2016

INDUCTION OF CELLULASE IN HIGH SOLIDS CULTIVATION OF *TRICHODERMA REESEI* FOR ENHANCED ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSE

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Digital Object Identifier: http://dx.doi.org/10.13023/ETD.2016.372

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INDUCTION OF CELLULASE IN HIGH SOLIDS CULTIVATION OF *TRICHODERMA REESEI* FOR ENHANCED ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSE

THESIS

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

By

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ABSTRACT OF THESIS

INDUCTION OF CELLULASE IN HIGH SOLIDS CULTIVATION OF *TRICHODERMA REESEI* FOR ENHANCED ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSE

This project aimed investigated cellulase in-situ production for large-scale on-farm production of lignocellulosic biofuel. Cellulase activity and glucose released by *T. reesei* with corn stover and wheat bran as co-substrates for solid state cultivation (SSC) were examined. Co-cultivation has previously increased *T. reesei* cellulase, but corn stover and wheat bran have not been co-cultivated (Dhillon, Oberoi et al. 2011). This work compared cellulase activity and glucose concentration of corn stover co-cultivated with 0-40% wheat bran in high solids. Samples with at least 20% wheat bran exhibited increased cellulase activity. However, the average glucose concentration without wheat bran was 3.29 g/L compared to 16.7 g/L with wheat bran.

Glucose released by *T. reesei* on pretreated corn stover with 0-40% wheat bran was compared at the optimal temperatures for fungal growth and for cellulase activity after SSC. Previous research has rarely used cellulase from SSC to hydrolyze lignocellulose. Following SSC of *T. reesei* at 30°C for seven days, samples were warmed to 50°C for five days. Glucose concentration increased to 12.1 and 32.7 g/L for samples with and without wheat bran. This strategy could reduce lignocellulosic fuel production costs by eliminating need for commercial cellulase and is promising for efficient cellulose hydrolysis.

KEYWORDS: *Trichoderma reesei*, lignocellulose, hydrolysis, solid state cultivation, cellulase

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July 17, 2016

Acknowledgments

I am incredibly grateful for the many people who have helped make this thesis possible. Dr. Sue Nokes has so thoughtfully provided much of her time and energy to encourage me during the inevitable ups and downs of data collection, decision making, and writing has been invaluable.

To my committee members, Dr. Michael Montross and Dr. Czarena Crofchek, thank you for your guidance in completing this research and for making classes enjoyable. Immense appreciation is due to Amanda Hickman, Mathew Ruwaya, and William Sympson. Their reminders to slow down and breathe prevented innumerable mistakes and illuminated many solutions. Their generous aid in the laboratory helped turn my experimental failures into successes. Thanks also to my fellow graduate students for struggling though classes with me and to Jayne White.

I have found so many wonderful friends at the University of Kentucky and much appreciation is due for their inspiring attitudes. As we move off to the far corners of the country, know that I am looking forward to celebrate your many achievements in life.

To my family, thank you for teaching me to be independent, brave, and resilient. I would not be here without you.

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Chapter One: Introduction

1.1 Overview of Biofuels

Currently, the vast majority of world energy resources used are derived from nonrenewable fossil fuels, such as petroleum and natural gas, which are limited in supply (EIA 2016). Globally, liquid fuel demand was expected to increase from 90 million barrels per day to 121 million barrels per day in 2040 (EIA 2016). By replacing petroleum with biofuel, it is possible to prolong availability of global petroleum reserves, while satisfying growing demand for energy (Tyson 1993, Brown and Brown 2012).

Extraction, processing, and burning of fossil fuels cause environmental problems including reduced air and water quality, leading to increased risks to human and animal health and increased risk of catastrophic climate changes (Brown and Brown 2012, Pachauri, Allen et al. 2014). Biofuels significantly reduced emissions with E95 fuels (a mixture of 95% biologically derived ethanol and 5% gasoline) producing 90% less CO_2, 67% less SO_2 and 14% less volatile organic carbon (VOC) emissions compared to gasoline which satisfied the Clean Air Act according to a Department of Energy total fuel cycle analysis (Tyson 1993). Furthermore, compared to traditional gasoline, biofuel produces less air pollution and is easier to separate from water in the event of an unintentional spill (Tyson 1993, Brown and Brown 2012, Thirmal and Dahman 2012). Additionally, certain agricultural practices such as reducing tillage, fertilizers, and other inputs can further reduce life cycle greenhouse gas (GHG) emissions of biofuels (Hill, Nelson et al. 2006, Jessup 2009).

Fluctuating prices in the global petroleum market have influenced consideration of alternative fuels (Selig, Vinzant et al. 2009, Brown and Brown 2012). Furthermore, dependence on foreign oil poses a national security threat as exemplified by the 1973 Organization of Petroleum Exporting Countries (OPEC) Embargo, the 1979 Oil Crisis, and the first Gulf War (Brown and Brown 2012).

Ethanol is a three carbon alcohol and is the traditional biofuel, but butanol has emerged as a biofuel of interest (Brown and Brown 2012). Butanol is a four

carbon, straight chained alcohol and is often synthesized through a petrochemical route as a precursor to paints, plastics, and polymers (Ezeji, Qureshi et al. 2003, Kehail and Brigham 2015). Butanol has advantages over the traditional biofuel, ethanol, due to its miscibility with both gasoline and diesel, higher energy content, lower vapor pressure, and nonhygroscopicity (Guo, Tang et al. 2012, Wang, Cao et al. 2013). Also, it is unnecessary to modify existing vehicular engines to allow for combustion of butanol (Thirmal and Dahman 2012).

1.2 Second-Generation Feedstocks

Efforts to produce biofuel from traditional edible feedstocks (e.g., corn starch, sugarcane, soybeans, and canola) (Jessup 2009) have been developed domestically and abroad, but create competition for cropland between energy and food (Hill, Nelson et al. 2006, Rathmann, Szklo et al. 2010). In a world with limited land resources, a growing population, and increasing energy demands (Moreno, Ibarra et al. 2015, EIA 2016), reserving sufficient cropland for food is critical to preventing a global food shortage (Brown and Brown 2012, Ma and Ruan 2015).

To address this problem, the United States government and other public and private entities have funded research into development of economical second-generation biofuels (Jessup 2009). Second generation biofuels are produced from lignocellulose found in dedicated energy crops, forest products, and agricultural residues (Wen, Wu et al. 2014, Moreno, Ibarra et al. 2015). Dedicated energy crops, such as miscanthus and switchgrass, are crops grown for the purpose of bioprocessing for energy production (Jessup 2009). Agricultural residues consist of non-edible portions of crops and are normally left in the field or burnt after harvest (Krishna, Reddy et al. 2001). Implementation of the Renewable Fuel Standard by the Energy Policy Act of 2005 and the Energy Independence and Security Act of 2007 contributed to increases in both American biofuel use and production (Jessup 2009, Brown and Brown 2012).

Lignocellulose is a renewable, agricultural material of which 10^9 tons are produced annually (Moreno, Ibarra et al. 2015, Taha, Shahsavari et al. 2015). Agricultural waste sources of lignocellulose include corn stover, wheat straw, and sugarcane bagasse, pea pod waste, rice straw, and others. As an abundant waste product, lignocellulosic biomass is cheap and attractive as feedstock (Wang, Cao et al. 2013, Moreno, Ibarra et al. 2015).

Lignocellulosic biomass is comprised of cellulose (35-50% w/w) interwoven with hemicellulose (20-35% w/w) and wrapped in lignin (5-30% w/w) (Brown and Brown 2003, Zhang and Lynd 2004). Cellulose molecules consist of 10,000-15,000 D-glucose units linearly connected by β(1→4) glycosidic bonds (Zhang and Lynd 2004, Nelson, Lehninger et al. 2008). β(1→4) glycosidic bonds make cellulose suitable for plant structural support and indigestible by humans and other non-ruminant animals (Zhang and Lynd 2004, Nelson, Lehninger et al. 2008). Cellulose exists in both crystalline and amorphous states, with the latter preferred for hydrolysis (Zhang and Lynd 2004).

Hemicellulose is a complex heteropolysaccharide with a lower molecular weight than cellulose (Hendriks and Zeeman 2009). It consists of hexoses (i.e., glucose, mannose, and galactose), pentoses (i.e., xylose and arabinose), deoxyhexoses, and sugar acids (Hendriks and Zeeman 2009, Taha, Shahsavari et al. 2015).

Lignin is composed of phenylpropane units, specifically the monomers coniferyl, sinapyl, and coumaryl alcohol (Brown and Brown 2003, Hendriks and Zeeman 2009). Lignin provides structural rigidity and protection from microbial attack and oxidative stress (Brown and Brown 2003, Hendriks and Zeeman 2009, Kumar, Barrett et al. 2009).

1.3 Choice of Substrates

Availability of feedstocks varied by region. Corn stover is the most abundant agricultural residue in the United States, Hungary, and China (Elshafei, Vega et al. 1991, Varga, Szengyel et al. 2002, Ma and Ruan 2015). Corn stover, which included the husks, leaves, cobs, and stalks normally left in the field, was

less expensive compared to corn grain and in 2008 was merely $55.12 per metric ton (compared to $256.28 per metric ton corn grain) (Qureshi, Saha et al. 2010). Furthermore, corn stover does not compete with the food supply for land (Wen, Wu et al. 2014, Ma and Ruan 2015).

When choosing a substrate, substrate composition should be considered in relation to downstream conditions such as hydrolyzing organism and pretreatment method. Juhasz et al. further illustrated this point when they tested enzyme production after seven days on different substrates using *T. reesei* RUT-C30 under liquid fermentation conditions (2005). Hydrolysis of steam pretreated corn stover yielded 59% of the theoretical glucose yield, compared to 33% for steam pretreated spruce and 53% for steam pretreated willow (Juhasz, Szengyel et al. 2005).

Singhania et al. analyzed cellulase production by *T. reesei* NRRL 11460 on 0.1 N sodium hydroxide pretreated sugarcane bagasse, cassava bagasse, wheat bran, and rice straw (2006). Sugarcane bagasse led to the highest cellulase activity (0.861 IU $^{mL-}$1) followed by wheat bran, cassava bagasse, and rice straw using the DNS method (Singhania, Sukumaran et al. 2006). The researchers evaluated cellulase activity at 24, 48, 72, 96, and 120 h and interestingly, peak cellulase activity occurred at different times for different substrates. For example, unpretreated and pretreated cassava bagasse peaked at 48 h, while 72 h was optimal for pretreated sugarcane bagasse and unpretreated wheat bran and 96 h was optimal for unpretreated sugarcane bagasse and pretreated wheat bran (Singhania, Sukumaran et al. 2006).

Deswal et al. also investigated solid substrate hydrolysis conditions using Fomitopsis sp. RCK2010 as the hydrolyzing organism (2011). The substrates investigated were wheat straw, rice straw, wheat bran, corn cobs, corn stover, mesquite (*Prosopis juliflora)*, and wild sage (*Lantana camera)*. Rice straw and wheat bran were pretreated with 0.5% (w/v) H_2SO_4 and 2.5% NaOH at 121°C for 15 min and the IUPAC protocol for measuring cellulase activity was used. When corn stover was used as a carbon source for Fomitopsis sp. RCK2010 under solid substrate cultivation, after 24 h enzyme activity was observed to be:

CMCase, 3.70 IU g^{-1}; FPase, 0.24 IU g^{-1}; and β-glucosidase, 2.77 IU g^{-1}. When the substrate was wheat bran the enzyme activity was 71.5 IU g^{-1} for CMCase, 3.27 IU g^{-1} for FPase, and 50.7 IU g^{-1} for β-glucosidase.

Researchers in Finland and Hungary grew *T. reesei* RUT-C30 on steam pretreated corn stover, spruce, willow, and Solka Floc and measured the filter paper activity using the IUPAC Measurement of Cellulase Activities method. After seven days of hydrolysis, filter paper activity per milliliter was 0.56 FPU mg^{-1} with steam pretreated willow, 0.52 FPU mg^{-1} with steam pretreated corn stover, and 0.45 FPU mg^{-1} with steam pretreated spruce (Juhasz, Szengyel et al. 2005).

Hydrolysis of multiple substrates has been investigated to a limited extent. Dhillon et al. investigated cellulase production by *T. reesei* RUT-C30 on ten gram 1-mm ground samples of solid state wheat bran, rice straw, cauliflower waste, kinnow pulp, and peapod waste (2011). DNS assays were performed on citrate buffer extracted samples after 96 h. Samples extracted from wheat bran produced the highest cellulase activity of 22.9 IU gds^{-1}, compared to 15.3, 15.7, 16.1, and 16.3 IU gds^{-1} for cauliflower waste, kinnow pulp, rice straw, and pea pod waste (Dhillon, Oberoi et al. 2011). When rice straw was combined with cauliflower waste, kinnow pulp, and wheat bran in 4:1 and 3:2 ratios, the cellulase production after 96 h increased significantly compared to either of the substrates alone with the 3:2 ratio being most favorable (Dhillon, Oberoi et al. 2011). For example, when rice straw and wheat bran were used combined as substrates, cellulase activity increased to 25.0 and 31.0 IU gds^{-1} for 4:1 and 3:2 ratio mixtures, respectively (Dhillon, Oberoi et al. 2011).

Camassola and Dhillon found that cellulase, β-glucosidase, endoglucanase, and xylanase activity was statistically significantly ($p<0.05$) increased when co-hydrolyzing sodium hydroxide and heat pretreated corn stover and wheat bran with *Penicillium echinulatum* as compared to either substrate alone (Camassola and Dillon 2007). For example, after two days when hydrolyzing pretreated sugarcane bagasse with wheat bran in 2:8 and 4:6 ratios, cellulase activity was 10.9 and 10.4 IU gds^{-1} day^{-1} compared to only 5.34 IU gds^{-1} day^{-1} for samples with only wheat bran (Camassola and Dillon 2007)

A similar technique, referred to as co-digestion, of using multiple substrates in solid-state anaerobic digestion has been effective in increasing methane yield relative to individual substrates alone (Yang, Xu et al. 2015). For example, when co-digesting corn stover with expired dog food yield of methane in liters per kilogram increased to 109-229% of yield with corn stover or expired dog food alone (Yang, Xu et al. 2015). Co-digesting lignocellulosic waste with high protein substrates may reduce the inhibitory effect of volatile fatty acids and ammonia in the high protein substrates (Yang, Xu et al. 2015).

1.4 Overview of Biomass Conversion to Fuel

Processing of biomass into biofuel consists of four primary stages: comminution and pretreatment, hydrolysis, fermentation, and distillation (Brown and Brown 2003; Taha, Shahsavari et al. 2015). A visual representation of this process can be found in Figure 1.

