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Enzyme-substrate interaction significantly affects activity loss during enzymatic hydrolyis of cellulose.

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ENZYME-SUBSTRATE INTERACTION SIGNIFICANTLY AFFECTS ACTIVITY LOSS DURING ENZYMATIC HYDROLYSIS OF CELLULOSE

By

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ABSTRACT

Rising concerns about dependency on fossil fuels in the 21st century has sparked a growth in research for renewable energy sources. One renewable energy production process of interest is the reduction of cellulose into fermentable sugars by means of enzymatic hydrolysis. The reaction requires a residence time on the order of seven or more days and usually does not achieve complete conversion. The slow reaction rate and incomplete conversion is generally attributed to loss of enzymatic activity during the reaction. Deactivation of the enzyme is classified here as either substrate related deactivation or nonspecific deactivation. The general term of nonspecific deactivation refers to any activity loss of the enzyme not attributed to interaction with substrate. Reasons for deactivation due to enzyme-substrate interaction are still uncertain and deactivation may possibly be attributed to factors such as poor desorption of enzyme from the substrate and product inhibition.

In this research, the nonspecific deactivation was quantified by activity measured following enzyme incubation in a substrate-free buffer for 2, 4, 8, 16, 24, 48, or 72 hours, followed by a second incubation of one hour with 2.0 grams of substrate. Testing for enzyme-substrate interaction was performed by adding an initial substrate load to the first incubation in the amount of 0.1, 0.2, or 0.4 grams, and then substrate was added during the second incubation to bring the total in all cases up to 2.0 grams. The amount of enzyme in the solution was held constant at 0.6 mL, for all cases. Two substrates of different crystallinity, filter paper (CrI = 45%) and dewaxed cotton (CrI = 90%) were studied here. The cellulase enzyme showed slight deactivation after incubating for

varying times during the initial incubation in a substrate-free buffer. Enzyme-substrate interactions also resulted in deactivation and generally contributed to more of the overall deactivation than did nonspecific deactivation. Deactivation was seen to depend on the initial incubation time, substrate load, and substrate type (crystallinity). There did not appear to be a consistent trend in relative percent deactivation for nonspecific deactivation and deactivation due to enzyme-substrate interaction for initial incubations less than 24 hours for either substrate, but the relative amount of nonspecific deactivation appeared to increase between 24 and 72 hours. However, the enzyme-substrate interaction still contributed to more than fifty percent of deactivation for all but one case. The lack of a trend prior to 24 hours is likely attributed to glucose concentrations that are within the range of error of the YSI analyzer.

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NOMENCLATURE

A_{non}	=	Activity of the nonspecific deactivation (2.0 grams of substrate incubated for one hour), grams of glucose per liter per gram of substrate added in the second incubation
$A_{t,non}$	=	Activity as the first incubation time changes, grams of glucose per liter per gram of substrate added in the second incubation
A_{sub}	=	Activity of a one hour incubation with substrate load equal to that added during the second incubation, grams of glucose per liter per gram of substrate added in the second incubation
$A_{t,sub}$	=	Activity with substrate interactions at a given time for initial incubation, grams of glucose per liter per gram of substrate added in the second incubation
C_1	=	Glucose concentration produced by the first incubation, grams of glucose per liter
C_2	=	Glucose concentration produced by second incubation, grams of glucose per liter
C_3	=	Glucose concentration change of control, grams of glucose per liter
P	=	Processivity, unitless
$P_{enz\text{-}sub}$	=	Portion of enzyme deactivation caused by enzyme-substrate interactions
$P_{nonspecific}$	=	Portion of enzyme deactivation not caused by substrate interactions
m	=	Amount of fresh substrate added in the second incubation, grams
VAA-CB	=	First product ‘anthranilic acid labeled cellobiose conjugate’ formation rate
VCB	=	Second product cellobiose formation rate

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I. INTRODUCTION

Rising concerns about dependency on fossil fuels has sparked a growth in research for renewable energy sources in the 21st century. One renewable energy production process of interest is the reduction of cellulose into fermentable sugars by means of enzymatic hydrolysis. Since cellulose accounts for about half of the organic material in the biosphere (Divne, 1994) this material can be a valuable resource. However, hydrolysis is inhibited by the slow kinetics of the reaction of the enzyme and substrate. The reaction requires a residence time on the order of seven or more days and usually does not achieve complete conversion. The slow reaction rate and incomplete conversion is generally attributed to enzyme deactivation and loss of activity. The loss of activity and deactivation of the enzyme has been widely investigated, but the reasons for deactivation and the exact mechanism are not well known.

Understanding how deactivation affects the process of enzymatic hydrolysis is important for design of an industrial-scale method to produce biomass-based fuels. The objective of this work is to quantify the relative degree of deactivation between two categories of deactivation. Deactivation of the enzyme is classified here as either substrate related deactivation or nonspecific deactivation. The general term of

nonspecific deactivation refers to any activity loss of the enzyme not attributed to interaction with substrate. This may include deactivation due to shear stress, liquid-gas interfacial effects, and thermal instability. While the exact mechanism for deactivation due to enzyme-substrate interaction is not known, deactivation may be attributed to factors such as poor desorption of enzyme from the substrate and product inhibition.

In order to quantify the relative degree of deactivation, enzymatic activity loss following incubation in a substrate-free buffer (nonspecific) was compared to activity loss following incubation with substrate. Tests were conducted with two different substrates to compare the effect of the degree of substrate crystallinity on activity loss from enzyme-substrate interactions. Incubation periods and substrate loadings were varied in order to characterize the degree of the effect of the substrate on activity loss. Activity was determined by measuring glucose release from substrate added during a brief, one-hour incubation period that followed the initial incubation periods.

II. BACKGROUND

A. Cellulose Substrate

Cellulose exists as a linear condensation polymer consisting of D-anhydroglucopyranose joined by β-1,4-glycosidic bonds; the repeating unit is anhydrocellobiose since adjacent anhydroglucose molecules are rotated 180° with respect to their neighbors. A schematic of the cellulose substrate is shown in Figure 2.1. Formation of one cellobiose (CB) unit, 1.04 nm in length and 0.53 nm in width, includes two glucose molecules. Three repeating cellobiose units form a single chain. Lee et al (2000) reported that elementary fibrils contain approximately 36 cellulose chains formed by hydrogen bonding and van der Waals forces; these elementary fibrils, 3.5 nm in diameter, compile into microfibrils with a diameter between ten and 30 nm. The microfibrils then form macrofibrils that span from 60 to 360 nm in diameter. The high degree of hydrogen bonding between the linear chains of the cellulose contributes to high stability and chemical resistance to change. The non-carbohydrate component, lignin, present in the cellulose causes highly polymeric characteristics due to the complex, cross-linking, polyphenolic structure. The lignin coats the cell wall and joins the cells together, protecting the cellulosic material.

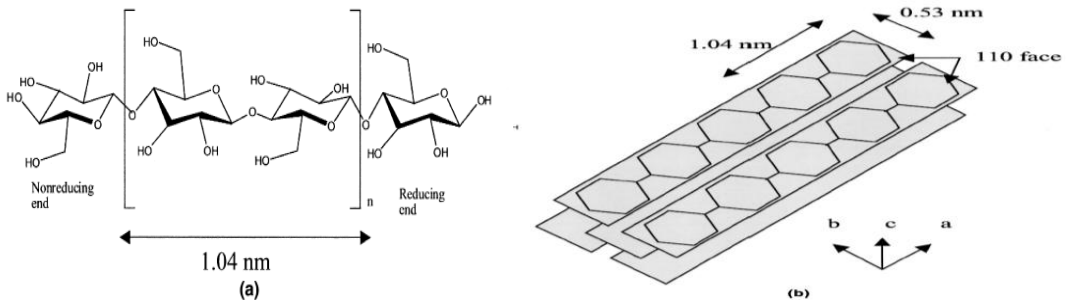

FIGURE 2.5 – a. Structure of cellulose featuring repeating β-1,4-linked anhydrocellobiose. b. Cellulose I crystal. The axes of the repeating unit (cellobiose) are: a = 0.817 nm, b = 1.04 nm, and c = 0.786 nm. The faces of the glucopyranose rings are parallel to the ab plane (110 face) of the crystal (Mosier et al, 1999; Zhang and Lynd, 2004).

During enzymatic hydrolysis, water breaks down the glycosidic bonds reducing the cellulose to a cellobiose repeating unit, $C_{12}H_{22}O_{11}$, and then into glucose, $C_6H_{12}O_6$. This reaction is described with Equation (2-1).

$$\text{Cellulose} \xrightarrow{\beta\text{-1,4glucanase}} \text{Cellobiose} \xrightarrow{\beta\text{-glucosidase}} \text{Glucose} \qquad (2\text{-}1)$$

Substrates including phosphoric acid swollen cellulose (PASC) (Haan et al, 2007), filter paper (Henrissat et al, 1985), avicel (Wood and Bhat, 1988), Solka-Floc (Bertrain and Dale, 1985; Fan et al, 1980; Lee et al, 1982; Sinitsyn et al, 1991), bacterial cellulose (BC) (Kipper et al, 2005; Jeoh et al, 2008), cotton fiber (Kleman-leyer et al, 1994; Lee et al, 2000) and bacterial microcrystalline cellulose (BMCC) (Bothwell et al, 1997; Carrad and Linder, 1999; Kipper et al, 2005) are utilized as pure cellulose in research. Amorphous celluloses compose PASC, while BMCC and cotton fiber contain almost pure crystalline cellulose. Blends of crystalline and amorphous cellulose form several substrates including filter paper, avicel, Solka-Floc and BC. Filter paper is a

more amorphous substrate, whereas the remaining three substrates are more crystalline (see crystalline index in Table II-I).

Table II-I outlines physical properties for some model substrates. The properties include the crystallinity index (CrI), specific surface area (SSA) determined by the Brunauer-Emmett-Teller (BET) procedure, number average of degree of polymerization (DP_N), and the fraction of reducing ends (F_{RE}). The SSA helps determine the accessibility of the enzyme to bind to the substrate to begin hydrolysis. The DP_N of cellulosic substrate determines the number of glucose monomers bonded together to form a polymer chain. The fraction of reducing ends, unitless, relates to the reciprocal of the DP_N. (Zhang and Lynd, 2004)

TABLE II-I

SUMMARY OF SOME PHYSICAL PROPERTIES OF MODEL CELLULOSIC SUBSTRATES
(*Zhang and Lynd, 2004*)

Substrate	**CrI**	**SSA (m^2/g)**	**DP_N**	**F_{RE} (%)**
Avicel	0.5 – 0.6	20	300	0.33
BC	0.76 – 0.95	200	2000	0.05
PASC	0 – 0.04	240	100	1.0
Cotton	0.81 – 0.95	NA	1000-3000	0.1 – 0.033
Filter Paper	0.45	NA	750	0.13
Wood Pulp	0.5 – 0.7	61 – 55	500 – 1500	0.06 – 0.2

A former concern for the slow reaction kinetics of enzymatic hydrolysis revolved around the heterogeneous structure of cellulose induced during the hydrolysis (Zhang et al, 1999; Valjamae et al, 1998). However, Yang et al (2006) reported that no change occurred in the reactivity of substrate during hydrolysis after removal of bound enzyme with alkali and the addition of fresh enzyme to restart the hydrolysis. Therefore, a reasonable expectation of an unchanging hydrolysis rate exists if no activity loss of the enzyme occurs, and the enzyme remains able to freely exchange from a bound state to a free state during hydrolysis.

