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QUANTIFYING CELLULASE IN HIGH-SOLIDS ENVIRONMENTS

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ABSTRACT OF THESIS

QUANTIFYING CELLULASE IN HIGH-SOLIDS ENVIRONMENTS

In recent years, fungal and bacterial cellulases have gained popularity for the conversion of lignocellulosic material to biofuels and biochemicals. This study investigated properties of fungal (*Trichoderma reesei*) and bacterial (*Clostridium thermocellum*) cellulases. Enzymatic hydrolysis was carried out with *T. reesei* using nine enzyme concentration and substrate combinations. Initial rates and extents of hydrolysis were determined from the progress curve of each combination. Inhibition occurred at the higher enzyme concentrations and higher solids concentrations. Mechanisms to explain the observed inhibition are discussed. Samples of *C. thermocellum* purified free cellulase after 98% hydrolysis were assayed to determine the total protein content (0.15 ± 0.08 mg/mL), the enzymatic activity (0.306 ± 0.173 IU/mL) and the cellulosome mass using the Peterson method for protein determination, the cellulase activity assay with phenol-sulfuric acid assay, and the indirect ELISA adapted for *C. thermocellum* cellulosomes, respectively. Issues regarding reproducibility and validity of these assays are discussed.

KEYWORDS: enzymatic hydrolysis, *T. reesei*, *C. thermocellum*, ELISA, cellulase activity

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THESIS

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The Graduate School
University of Kentucky
2008

QUANTIFYING CELLULASE IN HIGH-SOLIDS ENVIRONMENTS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Engineering
at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2008

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To my Momo,
I love you and miss you.

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CHAPTER ONE: INTRODUCTION

OVERVIEW OF THE LIGNOCELLULOSE TO ETHANOL PROCESS

Lignocellulosic biomass is an abundant source of renewable feedstock that can be converted into ethanol or other biobased products through hydrolysis and fermentation processes (Chang and Holtzapple, 2000; Moxley and Zhang, 2007; Reese, 1956). As the price of petroleum increases and the supply decreases, the search for alternative fuel sources intensifies. The new energy source must be economical and environmentally responsible in order to gain general public acceptance. One technology thought to be a partial solution to the petroleum crisis is lignocellulosic ethanol. Lignocellulose is plant biomass typically from a non-food source (such as agricultural residues or forestry wastes) that can be hydrolyzed to fermentable sugars, which are converted into ethanol via fermentation with yeast.

With more than one billion tons of lignocellulosic biomass produced annually in the United States alone (Chang, 2007; Perlack, 2005; Sticklen, 2007), technologies are emerging for the conversion of this waste material into useful and valuable products. The use of transportation fuels from biomass also reduces the accumulation of atmospheric greenhouse gases (Sticklen, 2007) because it is a carbon neutral process. The carbon dioxide released in the conversion process is consumed during the growth of the plants.

Lignocellulose (Figure 1.1) is composed of three main components: cellulose (30-50 wt%), hemicellulose (20-35 wt%) and lignin (5-30 wt%) (Zhang and Lynd, 2004) and is the material that forms the cell wall of plants (Chang, 2007).

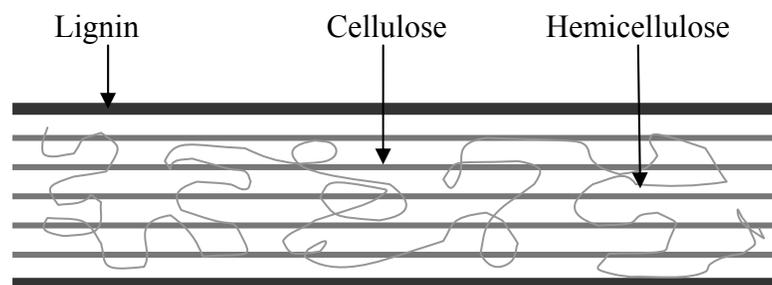


Figure 1.1. Cross-section of lignocellulose. The cellulose is embedded within the hemicellulose matrix and surrounded by an outer layer of lignin. The cross-section of lignocellulose can range from 7 to 30 nm.

Cellulose is a polysaccharide composed of linearly β -1,4-linked glucose residues. These linkages create a microcrystalline structure that is very stable due to the resulting inter- and intra-strand hydrogen bonds and van der Waals forces (Chang, 2007; Zhang and Lynd, 2004). The chains of glucose create microfibril bundles, which are embedded within hemicellulose and lignin and form the basis of the plant cell wall. The lignocellulosic structure, however, decreases the cellulose surface accessible for enzyme adsorption (Chang, 2007; Sticklen, 2007; Zhang and Lynd, 2004), thereby slowing hydrolysis. Cellulose accessibility is one of the top research priorities in the push to develop a process to convert lignocellulosic biomass into valuable products.

Hemicellulose is another polysaccharide found in lignocellulose. It is made up of a β -1,4-linked xylose backbone and can contain branches and hexose and pentose residues (Moxley and Zhang, 2007; Sticklen, 2007). Due to the various substitutions and branch-points of hemicellulose, its structure is more random and amorphous in comparison to cellulose (Sticklen, 2007). Hemicellulose is usually removed during pretreatment; however, it can be removed during hydrolysis but is generally not because the presence of pentoses restricts the rate of cellulose hydrolysis. Another disadvantage of hydrolyzing hemicellulose with the cellulose is that many of the microbes presently being studied to convert the sugars into ethanol are not capable of fermenting pentose sugars (Chang, 2007).

Lignin is the third major component of lignocellulose and is the most structurally complex. Its complexity reflects the three different components of lignin and the various structural characteristics they incorporate (Chang, 2007; Sticklen, 2007). Extensive cross-linking and structural heterogeneity impede disassembly of lignin into its components. Although a problem for hydrolyzing cellulose, the lignin layer of a plant cell wall is the plant's main defense against insect and microbial attack, which is a testament to its strength and durability (Brown, 2003; Chang, 2007; Sticklen, 2007).

Pretreatments are typically applied to the biomass to expand the lignin and hydrolyze the hemicellulose in order to gain access to the cellulose (Chang and Holtzapfel, 2000). Currently (2008) there are several competing pretreatments including dilute acid treatment, ammonia fiber explosion and sodium hydroxide treatment (Sticklen, 2007; Zhang and Lynd, 2004) that can be used, but none remove all the lignin. The majority of the cellulose remains intact, and some lignin also remains and can inhibit enzymatic hydrolysis. The cellulases can adsorb irreversibly to the lignin and thus become ineffective for hydrolyzing the cellulose (Xu et al., 2008).

Pretreatment is followed by hydrolysis, typically either dilute acid or enzymatic hydrolysis. The work reported in this thesis focused on enzymatic hydrolysis. Cellulase is the general term for any enzyme that has the ability to hydrolyze the polysaccharide cellulose. While many types of enzymes fall under the cellulase category, there are three major classifications: exoglucanase, endoglucanase and cellobiase. Each of these enzymes interacts with cellulose fibrils in a specific manner.

Exoglucanase is an enzyme with multiple functions. The hydrogen bonds that form within and between the cellulose chains create fibrils, which are responsible for the extreme rigidity of cellulose (Horton et al., 2006). One function of exoglucanase is to pull the fibrils apart into individual cellulose chains (Lynd et al., 2002; Schwarz, 2001). A second function of exoglucanase is to hydrolyze glucose monomers or cellobiose from the non-reducing ends of the individual cellulose chains (Lynd et al., 2002; Okazaki and Mooyoung, 1978). A non-reducing end is defined as a terminal residue in a polysaccharide chain that is locked into a glycosidic bond and is therefore unable to be oxidized (Horton et al., 2006).

Endoglucanase works to increase the rate at which exoglucanase can break down the cellulose. Endoglucanase hydrolyzes cellodextrins and oligosaccharides of various lengths from the cellulose chains (Lynd et al., 2002; Zhang and Lynd, 2006). This activity occurs in random locations, with the main purpose of creating as many non-reducing ends as possible (Gan et al., 2003; Schwarz, 2001). As more non-reducing ends are formed by endoglucanase activity, exoglucanase can further break down these oligosaccharides into glucose monomers.

Cellobiase is the third category of cellulase enzyme that works in conjunction with exoglucanase and endoglucanase. Cellobiase works solely on hydrolyzing cellobiose (a two-glucose sugar unit) into its two glucose monomers (Lau and Wong, 2001; Okazaki and Mooyoung, 1978). The overall rate of cellulose hydrolysis is thought to be controlled by cellobiase. Exoglucanase and endoglucanase are both inhibited by cellobiose (Lu et al., 2006; Okazaki and Mooyoung, 1978; Romaniec et al., 1993). Cellobiase activity is the rate-limiting step in this process because it controls the amount of cellobiose in close proximity to the exoglucanase and endoglucanase enzymes.

Cellulases can be produced by either fungi or bacteria during the fermentation process. Currently (2008) the *Trichoderma reesei* fungus has debatably become the most well-studied enzyme system (Lynd et al., 2002). However, scientists have recently shown more interest in the cellulase systems of bacteria.

CELLULASE ACTIVITY ASSAYS

Cellulose comes in many different forms and because of this heterogeneity in structure, it has proven difficult to standardize the measurement of various sources of cellulase activity (Ghose, 1987; Wood and Bhat, 1988). As a response to this difficulty, several assays have been developed to measure cellulase activity. Each assay has its own corresponding units of activity, which complicates the comparison of data between assays (Wood and Bhat, 1988). Most of the assays that have been developed fall into three main categories: (1) the accumulation of products, (2) the reduction in substrate quantity and (3) the change in the physical properties of substrates (Zhang et al., 2006b).

The majority of cellulase activity assays follow the accumulation of products over time (Wood and Bhat, 1988; Zhang et al., 2006b). Some assays measure reducing sugars, which are sugars with an aldehyde, ketone, hemiacetal or hemiketal group that are able to reduce oxidizing agents (Bruice, 2004), and some measure total sugars. The reducing sugar assays include the dinitrosalicylic acid (DNS) (Ghose, 1987) method and the Nelson-Somogyi (Nelson, 1944; Somogyi, 1952) method, two of the most common methods in use. These methods are robust enough to handle a relatively high sugar range and they have low interference from cellulase such that no dilutions and no protein removal are required. However, these methods may underestimate the cellulase activity when glucose is used as the standard and β -glucosidase is not in excess due to the weak stoichiometric relationship between the cellodextrins and the glucose standards. Other assays in this category include the ferricyanide method (Kidby and Davidson, 1973; Park and Johnson, 1949) and the BCA methods (Waffenschmidt and Jaenicke, 1987), which provide a higher sensitivity to reducing sugars but also experience a high interference from proteins so are not as applicable to these systems. The total sugars assays include the phenol-H₂SO₄ method (DuBois et al., 1956) and the anthrone-H₂SO₄ method (Roe, 1955; Viles and Silverman, 1949). These methods provide a strict stoichiometric relationship between the cellodextrins and the glucose standard. Also, there is little or no interference from protein. It has recently been reported that pure cellulose, such as cotton fiber, filter paper and Avicel, must be used for these methods because carbohydrate derivatives can greatly interfere in readings (Zhang et al., 2006b).

Other cellulase activity assays follow the reduction in weight of original substrate category (Zhang et al., 2006b) through gravimetric or chemical methods. These methods have tedious procedures and are not used often.

The third type of cellulase activity assay measures change in physical properties of substrates due to enzymatic action (Zhang et al., 2006b). Substrate properties that are measured in these assays are the uptake of alkali, fiber strength, structural integrity, turbidity and viscosity. Assays that measure swelling factor, fiber strength and structural integrity lack sensitivity and are not often used (Zhang et al., 2006b). Turbidity assays measure the reduction in absorbance due to particle suspension during the hydrolysis process, which allows the extent of hydrolysis to be monitored over a period of time. However, it is not an acceptable assay for measuring the initial hydrolysis rate for individual enzymes because this method does not measure the initial rate well. Viscosity assays provide a very sensitive measure of the initial hydrolysis rates for endoglucanases because a random break in a cellulose chain may cause a decrease in viscosity with little increase in reducing power (Wood and Bhat, 1988). The disadvantages of this type of assay are that they are difficult to automate and they rely on assumptions that are not always valid, and thus, are not often used (Ghose, 1987).

TOTAL PROTEIN ASSAYS

Total protein assays are used to quantify protein concentrations in samples. Since the cellulosome components are enzymes, and therefore proteins, this measurement provides an estimation of the amount of cellulase present. Three commonly used assays are the Folin phenol method as described by Lowry et al. (1951), a modification of the Folin phenol method as described by Peterson (1977) and the Coomassie Blue method as described by Bradford (1976). Each assay has application within certain situations.

The Lowry method measures total protein content through use of the Folin phenol reagent (Lowry et al., 1951; Peterson, 1977). This method can be used to measure protein in enzyme fractionations, protein in mixed tissue samples, protein in small amounts or very dilute samples, protein in samples with other colored substances and protein in antigen-antibody precipitates. The advantages associated with the Lowry method include increased sensitivity over methods used previously, easy adaptation for small-scale analyses and simplicity and precision of the assay. The disadvantages include variation in color intensity with different proteins, proportionality between the color and the protein concentration is not a strict relationship, interference caused by substances that may be in the sample solution and the lack of specificity for proteins (Lowry et al., 1951; Peterson, 1979). It has been reported that reducing agents (Lowry et al., 1951), detergents (Peterson, 1977) and ions, such as

potassium and magnesium (Bradford, 1976), can affect the accuracy of readings. Bradford (1976) also lists EDTA, Tris, thiol reagents and carbohydrates as potential interferences in the Lowry method.

The Peterson method is a simplification of the Lowry method, which allows it to be used in situations not applicable to the Lowry method. This method is capable of measuring total protein content even when substances that cause interference for the Lowry assay are present. The Peterson method is advantageous in that it can be used to measure soluble, membrane and proteolipid proteins in dilute solutions. It is also tolerant of nonionic and cationic detergents (Peterson, 1977). Other advantages of the Peterson modification of the Lowry method include the improvement of reagent stability, the capability of handling large sample volumes, the capability of isolating very small amounts (<1 µg) of protein even in the presence of interfering substances and the ability of conducting a microassay with the same reagents used in the standard assay (Peterson, 1979).

The Bradford method is one that has gained popularity recently due to its ease of performance and high sensitivity (Bradford, 1976; Pande and Murthy, 1994; Sapan et al., 1999). The process is relatively quick with the color-resulting reaction taking place in approximately two minutes and the stability of the reaction lasting for approximately one hour. It can be easily automated and used to assay large quantities of samples. Small effects may occur due to interfering substances such as Tris, EDTA, sucrose and trace amounts of detergent. However, these effects may be overcome by using the appropriate controls in a buffer solution (Bradford, 1976; Peterson, 1979). The principle idea behind this assay is the binding of a dye, Coomassie Brilliant Blue G-250, onto the protein present in a sample solution. The color formation is then measured by a spectrophotometer and the protein content calculated. A limitation of methods using this dye is the non-linearity of the standard curve, so only proteins that have been standardized with other methods, such as the Lowry, can be used (Peterson, 1979).

MICHAELIS-MENTEN KINETICS IN HOMOGENEOUS SYSTEMS

A homogeneous system is one in which the enzyme and reactant occur in the same phase. Kinetics of homogeneous enzyme-catalyzed reactions is typically described by the Michaelis-Menten equation:

$$v = \frac{V_{\max} S}{K_m + S} \quad (\text{Equation 1.1})$$

where $V_{\max} = \alpha E_0$, i.e. is proportional to the initial concentration of enzyme,

v = rate of reaction, (moles product/L/second),

α = proportionality constant,

K_m represents the substrate concentration when the reaction rate is equal to half of the maximum rate, (moles substrate/L), and

S is the concentration of substrate available to the enzymes (moles substrate/L) (Bailey and Ollis, 1986).