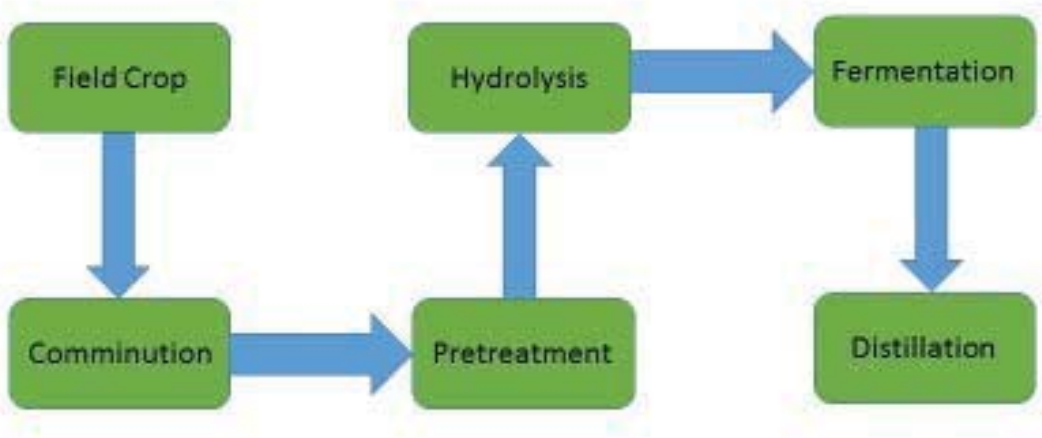


Figure 1. Overview of Biofuel Production Process.

1.4.1 Comminution

Comminution, or particle size reduction, is a highly energy intensive step in biofuels production (Li, Ruan et al. 2004). Comminution is frequently accomplished by grinding through a hammermill. It is correlated with increases in bulk density, flowability, and digestibility of biomass (Miao, Grift et al. 2011,

Hickman 2015). Particle size affects microbial kinetics and reducing particle size may improve cellulase activity and glucose yields (Pandey, Soccol et al. 2000, Li, Ruan et al. 2004). For example, Li, Ruan, et al. observed a 30% increase in glucose yield after 60 h when using 0.707 mm 0.3 N NaOH pretreated, rather than 2 mm (5.2 g L^{-1} and 4.0 g L^{-1}, respectively) (2004). Reduction of particle size does not, however, always improve cellulase yields; particle size reduction below 500 μm led to reduced cellulase activity yield compared to 500 μm particles when *T. reesei* NCIM 992 was the hydrolyzing organism over the same time period (Maurya, Singh et al. 2012). Energy input required to reach smaller particle sizes increases rapidly, therefore it is essential to optimize particle size in terms of yield and costs (Miao, Grift et al. 2011). Still, the comminution expense may be offset by reduced process expenditures in transportation and storage (Li, Ruan et al. 2004, Miao, Grift et al. 2011, Maurya, Singh et al. 2012).

Particle size for corn cobs and corn stover ranges in the literature from 0.5-3.0 mm, which is consistent with the particle size range in current biorefineries (Miao, Grift et al. 2011, Baral and Shah 2014).

1.4.2 Pretreatment

Prior to hydrolysis of lignocellulosic biomass, a pretreatment step can significantly increase the release of fermentable monosaccharides during hydrolysis by removing lignin and increasing biomass digestibility (Li, Ruan et al. 2004, Hendriks and Zeeman 2009, Kumar, Barrett et al. 2009, Moreno, Ibarra et al. 2015). Pretreatment can be costly, accounting for as much as a third of overall costs of biofuels production (Brown and Brown 2003), but promising research on reducing pretreatment costs is underway (Kumar, Barrett et al. 2009).

In addition to degrading lignin, pretreatment functions to increase the susceptibility of cellulose to enzymatic degradation during hydrolysis by increasing porosity, increasing surface area, and disrupting its crystalline structure (Li, Ruan et al. 2004, Zhang and Lynd 2004, Kumar, Barrett et al. 2009, Dionisi, Anderson et al. 2014, Moreno, Ibarra et al. 2015). Pretreatment which degrades lignin, but preserves cellulose and hemicellulose is desirable because

they can be hydrolyzed and fermented to valuable energy products (Kumar, Barrett et al. 2009, Moreno, Ibarra et al. 2015).Greater internal and external cellulose surface area allows cellulase enzymes to more easily hydrolyze β-glycosidic bonds (Zhang and Lynd 2004). A good pretreatment method avoids producing compounds which inhibit the hydrolyzing or fermenting organism (Kumar, Barrett et al. 2009). Energy input and cost of the chosen pretreatment method should be minimized (Li, Ruan et al. 2004, Kumar, Barrett et al. 2009, Baral and Shah 2014). Life cycle environmental impact is another area of concern and biochemical methods are typically less environmentally hazardous and have a lower energy requirement compared to chemical methods (Varga, Szengyel et al. 2002).

Existing chemical pretreatment options include acidic, alkaline peroxide, ammonia, ammonia fiber explosion (AFEX), basic, biological, steam explosion, hot water, treatment with organic solvent and others (Brown and Brown 2003, Li, Ruan et al. 2004, Selig, Vinzant et al. 2009, Moreno, Ibarra et al. 2015). Common pretreatment and hydrolysis methods produce weak acids, furan derivatives, and phenolic compounds which inhibit fermenting organisms such as *Clostridia* (Guo, Tang et al. 2012, Baral and Shah 2014).

1.4.3 Alkaline Pretreatment

Sodium hydroxide is the most frequently used base for alkaline pretreatment of biomass, although lime is also common (Mosier, Wyman et al. 2005, Modenbach 2013). Sodium hydroxide pretreatment causes swelling of lignocellulosic biomass, increased internal surface area, decreased crystallinity, and may reduce lignin in biomass by more than 95% (w/w of dry matter) (Varga, Szengyel et al. 2002, Li, Ruan et al. 2004, Modenbach 2013). The sodium hydroxide methods are convenient because they do not require special equipment, the reagents are less corrosive than acidic ones, high pressures are not required, and is relatively low cost (Modenbach 2013).

Various sodium hydroxide pretreatment methods have been investigated to optimize yields. Li, Ruan, et al. compared glucose yields after 60 h of

hydrolysis, using Spezyme CP as the source of cellulase, and using 2 mm ground corn stover pretreated with 0.1, 0.2, 0.3, and 1.0 N NaOH for 4 h as substrate (2004). The results showed that increasing sodium hydroxide concentration during pretreatment led to increased glucose yields (1.35, 3.71, 4.66, 5.60, and 6.25 g L^{-1}, respectively), but due to the cost of sodium hydroxide, the researchers recommended using 0.1-0.3 N (Li, Ruan et al. 2004).

Singhania et al. observed increased yields when hydrolyzing 0.1 N sodium hydroxide pretreated sugarcane bagasse (0.861 IU gds^{-1}) with *T. reesei* NRRL 11460 compared to unpretreated sugarcane bagasse (0.572 IU gds^{-1}) (Singhania, Sukumaran et al. 2006).

1.4.4 Thermal pretreatment

Knowledge is about the effect of high pressure and high temperature conditions on biomass as pretreatment for biofuel production and the effect varies among substrates (Bolado-Rodríguez, Toquero et al. 2016) Bolado-Rodríguez et al. autoclaved milled and dried wheat straw and sugarcane bagasse at 121°C for 60 min and measured degradation compounds released during autoclaving. For wheat straw, 1.48 g L^{-1} sugars and 9.88 g L^{-1} volatile solids were released, while for sugarcane bagasse, sugar released was not detectable and 10.43 g L^{-1} volatile solids were released (Bolado-Rodríguez, Toquero et al. 2016).

The effect of thermal pretreatment on anaerobic digestibility has been investigated (Bolado-Rodríguez, Toquero et al. 2016). Compared to dilute hydrochloric acid autoclaving, dilute sodium hydroxide autoclaving, and alkaline peroxide pretreatment methods, wheat straw and bagasse which had been autoclaved produced the highest methane yields with an increase to 62 and 58% of theoretical, respectively (Bolado-Rodríguez, Toquero et al. 2016). Studies which examined the effect of high pressure and high temperature conditions as a pretreatment of wheat bran and corn stover, the substrates in this study, were not found.

1.4.5 Hydrolysis

When producing lignocellulosic biofuels, hydrolysis, also known as saccharification, of cellulose is necessary before fermentation (Vintila, Kovacs et al. 2014). Hydrolysis breaks the β-glycosidic bonds in cellulose to release individual sugar monomers and allow for fermentation to proceed (Yoon, Ang et al. 2014).

Hydrolysis of pretreated biomass can be done chemically or biochemically. Chemical methods investigated include use of hydrochloric acid, sulfur dioxide, sulfuric acid, and chloride ionic liquid-containing catalytic acid (Von Sivers and Zacchi 1995, Binder and Raines 2010). Biochemical approaches use crude enzymes, bacteria, filamentous fungi, and/or yeast to perform hydrolysis (Elshafei, Vega et al. 1991, Awafo, Chahal et al. 1996, Pandey, Soccol et al. 2000, Li, Ruan et al. 2004).

Both chemical and biochemical hydrolysis methods present opportunities and challenges for researchers. For example, safety concerns and recycling difficulties with strong acids have limited implementation of these saccharification strategies (Binder and Raines 2010). For enzymatic hydrolysis, cellulose crystallinity, degree of polymerization, substrate moisture content, accessible surface area, and lignin content act as limitations (Zhang and Lynd 2004, Hendriks and Zeeman 2009).

Cellulase enzyme systems vary among organisms in effectiveness and mechanism. This is, in part, due to the diversity of organisms which produce cellulase, including bacteria and fungi (Ma and Ruan 2015). The following is a partial list of genera studied for cellulase production: *Trichoderma, Aspergillus, Phanerochaete, Penicillium, Humicola, Neospora, Chaetomium, Nectria,* and *Fomitopsis* (Deswal, Khasa et al. 2011, Dhillon, Oberoi et al. 2011, Wahid, Salleh et al. 2011). Many enzymes have been identified as part of cellulase systems (e.g., cellobiohydralase, β-glucosidase, xylanase, and endoglucanase) and the relative amount of enzyme produced varied between organisms. For example, after 96 h of solid state growth, samples extracted from cauliflower waste, kinnow pulp, rice straw, pea pod waste, and wheat bran each had higher cellulase

activity when *T. reesei* was used for hydrolysis (15.3-22.9 IU gds^{-1}) compared to *A. niger* (8.39-13.6 IU gds^{-1}), but samples hydrolyzed by *A. niger* had higher β-glucosidase activity (14.62-21.69 IU gds^{-1}) compared to *T. reesei* (10.82-13.58 IU gds^{-1}) (Dhillon, Oberoi et al. 2011).

1.4.6 In Situ Biochemical Hydrolysis

Biochemical hydrolysis can be very costly, but replacing purchasing enzymes with organisms grown onsite could reduce costs considerably (Wooley, Ruth et al. 1999, Moosavi-Nasab and Majdi-Nasab 2008, Lever, Ho et al. 2010, Wahid, Salleh et al. 2011, Vintila, Kovacs et al. 2014). In situ hydrolysis under solid state conditions, rather than liquid, may further reduce expenses (Wahid, Salleh et al. 2011). Solid state conditions are often simpler, less energy intensive, and less prone to contamination due to hyphal growth of filamentous fungi (Lever, Ho et al. 2010).

Solid state cultivation (SSC) is a method in which substrate is moistened to allow successful microbial growth, but without apparent free water (Awafo, Chahal et al. 1996, Yoon, Ang et al. 2014). 15% solids or more is generally considered solid state, though up to 60% has been investigated and 20-40% is typical (Gutierrez-Correa and Tengerdy 1997, Pandey, Soccol et al. 2000, Singhania, Sukumaran et al. 2006, Maurya, Singh et al. 2012). Furthermore, compared to submerged fermentation, SSC requires less water input, sterility, energy input, infrastructure requirement, and skilled labor; however, larger scale SSC presented additional problems as temperature, moisture, and other gradients arise which were not prevalent in submerged fermentation (Holker, Hofer et al. 2004, Sukumaran, Singhania et al. 2009).

Trichoderma species secrete a robust cellulase complex and commercial cellulases are frequently derived from Trichoderma (Zhang and Lynd 2004, Tian, Xie et al. 2015). As a filamentous fungi, *T. reesei* is capable of cultivation without free water and is thus, a good candidate for in situ solid state hydrolysis (Yoon, Ang et al. 2014). The T. reesei cellulase system has been well characterized and found to produce at least two cellobiohydralases, five endoglucanases, a β-

glucosidase, two xylanases, an α-L-arbinofluranosidase, an acetyl xylan esterase, a β-mannanase, and an α-glucuronidase using 2D electrophoresis (Kubicek 1992, Nogawa, Goto et al. 2001, Vinzant, Adney et al. 2001), with cellobiohydrolase I and II and endoglucanase II being the primary enzymes (Zhang and Lynd 2004).

Several mutant strains of *T. reesei* have been developed to manipulate cellulase production and efficacy (Dashtban, Buchkowski et al. 2011). *T. reesei* QMY-1, QM 9414, and MCG 80 (a descendent of RUT-C30) mutant cellulase systems were analyzed individually by Awafo et al. (1996). When 20 IU FPA g^{-1} of cellulase from each of these organisms was used to saccharify delignified wheat straw for 20 d, QMY-1 produced 235 IU g^{-1} cellulose; QM 9414 produced 235 IU g^{-1} cellulose; and MCG 80 produced 333 IU g^{-1} cellulose. When the glucose in the hydrolysate produced by *T. reesei* MCG 80 was measured, the concentration was about 45 g L^{-1} (Awafo, Chahal et al. 1996).

Low β-glucosidase production by *T. reesei* may be overcome by co-culturing with an organism which produces high levels of β-glucosidase. For example, Gutierrez-Correa and Tengerdy co-cultured *T. reesei* LM-UC4E1 and *Aspergillus phoenicus* QM329 on sodium hydroxide and autoclave pretreated sugarcane bagasse under solid state conditions (80% moisture content) (1997). The researchers found a synergistic effect in which both *T. reesei* and *A. phoenicus* produced more cellulase in the presence of the other. In fact, *T. reesei* LM-UC4 produced 5.3 IU g^{-1} cellulase in monoculture, but 13.4 IU g^{-1} in co-culture (Gutierrez-Correa and Tengerdy 1997). Dhillon et al. also observed a synergistic effect between *A. niger* BC-1 and *T. reesei* RUT-C30 when grown on pea pod waste, cauliflower waste, wheat bran, rice straw, and kinnow pulp (2011). For wheat bran colonized for 96 h, the filter paper activity measured was 13.6 IU gds^{-1} with *A. niger* BC-1, 22.9 IU gds^{-1} for *T. reesei* RUT-C30, and 24.2 IU gds^{-1} for a co-culture of *A. niger* BC-1 and *T. reesei* RUT-C30 (Dhillon, Oberoi et al. 2011). β-glucosidase activity on wheat bran after 96 h was 21.7 with *A. niger* BC-1, 13.6 with *T. reesei* RUT-C30, and 24.5 with a co-culture of *A. niger* BC-1 and *T. reesei* RUT-C30 (Dhillon, Oberoi et al. 2011).

Shrestha et al. cultivated T. reesei QM6a in co-culture with S. cerevisiae using wet-milled, sodium hydroxide and steam pretreated corn fiber as substrate (Shrestha, Khanal et al. 2009, Shrestha, Khanal et al. 2010). The research group obtained 12.8 g total sugar per 100 g corn fiber, but only about two grams of this was reducing sugar. The ethanol yield was 18% of the theoretical maximum. When the group used Phanerochaete chrysosporium and Gloeophyllum trabeum in place of *T. reesei* ethanol yield improved to 28% and 35%, respectively, of the theoretical maximum.