B. Biomass Conversion Process

A biomass conversion process consists of a series of steps shown as a schematic in Figure 2.2. For the ease of processing, milled feedstock of small particle size enters step one for pretreatment. Common milling methods include hammer mills and knife mills. The primary, costly components involve fractionation and enzymatic hydrolysis making up sixty percent of the total expense of producing ethanol from biomass (Nguyen and Saddler, 1991). Important future considerations in bioconversion consist of: development of high yield pretreatment procedure, a highly effective enzyme system, economical engineering techniques to maximize glucose yield, and microorganisms that efficiently convert multiple sugars to ethanol.

The process for breaking down cellulose polymers by enzymes into monomers, or glucose, defines the process of enzymatic hydrolysis. The addition of an enzyme follows the pretreatment process. The typical process of enzymatic hydrolysis of lignocellulosic material utilizes cellulase as a biocatalyst for conversion of cellulose to glucose.

Common cellulases to perform this process include fungi such as: *Fusarium solani, Clostridium thermocellum, Trichoderma reesei (T. reesei),* and *Trichoderma viride.* Any fungal cellulase complex frequently consists of endo-1,4-β-glucanase, exo-1,4-β-glucanase (cellobiohydrolase), exo-1,4-β-glucosidase, and cellobiase (β-glucosidase) (Gusakov et al, 1992). The basic mechanism for enzymatic hydrolysis follows four steps: (1) diffusion of the enzyme onto the surface of the substrate, (2) release of glucose from the cellulose polymer, (3) release of glucose into the bulk solution, and (4) diffusion of the enzyme into the bulk solution.

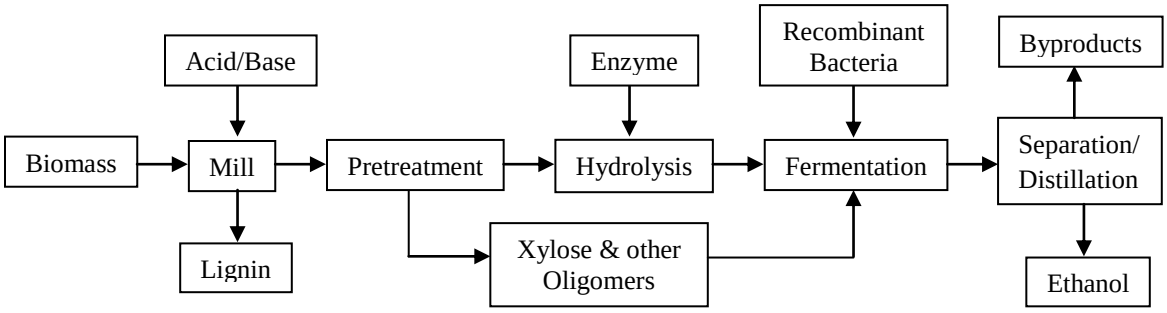

FIGURE 2.6 – Overall conversion process from biomass to ethanol.

C. Cellulase

The work here uses cellulase from *T. reesei*. T. *reesei* cellulases are composed of five endoglucanases (EGI – V), two cellobiohydrolases (CBHI – II), and β-glucosidase. Endoglucanases quickly decrease the degree of polymerization of substrate by fractioning the substrate. Exoglucanases release cellobiose from the substrate resulting in a gradual decrease in the degree of polymerization of cellulose. β-glucosidase hydrolyzes cellobiose to yield glucose. The composition of the three cellulases in the Spezyme CP are about: sixty percent CBHI, twenty percent CBHII, and twelve percent EGI. The

remaining compositions are unknown. The molecular weights of the proteins follow: 64,000 for CBHI; 53,000 for CBHII; 55,000 for EGI; 48,000 for EGII; and 25,000 for EGIII. The isoelectric points for CBHI, CBHII, and EGII are 3.6–3.9, 5.9, and 4.9, respectively (Medve et al, 1998).

D. Structure and Corresponding Function of Cellulases

Lee et al (2000) determined the structure of CBHI to be a "tadpole shaped enzyme" with a length of 18 nm and a width of 4 nm, by small-angle X-ray scattering. CBHI and CBHII contain a catalytic domain (CD) and a cellulose-binding domain (CBD). A glycosylated peptide links the domains together.

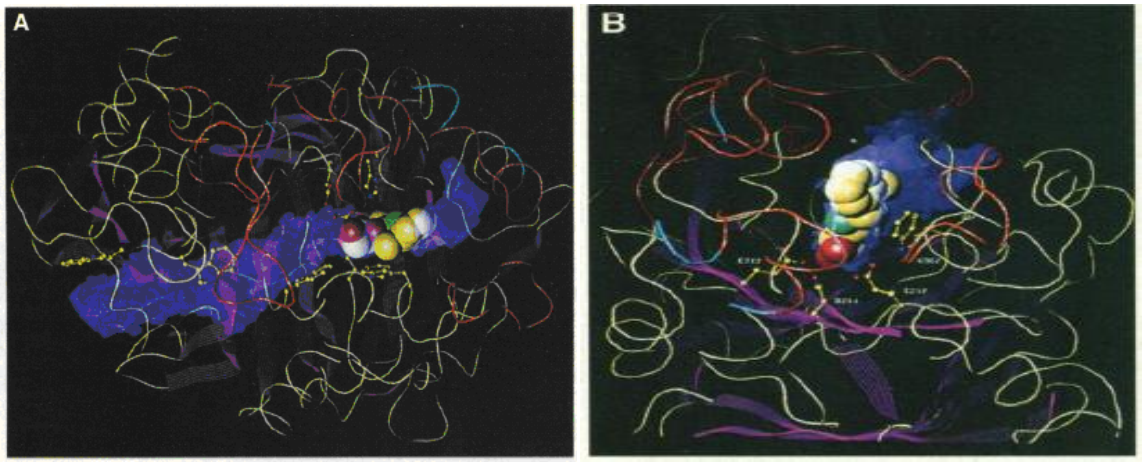

FIGURE 2.7 – The active site tunnel of CBHI drawn as a semi-transparent surface. The active site residues and ligand are included. The views are (A) orthogonal to the tunnel and (B) along the tunnel. The β sandwich is indicated by a magenta ribbon. The C_α trace is colored red to indicate the loops that are expected to be deleted in the related endoglucanase EGI of *T. reesei*. Because of low sequence identity, some loops are difficult to delimit precisely. Therefore these are in blue with red representing the most likely region to be deleted (*Divne et al, 1994*).

The catalytic domain in CBHI has dimensions of 6 x 5 x 4 nm. The CD consists of two large antiparallel beta sheets to from a beta sandwich. Four loops on the surface

tunnel are 4 nm long, with approximately seven glucosyl binding sites; refer to Figure 2.3. Divne et al (1994) proposed that the CBHI tunnel enables the remaining cellulose chain to stay attached to the enzyme after catalytic action, presuming the hydrolysis of cellulose by cellulase is processive. The CD of CBHII compares similarly to that of CBHI, but with only two loops and a length of 2 nm. The mechanism, however, for CBHII differs from CBHI. Divne et al (1994) expect that after production of cellobiose, the remaining cellulose chain either falls off the enzyme or threads further into the tunnel, thereby leading to another activity cycle. Although CBHI and CBHII belong to cellobiohydrolase, they work at different ends of the chain. CBHI is a strict exoglucanase (Boisset et al, 2000) and starts the hydrolysis at the reducing end of the cellulose chain (Barr et al, 1996; Nutt et al, 1998). Conversely, CBHII hydrolyzes the cellulose chain from the non-reducing end which consistently behaves like a more open and flexible active-site region (Zou et al, 1999; Varrot et al, 2003), and therefore acts as an endo-processive cellobiohydrolase (Boisset et al, 2003). Alternatively, Stahlberg et al (1993) claimed that *T. reesei* has no true exoglucanase since new reducing end groups on cellulose were observed following the hydrolysis of all cellulase components.

EGI and CBHI belong to the same family, and have significant homology, and 45 percent identity. The active site of CD in EGI differs as a groove rather than the tunnel of CBHI. This allow for glucan chains to cleave randomly into two shorter chains, resulting in a rapid degrease in degree of polymerization (Zhang and Lynd, 2004).

The generally hydrophobic cellulose binding domains of *T. reesei* contain only one ionizing amino acid side chain (Reinikainen et al, 1995). The CBD has no affinity toward soluble sugars. According to Zhang and Lynd (2004), the CBD of CBHI appears

as a small “wedge shaped fold” that exposes three aromatic residues on the hydrophobic cellulose binding surface, shown in Figure 2.4.

FIGURE 2.8 – The backbone structures of the CBDs from the CBHI from *T. reesei* (A) and the xylanse/cellobiohydrolase from *C.fimi* (B). The side chains of only those residues apparently involved in the interaction to cellulose are shown to demonstrate that, in spite of their different folding topologies and sizes, the binding faces of the two CBDs are very similar. Figure drawn using the program Molscript (*Kraulis, 1991*).

The spacing between the three aromatic residues compares similarly to the spacing of every other glucose ring on a glucan chain, which potentially helps the CBD recognize the binding site of carbohydrates. Positive entropy drives the binding of CBD to crystalline cellulose, revealing a unique thermodynamic binding force about carbohydrate binding proteins (Boraston et al, 2004). A noticeable release of structured water molecules occurs during the binding of CBD to cellulose (Creagh et al, 1996; Nimlos et al, 2007). Creagh et al (1996) argue that the release of water increases the entropy of the system. In the case of soluble saccharides, Creagh et al (1996) postulate the entropy change to be more than offset by the conformation restriction of the bound ligands leading to a net reduction in entropy. Boraston et al (2004) state that the molecular basis for the thermodynamic forces that drive protein-carbohydrate interaction remain a highly controversial topic, particularly with respect to the role of water molecules and the loss of entropy through conformation restrictions. Presently, a

possible mechanism to explain the binding force between CBD and cellulose is the accumulation of a number of individually weak hydrophobic interactions between the CBD and the hydrophobic (1, 0, 0) cellulose surface (Nimlos et al, 2007). Boraston et al (2004) concluded that hydrogen bonding was not responsible for the strong binding.

E. Processivity of Cellobiohydrolase

Previous description of the processivity of cellulase was derived from a structural basis, but no sound experiments support this theory (Kipper et al, 2005). After investigation of burst kinetics in the hydrolysis of fluorescence-labeled celluloses, Kipper et al (2005) reported that processivity values were 88±10, 42±10, and 34±2.0 cellobiose units for CBHI acting on labeled bacterial cellulose, bacterial microcrystalline cellulose, and endoglucanase-pretreated bacterial cellulose, respectively.

According to Kipper et al (2005), as an explanation of burst kinetics, processive cellobiohydrolase that released the fluorescent label as the first product from the chain end will not dissociate from the cellulose chain until the full processive cycle completes. Therefore, the factor of processivity minimally slows the second step, but the rate change allows for comparison to reveal the burst kinetics. The ratio of the second product cellobiose formation rate (VCB) and the first product 'anthranilic acid labeled cellobiose conjugate' formation rate (VAA-CB) characterizes processivity, P. Equation (2-2) defines the processivity.

$$P = \frac{VCB}{VAA\text{-}CB} \qquad (2\text{-}2)$$

Utilizing the ratio of produced CB to that of the sum of glucose and cellotriose as a measure of processivity, Medve et al (1998) found that processivity for CBHI to be approximately five to ten CB units on Avicel as a substrate. Von Ossowski et al (2003) found the processivity of CBHI to be 23 CB units while acting on BMCC.

F. Synergism

Synergism occurs when two cellulases from the same microorganism combine to yield a higher activity on the cellulose than when working separately. The ratio of the activity exhibited by mixtures of components to the sum of the activities of separate components defines the degree of synergism (DS). Zhang and Lynd (2004) summarize the following types of synergism: (1) endoglucanase and exoglucanase, (2) exoglucanase and exoglucanase, (3) endoglucanase and endoglucanase, (4) exoglucanase or endoglucanase and β-glucosidase.

