However, the solubilized sugars are relatively dilute because of the high volumes of water typically used (Hendriks and Zeeman 2009; Mosier et al. 2005).

Another pretreatment option is steam explosion, which uses high-temperature steam (220-270°C) to pressurize the reactor (Galbe and Zacchi 2007). The quick change in pressure when the pressure is released from the reactor causes the lignocellulose to explosively expand, disrupting the structure. The slightly acidic nature of the steam, along with the release of acetyl groups from lignocellulose also enhances hydrolysis of hemicellulose (Alvira et al. 2010). Lignin is also partially removed, leaving large pores that make the cellulose accessible to enzymes for subsequent enzymatic hydrolysis of the lignocellulose material (Jorgensen et al. 2007a). While steam production requires energy,

this pretreatment does not produce large streams of process water that later necessitates treatment.

The process of ammonia fiber expansion (AFEX) is similar to that of steam explosion, except the lignocellulose soaks in liquid ammonia in a pressurized reactor prior to rapid decompression. The explosion effectively breaks bonds between the lignin and hemicellulose fractions, allowing the material to expand and expose the cellulose (Jorgensen et al. 2007a). Although very little lignin and hemicellulose are removed, this pretreatment has been shown to be very effective for increasing the digestibility of the material (Alvira et al. 2010). Recycle of the ammonia would be required to make this option economically feasible (Balat et al. 2008).

Alkaline pretreatment using sodium hydroxide or calcium hydroxide causes the lignocellulose to swell, thereby increasing the surface area while reducing the degree of polymerization (DP) and crystallinity of the material (Balat et al. 2008; Galbe and Zacchi 2007; Hendriks and Zeeman 2009). Alkaline pretreatment is not as energy-intensive as some of the other pretreatment options because it can be performed at ambient temperatures and pressures, although longer reaction times may be needed to obtain the same level of digestibility offered by other forms of pretreatment (Jorgensen et al. 2007a). During alkaline pretreatment, very little of the saccharide fractions are solubilized, meaning that nearly 100% of the saccharides can be recovered during subsequent processing steps, which is desirable so full advantage can be taken of all the energy-rich components of lignocellulose. Sodium hydroxide and calcium hydroxide affect lignocellulose in essentially the same manner; however, sodium hydroxide has a higher reaction rate as compared to calcium hydroxide, which may take weeks to sufficiently pretreat lignocellulose instead of hours or days like sodium hydroxide. The lower reaction rate for calcium hydroxide may be due to its instability in water and its tendency to absorb carbon dioxide from the air to form calcium carbonate. For these reasons, sodium hydroxide pretreatment was selected for investigation in this current work.

1.1.2 Hydrolysis

Hydrolysis is the unit operation that depolymerizes the polysaccharide chains of cellulose and hemicellulose into fermentable oligosaccharides and/or monosaccharides. Acid hydrolysis and enzymatic hydrolysis are the two predominant methods for this operation. Acid hydrolysis typically uses dilute (4 wt %) or concentrated (60-90 wt %) sulfuric acid at high temperatures (120-200°C) and pressures (0.1-0.5 MPa) for up to 2 hrs (Kumar et al. 2009b) to break the cellulose chains into glucose. The challenges associated with this method include cost and production of compounds inhibitory to fermentation organisms. Some of the costs of using acid can be mitigated through acid recovery and by-product recovery. Gypsum is produced in large quantities during neutralization of the hydrolyzate with lime at the end of the process (Kumar et al. 2009b), which could be used to make building supplies like wallboard. Additionally, the remaining lignin can be burned for process heat. However, just like with dilute acid pretreatment, the production of inhibitory compounds like furfural, HMF, acetic acid, formic acid is possible (Balat et al. 2008), which impacts sugar recovery and ethanol yields.

Enzymatic hydrolysis uses cellulolytic enzymes produced from microorganisms to catalyze the depolymerization of cellulose into glucose oligomers, dimers, and monomers. This method is often used because enzymes provide a biological alternative to acid hydrolysis. The reaction conditions tend to be milder (pH of 4.8 and temperatures of ~50°C) and are not corrosive (Balat et al. 2008). However, enzyme costs still contribute significantly to the overall cost of lignocellulose conversion, even though extensive research in recent years has reduced their cost (Jorgensen et al. 2007a). Additionally, enzymatic saccharification can be much slower (on the order of days) than acid hydrolysis. Lignin and hemicellulose act as barriers to cellulose chains and hinder cellulase performance. Enzyme recycling and specialized enzyme cocktails can be used to overcome some of these limitations (Jorgensen et al. 2007a). Ultimately, enzymatic hydrolysis was chosen as the hydrolysis method of this current work because of the milder operating conditions and the ability to recover pentoses (since pentoses are retained during sodium hydroxide pretreatment) resulting from the hydrolysis of hemicellulose.

1.1.3 Fermentation and Product Recovery

The sugar-rich hydrolyzate obtained in the previous step can be fermented by microorganisms to produce ethanol. Even though fermentation is a well-established process, several challenges still limit its use for large-scale ethanol production. One of the biggest challenges associated with fermentation is effective use of sugars other than glucose. *Saccharomyces cerevisiae*, *Zymomonas mobilis* and modified *Escherichia coli* are the most common fermentative organisms used and have been studied intensively (Chang 2007; van Zyl et al. 2007), but all still lack the ability to effectively use both hexoses and pentoses for industrial production of ethanol. Additionally, the hydrolyzate obtained from lignocellulose typically does not contain as much sugar (~70 g/L) as compared to sugarcane or starch fermentations (>150 g/L) (Bayrock and Ingledew 2001; Brethauer and Wyman 2010). Subsequently, the alcohol concentrations are lower, making alcohol recovery one of the most expensive and energy-intensive operations of lignocellulose conversion.

This current work does not directly investigate fermentation or product recovery methods and includes only brief comments regarding these unit operations. However, readers are encouraged to refer to more detailed reviews that are available for fermentation (Balat et al. 2008; Brethauer and Wyman 2010; van Zyl et al. 2007), fermentation of sugars derived from hemicellulose (Girio et al. 2010; Saha 2003), and product separation and recovery (Balat et al. 2008; Huang et al. 2008).

This current work investigates several aspects associated with the conversion of lignocellulose, including pretreatment and enzymatic hydrolysis of lignocellulose at high-solids loadings, separation and recovery of purified sugar streams, and application of an existing kinetic model for the hydrolysis of lignocellulosic material. Chapters 2 and 3 provide extensive reviews of the use of high-solids loadings in pretreatment and enzymatic hydrolysis, respectively. Chapter 4 provides further details regarding sodium hydroxide pretreatment, state of the art technology for separating pentoses and hexoses and kinetic models developed for heterogeneous reactions. Chapter 5 outlines the objectives for this current work. Chapter 6 details the experimental work associated with characterization of effects of sodium hydroxide pretreatment conditions on

enzymatic hydrolysis performed at high-solids loadings. Chapter 7 discusses the calibration and validation of a kinetic model for the hydrolysis of lignocellulosic material (a heterogeneous reaction). Chapter 8 details the experimental work associated with separation of pentoses and hexoses using novel materials. Chapter 9 concludes this work with some final thoughts regarding the results of this work, as well as discussing potential future directions for this work.

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CHAPTER 2: THE USE OF HIGH-SOLIDS LOADINGS IN BIOMASS PRETREATMENT – A REVIEW¹

2.1 SUMMARY

The use of high-solids loadings ($\geq 15\%$ solids w/w) in the unit operations of lignocellulose conversion processes potentially offers many advantages over lower solids loadings, including increased sugar and ethanol concentrations and decreased production and capital costs. Since the term lignocellulosic materials refers to a wide range of feedstocks (agricultural and forestry residues, distillery by-products, and dedicated energy crops like grasses), the term “solids loading” here is defined by the amount of dry material that enters the process divided by the total mass of material and water added to the material. The goal of this paper is to provide a consolidated review of studies using a high-solids pretreatment step in the conversion process. Included in this review is a brief discussion of the limitations such as the lack of available water to promote mass transfer, increased substrate viscosity and increased concentration of inhibitors produced affecting pretreatment as well as, descriptions and findings of pretreatment studies performed at high solids, the latest reactor designs developed for pretreatment at bench- and pilot-scales to address some of the limitations, and high-solids pretreatment operations that have been scaled up and incorporated into demonstration facilities.

Keywords: high solids, lignocellulose conversion, pretreatment, pilot scale, high density

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2.2 INTRODUCTION

The production of commodity chemicals, (such as ethanol) from starch or lignocellulose, has a narrow profit margin. Studies utilizing low solids loadings ($\leq 5\%$ solids w/w) are numerous and helpful; however, improved efficiency has prompted new studies using high-solids loadings. Since the term lignocellulosic materials refers to a wide range of feedstocks (agricultural and forestry residues, distillery by-products, and dedicated energy crops like grasses), the term “solids loading” here is defined by the amount of dry material that enters the process divided by the total mass of material and water added to the material. Over the last few years, several studies have begun to investigate the effects of high-solids loadings ($\geq 15\%$ solids w/w) on different unit operations within the process stream (Hodge et al. 2008; Jorgensen et al. 2007b; Kristensen et al. 2009b; Lu et al. 2010; Zhang et al. 2010) as a means of improving the economics.

The main advantage of using high-solids loadings over low and moderate solids loadings is improved efficiency. Because there is a greater amount of biomass available in the reaction, higher sugar concentrations can be produced, which leads to increased ethanol concentrations (Hodge et al. 2008; Roche et al. 2009a). The conversion process is more environmentally friendly, as less water is consumed (Stickel et al. 2009; Um and Hanley 2008) under certain processing conditions. It should be noted that the water absorption capacity is a function of the lignocellulosic material, and significant water can be brought into the process, just through the selection of a particular type of material. However, some conversion processes have been developed to reduce process water and waste water by recovering and recycling liquid streams (Mohagheghi and Schell 2010; Stenberg et al. 1998). Capital and production costs are greatly reduced. Smaller reactors and equipment can be utilized for equivalent sugar and ethanol production. Energy usage for heating, cooling, mixing and ethanol distillation is reduced, which renders the overall conversion process more efficient on an energy basis.

Current technology has allowed the use of up to 30% solids content in the fermentation of starch, whereas only 15-20% solids in lignocellulose conversion has been handled at the pilot plant scale (Jorgensen 2009; Kristensen et al. 2009b). Zhang et al. (2010) estimate that a solids loading of approximately 30% lignocellulose should

translate to an ethanol yield of 5-10% (w/w). This yield is the minimum desired for the distillation process to be economical, as the energy requirement for distillation is significantly reduced for ethanol concentrations above 4% (Larsen et al. 2008). To achieve this minimum ethanol concentration, some studies show that at least 15% solids (dry matter) is required for enzymatic hydrolysis (Jorgensen et al. 2007b; Kristensen et al. 2009a), while others estimate that minimum to be about 20% (Larsen et al. 2008). Although data for high-solids pretreatment and hydrolysis are limited, it has been suggested that the combination of a high-solids pretreatment followed by high-solids hydrolysis has great potential at improving the process economics by increasing sugar and ethanol yields while decreasing capital costs (Hodge et al. 2008; Roche et al. 2009a). However, utilizing high-solids loadings in this conversion process is still relatively new, and more research is required to overcome certain challenges, like high concentrations of inhibitors and equipment mass transfer limitations that are not as apparent at the low and moderate solids loadings.

The goal of this review is to provide a consolidated source of information in regards to the latest advances in pretreatment technologies for high-solids operations. Following a brief discussion of limitations affecting pretreatments performed at high solids, various pretreatment studies performed with moderate and high-solids loadings are detailed and the latest reactor designs that address some of these limitations are discussed. Lastly, pretreatment operations that are known to have been successful at the pilot scale are summarized.

2.3 FACTORS LIMITING HIGH-SOLIDS PRETREATMENTS

Conventional pretreatments developed at lower solids loadings (5-10% solids) have long been shown to facilitate higher conversion of biomass into usable sugars compared to biomass which was not pretreated (Carvalho et al. 2008; Chen et al. 2009; Dadi et al. 2006; Galbe and Zacchi 2007; Schell et al. 1992; Schwald et al. 1989; Wyman et al. 2005a). Some pretreatments, like AFEX, have been developed to require very little water (as low as 10% has been reported) (Wyman et al. 2005b) and have been referred to as “dry” pretreatments. However, as more pretreatment options are investigated with increased solids loadings, several challenges become apparent. For example, as the

concentration of solid material increases, little to no free water may be available in the reactor (Kristensen et al. 2009b), which can limit the effectiveness of the chosen pretreatment. Actually, the type of biomass utilized can have a large effect on the amount of feedstock-associated water that enters the process, as well as on the way the solid and liquid phases interact. Water binds differently to the different fractions of lignocellulosic material. Hemicellulose tends to have a high water-holding capacity, while cellulose and lignin do not (Weber et al. 1993). Water plays an essential role in pretreatment reactions, aiding in chemical and enzymatic reactions, reducing the viscosity of the slurry by increasing the lubricity of the particles, providing a medium for solubilization of sugars and other compounds and for mass transfer by diffusion. Many of the limitations associated with pretreatments that were not initially developed to perform at high-solids loadings appear to be correlated with the lack of available water, which warrants further study in order to minimize these effects.

High-solids slurries tend to be very viscous with some being paste-like in nature (Jorgensen et al. 2007a; Knutsen and Liberatore 2010a). Pretreated corn stover at 20% insoluble solids can be formed into shapes that remain even after applied forces are removed (Stickel et al. 2009). However, particle shape and size have a significant impact on the viscosity of a slurry since these characteristics influence the particle networking and type of packing that take place within the slurry (Ehrhardt et al. 2010; Szijarto et al. 2011b; Viamajala et al. 2009). For example, fibrous particles from straw or corn stover can easily become entangled, creating a very complex network of particles, which interact very differently than more nonfibrous particles like wood chips and corn cobs. A reduction in particle size has been shown to reduce viscosity (Viamajala et al. 2009), although, size reduction may not be feasible in all cases due to the large energy requirement for milling or grinding (Miao et al. 2011). High viscosities are associated with challenges like mixing and material handling that must be addressed for high-solids pretreatments to be as effective as possible (Jorgensen et al. 2007a; Roche et al. 2009a). Energy demands increase as mixing becomes more difficult, which may counteract the benefits of using high-solids loadings. Reactors suitable for effective pretreatment of these complex networks of lignocellulosic materials are imperative, and designs implemented to overcome these limitations are discussed in a later section. Material

handling also becomes an issue because viscous materials are difficult to pump or pour, which may limit the pretreatments' applicability in a conventional continuous system. In the paper and pulping industry, the addition of additives like dimethylformamide to Kraft process black liquors has been investigated to reduce the viscosity of high-solids slurries (Llamas et al. 2007). The size, shape and concentration of particles, as well as the addition of additives, should be taken into consideration to keep viscosity from limiting the conversion process.

While pretreatments at high-solids loadings may be attractive for producing higher sugar concentrations, there is a risk for also producing higher concentrations of hydrolysis and fermentation inhibitors (Jorgensen et al. 2007b). Figure 2.1 shows some of the inhibitors that may be formed during the pretreatment of lignocellulose. It is well documented that dilute acid pretreatment leads to the production of degradation products like acetic acid, furfural, hydroxymethylfurfural (HMF) and phenolic compounds (Bjerre et al. 1996; Georgieva et al. 2008; Hodge et al. 2008; Vertes et al. 2010), which have been shown to inhibit the other downstream steps in the conversion process. In addition to the type and severity of the pretreatment, the composition of lignocellulosic material may also contribute to the variety of inhibitors produced. For example, the hemicellulose found in herbaceous biomass like agricultural residues is composed mainly of xylose, whereas in softwoods, the hemicellulose is composed of mainly mannose (Galbe and Zacchi 2007). Several studies have recently shown that sugars resulting from the hydrolysis of hemicellulose, like xylose, xylan and xylooligomers, have a significant impact on the conversion rates and yields of cellulase and β -glucosidase enzymes (Kim et al. 2011; Qing et al. 2010; Ximenes et al. 2011b). Pretreating agricultural residues under acidic conditions can lead to increased xylose yields, which can inhibit cellulase and β -glucosidase activity if these xylan hydrolysis products are not removed. However, inhibitor production is not limited to dilute acid pretreatment. Alkaline pretreatments performed at room temperature can produce aromatic compounds like furans, phenols (Klinke et al. 2004), low molecular weight acids (Knill and Kennedy 2003) and aldehyde compounds (Vertes et al. 2010). Ximenes et al. (2011a) and Kim et al. (2011) have reported a significant decrease in activity and even deactivation in some instances for cellulase and two types of β -glucosidase exposed to low concentrations (2-5 mg/mL) of

phenolic compounds. The enzymes were especially sensitive to the polyphenolic compound tannic acid. Tannins can be found in almost any part of the plant, so these findings are applicable to many biomass feedstocks. Optimization of pretreatment conditions to minimize inhibitor production, with consideration of the specific type and severity of the pretreatment and type and concentration of the biomass feedstock is necessary, as the combination of all of these variables is important when developing effective and efficient conversion processes (Figure 2.2).

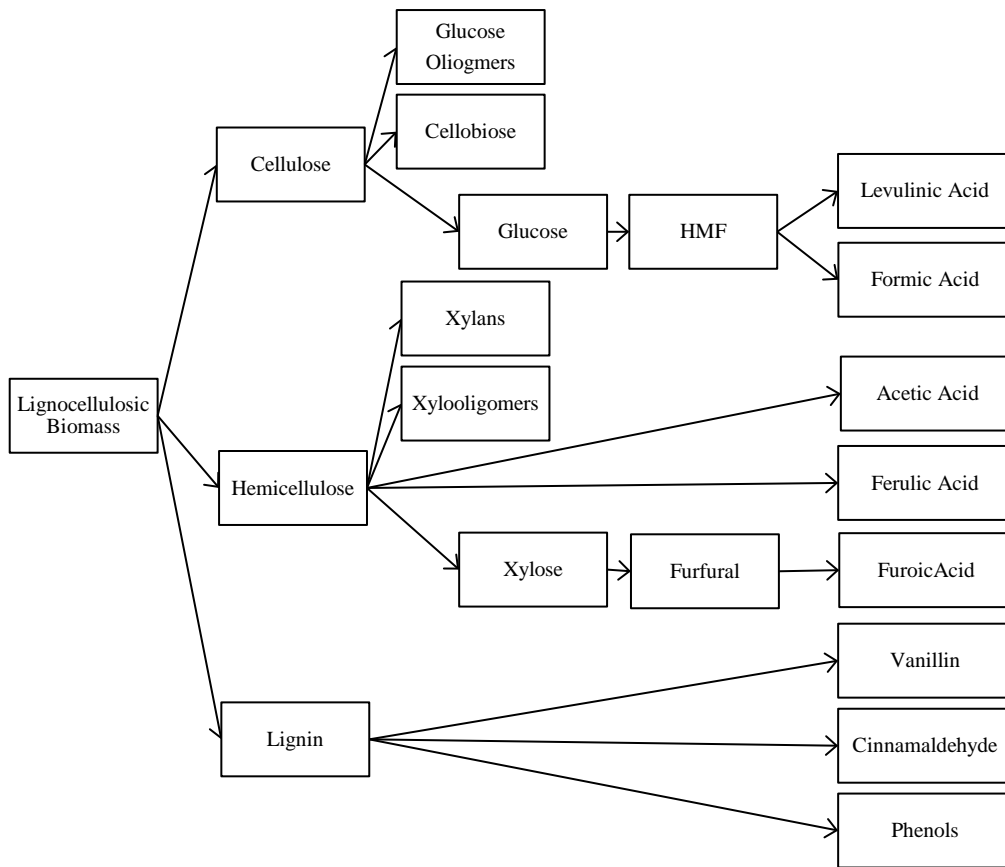


Figure 2.1. Schematic showing some of the products and potential inhibitors formed from the cellulose, hemicellulose and lignin fractions of lignocellulosic biomass during pretreatment.

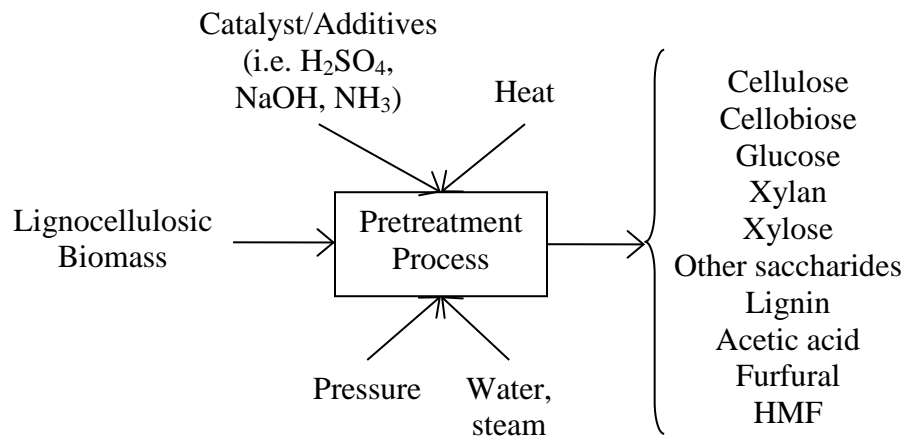


Figure 2.2. Schematic of a general pretreatment process.

2.4 PRETREATMENTS

The most important result of a pretreatment is that it enables maximum sugar yield following enzymatic hydrolysis and minimizes the loss of sugars and the formation of inhibitory products. Pretreatments facilitate the degradation of lignocellulose by modifying or removing lignin and/or hemicellulose, increasing the surface area or decreasing the particle size (Balat et al. 2008; Jorgensen et al. 2007a) so that cellulose is more accessible to enzymatic hydrolysis.

Numerous pretreatments have been developed, and each has its advantages and disadvantages, making it beneficial to tailor the pretreatment to the biomass source and desired end use. Table 2.1 shows the effects various pretreatments have on the different fractions of lignocellulosic material.

Table 2.1. Effects of various pretreatment methods on the three fractions of lignocellulosic material.

Pretreatment	Cellulose	Hemicellulose	Lignin	Other Effects
Dilute Acid	Very little solubilization	High solubilization	Condensation and precipitation	--
Liquid Hot Water (LHW)	Very little solubilization	High solubilization	Delignification	--
Steam Explosion	Slight degradation	Slight degradation	Redistribution	Increase in pore size
Biphasic CO ₂ -H ₂ O	Very little solubilization	High solubilization	--	Increase in surface area
SPORL	Slight degradation	Nearly complete solubilization	Partial delignification and sulfonation	Reduction in particle size
Alkaline	Reduction in DP and crystallinity	Partial hydrolysis	Some solubilization	Increase in surface area
AFEX	--	Disruption of bonds with lignin	Disruption of bonds with carbohydrates	--

DP = degree of polymerization

2.4.1 Acid Pretreatments

Pretreatments utilizing acids, especially dilute acid pretreatment, are the most commonly used pretreatment (Ehrhardt et al. 2010; Lloyd and Wyman 2005; Wyman et al. 2005a; Zhu et al. 2004). During acid pretreatment, hemicellulose hydrolyzes into its respective monosaccharides, while the lignin condenses and precipitates (Balat et al. 2008; Galbe and Zacchi 2007; Hendriks and Zeeman 2009). Dilute acid reagents like sulfuric and phosphoric acids at concentrations $\leq 4\%$ are typically utilized at elevated temperatures (140-200°C) for up to 1 hr (Galbe and Zacchi 2007). Sulfur dioxide has also been used as an acid catalyst in conjunction with steam pretreatment (Chandra et al. 2007). While acid pretreatment is effective in the breakdown of lignocellulosic material, it can result in many degradation products, like furfural, HMF and acetic acid (Vertes et al. 2010), which can be inhibitory in downstream processes. Other disadvantages associated with acid pretreatment include the loss of some fermentable sugars due to degradation, high costs of reactor materials which are resistant to corrosion, and the

additional cost of neutralizing the acid prior to downstream processing (Galbe and Zacchi 2007; Hendriks and Zeeman 2009; Mosier et al. 2005).

One of the earliest studies published regarding pretreatment at high-solids loadings was one utilizing SO₂ at 33% solids loading (Wayman et al. 1987). Aspen and corn stover were pretreated for 30 min at 160°C using 3% (w/w) SO₂ in a direct steam reactor. Solubilized hemicellulose sugar yields from aspen were ≥90% of the theoretical yield, with a significant reduction (25.5% to 5.6%) in soluble oligomer yield during pretreatment when compared to steam pretreatment without SO₂, which is a favorable result. The pretreatment of corn stover also resulted in solubilization of 79% of the hemicellulose sugars. Subsequent enzymatic hydrolysis and fermentation of aspen resulted in 91% and 73% theoretical glucose and ethanol yields, respectively, while corn stover resulted in 86.5% and 81% theoretical glucose and ethanol yields, respectively. One benefit of SO₂ over H₂SO₄ as an acid catalyst is that the pH is not lowered and the washing step between pretreatment and hydrolysis can be omitted without limiting enzymatic hydrolysis. SO₂ is also more compatible with stainless steel than H₂SO₄, and lignin may be better preserved, allowing for uses like heating and/or powering the conversion process or other higher value applications.

Another early pretreatment study utilizing high-solids loadings selected dilute sulfuric acid as a catalyst (Schell et al. 1992). The pretreatment consisted of two steps. The first step was soaking corn stover at 10% solids loading for 24 hr. The second step involved applying steam followed by flash cooling the corn stover. Although exact solids loading was not given, the researchers estimated that it was between 20 and 30%. Under the pretreatment conditions tested, the xylan was reduced by nearly 50% at the lower severities to nearly 100% at the higher severities. The subsequent glucose yields from enzymatic hydrolysis increased with increasing pretreatment severity from approximately 55% to 96% yield with the exception of the highest severity (77% glucose yield). It is possible the production of degradation products from these pretreatment conditions (180°C for 20 min) inhibited the enzymes digesting the corn stover. The authors also noted that optimization of pretreatment conditions for enzymatic hydrolysis may not optimize fermentation, since the presence of these degradation products (i.e. HMF,

furfural) are toxic to fermentative yeasts. The entire process should be evaluated as one system rather than optimizing each unit operation individually.

Continued research resulted in studies of a pilot-scale system (1 ton/day) capable of continuously pretreating corn stover at 20% solids loading (Schell et al. 2003). A range of temperature, acid concentration and retention times were studied and compared by a value known as the combined severity factor (CSF). The severity factor was developed as a means for combining the temperature of a reaction with the time spent at that temperature into a single value (Overend and Chornet 1987). It is used to rate processes as times and temperatures can be altered, while still maintaining a constant pretreatment severity. The CSF was further developed to include the pH at which the reaction takes place. It is used to facilitate the comparison among different pretreatment processes and conditions as it incorporates the pretreatment temperature, reaction time and the pH as follows:

$$\log R_o = \log \left(t \times e^{\left(\frac{T-100}{14.75} \right)} \right) - \text{pH} \quad \text{Equation 2.1}$$

where t is the reaction time in min and T is the temperature in °C (Galbe and Zacchi 2007; Kabel et al. 2007). It was determined that the optimum xylose yield (~70%) occurred for pretreatment conditions with a CSF in the range of 1.4-1.7. As the CSF increased above this range, xylose yields decreased, which was most likely the result of the monosaccharides forming degradation products like furfural. While CSF is a means of comparison among different pretreatment conditions, it does not necessarily provide an indication of the pretreatment effectiveness. Only a slight positive relationship was observed between CSF and cellulose conversion.

A percolation reactor designed by Zhu et al. (2004) was evaluated using 25% solids loading and an acid flowrate of 10 mL/min. It was observed that the acid exiting the reactor within the first several minutes had a higher pH than when the acid entered the reactor. The researchers attributed this pH change to the buffering capacity of the corn stover at high-solids loading. This same buffering capacity was also observed by Schell et al. (2003), where the main focus of the study was the production of xylose. While xylose yield increased with increasing time and temperature of pretreatment, the increased time also resulted in further dilution of the monosaccharides. Other

monosaccharides (glucose, arabinose, galactose and mannose) were also detected in the eluent. Further testing would be required to optimize the process for maximum sugar production, whether it is for a single desired monosaccharide or a combination.

In a later study (Zhu et al. 2005), it was determined that the optimum pretreatment conditions for corn stover in the percolation reactor were 170°C and 1.0% (w/w) acid applied at 10 mL/min. Mass balance closures accounted for $\geq 94\%$ of the xylose and glucose monosaccharides, with nearly 100% glucan digestibility. Two observations arose from the biomass pretreatment that may warrant further investigation. (1) Due to the axial position of the reactor, the corn stover at the inlet experiences a reaction time nearly double that of the corn stover at the outlet. (2) The CSF changed over the length of the reactor because of changes in the buffering capacity of the corn stover. These two issues led to a non-uniform pretreatment of the corn stover that may have several implications in the overall process. The corn stover located nearer to the inlet of the reactor is exposed to acids at lower pH for prolonged periods of time, thus potentially resulting in an increased production of degradation products. Furthermore, the corn stover nearer to the outlet of the reactor may not be fully converted to fermentable sugars since the acid is buffered and the reaction time is shorter

Acidic pretreatments typically remove the hemicellulose fraction by hydrolyzing it into its monosaccharide components, which facilitates enzymatic hydrolysis of the remaining cellulose. However, it has been shown that as reaction time, temperature, acid concentration or a combination of these three is increased beyond a certain point, xylose yield in the pretreatment liquor decreases. This decrease in xylose yield is typically attributed to xylose decomposing into other degradation products. Lu et al. (2009) observed similar trends. They reported xylose yields increasing when they increased the acid concentration or increased the reaction time. At a 2% acid concentration, xylose yields decreased with increasing reaction times. Acetic acid, HMF and furfural production were observed, but the concentrations were below inhibitory levels for yeast fermentation.

While sulfuric acid is most commonly used in dilute acid pretreatments, other organic acids, like fumaric acid and maleic acid, have been tested (Kootstra et al. 2009). Kootstra et al. (2009) measured glucose and xylose yields after pretreating wheat straw at

20% and 30% solids loadings with sulfuric, maleic and fumaric acids. Maleic and fumaric acids do not promote the reactions that lead to sugar degradation products (i.e. furfural and HMF) that often result from pretreatment with sulfuric acid. An additional benefit of these two acids over sulfuric acid is that the quality of the by-product stream changes from excessive amounts of gypsum to fertilizer or feed components. The acid to wheat straw ratio used was 5.17% (w/w), which is slightly higher than acid concentrations typically used in dilute acid pretreatment. For a given set of pretreatment conditions, glucose yields varied by up to as much as 30 percentage points among the three acid pretreatments. The xylose yields decreased with increasing solids loadings for sulfuric and maleic acid but increased slightly for the fumaric acid pretreatment. Additionally, furfural production was more significant for the sulfuric acid pretreatment than the other two treatments, which was expected based on the reaction mechanisms of the different acids. While the overall results for maleic acid were promising, price is a limiting factor, since maleic acid can cost at least ten times that of sulfuric acid.

Although the number of studies using acid pretreatment in a high-solids environment is limited, there appears to be an emerging consensus for the optimal conditions to utilize in dilute acid pretreatment (Table 2.2) to maximize glucose yields. Based on the conclusions of the high-solids studies reviewed, at solids loadings $\geq 20\%$, an acid concentration of 1% (w/w) at $\sim 180^\circ\text{C}$ with a reaction time ≤ 10 min resulted in optimal xylose yields from pretreatment and glucose yields from subsequent enzymatic hydrolysis (Lu et al. 2009; Schell et al. 2003; Schell et al. 1992; Zhu et al. 2004; Zhu et al. 2005).

Table 2.2. Conditions of optimal sugar yields from pretreatments utilizing high-solids loadings.

Pretreatment	Substrate	Solids Loading ^a	Residence Time	Temperature & Pressure	Other Conditions	% Sugar Yield ^b		Reference
						Glucose	Xylose	
<i>Acidic Pretreatments:</i>								
SO ₂ + Steam	Hardwood	33%	30 min	160°C, 0.5 MPa	3% (w/w) SO ₂	91.2%	91.6%	(Wayman et al. 1987)
SO ₂ + Steam	Corn stover	33%	30 min	160°C, 0.5 MPa	3% (w/w) SO ₂	86.5%	79.0%	(Wayman et al. 1987)
Steam	Corn fiber	70%	2 min	215°C	--	87%	40%	(Allen et al. 2001)
Dilute Acid + Steam	Corn stover	20-30% ^c	10 min	180°C	--	98%	NR ^d	(Schell et al. 1992)
Dilute Acid	Corn stover	20%	6.2 min	179°C	1.16% (w/w) acid	87%	70%	(Schell et al. 2003)
Dilute Acid	Corn stover	25% (v/v)	3 min	180°C	1% (w/w) acid, 10 mL/min	NR	73%	(Zhu et al. 2004)
Dilute Acid	Corn stover	25% (v/v)		170°C	1% (w/w) acid	98.7%	94%	(Zhu et al. 2005)
Organic Acids	Wheat straw	20%	30 min	150°C	5.17% (w/w) H ₂ SO ₄	>90%	80%	(Kootstra et al. 2009)
Liquid Hot Water (LHW)	Corn fiber	10%	2 min	215°C	--	93%	62%	(Allen et al. 2001)

Table 2.2, continued. Conditions of optimal sugar yields from pretreatments utilizing high-solids loadings.

Pretreatment	Substrate	Solids Loading ^a	Residence Time	Temperature & Pressure	Other Conditions	% Sugar Yield ^b		Reference
						Glucose	Xylose	
LHW	WDG	20% (w/v)	20 min	160°C	--	83%	50%	(Kim et al. 2008)
Hydrothermal	Wheat straw	25-40% ^e	6-12 min	195°C	--	94%	70%	(Petersen et al. 2009)
Acid-Catalyzed Hydrothermal	Rapeseed straw	20%	10 min	180°C	1% (w/w) acid	63.17%	75.12%	(Lu et al. 2009)
Biphasic CO ₂ -H ₂ O	Corn stover	20%	1 hr	160°C, 20 MPa	--	85%	10%	(Luterbacher et al. 2010)
Biphasic CO ₂ -H ₂ O	Switchgrass	20%	1 hr	160°C, 20 MPa	--	81%	13%	(Luterbacher et al. 2010)
Biphasic CO ₂ -H ₂ O	Hardwood	40%	1 hr	170°C, 20 MPa	--	73%	14%	(Luterbacher et al. 2010)
SPORL	Softwoods	20% (w/v)	30 min	180°C	8-10% (w/w) bisulfate + 1.8-3.7% (w/w) sulfuric acid	90%	76%	(Zhu et al. 2009)
SPORL	Hardwoods	20% (w/v)	30 min	180°C	4% (w/w) sodium bisulfite	89%	NR	(Wang et al. 2009)

Table 2.2, continued. Conditions of optimal sugar yields from pretreatments utilizing high-solids loadings.

Pretreatment	Substrate	Solids Loading ^a	Residence Time	Temperature & Pressure	Other Conditions	% Sugar Yield ^b		Reference
						Glucose	Xylose	
<i>Basic Pretreatments:</i>								
AFEX	DDGS	55%	5 min	70°C	--	68%	12.2%	(Kim et al. 2008)
NaOH	Rice straw	20%	3 hr		4% (w/w) NaOH	39.2%	NR	(Cheng et al. 2010)
Steam Explosion with NaOH and H ₂ O ₂	Corn stover	10%	24 hr	Room temperature	--	60%	NR	(Yang et al. 2010b)

^a Solids loading is indicated in (w/w) unless otherwise noted

^b Sugar yields are yields resulting from pretreatment and/or enzymatic hydrolysis

^c Solids concentration following 24 hr soaking in 1% (w/w) sulfuric acid at 10% solids loading

^d Not reported

^e Concentration of dry matter exiting continuous reactor

2.4.2 Alkaline Pretreatments

Lime and NaOH are common reagents used for alkaline pretreatments, which can be conducted over a wide range of operating conditions (Galbe and Zacchi 2007; Hendriks and Zeeman 2009; Jorgensen et al. 2007a; Mosier et al. 2005). Reaction time can vary from several minutes to days, while temperatures can range from ambient to 150°C (Galbe and Zacchi 2007; Jorgensen et al. 2007a). Alkaline pretreatment effectively increases the surface area by swelling the biomass particles and increasing carbohydrate accessibility to enzymes, while reducing the degree of polymerization (DP) and crystallinity of the cellulose fraction. The hemicellulose fraction can be partially hydrolyzed under strong alkaline conditions. The bonds between the lignin and carbohydrates are broken, and some lignin is solubilized (Balat et al. 2008; Galbe and Zacchi 2007; Hendriks and Zeeman 2009; Jorgensen et al. 2007a). Other advantages associated with this pretreatment over other pretreatments like dilute acid and AFEX are low cost, use of less caustic materials, and recoverable and recyclable reagents (Mosier et al. 2005). Alkaline pretreatments do not require specialized equipment, as the alkaline reagents typically used do not cause corrosion like dilute acids, and high pressures like those used in AFEX are not utilized. Drawbacks of alkaline pretreatments include a large number of inhibitors which can be produced at the harsher operating conditions (Hendriks and Zeeman 2009), and the effectiveness of these methods can be decreased with feedstocks with high levels of lignin, like woody biomass (Balat et al. 2008; Galbe and Zacchi 2007).

A study conducted by Cheng et al. (2010) compared the common reagents for alkaline pretreatment. For the lime pretreatment, a solids loading of 10% (w/w) and alkaline loadings of 0-10% were tested for reaction times of 1-3 hours at 95°C. The NaOH pretreatments were performed on 20% (w/w) solids with 0-4% alkaline loadings for 1-3 hours at 55°C. Delignification increased up to 27.0% and 23.1% for the lime and NaOH reagents, respectively, as reaction time and alkaline loading increased. The authors also reported an increase in enzymatic hydrolysis conversion with increasing alkaline loading, with a maximum glucose conversion of 48.5% and 39.2% for lime- and NaOH-pretreated solids, respectively. It should be noted that the solids were not washed between the pretreatment and enzymatic hydrolysis steps. A washing step is often used

in other pretreatment protocols, but it introduces another point where the biomass must be handled, resulting in loss of material. While washing the biomass post-pretreatment can remove inhibitors, it also removes any solubilized sugars, reducing the overall yield. However, a post-pretreatment washing step in this study did not significantly increase the glucose yield for the NaOH-pretreated solids. Even though the pretreatment conditions are not identical for the different reagents, the results have interesting implications. NaOH pretreatments are promising for high-solids pretreatments because glucose yields were similar to the yields produced from the harsher conditions of the lime pretreatment and because NaOH does not require a washing step after pretreatment.

2.4.3 Hydrothermal Pretreatments

Hydrothermal pretreatments utilize water at elevated temperatures to improve the conversion of lignocellulose. Several pretreatment technologies are included in this category, including steam, steam explosion and hydrothermolysis. Further details on each of these pretreatments are provided below.

Steam and steam explosion pretreatments offer short reaction times on the order of 1-5 min but also require high temperatures (160-240°C) and pressures (~1-3.5 MPa) (Galbe and Zacchi 2007; Jorgensen et al. 2007a). The high temperature of steam promotes the deacetylation of hemicellulose, resulting in acidic conditions that further catalyze the reaction (Alvira et al. 2010). These pretreatment conditions may produce degradation products from the cellulose and hemicellulose, while lignin is redistributed but not removed (Mosier et al. 2005). Temperature and pressure combinations should be carefully chosen to maximize accessibility for enzymes and minimize the degradation products, which can inhibit the enzymes and fermentative organisms in other downstream processes. Steam pretreatment has been proven to be effective on most types of lignocellulosic material, with the exception being softwoods. The hemicellulose fraction of softwoods contains few acetyl groups (Alvira et al. 2010). However, steam pretreatment is ideal if the desired end-products are fibers; feedstocks can be separated into individual fibers with minimal loss of material (Balat et al. 2008). Steam and steam explosion pretreatments are also advantageous because they increase pore size, allowing for better accessibility of the saccharides for hydrolysis, making this pretreatment a cost-

effective option for agricultural residues since steam is the reagent (Jorgensen et al. 2007a). The high energy content of the steam makes these pretreatments appropriate for use with high solids, as the amount of water added to the process can be reduced.

Hydrothermolysis, also known as liquid hot water (LHW) pretreatment can be used to hydrolyze lignocellulosic material. Like steam pretreatment, liquid water at elevated temperatures and pressures (180-230°C and 2.4-2.8 MPa) acts much like an acid, as the pH of the water at 220°C is about 5.5 (Allen et al. 2001; Mosier et al. 2005). Acetic acid, produced from deacetylation of the hemicellulose, also enhances the acid-catalyzed reactions. Under these conditions, LHW removes a significant portion of lignin (Mosier et al. 2005). Hemicellulose is also hydrolyzed into soluble sugars. However, pressure (~2.5 MPa) must be applied to keep the water in the liquid phase at the temperatures used (Mosier et al. 2005), requiring specialized equipment. Hydrothermolysis produces minimal inhibitors as compared to steam pretreatment and requires limited neutralization since no additional chemicals are used, but the overall concentration of soluble products tends to be lower than other pretreatments because a high volume of water is typically used (Hendriks and Zeeman 2009; Mosier et al. 2005). Based on the volume of water required for this pretreatment, solids loadings are limited to about 20%.

A study comparing steam and LHW pretreatments at high and moderate solids loadings was conducted by Allen et al (2001). However, a direct comparison is difficult to make, considering the steam pretreatment was performed at 50% (w/w) and 70% (w/w) corn fiber solids loadings, while the LHW pretreatment was performed at 10% (w/w) solids loadings, due to reactor volume limitations. This study determined that the reaction medium, steam or liquid water, directly impacted the solubility of the substrate, the capacity to recover C5 sugars and the downstream processes. For example, the LHW pretreatment resulted in 61% solubilization of the corn fiber, while steam pretreatment resulted in 44% and 37% solubilization for solids loadings of 50% and 70%, respectively. This trend of similar or decreasing yields for increasing solids loadings in pretreatment is not uncommon (Kootstra et al. 2009; Luterbacher et al. 2010). This same negative correlation was also reported for C5 and C6 sugar recoveries as the solids loadings increased. Much of the hemicellulose fraction either underwent a transformation and

reorganization within the insoluble portion of the corn fiber or degraded beyond useful monosaccharides at the higher solids loadings, resulting in a loss in fermentable C5 sugars. The final ethanol yield from the LHW pretreatment liquor was not impacted by the loss of C5 sugars; however, the rate of ethanol production from the liquid fraction (as compared to the simultaneous saccharification and fermentation of the pretreated corn fiber) was severely limited, likely because of solubilized inhibitory products. The liquid fraction produced from the steam pretreatment resulted in a reduced final fermentation rate and yield; however, the cause of the lower rate and yield is unknown because the inhibitor concentrations were similar to those found in the liquid fraction of the LHW pretreatment.

Another study utilizing LWH pretreatment with high-solids loadings was conducted by Kim et al. (2008), with a mixture of wet distillers' grains (WDG) and thin stillage as the biomass source at 13% to 30% solids loading. The by-products of the distilling process are typically used for nutritional supplements in the livestock and poultry industries; however, the high energy value of the residual sugars and fibers make these materials attractive as a feedstock for the production of energy or other high-value products. The LHW pretreatment did not degrade glucan or produce degradation products. Only 2.9% of the total glucan was converted to glucose during the pretreatment process, and no sugar degradation products were detected, which is a favorable characteristic of a pretreatment. In addition to the high-solids loading for the pretreatment process, the WDG and stillage mixture was subjected to high-solids enzymatic hydrolysis. The researchers report an increase in the glucose and xylose yields as solids loading for enzymatic hydrolysis increases to 20%, but the yields decrease at 30% solids loading. While the percentage glucose yields are comparable between the 13% and 30% solids loadings, the xylose yield is nearly double for the 30% solids loading. This increase can be explained by the fact that additional enzymes (xylanase and feruloyl esterase) were added to the mixture, which strengthens the argument that optimal enzyme mixtures may be required to reach the full potential of the biomass.

The Integrated Biomass Utilization System (IBUS) Project resulted in a continuous hydrothermal pretreatment reactor and process that is capable of processing wheat straw up to 100 kg/hr (Petersen et al. 2009). This process uses high temperatures

(185-205°C) and short residence times (6-12 min) to maximize both glucan and xylan recovery. The current process produces two process streams: the liquid fraction containing soluble xylan oligomers and degradation products and the solid fraction containing cellulose, insoluble hemicellulose and lignin. The solid fraction exits the reactor at approximately 25-40% DM. All pretreatment conditions studied except for one (205°C for 6 min) produced glucose recoveries $\geq 90\%$; however, hemicellulose recoveries covered a wide range (60-90%). Lower hemicellulose recovery is most likely due to the increase in production of degradation products at the higher severity pretreatment conditions, which was confirmed with further study of the inhibitors produced from the pretreated wheat straw (Thomsen et al. 2009).

2.4.4 Other/Combination Pretreatments

Other pretreatments utilized in high-solids studies do not fall into any one particular category, as some pretreatments combine multiple processes to selectively produce sugars. The results of these studies are presented below.

2.4.4.1 Biphasic CO₂-H₂O

Several pretreatment approaches utilize water with acid or base additions to initiate the breakdown of biomass. The biphasic CO₂-H₂O pretreatment offers many advantages by combining these two reagents in the pretreatment process. The supercritical points for water and CO₂ are 22.1 MPa at 373.9°C and 7.4 MPa at 31.1°C, respectively. Utilizing elevated temperatures and pressures, water remains in the liquid phase, acting much like a LHW pretreatment, and CO₂ is in its supercritical fluid phase. The addition of the CO₂ acts as an acid catalyst in the reaction (Luterbacher et al. 2010), while the CO₂ found in the supercritical phase has also been shown to have a swelling effect on biomass. Lastly, the reagents can be easily separated and reused, keeping costs low, as CO₂ is immiscible in water at atmospheric conditions (Kim and Hong 2001; Luterbacher et al. 2010). However, there is some additional capital costs associated with equipment suitable for pressurized systems.

The study performed by Luterbacher et al. (2010) is the first to combine this biphasic CO₂-H₂O pretreatment with high-solids loadings (40% w/w). It is also one of

the highest solids loadings reported for any pretreatment process. This pretreatment resulted in glucose yields above 70% for hardwoods and above 80% for switchgrass and corn stover, which are within ten percentage points from yields reported in other studies utilizing other leading pretreatment technologies (Luterbacher et al. 2010). These yields make this a promising pretreatment option, especially with good results from high-solids loadings and inexpensive chemical reagents. However, conditions should be optimized for different biomass feedstocks in order to limit the amount of degradation products produced in this process. Furfural and HMF were both produced in measurable quantities in this study. Not only are these products inhibitory to the downstream conversion processes, but the sugar yields are reduced when these products are formed.

2.4.4.2 Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose (SPORL)

SPORL is a recently developed, yet promising, process that combines a sulfite treatment of wood chips under acidic conditions with mechanical size reduction with disk refining (Zhu et al. 2011a; Zhu et al. 2009). This technique was specifically intended for the pretreatment of softwoods, for which other existing pretreatment technologies have had limited success in enhancing enzymatic hydrolysis yields. Conditions have since been investigated to include pretreatment of hardwoods (Wang et al. 2009). The SPORL process is a modification of the sulfite pulping process, which has been practiced at the industrial level for more than a century. The modifications made allow for nearly complete hemicellulose removal with minimal lignin condensation and removal. Some glucose is hydrolyzed in the process, but it is recovered at a later step. This pretreatment can be carried out with equipment (pulp digester and mechanical disk refiner) typically used in the pulp and paper industry. The pretreatment liquor can also be prepared and recovered with existing techniques, reducing costs associated with chemical needs and cleaning waste streams.

Zhu et al. (2009) investigated the combination of a sulfite treatment with mechanical size reduction by disk refining to enhance enzymatic hydrolysis of softwoods. This study was the first to establish this novel pretreatment process. Pretreatment conditions of spruce chips (20% w/v) that produced optimal cellulose conversion during enzymatic hydrolysis (>90%) was treatment with 8-10% bisulfite and 1.8-3.7% sulfuric

acid for 30 min at 180°C. Nearly all hemicellulose was removed, which exposed the underlying cellulose fraction to enzymatic attack. Additionally, furfural and HMF were produced in minimal concentrations, about 1 and 5 mg/g untreated wood, respectively.

In a later study performed by Wang et al. (2009), the SPORL process was expanded to include conditions appropriate for pretreatment of hardwood. At 20% (w/v), a bisulfite charge of 4% for 30 min at 180°C produced the highest glucose yield following enzymatic hydrolysis. Unlike the SPORL process for the softwoods, sulfuric acid was not necessary to maintain the acidic pH due to the high acetyl concentration of hardwoods. Several benefits were recognized by not having to supply additional acid to the reaction. SPORL, under these conditions, could avoid reactor corrosion and substrate neutralization for optimal enzymatic hydrolysis, as well as negligible production of inhibitory products like furfural and HMF. It is apparent from the results of these studies that the SPORL process is effective for the pretreatment of woody biomass, but further studies should be conducted to determine appropriate conditions prior to use with other lignocellulosic materials.

2.4.4.3 Ammonia Fiber Expansion (AFEX)

Ammonia fiber explosion (or expansion) techniques have, in general, been well investigated as a pretreatment option for lignocellulosic material (Galbe and Zacchi 2007; Jorgensen et al. 2007a; Kumar and Wyman 2009b). AFEX is a promising pretreatment option because it is effective in situations with high-solids content and the ammonia reagent can be recycled (Jorgensen et al. 2007a), which can help in the reduction of processing costs. This method has also been shown to be effective on corn stover and other agricultural residues (Balat et al. 2008). AFEX works by applying a pressure, which is released after a short reaction time to cause the “explosion” of the lignocellulosic components. Temperatures typically range from 70-100°C, with pressures of ~2 MPa and relatively short reaction times (5-10 min) (Galbe and Zacchi 2007; Kim et al. 2008). While the lignin and hemicellulose fractions are not removed, some lignin-carbohydrate bonds are broken, subsequently making the cellulose and the hemicellulose available for enzymatic hydrolysis (Jorgensen et al. 2007a). However, it has been reported that AFEX can lead to the production of some inhibitors such as furfural if the

processing conditions are not optimized for the material being pretreated (Jorgensen et al. 2007a).

Kim et al. (2008) conducted a study using AFEX to pretreat distiller's dried grains and soluble (DDGS) at high-solids loading (Table 2.2). The ammonia was applied at 0.8 g/g biomass, and the reaction was performed at 70°C for 5 min. This pretreatment significantly increased the rate at which the biomass was hydrolyzed as compared to untreated DDGS, and complete conversion was achieved by 72 hrs. In the study presented here, with the high-solids loading and ammonia recovery, the process is essentially a dry process, meaning the solids enter the reactor dry and leave the reactor dry. This aspect has interesting implications for the overall conversion process. The biomass can more easily be mixed to a desired solids loading for enzymatic hydrolysis or SSF, including higher solids loadings, thus reducing the amount of water needed in these downstream conversion processes. However, more research is necessary prior to utilization of biomass in this manner.

2.4.4.4 Steam Explosion Combined with NaOH and H₂O₂

The advantages of using NaOH and steam explosion individually as pretreatments were previously outline in the Alkaline Pretreatments section and the Hydrothermal Pretreatments section, respectively.

The combination of the steam explosion with the alkaline peroxide process allowed for the removal of hemicellulose and lignin, respectively (Yang et al. 2010b). The cellulose content of the corn stover was effectively increased from 37.5% in its raw state to 45.2% to 73.2% following steam explosion and alkaline peroxide pretreatment, when pretreatments were applied in that order (Table 2.2). A fed-batch process was also incorporated into the conversion process to gradually increase solids loading in enzymatic hydrolysis from the initial 12% to 30% at completion. This modification allowed for easier handling and mixing of the bulk material, while maintaining the viscosity at workable levels. Reducing-sugar yields increased from 90 g/L to 220 g/L at 12% and 30% solids loading, respectively. The combination of treatments used effectively removed lignin and hemicellulose and improved sugar conversion downstream.

2.5 REACTOR DESIGN FOR HIGH-SOLIDS PRETREATMENT

Reactors suitable for low to moderate solids loadings can limit the conversion process at high-solids loadings due to ineffective mixing, which can result in increased concentrations of localized inhibitors, poor heat and mass transfer and requiring excessive amounts of energy to operate. Other considerations that should be included in the reactor design are the types of biomass and the size of particles that will be treated (Jorgensen et al. 2007a). Some types of biomass, like straw and rice, contain silica that can cause wear on moving parts. Also, larger particle sizes are preferred so the ratio of energy consumed to energy produced is as small as possible, and the more particle size reduction needed, the more significant energy input needed. Reactors capable of handling high-solids loadings are being developed for research purposes and use at bench- and pilot-scales are reviewed below (Hsu et al. 1996; Jorgensen et al. 2007b; Zhu et al. 2004).

One of the earliest reactors designed specifically for high-solids pretreatment was proposed by Hsu et al. (1996) and with which they successfully pretreated biomass at a solids loading of 10-15% (w/w). The design is based on classic paddle-blender designs and consists of a custom-fabricated, 100 L horizontal shaft reactor intended for dilute acid pretreatment of biomass at high-solids loading at the pilot-scale. The reactor was constructed of Carpenter 20 Cb-3 stainless steel to accommodate dilute sulfuric acid at elevated temperatures and pressures (approximately 175°C and 1.1 MPa). The horizontal orientation is advantageous as it limits the amount of particle settling and dead mixing zones found in other types of reactors (Dasari et al. 2009), while the scraping action of the paddle design aids in maintaining a clear reactor surface ensuring maximum heat transfer from the reactor jacket to the slurry (Hsu et al. 1996). The horizontal orientation also takes advantage of free-fall mixing, reducing the effect viscosity has on mixing. Power input to operate the reactor can be reduced since lower paddle speeds can still provide adequate mixing as compared to a vertically oriented reactor.

Jorgensen et al. (2007b) reported using a reactor similar in design to Hsu et al. (1996). Their reactor was also placed in a horizontal orientation to utilize free-fall mixing. However, it is divided into five separate chambers with a total capacity of nearly

280 L. Each chamber is fitted with three paddles on a variable-speed, rotating shaft to aid in the mixing process. Although the solids loading for pretreatment was not reported, the wheat straw exiting the reactor was at 23-28% DM. Along with operating as a pretreatment reactor, it can double as a reactor for simultaneous saccharification and fermentation (SSF), so that the entire conversion process can be conducted within one reactor. This design is beneficial in that it reduces the overall capital costs by eliminating the need for multiple reactors.

A bench-scale percolation reactor was designed and tested by Zhu et al. (2004) for dilute acid pretreatment of corn stover. It was constructed using Monel tubing, since this material is resistant to corrosion by acid. The reactor can be operated at pressures approaching ~2 MPa and at solids loadings of 25% (v/v). The acid flows through a heating coil prior to entering the reactor for pretreatment at the desired temperature (160-180°C), while the effluent is cooled by a heat exchanger at the reactor exit. The flow rate of the dilute acid through the biomass can be controlled in order to optimize the saccharide yields while minimizing the production of inhibitory degradation products. This flow-through design also eliminates the potential problems associated with mixing a complex network of particles. The percolation reactor described by Zhu et al. (2004) has the advantage of operating in semi-batch mode, which provides several benefits to the dilute acid pretreatment process including: (1) Sugar products are discharged throughout the reaction process. By allowing the dilute acid to flow through the biomass, the pretreatment liquor contains fewer degradation products while the sugar yields are increased; (2) Larger amounts of lignin can be removed in semi-batch mode than in batch mode, which enhances cellulose availability in downstream processes; and (3) A packed bed reactor allows higher solids to liquid ratios, which can lead to increased sugar yields. It is worth noting that these benefits are specific to dilute acid pretreatment. Further study using the percolation reactor would be necessary to determine if these benefits transfer to other pretreatment regimes.

2.6 PILOT-SCALE OPERATIONS

Several pilot-scale operations have incorporated high-solids pretreatments into their conversion processes for research and development purposes. Some of the leading operations are discussed in further detail here.

In 2004, a demonstration plant designed by SEKAB E-Technology began operation in Sweden (S. Wännström, personal communication). This facility is the largest of its kind in Sweden (300-400 L/d bioethanol production capacity) and continues to be used as a development plant for industrial technology with a focus on bioethanol and biochemicals. The plant is fully equipped with all process steps from intake of the raw materials to the distillation of the final products and is designed to be flexible so that various kinds of feedstocks, pretreatments and other process concepts can be utilized for process optimization. The pretreatment system operates in a continuous mode at 25-40% solids loading under pretreatment conditions specifically selected for the available feedstock. For example, prior to the dilute acid pretreatment, the biomass can be conditioned with steam and/or acid (typically H₂SO₄ or SO₂) should it be necessary. Optimized procedures have been developed at this facility for both forestry and agricultural feedstocks. To date, SEKAB's demonstration plant has accumulated over 30,000 hours of operation, several patents and extensive knowledge for the production of ethanol from lignocellulose.

DONG Energy located in Denmark has a semi-continuous counter-current reactor that is capable of processing 100-1000 kg/hr and utilizing various pretreatments and feedstocks (Jorgensen et al. 2007a). This pilot plant is designed to test different pretreatment methods, to operate with larger particles and to operate at solids loadings up to 50% DM. It also has two separate pretreatment facilities for research purposes. One line is for research and development for continuous mode operation (≤ 100 kg/hr capacity), while the other is for mechanical development and scale-up (≤ 1 tonne/hr capacity) (Larsen et al. 2008). In 2009, DONG Energy opened a demonstration-scale operation in Kalundborg, Denmark. At this facility operated by Inbicon (a subsidiary of DONG Energy), the hydrothermal pretreatment of wheat straw is conducted at 30-40% solids loading. The pilot-scale facility is still used to optimize the process employed at the demonstration-scale facility.

The National Renewable Energy Laboratory (NREL) in the United States has a pilot-scale pretreatment reactor that operates at high-solids loadings and has been the source of pretreated biomass for many high-solids studies (Roche et al. 2009a; Schell et al. 2003; Schell et al. 1992). It is used for continuous, dilute acid pretreatment of $\leq 30\%$ solids loadings. Schell et al. (2003) provide a detailed description of the process. In August 2010, NREL completed the first phase in its Integrated Biorefinery Research Facility (IBRF). This expansion provides space for new pilot-scale biomass conversion equipment, including a continuous 1 ton/day horizontal pressure pretreatment reactor. This new facility will continue to be used as a research and development facility, studying various feedstocks and pretreatment options.

2.7 DIRECTION OF FUTURE WORK

In order to fully realize the advantages provided by pretreatment at high-solids loadings, several issues must be addressed. The efficiency and effectiveness of a pretreatment process not only depends on the pretreatment conditions, but also on the type of biomass entering the pretreatment process. The pretreatment type and severity must be considered in combination with the biomass type and concentration to produce the most accessible and highest yielding saccharides while limiting the inhibitors entering other downstream steps in the conversion process. Other factors to consider during pretreatment optimization is the cost of biomass, reagents and any specialized equipment and the best use of any potential by-products produced in the process. Additionally, reactor systems robust enough to withstand a range of pretreatment conditions (temperature, pressure, reagent concentrations) and biomass properties (concentration, particle size, composition) are needed, especially for large scale production.

2.8 CONCLUSIONS

The feasibility of lignocellulosic conversion would greatly improve if high-solids loadings could be used successfully in all the various unit operations. Increased sugar and ethanol yields combined with decreased capital and production costs and decreased water and power use contribute to a more efficient process compared to the conventional conversion process. As the benefits of utilizing high-solids loadings in the

lignocellulosic conversion process are realized, so too are the limitations. Issues associated with the lack of free water, the high viscosities and the increased production of inhibitors must be overcome in order to achieve economically viable sugar and ethanol yields. Researchers are tackling these problems on two fronts: reactor design and pretreatment optimization. Horizontal paddle reactors and percolation reactors have both been shown to be possible alternatives to standard reactor designs when it comes to high solids. The choice of pretreatment can also affect the effectiveness of the overall conversion process. The effort in optimizing these various pretreatment options for high solids is evident by the many studies discussed in this paper, but many questions still require answers before the full power of utilizing high solids is recognized.

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CHAPTER 3: ENZYMATIC HYDROLYSIS OF BIOMASS AT HIGH-SOLIDS LOADINGS – A REVIEW²

3.1 SUMMARY

Enzymatic hydrolysis is the unit operation in the lignocellulose conversion process that utilizes enzymes to depolymerize lignocellulosic biomass. The saccharide components released are the feedstock for fermentation. When performed at high-solids loadings ($\geq 15\%$ solids, w/w), enzymatic hydrolysis potentially offers many advantages over conversions performed at low- or moderate-solids loadings, including increased sugar and ethanol concentrations and decreased capital and operating costs.

The goal of this review is to provide a consolidated source of information on studies using high-solids loadings in enzymatic hydrolysis. Included in this review is a brief discussion of the limitations, such as a lack of available water, difficulty with mixing and handling, insufficient mass and heat transfer, and increased concentration of inhibitors, associated with the use of high solids, as well as descriptions and findings of studies that performed enzymatic hydrolysis at high-solids loadings. Reactors designed and/or equipped for improved handling of high-solids slurries are also discussed. Lastly, this review includes a brief discussion of some of the operations that have successfully scaled-up and implemented high-solids enzymatic hydrolysis at pilot- and demonstration-scale facilities.

Keywords: High-solids loadings; enzymatic hydrolysis; lignocellulose conversion; reactor design; corn stover; straw; woody biomass

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3.2 INTRODUCTION

Lignocellulose is the largest renewable source of carbon on the planet, as it is the main structural component of plants. Energy from lignocellulosic biomass has been tapped as one possible solution to decrease the United States' foreign dependence on petroleum, as well as serve as a more environmentally friendly source of energy. Lignocellulose can either be processed thermochemically or biochemically, depending on the desired product. The biorefinery concept is thought to be the desired model for biomass processing, where all of the biomass is exploited. The suite of products would be dictated by the market and selected to extract the greatest value possible out of lignocellulose (Figure 3.1).

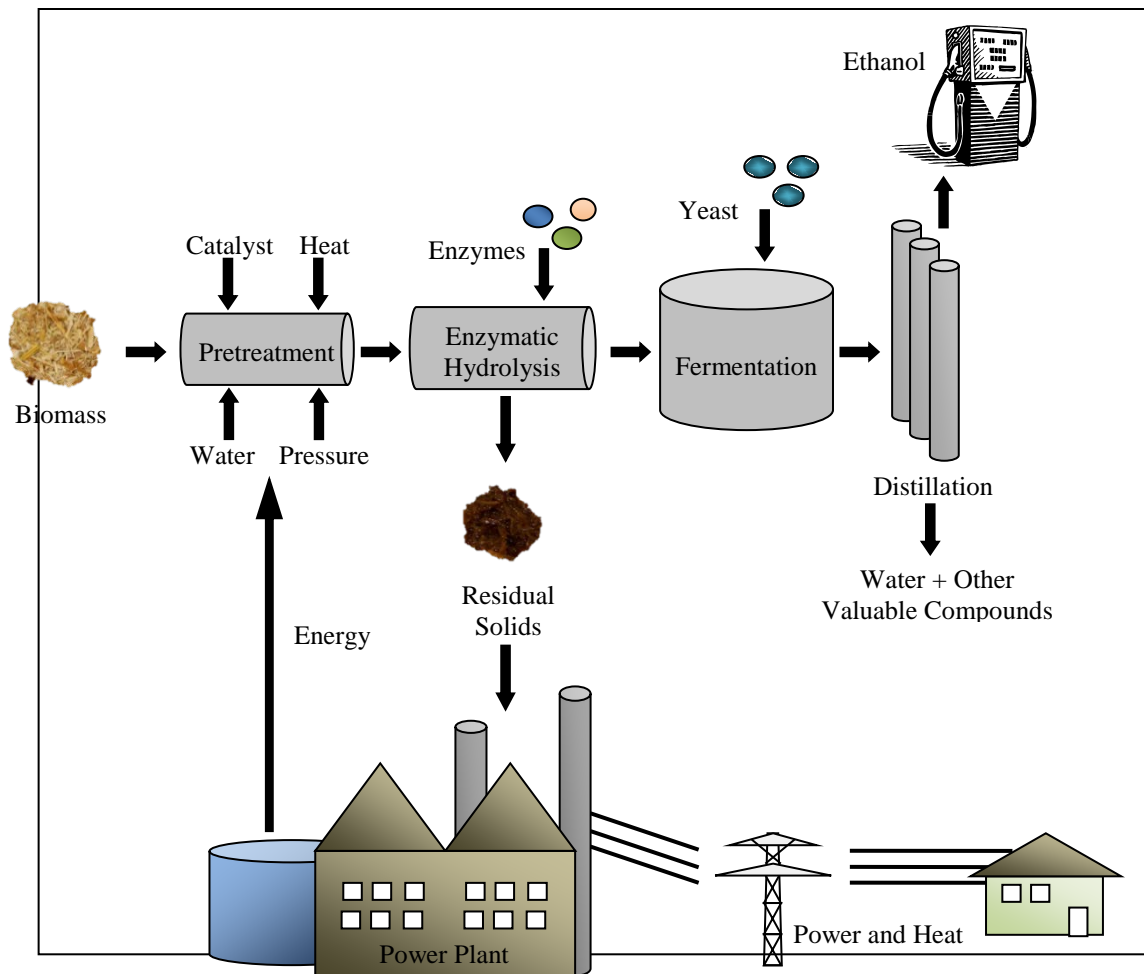


Figure 3.1. Schematic of the biorefinery concept. Lignocellulose enters the conversion process and undergoes pretreatment, enzymatic hydrolysis and fermentation. Distillation produces liquid transportation fuels, as well as other valuable products. The residual solids can be burned to produce energy that can be cycled back into the conversion process or shipped out to the grid for residential or commercial use.

Enzymatic hydrolysis of lignocellulose has long been studied as a method to depolymerize the biomass into fermentable sugars for conversion to biofuels and biochemicals, with a more recent focus on operating at high-solids loadings. It has been suggested that enzymatic hydrolysis conducted at high-solids loadings will be necessary to render the lignocellulosic conversion process more economically feasible. A process is considered “high solids” if the ratio of solids/liquid is such that very little to no free

water is present in the slurry (Hodge et al. 2009) or roughly a solids loadings $\geq 15\%$ (w/w).

Enzymatic hydrolysis performed at high-solids loadings offers several advantages over low- and moderate-solids loadings, the main one being final sugar concentrations are higher (Hodge et al. 2008; Roche et al. 2009a). In theory, higher sugar concentrations translate into higher ethanol concentrations, which could reduce energy use and costs associated with the distillation process (Humbird et al. 2010; Kristensen et al. 2009a). For the purpose of this paper, the term “concentration” refers to the amount of a component dissolved in a given volume of liquid, while the terms “yield” and “conversion” refer to the quantity of a product obtained expressed as a percentage of the theoretical maximum. Distillation is most economical when the ethanol concentration is $\geq 4\%$ (w/w). In order to obtain this ethanol yield, glucose yields must be at least 8% (w/w), which translated into a lignocellulose loading of $\geq 20\%$ (w/w) for enzymatic hydrolysis (Larsen et al. 2008). These estimates only account for conversion of cellulose; however, as improvements are made to hemicellulose conversion (hydrolysis and fermentation) technologies that work in combination with cellulose conversion, this initial solids loadings estimate may decrease. Another potential advantage is the reduction of capital and production costs. Smaller equipment and/or fewer reactors can be utilized to produce an equivalent output (Banerjee et al. 2010; Um and Hanley 2008). Fewer reactors also translate into reduced energy demands for heating, cooling and mixing (Kristensen et al. 2009a; Roche et al. 2009a), although the latter aspect may be a point of contention as increased solids makes effective mixing more difficult. Additionally, less water is needed, which reduces the cost of disposal or treatment of process water.

The goal for this review is to provide a consolidated source of information for the latest technological advances for managing enzymatic hydrolysis at high-solids loadings. Following a brief discussion of the factors limiting enzymatic hydrolysis at high solids, various aspects and approaches pertaining to hydrolysis operating conditions are detailed. Additionally, reactors designed to overcome some of the limitations associated with high-solids hydrolysis, as well as pilot- and demonstration-scale plants operating at high-solids loadings are discussed. Lastly, the authors comment on the envisioned direction for high-

solids hydrolysis research, as well as the necessary advances this technology must make to become commercially viable.

3.3 FACTORS LIMITING HIGH-SOLIDS ENZYMATIC HYDROLYSIS

As solids loading increases, challenges that were negligible in low-solid systems become more prominent, which has also been noted in high-solids pretreatment (Modenbach and Nokes 2012). One of the major challenges for enzymatic hydrolysis at high-solids loading is the lack of available water in the reactor. Water is essential to effective hydrolysis for two reasons: mass transfer and lubricity. Water increases the effectiveness of the enzymatic and chemical reactions, mainly by providing a medium for solubilizing and aiding in the mass transfer of products. Water also reduces the viscosity of the slurry by increasing the lubricity of the particles, which decreases the required shear stress necessary to produce a given shear rate, allowing lower power input for mixing (Hodge et al. 2009; Kristensen et al. 2009b). The physical and chemical properties of the specific biomass affect the way biomass absorbs water. As solids loadings approach 20% (w/w), the liquid fraction becomes fully absorbed into the biomass leaving little free water (Hodge et al. 2009). With lower amounts of free water, the apparent viscosity of the mixture increases, and consequently mixing and handling of material become more difficult.

Gervais et al. (1988) investigated the relationship between water content and water activity on microorganisms in a high-solids cellulose environment. No free water occurs when the matric potential of the substrate holds the water more tightly within its pores than the gravitational force acts on it. The water potential (= osmotic potential + matric potential) of the system is such that content affects mass transfer by limiting diffusion of products away from enzyme (Gervais et al. 1988). Not only can the enzymes release compounds from the biomass that are inhibitory to the organisms used in the fermentation step, but the sugar products they produce are known inhibitors in the enzymatic feedback mechanism (Gruno et al. 2004; Hodge et al. 2008; Holtzapple et al. 1990). For example, cellobiose inhibits the cellulase. Typically, cellulase is supplemented with β -glucosidase to reduce the inhibition by cellobiose. However, it has recently been shown that hydrolysis rates of cellulase and β -glucosidase are greatly

impacted by hemicellulose-derived products, like xylose, xylan and xylo-oligomers (Kim et al. 2011; Qing et al. 2010; Ximenes et al. 2011b). Pretreatment methods that do not remove these products or enzyme cocktails that include xylanases may have detrimental effects on glucose yields. While inhibition occurs at low solids, as well as at high solids, the increased concentration of inhibitors, in addition to the reduced mass transfer rate away from the enzyme, makes inhibition more apparent at high-solids loadings.

The challenges apparent at high solids are interrelated, so a less-than-ideal condition in one property exacerbates the negative effects of another property. For example, the substrates' physio/chemical properties affect the water retention value (WRV) of the biomass. A high WRV (due to high-solids content and the specific properties of the substrate) reduces the diffusion of inhibitors away from the enzymatic reaction, and increases the apparent viscosity of the mixture, thereby increasing the difficulty of stirring the mixture to assist with diffusion. Zhang et al. (2010) found that the energy required to mix increased one order of magnitude when they increased the solids loading of pretreated corn stover from 15% to 30% w/w (79.5 MJ/t slurry to 1009.2 MJ/t slurry, respectively) to produce 854.9 and 1723.2 MJ/t slurry of ethanol respectively. The higher solids loading did indeed achieve the goal of producing a higher concentration of ethanol in the broth; however, over half of the energy produced in the ethanol was consumed in the mixing to achieve the higher concentration of ethanol (compared to 9% of the energy needed to mix the system producing the lower concentration of ethanol).

While it is widely recognized that increasing the solids content in a conversion process increases product concentration (Gupta and Lee 2009), it is also widely recognized that the increase in yield is not linear with increasing initial solids content because yield (percent conversion) decreases with initial solids content (slope is a function of substrate type, pretreatment, and enzyme loading, among other things) (Kristensen et al. 2009b). In fact, this well-recognized challenge was observed so often that Kristensen et al. (2009b) coined the term *solids effect* to describe the persistence of a measured reduction in conversion when solids loadings are increased. The scientific community has yet to come to agreement as to the cause of the solids effect; however,

theories include substrate effects, product inhibition, water content and enzyme adsorption characteristics, just to name a few (Kristensen et al. 2009b).

Other challenges specific to high-solids enzymatic hydrolysis include long hydrolysis times. Enzymatic hydrolysis is typically thought to be the bottleneck of the entire conversion process in terms of both time and money, since the reaction time needed for most enzymes to convert lignocellulose into sufficient glucose concentrations for fermentation is on the order of days (usually ≥ 3 days). Long hydrolysis times can only be reduced so much by increasing enzyme loading. Recent studies have suggested that enzymes can overcrowd accessible cellulose sites, thus not reaching the full hydrolytic potential for the given enzyme loading (Bommarius et al. 2008; Xu and Ding 2007). Adjacent cellulose chains are $\sim 4\text{-}6$ Å apart, whereas the diameter of the cellulases is about 10-fold larger at about 45 Å (Figure 3.2). Furthermore, as in low-solids hydrolysis, the cost of the enzyme is also a limiting factor. Enzyme is typically added on a per weight of substrate basis. As the solids loading increases so must the amount of enzyme. While the cost of enzymes has decreased drastically over the years due to intense research developing cheaper production schemes, the cost is still at a level that makes this step in the conversion process one of the most expensive. Finding or developing enzymes with a high activity and inexpensive method of production would greatly benefit the entire conversion process. Moreover, it is also important to evaluate the economics when determining the balance between the loadings applied to the lignocellulose and the amount of time needed to reach sufficient glucose concentrations.

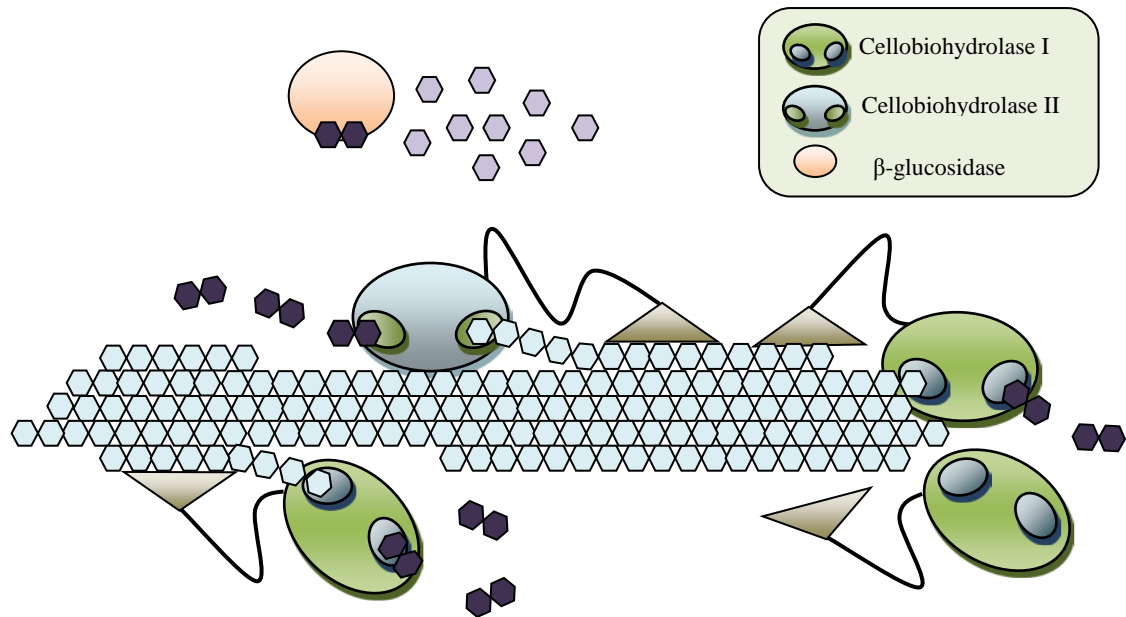


Figure 3.2. The processive movement of cellulases along a bundle of cellulose. The large dimensions of the cellulases cause overcrowding of the accessible cellulose chains.

3.4 IMPACTING RHEOLOGY OF HIGH-SOLIDS MIXTURES

Rheology is the branch of physics that deals with the deformation and flow of matter. At higher lignocellulose loadings, fundamental understanding of the rheology of these suspensions becomes a powerful tool in designing conversion equipment and processes (Ehrhardt et al. 2010; Knutsen and Liberatore 2009; Stickel et al. 2009; Viamajala et al. 2009). Factors which contribute to the rheological properties of a suspension include particle size distribution, particle aspect ratio, fiber flexibility (Knutsen and Liberatore 2009; Samaniuk et al. 2012) and physio/chemical properties of the substrate. Water retention value (WRV) of the substrate directly impacts the apparent viscosity of a suspension, affecting mixing and handling of the slurries (Rosgaard et al. 2007). For example, pretreated corn stover (PCS) slurries are considered “pourable” when yield stresses are at or below ~10 Pa or ~10% insoluble solids (Roche et al. 2009a; Stickel et al. 2009). Dilute acid PCS at 20% insoluble solids is a thick, paste-like substance that can be molded and formed into shapes that remain even after the applied forces are removed (Stickel et al. 2009). At even higher solids loadings (>30%), particles are not as lubricated because of the lack of free water, resulting in increased friction due

to particles interacting with both water and other particles. At this point, the mixture can no longer be called a slurry because it is unsaturated and acts more like a wet, granular substance. Substances with these varied rheological properties present many unique challenges in materials handling throughout a conversion process, particularly when continuous, industrial-scale processes are desired.