1.4.7 Culture Techniques

In this study, the hydrolyzing organism was *T. reesei* which was cultivated under solid state conditions. In past studies involving *T. reesei* grown in high-solids, *T. reesei* was subcultured on potato-dextrose agar (PDA) in Petri dishes prior to inoculation of substrate; often for seven days (Singhania, Sukumaran et al. 2006, Lever, Ho et al. 2010, Ma and Ruan 2015). The incubation temperatures for this part of these experiments were 30, 24, and 30°C, respectively. In other cases, the fungus was grown on PDA in Petri dishes for as few as five or six days at 30 and 28°C, respectively (Maurya, Singh et al. 2012, Vintila, Kovacs et al. 2014). Wahid et al. even reported Petri dishes with PDA being fully covered with *T. reesei* spores after only 5 days of incubation at 30^0C (Wahid, Salleh et al. 2011).

Once the microbial subculture sporulated on PDA, the fungus must be transferred to the substrate. Wahid et al. collected spores from their Petri dishes by washing the plates with five milliliters of a mineral salt solution and dislodging the spores into the solution with a sterile glass rod (2011). The spore suspension was filtered through Whatman No. 1 filter paper and washed repeatedly with mineral salt solution. The spore concentration was adjusted to 10^5-10^7 spores mL^{-1} after counting with a hemocytometer (Wahid, Salleh et al. 2011).

Researchers have inoculated liquid media, such as yeast malt broth, with *T. reesei* stock culture (Shrestha, Khanal et al. 2009, Shrestha, Ibanez et al. 2015). In these studies, stock cultures were thawed to room temperature and

transferred into flasks containing yeast malt broth. Researchers incubated cultures at 37^0C for seven days with shaking at 150 rpm. Shaking flasks during cultivation prevented temperature and nutrient gradients from arising in the media (Yoon, Ang et al. 2014).

Other researchers created a liquid media by extracting organisms from PDA plates by adding 0.1% (w/v) Tween-80 solution to Petri dish cultures; stirring to extract microorganisms; filtering the liquid through glass wool to remove mycelia; and adjusting the volume to achieve the desired spore concentration (10^7 mL^{-1} gds^{-1}) using a hemocytometer (Dhillon, Brar et al. 2011, Dhillon, Kaur et al. 2012). 10^7 spores gds^{-1} was also used for solid state cultivation by Kilikian, Afonso, et al. with *T. reesei* RUT-C30 other *Trichoderma*, and *Myceliophthora thermophile* (2014). The maximum cellulase activity after the 120 h hydrolysis period reported was 10.6 U gds^{-1} using a mixture of wheat bran and sugarcane bagasse at 80% moisture content with *M. thermophile*; the maximum cellulase activity for *T. reesei* RUT-C30 was 4.0 U gds^{-1} (2014). Lever et al. used 4 x 10^9 spores mL^{-1} to inoculate wheat straw with *T. reesei* QM9123 and obtained a maximum of 1.8 FPU gds^{-1} after 12 d (2010).

Preferred culture medium varied between organisms and even between strains. For example, Dashtban et al. found T. reesei strains QM9414 and RUT-C30 grew significantly more slowly in malt extract compared to strain QM6a ($p<0.001$). Furthermore, RUT-C30 grew faster in potato dextrose relative to both QM9414 and QM6a ($p<0.05$). These differences were determined optically after four days (Dashtban, Buchkowski et al. 2011).

Media which contained Tween-80, however, had higher cellulase activity than those without in one study with *T. reesei* RUT-C30 (Domingues, Queiroz et al. 2000). This is consistent with earlier work which found that culture media with 0.1% Tween-80 had enzyme yield up to 51.0% higher for some Trichoderma viride (Reese and Maguire 1969).

In another experiment, *Aspergillus niger* strain NS-2 was used to compare surface culture fermentation and SSF. Seven millimeter discs were cut from Petri dish cultures which were added to 250 mL flasks containing five grams of

sterilized wheat bran and water. The flasks were statically incubated at 30°C for 96 h (Bansal, Tewari et al. 2011). The researchers found that under solid state conditions, endo-glucanase activity was 333 IU gds^{-1} and β-glucosidase activity was 30 IU gds^{-1}. The researchers measured endo-β-1,4-glucanase, exo-β-1,4-glucanase, β-glucosidase, endo-β-1,4-xylanase, endo-β-1,4-mannanase, α-amylase, and glucoamylase activities. For all seven enzyme activities measured, the researchers found that enzyme activities from solid-state fermentations were higher in terms of micromoles sugar liberated per minute and the glucose yield lower compared to surface culture conditions. The researchers hypothesized that this may have been due to additional nutrients in the surface culture medium (Bansal, Tewari et al. 2011).

1.4.8 In Situ Enzyme Production with *T. reesei*

Trichoderma fungi, including *T. reesei* and *T. viride,* are known for secreting high levels of extracellular cellulase, with *T. reesei* being the best investigated (Singhania, Sukumaran et al. 2006, Dashtban, Buchkowski et al. 2011). *T. reesei* produces very little β-glucosidases. β-glucosidase hydrolyzes cellobiose into individual glucose units and thus, the relative lack of this enzyme is a limitation on complete cellulose hydrolyze by *T. reesei* (Awafo, Chahal et al. 1996, Juhasz, Szengyel et al. 2005, Yoon, Ang et al. 2014). Cellobiose accumulation also inhibits endoglucanases and exoglucanases of the *T. reesei* cellulase complex (Singhania, Sukumaran et al. 2010).

T. reesei has primarily been used under submerged cultivation conditions, but SSC has become an attractive alternative and has been investigated with wheat bran, sugar cane bagasse, corn stover, soybean bran, oil palm empty fruit bunches, and Ocimum gratissimum seed (Holker, Hofer et al. 2004, Singhania, Sukumaran et al. 2007, Wahid, Salleh et al. 2011).

1.4.9 Factors Affecting Yield

Fungal growth and enzyme production are dependent on temperature. The optimum temperature varies between fungi and may different for growth than

for enzyme production during SSF (Yoon, Ang et al. 2014). There must be sufficient heat to induce activation of cellulase, but not so warm as to cause denaturation (dos Santos, Abreu Filho et al. 2013). Many researchers have successfully grown *T. reesei* at 30°C on Petri dishes (Moosavi-Nasab and Majdi-Nasab 2008, Lever, Ho et al. 2010, Vintila, Kovacs et al. 2014); however, successful growth at 28°C has also been reported (Maurya, Singh et al. 2012).

Bone dry referred to the amount of matter in biomass less any water it holds. The amount of bone dry matter in air dried samples cannot be measured directly, but must be calculated. Moisture content is typically measured by use of a moisture analyzer or weighing, drying completely in an oven, and reweighing. Once the moisture content is determined, the mass of dry matter can be calculated by use of the following:

Equation 1. Moisture Content Equation.

$$m_{wb} = m_w/(m_w + m_{dm})$$

Where m_{wb} represents the total mass on a wet basis, m_w represents the mass of the water, and m_{dm} represents the mass of the bone dry matter in the sample. Culture medium moisture content impacts the functioning of the hydrolyzing and fermenting organisms and affects yields (Maurya, Singh et al. 2012). Sample moisture content must allow for dissolution of nutrients and absorption by the microorganism, but not high enough to impede air diffusion between particles or to increase contamination risk (Lever, Ho et al. 2010, Yoon, Ang et al. 2014). The optimal moisture content is dependent on the choice of substrate and microorganism, but for fungi under solid state conditions is generally in the range of 60-80% (Deswal, Khasa et al. 2011, Yoon et al., 2014). Maury et al. studied initial moisture content as a parameter for optimization of SSF using *T. reesei* (2012). This particular study found 70% moisture content with ten grams of wheat bran as substrate to produce the maximum cellulase activity (2.29 IU mL^{-1}) (Maurya, Singh et al. 2012).

T. reesei strain RUT-C30 was observed to produce maximum sugar (0.14 µmol glucose equivalents) at 72 h when grown in liquid culture media with D-lactose as the carbon source concentration of 1% (w/v) (Dashtban, Buchkowski

et al. 2011), while *T. reesei* QM9414 reached a maximum of 0.055 glucose equivalents at 120 h under the same conditions (Dashtban, Buchkowski et al. 2011). In the case of *T. reesei* RUT-C30, Dashtban et al. observed the cellulase activity decrease significantly after 72 h; for QM9414, observations were not performed after the maximum cellulase activity was observed (2011). Dhillon et al. found similar results: enzyme activity peaked at 96 h *T. reesei* at 22.89 IU gds $^{-1}$ in a 1:1 ratio with wheat bran (2011). Cellulases from other organisms have shown peak activity after 3-14 d (Deswal, Khasa et al. 2011, Tian, Xie et al. 2015).

Xie, Zhao, et al. investigated the effect of time and enzyme loading after cultivating *Trichoderma* strains G26, B-8, B-13, B-19, A6, and C1 on a 2:4:4 mixture of 100-mesh corn cob residue, wheat bran, and rice straw under solid state conditions for hydrolysis (2015). The maximum cellulase titer was observed after 96 h with *Trichoderma* strain G26 and was 41 IU, equivalent to 71 IU g^{-1}. When the solids loading was 12.5%, the measured glucose in the hydrolysate was 28, 46, and 52 g L^{-1} when the enzyme loading was 2.8, 6.5, and 10.2 IU mL^{-1}, respectively. When the solids loading was increased to 16.5%, the measured glucose increased to 53 and 57 g L^{-1} when the enzyme loading was 6.5 and 10.2 IU mL^{-1}, respectively (glucose concentration for 16.5% solids and 2.8 IU mL^{-1} enzyme load not given) (Xie, Zhao et al. 2015). Reducing sugar and glucose concentrations in the hydrolysate did not increase significantly between 48 and 96 hours, but did tend to increase over time (Xie, Zhao et al. 2015).

Mycelial growth, enzyme production, and transportation across cell membranes are affected by the pH of the medium (Deswal, Khasa et al. 2011). In general, researchers will not control pH throughout the experiment, but will adjust the initial pH to the desired value (Yoon, Ang et al. 2014). An initial medium pH of 5.0 was found to produce 2 IU $mL^{-}1$ of cellulase activity, compared to 1.5 IU mL^{-1} or less when the pH was 3.0, 4.0, 6.0, or 7.0 for *T. reesei* NCIM 992 on steam-pretreated, ground wheat bran (Maurya, Singh et al. 2012). *T. reesei* MCG 80 (Chahal 1985, Awafo, Chahal et al. 1996).

1.4.10 Hydrolysis with Fermented Material

Considine et al. cultivated *Penicillium capsulatum* under solid state conditions on beet pulp and extracted the enzyme (1988). The enzyme was then used transferred to beet pulp (8% w/v) at 50°C for saccharification. With this method, 76.8% hydrolysis was achieved when 11.0 cellulase units per gram were supplied and 49.5% when 2.8 cellulase units per gram were supplied (Considine, O'Rorke et al. 1988).

T. reesei is typically grown at 30°C (Gutierrez-Correa and Tengerdy 1997, Dashtban, Buchkowski et al. 2011, Wahid, Salleh et al. 2011, Vintila, Kovacs et al. 2014). Hydrolysis experiments which use cellulase, however, often used 50°C. For example, Li et al. used commercial cellulase (Spezyme CP) to hydrolyze sodium hydroxide pretreated 2 mm corn stover at 13% solids for five days at 50°C and obtained 1.35-6.25 g L^{-1} depending on the sodium hydroxide concentration during pretreatment (2004). Ma and Ruan found 50°C to be optimal for hydrolysis of corn stover by co-culture of *T. reesei* and *Coprinus comatus* in a bioreactor and reached 82% glucose yield (2015). The DNS method of measuring cellulase activity recommended 50°C because it is optimal for cellulase activity for *Trichoderma* (Ghose 1987).

1.4.11 Enzyme Extraction

Enzyme must be extracted from solid state samples before determining enzyme activity. Dhillon, Brar, et al. studied extraction strategies by removing one gram of dried apple pomace substrate without replacement every 24 h and mixing with 15 mL of differing extraction solutions (2012). The extraction solutions tested were 50 mol m^{-3} citrate buffer (pH 4.8), distilled water, milliQ water, 1 kg m^{-3} Tween-80, and 10.0 kg m^{-3} sodium chloride solution. Following 30 min of incubation at 200 rpm, the samples were centrifuged at 9000 x g for 15 min. The supernatant was decanted and the enzyme activity analyzed according to the methods by Ghose (1987). Filter paper activity was 51.6 IU g^{-1} for citrate buffer, 33.4 IU g^{-1} for distilled water, 31.7 IU g^{-1} for milliQ water, 35.7 IU g^{-1} for Tween-80 solution, and 45.5 IU g^{-1} for sodium chloride solution using the

substrate described with *A. niger* NRRL 567 as the hydrolyzing organism after 24 h (Dhillon, Brar et al. 2012).

In another experiment, Dhillon, Kaur et al. analyzed different enzyme extraction techniques by using different volumes of citrate buffer (10, 15, 20, and 25 mL gds^{-1}), buffer pH (4.0, 4.8, and 5.5), and shaking methods (incubator for 15 and 30 min, wrist action for 15 and 30 min, and vortex for 10 min) after 48 h of hydrolysis of apple pomace by *A. niger* NRRL 567 (Dhillon, Kaur et al. 2012). Extraction with 15 mL gds^{-1} citrate buffer led to the highest exoglucanase activity for all four treatment groups, compared to extraction with 10 or 25 mL gds^{-1}. Extraction with buffer of pH 4.0 led to measurement of significantly lower exoglucanase activity than extraction with pH 4.8 or 5.5 on four different sample types. There was not a significant difference between buffer with 4.8 and 5.5. Extraction with a wrist action shaker for 30 min produced significantly different results than extraction in a shaking incubator at 200 rpm for 30 min ($p<0.05$)

1.4.12 Cellulase Activity Measurement

Cellulase enzyme kinetics are difficult to elucidate because cellulases hydrolyze an insoluble substrate with variable composition (i.e., cellulose and hemicellulose) (Ghose 1987). Significant variation exists between cellulase systems from different organisms, and even cellulase systems from the same organism grown on different substrates (Awafo, Chahal et al. 1996, Pandey, Soccol et al. 2000, Maurya, Singh et al. 2012).

Differing methods of quantifying cellulase activities have evolved, which unfortunately, makes direct comparisons between some studies difficult (Ghose 1987). Measurement of cellulase activity is nevertheless, important because high cellulase activity is a strong indicator of the enzyme's ability to liberate sugar from lignocellulose. For example, a high cellulase activity may be accompanied by severe end-product inhibition limiting the enzyme's ability to saccharify in practice (Ghose 1987). In the case of *T. reesei* it is clear that end-product inhibition occurs, with glucose, cellobiose, and ethanol acting as noncompetitive inhibitors (Holtzapple, Cognata et al. 1990).

Despite the method being laborious and time-consuming, requiring large amounts of reagent, and presenting difficulties with reproducibility Cellulase activity assays are commonly performed according to the methods of Miller (1959) such as in Deswal et al. (2011), Dhillon et al. (2012) and Modenbach (2013) (Dashtban, Maki et al. 2010). This method has been widely accepted for use with *Trichoderma* cellulases, but may not be appropriate for use with cellulases produced by obligate anaerobes or other fungi (Ghose 1987).