Equation 1.1 describes a rectangular hyperbola, and the rate of reaction v is first order in substrate concentration at relatively low levels of S . This fact can be seen when S is much smaller than K_m , and Equation 1.1 reduces to $v = V_{\max} S / K_m$ or $v = \alpha S$, where α is a constant. As S increases, the order of the reaction decreases continuously from one to zero. For example, when S is greater than K_m , Equation 1.1 reduces to $v = V_{\max} S / S$, which is zero order. Another major outcome from Equation 1.1 is that the rate of reaction is proportional to the amount of enzyme present, which is true for some enzyme/substrate combinations; however, it is not applicable to all enzyme/substrate systems.

KINETICS IN HETEROGENEOUS SYSTEMS

A heterogeneous system results when the catalyst and substrate exist in different phases. For example, fungal cellulases are generally water soluble; however, lignocellulose is insoluble, and therefore, catalyst and substrate exist in different phases (Bailey and Ollis, 1986). The textbook theory for enzymes that adsorb to the surface of substrate is that as enzyme concentration is increased, a limiting rate is approached. This result is in contrast to the assumption for homogeneous systems that increased enzyme concentration proportionally increases the rate of reaction.

Solid substrates have a limited number of binding sites for the enzymes. Until the binding sites are all taken, the rate of reaction will increase with increased enzyme concentration. However, once all the binding sites are saturated, additional enzyme typically does not have any added effect on the reaction rate.

LIQUID AND LOW SOLIDS ENVIRONMENTS

Liquid and low-solids environments are composed of soluble or less than 10% insoluble components, respectively (Jorgensen et al., 2007). Currently (2008), these types of environments make up the majority of the systems used in hydrolysis and fermentation

processes. These systems are very easy to handle for conversion processes because the initial viscosities tend to be low and inhibition does not occur as quickly as with higher solids concentrations (Jorgensen et al., 2007). The fact that most assays used for quantifying enzymatic properties are developed for liquid and low-solids environments is another advantage of their use. One disadvantage, however, is that with low initial substrate, there is low product yield.

HIGH-SOLIDS ENVIRONMENTS

The production economics of commodity chemicals, such as ethanol, has a very narrow profit margin. Typically, as the initial substrate in the reactor increases, the product concentration in the reactor increases, hence, the more profitable the process (Jorgensen et al., 2007). For example, several years ago, the corn ethanol industry adopted very high gravity (VHG) fermentations, which are based on solids concentrations above 30% (Bayrock and Ingledew, 2001). The economics potentially improve in this environment because the resulting sugar concentration and subsequent final ethanol concentration will be higher. At high solids content (above 10-15% w/w) handling the processing stream becomes complicated. In addition, higher solids concentration can result in higher concentrations of inhibitors (Jorgensen et al., 2007).

INHIBITION

Many environmental factors are known to inhibit enzyme performance if the conditions are not optimized for the enzyme/substrate reaction. For example, pH, temperature, stir rate, chemical agents and irradiation are all factors that can potentially inhibit enzyme activity.

SUBSTRATE INHIBITION

It has been recognized for some time that lower cellulose conversion occurred at higher substrate concentrations, with both *C. thermocellum* (Lynd et al., 1989) and *T. reesei* (Valjamae et al., 2001). This lower cellulose conversion was consistent with a limiting factor other than cellulose (Lynd et al., 1989). A study in 2001 (Valjamae et al., 2001) hypothesized that an increased substrate concentration at a fixed enzyme load will also increase the average diffusion time for the enzymes to reach new substrate, effectively slowing the reaction rate.

ENZYME INHIBITION

Enzyme inhibition is defined as the state where maximum enzyme adsorption does not result in the maximum rate of hydrolysis. Some investigators have studied different enzyme concentrations on Avicel yet did not report enzyme inhibition (Steiner et al., 1988) presumably because the study was focused on cellulase adsorption and not rate of hydrolysis.

OBJECTIVES

This study investigated two potential sources of inhibition specific to heterogeneous systems, namely substrate concentration and enzyme concentration. Both *T. reesei* and *C. thermocellum* cellulases were investigated. The two objectives and associated hypotheses of this study are:

1. To quantify the apparent activity of fungal enzymes on lignocellulose as a function of enzyme concentration and cellulose content of the substrate.
2. To quantify the apparent activity of bacterial enzymes on lignocellulose and assess the reproducibility of the ELISA protocol developed to quantify bacterial cellulase mass concentrations.

LITERATURE CITED

- Bailey JE, Ollis, D.F. 1986. *Biochemical Engineering Fundamentals*. New York, NY: McGraw-Hill Publishing Company. 984 p.
- Bayrock DP, Ingledew WM. 2001. Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. *Journal of Industrial Microbiology & Biotechnology* 27(2):87-93.
- Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* 72(1-2):248-254.
- Brown RC. 2003. *Biorenewable resources: engineering new products from agriculture*: Blackwell Publishing Company. 286 p.
- Bruice PY. 2004. *Organic Chemistry*. Upper Saddle River, NJ: Prentice Hall.
- Chang MCY. 2007. Harnessing energy from plant biomass. *Current Opinion in Chemical Biology* 11(6):677-684.
- Chang VS, Holtzaple MT. 2000. Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology* 84-6:5-37.
- DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. 28(3):350-356.
- Gan Q, Allen SJ, Taylor G. 2003. Kinetic dynamics in heterogeneous enzymatic hydrolysis of cellulose: an overview, an experimental study and mathematical modelling. *Process Biochemistry* 38(7):1003-1018.
- Ghose TK. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry* 59(2):257-268.
- Horton HR, Moran, L.A., Scrimgeour, K.G., Perry, M. D., Rawn, J.D. 2006. *Principles of Biochemistry*. Upper Saddle River: Pearson Prentice Hall.
- Jorgensen H, Vibe-Pedersen J, Larsen J, Felby C. 2007. Liquefaction of lignocellulose at high-solids concentrations. *Biotechnology and Bioengineering* 96(5):862-870.
- Kidby DK, Davidson DJ. 1973. Convenient ferricyanide estimation of reducing sugars in nanomole range. *Analytical Biochemistry* 55(1):321-325.
- Lau ATY, Wong WKR. 2001. Purification and characterization of a major secretory cellobiase, Cba2, from *Cellulomonas biazotea*. *Protein Expression and Purification* 23(1):159-166.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193(1):265-275.

- Lu YP, Zhang YHP, Lynd LR. 2006. Enzyme-microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*. Proceedings of the National Academy of Sciences of the United States of America 103(44):16165-16169.
- Lynd LR, Grethlein HE, Wolkin RH. 1989. Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. Applied and Environmental Microbiology 55(12):3131-3139.
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiology and Molecular Biology Reviews 66(3):506-+.
- Moxley G, Zhang YHP. 2007. More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. Energy & Fuels 21:3684-3688.
- Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153(2):375-380.
- Okazaki M, Mooyoung M. 1978. Kinetics of enzymatic hydrolysis of cellulose - analytical description of a mechanistic model. Biotechnology and Bioengineering 20(5):637-663.
- Pande SV, Murthy MSR. 1994. A modified micro-Bradford procedure for elimination of interference from sodium dodecyl-sulfate, other detergents, and lipids. Analytical Biochemistry 220(2):424-426.
- Park JT, Johnson MJ. 1949. A submicrodetermination of glucose. Journal of Biological Chemistry 181(1):149-151.
- Perlack RD, Wright, L.L, Turhollow, A.F., Graham, R.L., Stokes, B.J., Erbach, D.C. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. Oak Ridge, TN: Oak Ridge National Laboratory. Report nr ORNL/TM-2005/66. 59 p.
- Peterson GL. 1977. Simplification of protein assay method of Lowry et al - which is more generally applicable. Analytical Biochemistry 83(2):346-356.
- Peterson GL. 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Analytical Biochemistry 100(2):201-220.
- Reese ET. 1956. Enzymatic hydrolysis of cellulose. Applied Microbiology 4(1):39-45.
- Roe JH. 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. Journal of Biological Chemistry 212(1):335-343.
- Romaniec MPM, Huskisson N, Barker P, Demain AL. 1993. Purification and properties of the *Clostridium thermocellum* Bglb gene-product expressed in *Escherichia coli*. Enzyme and Microbial Technology 15(5):393-400.

- Sapan CV, Lundblad RL, Price NC. 1999. Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry* 29:99-108.
- Schwarz WH. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology* 56(5-6):634-649.
- Somogyi M. 1952. Notes on sugar determination. *Journal of Biological Chemistry* 195(1):19-23.
- Steiner W, Sattler W, Esterbauer H. 1988. Adsorption of *Trichoderma reesei* cellulase on cellulose - experimental-data and their analysis by different equations. *Biotechnology and Bioengineering* 32(7):853-865.
- Sticklen MB. 2007. Feedstock crop genetic engineering for alcohol fuels. *Crop Science* 47:2238-2248.
- Valjamae P, Pettersson G, Johansson G. 2001. Mechanism of substrate inhibition in cellulose synergistic degradation. *European Journal of Biochemistry* 268(16):4520-4526.
- Viles FJ, Silverman L. 1949. Determination of starch and cellulose with anthrone. *Analytical Chemistry* 21(8):950-953.
- Waffenschmidt S, Jaenicke L. 1987. Assay of reducing sugars in the nanomole range with 2,2'-biquinoline. *Analytical Biochemistry* 165(2):337-340.
- Wood TM, Bhat KM. 1988. Methods for measuring cellulase activities. *Methods in Enzymology* 160:87-112.
- Xu F, Ding HS, Osborn D, Tejirian A, Brown K, Albano W, Sheehy N, Langston J. 2008. Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. *Journal of Molecular Catalysis B-Enzymatic* 51(1-2):42-48.
- Zhang YHP, Himmel ME, Mielenz JR. 2006b. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances* 24(5):452-481.
- Zhang YHP, Lynd LR. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnology and Bioengineering* 88(7):797-824.
- Zhang YHP, Lynd LR. 2006. A functionally based model for hydrolysis of cellulose by fungal cellulase. *Biotechnology and Bioengineering* 94(5):888-898.

CHAPTER TWO: SACCHARIFICATION OF LIGNOCELLULOSE AND ENZYME KINETICS USING FUNGAL CELLULASE

SUMMARY

Enzymatic hydrolysis was carried out for 72 hr using three cellulase concentrations (15, 40 and 60 FPU/g cellulose) and three substrates (Avicel, Avicel + xylan from oat spelt and corn stover). *T. reesei* cellulases supplemented with equal volumes of *A. niger* β -glucosidase were used in this study. Hydrolysis was followed over time, sampling with repeated measures. Initial rate and extent of hydrolysis was determined from each progress curve. Evidence of enzyme loading inhibition was observed because the lowest enzyme loading resulted in the highest rates and greatest extents of hydrolysis. Mechanisms to explain the observed phenomenon are discussed.

INTRODUCTION

Lignocellulosic biomass is the most abundant source of renewable material available. The technology exists to convert lignocellulose into ethanol or other biobased products thermochemically or biochemically through hydrolysis and fermentation processes (Chang and Holtzapfle, 2000; Moxley and Zhang, 2007; Reese, 1956).

As the need for alternative sources of fuel and environmental concerns heighten, the search for an economical and environmentally benign method for producing fuel intensifies. The current school of thought for the solution to this problem is converting lignocellulosic biomass, generally a waste product from the agricultural industry or material that can be grown on potential cropland, into fermentable sugars and then ethanol using microbes.

Lignocellulose is made up of three main components: cellulose, hemicellulose and lignin (Figure 2.1). Cellulose, although recalcitrant in its native form, can be broken down into fermentable sugars by some enzymes. Less than 20% of the glucan found in native cellulose can be broken down without some form of pretreatment (Zhang and Lynd, 2004). Hemicellulose is more random and more easily broken down into fermentable sugars if the correct enzymes are present (Sticklen, 2007). However, many of the microbes presently being studied are not capable of fermenting the pentose sugars that would result from the break down of hemicellulose (Chang, 2007). Lignin is another road block in the hydrolysis process. It blocks the hydrolytic enzymes from accessing the available cellulose and

hemicellulose. Pretreatment of the biomass is one method used to loosen the lignin before the hydrolysis process.

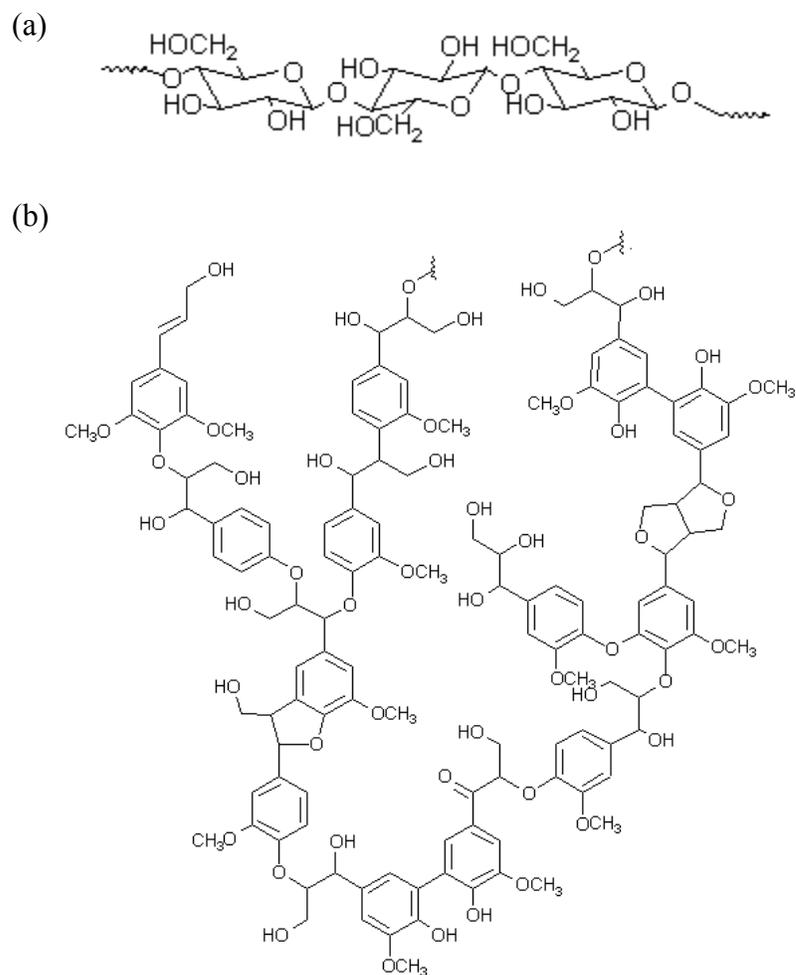


Figure 2.1. Structures of lignocellulose components. (a) Cellulose and (b) lignin are two of the main components found in lignocellulose. Cellulose figure adapted from Zhang and Lynd (2004), and lignin figure adapted from Adler (1977).