TABLE II-II

THE DS VALUES FOR DIFFERENT MODEL SUBSTRATES
(*Data from Zhang and Lynd, 2004*)

Model Substrate	**Bacterium Cellulose**	**Cotton**	**Avicel**	**Phosphoric acid – swollen cellulose**
DS	5 – 10	3.9 – 7.6	1.4 – 4.9	0.7 – 1.8

Table II-II gives the degrees of synergism for different model substrates. Hoshino et al (1997) observed higher DS as the crystallinity index increases. Cross-synergism, cellulase from different microorganisms, potentially hydrolyzes high crystalline cellulose more efficiently than typical synergism (Tarantili, et al, 1996). Converse and Optekar

(1993) reported the DS goes through a maximum as total enzyme concentration increases.

G. Product Inhibition

Cellobiose, an intermediate product, and/or glucose inhibit the celluloytic enzymes. Gregg et al (1996) found the inhibition to be competitive; Holtzapple et al (1984) disagreed and concluded non-competitive inhibition; and lastly, Gusakov and Sinitsyn (1992) reported inhibition as a combination of the two competitive types. Holtzapple et al (1990) discovered that free, adsorbed, and complexed forms of the enzyme species are subjected to inhibition in the process of cellulose hydrolysis. Gusakov and Sinitsyn (1992) stated that the enzyme/substrate ratio contributes substantially in deciding the extent of inhibition. Different product inhibition patterns depend on both the absolute enzyme concentration and the enzyme/substrate concentration ratio.

H. Mechanism for Enzyme Deactivation

Loss of enzymatic activity has been extensively studied by several groups to investigate several types of mechanisms. One mode of deactivation of the cellulase complex may be attributed to shear and interfacial effects. Kim et al (1981) reported that in a fine capillary reactor, about 60 percent of cellulase activity was lost when the enzyme was exposed to an air-liquid interface and shear rate of 850 s^{-1} for a period of four hours. However, Kim et al also reported that no deactivation occurred in the

absence of an air-liquid interface at the same shear rate. They also observed an activity loss of 16 percent with a shear rate as high as 4300 s^{-1} and no air-liquid interface. Similar results were found by Ganesh et al (2000) and Ghadge et al (2005). Thermal stability was not found to be a significant factor of deactivation, according to Eriksson et al (2002); they reported no deactivation of CBHI after a 96-hour incubation at 40°C and gentle mixing.

More factors must be considered for enzyme deactivation when a substrate is present. Binding reversibility of CBHI is of particular importance in describing the slow kinetics of enzymatic hydrolysis of cellulose. Essentially, the hydrolysis rate is hindered by a slow desorption process that is caused by the irreversible binding of CBHI to the substrate. Additionally, the non-desorbed CBHI impedes the accessibility of cellulose to other cellulase components. Howell and Mangat (1978) theorized that the cellobiohydrolases fraction always remained bound to the substrate during hydrolysis to explain the slow kinetics of the reaction. However, Howell and Mangat did not do any experiments to prove that negligible desorption of cellobiohydrolases from cellulose could possibly account for the reduction of hydrolysis rate. Ooshima et al (1991) inferred that the changing activity of cellulases was due to incomplete desorption of exoglucanases (cellobiohydrolases) which results in an increasing percentage of endoglucanases in the free-state cellulase complex. Their finding supported Howell and Mangat's hypothesis, though experiments with pure cellobiohydrolase should be performed to further validate findings. A study performed by Kyriacou et al (1988) with fractionated CHBI found, within experimental error, no desorption of CBHI from Solka-Floc after one-hour incubation following dilution at 5°C. This finding does not follow

the expected binding isotherms if indeed reversible binding existed. Further reports of irreversible binding of cellulose binding domain (CBD) of CBHI of filter paper substrate were given by Nidetzky et al (1994). They also concluded reversible binding exists for the catalytic domain (CD) of CBHI to filter paper. Ma et al (2008) provide another proof of incomplete desorption of CBHI from cellulose. Ong et al (1989) found when CBD is transplanted from *T. reesei* cellulases to another protein, apparent irreversible binding of the protein to cellulose was observed.

Though aforementioned work supports negligibly reversible binding of cellobiohydrolases to substrate, other works infer more apparent reversible binding. Carrard and Linder (1999) claimed that the binding of a recombinant CBD of CBHI was reversible while the binding of a recombinant CBD of cellobiohydrolase II (CBHII) was apparently irreversible. However, uncertainty remains in regards to whether the recombinant CBDs of cellobiohydrolases behave differently from native ones (Reinikainen et al, 1992). Bothwell et al (1997) performed an adsorption experiment of CBH1 at 50°C and also found reversible binding of CBH1, supported by similarities of adsorption and desorption isotherms. However, these experiments do not define the release of CBHI by product formation or reversible binding. Reversible binding is quite possible since no desorption of CBHI was seen from Solka-Floc following one-hour incubation at 5°C (Kyriacou et al, 1988).

Other potential factors for enzyme deactivation also have been studied. When removing products from the reaction, it was found that the hydrolysis rate still declined significantly (Howell and Mangat, 1978; Converse et al, 1988). The proved product inhibition is not the main reason for the hydrolysis rate reduction. Valjamae et al (1998)

showed initial hydrolysis rate decreases about 84 percent when the cellobiose product reached a concentration of 60μM. However, at the same concentration, the hydrolysis rate reduced less than ten percent compared to a control experiment with no initial cellobiose. This finding supports the theory that product inhibition is not a dominating factor in hydrolysis of cellulose.

Concerns also arose about the enzyme being entrapped in solid fibril or the solution within the cellulose, which could result in deactivation as well (Converse et al, 1988). However, for bacterial microcrystalline cellulose (BMCC), which has a high crystalline structure and a limited amount of intra-particle pores and inter-particulate voids (different from amorphous celluloses), the hydrolysis rate was still significantly reduced during the reaction (Valjamae et al, 1998). Therefore, the deactivation of enzyme due to entrapment in the pores also is not the main factor causing slow hydrolysis kinetics.

III. EXPERIMENTATION

A. Plan of Experimentation

Experiments were designed to identify the relative extents of deactivation of the enzyme due to nonspecific deactivation and enzyme-substrate interactions. The nonspecific deactivation was characterized by activity measured following enzyme incubation in a substrate-free buffer for 2, 4, 8, 16, 24, 48, or 72 hours, followed by a second incubation of one hour with 2.0 grams of substrate. Testing for enzyme-substrate interaction was performed by adding an initial substrate load to the first incubation in the amount of 0.1, 0.2, or 0.4 grams, and then the substrate was added during the second incubation to bring the total substrate in all cases up to 2.0 grams. The amount of enzyme was held constant at 0.6 ml for all cases. Two substrates of different crystallinity, filter paper (CrI = 45%) and dewaxed cotton (CrI = 90%) were studied here.

B. Materials

Substrates:

Johnson's® Pure cotton Balls (CrI = 90%)
Johnson and Johnson Consumer Products Co
Skillman, NJ 08558-9418 USA
Cotton grown and processed in USA

Fisherbrand® Filter Paper (CrI = 45%)
Qualitative P8
Fisher Scientific
Pittsburg, PA 15275 USA
Cat. No.: 09-795F

Made in U.K.

Enzyme:

Spezyme® CP
Genecor International
200 Merdian Center Boulevard
Rochester, NY 14606 USA
Made in Finland

0.05M citrate buffer with 4.8 pH, prepared according to NREL LAP procedure 006

Tetracycline (10mg/mL in 70% ethanol)

Cycloheximide (10mg/mL in DI Water)

C. Procedure

The reactions took place in 100mL volumes at 50°C and 150 rotations per minute in 300mL flasks. The temperature and agitation were controlled by an Innova 4230 incubator shaker (Figure 3.1). The pH of each batch was adjusted to 4.8 with a 0.05M citrate buffer. For prevention of bacterial growth, 3μL/mL of cycloheximide and 4μL/mL of tetracycline were added to the reaction vessel. The substrates tested were dewaxed cotton (crystalline index of 90%) and filter paper (crystalline index of 40%).

The following procedure was used for investigating the nonspecific deactivation of the enzyme. The cellulase was added to the aqueous buffer solution and was incubated for 2, 4, 8, 16, 24, 48, or 72 hours without substrate. After the first incubation, 2.0 grams of substrate was added for a second incubation of one hour. After the second incubation, a 1 mL sample was assayed for glucose concentration (C_2). The sample was heated to 90°C to deactivate the enzyme by a dry heatblock (Figure 3.2). The activity was expressed as glucose yield in the second incubation per amount of substrate added in the second incubation.

The following procedure was used for investigating deactivation due to the enzyme-substrate interaction. For the first incubation, enzyme and substrate loadings (ranging from 75 to300 filter paper units per gram of cellulose) are listed in Table III-I. The first incubations lasted for 2, 4, 8, 16, 24, 48, or 72 hours. At the end of the first incubation, 1mL of the solution was removed and assayed for glucose concentration (C_1). The sample then was heated to 90°C to deactivate the enzyme.

TABLE III-I

ENZYME AND SUBSTRATE LOADING IN FIRST INCUBATION

First Incubation	**75 FPU/g cellulose**	**150 FPU/g cellulose**	**300 FPU/g cellulose**
Substrate Loading	0.4g	0.2g	0.1g
Enzyme Loading	0.6mL	0.6mL	0.6mL

After the first incubation, fresh substrate (m) was added to achieve two percent (w/v) final substrate concentration, or 15 FPU/g cellulose (no additional enzyme is added). After the one hour second incubation, a 1 mL sample was assayed for glucose concentration (C_2). The substrate loading of the second incubation is given in Table III-II. The sample then was heated to 90°C to deactivate the enzyme.

TABLE III-II

SUBSTRATE LOADING IN SECOND INCUBATION

Second Incubation	**75 FPU/g cellulose**	**150 FPU/g cellulose**	**300 FPU/g cellulose**
Substrate Loading	1.6g	1.8g	1.9g

C_2 represents the total amount of glucose released during the first and second incubation. To deduct the glucose produced by the substrate loaded during the initial incubation from C_2, a control experiment was conducted. The control experiment contained the same amount of substrate as the first incubation but was carried out for the combined duration of the first and second incubation. The loading amounts of substrate in the control experiment are identified in Table III-III. The glucose increment of the control experiment is identified as C_3.

The cellulase activity from non-specific interactions is calculated as:

$$\frac{C_2}{m} \tag{3-1}$$

The cellulase activity from enzyme-substrate interactions is calculated as

$$\frac{C_2\text{-}C_1\text{-}C_3}{m} \tag{3-2}$$

TABLE III-III

ENZYME AND SUBSTRATE LOADING OF CONTROL EXPERIMENT

First Incubation	**75 FPU/g cellulose**	**150 FPU/g cellulose**	**300 FPU/g cellulose**
Substrate Loading	0.4g	0.2g	0.1g
Enzyme Loading	0.6mL	0.6mL	0.6mL
Second Incubation	**75 FPU/g cellulose**	**150 FPU/g cellulose**	**300 FPU/g cellulose**
Substrate Loading	0g	0g	0g
Enzyme Loading	0mL	0mL	0mL

After each test, the samples were cooled to room temperature for glucose measurement. The samples then were centrifuged using a GPR centrifuge (Figure 3.3) for ten minutes at 2000 rotations per minute in order to separate the un-dissolved solids

from the liquid. After centrifuging, the glucose concentration of the liquid hydrolyzatye was measured with the YSI 2700 Biochemistry Analyzer (Figure 3.4).