The reagent used in this protocol calls for 10.6 g of 3,5-dinitrosalicylic acid, 19.8 g of sodium hydroxide, 308 g of Rochelle salts (sodium potassium tartrate), 7.6 g of phenol melted at 50°C, and 8.3 g of sodium metabisulfite dissolved in 1416 mL of distilled water (Ghose 1987). This reagent will hereafter be referred to as DNS reagent. The Rochelle salts act to prevent solubilization of oxygen by the reagent by increasing ion concentration; phenol increases the color produced by the reaction; and sodium bisulfite stabilizes the color produced by phenol (Miller 1959, Teixeira, da Silva et al. 2012). Sodium hydroxide is used to produce the alkaline conditions required for a redox reaction to occur between the 3,5 dinitrosalicylic acid and reducing sugars to occur (Teixeira, da Silva et al. 2012). In an earlier study, Miller used sodium sulfite, rather than sodium metabisulfite, to measure cellulase activity (1959), but the most recently published NREL protocol for measuring cellulase, LAP-006, calls for sodium metabisulfite (Adney and Baker 2008). Other experiments have omitted both the phenol and sodium metabisulfite because of the toxicity of phenol (Saqib and Whitney 2011, Teixeira, da Silva et al. 2012). Early experiments which omitted Rochelle salts from the reagent found the color produced to be unstable due to oxidation of the sulfite (Miller 1959).

Teixera et al. studied the effect of amino acids on the measurement of reducing sugars using the DNS colorimetric method using the reagent specified by Ghose and the same reagent with phenol and sodium metabisulfite omitted (2012). The researchers found that the presence of cysteine, tryptophan, histidine, tyrosine, and hydroxyproline altered reducing sugar measurement when using DNS reagent lacking phenol and sodium metabisulfite (Teixeira, da

Silva et al. 2012). Experiments with the concentration of phenol (formally hydroxybenzene, C_6H_5OH) found using 0.2% phenol in the reagent led to five times the color intensity than when no phenol was used, but similar variation compared to 0.5% phenol (Miller 1959). Phenol is classified as a Class B poison by the US Department of Transportation. It is corrosive of living tissue causing serious burns, blindness if contacted with the eyes, and even death (Wallace 1991). Therefore, use of phenol should be avoided when possible.

The DNS method is appropriate when (i) samples each contain less than five milligrams of glucose; (ii) when glucose concentration is low (0.1 mg glucose may be added to each sample to increase measurement accuracy when concentration is too low); and (iii) when the sample is not acidic (Ghose 1987). For *T. reesei,* 0.05 M citrate buffer (pH 4.8) is used to solubilize the enzymes, centrifuging removes solids, and dinitrosalicylic acid stops the reaction (Coward-Kelly, Aiello-Mazzari et al. 2003, Adney and Baker 2008). The samples are then boiled in a water bath for 5 min (Ghose 1987, Adney and Baker 2008). Miller recommends boiling for 15 min to improve the color reaction (Miller 1959). Vigorous boiling, rather than gentle boiling improves color and increases reproducibility (Coward-Kelly, Aiello-Mazzari et al. 2003, Dashtban, Maki et al. 2010) A spectrophotometer is then used to determine the percent light transmittance at 540 nm. Sugar analysis can alternatively be performed by high performance liquid chromatography (HPLC) (Coward-Kelly, Aiello-Mazzari et al. 2003, Dashtban, Maki et al. 2010). As defined by Ghose, an international unit (IU) is defined as 1 μmol hydrolysis product min^{-1} (Ghose 1987).

1.5 Objectives

The ultimate goal of this project was to enable on-farm processing of lignocellulosic biomass by developing an economical method for hydrolyzing pretreated substrate. Pretreatment followed by enzymatic hydrolysis has been shown successful for biofuel production; however, the cost of commercial enzymes is prohibitive. Enzymes produced in situ may be less expensive if

produced in sufficient quantity and with sufficient activity to effectively saccharify substrate.

Many studies have investigated use of *T. reesei* in liquid fermentation to produce enzymes for in-situ hydrolysis; however, this study investigated production of cellulases in high solids fermentation as a method for increasing the concentration of cellulases and glucose in the fermentation broth. *T. reesei* requires nutrients in addition to carbon for growth, but a complex media would not be economical for on-farm bioconversion. Wheat bran contains more nutrients than corn stover, but it is also more expensive. The objectives of this study were to determine:

1). The minimal amount of wheat bran that could be mixed with both unpretreated and pretreated corn stover to result in *T. reesei* growth sufficient to increase production of cellulases for saccharification of the substrates and

2). If raising the temperature during hydrolysis would inhibit fungal growth, encourage cellulose activity, and improve glucose yields (Figure 2).

The first objective determined the cellulase production by *T. reesei* RUT-C30 under varying ratios of unpretreated and 0.2 N NaOH pretreated corn stover to wheat bran after seven days. Solid state cultivation methods were used with 25% solids loading.

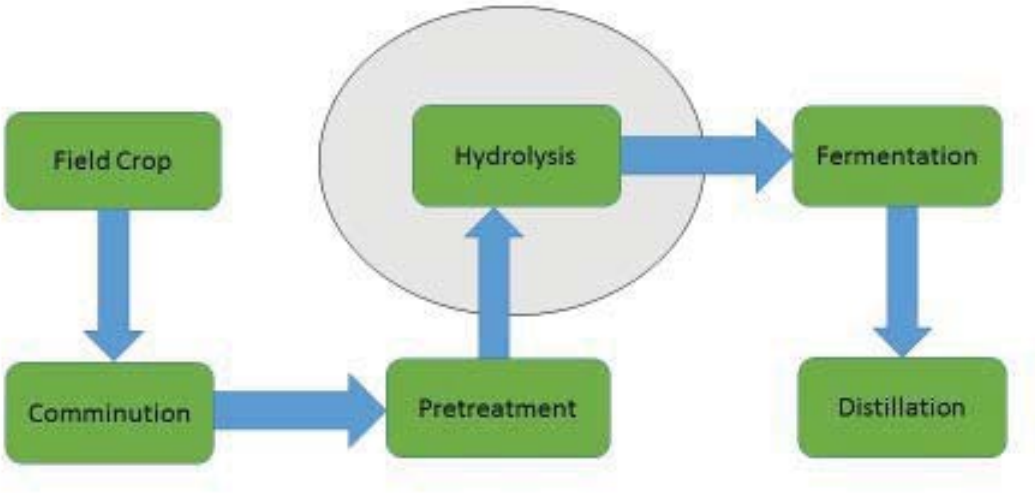


Figure 2. Focus of Objective I and II.
The first objective of this thesis focuses on enzyme production for hydrolysis in the biofuel production process.

SSC of corn stover and wheat bran has not produced glucose concentrations sufficient for efficient fermentation for biofuel production. The second objective determined whether cellulase produced by *T. reesei* RUT-C30 during SSC on corn stover and wheat bran mixtures would efficiently hydrolyze the cultivated residues if temperatures were manipulated to promote cellulase activity (50°C) rather than fungal growth (30°C).

Chapter Two: Materials and Methods

2.1 Objective I

For Objective I, the stages in methodology were collection of biomass from field, comminution, sodium hydroxide pretreatment if applicable, sterilization of biomass, inoculation with *T. reesei* RUT-C30, incubation, centrifugation and liquid extraction, and analysis of enzyme activity and glucose concentration. An illustration of the steps in Objective I is given in Figure 2.

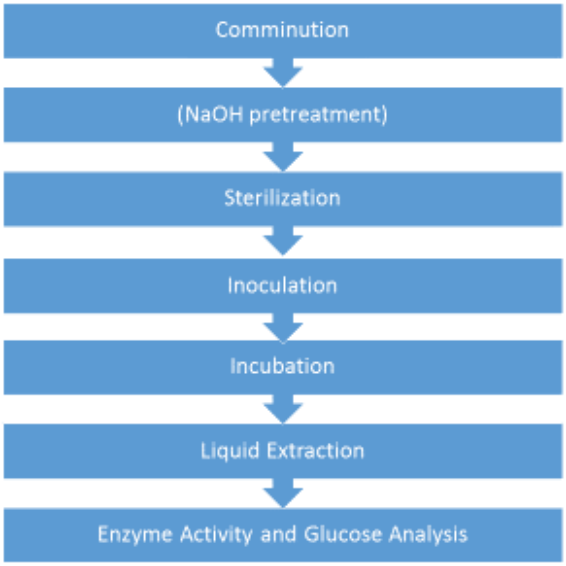


Figure 2. Experimental Methodology Schematic for Objective I.
The schematic illustrates the steps in the experimental process each sample.

2.1.1 Feedstock Specifications

Corn stover was obtained from Woodford County Animal Research Center, Woodford County, KY in September 2010. The corn was planted using conventional tilling practices in April 2010. The biomass was prepared for laboratory use by drying at 45°C for 24 h. Before use, the corn stover was ground so that particles would pass through a 5 mm screen using a hammermill manufactured by C.S. Bell Co. (Tiffin, OH).

The wheat bran used in this experiment was ordered from Honeyville Inc. (Brigham City, UT) in a 50 lb. bag.

2.1.2 Pretreatment of Corn Stover

Four grams of sodium hydroxide was dissolved in 500 mL water. The equivalent of fifty grams bone dry corn stover was added to the solution and stirred. The amount of wet corn stover to add was calculated using an OHAUS MB35 Moisture Analyzer to analyze a 0.5-0.6 g sample (Serial Number: 1128123601; OHAUS Corporation, Parsippany, NJ) using Equation 1. The density of water was assumed to be 1.0 g mL^{-1}. The mixture was stirred every 15 min for 2 h and stored at room temperature in accordance with methodology outlined by Modenbach (2013). Following pretreatment, samples were washed with reverse osmosis (RO) water over a vacuum filter until neutral pH was achieved as determined using pH paper. The wet biomass samples were thoroughly stirred and manually squeezed to remove excess moisture prior to pH testing. The paper was allowed to rest in the samples before interpretation. Samples were air dried to 9-25% solids content (w/w, wet basis). The range was due to variation in drying time and humidity.

2.1.3 Preparation of Biomass Samples

Samples were prepared in 500 mL Erlenmeyer flasks. Samples contained 10, 9, 8, 7, and 6 g bone dry corn stover and were supplemented with 0, 1, 2, 3, and 4 g bone dry wheat bran, respectively (i.e., 10:0, 9:1, 8:2, 7:3, 6:4 corn stover to wheat bran ratios). The moisture content of the corn stover was determined by using an OHAUS MB35 Moisture Analyzer to analyze a 0.5-0.6 g sample and Equation 1. The density of water was assumed to be 1.0 g mL^{-1}. A diagram of the sample types is shown in Figure 3. Samples were either pretreated as described in the previous section or unpretreated. Three samples of each of the five ratios listed was prepared using pretreated and unpretreated corn stover (30 samples total).

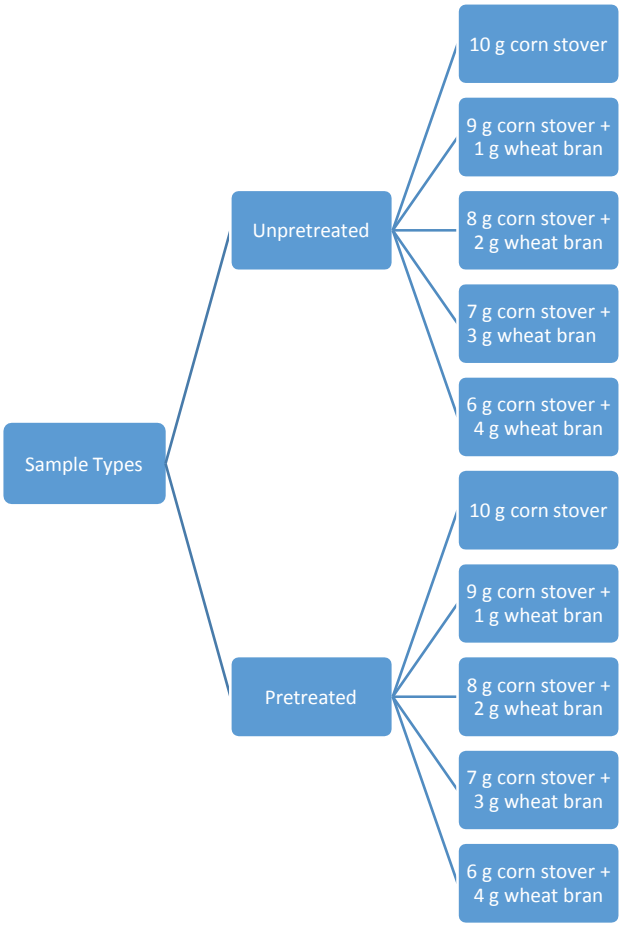


Figure 3. Schematic of Objective I Sample Types.

The samples were mixed thoroughly to achieve a uniform composition. The samples were sterilized by autoclave at 121°C and 15 psi for 90 min and cooled to room temperature. The autoclave used in this experiment was an Amsco® Lab 250 Steam Sterilizer manufactured by STERIS Corporation (Mentor, OH).

Modified Mandel's media was used as a nutrient supplement for biomass samples. The composition of the media is given in Table 1 (Moosavi-Nasab and Majdi-Nasab 2008). Glucose and sugar beet pulp were omitted from the media. The pH of the media was adjusted to 4.8 using 1 N NaOH. The media was sterilized by autoclave at 121^0C and 15 psi for 30 min and cooled to room temperature.

Table 1. Composition of nutrient media used to supplement biomass samples.

	Concentration	Unit
KH_2PO_4	2.0	g L^{-1}
$CaCl_2•2H_2O$	0.4	g L^{-1}
$MgSO_4•7H_2O$	0.3	g L^{-1}
$(NH_4)_2SO_4$	1.4	g L^{-1}
Urea	0.3	g L^{-1}
Peptone	2.0	g L^{-1}
Tween-80	2.0	mL L^{-1}
$FeSO_4•7H_2O$	5.0	mg L^{-1}
$MnSO_4•H_2O$	16	mg L^{-1}
$ZnSO_4•7H_2O$	14	mg L^{-1}
$CoCl_2$	20	mg L^{-1}

Using the measured moisture content of the wheat bran and corn stover, the moisture content of each sample was adjusted to 75% using modified Mandel's media. Moisture losses and gains during autoclaving were accounted for when adjusting the initial moisture content.

2.1.4 Cultivation of *T. reesei*

T. reesei strain RUT-C30 was obtained from ATCC (#56765, Manassas, VA) and stored at -80°C until needed. Potato dextrose agar (PDA) plates were used to prepare inoculum. PDA was prepared by dissolving 4.0 g potato starch,
20.0 g dextrose, and 15.0 g agar per liter of solution in distilled water. The solution was sterilized by autoclaving for 30 min at 121˚C at 15 psi. After cooling
to lukewarm temperature, the solution was transferred to Petri dishes and allowed to gelatinize under an ethanol-sterilized laminar hood. The Petri dishes were inoculated by transferring the thawed cultures from the vial to the center

of the Petri dish using a sterile syringe or by transferring spores from a colonized Petri dish. Petri dish cultures were covered and allowed to grow until fully sporulated, about seven days, at 30°C in a static incubator. Petri dish cultures were stored at -4°C until needed. A picture of a PDA plate sporulated with *T. reesei* RUT-C30 can be found in Appendix B.