Several rheological models of interest, like the Bingham, Herschel-Buckley, Power Law, Wildemuth-Williams and Casson models (Dibble et al. 2011; Ehrhardt et al. 2010; Roche et al. 2009a; Um and Hanley 2008; Viamajala et al. 2009), have been developed to describe the non-Newtonian behavior of these types of systems, but discussion of these models is beyond the scope of this paper.

Um and Hanley (2008) analyzed rheological properties of high solids (10-20% w/v) enzymatically hydrolyzed slurries of the model cellulose feedstock Solka Floc, a delignified spruce pulp. Commercially-available *Trichoderma longibrachiatum*-sourced enzymes (30 FPU/g cellulose supplemented with β -glucosidase) were evaluated at 10, 15 and 20% solids loadings. The enzymatic suspensions exhibited a pseudoplastic behavior overall, with viscosities ranging from 0.04 to 0.01, 0.23 to 0.03, and 0.29 to 0.04 Pa·s for substrate concentrations of 10, 15 and 20% (respectively) initial solids measured at 50°C. As the hydrolysis progressed, a decrease in viscosity was observed for all solids loadings (dropping by approximately half in 3 hours). Zhang et al. (2010) showed the same trend with high-solids steam exploded corn stover. Several studies using dilute acid-pretreated corn stover also observed a reduction in yield stress (and therefore viscosity) as solids loadings in enzymatic hydrolysis decreased (Figure 3.3) (Dibble et al. 2011; Ehrhardt et al. 2010; Knutsen and Liberatore 2009; Roche et al. 2009a; Viamajala et al. 2009).

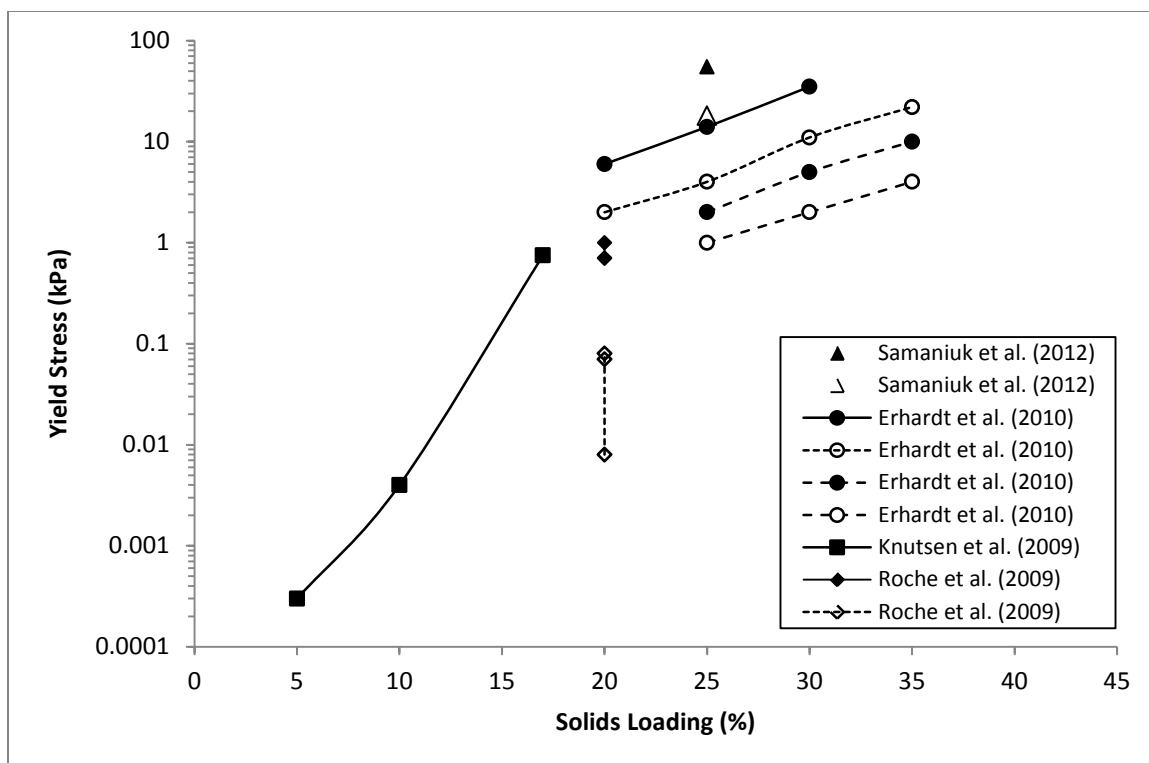


Figure 3.3. Yield stress measurements as a function of solids loadings from studies investigating rheological properties of dilute acid-pretreated corn stover (used in all studies, except Samaniuk et al. (2012), who used untreated corn stover). Additional yield stress measurement conditions include addition of 2% carboxymethyl cellulose (CMC) to untreated corn stover (Samaniuk et al. 2012), elevated temperatures (Ehrhardt et al. 2010), solids loadings at 25°C (Knutsen and Liberatore 2009), and enzymatic hydrolysis at 0 and 24 hr for different enzyme loadings (Roche et al. 2009a). Yield stress was measured by parallel plate flow and vane-in-cup geometries (Knutsen and Liberatore 2009; Roche et al. 2009a) or torque rheometry (Ehrhardt et al. 2010; Samaniuk et al. 2012).

Additionally, Roche et al. (2009a) found that at 20% solids, >40% conversion was necessary for the slurry to become pourable. They also reported a distinct difference between PCS that was enzymatically hydrolyzed as compared to PCS that was just diluted. The yield stress for diluted PCS is higher by a full order of magnitude than that of hydrolyzed PCS at corresponding particle volume fractions. Although specific mechanisms for this difference were not investigated, one theory is that the enzymes alter the particles during hydrolysis, converting them from complex networks of material with distinct liquid and solid phases, to a homogeneous slurry as the liquid and solid phases become indistinguishable.

Particle size affects the rheological properties of the suspensions, directly impacting mixing and pumping costs (Dibble et al. 2011). Viamajala et al. (2009) found that smaller particle sizes resulted in smaller apparent viscosities under equivalent conditions. Mechanical pretreatment is often utilized to reduce particle size to make the rheological properties more favorable for other steps downstream in the process. However, temperature and acid concentration in dilute acid pretreatment directly affect yield stress of a slurry, possibly as a result of a reduction in particle size, as well as enhancing enzymatic hydrolysis due to the modification of the surface chemistry of the particles (Dibble et al. 2011; Ehrhardt et al. 2010). While a reduction in particle size lowers viscosity, as well as increases conversion efficiency, the manner in which the size reduction occurs is also important. Size reduction via pretreatment provides better digestibility and a reduced yield stress as compared to mechanical size reduction, which did not significantly impact either property (Dibble et al. 2011). In some cases, the pretreatment, like dilute acid pretreatment, hydrothermal pretreatment or SPORL (sulfite pretreatment to overcome recalcitrance of lignocelluloses) performed prior to the hydrolysis step alters the structure of the biomass significantly so that liquefaction occurs quickly upon addition of the enzymes and mixing can resume (Jorgensen et al. 2007b; Zhu et al. 2011a). However, in most cases, the solid fraction is still a complex network of fibrous material (Ehrhardt et al. 2010; Szijarto et al. 2011b; Viamajala et al. 2009). Sufficient mixing is required for timely hydrolysis of the biomass, and traditional mixing methods like stirred-tank reactors with impellers require excessive power and shaking does not provide adequate mixing. Several mixing alternatives are discussed in a later section.

The pulp and paper industry has long used additives to modify rheological properties of lignocellulosic slurries (Samaniuk et al. 2012). Knutsen and Liberatore (2010b) found that the most effective additive groups (in descending order) to reduce yield stress were surfactants, additives with polar head groups, additives with hydrophobic tails, unmodified protein and polymers. CTAB (cetyl trimethylammonium bromide) and CPCl (cetylpyridinium chloride), both surfactants, were two of the most effective additives for reducing yield stress. Samaniuk et al. (2012) used water soluble polymers (WSPs) like carboxymethyl cellulose (CMC), polyethylene oxide (PEO) and

polyacrylamide (PAM), to modify rheological properties of lignocellulosic slurries. Additives like CMC reduced the friction between cellulose surfaces, making it easier to mix high-solids suspensions. The addition of 2% CMC reduced the yield stress by ~67% from 55 kPa to ~18 kPa. A four-fold increase in CMC resulted in reducing by another 50%. They also found that a lower degree of substitution for CMC had a positive impact on the yield stress; however, this trend was more apparent at higher CMC loadings. Furthermore, a reduction in yield stress was observed as the molecular weights of the WSPs increased up to a certain point. For example, yield stress decreased with the addition of 600 kDa, as well as 2000 kDa, PEO, but no further change in yield stress was observed with the addition of 7000 kDa PEO. Several other additives were screened by monitoring the reduction in torque as measured by a torque rheometer to determine whether they warranted further investigation. Fly ash and microcrystalline cellulose were evaluated as possible additives, but their impact was limited. The surfactant Polysorbate 80 reduced the yield stress by 36% but required high concentrations (10%). Guar gum, hydroxypropyl methyl cellulose (HPMC), a guar gum-xanthan gum mixture and a guar gum-HPMC mixture were all more effective than CMC, where guar gum and the two mixtures containing guar gum resulted in the highest reduction in torque (~80%). The addition of additives may be costly, but like the pulp and paper industry, it may become economically feasible to utilize such methods of modification for high-solids conversion processes. It is important, however, that these additives be as inexpensive as possible and do not negatively impact the conversion process by inhibiting the hydrolytic enzymes or fermentative organisms.

3.5 IMPACTING ENZYMATIC HYDROLYSIS RATE AND EXTENT

The term “lignocellulosic biomass” refers to many different types of biomass, including forestry and agricultural residues (woody biomass, straw, stover), fermentation by-products (DDGS) and dedicated energy crops (grasses), just to name a few. Each type of lignocellulosic material is slightly different in regards to composition, resulting in unique challenges in the enzymatic hydrolysis step of the conversion process. The following sections are organized based on various aspects in need of consideration during the conversion of lignocellulose and highlight some of the challenges and breakthroughs

associated with enzymatic hydrolysis performed at high-solids loadings for different types of biomass. It is important to note that while each of these processing approaches are discussed individually, it is often difficult to separate out the combined effects of multiple process conditions.

Furthermore, when determining cellulose conversion, it is important to note that the standard method of calculating conversions as described by (Brown and Torget 1996) can grossly overestimate actual conversion for high-solids systems. In some instances, conversions can be overestimated by up to 36% (Kristensen et al. 2009a). Determining cellulose conversion in high-solids systems can become very complicated, but several studies have proposed new methods for determining cellulose conversion (Kristensen et al. 2009a; Zhang et al. 2007; Zhu et al. 2011b) under these high solids operating conditions. The standard method for conversion calculations typically compares the amount of glucose measured in the hydrolyzate (the liquid fraction) to the potential glucose found in the biomass (the solid fraction). This method requires the assumption that all components have a consistent density throughout the reaction and that it is approximately equal to that of water. As solids loadings increase, this assumption no longer remains valid, resulting in overestimated conversions.

3.5.1 Biomass Processing

Enzymatic hydrolysis is an intermediate step in the conversion process, and while producing high sugar yields is favorable, the resulting hydrolyzate must be subsequently capable of supporting fermentative organisms while they produce biofuels. Some of the more expensive steps in substrate preparation are washing the substrate following pretreatment and detoxifying the hydrolyzate produced during enzymatic hydrolysis. It is likely that for industrial processes unwashed, whole slurries (liquid + solids) from pretreatment will be used in enzymatic hydrolysis (Hodge et al. 2008), indicating a need for robust enzymes capable of maintaining their activity in the presence of possible inhibitors and degradation products or developing pretreatments that do not produce such products. Furthermore, the cost of hydrolyzate detoxification alone can be up to 22% of the total ethanol production cost (Lau et al. 2008).

Several studies have investigated the effects of eliminating washing and/or detoxifying steps in the lignocellulose conversion process, with some promising results. Hodge et al. (2008) studied the effects of soluble and insoluble inhibitors on enzymatic hydrolysis by comparing the glucose yields produced from a washed pretreated substrate (which introduces only potentially insoluble inhibitors into the hydrolysis reaction since all soluble inhibitors are washed away) and an unwashed whole slurry substrate (which introduces both potentially soluble and insoluble inhibitors to the hydrolysis reaction). However, to maintain the high-solids loading and modify the pH, the solid and liquid fractions were separated, the liquid fraction pH was adjusted, and the two fractions were combined. Should the whole slurry be used at the industrial scale (as this study states in its rationalization for this work), this method of pH modification may not be feasible. This challenge is just one of many that must be solved prior to implementing a complete conversion process. Regardless, this study utilized an insoluble solids loading of 5-13% (~9-24% total solids loading) and relatively low enzyme loadings (<20 FPU/g cellulose). Based on the glucose production from hydrolysis, the authors suggested that the limitations due to mass diffusion are more prevalent than the sugar inhibition beyond a specific solid content. For instance, sugar inhibition would result in a “leveling-off” of the hydrolysis rate, much like what would be seen in a typical hydrolysis curve. However, a sharp decrease in the hydrolysis rate was reported here. Using the washed substrate, this decrease is not prevalent until ~20% insoluble solids loadings are reached, where convective mixing and available water are negligible, likely indicating the point of mass transfer limitations. This decrease occurs at much lower solids loadings (<10% insoluble solids) for unwashed substrate, indicating that the soluble components contributed to a higher rate of enzyme inhibition or limited mass transfer by reducing the amount of water available for reaction. (Further discussion on the restriction of water can be found in Section 4.4 Solids Effects.)

Pristavka et al. (2000) also conducted enzymatic hydrolysis studies with SO₂-catalyzed steam exploded willow. These studies were concerned with simplifying the conversion process by neglecting to wash the pretreated willow between the pretreatment and hydrolysis steps and eliminating mechanical stirring of the biomass slurry. The reason for eliminating the washing step was two-fold. First, less water would be used in

the conversion process, making the process more economical and more environmentally friendly. Secondly, washing usually leads to the solubilization and removal of a significant portion of sugars. These sugars ultimately end up accumulating in wastewater, resulting in an expensive processing step to recover them and/or treating the water. The high-solids loadings (up to 25% ODM (organic dry matter)) used in this study would make mechanical stirring of the slurry extremely energy intensive, so it was removed. With these process modifications, a lower degree of conversion was observed as compared to biomass that was washed prior to hydrolysis (53% vs. 74%). However, the degree of cellulose conversion increased to >95% when the pH of the unwashed, pretreated willow was adjusted with solid NaOH to the optimal pH of the enzymes. The significant increase in conversion following pH adjustment highlights the importance of maintaining optimal hydrolysis conditions for the enzymes, even if that means finding new, inexpensive and less resource-intensive methods of doing so.

Lu et al. (2010) investigated the effects (post-pretreatment) washed substrate had on enzymatic hydrolysis and fermentation. Using steam-exploded corn stover, substantial differences in conversion efficiencies were not observed for washed and unwashed substrates up to a solids loading of 30% (w/w). However, closer examination of the conversion calculations revealed differences between washed and unwashed substrates, since conversions were based on water insoluble solids and not total solids content. (Essentially the denominators were different for the two treatments.) Additionally, the pH of the unwashed corn stover was not adjusted prior to addition of enzymes and buffer at pH 4.8. Cellulose conversion remained fairly consistent (70-75%) for all solids loadings, although glucose content was higher for the washed substrate than the unwashed substrate. Ethanol production was also independent of solids loading (up to 30% w/w) for the water-washed corn stover, reaching 92-94% of theoretical yield. However, the results were quite different for the unwashed substrate. At the lower solids loadings studied (10-15% w/w), ethanol production fell to 88% and 86%, respectively, and decreased as the solids loading increased, until no ethanol could be measured ($\geq 25\%$ solids loading). The levels of acetic acid and furfural measured at the higher solids loading reached inhibitory concentrations. Inclusion of the water-washing step following

pretreatment appears to eliminate the need for another costly detoxification step following enzymatic hydrolysis for steam-exploded corn stover.

In contrast to this study, others report contradicting results regarding the wash step (Lau and Dale 2009; Lau et al. 2008). Lau et al. (2008) reported that when AFEX-pretreated corn stover was fermented following enzymatic hydrolysis at 18% (w/w) solids loading, the ethanol yield of ~93%, even though the solids loading during hydrolysis and glucose concentration before fermentation were similar to those reported in Lu et al. (2010) who reported a 68% ethanol yield. While these results are so different, it should be noted that different pretreatments, as well as fermentative organisms were used (*E. coli* vs. *S. cerevisiae*, respectively), making it difficult to directly compare these fermentation results. However, Lau and Dale (2009) achieved higher ethanol production rates fermenting unwashed substrates (~0.17 g/L/hr as compared to 0.12 g/L/hr for washed substrate) with *S. cerevisiae* 424A (LNH-ST) (a genetically modified strain for improved xylose fermentation), suggesting that the elimination of the washing step following pretreatment, and with no adjustments made to the pH prior to hydrolysis, is beneficial for fermentation under the conditions examined in this study. Ethanol concentration from unwashed substrate was 40 g/L (no data given for washed substrate). Xylose metabolism from the genetically modified strain is likely the largest contributing factor to the discrepancy in reported ethanol yields, but it was also reported that the this strain of *S. cerevisiae* performed similarly on washed substrate as compared to unwashed substrate. This study suggests that the washing step can be eliminated without any loss in ethanol yield. Contradictory results indicate the need for further study of this issue, or at the very least, optimization studies under specific process conditions.

In another study, LHW-pretreated sweet sorghum bagasse was hydrolyzed at 15-30% solids (w/v) with either 20 or 30 FPU/g glucan cellulase (Wang et al. 2012). Washing the substrate prior to hydrolysis also did not improve the conversion rates. Washed substrate yielded 63.2 g/L of sugar, whereas the unwashed substrate resulted in a sugar concentration of 66.1 g/L. It was suggested, although not verified, that the washing step actually removed some of the smaller cellulose particles that may have been easier to hydrolyze than larger cellulose particles.

The inconclusive results of these studies illustrate the complexity of defining appropriate processing conditions that work in all situations. Operating conditions must be chosen carefully in order to realize the full potential of using lignocellulose as a valuable energy source. Table 3.1 illustrates the wide variety of operating conditions that have been studied with regards to high-solids loadings enzymatic hydrolysis. Depending on various factors, like substrate choice, pretreatment conditions and hydrolysis conditions, it may be possible to eliminate certain steps like washing pretreated substrate or detoxifying hydrolyzate prior to fermentation, thus simplifying the overall conversion process. However, elimination of these steps may present new problems that must be solved. For instance, should the washing step following pretreatment be eliminated, it may be necessary to adjust the pH in another manner so the hydrolytic enzymes can work most effectively.

Table 3.1. Conditions of conversions from enzymatic hydrolysis using high-solids loadings.

Substrate	Solids Loading ^a	Pretreatment	Enzyme Loading	Hydrolysis Conditions	% Conversion	Reference
Woody Biomass						
Aspen wood chips	10% (w/v)	Steam + SO ₂	17 FPU/g solids + β -glucosidase	96 hr at 45°C and 140 rpm	100%	(Schwald et al. 1989)
Willow	21%	SO ₂ steam explosion	42 FPU/g DM + pectinase + β -glucosidase	24 hr at 50°C	100%	(Pristavka et al. 2000)
Olive tree pruning biomass	20% (w/v)	Liquid hot water	15 FPU/g solids	72 hr at 50°C and 150 rpm	64%	(Cara et al. 2007)
	30% (w/v)				50%	
	20% (w/v)	Steam explosion			55%	
	30% (w/v)	40%				
Mixed hardwood chips	20%	Green liquor	20 FPU/g cellulose + β -glucosidase + xylanase	48 hr at 50°C and 90 rpm	63%	(Xue et al. 2012)
Hardwood pulp	20%	--	20 FPU/g cellulose + 80 CBU/g cellulose	96 hr at 50°C and 20 rpm (peg mixer)	80%	(Zhang et al. 2009)
Poplar	20%	Organosolv			83%	
Poplar	20%	Steam explosion	NR	48 hr at 50°C	44%	(Di Risio et al. 2011)

Table 3.1, continued. Conditions of conversions from enzymatic hydrolysis using high-solids loadings.

Substrate	Solids Loading ^a	Pretreatment	Enzyme Loading	Hydrolysis Conditions	% Conversion	Reference
<i>Agricultural Residues</i>						
Corn stover	28%	Dilute acid	22 FPU/g cellulose	168 hr at 45°C and 130 rpm	73%	(Hodge et al. 2008)
Corn stover	12-15%	Dilute acid	10.7 FPU/g cellulose	266 hr at 45°C and 400 rpm	~80%	(Hodge et al. 2009)
Corn stover	20%	Dilute acid	12 FPU/g cellulose	168 hr at 48°C and 4 rpm	77%	(Knutsen and Liberatore 2010b)
Corn stover	10%	Ethanol Steam	5 FPU/g cellulose + 5 CBU/g cellulose	72 hr at 50°C and 150 rpm	51% 66%	(Chandra et al. 2011)
Corn stover	30% (w/v) 1-stage hydrolysis	Steam explosion	30 FPU/g cellulose	72 hr at 50°C and 150 rpm	60%	(Yang et al. 2011)
	30% (w/v) 3-stage hydrolysis			30 hr at 50°C and 150 rpm	81%	
Corn stover	15%	Steam explosion	20 FPU/g solids	96 hr at 50°C and 220 rpm	75%	(Lu et al. 2010)
	20%				74%	
	25%				74%	
	30%				73%	

Table 3.1, continued. Conditions of conversions from enzymatic hydrolysis using high-solids loadings.

Substrate	Solids Loading ^a	Pretreatment	Enzyme Loading	Hydrolysis Conditions	% Conversion	Reference
Corn stover	20%	Screw extrusion at 100 rpm and 100°C	15 FPU/g cellulose	96 hr at 70°C and 150 rpm	29%	(Zambare et al. 2011)
Corn stover	16.2%	Washed AFEX	15 FPU/g cellulose +	96 hr at 50°C and 250 rpm	NR	(Lau and Dale 2009)
	17.6%	Unwashed AFEX	32 <i>p</i> NPGU/g cellulose + xylanase + pectinase		NR	
Corn stover	18%	AFEX	15 FPU/g cellulose + 32 <i>p</i> NPGU/g cellulose + xylanase + pectinase	96 hr at 50°C and 250 rpm	NR	(Lau et al. 2010)
Corn stover and DDGS	18%	AFEX	15 FPU/g cellulose + 64 <i>p</i> NPGU/g cellulose	144 hr at 50°C and 200 rpm	>95%	(Lau et al. 2008)

Table 3.1, continued. Conditions of conversions from enzymatic hydrolysis using high-solids loadings.

Substrate	Solids Loading ^a	Pretreatment	Enzyme Loading	Hydrolysis Conditions	% Conversion	Reference
DDGS	20%	AFEX	15 FPU/g cellulose + 40 U β -glucosidase/g cellulose + 40 U xylanase/g cellulose + 1.2 U FAE/g solids	72 hr at 50°C	75%	(Dien et al. 2008)
Barley straw	10%	Steam	7.5 FPU/g solids + 13 CBU/g solids	72 hr at 50°C	73%	(Rosgaard et al. 2007)
	15%				81%	
	5 + 5 + 5%				65%	
	10 + 5%				68%	
Barley straw	15% (w/v)	Steam explosion	7 FPU/g solids + 8.4 IU β -glucosidase/g solids + 72 U xylanase/g solids	120 hr at 50°C and 150 rpm	59%	(Garcia-Aparicio et al. 2011)
Wheat straw	20%	Steam	7 FPU/g DM + β -glucosidase	96 hr at 50°C and 6.6 rpm	60%	(Jorgensen et al. 2007b)
	30%				42%	
	40%				35%	
Wheat straw	30%	Steam	5 FPU/g DM + β -glucosidase	96 hr at 50°C and 6.6 rpm	41%	(Jorgensen 2009)

Table 3.1, continued. Conditions of conversions from enzymatic hydrolysis using high-solids loadings.

Substrate	Solids Loading ^a	Pretreatment	Enzyme Loading	Hydrolysis Conditions	% Conversion	Reference
Wheat straw	9 + 8 + 7 + 6% (w/v)	NaOH	9.6 FPU/g solids	144 hr at 50°C and 120 rpm	39%	(Zhang et al. 2012)
Rye straw	17.5% 17%	Soda pulp LHW	13 FPU/g cellulose + 35 CBU/g cellulose	48 hr at 45°C and 120 rpm	40% 65%	(Ingram et al. 2011)
<i>Other Biomass</i>						
Creeping wild ryegrass	20%	Dilute acid	150 FPU/g cellulose + 150 CBU/g cellulose	NR	90%	(Quiroga et al. 2010)
Prairie cord grass	20%	Screw extrusion at 100 rpm and 100°C	15 FPU/g cellulose	96 hr at 70°C and 150 rpm	47%	(Zambare et al. 2011)
Sugarcane bagasse	9 + 8 + 7 + 6% (w/v)	NaOH	9.6 FPU/g solids	144 hr at 50°C and 120 rpm	55%	(Zhang et al. 2012)
Sweet sorghum bagasse	20% (w/v)	LHW	30 FPU/g cellulose	72 hr at 50°C and 100 rpm + 0.175 mL Tween80/g solids	60%	(Wang et al. 2012)

Table 3.1, continued. Conditions of conversions from enzymatic hydrolysis using high-solids loadings.

Substrate	Solids Loading ^a	Pretreatment	Enzyme Loading	Hydrolysis Conditions	% Conversion	Reference
Cassava bagasse	15% (w/v)	Dilute acid	20 FPU/g DM	72 hr at 50°C	65%	(Ma et al. 2011)
	20% (w/v)				56%	
	25% (w/v)				50%	
	10 + 7.5 + 7.5% (w/v)				74%	
Filter paper	15%	--	10 FPU/g DM + β -glucosidase	96 hr at 50°C and 60 rpm	48%	(Kristensen et al. 2009b)
	20%				44%	
	35%			96 hr at 50°C and 6 rpm	33%	
Bacterial cellulose	20%	--	10 mg cellulase + 10 mg β -glucosidase/g cellulose	72 hr at 50°C	85%	(Roberts et al. 2011)
Solka Floc	28%	--	18 FPU/g cellulose	120 hr at 50°C	33%	(Lavenson et al. 2012)

^aSolids loadings are reported on (w/w) wet basis unless otherwise noted.

Abbreviations: NR, Not reported; DDGS, Distiller's dried grains and solubles; AFEX, Ammonia fiber explosion; LHW, Liquid hot water; FPU, Filter paper unit; CBU, Cellobiase unit; *p*NPGU, p-nitrophenyl- β -D-galactopyranoside unit; U or IU, International unit; FAE, Feruloyl esterase; DM, Dry matter

3.5.2 Feeding Strategies

Fed-batch feeding schemes have been investigated as an alternative method of achieving high-solids loadings in enzymatic hydrolysis (Chandra et al. 2011; Hodge et al. 2009; Rosgaard et al. 2007; Yang et al. 2011) because of some of the advantages it offers over single feeding schemes. For instance, the initial viscosity is lower, so diffusion and mixing limitations can be minimized or altogether avoided. A fed-batch feeding regime also allows time for the slurry to liquefy before adding additional solids, which maintains a level of free water that is available for the reaction process and for diffusion (away from the enzymes) of potentially inhibitory products that result from the hydrolysis reaction. However, when a fed-batch approach is selected, one must consider how and when to add substrate, as well as enzymes, to the reaction in order to maintain high rates of conversion. Table 3.2 illustrates the variety of substrate and enzyme application rates used in fed-batch studies.

Table 3.2. Substrate and enzyme application times for fed-batch hydrolysis.

Substrate	Pretreatment	Substrate Additions	Total Solids Loading ^a	Time of Additions	Duration of Hydrolysis	Enzyme Application ^b	% Conversion	Reference
Corn stover	Dilute acid	variable - maintained 15% insoluble solids	25%	approximately every 24 hr	288 hr	proportional	~80%	(Hodge et al. 2009)
Corn stover	Steam	2.5% + 2.5% + 2.5% + 2.5%	10%	0, 3, 6, 9 hr	72 hr	whole	60%	(Chandra et al. 2011)
			10%	0, 24, 48, 72 hr	192 hr	whole	62%	
Corn stover	Steam explosion	12% + 6% + 6% + 6%	30%	0, 12, 36, 60 hr	144 hr	proportional	60%	(Yang et al. 2011)
Wheat straw	NaOH	9% + 8% + 7% + 6%	30%	0, 8, 24, 48 hr	144 hr	whole	35%	(Zhang et al. 2012)
Barley straw	Steam	5% + 5% + 5%	15%	0, 6, 24 hr	72 hr	proportional	64%	(Rosgaard et al. 2007)
		10% + 5%	15%	0, 24 hr			69%	
		5% + 5% + 5%	15%	0, 6, 24 hr	whole	65%		
		10% + 5%	15%	0, 24 hr	68%			

Table 3.2, continued. Substrate and enzyme application times for fed-batch hydrolysis.

Substrate	Pretreatment	Substrate Additions	Total Solids Loading ^a	Time of Additions	Duration of Hydrolysis	Enzyme Application ^b	% Conversion	Reference
Sweet sorghum bagasse	LHW	7.5% + 3.75% + 3.75%	15%	0, 24, 48 hr	120 hr	proportional	59%	(Wang et al. 2012)
		10% + 5% + 5%	20%				60%	
		15% + 7.5% + 7.5%	30%				54%	
Cassava bagasse	Dilute acid	10% + 7.5% + 7.5%	25%	0, 6, 12 hr	72 hr	proportional	84%	(Ma et al. 2011)
						whole	74%	
Sugarcane bagasse	NaOH	9% + 8% + 7% + 6%	30%	0, 8, 24, 48 hr	144 hr	whole	51%	(Zhang et al. 2012)

^aTotal solids loadings are based on the amount of total insoluble solids had all substrate been added initially

^bEnzyme application is based on when the enzyme was applied to the system: 'whole' denotes one enzyme application added initially; 'proportional' denotes that enzyme was applied with each substrate application

Hodge et al. (2009) conducted a study in which the fed-batch approach was utilized in order to achieve a final insoluble solids content of 15% (w/w) (equivalent to a 25% (w/w) initial solids loading). This solids loading was the upper limit of unhydrolyzed pretreated corn stover that could be effectively mixed in the stirred tank reactors (STRs) available to the researchers. High cellulose conversion (>80% cellulose conversion) was reported; however, the reaction time was more than double the typical hydrolysis reaction time (168 hrs vs. 72 hrs). The extended time problem may be overcome through the use of higher enzyme loadings or enzymes that can tolerate higher sugar concentrations. The enzyme loading used in this study was 10.7 FPU/g cellulose, a relatively low loading, and it was applied proportionally with each addition of substrate. A study conducted by Yang et al. (2011) obtained a similar cellulose conversion (70.6%), with a higher solids loading (30%), an enzyme loading almost twice (20 FPU/g cellulose) that used in the former study and with a much shorter reaction time (30 hrs). Both studies attribute the high conversion rate, at least in part, to the fact that the substrates were washed prior to hydrolysis, possibly eliminating any potential inhibitory products that resulted from the pretreatments. The latter study also supplemented fresh enzyme with each addition of new biomass, which increased the final enzyme loading from 10 to 15 FPU/g cellulose. The fresh enzyme may have also enhanced the glucose yield, replacing the enzyme that may be non-productively bound to the lignin or deactivated by extended hydrolysis times.

Zhang et al. (2012) studied another fed-batch approach for the conversion of NaOH-pretreated sugarcane bagasse and wheat straw. Pretreated biomass was fed into the reactor at 9%, 8%, 7%, and 6% solids over the course of 48 hrs to achieve a final solids loading of 30% (w/v). All enzymes were added with the first addition of lignocellulose. Glucose conversion from wheat straw reached a maximum (~60%) after the first feeding, but decreased with each successive feeding. The higher rate of conversion was likely due to the low solids loading and high enzyme loading at the beginning of the reaction. With each successive feeding, the enzyme: substrate ratio decreased. After 72 hr of hydrolysis, the conversion began to level off, resulting in a final glucose conversion of 39%. A slightly different conversion profile was observed

with the bagasse. The conversion continued to increase over the course of the hydrolysis reaction, with the exception of the last feeding time (6% solids at 48 hr). The final feeding resulted in a sharp decrease in conversion, but it recovered within 24 hr following the feeding, leading to an increase in conversion over the batch. The final glucose conversion of the sugarcane bagasse was 55%. Differences in the way the pretreatment affected the lignocellulose may have led to the different glucose yields between the two substrates. It was reported that the pretreatment caused the surface of the two substrates to become rough and fragmented as lignin was removed, allowing for better access to the cellulose; however, the bagasse appeared to have a rougher, more fragmented surface than the wheat straw. Following 144 hr of hydrolysis, the surfaces were relatively smooth as compared to the start of the hydrolysis.

Wang et al. (2012) considered the use of a fed-batch feeding scheme. Initially, the reactors were charged with half of the final solids loading, followed by two additional feedings at 24 and 48 hr of one-fourth of the final solids loading. The system containing 30% solids achieved the highest final sugar concentration with nearly 115 g/L. Even with the fed-batch system, the conversion decreased with increasing solids loadings; however, the conversion of the 30% solids reaction was only 5% less than the systems at 15% and 20% solids (55% vs. ~60%, respectively).

Fed-batch was utilized by Ma et al. (2011) to achieve a 25% (w/v) solids loading. Enzymes were added either all at once at the beginning of the reaction or with each addition of the dilute acid pretreated cassava bagasse. At this solids loading, the batch reaction reached ~50% conversion, whereas the fed-batches with a single enzyme addition and multiple enzyme additions achieved ~75% and 84% conversion, respectively. These results are similar to those reported in other fed-batch studies (Hodge et al. 2009; Yang et al. 2011), indicating that under the right conditions fed-batch systems may be a plausible solution for achieving higher conversion rates for hydrolysis performed at high-solids loadings.

Rosgaard et al. (2007) investigated several different regimes for batch and fed-batch hydrolysis, including variations of sequential addition of substrate as well as substrate plus fresh enzyme. The addition of fresh enzyme with each substrate addition maintained a constant enzyme: substrate ratio throughout the whole reaction, as opposed

to the other fed-batch feeding schemes where all the enzyme was added in one application. In these cases, the effective enzyme: substrate ratio decreased with each subsequent addition of substrate. Not surprisingly, the fed-batch schemes that received the full enzyme application at the start of the reaction produced higher glucose yields during the first few hours as compared to the fed-batch reactions that received fresh enzyme with each substrate addition. However, the extent of the hydrolysis reaction was not affected by the method of enzyme application as the final glucose concentrations were not different for the fed-batch reactions with and without additional enzyme applications (62-67 g/L). Furthermore, lower viscosity is often touted as an advantage of fed-batch systems over batch systems because mixing becomes easier as viscosity decreases. The viscosities of the fed-batch systems in this study were lower than in the batch systems, but no benefits were observed in regards to glucose production as the batch system at 15% solids resulted in higher glucose production (78 g/L) after 72 hr hydrolysis. Final glucose concentrations of the fed-batch systems, though, were impacted by each addition of substrate. Hydrolysis rates decreased and never fully recovered, resulting in lower final yields than the batch systems.

Additionally, Chandra et al. (2011) reported on a fed-batch approach at a moderate solids loading that did not perform as well as a single stage feeding approach. The total solids loadings achieved for both feeding schemes was 10%. Two enzyme loadings were tested (5 and 60 FPU/g cellulose), and at both loadings, the batch reaction produced the higher yields, approximately 66% and 90% for steam-pretreated corn stover, respectively. However, when the solids are fed at 24 hr intervals, the respective yields are lower (approximately 55% and 80%) and the hydrolysis rates slower. The authors suggest these reductions in yields and rates are the result of non-productive binding of enzyme to xylan or lignin fractions of the substrate or the inability of the enzyme to desorb from partially hydrolyzed substrate and find accessible cellulose sites in the fresh substrate. Free protein measurements taken at 72 hr indicate that 50-70% of the cellulase was still adsorbed to the substrate for both enzyme loadings, while the cellulose conversion ceased. The lower hydrolysis rate at the higher enzyme loading seems to indicate that the enzymes are saturating the accessible cellulose sites, thus

reaching a maximum hydrolysis rate that is lower than that of the batch reaction when all the accessible cellulose sites are available at once.

The results of fed-batch feeding schemes are currently still inconclusive, as indicated by the preceding studies, making the decision to use a fed-batch approach unclear. Many advantages are realized regarding the use of fed-batch systems, but questions persist. For instance, at what point in the reaction should subsequent additions of substrate be applied to maintain a high rate of conversion? Should enzymes be added in a single application, as a supplement to the original application, or proportionally to the substrate? Does the benefit of reduced viscosity make a difference in energy consumption during the conversion process to overcome the potentially reduced sugar yield that may result from the fed-batch as compared to the batch system?

3.5.3 Effects of Enzyme Synergism

Enzymatic hydrolysis, especially at high-solids loading, has been identified as the largest impediment to achieving high yields in a timely manner in the lignocellulose to ethanol conversion process, mainly because a significant portion of sugars produced are in oligomeric or polymeric form, which cannot be used in the fermentation process. Several studies have investigated this issue from the perspective of the enzyme (Table 3.1), experimenting with enzyme supplementation (in addition to cellulase) and alternative organism sources for cellulase (Dien et al. 2008; Lau and Dale 2009; Lau et al. 2010; Zambare et al. 2011). Supplementing cellulase with β -glucosidase has long been used to minimize end-product inhibition of the cellulase and achieve higher conversions. Lau et al. (2010) investigated the use of several different enzymes other than cellulase and β -glucosidase to enhance the conversion of lignocellulose. Their enzyme cocktail included xylanase and pectinase to target the hemicellulose that acts as a barrier to cellulose if not removed during pretreatment. The focus of this work was on the fermentation step, so the details regarding the enzymatic hydrolysis are limited. However, the hydrolyzates produced from AFEX-pretreated corn stover with these enzyme cocktails were able to produce 40 g/L (5.1% v/v) of ethanol with *Saccharomyces cerevisiae*.

Another study investigated the effects of supplementing the typical cellulase and β -glucosidase enzyme cocktail with xylanase on the hydrolysis of steam-exploded barley straw (Garcia-Aparicio et al. 2011). The addition of the xylanase to the enzyme mixture enhanced the conversion rate of the cellulose, especially at low solids loading and early in the hydrolysis reaction. Conversion at higher solids loadings may be reduced by the higher concentration of xylooligomers produced with the addition of xylanases, as has recently been shown (Qing et al. 2010). However, the xylanase used in the supplementation study did contain some β -xylosidase activity, which, if present, might counteract the inhibition caused by xylooligomers. The positive effects of the xylanase addition reported in this study support the idea that overall enzyme loadings could be reduced if better conversion is achieved by incorporating an array of different enzymes. However, a different study conducted by Di Risio et al. (2011) also evaluated various enzyme cocktails made from commercially-available enzyme solutions. All three cocktails assessed consisted of the same base solution: cellulase and β -glucosidase. Each solution was supplemented with a third commercial enzyme solution with different active components: cellulase + xylanase, cellulase + xylanase + β -glucosidase, and xylanase. The highest glucose yields (44%) resulted from the enzyme cocktail consisting of the base solution supplemented with the commercial solution containing cellulase + xylanase + β -glucosidase activity. Surprisingly, the enzyme solution supplemented with the enzyme promoted as a “xylanase” actually yielded significantly less xylose than the other two enzyme solutions (39% as compared with 54% and 85%). However, there is no indication that the xylanase activity of this commercial product was independently verified prior to use. Glucose yields ranged from 32%-42%.

Taking it a step further, another group studied the effects of various addition schemes and enzyme loadings using an enzyme cocktail containing cellulase, β -glucosidase and xylanase on the hydrolysis of mixed hardwood chip pulps (Xue et al. 2012). The enzyme cocktails consisted of fungal cellulase (C), xylanases (X) and β -glucosidase (B) solutions mixed in the ratio of 10:3:3 (by volume). The mixtures were added to the substrate in the following manners: (1) cellulase, xylanases and β -glucosidase was mixed with substrate at the desired solids loading (CXB); (2) cellulase was added to 5% solids, pressed or filtered to obtain the desired solids loading, and

hydrolyzed for a period of time before the xylanases and β -glucosidase mixture was added (C+XB); and (3) half of the cellulase was added to 5% solids, pressed or filtered to obtain the desired solids loading, and hydrolyzed for a period of time before the cellulase (half dose), xylanases and β -glucosidase mixture was added (C+CXB). With the CXB mixture, a decrease in conversion was observed with an increase in solids loading. Enzyme loading also plays an important role in the optimization of biomass conversion. For example, with the CXB enzyme mixture, the difference in sugar yields decreased with increased enzyme loadings. At 40 FPU/g solids, conversion decreased from 70% to 68% for 5% and 20% solids loading, respectively, which represents no significant difference in conversion. However, at 5 FPU/g solids, conversion decreased from 40% to 19% for 5% and 20% solids loadings, respectively. The authors hypothesized the decreased conversion was the result of ineffective mixing of the enzyme mixture with the substrate as the solids loadings increased. Based on this hypothesis, the authors added the enzyme to a low solids mixture, allowing time for the enzymes to adsorb to the substrate, before filtering off 80% of the liquid to obtain 20% solids loadings. Enzyme activity was tested following filtration to determine whether any enzyme was lost during this process. Cellulase activity registered at 80% of the original activity, whereas only 20% of the xylanases activity was retained. This observation resulted in the modified application of the enzyme mixture (C+XB). At 20% solids and 20 FPU/g solids, sugar conversion increased from 44% for the CXB mixture to 59% for the C+XB mixture. Sugar concentrations increased from 84 g/L to 114 g/L. This modified enzyme application process was also beneficial at low solids loadings (5%), increasing conversion from 19% with CXB to 38% with C+XB. Taking this enzyme application process one step further, additional cellulase was added with the xylanases and β -glucosidase mixture (C+CXB). In this instance, although the sugar concentration increased to 121 g/L glucose (63% conversion), the conversion at 20% solids was similar to that at 5% solids at all enzyme loadings tested. These experiments indicate the importance of determining enzyme mixtures and application schemes that provide the optimal sugar yields and concentrations for the conversion process.

Along with the feeding scheme and the enzyme loading, the type of enzyme used can have a significant impact on the liquefaction of biomass. The term “cellulase” can

refer to a wide variety of enzymes, and commercially available enzymes can often be a crude mixture of enzymes (i.e. *T. reesei* cellulase that is commonly used in hydrolysis studies). To be more specific, for example, the *T. reesei* “cellulase” can refer to a mixture of cellobiohydrolases (CBH), endoglucanases (EG), xylanases (XYLs), and β -glucosidase, among other enzyme components. Using an array of CBHs, EGs, XYLs and a β -glucosidase, both individually and in combination, Szijarto et al. (2011b) assessed the enzymes on their ability to liquefy hydrothermally pretreated wheat straw. For the *T. reesei* components, it was determined that the EGs (especially Cel5A) were the most important in liquefying lignocellulose. This enzyme alone reduced the viscosity of the slurry by nearly 90%. The CBHs and XYLs had little to no effect on the viscosity, even though the sugar production was similar to that of some of the EGs. Furthermore, a mixture of enzymes produced the highest sugar yields, even though the viscosity was reduced by only about 82%, indicating that the amount of sugar hydrolyzed is not the main factor in reducing viscosity, but that the sites at which the polysaccharides are cleaved is more important.

Since enzymes play such a vital role in the conversion of lignocellulose, much of the process integration depends on these biological catalysts. For instance, a balance must be struck between the enzyme loading used and enzyme cost. High enzyme loadings not only increase the total cost, but as discussed in the introduction, studies suggest that enzymes are overcrowding accessible cellulose chains, thus reducing the rate at which cellulose is hydrolyzed. One such study was conducted by Olsen et al. (2011). At a solids loading of 29% (w/w) pretreated corn stover, a range of enzyme loadings (5-83 FPU/g cellulose) were evaluated for hydrolysis yields. At enzyme loadings >66 FPU/g cellulose, the hydrolysis curves started to coincide. It was suggested that the lack of improvement in hydrolysis rate and conversion was due to the substrate being completely saturated with enzymes bound to all the accessible sites. High enzyme loadings also do not make sense economically. Based on a techno-economic model of the bioethanol conversion process, an optimum total solids loading of about 20% with an enzyme loading of 20 mg/g solids (8.8 FPU/g solids) was determined to produce the minimum ethanol selling price with currently available, commercial enzymes (Humbird et al. 2010). This model evaluated the cost of production at 2007 enzyme production

costs (\$0.35/gal), as well as the enzyme production cost projected by the Multi-Year Program Plan (MYPP) from the DOE's Office of Biomass Program for 2012 (\$0.12/gal) (United States Department of Energy Office of the Biomass Program 2011). At the lower enzyme production cost, solids loadings could potentially be increased up to 26% and remain economically viable. In the time since this study was published, the MYPP re-evaluated the cost of enzyme production and the current projection for 2012 was fairly consistent with the "high" cost of enzyme production reported in the study at \$0.34/gal of ethanol (2007\$). Under the assumptions made constructing this model, 20% solids loading remains the maximum that is economically feasible for the ethanol production process.

Zhang et al. (2009) evaluated enzyme loading to determine the effect it had on glucose concentration. A 50% reduction in enzyme loading decreased the glucose concentration by only 21%. The implication of this observation is that enzyme loading can be optimized to provide the maximum concentration at the lowest unit cost. For example, it may not be worth converting an extra 5% of glucose if it accounts for ~15% of the total enzyme cost unless the return on the extra glucose recovers the cost of the additional enzyme.

While the cellulase system of *T. reesei* is one of the most commonly studied enzyme systems, other organisms also produce cellulolytic enzymes that could potentially impart superior activity under certain conditions. Ingram et al. (2011) compared the conversion efficiencies of enzymes from two different organisms, *T. reesei* and a genetically-modified (for increased cellulase production) strain of *Penicillium janthinellum*. Enzyme mixtures from both organisms contained cellulases, β -glucosidases and xylanase activity. With the cellulase from *T. reesei*, an increase in glucose concentration as biomass loading increased was observed for the organosolv and the LHW-pretreated rye straw. After 48 hrs of hydrolysis at 17.5% solids, the *P. janthinellum* cellulase converted 72% of the soda-pretreated rye straw. Higher enzyme loadings of *P. janthinellum* cellulase were necessary to achieve the same level of conversion produced by the *T. reesei* cellulase (27 FPU/g cellulose vs. 13 FPU/g cellulose); however, the *P. janthinellum* cellulase appeared to be more tolerant to changes in pH. This study highlights the fact that the conversion process is dependent on many

factors, including, but not limited to, the type of biomass, the conditions of the pretreatment, and the source of enzymes.

In another study partially purified cellulase from the thermostable *Geobacillus* R7 was evaluated as an alternative cellulase source (Zambare et al. 2011). For short hydrolysis times (36 hr), the *Geobacillus* cellulase was comparable to a commercial enzyme preparation. However, for hydrolysis of pretreated prairie cord grass using this cellulase, the glucose recovery at 96 hrs for solids loadings $\geq 10\%$ was between 46.2% and 48.7%. It does not appear that the solids loading had much of an impact on conversion of the prairie cord grass; although the conversion of cellulose into glucose utilizing the *Geobacillus* R7 cellulase was better than the conversion of the pretreated corn stover at 27%-31%. *Geobacillus* R7 also has the added benefit of being ethanologenic. During the hydrolysis, *Geobacillus* R7 produced a small amount of ethanol (0.035 g/L) from the pretreated prairie cord grass, which has possible implications for consolidated bioprocessing of lignocellulose materials. Subsequent fermentation of the hydrolyzate with *S. cerevisiae* resulted in an ethanol production of 7.8 g/L (or 0.47 g ethanol/g glucose) for the 20% solids loading of prairie cord grass.

Lastly, Matano et al. (2012) engineered fermentative yeast to express three different types of cellulase on its surface. This yeast was subsequently evaluated in SSF processes utilizing 25% (w/v) pretreated rice straw. Initially, a control yeast strain was supplemented with a commercial cellulase (100 FPU/g biomass). This combination resulted in an ethanol yield of 80% and liquefaction after 72 hr. When combined with the modified yeast strain, the commercial cellulase loading could be reduced to 10 FPU/g biomass and produce the same ethanol yield (79%). Further study showed that a maximum ethanol concentration (43.1 g/L) was obtained following a 2 hr liquefaction period prior to the addition of the modified yeast, corresponding to an ethanol yield of 89%. Residual glucose was reduced by an order of magnitude with the modified strain (16 g/L to 1.6 g/L). The authors hypothesized that the close proximity of the cellulases on the surface of the yeast provided a synergistic effect that resulted in an increased hydrolysis of cellulose. As commercial enzymes are still a relatively large portion of the overall cost of the conversion process, the ability to reduce the commercial enzyme

loading and replace it with an organism capable of both the hydrolysis and fermentation is very attractive.

3.5.4 Solids Effect

For conversion of lignocellulose into usable and valuable products, it makes economical sense to utilize locally-available biomass, as shipping biomass over long distances greatly reduces the beneficial impacts. Cara et al. (2007) studied the conversion of olive tree pruning biomass (consisting of leaves and thin branches) up to 30% (w/v) solids loadings. The final glucose concentrations increased with increasing solids loading, achieving 61 g/L and 52 g/L glucose at 30% solids loading of the liquid hot water (LHW) pretreated biomass and steam exploded biomass, respectively. However, the conversions of the LHW-pretreated biomass decreased nearly linearly from 76.2% at 2% solids to 49.9% at 30% solids. Conversions of the SE-pretreated biomass held steady between 60% and 63% up to 10% solids loading before decreasing to 39.6% at 30% solids. In a different study, the researchers also observed that the glucose concentration decreased as the solids loading was increased beyond 10% solids for the soda pretreated rye straw (Ingram et al. 2011). The overall conversion of cellulose decreased from ~65% to 40% as solids loadings increased from ~10% to 17.5%. This result is not unusual, as most studies performed at high-solids loadings sacrifice conversion for a more concentrated glucose product (Cara et al. 2007; Jorgensen et al. 2007b; Kristensen et al. 2009b).

Kristensen et al. (2009b) also studied four mechanisms that possibly contribute to the so-called solids effect: (1) compositional and substrate effects, (2) product inhibition, (3) water concentration, and (4) cellulase adsorption. These mechanisms were studied with filter paper, which is essentially a pure cellulose substrate. The researchers observed the same decreasing trend in conversion as solids increased using the filter paper, much like that observed with lignocellulose. Therefore, it was concluded that lignin, which is absent in filter paper, is likely not the reason for the solids effect. Study of the second mechanism, product inhibition, resulted in significantly different conversions after 48 hours of hydrolysis for 5% DM and 20% DM (64.5% vs. 38.6% or 30 g/L vs. 86 g/L, respectively). However, the final conversions for these solids loadings

with an additional 50 g/L glucose added resulted in fairly similar conversions (29.7% and 26.3% or 64 g/L vs. 109 g/L for 5% DM + 50 g/L glucose and 20% DM + 50 g/L glucose, respectively). This experiment did not elucidate the exact reason for the observed similar conversions, but two hypotheses were offered. It was suggested that other components in the hydrolysis mask the product inhibition or that enzymes are inhibited similarly once a certain glucose concentration is reached.

Kristensen et al. (2009b) next attempted to quantify the effects of water on the hydrolysis reaction. Water content was decreased by 25% and replaced by oleyl alcohol. The alcohol allowed the viscosity of the slurry to remain constant, thus removing the effects of the viscosity, while the water to solids (or enzyme) ratio was altered. With this decrease in water, a 5% decrease in glucose yield was observed. However, increasing the solids content from 20% to 25% (which is essentially equivalent to a 25% reduction in water), typically decreases glucose yields by $\geq 12\%$. The authors argue this discrepancy in glucose reduction indicates that lower water content is apparently not the limiting factor responsible for the solids effect.

Lastly, cellulase adsorption was investigated as a possible source of the solids effect (Kristensen et al. 2009b). Cellulase adsorption to filter paper was determined by measuring the total nitrogen content of the biomass after 24 hr of hydrolysis. The amount of adsorbed cellulase measured was halved (40% to 17%) as solids loading increased from 5% to 25%. At the same time, conversion was reduced from $\sim 60\%$ to $< 50\%$. A strong correlation between decreasing adsorption and conversion was observed, indicating that cellulase is not effectively adsorbing onto cellulose causing a decrease in yield. The authors hypothesize that increasing concentrations of glucose and cellobiose inhibit the adsorption of enzymes. Knowledge of the mechanisms of high-solids product inhibition and the mechanisms of high-solids enzyme adsorption inhibition can provide the key to improving the overall conversion process, thus unlocking the full potential of high-solids conversions.

In contrast to the previous study, Roberts et al. (2011) investigated the interactions of water with biomass at high-solids loading without maintaining a constant viscosity. Time domain NMR was used to measure the transverse (or spin-spin) relaxation times (T_2) of protons in water molecules to indicate the extent of water

constraint (or degree to which water is tightly bound to biomass). Essentially, the nuclei of water molecules that are tightly bound have a shorter relaxation time than nuclei that are less tightly bound. By measuring these relaxation times, constraint can be determined. It was found that water was more tightly bound as solids loadings increased, suggesting that an indirect relationship between water constraint and yield exists. However, the relaxation time of the primary bound water (water that interacts directly with the surface of the cellulose) was constant regardless of the solids loading. Interactions at the water-solids interface appear to remain constant, suggesting the chemistry at the surface of the cellulose does not change as water content changes. These results further suggest that the water primarily interacts with the cellulose, and the impact of the solute is minimized. However, these studies were conducted with bacterial cellulose, a substrate that is essentially pure cellulose. It is unclear whether cellulose derived from pretreated lignocellulose would interact with water in a similar manner or to what extent the type of pretreatment may affect these cellulose-water interactions. With the addition of excess glucose or mannose to 5% solids, the hydrolysis rate reduced to one similar to 15% solids loading. The authors hypothesize that the negative effects on the hydrolysis rate are caused by water constraint as opposed to the monosaccharides impacting the enzyme activity. It is also possible that the lack of available water limited the uniform distribution of synergistic enzymes, thus hindering the hydrolysis rate. Also, in contrast to the previous study, the results presented in this study indicate that water (or the lack of it) has a great impact on the overall hydrolysis rate. Even though the addition of oleyl alcohol in the former study reduced the water content in the reaction, the constant viscosity helped maintain adequate mixing and therefore did not limit the diffusion of enzymes throughout the suspension. While these studies draw conflicting conclusions on the effect of water on lignocellulose conversion, they do highlight the need for effective mixing. Adequate mixing was provided in the former study, even with a low water: substrate ratio because of the low viscosity afforded by the addition of alcohol, whereas the latter study simply reduced the water: substrate ratio without regard for the viscosity. These studies also highlight the difficulty of quantifying and assigning the challenges of operating at high solids to any one factor (lack of water, high viscosity, adequate mixing, etc.) when all these factors are so interrelated.

3.5.5 Effect of Viscosity on Mixing

High viscosity of high-solids slurries is another hurdle that must be overcome. Much of the previous discussion (i.e. effects of enzymes on liquefaction and solids loadings) also affects the rheology, but this section discusses specific viscosity modifiers and their effects on enzymatic hydrolysis. Ineffective mixing increases the limitations associated with mass transfer, including removal of local inhibitors and hydrolysis products and transfer of heat throughout the reactor. The pulp and paper industry has long been using viscosity modifiers to enhance the processability of fibrous slurries (Knutsen and Liberatore 2010b), much like the types of slurries produced by lignocellulose materials prevalent in the conversion to biofuels and biochemicals. One study (Knutsen and Liberatore 2010b) investigated the use of 18 different chemical additives and evaluated the effects on the slurry rheology and hydrolysis rates. Several surfactants added to lignocellulosic slurries at 2% (w/w), including CPCI, CTAB, sodium dodecylbenzene sulfonate (NaDBS) and sodium dodecyl sulfonate (SDS), positively affected the rheological properties of the slurry by reducing the viscosity by nearly four-fold as compared to the viscosity of the unmodified slurry. Although slight decreases in the extent of the hydrolysis reactions were observed, only the CPCI and the CTAB did not reduce hydrolysis rates. Additionally, Ma et al. (2011) tested the surfactant Tween-80 and found that it did not produce a significant increase in conversion at a 10% solids loading to warrant its use. However, at 25% solids loading, the addition of the surfactant (2 g/L) increased cellulose conversion by 30%. Contrary to what Kristensen et al. (2009b) said, the inhibition caused by non-productive binding of the enzyme to lignin does not seem to have as large of an effect at low solids as it does at high solids. These results show some promise in modifying viscosity properties of lignocellulose slurries; however, more work is warranted to understand the mechanism by which these surfactants work, as well as determining the economical value of the use of such additives.

Another approach to reducing viscosity is to raise the temperature at which the hydrolysis reaction takes place (Szijarto et al. 2011a). In order to work at higher temperatures, enzymes that can tolerate the increased temperatures must be used. It has

been shown that EGs from more thermotolerant organisms worked better at reducing the viscosity of a lignocellulose slurry, while other types of enzymes appeared to have little effect (Szijarto et al. 2011a). *T. aurantiacus* proved to be more thermotolerant than *A. thermophilum*, as the *T. aurantiacus* EG continued to reduce the viscosity at temperatures up to 75°C. *A. thermophilum* enzymes were less active above 65°C, resulting in a reduced effect on the viscosity. The ability to use alternate sources of cellulase enzymes illustrates the number of reaction condition variables (i.e. temperature, components in enzyme cocktail, and solids content in slurry) open to modification.

The method of mixing the slurry can also have a substantial impact on the conversion of lignocellulose. For example, Zhang et al. (2009) observed a significantly reduced liquefaction time when comparing hydrolysis at high solids (17-20% w/w) performed in shake flasks with a lab-scale peg mixer. Peg mixers are commonly used in the pulp and paper industry, which routinely utilizes solids loadings up to 35% (Zhang et al. 2009). (Readers are referred to the section entitled “Reactor design for enzymatic hydrolysis at high solids” for more details on the peg mixer.) Liquefaction occurred after 1 hr of hydrolysis in the peg mixer, whereas the shake flask required 40 hr. The decrease in liquefaction time can most likely be attributed to the effective mixing provided by the peg mixer and the breaking down of the large fiber network that tends to occur as solids loadings surpass 8%. At 20% (w/w) solids loadings, hydrolysis performed in the peg mixer resulted in 144 g/L and 158 g/L of glucose from unbleached hardwood and Organosolv pretreated poplar, respectively. These concentrations are the highest glucose concentrations achieved known to the authors at the time of writing this review.

One of the highest solids loadings in enzymatic hydrolysis reported to date is 40% (w/w) (Jorgensen 2009; Jorgensen et al. 2007b). A horizontally-oriented rotating drum was utilized as the reactor in these studies in order to effectively mix the solids. The studies found that cellulose and hemicellulose conversion decreased from ~90% to ~33% and ~70% to 35%, respectively, with the increase in solids loading from 2% to 40%, but the reactor was providing adequate mixing as evidenced by the conversion of lignocellulose into fermentable saccharides (86 g glucose/kg at 40% solids) (Jorgensen et al. 2007b). At 40% solids, liquefaction occurred after only 4 hrs. The viscosity was still high, as the slurry turned into a thick, clay-like paste and remained as a thick paste

following 96 hrs of hydrolysis. Additionally, the reactor was a very energy efficient solution to the mixing problem. Mixing speed did not affect the liquefaction time, so relatively low speeds (6.6 rpm) could be used. It was also shown that ethanol could be produced in the same rotating drum reactor from the resulting slurries, where the highest ethanol yield (48 g/kg DM) reported was from the slurry at 35% solids. Even at reduced enzyme loadings (5 FPU/g DM supplemented with β -glucosidase at a 5:1 loading), ~40% conversion for both cellulose and hemicellulose can be achieved at 30% solids loading (Jorgensen 2009). These results suggest using one reactor for all processing steps in the conversion of lignocellulose, with the implication that capital and equipment costs can potentially be greatly reduced as both the number of reactors and amount of enzyme used decreases. However, with the yield penalty for conversion at higher solids loadings being high, a full techno-economic analysis would be needed to fully validate such a system operating under the given conditions.

3.5.6 Tools and Methods for Measuring the Progress of Enzymatic Hydrolysis at High-Solids Loadings

As more and more interest is expressed in the use of high-solids loadings in the conversion of lignocellulose, it is also important that tools are available to properly measure and study the progress of the hydrolysis reaction. Calorimetry has been studied as a new tool for determining enzymatic kinetics of high-solids loadings in hydrolysis (Olsen et al. 2011). It provides higher sensitivity than HPLC in the early stages of the hydrolysis, making calorimetry a useful tool to evaluate initial rates of hydrolysis. Avicel showed that enzyme hydrolysis slowed when enzyme loading of >30 FPU/g cellulose were used. It is believed that this reduction in rate is due to the lack of available binding sites on the cellulose, as illustrated by the heat-flow curves converging upon a single value, regardless of the enzyme loading.

Lavenson et al. (2012) also implemented the use of new tools to monitor liquefaction and the extent of hydrolysis of cellulose. Liquefaction and the spatial homogeneity of the enzyme distribution in Solka-Floc suspensions (28% w/w) were monitored with magnetic resonance imaging (MRI). The MRI signal is proportional to the amount of free water in the reaction, which correlates to the degree of liquefaction in

the system. Additionally, a penetrometer was used to monitor the mechanical strength of the suspension. Measurements were taken on two hydrolysis systems, where one contained a mixed Solka-Floc and enzyme suspension and the other contained a Solka-Floc suspension that received an application of enzyme but no mixing. Mechanical strength of the mixed suspension decreased by 20% of the initial strength after ~30 hrs, as compared to ~170 hrs for the unmixed suspension. Based on the MRI results, the mixed samples did not show a spatial gradient, indicating uniform liquefaction when the enzyme and substrate are initially well-mixed. The unmixed samples showed a slow change in spatial gradients, which were attributed to ineffective diffusion of the enzyme to the substrate. Since liquefaction occurs nearly six times faster for the mixed samples, it is not surprising that higher final glucose concentrations are also obtained as compared to the unmixed samples and in much less time. For example, the mixed suspension reached ~75 g/L glucose in only ~120 hrs, whereas the unmixed suspension produced only ~50 g/L in 300 hrs. Furthermore, adequate initial mixing of the enzyme and substrate resulted in an initial rate of hydrolysis an order of magnitude higher (1.8 g/L/hr as compared to 0.21 g/L/hr).

3.6 REACTOR DESIGN FOR ENZYMATIC HYDROLYSIS AT HIGH SOLIDS

Several groups studying the use of high-solids loadings for enzymatic hydrolysis have embraced a horizontal orientation of the reactor (Dasari et al. 2009; Jorgensen et al. 2007b; Larsen et al. 2008; Roche et al. 2009b). Gravitational or free-fall mixing provides many advantages over typical vertical stirred tank reactors and are used in other industrial processes that require mixing highly viscous slurries, like peanut butter, ketchup and concrete (Dasari et al. 2009; Roche et al. 2009b). The horizontal orientation minimizes particle settling and local accumulation of reaction products within the reactor, as well as ensuring better enzyme distribution. These types of reactors are also easily scalable from bench-scale to pilot-scale and larger. Power requirements are lower for horizontal reactors equipped with paddles over vertical stirred tank reactors that provide the same level of effective mixing (Dasari et al. 2009).

Roche et al. (2009b) employed free-fall mixing in their design for bench-scale reactors for enzymatic hydrolysis. Polypropylene bottles (125 mL and 250 mL) were

placed on a roller apparatus in a horizontal orientation. The roller apparatus and bottles were placed in an incubator for temperature control during enzymatic hydrolysis. This roller-bottle system produced results comparable to shake flasks when utilizing intermittent hand mixing, especially following enzyme addition and prior to sampling, for up to 30% solids (data not shown). At 20% solids loading, these two mixing schemes resulted in 80-85% cellulose conversion. The roller-bottle reactors eliminated the human component of mixing, resulting in more consistent mixing and better enzyme and reaction product distribution.

Hydrolysis studies conducted by Dasari et al. (2009) utilized a horizontal reactor of intermediate capacity (8 L). The reactor was constructed from a cylinder made of Pyrex glass with aluminum lids fitted over the ends. An adjustable speed, rotating shaft with rubber-tipped, stainless steel blades attached was inserted into the reactor. Three sampling ports were located along the length of the reactor. Hydrolysis studies comparing the horizontal reactor to shake flasks found, at 25% solids loading, approximately 10% more glucose was produced in the horizontal reactor.

Jorgensen et al. (2007b) developed a reactor for use in pretreatment and enzymatic hydrolysis processes with a total volume of 280 L. Several features have been implemented into the pilot-scale drum reactor, as well as the smaller glass reactor, to address issues associated with high-solids loadings. The horizontal orientation of the reactors takes advantage of free-fall mixing, eliminating the need for mechanical mixing. Evaluation of a range of mixing speeds (3.3-11.5 rpm) by Jorgensen et al. (2007b) resulted in no significant differences in cellulose conversion over the tested range, so energy input for mixing is significantly reduced as compared to vertically oriented stirred tank reactors. In addition to free-fall mixing, a rotating shaft affixed with paddles supplies additional mixing capabilities, as the shaft in the pilot-scale reactor can be programmed to change rotational direction two times per minute. The paddles also provide a scraping action that removes lignocellulosic material from the reactor walls, improving heat transfer between the reactor and the biomass.

The Integrated Biomass Utilization System (IBUS) Project coordinated by DONG Energy in Denmark also utilizes free-fall reactors. DONG Energy has free-fall reactors in a variety of sizes for research and development purposes (400 L) and has successfully

scaled one up to a capacity of 11,000 L (Jorgensen et al. 2007a; Larsen et al. 2008). These reactors routinely operate at approximately 40% solids loading. Larger particle sizes can be used, since the mechanical work of the mixing helps tear biomass fibers and particles apart (Larsen et al. 2008). This tearing action also increases the surface area of the lignocellulose, resulting in increased enzyme accessibility to the cellulose and hemicellulose.

While most reactors implemented for high-solids enzymatic hydrolysis have employed some form of free-fall mixing, Zhang et al. (2010) investigated the effects of a helical impeller in a vertical reactor on SSF at solids loadings up to 30% (w/w) and compared it to a typical Rushton (paddle) impeller (Figure 3.4a-b). Helical impellers are suggested for use in highly viscous, non-Newtonian fluid agitation, which describes high-solids biomass slurries. The helical impeller performed better than the Rushton impeller with regard to every aspect tested. The feeding rate of lignocellulose into the reactor was adjusted so that a liquefied slurry could be maintained throughout the feeding period. The helical impeller provided better mixing, as the feeding period was completed more than 2 hr sooner than that of the Rushton impeller. The helical impeller also resulted in higher ethanol concentration (51.0 g/L vs. 43.9 g/L) and productivity, as well as consuming less power. At 30% solids (prior to inoculation with the fermentative organism), the Rushton impeller required nearly 40 W/kg corn stover (CS) before decreasing to ~29 W/kg CS after 72 hr of saccharification and fermentation. The helical impeller required ~8 W/kg CS and ~1 W/kg CS prior to inoculation and after 72 hr, respectively. (It should be noted that the stirring rates for the two impellers were different; however, the power requirements were normalized based on the “no-load” power consumption for each impeller.) Lastly, the mixing efficiency of the helical impeller was superior to the Rushton impeller. The geometry of the impeller can play a significant role in effectively mixing biomass slurries. Other geometries tested by Wang et al. include a plate-and-frame impeller and a double-curved-blade impeller (Figure 3.4c-d). The impellers were tested at various speeds and 100 rpm resulted in the best conversion efficiencies for both geometries. However, the plate-and-frame impeller achieved a higher conversion than the double-curved-blade impeller by nearly 18%, indicating that the geometry of the impeller can have an effect on the hydrolysis. The

authors suggested that the plate-and-frame impeller provides a more consistent mixing regime at every depth in the reactor, whereas the axial flow induced by the double-curved-blade impeller is a function of the distance from the blades.

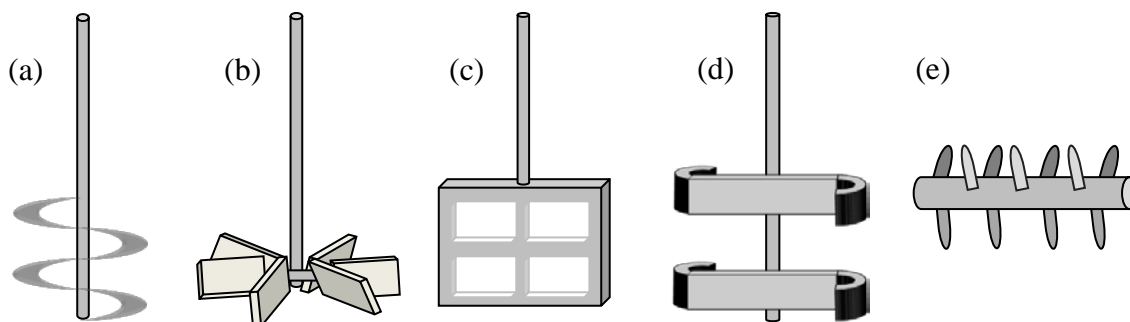


Figure 3.4. Different types of impellers studied for use with high-solids enzymatic hydrolysis. (a) Helical impeller, (b) Rushton impeller, (c) plate-and-frame impeller, (d) double-curved-blade impeller, and (e) peg mixer.

Another study investigated the use of a peg mixer (Figure 3.4e) for enzymatic hydrolysis at high-solids loadings (Zhang et al. 2009). The mixer used in this study was a 9 L reactor fitted with a rotating shaft with pegs extending out radially. The time for liquefaction of 20% (w/w) of unbleached hardwood pulp was significantly reduced when comparing shake flasks to the peg mixer (40 hr vs. 1 hr). The benefit of this mixer is that it has been proven effective with lignocellulosic material. High-solids enzymatic hydrolysis is just another application for the peg mixer.

From the various aforementioned reactors utilized with high-solids enzymatic hydrolysis reactions, there are several suggestions to improve the mixing of highly viscous slurries. Free-fall mixing relies on gravity to effectively mix the slurry, which consumes less energy than a stirred tank reactor providing a similar degree of mixing. An effective mixing regime can greatly depend on the impeller geometry, as the shape of an impeller can cause large differences in speed and shear effects at various impeller-slurry interfaces throughout the reactor. High shear rates have been shown to disrupt the adsorption of cellulase onto biomass or to even cause the denaturation of cellulase (Cao and Tan 2004; Kaya et al. 1996). Lastly, technology should be borrowed from other applications, where possible. For instance, peg mixers are a “tried-and-true” technology

that is commonly used in the long-established pulp and paper industry. All of these ideas have shown some promise but require more study and fine-tuning before being implemented into the lignocellulose conversion process.

3.7 PILOT AND DEMONSTRATION-SCALE OPERATIONS

Several plants operating at pilot- and demonstration -scale level have recently come online. These installations will help the industry gain valuable insights and improve upon the challenges and limitations that are not recognized at the laboratory scale.

One such pilot plant constructed in Denmark is operated by Inbicon (a subsidiary of DONG Energy), with a distillation capacity of ~1 ton fermentation broth/hr. Additionally, in 2010, Inbicon opened its demonstration-scale plant that is capable of producing 5.3 million liters of ethanol each year. Enzymatic hydrolysis is performed here at 25-30% (w/w) solids content with a relatively low enzyme loading of 3-6 FPU/g DM. However, the plant is capable of handling up to 40% (w/w) solids in any of its process streams (Jorgensen et al. 2007a; Larsen et al. 2008). Since this operation is also used for developmental purposes, they have reactors that range from 400 L up to 11,000 L. Additionally, pretreatment and fermentation are performed at high-solids loadings, 20-40% and ~18% DM, respectively. At the end of the conversion process, the remaining lignin-rich material (40-95% DM) is burned to produce heat and electricity that can be cycled back into the conversion operation.

The National Renewable Energy Laboratory (Golden, CO, USA) recently expanded their lignocellulose processing facilities to achieve a capacity of 4,000 L and to operate at solids loading of $\geq 20\%$ (w/w) (National Renewable Energy Laboratory). The conversion process is designed as a semi-continuous operation with pretreatment occurring in horizontal reactors with paddles, taking advantage of the reduced energy inputs required with free-fall mixing of lignocellulose. Following liquefaction at ~24-30 hrs, the slurry is pumped into vertical, stirred tank reactors to complete the enzymatic hydrolysis of the material. This operation is capable of processing about 0.5 to 1 ton dry biomass into ethanol each day.

3.8 DIRECTION OF FUTURE WORK

In order to fully realize the benefits of operating enzymatic hydrolysis at high solids, several issues must be addressed. There are many variables associated with enzymatic hydrolysis that can affect the efficiency of the conversion, including (but not limited to) biomass source, pretreatment method, enzyme source and enzyme mixture. Each of these components must be considered when designing a process for lignocellulose conversion, which makes optimal processing conditions difficult to devise. Further study for the optimization of glucose yields, especially in regards to the use of fed-batch systems, enzyme supplementation, washing and detoxification steps, and additives, both individually and in combination, is still very much needed. It is also important that a better understanding of some of the mechanisms that seem to have the greatest impacts on the conversion process is achieved. Robust reactors capable of effectively mixing biomass slurries to minimize end-product inhibition and heat and mass transfer limitations are needed. Additionally, the cost of enzymes, biomass and any necessary specialty equipment, as well as the best uses for any potential by-products produced in the conversion process, should be considered in the design stages.

3.9 CONCLUSIONS

Recent national and international focus on producing biofuels and chemicals from lignocellulose has led to significant research on the development and optimization of effective conversion processes. Several definitive conclusions regarding enzymatic hydrolysis performed at high-solids loadings can be made following a thorough review of the available literature on this topic:

- Free-fall mixing is effective. The advantages of this type of mixing system are numerous, and it has been employed successfully in other industrial processes.
- The solids effect is real. Although, the exact cause of this phenomenon has not been determined, there are several hypotheses that have been suggested, including

- lower cellulase adsorption (increased concentrations of glucose and cellobiose have been shown to inhibit the adsorption of enzymes onto cellulose);
 - product inhibition of enzymes occurs earlier because of the higher concentration of products;
 - inadequate mixing, which can emphasize diffusional limitations exacerbating product inhibition and access of enzyme to substrate;
 - interaction of water with substrate (water has been shown to be more tightly bound to lignocellulose as the solids loadings increase, thus less water is available to the enzymes to perform the hydrolysis reaction).
- Contradictory evidence continues to raise questions regarding the lignocellulose conversion process. For example, some studies have shown that washing solids following pretreatment can enhance sugar production and fermentation, while others have found the opposite to be true. Additionally, arguments persist regarding the effects water has on the overall conversion process. Lastly, as long as enzyme cost remains a large portion of the overall conversion cost, enzymes also demand further attention, especially with regards to proper loadings and combinations.
 - Fed-batch systems are worth investigating. While there have been some conflicting results, many studies show overwhelming support for conducting high-solids operations as a fed-batch system.
 - The use of additives to reduce slurry viscosity has achieved some success at the lab-scale. However, the economics of the use of additives on an industrial-scale should be validated prior to implementation at that level.

The use of high-solids operations would make biofuels produced from the conversion of lignocellulose more economical and more price-competitive with petroleum. Increasing sugar and ethanol yields while reducing capital and production costs, lowering energy demands and lowering water requirements will contribute to a more economically feasible process as compared to one operated at low- or moderate-solids loadings. Despite all the benefits of operating at high solids, the process remains restricted due primarily to the lack of available water within the culture, high viscosities,

which translate to difficulties with mixing and handling, and increased concentration of inhibitors, which extends reaction times and increases enzyme costs. Researchers are attacking these issues from many angles, experimenting with different pretreatment methods and various enzyme sources and cocktails, while modifying operating conditions and slurry properties. Although there has been some success at performing enzymatic hydrolysis at high solids at the pilot and demonstration scale, many questions must be resolved before the full potential of high-solids lignocellulose conversion will be realized.

3.10 ACKNOWLEDGMENTS

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CHAPTER 4: LITERATURE REVIEW

4.1 STRUCTURE OF LIGNOCELLULOSE

Lignocellulose is composed of three main fractions: cellulose, hemicellulose and lignin. Cellulose typically makes up the largest portion of these fractions at about 30-50% for herbaceous crops and about 40-50% for woody crops. Cellulose is a linear polymer, formed from β -1,4-linked glucose units (Figure 4.1a) and can contain up to 15,000 of these monomers (O'Sullivan 1997; Sticklen 2007). The polymer chains bundle together into microfibrils. In its native form, cellulose is relatively recalcitrant to depolymerization, stabilized by the inter- and intra-strand hydrogen bonding and the resulting van der Waals forces (Chang 2007; Zhang and Lynd 2004). However, through a combination of pretreatment and enzymatic hydrolysis, cellulose can be depolymerized into fermentable sugars.