D. Equipment

FIGURE 3.5 – Innova Incubator

Innova 4230
New Brunswick Scientific Co., Inc.
Edison, NJ USA
Serial No.: 101028846
Mfg No.: M1233-0001

FIGURE 3.6 – VWR Dry Heatblock

VWR Analog Dry Heatblock
HBNRY Trobmner LLC USA
Serial No.: 090217013
Model: 949310
Cat. No.: 12621-104

FIGURE 3.7 – GPR Centrifuge

GPR Centrifuge
Beckman Instruments, Inc.
SP INCO Division
1050 Page Mill Road
Palo Alto, CA 94304 USA
Serial No.: 1C014
Cat. No.: 349702

FIGURE 3.8 – YSI 2700

YSI 2700 Select Biochemistry Analyzer
Yellow Springs Instrument Co., Inc.
Yellow Springs, OH 45387-0279 USA
Serial No.: 95H36904
Model: 2700-D Biochem

IV. RESULTS AND DISCUSSION

A. Nonspecific Deactivation

The cellulase enzyme showed slight deactivation after incubating for varying times during the initial incubation (Figure 4.1). The general standard to measure enzyme activity is based on reactivity towards filter paper, but the activity towards dewaxed cotton was also investigated and results can be found in Appendix 2.

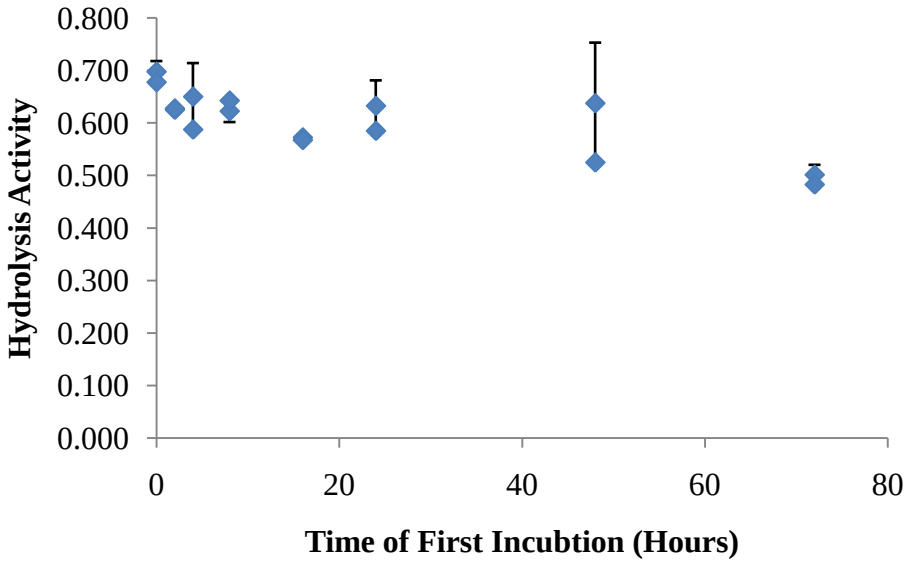

FIGURE 4.25 – Activity of filter paper for nonspecific deactivation with varying time of first incubation.

The activity trend indicates the enzyme will deactivate even without the presence of the substrate. Therefore, the environment where the cellulase resides causes deactivation independent from the presence of substrate; this process is termed here

nonspecific deactivation. This concept is supported by Kim et al (1981), Ganesh et al (2000), and Ghadge et al (2005) who reported enzyme deactivation due to shear stresses and liquid-air interfacial effects without substrate present.

Investigating the activities of two substrates (Appendix 3) showed nonspecific deactivation for both substrates. Activity is higher towards filter paper due to the difference in factors such as the degree of polymerization and crystallinity index between the two substrates. (The DP_N and CrI for filter paper are 750 and 0.45, respectively. The DP_N and CrI of dewaxed cotton are 1000–3000 and 0.81–0.95, respectively.) This difference indicates that deactivation may also be due to enzyme-substrate interactions.

B. Deactivation Related to Enzyme-Substrate Interaction

Figures 4.2, 4.3, and 4.4 compare the activity trends of hydrolysis of the filter paper and dewaxed cotton cellulose with first incubation substrate loadings of 0.1, 0.2, and 0.4 grams, respectively. The decrease in activity shown here is due to the combined effect of both enzyme-substrate interactions and nonspecific deactivation. The enzyme-substrate interaction (that occurred during the initial incubation period) hindered the total activity (measured after the second incubation) more than did nonspecific deactivation activity (the activities appear to decrease more than in Figure 4.1). The final filter paper activity from the nonspecific deactivation was approximately 0.5 grams of glucose per liter per gram of substrate added in the second incubation (Figure 4.1), compared to 0.35, 0.28, and 0.12 for the initial substrate loads of 0.1, 0.2, and 0.4 grams, respectively (Figures 4.2 – 4.4). These figures indicate overall deactivation depends on the initial

incubation time, substrate load, and substrate type (crystallinity), which are quantified in more detail below.

The enzyme-substrate interaction during the initial incubation caused a greater overall decrease in activity from an initial incubation time of two hours to 72 hours than what was caused by nonspecific deactivation. The filter paper activity decreased by 0.37, 0.47, and 0.63 grams of glucose per liter per gram of substrate added in the second incubation for an initial substrate load of 0.1, 0.2, and 0.4 grams, respectively. The dewaxed cotton activity decreased by 0.08, 0.17, and 0.09 grams of glucose per liter per gram of substrate added in the second incubation for an initial substrate load of 0.1, 0.2, and 0.4 grams, respectively. These trends generally indicate that activity varied inversely with initial incubation time.

Comparison of Figures 4.2 through 4.4 indicates the enzyme-substrate interaction is a function of initial substrate load. This relationship is exhibited by the trend of increasing activity loss towards filter paper as initial substrate load increases. The loss of filter paper activity increased by 0.10 grams of glucose per liter per substrate added in the second incubation as the initial substrate load doubled, and then activity decreased by 0.26 when the initial substrate load was increased from 0.1 to 0.4 grams. The relationship for dewaxed cotton is less obvious because of the low starting activity. However, there is a noticeable increase of activity loss of 0.09 gram of glucose per liter per substrate added in the second incubation as the initial substrate load was doubled. The increase in activity loss as more substrate was introduced during the initial incubation indicates that in addition to activity losses due to the incubating environment, activity loss also strongly depends on enzyme-substrate interactions. The activity then also varied inversely with the

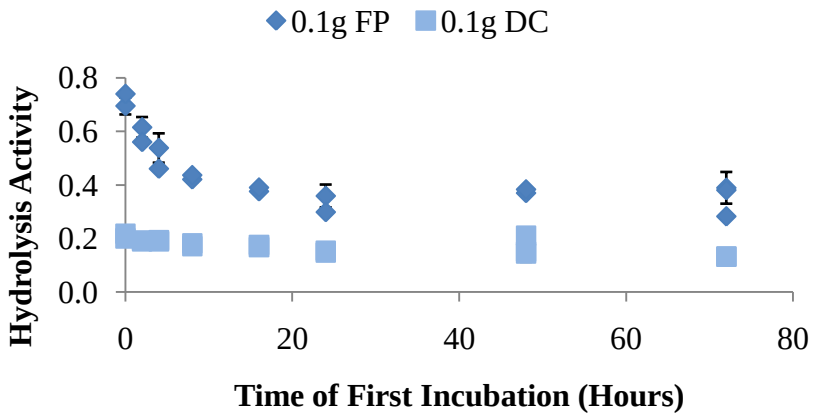

FIGURE 4.26 – Filter paper and dewaxed cotton substrate activity comparison for 300 FPU/g, or 0.1 grams of initial substrate load. (Activity is g glucose/L/g substrate)

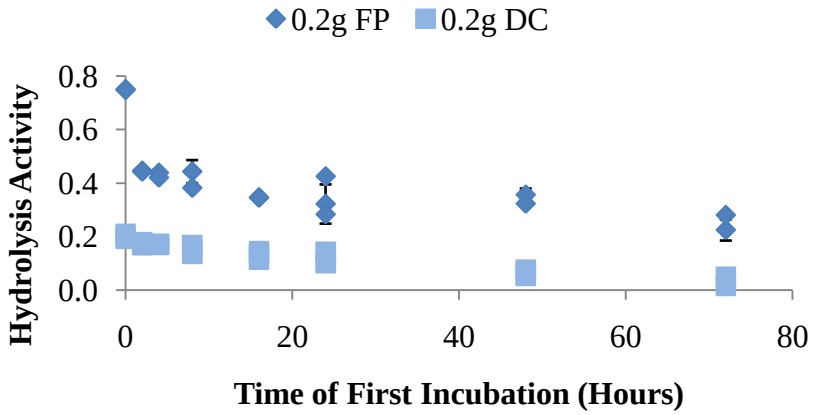

FIGURE 4.27 – Filter paper and dewaxed cotton substrate activity comparison for 150 FPU/g, or 0.2 grams of initial substrate load. (Activity is g glucose/L/g substrate)

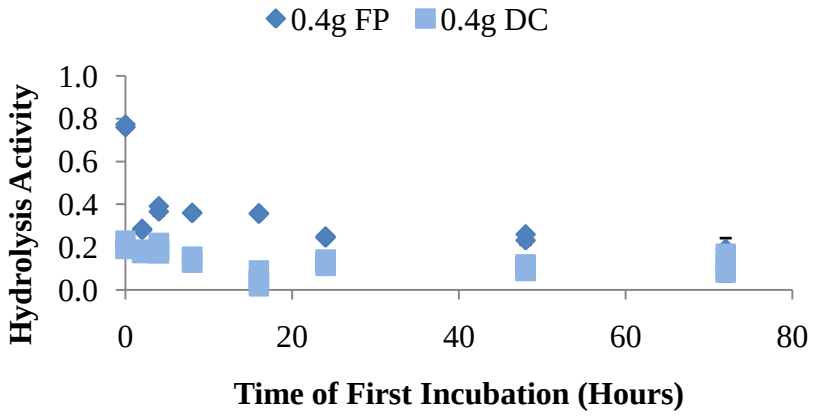

FIGURE 4.28 – Filter paper and dewaxed cotton substrate activity comparison for 75 FPU/g, or 0.4 grams of initial substrate load. (Activity is g glucose/L/g substrate)

amount of initial substrate load.

Finally, the dependence of activity based on the specific substrate is also noticeable by comparison of Figures 4.2 through 4.4. There was a greater decrease in activity towards filter paper than dewaxed cotton as the initial incubation time increased. The filter paper activity gradually approached that of the dewaxed cotton and even reached it at an initial incubation time of 72 hours at a loading of 0.4 grams of substrate, the highest substrate load tested. This decrease in activity was approximately 0.23 grams of glucose per liter per gram of substrate added in the second incubation. The differences in activity loss between the filter paper and dewaxed cotton can be attributed to the differences in crystallinity index. The more highly crystalline material, dewaxed cotton, is harder to digest in general so the activity started low and remained low regardless of the incubation time.

C. Relative Deactivation of Enzyme

Both nonspecific deactivation and enzyme-substrate interactions contribute to the overall deactivation of the enzyme. The percentage of nonspecific deactivation is calculated using Equation (4-1):

$$P_{nonspecific} = \frac{1 - \frac{A_{t,non}}{A_{non}}}{1 - \frac{A_{t,sub}}{A_{sub}}} \qquad (4\text{-}1)$$

where $P_{nonspecific}$ is the percentage of enzyme deactivation not caused by substrate interactions, A_{non} is the activity of the nonspecific deactivation (2.0 grams of substrate incubated for one hour), $A_{t,non}$ is the activity at a given time for incubation time, A_{sub} is

the activity of a one hour incubation with substrate load equal to that added during the second incubation, and $A_{t,sub}$ is the activity with substrate interactions at a given time for initial incubation. The enzyme-substrate interaction makes up the remaining deactivation, given by Equation (4-2):

$$P_{enz\text{-}sub}=1 - P_{nonspecific} \tag{4-2}$$

where $P_{enz\text{-}sub}$ is the portion of enzyme deactivation caused by enzyme-substrate interactions.