One hydrolytic enzyme system that has received a great deal of attention for the biomass conversion process is from the fungus *Trichoderma reesei*. It is one of the most well-known and well-studied cellulase-producing organism because it is known to secrete high levels of cellulase activity (Rosgaard et al., 2007; Zhang and Lynd, 2004). Cellulases from *T. reesei* have become the industry standard (Lynd et al., 2002). *T. reesei* also produces a low level of β -glucosidase; however in most studies, commercially produced *T. reesei* cellulases are supplemented with additional β -glucosidase, usually produced by *Aspergillus niger* (Rosgaard et al., 2007).

In the search for an efficient process for converting lignocellulosic material into fermentable sugars for biofuel production, various enzyme concentrations applied to cellulosic substrates have been studied. The present study was conducted to determine how increased enzyme concentrations affect the initial rate and the extent of enzymatic hydrolysis on three different substrates. A secondary objective was to determine whether enzymatic hydrolysis was more closely correlated with the enzyme:cellulose ratio or the enzyme:substrate ratio given that lignocellulosic composition can vary within an experiment if the plant cell walls have been genetically altered.

MATERIALS AND METHODS

ENZYMES

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

SUBSTRATES

Three different substrates were used in the enzymatic hydrolysis. Avicel was obtained from FMC Corp. (PH105, Philadelphia, PA), xylan from oat spelts was obtained from Sigma (X0627, St. Louis, MO) and corn stover was obtained from the University of Kentucky Animal Research Center (Lexington, KY). The average cellulose content for corn stover was assumed to be 37.5% (Lee et al., 2007).

PETERSON METHOD FOR PROTEIN DETERMINATION

The Peterson method was conducted according to the protocol provided with the total protein kit (TP0300, Sigma, St. Louis, MO). Standard curve tubes were prepared in triplicate

and ranged from 0-400 $\mu\text{g}/\text{mL}$ in 100 $\mu\text{g}/\text{mL}$ intervals, including 50 $\mu\text{g}/\text{mL}$. Sample tubes of cellulase and β -glucosidase were also prepared in triplicate. To all tubes, 1.0 mL of the Lowry reagent solution was added and mixed well. The tubes were incubated at room temperature for 20 min. A 0.5 mL aliquot of Folin and Ciocalteu's phenol reagent working solution was added to each tube and rapidly and immediately mixed. The tubes were again incubated for 30 min to allow the color to develop. The absorbance was measured at 750 nm against the 0 $\mu\text{g}/\text{mL}$ standard protein blank.

CELLULASE ACTIVITY ASSAY

The cellulase activity assay was conducted as outlined by the NREL LAP-006 (Adney, 1996), with a few modifications. Cellulase was diluted with 0.05 M Na-citrate buffer at a pH of 4.8 so that the final volume was 1.0 mL. Three replications of three dilutions were used. An additional 2.0 mL of 0.05 M Na-citrate buffer was added to each of the sample test tubes, as well as to the blank test tubes. Two filter paper strips (1 cm x 6 cm; approximately 50 mg) were added to all sample and blank test tubes. All tubes were incubated in a water bath for 1.0 hr at 50°C. After incubation, the tubes were immediately placed in an ice bath to stop the hydrolysis reaction. One milliliter of appropriately diluted enzyme solution was added to the respective blank tubes. The tubes were vortexed and poured into 2.0 mL o-ring centrifuge tubes. The samples were centrifuged at 6000 rpm for 20 min. The soluble sugar content in the supernatant was then determined using the phenol-sulfuric acid assay (DuBois et al., 1956).

Cellulase activity was calculated by comparing the sugar concentrations to a standard curve. This assay required concentrated H_2SO_4 , 5% phenol solution and 100 mg/L glucose standard. The glucose standard curve tubes were prepared by diluting the stock solution to range from 0-100 mg/L. A 0.5 mL aliquot of the diluted glucose standard was added to each respective tube along with 0.5 mL of 5% phenol solution. Likewise, 0.5 mL of the sample supernatant was added to each respective tube along with 0.5 mL of 5% phenol solution. All tubes were vortexed to thoroughly mix the contents. A 2.5 mL aliquot of concentrated H_2SO_4 was added to each tube. The tubes were immediately sealed and mixed. The tubes were incubated for 30 min at room temperature before reading the absorbance at 485 nm.

ENZYMATIC HYDROLYSIS

The enzymatic hydrolysis was conducted as outlined by the NREL LAP-Enzymatic Saccharification of Lignocellulosic Biomass (Brown and Torget, 1996), with slight modifications. Each hydrolysis flask contained 200 mL of the hydrolysis solution. The hydrolysis solution was composed of 100 mL of 0.1 M Na-citrate buffer and 100 μ L of 2.0% NaN_3 to prevent microbial growth. Enough substrate (dry weight basis) was added such that 1.0% of the total hydrolysis solution volume was cellulose. After determining the amount of enzyme solution necessary to achieve the desired concentration, deionized water was added to bring the working volume up to 200 mL. The enzyme solution consisted of equal parts (v/v) of cellulase and β -glucosidase, and *T. reesei* cellulase volume determined β -glucosidase volume. Blank test tubes contained the same concentrations of each hydrolysis solution component, with the exception of the enzyme solution, but with a 3.0 mL working volume.

All components of the hydrolysis solution, with the exception of the enzyme solution, were added to the flasks and placed in a 50°C incubator and allowed to equilibrate. The appropriate amount of enzyme solution was added to each of the hydrolysis flasks. Two milliliter samples were collected from each flask and the corresponding blank test tubes were removed from the incubator and placed in a cold water bath. The appropriate amount of enzyme solution was added to each blank test tube, vortexed and poured into a 2.0 mL o-ring centrifuge tube. All samples and blanks were boiled for 5.0 min to denature the enzyme and placed in a -40°C freezer for later analysis. After thawing, the concentration of soluble sugars was determined by the phenol-sulfuric acid method as outlined by DuBois et al. (1956).

EXPERIMENTAL DESIGN

Three enzyme concentrations (NREL standard of 15 FPU/g cellulose, 40 FPU/g cellulose and saturated conditions of 60 FPU/g cellulose) were studied on three different substrates to determine if enzyme concentration had any effect on the initial rate or the extent of hydrolysis. The enzyme concentrations were applied to the 27 hydrolysis flasks in a generalized randomized complete block design (block = substrate). An experimental unit was a hydrolysis flask with a given enzyme treatment. A 2.0 mL aliquot was taken from each flask at various times (5, 15, 30, 60 min, 2, 6, 24, 48, 72 hr) in order to calculate the initial hydrolysis rate and to measure the extent of hydrolysis. Soluble sugar (glucose equivalents) concentration was measured.

The soluble sugar concentrations were used to create time course hydrolysis curves for each of the substrate and enzyme concentration combinations. The initial rate of hydrolysis (v_0) was determined by fitting a line to the points from the portion of the hydrolysis curve where the slope was greater than zero. The groups of points with the greatest slopes were selected for the initial rates. Extents of hydrolysis were determined from the portion of the hydrolysis curve where the slope approaches zero. These points were averaged and considered the extent of hydrolysis.

STATISTICAL ANALYSIS

The data was analyzed as a 3x3 factorial in a generalized randomized complete block design (substrate = block) using PROC GLM of SAS to determine if any differences in initial rate of hydrolysis and extent of hydrolysis existed. If differences existed, least squares means were computed and all possible pairwise comparisons were made among hydrolysis conditions.

RESULTS

PETERSON METHOD FOR PROTEIN DETERMINATION

Each sample of cellulase contained 149.5 (\pm 5.9) mg/mL of protein, and the β -glucosidase contained 178.2 (\pm 2.8) mg/mL of protein (Table 2.1).

Table 2.1. Characteristics of the *T. reesei* cellulase and the *A. niger* β -glucosidase used in enzymatic hydrolysis. Equal parts of cellulase and β -glucosidase were added to each hydrolysis flask.

Enzyme	Protein Content (\pm Std Error) (mg/mL)	Cellulase Activity (FPU/mL)	Specific Activity (FPU/mg)
<i>T. reesei</i> 1	145.5 (\pm 1.8)	16.44	0.11
<i>T. reesei</i> 2	149.5 (\pm 1.9)	20.90	0.14
<i>T. reesei</i> 3	153.6 (\pm 4.9)	16.44	0.11
<i>A. niger</i>	178.2 (\pm 1.6)	--	--

CELLULASE ACTIVITY ASSAY

T. reesei cellulase activities can be found in Table 2.1. Activity was used to calculate the volume of cellulase needed for the various levels of enzyme concentration for the enzymatic hydrolysis.

ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis was performed on three different substrates (Avicel, Avicel + xylan, corn stover) at three different enzyme levels (15, 40, 60 FPU/g cellulose) for 72 hours. Samples were collected at several times throughout the hydrolysis period and analyzed for soluble sugar content. Figure 2.2a-c shows the soluble sugar content over time during the hydrolysis period. From these hydrolysis curves, the initial rates and the extents of hydrolysis were determined (Table 2.2).

Table 2.2. Enzymatic hydrolysis summary.

Substrate	[Cellulose] (g/L)	[S] (g/L)	[E] (FPU/g cellulose)	[E] (FPU/mL enzyme)	[E] (mL/L)	Vo (\pm Std Error) [†] (g/L hr)	Extent (\pm Std Error) (g/L)
Avicel	10.0	10.0	15.0	16.4	9.1	1.78 (\pm 0.04) ^c	6.06 (\pm 0.50)
	10.0	10.0	40.0	16.4	24.3	1.42 (\pm 0.01) ^d	4.51 (\pm 0.93)
	10.0	10.0	60.0	16.4	36.5	0.69 (\pm 0.04) ^e	4.71 (\pm 0.93)
Avicel + Xylan	10.0	16.3	15.0	20.9	7.2	2.50 (\pm 0.09) ^b	8.16 (\pm 0.73)
	10.0	16.3	40.0	20.9	19.6	2.84 (\pm 0.25) ^a	10.07 (\pm 0.60)
	10.0	16.3	60.0	20.9	28.7	2.01 (\pm 0.16) ^c	9.13 (\pm 0.94)
Corn Stover	10.0	28.8	15.0	16.4	9.1	0.32 (\pm 0.04) ^{f,g}	2.15 (\pm 0.20)
	10.0	28.8	40.0	16.4	24.3	0.58 (\pm 0.06) ^{e,f}	2.99 (\pm 0.20)
	10.0	28.8	60.0	16.4	36.5	0.15 (\pm 0.01) ^g	3.24 (\pm 0.21)

[†]Initial rates (Vo) with like letters are considered to be statistically the same at $\alpha=0.05$.

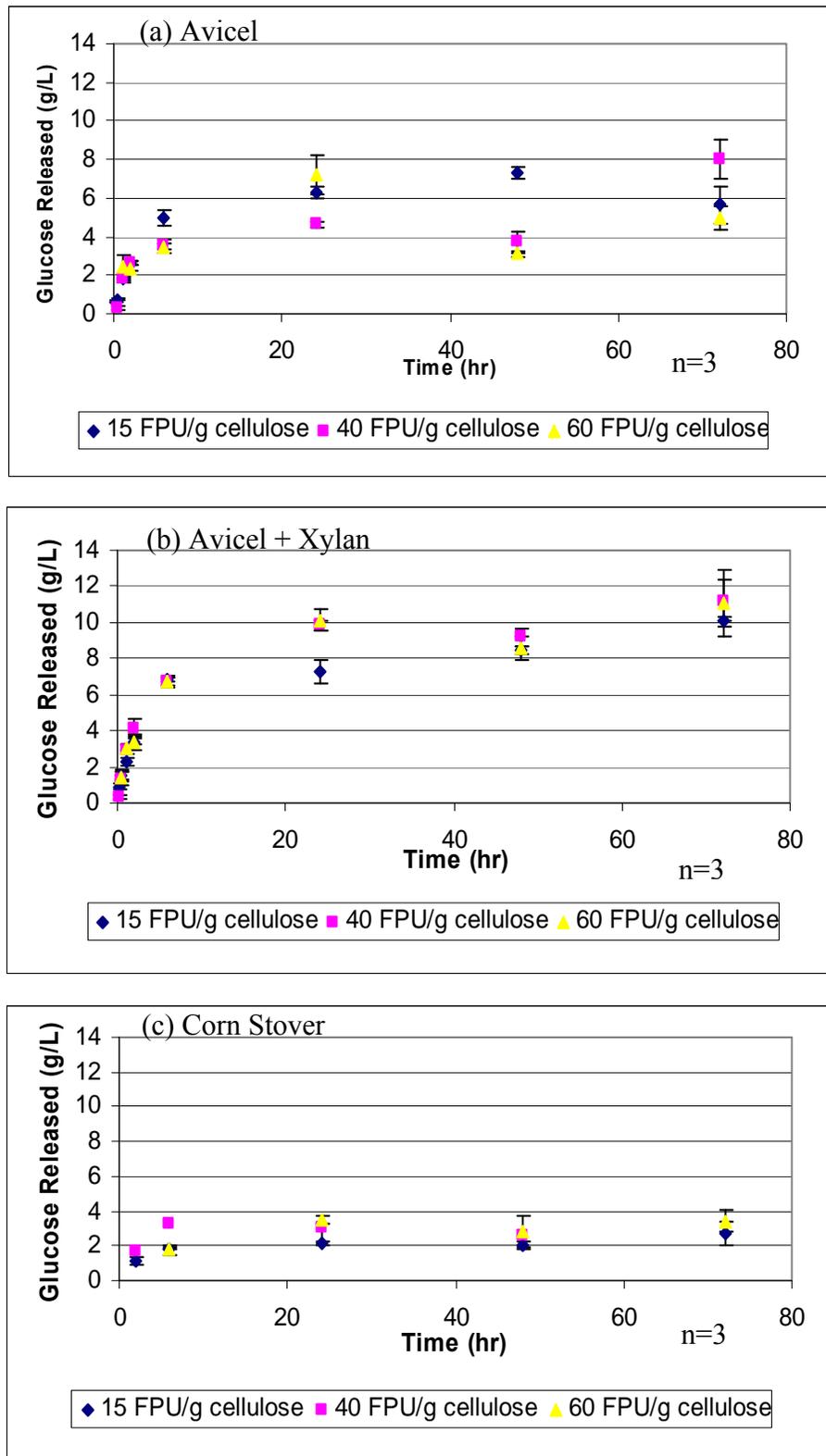


Figure 2.2. Hydrolysis curves. The soluble sugar content in each hydrolysis flask was recorded throughout the hydrolysis period. Enzyme concentrations are 15, 40 and 60 FPU/g cellulose and the substrates are (a) Avicel, (b) Avicel + xylan and (c) corn stover.

Figure 2.3 compares the initial rates for each treatment. The initial rates for Avicel + xylan and corn stover exhibited similar trends. The treatments with 40 FPU/g cellulose had the highest initial rate, followed by the treatments with 15 FPU/g cellulose and 60 FPU/g cellulose, respectively. However, for the treatments with Avicel, it shows that as the enzyme concentration increases, the initial rate decreases.