Under an ethanol-washed laminar hood, five discs of five millimeter diameter were extracted from a PDA plate colonized with *T. reesei* RUT-C30 using a flame-sterilized cork borer and transferred to each sample. The flasks were covered and the contents gently mixed by rolling the flask before placing in a static incubator at 30°C for seven days. Pictures of samples after seven days can be found in Appendix C.

2.1.5 Sample Extraction

The contents were stored in a static incubator at 30°C for seven days. To extract cellulase, 35-75 mL 0.05 M sodium citrate buffer with 1% Tween-80 was measured and then added. The volume of added buffer was recorded for each sample. The samples were placed in an incubator with shaking at 180 rpm for 1 h at 30°C, then centrifuged at 4,000 rpm for 20 min. The cellulase activity of the supernatant was measured. Samples were stored at -40°C when necessary. The supernatant was used for cellulase activity and glucose analysis.

2.1.6 Cellulase Activity Assay

Cellulase activity was determined using the cellulase activity assay methods established by NREL LAP-006 (Adney and Baker 2008). Cellulase activity was measured in international units per milliliter. An international unit was defined as millimole glucose released per minute (i.e., mmol min^{-1}).

Samples prepared according to the previous section were completely thawed and solutions with 100, 80, 60, 40, and 20% extraction sample were

prepared by mixing with 0.05 M sodium citrate buffer. The recipe for the sodium citrate buffer in this experiment can be found in Appendix A.

Enzyme blanks were prepared by adding 0.5 mL sample solution to 1.0

mL 0.05 M sodium citrate buffer in 15 mL centrifuge tubes. Enzyme unknowns were prepared by adding 0.5 mL sample solution to 1.0 mL 0.05 M sodium citrate buffer in 15 mL centrifuge tubes containing 1.0 x 6.0 Whatmann No. 2 filter paper stripes. These were prepared in triplicate.

Glucose standards were prepared in triplicate by adding 0.310, 0.245, 0.180, 0.115, and 0.050 mL 10 g L^{-1} glucose solution to 1.190, 1.255, 1.320, 1.385, and 1.450 mL 0.05 M sodium citrate buffer in 15 mL centrifuge tubes.

Controls were prepared in triplicate by adding 1.5 mL 0.05 M sodium citrate to 15 mL centrifuge tubes, three of which contained a 1.0 x 6.0 Whatmann No. 2 filter paper strip and three which did not.

Sample solutions, glucose standards, controls, and enzyme blanks and unknowns without enzyme solution were covered and placed in a 50°C water bath. After ten minutes, 0.5 mL of each sample solution was added to three enzyme blank and three enzyme unknown centrifuge tubes. The tubes were covered again and then placed back in the 50°C water bath for exactly 60 min.

After exactly 60 min, 3.0 mL DNS reagent (see Appendix A) was added to all glucose standards, enzyme blanks, enzyme unknowns, and controls. The tubes were placed in a 100°C water bath for 5 min to allow for color change, cooled to room temperature, and then centrifuged for 10 min at 4000 rpm. Samples were then prepared for spectrophotometric reading by diluting 200 µL with 2.500 mL RO water and thoroughly mixing. In some instances, the dilution was adjusted to 100 µL of sample with 2.600 mL RO water due to very high light absorbance by the samples. This difference is noted where appropriate in the data in Appendix D. Light absorbance at 540 nm was then measured using a spectrophotometer. The spectrophotometer used in this experiment was a Spectronic Genesys 2 obtained from Thermo Fisher Scientific.

The average light absorbance for each glucose standard was plotted against the known glucose concentrations in milligrams per half milliliter. A linear function was created. The average absorbance of enzyme blank for each sample solution was calculated and subtracted from each corresponding

enzyme solution. The difference between the absorbance at 540 nm was used to calculate the glucose released by the enzyme. The glucose released was used to calculate the International Units (IU) present in each milliliter of enzyme sample for each concentration. An IU was defined as the amount of enzyme which released one micromole of glucose per minute. In cases where the cellulase activity was calculated to be negative, the value was assumed zero. The raw data is presented in Appendix D. A sample calculation can be found in Appendix E.

2.1.7 Glucose Concentration Measurement

An YSI 2900D Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, Ohio) was used to determine the final glucose concentration. The sensitivity of the instrument was 0.05-25.0 g L^{-1}. The concentration of each sample was adjusted to obtain the glucose concentration of biomass samples before dilution during liquid extraction. For calculation purposes, by weight, corn stover was assumed to be 53% cellulose, 15% hemicellulose, 16% lignin, and 16% other material, consistent with “The U.S. Department of Energy Biofuels Research Program” (Bull 1991).

2.1.8 Statistical Analysis

Statistical analysis was performed using SAS 9.4. Figures were produced using Sigma plot 12.3. An analysis of variance (ANOVA) was used to analyze this experiment. The dependent variables, glucose concentration and cellulase activity, were analyzed as main effects (Montgomery 2013). Pretreatment status of corn stover, amount of wheat bran, and the interaction between pretreatment and wheat bran amount were examined. Tukey’s Range Test was used to determine the significance of differences between means and control experiment-wise error (Montgomery 2013). The relevant SAS code can be found in Appendix G.

2.2 Objective II

Five gram samples were prepared in triplicate which contained wheat bran mixed with pretreated corn stover with 0, 20, and 40% wheat bran. The samples were prepared just as in Objective I with the exceptions of using half of the substrate, three rather than five PDA discs for inoculation, and 250 mL Erlenmeyer flasks instead of 500 mL flasks. After seven days of SSC, flasks were transferred to a 50°C incubator for five days for hydrolysis. Controls were prepared in triplicate exactly as above, but were kept at 30°C during the five day hydrolysis period. Samples were extracted for YSI analysis as in Objective I, using 15-25 mL 0.05 M sodium citrate buffer for extraction. Figure 4 provides a schematic diagram of the experimental set-up.

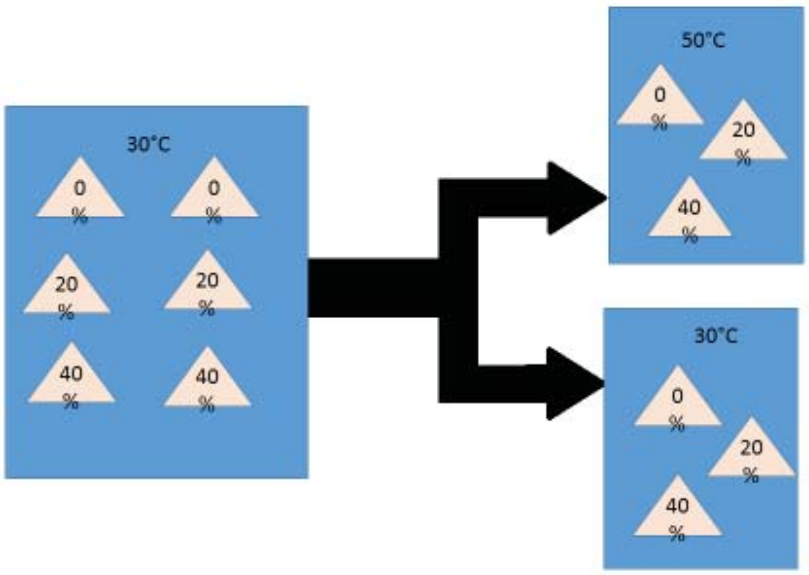


Figure 4. Schematic of Objective II Experiment.
The percentages refer to the percentage of wheat bran in the five gram pretreated corn stover samples.

Statistical analysis was performed using SAS 9.4. Figures were produced using Sigma Plot 12.3. Analysis of variance (ANOVA) was used to analyze the effect of the hydrolysis period on final glucose concentration (Montgomery 2013). Glucose concentration, was analyzed as the main effect. Supplementation level of wheat bran was examined. The results were separately compared to the glucose concentrations produced in the controls

and to mean glucose concentrations in Objective I. Tukey's Range Test was used to determine the significance of differences between means and control Type I experiment-wise error (Montgomery 2013). The relevant SAS code can be found in Appendix G.

Samples with only pretreated corn stover and with pretreated corn stover supplemented with 40% wheat bran were used to collect preliminary cellulase activity data. The DNS method described in Objective I was used (Adney and Baker 2008). Due to the small sample size, only a limited number of cellulase concentrations (1-3) per sample could be analyzed.

Chapter Three: Results and Discussion

3.1 Objective I

The range of mean cellulase activities for samples with unpretreated corn stover ranged from 0.57 to 1.06 IU mL^{-1}, or 1.70 to 3.19 IU gds^{-1}, while the range for samples with pretreated corn stover was 0.00 to 1.41 IU mL^{-1}, or 0.00 to 4.22 IU gds^{-1}. The highest cellulase activity of the unpretreated corn stover samples contained six grams of corn stover and four grams of wheat bran. For samples with pretreated corn stover, the maximum was observed when there were seven grams of corn stover and three grams of wheat bran. A boxplot of the distribution of enzyme activity by pretreatment status can be found in Figure 6. The average cellulase activities and standard deviation of the means for unpretreated and pretreated corn stover samples can be found in Table 2 and Table 3 and the data are illustrated in Figure 6. Detailed results of Objective I can be found in Appendix C.

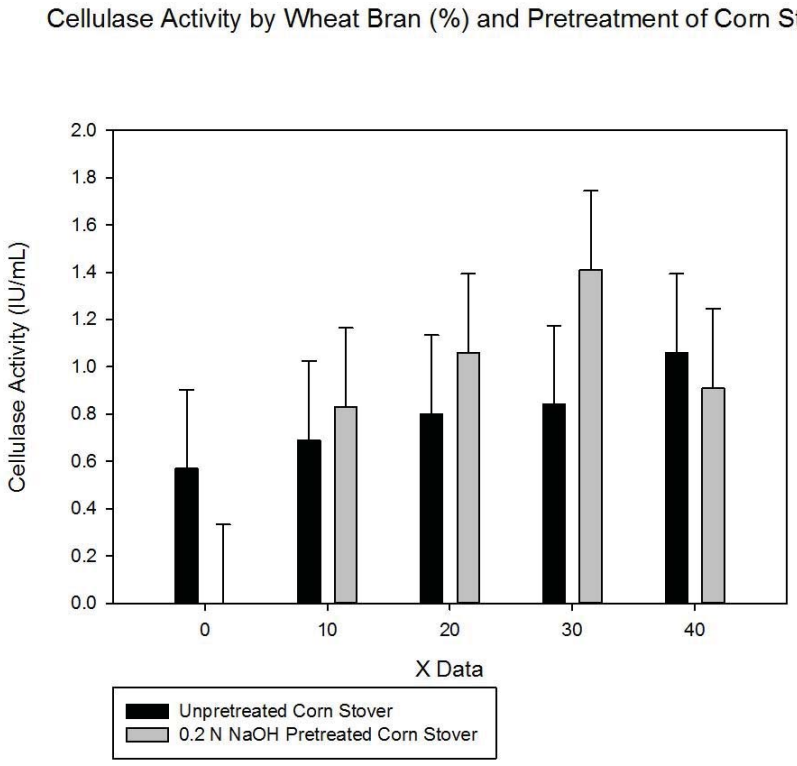


Figure 5. Bar Graph of Objective I Cellulase Activity Data. Error bars represent the root meant square error of the data (0.334).

Table 2. Mean cellulase activity of wheat bran and unpretreated corn stover samples.

Unpretreated corn stover (g)	Wheat bran (g)	Cellulase Activity ± Standard Deviation (IU mL^{-1})	Cellulase Activity ± Standard Deviation (IU gds^{-1})
6	4	1.06±0.30	3.19±0.89
7	3	0.84±0.33	2.51±0.99
8	2	0.80±0.20	2.40±0.60
9	1	0.69±0.26	2.06±0.77
10	0	0.57±0.33	1.70±0.97

Table 3. Mean cellulase activity of wheat bran and 0.2 N NaOH pretreated corn stover mixtures.

Pretreated corn stover (g)	Wheat bran (g)	Cellulase Activity ± Standard Deviation (IU mL^{-1})	Cellulase Activity± Standard Deviation (IU gds^{-1})
6	4	0.91±0.47	2.73±1.40
7	3	1.41±0.46	4.22±1.38
8	2	1.06±0.50	3.19±1.50
9	1	0.83±0.18	2.50±0.54
10	0	0.00±0.00	0.00±0.00

The effect of pretreatment on cellulose activity was not statistically significant ($p>0.05$) as shown in Table 4 and illustrated in Figure 6.

Table 4. Summary of ANOVA of enzyme activity.
Summary of ANOVA for effect of pretreatment ("pt"), wheat bran amount ("wb"), and interaction of pretreatment and wheat bran amount ("pt*wb") on enzyme activity.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
pt	1	0.0208	0.0208	0.19	0.67
wb	4	2.547	0.636	5.69	0.0032
pt*wb	4	1.12	0.279	2.50	0.075

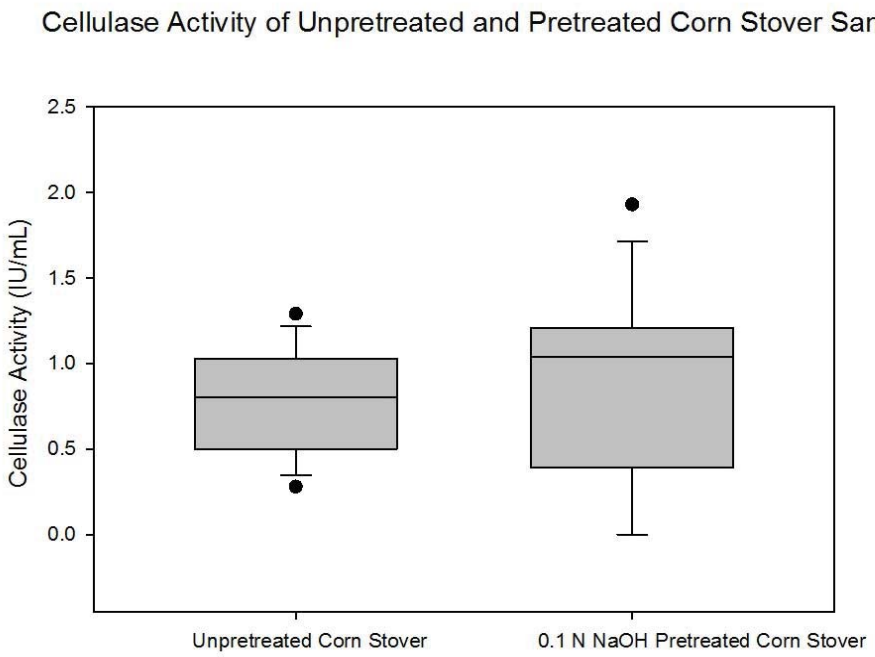


Figure 6. Distribution of enzyme activity by pretreatment.
The box plots illustrate means, upper and lower quartiles, upper, and upper and lower limits by pretreatment status of corn stover.