Figures 4.5 through 4.7 show the relative percent of deactivation distributed between nonspecific deactivation ($P_{nonspecific}$) and enzyme-substrate interactions ($P_{enz\text{-}sub}$) for filter paper. Figures 4.8 through 4.10 show the same relation for the dewaxed cotton substrate. There does not appear to be a consistent trend for initial incubations less than 24 hours for either substrate. During these time increments, the low amount of glucose released is on the order of the range of error for YSI measurements. This range of error explains the unexpected data points collected at an initial incubation of 16 hours; at this point, the nonspecific deactivation activity (Appendix 2) is practically the same as the activity for 2.0 grams of substrate incubated for one hour which generates the low percentage of relative deactivation due to nonspecific deactivation. After 24 hours, the relative amount of nonspecific deactivation generally appears to have increased for filter paper as the initial incubation time increased. Due to initial low activity of dewaxed cotton, the time until the measured glucose concentration is above the range of error of the YSI is even longer than 24 hours. It appears that the contribution of nonspecific deactivation also increased with dewaxed cotton at times as long as 72 hours.

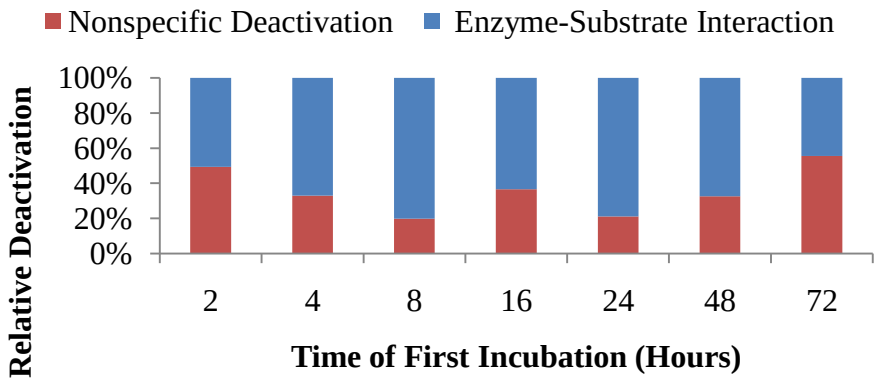

FIGURE 4.29 – Relative percent of deactivation for loading of 0.1 grams of filter paper substrate for varying times of the initial incubation.

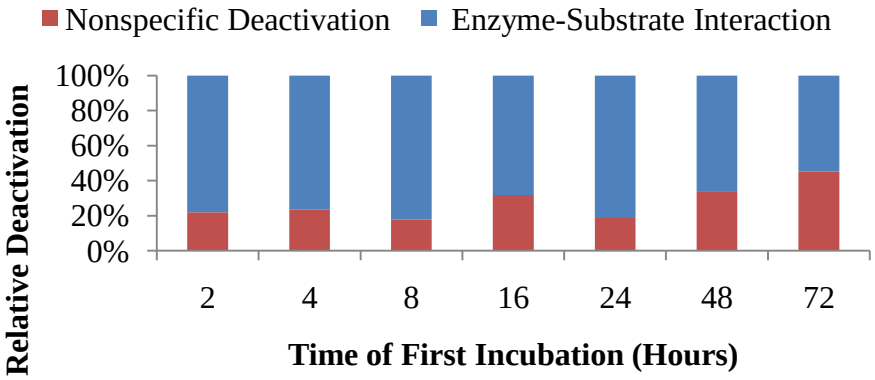

FIGURE 4.30 – Relative percent of deactivation for loading of 0.2 grams of filter paper substrate for varying times of the initial incubation.

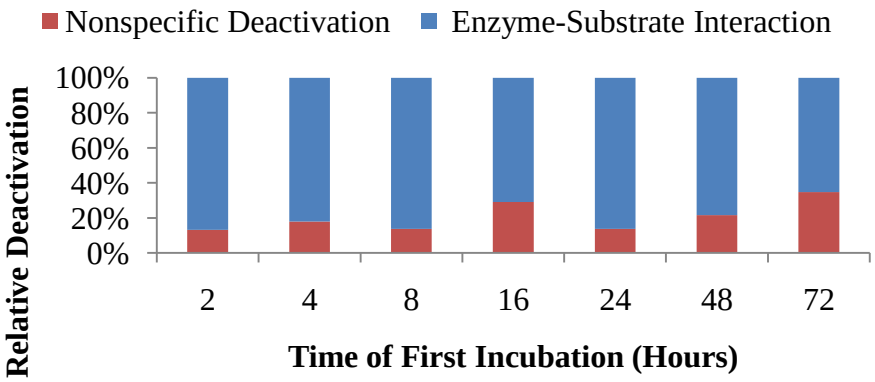

FIGURE 4.31 – Relative percent of deactivation for loading of 0.4 grams of filter paper substrate for varying times of the initial incubation.

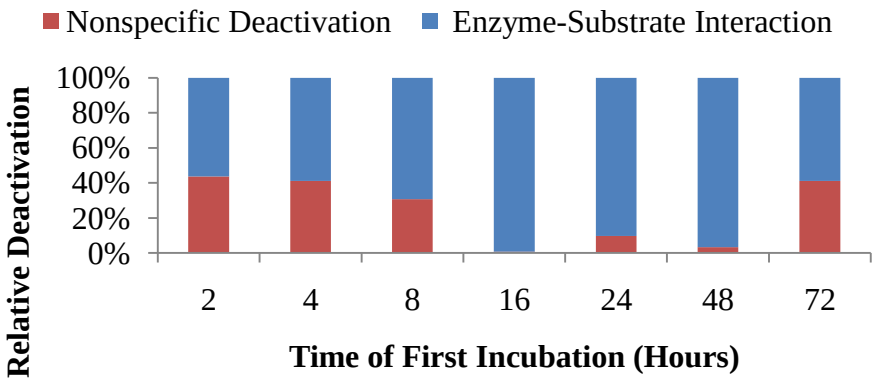

FIGURE 4.32 – Relative percent of deactivation for loading of 0.1 grams of dewaxed cotton substrate for varying times of the initial incubation.

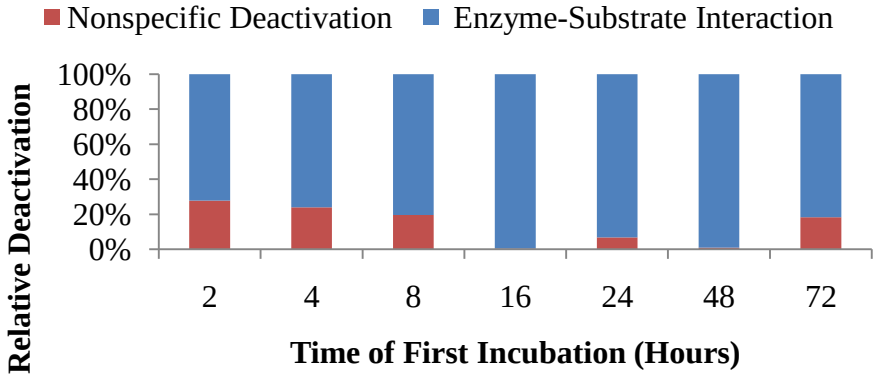

FIGURE 4.33 – Relative percent of deactivation for loading of 0.2 grams of dewaxed cotton substrate for varying time of the initial incubation.

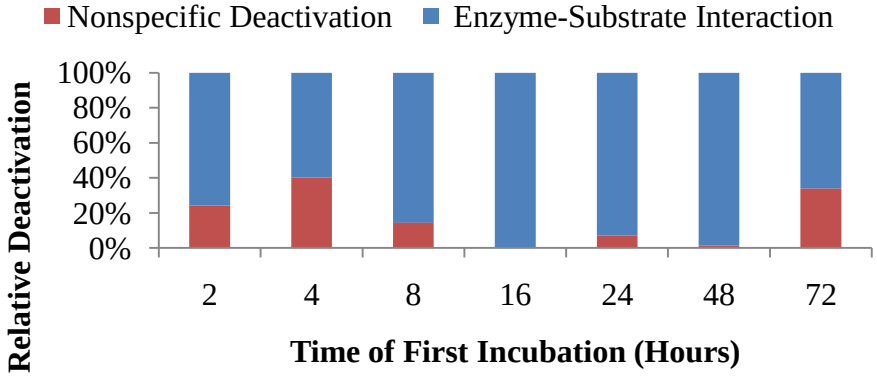

FIGURE 4.34 – Relative percent of deactivation for loading of 0.4 grams of dewaxed cotton substrate for varying times of the initial incubation.

The increasing nonspecific deactivation contribution after 24 hours corresponds to the activity trends shown previously. The nonspecific deactivation activity (Figure 4.1) appears to be steadily decreasing as time increases while the hydrolysis activity with initial substrate loads (Figures 4.2 through 4.4) tends to level off after the 24 hour initial incubation.

Figures 4.11 through 4.17 show the relative percent of deactivation as the initial filter paper substrate load increased for the varying first incubation times (2-72 hours). Figures 4.18 through 4.24 show the same relation for the dewaxed cotton substrate.

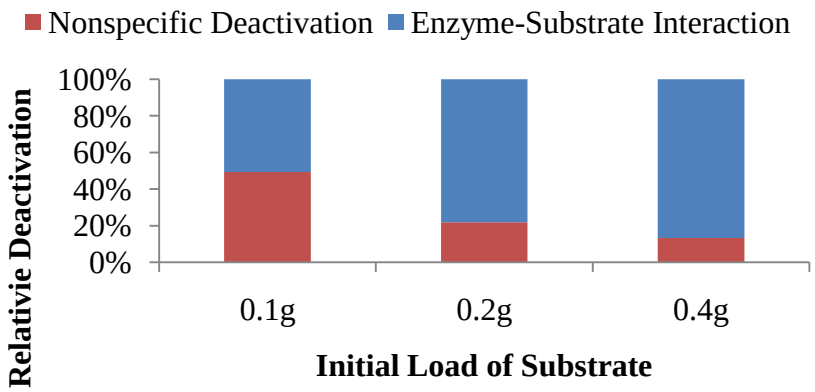

FIGURE 4.35 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 2 hours.

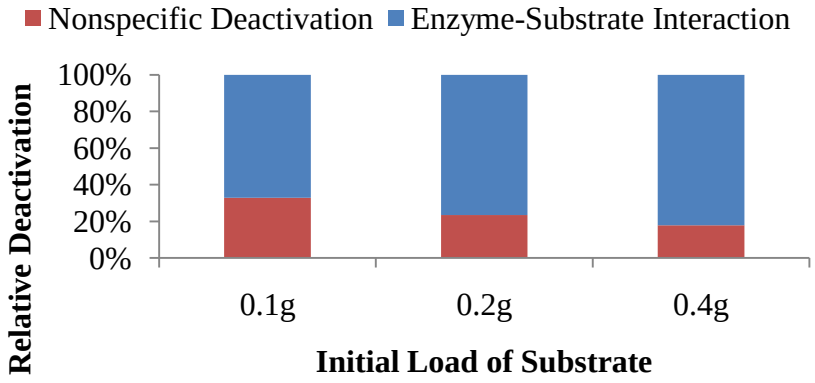

FIGURE 4.36 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 4 hours.