Hemicellulose, another carbohydrate polymer that makes up about 20-30% of lignocellulose (Girio et al. 2010), is more randomly assembled and structurally more complex than cellulose (Figure 4.1b) since it can be composed of several different types of sugars compared to only glucose for cellulose. Hemicellulose is primarily comprised of xylan or glucomannan chains, intermixed with other components like hexose (glucose, mannose and galactose) and pentose (xylose and arabinose) sugars and uronic acids (glucuronic and galacturonic acids) (Hendriks and Zeeman 2009). The variety and amount of each component is dependent on the lignocellulose source. For example, glucuronoxylans form a major portion of hemicellulose in hardwoods, while galactoglucomannans account for a large portion of the hemicellulose of softwoods (Girio et al. 2010). Unlike cellulose, hemicellulose is a branched polymer (Moxley and Zhang 2007; Sticklen 2007). The hemicellulose often associated with agricultural residues (corn stover, wheat straw) contains branch points formed by arabinose and glucose chains substituted along the β -1,4-linked xylose backbone. Cellulose is embedded within the hemicellulose matrix, which acts as a connection between the cellulose and lignin fractions. Hemicellulose also helps provide rigidity to the lignocellulose structure (Hendriks and Zeeman 2009).

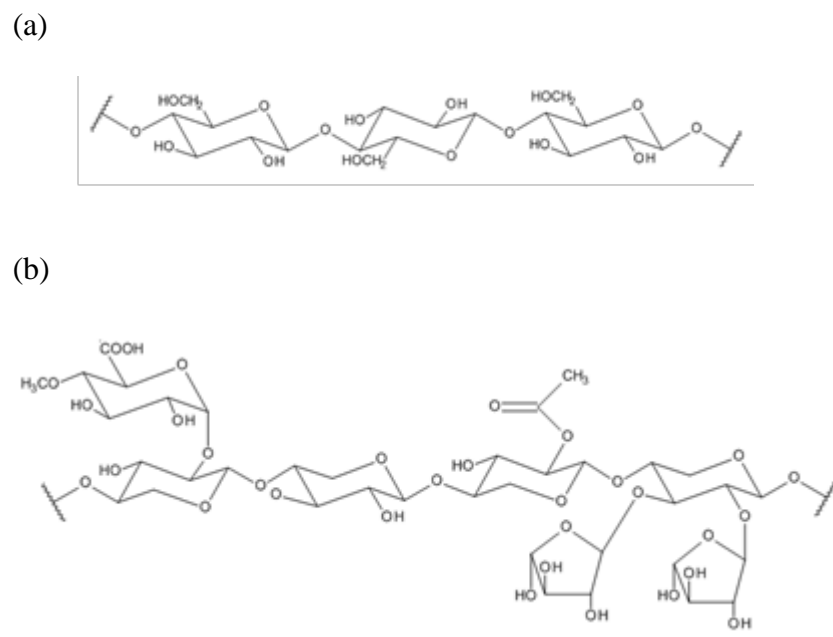


Figure 4.1. Chemical structures of (a) cellulose and (b) hemicellulose. The xylan backbone contains various side chains and branch points, including glucose, arabinose and acetate, making this carbohydrate chain more complex and variable than the linear cellulose chain. Figures adapted from Menon and Rao (2012).

Lignin is a complex, phenolic polymer (Figure 4.2) that acts as a protective barrier encasing cellulose and hemicellulose. It also provides structural support and aids in the transport of water within the plant (Buranov and Mazza 2008; Grabber 2005; Petridis et al. 2011). However, by nature's design, lignin is a major obstacle in the degradation of cellulose and hemicellulose, with its irregular linkages and non-repetitive order of components. The complexity of this polymer is due to three monolignol components, including *p*-coumaryl, coniferyl and sinapyl alcohols and many of their derivatives, polymerizing into an irregular network via a number of different linkages (β -O-4, α -O-4, β -5, β -1, 5-5, 4-O-5 and β - β linkages). Once integrated into the lignin polymer, these monolignols are referred to as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties, respectively (Buranov and Mazza 2008; Grabber 2005; Zhao et al. 2012). The ratio of H:G:S constituents of the lignin structure can vary depending on the source of the lignocellulose (Adler 1977). For example, corn stover typically contains

about 7-23% lignin (Cheng 2010; Lee et al. 2007) that is comprised of H:G:S constituents in a ratio of 4:35:61 (Table 4.2) (Grabber 2005). However, softwoods are comprised of nearly 30% lignin and have a much different H:G:S ratio of 5:93.5:1.5 (Adler 1977). Rice straw has a more balanced proportion of these units in terms of guaiacyl and syringyl moieties, with an H:G:S ratio of 15:45:40. Additionally, syringyl content has been linked to a plant's resistance to fungal infection (Buranov and Mazza 2008). It is important to note that the combination of these constituents is not only critical to the structure of the plant, but also has implications for tailoring conversion process steps to achieve optimum product yield.

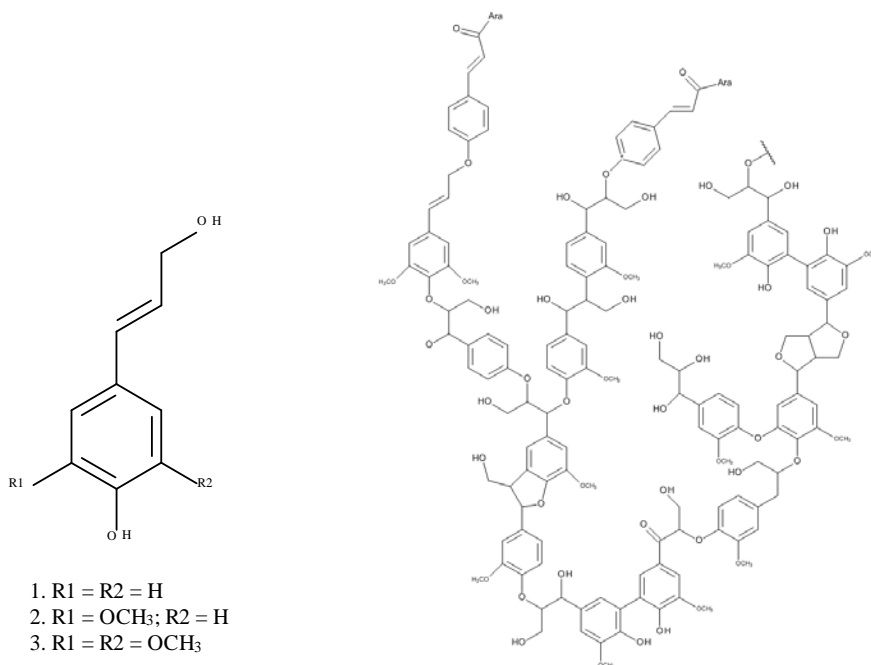


Figure 4.2. Lignin (right) is a complex structure composed from three main monolignol components (left) being (1) *p*-coumaryl, (2) coniferyl and (3) sinapyl alcohols. Possible sites for linking lignin to hemicellulose are denoted by ‘Ara’ (which represents arabinose). Figures adapted from Adler (1977) and Buranov and Mazza (2008).

Lignin is attached to hemicellulose through a structure called the lignin-carbohydrate complex (LCC) (Buranov and Mazza 2008; Grabber 2005). In herbaceous biomass, the LCC is composed of a phenolic lignin unit linked to an arabinoxylan by ferulic acid (Figure 4.3), which reportedly varies from the type of LCCs present in woody biomass. Additionally, the location of the ferulic acid has shown slight differences

depending on the lignocellulose source. For example, in wheat bran the ferulic acid forms an ester linkage with the second carbon of an arabinose branch-point off a xylan backbone, whereas the ester linkage occurs on the third carbon in bagasse and on the fifth carbon for grasses. It is believed that the ferulic acid acts as an anchoring point for the lignin into cell walls in the early stages of lignification, and have a significant impact on the ability to hydrolyze the carbohydrate fractions (Wu et al. 2011). Grabber (2005) compared biomass with 4.5 g/kg with 15.9 g/kg ferulate cross-linkages and found biomass with only 4.5 g/kg ferulate cross-linkages produced 46% and 20% more sugar after 6 hr and 72 hr hydrolysis, respectively, indicating that the reduction in ferulate cross-linking significantly impacted both the initial rate and the extent of hydrolysis of the modified biomass. However, the degree of ferulate cross-linking is a function of the type of biomass and is not something that can be controlled, unless it is manipulated through genetic modifications of the plant.

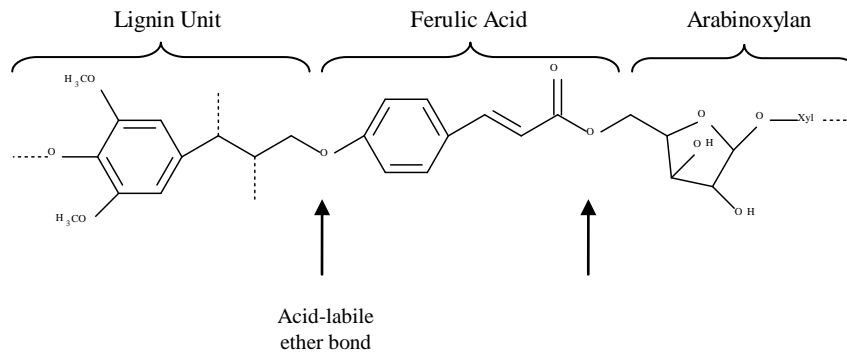


Figure 4.3. The lignin-carbohydrate complex. Ferulic acid links the phenolic lignin unit with an arabinoxylan chain. The resulting ether and ester bonds are susceptible to acid or alkali as denoted in the figure above. Figure adapted from Buranov and Mazza (2008).

4.2 EFFECTS OF SODIUM HYDROXIDE PRETREATMENT ON STRUCTURAL COMPONENTS OF LIGNOCELLULOSE

Alkaline pretreatment with sodium hydroxide is a viable, low-cost option for modifying the structure of lignocellulose prior to hydrolysis and fermentation of the carbohydrate fractions. It can be performed using a wide range of operating conditions (Hendriks and Zeeman 2009; Modenbach and Nokes 2012; Mosier et al. 2005). For instance, reaction times can be as short as a few minutes or on the order of hours or days, with temperature ranging from ambient to 150°C (Galbe and Zacchi 2007; Jorgensen et al. 2007a). Using sodium hydroxide for pretreatment is also advantageous over other pretreatments, like dilute acid and ammonia fiber expansion (AFEX). The alkaline reagents are less caustic than dilute acid, and alkaline pretreatment can be performed at ambient pressure, unlike AFEX, eliminating the need for specialized corrosion-resistant equipment or that can withstand high pressures (Mosier et al. 2005). It is also possible to recover and recycle alkaline reagents, potentially reducing costs associated with pretreatment (Mosier et al. 2005).

Alkaline pretreatment can play an important role in the conversion of lignocellulose. With a narrow profit margin for commodity chemicals like ethanol, it is imperative to develop a conversion process that can be integrated into a biorefinery concept. A biorefinery, modeled after the traditional petroleum refinery, should be capable of economically producing a variety of valuable and useful products, including liquid transportation fuels, commodity chemicals and precursory chemical building blocks. Pretreatments using dilute acid and liquid hot water tend to remove the hemicellulose fraction, eliminating a potentially valuable energy stream. Xylose, the predominant carbohydrate found in hemicellulose of herbaceous biomass, can either be fermented by organisms capable of utilizing pentoses or be converted into other chemical building blocks like xylitol and glycerol (Werpy and Peterson 2004). Residual solids (like lignin) produced from alkaline pretreatment can even be used to generate a number of other products. For example, lignin and/or its components can be used as a solid fuel source that can be burned to produce heat and electricity for the biorefinery or distributed to the grid for residential or commercial use (Jorgensen et al. 2007a; Ragauskas et al. 2006); as a component of phenolic powder resins, polyurethane foams, epoxy resins, or

biodispersants (Kadam et al. 2008; Lora and Glasser 2002); or as valuable food and industrial products like vanillin, ferulic acid or vinyl guaiacol (Buranov and Mazza 2008).

4.2.1 Mechanism of Sodium Hydroxide Pretreatment

The ferulic acid linkage between the lignin and the hemicellulose fractions is the point of reaction during NaOH pretreatment (Buranov and Mazza 2008). The ester bond between the ferulic acid and the carbohydrate is highly susceptible to alkali degradation, as the hydroxide ion (dissociated from NaOH) increases the rate at which the hydrolysis reaction occurs as compared to water (Bruice 2004). The mechanism of alkaline pretreatment (Figure 4.4) is such that the hydroxide ion attacks the carbon of the ester bond (step 1), whether between the lignin and carbohydrate or even between two lignin components or two carbohydrate components. A tetrahedral intermediate forms (step 2), but quickly collapses when a negatively-charged oxygen atom expels an alkoxide ($-\text{OCH}_3$) from the carboxylic acid (step 3). In a very fast reaction, the resulting alkoxide acts as a base, deprotonating the carboxylic acid (step 4). The result is the irreversible hydrolysis of the ester bond, weakening the structural integrity of the lignocellulose.

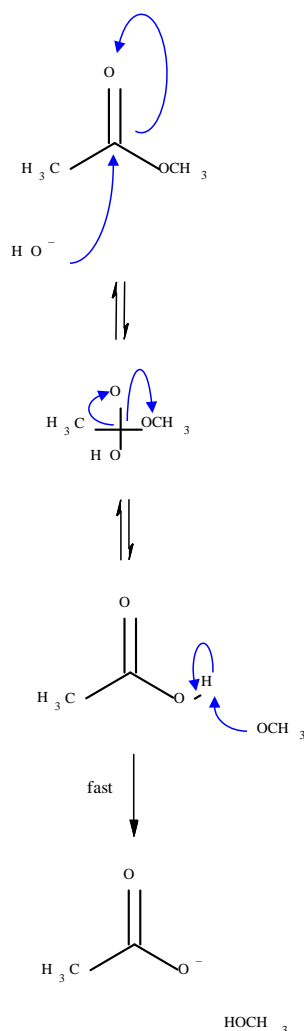


Figure 4.4. Mechanism of base hydrolysis of an ester bond. The hydroxide ion attacks the C of the C=O. A tetrahedral intermediate forms but immediately collapses as an alkoxide leaves the carboxylic acid. In a very fast reaction, the alkoxide acts as a base and deprotonates the carboxylic acid. Arrows pointing from molecular components to other components or bonds indicate movement of electrons. Figure adapted from Carey (2000).

4.2.2 Structural Changes Associated with Sodium Hydroxide Pretreatment

Pretreatment with sodium hydroxide results in several structural modifications of lignocellulose beneficial for enzymatic hydrolysis. Bonds linking the protective lignin barrier with hemicellulose are broken. Depending on the pretreatment conditions, lignin is partially or totally solubilized, and degradation of the hemicellulose fraction may occur. Sodium hydroxide pretreatment also swells the lignocellulose particles, leading to an increase in surface area and greater accessibility to the cellulose fraction.

Additionally, a decrease in the degree of polymerization and crystallinity of the cellulose is likely, increasing the enzymatic digestibility of the polysaccharide.

Solids loading of the system can also have significant impacts on the effectiveness of the pretreatment. At low solids loadings (<10%), where most conventional pretreatments have been developed, pretreatment processes have been shown to facilitate conversion of biomass into fermentable sugars (Modenbach and Nokes 2012). However, these systems with higher water contents also require higher costs for handling and removing excess water and neutralizing the material prior to subsequent conversion steps. There is also the concern of treating a large effluent of wastewater, especially in instances where recycling and/or recovery of the pretreatment chemical are not possible. Some research has been conducted using NaOH pretreatment with higher solids loadings (Cheng et al. 2010; Cui et al. 2012) as a way to reduce the water requirements necessary during pretreatment. At high-solids loadings (>15%), many challenges that are not apparent with low solids loadings emerge. For instance, there may be little to no free water in the reactor, which could significantly impact the effectiveness of the pretreatment (Kristensen et al. 2009b), since water aids in chemical reactions, reduces the viscosity of the slurry by increasing the lubricity of the particles, provides a medium for mass transfer by diffusion of the NaOH to the lignocellulose material and improves the handling capability of the bulk material (Modenbach and Nokes 2012). While it is not possible to give a definitive recommendation for the optimal moisture content during NaOH pretreatment without further study, it is possible to say that many factors must be considered when choosing pretreatment conditions in order to obtain an optimal sugar recovery and yield in subsequent processing steps.

4.2.2.1 Structural Changes of Lignin

Lignin is the main component of lignocellulose affected by NaOH pretreatment, and the pulp and paper industry have long taken advantage of alkaline delignification in the Kraft process used in paper-making (Zhao et al. 2012). The Kraft process uses NaOH at elevated temperatures (160°C-170°C) together with sodium sulfide to remove lignin and produce cellulose fibers from woody biomass (Hamaguchi et al. 2012; Wu et al. 2011). However, by-products of the Kraft process, like sulfur compounds and

chlorinated compounds (Kadam et al. 2008), may have negative effects on other downstream processes in the conversion process. Additional processing to remove these compounds, as well as the wastewater treatment that would be required, can complicate pretreatment processes that directly mimic the Kraft process. Sodium hydroxide alone is capable of removing lignin from lignocellulose of hardwood and herbaceous biomass, much simplifying the process. However, as alkaline pretreatment is developed for use prior to enzymatic hydrolysis in the conversion of lignocellulose into transportation fuels and other chemicals, researchers are more closely investigating the effects of alkaline pretreatment conditions on the delignification of biomass.

The alkaline pretreatment reacts with the ester bonds linking the lignin to the hemicellulose in the LCC network. As these bonds are broken, the LCC networking is disrupted, allowing lignin components to be solubilized. Duguid et al. (2009) saw <2% reduction in lignin in corn stover when pretreated with 5.8 g NaOH/100 g biomass at room temperature for 2 hr, whereas Chen et al. (2009) observed 73.9% lignin removal in corn stover when pretreated with 16 g NaOH/100 g biomass at 121°C for 30 min (Table 4.1). Alkaline pretreatment can also cause xylan solubilization, especially where xylan is associated with the LCC complex (Cui et al. 2012). For instance, Cui et al. (2012) reported up to 34% loss of xylan coupled with 22% lignin degradation during pretreatment with 5 g NaOH/100 g biomass and 75% moisture content for 90 days. It has been hypothesized that disruption of this cross-linking enhances enzyme adsorption and enzyme effectiveness by reducing inhibition of xylooligomers and unproductive binding with lignin (Kim and Holtzapple 2006; Kumar et al. 2009a; Wu et al. 2011). Removal of lignin by NaOH often leads to the release of acetyl groups and uronic acid substitutions, which can enhance the digestibility of cellulose and hemicellulose (Cui et al. 2012; Kumar et al. 2009a; Wan et al. 2011). However, hydrolytic enzymes can be inhibited by some of these degradation products, like xylooligomers (Qing et al. 2010), organic acids, phenols (Kim et al. 2011), furfural and hydroxymethyl furfural (HMF) (Hodge et al. 2008), making the selection of process conditions, like alkaline loading, moisture content, temperature and time, extremely important. Balance is the key to achieving optimal lignin removal, while limiting the production of inhibitory compounds. For instance, Cui et al. (2012) found that delignification was influenced by NaOH

loading, time and moisture content during long-term wet-storage of corn stover. Addition of 2-5 g NaOH/100 g biomass increased lignin degradation by ~10-25% over a 90 day storage period; however, most of this lignin degradation occurred within the first five days of storage. A higher loss in xylan (up to 34%) was also observed with the increase in lignin degradation. Wan et al. (2011) also observed a sharp increase in xylan degradation with an increase in lignin degradation. As NaOH loading increased from 4 g NaOH/100 g biomass to 40 g NaOH/100 g biomass, lignin degradation increased moderately from ~7% to ~15%, but xylan removal increased from 5% to nearly 50% over the same NaOH loadings. Although no inhibition was observed during enzymatic hydrolysis and inhibitor concentrations were not measured, the presence of inhibitory compounds from the degradation of xylan is possible. However, they were likely removed during the washing and neutralizing of the soybean straw prior to use in the hydrolysis reaction.

Table 4.1. Effects of pretreatment conditions on biomass composition and enzymatic hydrolysis of cellulose to glucose.

Substrate	Pretreatment Conditions			Biomass Composition				Glucose Conversion ^a	Glucose Yield (mg/g substrate) ^b	Ref.
	NaOH Loading (g NaOH/100 g biomass)	Time (hr)	Temp (°C)	Glucan	Xylan	Lignin	Delignification			
<i>Woody Biomass</i>										
Poplar	-	-	-	43.8%	14.9%	29.1%	-	-	-	Gupta and Lee (2010)
	15	24	60	40.2%	13.2%	21.7%	25.4%	22.0%	98	
	50			41.8%	8.1%	23.1%	20.6%	25.6%	119	
Mixed hardwood	-	-	-	42.8%	14.6%	23.9%	-	-	-	Sills and Gossett (2012)
	10	24	25	48.1%	15.2%	21.3%	10.9%	51.0%	272	
	20			49.4%	15.3%	20.4%	14.6%	53.0%	291	
Birch	-	-	-	41.0%	27.9%	29.7%	-	-	-	Mirahmadi et al. (2010)
	7	2	100	56.1%	8.0%	25.2%	15.0%	80.0%	498	
Spruce	-	-	-	43.0%	20.8%	28.8%	-	-	-	Mirahmadi et al. (2010)
	7	2	5	50.0%	15.9%	28.2%	2.1%	35.0%	194	
Spruce	-	-	-	49.8%	5.4%	30.6%	-	-	-	Zhao et al. (2008)
	140	24	-15	54.1%	3.8%	29.0%	19.0%	61.0%	366	
			23	53.4%	3.8%	29.2%	18.7%	18.0%	107	
		2	60	53.2%	3.7%	28.8%	19.5%	19.0%	112	

Table 4.1., continued. Effects of pretreatment conditions on biomass composition and enzymatic hydrolysis of cellulose to glucose.

Substrate	— Pretreatment Conditions —			— Biomass Composition —				Glucose Conversion ^a	Glucose Yield (mg/g substrate) ^b	Ref.
	NaOH Loading (g NaOH/100 g biomass)	Time (hr)	Temp (°C)	Glucan	Xylan	Lignin	Delignification			
<i>Herbaceous Biomass</i>										
Corn stover	-	-	-	NR^c	NR	NR	-	-	-	Cui et al. (2012)
	2	90 d	23	NR	NR	NR	11.6%	20.7%	NR	
	3.5			NR	NR	NR	20.2%	25.7%	NR	
	5			NR	NR	NR	22.1%	37.4%	NR	
Corn stover	-	-	-	36.0%	21.0%	23.0%	-	-	-	Duguid et al. (2009)
	2.9	2	room	NR	NR	NR	<2%	NR	180	
	5.8			NR	NR	NR	<2%	NR	240	
Corn stover	-	-	-	34.6%	21.8%	17.7%	-	-	-	Gupta and Lee (2010)
	10	24	60	31.0%	17.4%	6.3%	64.4%	82.0%	282	
	15			30.1%	16.2%	4.9%	72.3%	93.8%	313	
	50			27.9%	7.6%	3.3%	81.4%	99.8%	309	
Corn stover	-	-	-	36.2%	20.1%	21.2%	-	-	-	Zhang et al. (2011)
	2.5	9	21	NR	NR	NR	18.0%	NR	200	
	5			NR	NR	NR	31.0%	NR	225	
	10			NR	NR	NR	48.0%	80.0%	320	
Corn stover	-	-	-	39.2%	23.2%	13.5%	-	-	-	Sills and Gossett (2012)
	10	24	25	45.7%	27.2%	11.2%	17.0%	67.0%	340	
	20			50.9%	27.4%	7.7%	43.0%	80.0%	452	
Corn stover	-	-	-	38.7%	21.7%	19.3%	-	-	-	Chen et al. (2009)
	16	0.5	120	64.1%	24.6%	8.6%	55.4%	80.0%	569	

Table 4.1., continued. Effects of pretreatment conditions on biomass composition and enzymatic hydrolysis of cellulose to glucose.

Substrate	— Pretreatment Conditions —			— Biomass Composition —				Glucose Conversion ^a	Glucose Yield (mg/g substrate) ^b	Ref.
	NaOH Loading (g NaOH/100 g biomass)	Time (hr)	Temp (°C)	Glucan	Xylan	Lignin	Delignification			
Wheat straw	-	-	-	36.0%	26.0%	7.6%	-	-	-	McIntosh and Vancov (2011)
	7.5	1.5	60	NR	NR	NR	23.0%	NR	275	
	10			NR	NR	NR	35.0%	NR	290	
	20			NR	NR	NR	42.0%	NR	350	
Soybean straw	-	-	-	34.1%	11.4%	21.6%	-	-	-	Wan et al. (2011)
	4	24	24	NR	NR	NR	8.0%	47.0%	NR	
	12			NR	NR	NR	10.0%	50.0%	NR	
	20			NR	NR	NR	12.0%	52.0%	NR	
	40			NR	NR	NR	14.8%	64.6%	NR	
Rice straw	-	-	-	36.3%	19.5%	17.6%	-	-	-	Cheng et al. (2010)
	4	2	55	32.5%	22.4%	14.0%	19.2%	36.3%	118.1	
	4 ^d	3		32.8%	22.4%	13.3%	23.1%	39.2%	142.3	
Sweet sorghum bagasse	-	-	-	38.7%	22.6%	15.4%	-	-	-	Wu et al. (2011)
	40	2	25	NR	NR	NR	66.0%	92.0%	NR	
	80			NR	NR	NR	76.0%	95.0%	NR	
	200			NR	NR	NR	80.0%	99.0%	NR	

Table 4.1., continued. Effects of pretreatment conditions on biomass composition and enzymatic hydrolysis of cellulose to glucose.

Substrate	— Pretreatment Conditions —			— Biomass Composition —				Glucose Conversion ^a	Glucose Yield (mg/g substrate) ^b	Ref.
	NaOH Loading (g NaOH/100 g biomass)	Time (hr)	Temp (°C)	Glucan	Xylan	Lignin	Delignification			
Switchgrass	-	-	-	32.0%	17.9%	21.4%	-	-	-	Xu et al. (2010)
	20	96	21	NR	NR	NR	63.0%	74.0%	262.9	
		12	50	NR	NR	NR	71.0%	77.7%	276.1	
Switchgrass	-	-	-	38.7%	22.6%	21.1%	-	-	-	Sills and Gossett (2012)
	10	24	25	43.3%	23.1%	16.6%	12.2%	47.0%	226	
	20			48.7%	23.5%	14.1%	25.4%	61.0%	330	

^aGlucose conversion is the ratio of the amount of glucose released during enzymatic hydrolysis to the theoretical amount of glucose available

^bGlucose yield is the ratio of the mass of glucose released during enzymatic hydrolysis to the mass of the initial biomass

^cNR = not reported

^dBiomass pretreated under indicated conditions was not washed prior to enzymatic hydrolysis

Values in **bold** denote composition of raw biomass prior to pretreatment

Theoretical glucan-to-glucose yield (in mg glucose/g biomass) can be calculated by: Glucan % × 100 g biomass × 1.11 g glucose/g glucan × 1000 mg/g

Theoretical glucan-to-ethanol yield (in g ethanol/g biomass) can be calculated by: Glucan % × 100 g biomass × 1.11 g glucose/g glucan × 0.511 g ethanol/g glucan

As mentioned previously, lignin structures vary with different sources of lignocellulose, which means that NaOH pretreatment works more effectively on some sources of biomass than others. Shimizu et al. (2012) investigated the effects of NaOH on the degradation of the β -O-4 bonds between model lignin dimers. They reacted guaiacyl-guaiacyl (G-G), guaiacyl-syringyl (G-S), syringyl-guaiacyl (S-G) and syringyl-syringyl (S-S) dimers with a 1 M NaOH solution at 40-70°C for 3-7 hr and found that the compounds containing a syringyl unit reacted more readily in the alkaline solution as compared to G-G dimers. The orientation of the dimer components also affected the rate of degradation, as the S-G and G-S dimers did not degrade at an equivalent rate. The order of the rates of degradation was determined as follows: S-S > G-S > S-G > G-G, where S-S degraded nearly 7.5-fold faster than G-G at 130°C. As with these model lignin compounds, real sources of lignocellulose containing a higher proportion of syringyl units is more easily delignified. Lignin from hardwoods is composed of ~7-40 times more syringyl units than lignin from softwoods (Adler 1977), making hardwoods more susceptible to alkaline pretreatment than softwoods (Shimizu et al. 2012). Rice straw, bagasse and some grasses, which tend to have S-G ratios more similar to hardwoods than to softwoods, have shown significant lignin removal following alkaline (NaOH) pretreatment at short reaction times and moderate temperatures (data not given) (Wu et al. 2011). These sources of lignocellulose also contain high levels of syringyl units (10-65%) in the lignin fraction (Adler 1977).

Table 4.2. Typical ratios of lignin moieties found in various biomass sources. Ratios compiled from Buranov and Mazza (2008) and Lapierre et al. (1995).

Biomass	H:G:S
<i>Woody Biomass</i>	
Poplar	0:37:63
Oak	0:32:68
Birch	0:22:78
Spruce	2:98:0
Pine	18:82:0
<i>Herbaceous Biomass</i>	
Corn	4:35:61
Wheat	5:49:46
Rice	15:45:40
Flax	4:67:29

4.2.2.2 Degradation of Cellulose

Alkaline degradation of cellulose is dependent on several factors, including the nature and concentration of the alkali, the nature and origin of the cellulose and temperature (Ciolacu and Popa 2005; Fengel et al. 1995; Knill and Kennedy 2003). At relatively low temperatures (<100 °C) and low alkali concentrations (<4%), structural changes for cellulose are insignificant, as glycosidic β (1, 4) linkages are alkali stable under these conditions (Knill and Kennedy 2003). Kim and Holtzapple (2006) reported no significant structural changes or degradation to cellulose pretreatment with 50 g $\text{Ca}(\text{OH})_2/100$ g biomass at low temperatures (25°C-55°C), even for extended pretreatment times up to 16 weeks. Another study (Cui et al. 2012) reported that long-term storage (90 days) of wet corn stover without the addition of NaOH resulted in ~10% loss of cellulose; however, storage with the application of 2 g NaOH/100 g biomass caused only ~5% degradation of cellulose. The addition of NaOH likely made the environmental conditions unfavorable for microorganisms that would have grown on the cellulose, thus protecting it from microbial degradation. These conditions (low alkali concentrations and low to moderate temperatures) are favorable for lignocellulose pretreatment because lignin is affected, but most of the cellulose remains unaltered and available for hydrolysis into fermentable carbohydrates.

At higher alkali concentrations (>6%), many structural and morphological changes begin to occur in cellulose. As alkali concentrations increase, crystalline structures (regions of highly ordered polymer chains interspersed with more amorphous regions) begin to swell. The swelling starts first in amorphous regions, followed by the crystalline region. The degree of polymerization (DP) and the degree of crystallinity (CrI; crystallinity index) decrease with increasing alkali concentration (Eronen et al. 2009; Mittal et al. 2011). Ciolacu and Popa (2005) studied the structural changes of microcrystalline cellulose, cellulose linters (secondary growth of short, thick-walled fibers produced by cotton) and spruce pulp treated with several alkali concentrations (0-18% NaOH). At 18% NaOH, they observed similar reductions (~19%) in both the DP and CrI for microcrystalline cellulose and cellulose linters as compared to treatment without the addition of alkali, whereas the DP and CrI of spruce pulp were reduced by 27% and 36%, respectively. These structural changes are advantageous for the conversion of lignocellulose into fermentable sugars because enzymatic hydrolysis is enhanced as amorphous regions of cellulose are more easily digested by cellulolytic enzymes.

Additionally, increased alkali concentrations can lead to partial or total transformation of cellulose I to cellulose II through a process known as mercerization (Ciolacu and Popa 2005; Eronen et al. 2009). Cellulose I is natural cellulose produced by bacteria, algae and higher-order plants, where the cellulose chains are parallel to one another (Figure 4.5). Cellulose II is a form of regenerated cellulose in which the chains lie antiparallel to one another (O'Sullivan 1997). This transformation begins at NaOH concentrations of about 7.5%-10% and 10%-12.5% for spruce pulp and cotton linters, respectively (Ciolacu and Popa 2005). At these concentrations, the cellulose lattice-work swells as intermolecular hydrogen bonds are broken and chain conformations are altered, resulting in amorphous regions. Cleavage of intramolecular hydrogen bonds further degrades the structural regularity of the crystalline regions of cellulose, subsequently reducing the DP and the crystallinity of the cellulose. Eronen et al. (2009) used Raman spectroscopy to show the structural changes that resulted from breaking these hydrogen bonds. They also reported that AFM imaging revealed that cellulose II appeared to be more granular as compared to cellulose I, indicating that transformation from cellulose I

to cellulose II not only changes the chemical structure but also the physical appearance of the cellulose.

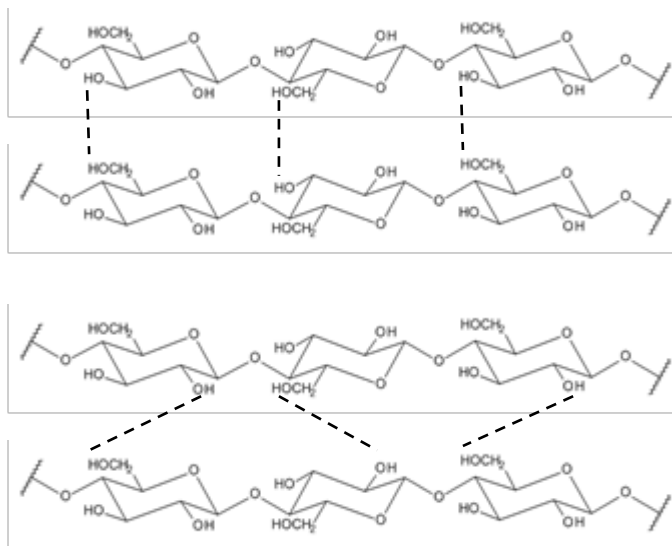


Figure 4.5. Schematic of two cellulose polymorphs (a) cellulose I and (b) cellulose II. The dotted lines indicate possible hydrogen bonds between neighboring strands of cellulose.

The nature and the origin of the cellulose plays a significant role in structural changes caused by alkaline treatment, as evidenced by the differences in reduction of DP and CrI in pure cellulose substrates (microcrystalline cellulose and cellulose linters) as compared to lignocellulose (spruce pulp) substrates discussed previously. Additionally, Ishikura et al. (2010) reported longitudinal contraction and changes in mechanical properties of wood; however, lattice transformations typical of alkali-treated cotton fibers were not observed. It was hypothesized that the lignin matrix likely prevented sufficient swelling of the cellulose fibers and crystallites that leads to lattice transformations. However, some pockets of swelling do occur and result in regions of amorphous cellulose where crystalline cellulose was previously, since the fibers could not return to the crystalline structure upon removal of the NaOH. Degradation of cellulose treated with NaOH is also dependent on the initial DP of the cellulose. Mittal et al. (2011) reported that cellulose sources with greater initial DP were not solubilized as readily as cellulose

sources with lower initial DP. For example, Avicel, which had a lower DP (~90) than other cellulose sources investigated, released nearly 20% of its cellulose when treated with 1926 g NaOH/100 g cellulose at 25°C for 2 hr. Cellulose was more easily solubilized for Avicel fractions with a DP <40. Conversely, cotton linters, which had an initial DP of 600, experienced very little cellulose solubilization during the NaOH treatment. Comparatively, the DP of corn stover is reported to be ~7,000, much higher than other sources of pure cellulose (Kumar et al. 2009a).

The temperature at which the alkaline treatment of cellulose is conducted can also cause significant changes to the structure. Low to moderate temperatures (<100°C) are preferential for alkaline pretreatment prior to the conversion of lignocellulose to fermentable sugars, since cellulose is affected very little at these temperatures, as mentioned earlier. However, at higher temperatures (>100°C), cellulose is more likely to undergo significant degradation and structural changes. Boiling cellulose in a NaOH solution can lead to a reaction known as “peeling” or “unzipping”, where reducing ends of the cellulose chain are subjected to β -alkoxy-carbonyl elimination. The resulting products are a glucoisosaccharinic acid and another reducing end that propagates the peeling reaction (Knill and Kennedy 2003; Machell et al. 1957), with an average of nearly 50 glucose molecules removed before termination occurs (Whistler and Bemiller 1958). However, some reducing ends may remain stable if they are inaccessible to the alkali due to the nature of the cellulose, leading to the termination of further degradation. At even higher temperatures (>170°C), hydrolysis or alkaline scission can occur at random locations along the cellulose chain (Knill and Kennedy 2003). This hydrolysis can lead to new reducing ends that are vulnerable to degradation. Peeling, termination and scission tend to occur in anaerobic conditions; however, under oxidized conditions, carbonyl groups are often hydrolyzed. More specifically, carbonyl groups located at any position along the cellulose chain (except those positioned as an end group) are extremely alkali labile even under mild conditions, and nearly all cellulose molecules containing these carbonyl groups are hydrolyzed (Knill and Kennedy 2003), indicating that alkaline pretreatment may be more effective in an oxidative environment.

4.2.2.3 Changes to Hemicellulose

Hemicellulose, with its branched and somewhat irregular structure, tends to be the most sensitive of the three lignocellulose fractions to changes in pretreatment conditions (Chandra et al. 2007). In dilute alkaline pretreatment conditions, hemicellulose remains mostly intact with the cellulose fraction (Chandra et al. 2007; Varga et al. 2002); however, some studies have shown that hemicellulose can be solubilized as NaOH concentrations increase. For example, both Varga et al. (2002) and Li et al. (2004) found that hemicellulose content in the solid fraction was reduced by more than 60% when pretreating lignocellulose with 10% NaOH. Solubilization of hemicellulose in these more severe pretreatment conditions can also lead to further degradation of sugars into furfural and HMF, two components known for their inhibitory effects on fermentation (Chandra et al. 2007). Additional changes occurring during the alkaline pretreatment of lignocellulose that have been noted include saponification of the ester bonds that link hemicellulose to other lignocellulosic components, removal of acetyl and uronic acid substitutions on hemicellulose and the formation of salts both in solution and incorporated into the lignocellulose (Carvalho et al. 2008).

4.2.3 Limitations of Sodium Hydroxide in Pretreatment

Currently, pretreatments are typically chosen in such a way as to limit inhibitor production while optimizing glucose retention for subsequent processing steps. Even though progress has been made through supplementing cellulases with xylanases during enzymatic hydrolysis and genetically modifying fermentation organisms, glucose is still the favored feedstock of existing fermentation technology. One limitation of sodium hydroxide pretreatment is that in mild operating conditions, this pretreatment requires long reaction times, usually on the order of hours or days (Balat et al. 2008). Also, cellulose and hemicellulose are left relatively intact, while only the lignin is modified (Chandra et al. 2007). Not only can hemicellulose act as a barrier if left in the solid fraction with cellulose, but any portions that are solubilized during pretreatment can act as inhibitors to the cellulase enzymes used in enzymatic hydrolysis (Qing et al. 2010). However, other pretreatments like dilute acid and liquid hot water simply solubilize the hemicellulose fraction and discard it with the waste stream, essentially eliminating a large

portion of potential energy. If harsher conditions are used to remove more of the hemicellulose in sodium hydroxide pretreatment, then not only does the potential carbohydrate yield decrease, but solubilized hemicellulose components can be degraded further into furan derivatives and their acids (furfural, HMF, formic acid and levulinic acid), which are inhibitory to fermentative organisms at concentrations as low as 1 g/L (Cantarella et al. 2004). Since the main mechanism of this pretreatment is delignification of biomass, it is most effective on herbaceous biomass. Woody biomass or biomass high in lignin reduces the usefulness of the sodium hydroxide pretreatment (Balat et al. 2008; Galbe and Zacchi 2007). Additionally, lignin may not be completely solubilized, but simply redistributed and condensed onto the cellulose, eliminating any positive effects from structural changes associated with lignin removal and swelling of the biomass (Hendriks and Zeeman 2009). Lastly, not all of the sodium hydroxide can be recovered and recycled like in alkaline pretreatment with lime. Some of the sodium hydroxide is consumed during the pretreatment, being incorporated into the biomass as salts (Balat et al. 2008; Carvalheiro et al. 2008; Mosier et al. 2005).

4.3 INHIBITORS

One of the major challenges associated with the processing of lignocellulose for conversion into other products is the unintentional production of inhibitors during the unit operations upstream of fermentation. Both pretreatment and hydrolysis processes are known to produce compounds inhibitory to subsequent processes when performed under certain conditions (Elander et al. 2009; Hodge et al. 2008; Holtzapple et al. 1990; Kim et al. 2011; Palmqvist and Hahn-Hagerdal 2000; Qing et al. 2010; Thomsen et al. 2009; Ximenes et al. 2011b). Limiting the production of inhibitors not only provides a better environment for the enzymes and fermentative organisms to perform at their optima, but it also limits the amount of fermentable sugars lost to degradation products. Both of these aspects have a significant impact on final useful product yields and ultimate feasibility of the process.

Each pretreatment method has its own advantages and disadvantages, especially in terms of production of inhibitors. The duration, pH and temperature of the pretreatment, as well as the lignocellulose material being pretreated, all contribute to the

types of inhibitors that are produced (Vertes et al. 2010). Figure 2.1 illustrates the variety and origins of just a few examples of degradation products formed during pretreatment processes. Furfural and hydroxymethyl furfural (HMF) are two of the most common inhibitors produced from the degradation of pentoses and hexoses, respectively, under acidic conditions (Klinke et al. 2004). Solubilization of lignin can lead to the production of phenolic degradation products (Ximenes et al. 2011b), like vanillin and guaiacol, as well as organic acids, like acetic and formic acids (Vertes et al. 2010).

Inhibitors produced during pretreatment and enzymatic hydrolysis can greatly impact enzyme activity, slowing down the rate and/or extent of hydrolysis. Monosaccharides (glucose, xylose) and oligosaccharides (glucooligomers, xylooligomers) produced from the solubilization of cellulose and hemicellulose under certain pretreatment conditions have been shown to be inhibitory to cellulolytic enzymes (Kim et al. 2011; Qing et al. 2010). Even though the goal of enzymatic hydrolysis in the conversion process is to break down the cellulose into glucose to be used in fermentation, glucose and cellobiose produced from the catalytic activity of the enzymes are also known inhibitors of the cellulase enzymes due to product inhibition inherent in enzymes. For instance, Holtzapple et al. (1990) reported that cellulase retained only 37% of its activity when subjected to a 55% glucose solution. Cellobiose is a strong inhibitor of cellulase, so β -glucosidase is often used to supplement the cellulase to reduce the inhibitory effects of cellobiose. Additionally, phenol-based compounds also inhibit cellulase enzymes. In one study, it was shown that vanillin at a concentration of 10 g/L reduced cellulose hydrolysis of wet cake by 50% (Ximenes et al. 2011b). Enzymatic hydrolysis is already considered the bottleneck of the lignocellulose conversion process. Production of compounds that could retard the rate of hydrolysis any further is highly undesirable.

Fermentative organisms are also highly susceptible to inhibitory compounds produced during the degradation of lignocellulosic material. Sugar degradation products furfural and HMF can affect cell growth and ethanol production rates at relatively low concentrations. At ~4 g/L, HMF can increase the lag phase prior to growth and metabolic activities of *Saccharomyces cerevisiae*. Slightly higher concentrations of HMF (15 g/L) can completely inhibit growth and product formation of the yeast (Vertes et al.

2010). Um and van Walsum (2012) reported a production of ~18 g/L HMF resulting from a dilute acid pretreatment performed at 185°C for 35 min with 0.7% (w/v) sulfuric acid on 8% solids. However, the effects of inhibitors can be cumulative, and complete inhibition can occur at even lower concentrations when multiple inhibitors are present in combination (Klinke et al. 2004). Phenols, like vanillin, can be inhibitory at still lower concentrations (~1.5 g/L) (Palmqvist and Hahn-Hagerdal 2000; Vertes et al. 2010). Organic acids, like acetic and formic, tend to have a lesser effect on *S. cerevisiae* than some bacterial fermentative organisms, although the growth of the yeast can be impacted (Vertes et al. 2010).

4.4 MODELING OF HETEROGENEOUS SYSTEMS

Modeling has been used for many years to predict or to gain a better understanding of enzymatic hydrolysis of cellulose. Many existing kinetic models are based on the Michaelis-Menten equation. However, one of the underlying assumptions for deriving this equation is that the reaction is homogeneous (single-phase) in nature (Fan and Lee 1983; Xu and Ding 2007). Enzymatic hydrolysis of cellulose is, in fact, a heterogeneous reaction because the cellulase is soluble whereas the cellulose is insoluble. Therefore, the enzyme and the substrate are found in two different phases, making this reaction heterogeneous.

While models that consider the heterogeneous nature of the reaction have been developed in recent years, it is interesting to note two older studies that recognized the problem of heterogeneity (Fan and Lee 1983; Huang 1975). However, these studies then made assumptions that essentially resulted in models constructed for homogeneous systems.

More recently, Valjamae et al. (2003) applied fractal kinetics to the enzymatic hydrolysis of cellulose. Fractal kinetics had previously proven to be effective in modeling chemical reactions that are diffusion-limited or dimensionally-restricted (Movagarnejad et al. 2000; Valjamae et al. 2003), both of which can be used to describe the hydrolysis reaction of lignocellulose. Dimensional restriction is very probable in the case of cellulose hydrolysis, since once attached, the exoglucanase enzyme proceeds along the cellulose fibril in one direction. In fractal kinetics, the rate coefficient is time-

dependent and decreases with time. It is possible that dimensional restriction is responsible, at least in part, for the decrease seen in reaction rates. Diffusion limitations may also play a part in the reduction of the reaction rate. The availability of water in these processes, especially as solids loadings are increased, can impact the effectiveness of these conversions. Selig et al. (2012) found that all solids (soluble and insoluble) constrain water in such a way that it is not as readily available for the reaction, with soluble components constraining water more tightly than insoluble solids. It is hypothesized that as the reaction progresses, more solubilized species are present, impacting the availability of water and the environment of the system in a way that is detrimental to the overall reaction rate. The results of the work by Selig et al. (2012) further support the incorporation of a fractal component in lignocellulose hydrolysis models.

In 2007, Xu and Ding (Xu and Ding 2007) returned to the idea of fractal kinetics, but they also incorporated the concept of “jamming” into their model. (See Chapter 7 for model equations.) They contend that the size of the cellulase enzymes is large in comparison to the distance between individual cellulose chains, so as the enzymes bind to available active sites, they block other’s active sites (Figure 4.8). The enzymes essentially cause a traffic jam, decreasing the rate at which the cellulose is broken down. Xu and Ding recognize the fact that their model is not complete and could greatly benefit from some improvements, but it is a good first attempt at explaining the fractal kinetics and jamming concepts. Also, they use Avicel, a model cellulose, for the experimental portion of this study. Using a more realistic substrate like pretreated corn stover to validate the mathematical model presented herein could provide great insights into the adequacy of the proposed hydrolysis mechanism.

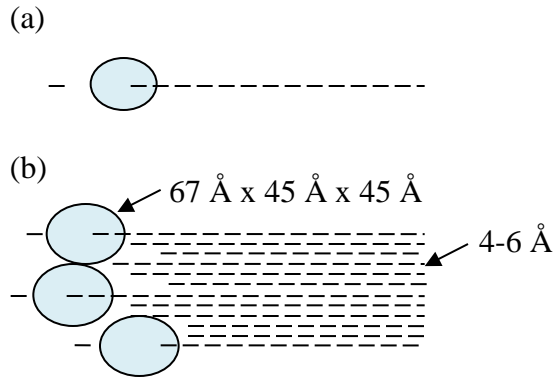


Figure 4.6. (a) Schematic of fractal kinetics. The enzyme (ellipsoid) attaches to the cellulose chain (dashed line), hydrolyzing one glucose monomer at a time. (b) Schematic of jammed kinetics. The size of the enzymes may overcrowd the cellulose chains. Figure adapted from Xu and Ding (2007).

Bommarius and coworkers (2008) use the fractal and jamming model proposed by Xu and Ding (2007) to fit their experimental hydrolysis data, performed at 10% (w/v) cellulose and enzyme loadings of 0.125-62.5 FPU/g cellulose. However, they were studying the effects of pretreatment methods on cellulose and, therefore, also used a model substrate (Avicel). They concluded that as enzyme concentrations increase jamming plays a larger role than either the fractal kinetics or the pretreatment method. As the reaction progresses, fewer sites are available for the enzymes to adsorb to without being spatially hindered by other enzymes.

To date, no studies have been found that fit the fractal and jamming models proposed by Xu and Ding (2007) to data collected from the enzymatic hydrolysis of lignocellulosic material. This current work tested the fractal and jamming models on a more realistic substrate that would be used in large-scale production of biofuels or for other biobased products.

4.5 SEPARATION TECHNOLOGY FOR MONOSACCHARIDE RECOVERY

4.5.1 Current Technology for Monosaccharide Recovery

Methods and processes employed in the separation of components of mixtures vary across a wide range (Table 4.3), but most used for the separation of saccharides tend to fall within three main categories. Extraction (Huang et al. 2008; Ragauskas et al. 2006), membrane filtration (Novalin and Zweckmair 2009; Sanz and Martinez-Castro 2007) and chromatographic separation technologies (Cano et al. 2006; Swallow and Low 2002) have been developed and modified to fit many different situations. The following is provided as a very brief review of select separations technologies and is not meant to be all inclusive.

Table 4.3. Various methods used for separation of components from a mixture.

Method of Separation	Mechanism of Separation
Adsorption	Adhesion of components to a surface
Centrifugation	Differences in density between components
Chromatography	Interaction of components with chromatographic material based on size of the component, affinity of the component for the material, or ion exchange between the component and the material
Distillation	Difference in boiling points of components
Extraction	Use of one substance to solubilize certain components from another substance
Flocculation	Promotion of clumping of solid particles followed by precipitation
Filtration (micro-, ultra-, nano-)	Size
Sieving	Size

Pretreatments are the most prominent form of sugar extraction used in the processing of lignocellulosic material prior to conversion to biofuels. Huang et al. (2008) reviewed several pretreatment options, including steam-explosion based extraction, alkaline extraction, and liquid hot water extraction, that successfully extract

hemicellulose components from biomass. Pretreatments have also been combined with other separation technologies in order to isolate specific carbohydrate fractions. For instance, a 2006 study reports that a twin-screw extruder in combination with ultrafiltration holds great potential for the extraction of hemicellulose components (oligosaccharides, polysaccharides) (Ragauskas et al. 2006). Another combined process includes an alkaline pretreatment system and a nanofiltration membrane that is capable of separating xylooligomers with a molecular weight as low as 200 (Huang et al. 2008). However, neither of these methods is effective for separating monosaccharides from solution. The pretreatment conditions must be severe enough to remove the hemicellulose fraction from lignocellulose, but mild enough to avoid complete solubilization of hemicellulose into its monomer components.

Membrane filtration is often utilized as a sample preparation technique (Sanz and Martinez-Castro 2007), but it is also used to separate valuable substances from liquid fractions in biorefineries (Novalin and Zweckmair 2009). Samples are typically filtered prior to entering the chromatography column in order to remove any insoluble materials that may cause blockages. Sample preparation techniques can be applied to carbohydrate fractionation; however, these techniques are tedious and lack automatization (Sanz and Martinez-Castro 2007). For carbohydrate fractionation applications, ultra- and nanofiltration has become more popular recently. However, these filtration methods are limited to separating oligosaccharides from polysaccharides (Sanz and Martinez-Castro 2007). One study reviewed by Sanz and Martinez-Castro (2007) shows that a combination of ultra- and nanofiltration membranes produced promising results for purifying and concentrating oligosaccharides from chicory rootstock. Nanofiltration has also been applied to the separation of hemicelluloses from concentrated alkaline process liquors (Schlesinger et al. 2006). Plasticized liquid membranes have been used to separate fructose from glucose for the production of high fructose corn syrup (Sanz and Martinez-Castro 2007). These types of membranes had previously proven successful in the sugar industry by separating sugars relatively close in molecular weights. Plasticized liquid membranes are often utilized in separating sucrose, glucose and fructose from molasses, sugar cane and sugar beet juice samples (Sanz and Martinez-Castro 2007). While membrane filtration technology is getting better at separating sugars at smaller

molecular weights, membrane filters are still not selective for any characteristic other than size, making monosaccharides like glucose (MW = 180 g/mol) and xylose (MW = 150 g/mol) nearly indistinguishable. In order to collect a specific hydrolyzate component, other technologies must be explored.

Chromatographic separation is a technology that allows for a higher degree of specificity as compared to filtration. Several different types of chromatography columns have been developed for carbohydrate separation, including gel permeation, reverse-phase, silica, amino-bonded silica and fixed ion resin columns (Sanz and Martinez-Castro 2007; Swallow and Low 2002). Size exclusion chromatography is widely used for the fractionation and analysis of molecular weight distributions of polysaccharides of industrial or biochemical importance (Churms 1996b) because it is easily automated and environmentally friendly (Sanz and Martinez-Castro 2007). Unfortunately, similarly sized components are eluted at approximately the same time.

One commercial-scale process that separates sugars from a complex mixture is the honey industry. The honey industry analyzes the carbohydrate constituents by HPLC. These columns are typically packed with amine-modified silica (Cano et al. 2006). While amine-modified silica is used because of its relatively low cost and high capacity for carbohydrate analytes (Churms 1996a), this method only allows identification and quantification of some of the carbohydrates (Cano et al. 2006). Also, this method of separation is limited to laboratory scale; it has not been applied to separation at the industrial level. At the industrial scale, the separation of glucose and fructose occurs with the use of sulfonated cross-linked styrene divinylbenzene cation exchange resins in the calcium form (Lei et al. 2010; Luz et al. 2008; Vankova et al. 2010). Carbon fractionation is another method used to separate out sugars from complex matrices, like honey. Activated charcoal and ethanol gradients have successfully separated monosaccharides from honey (Ruiz-Matute et al. 2008; Sanz and Martinez-Castro 2007). While each of these options has their own advantages, many of these options poorly resolve similarly structured carbohydrates and are less efficient at ambient temperatures (Swallow and Low 2002), which limits their use for separation of glucose or xylose from other hydrolyzate constituents.

Much research is being conducted in the area of separating sugars that result from biomass hydrolyzate. Wooley et al. (1998) studied the use of a simulated moving bed (SMB) ion exchange chromatography process to separate and purify hydrolyzate sugars from other components like sulfuric and acetic acid. A commercially available adsorbent, Dowex 99, was used in the SMB, and it was shown that this method could effectively isolate the sugars from the other impurities in the hydrolyzate. However, all the sugars eluted at approximately the same time, so separation among sugars did not occur. Xie et al. (2005a) also saw the same results using two different adsorbent materials (Xie et al. 2005b); the sugars were collected as a “center cut” since they eluted at the same time. Lei et al. (2010) extended the search for an adsorbent material that effectively fractionated hydrolyzate sugars by characterizing the adsorption behavior of glucose, arabinose and xylose on five different cation exchange resins. They found that as cross-linking decreases, the separation of these three sugars is much better. The Ca^{2+} ion loaded resin also provided the best separation of these sugars over K^{+} and Fe^{3+} ion loaded resins. However, the degree to which these sugars are resolved is not clear since isotherms for each monosaccharide were determined individually, and selectivity factors were calculated to determine each material’s ability to separate the monosaccharides.

In the last couple of decades, molecularly imprinted polymers (MIPs) have been explored as a means to achieve a more specific separation. MIPs have become a well-developed tool for complex separation processes (Sanz and Martinez-Castro 2007), such as the separation of dyes, vitamins, nucleotide bases and other components that are typically difficult to separate (Li and Li 2007; Wizeman and Kofinas 2001). MIPs are becoming more popular because they are tailor-made for specific separations. For example, Wizeman and coworkers (2001) developed a novel MIP that was capable of binding glucose. The results showed that the mass of glucose binding was significantly higher on the imprinted material as opposed to the non-imprinted material and that glucose binding increases and fructose binding decreases as cross-linking within the material increases. This concept is constantly being expanded upon as more imprinting techniques, imprinting molecules and imprinted materials are researched and developed.

4.5.2 Benefits of Sugar Separations

There are several benefits associated with separating the C5 sugars from the C6 sugars prior to fermentation. *Saccharomyces cerevisiae*, the most common fermentative organism currently used, is unable to utilize pentoses without being genetically modified (Girio et al. 2010; Ho et al. 1998). Xylose is essentially wasted upon entering the fermentation process since *S. cerevisiae* does not have the proper metabolic pathways to process it. This process stream can either be fed into a co-fermentation reaction scheme that contains an organism capable of converting pentoses to ethanol or the C5 sugars can be utilized as building blocks (Figure 4.6) for commodity or high-value chemicals (Carvalho et al. 2008; Kadam et al. 2008). By diverting this energy-rich stream to other higher value bioproducts, biorefineries become more viable and more competitive with petroleum refineries.

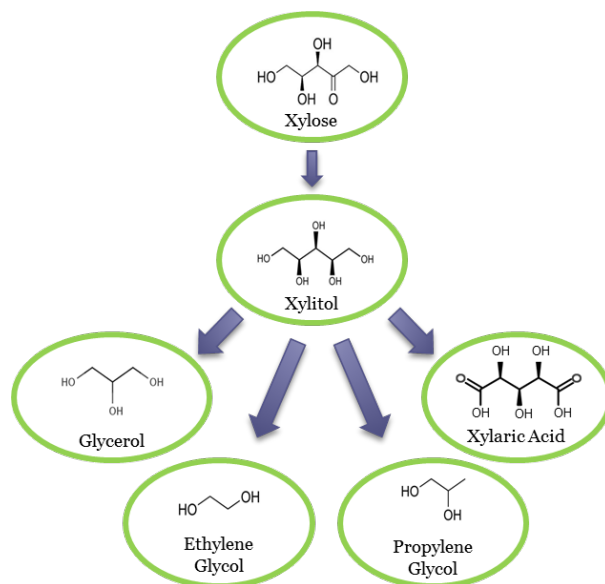


Figure 4.7. Chemicals that can be produced from xylose that are used commercially and produced at the commodity scale volume. (Adapted from Werpy and Peterson (2004)).

4.5.3 Liquid Chromatography

Liquid chromatography is an important tool in preparative chemistry. It is often used to separate a particular compound from a mixture of compounds prior to further use (Belter et al. 1998; Harrison et al. 2003). Liquid chromatography involves applying a

liquid sample to an adsorbent material, or stationary phase, and following it with another liquid, or mobile, phase. As compounds flow past the stationary phase, they interact with the adsorbent based on the compound's own properties. The strength of interaction will cause the compounds to separate from one another and exit the column at different times (Figure 4.7). Fractions of the exiting mobile phase containing the separated compounds can then be collected.

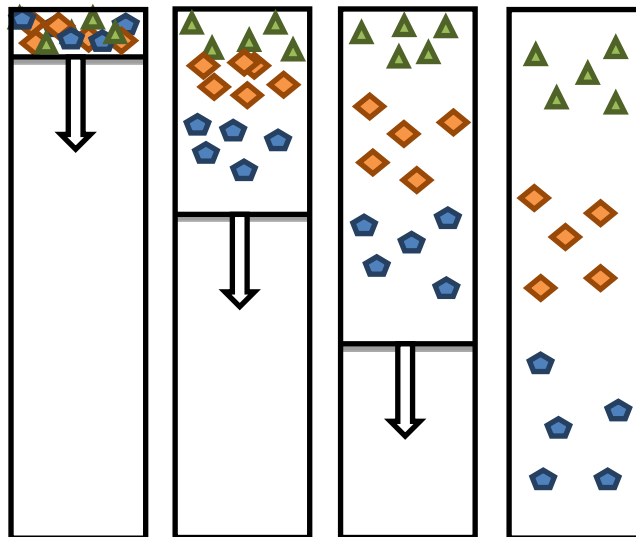


Figure 4.8. Illustration of column chromatography. Over time, as the mobile phase runs through the column, the solutes separate from each other based on how they interact with the stationary phase. Figure adapted from Harrison et al. (2003).

The type of adsorbent material and its characteristics are very closely tied to its ability to separate a mixture of compounds. Silica is compatible with water and organic solvents and works well with hydrophilic compounds (Harrison et al. 2003). Silica particles are also capable of having a large surface area, which is important for adsorption, while maintaining a small particle size. However, particle size must be taken into consideration when designing a chromatography process. A small, uniform particle size is critical for adequate separation, but the pressure drop across the packed bed increases with a decrease in particle size. Separation resolution and pressure drop must be balanced in order to get the most effective separation possible. Mesoporous silica nanoparticles are one class of materials that can be used for liquid chromatography.

These materials can be tailor-made to give various structural characteristics (i.e. particle size, pore size, surface area) based on the conditions used for synthesis (i.e. temperature, pH, surfactants) (Bogush et al. 1988; Wu et al. 2013).

This current work investigated the use of silica nanoparticles synthesized by different methods for their effectiveness of selectively separating specific monosaccharides from mixtures. Both bulk adsorption and liquid chromatography techniques were studied.

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CHAPTER 5: PROJECT OBJECTIVES

The overall goal of this research was to improve the efficiency and effectiveness of the lignocellulose conversion process through a more basic understanding of pretreatment and enzymatic hydrolysis at high solids including kinetic modeling and separation and recovery of the glucose stream.

The impact of high-solids loadings on the effectiveness of sodium hydroxide pretreatment and enzymatic hydrolysis of corn stover were investigated in this study. High-solids loadings have been receiving much attention recently as a solution to increasing saccharide and ethanol yields from lignocellulosic feedstocks. It was hypothesized that high-solids pretreatment followed by high-solids enzymatic hydrolysis would result in characteristically different behaviors from when one or both of these subsequent processes are performed at low-solids loadings. The goal of this study was two-fold: (1) investigate existing methods of pretreatment and enzymatic hydrolysis for use at high solids and (2) determine the effects of sodium hydroxide pretreatment conditions performed at high-solids loadings on saccharide yields from enzymatic hydrolysis performed at low- and high-solids loadings to gain an understanding of the inhibition observed in high-solids conversion processes. Corn stover was used in this project because in addition to being recognized as a potential feedstock for biofuel and biochemical production, it is a by-product of a major production crop in the state of Kentucky. Sodium hydroxide pretreatment was selected for use in this work for several reasons. Sodium hydroxide pretreatment does not produce as many types of inhibitors as other pretreatment options. Lignin can be solubilized by this pretreatment but typically is not under the conditions chosen for this work. Hemicellulose also remains intact, very nearly eliminating the possibility of producing sugar degradation products. Hemicellulose can then be fractionated from cellulose and recovered as a separate processing stream in a useful form that can be diverted for use as an industrial feedstock for other chemical or biochemical processes. Other pretreatments, like dilute acid pretreatment, can separate hemicellulose from cellulose, but it is solubilized and not in a form that can be readily used.

The data from the previous experiments were used to test the robustness of an enzyme kinetics model that included fractal kinetics and jamming effects as a means to explain the decline in the rate of reaction of enzymatic hydrolysis of high solids. This current work was the first that used data collected from the enzymatic hydrolysis of lignocellulosic material to fit models that included fractal kinetics and jamming effects. It was hypothesized that the addition of one or both of these additional parameters would provide a better fit (and therefore a better description of the complex, heterogeneous reaction of enzymatic hydrolysis of lignocellulose) than the classical Michaelis-Menten kinetics model. Evaluation of this model with a real lignocellulosic substrate will provide insight into how well the mechanisms involved in high-solids hydrolysis are understood, which could lead to an improved understanding of the enzyme-substrate interactions and glucose yields from high-solids conversion processes.

The purpose of the final study was to develop effective separation techniques for saccharides produced from enzymatic hydrolysis of lignocellulosic material. It was hypothesized that the development of a new imprinting technique used in the synthesis of imprinted silicate materials could selectively separate and recover specific monosaccharides produced during enzymatic hydrolysis of lignocellulose. Specifically for this work, the recovery of hydrolyzate sugars using newly developed, molecularly-imprinted materials in bulk and solid phase extraction were quantified. The overall project was a collaborative, multidisciplinary effort among several groups (Biosystems and Agricultural Engineering, Chemical Engineering and Material Science and Occupational and Environmental Health) and universities (University of Kentucky and University of Iowa). The portion of this current work contributing to the overall project was the testing of the new materials in real hydrolyzate solutions. Successful separation of pentose (predominately xylose) and hexose (glucose) saccharides found in hydrolyzate would allow for the development and improvement of biorefinery processes that exploit every component of lignocellulose, expanding the range of products to more closely resemble those of a standard petroleum refinery.

CHAPTER 6: THE IMPACT OF HIGH-SOLIDS LOADINGS ON THE EFFECTIVENESS OF SODIUM HYDROXIDE PRETREATMENT AND SUBSEQUENT ENZYMATIC HYDROLYSIS OF CORN STOVER

6.1 SUMMARY

Sodium hydroxide (NaOH) pretreatment and subsequent enzymatic hydrolysis were performed at high-solids loadings using corn stover as the substrate. Factors investigated included duration of pretreatment at different temperatures and NaOH loadings, as well as hydrolysis solids and enzyme loadings. Durations of <120 min at low to moderate temperatures (25°C-70°C) did not have significant effects on the subsequent composition of corn stover when pretreated at 20% (w/w) solids. However, while the post-treatment composition was essentially equivalent for all time and temperature combinations tested, the structure of the components was likely affected by the pretreatment, as differences in subsequent cellulose conversions were observed. At high-solids loadings, cellulose conversions ranged from ~28-37% for corn stover pretreated at 25°C, whereas conversions were ~5-8% for corn stover pretreated at 70°C. Additionally, when enzyme loadings were investigated, cellulose conversions decreased to ~4% at the median enzyme loading before increasing as high as ~37% for the highest enzyme loading for corn stover pretreated at 25°C. However, conversion of corn stover pretreated at 70°C was not significantly different among the subsequent enzyme loadings tested and ranged from 5%-8%.

NaOH loading during pretreatment was examined to determine its effects on the post-treatment composition of biomass, as well as cellulose conversion efficacy in the subsequent enzymatic hydrolysis step. Increased NaOH loadings improved the cellulose content of the corn stover compared to the raw corn stover for the 2 hr pretreatment from 38% to 49% by reducing other components like ash, lignin and other unquantified components. NaOH loadings up to 10 g NaOH/100 g corn stover during the 24 hr pretreatment increased percent cellulose content from 37.9% to 46.6% but at higher NaOH loadings the percent cellulose content did not increase by as much. At a NaOH loading of 20 g NaOH/100 g corn stover, xylose content decreased for the 24 hr pretreatment. The degradation of xylose is of concern because it could result in an increased concentration of inhibitory products. Even with modifications made to the corn

stover composition, cellulose conversion of the corn stover pretreated for 2 hr decreased from 5.7% to 0.6% with NaOH loadings increasing from 4 to 20 g NaOH/100 g corn stover when hydrolyzed at 20% (w/w). The same trend was observed at low-solids loadings; however, the conversions were significantly higher (40.6% to 21.6%). Low conversions (<9%) were also observed for corn stover pretreated for 24 hr and hydrolyzed at high-solids loadings. Even though the corn stover was water-washed following pretreatment, no additional measures were taken to adjust the pH of the corn stover prior to use in enzymatic hydrolysis. Inadequate neutralization of the corn stover following pretreatment is a likely cause of the reduced conversions.

Flushing of the hydrolyzate and reusing of the substrate was also studied as a method for reducing inhibitory compounds affecting hydrolysis in order to increase overall glucose yields. Glucose conversions increased from 37%-49% for conventional batch reactions up to 73%-99% for flushed reactions. While conversion of the unwashed PCS was not as high as that of the washed PCS, the unwashed PCS with flushed hydrolysis still achieved significantly higher glucose concentrations than that of the washed PCS in conventional batch hydrolysis (73 g/L vs. 48 g/L) with an enzyme loading of 15 FPU/g solids. It can be inferred from this study that flushing of the PCS throughout the hydrolysis reaction eliminates the need to wash the pretreated biomass prior to enzymatic hydrolysis, thus reducing the amount of process water required.

6.2 INTRODUCTION

Lignocellulose can provide an abundant and renewable source of energy. While conversion of lignocellulose into liquid fuels is not a new idea, the use of high-solids loadings in the unit steps of pretreatment and subsequent enzymatic hydrolysis is relatively recent (Jorgensen et al. 2007b; Kristensen et al. 2009b; Pristavka et al. 2000). Systems are considered to be “high solids” at solids loadings $\geq 15\%$ (w/w). The advantages of operating at high solids are increased sugar and ethanol concentrations and reduced capital and operating costs (Banerjee et al. 2010; Hodge et al. 2008; Humbird et al. 2010). However, at this level of solids loadings, several challenges emerge that are not as apparent at low- or moderate-solids loadings. For example, the lack of available water in the system and inadequate mixing of the solids can limit heat and mass transfer.

In pretreatment, these limitations can lead to temperature gradients that may result in non-uniform treatment of biomass. In hydrolysis, these limitations can lead to regions of sub-optimal temperatures and pockets of increased inhibitor concentrations, both of which are detrimental to enzyme activity. Additionally, while interest in the use of high-solids loadings for pretreatment or enzymatic hydrolysis is increasing, few investigations into the operation of the combination of these two processing steps at high-solids loadings are available (Larsen et al. 2008; Lau and Dale 2009).

Pretreatment with sodium hydroxide reportedly results in several structural modifications of lignocellulose that are beneficial for enzymatic hydrolysis (Cheng et al. 2010; Cui et al. 2012; Xu et al. 2010). Bonds linking the protective lignin barrier with hemicellulose are broken. Depending on the pretreatment conditions, lignin is partially or totally solubilized, and degradation of the hemicellulose fraction may occur. Sodium hydroxide pretreatment also swells the lignocellulose particles, leading to an increase in surface area and greater accessibility to the cellulose fraction (Hendriks and Zeeman 2009). Additionally, a decrease in the degree of polymerization and crystallinity of the cellulose is likely, increasing the enzymatic digestibility of the polysaccharide (Eronen et al. 2009; Mittal et al. 2011).

Enzymatic hydrolysis is often identified as a bottleneck in the lignocellulose conversion process (Jorgensen et al. 2007a). The release rate of glucose is not constant. The initial rates tend to be very quick; however, glucose released slows as the reaction progresses. The use of high-solids loadings in enzymatic hydrolysis has aided in producing a more concentrated glucose product, but the reduction in glucose release rate is still observed, likely caused by the inhibition of enzymes by glucose and other inhibitory products. Simultaneous saccharification and fermentation is one method that has been studied extensively to alleviate inhibition of enzymes by these products; however, the optimum conditions for the hydrolytic enzymes and the fermentative organisms are not identical, thereby causing some loss of efficiency. Another method that has recently been suggested for high-solids systems is flushing of the hydrolyzate to reuse the substrate (Yang et al. 2010a). This method could remove potentially inhibitory products from the reactor, relieving some of the stress on the enzymes, thereby boosting the rate of glucose release and using the biomass more effectively.

The objective of this study was to identify and characterize the effects of NaOH pretreatment on the post-pretreatment composition of biomass and the performance of the subsequent enzymatic hydrolysis when both processing steps are performed at high-solids loadings. A relatively new enzymatic hydrolysis design employing high-solids loadings with hydrolyzate flushing and substrate reuse was also investigated to determine its effectiveness at removing inhibitory products while maintaining a consistent rate of glucose production. The hypothesis was that substrate reuse would allow the solids effect to be mitigated.

6.3 MATERIALS AND METHODS

6.3.1 Enzyme

The enzyme system consisted of crude cellulase liquid from *T. reesei* (Celluclast 1.5L) supplemented with β -glucosidase from *A. niger* (Novozyme 188). Both enzymes were purchased from Sigma (St. Louis, MO).

6.3.2 Substrate

Corn stover collected directly from the field at the Woodford County Animal Research Center in Woodford County, KY in September 2010 was used as the substrate. The corn (P1253 Pioneer) had been planted using conventional tillage practices in April 2010. Stover is composed of material other than grain (MOG). After collection, the samples were prepared for laboratory storage by drying at 45°C and grinding through a hammer mill with a 5 mm screen.

6.3.3 Peterson Method for Protein Determination

The Peterson method was conducted according to the protocol provided with the total protein kit purchased from Sigma (TP0300; St. Louis, MO). The standard curve ranged from 0-400 $\mu\text{g}/\text{mL}$ at 100 $\mu\text{g}/\text{mL}$ intervals, and included 50 $\mu\text{g}/\text{mL}$. Sample tubes with the *T. reesei* cellulase and *A. niger* β -glucosidase of varying activities (listed below) were prepared in triplicate. To all tubes, 1.0 mL of the Lowry reagent was added and mixed. The tubes were incubated for 20 min at room temperature. Folin and Ciocalteu's phenol reagent (0.5 mL) was added to each tube and immediately and rapidly mixed.

The tubes were then incubated for 30 min at room temperature to allow color to develop. The absorbance was measured at 750 nm against the 0 µg/mL protein blank.

6.3.4 Cellulase Activity Assay

The cellulase activity assay was conducted as outlined by the NREL LAP-006 (Adney and Baker 1996). Standard curve tubes using 10 g/L stock glucose solution were prepared in triplicate and ranged from 0-10 mg/mL. A filter paper strip (1 cm × 6 cm; approximately 50 mg) and 1.0 mL of the Na-citrate buffer were added to all the sample test tubes. All tubes were equilibrated to 50°C in a water bath. Cellulase samples prepared at different initial activities were diluted in 0.05 M Na-citrate buffer at pH 4.8 so that the final volume was 0.5 mL and was added to each of the sample test tubes. Five cellulase dilutions were tested in triplicate, as well as a substrate blank and an enzyme blank. All tubes were incubated at 50°C for 1 hr, at which time 3.0 mL DNS reagent was added to stop the hydrolysis reaction. The tubes were placed in a 93°C water bath for 15 min to allow for color formation. The tubes were cooled in an ice water bath prior to vortexing and centrifuging the samples for 10 min at 6000 rpm. A 0.2 mL aliquot of the assay solution was diluted in 2.5 mL DI water, and the absorbance was measured at 540 nm against the 0 mg/mL glucose standard blank.

Cellulase activity was determined by comparing the sugar concentrations of the sample tubes to the standard curve. The amount of sugar released was plotted against the enzyme concentration (log scale). The enzyme concentration that released 2.0 mg glucose was estimated, and cellulase activity was calculated using the following equation

$$\text{Filter paper activity (FPU/mL)} = \frac{0.37}{[\text{E}] \text{ releasing 2.0 mg glucose}} \quad \text{Equation 6.1}$$

where [E] is the enzyme concentration. The activity is expressed in filter paper units (FPU)/mL of original enzyme solution, where 1 FPU/mL is the amount of enzyme required to release 1 µmol reducing sugar/min.

6.3.5 Composition of Corn Stover

Laboratory Analytical Procedures (LAP) established by the National Renewable Energy Laboratory (NREL) were used to determine total solids, structural carbohydrates, soluble- and insoluble lignin and ash of raw and pretreated biomass (Sluiter et al. 2005; Sluiter 2008a; Sluiter 2008b). HPLC was used to measure the sugars derived from cellulose and hemicellulose (glucose, xylose, arabinose, mannose and galactose). A Dionex U3000 HPLC system was equipped with a Bio-Rad Aminex HPX-87P column and Micro-Guard de-ashing column and operated at 78°C with deionized water as the mobile phase at a flow rate of 0.45 mL/min. The sample components were detected with a Shodex-101 refractive index detector.

6.3.6 Pretreatment of Corn Stover

Sodium hydroxide pretreatment was performed according to Duguid et al. (2009) with some modifications. Ten gram samples of dried, ground corn stover were placed in 500 mL Erlenmeyer flasks. The dry samples were autoclaved on a liquid cycle at 121°C for 30 min to ensure no loss of biomass due to microbial contamination. The flasks were allowed to cool to room temperature prior to equilibration at the selected pretreatment temperature. Following equilibration, 50 mL 0.2 N NaOH was added to each flask to obtain a solids loading of 20% (w/v), unless otherwise stated. The samples were incubated at the treatment temperature (see below) for a selected pretreatment time while being mixed at 150 rpm. The pretreated corn stover (PCS) was washed with DI water (3-5 volumes) and vacuum filtered. The pH of the corn stover was adjusted to the desired pH (see below) with concentrated acetic acid during the washing process. The samples were dried in a 45°C oven for 24 hr. The solids content was determined by drying samples at 105°C for 24 hr. The treated corn stover was stored at 4°C until further use, typically 24 hours or less.

6.3.7 Enzymatic Hydrolysis

The hydrolysis was performed according to an NREL-LAP (Selig 2008), with some modifications. Pretreated biomass was added at the desired solids loading on a weight basis. Cellulase was added to achieve an appropriate enzyme loading and was

supplemented with β -glucosidase at ratio of 2:1CBU/g biomass to FPU/g biomass. Following hydrolysis, the samples were immediately transferred to a boiling water bath for 5 min to denature the enzymes and then in an ice bath to cool. Samples of the slurries were collected and placed in 15 mL centrifuge tubes. The slurry samples were diluted 10-fold with DI water, mixed well, and centrifuged. Samples of the liquid fraction were then collected, diluted and analyzed by HPLC, according to the procedure outlined above (See 6.3.5 Composition of Corn Stover).