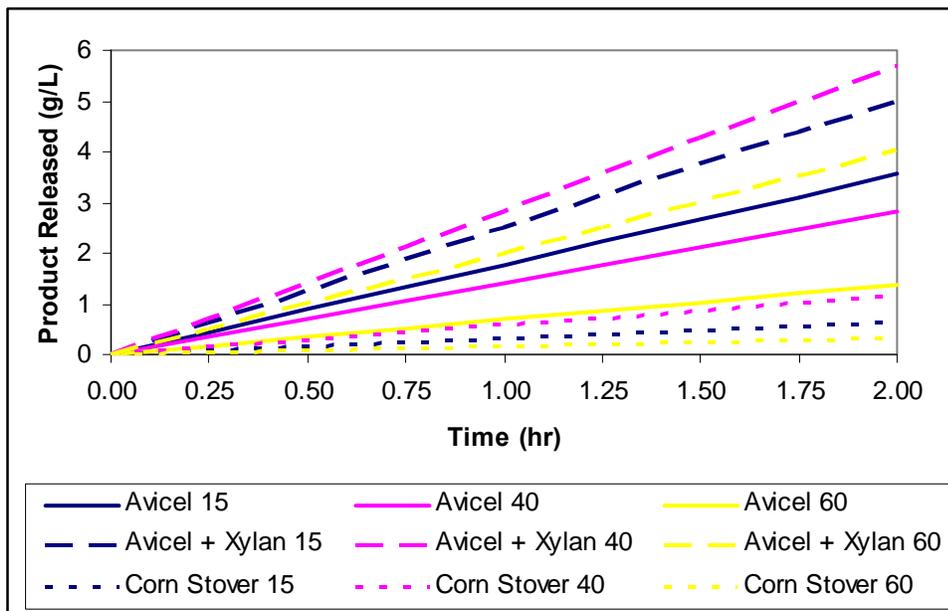


Figure 2.3. Initial rates of hydrolysis. The initial rates for Avicel + xylan and corn stover are exhibiting similar trends for the three enzyme concentrations, whereas the initial rates for Avicel show that as the enzyme concentrations increase, the initial rates decrease. The enzyme concentrations, 15, 40 and 60 are 15, 40 and 60 FPU/g cellulose, respectively.

Figure 2.4 shows the extent of soluble sugar content after 72 hours of hydrolysis. While the treatments with Avicel + xylan were not significantly different from each other with respect to glucose released at three enzyme concentrations, significantly more glucose was released from the Avicel + xylan treatments than any other substrate and enzyme concentration combinations. The Avicel + xylan treatments produced a minimum glucose release of about 8 g/L, where the Avicel and corn stover treatments produced a maximum glucose release of about 6 g/L and 3 g/L, respectively.

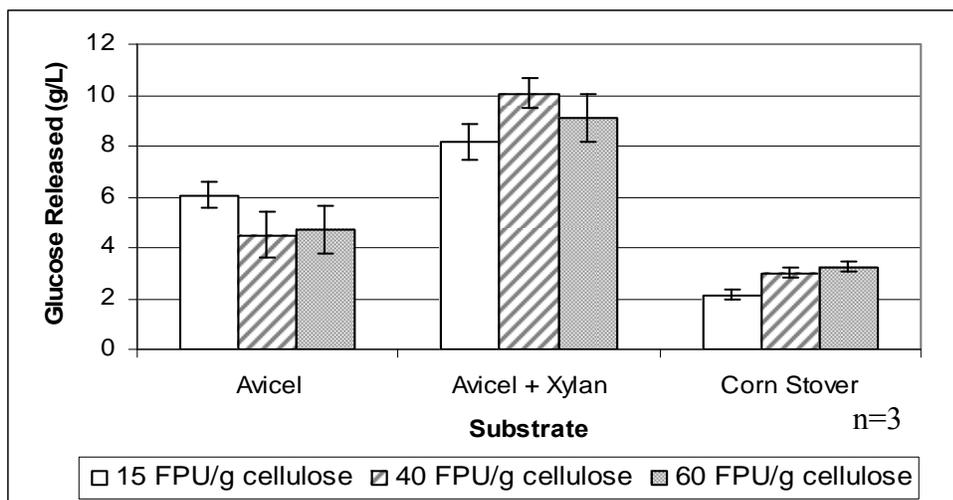


Figure 2.4. Extent of hydrolysis after 72 hours for three substrates. While the Avicel + xylan treatments were not significantly different from each other, more glucose was released from these treatments than from the Avicel or corn stover treatments.

Figure 2.5 shows the soluble sugar content after 72 hours of hydrolysis on a per gram of cellulose basis. The Avicel + xylan treatments produced at least 0.8 g glucose for every gram of cellulose present in the hydrolysis flask and reached a maximum glucose release of approximately 1 g for each gram of cellulose present for the higher enzyme concentrations. Both the Avicel and the corn stover treatments produced lower glucose concentrations.

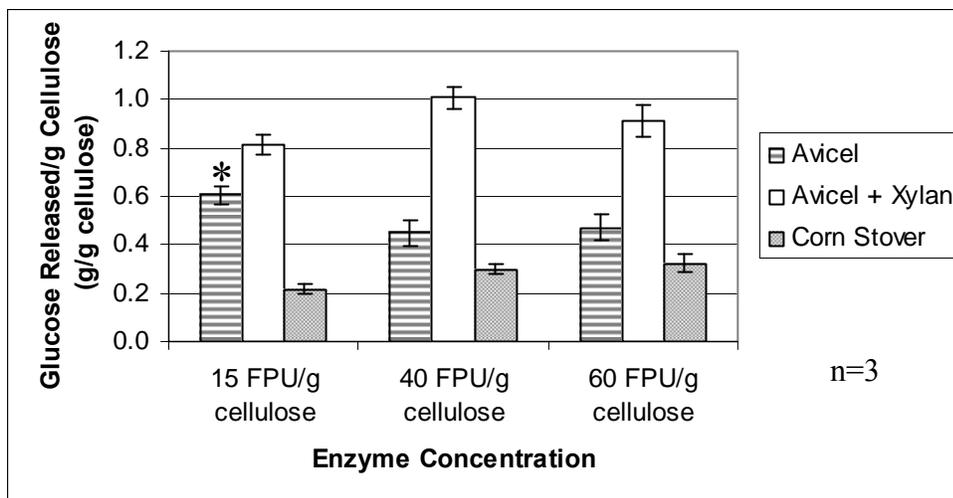


Figure 2.5. Soluble sugar released after 72 hours of hydrolysis on a per gram cellulose basis at three different enzyme concentrations for the three substrates. The column marked with an asterisk (*) is significantly different from all other substrate and enzyme concentration combinations at $\alpha=0.05$.

Figure 2.6 shows the soluble sugar content after 72 hours of hydrolysis on a per gram of substrate basis. When normalizing the glucose released per gram of substrate, corn stover produced significantly lower glucose than the other two substrates with approximately 0.1 g glucose/g substrate for all three enzyme concentrations.

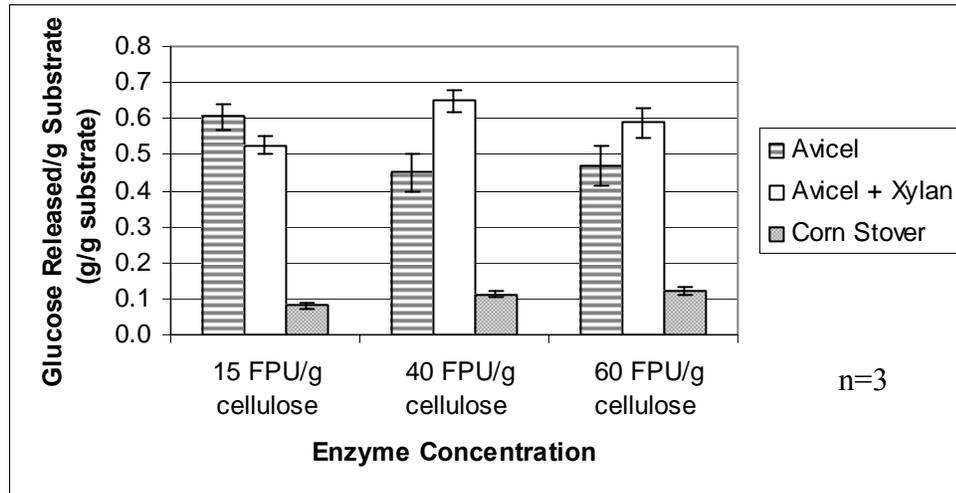


Figure 2.6. Soluble sugar released after 72 hours of hydrolysis on a per gram substrate basis at three different enzyme concentrations for three substrates. Enzyme concentrations are 15, 40 and 60 FPU/g cellulose and the substrates are Avicel, Avicel + xylan and corn stover. There were no statistically significant treatment differences within substrate groupings.

Figure 2.7 compares the extent of hydrolysis on a per gram of cellulose basis to a per gram of substrate basis. It was determined that the Avicel + xylan and the corn stover comparisons at all enzyme concentrations are significantly different when normalizing the results per gram of cellulose or gram substrate. Avicel, as it was expected to be, was the same on either basis because Avicel is “pure” cellulose.

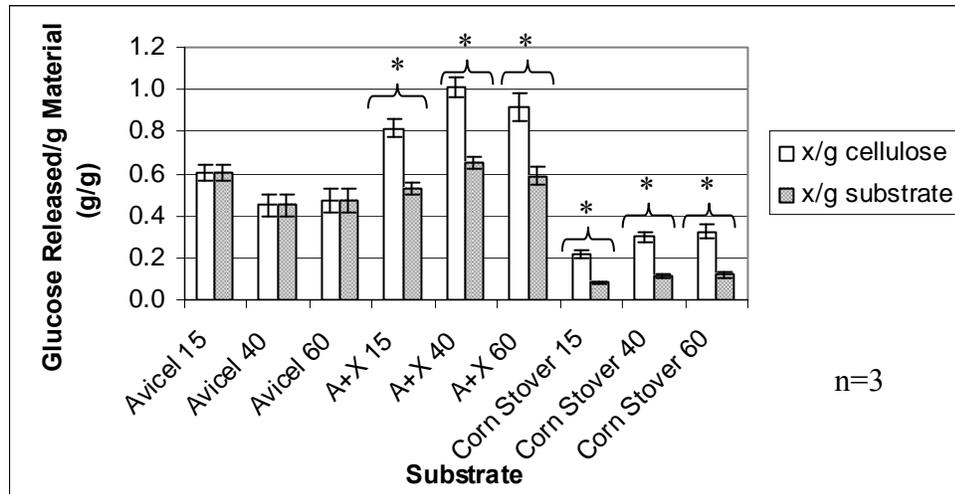


Figure 2.7. Comparison of extent of hydrolysis on a per gram cellulose basis to extent of hydrolysis on a per gram of substrate basis. Columns are denoted by type of substrate and enzyme concentration (where 15, 40 and 60 = FPU/g cellulose). Pairs of columns marked with asterisks (*) are significantly different from each other at $\alpha=0.05$.

DISCUSSION

The initial rates of hydrolysis for Avicel + xylan and corn stover exhibited the same trends. The treatments with the 40 FPU/g cellulose produced the highest amount of glucose released, followed by the treatments with 15 FPU/g cellulose and 60 FPU/g cellulose, respectively. In contrast to the other two substrates, the treatments with Avicel showed that as the enzyme concentration increased, the initial rate of hydrolysis decreased. This phenomenon is potentially due to inhibition caused by higher enzyme loadings. The National Renewable Energy Lab (NREL) recommends an enzyme loading of 15 FPU/g cellulose. The increased levels of enzyme could be causing inhibition from overcrowding, or “jamming” effects (Xu and Ding, 2007). Overcrowding or jamming is when enzymes cannot access the cellulose because other enzymes block potential binding sites. It is also more likely at higher solids concentrations for the cellulases to bind to other non-cellulose components in the substrate, thus resulting in non-productive binding or irreversible adsorption (Zhang and Lynd, 2004). Overcrowding and non-productive binding can greatly reduce the cellulase activity breaking down cellulose.

The extent of hydrolysis showed some inhibition occurring at the higher enzyme concentrations for all substrates. For Avicel, 15 FPU/g cellulose produced the highest extent and was significantly different from 40 and 60 FPU/g cellulose treatments. For Avicel + xylan, the highest extent occurred at 40 FPU/g cellulose, followed by 60 FPU/g cellulose and 15 FPU/g cellulose, respectively. For corn stover, none of the enzyme concentrations were significantly different. The same reasons stated above concerning inhibition of the initial rates are applicable concerning the inhibition of the extent of hydrolysis for Avicel.

When considering the extent of hydrolysis, corn stover produced very little soluble sugars at all enzyme loadings as compared to the other two substrates. The literature suggests that without pretreatment, less than 20% cellulose is accessible to the enzymes, whereas with some pretreatment, nearly 100% of the cellulose is made accessible (Zhang and Lynd, 2004). The conversion measured for corn stover matches what the literature suggests for corn stover without pretreatment. With less accessible cellulose and more lignin and hemicellulose present, the occurrence of non-productive binding could increase to the point of decreased sugar production.

The inhibition seen in the Avicel and Avicel + xylan treatments may be described using fractal kinetics with jamming. The classical derivation of Michaelis-Menten kinetics relies on the law of mass-action, which assumes that the reactant depletion rate is proportional

to the product of the two reactant concentrations (representing the probability of collision of the two reactants). The rate of collision depends on the distance between reactant molecules, which is assumed to follow Fick's Law for diffusion, if there is no mixing (Xu and Ding, 2007).

Heterogeneous systems follow non-Fickian diffusion, which can result in non-classical kinetic behavior. Xu and Ding (2007) hypothesized that fractal kinetics was more descriptive of heterogeneous systems, where the order of the reaction is a fraction. They described the kinetics with the following equations:

$$\frac{k_2[E]t^{1-f}}{1-f} = [P] - K_m \ln\left(1 - \frac{[P]}{[S]}\right) \quad (\text{Equation 2.1})$$

$$\frac{\log([E]_a/[E]_b)}{\log(t_b/t_a)} = 1 - f \quad (\text{Equation 2.2})$$

Incorporating the fractal kinetics described the system well, as long as no enzyme inhibition was present. Xu and Ding (2007) state that the relationship

$$\frac{[E]_a}{[E]_b} < \frac{t_b}{t_a}, \quad \frac{\log([E]_a/[E]_b)}{\log(t_b/t_a)} > 1 \quad (\text{Equation 2.3})$$

is unique for overcrowded or "jammed" enzymes. When the jamming factor is incorporated into the equations above, they found the following equations:

$$\left(1 - \frac{[E]}{j[S]}\right) \frac{k_2[E]t^{1-f}}{1-f} = [P] - K_m \ln\left(1 - \frac{[P]}{[S]}\right) \quad (\text{Equation 2.4})$$

$$\left(1 - \frac{[E]_a}{j[S]}\right) [E]_a t_a^{1-f} = \left(1 - \frac{[E]_b}{j[S]}\right) [E]_b t_b^{1-f} \quad (\text{Equation 2.5})$$

Figure 2.8 demonstrates the differences in progress curves when considering classical, fractal and jammed kinetic profiles.