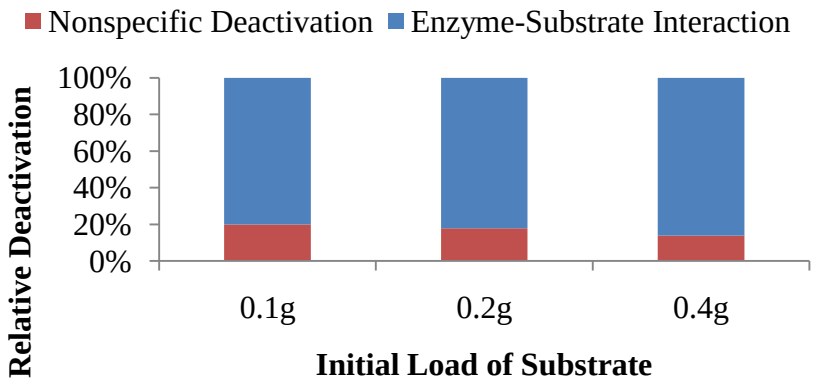

FIGURE 4.37 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 8 hours.

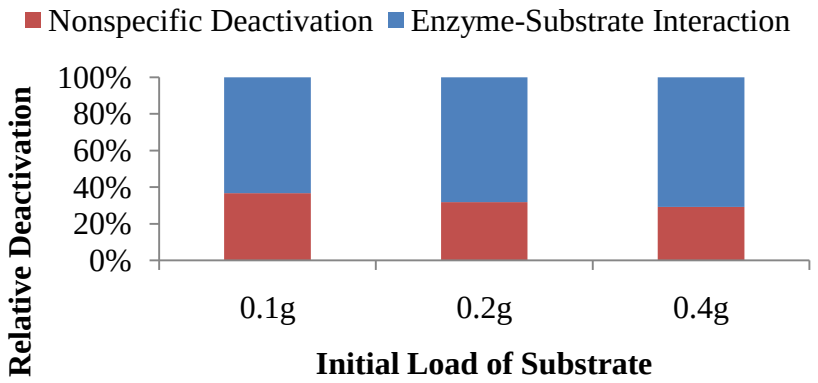

FIGURE 4.38 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 16 hours.

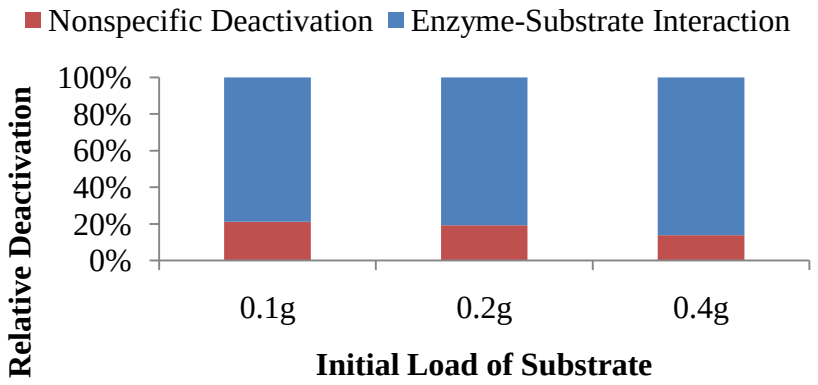

FIGURE 4.39 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 24 hours.

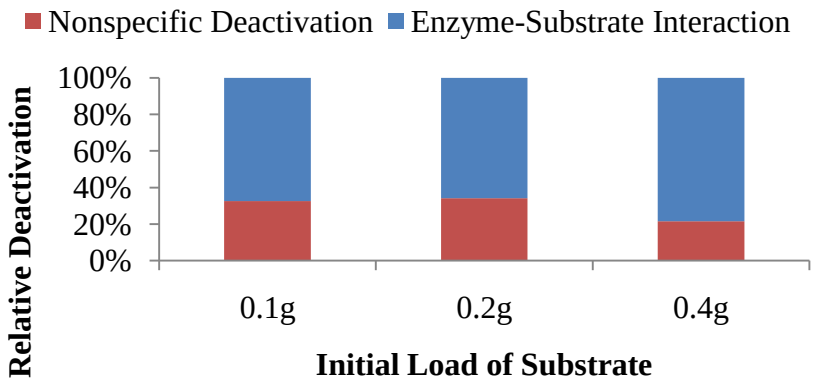

FIGURE 4.40 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 48 hours.

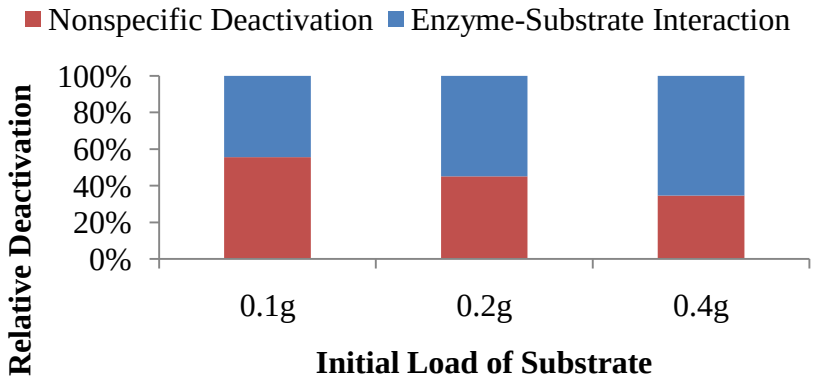

FIGURE 4.41 – Relative percent of deactivation for filter paper substrate for an initial incubation time of 72 hours.

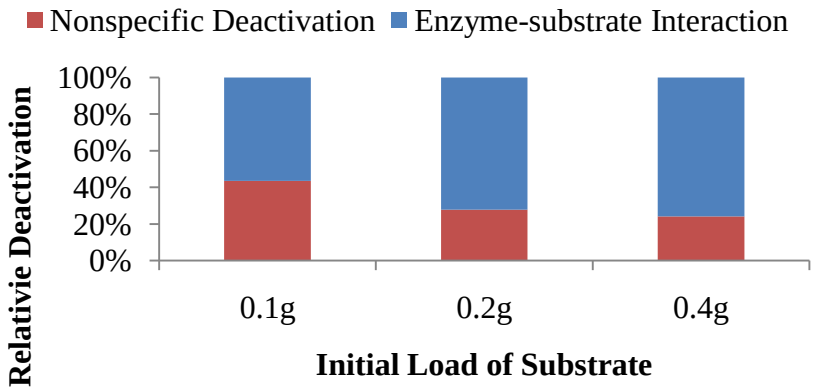

FIGURE 4.42 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 2 hours.

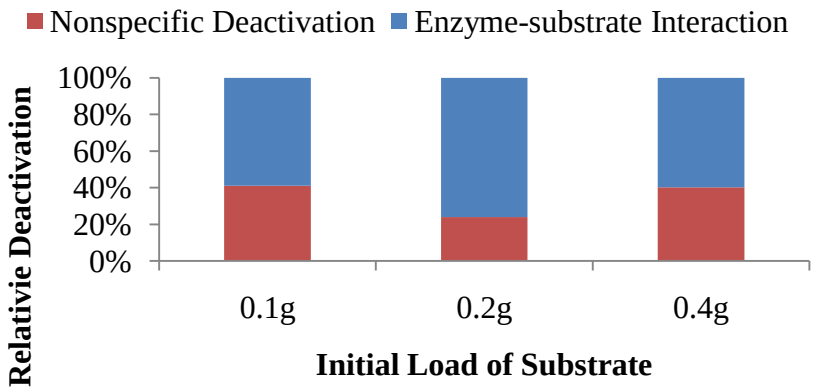

FIGURE 4.43 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 4 hours.

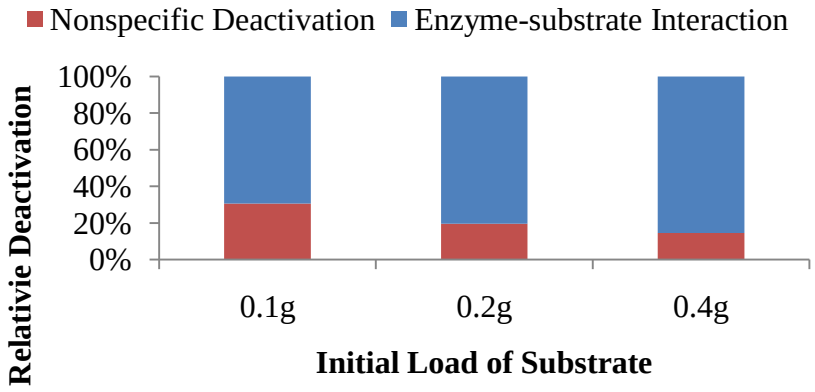

FIGURE 4.44 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 8 hours.

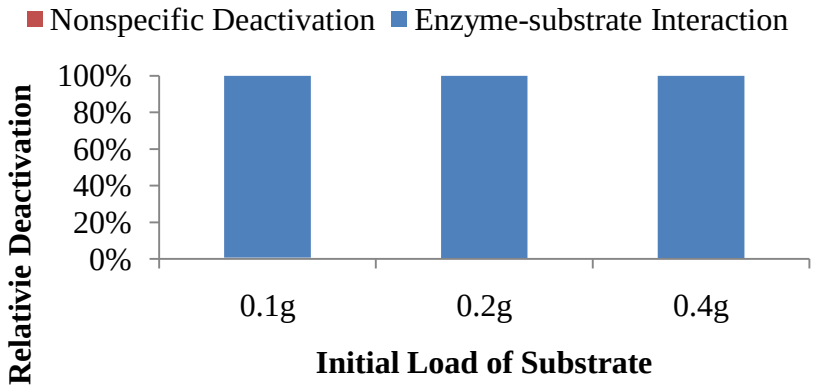

FIGURE 4.45 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 16 hours.

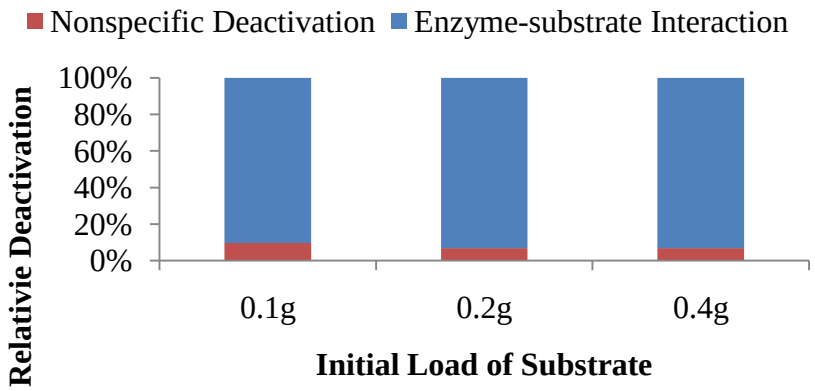

FIGURE 4.46 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 24 hours.

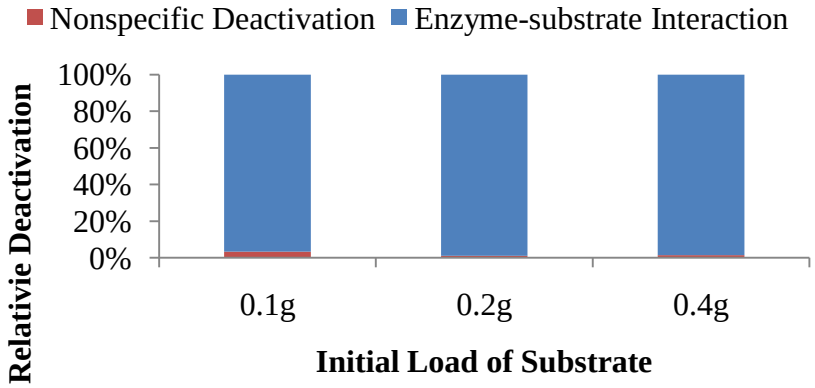

FIGURE 4.47 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 48 hours.