6.3.8 Calculation of Cellulose Conversion

Cellulose conversion is typically calculated as the ratio between the amount of glucose (and sometimes cellobiose) released during hydrolysis to the theoretical amount of glucose that could be released during hydrolysis. This calculation is based upon several assumptions: (1) the specific gravity of all components in the reaction are the same (1.0 g/mL), (2) the volume of the liquid is equivalent to the volume of the hydrolysis slurry, and (3) the volume of the liquid remains constant throughout the entire reaction. However, these assumptions do not necessarily hold true at high-solids loadings. Cellulose conversions were calculated according to (Kristensen et al. 2009a). Following enzymatic hydrolysis at high solids, the sample was diluted 10 times, which allows cellulose conversions to be calculated with the following equation

$$\% \textit{ cellulose conversion} = \frac{\frac{m_{ws} - m_{is}}{SG_{aq.phase}} \times ([Glu] + 1.056 \times [Cel])}{1.111 \times m_{cs} \times F_c \times DM} \times 100\% \quad \text{Equation 6.2}$$

where m_{ws} , m_{is} and m_{cs} are the mass of the whole slurry (in grams), the mass of the insoluble solids after hydrolysis (in grams) and the mass of the corn stover (in grams), respectively, [Glc] and [Cel] are the glucose and cellobiose concentrations (in g/L), respectively, 1.056 and 1.111 are conversion factors accounting for the water molecule required to hydrolyze glucose and cellobiose from cellulose, F_c is the fraction of cellulose in the corn stover, and DM is the initial dry matter solids loading.

6.3.9 Experimental Design

6.3.9.1 Low-Solids Loading in Pretreatment

One pretreatment preparation followed that of Sills and Gossett (2012), with slight modifications (Figure 6.1). Briefly, 10 g samples of corn stover were measured and placed in 500 mL Erlenmeyer flasks. The flasks were equilibrated to 25°C prior to the addition of 0.25 N NaOH (20 g NaOH/100 g CS) to obtain a solids loading of 5% (w/v). Samples were incubated for 24 hr with shaking at 200 rpm. Following pretreatment, the corn stover was washed with approximately 500-600 mL DI water and vacuum filtered. The PCS was divided into two portions. One portion was reserved for low-solids enzymatic hydrolysis (2.5 and 5% solids (w/w)), and the other was dried at 45°C for 24 hr prior to enzymatic hydrolysis at low (5% solids) and high solids (20% solids). This method was selected to match the low-solids hydrolysis conditions of Sills and Gossett (2012) and the low- and high-solids hydrolysis conditions of this current study, as well as to determine whether drying the biomass following pretreatment affected the subsequent hydrolysis of the material. Samples were collected from the dried PCS for compositional analysis.

Statistical Analysis. The data were analyzed in a completely randomized design using PROC GLM of SAS to determine whether any differences in cellulose or hemicellulose conversion existed. If differences existed, least squares means were computed, and all possible pairwise comparisons were made among the treatments.

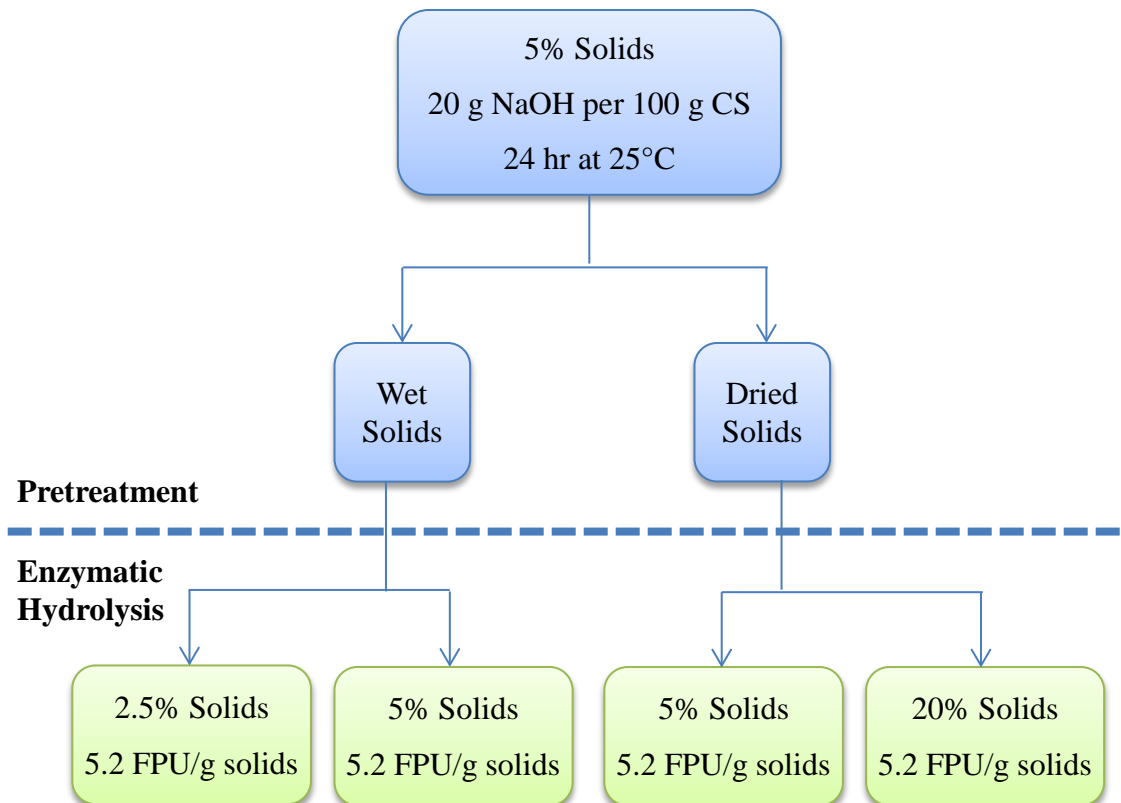


Figure 6.1. Process conditions for the investigation into the effects of drying PCS on glucose yields from subsequent enzymatic hydrolysis.

6.3.9.2 Effects of Pretreatment and Enzymatic Hydrolysis Conditions

Effects of pretreatment time and temperature. Corn stover was pretreated at various times and temperatures to determine their effects on corn stover composition and enzymatic digestibility (Figure 6.2). In these tests, pretreatment was conducted at two temperatures (25°C and 70°C) over four times (30, 60, 90 and 120 min). Pretreatment conditions were intentionally mild to minimize loss of structural carbohydrates and to minimize the production of potentially inhibitory products. PCS was washed with 3 volumes DI H₂O, vacuum filtered and dried. Samples of dried PCS were collected to determine composition.

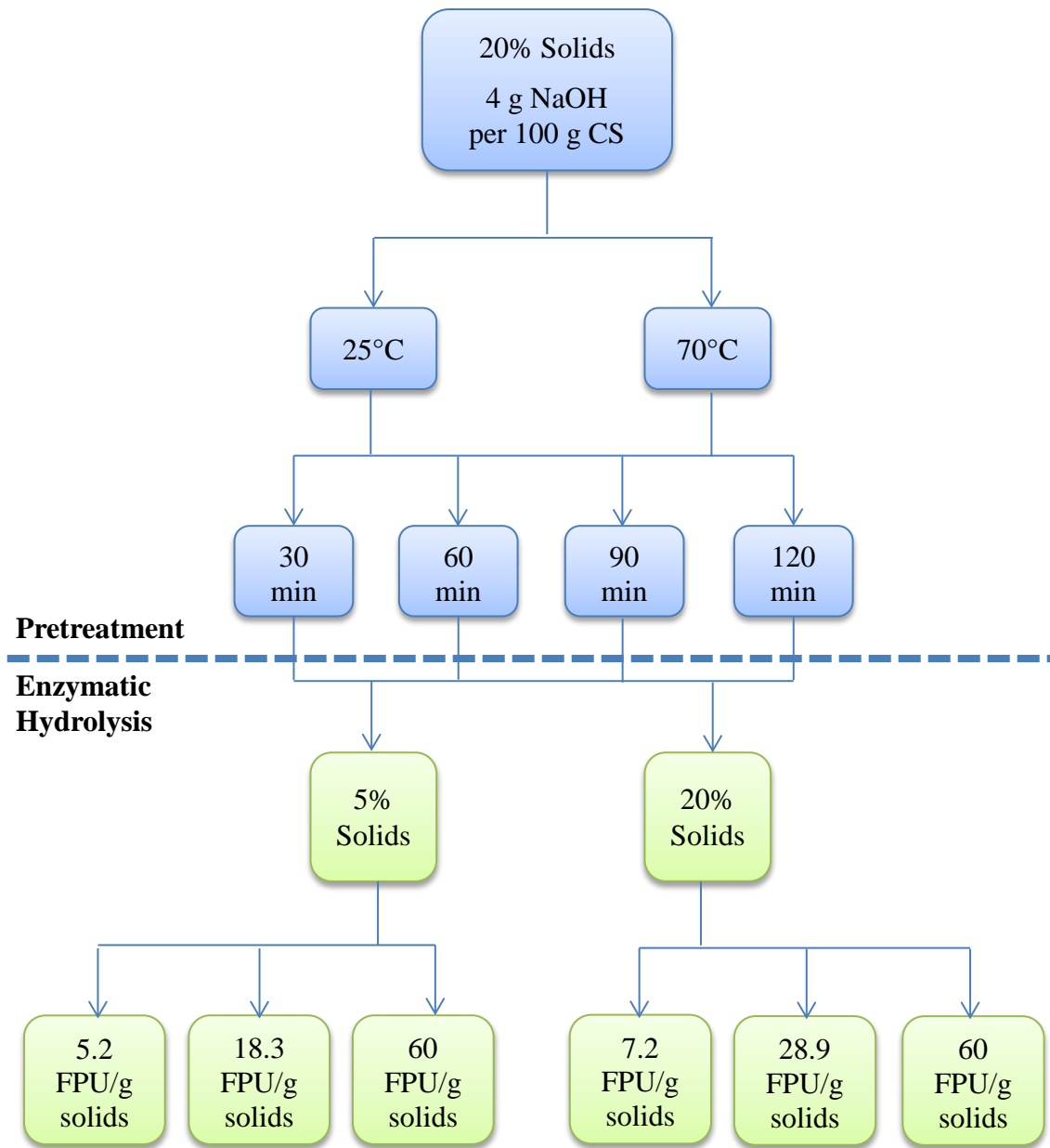


Figure 6.2. Process conditions for the investigation into pretreatment and enzymatic hydrolysis performed at high-solids loadings and their impact on glucose yields.

Effects of enzymatic hydrolysis solids loadings and enzyme loadings. PCS was then enzymatically hydrolyzed at two solids loadings (5% or “low solids” and 20% or “high solids”; 0.25 g PCS samples) and three enzyme loadings. All hydrolysis samples were analyzed by HPLC, according to the procedure outlined above (See 6.3.5 Composition of Corn Stover).

Statistical Analysis. The data were analyzed as a 8×2 factorial with nested variables in a generalized randomized complete block design (pretreatment time × temperature = block) using PROC GLM of SAS to determine whether any differences in cellulose conversion existed. If differences existed, least squares means were computed, and all possible pairwise comparisons were made among the combinations of pretreatment and hydrolysis conditions.

6.3.9.3 NaOH Loading in Pretreatment

Additionally, the effects of NaOH loading during pretreatment were investigated (Figure 6.3). Corn stover samples were prepared as above. Following equilibration at 25°C, 50 mL of NaOH solution (0.2 N, 0.5 N or 1.0 N to achieve a NaOH loading of 4, 10 or 20 g NaOH/100 g CS) were added to each flask to obtain a solids loading of 20% (w/v). The samples were incubated for either 2 or 24 hr while shaking at 150 rpm. The preparation of the pretreated corn stover continued as outlined above. Enzymatic hydrolysis was subsequently performed at solids loadings of 5% and 20% (w/w) and an [E] = 5.2 FPU/g solids.

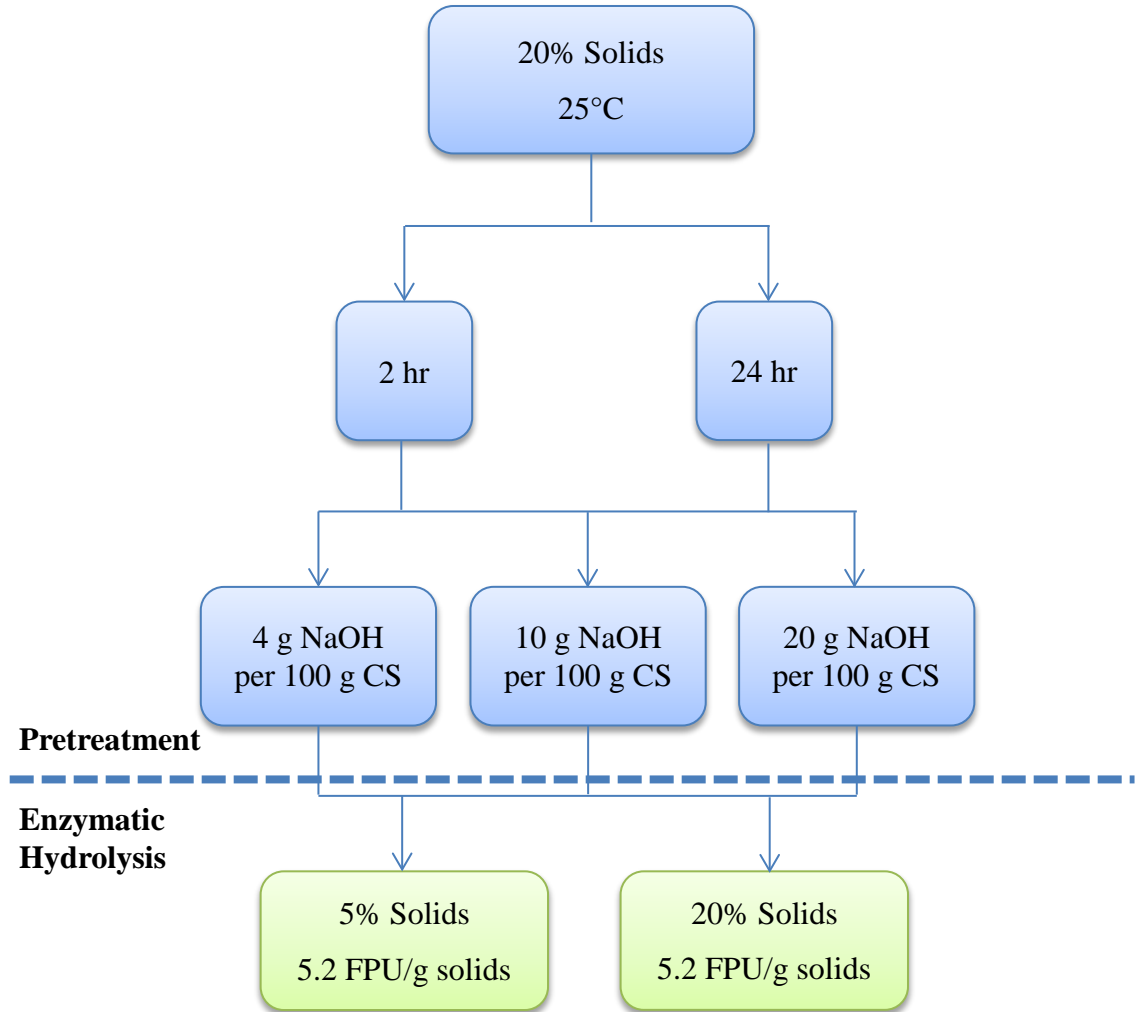


Figure 6.3. Process conditions for the investigation into the impact of NaOH loading in pretreatment on glucose yields in the subsequent enzymatic hydrolysis.

Statistical Analysis. The data were analyzed as a $2 \times 3 \times 2$ factorial in a generalized randomized complete block design (pretreatment time = block) using PROC GLM of SAS to determine whether any differences in cellulose conversion existed. If differences existed, least squares means were computed, and all possible pairwise comparisons were made among the combinations of pretreatment and hydrolysis conditions.

6.3.9.4 *Hydrolyzate Flushing and Substrate Reuse*

Hydrolyzate flushing and substrate reuse experiments were performed in order to investigate the effects soluble end-products have on enzyme performance. Preliminary tests were conducted with corn stover pretreated at 10% (w/v) solids with 10 g NaOH/100 g CS for 24 hr at 25°C. Washed PCS was weighed and placed into columns (10 cm x 2.5 cm I.D. fitted with a porous polyethylene filter disc) for enzymatic hydrolysis. The columns were fitted with three-way valves at each end to allow for addition of fresh buffer through the top and collection of samples from the bottom. Hydrolysis was performed at 20% (w/w) solids with a working volume of 10 mL and an enzyme loading of 15 or 60 FPU/g solids. Control samples were incubated in batch (without interruption) for 72 hr. Hydrolyzate samples were collected by flushing the biomass every 24 hr over the 72 hr hydrolysis period. Flushing was performed by adding 10 mL of 0.05 M sodium citrate buffer through the input valve located at the top of the column with a syringe. A 10 mL sample was then collected from the outlet valve at the bottom of the column, which forced the buffer to move through the biomass, collecting and removing solubilized products. Hydrolyzate samples were boiled for 5 min to denature the enzymes before being stored at -45°C until analysis. Samples were analyzed for glucose content using a YSI 7100 Multiparameter Bioanalytical System (MBS; YSI Incorporated, Yellow Springs, OH).

Additional hydrolyzate flushing and substrate reuse experiments were performed using PCS that was either washed (WPCS) or unwashed (UPCS). The washing process consisted of neutralization through the addition of glacial acetic acid followed by washing with 5 volumes of DI water and vacuum filtration. UPCS was also neutralized with glacial acetic acid before removal of any excess liquid via vacuum filtration. All pretreated corn stover was dried at 45°C for 24 hr. The solids content was determined by drying samples at 105°C for 24 hr. Pretreated corn stover was stored at 4°C until all hydrolyzate flushing experiments were completed (typically within 6 weeks). Enzymatic hydrolysis was performed as outlined above in the preliminary tests with and without enzyme supplementation during the flushing of the columns. For those samples that received additional enzyme, buffer used in the flushing procedure was supplemented with an enzyme loading of 2.5 FPU/g solids. Samples of the liquid fraction were then

collected, diluted and analyzed by HPLC, according to the procedure outlined above (See 6.3.5 Composition of Corn Stover).

Statistical Analysis. The data were analyzed as a 2×2×3 factorial in a generalized randomized complete block design (washing treatment = block) using PROC GLM of SAS to determine whether any differences in glucose released existed. If differences existed, least squares means were computed, and all possible pairwise comparisons were made for all treatment combinations.

6.4 RESULTS

6.4.1 Enzymes

Multiple sources of cellulase and β -glucosidase were used throughout the course of this study. Table 6.1 shows the protein content, measured activity over time and the specific activity for each enzyme source utilized. Activity 2 was measured approximately 14 months after Activity 1. The reduction in activity emphasizes the point that enzyme activity is a dynamic characteristic of the enzyme.

Table 6.1. Characteristics of the Celluclast 1.5L and the Novozyme 188 used in the enzymatic hydrolysis studies.

Enzyme	Protein Content (\pm Std Dev) (mg/mL)	Activity 1 (FPU/mL or CBU/mL) ^a	Activity 2 (FPU/mL or CBU/mL)	Specific Activity (FPU/mg protein or CBU/mg)
<i>T. reesei</i> 1	169.3 (\pm 2.1)	82.2	40.2	0.24 ^b
<i>T. reesei</i> 2	181.8 (\pm 6.3)	105.7	32.7	0.18 ^b
<i>T. reesei</i> 3	175.2 (\pm 16.6)	59.7	--	0.34
<i>T. reesei</i> 4	181.7 (\pm 22.1)	68.5	--	0.38
<i>T. reesei</i> 5	201.7 (\pm 10.1)	73.3	--	0.36
<i>A. niger</i> 1	262.4 (\pm 8.5)	65.6	--	0.25
<i>A. niger</i> 2	282.4 (\pm 17.5)	76.0	--	0.27

^aCellulase activity is measured in FPU/mL and β -glucosidase activity is measured in CBU/mL.

^bSpecific activity is calculated using Activity 2 where available

6.4.2 Effects of Low-Solids Loading in Pretreatment

From a practical standpoint, in order to perform enzymatic hydrolysis with high-solids loadings, the biomass following pretreatment had to be dried to remove any excess water and achieve appropriate solids loadings. The effect of this drying is shown in the next figure. Figure 6.4 presents the cellulose and hemicellulose conversion obtained using different solids loadings in hydrolysis, where the corn stover in the reactors containing 2.5% and 5% solids was still wet and 5% and 20% had been dried. It was possible to operate at 5% solids using either wet or dry corn stover, so both forms were hydrolyzed to determine whether drying affected the conversions. As Figure 6.4 indicated, there are no significant differences in conversion of cellulose or hemicellulose that can be attributed to drying the corn stover at 45°C for 24 hr based on the conversions obtained from hydrolyzing wet and dry PCS at 5% solids.

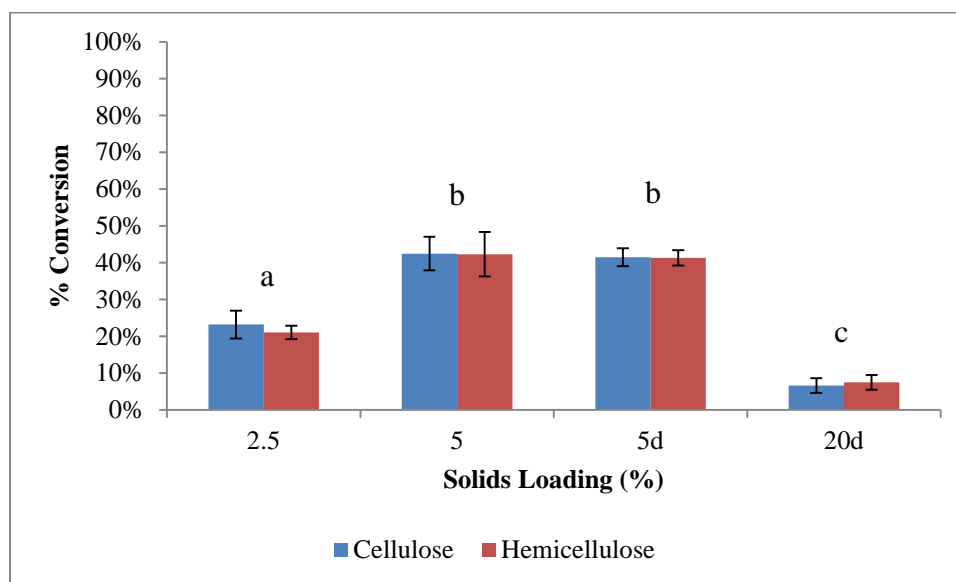


Figure 6.4. Conversion of cellulose and hemicellulose from corn stover pretreated at 5% (w/v) and 25°C for 24 hr with a NaOH loading of 20 g NaOH/100 g CS. Hydrolysis was performed with an enzyme loading of 5.2 FPU/g solids on both wet and dry solids, where the dried solids are indicated with the letter 'd'. Error bars represent standard deviation of three replicates. Column groupings with the same letters are not significantly different from one another. (See Figure 6.1 for experimental conditions.)



Figure 6.5. (From left to right) Raw corn stover, NaOH-pretreated corn stover, and solid fractions following enzymatic hydrolysis performed at 5% and 20% (w/w) solids and $[E] = 60$ FPU/g solids.

6.4.3.1 Characterization of Raw and Pretreated Corn Stover

Compositional analysis performed on raw corn stover resulted in cellulose, hemicellulose, lignin and ash fractions of 37.9%, 21.6%, 21.1% and 4.8%, respectively, with the remaining portion accounting for components that were not quantified (Appendix A.1). Figure 6.6 contains the composition of raw and NaOH-pretreated corn stover at all time and temperature combinations. The NaOH pretreatment appears to mostly affect the acid soluble lignin and the ash fractions, as well as the fraction of components that were not quantified. The acid soluble lignin was reduced by 14.3%-23.9%, while the ash was reduced by 31.5%-46.3% (Appendix A.1). However, there does not appear to be much difference in the overall composition among the various pretreatment conditions.

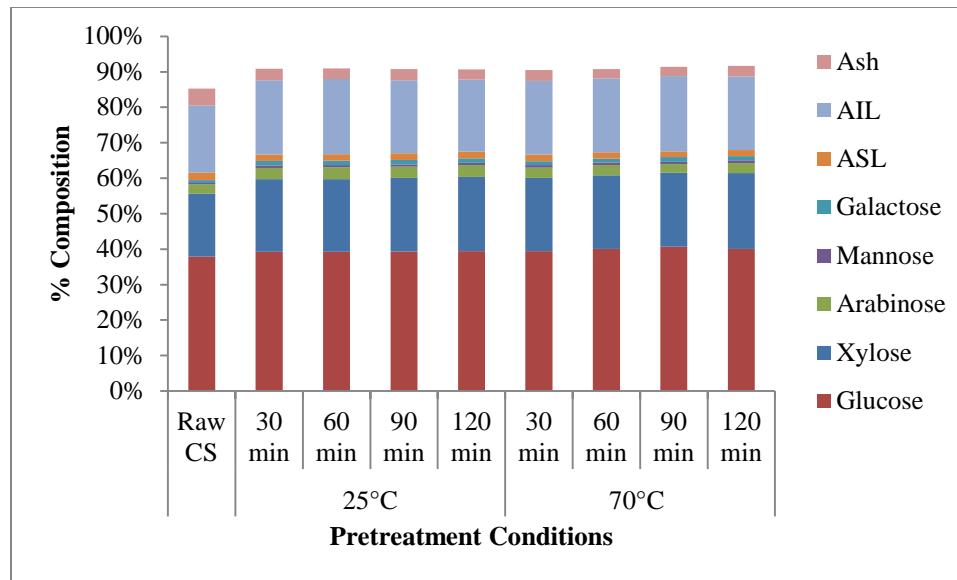


Figure 6.6. Composition of raw and pretreated corn stover. Results are calculated as % oven dried material.

Figure 6.7 shows the composition of pretreated corn stover with and without the addition of NaOH (referred to from this point on as “untreated”). A slight loss of glucose and arabinose (1.3%-1.8% and 4.3%-34.3%, respectively) was observed in the untreated corn stover when compared to the NaOH-pretreated corn stover. It should also be noted that while acid soluble lignin and ash were removed from the untreated corn stover, it was less than the amounts removed from the NaOH-pretreated corn stover. Lastly, 90.5%-91.7% of the NaOH-pretreated corn stover and 84.0%-88.1% of the untreated corn stover is accounted for in the quantified component fractions. Based on the percent total of the measured components calculated, the NaOH pretreatment removes more of the unquantified components than the pretreatment lacking NaOH.

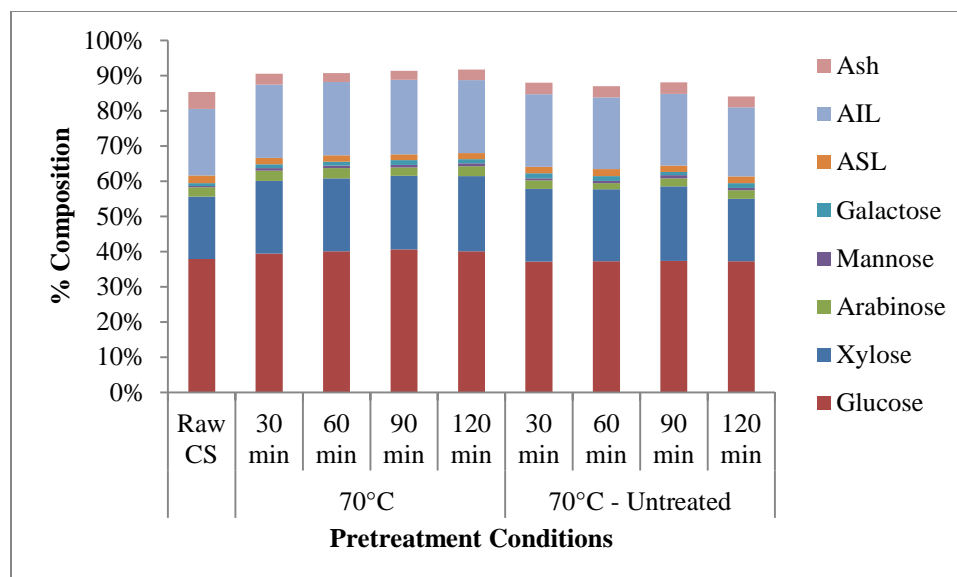


Figure 6.7. Composition of NaOH-pretreated and untreated corn stover. Results are calculated as % oven dried material.

6.4.3.2 Solids Loading in Enzymatic Hydrolysis

Figure 6.8 shows the conversion of cellulose achieved for material pretreated at 25°C for up to 2 hr and enzymatically hydrolyzed at low- and high-solids loadings. Conversion was consistent among all pretreatment conditions for hydrolysis performed at low- and high-solids loadings. At 5% solids, conversion yields fell between 27% and 33%. Conversions for the 20% solids reactions were fairly similar to those of the 5% solids, with cellulose conversions ranging from 28%-37%. Corn stover pretreated for 30 min and hydrolyzed at 5% solids did not reach the same level of conversion as the corn stover pretreated for 120 min and hydrolyzed at 20% solids, resulting in the only pair of significantly different treatments.

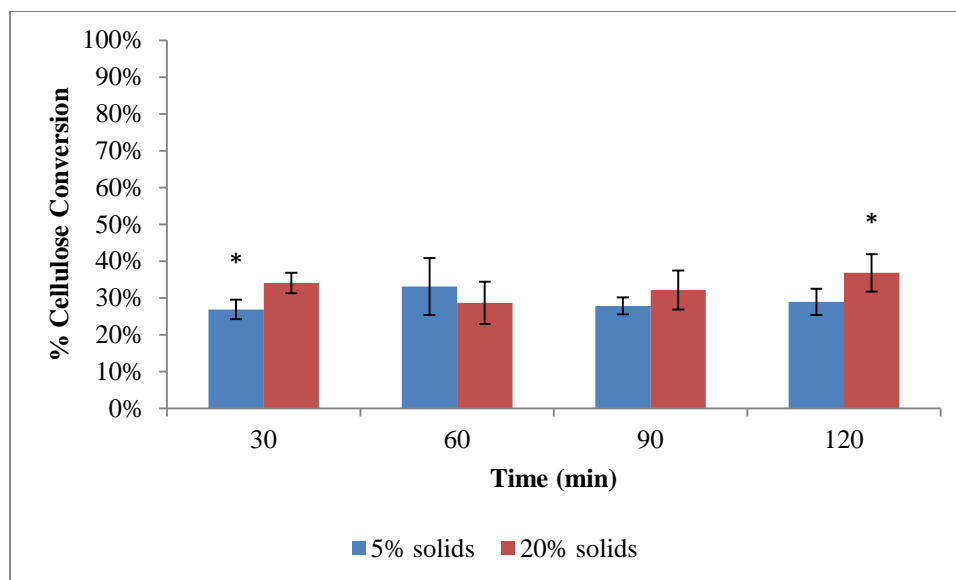


Figure 6.8. Conversion of cellulose pretreated at 25°C for up to 2 hr and enzymatically hydrolyzed at low- and high-solids loadings with [E] = 60 FPU/g solids. Error bars represent the standard deviation of three replicates. Bars labeled with asterisks (*) are significantly different from each other. (See Figure 6.2 for experimental conditions.)

Conversions for corn stover pretreated at 70°C showed a much different trend as compared to pretreatment at 25°C (Figure 6.9). Corn stover hydrolyzed at low-solids loadings achieved conversions ~2-3 times higher than corn stover pretreated under corresponding times at 25°C. For instance, the 90 min pretreatment resulted in cellulose conversion of 28% and 85% for pretreatment temperatures of 25°C and 70°C, respectively (comparing Figure 6.8 (25°C) to Figure 6.9 (70°C)). Hydrolysis performed with high-solids loadings of corn stover pretreated at 70°C was not very productive, with all conversions <8%. Corn stover pretreated at the high temperature produced 3.7-6.7 times less glucose than corresponding samples pretreated at the low temperature.

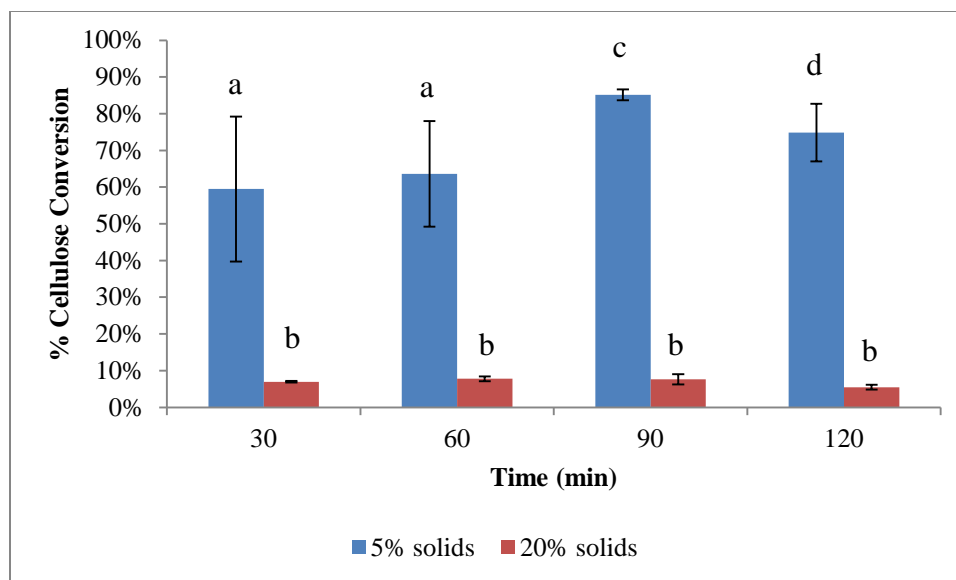


Figure 6.9. Conversion of cellulose pretreated at 70°C for up to 2 hr and enzymatically hydrolyzed at low- and high-solids loadings with [E] = 60 FPU/g solids. Error bars represent the standard deviation of three replicates. Bars with the same letter are not significantly different. (See Figure 6.2 for experimental conditions.)

6.4.3.3 Enzyme Loading

Figure 6.10 quantifies the conversion of cellulose pretreated at 25°C during four sampling times over the course of 2 hrs and subsequently enzymatically hydrolyzed at 5% (w/w) solids comparing three enzyme loadings. Within an enzyme loading, pretreatment times showed no difference in conversion. Conversions ranged from ~27-33% at 60 FPU/g solids to ~34-41% at 5.2 FPU/g solids. Additionally, for the 30, 60 and 90 min pretreatments, enzyme loading does not appear to have any effect on the cellulose conversion, as the conversion remains stagnant as enzyme loading increases. Similarly, for the 120 min pretreatment, a slight decrease in conversion is observed as the enzyme loading increases from 5.2 to 60 FPU/g solids. For instance, the conversions at the 120 min pretreatment decreased from nearly 41% at 5.2 FPU/g solids to ~29% at 60 FPU/g solids.

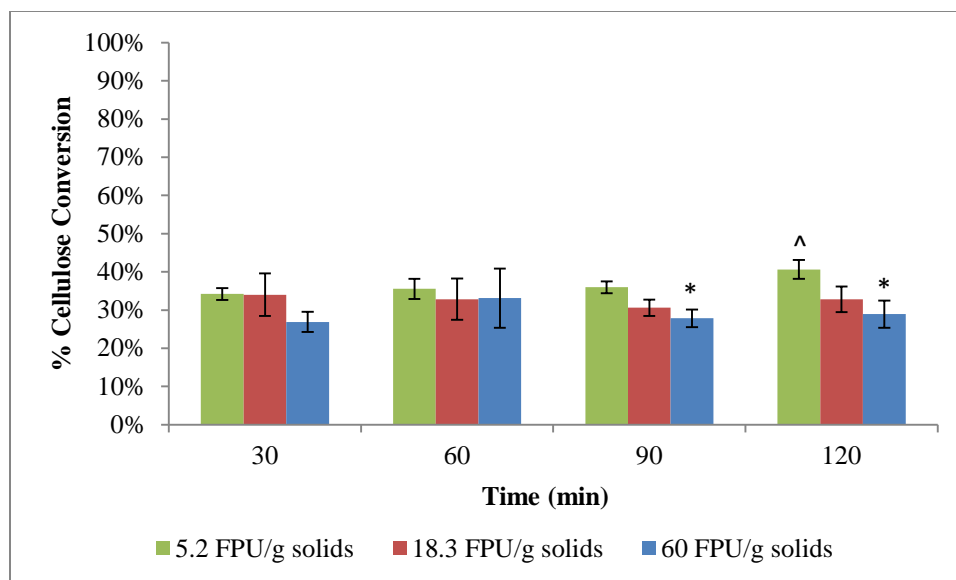


Figure 6.10. Conversion of cellulose pretreated at 25°C for up to 2 hrs and enzymatically hydrolyzed at low-solids loadings (5% w/w) with the enzyme loadings shown in the legend. Error bars represent the standard deviation of three replicates. Columns labeled with an asterisk (*) are significantly different from those labeled with a caret (^). (See Figure 6.2 for experimental conditions.)

For 25°C PCS hydrolyzed at 20% (w/w) solids loading (Figure 6.11), the overall conversions were lower than those observed at low solids hydrolysis (Figure 6.10), with the exception of the 60 FPU/g solid treatment. The highest enzyme loading also resulted in conversions that were >3 and >7 times higher than enzyme loadings of 7.2 and 28.9 FPU/g solids, respectively. Inexplicably, the lowest conversions resulted from the intermediate enzyme loading. The reactions performed at 60 FPU/g solids resulted in conversions (28%-37%) similar to those observed in this study at low-solids hydrolysis independent of enzyme loadings (27%-41% conversion).

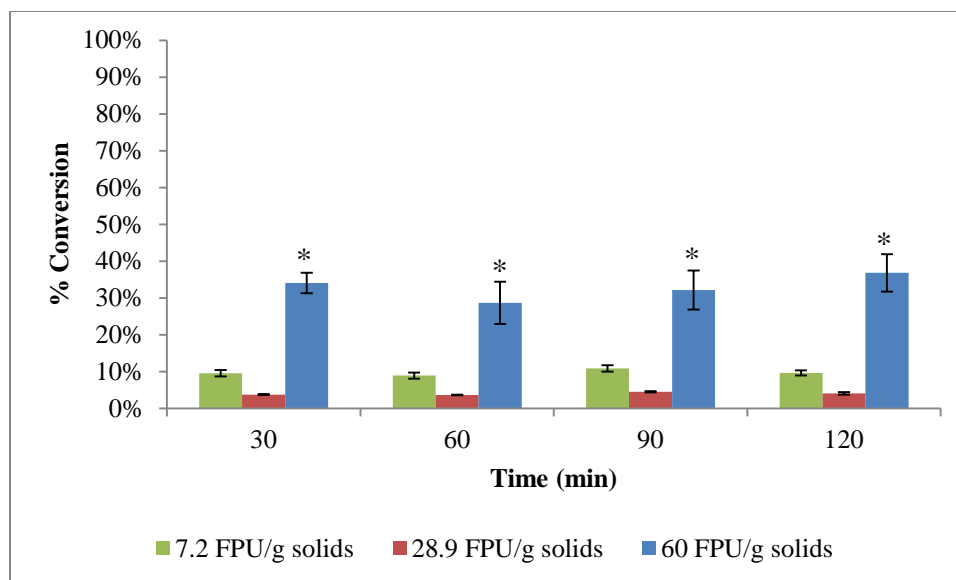


Figure 6.11. Conversion of cellulose pretreated at 25°C for up to 2 hrs and enzymatically hydrolyzed at high-solids loadings (20% w/w) with the enzyme loadings shown in the legend. Error bars represent the standard deviation of three replicates. Columns denoted with asterisks (*) are significantly different from those not marked. Columns not marked are not significantly different from other unmarked columns. (See Figure 6.2 for experimental conditions.)

Figure 6.12 shows the cellulose conversion of corn stover pretreated at 70°C over the course of 2 hrs and enzymatically hydrolyzed at 5% (w/w) solids loadings. At the 30 and 60 min pretreatments, the conversions increase from ~25% to ~60% as the enzyme loading is increased from 5.2 to 60 FPU/g solids. The increase in conversion is even larger for the 90 and 120 min pretreatments. This trend is the inverse of that observed for the identical treatment at 25°C. Furthermore, the overall conversions at the moderate and high enzyme loadings are greater for the corn stover pretreated at 70°C as compared to that pretreated at 25°C with subsequent low-solids hydrolysis (Figure 6.12 compared with Figure 6.10).

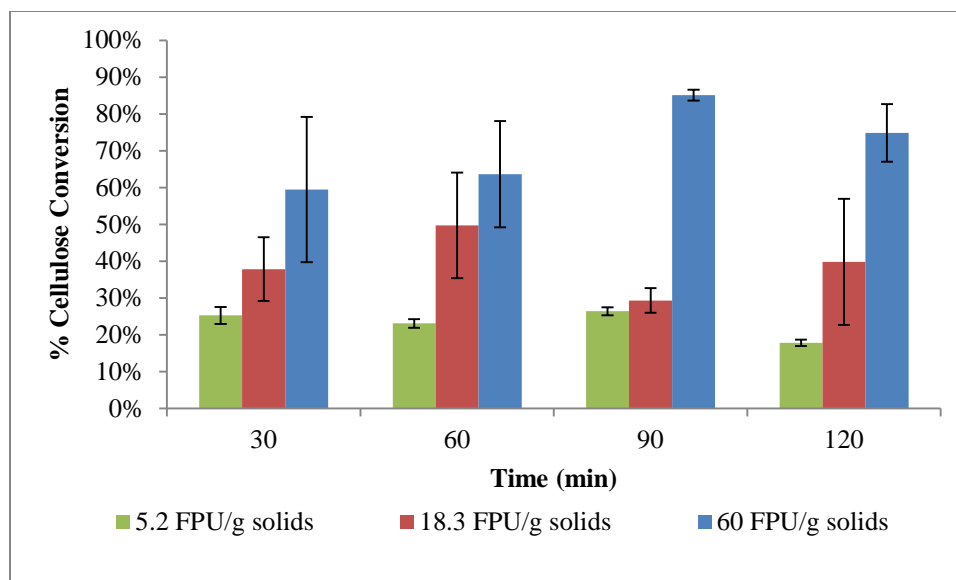


Figure 6.12. Conversion of cellulose pretreated at 70°C for up to 2 hrs and enzymatically hydrolyzed at low-solids loadings (5% w/w) with the enzyme loadings shown in the legend. Error bars represent the standard deviation of three replicates. (See Figure 6.2 for experimental conditions.)

Figure 6.13 presents corn stover pretreated at 70°C and subsequently hydrolyzed at 20% (w/w) solids. The conversions ranged from 5.5-8.1% and were relatively independent of pretreatment time or enzyme loading. This trend is different from that observed for the 25°C PCS hydrolyzed at high solids (Figure 6.11), where the cellulose conversion increased sharply for the 60 FPU/g solids enzyme loading.

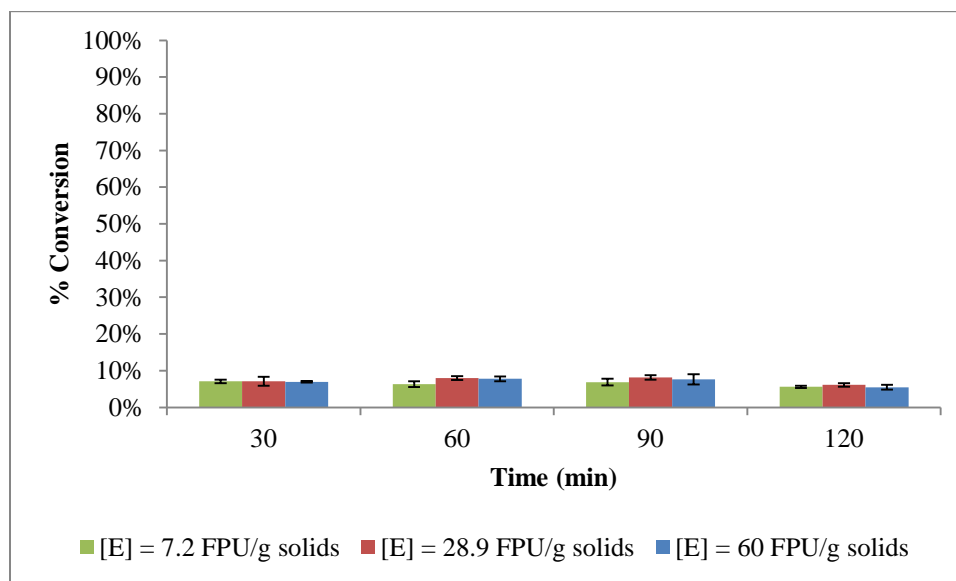


Figure 6.13. Conversion of cellulose pretreated at 70°C for up to 2 hrs and enzymatically hydrolyzed at high-solids loadings (20% w/w) with the enzyme loadings shown in the legend. Error bars represent the standard deviation of three replicates. (See Figure 6.2 for experimental conditions.)

6.4.4 NaOH Loading in Pretreatment

6.4.4.1 Characterization of Pretreated Corn Stover

Figure 6.14 shows the composition of pretreated corn stover at pretreatment times up to 2 hours and three different NaOH loadings. Pretreatment with NaOH effectively increased cellulose content 4.2%-29.4% for all reaction conditions investigated, with the most effective pretreatment occurring at 20 g/100 g CS for 2 hr. Reduction in acid soluble lignin content (and the unquantified components) was observed in all samples, as well as some minor sugars (mannose and galactose) and ash under some pretreatment conditions. The most severe pretreatment (20 g NaOH/100 g CS for 24 hr) resulted in a reduction of xylan content.

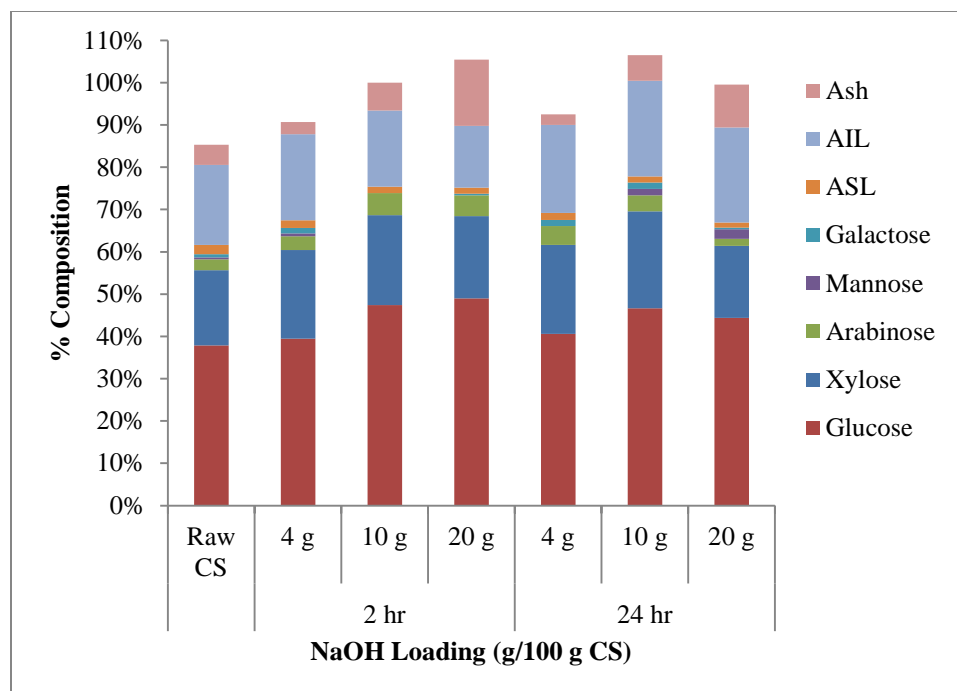


Figure 6.14. Composition of raw and pretreated corn stover. Results are calculated as % oven dried material. Pretreatment was performed at 20% (w/v) solids for either 2 or 24 hr at 25°C. Sums of components may not equal 100% due to some components not being quantified during this analysis.

6.4.4.2 Solids Loading in Enzymatic Hydrolysis

Figure 6.15 and Figure 6.16 show the conversion of cellulose and hemicellulose for corn stover pretreated at various NaOH loadings for 2 hr at 25°C and hydrolyzed at low and high solids, respectively. For both hydrolysis solids loadings, there is an apparent decrease in conversion of cellulose from 40.6% to 21.5% and 5.7% to 0.6% at low and high-solids loadings, respectively. Hemicellulose displayed a similar trend, decreasing from 35.1% to 12.7% and 3.6% to 0% for low and high-solids loadings, respectively. At the lowest NaOH loading, the conversion of cellulose was nearly 7 times greater for the lower hydrolysis solids loading than for the higher solids loading, and these disparities only increased as NaOH loading increased to 20 g NaOH/100 g CS.

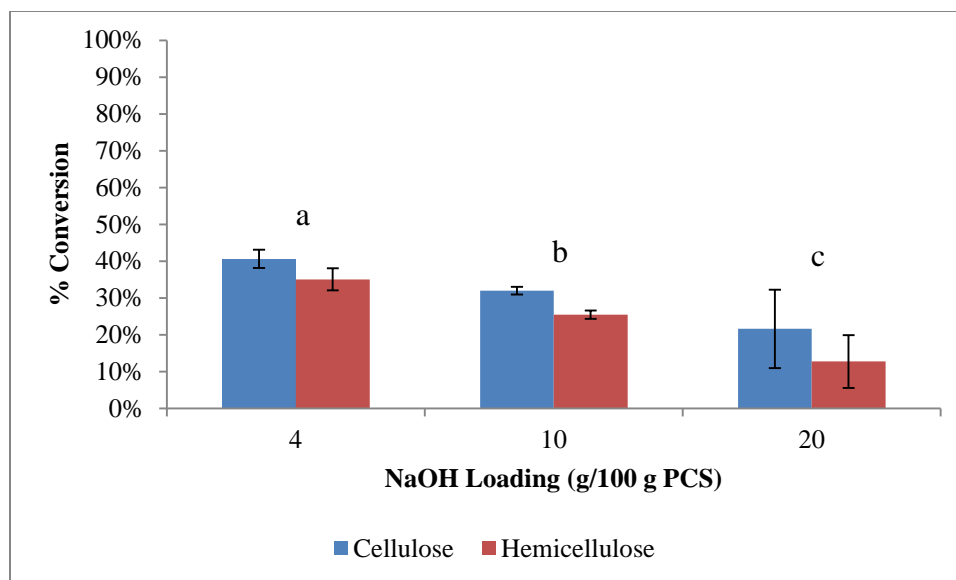


Figure 6.15. Conversion of cellulose pretreated with three NaOH loadings for 2 hr and enzymatically hydrolyzed at low-solids (5% w/w) with an [E] = 5.2 FPU/g solids. Pretreatment was performed at 20% (w/v) solids. Column groupings denoted with the same letters are not significantly different from one another. (See Figure 6.3 for experimental conditions.)

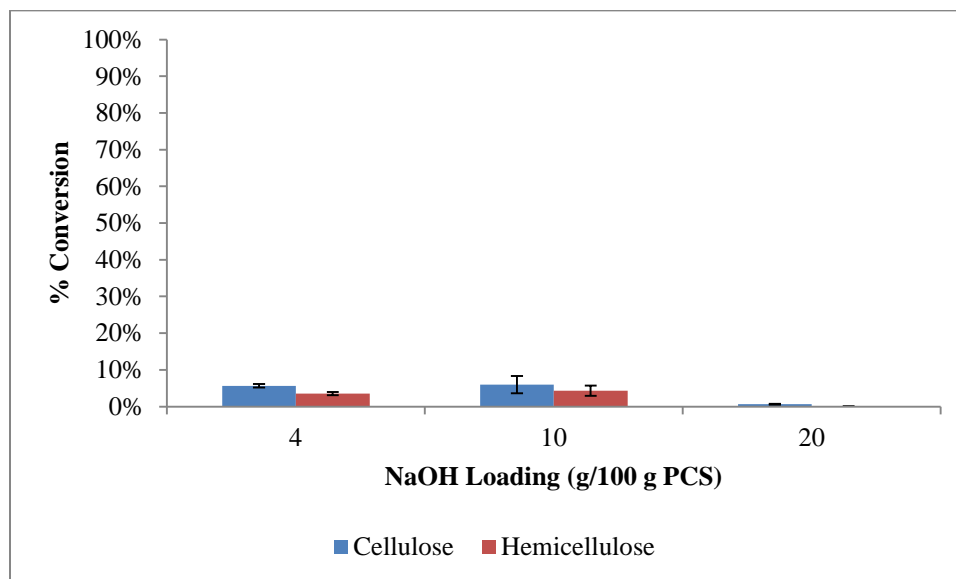


Figure 6.16. Conversion of cellulose pretreated with three NaOH loadings for 2 hr and enzymatically hydrolyzed at high solids (20% w/w) with an [E] = 5.2 FPU/g solids. Pretreatment was performed at 20% (w/v) solids. (See Figure 6.3 for experimental conditions.)

Figure 6.17 and Figure 6.18 show the conversion of cellulose and hemicellulose for corn stover pretreated at three NaOH loadings for 24 hr at 25°C and hydrolyzed at low and high solids, respectively. In contrast to the trends observed for the 2 hr pretreatments, the conversion of cellulose increased from 20% to ~63% with increasing NaOH loading for the hydrolysis performed at low-solids loading. The conversion of hemicellulose did not follow this same trend, as it achieved a maximum conversion (~55%) at 10 g NaOH/100 g CS. For the hydrolysis performed at high-solids loadings, the conversions are more than 5-fold smaller than the conversions observed at low-solids loadings.

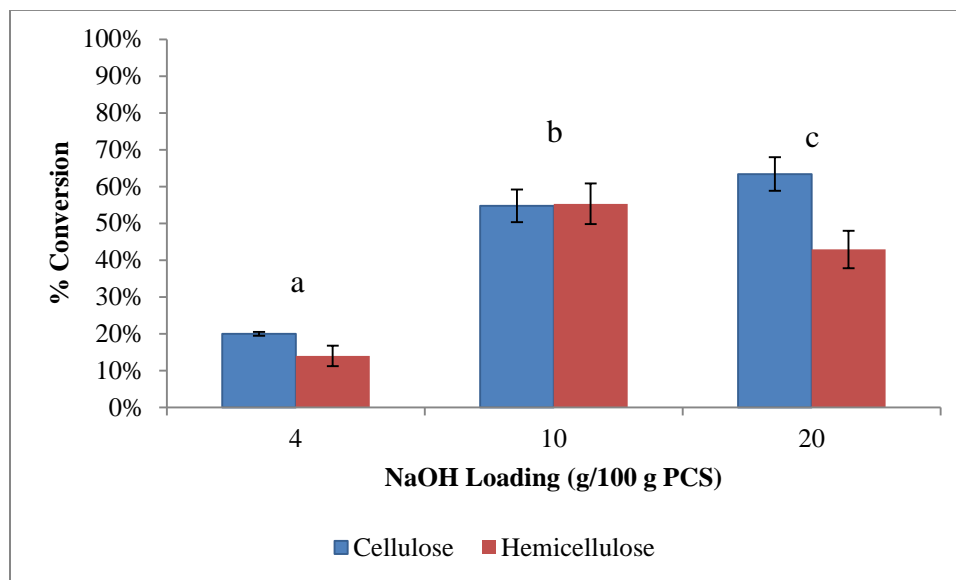


Figure 6.17. Conversion of cellulose pretreated for 24 hr and enzymatically hydrolyzed at low-solids (5% w/w) with an [E] = 5.2 FPU/g solids. Pretreatment was performed at 20% (w/v) solids. Column groupings denoted with the same letters are not significantly different from one another. (See Figure 6.3 for experimental conditions.)

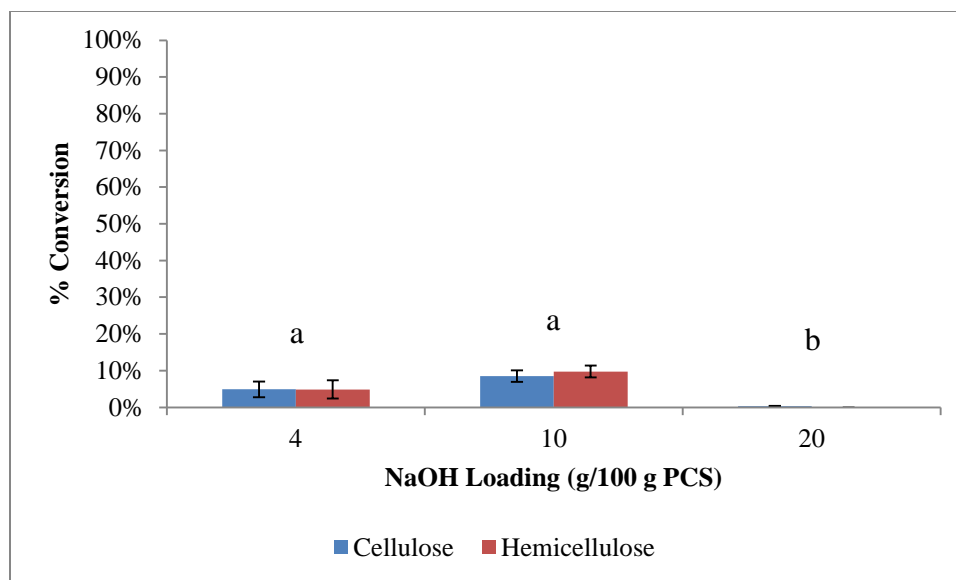


Figure 6.18. Conversion of cellulose pretreated for 24 hr and enzymatically hydrolyzed at high solids (20% w/w) with an [E] = 5.2 FPU/g solids. Pretreatment was performed at 20% (w/v) solids. Column groupings denoted with the same letters are not significantly different from one another. (See Figure 6.3 for experimental conditions.)

6.4.5 Hydrolyzate Flushing and Substrate Reuse

Washing corn stover following the pretreatment process appears to affect its composition (Figure 6.19). The largest difference between the washed and unwashed PCS is the amount of ash that remains in the unwashed sample. Nearly five times the amount of ash was measured in the unwashed sample than the washed sample.

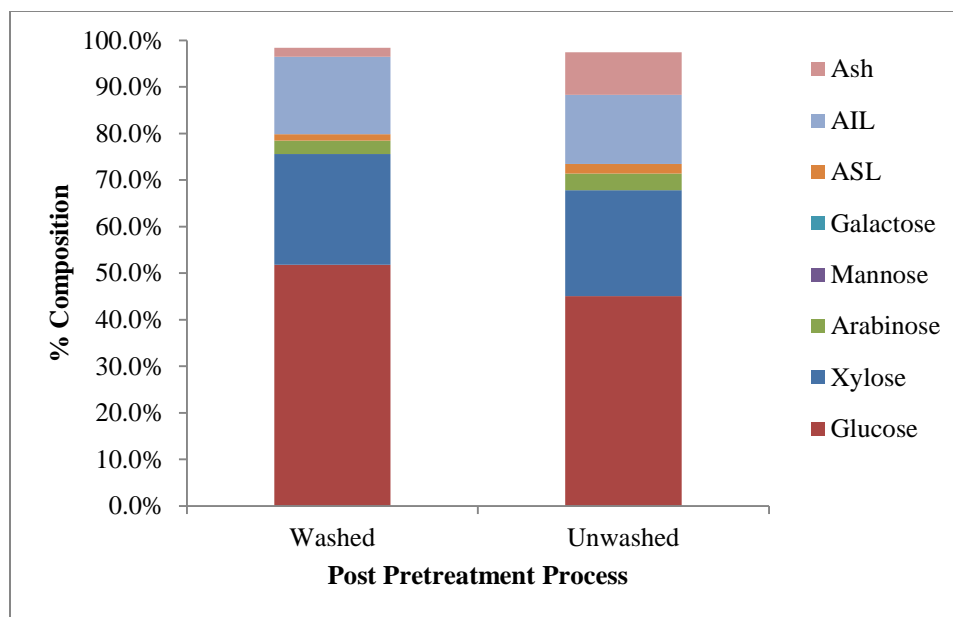


Figure 6.19. Composition of pretreated corn stover that was either washed or unwashed following pretreatment. Results are calculated as % oven dried material. Pretreatment was performed at 10% (w/v) solids pretreated with 10 g NaOH/100 g CS for 24 hr at 25°C.

Figure 6.20 shows the glucose concentrations obtained from enzymatic hydrolysis with the 15 FPU/g solids enzyme loading on washed and unwashed PCS following each hydrolyzate flushing cycle. The columns flushed every 24 hr of the 72 hr hydrolysis period produced significantly more glucose than those operated under conventional batch conditions for the full 72 hr (control). It is also evident from this figure that the majority of the glucose produced in the conventional batch system is likely hydrolyzed within the first 24 hr of the hydrolysis reaction, since there is very little or no difference between the glucose concentration for the 72 hr hydrolysis and the first 24 hr of the flushed systems. This result is not altogether surprising, considering the hydrolysis rate is fastest within the initial hours of the reaction (data not shown). Washing the corn stover following pretreatment did not show consistent improvement of glucose yields from hydrolysis. For instance, washed PCS in the control system released more glucose than unwashed PCS when the reaction was not supplemented with additional enzyme. However, washing the corn stover did not seem to improve the amount of glucose released in the control system when the reactions were supplemented with enzyme. Supplementation

with additional enzyme improved the amount of glucose released from 73 g/L to 97 g/L for the unwashed, flushed PCS samples.

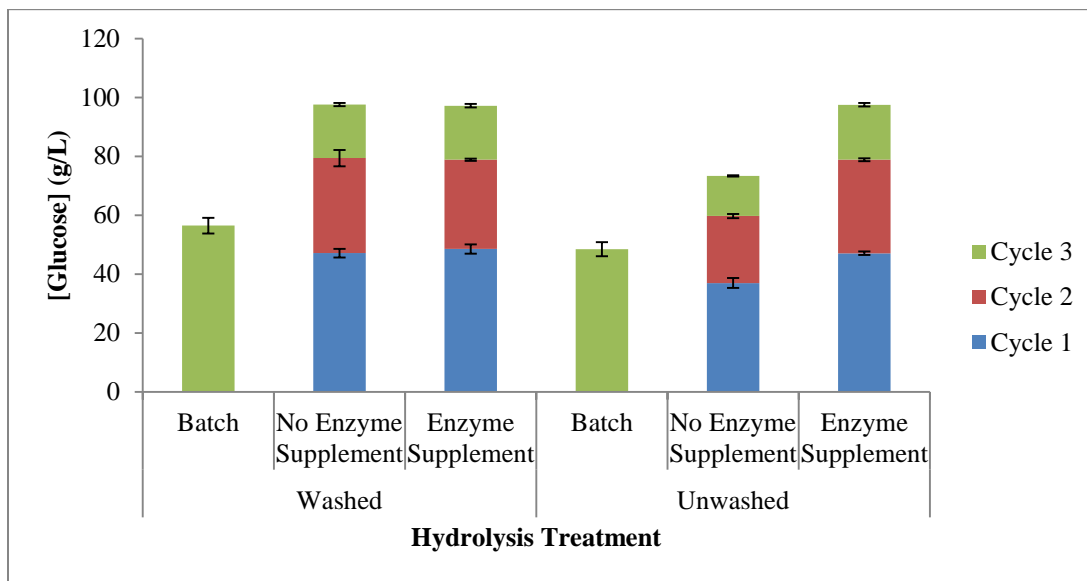


Figure 6.20. Glucose production from enzymatic hydrolysis performed for 72 hr under conventional batch conditions (control) or with flushing of hydrolyzate and reuse of the substrate. Corn stover was pretreated at 10% (w/v) solids with a loading of 10 g NaOH/100 g CS for 24 hr at 25°C. Enzymatic hydrolysis was performed at 20% (w/w) solids on washed PCS. Initial enzyme loading was 15 FPU/g solids. Each flushing cycle contained an additional enzyme loading of 2.5 FPU/g solids for the samples that received enzyme supplementation. Bars labeled with the same letter show no statistical difference.

Glucose yields from washed and unwashed PCS hydrolyzed with 60 FPU/g solids enzyme loading following each flushing cycle are shown in Figure 6.21. The higher enzyme loading resulted in higher overall glucose yields for the flushed samples compared to the flushed hydrolysis performed with 15 FPU/g solids enzyme loadings. Removal of the hydrolyzate shows significant improvement in glucose yields compared to the conventional batch hydrolysis, increasing yields by more than 47% in all cases. Many of the same trends observed with the 15 FPU/g solids enzyme loading are also seen here. For instance, washed PCS in the control system released more glucose than unwashed PCS when the reaction was not supplemented with additional enzyme. However, washing the corn stover did not seem to improve the amount of glucose released in the control system when the reactions were supplemented with enzyme.

Supplementation with enzyme improved the amount of glucose released from 85 g/L to 103 g/L for the unwashed, flushed PCS sample. The washed, flushed PCS sample was the most easily digested, releasing 113 g/L glucose.

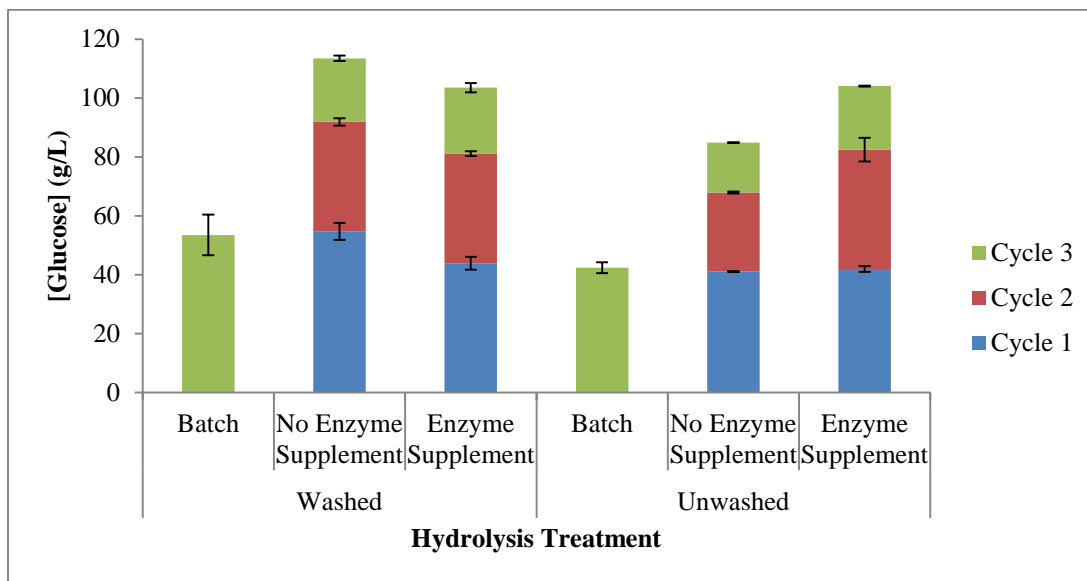


Figure 6.21. Glucose production from enzymatic hydrolysis performed for 72 hr under conventional batch conditions or with flushing of hydrolyzate and reuse of the substrate. Corn stover was pretreated at 10% (w/v) solids with 10 g NaOH/100 g CS for 24 hr at 25°C. Enzymatic hydrolysis was performed at 20% (w/w) solids on washed PCS. Initial enzyme loading was 60 FPU/g solids. Each flushing cycle contained an additional enzyme loading of 2.5 FPU/g solids for the samples that received enzyme supplementation. Bars labeled with the same letter show no statistical difference.

While higher total glucose concentrations were achieved when the hydrolyzate was flushed, it is apparent from Figure 6.20 and Figure 6.21 that the rate of glucose production slowed with each successive cycle. For washed PCS with no enzyme supplementation, independent of enzyme loading, the glucose production during cycle 2 and 3 were 68% and 39% of cycle 1, respectively. However, for the flushed hydrolysis performed with the high enzyme loading and receiving additional enzyme supplementation, this reduction in rate was not apparent until the last cycle (i.e. cycle 1 and 2 produced approximately the same amount of glucose).

Flushing the hydrolyzate and reusing the substrate increased cellulose conversion significantly compared to conventional batch hydrolysis for both the washed and

unwashed PCS (Figure 6.22). For the washed PCS receiving no enzyme supplementation, conversion increased 1.7 and 1.9 times for 15 and 60 FPU/g solids enzyme loadings, respectively, where the higher enzyme loading achieved nearly complete conversion of cellulose. For the unwashed PCS receiving no enzyme supplementation, conversion increased 1.6 and 2.0 times for 15 and 60 FPU/g solids enzyme loadings, respectively. Supplementing with additional enzyme resulted in slightly larger increases for each case. Interestingly, for all cases, the 15 FPU/g solids enzyme loading produced the same or higher conversion in the conventional batch (control) hydrolysis reaction than the 60 FPU/g solids enzyme loading. However, the opposite was observed for the flushed samples. The 60 FPU/g solids enzyme loading produced the higher conversion.

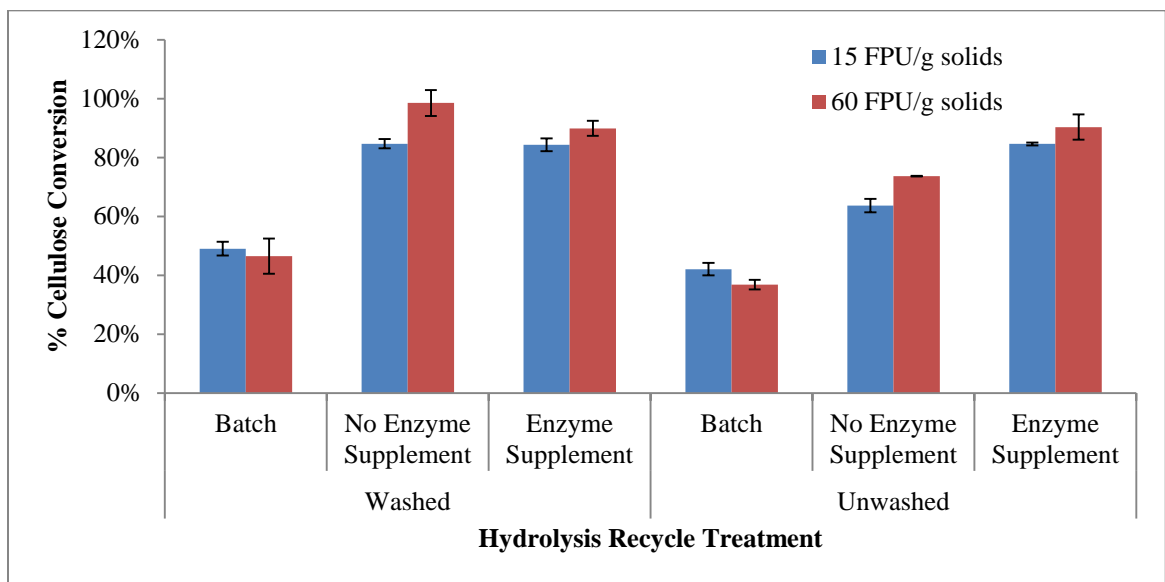


Figure 6.22. Conversion of cellulose for PCS hydrolyzed for 72 hr under conventional batch conditions or with flushing of hydrolyzate and reuse of the substrate. Corn stover was pretreated at 10% (w/v) solids with 10 g NaOH/100 g CS for 24 hr at 25°C. Enzymatic hydrolysis was performed at 20% (w/w) solids on washed PCS. Initial enzyme loading was either 15 or 60 FPU/g solids. Each flushing cycle contained an additional enzyme loading of 2.5 FPU/g solids for the samples that received enzyme supplementation.

6.5 DISCUSSION

6.5.1 Low-Solids Loading in Pretreatment

Solids loadings in successive pretreatment and hydrolysis operations can affect the overall effectiveness of the conversion process. Increased solids loadings in pretreatment do not appear to negatively impact cellulose conversion for low-solids hydrolysis systems. For example, low-solids pretreatment followed by low-solids hydrolysis resulted in ~40% cellulose conversion, whereas high-solids pretreatment followed by low-solids hydrolysis resulted in ~60% cellulose conversion. However, increased solids loadings in hydrolysis appear to have a large negative impact on cellulose conversion, irrespective of solids loadings in pretreatment, with the low-solids/high-solids and the high-solids/high-solids conversion schemes resulting in ~8% and 0% cellulose conversion, respectively.

6.5.2 Pretreatment Time and Temperature

Sodium hydroxide alters the lignocellulosic structure by breaking bonds between the lignin and carbohydrates, specifically the ester bonds within the xylan backbone and that link the hemicellulose to the lignin (Sills and Gossett 2012). In this process, some lignin can be solubilized (Balat et al. 2008; Galbe and Zacchi 2007; Hendriks and Zeeman 2009; Jorgensen et al. 2007a), as well as some of the hemicellulose. Duguid et al. (2009) saw <2% reduction in lignin with a 5.8 g NaOH/100 g biomass pretreatment at 25°C, whereas Chen et al. (2009) observed 73.9% lignin removal with a 16 g NaOH/100 g biomass treatment at 25°C. However, this present study observed an apparent increase in total lignin of up to 9% in some cases, likely caused by the loss of components other than lignin (Figure 6.6 and Figure 6.7). These results indicate that the chosen pretreatment time and temperature combinations may not have been adequate for the removal of lignin, since final biomass composition is dependent on the selected pretreatment conditions and the type of substrate (Table 4.1). As mentioned earlier, these direct comparisons of substrate composition are complicated by the fact that the denominator changes as the treatment changes. However, it is hypothesized that the tested pretreatment conditions may have instead caused the rearrangement of lignin components, enabling enzymatic hydrolysis to proceed.

It is not unusual to observe a reduction in cellulose conversion to glucose (percent of theoretical) as solids loadings are increased in enzymatic hydrolysis. This decrease in conversion yields with increasing solids loadings is referred to as the *solids effect* and is an undesirable characteristic that negates the advantages of working at high solids (Cara et al. 2007; Jorgensen et al. 2007b; Kristensen et al. 2009b). However, final glucose concentrations tend to increase with an increase in solids loading, even as conversion decreases, due to the change in total solids loading. To date, most studies sacrifice conversion for a more concentrated glucose product; although, determining the cause of the solids effect may lead to improved conversion efficiencies. In this current study, the conversion of cellulose (the percent of cellulose released as glucose) decreased as expected with increasing solids loadings. However, the apparent glucose concentration (g glucose/L) also decreased with increasing solids loadings, indicating additional problems that are not apparent in other studies arising during pretreatment and enzymatic hydrolysis. The observed inhibition could likely be due to a number of factors, including by-products of the neutralization process, increased inhibitor concentrations and mass transfer limitations.

The NaOH pretreatment is a strongly alkaline processing step. The pH of this treatment is ~13-14, much higher than the optimal pH (~5) of the enzymes. Neutralization of the biomass following pretreatment is crucial for the optimal performance of enzymes during hydrolysis. In many instances, large volumes of water (10-20 volumes) are used to rinse the pretreated biomass (Banerjee et al. 1995; Cheng et al. 2010; Sills and Gossett 2012). One of the reasons for working with high-solids loadings is to reduce the amount of water consumed in the conversion process. Washing biomass until a neutral pH is obtained is counterproductive to reaching this goal. In this study, neutralization occurred with the addition of glacial acetic acid and washing of the solids with less water (3-5 volumes) compared to other studies. When acids react with bases, the main products that form are water and salts, which in this case would be sodium acetate. Sub-optimal pH and residual salts are possible causes for the reduced glucose production in enzymatic hydrolysis. One study found that production of cellulase by *Bacillus coagulans* and the subsequent hydrolysis of cellulose slowed as acetate concentration increased (Romsaiyud et al. 2009). Cellulase production and

cellulose hydrolysis were still measurable at an acetate concentration of 10 mmol/L, but cellulase production slowed by as much as 75% at 30 mmol/L acetate and was completely eliminated at 60 mmol/L acetate.

It is also possible that the combination of high-solids loadings in both pretreatment and enzymatic hydrolysis led to increased inhibitor yields. NaOH pretreatment typically results in partial to total solubilization of lignin and hemicellulose, depending on the severity of the pretreatment conditions; although very little solubilization of these fractions was observed in this current work. Removal of lignin by NaOH often leads to the release of acetyl groups and uronic acid substitutions, which can enhance the digestibility of cellulose and hemicellulose (Cui et al. 2012; Kumar et al. 2009a; Wan et al. 2011). However, hydrolytic enzymes can be inhibited by some of these degradation products, making the selection of process conditions, like alkaline loading, moisture content, temperature and time, extremely important. Balance is the key to achieving optimal lignin removal, while limiting the production of inhibitory compounds. For instance, Cui et al. (2012) found that delignification was influenced by NaOH loading, time and moisture content during long-term wet-storage of corn stover. Addition of 2-5% (w/w) NaOH increased lignin degradation by ~10-25% over a 90-d storage period; however, most of this lignin degradation occurred within the first five days of storage. A higher loss in xylan (up to 34%) was observed concurrently with the increase in lignin degradation. Wan et al. (2011) also observed a sharp increase in xylan degradation with an increase in lignin degradation. As NaOH loading increased from 4% to 40% (w/w), lignin degradation increased moderately from ~7% to ~15%, but xylan removal increased from 5% to nearly 50% over the same NaOH loadings. Although no inhibition was observed during enzymatic hydrolysis and inhibitor concentrations were not measured, the presence of inhibitory compounds from the degradation of xylan is possible. However, they were likely removed during the washing and neutralizing of the soybean straw prior to use in the hydrolysis reaction.