As illustrated in Table 4, the effect of pretreatment and the interaction between pretreatment and wheat bran supplementation was not significant, while the effect of wheat bran supplementation was statistically significant (p=0.0184). Figure 7 gives a visual representation of the distribution of glucose concentration by wheat bran supplementation level. Looking more closely at the results using Tukey's Range Test, we can conclude that there is a statistically significant difference between the enzyme activities of samples without wheat bran and those supplemented with at least 20% wheat bran (see Table 5). This is consistent with results from previous experiments with *T. reesei* RUT-C30 which saw increased cellulase activity in samples with mixtures of rice straw and other substrates compared to the individual substrates (Dhillon, Oberoi et al. 2011). It is also consistent with previous research which has found supplementing lignocellulosic substrates with wheat bran during hydrolysis was *P. echinulatum* and with a co-culture of *T. reesei* and *A. oryzae* to increase cellulase and other enzyme yields (Camassola and Dillon 2007, Brijwani, Oberoi et al. 2010).

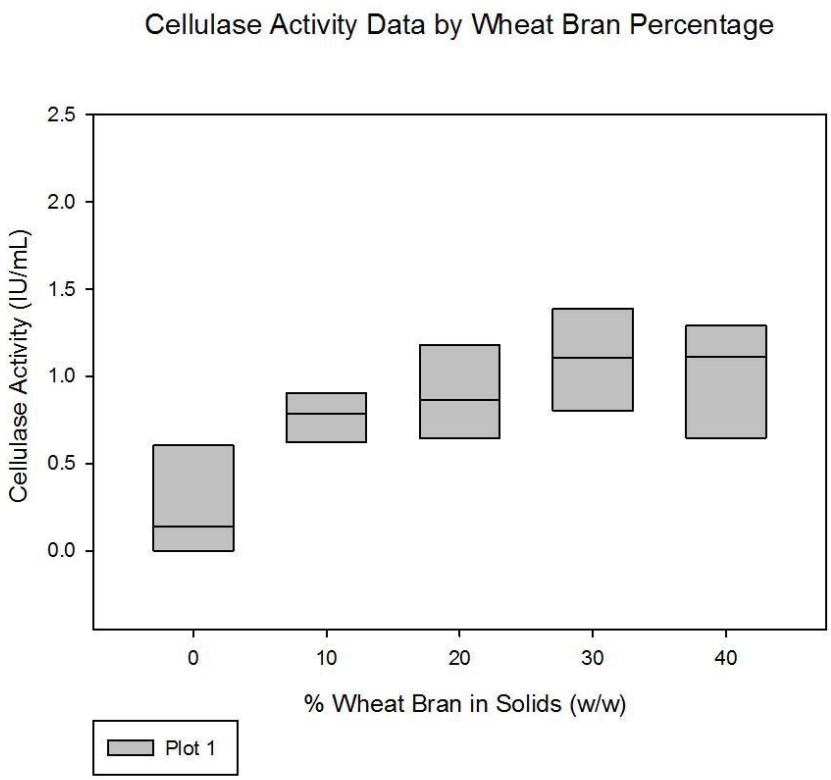


Figure 7. Distribution of enzyme activity by wheat bran amount.
The box plots illustrate means, upper and lower quartiles, and upper and lower limits of enzyme activity.

Table 5. Tukey groupings of samples by wheat bran supplementation level.
The abbreviation "wb" referred to grams of wheat bran in a ten gram sample. The mean referred to average cellulase activity (IU mL^{-1}) for samples with the corresponding amount of wheat bran. "N" referred to sample size.

Means with the same letter are not significantly different.				
Tukey Grouping		**Mean**	**N**	**wb**
	A	1.123	6	3
	A	0.987	6	4
	A	0.932	6	2
B	A	0.760	6	1
B		0.283	6	0

The mean glucose concentrations by wheat bran supplementation level after seven days of samples with unpretreated corn stover ranged from 1.94 to 15.95 g L^{-1}, while the range for pretreated corn stover samples was 2.17 to 17.59

g L^{-1}. In each case, the lowest concentration was observed in samples with 30% wheat bran and the highest in samples without wheat bran. Table 6 and Table 7 give the mean glucose concentration and standard deviation for each wheat bran supplementation level for unpretreated and pretreated corn stover samples, respectively. Figure 8 depicts the final glucose concentration of samples by pretreatment of corn stover and wheat bran supplementation percentage.

Table 6. Average glucose concentrations of unpretreated corn stover samples in Objective I.

Unpretreated corn stover (g)	Wheat bran (g)	Glucose ± Standard Deviation (g L^{-1})
6	4	2.35±0.32
7	3	1.94±0.27
8	2	4.94±1.90
9	1	4.77±1.04
10	0	15.95±3.75

Table 7. Average glucose concentration of 0.2 N NaOH pretreated corn stover samples in Objective I.

Pretreated corn stover (g)	Wheat bran (g)	Glucose ± Standard Deviation (g L^{-1})
6	4	2.87±1.43
7	3	2.17±1.64
8	2	3.30±0.25
9	1	4.01±1.88
10	0	17.59±3.12

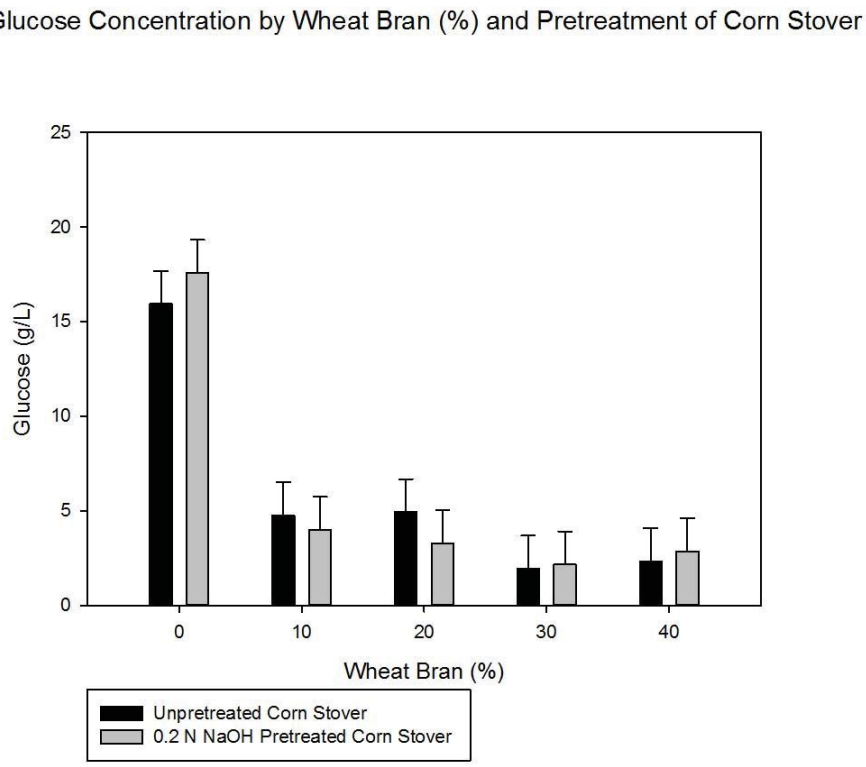


Figure 8. Objective I Final Glucose Concentration. Error bars represent the root mean square error (1.74).

Figure 9 provides an illustration of the distribution of glucose concentration of samples by wheat bran supplementation level. Because glucose is a product of cellulase, it was expected that enzyme activity and glucose concentration would be positively correlated; however, samples with only pretreated corn stover presented no measureable cellulase activity (mean 0.00 IU gds^{-1}) and the highest glucose concentration (mean 15.95 IU mL^{-1}), while samples with the highest mean cellulase activity (1.06 IU mL^{-1}) had only 3.30 g L^{-1} mean glucose.

Figure 9. Distribution of glucose concentration by wheat bran supplementation level.
The box plot illustrates the means, upper and lower quartiles, and upper and lower limits of glucose concentration.

The effect of pretreating corn stover and supplementing corn stover with wheat bran on glucose concentration was statistically significant with $p=0.0472$ and $p<0.0001$, respectively. The effect of the interaction of pretreatment of corn stover and wheat bran supplementation was statistically insignificant (see Table 8). Tukey's Range Test was used to analyze difference between means of samples supplemented with different amounts of wheat bran. As shown in Table 9, there is 95% certainty that samples without wheat bran had a statistically significantly different mean glucose concentration after seven days than all samples which had been supplemented with wheat bran. It is interesting that final samples with only corn stover had the lowest cellulase activity, but the highest glucose concentration.

It is possible that the cellulase activity measured by the DNS method does not reflect the activity of all cellulase enzymes and that the concentration of cellulase measured was too low for measurement. For example, Juhasz et al. found that the filter paper activity after seven days of hydrolysis by *T. reesei* RUT-C30 was 0.52 FPU mg^{-1}, but the endoglucanase activity was 117 FPU mg^{-1}, exoglucanase I activity was 3.8 FPU mg^{-1}, cellobiohydralase I activity was 17.5 FPU mg^{-1}, and β-glucosidase activity was 4.7 FPU mg^{-1} (Juhasz, Szengyel et al. 2005). The exoglucanase I and cellobiohydralase I activities were measured with 4-methylumbelliferyl (MeUmb) β-D-lactosides, while the endoglucanase was measured using hydroxyethylcellulose (HEC) (Bailey, Biely et al. 1992, Juhasz, Szengyel et al. 2005). MeUmb β-D-lactosides are chromophoric glycosides which are not acted on by endoglucanases, but are reliably acted upon by cellobiohydralase I (van Tilbeurgh, Claeyssens et al. 1982). The HEC assay is very similar to the DNS method, but uses HEC instead of filter paper strips. HEC is a soluble cellulose, unlike the crystalline cellulose in filter paper, and is degraded by endoglucanase, but not cellobiohydrase (Bailey 1981)

Leisola and Linko found that the DNS method developed by Ghose and suggested by IUPAC produced low enzyme activity results when the product was cellobiose (Leisola and Linko 1976, Ghose 1987). Because *T. reesei* produces a low β-glucosidase titer, it is expected that the hydrolysate contained high cellobiose and that the results of the DNS assay may not fully reflect the enzyme activity (Gutierrez-Correa and Tengerdy 1997, Dhillon, Oberoi et al. 2011). Furthermore, the presence of cellobiose may have reduced the reducing sugar concentration to below detectable levels in samples with only pretreated corn stover. Ghose acknowledged that the cellulose azure assay developed by Leisola and Linko may have application in addition to the DNS assay (Leisola and Linko 1976, Ghose 1987). Rather than measuring the concentration of reducing sugars as in the DNS assay, the cellulose azure assay measures the amount of solubilized cellulose and does not depend on the relative concentration of cellobiose and glucose (Leisola and Linko 1976).

Table 8. Summary of ANOVA of glucose concentration.
Summary of ANOVA for effect of pretreatment ("pt"), wheat bran supplementation ("wb", in grams), and interaction of pretreatment and wheat bran supplementation ("pt*wb") on glucose concentration.

Source	DF	Anova SS	Mean Square	F Value	Pr > F
pt	1	12.37	12.37	4.54	0.0472
wb	4	1029	257.1	94.31	<.0001
pt*wb	4	22.31	5.578	2.05	0.1308

Table 9. Results of Tukey's grouping for glucose concentration.
The abbreviation "wb" refers to the amount of wheat bran in each sample in the group. The mean refers to the average glucose concentration (g L^{-1}) of samples with the corresponding amount of wheat bran.

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**wb**
A	17.59	6	0
B	4.39	6	1
B	4.12	6	2
B	2.61	6	4
B	2.05	6	3

3.2 Objective II

The mean glucose concentrations of samples stored at 50°C for five days after one week of SSC was 32.7, 14.0, and 10.1 g L^{-1} (196, 84.0, and 60.6 mg gds^{-1}) for samples with 0, 20, and 40% wheat bran (see Figure 9 and Figure 10). The raw data can be found in Appendix F. Compared to 0.88, 0.59, and 3.40 g L^{-1} (7.0, 3.5, and 20.4 mg gds^{-1}) for the control samples (stored at 30°C for five days after one week of SSC), the effect of the temperature change was significant with $p<0.0001$ (Table 11). The effect of changing the percentage of wheat bran in the sample and the interaction between the wheat bran percentage and temperature were also significant with $p<0.0001$ and $p=0.0004$, respectively.

Table 10. Summary of Objective II results.
Corn stover was pretreated with 0.2 N sodium hydroxide for two hours at room temperature.

Pretreated corn stover (g)	Wheat bran (g)	Temperature during days 8-12 (°C)	Mean Glucose Concentration ± Standard Deviation (g L^{-1})
3	2	50	10.1±1.9
4	1	50	14.0±1.2
5	0	50	32.7±7.2
3	2	30	0.88±0.48
4	1	30	0.59±0.31
5	0	30	3.40±2.38

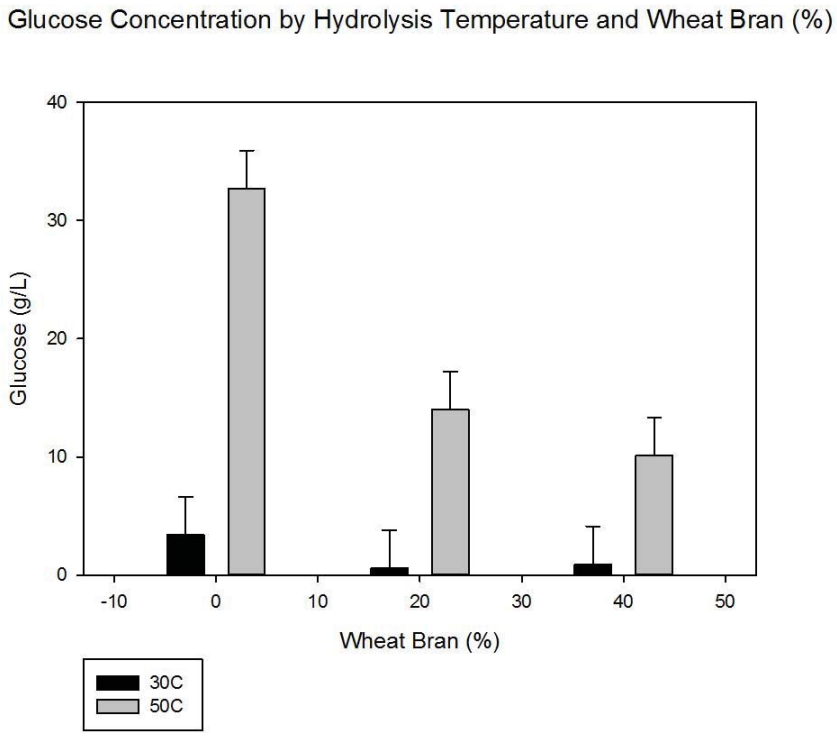


Figure 10. Bar Graph of Final Glucose Concentrations in Objective II. The error bars represent the root mean square (3.22) of the data depicted.