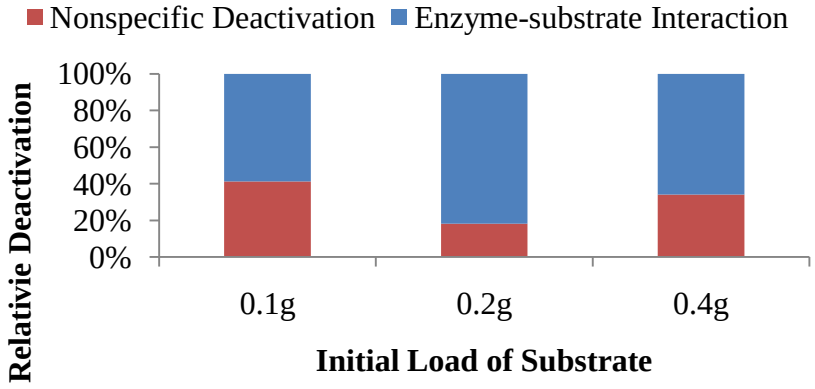

FIGURE 4.48 – Relative percent of deactivation for dewaxed cotton substrate for an initial incubation time of 72 hours.

For filter paper, it appears that as the substrate load increased for a constant initial incubation time, a larger contribution to overall deactivation was caused by enzyme-substrate interactions. However, the relative percent of nonspecific deactivation was higher for the longer initial incubation times than for the shorter times, specifically comparing an initial incubation time of 72 hours to an initial incubation time of two hours (Figures 4.17 and 4.11). The percent of deactivation caused by nonspecific deactivation increases from an initial incubation of two hours to 72 hours as follows: 50 to 56, 23 to 45, and 18 to 35 percent for initial substrate loads of 0.1, 0.2, and 0.4 grams, respectively.

For the dewaxed cotton substrate, there does not seem to be a strong trend, which again is attributed to the low activity towards the higher crystalline material and, hence, the glucose measurements are in the range of error of the YSI. The figures also show that the deactivation due to enzyme-substrate interaction caused more than 50 percent of the total deactivation for all cases but the case of an initial substrate load of 0.1g of filter paper and an initial incubation of 72 hours (44%).

V. CONCLUSIONS

The following conclusions are valid only for the conditions described in the Experimentation chapter and are specific to the substrate, incubation conditions, and enzyme used. (1) The cellulase enzyme studied here loses activity due to nonspecific deactivation caused by the incubating environment and due to enzyme-substrate interactions during the hydrolysis of cellulose. (2) The deactivation of the enzyme due to enzyme-substrate interactions is a function of incubation time, amount of initial substrate load, and the type of substrate. (3) As initial incubation time increases, activity of the enzyme decreases. (4) As initial substrate loading increases, activity of the enzyme generally decreases. (5) The activity of the enzyme depends on the substrate crystallinity. Activity was higher towards the substrate with lower crystallinity. (6) The deactivation caused by enzyme-substrate interaction contributes more to the activity loss that do nonspecific interactions during enzymatic hydrolysis.

VI. RECOMMENDATIONS

Since enzymatic hydrolysis requires several days to maximize extent of conversion, one recommendation is to continue the same experimentation to longer incubation periods, on the order of several days. This change would allow observations on how long the activity requires to level off, the trend (if one exists) in the relationship between nonspecific deactivation and enzyme-substrate interaction, at longer initial incubation times.

To increases the glucose magnitudes above the error of readings by the YSI Biochemistry Analyzer, increase the amount of substrate loading in the initial incubation, and perhaps in the second incubation as well. The higher initial substrate load would also serve to generate more data which may help show a better trend in the relationship between the enzyme-substrate interaction and the amount of substrate.

Finally, research should be conducted to investigate the enzyme-substrate interaction with substrates that have lower cyrstallinities than dewaxed cotton (CrI = 90%). Collecting these data could clarify the relationship between enzyme-substrate interaction and substrate crystallinity.

APPENDIX 1: RAW DATA

TABLE A1-1

NONSPECIFIC DEACTIVATION GLUCOSE MEASUREMENTS FOR FILTER PAPER SUBSTRATE

First Incubation Time, hours	Glucose Measurement, g glucose/L	
2	1.25, 1.25	1.25, 1.26
4	1.30, 1.30	1.17, 1.18
8	1.24, 1.25	1.28, 1.29
16	1.14, 1.15	1.13, 1.14
24	1.26, 1.27	1.17, 1.17
48	1.27, 1.28	1.04, 1.06
72	1.00, 1.01	0.96, 0.97

TABLE A1-2

0.1 GRAMS OF FILTER PAPER LOADING FOR INITIAL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Incubation	Second Incubation
2	0.331, 0.337	1.66, 1.65
	0.342, 0.344	1.56, 1.56
4	0.450, 0.462	1.56, 1.55
	0.555, 0.551	1.51, 1.50
8	0.736, 0.726	1.56, 1.55
	0.746, 0.736	1.60, 1.59
16	0.776, 0.764	1.52, 1.51
	0.779, 0.767	1.55, 1.54
24	0.844, 0.830	1.53, 1.54
	0.844, 0.829	1.43, 1.41
48	0.940, 0.940	1.67, 1.67
	0.954, 0.955	1.71, 1.71
72	0.825, 0.826	1.58, 1.58
	0.972, 0.972	1.29, 1.29
	0.738, 0.738	1.71, 1.71

TABLE A1-3

0.1 GRAMS OF FILTER PAPER LOADING FOR
CONTROL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Control	Second Control
2	0.331, 0.330	0.493, 0.472
	0.325, 0.320	0.483, 0.469
4	0.537, 0.547	0.630, 0.610
	0.545, 0.548	0.612, 0.613
8	0.731, 0.731	0.761, 0.758
	0.737, 0.737	0.783, 0.733
16	0.763, 0.763	0.800, 0.789
	0.772, 0.771	0.809, 0.797
24	0.839, 0.839	0.867, 0.859
	0.831, 0.819	0.861, 0.838
	0.843, 0.843	0.867, 0.589
48	0.908, 0.912	0.895, 0.905
	0.915, 0.893	0.966, 0.951
	0.913, 0.921	0.941, 0.949
72	0.943, 0.955	0.966, 0.974
	0.979, 0.981	0.989, 1.00
	0.964, 0.977	0.974, 0.986

TABLE A1-4

0.2 GRAMS OF FILTER PAPER LOADING FOR
INITIAL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Incubation	Second Incubation
2	0.460, 0.471	1.51, 1.54
	0.476, 0.488	1.53, 1.54
4	0.836, 0.852	1.78, 1.79
	0.828, 0.845	1.74, 1.75
8	1.25, 1.24	2.13, 2.13
	1.25, 1.27	2.04, 2.03
16	1.66, 1.66	2.34, 2.34
	1.51, 1.51	2.19, 2,19
24	1.58, 1.59	2.24, 2.25
	1.77, 1.77	2.35, 2.37
	1.71, 1.71	2.55, 2.56
48	1.71, 1.71	2.38, 2.35
	1.51, 1.51	2.12, 2.09
72	1.82, 1.82	2.25, 2.21
	1.79, 1.81	2.33, 2.29

TABLE A1-5

0.2 GRAMS OF FILTER PAPER LOADING FOR
CONTROL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Control	Second Control
2	0.488, 0.501	0.756, 0.770
	0.477, 0.492	0.721, 0.738
4	0.902, 0.914	1.02, 1.04
	0.873, 0.884	1.05, 1.07
8	1.28, 1.29	1.36, 1.39
	1.23, 1.24	1.31, 1.33
16	1.42, 1.43	1.49, 1.52
	1.44, 1.45	1.47, 1.49
24	1.55, 1.56	1.65, 1.66
	1.54, 1.53	1.62, 1.60
	1.57, 1.57	1.63, 1.64
48	1.78, 1.80	1.82, 1.83
	1.73, 1.73	1.73, 1.74
	1.83, 1.88	1.80, 1.81
72	1.91, 1.95	1.92, 1.93
	1.93, 1.94	1.90, 1.92
	1.90, 1.93	1.93, 1.93

TABLE A1-6

0.4 GRAMS OF FILTER PAPER LOADING FOR
INITIAL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Incubation	Second Incubation
2	0.820, 0.823	1.68, 1.68
	0.810, 0.813	1.66, 1.66
4	1.49, 1.50	2.32, 2.32
	1.46, 1.48	2.34, 2.33
8	2.17, 2.21	2.84, 2.82
	2.14, 2.19	2.80, 2.81
16	2.77, 2.84	3.41, 3.43
	2.70, 2.80	3.37, 3.37
24	3.13, 3.15	3.60, 3.60
	3.16, 3.14	3.62, 3.62
48	3.50, 3.51	3.88, 3.87
	3.46, 3.46	3.88, 3.87
72	3.78, 3.76	4.13, 4.11
	3.67, 3.65	3.90, 3.89

TABLE A1-7

0.4 GRAMS OF FILTER PAPER LOADING FOR
CONTROL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Control	Second Control
2	0.785, 0.784	1.22, 1.29
	0.849, 0.843	1.19, 1.17
4	1.51, 1.49	1.77, 1.74
	1.56, 1.55	1.78, 1.78
8	2.23, 2.21	2.28, 2.28
	2.27, 2.25	2.33, 2.33
16	2.91, 2.90	2.98, 2.98
	3.00, 2.95	2.98, 2.99
24	2.89, 2.92	3.21, 3.23
	2.55, 2.57	2.87, 2.89
	3.10, 3.08	3.16, 3.16
48	3.54, 3.53	3.47, 3.48
	3.26, 3.21	3.22, 3.21
	3.43, 3.44	3.42, 3.43
72	3.64, 3.67	3.67, 3.67
	3.38, 3.40	3.38, 3.41
	3.57, 3.61	3.70, 3.70

TABLE A1-8

NONSPECIFIC DEACTIVATION GLUCOSE
MEASUREMENTS FOR DEWAXED COTTON SUBSTRATE

First Incubation Time, hours	Glucose Measurement, g glucose/L		
2	0.399, 0.397	0.389, 0.392	
4	0.387, 0.385	0.403, 0.403	
8	0.423, 0.420	0.358, 0.357	
16	0.407, 0.407	0.392, 0.375	0.436, 0.434
24	0.391, 0.392	0.401, 0.371	0.417, 0.416
48	0.422, 0.423	0.386, 0.361	0.423, 0.423
72	0.396, 0.370	0.323, 0.325	

TABLE A1-9

0.1 GRAMS OF DEWAXED COTTON LOADING FOR
INITIAL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Incubation	Second Incubation
2	0.039, 0.049	0.427, 0.414
	0.044, 0.049	0.441, 0.419
	0.043, 0.046	0.433, 0.423
4	0.069, 0.065	0.463, 0.433
	0.090, 0.067	0.433, 0.468
8	0.116, 0.109	0.466, 0.480
	0.111, 0.103	0.449, 0443
16	0.185, 0.200	0.514, 0.517
	0.192, 0.192	0.539, 0.521
24	0.222, 0.218	0.523, 0.502
	0.232, 0.226	0.545, 0.524
48	0.313, 0.305	0.605, 0.583
	0.313, 0.275	0.576, 0.556
	0.388, 0.347	0.755, 0.730
72	0.351, 0.341	0.625, 0.603
	0.348, 0.337	0.621, 0.597

TABLE A1-10

0.1 GRAMS OF DEWAXED COTTON LOADING FOR
CONTROL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Control	Second Control
2	0.034, 0.036	0.053, 0.053
	0.044, 0.046	0.062, 0.060
4	0.072, 0.074	0.087, 0.078
	0.072, 0.066	0.087, 0.087
8	0.120, 0.114	0.135, 0.128
	0.119, 0.112	0.135, 0.124
16	0.205, 0.196	0.204, 0.195
	0.197, 0.187	0.206, 0.198
24	0.234, 0.243	0.245, 0.248
	0.244, 0.219	0.273, 0.249
	0.245, 0.230	0.195, 0.195
48	0.338, 0.347	0.325, 0.342
	0.335, 0.320	0.344, 0.310
	0.303, 0.302	0.163, 0.156
72	0.366, 0.357	0.382, 0.366
	0.383, 0.343	0.400, 0.380
	0.370, 0.359	0.382, 0.369