The temperature at which the pretreatment is performed may also affect the sugar yield from hydrolysis. The pretreatment conditions investigated in this current study were intentionally chosen to be mild to limit the loss of hemicellulose sugars. Additionally, low to moderate temperatures (<100°C) are preferential for alkaline

pretreatment prior to the conversion of lignocellulose to fermentable sugars, since cellulose is affected very little at these temperatures. The lignocellulose may have undergone subtle changes that were not detected in the compositional analysis but became evident following enzymatic hydrolysis when higher cellulose conversions were achieved for corn stover pretreated at a higher temperature (Figure 6.8 and Figure 6.9). For example, bonds may have been broken and the components condensed onto the lignocellulose without becoming soluble, which could have exposed cellulose but not changed the overall composition of the material. However, the increase in cellulose conversion was only observed for PCS hydrolyzed at low solids. Sweet sorghum bagasse has been shown to have significant lignin removal following alkaline (NaOH) pretreatment at short reaction times and moderate temperatures. Wu et al. (2011) reported that an increase in temperature from 25°C to 50°C resulted in the delignification of sweet sorghum bagasse to increase from 65% to 90% when pretreated with a NaOH loading of 40 g NaOH/100 g biomass for 2 hr. The glucose recovery was not impacted by the change in pretreatment temperature. Xu et al. (2010) also observed an improved sugar yield when pretreating with higher temperatures. By changing the pretreatment temperature from 50°C to 121°C, lignin content was reduced by 25% and 35%, respectively, which resulted in the glucose yield nearly doubling for switchgrass pretreated with the increased temperature (~90 mg/g biomass vs. ~175 mg/g biomass). In the current study, a change in lignin content was not measured between the two pretreatment temperatures tested, but it is possible that bonds between the lignin and the hemicellulose were broken, allowing access to the cellulose, before solubilized lignin compounds condensed back onto the solids.

6.5.3 NaOH Loading in Pretreatment

Alkaline degradation of cellulose is dependent on several factors, including the nature and concentration of the alkali, the nature and origin of the cellulose and the reaction temperature (Ciolacu and Popa 2005; Fengel et al. 1995; Knill and Kennedy 2003). At relatively low temperatures (<100 °C) and low alkali concentrations (<4%), structural changes for cellulose are insignificant, as glycosidic β -(1, 4) linkages are alkali stable under these conditions (Knill and Kennedy 2003). In this current study, the

composition of corn stover pretreated with low NaOH loadings (4 g NaOH/100 g CS) was not significantly different when pretreatment times were extended from 2 hr to 24 hr, indicating the stability of the cellulose under the conditions investigated. Kim and Holtzaple (2006) reported no significant structural changes or degradation to cellulose pretreatment using 5% (w/v) lime (50 g Ca(OH)₂/100 g biomass) at low temperatures (25°C-55°C), even for extended pretreatment times up to 16 weeks. Another study (Cui et al. 2012) reported that long-term storage (90 days) of wet corn stover without the addition of NaOH resulted in ~10% loss of cellulose; however, storage with the application of 2% NaOH caused only ~5% degradation of cellulose. The addition of NaOH likely made the environmental conditions unfavorable for microorganisms that would have grown on the cellulose, thus protecting it from microbial degradation. These conditions (low alkali concentrations and low to moderate temperatures) are favorable for lignocellulose pretreatment because lignin is affected, but most of the cellulose remains unaltered and available for hydrolysis into fermentable carbohydrates.

At higher alkali concentrations (>6%), many structural and morphological changes begin to occur in cellulose. As alkali concentrations increase, crystallite structures (regions of highly ordered polymer chains interspersed with more amorphous regions) begin to swell. The swelling starts first in amorphous regions, followed by the crystalline region. The degree of polymerization (DP) and the degree of crystallinity (CrI; crystallinity index) decrease with increasing alkali concentration (Eronen et al. 2009; Mittal et al. 2011). In this current study, conversion of corn stover pretreated for 24 hr increased with increasing NaOH loadings when hydrolyzed at low solids loadings (Figure 6.17). An apparent change in composition was observed at the higher NaOH loadings. For example, carbohydrates that predominately comprise cellulose and hemicellulose were lower for corn stover pretreated for 24 hr with a loading of 20 g NaOH/100 g CS than corn stover pretreated for 2 hr with the same NaOH loading, as well as corn stover pretreated for 24 hr with half the NaOH loading. Ciolacu and Popa (2005) studied the structural changes of microcrystalline cellulose, cellulose linters (secondary growth of short, thick-walled fibers produced by cotton) and spruce pulp treated with several alkali concentrations (0-18% NaOH). At 8.5% NaOH (which is similar to the highest NaOH loading in the current study), they observed reductions in the

DP (6.6%-18.2%) and CrI (7.5%-10.0%) for all three substrates tested when compared to cellulose treated without the addition of NaOH. These structural changes are advantageous for the conversion of lignocellulose into fermentable sugars because enzymatic hydrolysis is enhanced as amorphous regions of cellulose are more easily digested by cellulolytic enzymes.

Lignin content varied with NaOH loading; however, with the NREL biomass composition analysis, it is difficult to say with certainty that the component either increased or decreased relative to another sample treated under different conditions. The corn stover was treated in large batches, and samples of equivalent mass were collected from the batches for compositional analysis. The amount of solids recovered from pretreatment was not measured, and without that information, it is difficult to directly compare between treatments since the numerators and denominators change from sample to sample. For instance, 1 g of untreated, raw corn stover is not that same as 1 g of corn stover pretreated with 4 g NaOH/100 g biomass, which is not the same as 1 g of corn stover pretreated with 20 g NaOH/100 g biomass.

Percent ash content for a given mass of PCS increased with increasing NaOH loading. Ash is the residue remaining after lignocellulose is ignited at 575°C and can consist of minerals such as aluminum, calcium and sodium (Lee et al. 2007). The higher NaOH loadings likely contributed to the higher apparent ash content of the PCS if residual NaOH was still present following washing with DI water.

Even with modifications made to the corn stover composition, cellulose conversion of the corn stover pretreated for 2 hr decreased from 5.7% to 0.6% with NaOH loadings increasing from 4 to 20 g NaOH/100 g corn stover when hydrolyzed at 20% (w/w). The same trend was observed for corn stover hydrolyzed at low-solids loadings; however, the conversions were significantly higher. Low conversions (<9%) were also observed for corn stover pretreated for 24 hr and hydrolyzed at high-solids loadings. Inadequate neutralization of the corn stover following pretreatment is a possible cause of the reduced conversions. However, inadequate neutralization does not account for the higher conversions observed for the corn stover pretreated for 24 hr and hydrolyzed at low-solids loadings. It is likely that the buffer used in enzymatic hydrolysis was able to counteract the high pH of the PCS in this case, allowing enzymatic

hydrolysis to proceed, whereas the buffer may not have been adequate in regulating the pH for high-solids loadings. Additionally, the increase in cellulose conversion observed for corn stover pretreated for 24 hr and hydrolyzed at low-solids loadings as NaOH loadings increased does not agree with the decreasing conversion of the corn stover pretreated for only 2 hr. The additional compositional modifications that resulted from the extended pretreatment time, in combination with the adequate buffering capacity for low-solids loadings, is a possible cause for the improved conversions.

6.5.4 Hydrolyzate Flushing and Substrate Reuse

Many studies have been conducted to examine the potential of recycling enzyme to reduce the cost of the hydrolysis operation and limit inhibition of the enzymes (Gregg and Saddler 1996; Lee et al. 1995; Qi et al. 2011; Qi et al. 2012; Tu et al. 2007; Tu et al. 2009), but few have looked at the possibility of reusing the substrate as a way to capitalize on the full energy potential contained in biomass when working at high-solids loadings (Yang et al. 2010a; Yang et al. 2011). One study investigated the use of hydrolyzate flushing and substrate reuse to not only improve cellulose conversion for high-solids systems but to also reduce the retention time (Yang et al. 2011). Steam-exploded corn stover was hydrolyzed with an enzyme loading of 15 FPU/g cellulose for 24 hr in three stages. Flushing of the hydrolyzate occurred after 9, 18 and 24 hr. Conventional batch hydrolysis conducted at 30% (w/v) solids for 72 hrs resulted in 45% cellulose conversion. Flushing of the hydrolyzate and reusing of the solids resulted in 71% conversion after only 24 hr. In other words, flushing the hydrolyzate increased the cellulose conversion by ~1.5 times in only one third the amount of time compared to conventional batch hydrolysis. In this current study, flushing increased cellulose conversion of NaOH-pretreated corn stover by 1.5-2 times over 72 hr compared to conventional batch hydrolysis.

It was hypothesized that flushing the hydrolyzate may eliminate the need for washing lignocellulose material following pretreatment by reducing inhibitory components that may be present, thus reducing the amount of process water required. However, washing the corn stover following pretreatment appeared to affect its composition Figure 6.19. This compositional difference has implications for the

subsequent enzymatic hydrolysis, especially when dealing with high-solids loadings. When considered on a weight basis (total solids), the washed PCS has a higher *cellulose loading* than the unwashed PCS. Since the total solids were equivalent for hydrolysis reactions containing washed and unwashed PCS and the enzyme was applied based on the total solids content, the reactors containing the washed PCS would have a lower enzyme: cellulose ratio than the reactors containing unwashed PCS, which could potentially result in a reduced cellulose conversion. The data presented in this current work do not substantiate this claim, since washed PCS released just as much, if not more glucose than unwashed PCS. At the scale this experiment was performed, the difference in cellulose content for washed and unwashed PCS was not substantial. However, it is still a concern that should be addressed, especially at larger scales where discrepancies between cellulose content may be much more significant.

Flushing the hydrolyzate and reusing the substrate has been shown to improve glucose yields of enzymatic hydrolysis performed at high-solids loadings compared to conventional batch hydrolysis. However, with each successive cycle, the rate of glucose production was reduced. Several factors may play a role in reducing the conversion rate, including non-reversible and/or unproductive binding of enzyme to the substrate, removal of the more easily converted cellulose fraction (amorphous regions vs. crystalline regions), a lack of new, accessible cellulose chains and/or loss of enzyme during flushing of the reactor. Although results are inconclusive, many studies suggest that cellulase may bind to lignin irreversibly, rendering it ineffective in converting cellulose to glucose (Converse et al. 1990; Kumar and Wyman 2009a; Kumar and Wyman 2009b; Qi et al. 2011; Tu et al. 2009). Amorphous regions and solubilized saccharide chains are more easily converted than crystalline regions of the cellulose. As hydrolysis progresses, these regions are depleted and the rate of glucose production is impacted. Additionally, as the rate slows, the number of new cellulose chains accessible to the enzymes is limited and overcrowding of the enzyme can occur, further impacting glucose production. It is also possible that the flushing process removes a significant portion of the solubilized enzyme, specifically the β -glucosidase, and especially in cases where additional enzyme has not been reapplied to the hydrolysis reaction following each flushing cycle. The removal of β -glucosidase could lead to the inhibition of cellulase due to accumulation of

cellobiose, resulting in a lower glucose yield in each successive cycle. Cellobiose was measured in some of the hydrolysis samples (data not shown), which is a potential source of inhibition.

However, not all treatments resulted in a reduction of glucose production with each successive cycle. The high enzyme loading in conjunction with additional enzyme supplementation for each cycle maintained a steady production of glucose through cycle 1 and 2 that was not observed in hydrolysis without enzyme supplementation. The reduction in production rate was not observed until cycle 3 when sufficient amounts of enzyme were likely either removed or deactivated, resulting in that reduction. The same trend was not observed, however, with the lower enzyme loading. It is likely that the lower initial enzyme loading was not sufficient to fully saturate all accessible cellulose sites. Even with the additional enzyme supplementation, the accessible sites were likely never fully saturated, resulting in a reduction of glucose production rate with each successive cycle as enzyme was either removed or deactivated.

The difference seen in washed and unwashed samples was not as apparent in samples receiving additional enzyme supplementation during each successive flushing cycle compared to those with no enzyme supplementation, even though the extra enzyme applications were very low (2.5 FPU/g solids). The fresh enzyme likely replenished enzyme activity lost to deactivation or denaturation of enzyme from the initial dosage during the first cycle or removed during the flushing process.

There are also implications for large-scale processing of biomass. Flushing of the hydrolyzate could eliminate the need for washing pretreated biomass prior to enzymatic hydrolysis, reducing the process water demand, as well as the amount of wastewater that would require treatment. It is important that the process water demand be minimized for any process to be considered environmentally friendly and economically feasible (Mohagheghi and Schell 2010). For the low enzyme loading, the unwashed hydrolysis receiving doses of 2.5 FPU/g solid supplemental enzyme during each flushing cycle performed just as well as the washed PCS that was not supplied with additional enzyme doses during flushing, achieving nearly 85% conversion. In each case, the conversion was substantially higher than that of the conventional batch reactions. The hydrolyzate flushing method may also potentially be used as a way to reduce the retention time

necessary to produce high cellulose conversions. Optimization of the flushing time can ensure that the rate of glucose production remains high (i.e. the process remains in the first-order section of the hydrolysis curve).

Reduced enzyme loadings may also be a possibility with flushing of the hydrolyzate. In this study, a four-fold increase in enzyme loading resulted in only a slight increase in overall conversion. The return for using the higher enzyme loading was not recognized, especially since enzyme cost is such a large portion of the overall conversion cost. The high cost of the enzyme can make high enzyme loadings prohibitive at an industrial scale (Weiss et al. 2013).

One limitation of using a flushing process to mitigate the production of inhibitors is dilution of the final product, which may negate some of the advantages of working at high-solids loadings. However, it is likely that a larger total saccharide mass would be achieved. A complete techno-economic analysis would be necessary to validate the use of hydrolyzate flushing and substrate reuse (with possible enzyme supplementation) over conventional batch hydrolysis at either low- or high-solids loadings.

Water plays a critical role in the hydrolysis reaction. Water availability can impact the effectiveness of process designs. It is therefore essential to understand the true impact of processing parameters on water activity and availability for the overall process to be economically viable. Selig et al. (2012) hypothesized that soluble species have a significant negative effect on the availability of water, which consequently negatively affects enzyme systems. They found that soluble species did, in fact, reduce the availability of water, resulting in lower cellulose conversion. Conversely, increasing non-hydrolysable insoluble solids did not appear to affect the overall cellulose conversion. These soluble species include not only solubilized sugars resulting from the hydrolysis process, but also the soluble enzyme systems and other components found in commercial cellulase preparations, like fermentation by-products, stabilizers and preservatives. Additional evidence supporting this hypothesis was provided by purifying commercial cellulase preparations and applying different enzyme loadings (1-50 mg protein/g cellulose) to 5% and 20% initial dry solids loadings (Sigmacel 50 cellulose). Cellulose conversion for the two solids loadings increased with increasing enzyme loadings, indicating that the soluble enzyme systems may become inhibitory at higher

enzyme concentrations. Furthermore, upon addition of increasingly concentrated non-enzymatic components that were previously removed during purification of the commercial enzyme preparation, a significant decline in cellulose conversion was observed. The authors also investigated some common compounds used as preservatives in commercial enzyme preparations (glycerol and sorbitol). It was shown that these preservatives negatively impacted cellulose conversion (5% solids loading), reducing conversion of pure cellulose by 15% when sorbitol was present at 25 mg/mL of hydrolyzate liquid (500 mg sorbitol/g cellulose) compared to when no sorbitol was present. Conversion of pure cellulose was reduced by as much as 40% when sorbitol was present at 160 mg/mL hydrolyzate liquid (3,200 mg/g cellulose). This sorbitol concentration would be equivalent to having an enzyme loading ~6 times higher than the highest enzyme loading examined in this current work under the same solids loading.

According to another study that characterized multiple commercial enzyme preparations (Nieves et al. 1998), the cellulase system produced from *T. reesei* and marketed as Celluclast 1.5L (the enzyme preparation used in this current work) contained ~280 mg sorbitol/mL enzyme preparation. The protein content and the specific activity of the commercial cellulase preparation surveyed in the Nieves et al. (1998) study were very similar to the measurements obtained in this current work (Table 6.2). Assuming a similar sorbitol concentration as reported in the Nieves et al. (1998) study in the enzyme preparation used in this current work, that translates to an application of ~120-500 mg sorbitol/g cellulose for enzyme applications of 15-60 FPU/g solids (82-328 mg protein/g cellulose) on 5% solids loadings (Figure 6.23). Increasing the sorbitol concentration from 120 mg/g cellulose to 500 mg/g cellulose in combination with the enzyme (protein) concentration from 82 mg/g cellulose to 328 mg/g cellulose reduced the conversion of cellulose in PCS by nearly 70% and 30% at 5% and 20% solids loadings, respectively. The unintentional application of high sorbitol concentrations could explain the low cellulose conversions observed in the hydrolysis studies presented here, as well as the decrease in conversion observed with the data presented in chapter 7.

Table 6.2. Characteristics of commercially available cellulase preparations.

	Protein Content (mg/mL enzyme preparation)	Specific Activity (FPU/mg protein)
Modenbach et al. (2012)	182	0.38
Nieves et al. (1998)	166	0.37

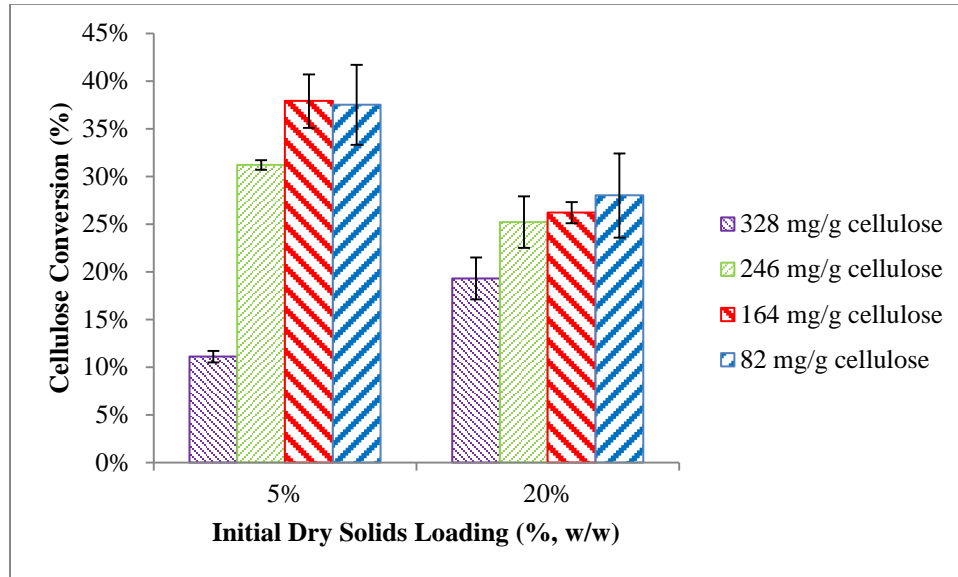


Figure 6.23. Cellulose conversion of PCS at 5% and 20% solids loadings at four different enzyme concentrations. Pretreatment was performed at 10% solids loadings with 10 g NaOH/100 g CS at 25°C for 24 hr. Hydrolysis was performed for 96 hr.

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CHAPTER 7: MODELING HETEROGENOUS ENZYMATIC CELLULOLYTIC HYDROLYSIS REACTIONS USING THE INTEGRATED FORM OF THE MICHAELIS-MENTEN EQUATION

7.1 SUMMARY

Experimental cellulose hydrolysis data were collected for five solids loadings (2%, 5%, 10%, 15%, and 20%) and four cellulase loadings (15, 30, 45 and 60 FPU/g solids) over 96 hr. Kinetic parameters K_m and V_m were determined by simultaneously fitting the integrated form of the Michaelis-Menten kinetics model to these experimental data. Lambert's ω function was used to solve the integrated equation because of the implicit nature of the resulting equation. Due to the heterogeneous nature of the cellulose hydrolysis reaction, non-classical kinetic characteristics, like fractal kinetics, may be encountered. Additionally, the large size of the enzymes relative to the reactive surface of the cellulose chain may lead to overcrowding or jamming of the system, affecting the rate of reaction. Additional parameters to represent the kinetic effects of fractal kinetics (f) and for jammed enzymes (j) were included in the modeling.

7.2 INTRODUCTION

The conversion of lignocellulosic material to valuable products is a very promising process since cellulose is an abundant and renewable source of energy. Developing an economically viable commercial process will undoubtedly require issues related to its optimization to be solved (Gusakov et al. 1985). Modeling the hydrolysis reaction enables researchers to understand and predict the course of the reaction at any given point, providing a very powerful tool to the industry. To date, many models have been proposed for the enzymatic hydrolysis operation based on enzyme kinetics and knowledge of the mechanism of the cellulolytic enzymes (Bansal et al. 2009; Converse and Optekar 1993; Fan and Lee 1983; Gan et al. 2003; Kadam et al. 2004; Nidetzky and Steiner 1993; Zhang and Lynd 2006). Modelers have incorporated concepts like enzyme adsorption onto the substrate, the synergism between various enzyme components, and inhibition caused by degradation products from pretreatment and/or end-products from hydrolysis to improve the model's ability to describe the progress of the hydrolysis reaction. However, the complex nature of the interactions between cellulose and

cellulase makes it difficult to capture the full scope of the reaction in a simplistic, easy-to-use mathematical model.

Classical Michaelis-Menten kinetics have been the basis for many kinetic models and have generally described the process adequately. However, some of the underlying assumptions of the Michaelis-Menten model do not hold true for the hydrolysis of lignocellulose. A major assumption of Michaelis-Menten kinetics is that the reaction is homogeneous in nature (Xu and Ding 2007), meaning that the enzyme and substrate are in the same phase (i.e. soluble enzyme and soluble substrate). Additionally, the kinetics of homogenous reactions are based on classical mass-action law, or Fickian diffusion. The depletion rate of the substrate is proportional to the probability of a collision between a substrate molecule and enzyme in a three-dimensional space, leading to a reaction. This probability is a function of the diffusion of the reactants through the solution (Xu and Ding 2007).

The lignocellulose hydrolysis reaction is initially completely heterogeneous in nature (Bansal et al. 2009; Valjamae et al. 1998), meaning that the enzyme and substrate are in two different phases (i.e. soluble enzyme, insoluble substrate). Figure 8.1 illustrates some other examples of heterogeneous reactions and the interfaces at which the enzymes catalyze reactions. The enzyme must diffuse through the mixture and adsorb onto the substrate at an available reaction site. This interfacial reaction is one cause of deviation from classical Michaelis-Menten kinetics. As the reaction progresses, the adsorbed enzymes act upon the insoluble polysaccharides (cellulose, hemicellulose) in a processive manner to produce soluble saccharide species (mono-, di-, tri-, oligosaccharides). The processive movement of the cellulase results in a reaction that is dimensionally restricted; the enzyme can only move in one direction until it reaches the end of the cellulose chain. It is no longer necessary for that cellulase to collide with a substrate molecule to catalyze successive reactions. Restricted dimensionality is another cause of deviation from classical Michaelis-Menten kinetics. Furthermore, the system contains both soluble and insoluble forms of the substrate, resulting in a system that has aspects of both homogeneous and heterogeneous reactions.

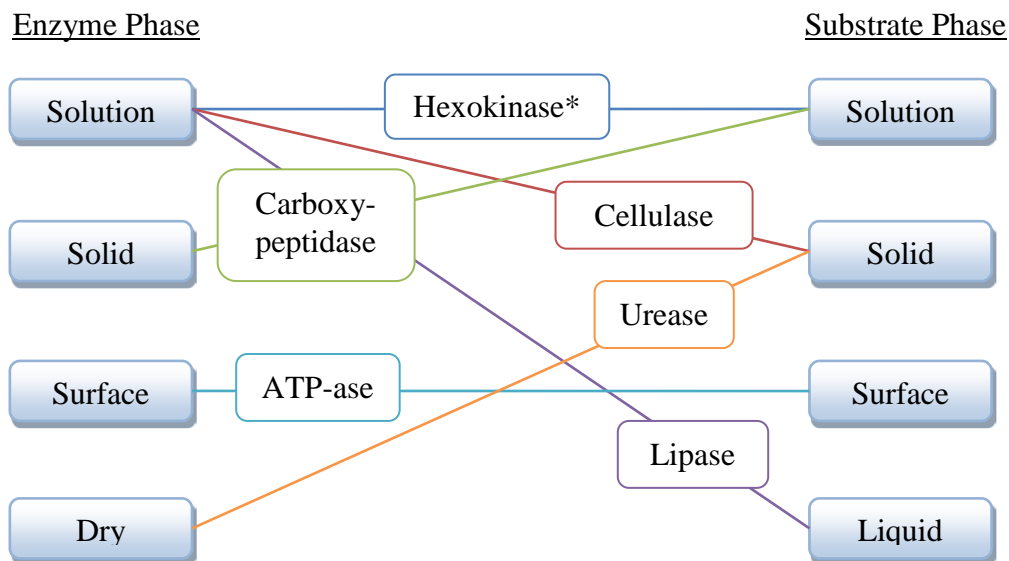


Figure 7.1. Examples of enzymes that catalyze heterogeneous reactions and the interfaces at which they work. The reaction marked with an asterisk (*) denotes a classical homogeneous reaction. Figure adapted from McLaren and Packer (1970).

The development of fractal kinetics has been useful for describing diffusion-limited reactions (Kopelman 1988; Wang and Feng 2010; Xu and Ding 2007; Yao et al. 2011). Diffusion limitations may result from the heterogeneity of the reaction, as well as high-solids loadings, like those investigated in this current work, where the availability of free water is limited. Dimensional restriction can also trigger non-classical kinetic behaviors, prompting the use of fractal kinetics.

The jamming effect is another kinetic behavior investigated in this work. Insoluble substrates in heterogeneous reactions are often packed with fixed intermolecular distances (Bommarius et al. 2008; Xu and Ding 2007). For example, the cellulose fraction of lignocellulose is actually composed of cellulose bundles, called fibrils, where multiple cellulose chains are packed together in a parallel fashion. When the cross-section of an enzyme is larger than the distance between these fixed strands, the adsorbed enzyme may actually block adjacent strands of cellulose (Figure 3.2). This behavior is very much like a traffic jam, where large vehicles block adjacent lanes. When the enzymes reach a critical level, the cellulase can overcrowd available cellulose chains, effectively reducing the rate of the reaction.

7.3 MATERIALS AND METHODS

7.3.1 Enzyme

The enzyme system consisted of crude cellulase liquid from *T. reesei* (Celluclast 1.5L) supplemented with β -glucosidase from *A. niger* (Novozyme 188). Both enzymes were purchased from Sigma (St. Louis, MO).

7.3.2 Substrate

Corn stover collected directly from the field at the Woodford County Animal Research Center in Woodford County, KY in September 2010 was used as the substrate. The corn (P1253 Pioneer) had been planted using conventional tillage practices in April 2010. Stover is composed of material other than grain (MOG). After collection, the samples were prepared for laboratory storage by drying at 45°C and grinding through a hammer mill with a 5 mm screen.

7.3.3 Pretreatment of Corn Stover

Sodium hydroxide pretreatment was performed according to Duguid et al. (2009) with some modifications. Dried, ground corn stover was placed in 500 mL Erlenmeyer flasks. The dry samples were autoclaved on a liquid cycle at 121°C for 30 min to ensure no loss of biomass due to microbial contamination. The flasks were allowed to cool to room temperature prior to equilibration at the selected pretreatment temperature. Following equilibration, a sodium hydroxide solution was added to the flasks to obtain a solids loading of 10% (w/v) and 10 g NaOH/100 g CS. The flasks were incubated at 25°C for 24 hr. The pretreated corn stover (PCS) was neutralized by washing with 5 volumes of DI H₂O and vacuum filtered before drying at 45°C for 24 hr. Samples were collected to determine composition of corn stover. PCS was stored at 4°C.

7.3.4 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to an NREL-LAP (Selig 2008), with some modifications. Pretreated biomass was added at the desired solids loading (2%, 5%, 10%, 15%, or 20% w/w). Cellulase was added to achieve an appropriate

enzyme loading (15, 30, 45 or 60 FPU/g solids) and was supplemented with β -glucosidase at ratio of 2:1CBU/g biomass to FPU/g biomass. Samples were collected at predetermined times over the course of 96 hr. Following hydrolysis, the samples were immediately transferred to a boiling water bath for 5 min to denature the enzymes. The samples were placed in an ice bath to cool. Slurries were transferred to 15 mL centrifuge tubes and diluted 10-fold with DI water, mixed well, and centrifuged. Samples of the liquid fraction were then collected, diluted and syringe-filtered (0.2 μ m) prior to analysis by HPLC to measure the sugars derived from cellulose and hemicellulose (glucose, cellobiose, xylose, arabinose, mannose and galactose). A Dionex U3000 HPLC system was equipped with a Bio-Rad Aminex HPX-87P column and Micro-Guard de-ashing column and operated at 78°C with deionized water as the mobile phase at a flow rate of 0.45 mL/min. The sample components were detected with a Shodex-101 refractive index detector.

7.3.5 Model Development

A model was developed based on the work of Xu and Ding (2007) that incorporated parameters for fractal kinetics and jamming characteristics to describe the hydrolysis of pretreated lignocellulose by cellulolytic enzymes. The product time course profiles generated were used to fit the kinetic parameters.

7.3.5.1 Classical Michaelis-Menten Kinetics

The basis of this model was the classical Michaelis-Menten kinetic scheme, where the enzyme (E) binds to the substrate (S) to form an enzyme-substrate complex (ES) before the enzyme releases the newly formed product (P).



Rate equations for product formation or substrate consumption can then be derived from Equation 7.1 as

$$v = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{v_m[S]}{K_m + [S]} \quad \text{Equation 7.2,}$$

where $V_m = k_2[E_o]$ and $K_m = (k_{-1} + k_2)/k_1$. Full derivations, including necessary assumptions, can be found in any basic enzymatic kinetics text (Bailey and Ollis 1986; Shuler and Kargi 2002). Integration of Equation 7.2 in terms of substrate leads to

$$V_m t = [S_o] - [S] + K_m \ln \frac{[S_o]}{[S]} \quad \text{Equation 7.3.}$$

The implicit nature of this equation means that the substrate concentration cannot be solved for directly at any given time and so must be numerically approximated (Goudar et al. 1999). However, a closed solution for [S]

$$[S] = K_m \omega \left[\frac{[S_o]}{K_m} \exp \left(\frac{[S_o] - V_m t}{K_m} \right) \right] \quad \text{Equation 7.4}$$

proposed by Schnell and Mendoza (1997) allows the use of Lambert's ω function as a solution to the transcendental equation

$$\omega(x) \exp(\omega(x)) = x \quad \text{Equation 7.5.}$$

The substrate concentration at any given time can also be determined from the relationship between the substrate and product, which can be written as

$$[S] = [S_o] - ([P] - [P_o]) \quad \text{Equation 7.6,}$$

assuming the initial substrate concentration and the product concentration are known. Alternatively, if the product concentration is desired, Equation 7.4 can be substituted into Equation 7.6 and rearranged to become

$$[P] = [P_o] + [S_o] - K_m \omega \left[\frac{[S_o]}{K_m} \exp \left(\frac{[S_o] - V_m t}{K_m} \right) \right] \quad \text{Equation 7.7.}$$

Equation 7.7 may still appear to be extremely cumbersome and of not much benefit, but highly accurate algorithms have been published to solve Equation 7.5 (Barry et al. 1995; Corless et al. 1996; Fritsch et al. 1973). With the use of computational software packages, like MATLAB, the kinetic parameters K_m and V_m can be determined by fitting Equation 7.7 with experimental data. Examples of the MATLAB code used can be found in Appendix C.

7.3.5.2 Fractal Michaelis-Menten Kinetics

Michaelis-Menten kinetics are based on the underlying assumption of a homogeneous reaction, where the enzyme and the substrate are in the same phase. Hydrolysis of cellulose by cellulolytic enzymes are often modeled using classical Michaelis-Menten kinetics even though it is a heterogeneous reaction with the enzyme and substrate in different phases. For example, lignocellulose is an insoluble substrate (solid phase), but the cellulolytic enzymes are soluble (liquid phase). Generally speaking, homogeneous reactions typically have rates that are proportional to the diffusion constant, since the substrate and enzyme in three-dimensional space must come together in the correct orientation before the reaction can take place. Heterogeneous reactions tend to occur at interfaces, which can trigger some non-classical kinetic behaviors (Kopelman 1988; Xu and Ding 2007), especially where diffusion limitations exist. Adsorption of cellulase onto the cellulose chain followed by the processive movement of the cellulase along the chain reduces the number of dimensions of the reaction from three to one (Bommarius et al. 2008), which can incite fractal kinetic behavior.

Xu and Ding (2007) proposed a new model for cellulose hydrolysis that included a fractal term to account for the heterogeneous nature of the reaction. The integrated form of that model can be written as

$$\frac{V_m t^{1-f}}{1-f} = [S_o] - [S] + K_m \ln \frac{[S_o]}{[S]} \quad \text{Equation 7.8.}$$

The fractal component, f , is a non-integer kinetic order that accounts for the time dependence of the rate coefficient (Kopelman 1988), resulting from the restrictions

imparted by the heterogeneous nature of the reaction. Rearranging Equation 7.8 and substituting it into Equation 7.7 gives

$$[P] = [P_o] + [S_o] - K_m \omega \left[\frac{[S_o]}{K_m} \exp\left(\frac{[S_o] - v_m t^{1-f}}{K_m}\right) \right] \quad \text{Equation 7.9,}$$

which can be implemented into the MATLAB program discussed previously to determine the kinetic parameters.

7.3.5.3 Jammed Michaelis-Menten Kinetics

It has also been proposed that the decrease in the rate of the hydrolysis reaction could be the result of enzymes blocking adjacent active sites on the substrate due to the physical dimensions of the enzyme compared to the cellulose chains. For instance, cellulose is composed of tightly packed polymers of glucose spaced ~4-6 Å apart, whereas the diameter of the forward surface of the cellulase is about 10 times greater (~45 Å) (Xu and Ding 2007). It is easy to see with this discrepancy in size between the enzyme and substrate how the adsorption of enzymes can be slowed by overcrowding the substrate, thus leading to a slower overall reaction rate.

To account for this possibility, the following model was proposed (Xu and Ding 2007):

$$\left(1 - \frac{[E_o]}{j[S_o]}\right) V_m t = [S_o] - [S] + K_m \ln \frac{[S_o]}{[S]} \quad \text{Equation 7.10.}$$

Once the enzyme loading reaches a critical concentration, $[E_o] \geq j[S_o]$, the jamming effect becomes evident. However, as long as the enzyme loading is well below this critical value, $[E_o] \ll j[S_o]$, the jamming effects should not significantly impact the reaction rate.

7.3.5.4 Jammed, Fractal Michaelis-Menten Kinetics

These reactions may also exhibit both fractal characteristics and jamming effects. The model combining these two effects can be expressed as

$$\left(1 - \frac{[E_o]}{j[S_o]}\right) \frac{v_m t^{1-f}}{1-f} = [S_o] - [S] + K_m \ln \frac{[S_o]}{[S]} \quad \text{Equation 7.11.}$$

7.3.5.5 Solid Substrate Kinetics

Enzymes that act on insoluble substrates may demonstrate kinetic characteristics much different from those acting on soluble substrates (as described in the previous sections) (Blanch and Clark 1995; McLaren and Packer 1970). In this instance, the number of available reaction sites may be limited since cellulolytic enzymes can only adsorb at specific locations on the cellulose chain, resulting in an apparent enzyme concentration that is in excess ($[E] \gg [S]$).

The reaction scheme for this type of situation is still represented by Equation 7.1, but the derivation of the initial rate of the reaction (shown below) yields a slightly different result. Assuming that the reaction has reached steady state ($\frac{d[ES]}{dt} = 0$), the equilibrium rate expression can be written as

$$k_1([E_o] - [ES])([S_o] - [ES]) = k_{-1}[ES] + k_2[ES] \quad \text{Equation 7.12,}$$

where $E = E_o - ES$ and $S = S_o - ES$. Expanding the left hand side gives

$$k_1[E_o]([S_o] - [ES]) - k_1[ES]([S_o] - [ES]) = k_{-1}[ES] + k_2[ES] \quad \text{Equation 7.13.}$$

With soluble enzyme in excess ($[E_o] \gg \gg [ES]$), then $[E_o] - [ES] \cong [E_o]$, allowing the simplification of Equation 7.13 to

$$k_1[E_o]([S_o] - [ES]) = k_{-1}[ES] + k_2[ES] \quad \text{Equation 7.14.}$$

Further rearrangement and solving for ES gives

$$k_1[E_o][S_o] - k_1[E_o][ES] = k_{-1}[ES] + k_2[ES] \quad \text{Equation 7.15}$$

$$k_1[E_o][S_o] = (k_{-1} + k_2[ES] + k_1[E_o][ES]) = (k_{-1} + k_2 + k_1[E_o][ES]) \quad \text{Equation 7.16}$$

$$\frac{k_1[E_o][S_o]}{k_{-1} + k_2 + k_1[E_o]} = [ES] \quad \text{Equation 7.17}$$

$$\frac{[E_o][S_o]}{\frac{k_{-1} + k_2}{k_1} + [E_o]} = [ES] \quad \text{Equation 7.18}$$

$$\frac{[E_o][S_o]}{K_m + [E_o]} = [ES] \quad \text{Equation 7.19.}$$

Substituting Equation 7.19 into the rate expression gives

$$v_o = k_2[ES] = k_2 \frac{[E_o][S_o]}{K_m + [E_o]} \quad \text{Equation 7.20.}$$

where $k_2E_o = V_m$. The velocity then becomes

$$v_o = \frac{V_m[S_o]}{K_m + [E_o]} \quad \text{Equation 7.21}$$

$$v = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{V_m[S]}{K_m + [E_o]} \quad \text{Equation 7.22,}$$

which resembles the classical Michaelis-Menten form, except with the enzyme and substrate interchanged in the denominator. For clarity, this model will be referred to as the *modified* Michaelis-Menten model. The integrated form of Equation 7.22 is much simpler than the classical Michaelis-Menten equation and does not require the use of the Lambert's ω function to solve it.

$$[P] = \frac{V_m t [S]}{K_m + [E_o]} \quad \text{Equation 7.23}$$

The fractal kinetics and jamming components can be applied to the integrated form of Equation 7.23 much like they were incorporated to the classical Michaelis-Menten model previously.

$$[P] = \frac{V_m t^{1-f} [S]}{K_m + [E_o]} \quad \text{Equation 7.24}$$

$$[P] = \frac{\left(1 - \frac{[E_o]}{j[S_o]}\right) V_m t [S]}{K_m + [E_o]} \quad \text{Equation 7.25}$$

The kinetic parameters in the models, K_m , V_m , f and j , were simultaneously fit to the experimentally measured glucose released from the enzymatic hydrolysis of pretreated corn stover using the *lsqcurvefit* function in MATLAB by minimizing the least squares estimates between the modeled and measured glucose concentrations. A single value for each of the model parameters K_m , V_m , f and j was determined across experiments by simultaneously fitting the models with the respective parameters to hydrolysis data collected as a function of solids loading. MATLAB code developed for these models can be found in Appendix C.

7.4 STATISTICAL ANALYSIS

The data were analyzed as a 4×5 factorial in a generalized randomized complete block design (solids loading = block) using PROC GLM of SAS to determine whether any differences in initial rate of hydrolysis or extent of hydrolysis existed. If differences existed, least squares means were computed, and all possible pairwise comparisons were made among hydrolysis conditions. SAS input code can be found in Appendix E.

7.5 RESULTS

7.5.1 Enzymatic Hydrolysis

7.5.1.1 Effect of Initial Substrate Concentration

Figure 7.2 shows the initial rates of the hydrolysis reactions performed at the five solids loadings investigated and an enzyme loading of 15 FPU/g solids. The initial rates

were determined from the slope of the hydrolysis curve over the first hour of the hydrolysis reaction. The initial rates of hydrolysis reactions performed at the other enzyme loadings (30, 45 and 60 FPU/g solids) can be found in Appendix D. The initial rates increased from 1.1 to 12.5 g glucose/L-hr as the solids loadings increase from 2% to 20%, respectively.

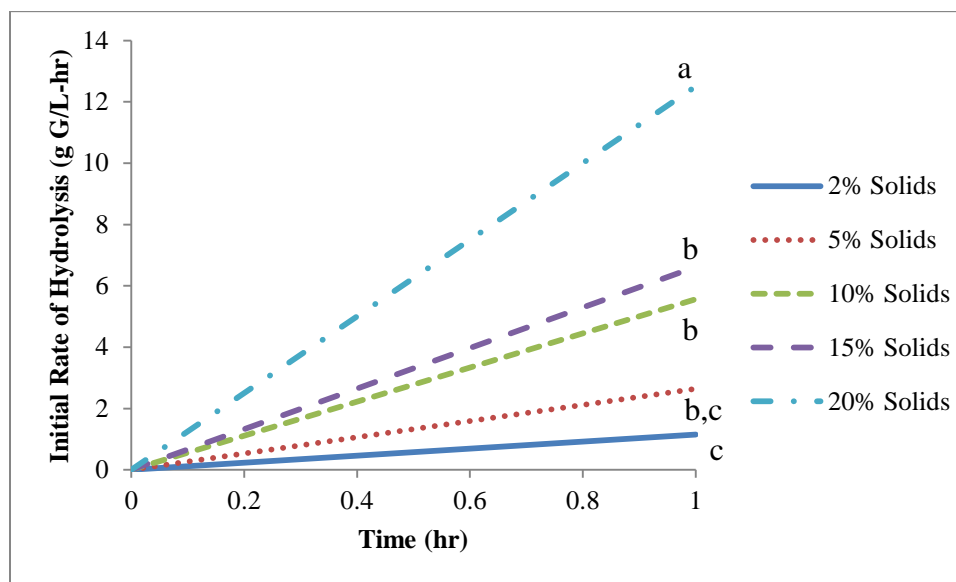


Figure 7.2. Initial rates of hydrolysis. Hydrolysis was performed at the various solids loadings indicated with an enzyme loading of 15 FPU/g solids for 96 hr. Initial rates were determined manually from the first hour of hydrolysis. Rates with the same letter are statistically the same at $\alpha=0.05$.

The extent of glucose released after 72 hr hydrolysis is shown in Figure 7.3 for the five solids loadings investigated hydrolyzed with an enzyme loading of 15 FPU/g solids. The extents of reaction for the other enzyme loadings can be found in Appendix D. The glucose released increased from 3.3 g glucose/L to ~19 g glucose/L with increasing solids loadings up to 10% solids and remained steady at ~19 g glucose/L for higher solids loadings.

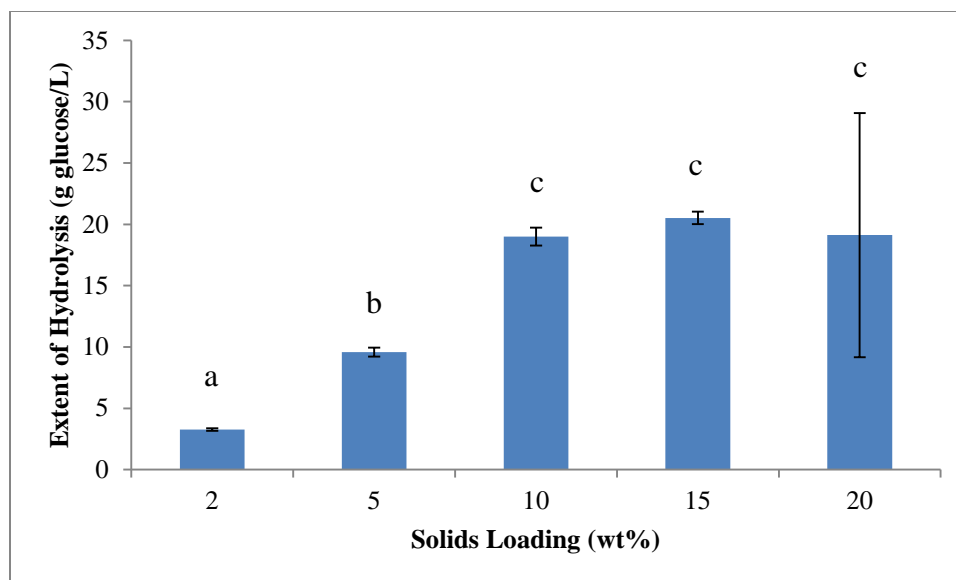


Figure 7.3. Extent of glucose released after 96 hr of hydrolysis. Columns labeled with the same letter are statistically the same at $\alpha=0.05$.

7.5.1.2 Effect of Initial Enzyme Concentration

The enzyme utilization efficiency illustrates the amount of glucose a given amount of enzyme can release. It is apparent from Figure 7.4 that enzyme loading has a negative effect on the enzyme utilization efficiency. For instance, an enzyme loading of 15 FPU/g solids at 5% solids (1974 mg protein/L) produced ~220 mg glucose/mg protein. Increasing the enzyme loading four-fold to 60 FPU/g solids (7895 mg protein/L) reduced the glucose production by more than 10-fold to 18 mg glucose/mg protein. Furthermore, a four-fold increase in solids loading from 5% to 20% with an enzyme loading of 15 FPU/g solids (7895 mg protein/L) resulted in a glucose production of 40 mg glucose/mg protein. The enzyme utilization efficiency was only two times greater even though the solids loading was four times higher (at equivalent protein concentrations). Additionally, the enzyme utilization efficiency was ~5.5 times less at 20% solids loading compared to 5% solids loading even with an equivalent enzyme: substrate ratio.

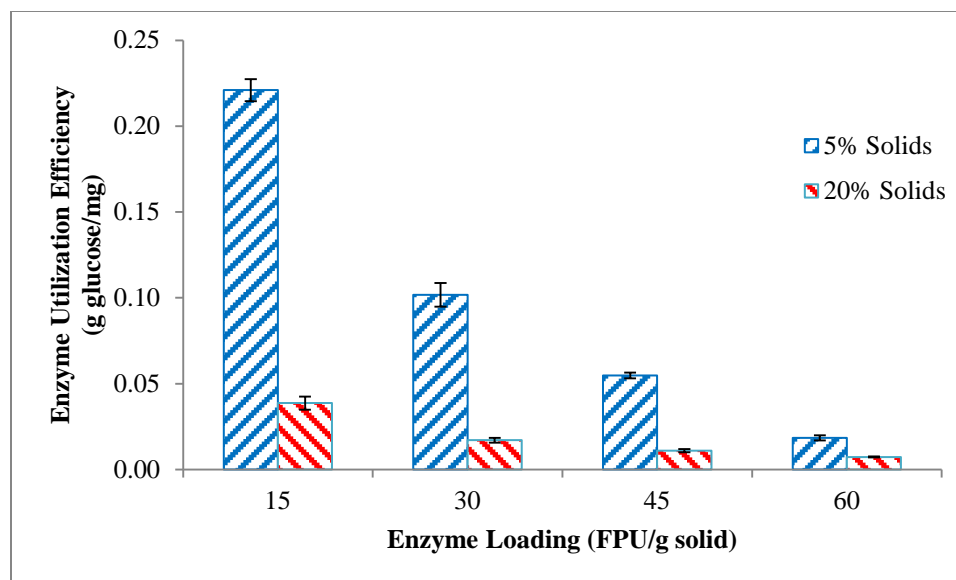


Figure 7.4. Effect of the enzyme loading on the enzyme utilization efficiency. Hydrolysis was performed for 96 hrs at 5% and 20% solids loadings and four enzyme loadings.

7.5.2 Model Analysis and Parameter Determination

The integrated form of the classical Michaelis-Menten kinetics model was used as a base model, upon which other parameters (fractal and/or jamming parameters) were incorporated to describe the time course hydrolysis curves. For conciseness, a representative data set (data from hydrolysis using 15 FPU/g solids) was chosen to present the resulting parameters in this chapter. Additional sets of data and the associated kinetic parameters for other enzyme loadings (30, 45 and 60 FPU/g solids) can be found in Appendix D.

Following Goudar et al. (1999), the initial substrate concentration was set to $[P_{\infty}]$, which was the average glucose released at the full extent of the reaction. This value was chosen to represent the accessible cellulose in the lignocellulose. Additionally, lower and upper bounds were placed on the kinetic parameters during the *lscurvefit* process. For example, the constraint that none of the estimated parameters may be negative was set. A negative K_m and V_m would imply that the reaction is going in reverse; cellulose would be polymerized instead of hydrolyzed. Therefore, the lower bound for all four parameters was set to 0. In addition, by definition K_m cannot realistically be larger than the substrate concentration if a V_m is determined for the reaction. Therefore the upper bound for K_m

was set to $[S_o]$. Fractal kinetics infers a non-integer reaction order, so an upper bound for f was set to 1 such that only non-integer values could be fit. In the instance that $f = 0$, then the fractal model would reduce to the Michaelis-Menten kinetics model. Likewise, as the jamming term $\left(1 - \frac{[E_o]}{j[S_o]}\right) \rightarrow 1$, then jamming becomes negligible, and the model would reduce to the Michaelis-Menten model. Lastly, the initial product concentration ($[P_o]$) was assumed to be 0.

Figure 7.5 shows the correlations between the observed and the predicted rate of product (glucose) formed for each of the four models examined using the classical Michaelis-Menten model (based on Equation 7.7).

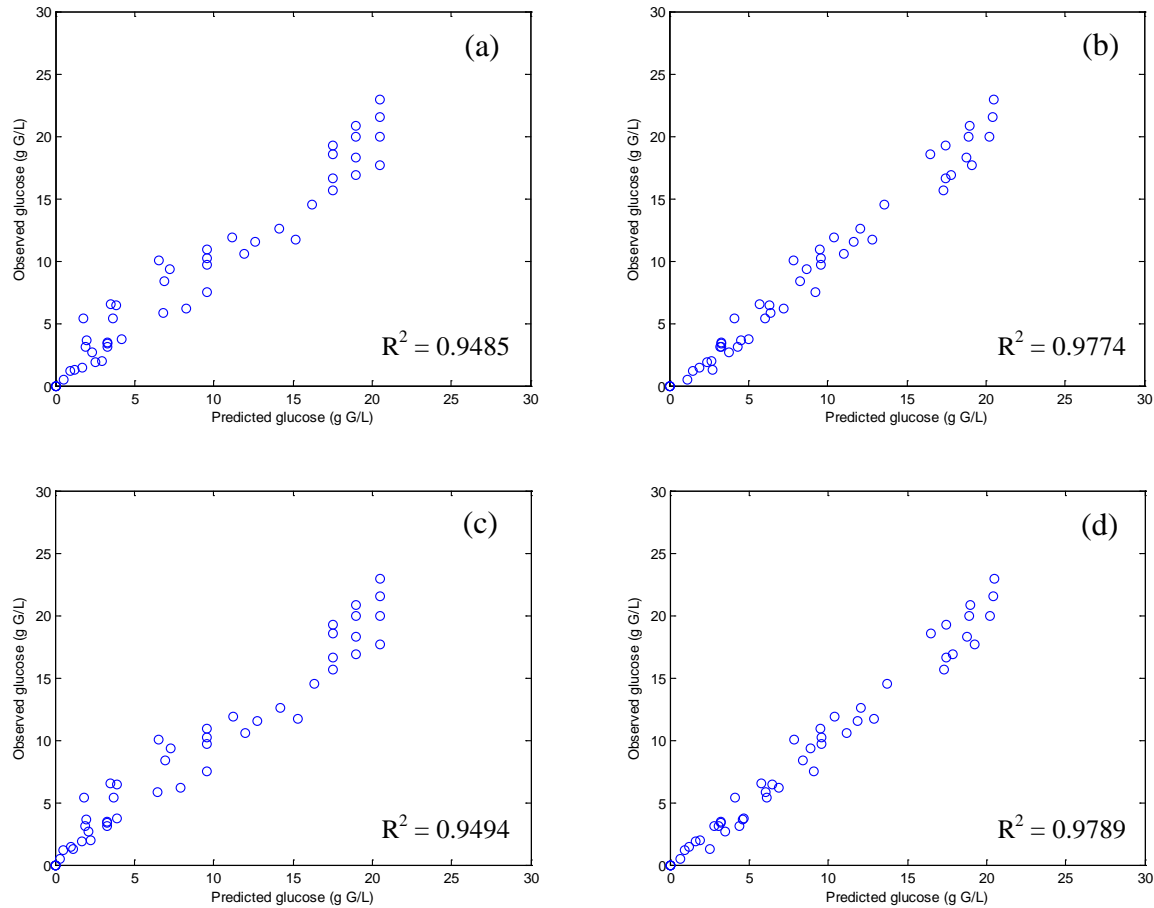


Figure 7.5. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 15 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components.

Table 7.1 lists the kinetic parameters estimated from fitting experimental hydrolysis data to the classical Michaelis-Menten models discussed previously. The estimated dissociation constant (K_m) is equivalent for all four models. However, it should be noted that this value is the upper bound set for this parameter within the model. Additionally, the estimated fractal (f) parameter was equivalent for the two models that have that parameter. The maximum velocity (V_m) and jamming (j) parameters varied slightly with each model.

F-statistics were calculated to determine whether the slope and intercept of the line fitting the predicted vs. observed glucose concentrations were statistically different from 1.0 and 0.0, respectively. The null hypothesis will not be rejected if the calculated F-statistic is smaller than the critical F-statistic for the given degrees of freedom ($df = 2, 48$), indicating that the model has some merit in fitting the data. Smaller values of F mean the model is a good fit (Haefner 2005). Calculated F values for each model considered indicated that the null hypothesis could not be rejected and that the models had merit in fitting the experimental data (data not shown). Even though F-statistics provide a better indication of fit, the decision to fail to reject the null hypothesis led to the use of R^2 values to evaluate the models.

The R^2 value gives an indication of the fit of the predicted glucose concentrations to the observed glucose concentrations. A value closer to 1.0 indicates a better fit. Predicting the glucose concentrations using the classical Michaelis-Menten model provides a relatively good fit ($R^2 = 0.9485$) when compared to the observed glucose concentrations, even though the underlying assumptions are not valid for hydrolysis of cellulose. Inclusion of the fractal parameter improved the fit of the model ($R^2 = 0.9774$). Inclusion of both the fractal and the jamming parameters only improved the fit slightly compared to the model with only the fractal parameter, indicating that the jamming parameter does not adequately describe the cellulose hydrolysis reaction at enzyme loading of 15 FPU/g solids.

Table 7.1. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 15 FPU/g solids using the classical Michaelis-Menten kinetics model.

Model	K_m (g/L)	V_m (g/L-hr)	f	j	R^2
MM	20.50	8.03	--	--	0.9485
MM+f	20.50	7.35	0.47	--	0.9774
MM+j	20.50	8.94	--	8.63	0.9494
MM+f+j	20.50	8.17	0.47	8.83	0.9789

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

The correlations between the observed and the predicted glucose released for each of the four models based on the modified Michaelis-Menten model are shown in Figure 7.6. Upon inspection of these figures, it is apparent that the modified Michaelis-Menten model with no additional parameters, as well as the model with the jamming parameter are not linear, indicating a very poor fit using these two models. The poor fit is further substantiated by the R^2 values; both models resulted in an R^2 that is < 0.3 (Table 7.2). However, the two models that include the fractal parameter fit the data equally well ($R^2 = 0.9620$), indicating that the jamming parameter is again inadequate in describing the cellulose hydrolysis reaction at an enzyme loading of 15 FPU/g solids.

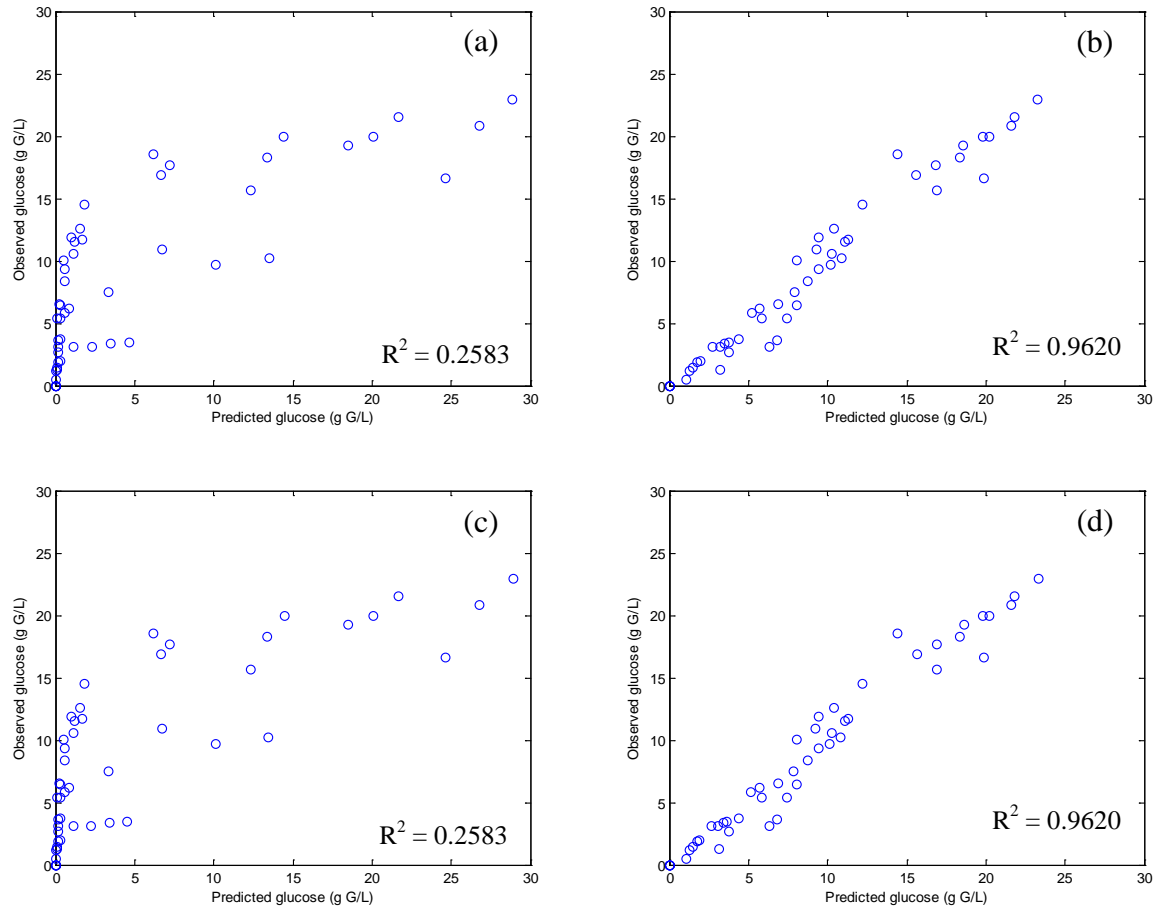


Figure 7.6. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 15 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components.

Table 7.2 lists the kinetic parameters estimated from fitting experimental hydrolysis data to the modified Michaelis-Menten models (based on Equation 7.23) discussed previously. The estimated parameters varied more with the models based on the modified Michaelis-Menten than compared to the classical Michaelis-Menten models. These values for K_m were on the same order of magnitude as the previous models, but ranged from 13.8 to 20.0. These parameter estimates did not converge at the upper bound set by the model. The values of V_m appear to depend on whether the fractal component was included. For models without the fractal component, the V_m was an order of magnitude lower than compared to the models with the fractal component. However, the models with the fractal component fit the data better.

Table 7.2. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 15 FPU/g solids using the modified Michaelis-Menten kinetics model.

	K_m (FPU/g solids)	V_m (g/L-hr)	f	j	R^2
MM	20.01	0.51	--	--	0.2583
MM+f	17.40	2.96	0.77	--	0.9620
MM+j	13.83	0.43	--	149.54	0.2583
MM+f+j	17.09	2.95	0.77	121.88	0.9620

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

Figure 7.7 and Figure 7.8 shows the experimental hydrolysis data for different levels of solids loadings with the curves generated from the estimated parameters. The calculated fractal reaction profile (Figure 7.7b) showed better agreement to the observed data compared to the classical Michaelis-Menten profile (Figure 7.7a), especially in the transition phase from first-order to zero-order kinetics.

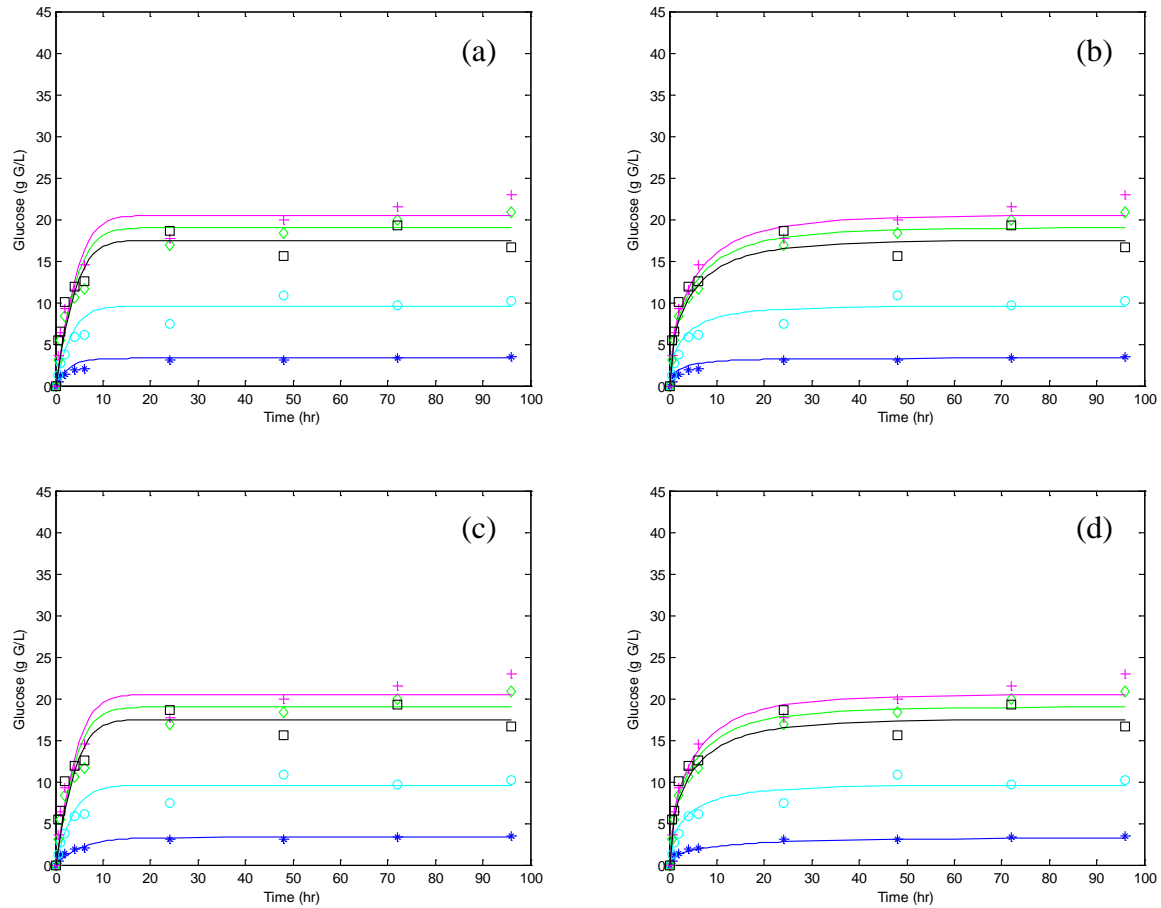


Figure 7.7. PSC hydrolysis by *T. reesei* cellulase at 15 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components. (Symbols: ‘blue *’ 2% solids; ‘cyan o’ 5% solids; ‘green \diamond ’ 10% solids; ‘magenta +’ 15% solids; ‘black \square ’ 20% solids; solid lines are model predictions)

The modified Michaelis-Menten model resulted in a much different profile curve compared to the classical Michaelis-Menten model. The hydrolysis profile curves produced by the base model and the jammed model (Figure 7.8a, c) do not have the characteristic shape of a hydrolysis curve; the rate does not appear to decrease with time for these models. The fractal models do have this characteristic shape; however, the tendency for these curves is to continue to increase with time. These profiles do not appear to “level off” at the extended hydrolysis times to the same extent as the classical Michaelis-Menten models.

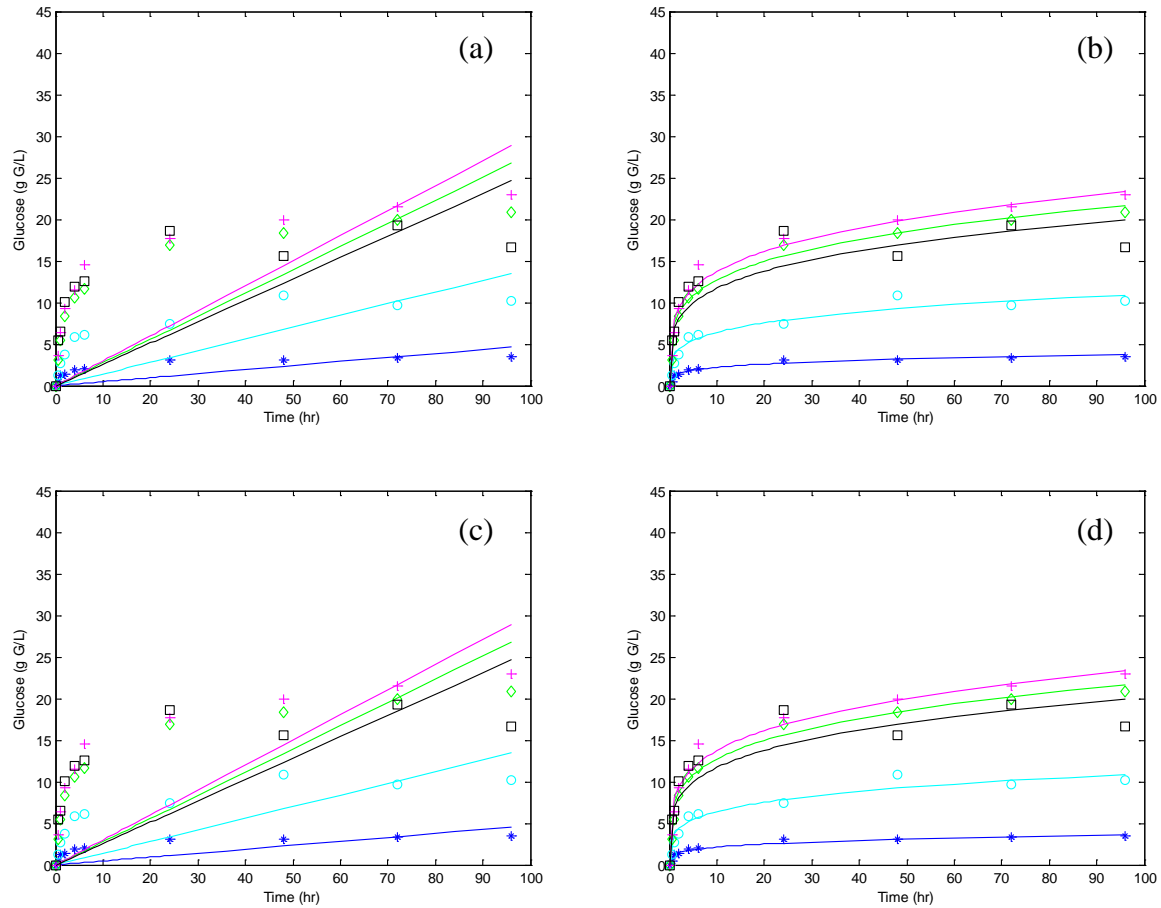


Figure 7.8. PSC hydrolysis by *T. reesei* cellulase at 15 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components. (Symbols: ‘blue *’ 2% solids; ‘cyan ○’ 5% solids; ‘green ◇’ 10% solids; ‘magenta +’ 15% solids; ‘black □’ 20% solids; solid lines are model predictions)

7.6 DISCUSSION

7.6.1 Effects of Initial Substrate and Enzyme Loading on Enzymatic Hydrolysis

For the lignocellulose conversion process to be economically feasible, a balance must be struck between initial substrate concentration and enzyme loading to produce maximal glucose yields with minimal inputs. A commonly observed characteristic of hydrolysis reactions is that higher solids loadings in the conversion process tend to lead to higher initial rates (Gan et al. 2003) and higher final product concentrations (Gupta and Lee 2009; Kristensen et al. 2009b) but a lower percent conversion. That trend was only partially observed in this current study. An increase in solids loadings with an enzyme loading of 15 FPU/g solids resulted in an increase in glucose released only up to 10% solids loading. The final glucose concentration did not change at solids loadings higher than 10%. Similar trends were observed with enzyme loadings of 30 and 45 FPU/g solids, except glucose concentrations increased with solids loadings up to 15% and remained unchanged at 20% solids (data shown in Appendix D). At the highest enzyme loading, 60 FPU/g solids, the glucose concentration increased with solids loadings up to 15% but decreased for 20% solids. Initial rates did not strictly follow the expected trend, either.

Enzyme loading also impacted final glucose concentrations. Enzyme efficiency (defined as the amount of glucose released for a given amount of enzyme, mg glucose/mg protein) decreased significantly with increasing enzyme loadings, as seen in Figure 7.4. Essentially, higher enzyme loadings did not result in a proportionally higher glucose concentration, meaning that the enzyme efficiency was greatly diminished at higher enzyme loadings. Gan et al. (2003) also reported a decrease in enzyme efficiency from 68 mg glucose/mg protein to 27 mg glucose/mg protein when the enzyme loading increased from 100 mg/L to 500 mg/L at 2% solids (α -cellulose fibers from wood pulp) loading. It is hypothesized that increasing solids and/or enzyme loadings significantly impacts the availability of free water in the system, to the detriment of the hydrolysis reaction, as discussed in Chapter 6.

7.6.2 Determination of Kinetic Parameters

An experimental and theoretical analysis of enzymatic hydrolysis using the classical Michaelis-Menten kinetics model and a modified (for insoluble substrates) Michaelis-Menten model was conducted. Nidetzky and Steiner (1993) also estimated kinetic parameters associated with the hydrolysis of cellulose. They reported a value for K_m of 37.6 FPU/g solids and 6.3 FPU/g solids for cellulose that is easily or more difficult to hydrolyze. It should be noted that the substrate used in their study was microcrystalline cellulose with particle sizes of approximately 50 μm . These values of K_m are in the same range determined with the modified Michaelis-Menten models in this current work for lignocellulose with particle sizes of < 5 mm. However, the maximum velocities of 2,680 g/L-hr and 240 g/L-hr for easily and more difficult hydrolyzed cellulose reported by Nidetzky and Steiner (1993) were much higher than those determined in this current work at 0.4 to 9.0 g/L-hr. Okazaki and Mooyoung (1978) also reported values for K_m ranging from 3.8 to 7.4 g/L using purified endoglucanase from *Trichoderma viride* to hydrolyze CMC (carboxymethylcellulose). These values are 2-5 times smaller than those determined in this current work with the classical Michaelis-Menten models, likely due to the lack of synergistic effects associated with non-purified cellulase systems.

Additional parameters were incorporated to each of these models to determine whether effects such as fractal kinetics or jamming of the enzyme could explain the reduction in the hydrolysis reaction rate over time. In both instances, inclusion of the fractal kinetics component improved the model's ability to fit the experimental hydrolysis data, indicating that the heterogeneity of the reaction should be accounted for in the model.

Valjamae et al. (2003) investigated the use of fractal kinetics to model the hydrolysis of bacterial cellulose by purified *T. reesei* cellulase (Cel7A). They observed a fractal parameter, f , ranging from 0.35-0.6 for solids loadings of 2 mg/mL to 0.25 mg/mL ($<1\%$ w/w solids loadings). This range is consistent with the fractal component determined by Xu and Ding (2007). Modeling the hydrolysis of phosphoric acid swollen cellulose (PASC) resulted in an $f = 0.44$. The solids loading in the Xu and Ding (2007) study was $<1\%$ (w/w). Wang and Feng (2010) modeled the hydrolysis of acid-pretreated

Avicel performed at a solids loading of ~10% (w/w) with an enzyme loading of 37.5 FPU/g cellulose. The resulting fractal component was 0.42. Furthermore, they observed that the fractal component appeared to increase with increasing enzyme loading. This trend was not observed in this current work. In fact, there did not appear to be any real relationship between enzyme loading and the fractal component (data not shown). A final study to incorporate a fractal component into a hydrolysis model reported an f of ~0.55 for hydrolysis of dilute acid PCS performed at 20% solids loading (Yao et al. 2011). These values are all fairly consistent with the values determined by the classical Michaelis-Menten models analyzed in this current work (0.41-0.52). However, the Michaelis-Menten models modified for insoluble solids resulted in higher values of f (0.77-0.85).

The term $\left(1 - \frac{[E_o]}{j[S_o]}\right)$ describes the degree of jamming that occurs in the hydrolysis reaction. The relationship between the enzyme and substrate concentration determines whether jamming significantly impacts the reaction rate. For instance, when $[E_o] \gg j[S_o]$, jamming is expected to occur. From this relationship, one could infer that a smaller jamming parameter value would indicate a system impacted by jammed enzymes. Xu and Ding (2007) reported a jamming parameter value of 4.4×10^{-5} for hydrolysis performed at 2 g/L cellulose with an enzyme concentration of 0.1-0.2 μM . Another study found that the jamming parameter increased from 0 to 0.093 when enzyme concentrations increased from 2.3 μM to 1.19 mM (assuming an enzyme molecular weight of ~61.5 kDa). Jamming parameter values determined in this current work were several orders of magnitude larger at 8.6 and 149.5 for the classical and modified Michaelis-Menten models, respectively. It is clear from Figure 7.7c and Figure 7.8c that the addition of the jamming parameter did not improve the fit of the predicted hydrolysis curves to the experimental hydrolysis data, indicating that jamming was not likely under the conditions tested in this study.

Although the assumptions associated with the classical Michaelis-Menten kinetics model are not valid for the hydrolysis of cellulose, it can still be used as a good first estimate of the kinetic parameters. The cellulose hydrolysis reaction begins as a completely heterogeneous reaction and slowly transforms into a system with both heterogeneous and homogeneous components as time progresses. The concentration of

solubilized oligosaccharides increases as the enzymes act upon the cellulose polymers, reestablishing the validity of the assumptions associated with the classical Michaelis-Menten kinetics model, which is presumably the reason the classical Michaelis-Menten model with the fractal component (a model based upon homogeneous reaction schemes with a component that accounts for the heterogeneity of the reaction) provided the best fit (based on the R^2 values) of the experimental hydrolysis data under the conditions investigated.

There are still some limitations to these models associated with the assumptions made for simplification. The upper bounds set for the dissociation constant, K_m , appear to impact the fitting of the classical Michaelis-Menten models. The estimated values for K_m are equivalent to the upper bound. When this upper bound is removed, the estimated K_m is several orders of magnitude larger than the initial substrate concentration. Upon inspection of the hydrolysis progress curves, a K_m of that large a magnitude does not seem reasonable.

The kinetic parameters K_m and V_m were determined; however, it is difficult to decouple these two values with the numerical method used to fit these parameters. This issue arises due to the highly dependent relationship between these two parameters and is only apparent under certain conditions. For instance, for very small values of K_m , values of V_m can change very quickly. For very large values of K_m , values of V_m become more constant, often causing the estimated parameters to approach the upper boundaries set. These limitations of the numerical method and the highly dependent relationship between the two parameters can result in instability in the estimated parameters.

The assumption that S_o is equivalent to P_∞ does not capture the solid substrate traits associated with the hydrolysis of lignocellulose, since P_∞ only accounts for the substrate that is solubilized. By making this assumption, the mechanism of the enzymatic hydrolysis reaction has been disregarded. For instance, the kinetics of this type of reaction depend on the nature of the enzymes, the structure of the substrate, and the physical interactions between the enzyme and substrate (Fan and Lee 1983). Others have taken the mechanistic approach to modeling the hydrolysis of cellulose (Fan and Lee 1983; Gan et al. 2003; Igarashi et al. 2011; Levine et al. 2010; Okazaki and Mooyoung

1978) and exploring some of these mechanistic concepts could improve the assumptions associated with the model presented in this current work.

Additionally, the initial product concentration, $[P_0]$, was assumed to be negligible. However, this assumption may not be valid since the enzyme solution may have some residual sugars present from the industrial fermentation production. These residual sugars could be impacting the measured glucose concentrations, especially at higher enzyme loadings, where the inadvertent application of these residual sugars would be higher. Nieves et al. (1998) reported that the Celluclast 1.5L commercial cellulase preparation contained 8.0 mg glucose/mL of enzyme preparation. Assuming the cellulase preparation used in this current work has a similar residual glucose concentration, it translates into an initial glucose concentration of ~4-15 mg/g cellulose, depending on the enzyme loading. The glucose concentrations for each measured time point were normalized based on the amount of glucose measured in the samples collected at time $t = 0$ to justify the $[P_0] = 0$ assumption.

Lastly, the units of some of the input variables, especially the enzyme concentration (FPU/g solids) are not typically used for modeling, making it difficult to compare the kinetic parameters determined in this current work to those from other works.

Table 7.3. Nomenclature.