Table 11. Summary of ANOVA of Objective II results.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
temp	1	1353	1353	130.2	<.0001
wb	2	553.8	276.9	26.64	<.0001
temp*wb	2	336.9	168.5	16.21	0.0004

Assuming that reducing sample size did not significantly affect the enzyme load at the end of the initial seven day cultivation period, the initial enzyme loading for corn stover only samples would have been 0 IU mL^{-1}. Therefore, this cannot explain the significantly different glucose concentration (32.7 g L^{-1}) observed in these samples after the five day hydrolysis period. Preliminary data collected after the five day hydrolysis period indicated that the cellulase activity for samples with only pretreated corn stover and with 40% wheat bran was 0 IU mL^{-1}. The complete data can be found in Appendix H. As previously stated, the DNS method may not be the most appropriate cellulase assay and performance of other assays may provide a fuller explanation for the differences in glucose concentration.

When Sukumaran et al. hydrolyzed alkali pretreated water hyacinth, rice straw, and sugar cane bagasse with *T. reesei* RUT-C30 and *A. niger* MTCC 7956 for 40 h with the same enzyme loading and obtained 14.2, 26.3, and 17.8 g L^{-1} glucose, respectively (Sukumaran, Singhania et al. 2009). It is plausible that substrate is a more important predictor of final glucose concentration than enzyme loading.

Tukey groupings were used to control the Type I experiment-wise error (i.e., accepting a false positive across the entire experiment) of the effect of temperature during days 8-12 (Table 12). The mean glucose concentration of all samples stored at 50°C during the second phase was 18.9 g L^{-1}, compared to only 1.62 g L^{-1} for samples stored at 30°C. With at least 95% confidence, there is certainty that the mean glucose concentrations are different. Tukey groupings were used for the same purpose in analyzing the effect of wheat bran percentage in the original sample. With at least 95% confidence, there is certainty that the mean glucose concentration of samples with only corn stover (19.0 g L^{-1}) was

different than the mean glucose concentration of samples with both corn stover and wheat bran (7.31 and 5.50 g L^{-1}) (Table 13). This is in accordance with data collected by Ma and Ruan who found maximum glucose production on homogenized corn stover by *T. reesei* and *Coprinus comatus* with 5% solids occurred at 50°C (82% theoretical) as compared to 40, 45, and 55°C (Ma and Ruan 2015).

Table 12. Tukey Groupings by Temperature for Objective II.

Mean referred to the mean glucose concentration (g L^{-1}) of samples stored at the temperature (°C) indicated in the "temp" column. "N" referred to the number of samples.

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	temp
A	18.96	9	50
B	1.622	9	30

Table 13. Tukey Groupings by Wheat Bran Percent for Objective II.

Mean referred to mean glucose concentration (g L^{-1}) of samples with wheat bran percentage indicated in "wb" column. "N" referred to number of samples.

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**wb**
A	18.07	6	0
B	7.310	6	20
B	5.500	6	40

Other researchers also used 30°C for yeast cultivation and 50°C for hydrolysis to obtain glucose. Rana et al. cultivated *T. reesei* and *Aspergillus saccharolyticus* and isolated the cellulase enzymes in nutrient media before hydrolyzing 5% solids mixture of steam exploded corn stover and loblolly pine for 72 h (Rana, Eckard et al. 2014). Separate fermentation was performed with *Saccharomyces cerevisiae* and 70% theoretical ethanol yield was achieved, indicating efficient hydrolysis of the substrate (Rana, Eckard et al. 2014).

Previous researchers used commercial cellulase (Spezyme CP) to hydrolyze for 2-mm ground corn stover which had been pretreated with 0.1 N NaOH for four hours with stirring every 30 min and obtained 1.35 g L^{-1} glucose or 104 mg gds^{-1} (Li, Ruan et al. 2004). The hydrolysis proceeded for five days and

100 Genencor cellulase units (GCU) g^{-1} was used as the enzyme loading. Glucose yield was lower for samples hydrolyzed with a lower enzyme load (e.g., glucose yield was 20% higher for samples loaded with 100 GCU g^{-1} compared to samples loaded with 20 GCU g^{-1}) (Li, Ruan et al. 2004). Because the enzyme loading in this experiment is difficult to replicate, it is possible that the increased glucose yield (g gds^{-1}) are due to higher enzyme loading than used in this experiment. Furthermore, by replacing the commercial enzyme with in situ SSC of *T. reesei* RUT-C30, overall production costs would be reduced substantially using the methods in this experiment.

The final glucose concentration of samples in Objective I and those stored at 50°C during the second phase in Objective II were compared and is depicted in Figure 11. It was assumed that the different sample sizes did not change the effect of the percentage of wheat bran. With $p<0.0001$, there is statistical certainty that the wheat bran percentage of samples and additional hydrolysis phase affected final glucose concentrations (see Table 14). The interaction between the effects of wheat bran percentage and additional hydrolysis time was not statistically significant.

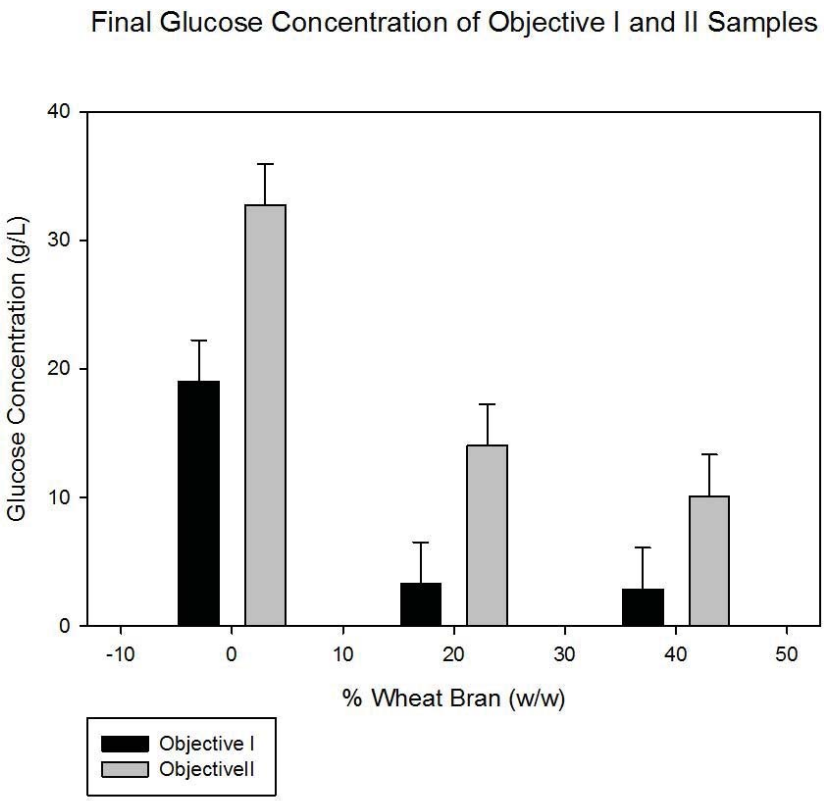


Figure 11. Final Glucose Concentration in Objective I and II.
All samples represented used 0.2 N NaOH pretreated corn stover. Samples in Objective I and II were stored at 30°C for seven days. Objective II samples were subsequently stored at 50°C for five days.

Table 14. Summary of ANOVA for Objective I and Objective II Comparison.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
wb	2	1367	683.8	66.94	<.0001
time	1	496.4	496.4	48.60	<.0001
wb*time	2	29.48	14.74	1.44	0.2744

Tukey groupings were used to prevent declaring false positives when comparing final glucose concentrations from Objective I and II. Table 15 shows that the mean glucose concentration for samples with only corn stover was statistically significantly different than the mean glucose concentrations for samples with 20 and 40% wheat bran with at least 95% certainty. The minimum significant difference was 4.92 g L^{-1}.

Table 16 shows the Tukey groupings for the effect of time elapsed since initial inoculation on glucose concentration. The minimum significant difference in glucose concentration was 3.28 g L^{-1}. With a mean glucose concentration of 8.47 g L^{-1} for samples which did not undergo a second hydrolysis phase and 19.0 g L^{-1} for those that did, there is more than 95% statistical certainty that the effect of the second hydrolysis phase was significant.

Table 15. Tukey Groupings for Wheat Bran Percentage for Objective I and II comparison.
Mean referred to the mean glucose concentration (g L^{-1}) with the corresponding wheat bran percentage indicated in the “wb” column. “N” referred to the number of repetitions included in the calculation.

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**wb**
A	25.98	6	0
B	8.685	6	20
B	6.497	6	40

Table 16. Tukey Groupings for Comparison of Samples by Time.
Mean referred to the mean glucose concentration (g L^{-1}) of samples which had been stored for the number of days indicated in the “time” column. After seven days, temperature was increased. “N” referred to the number of samples included in the calculation.

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**time**
A	18.97	9	12
B	8.470	9	7

In Objective I, all samples with only pretreated corn stover presented 0.00 IU mL^{-1} cellulase activity after seven days. Assuming that there was no change in enzyme activity as a result of decreasing the scale in Objective II, one would not expect a high concentration of glucose in samples which were not supplemented with wheat bran. Despite this, samples with only pretreated corn stover had the highest observed glucose concentration in both Objective I and II. Preliminary data from samples in Objective II with only pretreated corn stover and with pretreated corn stover and 40% wheat bran indicated 0.00 IU mL^{-1}. As discussed in Objective I, the DNS assay may not be the best assay for measuring cellulase

in this experiment due to probable high cellobiose concentration and the cellulase azure assay or the hydoxyethylcellulose assay may be more appropriate due to the necessarily high dilution of samples and likely high cellobiose concentration (Leisola and Linko 1976, Ghose 1987, Bailey, Biely et al. 1992).

Chapter Four: Conclusions

Objective I aimed to increase cellulase activity by supplementing corn stover with wheat bran for enzyme production using *T. reesei* RUT-C30 with SSC with an overall goal to increase glucose production for subsequent fermentation. Adding at least 20% of wheat bran was found to be a viable way to increase cellulase production. However, addition of any wheat bran had a negative impact on glucose yields.

The second objective aimed to increase glucose yields by increasing the temperature of the substrates to 50°C for five days after seven days of cultivation at 30°C. This method was successful with an average glucose concentration above 30 g L^{-1} for samples with only pretreated corn stover that underwent the additional hydrolysis period compared to 18 g L^{-1} for samples with only pretreated corn stover that did not undergo the additional hydrolysis period.

Furthermore, the increase in temperature was indeed necessary as the glucose concentration of samples with only pretreated corn stover stored at 30°C was only 3.48 g L^{-1} Samples with added wheat bran had a significantly lower glucose concentration compared to corn stover only samples. Pretreatment increased glucose yields.

Adding a second hydrolysis period at 50°C after cultivation at 30°C and using only pretreated corn stover is recommended for SSC with *T. reesei* RUT- C30. Having an enzyme production and hydrolysis phase where the temperature is changed from the ideal temperature for the organism to the ideal temperature for the enzyme would be relatively simple for a large-scale, on-farm process.

The high glucose concentration of samples with only pretreated corn stover cannot be explained by the cellulase activity measurement in this experiment. Performance of alternative cellulase activity assays, such as the cellulose azure assay and the HEC assay, may explain the discrepancy (Leisola and Linko 1976, Ghose 1987).

Chapter Five: Future Work

In 2012, the average cost of wheat bran in Kansas City, MO was $173 per ton (Capehart 2016). In contrast, the cost of corn stover was estimated at only $55 per ton (Qureshi, Saha et al. 2010). Because of this difference in cost, it is therefore, recommended to use as little wheat bran as is required to sufficiently increase cellulase yield if cellulase is the targeted end product. Reduction in competition between food and fuel is another reason to reduce wheat bran input relative to corn stover (Brown and Brown 2012). This study found that when corn stover was supplemented with 20, 30, or 40% wheat bran, the cellulase activity of *T. reesei* RUT-C30 was significantly higher than if there was no wheat bran supplementation. There was no statistical difference between samples with 20, 30, or 40% wheat bran. Therefore, repeated experiments could further elucidate the optimal wheat bran supplementation level. Increasing the amount of wheat bran could prove worthwhile if cost savings on commercial enzymes are significant.

During the course of this experiment, it was noticed that the amount of fungus visually observed varied between replicates and this likely affected the cellulase production. While the seven day incubation period was sufficient for proliferation of *T. reesei* in most cases; in unreported work, some samples appeared to overcome a lag phase after the seven day incubation period. Future work might prolong the hydrolysis period to obtain more consistent results considering that some researchers have reported observing a plateau in cellulase production (Deswal, Khasa et al. 2011). The optimal incubation time may vary depending on the ratio of corn stover to wheat bran given previous experiments which found different optimal incubation times for different substrates (Singhania, Sukumaran et al. 2006). Increasing sample size is likely to affect proliferation of fungus as well and increasing the scale of this study could produce different and interesting results.

The effect of sterilizing the corn stover and wheat bran by autoclave was unknown. Differences in mass before and after use of the autoclave were

recorded and assumed to be due to changes in water content. However, whether the mass increased or decreased varied and it remains unknown whether structural changes occurred during the high pressure and high heat process. Past work has shown that pretreatment by autoclave of wheat straw and sugarcane bagasse is effective for anaerobic digestion. However, the effect was significantly different on wheat straw and sugarcane bagasse, so a similar study on corn stover and wheat straw is recommended. (Bolado-Rodríguez, Toquero et al. 2016).

Further experimentation with having separate enzyme production and hydrolysis phases during SSC is recommended. The relatively simple method of changing the storage temperature has promise to dramatically increase in situ glucose production and to simplify subsequent biofuel production. Previous experiments have indicated that *T. reesei* produces little β-glucosidase, an enzyme which breaks down cellobiose into its glucose dimers (Dhillon, Oberoi et al. 2011). *Aspergillus niger* produces cellulase with high β-glucosidase activity which has been shown to release up to 92% of glucose from wheat bran at 10% solids loading and with five 7 mm diameter discs of *A. niger* colonies from PDA plates (Bansal, Tewari et al. 2011). Therefore, future work using separate enzyme production and hydrolysis phases with *T. reesei* and *A. niger* are recommended as a way to increase cellulase efficiency.

Ghose has established an additional assay to measure endoglucanase activity with HEC in conjunction with the cellulase activity assay (Ghose 1987). Further research into the endoglucanase activity of samples produced using the methods in this experiment is recommended as a way to explain the high glucose concentration in samples containing only corn stover which had a low apparent cellulase activity.

Appendices

Appendix A. Reagent Recipes.

One molar sodium citrate buffer was prepared by mixing the following:

Ingredient	Quantity
RO Water	750 mL
Sodium Citrate Monohydrate	210 g
Sodium Hydroxide	50-60 g, as needed to obtain pH 4.3

The buffer was diluted to 0.05 M and the pH adjusted to pH 4.8 for use in Objective I. In Objective II, the solution was diluted to 0.1 M and the pH adjusted to 4.8

Preparation of DNS reagent was prepared using multiple steps. First, the following ingredients were mixed:

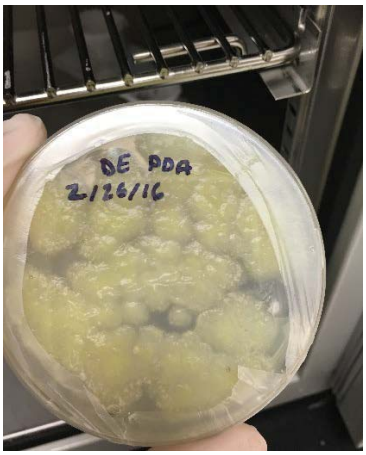
Ingredient	Quantity
3,5 dinitrosalicylic acid	10.6 g
Sodium Hydroxide	19.8 g
RO water	1416 mL

After the above was dissolved completely, the following was added:

Rochelle salts (sodium potassium tartrate)	306 g
Phenol	7.6 mL
Sodium metabisulfite	8.3 g

The pH was checked by adding 0.1 N HCL to a three milliliter sample of the reagent until the pH of the phenolphthalein endpoint was reached. If more than 5- 6 mL of hydrochloric acid was require, sodium hydroxide was added in accordance with the IUPAC protocol (Adney and Baker 2008).