TABLE A1-11

0.2 GRAMS OF DEWAXED COTTON LOADING FOR
INITIAL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Incubation	Second Incubation
2	0.076, 0.079	0.428, 0.424
	0.082, 0.087	0.445, 0.428
	0.066, 0.062	0.398, 0.391
4	0.128, 0.118	0.472, 0.465
	0.115, 0.105	0.453, 0.444
8	0.213, 0.198	0.479, 0.469
	0.246, 0.228	0.569, 0.559
16	0.447, 0.419	0.696, 0.697
	0.384, 0.347	0.604, 0.578
	0.371, 0.374	0.656, 0.656
24	0.526, 0.530	0.808, 0.804
	0.489, 0.485	0.689, 0.687
48	0.592, 0.530	0.740, 0.736
	0.540, 0.532	0.644, 0.643
72	0.893, 0.901	1.00, 1.00
	0.803, 0.804	0.845, 0.845

TABLE A1-12

0.2 GRAMS OF DEWAXED COTTON LOADING FOR
CONTROL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Control	Second Control
2	0.066, 0.074	0.098, 0.102
	0.076, 0.075	0.103, 0.102
4	0.131, 0.126	0.164, 0.158
	0.117, 0.131	0.157, 0.160
8	0.218, 0.218	0.241, 0.239
	0.233, 0.232	0.260, 0.257
16	0.382, 0.376	0.377, 0.376
	0.432, 0.421	0.467, 0.440
	0.417, 0.411	0.458, 0.442
24	0.448, 0.445	0.475, 0.460
	0.581, 0.542	0.590, 0.570
48	0.605, 0.605	0.662, 0.608
	0.906, 0.877	0.811, 0.783
	0.651, 0.650	0.655, 0.654
72	0.701, 0.698	0.723, 0.711
	0.970, 0.964	0.943, 0.948
	0.759, 0.755	0.783, 0.769

TABLE A1-13

0.4 GRAMS OF DEWAXED COTTON LOADING FOR
INITIAL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Incubation	Second Incubation
2	0.156, 0.164	0.478, 0.485
	0.125, 0.126	0.468, 0.457
	0.148, 0.151	0.461, 0.462
4	0.276, 0.273	0.581, 0.603
	0.186, 0.186	0.581, 0.581
	0.233, 0.240	0.577, 0.585
8	0.395, 0.395	0.691, 0.696
	0.416, 0.417	0.666, 0.672
16	0.703, 0.707	0.862, 0.869
	0.789, 0.785	1.02, 1.02
	0.697, 0.703	0.862, 0.868
	0.750, 0.753	0.866, 0.867
24	0.990, 1.00	1.05, 1.05
	0.687, 0.678	0.876, 0.867
	0.721, 0.733	0.964, 0.964
48	0.990, 1.00	1.19, 1.19
	0.868, 0.869	1.02, 1.01
	1.01, 1.02	1.20, 1.20
	0.974, 0.987	1.16, 1.16
72	1.22, 1.23	1.37, 1.37
	1.20, 1.19	1.41, 1.42
	1.44, 1.15	1.58, 1.58
	1.19, 1.18	1.34, 1.34

TABLE A1-14

0.4 GRAMS OF DEWAXED COTTON LOADING FOR
CONTROL INCUBATION GLUCOSE MEASUREMENT

First Incubation Time, hours	Glucose Measurement, g glucose/L	
	First Control	Second Control
2	0.148, 0.166	0.202, 0.211
	0.143, 0.151	0.172, 0.172
4	0.267, 0.264	0.319, 0.321
	0.233, 0.235	0.262, 0.278
8	0.469, 0.471	0.522, 0.528
	0.408, 0.409	0.451, 0.453
16	0.484, 0.505	0.664, 0.661
	0.614, 0.623	0.625, 0.625
24	0.756, 0.762	0.778, 0.784
	0.742, 0.747	0.742, 0.745
48	1.04, 1.05	1.05, 1.06
	1.04, 1.05	1.04, 1.05
72	1.27, 1.28	1.28, 1.29
	1.28, 1.29	1.30, 1.31

TABLE A1-15

MAXIMUM GLUCOSE YIELD POSSIBLE FOR 1 HOUR INCUBATION
OF FILTER PAPER SUBSTRATE

Amount of Substrate, g	Glucose Measurement, g glucose/L	
	First Sample	Second Sample
2.0	1.39, 1.40	1.35, 1.36
1.9	1.31, 1.33	1.37, 1.44
1.8	1.35, 1.34	1.36, 1.34
1.6	1.23, 1.20	1.23, 1.24

TABLE A1-16

MAXIMUM GLUCOSE YIELD POSSIBLE FOR 1 HOUR INCUBATION
OF DEWAXED COTTON SUBSTRATE

Amount of Substrate, g	Glucose Measurement, g glucose/L	
	First Sample	Second Sample
2.0	0.411, 0.417	0.407, 0.401
1.9	0.380, 0.381	0.417, 0.410
1.8	0.326, 0.366	0.382, 0.375
1.6	0.276, 0.366	0.395, 0.340

APPENDIX 2: FILTER PAPER AND DEWAXED COTTON ACTIVITY

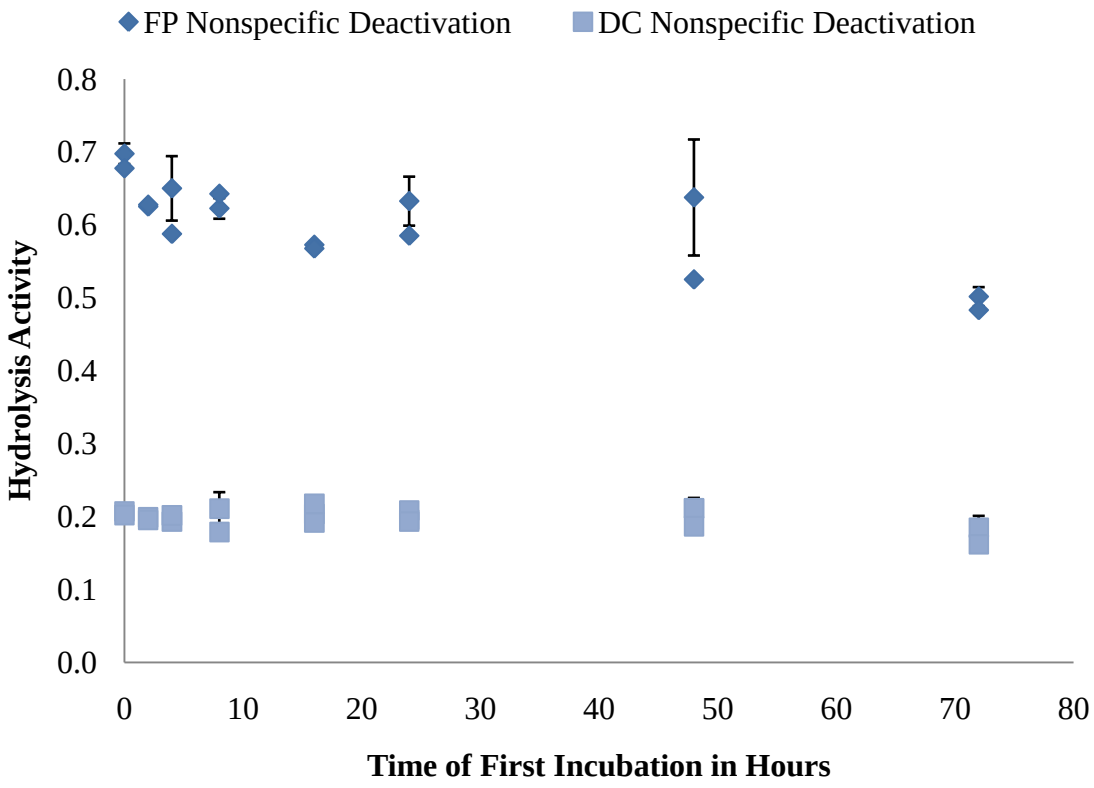

FIGURE A3.1 – Activity comparison of filter paper and dewaxed cotton for nonspecific deactivation with varying time of first incubation.

APPENDIX 3: SAMPLE CALCULATION

The following sample calculation is for determining activity of filter paper:

Data Needed

Experiment	*Glucose Concentration (g/L)*	
2.0g incubated for 1 hour	1.39, 1.40	1.35, 1.36
2.0g incubated for after nonspecific deactivation	1.25, 1.25	1.25, 1.26
1.9g incubated for 1 hour	1.31, 1.33	1.37, 1.44
0.1g incubated for 2 hours (C_1)	0.331, 0.337	0.342, 0.344
1.9g incubated after C_1 for 1 hour (C_2)	1.66, 1.65	1.56, 1.56
0.1g incubated for 2 hour (Control 1)	0.331, 0.330	0.325, 0.320
0.1g incubated for 3 hour (Control 2)	0.493, 0.472	0.483, 0.469

Calculate A_{non}

Average data for 2.0g incubated for 1 hour: $\frac{1.39+1.40}{2} = 1.40$ and $\frac{1.35+1.36}{2} = 1.36$

Divide averages by amount of substrate (2.0g): $\frac{1.40}{2}$=0.698 and $\frac{1.36}{2}$=0.678

Average these values to get A_{non}: $A_{non}=\frac{0.700+0.68}{2}=0.688\frac{\text{g glucose}}{\text{L·g substrate}}$

Calculate A_{sub}

Average data of 1.9g incubated for 1 hour: $\frac{1.31+1.33}{2}$=1.32 and $\frac{1.37+1.44}{2}$=1.41

Divide by amount of substrate (1.9g): $\frac{1.32}{1.9}$=0.695 and $\frac{1.41}{1.9}$=0.742

Average these to get A_{sub}: $\frac{0.695+0.742}{2}=0.717\frac{\text{g glucose}}{\text{L·g substrate}}$

Calculate $A_{t,non}$

Average data for 2.0g incubated after 2-hour nonspecific deactivation: $\frac{1.25+1.25}{2}$=1.25 and $\frac{1.25+1.26}{2}$=1.26

Divide by amount of substrate (2.0g): $\frac{1.25}{2}$=0.625 and $\frac{1.25}{2}$=0.628

Average to get $A_{t,non}$: $A_{t,non}=\frac{0.625+0.628}{2}=0.626\ \frac{\text{g glucose}}{\text{L·g substrate}}$

Calculate activity for 2-hour initial incubation and initial substrate load of 0.1g ($A_{t,sub}$)

Average data for C_1: $\frac{0.331+0.337}{2}$=0.334 and $\frac{0.342+0.344}{2}$=0.343

Average data for C_2: $\frac{1.66+1.65}{2}$=1.66 and $\frac{1.56+1.56}{2}$=1.56

Calculate control difference (C_3): $\frac{0.493+0.472+0.483+0.469}{4}-\frac{0.331+0.330+0.325+0.320}{4}=0.153$

Activity is: $A_{t,sub}=\frac{1}{2}\left(\frac{1.t6-0.334-0.153}{1.9}+\frac{1.56-0.343-0.153}{1.9}\right)=0.588\ \frac{\text{g glucose}}{\text{L·g substrate}}$

Calculate $P_{nonspecific}$

$$P_{nonspecific}=\frac{1-\frac{A_{t,non}}{A_{non}}}{1-\frac{A_{t,sub}}{A_{sub}}}=\frac{1-\frac{0.626}{0.689}}{1-\frac{0.588}{0.717}}=0.493$$

Calculate $P_{enz\text{-}sub}$

$$P_{enz\text{-}sub}=1-P_{nonspecific}=1-0.493=0.507$$

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