Symbol	Definition
[E]	Enzyme concentration (FPU/g solids)
[E _o]	Initial enzyme concentration (FPU/g solids)
[P]	Product concentration (g/L)
[P _∞]	Product concentration at t = ∞ (g/L)
[P _o]	Initial product concentration (g/L)
[S]	Substrate concentration (g/L)
[S _o]	Initial substrate concentration (g/L)
E	Enzyme
ES	Enzyme-substrate complex
f	Fractal parameter
j	Jamming parameter
k ₁	Rate constant (hr ⁻¹)
k ₋₁	Rate constant (hr ⁻¹)
k ₂	Rate constant (hr ⁻¹)
K _m	Dissociation constant (g/L or FPU/g solids)
P	Product
S	Substrate
t	Time (hr)
v	Velocity (g/L-hr)
V _m	Maximum velocity (g/L-hr)
v _o	Initial velocity (g/L-hr)
ω	Lambert's function

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CHAPTER 8: EVALUATION OF MESOPOROUS SILICA MATERIALS FOR THE SEPARATION AND RECOVERY OF MONOSACCHARIDES IN HYDROLYZATE OF PRETERATED CORN STOVER

8.1 SUMMARY

Mesoporous silica materials (2 to 50 nm pore diameters) synthesized with three different methods were evaluated, using both liquid chromatography and bulk adsorption, for their effectiveness in selectively separating specific monosaccharides from solution. A novel synthesis technique termed microphase-directed molecularly imprinting (MDMI) was used to produce non-imprinted, glucose-imprinted and xylose-imprinted silica materials. Liquid chromatography was performed with each of these materials, as well as a commercially available, amine functionalized silica material, using both a prepared glucose and xylose solution and an enzymatically-produced hydrolyzate. Some separation of the glucose and xylose peaks was observed when eluting the prepared sugar solution through the commercially available material but not the hydrolyzate. However, no separation was apparent for any of the MDMI materials using either the prepared sugar solution or the hydrolyzate. Many factors (i.e. stationary phase, mobile phase, temperature, pH) affect elution profiles, making the development of liquid chromatography protocols complex. Different mobile phases (pH 5 buffer at room temperature, pH 5 buffer at room temperature followed by buffer at 50°C, and 90:10 acetonitrile-water mixture) were also evaluated for their effect on the elution profiles of glucose. The acetonitrile-water mobile phase resulted in a shorter, broader peak compared to the pH 5 buffer, but the peak maximum was not substantially shifted in either direction, so separation was not improved.

Another class of mesoporous silica materials was synthesized using the well-established Santa Barbara Acid (SBA) method. This method was used to produce materials with different sized pores in order to evaluate the effect that pore size has on the diffusion and separation of monosaccharides. No change in the glucose peaks was apparent with the pH 5 buffers, but the peak shifted to the right as the pore size increased when using the acetonitrile-water mixture as the mobile phase.

Fluorescently-tagged dextrans (MW = 40,000) were added to a prepared glucose solution to compare the elution profiles of large and small saccharide species. The

normalized elution profiles for these two species overlapped, indicating that the glucose was not interacting with the mesoporous silica material any differently than the larger dextrans.

Lastly, mesoporous silica materials were synthesized using the established Stöber method and bulk adsorption of selected monosaccharides was evaluated. Glucose and xylose concentrations in enzymatically-produced hydrolyzate were measured before and after mixing hydrolyzate with the silica particles for 24 hr. The non-imprinted particles adsorbed small, but similar amounts of both glucose and xylose. The glucose-imprinted materials adsorbed four times more glucose than xylose from the hydrolyzate, indicating that the synthesis method coupled with the novel imprinting technique could selectively adsorb (and separate) a desired monosaccharide from solution.

8.2 INTRODUCTION

The abundance of lignocellulose, and the energy-rich saccharides that comprise the material, make it a leading candidate as a feedstock for energy production in biorefineries. One of the primary products from these refineries will likely be liquid transportation fuels like ethanol or butanol, but for long-term economic viability these refineries will also be required to produce a range of products similar to that of traditional petroleum refineries (Kadam et al. 2008; Menon and Rao 2012). To be as efficient as possible, all components of the lignocellulose material must be utilized, including the saccharides of the hemicellulose fraction and the phenolic compounds of the lignin. The lignin has traditionally been fractionated and recovered for heat and power generation for the unit operations of the conversion process. However, hemicellulose is composed of many different types of saccharides, including the five-carbon (C5) sugars xylose and arabinose and the six-carbon (C6) sugars glucose, mannose and galactose. These C5 sugars have deleterious effects on traditional glucose-based fermentation processes since C5 sugars are not metabolized as easily and may even be toxic to the yeast. The C5 sugars, especially xylose, can be better utilized as building blocks for other commodity or high-value chemicals (Figure 4.6) (Carvalho et al. 2008; Kadam et al. 2008). Separation and recovery of this energy-rich stream of C5 sugars (Figure 8.1) is a

promising method for improving the economic viability and competitiveness of biorefineries with traditional petroleum refineries.

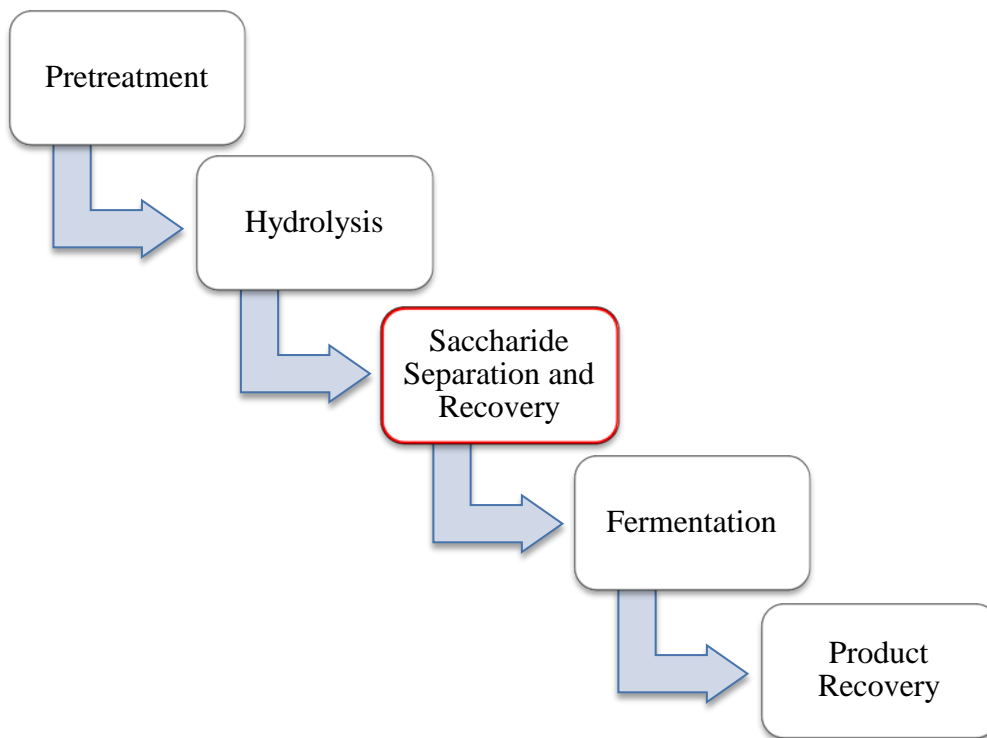


Figure 8.1. Unit operations typical of the lignocellulose conversion process with the proposed addition of saccharide separation and recovery.

Molecularly imprinted polymers (MIPs) are of interest as a means to achieve more precise separations for complex separation processes (Sanz and Martinez-Castro, 2007). Separation processes that use MIPs include the separation of dyes, vitamins, nucleotide bases and other components that are typically difficult to separate (Wizeman and Kofinas, 2001; Li and Li, 2007). MIPs are tailor-made for specific separations. For example, Wizeman and coworkers (2001) developed a novel MIP that was capable of binding glucose. The imprinted materials were synthesized with the cross-linking polymer ethylene glycol diglycidyl ether (EDGE) and imprinted with the templating molecule glucose phosphate monosodium salt (GPS). The results showed that the mass of glucose binding was significantly higher on the imprinted material as opposed to the non-imprinted material (0.56 g glucose/g material using 1.5 mol% GPS vs. 0.18 g

glucose/g material, respectively) and that glucose binding increased from 0.48 to 0.56 g glucose/g material and fructose binding decreased from 0.34 to 0.23 g fructose/g material as the amount of templating molecules used in the synthesis of the material increased. The molecular imprinting of polymers is well-established; however, polymers are less thermally, chemically and mechanically stable and more susceptible to solvent degradation than ceramic (silica) material (Tan and Rankin 2005). For these reasons, molecular imprinting of silica material was examined in this current work.

The concept of synthesizing tailor-made imprinted materials for specific applications is constantly being expanded upon as more imprinting techniques, imprinting molecules and imprinted materials are developed. Mesoporous (2 to 50 nm diameter pores) silicate material can be imprinted by cationic surfactants used as templates (Wu et al. 2013) to produce tailor-made separation materials specific to a single type of molecule. Silica is attractive for these imprinted materials because the synthesis conditions (i.e. reaction temperature, pH, silica source, surfactant type) can be customized to produce materials with various morphologies like spheres, fibers and tubules (Wu et al. 2013; Zhao et al. 2000), as well as various pore structures, like hexagonal, cubic and wormhole-like pores (Sang and Coppens 2011; Wu et al. 2013).

Microphase-directed molecular imprinting (MDMI) is a relatively new approach to molecular imprinting where specific molecular shapes are imprinted on silica and provide site selectivity only for molecules matching the initial imprinting molecule (Figure 8.2). MDMI uses surfactant imprinting molecules (SIMs) and cationic surfactants, which when combined form mixed micelles (Figure 8.3). Surfactant templating creates high surface area, silicate material with uniform mesopores (Meynen et al. 2007). The SIMs are composed of functionalized surfactants whose headgroup imprints sites complementary to the molecule of interest. In this current work, the molecules of interest are the specific monosaccharides glucose and xylose, and the functionalized surfactants have the respective saccharide headgroups (either glucose or xylose). Removal of the mixed micelle from the silica material with an ethanol-HCl solution results in an imprinted site, which is specific for the saccharide headgroup of the surfactant-imprinting molecule. The silica materials should selectively adsorb only those molecules that match the configuration of the –OH groups in the binding sites.

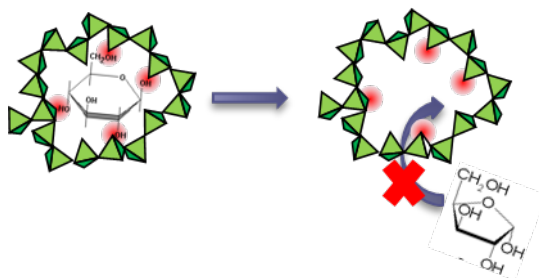


Figure 8.2. Traditional molecular imprinting processes of silica (green) around a molecule of interest, like a six-carbon monosaccharide. The sugar –OH groups create binding sites (red) that do not match an undesired five-carbon monosaccharide, leading to selectivity of the six-carbon monosaccharide.

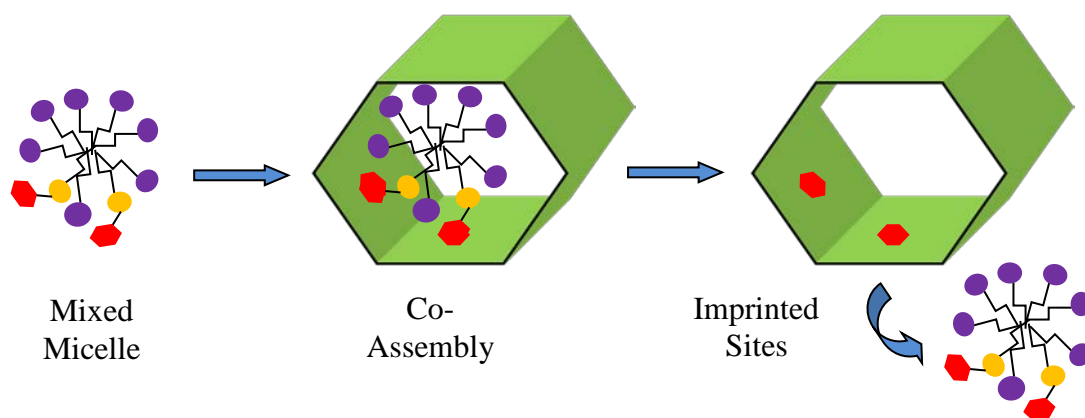


Figure 8.3. Microphase-directed molecular imprinting of silica (green) by mixed micelles, consisting of surfactant (purple) and surfactant imprinting molecules (yellow + red). Removal of the mixed micelle creates selectively imprinted sites.

Santa Barbara Acid (SBA) materials are a class of highly-ordered, mesoporous silica materials that are well-defined and characterized (Meynen et al. 2007). These materials are highly tunable, and different pore structures can be developed depending on the synthesis conditions (Meynen et al. 2007). For instance, SBA-15 materials (used in

this current work) have a two-dimensional hexagonal pore structure, but other synthesis conditions can result in three-dimensional hexagonal pores, cubic pores or cubic cage-structured pores (Meynen et al. 2007). SBA materials have many industrial applications, including use as structural scaffolding for immobilizing proteins on sensors (Sang and Coppens 2011), thin-film composite materials for ceramic coatings (Aksay et al. 1996), and optics and electronics (Lee et al. 1997). The imprinting technique described previously was coupled with SBA-15 materials, since the synthesis techniques for these silica materials are well-established.

Stöber particles are another well-established class of silica materials synthesized using the method of Stöber et al. (1968). The Stöber method has been widely investigated, and excellent control is exhibited in the production of both silica and non-silica particles (Wu et al. 2013). These particles are monodisperse, spherical particles with a diameter on the order of ~500 to 2000 nm. This method can be coupled with the imprinting technique described previously to produce imprinted particles of uniform size and shape. Once imprinted, the particles can be used for molecule-specific chromatographic separation and recovery. Size and shape uniformity, which is important for maintaining appropriate flow rates and pressures, is desirable for chromatographic separation applications.

8.3 MATERIALS AND METHODS

8.3.1 Chemicals and Materials

All chemicals were reagent-grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. A NH₂ spherical silica gel (SSG) was purchased from Sorbent Technologies (Atlanta, GA). Fluorescent dextrans (FITC-dextran 40) with a molecular weight of 40 kDa were purchased from TdB Consulting AB (Uppsala, Spain). Commercially-available cellulase from *T. reesei* (Celluclast 1.5L) and β -glucosidase from *A. niger* (Novozyme 188) were purchased from Sigma-Aldrich (St. Louis, MO).

Corn stover (CS) was collected directly from the field at the Woodford County Animal Research Center in Woodford County, KY in September 2010. The corn (P1253 Pioneer) had been planted using conventional tillage practices in April 2010. The stover was collected from the field by hand after grain harvest and was composed of plant

material other than grain (MOG; whole stalks, including stems and leaves). After collection, the samples were prepared for laboratory storage by drying at 45°C and grinding through a hammer mill with a 5 mm screen.

8.3.2 Pretreatment of Corn Stover

Pretreatment of the corn stover was conducted according to Duguid et al (2009), with slight modifications. Corn stover (10% w/w) was pretreated with 8 g NaOH/100 g CS for 2 hr at room temperature with manual stirring every 15 min. All samples were vacuum-filtered and washed with 3 volumes of DI water. Samples were then transferred into 500 mL Erlenmeyer flasks and prepared for enzymatic hydrolysis.

8.3.3 Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted according to the NREL LAP-009 (Brown and Torget 1996), with slight modifications. The appropriate volumes of 0.1 M Na-citrate buffer and 2.0% NaN₃ were added to each hydrolysis flask. Enough substrate was added to reach a solids loading of 5% (w/w). The pH of each flask was adjusted to 4.8 using concentrated HCl. After determining the amount of enzyme solution necessary to achieve 15 FPU/g cellulose, DI water was added to the flask to bring the working volume up to 100 mL. The enzyme solution consisted of cellulase and β-glucosidase at a 1:2 FPU to CBU ratio. Results from previous studies have shown that by adding β-glucosidase, the enzymatic activity is sufficient to avoid inhibition of the cellulase caused by cellobiose (Elander et al. 2009; Kumar and Wyman 2009c; Yang et al. 2006).

All components of the hydrolysis solution, with the exception of the enzyme solution, were added to the flasks and allowed to equilibrate in a 50°C incubator. The enzymes were added, and the hydrolysis flasks were incubated for 72 hrs. Following the hydrolysis period, samples were collected and placed in a boiling water bath for 5 min to denature the enzymes, which was immediately followed by an ice water bath. The samples were centrifuged and aliquots of the supernatant were placed in a -45°C freezer for later use. After thawing, the concentrations of glucose and xylose were determined using a YSI MBS 7100 Analyzer (YSI Incorporated, Yellow Springs, OH).

8.3.4 Material Synthesis

8.3.4.1 Microphase-Directed Molecularly Imprinted (MDMI) Silica Particles

Non-imprinted, glucose-imprinted and xylose-imprinted MDMI silica particles were produced. To synthesize the material, 0.01 M HCl was added to a flask. The surfactant cetyl triethylammonium bromide (CTAB) was added and stirred vigorously. For the imprinted materials, the appropriate sugar surfactant [octyl- β -D-glucopyranoside (C_8G_1) for glucose-imprinted and octyl- β -D-xylanofuranoside (C_8X_1) for xylose-imprinted] was also added to the mixture. The flasks containing the mixtures were placed in a 50°C water bath for 30 min and stirred continuously. The required amount of silica precursor (tetramethoxysilane, TMOS) was added to the mixture, and stirring continued for an additional 20 min. The mixtures were then exposed to a gentle vacuum to remove any remaining methanol, and poured into petri dishes. The petri dishes were placed in an ammonium bath in an oven set to 50°C for 24 hrs to encourage gelation of the materials. The petri dishes were removed from the ammonium bath and placed back in the oven for a 9 d aging period. After the aging period, the materials were ground and placed into a 250 mL flask. To extract the surfactants from the materials, 210 mL of 200 proof ethanol and 7 mL of 12.1 M HCl was added to each flask and stirred for 24 hrs. The materials were filtered and returned to the appropriate flask. This extraction process was repeated two more times. After the final filtration, the materials were dried at 50°C for 24 hrs.

Particle Characterization. Pore diameter and surface area were measured by nitrogen adsorption at 77 K (Micromeritics Tristar 3000). Before analysis, samples were degassed at 120°C for a minimum of 4 hr under flowing nitrogen gas (Bhambhan et al. 1972). Specific surface area was estimated using the Brunauer, Emmett and Teller (BET) theory (Brunauer et al. 1938). Pore diameter was estimated as the peak in the pore size distribution determined by the Barrett, Joyner and Halenda (BJH) method (Barrett et al. 1951).

8.3.4.2 SBA-15 Silica Particles

Non-imprinted SBA-15 silica material was prepared according to Sang and Coppens (2011) and obtained from Daniel Schlipf³. At 40°C, 120 g 2M HCl was mixed with 4 g block-copolymer P123 in DI H₂O for 2 hrs. Nine grams of TEOS was slowly dropped into the mixture and rapidly mixed for 10 min. The mixture was kept static at 40°C for 24 hrs, followed by hydrothermal aging at a previously selected temperature (50°C, 75°C, 100°C or 150°C) for 48 hr. After aging the materials, the solids were filtered and washed several times with DI H₂O. The materials were allowed to dry overnight at 100°C before being calcined at 540°C for 24 hrs. Characterization of the materials was immediately conducted by performing x-ray diffraction (XRD) and nitrogen adsorption analyses on the materials.

8.3.4.3 Silica Stöber Particles

Non-imprinted and glucose-imprinted Stöber particles were prepared according to Stober et al. (1968) and obtained from Suvid Joshi⁴. Briefly, 58.22 g ethanol, 9.8 mL concentrated ammonia and 10.8 g DIUF water were mixed. After stirring the solution for 15 min, 5.26 g tetraethoxysilane (TEOS) was added and mixed vigorously. Twenty milligrams of imprinting surfactants (CTAB or a 1:1 mass ratio of CTAB:C₈G₁) were added exactly 1 min after the TEOS. Particle precipitation was already apparent as per the increased turbidity of the mixture. The mixture was stirred for 24 hr at room temperature to allow solidification of the silica. The particles were recovered by centrifugation and aged in an oven at 50°C for 24 hr to promote additional solidification and interactions with the surfactants. Aged particles were washed three times in ethanol with sonication and centrifugation to remove the surfactant, followed by three washes in DIUF water with sonication and centrifugation until the pH of the supernatant was neutral, as indicated with pH paper. Finally, the particles were dried at 50°C for 24 hr.

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8.3.5 Liquid Chromatography

8.3.5.1 Cartridge Preparation

Empty 3 mL cartridges (Machery-Nagel, Bethlehem, PA) fitted with polyethylene frits were packed with 250 mg of the synthesized silica materials or 500 mg of a commercially-available amine-functionalized, spherical silica gel material and conditioned with 3 volumes of the appropriate mobile phase. Following conditioning, the cartridges were wrapped with parafilm and stored in a zip-top bag for at least 24 hrs prior to use to thoroughly wet the material. One additional volume of the mobile phase was applied to the cartridge immediately prior to use to ensure all void spaces were saturated with buffer.

8.3.5.2 Chromatographic Separation of Selected Monosaccharides

A prepared sugar solution, pH 5 buffer containing 125 g/L each of both glucose and xylose (G + X), or hydrolyzate from pretreated corn stover (PCS) was applied to the cartridge. The volume of the sample was chosen such that the mass of the sugar applied to the cartridge was 5-10% of the mass of the adsorbent material. Next, the mobile phase, and if necessary, pressure was applied to the cartridge. Fractions of the eluent were collected using a Bio-Rad Model 2110 Fraction Collector (Bio-Rad, Hercules, CA). Each fraction sample was analyzed on a YSI MBS 7100, and chromatograms for each monosaccharide were created.

8.3.6 Experimental Design

8.3.6.1 Separation and Recovery of Monosaccharides Using Liquid Chromatography

Effects of particle imprinting on separation and recovery of selected monosaccharides. Liquid chromatography was used to evaluate the effectiveness of non-imprinted, glucose-imprinted and xylose-imprinted MDMI silica materials in separation and recovery of selected monosaccharides (glucose and xylose). These materials were compared to a commercially-available silica gel that had no specificity for either monosaccharide. A prepared sugar solution (pH 5 buffer containing 125 g/L each glucose and xylose) and hydrolyzate (from enzymatic hydrolysis of pretreated corn

stover) were used for the analysis. The mobile phase was an 80:20 acetonitrile-water mixture.

Effects of the mobile phase on separation and recovery of selected monosaccharides. To evaluate different mobile phase options, cartridges were packed with 250 mg of the non-imprinted MDMI silica material and conditioned with 3 volumes of pH 5 buffer. Non-imprinted MDMI material was selected since any differences in chromatogram profiles would be due to the effects of the mobile phase on the glucose. Following conditioning, the cartridges were wrapped with parafilm and stored in a zip-top bag for at least 24 hrs before use. One additional volume of pH 5 buffer was applied to the cartridge immediately prior to use to ensure all void spaces were saturated with buffer. A glucose sugar solution (100 g/L) was applied to the cartridge such that the mass of the sugar applied to the cartridge was 5% of the mass of the adsorbent material. Next, one of three mobile phases (pH 5 buffer; pH 5 buffer at room temperature and 50°C; and acetonitrile-water (90:10)) was applied to the cartridge, and fractions of the eluent were collected. pH 5 buffer was selected to mimic pH conditions of enzymatic hydrolyzate. The increased temperature was selected to promote recovery of any glucose that may have been adsorbed to the material. Acetonitrile-water mixtures are common chromatographic mobile phases. The fraction samples were analyzed for glucose content using a YSI 7100 MBS, and a chromatogram for the glucose was created. The acetonitrile-water (90:10) mobile phase was also tested with the G + X sugar solution containing 100 g/L each of glucose and xylose in pH 5 buffer.

Effects of pore size on separation and recovery of selected monosaccharides. SBA-15 materials were aged at different temperatures to produce silica materials with various average pore sizes. Three pore sizes were examined with regards to ability to separate selected monosaccharides from solution. Liquid chromatography was performed as described above. A glucose sugar solution (100 g/L) was applied to the cartridge such that the mass of the sugar applied to the cartridge was 5% of the mass of the adsorbent material. Collected fractions were analyzed, and chromatograms for glucose elution were produced. Three different mobile phases were tested: pH 5 buffer, pH 5 buffer at room temperature followed by pH 5 buffer at 50°C, and 90:10 acetonitrile: water. The

acetonitrile-water (90:10) mobile phase was also tested with the G + X sugar solution containing 100 g/L each of glucose and xylose in pH 5 buffer.

Characterization of column features using fluorescently-tagged dextrans. The dead volume and the glucose adsorption ability of the SBA-15 packed cartridges were quantified. A glucose (100 g/L) and fluorescent dextran (FD40; 2200 µg/mL) solution was applied to the cartridge such that the mass of the sugar applied to the cartridge was 5% of the mass of the adsorbent material. Next, the mobile phase (pH 5 buffer) was applied to the cartridge, and fractions of the eluent were collected. The fraction samples were analyzed for glucose content using a YSI 7100 MBS and the FD40 content using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., Santa Clara, CA). A chromatogram for the each of the compounds was created.

8.3.6.2 Bulk Adsorption of Glucose from Real Hydrolyzate

Glucose-imprinted and non-imprinted Stöber particles were evaluated for their ability to selectively separate glucose from other sugars present in hydrolyzate produced from enzymatic hydrolysis of pretreated corn stover. Stöber particles were produced according to Stober et al. (1968). Glucose-imprinting was achieved by using a 1:1 ratio of CTAB to C8G1 surfactant templating molecules.

Hydrolyzate was prepared as previously outlined, with slight modifications. The corn stover was pretreated at 10% (w/v) solids using 10 g NaOH/100 g corn stover for 24 hr at 25°C. Enzymatic hydrolysis was performed at 5% (w/w) solids with an [E] = 30 FPU/g solids (and 60 CBU/g solids). The hydrolyzate was collected and stored at -45°C until needed. Upon thawing, the hydrolyzate was diluted to 5 different concentrations prior to addition to the Stöber particles.

Bulk adsorption samples were prepared by mixing 50 mg particles with 1 mL of DI H₂O in 4.5 mL Wheaton vials for 24 hr. The samples were then transferred into centrifuge tubes and centrifuged at 13,300 rpm for 1 min. The water was decanted before the particles were re-suspended into 1 mL of the prepared hydrolyzate and transferred back to the Wheaton vials. The samples were stirred for an additional 24 hr. The samples were centrifuged again at 13,300 rpm for 8 min. The supernatant was collected, syringe filtered (0.2 µm) and analyzed by HPLC. The sugars derived from cellulose and

hemicellulose (glucose, xylose, arabinose, mannose and galactose) were measured using a Dionex U3000 HPLC system equipped with a Bio-Rad Aminex HPX-87P column and a deashing Micro-Guard column and operated at 78°C with deionized water as the mobile phase at a flow rate of 0.6 mL/min. The sample components were detected with a Shodex-101 refractive index detector.

Statistical Analysis. The data were analyzed with t-tests to determine whether any significant differences existed among the means of adsorbed monosaccharides.

8.4 RESULTS

8.4.1 Separation and Recovery of Monosaccharides Using Liquid Chromatography

8.4.1.1 Effects of Imprinted Particles

All materials were characterized prior to use. Pore size and surface area was determined for all synthesized materials using nitrogen adsorption (Table 8.1). XRD was performed to ensure the materials had the expected structure (data not shown).

Table 8.1. Characteristics of the MDMI silica materials.

Material	Pore Size (nm)	Surface Area (m ² /g)
Commercial	7.0 [†]	500 [†]
Non-imprinted	6.8	922
Glucose-imprinted	3.8	995
Xylose-imprinted	8.2	916

[†] Provided by Sorbtech (http://www.sorbtech.com/catalog.aspx?family_id=181#topofpage)

Table 8.2 contains the concentrations of glucose and xylose found in both the prepared sugar solution and the hydrolyzate from PCS used to evaluate the ability of each of the four adsorbent materials to separate these two monosaccharides. While the concentrations for the prepared sugar solution were high, the sample volume was selected such that the total mass of the sugars applied to the cartridges was only 5-10% of the adsorbent mass.

Table 8.2. Concentrations of monosaccharides in a model and a real hydrolyzate.

Saccharide	Sugar Solution	Hydrolyzate		
	Concentration (g/L)	Actual Yield (g/L)	Theoretical Yield ^a (g/L)	Percent Yield ^b
Glucose	125	7.3	16.9	43.2%
Xylose	125	3.1	8.0	38.8%

^aTheoretical yield of glucose and xylose was determined according NREL LAP-019 “Determination of Structural Carbohydrates and Lignin in Biomass” (Sluiter et al. 2005).

^bPercent yield = Actual yield/Theoretical yield

Chromatograms (Figure 8.4) and recovery profiles (Figure 8.5) from the application of the glucose + xylose sugar solution were produced for each of the four adsorbent materials. For the commercial material, some separation of the two peaks is apparent, although it is not complete separation. No apparent separation was observed for any of the MDMI silica materials. For each of the four materials tested, at least 80% and 90% of the glucose and xylose, respectively, loaded onto the materials was recovered in the fractions.

Chromatograms (Figure 8.6) and recovery profiles (Figure 8.7) from the application of the hydrolyzate from PCS were produced for each of the four adsorbent materials. No separation was apparent for any of the materials, including the commercial material. Recovery for glucose and xylose loaded onto the material was closer to 100%.

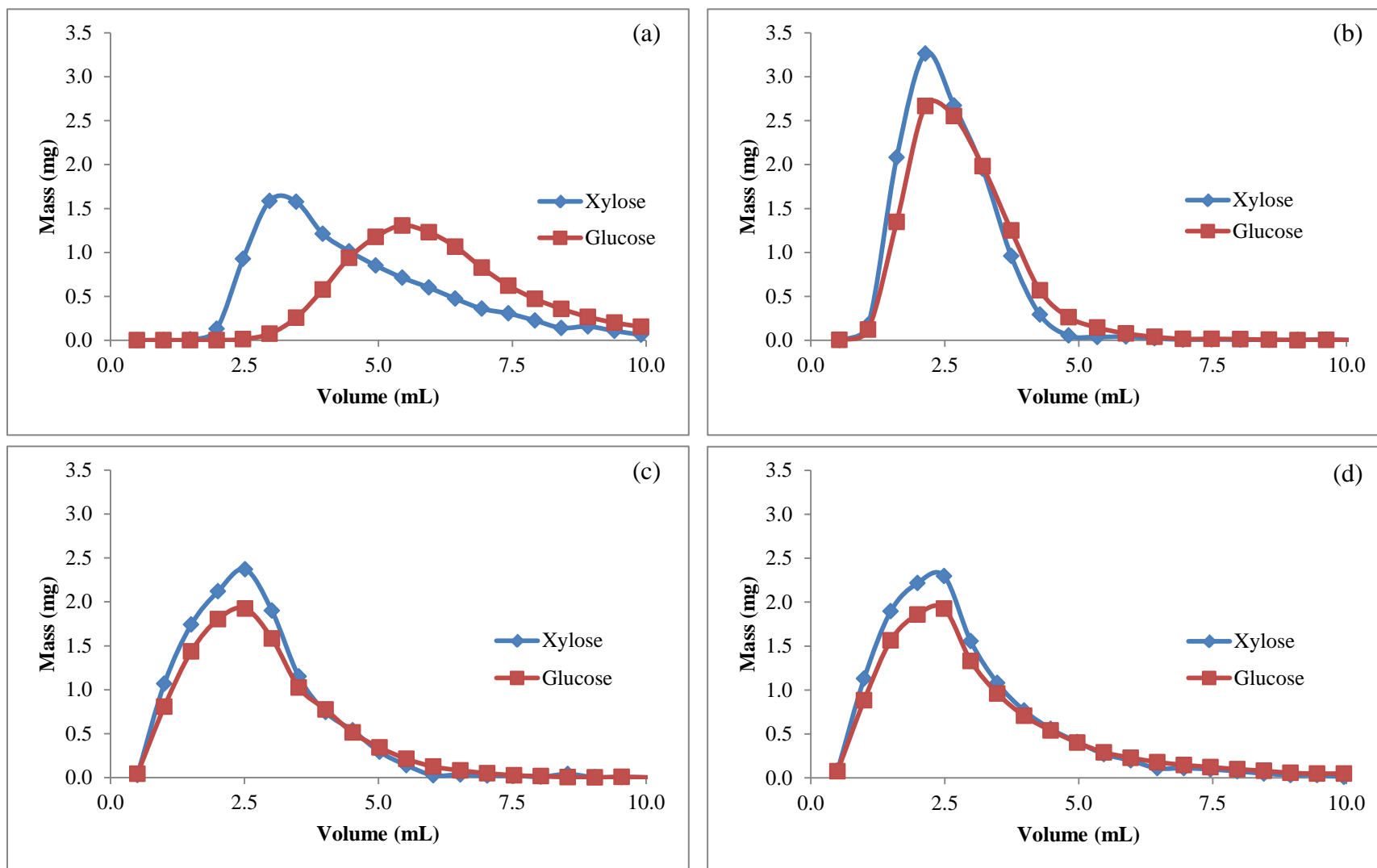


Figure 8.4. Chromatograms produced from the application of glucose + xylose sugar solution to cartridges containing four different adsorbent materials. NH₂ Spherical Silica gel (a); Non-imprinted MDMI Silica (b); Glucose-imprinted MDMI Silica (c); Xylose-imprinted MDMI Silica (d).

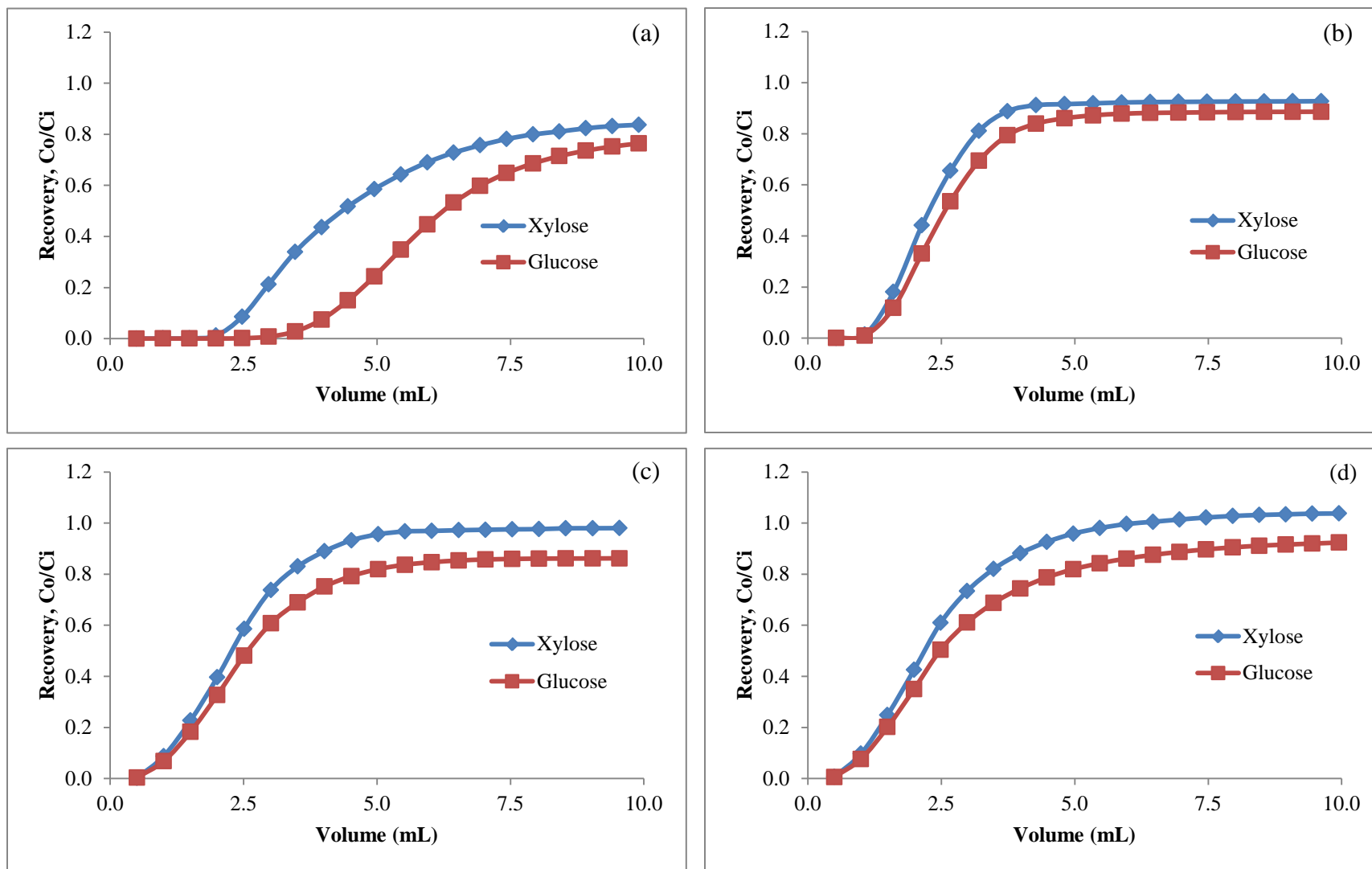


Figure 8.5. Recovery profiles produced from the application of glucose + xylose sugar solution to cartridges containing four different adsorbent materials. NH₂ Spherical Silica gel (a); Non-imprinted MDMI Silica (b); Glucose-imprinted MDMI Silica (c); Xylose-imprinted MDMI Silica (d).

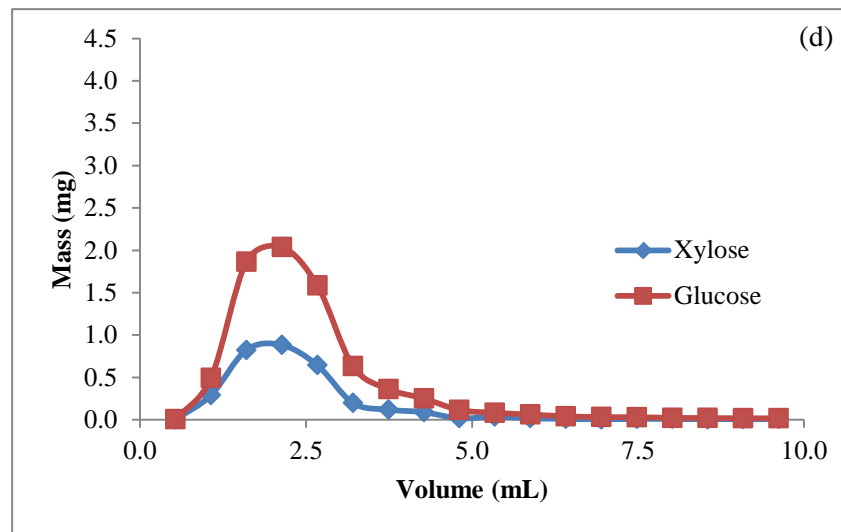
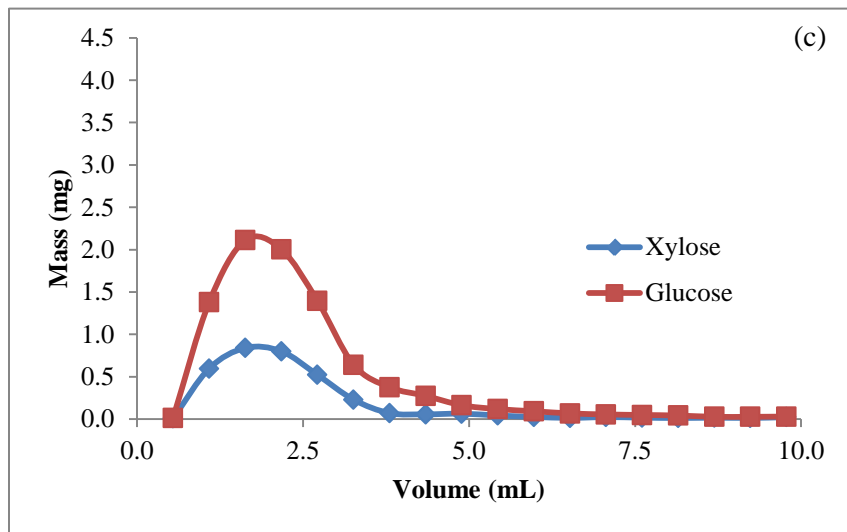
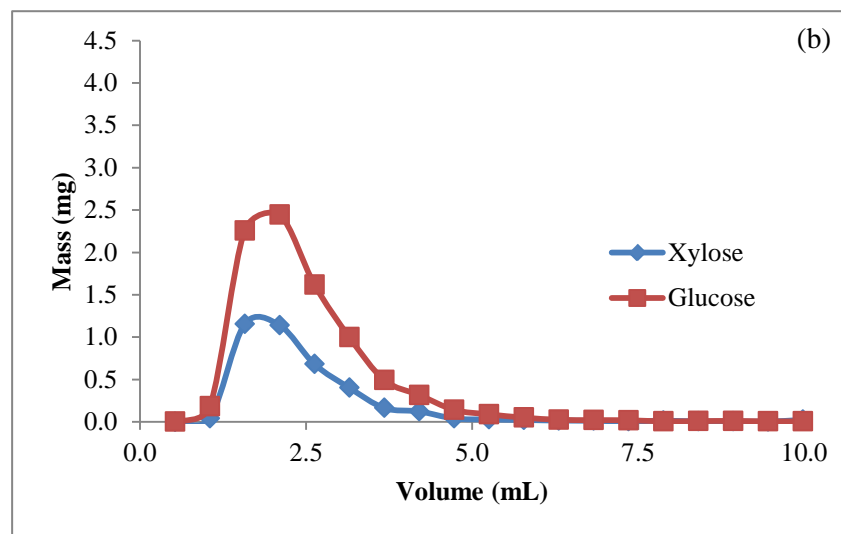
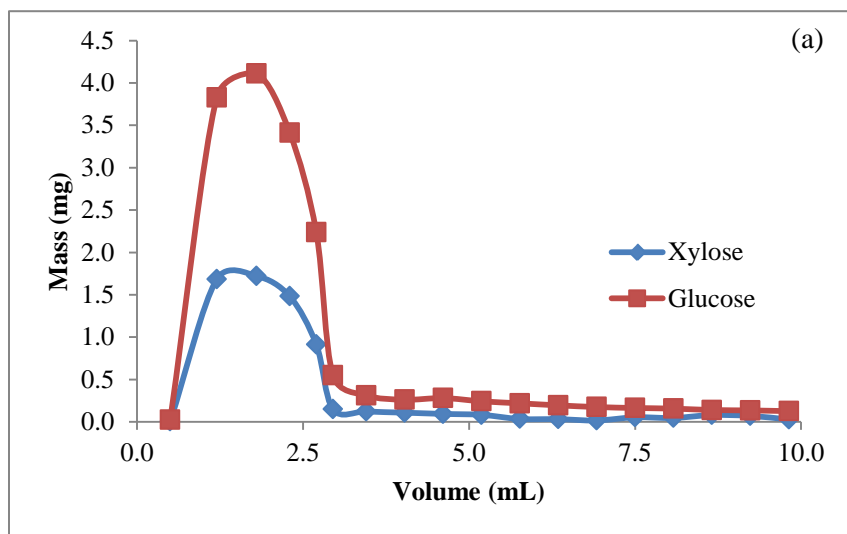


Figure 8.6. Chromatograms produced from the application of hydrolyzate from PCS to cartridges containing four different adsorbent materials. NH₂ Spherical Silica gel (a); Non-imprinted MDMI Silica (b); Glucose-imprinted MDMI Silica (c); Xylose-imprinted MDMI Silica (d).

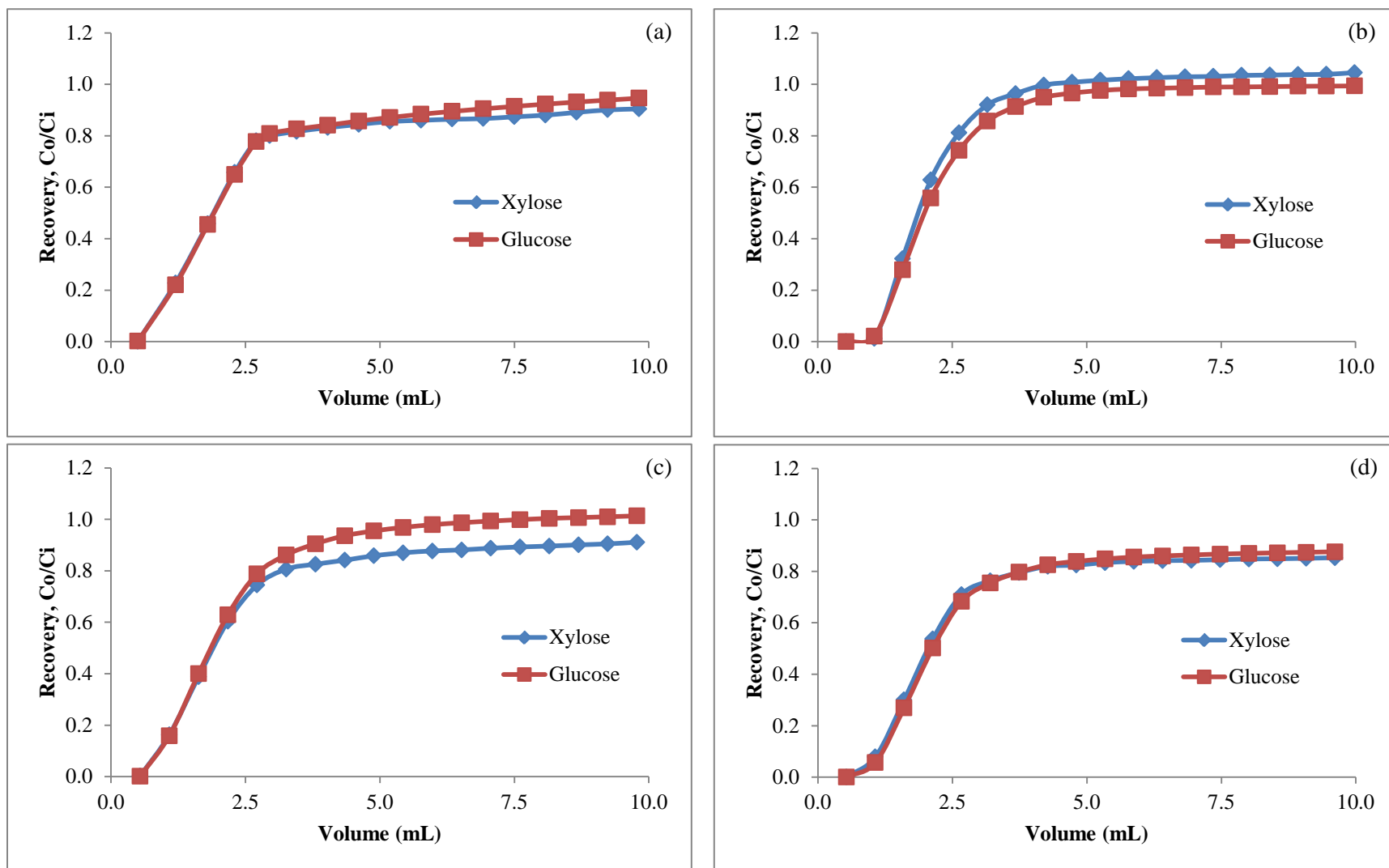


Figure 8.7. Recovery profiles produced from the application of hydrolyzate from PCS to cartridges containing four different adsorbent materials. NH₂ Spherical Silica gel (a); Non-imprinted MDMI Silica (b); Glucose-imprinted MDMI Silica (c); Xylose-imprinted MDMI Silica (d).

8.4.1.2 *Effects of Mobile Phases*

Three different mobile phases were evaluated to attempt to enhance separation: pH 5 buffer at room temperature, pH 5 buffer at room temperature followed by pH 5 buffer at 50°C, and acetonitrile-water (90:10). The glucose chromatograms and recovery profiles for the different mobile phases are shown in Figure 8.8. The glucose peaks for the two pH 5 buffers are essentially identical. The increased temperature of the pH 5 buffer did not promote any additional recovery of glucose, as seen by the lack of an apparent peak. The acetonitrile-water mobile phase did appear to affect the elution profile of the glucose. The maximum elution occurs at the same point as the pH 5 buffer mobile phases, but the peak height is about a quarter of the height of the pH 5 buffer peaks. The acetonitrile-water peak also has a long lagging tail, indicating a longer interaction with the adsorbent material.

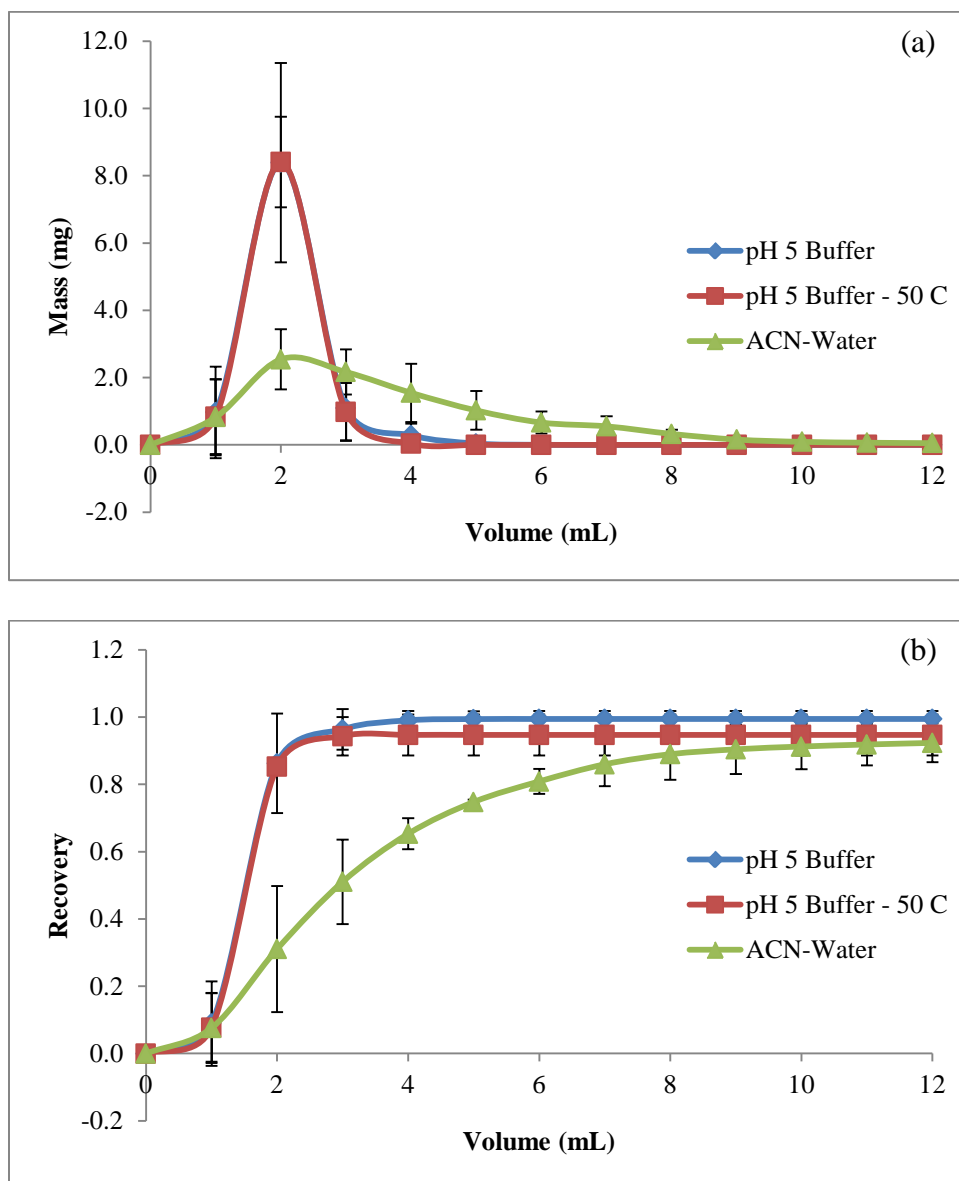


Figure 8.8. Chromatograms (a) and recovery profiles (b) produced from eluting a prepared glucose sugar solution (100 g/L) with different mobile phases. The adsorbent material was non-imprinted MDMI silica. The error bars indicate the standard deviation of two replicates.

8.4.1.3 Effects of Pore Size

It was hypothesized that the smaller pore size of the MDMI materials might hinder the monosaccharides from interacting with the imprinted spots and therefore reduces the ability of the materials to adsorb and separate out the desired

monosaccharides. SBA-15 materials were chosen because the pore sizes are highly tunable depending on the temperature used in an aging step during synthesis. Increasing the aging temperature from 50°C to 150°C increased the pore size from 6.5 nm to 10.4 nm, as seen in Table 8.3.

Table 8.3. Characteristics of the SBA-15-xx^a silica materials.		
Material	Pore Size (nm)	Surface Area (m ² /g)
Non-imprinted MDMI Silica	2.9	902.1
SBA-15-50	6.5	752.5
SBA-15-75	8.3	700.0
SBA-15-100	9.6	630.1

^aThe xx values indicate the temperature at which the SBA-15 materials were aged.

Figure 8.9, Figure 8.10 and Figure 8.11 show the glucose chromatograms and recovery profiles for SBA-15 materials aged at 50°C, 75C and 100°C, respectively. The profiles for the different mobile phases follow similar trends to the previous experiment using MDMI materials. The pH 5 buffers produced similar profiles, and the peak resulting from the acetonitrile-water mobile phase is much shorter and broader.

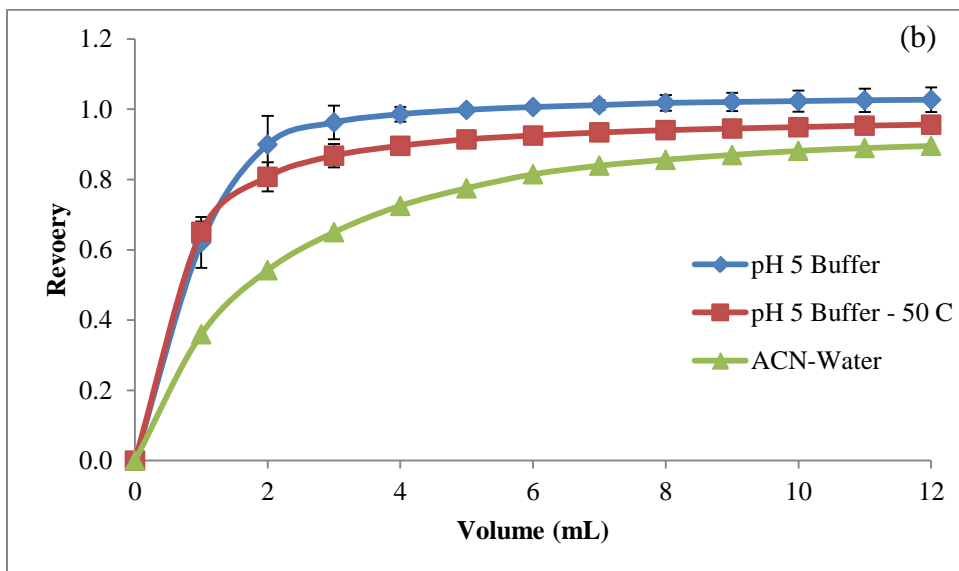
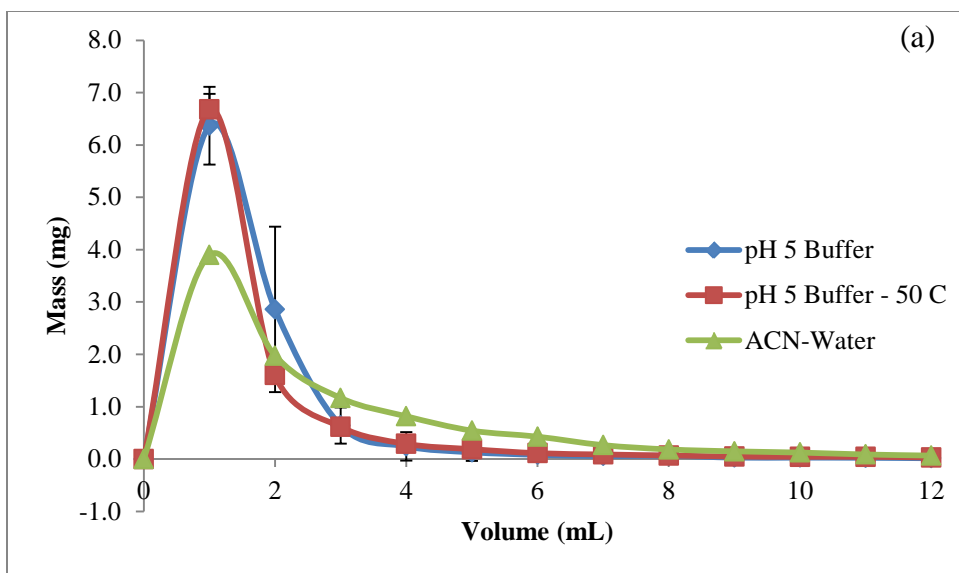


Figure 8.9. Glucose chromatograms (a) and recovery profiles (b) produced eluting a prepared glucose solution (100 g/L) using different mobile phases. The adsorbent material was SBA-15 materials aged at 50°C. The error bars indicate the standard deviation of two replicates. No error bars could be determined for the acetonitrile-water mobile phase, since only one run was conducted.

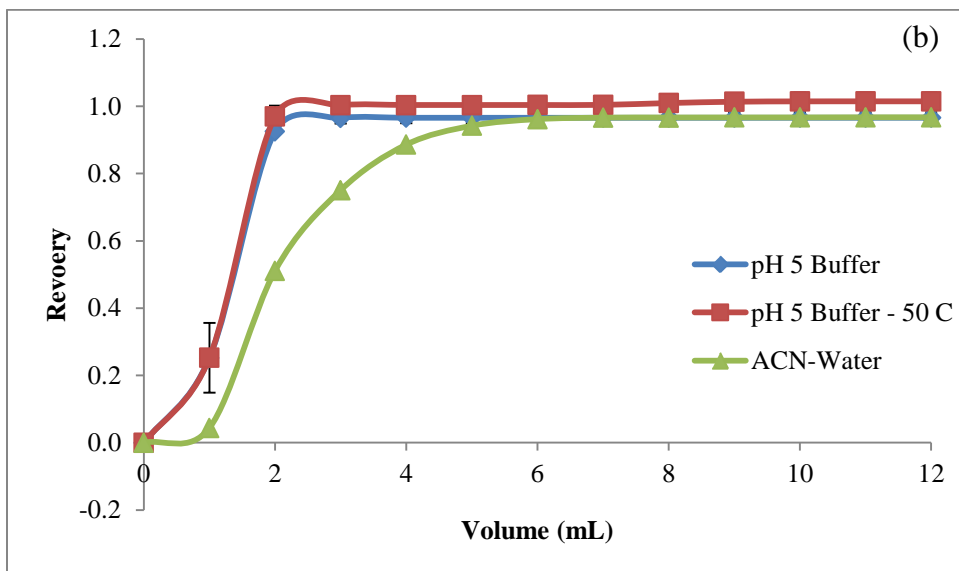
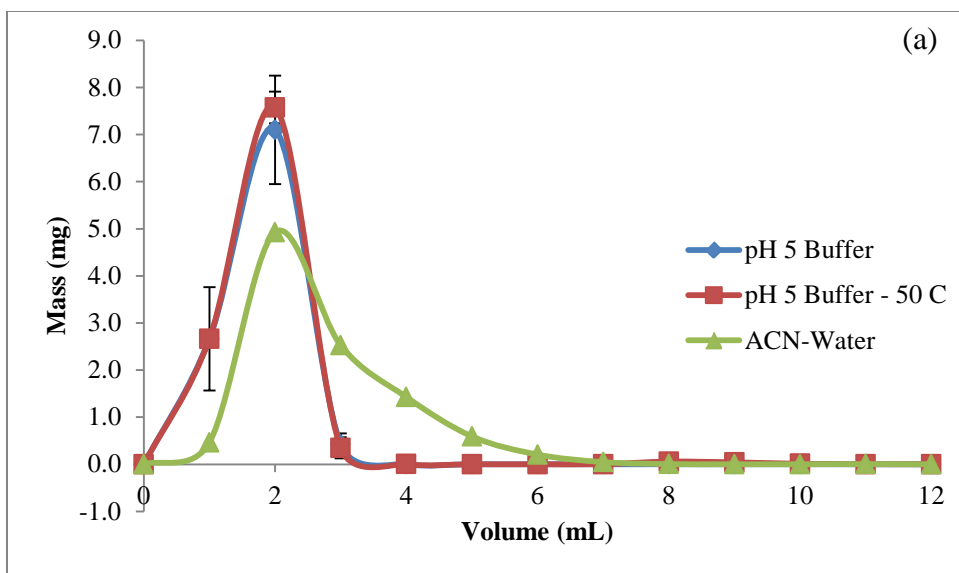


Figure 8.10. Glucose chromatograms (a) and recovery profiles (b) produced eluting a prepared glucose solution (100 g/L) using different mobile phases. The adsorbent material was SBA-15 materials aged at 75°C. The error bars indicate the standard deviation of two replicates. No error bars could be determined for the acetonitrile-water mobile phase, since only one run was conducted.

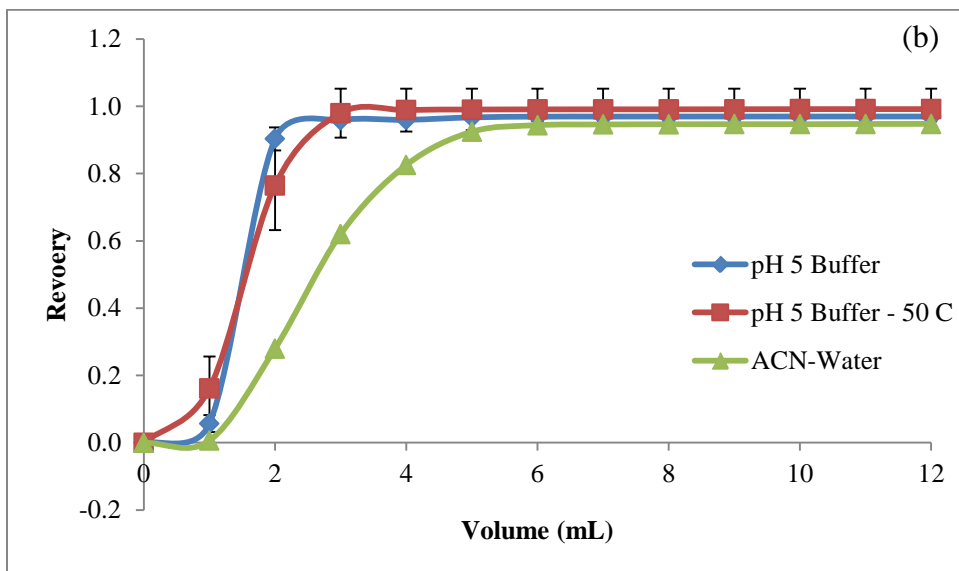
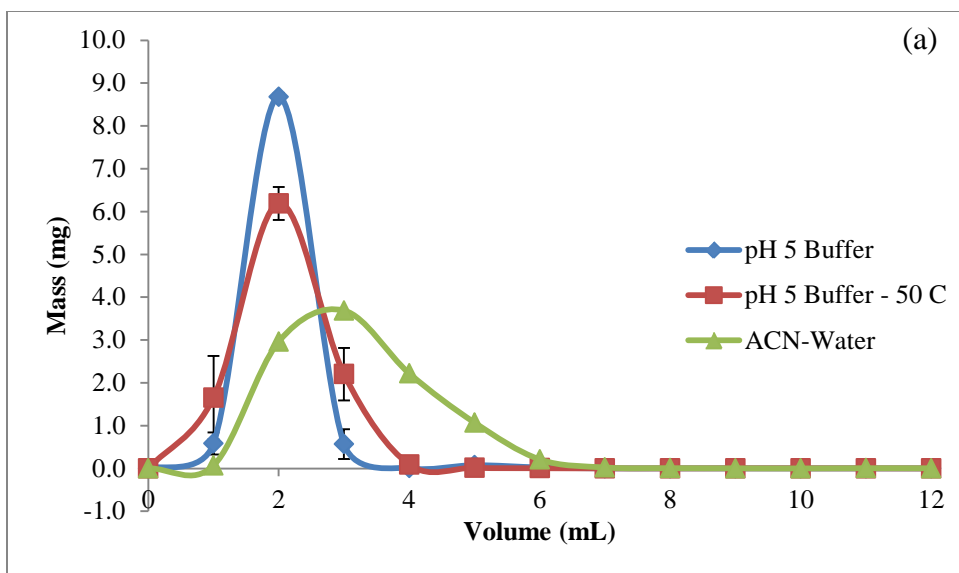


Figure 8.11. Glucose chromatograms (a) and recovery profiles (b) produced eluting a prepared glucose solution (100 g/L) using different mobile phases. The adsorbent material was SBA-15 materials aged at 100°C. The error bars indicate the standard deviation of two replicates. No error bars could be determined for the acetonitrile-water mobile phase, since only one run was conducted.

Additionally, the acetonitrile-water mobile phase was examined using a prepared glucose and xylose solution (100 g/L each) to determine if the larger pore sizes affected the interaction of the two sugars with the adsorbent material. Figure 8.12 and Figure 8.13 show the chromatograms and recovery profiles for SBA-15 materials aged at 50°C and 100°C, respectively. Although there was no apparent separation between the glucose and

xylose with either material, the peaks were broader than when the prepared sugar solution containing only glucose was applied to the columns.

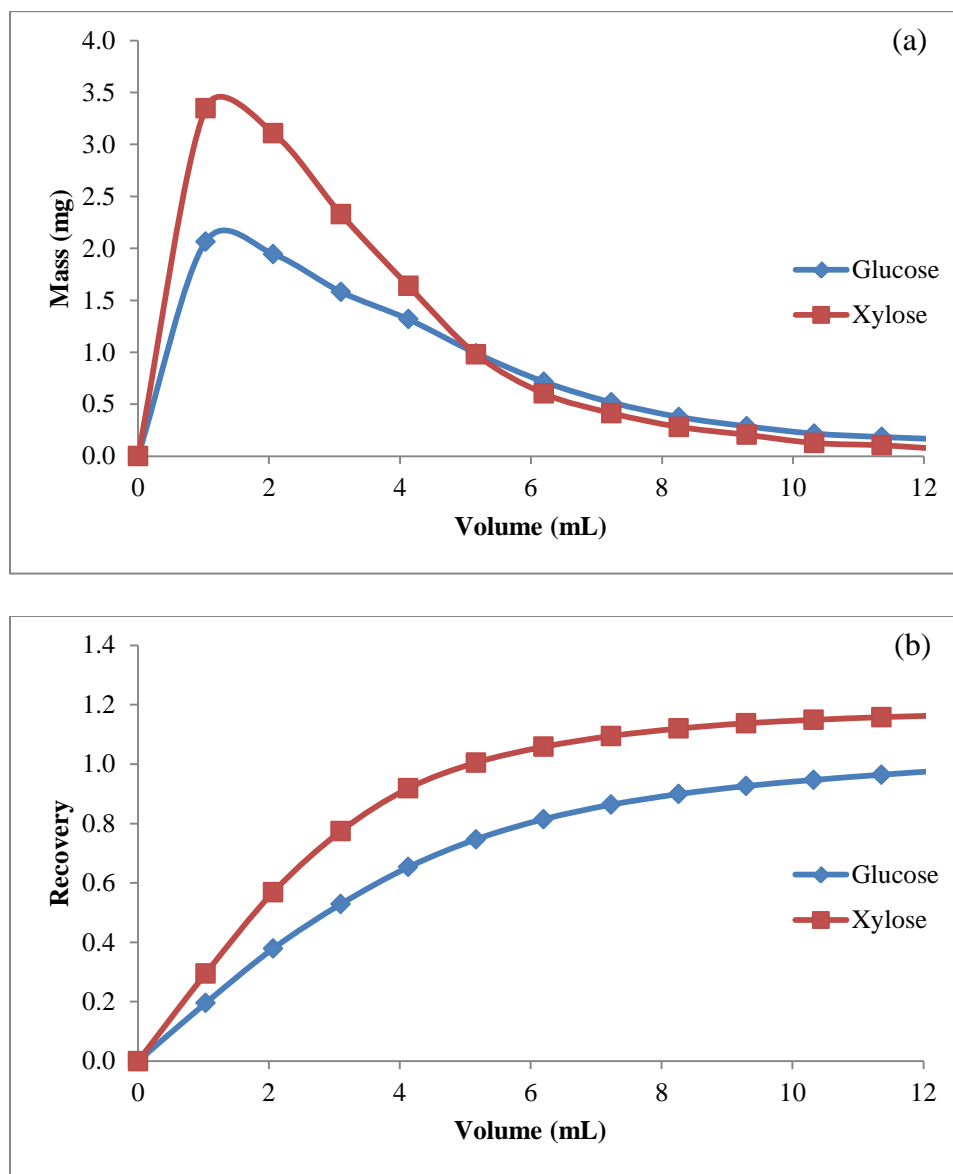


Figure 8.12. Chromatograms (a) and recovery profiles (b) produced eluting a prepared glucose and xylose solution (100 g/L each) using 90:10 acetonitrile: water as the mobile phase. The adsorbent material was SBA-15 materials aged at 50°C.

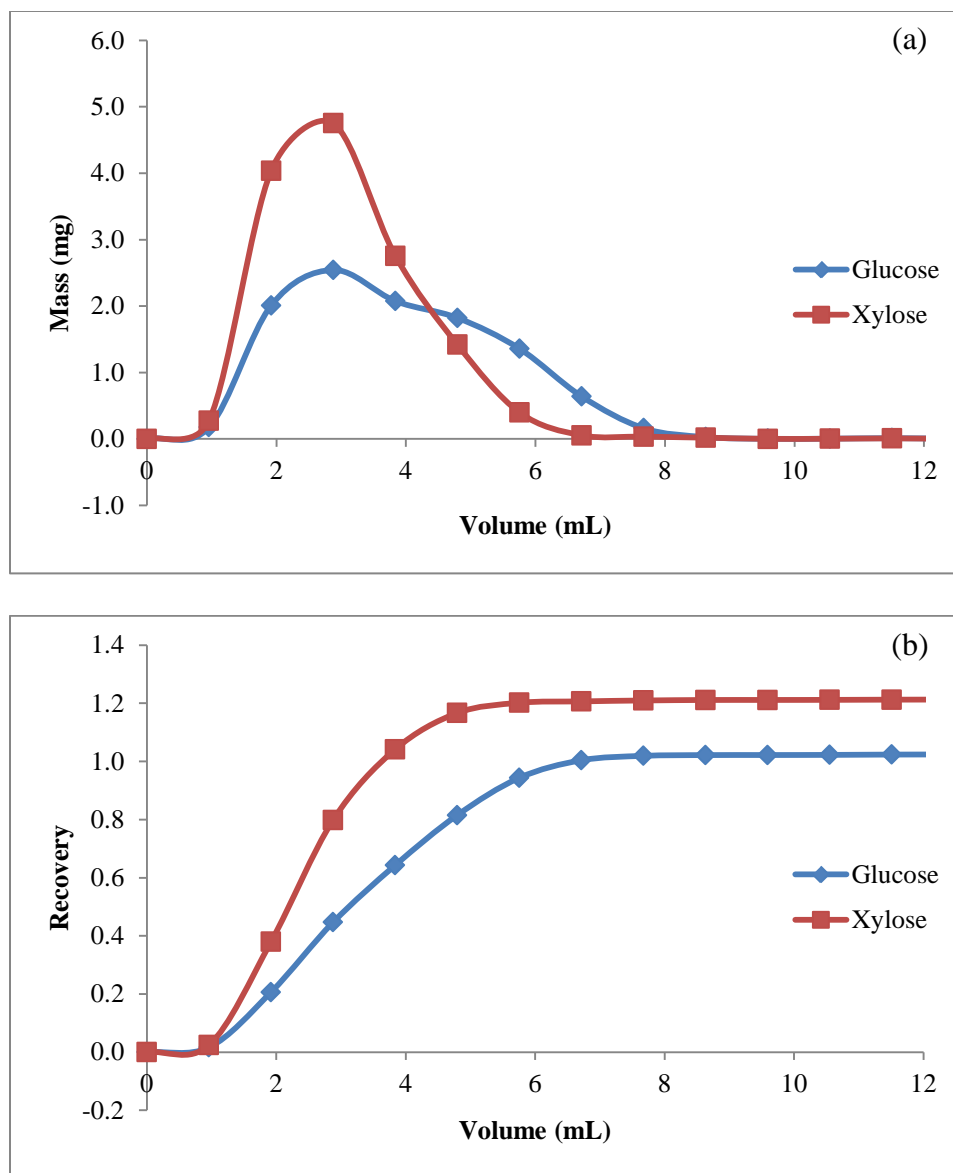


Figure 8.13. Chromatograms (a) and recovery profiles (b) produced eluting a prepared glucose and xylose solution (100 g/L each) using 90:10 acetonitrile: water as the mobile phase. The adsorbent material was SBA-15 materials aged at 100°C.

8.4.1.4 Characterization of Column Features

Large fluorescently-tagged dextrans (FD40) were used to examine whether any interaction was occurring between the monosaccharides and the adsorbent materials. The dextrans had a molecular weight of about 40 kDa much larger than that of glucose (MW = 180 g/mol). It was hypothesized that at least some separation of the elution peaks would be apparent due to differences in sizes. The smaller glucose molecules would

elute later than the larger fluorescent dextrans if the smaller molecules were in fact interacting with the pores of the adsorbent material. However, upon inspection of the chromatogram in Figure 8.14, no separation between the two peaks is apparent, indicating that the glucose is likely not interacting with the adsorbent materials.

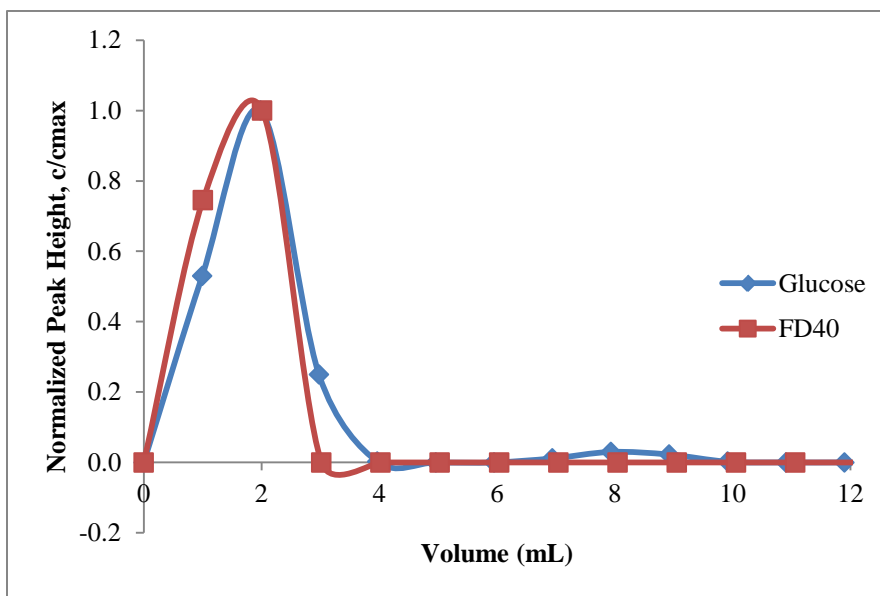


Figure 8.14. Chromatogram of glucose (MW = 180) and a fluorescently-tagged dextran (MW ~ 40,000). The peaks have been normalized based on the maximum concentration of each respective component for ease of comparison. The peaks show no apparent separation, indicating that the glucose molecules do not interact with the adsorbent material any differently than the larger molecules. Non-imprinted SBA-15 materials aged at 50°C were used as the stationary phase.

8.4.2 Bulk Adsorption of Glucose from Real Hydrolyzate

Non-imprinted and glucose-imprinted particles synthesized by the Stöber method were evaluated for their ability to selectively adsorb glucose and/or xylose from hydrolyzate. The non-imprinted particles did not selectively adsorb either monosaccharide in significant quantities (6.8 mg glucose and 6.5 mg xylose per g particles). However, the glucose-imprinted material selectively adsorbed four times more glucose than xylose (34.4 mg glucose and 8.7 mg xylose per g particles).

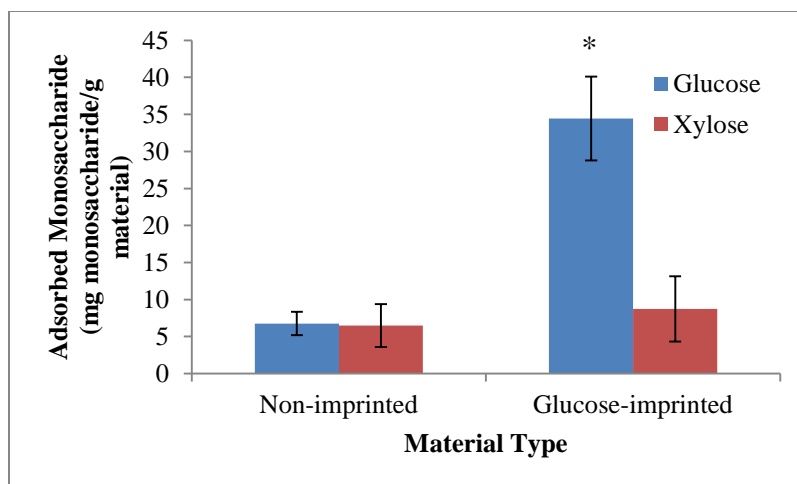


Figure 8.15. Glucose and xylose adsorption by non-imprinted and glucose-imprinted Stöber particles. Bars marked with an asterisk (*) are significantly different from the other samples.