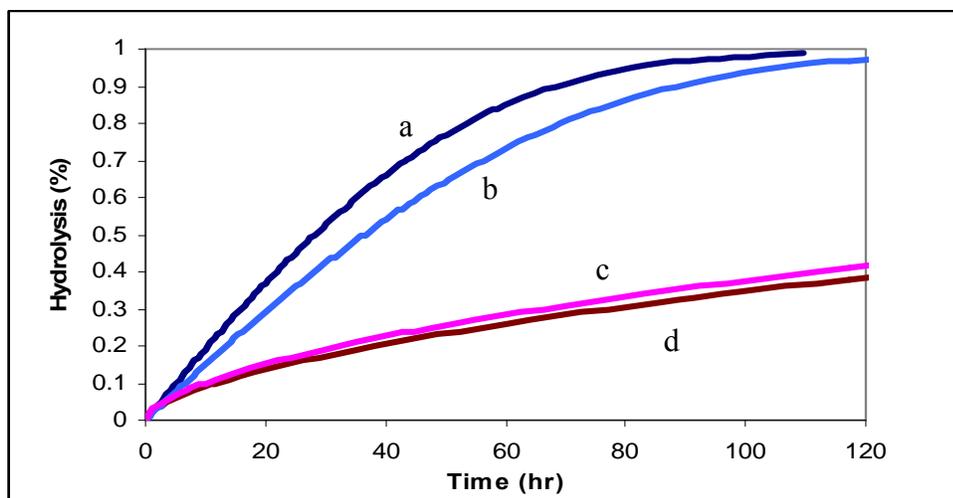


Figure 2.8. Simulated classical, fractal and jammed kinetic profiles. (a) Classical Michaelis-Menten kinetics, (b) jammed Michaelis-Menten kinetics, (c) fractal Michaelis-Menten kinetics based on Equation 2.1 and (d) jammed, fractal Michaelis-Menten kinetics based on Equation 2.4. Simulation conditions: $[S] = 10$ mM, $K_m = 5$ mM, $k_2 = 3000$ hr⁻¹, $[E] = 0.1$ μ m, $f = 0.4$, $j = 0.000044$.

A secondary objective of this study was to determine if calculating enzyme loadings on a per gram cellulose basis or on a per gram substrate basis is equally effective, given that the composition of lignocellulose can vary from treatment to treatment, even with the same substrate if the cell wall has been genetically modified. Based on the findings of this study, loadings should be calculated on a per gram of cellulose basis because the results may differ significantly if the amount of cellulose is different, even if the amount of substrate is kept constant.

LITERATURE CITED

- Adler E. 1977. Lignin Chemistry - Past, Present and Future. *Wood Science and Technology* 11(3):169-218.
- Adney B, Baker, J. 1996. Measurement of cellulase activities. Golden, CO: National Renewable Energy Laboratory. Report nr NREL/TP-501-42628.
- Brown L, Torget R. 1996. Enzymatic saccharification of lignocellulosic biomass. Golden, CO: National Renewable Energy Laboratory. Report nr LAP 009.
- Chang MCY. 2007. Harnessing energy from plant biomass. *Current Opinion in Chemical Biology* 11(6):677-684.
- Chang VS, Holtzaple MT. 2000. Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology* 84-6:5-37.
- DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *28(3):350-356*.
- Lee D, Owens VN, Boe A, Jeranyama P. 2007. Composition of herbaceous biomass feedstocks. Brookings, SD: North Central Sun Grant Center. 16 p.
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* 66(3):506-+.
- Moxley G, Zhang YHP. 2007. More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. *Energy & Fuels* 21:3684-3688.
- Reese ET. 1956. Enzymatic hydrolysis of cellulose. *Applied Microbiology* 4(1):39-45.
- Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS. 2007. Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates. *Biotechnology Progress* 23:1270-1276.
- Sticklen MB. 2007. Feedstock crop genetic engineering for alcohol fuels. *Crop Science* 47:2238-2248.
- Xu F, Ding HS. 2007. A new kinetic model for heterogeneous (or spatially confined) enzymatic catalysis: Contributions from the fractal and jamming (overcrowding) effects. *Applied Catalysis a-General* 317(1):70-81.
- Zhang YHP, Lynd LR. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnology and Bioengineering* 88(7):797-824.

CHAPTER THREE: ASSAYING CELLULASE ACTIVITY OF BACTERIA GROWN IN SOLID SUBSTRATE CULTIVATION

SUMMARY

The cellulosome is an elaborate, extracellular, enzymatic complex that very efficiently breaks down cellulose into smaller sugars that can be transported and used in *Clostridium thermocellum* as its carbon source. Because *C. thermocellum* is capable of hydrolyzing lignocellulose, it is potentially an excellent candidate for the commercial conversion of biomass into biofuels. Several types of assays must be conducted in order to quantify properties of the cellulolytic protein produced by this organism. It is necessary to measure both a cellulosome mass and an enzymatic activity associated with a particular sample. Assays such as the Lowry, Bradford or Peterson methods can determine the total protein concentration of a sample, whereas an indirect ELISA method has been developed to quantify cellulase-specific protein mass concentrations in liquid fermentations. Once the mass is determined, researchers must then measure the cellulase activity by conducting a phenol-sulfuric acid assay or a DNS filter paper assay. The objective of this study is to investigate the use and reproducibility of these assays in high-substrate density systems, such as seen in biomass-biofuel production and in solid substrate cultivations. The confidence in the validity and the reproducibility of these assays will allow the researchers to address issues involving the relationship between the cellulosome mass concentration and the cellulase activity.

INTRODUCTION

CLOSTRIDIUM THERMOCELLUM

Clostridium thermocellum is a moderately thermophilic anaerobe that thrives in temperatures near 60°C. This microbe is highly specialized for growth on cellulose and cellodextrins but does not grow as rapidly on glucose or fructose (Schwarz, 2001; Zhang and Lynd, 2005). *C. thermocellum* is an anaerobe with limited ATP production, but ATP is utilized for cell growth and maintenance, as well as for cellulase synthesis (Lynd and Zhang, 2002; Zhang and Lynd, 2005). In order to overcome the high ATP demands for cellulase synthesis and the limited supply of ATP, *C. thermocellum* meets its high energy demands by hydrolyzing cellulose. This organism produces several different types of cellulase components that work cooperatively to efficiently degrade cellulose (Lamed et al., 1985). *C. thermocellum* is therefore a potentially excellent candidate for the commercial conversion of

biomass into disaccharides, which can either be fermented by *C. thermocellum* into ethanol or enzymatically converted to monosaccharides for conversion to other biofuels and biochemicals (Bothun et al., 2004; Schwarz, 2001).

CELLULOSOMES AND CELLULASE MECHANISMS

The cellulase complex of *C. thermocellum* is known as a cellulosome. The cellulosome (Figure 3.1) is an elaborate, extracellular complex attached to the cell wall that efficiently breaks down cellulose into smaller polysaccharides, which can then be transported into *C. thermocellum* and used as its carbon source (Bhat and Wood, 1992; Schwarz, 2001). Figure 3.2 details the general structure of bacterial cellulosomes.

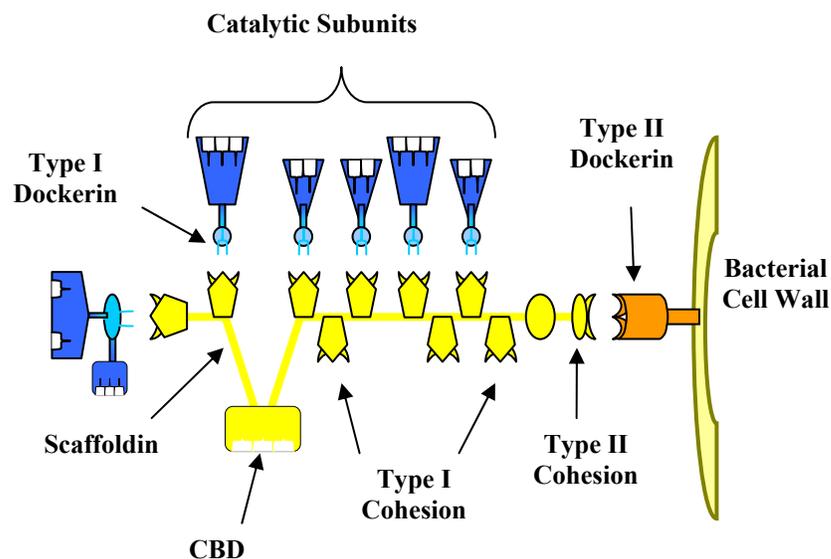


Figure 3.1. The cellulosome. The dockerin units on the catalytic subunits interact with the cohesion units, which results in an extracellular cellulase complex for *C. thermocellum*. Figure adapted from Gilbert (2007).

The cellulosome is composed of non-catalytic protein scaffolding, known as the scaffoldin, upon which the catalytic components sit. The catalytic subunits have dockerin modules that attach to the cohesion modules on the scaffoldin (Gilbert, 2007). There is also a cellulose binding domain (CBD) located on the scaffoldin (Figure 3.1). The CBD binds to the cellulose and keeps it in close proximity to the cellulosome components. The various catalytic subunits then break down the glycosidic bonds of the cellulose to form smaller sugars that *C. thermocellum* can use. The cellulosome of *C. thermocellum* is known to have many different types of catalytic subunits, which have been located and confirmed through genetic sequencing. *C. thermocellum* has multiple genes for both exoglucanase and endoglucanase, as well as genes for lichenase, chitinase, mannase, xylanase and cellobiase (Schwarz, 2001). This combination of various catalytic subunits increases the efficiency of the cellulosome. According to Zhang and Lynd (2005), *C. thermocellum* assimilates cellodextrins with an average length of four residues ($n \approx 4$). The ratio of energy used to energy gained by transporting larger cellodextrins ($n > 2$) is more favorable than if cellobiose or glucose were assimilated. However, even when cellulose is unavailable, it is possible for the *C. thermocellum* cellulosome to hydrolyze other types of sugars that may be available instead.

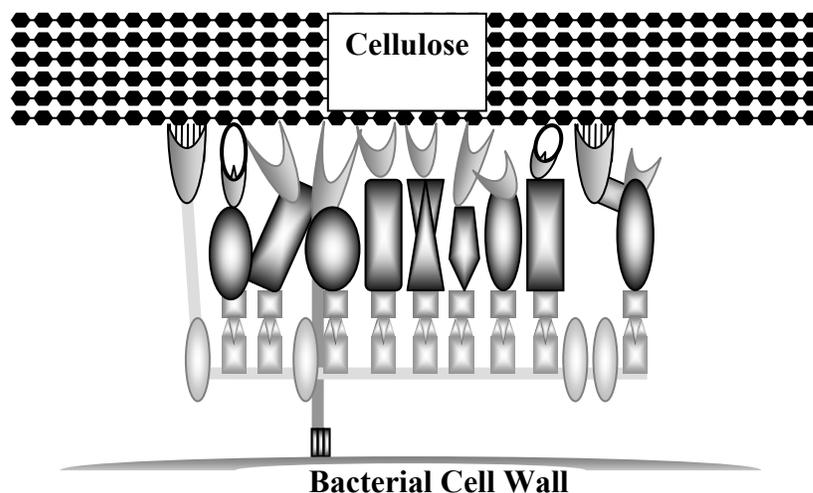


Figure 3.2. The structure of a hypothetical cellulosome. This structure is modeled on data collected from *Clostridium cellulovorans*. The catalytic subunits are drawn as crescents, where the cellulose binding domains (CBD) are drawn as crescents with teeth. The CBD holds onto the cellulose while the catalytic subunits break the glycosidic bonds. Figure adapted from Schwarz (2001).

The cellulosome offers several advantages for cellulose hydrolysis (Schwarz, 2001). One advantage is the increased synergism between the enzymes and the substrate due to the correct ratio of cellulolytic components. The correct ratio of components ensures that as long as cellulose is available, hydrolysis can occur with very little end-product inhibition. Another advantage of the cellulosome is the physical spacing between components prevents nonproductive adsorption onto cellulose. Nonproductive adsorption decreases the efficiency of the cellulosome. Correct spacing between catalytic components ensures that each available active site can be used to break down the cellulose. A third advantage of the cellulosome is that the entire complex is bound to a single site as opposed to components that must be bound to specific sites. All catalytic components necessary to hydrolyze the cellulose are located in close proximity to each other.

ELISA

Enzyme-linked immunosorbent assays (ELISA) have been used as early as the mid-1980s to quantify specific cellulase components of various *Trichoderma* species (Kolbe and Kubicek, 1990; Oh et al., 1986). Nieves and coworkers (1995) also used an ELISA to detect the presence of specific cellulase components of *Acidothermus cellulolyticus* and *Thermomonospora fusca*. The cellulases measured in the aforementioned studies are not cell-associated, but the *C. thermocellum* cellulosome is attached to the cell. Zhang and Lynd (2003) developed an ELISA in order to quantify the cellulosome mass of *C. thermocellum* independently of the cell mass.

The main use of the enzyme-linked immunosorbent assay (ELISA) is to measure the concentration of a desired reactant (Crowther, 1995; Zhang and Lynd, 2003). This assay has many advantages (Crowther, 1995) for use in high-solids environments. The main advantage is that the ELISA is specific to one set of nucleotides, and therefore can accurately separate the cellulosome from other proteins in the cell. In addition, an ELISA assay is easily automated to be a high-throughput assay. An ELISA is generally one of four different types: direct, indirect, sandwich or competition (Crowther, 1995).

Since *C. thermocellum* has cellulosomes that contain enzymes, total protein assays quantify both the cell mass and cellulosome mass. The indirect ELISA is used to detect a specific antibody, which indirectly measures the protein of interest. Zhang and coworkers (2003) used the indirect ELISA to quantify the cellulase mass concentration of *C. thermocellum* independently of the cell mass concentration. The ELISA allows the

specificity of the assay to be controlled by the antigen that is attached to the solid phase, i.e. the denatured cellulosome that is attached to the well in the plate (Crowther, 1995).

The basic reaction scheme of an indirect ELISA includes four (Figure 3.3) key steps (Crowther, 1995). The antigen is attached to the solid phase, which in the present study is the cellulosome and the plastic, 96-well plate, respectively. The primary antibody is added and reacts with the antigen. The ELISA is also effective in measuring the cellulosome mass concentration rather than total protein concentration because the antibodies used in the assay were specific to the cohesion domain of the scaffoldin protein from *C. thermocellum* (Zhang and Lynd, 2003). This specificity guarantees that only the cellulosome protein is accounted for in the color change. Then, the secondary antibody, which is an antibody labeled with an enzyme such as horseradish peroxidase, alkaline phosphatase, or β -galactosidase, is added to react with the primary antibody. Finally, a color-forming agent is added. As it reacts with the enzyme on the secondary antibody, the solution in the well changes color. The color change is read, and the cellulase concentration is determined by using a standard curve previously determined.

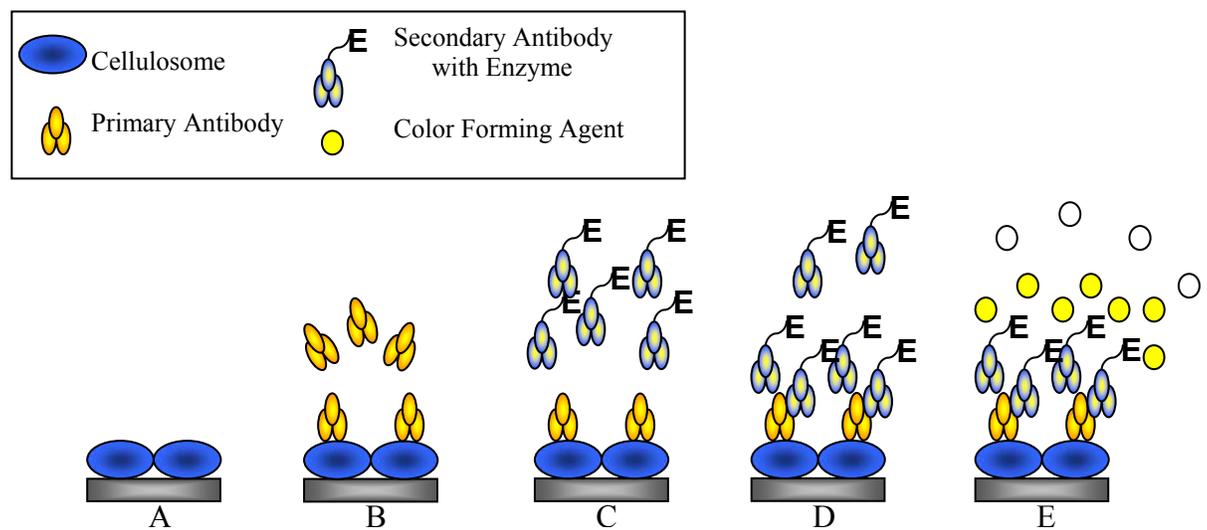


Figure 3.3. The steps of the indirect ELISA process. (A) The antigen (denatured cellulosome) is adsorbed to the solid phase (96-well plate). (B) The primary antibody is added and attaches to the antigen. Excess antibody is removed by washing. (C-D) The secondary antibody is added and reacts with the primary antibody. Excess antibody is removed by washing. (E) A color-forming agent is added, which reacts with the enzyme on the secondary antibody. The color change that occurs can be read by a spectrophotometer. Figure adapted from Crowther (2001).