8.5 DISCUSSION

8.5.1 Separation and Recovery of Monosaccharides Using Liquid Chromatography

8.5.1.1 Effects of Imprinted Particles

From this study of the MDMI silica materials, it is hypothesized that the similarity between the glucose and xylose chromatograms is the result of the two monosaccharides interacting with the adsorbent materials in the same way (Figure 8.16) or not interacting with the material at all (Figure 8.17). For instance, it may be possible that the monosaccharides bind indiscriminately to the silica material, regardless of the type of saccharide or the specificity and availability of imprinted sites. It may also be possible that the monosaccharides are indiscriminately binding to imprinted sites, regardless of the site specificity. Monosaccharides are difficult to separate because the structures are so similar, possibly only differing by the position of a single hydroxyl (–OH) group. Although glucose and xylose differ in the number of carbons, the structures may still be too similar for the imprinted sites to selectively choose the correct sugar. Another alternative is that the monosaccharides are not interacting with the adsorbent material at all, especially if the pores are not adequately sized and/or the saccharides are hydrated. A glucose molecule is about 1 nm in diameter. The pores of the glucose-imprinted MDMI materials are only 3.8 nm on average. Even though the pores are large enough for the glucose molecules to enter, the small pore size may impose some diffusion limitations,

which would impact the effective binding the monomers to the imprinted sites. Additionally, glucose-water interactions are strong (or at least comparable in strength to the interactions of the glucose with the functionalized sites in the imprinted silica). Water molecules may be interfering with the –OH groups that need to interact with the functionalized groups in the imprinted sites, thus reducing the ability of the sugar to interact with the adsorbent material. Evaluating the elution profiles with the fluorescently-tagged dextrans later indicated that the monosaccharides were, in fact, not interacting with the materials.

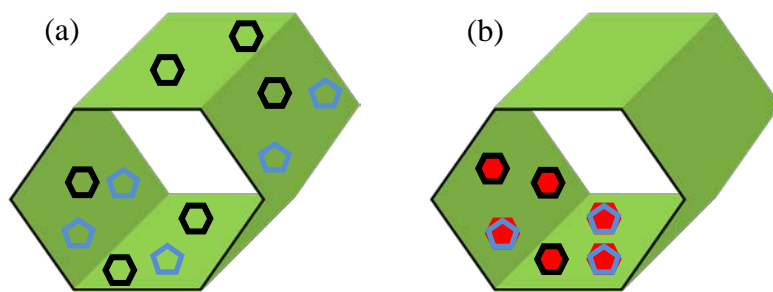


Figure 8.16. C5 and C6 sugars may be (a) binding indiscriminately to the silica material or (b) binding indiscriminately to the glucose-imprinted sites in the silica material. Black hexagons = C6 sugars; blue pentagons = C5 sugars; red hexagons = glucose-imprinted sites.

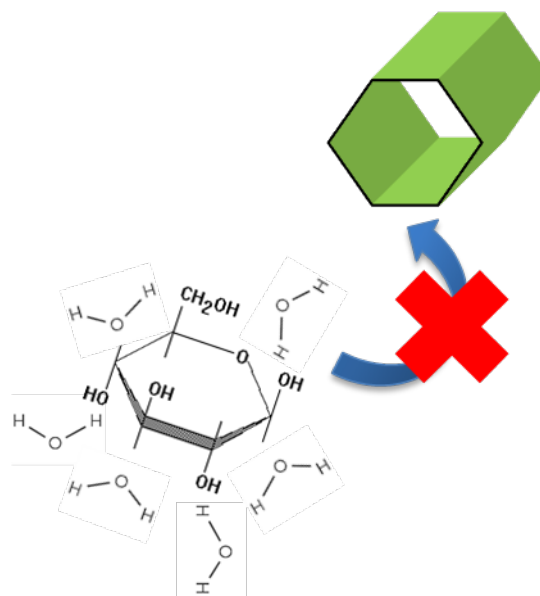


Figure 8.17. C5 and C6 may not interact with the MDMI materials at all. It is possible the pores are not adequately sized, especially if the sugars are hydrated. The types of saccharide species present in solution may also lead to no interaction between the sugars and the MDMI materials.

Although some separation of the glucose and xylose peaks was observed with the prepared sugar solution and the commercial material, this separation was not apparent with the real hydrolyzate. It is possible that the prepared sugar solution was “clean” as compared to the hydrolyzate sample, and some of the other constituents in the hydrolyzate interfered with the glucose and xylose interacting with the adsorbent material. Hydrolyzate may contain a variety of components, including enzymes, soluble saccharide species (mono-, di-, tri-, and oligosaccharides) and phenolic compounds from lignin degradation. It is likely that the lack of separation is due to the hydrolyzate fouling the commercial material (Figure 8.18).

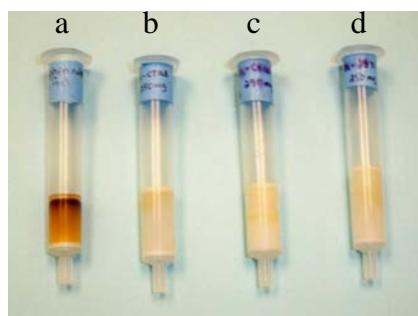


Figure 8.18. Cartridges following the application of the hydrolyzate from PCS and collection of the fractions. The cartridges contain (a) commercial, (b) non-imprinted, (c) glucose-imprinted and (d) xylose-imprinted materials.

8.5.1.2 Effects of Mobile Phases

The pH 5 buffer at room temperature was chosen as a mobile phase in this study because it mimics conditions that are common for enzymatically produced hydrolyzate. This case represents the minimum requirements necessary for performing a chromatographic separation of the hydrolyzate sugars (i.e. no additional chemical or energy requirements are necessary to conduct the separation). The pH 5 buffer at room temperature followed by pH 5 buffer at 50°C was selected because it has been shown that increased mobile phase temperatures can promote recovery of any bound saccharides (Kuhn and Maugeri 2010). Acetonitrile-water (90:10) has been shown to separate carbohydrates on other chromatography columns (Bio-Rad). A prepared glucose solution was chosen for this work to simplify the system; it was assumed that the mobile phase would cause a shift in the glucose peak if it had any effect on the saccharide elution.

The acetonitrile-water mobile phase resulted in the biggest change of the glucose chromatography profiles. This mobile phase (70:30 acetonitrile-water) has been used with an Aminex disaccharide column to separate a variety of saccharides from one another in ice cream samples (Bio-Rad), but the type of interaction between the saccharides and the stationary phase with that combination is different from the one in this current work. For example, Aminex carbohydrate columns use a combination of size

exclusion and ligand exchange mechanisms to separate the various saccharide species. In this current work, the monosaccharides interact with hydroxyl groups within the imprinted sites. However, the broad peak and long lagging tail with the MDMI silica material and the acetonitrile-water mobile phase is not desirable for chromatographic separations. If these materials prove to be a viable option for chromatographic separation, further development of the chromatographic separation procedure used in this current study will likely be required to select the best mobile phase for the separation of carbohydrates. Cano et al. (2006) examined a ternary mobile phase mixture of acetonitrile, water and ethyl acetate in various proportions. A 50:10:40 (v:v:v) mixture of acetonitrile-water-ethyl acetate increased the difference in elution times of glucose and xylose from honey. The glucose and xylose eluted at approximately 4 and 9 min, respectively. Elution times for glucose and xylose from honey were approximately 3 and 5 min using an acetonitrile-water (80:20) mobile phase following the official procedure developed by the Harmonized Methods of European Honey Commission.

8.5.1.3 Effects of Pore Size

It was hypothesized that the small pore size of the non-imprinted MDMI silica material (2.9 nm) was hindering the glucose molecules from entering the pores and interacting the imprinted sites. A glucose molecule is about 1 nm in diameter. Even though the pore size of this material was almost three times larger than a glucose molecule, any interaction between glucose and water may increase the apparent size of the glucose molecule enough so that it is unable to enter the pores. Increasing the pore size (by using the SBA-15 materials) could improve the probability that the glucose molecules are able to reach the imprinted sites, which is evidenced by the shift in the glucose peaks when using the acetonitrile-water mobile phase. As the pore size increased, the peak shifts further to the right, indicating that the glucose is interacting with the stationary phases for longer periods of time. For example, when the pore size is 6.5 nm, the glucose peak reaches a maximum after 1 mL of solution is eluted, but a pore size of 9.6 nm resulted in a peak maximum occurring after about 3 mL of solution has eluted.

8.5.1.4 Characterization of Column Features

The addition of the fluorescently-tagged dextrans (MW = 40 kDa) to the prepared glucose solution allowed for the comparison of the elution profiles between large and small saccharide species. In theory, the larger species should elute first followed by the smaller species at some later time, indicating that the smaller species was interacting with the stationary phase (i.e. diffusing in and out of the pores, adsorbing to the imprinted sites). However, the elution profiles for the two species were nearly identical, meaning that the two species were interacting with the stationary phase in similar ways. Chromatographic separation is impacted by several factors, like component interaction with the stationary phase due to chemical composition, diffusion characteristics or bed height; and component interaction with the mobile phase due to the chemical composition or flowrate. The correct combination of all these factors is essential to separating the desired components.

8.5.2 Bulk Adsorption of Glucose from Real Hydrolyzate

Stöber particles molecularly imprinted with glucose-binding sites were used to evaluate the effectiveness of selectively separating glucose from other monosaccharides and components found in enzymatically-produced hydrolyzate. This separation technology has potential applications for purifying sugar streams for optimal use of all energy-rich fractions of lignocellulose in biorefineries. Monosaccharide structures are very similar, possibly only differing by a single carbon atom or orientation of a hydroxyl group, making it difficult to selectively separate a single type of monosaccharide from solution at an industrial scale. The results observed in this current work indicate that the glucose-imprinted materials synthesized were capable of selectively separating glucose from other monosaccharides found in hydrolyzate. It is hypothesized that the uniformity of the particles, as well as the extended retention time (24 hrs) allowed for better interaction between the monosaccharides and the imprinted materials, resulting in better adsorption of the desired monosaccharides. This imprinting technology could be developed further with other monosaccharide templating molecules, like xylose, such that the materials could be synthesized with specific monosaccharide-binding sites and other desired purified sugar streams could be obtained.

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CHAPTER 9: CONCLUSIONS AND FUTURE DIRECTIONS

The use of second generation feedstocks for valuable products, like energy-dense liquid transportation fuels, pharmaceuticals and commodity chemicals, still requires much development in the conversion process to become commercially viable. It will be necessary to integrate the lignocellulose conversion process with the concept of the biorefinery for it to be economically viable and competitive with the traditional petroleum refinery. This current work investigated several aspects associated with the conversion of lignocellulose, including pretreatment and enzymatic hydrolysis of lignocellulose at high-solids loadings, separation and recovery of purified sugar streams, and the application of an existing kinetic model for the hydrolysis of lignocellulose material.

9.1 SODIUM HYDROXIDE PRETREATMENT AND THE SUBSEQUENT ENZYMATICAL HYDROLYSIS OF HIGH-SOLIDS LOADINGS

Sodium hydroxide (NaOH) pretreatment and the subsequent enzymatic hydrolysis were performed at high-solids loadings using corn stover as the substrate. Several factors associated with these two processes were investigated, including the duration of pretreatment at different temperatures and NaOH loadings, hydrolysis solids loadings and enzyme loadings. Relatively mild pretreatment conditions were intentionally chosen in order to avoid the production of compounds that are known inhibitors of the enzymes and fermentation organisms used in downstream processes. These conditions did not have significant effects on the subsequent composition of the corn stover when pretreated at 20% (w/w) solids loadings. However, the structure of the components was likely affected by the pretreatment since differences in cellulose conversions were observed.

NaOH loadings examined effectively increased the cellulose content of the corn stover by removing other non-cellulose components like ash, lignin and other unquantified components, with the exception of the most severe (highest NaOH loading, longest pretreatment time). The most severe pretreatment conditions resulted in the loss of some of the hemicellulose fraction, which is a concern since xylose (the main component of corn stover hemicellulose) is lost as a potentially viable feedstock, as well as degrading into a number of compounds that are inhibitory to downstream processes.

Flushing the hydrolyzate and reusing the substrate was also shown to increase overall glucose yields. It can be inferred from this study that flushing the pretreated corn stover throughout the hydrolysis reaction eliminates the need to wash the pretreated biomass prior to enzymatic hydrolysis, thereby reducing the amount of process water required.

9.1.1 Future Direction

Further study into NaOH pretreatment and enzymatic hydrolysis conditions used for processing high-solids loadings of lignocellulose is warranted to advance the understanding of the mechanisms involved, as well as improve the conversion of cellulose. One modification that can be made to the process is the use of horizontal roller bottles or reactors instead of shake flasks. The horizontal reactors have been shown to improve cellulose conversion at high-solids loadings since it promotes a more uniform mixing scheme without significantly increasing the required energy inputs (Dasari et al. 2009; Jorgensen et al. 2007b; Larsen et al. 2008; Roche et al. 2009b). Additionally, the fermentability of the hydrolyzate produced with high-solids loadings should be evaluated. High-solids loadings in the pretreatment and enzymatic hydrolysis processes may result in higher concentrations of inhibitory concentrations. Improved glucose yields are of little value if the ethanol yields are not significantly improved due to high inhibitor concentrations in the hydrolyzate.

Flushing the hydrolyzate and reusing the substrate is still a relatively new concept for improving conversion efficiency of cellulose. Many factors associated with this method still warrant investigation and development. Application and supplementation rates of enzymes should be examined to optimize the cellulose conversion in the flushing scheme. Timing of the flushing cycles is also important to maximize the rate of cellulose conversion and reduce the reaction time of the enzymatic hydrolysis step. Further study of the effect of water activity on the enzymatic hydrolysis reaction could lead to a better understanding of the reaction mechanism and other viable options for improving cellulose conversion. More importantly, an economic analysis should be conducted to determine the validity and efficacy of using a flushing method, both with and without

enzyme supplementation, compared to using the conventional batch hydrolysis at low- and high-solids loadings.

9.2 APPLICATION OF THE INTEGRATED MICHAELIS-MENTEN EQUATION FOR MODELING HETEROGENEOUS ENZYMATIC HYDROLYSIS REACTIONS

An experimental and theoretical analysis of enzymatic hydrolysis using the classical Michaelis-Menten kinetics model and a modified Michaelis-Menten model for insoluble substrates was conducted. Kinetic parameters K_m and V_m were determined by simultaneously fitting the integrated Michaelis-Menten model using MATLAB. The implicit nature of the integrated form of the Michaelis-Menten equation necessitated the use of Lambert's ω function. In addition to the kinetic parameters, two other parameters were incorporated into the model and evaluated both individually and together. The fractal component was added to describe the fractal kinetic characteristics that may occur due to the heterogeneous nature of the enzymatic hydrolysis of cellulose. The jamming parameter was evaluated to determine whether the size of the cellulase was causing the reduction in the reaction rate by overcrowding the available cellulose sites. Incorporation of the fractal component into the models improved the fit of the model to the experimental hydrolysis data, indicating that the heterogeneous nature of the reaction does impact the rate of the reaction.

9.2.1 Future Direction

One of the major assumptions associated with the Michaelis-Menten model is that the reaction is homogeneous in nature. That assumption is not valid for the hydrolysis of cellulose since the enzymes and substrates are in two different phases (i.e. heterogeneous reaction). Two approaches were investigated for describing the heterogeneous nature of the reaction: (1) a fractal parameter was incorporated into the traditional Michaelis-Menten model and (2) a modified Michaelis-Menten model adapted for use with insoluble substrates and evaluated both with and without the fractal parameter. The traditional Michaelis-Menten model and the two other approaches for heterogeneous reactions all assumed one type of reaction or the other (homogeneous vs. heterogeneous); however, the enzymatic hydrolysis of lignocellulose begins as (nearly) completely

heterogeneous in nature before shifting to a partially heterogeneous, partially homogeneous reaction. As the cellulase acts on the cellulose, some soluble saccharide species (mono-, di-, tri- and oligosaccharides) are released by the enzymes, resulting in enzymes and substrates that are in the same phase (i.e. homogeneous reaction). These soluble species are acted upon by the enzymes until only monosaccharides remain. This transition from one type of reaction (heterogeneous) to a combination of the two types (heterogeneous + homogeneous) is not incorporated into the models examined in this current work.

Additionally, some of the assumptions made concerning the kinetic parameters and the units associated with the input variables may warrant reevaluation. For instance, the upper bounds placed on the dissociation constant, K_m , may have limited the ability of the model to fit the experimental hydrolysis data since the fitted value for K_m was consistently the upper bound set when evaluating the traditional Michaelis-Menten models. The units associated with the input variables, especially the enzyme concentration, are not typical of most models. The enzyme loading is measured by its activity and given in terms of FPU/g solids. Most modeling studies use true concentration units like mM or g/L for enzyme inputs. However, the commercial enzyme preparation used in this work is actually a combination of multiple types of enzymes that fall in the cellulase category. To convert the enzyme loading used in this work from activity units to concentration units would require the assumptions that only a single enzyme type was used and that each enzyme molecule was equally active. This inconsistency with other studies, while not inaccurate, makes comparisons between the kinetic parameters found in this work with others difficult. Conversion of the activity units to concentration units would allow for easier comparison to other fitted kinetic parameters found in literature.

9.3 THE SEPARATION AND RECOVERY OF MONOSACCHARIDES USING MESOPOROUS SILICA MATERIALS

Imprinted mesoporous silica materials were synthesized using three different methods (one novel and two established synthesis methods) and evaluated by liquid chromatography or bulk adsorption for effective and selective separation of specific

monosaccharides from solution. No separation of glucose and xylose was observed during liquid chromatography of either a prepared sugar solution or hydrolyzate produced from enzymatically hydrolyzed lignocellulose, which was likely due to the difficulty in developing chromatographic methods. Many factors, like pH, temperature and mobile phase composition, affect the effectiveness of chromatographic separation. Multiple mobile phases were evaluated but further study is still warranted to find the optimal separation method.

Synthesis of the imprinted silica materials with the established Stöber method produced uniformly sized particles. This feature is desirable for chromatographic separations. Bulk adsorption tests showed that the glucose-imprinted particles were successful at selectively adsorbing glucose from hydrolyzate. Chromatographic separation was not evaluated for this material in this current work; although, its investigation would be warranted as a possible application at the industrial scale.

9.3.1 Future Direction

Developing new chromatographic separation techniques can be time-consuming and cumbersome. Many factors affect the effective separation of components from solution, and each one requires thorough evaluation. Synthesis of novel mesoporous silica materials for selective separation of specific monosaccharides has many applications, especially in the biofuels arena, where all components of lignocellulose must be exploited for the conversion process to be economical. Even though the materials synthesized with a novel imprinting technique in this current work have been shown to selectively separate glucose from solution in bulk adsorption applications, many other aspects of the operation must be developed further prior to incorporation into the lignocellulose conversion process. For instance, once the monosaccharides are adsorbed to the material, they must be recovered for use in downstream processes (fermentation, conversion). Appropriate conditions for desorption of the saccharides such that they are in a usable form and in a solution that is not inhibitory to fermentative organisms must be considered and evaluated. Should the materials be used in a chromatographic separation application, optimal methods must be devised, including bed height of column,

temperature and pH of mobile phase, and chemical composition of mobile phase, to name a few of the impacting factors.

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APPENDIX A: COMPOSITION OF PRETREATED CORN STOVER

A.1 PRETREATMENT TIME AND TEMPERATURE STUDY

Table A.1. Composition of corn stover pretreated at 20% (w/v) solids using different pretreatment times and temperatures.

Biomass Pretreatment Conditions													
Raw CS	25°C				70°C				70°C - Untreated				
	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	
Glu	37.9 (0.5)	39.2 (0.3)	39.2 (0.4)	39.3 (1.4)	39.5 (1.2)	39.4 (0.7)	40.1 (2.1)	40.7 (1.3)	40.1 (0.7)	37.2 (0.2)	37.3 (0.7)	37.4 (0.2)	37.3 (0.2)
Xyl	17.8 (0.4)	20.5 (0.4)	20.5 (0.0)	20.8 (0.6)	20.9 (0.5)	20.6 (0.4)	20.7 (1.0)	20.9 (0.4)	21.4 (0.3)	20.6 (0.1)	20.5 (0.0)	21.2 (0.0)	17.7 (4.1)
Ara	2.6 (0.1)	3.1 (0.3)	3.3 (0.1)	3.1 (0.5)	3.3 (0.5)	3.0 (0.2)	2.9 (0.3)	2.4 (0.3)	2.8 (0.5)	2.5 (0.2)	1.7 (0.6)	2.2 (0.2)	2.5 (0.1)
Man	0.4 (0.0)	0.7 (0.0)	0.7 (0.0)	0.7 (0.0)	0.6 (0.4)	0.7 (0.0)	0.7 (0.0)	0.7 (0.1)	0.8 (0.1)	0.5 (0.0)	0.6 (0.0)	0.9 (0.0)	0.6 (0.4)
Gal	0.8 (0.1)	1.2 (0.0)	1.3 (0.1)	1.3 (0.0)	1.3 (0.1)	1.1 (0.1)	1.1 (0.1)	1.3 (0.0)	1.2 (0.2)	1.4 (0.0)	1.4 (0.0)	0.9 (0.4)	1.4 (0.1)
AIL	18.9 (0.3)	20.9 (0.4)	21.2 (0.6)	20.6 (1.1)	20.3 (0.2)	20.8 (1.2)	20.8 (1.2)	21.2 (0.5)	20.7 (0.2)	20.6 (0.7)	20.4 (0.1)	20.3 (0.2)	19.7 (0.2)
ASL	2.2 (0.0)	1.9 (0.1)	1.8 (0.1)	1.8 (0.1)	1.8 (0.1)	1.8 (0.0)	1.8 (0.1)	1.7 (0.1)	1.7 (0.1)	1.9 (0.0)	1.9 (0.0)	1.9 (0.0)	1.9 (0.0)
Ash	4.8 (0.2)	3.3 (0.1)	3.0 (0.2)	3.1 (0.1)	2.9 (0.4)	3.0 (0.5)	2.6 (0.5)	2.6 (0.6)	3.0 (0.2)	3.3 (0.1)	3.2 (0.2)	3.3 (0.0)	3.0 (0.0)
Other	14.7	9.1	9.1	9.3	9.3	9.5	9.3	8.6	8.3	11.9	13.6	11.9	16.0

Abbreviations: CS = corn stover; Glu = glucose; Xyl = xylose; Ara = arabinose; Man = mannose; Gal = galactose; AIL = acid insoluble lignin; ASL = acid soluble lignin

A.2 NaOH LOADING IN PRETREATMENT STUDY

Table A.2. Composition of raw and pretreated corn stover. Results are calculated as % (w/w) oven dried material. Pretreatment was performed at 20% (w/v) solids for either 2 or 24 hr at 25°C.

Time (hr)	--	2			24		
NaOH Loading (g/100 g CS)	--	4	10	20	4	10	20
Glu	37.9 (0.5)	39.5 (1.1)	47.4 (2.3)	49.0 (2.0)	40.6 (9.4)	46.6 (6.4)	44.4 (1.1)
Xyl	17.8 (0.4)	20.9 (0.5)	21.2 (1.5)	19.4 (0.8)	21.0 (6.1)	23.0 (4.1)	17.0 (0.3)
Ara	2.6 (0.1)	3.3 (0.4)	5.2 (1.5)	4.9 (0.3)	4.4 (0.4)	3.8 (3.0)	1.7 (0.5)
Man	0.4 (0.0)	0.6 (0.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.5 (1.4)	2.3 (0.3)
Gal	0.8 (0.1)	1.3 (0.1)	0.0 (0.0)	0.4 (0.7)	1.5 (0.2)	1.5 (1.3)	0.3 (0.1)
AIL	18.9 (0.3)	20.3 (0.4)	18.0 (0.3)	14.7 (1.3)	20.8 (1.1)	22.6 (6.6)	22.5 (5.4)
ASL	2.2 (0.0)	1.8 (0.1)	1.5 (0.2)	1.4 (0.1)	1.6 (0.1)	1.4 (0.2)	1.2 (0.0)
Ash	4.8 (0.2)	2.9 (0.4)	6.6 (0.9)	15.6 (4.7)	2.4 (0.2)	6.1 (0.9)	10.1 (1.2)
Other	14.7	9.3	0.0	0.0	7.5	0.0	0.4

Abbreviations: CS = corn stover; Glu = glucose; Xyl = xylose; Ara = arabinose; Man = mannose; Gal = galactose; AIL = acid insoluble lignin; ASL = acid soluble lignin

A.3 HYDROLYZATE FLUSHING AND SUBSTRATE REUSE STUDY

Table A.3. Composition of raw and pretreated corn stover receiving different post-treatment processes. Results are calculated as % (w/w) oven dried material. Pretreatment was performed at 10% (w/v) solids 24 hr at 25°C.

	Raw CS	Washed ^a	Unwashed ^a
Glu	37.9 (1.5)	51.8 (1.3)	45.1 (0.3)
Xyl	17.8 (0.3)	23.7 (0.1)	22.8 (0.1)
Ara	2.6 (2.3)	2.9 (0.1)	3.6 (0.1)
Man	0.4 (0.0)	0.0 (0.0)	0.0 (0.0)
Gal	0.8 (0.0)	0.0 (0.0)	0.0 (0.0)
AIL	8.9 (0.1)	16.6 (0.4)	14.9 (0.3)
ASL	2.2 (0.1)	1.4 (0.0)	2.0 (0.0)
Ash	4.8 (0.4)	1.9 (0.1)	9.1 (0.6)
Other	14.7	1.6	2.6

Abbreviations: CS = corn stover; Glu = glucose; Xyl = xylose; Ara = arabinose; Man = mannose; Gal = galactose; AIL = acid insoluble lignin; ASL = acid soluble lignin

^aPost-treatment conditions: ‘Washed’, neutralized with glacial acetic acid and washed with 5 volumes of deionized water; ‘Unwashed’, neutralized with glacial acetic acid and excess liquid removed with vacuum filtration.

APPENDIX B: SACCHARIDE CONCENTRATIONS FROM ENZYMATIC HYDROLYSIS

B.1 PRETREATMENT TIME AND TEMPERATURE STUDY

Table B.1. Saccharide concentrations produced from enzymatic hydrolysis of PCS. Pretreatment was performed at 25°C.

Pretreatment Time (min)	Solids Loading (%)	Enzyme Loading (FPU/g solids)	Saccharide Concentration (mg X/g DM)					
			Glucose	Cellobiose	Xylose	Arabinose	Mannose	Galactose
30	5	5.2	143.90 (6.59)	0.04 (0.04)	35.88 (2.42)	10.29 (1.29)	11.90 (2.08)	13.07 (1.14)
		18.3	129.31 (4.51)	13.07 (22.65)	31.54 (0.91)	7.61 (1.08)	3.80 (1.80)	3.58 (1.07)
		60	112.21 (11.83)	0.90 (1.57)	21.31 (2.76)	8.19 (2.91)	2.30 (1.93)	10.28 (3.52)
	20	7.2	27.92 (1.35)	11.71 (4.65)	7.78 (0.20)	3.38 (0.24)	0.85 (0.07)	0.78 (0.07)
		28.9	15.81 (0.58)	0.00 (0.00)	4.47 (1.07)	2.54 (2.53)	0.00 (0.00)	0.57 (0.06)
		60	34.71 (0.95)	102.90 (10.45)	8.88 (1.12)	1.28 (0.63)	0.40 (0.08)	6.12 (1.33)
60	5	5.2	149.51 (10.91)	0.06 (0.11)	40.37 (1.63)	11.94 (1.25)	12.27 (0.87)	13.54 (1.68)
		18.3	129.18 (18.01)	8.53 (14.77)	29.90 (2.87)	7.31 (1.09)	3.55 (0.93)	2.93 (0.53)
		60	139.32 (32.60)	0.00 (0.00)	25.28 (3.68)	9.03 (2.15)	5.35 (1.98)	7.81 (5.03)
	20	7.2	29.74 (3.88)	7.41 (0.59)	8.44 (0.81)	3.72 (0.080)	2.08 (1.09)	1.18 (0.34)
		28.9	15.37 (0.33)	0.00 (0.00)	4.10 (1.02)	2.34 (0.22)	0.00 (0.00)	1.09 (0.23)
		60	37.16 (2.80)	79.30 (25.25)	8.10 (1.39)	3.79 (2.28)	2.32 (1.76)	3.92 (2.00)
90	5	5.2	151.19 (6.45)	0.16 (0.14)	40.52 (2.04)	10.88 (1.48)	12.27 (1.42)	12.99 (1.33)
		18.3	120.87 (5.73)	7.53 (13.03)	31.23 (2.88)	6.15 (1.30)	4.71 (1.81)	3.56 (0.86)
		60	117.18 (9.64)	0.00 (0.00)	23.96 (5.89)	9.58 (2.46)	2.80 (1.57)	10.74 (6.09)
	20	7.2	37.27 (4.22)	7.95 (0.84)	10.26 (1.01)	4.12 (0.84)	1.78 (0.83)	1.59 (0.20)
		28.9	18.95 (0.83)	0.01 (0.02)	6.00 (0.65)	0.78 (1.36)	0.00 (0.00)	1.21 (0.51)
		60	37.07 (1.73)	93.09 (21.59)	9.30 (1.90)	2.69 (2.19)	0.95 (0.72)	5.00 (1.42)
120	5	5.2	170.90 (10.47)	0.08 (0.13)	45.11 (3.88)	11.23 (1.33)	14.37 (1.85)	14.59 (0.82)
		18.3	132.05 (16.75)	5.71 (9.89)	34.35 (4.61)	7.84 (0.59)	5.63 (3.28)	4.49 (1.84)
		60	121.72 (15.01)	0.00 (0.00)	23.74 (4.32)	11.39 (2.22)	2.49 (1.73)	11.09 (4.31)
	20	7.2	31.68 (2.26)	8.39 (2.35)	8.75 (0.25)	3.54 (0.34)	1.61 (0.97)	0.86 (0.53)
		28.9	17.10 (1.36)	0.00 (0.00)	4.87 (1.00)	3.78 (2.19)	0.00 (0.00)	1.12 (0.61)
		60	37.61 (3.55)	111.28 (17.20)	9.82 (1.67)	2.51 (2.45)	0.92 (0.80)	4.99 (1.94)

B.2 NaOH LOADING IN PRETREATMENT STUDY

Table B.2. Saccharide concentrations produced from enzymatic hydrolysis of PCS treated at various NaOH loadings. Hydrolysis was performed at 5% (w/w) solids and [E] = 5.2 FPU/g solids for 72 hr at 50°C.

Pretreatment Time (hr)	NaOH Loading (g/100 g PCS)	Saccharide Concentration (mg X/g DM)					
		Glucose	Cellobiose	Xylose	Arabinose	Mannose	Galactose
2	4	170.90 (±10.47)	0.08 (±0.13)	45.11 (±3.88)	11.23 (±1.33)	14.37 (±1.85)	14.59 (±0.82)
	10	134.35 (±4.35)	0.14 (±0.02)	55.10 (±2.41)	0.51 (±0.89)	3.33 (±0.89)	3.28 (±0.40)
	20	89.35 (±46.00)	1.50 (±1.28)	27.49 (±19.29)	0.00 (±0.00)	3.29 (±1.66)	0.36 (±0.02)
24	4	84.01 (±2.27)	0.00 (±0.00)	23.18 (±4.90)	5.48 (±1.02)	2.41 (±0.20)	3.02 (±1.37)
	10	229.71 (±18.36)	0.63 (±0.32)	112.40 (±11.68)	13.84 (±2.80)	5.48 (±1.32)	3.69 (±2.48)
	20	264.59 (±18.89)	2.14 (±0.54)	88.36 (±7.92)	9.63 (±1.02)	4.46 (±1.22)	2.57 (±2.46)

Table B.3. Saccharide concentrations produced from enzymatic hydrolysis of PCS treated at various NaOH loadings. Hydrolysis was performed at 20% (w/w) solids and [E] = 5.2 FPU/g solids for 72 hr at 50°C.

Pretreatment Time (hr)	NaOH Loading (g/100 g PCS)	Saccharide Concentration (mg X/g DM)					
		Glucose	Cellobiose	Xylose	Arabinose	Mannose	Galactose
2	4	29.08 (±2.05)	7.70 (±2.16)	8.04 (±0.23)	3.25 (±0.32)	1.48 (±0.89)	0.79 (±0.49)
	10	21.59 (±7.61)	1.66 (±1.63)	6.77 (±2.10)	0.00 (±0.00)	0.42 (±0.25)	0.45 (±0.14)
	20	1.62 (±0.34)	0.72 (±0.21)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.03 (±0.04)
24	4	18.73 (±8.30)	0.05 (±0.09)	6.38 (±3.38)	1.43 (±0.80)	0.16 (±0.28)	0.50 (±0.38)
	10	32.93 (±6.03)	0.00 (±0.00)	13.96 (±2.58)	2.52 (±0.37)	0.00 (±0.00)	0.53 (±0.46)
	20	0.59 (±0.06)	0.64 (±0.13)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)

B.3 HYDROLYZATE FLUSHING AND SUBSTRATE REUSE STUDY

Table B.4. Glucose concentrations obtained from flushing the hydrolyzate and reusing the substrate.

Enzyme Supplementation	Post-Pretreatment Processing	Hydrolysis Treatment	Glucose Concentration (g/L)				
			Cycle 1	Cycle 2	Cycle 3	Total	
No	Washed	Batch	0.00 (--)	0.00 (--)	57.86 (3.60)	57.86 (3.60)	
		Flushed	47.11 (1.46)	32.28 (2.75)	18.15 (0.56)	97.54 (1.85)	
	Unwashed	Batch	0.00 (--)	0.00 (--)	46.91 (2.88)	46.91 (2.88)	
		Flushed	36.95 (1.66)	22.75 (0.72)	13.62 (0.23)	73.31 (2.61)	
	Yes	Washed	Batch	0.00 (--)	0.00 (--)	55.00 (0.75)	55.00 (0.75)
			Flushed	48.49 (1.56)	30.37 (0.37)	18.30 (0.59)	97.17 (2.52)
Yes	Unwashed	Batch	0.00 (--)	0.00 (--)	49.95 (0.48)	49.95 (0.48)	
		Flushed	47.08 (0.64)	31.76 (0.50)	18.64 (0.60)	97.48 (0.55)	
No	Washed	Batch	0.00 (--)	0.00 (--)	59.14(3.87)	59.14 (3.87)	
		Flushed	54.69 (2.88)	37.18 (1.24)	21.59 (0.94)	113.46 (5.06)	
	Unwashed	Batch	0.00 (--)	0.00 (--)	41.48 (0.65)	41.48 (0.65)	
		Flushed	41.06 (0.08)	26.81 (0.32)	16.99 (0.08)	84.86 (0.16)	
	Yes	Washed	Batch	0.00 (--)	0.00 (--)	47.85 (0.01)	47.85 (0.01)
			Flushed	43.84 (2.17)	37.29 (0.83)	22.41 (1.58)	103.54 (2.92)
Yes	Unwashed	Batch	0.00 (--)	0.00 (--)	43.23 (2.63)	43.23 (2.63)	
		Flushed	41.95 (0.99)	40.49 (4.05)	21.60 (0.12)	104.04 (4.92)	

Table B.5. Cellulose conversion achieved from flushing the hydrolyzate and reusing the substrate.

Enzyme Supplementation	Post-Pretreatment Processing	Hydrolysis Treatment	Cellulose Conversion (%)				
			Cycle 1	Cycle 2	Cycle 3	Total	
No	Washed	Batch	0.0 (--)	0.0 (--)	50.2 (3.1)	50.2 (3.1)	
		Flushed	40.9 (1.3)	28.0 (2.4)	15.8 (0.5)	84.7 (1.6)	
	Unwashed	Batch	0.0 (--)	0.0 (--)	46.9 (2.9)	46.9 (2.9)	
		Flushed	36.9 (1.7)	22.7 (0.7)	13.6 (0.2)	73.2 (2.6)	
	Yes	Washed	Batch	0.0 (--)	0.0 (--)	47.7 (0.7)	47.7 (0.7)
			Flushed	42.1 (1.4)	26.4 (0.3)	15.9 (0.5)	84.3 (2.2)
Unwashed		Batch	0.0 (--)	0.0 (--)	43.4 (0.4)	43.4 (0.4)	
		Flushed	40.9 (0.6)	27.6 (0.4)	16.2 (0.5)	84.6 (0.5)	
No	Washed	Batch	0.0 (--)	0.0 (--)	51.3 (3.4)	51.3 (3.4)	
		Flushed	47.5 (2.5)	32.3 (1.1)	18.7 (0.8)	98.5 (4.4)	
	Unwashed	Batch	0.0 (--)	0.0 (--)	41.4 (0.7)	41.4 (0.7)	
		Flushed	41.0 (0.1)	26.8 (0.3)	17.0 (0.1)	84.8 (0.2)	
	Yes	Washed	Batch	0.0 (--)	0.0 (--)	41.5 (0.0)	41.5 (0.0)
			Flushed	38.1 (1.9)	32.4 (0.7)	19.5 (1.4)	89.9 (2.5)
Unwashed		Batch	0.0 (--)	0.0 (--)	37.5 (2.3)	37.5 (2.3)	
		Flushed	36.4 (0.9)	35.1 (3.5)	18.8 (0.1)	90.3 (4.3)	

APPENDIX C: MATLAB CODE

MATLAB Code for Traditional and Modified Michaelis-Menten Models

```
clear all
close all
clc

global S0
global E0

% Data in D is time (hr) and product (g G/L) for
% E = 15, 30, 45, and 60 FPU/g solids
D = [0 0.000 0.000 0.000 0.000;
0.5 0.468 0.556 0.674 5.142;
1 1.201 1.175 1.281 6.794;
2 1.440 1.945 1.960 7.320;
4 1.888 2.345 2.209 7.999;
6 2.007 2.401 2.315 7.310;
24 3.084 3.304 3.607 9.383;
48 3.138 4.036 3.801 6.325;
72 3.384 4.277 3.358 5.869;
96 3.458 4.170 6.029 5.509;
0 0.000 0.000 0.000 0.000;
0.5 1.300 2.210 1.877 0.593;
1 2.656 5.474 2.873 2.905;
2 3.715 6.608 3.950 3.136;
4 5.827 8.278 5.231 3.253;
6 6.156 10.287 5.463 3.040;
24 7.499 4.632 5.993 3.345;
48 10.894 6.140 6.358 3.035;
72 9.665 6.504 7.636 2.514;
96 10.238 7.190 7.218 3.057;
0 0.000 0.000 0.000 0.000;
0.5 3.107 2.533 4.736 7.112;
1 5.399 3.812 6.946 9.469;
2 8.380 7.816 8.635 11.985;
4 10.529 11.222 9.360 13.626;
6 11.676 11.995 -2.812 12.820;
24 16.876 16.699 13.864 14.149;
48 18.319 12.439 14.786 15.281;
72 19.918 14.004 14.085 15.777;
96 20.862 14.178 14.251 15.367;
0 0.000 0.000 0.000 0.000;
0.5 3.672 4.961 4.511 4.624;
1 6.439 7.577 8.947 10.016;
2 9.305 10.448 11.551 14.718;
4 11.535 13.271 14.288 20.018;
6 14.551 15.215 15.289 21.172;
24 17.663 17.651 19.526 25.897;
48 19.960 19.130 22.996 31.798;
72 21.560 20.203 24.794 23.337;
96 22.897 21.421 25.708 37.246;
0 0.000 0.000 0.000 0.000;
0.5 5.428 8.444 4.706 4.432;
```

```

1  6.496  14.407  7.142  5.761;
2  10.054 17.509 11.947  9.113;
4  11.846 20.920 15.945 13.800;
6  12.591 20.477 19.118 13.441;
24 18.571 24.545 21.701 15.813;
48 15.635 26.703 22.798 17.289;
72 19.265 24.287 22.490 17.107;
96 16.603 22.727 20.526 18.662];

% Data in dPinf is delta P infinity (g G/L)
dPinf=[0 3.3 3.9 4.2 6.8;
0.5 3.3 3.9 4.2 6.8;
1 3.3 3.9 4.2 6.8;
2 3.3 3.9 4.2 6.8;
4 3.3 3.9 4.2 6.8;
6 3.3 3.9 4.2 6.8;
24 3.3 3.9 4.2 6.8;
48 3.3 3.9 4.2 6.8;
72 3.3 3.9 4.2 6.8;
96 3.3 3.9 4.2 6.8;
0 9.6 6.1 6.8 3.0;
0.5 9.6 6.1 6.8 3.0;
1 9.6 6.1 6.8 3.0;
2 9.6 6.1 6.8 3.0;
4 9.6 6.1 6.8 3.0;
6 9.6 6.1 6.8 3.0;
24 9.6 6.1 6.8 3.0;
48 9.6 6.1 6.8 3.0;
72 9.6 6.1 6.8 3.0;
96 9.6 6.1 6.8 3.0;
0 19.0 14.3 14.2 15.1;
0.5 19.0 14.3 14.2 15.1;
1 19.0 14.3 14.2 15.1;
2 19.0 14.3 14.2 15.1;
4 19.0 14.3 14.2 15.1;
6 19.0 14.3 14.2 15.1;
24 19.0 14.3 14.2 15.1;
48 19.0 14.3 14.2 15.1;
72 19.0 14.3 14.2 15.1;
96 19.0 14.3 14.2 15.1;
0 20.5 19.6 23.3 29.6;
0.5 20.5 19.6 23.3 29.6;
1 20.5 19.6 23.3 29.6;
2 20.5 19.6 23.3 29.6;
4 20.5 19.6 23.3 29.6;
6 20.5 19.6 23.3 29.6;
24 20.5 19.6 23.3 29.6;
48 20.5 19.6 23.3 29.6;
72 20.5 19.6 23.3 29.6;
96 20.5 19.6 23.3 29.6;
0 17.5 24.6 21.9 17.2;
0.5 17.5 24.6 21.9 17.2;

```

```

1  17.5    24.6    21.9    17.2;
2  17.5    24.6    21.9    17.2;
4  17.5    24.6    21.9    17.2;
6  17.5    24.6    21.9    17.2;
24 17.5    24.6    21.9    17.2;
48 17.5    24.6    21.9    17.2;
72 17.5    24.6    21.9    17.2;
96 17.5    24.6    21.9    17.2];

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

% Solve the integrated MM equation for the combined data for the
% given enzyme loading, which returns a value for Ka and Vm

% TRADITIONAL MICHAELIS-MENTEN

% Define variables
for i=1:n(:,2)-1;
t=D(:,1); % time (hr)
Pt=D(:,i+1); % product (g G/L)
S0=dPinf(:,i+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)
E0=E0(:,i);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
    % upper bounds set for Ka based on max dPinf
Kaub=Kaub(:,i);

% Set initial guesses for parameters
Ka=10;
Vm=10;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm]';
F=MMintegrated(x0, xdata);

% Use least squares estimates to find "best fit" for Ka and Vm
lb=[0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-12, 'TolX', 1e-12,...
    'MaxFunEvals', 10000);
[x(:,i), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
    (@(x0,xdata) MMintegrated(x0,xdata),x0,xdata,ydata,lb,ub,options)

% Use new parameters to predict P
H=MMintegrated(x(:,i),xdata);

```

```

% Calculate F statistic
Hones=[ones(size(H)) H];
b1(:,i)=regress(Pt,Hones)

n=length(Pt);
xbar1=mean(Hones(:,2)); % mean of predicted product values
yhat1=(mean(Pt)+b1(2).*(Hones(:,2)-xbar1));
    % calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat1).^2))/(n-2); % standard error
fstat(:,i)=((n-2)*(n*b1(1)^2+2*n*xbar1*b1(1)*(b1(2)-1)...
    +sum(Hones(:,2).^2*(b1(2)-1)^2))/(2*n*S^2) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; % sums of squares of observed
R2(:,i)=1-(resnorm/SST)

% Calculate 95% confidence intervals for parameters
ci=nlparci(x(:,i),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(H,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 25 0 25])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), H(1:10),'b-',
xdata(1:10),Pt(1:10),'b*',xdata(11:20),...
    H(11:20),'c-', xdata(11:20),Pt(11:20),'co',...
    xdata(21:30), H(21:30),'g-', xdata(21:30),Pt(21:30),'gd',...
    xdata(31:40), H(31:40),'m-', xdata(31:40),Pt(31:40),'m+',...
    xdata(41:50), H(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

% Determine the number of rows D(1) and columns D(2) that are in the
% data
n=size(D);

% INSOLUBLE SOLIDS (HIGH SOLIDS) MICHAELIS-MENTEN

% Define variables
for j=1:n(:,2)-1;
t=D(:,1); % time (hr)
Pt=D(:,j+1); % product (g G/L)
S0=dPinf(:,j+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)

```



```

E0=E0(:,j);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
      % upper bounds set for Ka based on max dPinf
Kaub=Kaub(:,j);

% Set initial guesses for parameters
Ka=10;
Vm=10;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm]';
A=MMintegrated_E(x0, xdata);

% Use least squares estimates to find "best fit" for Ka and Vm
lb=[0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-12, 'TolX', 1e-12,...
                'MaxFunEvals', 10000);
[x2(:,j), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
    (@MMintegrated_E,x0,xdata,ydata,lb,ub, options)

k2(:,j)=x2(2)./S0; % rate constant, k2

% Use new parameters to predict P
C=MMintegrated_E(x2(:,j),xdata);

% Calculate F statistic
Cones=[ones(size(C)) C];
b2(:,j)=regress(Pt,Cones)

n=length(Pt);
xbar2=mean(Cones(:,2)); % mean of predicted product values
yhat2=(mean(Pt)+b2(2).*(Cones(:,2)-xbar2));
      % calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat2).^2))/(n-2); % standard error
fstat2(:,j)=(n-2)*(n*b2(1)^2+2*n*xbar2*b2(1)*(b2(2)-1)...
            +sum((Cones(:,2).^2)*(b2(2)-1)^2))/(2*n*S^2) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; % sums of squares of observed
Rsq(:,j)=1-(resnorm/SST)

% Calculate 95% confidence intervals for parameters
ci=nlparci(x2(:,j),residual,'jacobian',jacobian)

```

```

% Plot predicted vs. observed values
figure
plot(C,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 30 0 30])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), C(1:10),'b-', xdata(1:10),Pt(1:10),'b*',...
     xdata(11:20), C(11:20),'c-', xdata(11:20),Pt(11:20),'co',...
     xdata(21:30), C(21:30),'g-', xdata(21:30),Pt(21:30),'gd',...
     xdata(31:40), C(31:40),'m-', xdata(31:40),Pt(31:40),'m+',...
     xdata(41:50), C(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

```

```

function F = MMintegrated(x0, xdata)

% MMintegrated solves the integrated Michaelis-Menten equation for Pt
% (equation 8 Goudar et al, Buichimica et Biophysica Acta 1429(1999)
% 377-383)

global S0

P0=0; % initial product concentration (g G/L)
omega=(S0./x0(1)).*exp((S0-x0(2).*xdata)./x0(1));
z=lambertw(omega);
F=P0+S0-x0(1).*z; % product concentration at time, t (g G/L)

end

```

```
function A = MMintegrated_E(x0, xdata)

% MMintegrated_E solves the integrated Michaelis-Menten equation for
% Pt (equation 8 Goudar et al, Buichimica et Biophysica Acta
% 1429(1999) 377-383)

global S0
global E0

P0=0; % initial product concentration (g G/L)

A=((xdata.*x0(2).*S0)./(x0(1)+E0));
    % product concentration at time, t (g G/L)

end
```

MATLAB Code for Model with Fractal Parameter

```
clear all
close all
clc

global S0
global E0

% Data in D is time (hr) and product (g G/L) for
% E = 15, 30, 45, and 60 FPU/g solids
D = [0 0.000 0.000 0.000 0.000;
0.5 0.468 0.556 0.674 5.142;
1 1.201 1.175 1.281 6.794;
2 1.440 1.945 1.960 7.320;
4 1.888 2.345 2.209 7.999;
6 2.007 2.401 2.315 7.310;
24 3.084 3.304 3.607 9.383;
48 3.138 4.036 3.801 6.325;
72 3.384 4.277 3.358 5.869;
96 3.458 4.170 6.029 5.509;
0 0.000 0.000 0.000 0.000;
0.5 1.300 2.210 1.877 0.593;
1 2.656 5.474 2.873 2.905;
2 3.715 6.608 3.950 3.136;
4 5.827 8.278 5.231 3.253;
6 6.156 10.287 5.463 3.040;
24 7.499 4.632 5.993 3.345;
48 10.894 6.140 6.358 3.035;
72 9.665 6.504 7.636 2.514;
96 10.238 7.190 7.218 3.057;
0 0.000 0.000 0.000 0.000;
0.5 3.107 2.533 4.736 7.112;
1 5.399 3.812 6.946 9.469;
2 8.380 7.816 8.635 11.985;
4 10.529 11.222 9.360 13.626;
6 11.676 11.995 -2.812 12.820;
24 16.876 16.699 13.864 14.149;
48 18.319 12.439 14.786 15.281;
72 19.918 14.004 14.085 15.777;
96 20.862 14.178 14.251 15.367;
0 0.000 0.000 0.000 0.000;
0.5 3.672 4.961 4.511 4.624;
1 6.439 7.577 8.947 10.016;
2 9.305 10.448 11.551 14.718;
4 11.535 13.271 14.288 20.018;
6 14.551 15.215 15.289 21.172;
24 17.663 17.651 19.526 25.897;
48 19.960 19.130 22.996 31.798;
72 21.560 20.203 24.794 23.337;
96 22.897 21.421 25.708 37.246;
0 0.000 0.000 0.000 0.000;
0.5 5.428 8.444 4.706 4.432;
```

```

1  6.496  14.407  7.142  5.761;
2  10.054 17.509 11.947  9.113;
4  11.846 20.920 15.945 13.800;
6  12.591 20.477 19.118 13.441;
24 18.571 24.545 21.701 15.813;
48 15.635 26.703 22.798 17.289;
72 19.265 24.287 22.490 17.107;
96 16.603 22.727 20.526 18.662];

% Data in dPinf is delta P infinity (g G/L)
dPinf=[0 3.3 3.9 4.2 6.8;
0.5 3.3 3.9 4.2 6.8;
1 3.3 3.9 4.2 6.8;
2 3.3 3.9 4.2 6.8;
4 3.3 3.9 4.2 6.8;
6 3.3 3.9 4.2 6.8;
24 3.3 3.9 4.2 6.8;
48 3.3 3.9 4.2 6.8;
72 3.3 3.9 4.2 6.8;
96 3.3 3.9 4.2 6.8;
0 9.6 6.1 6.8 3.0;
0.5 9.6 6.1 6.8 3.0;
1 9.6 6.1 6.8 3.0;
2 9.6 6.1 6.8 3.0;
4 9.6 6.1 6.8 3.0;
6 9.6 6.1 6.8 3.0;
24 9.6 6.1 6.8 3.0;
48 9.6 6.1 6.8 3.0;
72 9.6 6.1 6.8 3.0;
96 9.6 6.1 6.8 3.0;
0 19.0 14.3 14.2 15.1;
0.5 19.0 14.3 14.2 15.1;
1 19.0 14.3 14.2 15.1;
2 19.0 14.3 14.2 15.1;
4 19.0 14.3 14.2 15.1;
6 19.0 14.3 14.2 15.1;
24 19.0 14.3 14.2 15.1;
48 19.0 14.3 14.2 15.1;
72 19.0 14.3 14.2 15.1;
96 19.0 14.3 14.2 15.1;
0 20.5 19.6 23.3 29.6;
0.5 20.5 19.6 23.3 29.6;
1 20.5 19.6 23.3 29.6;
2 20.5 19.6 23.3 29.6;
4 20.5 19.6 23.3 29.6;
6 20.5 19.6 23.3 29.6;
24 20.5 19.6 23.3 29.6;
48 20.5 19.6 23.3 29.6;
72 20.5 19.6 23.3 29.6;
96 20.5 19.6 23.3 29.6;
0 17.5 24.6 21.9 17.2;
0.5 17.5 24.6 21.9 17.2;

```

```

1  17.5    24.6    21.9    17.2;
2  17.5    24.6    21.9    17.2;
4  17.5    24.6    21.9    17.2;
6  17.5    24.6    21.9    17.2;
24 17.5    24.6    21.9    17.2;
48 17.5    24.6    21.9    17.2;
72 17.5    24.6    21.9    17.2;
96 17.5    24.6    21.9    17.2];

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

% Solve the integrated MM equation for the combined data for the
given
% enzyme loading, which returns a value for Ka, Vm and the fractal
% component, f

% TRADITIONAL MICHAELIS-MENTEN

% Define variables
for i=1:n(:,2)-1;
t=D(:,1); % time (hr)
Pt=D(:,i+1); % product (g G/L)
S0=dPinf(:,i+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)
E0=E0(:,i);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
Kaub=Kaub(:,i);

% Set initial guesses for parameters
Ka=10;
Vm=10;
f=0.5;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm f]';
F=MMintegrated_f(x0, xdata);

% Use least squares estimates to find "best fit" for Ka and Vm
lb=[0 0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf 1]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-12, 'TolX', 1e-12,...
'MaxFunEvals', 10000);
[x(:,i), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
(@(x0,xdata)
MMintegrated_f(x0,xdata),x0,xdata,ydata,lb,ub,options)

```

```

% Use new parameters to predict P
H=MMintegrated_f(x(:,i),xdata);

% Calculate F statistic
Hones=[ones(size(H)) H];
b1(:,i)=regress(Pt,Hones)

n=length(Pt);
xbar1=mean(Hones(:,2)); % mean of predicted product values
yhat1=(mean(Pt)+b1(2).*(Hones(:,2)-xbar1));
% calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat1).^2))/(n-2); % standard error
fstat(:,i)=(n-2)*(n*b1(1)^2+2*n*xbar1*b1(1)*(b1(2)-1)...
+sum(Hones(:,2).^2*(b1(2)-1)^2))/(2*n*S^2) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; % sums of squares of observed
R2(:,i)=1-(resnorm/SST)

% Calculate 95% confidence intervals for parameters
ci=nlparci(x(:,i),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(H,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 25 0 25])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), H(1:10),'b-',
xdata(1:10),Pt(1:10),'b*',xdata(11:20),...
H(11:20),'c-', xdata(11:20),Pt(11:20),'co',xdata(21:30),...
H(21:30),'g-', xdata(21:30),Pt(21:30),'gd',xdata(31:40),...
H(31:40),'m-', xdata(31:40),Pt(31:40),'m+',xdata(41:50),...
H(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

% INSOLUBLE SOLIDS (HIGH SOLIDS) MICHAELIS-MENTEN

% Define variables
for j=1:n(:,2)-1;
t=D(:,1); %time (hr)

```

```

Pt=D(:,j+1); %product (g G/L)
S0=dPinf(:,j+1); %initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; %inital enzyme loading (FPU/g solids)
E0=E0(:,j);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
Kaub=Kaub(:,j);

% Set initial guesses for parameters
Ka=10;
Vm=10;
f=0.5;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm f]';
A=MMintegrated_E_f(x0, xdata);

% Use least squares estimates to find "best fit" for Ka, Vm and f
lb=[0 0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf 1]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-13, 'TolX', 1e-13,...
    'MaxFunEvals', 10000);
[x2(:,j), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
    (@MMintegrated_E_f,x0,xdata,ydata,lb,ub, options)

k2(:,j)=x2(2)./S0; % rate constant, k2

% Use new parameters to predict P
C=MMintegrated_E_f(x2(:,j),xdata);

%Calculate F statistic
Cones=[ones(size(C)) C];
b2(:,j)=regress(Pt,Cones)

n=length(Pt);
xbar2=mean(Cones(:,2)); % mean of predicted product values
yhat2=(mean(Pt)+b2(2).*(Cones(:,2)-xbar2));
% calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat2).^2))/(n-2); % standard error
fstat2(:,j)=((n-2)*(n*b2(1)^2+2*n*xbar2*b2(1)*(b2(2)-1)...
    +(sum((Cones(:,2).^2)*(b2(2)-1)^2)))/(2*n*S^2)) % F-statistic

%Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; % sums of squares of observed
Rsq(:,j)=1-(resnorm/SST)

```



```

% Calculate 95% confidence intervals for parameters
ci=nlparci(x2(:,j),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(C,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 30 0 30])

% Plot hydrolysis progress curves with predicted and observed values
figure
% subplot(2,2,j)
plot(xdata(1:10), C(1:10),'b-',
xdata(1:10),Pt(1:10),'b*',xdata(11:20),...
C(11:20),'c-', xdata(11:20),Pt(11:20),'co',...
xdata(21:30), C(21:30),'g-', xdata(21:30),Pt(21:30),'gd',...
xdata(31:40), C(31:40),'m-', xdata(31:40),Pt(31:40),'m+',...
xdata(41:50), C(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

```

```

function F = MMintegrated_f(x0, xdata)

% MMintegrated_f solves the integrated Michaelis-Menten equation with
% a fractal component for Pt (equation 8 Goudar et al, Biuchimica et
% Biophysica Acta 1429(1999) 377-383)

global S0

P0=0; % inital product concentration (g G/L)
Vmtf=(x0(2).*xdata.^(1-x0(3)))./(1-x0(3));
% incorporation of fractal component
omega=(S0./x0(1)).*exp((S0-Vmtf)./x0(1));
z=lambertw(omega);
F= P0+S0-x0(1).*z; % product concentration at time, t (g G/L)

end

```

```

function A = MMintegrated_E_f(x0, xdata)

% MMintegrated_E_f solves the integrated Michaelis-Menten equation
% modified for insoluble solids with a fractal component for Pt
% (equation 8 Goudar et al, Buichimica et Biophysica Acta 1429(1999)
% 377-383)

global S0
global E0

P0=0; % inital product concentration (g G/L)

Vmtf=(x0(2).*xdata.^(1-x0(3)))./(1-x0(3));
% incorporation of fractal component
A=((Vmtf.*S0)./(x0(1)+E0)); % product concentration at time, t (g
G/L)

end

```

MATLAB Code for Model with Jamming Parameter

```
clear all
close all
clc

global S0
global E0

% Data in D is time (hr) and product (g G/L) for
% E = 15, 30, 45, and 60 FPU/g solids
D = [0 0.000 0.000 0.000 0.000;
0.5 0.468 0.556 0.674 5.142;
1 1.201 1.175 1.281 6.794;
2 1.440 1.945 1.960 7.320;
4 1.888 2.345 2.209 7.999;
6 2.007 2.401 2.315 7.310;
24 3.084 3.304 3.607 9.383;
48 3.138 4.036 3.801 6.325;
72 3.384 4.277 3.358 5.869;
96 3.458 4.170 6.029 5.509;
0 0.000 0.000 0.000 0.000;
0.5 1.300 2.210 1.877 0.593;
1 2.656 5.474 2.873 2.905;
2 3.715 6.608 3.950 3.136;
4 5.827 8.278 5.231 3.253;
6 6.156 10.287 5.463 3.040;
24 7.499 4.632 5.993 3.345;
48 10.894 6.140 6.358 3.035;
72 9.665 6.504 7.636 2.514;
96 10.238 7.190 7.218 3.057;
0 0.000 0.000 0.000 0.000;
0.5 3.107 2.533 4.736 7.112;
1 5.399 3.812 6.946 9.469;
2 8.380 7.816 8.635 11.985;
4 10.529 11.222 9.360 13.626;
6 11.676 11.995 -2.812 12.820;
24 16.876 16.699 13.864 14.149;
48 18.319 12.439 14.786 15.281;
72 19.918 14.004 14.085 15.777;
96 20.862 14.178 14.251 15.367;
0 0.000 0.000 0.000 0.000;
0.5 3.672 4.961 4.511 4.624;
1 6.439 7.577 8.947 10.016;
2 9.305 10.448 11.551 14.718;
4 11.535 13.271 14.288 20.018;
6 14.551 15.215 15.289 21.172;
24 17.663 17.651 19.526 25.897;
48 19.960 19.130 22.996 31.798;
72 21.560 20.203 24.794 23.337;
96 22.897 21.421 25.708 37.246;
0 0.000 0.000 0.000 0.000;
0.5 5.428 8.444 4.706 4.432;
```

```

1   6.496   14.407   7.142   5.761;
2   10.054  17.509   11.947  9.113;
4   11.846  20.920   15.945  13.800;
6   12.591  20.477   19.118  13.441;
24  18.571  24.545   21.701  15.813;
48  15.635  26.703   22.798  17.289;
72  19.265  24.287   22.490  17.107;
96  16.603  22.727   20.526  18.662];

% Data in dPinf is delta P infinity (g G/L)
dPinf=[0   3.3 3.9 4.2 6.8;
0.5 3.3 3.9 4.2 6.8;
1   3.3 3.9 4.2 6.8;
2   3.3 3.9 4.2 6.8;
4   3.3 3.9 4.2 6.8;
6   3.3 3.9 4.2 6.8;
24  3.3 3.9 4.2 6.8;
48  3.3 3.9 4.2 6.8;
72  3.3 3.9 4.2 6.8;
96  3.3 3.9 4.2 6.8;
0   9.6 6.1 6.8 3.0;
0.5 9.6 6.1 6.8 3.0;
1   9.6 6.1 6.8 3.0;
2   9.6 6.1 6.8 3.0;
4   9.6 6.1 6.8 3.0;
6   9.6 6.1 6.8 3.0;
24  9.6 6.1 6.8 3.0;
48  9.6 6.1 6.8 3.0;
72  9.6 6.1 6.8 3.0;
96  9.6 6.1 6.8 3.0;
0   19.0   14.3   14.2   15.1;
0.5 19.0   14.3   14.2   15.1;
1   19.0   14.3   14.2   15.1;
2   19.0   14.3   14.2   15.1;
4   19.0   14.3   14.2   15.1;
6   19.0   14.3   14.2   15.1;
24  19.0   14.3   14.2   15.1;
48  19.0   14.3   14.2   15.1;
72  19.0   14.3   14.2   15.1;
96  19.0   14.3   14.2   15.1;
0   20.5   19.6   23.3   29.6;
0.5 20.5   19.6   23.3   29.6;
1   20.5   19.6   23.3   29.6;
2   20.5   19.6   23.3   29.6;
4   20.5   19.6   23.3   29.6;
6   20.5   19.6   23.3   29.6;
24  20.5   19.6   23.3   29.6;
48  20.5   19.6   23.3   29.6;
72  20.5   19.6   23.3   29.6;
96  20.5   19.6   23.3   29.6;
0   17.5   24.6   21.9   17.2;
0.5 17.5   24.6   21.9   17.2;

```

```

1  17.5    24.6    21.9    17.2;
2  17.5    24.6    21.9    17.2;
4  17.5    24.6    21.9    17.2;
6  17.5    24.6    21.9    17.2;
24 17.5    24.6    21.9    17.2;
48 17.5    24.6    21.9    17.2;
72 17.5    24.6    21.9    17.2;
96 17.5    24.6    21.9    17.2];

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

% Solve the integrated MM equation for the combined data for the
given
% enzyme loading, which returns a value for Ka, Vm and the jamming
% component, J

% TRADITIONAL MICHAELIS-MENTEN

% Define variables
for i=1:n(:,2)-1;
t=D(:,1); % time (hr)
Pt=D(:,i+1); % product (g G/L)
S0=dPinf(:,i+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)
E0=E0(:,i);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
Kaub=Kaub(:,i);

%set initial guesses for parameters
Ka=10;
Vm=10;
J=10;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm J]';
F=MMintegrated_j(x0, xdata);

% Use least squares estimates to find "best fit" for Ka, Vm and J
lb=[0 0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf inf]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-12, 'TolX', 1e-12,...
'MaxFunEvals', 10000);
[x(:,i), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
(@(x0,xdata)
MMintegrated_j(x0,xdata),x0,xdata,ydata,lb,ub,options)

```

```

% Use new parameters to predict P
H=MMintegrated_j(x(:,i),xdata);

% Calculate F statistic
Hones=[ones(size(H)) H];
b1(:,i)=regress(Pt,Hones)

n=length(Pt);
xbar1=mean(Hones(:,2)); % mean of predicted product values
yhat1=(mean(Pt)+b1(2).*(Hones(:,2)-xbar1));
% calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat1).^2))/(n-2); % standard error
fstat(:,i)=(n-2)*(n*b1(1)^2+2*n*xbar1*b1(1)*(b1(2)-1)...
+sum(Hones(:,2).^2*(b1(2)-1)^2))/(2*n*S^2) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; %Sums of squares of observed
R2(:,i)=1-(resnorm/SST)

% Calculate 95% confidence intervals for parameters
ci=nlparci(x(:,i),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(H,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 25 0 25])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), H(1:10),'b-',
xdata(1:10),Pt(1:10),'b*',xdata(11:20),...
H(11:20),'c-', xdata(11:20),Pt(11:20),'co',...
xdata(21:30), H(21:30),'g-', xdata(21:30),Pt(21:30),'gd',...
xdata(31:40), H(31:40),'m-', xdata(31:40),Pt(31:40),'m+',...
xdata(41:50), H(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

%INSOLUBLE SOLIDS (HIGH SOLIDS) MICHAELIS-MENTEN

for j=1:n(:,2)-1;
t=D(:,1); % time (hr)

```

```

Pt=D(:,j+1); % product (g G/L)
S0=dPinf(:,j+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)
E0=E0(:,j);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
Kaub=Kaub(:,j);

% Set initial guesses for parameters
Ka=10;
Vm=10;
J=10;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm J]';
A=MMintegrated_E_j(x0, xdata);

% Use least squares estimates to find "best fit" for Ka, Vm and J
lb=[0 0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf inf]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-13, 'TolX', 1e-13,...
    'MaxFunEvals', 10000);
[x2(:,j), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
    (@MMintegrated_E_j,x0,xdata,ydata,lb,ub, options)

k2(:,j)=x2(2)./S0;

% Use new parameters to predict P
C=MMintegrated_E_j(x2(:,j),xdata);

% Calculate F statistic
Cones=[ones(size(C)) C];
b2(:,j)=regress(Pt,Cones)

n=length(Pt);
xbar2=mean(Cones(:,2)); % mean of predicted product values
yhat2=(mean(Pt)+b2(2).*(Cones(:,2)-xbar2));
% calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat2).^2))/(n-2); % standard error
fstat(:,j)=((n-2)*(n*b2(1)^2+2*n*xbar2*b2(1)*(b2(2)-1)...
    +(sum((Cones(:,2).^2)*(b2(2)-1)^2)))/(2*n*S^2)) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; % sums of squares of observed
R2(:,j)=1-(resnorm/SST)

```

```

% Calculate 95% confidence intervals for parameters
ci=nlparci(x2(:,j),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(C,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 30 0 30])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), C(1:10),'b-', xdata(1:10),Pt(1:10),'b*',...
      xdata(11:20), C(11:20),'c-', xdata(11:20),Pt(11:20),'co',...
      xdata(21:30), C(21:30),'g-', xdata(21:30),Pt(21:30),'gd',...
      xdata(31:40), C(31:40),'m-', xdata(31:40),Pt(31:40),'m+',...
      xdata(41:50), C(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

```

```

function F = MMintegrated_j(x0, xdata)

% MMintegrated_j solves the integrated Michaelis-Menten equation with
% a jamming component for Pt (equation 8 Goudar et al, Biuchimica et
% Biophysica Acta 1429(1999) 377-383)

global S0
global E0

P0=0; % initial product concentration (g G/L)

Vmtj=(1-(E0./(x0(3).*S0))).*x0(2).*xdata;
% incorporation of jamming component
omega=(S0./x0(1)).*exp((S0-Vmtj)./x0(1));
z=lambertw(omega);
F= P0+S0-x0(1).*z; % product concentration at time, t (g G/L)

end

```



```

function A = MMintegrated_E_j(x0, xdata)

% MMintegrated_E_j solves the integrated Michaelis-Menten equation
% modified for insoluble solids with a jamming component for Pt
% (equation 8 Goudar et al, Buichimica et Biophysica Acta 1429(1999)
% 377-383)

global S0
global E0

P0=0; % initial product concentration (g G/L)

Vmtj=(1-(E0./(x0(3).*S0))).*x0(2).*xdata;
% incorporation of jamming component
A=((Vmtj.*S0)./(x0(1)+E0)); % product concentration at time, t (g
G/L)

end

```

MATLAB Code for Models with Fractal + Jamming Parameters

```
clear all
close all
clc

global S0
global E0

% Data in D is time (hr) and product (g G/L) for
% E = 15, 30, 45, and 60 FPU/g solids
D = [0 0.000 0.000 0.000 0.000;
0.5 0.468 0.556 0.674 5.142;
1 1.201 1.175 1.281 6.794;
2 1.440 1.945 1.960 7.320;
4 1.888 2.345 2.209 7.999;
6 2.007 2.401 2.315 7.310;
24 3.084 3.304 3.607 9.383;
48 3.138 4.036 3.801 6.325;
72 3.384 4.277 3.358 5.869;
96 3.458 4.170 6.029 5.509;
0 0.000 0.000 0.000 0.000;
0.5 1.300 2.210 1.877 0.593;
1 2.656 5.474 2.873 2.905;
2 3.715 6.608 3.950 3.136;
4 5.827 8.278 5.231 3.253;
6 6.156 10.287 5.463 3.040;
24 7.499 4.632 5.993 3.345;
48 10.894 6.140 6.358 3.035;
72 9.665 6.504 7.636 2.514;
96 10.238 7.190 7.218 3.057;
0 0.000 0.000 0.000 0.000;
0.5 3.107 2.533 4.736 7.112;
1 5.399 3.812 6.946 9.469;
2 8.380 7.816 8.635 11.985;
4 10.529 11.222 9.360 13.626;
6 11.676 11.995 -2.812 12.820;
24 16.876 16.699 13.864 14.149;
48 18.319 12.439 14.786 15.281;
72 19.918 14.004 14.085 15.777;
96 20.862 14.178 14.251 15.367;
0 0.000 0.000 0.000 0.000;
0.5 3.672 4.961 4.511 4.624;
1 6.439 7.577 8.947 10.016;
2 9.305 10.448 11.551 14.718;
4 11.535 13.271 14.288 20.018;
6 14.551 15.215 15.289 21.172;
24 17.663 17.651 19.526 25.897;
48 19.960 19.130 22.996 31.798;
72 21.560 20.203 24.794 23.337;
96 22.897 21.421 25.708 37.246;
0 0.000 0.000 0.000 0.000;
0.5 5.428 8.444 4.706 4.432;
```

```

1  6.496  14.407  7.142  5.761;
2  10.054 17.509 11.947  9.113;
4  11.846 20.920 15.945 13.800;
6  12.591 20.477 19.118 13.441;
24 18.571 24.545 21.701 15.813;
48 15.635 26.703 22.798 17.289;
72 19.265 24.287 22.490 17.107;
96 16.603 22.727 20.526 18.662];

% Data in dPinf is delta P infinity (g G/L)
dPinf=[0 3.3 3.9 4.2 6.8;
0.5 3.3 3.9 4.2 6.8;
1 3.3 3.9 4.2 6.8;
2 3.3 3.9 4.2 6.8;
4 3.3 3.9 4.2 6.8;
6 3.3 3.9 4.2 6.8;
24 3.3 3.9 4.2 6.8;
48 3.3 3.9 4.2 6.8;
72 3.3 3.9 4.2 6.8;
96 3.3 3.9 4.2 6.8;
0 9.6 6.1 6.8 3.0;
0.5 9.6 6.1 6.8 3.0;
1 9.6 6.1 6.8 3.0;
2 9.6 6.1 6.8 3.0;
4 9.6 6.1 6.8 3.0;
6 9.6 6.1 6.8 3.0;
24 9.6 6.1 6.8 3.0;
48 9.6 6.1 6.8 3.0;
72 9.6 6.1 6.8 3.0;
96 9.6 6.1 6.8 3.0;
0 19.0 14.3 14.2 15.1;
0.5 19.0 14.3 14.2 15.1;
1 19.0 14.3 14.2 15.1;
2 19.0 14.3 14.2 15.1;
4 19.0 14.3 14.2 15.1;
6 19.0 14.3 14.2 15.1;
24 19.0 14.3 14.2 15.1;
48 19.0 14.3 14.2 15.1;
72 19.0 14.3 14.2 15.1;
96 19.0 14.3 14.2 15.1;
0 20.5 19.6 23.3 29.6;
0.5 20.5 19.6 23.3 29.6;
1 20.5 19.6 23.3 29.6;
2 20.5 19.6 23.3 29.6;
4 20.5 19.6 23.3 29.6;
6 20.5 19.6 23.3 29.6;
24 20.5 19.6 23.3 29.6;
48 20.5 19.6 23.3 29.6;
72 20.5 19.6 23.3 29.6;
96 20.5 19.6 23.3 29.6;
0 17.5 24.6 21.9 17.2;
0.5 17.5 24.6 21.9 17.2;

```

```

1  17.5    24.6    21.9    17.2;
2  17.5    24.6    21.9    17.2;
4  17.5    24.6    21.9    17.2;
6  17.5    24.6    21.9    17.2;
24 17.5    24.6    21.9    17.2;
48 17.5    24.6    21.9    17.2;
72 17.5    24.6    21.9    17.2;
96 17.5    24.6    21.9    17.2];

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

% Solve the integrated MM equation for the combined data for the
% given enzyme loading, which returns a value for Ka, Vm, the fractal
% component f and the jamming component J

%TRADITIONAL MICHAELIS-MENTEN

% Define variables
for i=1:n(:,2)-1;
t=D(:,1); % time (hr)
Pt=D(:,i+1); % product (g G/L)
S0=dPinf(:,i+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)
E0=E0(:,i);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
Kaub=Kaub(:,i);

% Set initial guesses for parameters
Ka=10;
Vm=10;
f=0.5;
J=10;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm f J]';
F=MMintegrated_fj(x0, xdata);

% Use least squares estimates to find "best fit" for Ka, Vm, f and J
lb=[0 0 0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf 1 inf]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-12, 'TolX', 1e-12,...
'MaxFunEvals', 10000);
[x(:,i), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
(@(x0,xdata)
MMintegrated_fj(x0,xdata),x0,xdata,ydata,lb,ub,options)

```

```

% Use new parameters to predict P
H=MMintegrated_fj(x(:,i),xdata);

% Calculate F statistic
Hones=[ones(size(H)) H];
b1(:,i)=regress(Pt,Hones)

n=length(Pt);
xbar1=mean(Hones(:,2)); % mean of predicted product values
yhat1=(mean(Pt)+b1(2).*(Hones(:,2)-xbar1));
% calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat1).^2))/(n-2); % standard error
fstat(:,i)=((n-2)*(n*b1(1)^2+2*n*xbar1*b1(1)*(b1(2)-1)...
            +sum(Hones(:,2).^2*(b1(2)-1)^2)))/(2*n*S^2) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; %Sums of squares of observed
R2(:,i)=1-(resnorm/SST)

% Calculate 95% confidence intervals for parameters
ci=nlparci(x(:,i),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(H,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 25 0 25])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), H(1:10),'b-',
xdata(1:10),Pt(1:10),'b*',xdata(11:20),...
     H(11:20),'c-', xdata(11:20),Pt(11:20),'co',xdata(21:30),...
     H(21:30),'g-', xdata(21:30),Pt(21:30),'gd',xdata(31:40),...
     H(31:40),'m-', xdata(31:40),Pt(31:40),'m+',xdata(41:50),...
     H(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

% Determine the number of rows D(1) and columns D(2) that are in the
data
n=size(D);

%INSOLUBLE SOLIDS (HIGH SOLIDS) MICHAELIS-MENTEN

% Define variables
for j=1:n(:,2)-1;
t=D(:,1); % time (hr)

```

```

Pt=D(:,j+1); % product (g G/L)
S0=dPinf(:,j+1); % initial substrate loading (or delta P inf) (g G/L)
E0=[15 30 45 60]; % initial enzyme loading (FPU/g solids)
E0=E0(:,j);
P0=0; % initial product concentration (g G/L)
Kaub=[20.5 24.6 23.3 29.6];
Kaub=Kaub(:,j);

% Set initial guesses for parameters
Ka=10;
Vm=10;
f=0.5;
J=10;

% Pass information to function
xdata=t;
ydata=Pt;
x0=[Ka Vm f J]';
A=MMintegrated_E_fj(x0, xdata);

% Use least squares estimates to find "best fit" for Ka, Vm, f and J
lb=[0 0 0 0]; % lower bounds on Ka and Vm fitting
ub=[Kaub inf 1 inf]; % upper bounds on Ka and Vm fitting
options=optimset('MaxIter', 10000, 'TolFun', 1e-13, 'TolX', 1e-13,...
    'MaxFunEvals', 10000);
[x2(:,j), resnorm,
residual,exitflag,output,lambda,jacobian]=lsqcurvefit...
    (@MMintegrated_E_fj,x0,xdata,ydata,lb,ub, options)

k2(:,j)=x2(2)./S0;

% Use new parameters to predict P
C=MMintegrated_E_fj(x2(:,j),xdata);

% Calculate F statistic
Cones=[ones(size(C)) C];
b2(:,j)=regress(Pt,Cones)

n=length(Pt);
xbar2=mean(Cones(:,2)); % mean of predicted product values
yhat2=(mean(Pt)+b2(2).*(Cones(:,2)-xbar2));
    % calculated yhat2 (residual) for S-squared equation
S=(sum((Pt-yhat2).^2))/(n-2); % standard error
fstat(:,j)=(n-2)*(n*b2(1)^2+2*n*xbar2*b2(1)*(b2(2)-1)...
    +(sum((Cones(:,2).^2)*(b2(2)-1)^2)))/(2*n*S^2) % F-statistic

% Calculate R-squared of predicted vs. observed
CT=(sum(Pt)^2)/n;
SST=(Pt'*Pt)-CT; % sums of squares of observed
R2(:,j)=1-(resnorm/SST)

```

```

% Calculate 95% confidence intervals for parameters
ci=nlparci(x2(:,j),residual,'jacobian',jacobian)

% Plot predicted vs. observed values
figure
plot(C,Pt,'o')
xlabel('Predicted glucose (g G/L)')
ylabel('Observed glucose (g G/L)')
axis([0 25 0 25])

% Plot hydrolysis progress curves with predicted and observed values
figure
plot(xdata(1:10), C(1:10),'b-', xdata(1:10),Pt(1:10),'b*',...
      xdata(11:20), C(11:20),'c-', xdata(11:20),Pt(11:20),'co',...
      xdata(21:30), C(21:30),'g-', xdata(21:30),Pt(21:30),'gd',...
      xdata(31:40), C(31:40),'m-', xdata(31:40),Pt(31:40),'m+',...
      xdata(41:50), C(41:50),'k-', xdata(41:50),Pt(41:50),'ks')
xlabel('Time (hr)')
ylabel('Glucose (g G/L)')
end

```

```

function F = MMintegrated_fj(x0, xdata)

% MMintegrated_fj solves the integrated Michaelis-Menten equation
% with the fractal and jamming components for Pt (equation 8 Goudar
% et al, Buichimica et Biophysica Acta 1429(1999) 377-383)

global S0
global E0

P0=0; % initial product concentration (g G/L)

Vmtfj=(1-(E0./(x0(4).*S0))).*(x0(2).*xdata.^(1-x0(3)))./(1-x0(3));
% incorporation of fractal and jamming components
omega=(S0./x0(1)).*exp((S0-Vmtfj)./x0(1));
z=lambertw(omega);
F= P0+S0-x0(1).*z; % product concentration at time, t (g G/L)

end

```

```

function A = MMintegrated_E_fj(x0, xdata)

% MMintegrated_E_fj solves the integrated Michaelis-Menten equation
% modified for insoluble solids with the fractal and jamming
% components for Pt (equation 8 Goudar et al, Buichimica et
% Biophysica Acta 1429(1999) 377-383)

global S0
global E0

P0=0; % initial product concentration (g G/L)

Vmtfj=(1-(E0./(x0(4).*S0))).*(x0(2).*xdata.^(1-x0(3)))./(1-x0(3));
% incorporation of fractal and jamming components
A=((Vmtfj.*S0)./(x0(1)+E0));
% product concentration at time, t (g G/L)

end

```


APPENDIX D: MODEL FIGURES AND KINETIC PARAMETERS

D.1 ENZYMATIC HYDROLYSIS

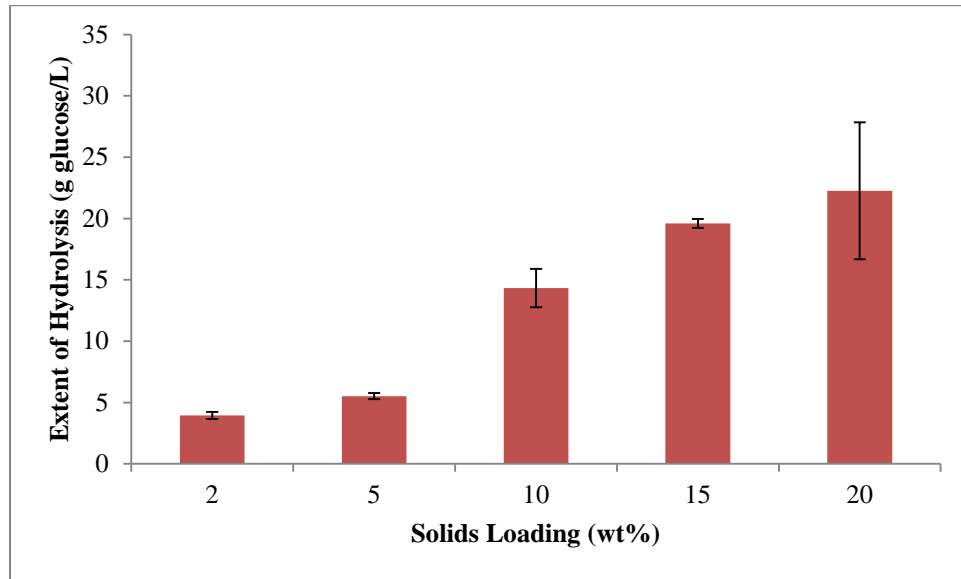


Figure D.1. Extent of glucose released after 96 hr of hydrolysis with 30 FPU/g solids. Columns labeled with the same letter are statistically the same at $\alpha=0.05$.

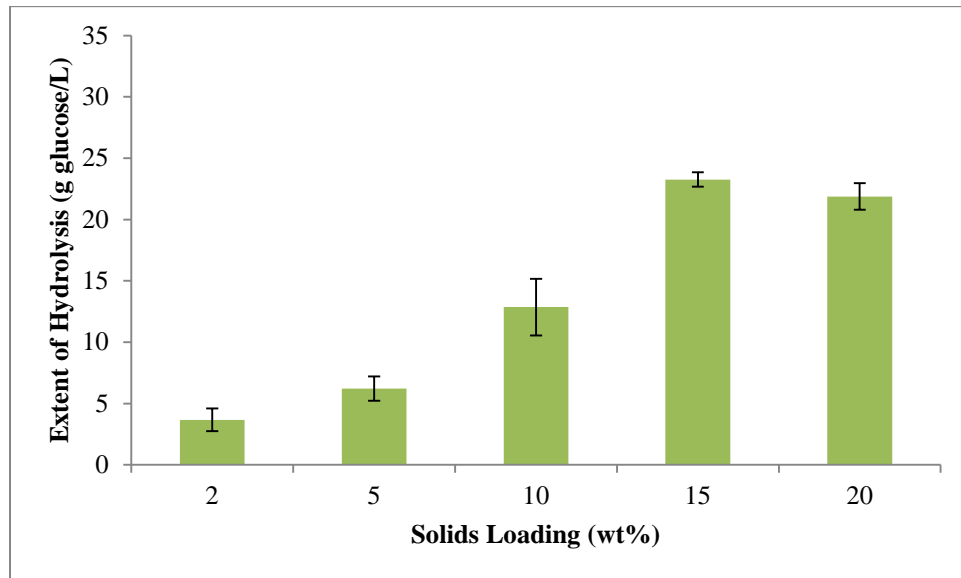


Figure D.2. Extent of glucose released after 96 hr of hydrolysis with 45 FPU/g solids. Columns labeled with the same letter are statistically the same at $\alpha=0.05$.

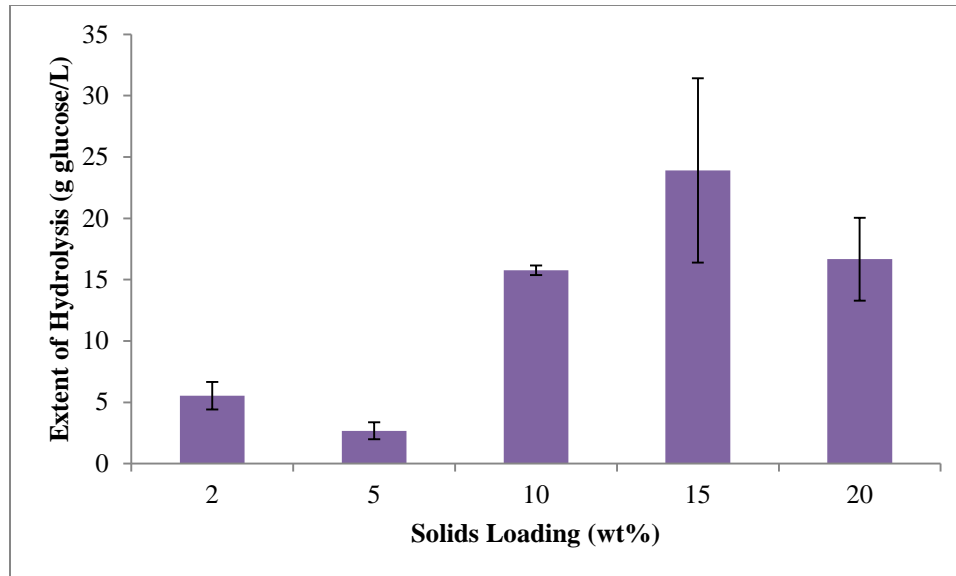


Figure D.3. Extent of glucose released after 96 hr of hydrolysis with 60 FPU/g solids. Columns labeled with the same letter are statistically the same at $\alpha=0.05$.

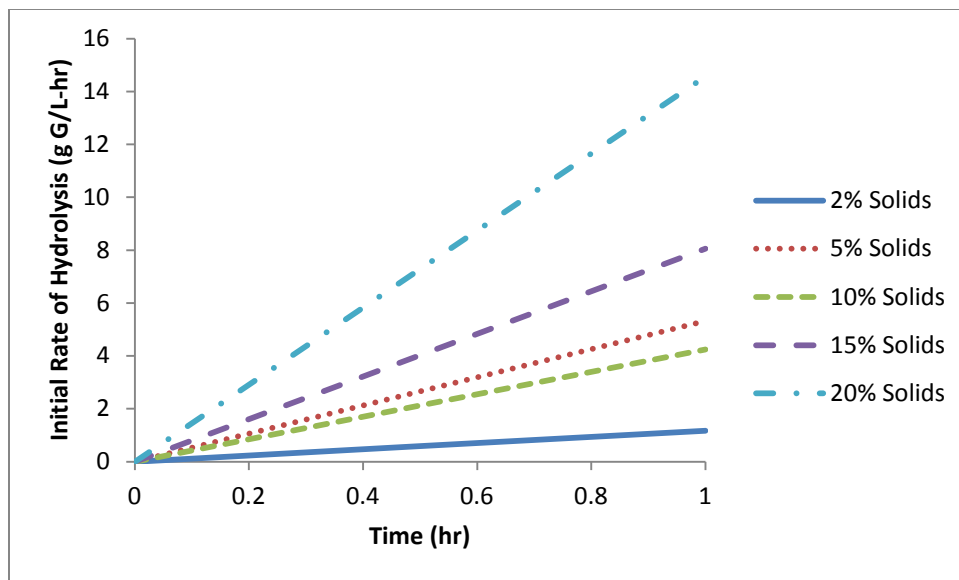


Figure D.4. Initial rates of hydrolysis. Hydrolysis was performed at the various solids loadings indicated with an enzyme loading of 30 FPU/g solids for 96 hr. Initial rates were determined manually from the first hour of hydrolysis. Rates with the same letter are statistically the same at $\alpha=0.05$.

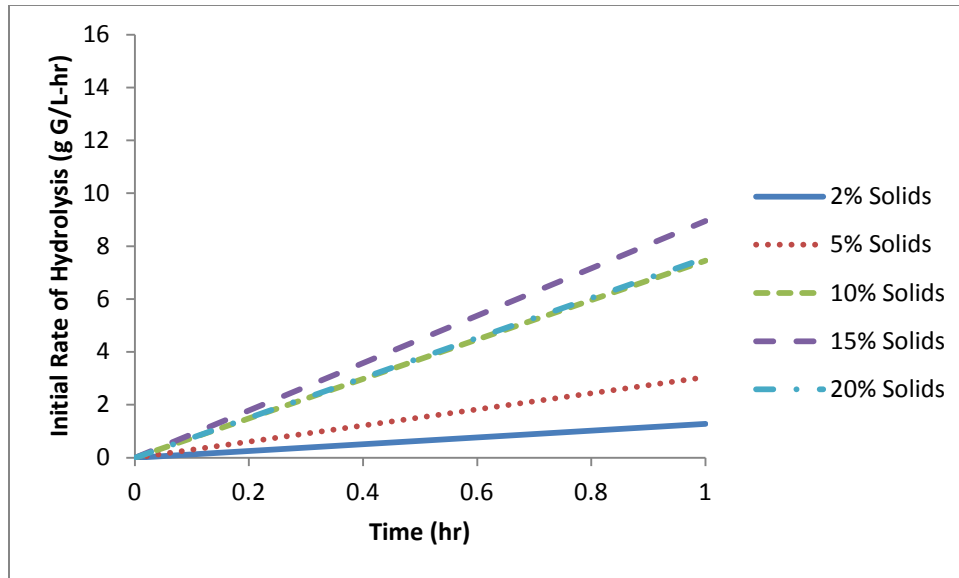


Figure D.5. Initial rates of hydrolysis. Hydrolysis was performed at the various solids loadings indicated with an enzyme loading of 45 FPU/g solids for 96 hr. Initial rates were determined manually from the first hour of hydrolysis. Rates with the same letter are statistically the same at $\alpha=0.05$.

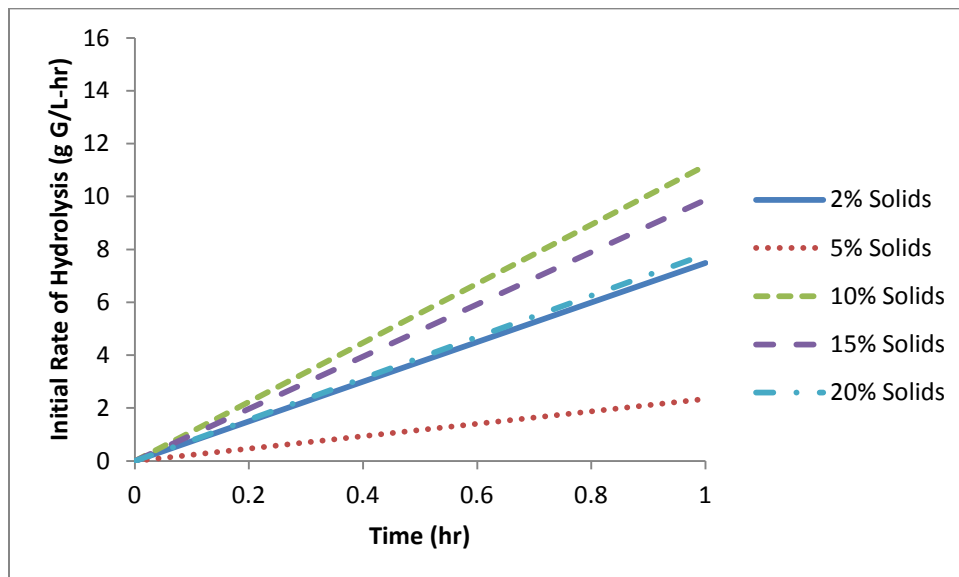


Figure D.6. Initial rates of hydrolysis. Hydrolysis was performed at the various solids loadings indicated with an enzyme loading of 60 FPU/g solids for 96 hr. Initial rates were determined manually from the first hour of hydrolysis. Rates with the same letter are statistically the same at $\alpha=0.05$.

D.2 MODEL PARAMETERS AND FIGURES

Table D.1. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 30 FPU/g solids using the classical Michaelis-Menten kinetic models.

	K_m (g/L)	V_m (g/L-hr)	f	j	R^2
MM	24.60	19.29	--	--	0.9327
MM+f	24.60	14.06	0.41	--	0.9513
MM+j	24.60	20.51	--	30.38	0.9329
MM+f+j	24.60	14.80	0.41	32.56	0.9516

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

Table D.2. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 30 FPU/g solids using the modified Michaelis-Menten kinetic models.

	K_m (FPU/g solids)	V_m (g/L-hr)	f	j	R^2
MM	23.47	0.77	--	--	-0.0753
MM+f	19.14	4.11	0.85	--	0.9150
MM+j	18.59	0.70	--	65722	-0.0753
MM+f+j	18.14	4.03	0.85	74228	0.9150

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

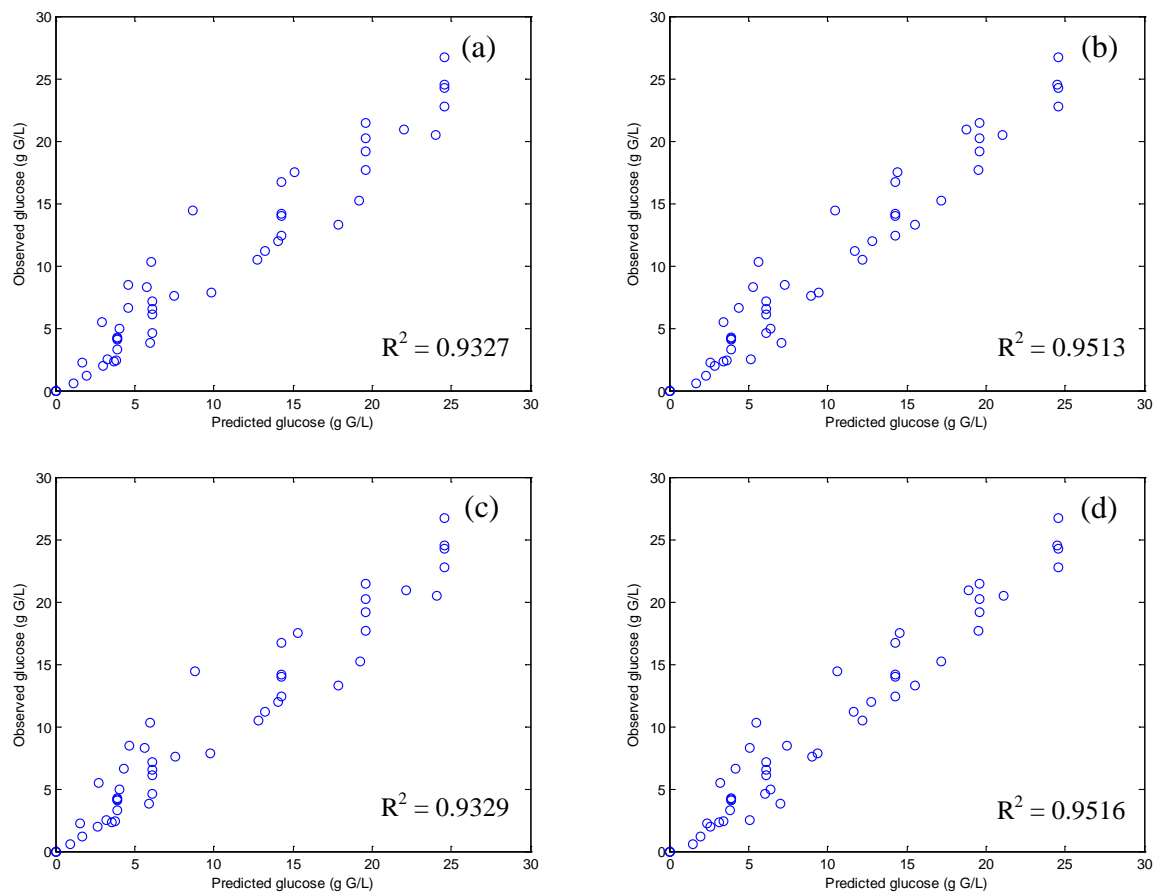


Figure D.7. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 30 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components.

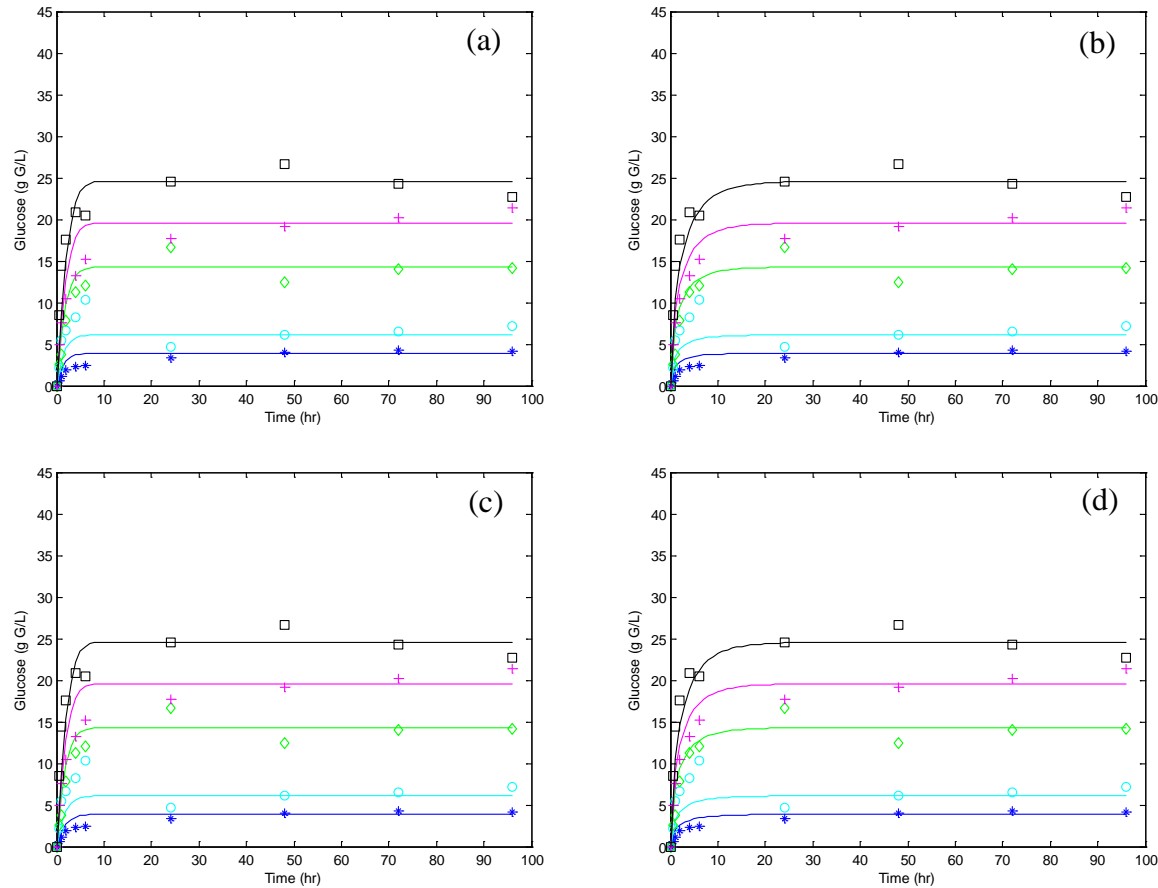


Figure D.8. PSC hydrolysis by *T. reesei* cellulase at 30 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components. (Symbols: ‘blue *’ 2% solids; ‘cyan o’ 5% solids; ‘green \diamond ’ 10% solids; ‘magenta +’ 15% solids; ‘black \square ’ 20% solids)

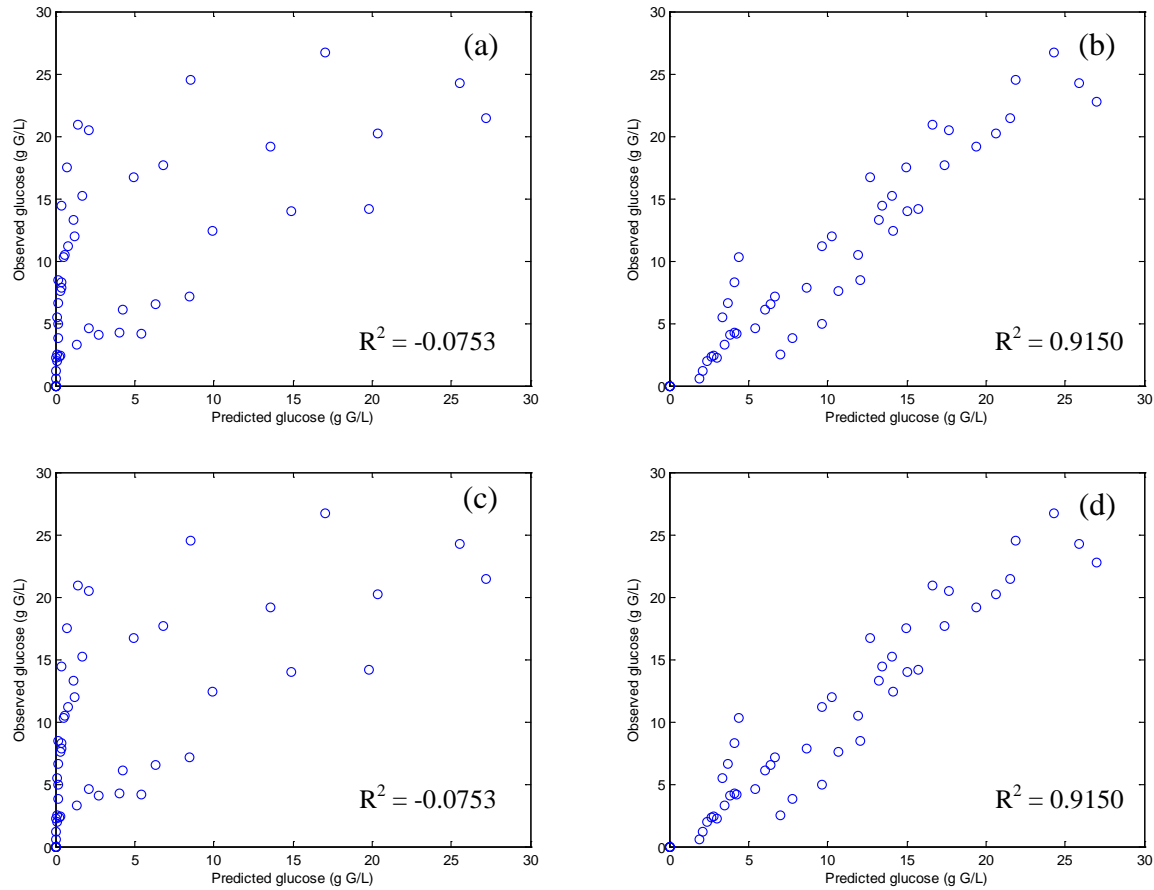


Figure D.9. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 30 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components.

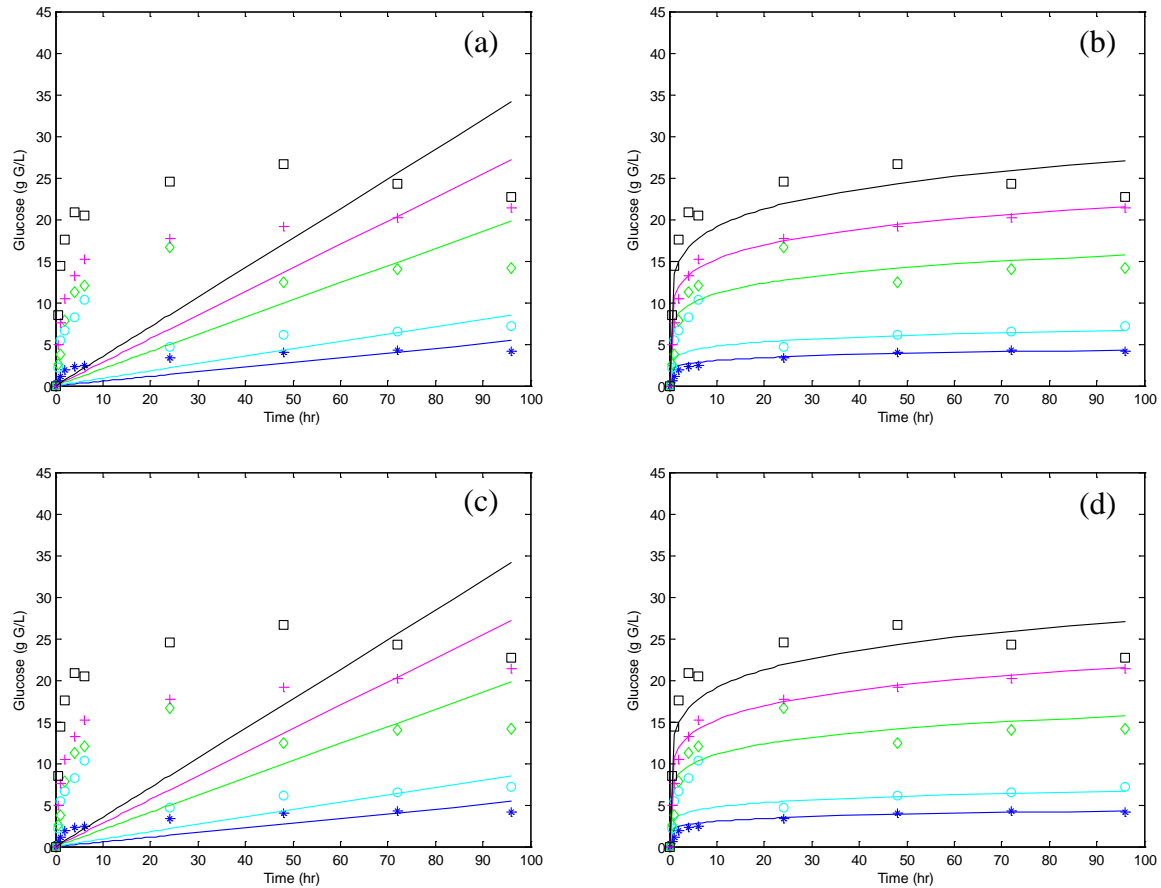


Figure D.10. PSC hydrolysis by *T. reesei* cellulase at 30 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components. (Symbols: 'blue *' 2% solids; 'cyan ○' 5% solids; 'green ◇' 10% solids; 'magenta +' 15% solids; 'black □' 20% solids)

Table D.3. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 45 FPU/g solids using the classical Michaelis-Menten kinetic models.

	K_m (g/L)	V_m (g/L-hr)	f	j	R^2
MM	23.30	10.39	--	--	0.8769
MM+f	23.30	8.60	0.52	--	0.9183
MM+j	23.30	11.94	--	17.91	0.8790
MM+f+j	23.30	9.84	0.53	19.42	0.9218

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

Table D.4. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 45 FPU/g solids using the modified Michaelis-Menten kinetic models.

	K_m (FPU/g solids)	V_m (g/L-hr)	f	j	R^2
MM	22.40	0.98	--	--	0.2259
MM+f	17.29	5.61	0.79	--	0.9103
MM+j	21.52	0.97	--	79585	0.2259
MM+f+j	10.61	5.06	0.79	229.35	0.9104

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

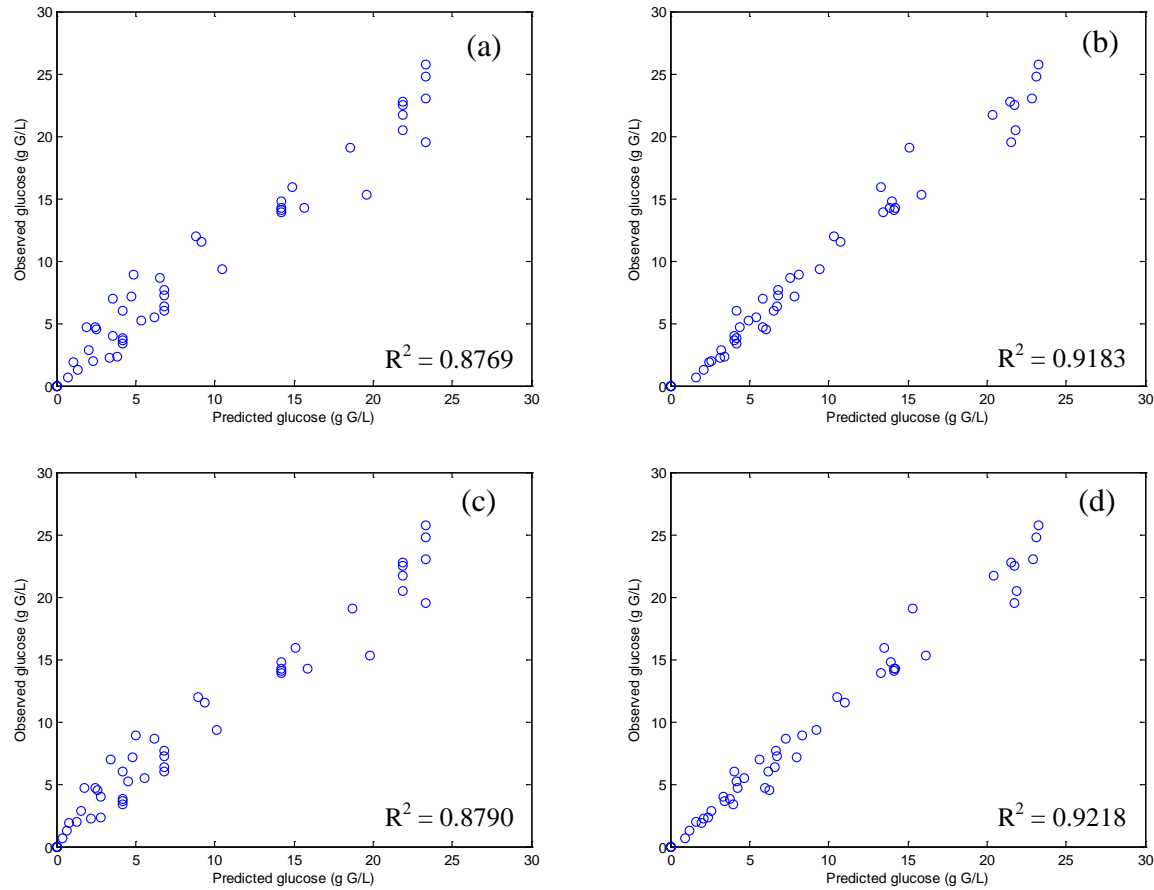


Figure D.11. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 45 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components.

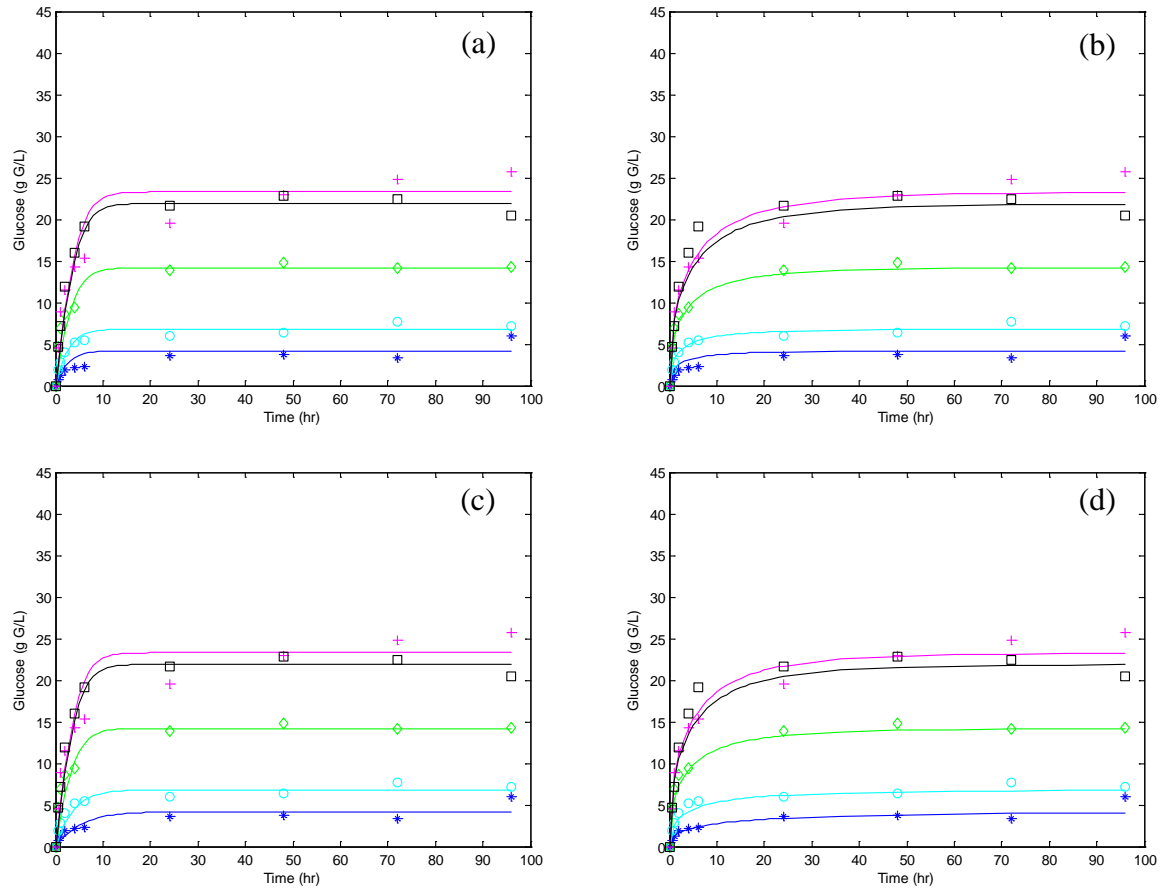


Figure D.12. PSC hydrolysis by *T. reesei* cellulase at 45 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components. (Symbols: ‘blue *’ 2% solids; ‘cyan o’ 5% solids; ‘green \diamond ’ 10% solids; ‘magenta +’ 15% solids; ‘black \square ’ 20% solids)

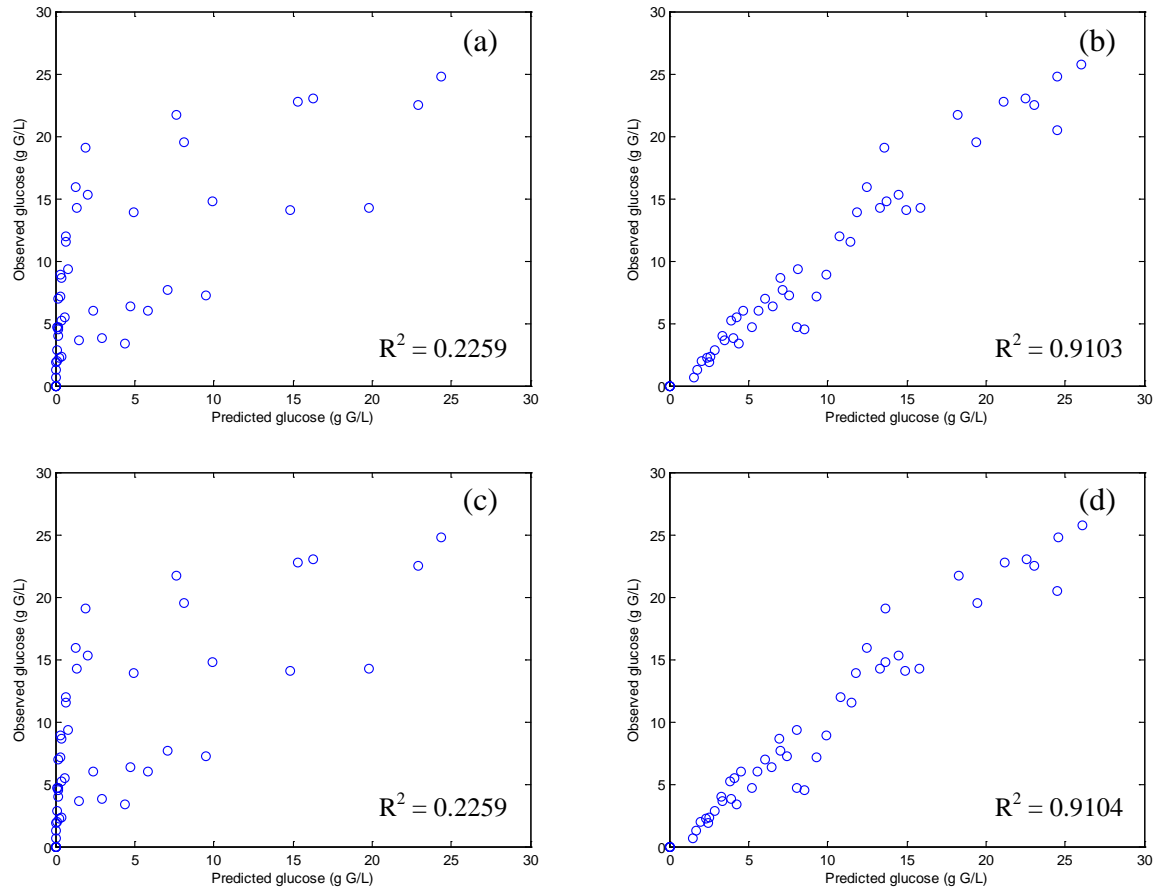


Figure D.13. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 45 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components.

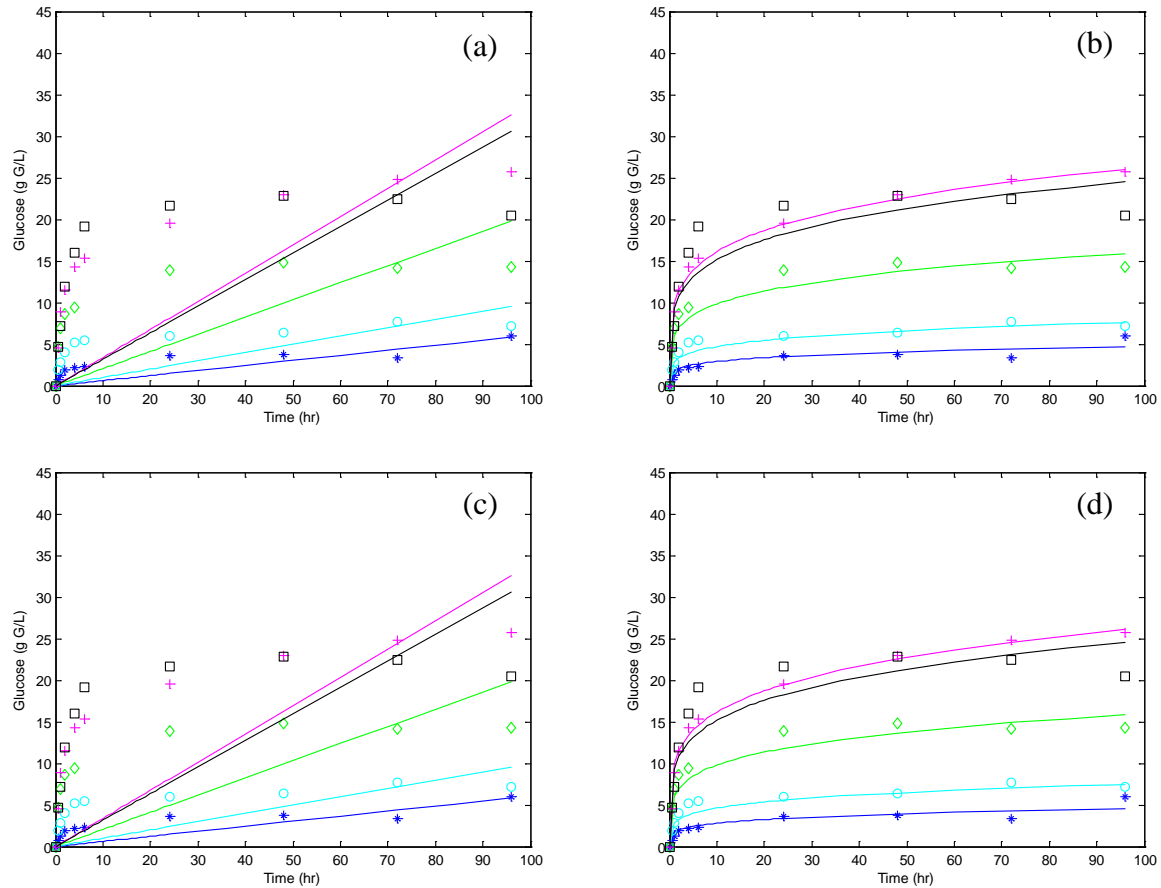


Figure D.14. PSC hydrolysis by *T. reesei* cellulase at 45 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components. (Symbols: 'blue *' 2% solids; 'cyan ○' 5% solids; 'green ◇' 10% solids; 'magenta +' 15% solids; 'black □' 20% solids)

Table D.5. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 60 FPU/g solids using the classical Michaelis-Menten kinetic models.

	K_m (g/L)	V_m (g/L-hr)	f	j	R^2
MM	25.54	15.54	--	--	0.9138
MM+f	5.81	5.90	0.51	--	0.9476
MM+j	25.53	15.67	--	48435	0.9138
MM+f+j	5.81	5.90	0.51	43463	0.9476

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

Table D.6. Kinetic parameters of PCS hydrolysis by *T. reesei* cellulase at 60 FPU/g solids using the modified Michaelis-Menten kinetic models.

	K_m (FPU/g solids)	V_m (g/L-hr)	f	j	R^2
MM	27.70	1.30	--	--	0.1147
MM+f	6.47	5.95	0.81	--	0.9026
MM+j	26.48	1.31	--	136.79	0.1150
MM+f+j	13.93	6.62	0.81	107990	0.9026

Abbreviations: MM, Michaelis-Menten; MM+f, Michaelis-Menten with fractal component; MM+j, Michaelis-Menten with jamming component; MM+f+j, Michaelis-Menten with fractal and jamming components

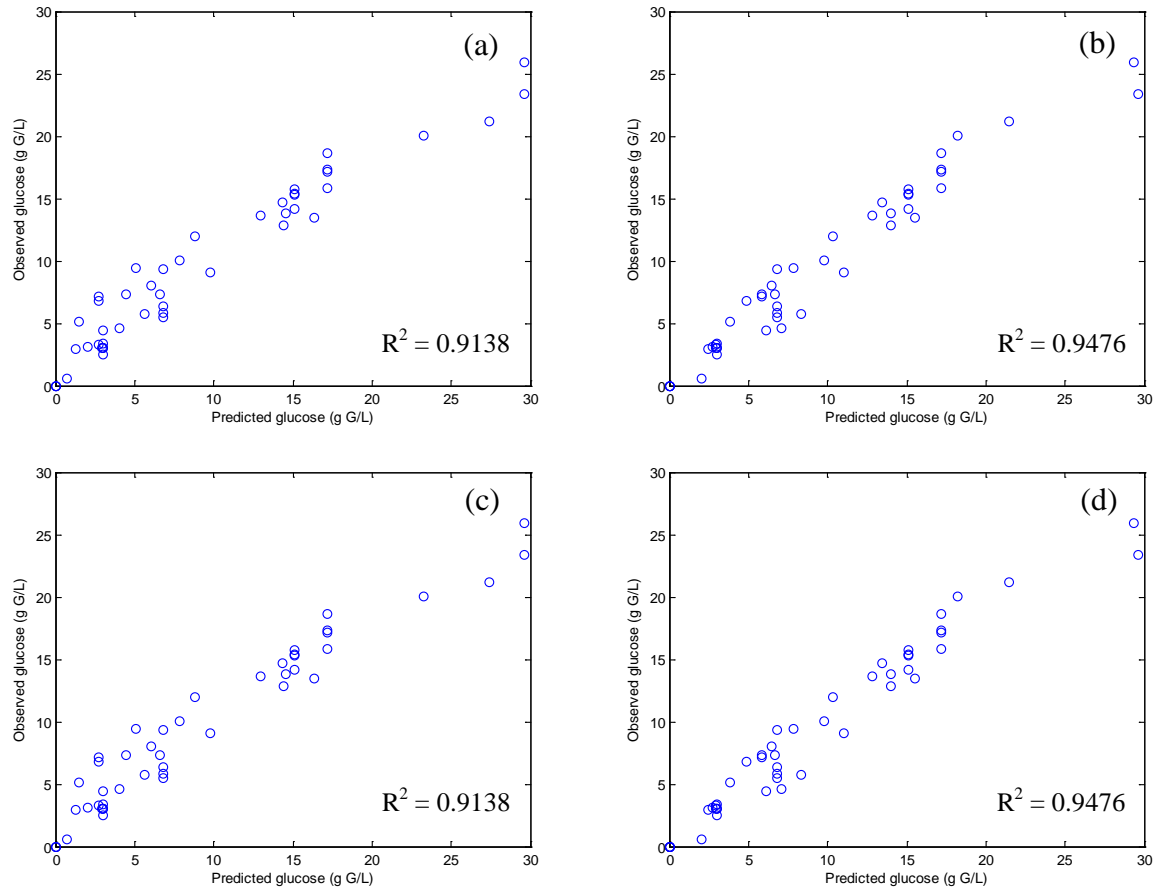


Figure D.15. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 60 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components.

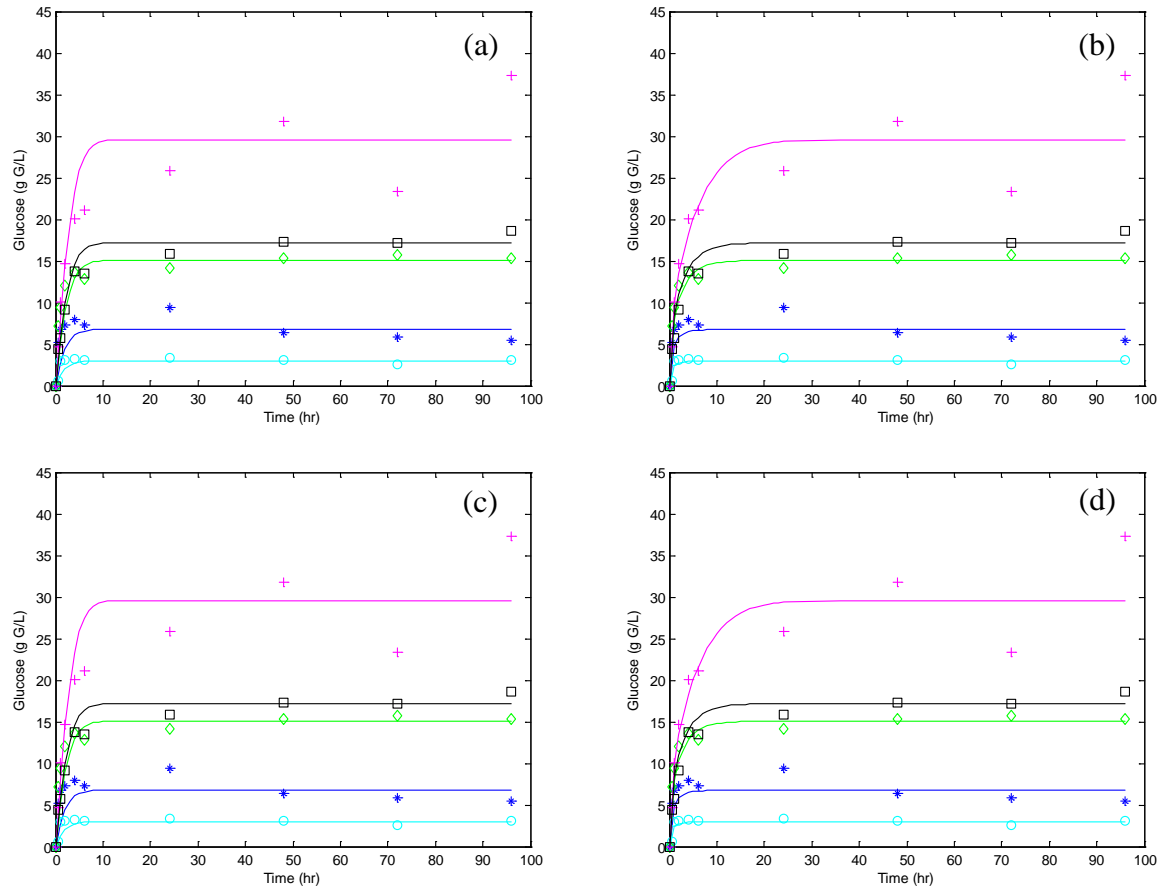


Figure D.16. PSC hydrolysis by *T. reesei* cellulase at 60 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model; (b) the Michaelis-Menten model with a fractal component; (c) the Michaelis-Menten model with a jamming component; and (d) the Michaelis-Menten model with fractal + jamming components. (Symbols: ‘blue *’ 2% solids; ‘cyan o’ 5% solids; ‘green \diamond ’ 10% solids; ‘magenta +’ 15% solids; ‘black \square ’ 20% solids)

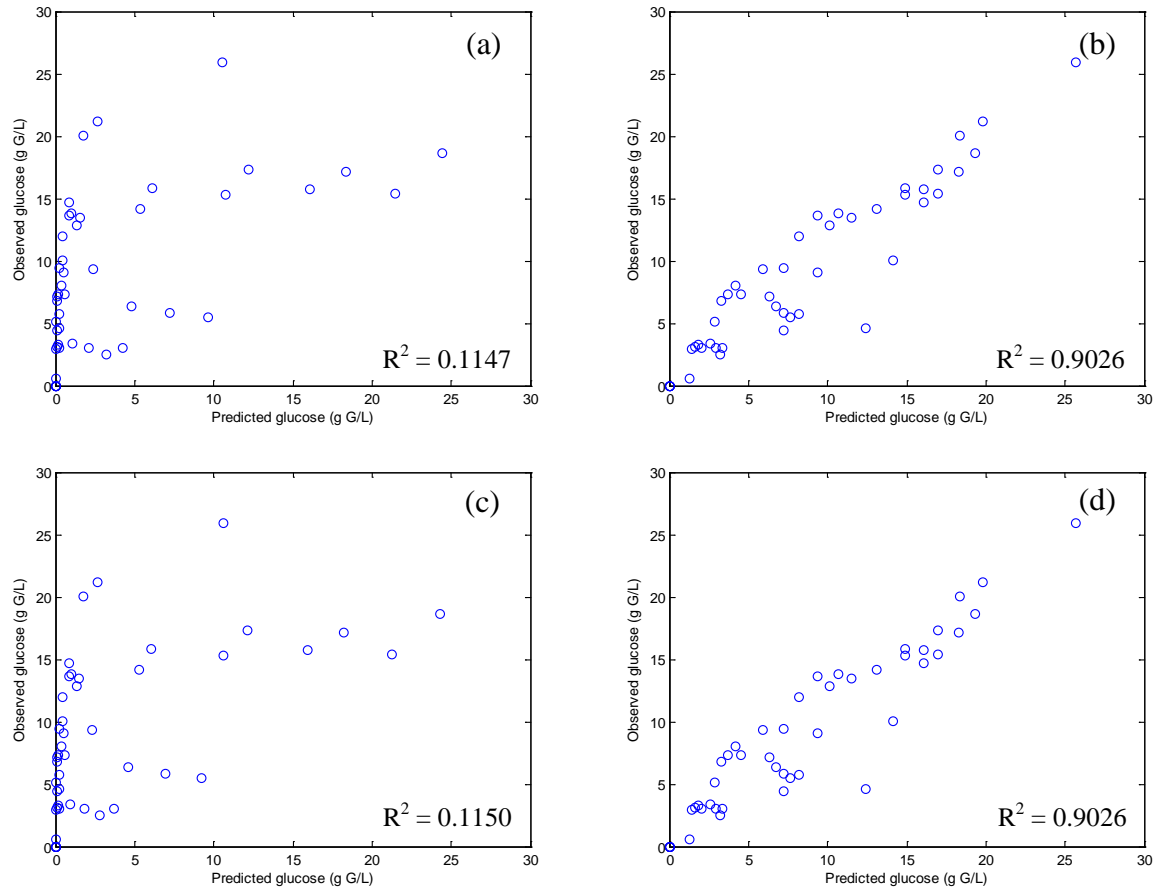


Figure D.17. Correlation between predicted and observed PCS hydrolysis by *T. reesei* cellulase at 60 FPU/g solids. Experimental hydrolysis data were used to fit the kinetic parameters of (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components.

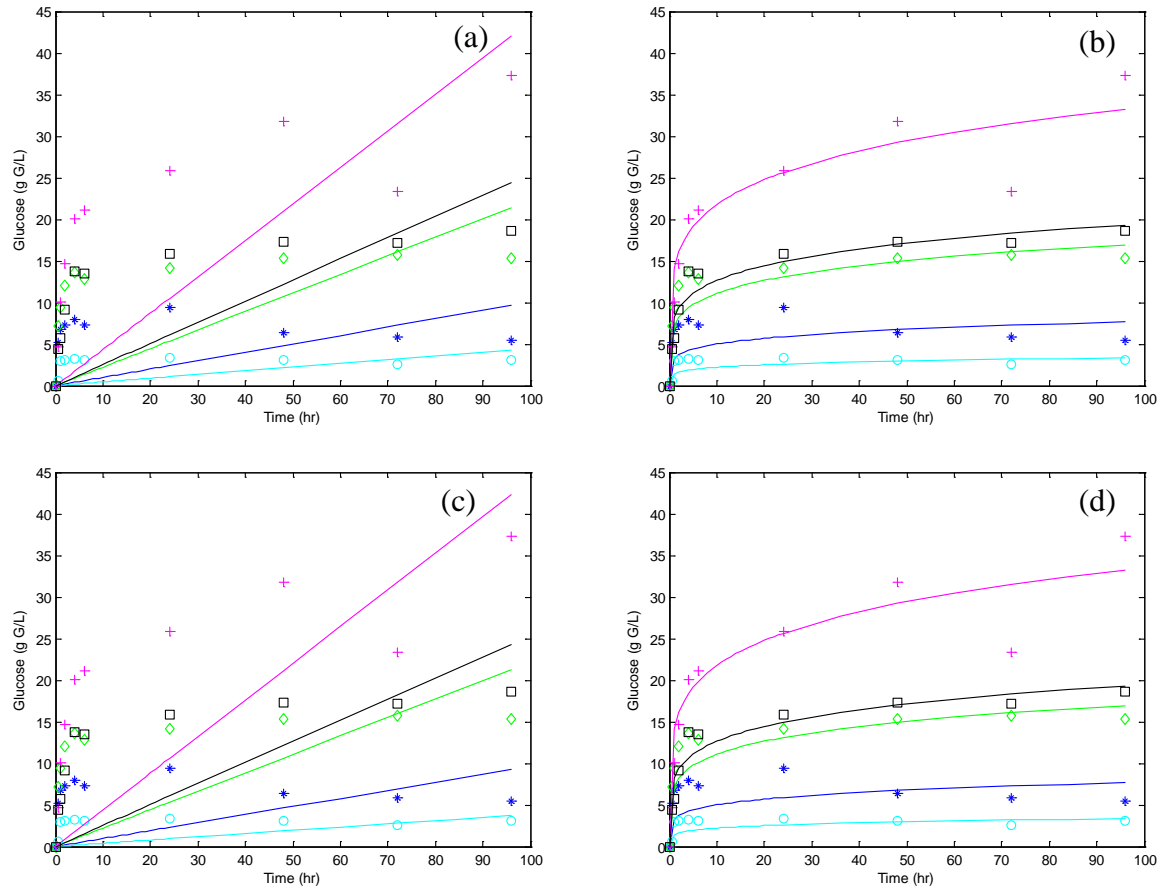


Figure D.18. PSC hydrolysis by *T. reesei* cellulase at 60 FPU/g solids. Experimental hydrolysis data are fitted with (a) the classical Michaelis-Menten model modified for insoluble substrates; (b) the modified Michaelis-Menten model with a fractal component; (c) the modified Michaelis-Menten model with a jamming component; and (d) the modified Michaelis-Menten model with fractal + jamming components. (Symbols: 'blue *' 2% solids; 'cyan o' 5% solids; 'green \diamond ' 10% solids; 'magenta +' 15% solids; 'black \square ' 20% solids)

APPENDIX E: SAS CODE

E.1 EFFECTS OF PRETREATMENT AND ENZYMATIC HYDROLYSIS CONDITIONS

```
ODS PDF FILE ="\\Client\F$\exp1conv.pdf";
```

```
DM 'CLEAR LOG';  
DM 'CLEAR OUTPUT';
```

```
DATA LOW_SOLIDS_HYDROLYSIS;  
INPUT BLOCK $ SOLIDS ENZYME GLU @@;  
DATALINES;
```

A	5	5.2	0.326	A	5	5.2	0.343	A	5	5.2	0.357
A	5	18.3	0.403	A	5	18.3	0.319	A	5	18.3	0.298
A	5	60	0.250	A	5	60	0.257	A	5	60	0.299
A	20	7.2	0.090	A	20	7.2	0.092	A	20	7.2	0.106
A	20	28.9	0.038	A	20	28.9	0.036	A	20	28.9	0.039
A	20	60	0.353	A	20	60	0.308	A	20	60	0.360
B	5	5.2	0.353	B	5	5.2	0.330	B	5	5.2	0.383
B	5	18.3	0.267	B	5	18.3	0.367	B	5	18.3	0.352
B	5	60	0.417	B	5	60	0.308	B	5	60	0.267
B	20	7.2	0.087	B	20	7.2	0.098	B	20	7.2	0.082
B	20	28.9	0.037	B	20	28.9	0.036	B	20	28.9	0.036
B	20	60	0.224	B	20	60	0.336	B	20	60	0.302
C	5	5.2	0.342	C	5	5.2	0.369	C	5	5.2	0.368
C	5	18.3	0.330	C	5	18.3	0.300	C	5	18.3	0.288
C	5	60	0.252	C	5	60	0.289	C	5	60	0.294
C	20	7.2	0.118	C	20	7.2	0.106	C	20	7.2	0.101
C	20	28.9	0.043	C	20	28.9	0.047	C	20	28.9	0.045
C	20	60	0.350	C	20	60	0.354	C	20	60	0.260
D	5	5.2	0.388	D	5	5.2	0.397	D	5	5.2	0.434
D	5	18.3	0.331	D	5	18.3	0.360	D	5	18.3	0.293
D	5	60	0.320	D	5	60	0.250	D	5	60	0.297
D	20	7.2	0.101	D	20	7.2	0.099	D	20	7.2	0.089
D	20	28.9	0.044	D	20	28.9	0.040	D	20	28.9	0.038
D	20	60	0.343	D	20	60	0.336	D	20	60	0.427
E	5	5.2	0.273	E	5	5.2	0.257	E	5	5.2	0.228
E	5	18.3	0.468	E	5	18.3	0.372	E	5	18.3	0.295
E	5	60	0.707	E	5	60	0.711	E	5	60	0.367
E	20	7.2	0.075	E	20	7.2	0.066	E	20	7.2	0.072
E	20	28.9	0.070	E	20	28.9	0.060	E	20	28.9	0.084
E	20	60	0.072	E	20	60	0.068	E	20	60	0.069
F	5	5.2	0.221	F	5	5.2	0.244	F	5	5.2	0.228
F	5	18.3	0.468	F	5	18.3	0.653	F	5	18.3	0.370
F	5	60	0.584	F	5	60	0.799	F	5	60	0.525
F	20	7.2	0.054	F	20	7.2	0.065	F	20	7.2	0.069

F	20	28.9	0.074	F	20	28.9	0.082	F	20	28.9	0.083
F	20	60	0.077	F	20	60	0.072	F	20	60	0.084
G	5	5.2	0.258	G	5	5.2	0.257	G	5	5.2	0.276
G	5	18.3	0.280	G	5	18.3	0.268	G	5	18.3	0.331
G	5	60	0.868	G	5	60	0.847	G	5	60	0.839
G	20	7.2	0.062	G	20	7.2	0.066	G	20	7.2	0.079
G	20	28.9	0.081	G	20	28.9	0.075	G	20	28.9	0.087
G	20	60	0.080	G	20	60	0.061	G	20	60	0.087
H	5	5.2	0.173	H	5	5.2	0.188	H	5	5.2	0.173
H	5	18.3	0.595	H	5	18.3	0.292	H	5	18.3	0.306
H	5	60	0.718	H	5	60	0.690	H	5	60	0.837
H	20	7.2	0.058	H	20	7.2	0.057	H	20	7.2	0.052
H	20	28.9	0.066	H	20	28.9	0.059	H	20	28.9	0.058
H	20	60	0.062	H	20	60	0.055	H	20	60	0.048

RUN;

```

PROC GLM DATA = LOW_SOLIDS_HYDROLYSIS;
CLASS BLOCK SOLIDS ENZYME;
MODEL GLU = BLOCK SOLIDS SOLIDS(ENZYME) BLOCK*SOLIDS
            BLOCK*SOLIDS(ENZYME) SOLIDS*SOLIDS(ENZYME)
            BLOCK*SOLIDS*SOLIDS(ENZYME);
MEANS BLOCK SOLIDS SOLIDS(ENZYME) BLOCK*SOLIDS
        BLOCK*SOLIDS(ENZYME) SOLIDS*SOLIDS(ENZYME)
        BLOCK*SOLIDS*SOLIDS(ENZYME)/TUKEY;
LSMEANS BLOCK SOLIDS SOLIDS(ENZYME) BLOCK*SOLIDS
          BLOCK*SOLIDS(ENZYME) SOLIDS*SOLIDS(ENZYME)
          BLOCK*SOLIDS*SOLIDS(ENZYME)/PDIFF;
RUN;
```

ODS PDF CLOSE;

E.2 NAOH LOADING IN PRETREATMENT

ODS PDF FILE = "\\Client\F\$\NaOHexp.pdf";

DM 'CLEAR LOG';
DM 'CLEAR OUTPUT';

DATA NAOH_LOADING;
INPUT PTRT_TIME NAOH EH_SOLIDS GLU @@;
DATALINES;

2	4	5	0.163	2	4	5	0.167	2	4	5	0.183
2	4	20	0.028	2	4	20	0.031	2	4	20	0.028
2	10	5	0.131	2	10	5	0.133	2	10	5	0.139
2	10	20	0.019	2	10	20	0.019	2	10	20	0.033
2	20	5	0.037	2	20	5	0.122	2	20	5	0.110
2	20	20	0.001	2	20	20	0.002	2	20	20	0.002
24	4	5	0.085	24	4	5	0.081	24	4	5	0.086
24	4	20	0.026	24	4	20	0.010	24	4	20	0.026
24	10	5	0.215	24	10	5	0.250	24	10	5	0.224
24	10	20	0.041	24	10	20	0.038	24	10	20	0.028
24	20	5	0.243	24	20	5	0.278	24	20	5	0.273
24	20	20	0.001	24	20	20	0.001	24	20	20	0.001

RUN;

PROC GLM DATA = NAOH_LOADING;
CLASS PTRT_TIME NAOH EH_SOLIDS;
MODEL GLU = PTRT_TIME NAOH EH_SOLIDS PTRT_TIME*NAOH
PTRT_TIME*EH_SOLIDS NAOH*EH_SOLIDS
PTRT_TIME*NAOH*EH_SOLIDS;
MEANS PTRT_TIME NAOH EH_SOLIDS PTRT_TIME*NAOH
PTRT_TIME*EH_SOLIDS NAOH*EH_SOLIDS
PTRT_TIME*NAOH*EH_SOLIDS/TUKEY;
LSMEANS PTRT_TIME NAOH EH_SOLIDS PTRT_TIME*NAOH
PTRT_TIME*EH_SOLIDS NAOH*EH_SOLIDS
PTRT_TIME*NAOH*EH_SOLIDS/PDIFF;
RUN;

ODS PDF CLOSE;

E.3 HYDROLYZATE FLUSHING AND SUBSTRATE RECYCLE

ODS PDF FILE = "\\Client\F\$\flush.pdf";

DM 'CLEAR LOG';
DM 'CLEAR OUTPUT';

DATA HYDROLYSIS;
INPUT TREATMENTS \$ ENZ HYD \$ GLU @@;
DATALINES;

W	15	B	60.41	W	15	B	55.32
W	15	B	54.47	W	15	B	55.53
W	15	FNS	96.23	W	15	FNS	98.85
W	15	FS	98.95	W	15	FS	95.38
W	60	B	56.40	W	60	B	61.87
W	60	B	47.85	W	60	B	47.85
W	60	FNS	117.04	W	60	FNS	109.89
W	60	FS	105.61	W	60	FS	101.48
UW	15	B	48.95	UW	15	B	44.88
UW	15	B	49.61	UW	15	B	50.29
UW	15	FNS	75.16	UW	15	FNS	71.47
UW	15	FS	97.87	UW	15	FS	97.09
UW	60	B	41.94	UW	60	B	41.02
UW	60	B	41.37	UW	60	B	45.09
UW	60	FNS	84.98	UW	60	FNS	84.75
UW	60	FS	107.52	UW	60	FS	100.57

RUN;

PROC GLM DATA = HYDROLYSIS;
CLASS TREATMENTS ENZ HYD;
MODEL GLU = TREATMENTS ENZ HYD TREATMENTS*ENZ
TREATMENTS*HYD ENZ*HYD TREATMENTS*ENZ*HYD;
MEANS TREATMENTS ENZ HYD TREATMENTS*ENZ TREATMENTS*HYD
ENZ*HYD TREATMENTS*ENZ*HYD/TUKEY;
LSMEANS TREATMENTS ENZ HYD TREATMENTS*ENZ TREATMENTS*HYD
ENZ*HYD TREATMENTS*ENZ*HYD/PDIFF;
RUN;

ODS PDF CLOSE;

E.4 INITIAL RATE OF HYDROLYSIS FOR MODEL DATA

ODS PDF FILE = "\\Client\F\$\initialrate.pdf";

DM 'CLEAR LOG';

DM 'CLEAR OUTPUT';

DATA INITIAL_RATE;

INPUT ENZ SUB RATE@@;

DATALINES;

15	2	1.102	15	2	1.172	15	2	1.170
30	2	1.048	30	2	1.248	30	2	1.192
45	2	1.328	45	2	1.298	45	2	1.192
60	2	7.464	60	2	7.342	60	2	7.668
15	5	2.645	15	5	2.710	15	5	2.585
30	5	5.670	30	5	6.430	30	5	3.870
45	5	3.005	45	5	3.205	45	5	2.940
60	5	1.895	60	5	2.385	60	5	2.745
15	10	5.390	15	10	6.110	15	10	5.190
30	10	6.560	30	10	3.720	30	10	2.450
45	10	7.780	45	10	7.870	45	10	6.710
60	10	10.490	60	10	12.990	60	10	10.030
15	15	6.255	15	15	6.675	15	15	6.915
30	15	8.565	30	15	8.055	30	15	7.530
45	15	9.075	45	15	8.925	45	15	8.865
60	15	9.840	60	15	10.305	60	15	9.450
15	20	23.200	15	20	6.340	15	20	7.960
30	20	15.800	30	20	9.660	30	20	18.240
45	20	7.300	45	20	7.840	45	20	7.500
60	20	6.840	60	20	10.480	0	20	6.100

RUN;

PROC GLM DATA = INITIAL_RATE;

CLASS ENZ SUB;

MODEL RATE=ENZ SUB ENZ*SUB;

MEANS ENZ SUB ENZ*SUB/TUKEY;

LSMEANS ENZ SUB ENZ*SUB/PDIFF;

RUN;

ODS PDF CLOSE

E.5 EXTENT OF HYDROLYSIS FOR MODEL DATA

ODS PDF FILE = "\\Client\F\$\extent.pdf";

DM 'CLEAR LOG';

DM 'CLEAR OUTPUT';

DATA EXTENT;

INPUT ENZ SUB GLU@@;

DATALINES;

15	2	3.199	15	2	3.216	15	2	3.384
15	5	9.995	15	5	9.357	15	5	9.370
15	10	19.666	15	10	19.098	15	10	18.217
15	15	20.611	15	15	20.980	15	15	19.969
15	20	29.141	15	20	9.246	15	20	18.965
30	2	3.621	30	2	4.101	30	2	4.118
30	5	5.714	30	5	5.596	30	5	5.258
30	10	16.113	30	10	13.164	30	10	13.714
30	15	19.296	30	15	20.009	30	15	19.498
30	20	16.422	30	20	22.798	30	20	27.539
45	2	2.598	45	2	4.129	45	2	4.283
45	5	6.894	45	5	5.088	45	5	6.684
45	10	10.198	45	10	14.288	45	10	14.084
45	15	23.814	45	15	23.313	45	15	22.642
45	20	22.474	45	20	22.527	45	20	20.636
60	2	5.627	60	2	6.603	60	2	4.361
60	5	2.893	60	5	3.225	60	5	1.896
60	10	15.631	60	10	16.213	60	10	15.463
60	15	22.496	60	15	17.206	60	15	32.010
60	20	12.955	60	20	19.538	60	20	17.513

RUN;

PROC GLM DATA = EXTENT;

CLASS ENZ SUB;

MODEL GLU=ENZ SUB ENZ*SUB;

MEANS ENZ SUB ENZ*SUB/TUKEY;

LSMEANS ENZ SUB ENZ*SUB/PDIFF;

RUN;

ODS PDF CLOSE;

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Place of Birth: Baton Rouge, Louisiana

Educational Institutions Attended and Degrees Awarded

Masters of Science Biosystems and Agricultural Engineering, University of Kentucky, Lexington, KY (2008).

Bachelors of Science Biological and Agricultural Engineering, Louisiana State University, Baton Rouge, LA (2006), Magna Cum Laude.

Professional Positions

Graduate Research Assistant, Department of Biosystems and Agricultural Engineering, University of Kentucky (August 2011-August 2013).

National Science Foundation Fellow, Department of Biosystems and Agricultural Engineering, University of Kentucky (August 2008-July 2011).

Graduate Research Assistant, Department of Biosystems and Agricultural Engineering, University of Kentucky (August 2006-August 2008).

Undergraduate Research Assistant, Department of Biological and Agricultural Engineering, Louisiana State University (June 2003-July 2006).

Scholastic and Professional Honors

New Faces of ASABE (2011)

2010 LSU Chancellor's Sesquicentennial Service Award (2010)

National Science Foundation Fellowship (2008-2011)

Donald W. Clayton Engineering Excellence Award for Outstanding Undergraduate Students (2006)

Carl H. Thomas Memorial Scholarship (2005)

Blue Angels' Alumni Association Scholarship (2004, 2005)

LSU Centennial Scholarship (2002-2006)

Pegues Scholarship (2002-2006)

TOPS Scholarship – Honors Award (2002-2006)

LSU Dean's List (4 semesters)

LSU Chancellor's List (3 semesters)

Professional Publications and Presentations

Modenbach, A.A., Nokes, S.E. Towards improving enzymatic hydrolysis of sodium hydroxide pretreated corn stover at high-solids loadings. *Presented at the American Society of Agricultural and Biological Engineering Annual International Meeting, Kansas City, MO, 2013.*

Gray, K., Nokes, S., Montross, M., **Modenbach, A.,** Jackson, J. Investigation of alkaline hydrogen peroxide pretreatment for its use in an on-farm butanol bioprocessing facility. *Presented at the American Society of Agricultural and Biological Engineering Annual International Meeting, Kansas City, MO, 2013.*

Modenbach, A.A., Nokes, S.E. 2013. Effects of sodium hydroxide pretreatment on structural components of biomass. *Transactions of the ASABE.* (In review).

Modenbach, A.A., Nokes, S.E. 2012. Enzymatic hydrolysis of biomass at high-solids loadings – A review. *Biomass and Bioenergy.* 56: 526-544.

Modenbach, A., Nokes, S., Montross, M., Knutson, B. 2012. Characterization of soluble and insoluble inhibitor effects on enzymatic hydrolysis at high solids using pretreated corn stover. *Presented at the American Society of Agricultural and Biological Engineering Annual International Meeting, Dallas, TX, 2012.*

Modenbach, A.A., Nokes, S.E. 2011. The use of high-solids loadings in biomass pretreatment – A review. *Biotechnology and Bioengineering.* 109(6): 1430-1442.

Modenbach, A., Nokes, S., Knutson, B., Rankin, S. 2011. Recovery of a Purified Stream of C5 Sugars from Lignocellulosic Hydrolyzate Using Microphase-Directed Imprinted Materials. *Presented at the American Society of Agricultural and Biological Engineering Annual International Meeting, Louisville, KY, 2011.*

Modenbach, A., Nokes, S., Knutson, B., Rankin, S. 2011. Recovery of a Purified Stream of C5 Sugars from Lignocellulosic Hydrolyzate Using Microphase-Directed

- Imprinted Materials. *Presented at the S-1041 Annual Symposium, Stillwater, OK, 2011.*
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- Abadie, A.,** Nokes, S.E., Chen, W. 2008. Quantifying Cellulase in High-Solid Systems. *Presented at the American Society of Agricultural and Biological Engineers Annual International Meeting, Providence, RI, 2008.*
- Abadie, A.,** Christy, A., Jones, J., Wang, J., Lima, M. 2008. Longitudinal survey of female faculty in biological and agricultural engineering. *Transactions of the ASABE.*
- Schramm, R., **Abadie, A.,** Hua, N., Xu, Z., Lima, M. 2007. Fractionation of the rice bran layer and quantification of vitamin E, oryzanol, protein, and rice bran saccharide. *Journal of Biological Engineering.* 1:9.
- Abadie, A.,** Bridges, T.K., Lima, M. 2006. Minimizing the Environmental Impact of Construction Materials in Playground Design. *Presented at the American Society of Agricultural and Biological Engineers Annual International Meeting Undergraduate Poster Competition, Portland, OR, 2006.*
- Abadie, A.,** Bridges, T.K., Qureshi, T., Lima, M. 2006. Minimizing the Environmental Impact of Construction Materials in Playground Design. *Presented at the Environmental Protection Agency's P3: People, Prosperity, and the Planet Student Design Competition for Sustainability, Washington, D.C., 2006.*
- Abadie, A.,** Lima, M. 2005. The Quantification and Extraction of an Anti-tumor Compound from Rice Bran. *Presented at the American Society of Agricultural Engineers Annual International Meeting Undergraduate Poster Competition, Tampa, FL, 2005.*

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