The ELISA is used to quantify the cellulosome mass concentration present in a sample, whereas cellulase activity assays are used to measure the degree to which the cellulase is effectively breaking glycosidic bonds. Cellulase may be present in the sample, but may not perform optimally if the saccharification conditions are not adequate. Both types of assay are required to determine the combined effect of the quantity and the activity of cellulase in systems where the cellulase is produced by the microbe and not added from commercial enzymes

According to Zhang and Lynd (2003), they have successfully developed an ELISA method that quantifies cellulase mass concentration for liquid cultivation. The objective of this study was to explore the ability to transfer the ELISA developed by Zhang and Lynd for liquid cultivation to solid substrate cultivation. Our hypothesis was that if the ELISA is accurate in quantifying cellulosome mass from liquid systems, the method can be adapted to quantify cellulosome mass from high-solids environments.

MATERIALS AND METHODS

AMORPHOUS CELLULOSE

Amorphous cellulose was prepared as described by Zhang et al. (2006a) with some modifications. Microcrystalline cellulose (0.2 g, Avicel PH-105, FMC Corp, Philadelphia, PA) and 0.6 mL distilled water were added to a 20 mL centrifuge tube. Ten milliliters of ice-cold H_3PO_4 (86.5%) was slowly added and vigorously stirred, with special attention to mixing prior to the addition of the last 2.0 mL of H_3PO_4 . The suspension was placed on ice for 1.0 hour with occasional mixing. After the hour in the ice bath, 40 mL of ice-cold distilled water was added to the centrifuge tube in 10 mL intervals with vigorous shaking between additions. The cellulose suspension was centrifuged with a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Dupont Instruments, Wilmington, DE) at 5000g and 4°C for 20 min. The pellet was resuspended in ice-cold water and centrifuged at 7000g for 10 min. The supernatant, which contained phosphoric acid, was discarded. This washing process was repeated three times. Any remaining phosphoric acid was neutralized by adding 0.5 mL of 2.0 M Na_2CO_3 . The pellet was resuspended in 45 mL of ice-cold water and centrifuged. This washing process was repeated until the pH was in the range of 5-7 (approximately three washings). The amorphous cellulose suspension was stored at 4°C with the addition of a small amount of sodium azide to discourage microbial growth (Zhang et al., 2006a).

CELLULASE FRACTIONS

Cell culture preparation. *C. thermocellum* ATCC 27405 was obtained from American Type Culture Collection (Rockville, MD) and re-isolated as previously described (Erbeznik et al., 1997). The organism was grown in basal medium that contained (per liter): 1530 mg Na₂HPO₄, 1500 mg KH₂PO₄, 500 mg NH₄Cl₂, 500 mg (NH₄)₂SO₄, 90 mg MgCl₂·6H₂O, 30 mg CaCl₂, 4000 mg yeast extract, 10 mL standard vitamins, 5.0 mL modified metals, 500 mg cysteine hydrochloride, 1.0 mL resazurin and 4000 mg sodium carbonate. The medium pH was adjusted to 6.7 with NaOH and was maintained under a 100% carbon dioxide atmosphere. The vitamin solution contained (per liter of distilled water): 100 mg Pyridoxamine 2 HCL, 200 mg Riboflavin, 200 mg Thiamine HCL, 200 mg Nicotinamide, 200 mg CaD Pantotheinate, 100 mg Lipoic acid, 10 mg p-aminobezoic acid, 5.0 mg Folic acid, 5.0 mg Biotin, 5.0 mg Cobalamin (Co B₁₂), 100 mg Pyridoxal HCL and 100 mg Pyridoxine. The modified metal solution contained (per liter of distilled water): 500 mg Na₄EDTA, 200 mg FeSO₄·7H₂O, 10 mg ZnSO₄·7H₂O, 200 mg MnCl₂·4H₂O, 20 mg H₃BO₃, 20 mg CoCl₂·6H₂O, 1.0 mg CuCl₂·2H₂O, 2.0 mg NiCl₂·6H₂O, 3.0 mg Na₂MoO₄·2H₂O, 10 mg Na₂WO₄·2H₂O and 1.0 mg Na₂SeO₃. Avicel was prepared separately and added to provide a final concentration of 4 g/L after the medium was sterilized.

C. thermocellum was grown in two batch reactors with 10 g/L cellulose until >98% and of the substrate was hydrolyzed. As depicted in Figure 3.4, the solution from the first reactor was centrifuged and the supernatant collected. The pellet was stored at -80°C for later cellulase fractionation. The supernatant contained crude free cellulase at 98% hydrolysis (CFC98). This solution was purified as described later in order to obtain purified free cellulase, 98% hydrolysis (PFC98). The pellet was then fractionated into crude pellet cellulase, 98% hydrolysis (CPC98) and free pellet cellulase, 98% hydrolysis (PPC98) as described later.

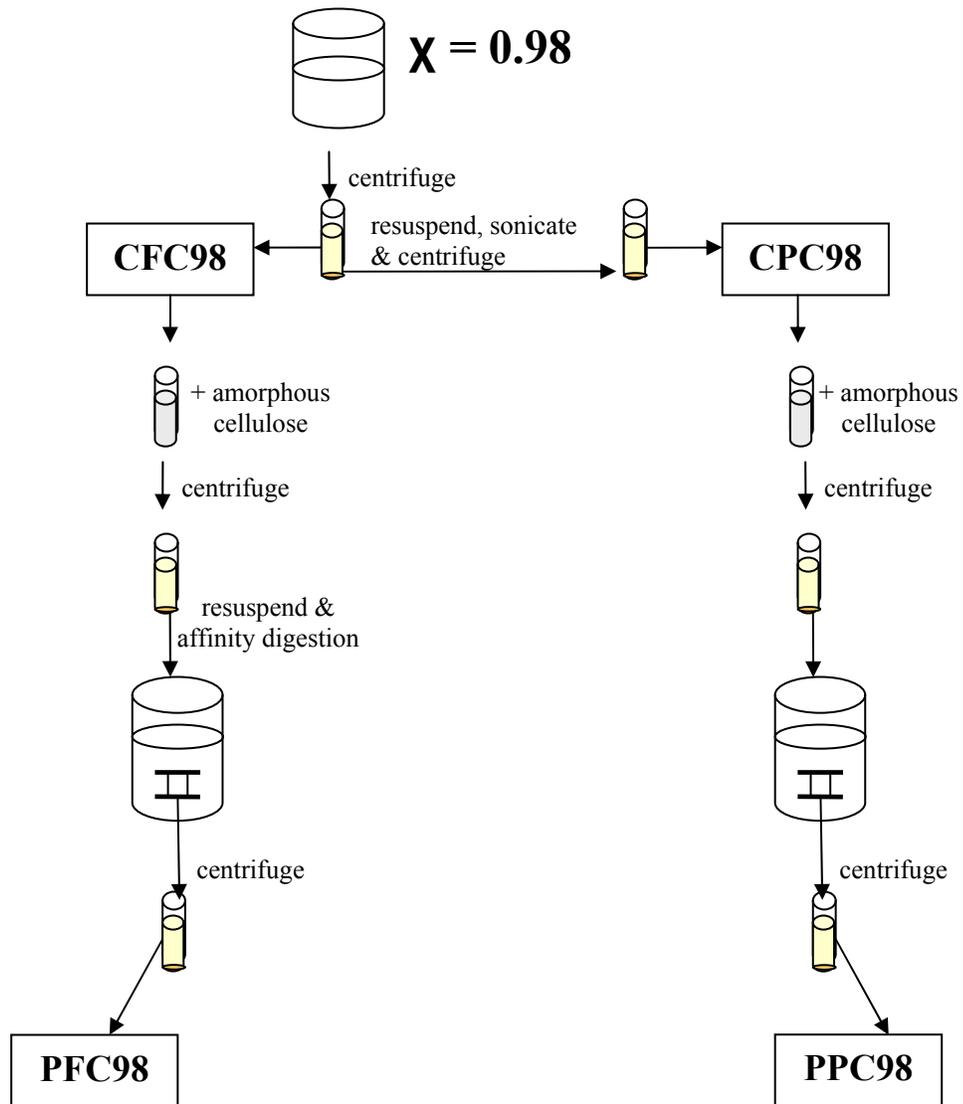


Figure 3.4. Cellulase fractions obtained with 98% hydrolysis. CFC98 = crude free cellulase, PFC98 = purified free cellulase, CPC98 = crude pellet cellulase, PPC98 = purified pellet cellulase.

AFFINITY DIGESTION

Purified cellulase fractions were obtained by affinity digestion as described by Morag et al. (1992) and Zhang and Lynd (2003). Amorphous cellulose was added to the various fractions of cellulase and allowed to incubate at 4°C and 100 rpm overnight. After incubation, the suspension was centrifuged at approximately 14300g and 4°C for 30 min. The pellet was resuspended in 50 mL dialysis buffer (50 mM Tris buffer, 10 mM CaCl₂, 5.0 mM DTT, pH 7.0) and placed in dialysis sacs (Spectra/Por, MWCO 6-8000, Rancho Dominguez, CA). The dialysis sacs were placed in a distilled water bath and incubated at 55°C and 140 rpm for approximately 5.0 hours. The water was changed every 0.5 hours to avoid cellulase inhibition by hydrolysis products. When the suspension in the dialysis sacs turned transparent, they were removed from the water bath, and the contents of the sacs were emptied into a centrifuge tube. The suspension was centrifuged at 5000g for 20 min. The resulting supernatant was considered “purified cellulase.”

CELLULASE ACTIVITY ASSAY

The cellulase activity was measured as described by Zhang and Lynd (2003). The cellulase fraction to be assayed was diluted with 50 mM Tris buffer and 10 mM CaCl₂ at a pH of 7.0 so that the final volume was 1.5 mL. Three replications of three dilutions were made (Figure 3.5). The enzymatic reaction occurred in a buffer that consisted of 50 mM Tris, 3.0 g/L NaN₃, 10 mM CaCl₂, 20 mM DTT and 40 g/L Avicel PH-105. A 1.5 mL aliquot of the buffer was added to each of the test tubes with the 1.5 mL of diluted cellulase. A 1.5 mL aliquot of the buffer was also added to the tubes that would later become the blanks for each cellulase dilution. All tubes were incubated for 1.0 hr at 60°C with continuous shaking. After incubation, the tubes designated as the blanks had 1.5 mL of diluted cellulase solution added and were immediately placed in an ice bath to halt hydrolysis. The tubes were vortexed and poured into 2.0 mL o-ring centrifuge tubes and centrifuged at 6000 rpm for 10 min. The soluble sugar content in the supernatant was then determined using the phenol-sulfuric acid assay.

The phenol-sulfuric acid method was conducted as outlined by DuBois et al. (1956) with slight modifications and used to measure total soluble sugar concentrations. Cellulase activity was calculated from the sugar concentration released. This assay requires concentrated H₂SO₄, 5% phenol solution and 100 mg/L glucose standard. The glucose standard curve tubes were prepared by diluting the stock solution to range from 0-100 mg/L.

A 0.5 mL aliquot of the diluted glucose standard was added to each respective tube along with 0.5 mL of 5% phenol solution (Figure 3.5). Likewise, 0.5 mL of the sample supernatant was added to each respective tube along with 0.5 mL of 5% phenol solution. All tubes were vortexed to thoroughly mix the contents. A 2.5 mL aliquot of concentrated H₂SO₄ was added to each tube. The tubes were immediately sealed and mixed. The tubes were incubated for 30 min at room temperature before transferring to a cuvette and reading the absorbance at 485 nm.

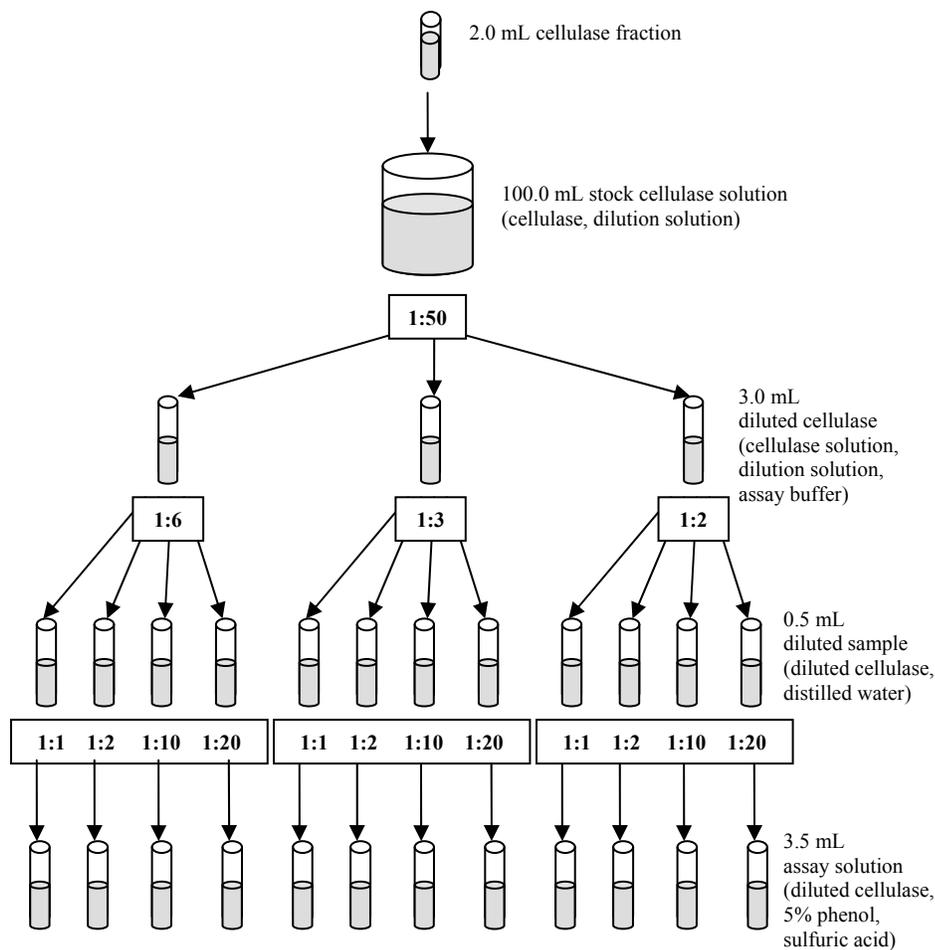


Figure 3.5. Dilution chart for the phenol-sulfuric acid assay.

TOTAL PROTEIN ASSAYS

Peterson Method

The Peterson method was conducted according to the protocol provided with the total protein kit (TP0300, Sigma, St. Louis, MO), (Figure 3.6). Standard curve tubes were prepared in triplicate and ranged from 0-400 $\mu\text{g}/\text{mL}$ in 100 $\mu\text{g}/\text{mL}$ intervals, including 50 $\mu\text{g}/\text{mL}$. Sample tubes were also prepared in triplicate and were diluted to 1.0 mL with water. One milliliter of the Lowry reagent solution was added to each tube and mixed well. The tubes were incubated at room temperature for 20 min. A 0.5 mL aliquot of Folin and Ciocalteu's phenol reagent (Folin and Ciocalteu, 1927) working solution was added to each tube and rapidly and immediately mixed. The tubes were again incubated for 30 min to allow the color to develop. The absorbance was measured at 750 nm against the 0 $\mu\text{g}/\text{mL}$ standard protein blank.

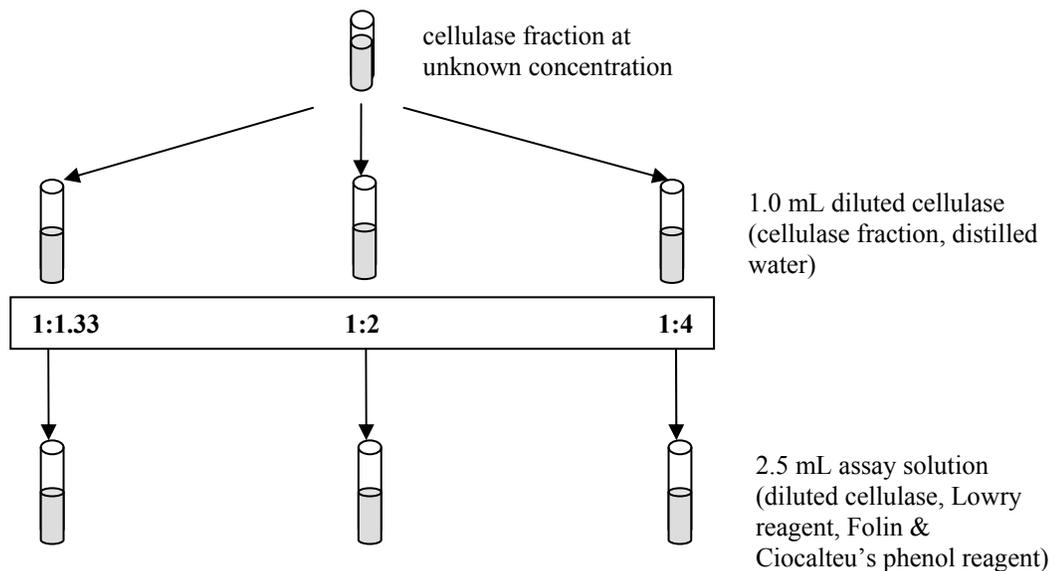


Figure 3.6. Dilution chart for the Peterson method for total protein determination.

Lowry Method

Sample protein concentrations were determined as outlined by Lowry et al. (1951) using bovine serum albumin (BSA) as the standard. Lowry 1 was prepared by adding 20 g Na_2CO_3 into 1.0 L of 0.1 M NaOH. Copper reagent consisted of 300 mL of Lowry 1, 3.0 mL of 4% (w/v) sodium tartarate and 3.0 mL of 2% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. These three reagents must be added in the stated order. Phenol reagent consisted of 30.0 mL of 2.0 N Folin-Ciocalteu acid and 30.0 mL of distilled water. Both the copper reagent and the phenol reagent are unstable in light and should be made right before use. Also, 0.2 N NaOH was used to lyse the cells and 2000 mg/L BSA (Sigma A-4503, Fraction V) was used as the standard.

Standard curve tubes were prepared in triplicate and ranged from 0-2000 mg/L in 400 mg/L intervals. Sample tubes were also prepared in triplicate with a 125 μL aliquot of the protein sample. To all tubes, 125 μL of 0.2 N NaOH was added and placed in a boiling water bath for 15-20 min. After the tubes cooled, 3.75 mL of the copper reagent was added and mixed. The tubes were incubated at 39°C for 45 min. A 0.75 mL aliquot of the phenol reagent is added and mixed in each tube. The tubes were incubated at room temperature for 30 min in the dark. The absorbance was measured at 660 nm against the 0 mg/L BSA blank.

ELISA

Cellulase concentration was measured by indirect ELISA (Crowther, 1995; Zhang and Lynd, 2003). Carbonate bicarbonate buffer (CBB), used for antigen dilution, was prepared as a 50 mM carbonate/bicarbonate solution (Sigma C3041) with a pH of 9.8. Phosphate-buffered saline (PBS) contained 10 mM phosphate buffer and 150 mM NaCl at a pH of 7.4 with 0.05% Tween-20 (PBST), which was used to wash the wells. Blocking buffer (BB) consisted of 0.25% BSA, 0.05% Tween-20 and 0.05% NaN_3 in PBS. Primary antibody (Genosys Co., Woodlands, TX) was diluted to 1:800 using the BB. The secondary antibody, or conjugate, was whole molecule anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma A3687), which was diluted to 1:4000 with BB. Alkaline phosphatase substrate solution consisted of 1.0 mg/mL *p*-nitrophenol phosphate (pNPP) (Sigma N2765) and 0.5 mM MgCl_2 in 10% diethanolamine with a pH adjusted to 9.8 by concentrated HCl. Stopping reagent, used to stop color formation, consisted of 2.0 M Na_2CO_3 . All assays were conducted in flat-bottomed, 96-well Nunc-Immuno™ PolySorp™ surface plates (Roskilde, Denmark).

Samples to be assayed were prepared by mixing 0.2 mL of the cellulase samples and 0.245 mL of EDTA denaturing buffer, which consisted of 0.07 M NaCl, 7.0 mM EDTA, 0.28 M NaOH, 0.116 M DTT and 7.0 g/L cellobiose. This mixture was boiled for exactly 10 min. After cooling, the pH of all the sample mixtures was adjusted to 9.6 using 0.8 M HCl. The solution was then diluted to 2.0 $\mu\text{g/mL}$ using CBB. Further dilutions were made to the samples to achieve concentrations ranging from 0-2.0 $\mu\text{g/mL}$ in 0.25 $\mu\text{g/mL}$ intervals. After adding 50 μL of CBB to each well, 50 μL of the diluted cellulase mixtures was pipetted into each respective well. The plate was incubated for 2.0 hrs at 37°C in a rotary shaker set at 60 rpm to induce antibody coating of the well. After incubation, the plate was washed four times with distilled water (Original Wellwash 4 Mk2, Fisher Scientific, Waltham, MA) and then flip dried. A 0.2 mL aliquot of BB was adding to each well to prevent nonspecific binding. The plate was incubated at 37°C for 0.5 hr with rotary shaking set to 60 rpm and then washed four times with distilled water and flip dried. A 50 μL volume of diluted primary antibody solution was added to each well. The plate was incubated at 37°C for 1.5 hr with rotary shaking set to 60 rpm and washed four times with PBST, then four times with distilled water and flip dried. A 50 μL volume of diluted secondary antibody solution was added to each well. The plate was incubated at 37°C for 1.5 hr with rotary shaking set to 60 rpm and washed four times with PBST, then four times with distilled water and flip dried. A 100 μL aliquot of freshly made substrate was added to each well. The plate was incubated at room temperature for approximately 20 min, allowing color to develop. The reaction was stopped by adding 50 μL of stopping reagent to each well. Absorbance readings were taken by a μQuant spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT) at 450 nm and with a reference filter at 750 nm and air as a blank.

RESULTS

Cellulase fractions were collected from supernatant (approximately 2 L) and pellet samples. The supernatant contained crude free cellulase (CFC98). After affinity digestion, approximately 48 mL of purified free cellulase (PFC98) was collected. The pellet that resulted from the batch culture was used to collect the crude pellet cellulase (CPC98). Ten milliliters of resuspended pellet were French pressed and approximately 9 mL of pellet cellulase was collected.

CELLULASE ACTIVITY ASSAY

The cellulase activity was determined for three dilutions of the PFC98 fraction. The three dilutions had dilution factors of 300, 150 and 100. The cellulase activities were 0.306 ± 0.173 , 0.236 ± 0.226 and 0.108 ± 0.068 IU/mL, respectively.

TOTAL PROTEIN

The total protein content of the PFC98 and PPC98 fractions, determined using the Lowry method, contained 0.617 mg/mL and 0.143 mg/mL of protein, respectively.

The total protein content of the PFC98 and the CPC98 fractions, determined using the Peterson method contained 0.15 ± 0.08 mg/mL and 52.19 ± 28.00 mg/mL of protein, respectively.

ELISA

To estimate the cellulase content in the PFC98 fraction, the standard curve was recalculated using the protein content found from the Peterson method for protein determination for the CPC98 fraction and assuming that after purification, the PPC98 fraction would be 80% of the crude fraction. From these assumptions, the estimated content was 5.3 g of cellulase.

DISCUSSION

The cellulase assayed in the present study was 12 times less active per mL than that used in the Zhang and Lynd study (2003); however, the protein content was also lower in the present study. The lower protein content is likely the reason for the lower activity reported here because it could also indicate lower cellulase content.

In the present study, as the enzyme concentration increased, the activity decreased. This trend was similar to that seen using the *T. reesei* cellulase in Chapter 2.

According to Zhang and Lynd (2003), they used the Bradford method for protein determination for the original batch culture supernatant and the Peterson method for protein determination for the original batch culture pellet. They obtained 1.547 mg/mL and 0.255 mg/mL of protein for the PFC98 and CPC98 fractions, respectively. The present study was unable to replicate these results. The protein content Zhang and Lynd were able to produce for PFC98 was 10-fold higher and CPC98 was 204-fold smaller than the protein produced in the present study. The discrepancy in results may be the result from the use of different

protein determination methods. The present study used the Peterson method for all protein determination.

Zhang and Lynd (2003) reported that their PFC98 fraction contained 43 mg of cellulase, which was more than 120-fold smaller than the value estimated in the present study. However, the PPC98 was estimated at 80% of the crude fraction, which is a high assumption. It is more likely that after purification, there is less than 80% recovery of protein.

The ELISA results produced in the Zhang and Lynd (2003) study were unable to be replicated in the present study. One reason for this discrepancy may be because the ELISA protocol was not optimized for capacity of cellulase binding in the wells for the present study. Considering the differences found in the protein content, it is possible that overcrowding or jamming effects may be affecting the adsorption of cellulase protein to the antigen. With lower enzyme concentrations in the well, an accurate measurement may not have been taken, thus affecting the ELISA results.

LITERATURE CITED

- Bhat KM, Wood TM. 1992. The cellulase of the anaerobic bacterium *Clostridium thermocellum* - isolation, dissociation, and reassociation of the cellulosome. *Carbohydrate Research* 227:293-300.
- Bothun GD, Knutson BL, Berberich JA, Strobel HJ, Nokes SE. 2004. Metabolic selectivity and growth of *Clostridium thermocellum* in continuous culture under elevated hydrostatic pressure. *Applied Microbiology and Biotechnology* 65(2):149-157.
- Crowther JR. 1995. ELISA: Theory and Practice. Walker JM, editor. Totowa: Humana Press, Inc. 256 p.
- Crowther JR. 2001. The ELISA Guidebook. Totowa, NJ: Humana Press. 436 p.
- DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *J Biol Chem* 28(3):350-356.
- Erbeznik M, Jones CR, Dawson KA, Strobel HJ. 1997. *Clostridium thermocellum* JW20 (ATCC 31549) is a coculture with *Thermoanaerobacter ethanolicus*. *Applied and Environmental Microbiology* 63(7):2949-2951.
- Folin O, Ciocalteu V. 1927. On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry* 73(2):627-650.
- Gilbert HJ. 2007. Cellulosomes: microbial nanomachines that display plasticity in quaternary structure. *Molecular Microbiology* 63(6):1568-1576.
- Kolbe J, Kubicek CP. 1990. Quantification and identification of the main components of the *Trichoderma* cellulase complex with monoclonal-antibodies using an enzyme-linked-immunosorbent-assay (ELISA). *Applied Microbiology and Biotechnology* 34(1):26-30.
- Lamed R, Kenig R, Setter E, Bayer EA. 1985. Major characteristics of the cellulolytic system of *Clostridium thermocellum* coincide with those of the purified cellulosome. *Enzyme and Microbial Technology* 7(1):37-41.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193(1):265-275.
- Lynd LR, Zhang YH. 2002. Quantitative determination of cellulase concentration as distinct from cell concentration in studies of microbial cellulose utilization: Analytical framework and methodological approach. *Biotechnology and Bioengineering* 77(4):467-475.
- Morag E, Bayer EA, Lamed R. 1992. Affinity digestion for the near-total recovery of purified cellulosome from *Clostridium thermocellum*. *Enzyme and Microbial Technology* 14(4):289-292.

- Nieves RA, Chou YC, Himmel ME, Thomas SR. 1995. Quantitation of *Acidothermus cellulolyticus* E1 endoglucanase and *Thermomonospora fusca* E(3) exoglucanase using enzyme-linked-immunosorbent-assay (ELISA). *Applied Biochemistry and Biotechnology* 51-2:211-223.
- Oh TK, Kim SH, Park KH. 1986. Determination of cellobiohydrolase from culture filtrate of *Trichoderma viride* by the method of single immunodiffusion and enzyme-linked-immunosorbent-assay. *Biotechnology Letters* 8(6):403-406.
- Schwarz WH. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology* 56(5-6):634-649.
- Zhang YH, Lynd LR. 2003. Quantification of cell and cellulase mass concentrations during anaerobic cellulose fermentation: Development of an enzyme-linked immunosorbent assay-based method with application to *Clostridium thermocellum* batch cultures. *Analytical Chemistry* 75(2):219-227.
- Zhang YHP, Cui JB, Lynd LR, Kuang LR. 2006a. A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: Evidence from enzymatic hydrolysis and supramolecular structure. *Biomacromolecules* 7(2):644-648.
- Zhang YHP, Lynd LR. 2005. Cellulose utilization by *Clostridium thermocellum*: bioenergetics and hydrolysis product assimilation. *Proceedings of the National Academy of Sciences of the United States of America* 102(20):7321-7325.

CHAPTER FOUR: FUTURE WORK

There are several areas in which the studies presented in this work can be extended. The corn stover used for the hydrolysis by fungal cellulases could be pretreated in some manner other than simple milling procedures. Other pretreatment options could include dilute acid treatment, ammonia fiber explosion, or sodium hydroxide treatment. Increased cellulose accessibility could potentially result in increased soluble sugar content. With different pretreatments, all advantages and disadvantages must be considered, especially from economical and biochemical standpoints; however, this does not represent novel work.

Another area that can be further explored is the use of HPLC for sugar evaluation instead of the phenol-sulfuric acid assay. While the phenol-sulfuric acid assay measures total soluble sugars, HPLC offers a more detailed analysis and can assess the types of soluble sugars present in a sample. More information about the products of hydrolysis can potentially lead to a better understanding of the mechanism and result in the optimization of the hydrolysis process. Knowledge of the sugars produced could also benefit downstream in the fermentation process.

While the ELISA has been beneficial in many ways for cellulase quantification, there are still some parameters that warrant further investigation. For example, the specific well loadings have not been optimized for the plate used in this study. It is possible that overcrowding or jamming effects may be affecting the adsorption of antigen to the solid phase. With lower antigen concentrations in the well, there is the potential for lower enzyme concentrations in the well and would therefore not produce an accurate measurement. A better understanding of the binding capacity for these plates and antigen concentrations can potentially lead to more accurate measurements.

In addition to using the four cellulase fractions produced from the batch of *C. thermocellum* after 98% hydrolysis, using eight cellulase fractions (Figure 4.1) produced after allowing only 80% completion of hydrolysis could provide a better insight into the capabilities of *C. thermocellum* cellulase in high-solids environments. The environment in the 80% hydrolysis reactor is a closer approximation than the 98% hydrolysis system to the environment of a high-solids system. The eight fractions of cellulase should be studied because they represent all possible levels of cellulase adsorption to substrate and microbe. Also, the assays used in quantifying enzymatic properties must be adapted for use in high-

solids environments in order to ensure accurate measurements. Progress in these areas will allow for advancements in solid substrate cultivation for biofuel and biochemical production.

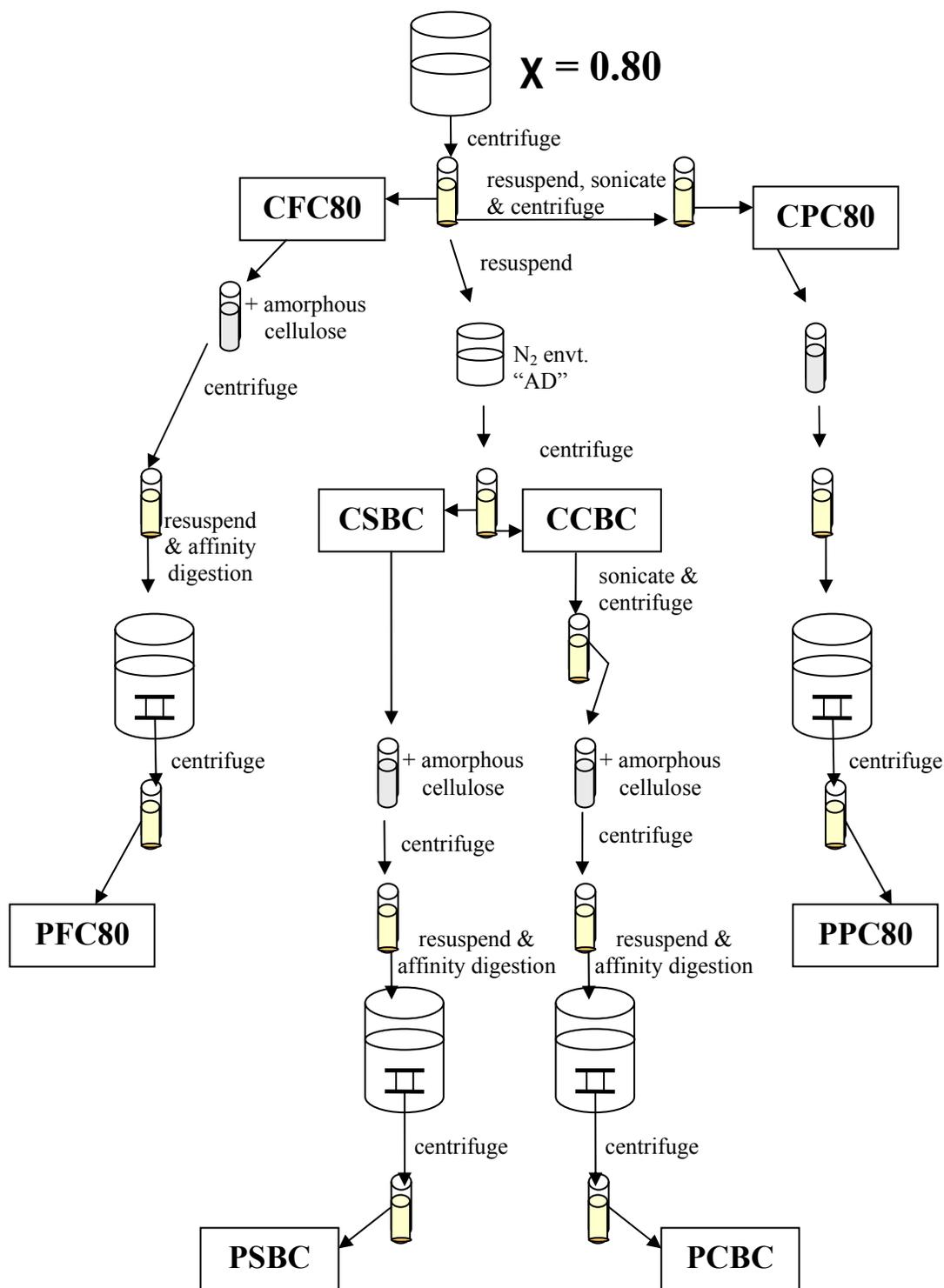


Figure 4.1. Cellulase fractions obtained with 80% hydrolysis. CFC80 = crude free cellulase, PFC80 = purified free cellulase, CPC80 = crude pellet cellulase, PPC80 = purified pellet cellulase, CSBC = crude substrate-bound cellulase, PSBC = purified substrate-bound cellulase, CCBC = crude cell-bound cellulase, PCBC = purified cell-bound cellulase.

REFERENCES

- Adler E. 1977. Lignin Chemistry - Past, Present and Future. *Wood Science and Technology* 11(3):169-218.
- Adney B, Baker, J. 1996. Measurement of cellulase activities. Golden, CO: National Renewable Energy Laboratory. Report nr NREL/TP-501-42628.
- Bailey JE, Ollis DF. 1986. *Biochemical Engineering Fundamentals*. New York, NY: McGraw-Hill Publishing Company. 984 p.
- Bayrock DP, Ingledew WM. 2001. Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. *Journal of Industrial Microbiology & Biotechnology* 27(2):87-93.
- Bhat KM, Wood TM. 1992. The cellulase of the anaerobic bacterium *Clostridium thermocellum* - isolation, dissociation, and reassociation of the cellulosome. *Carbohydrate Research* 227:293-300.
- Bothun GD, Knutson BL, Berberich JA, Strobel HJ, Nokes SE. 2004. Metabolic selectivity and growth of *Clostridium thermocellum* in continuous culture under elevated hydrostatic pressure. *Applied Microbiology and Biotechnology* 65(2):149-157.
- Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* 72(1-2):248-254.
- Brown L, Torget R. 1996. Enzymatic saccharification of lignocellulosic biomass. Golden, CO: National Renewable Energy Laboratory. Report nr LAP 009.
- Brown RC. 2003. *Biorenewable resources: engineering new products from agriculture*: Blackwell Publishing Company. 286 p.
- Bruice PY. 2004. *Organic Chemistry*. Upper Saddle River, NJ: Prentice Hall.
- Chang MCY. 2007. Harnessing energy from plant biomass. *Current Opinion in Chemical Biology* 11(6):677-684.
- Chang VS, Holtzapple MT. 2000. Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology* 84-6:5-37.
- Crowther JR. 1995. *ELISA: Theory and Practice*. Walker JM, editor. Totowa: Humana Press, Inc. 256 p.
- Crowther JR. 2001. *The ELISA Guidebook*. Totowa, NJ: Humana Press. 436 p.
- DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Journal of Biological Chemistry* 28(3):350-356.

- Erbeznik M, Jones CR, Dawson KA, Strobel HJ. 1997. *Clostridium thermocellum* JW20 (ATCC 31549) is a coculture with *Thermoanaerobacter ethanolicus*. Applied and Environmental Microbiology 63(7):2949-2951.
- Folin O, Ciocalteu V. 1927. On tyrosine and tryptophane determinations in proteins. Journal of Biological Chemistry 73(2):627-650.
- Gan Q, Allen SJ, Taylor G. 2003. Kinetic dynamics in heterogeneous enzymatic hydrolysis of cellulose: an overview, an experimental study and mathematical modelling. Process Biochemistry 38(7):1003-1018.
- Ghose TK. 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59(2):257-268.
- Gilbert HJ. 2007. Cellulosomes: microbial nanomachines that display plasticity in quaternary structure. Molecular Microbiology 63(6):1568-1576.
- Horton HR, Moran LA, Scrimgeour KG, Perry MD, Rawn JD. 2006. Principles of Biochemistry. Upper Saddle River: Pearson Prentice Hall.
- Jorgensen H, Vibe-Pedersen J, Larsen J, Felby C. 2007. Liquefaction of lignocellulose at high-solids concentrations. Biotechnology and Bioengineering 96(5):862-870.
- Kidby DK, Davidson DJ. 1973. Convenient ferricyanide estimation of reducing sugars in nanomole range. Analytical Biochemistry 55(1):321-325.
- Kolbe J, Kubicek CP. 1990. Quantification and identification of the main components of the *Trichoderma* cellulase complex with monoclonal-antibodies using an enzyme-linked-immunosorbent-assay (ELISA). Applied Microbiology and Biotechnology 34(1):26-30.
- Lamed R, Kenig R, Setter E, Bayer EA. 1985. Major characteristics of the cellulolytic system of *Clostridium thermocellum* coincide with those of the purified cellulosome. Enzyme and Microbial Technology 7(1):37-41.
- Lau ATY, Wong WKR. 2001. Purification and characterization of a major secretory cellobiase, Cba2, from *Cellulomonas biazotea*. Protein Expression and Purification 23(1):159-166.
- Lee D, Owens VN, Boe A, Jeranyama P. 2007. Composition of herbaceous biomass feedstocks. Brookings, SD: North Central Sun Grant Center. 16 p.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193(1):265-275.
- Lu YP, Zhang YHP, Lynd LR. 2006. Enzyme-microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*. Proceedings of the National Academy of Sciences of the United States of America 103(44):16165-16169.

- Lynd LR, Grethlein HE, Wolkin RH. 1989. Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. *Applied and Environmental Microbiology* 55(12):3131-3139.
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* 66(3):506-+.
- Lynd LR, Zhang YH. 2002. Quantitative determination of cellulase concentration as distinct from cell concentration in studies of microbial cellulose utilization: Analytical framework and methodological approach. *Biotechnology and Bioengineering* 77(4):467-475.
- Morag E, Bayer EA, Lamed R. 1992. Affinity digestion for the near-total recovery of purified cellulosome from *Clostridium thermocellum*. *Enzyme and Microbial Technology* 14(4):289-292.
- Moxley G, Zhang YHP. 2007. More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. *Energy & Fuels* 21:3684-3688.
- Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* 153(2):375-380.
- Nieves RA, Chou YC, Himmel ME, Thomas SR. 1995. Quantitation of *Acidothermus cellulolyticus* E1 endoglucanase and *Thermomonospora fusca* E(3) exoglucanase using enzyme-linked-immunosorbent-assay (ELISA). *Applied Biochemistry and Biotechnology* 51-2:211-223.
- Oh TK, Kim SH, Park KH. 1986. Determination of cellobiohydrolase from culture filtrate of *Trichoderma viride* by the method of single immunodiffusion and enzyme-linked-immunosorbent-assay. *Biotechnology Letters* 8(6):403-406.
- Okazaki M, Mooyoung M. 1978. Kinetics of enzymatic-hydrolysis of cellulose - analytical description of a mechanistic model. *Biotechnology and Bioengineering* 20(5):637-663.
- Pande SV, Murthy MSR. 1994. A modified micro-Bradford procedure for elimination of interference from sodium dodecyl-sulfate, other detergents, and lipids. *Analytical Biochemistry* 220(2):424-426.
- Park JT, Johnson MJ. 1949. A submicrodetermination of glucose. *Journal of Biological Chemistry* 181(1):149-151.
- Perlack RD, Wright, L.L, Turhollow, A.F., Graham, R.L., Stokes, B.J., Erbach, D.C. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. Oak Ridge, TN: Oak Ridge National Laboratory. Report nr ORNL/TM-2005/66. 59 p.

- Peterson GL. 1977. Simplification of protein assay method of Lowry et al - which is more generally applicable. *Analytical Biochemistry* 83(2):346-356.
- Peterson GL. 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Analytical Biochemistry* 100(2):201-220.
- Reese ET. 1956. Enzymatic hydrolysis of cellulose. *Applied Microbiology* 4(1):39-45.
- Roe JH. 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. *Journal of Biological Chemistry* 212(1):335-343.
- Romaniec MPM, Huskisson N, Barker P, Demain AL. 1993. Purification and properties of the *Clostridium thermocellum* Bglb gene-product expressed in *Escherichia coli*. *Enzyme and Microbial Technology* 15(5):393-400.
- Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS. 2007. Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates. *Biotechnology Progress* 23:1270-1276.
- Sapan CV, Lundblad RL, Price NC. 1999. Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry* 29:99-108.
- Schwarz WH. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology* 56(5-6):634-649.
- Somogyi M. 1952. Notes on sugar determination. *Journal of Biological Chemistry* 195(1):19-23.
- Steiner W, Sattler W, Esterbauer H. 1988. Adsorption of *Trichoderma reesei* cellulase on cellulose - experimental-data and their analysis by different equations. *Biotechnology and Bioengineering* 32(7):853-865.
- Sticklen MB. 2007. Feedstock crop genetic engineering for alcohol fuels. *Crop Science* 47:2238-2248.
- Valjamae P, Pettersson G, Johansson G. 2001. Mechanism of substrate inhibition in cellulose synergistic degradation. *European Journal of Biochemistry* 268(16):4520-4526.
- Viles FJ, Silverman L. 1949. Determination of starch and cellulose with anthrone. *Analytical Chemistry* 21(8):950-953.
- Waffenschmidt S, Jaenicke L. 1987. Assay of reducing sugars in the nanomole range with 2,2'-bicinehoninate. *Analytical Biochemistry* 165(2):337-340.
- Wood TM, Bhat KM. 1988. Methods for measuring cellulase activities. *Methods in Enzymology* 160:87-112.
- Xu F, Ding HS. 2007. A new kinetic model for heterogeneous (or spatially confined) enzymatic catalysis: Contributions from the fractal and jamming (overcrowding) effects. *Applied Catalysis a-General* 317(1):70-81.

- Xu F, Ding HS, Osborn D, Tejirian A, Brown K, Albano W, Sheehy N, Langston J. 2008. Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. *Journal of Molecular Catalysis B-Enzymatic* 51(1-2):42-48.
- Zhang YH, Lynd LR. 2003. Quantification of cell and cellulase mass concentrations during anaerobic cellulose fermentation: Development of an enzyme-linked immunosorbent assay-based method with application to *Clostridium thermocellum* batch cultures. *Analytical Chemistry* 75(2):219-227.
- Zhang YHP, Cui JB, Lynd LR, Kuang LR. 2006a. A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: Evidence from enzymatic hydrolysis and supramolecular structure. *Biomacromolecules* 7(2):644-648.
- Zhang YHP, Himmel ME, Mielenz JR. 2006b. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances* 24(5):452-481.
- Zhang YHP, Lynd LR. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnology and Bioengineering* 88(7):797-824.
- Zhang YHP, Lynd LR. 2005. Cellulose utilization by *Clostridium thermocellum*: bioenergetics and hydrolysis product assimilation. *Proceedings of the National Academy of Sciences of the United States of America* 102(20):7321-7325.
- Zhang YHP, Lynd LR. 2006. A functionally based model for hydrolysis of cellulose by fungal cellulase. *Biotechnology and Bioengineering* 94(5):888-898.

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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 Meeting, Providence, RI, 2008.*
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