Appendix B. Photographs of Fungal Cultures



PDA plate with sporulated *T. reesei* RUT-C30.

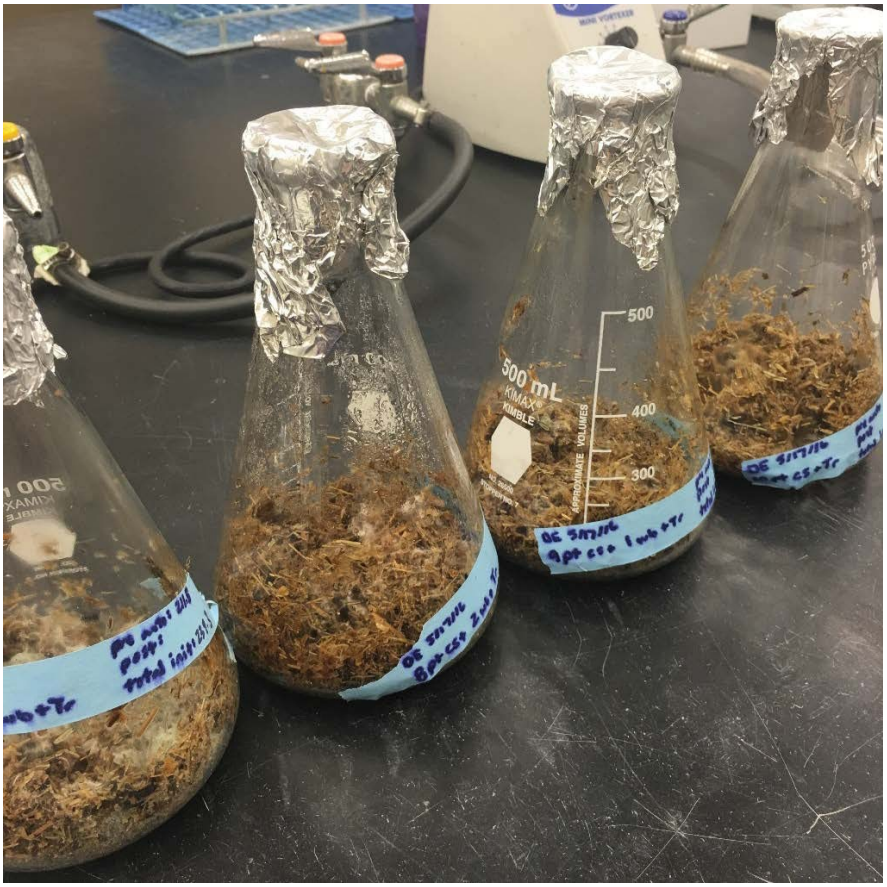
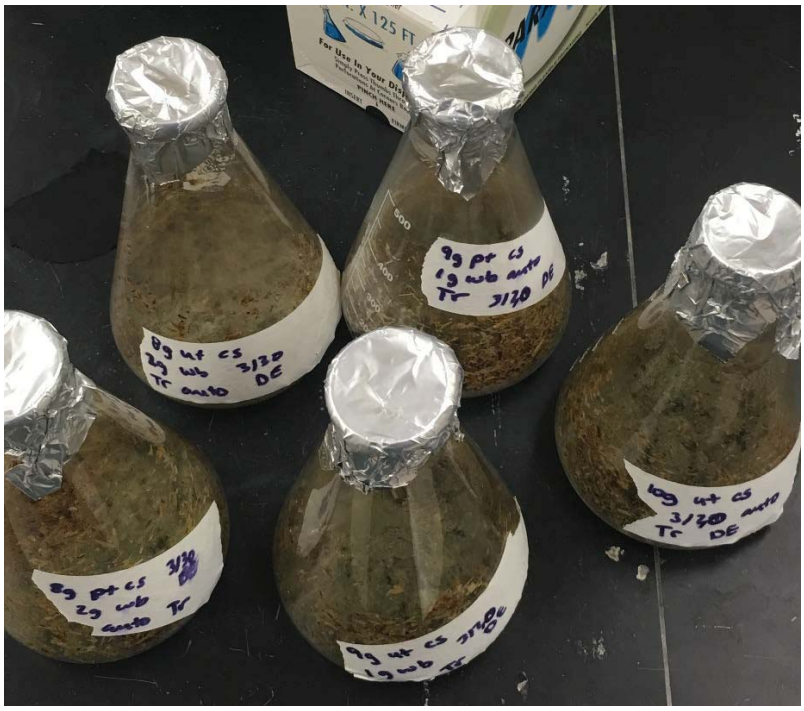
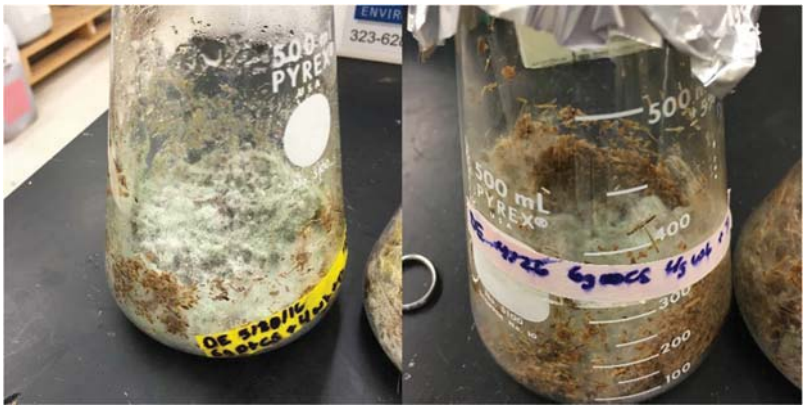


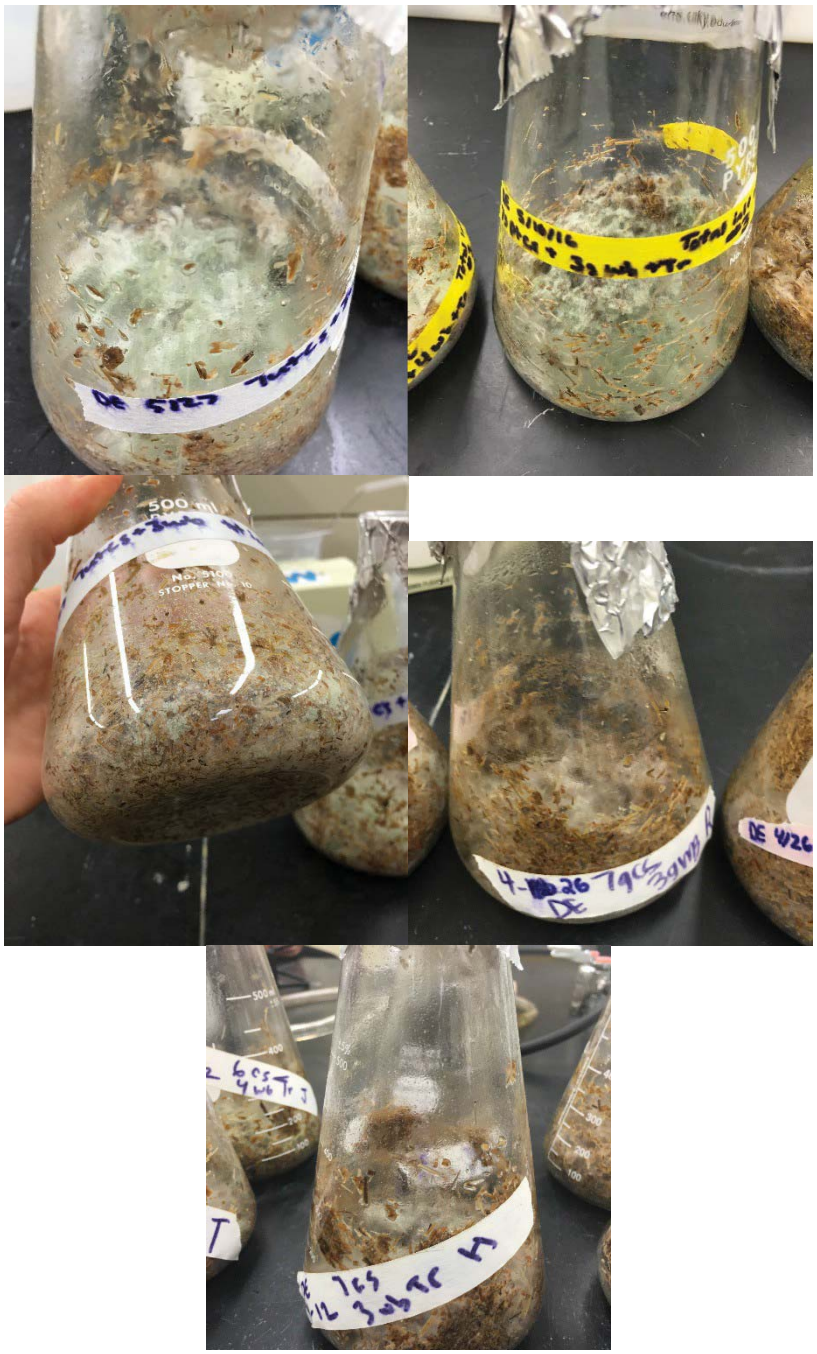
Photo line-up of samples with 7, 8, 9, and 10 g pretreated corn stover and 3, 2, 1, and 0 g of wheat bran (left to right) after seven days of incubation. Notice the apparent difference in colonization by *T. reesei* RUT-C30 between the far left sample and the far right.



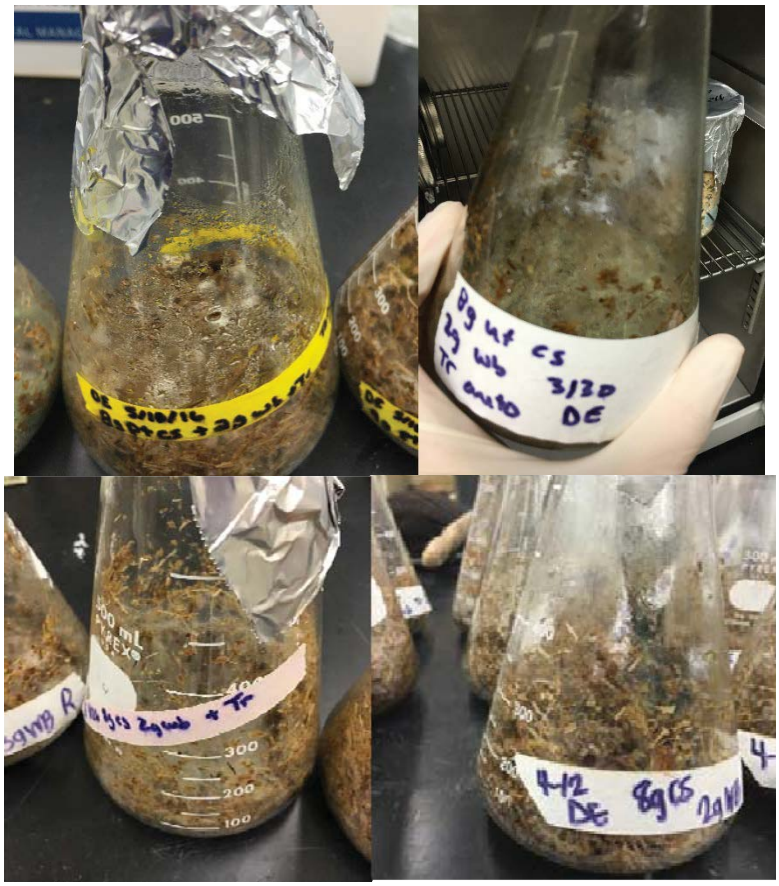
Picture of an array of unpretreated and pretreated corn stover samples after seven days of growth. Counterclockwise from top left, the samples contain eight grams unpretreated corn stover with two grams wheat bran; nine grams pretreated corn stover with one gram of wheat bran; ten grams of unpretreated corn stover; nine grams of unpretreated corn stover with one gram of wheat bran; and eight grams of pretreated corn stover with two grams of wheat bran.



Pictures of samples with six grams of corn stover and four grams of wheat bran. The sample on the left used pretreated corn stover, while the sample on the right contained unpretreated corn stover. Notice the increased growth of the pretreated corn stover sample.

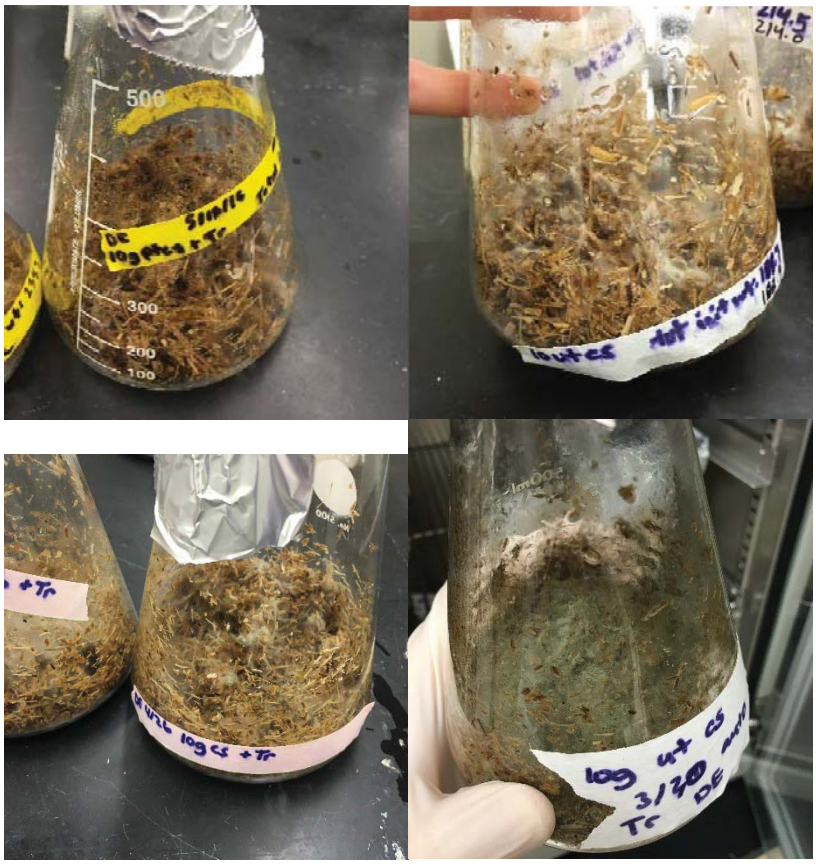


Pictures of samples with seven grams of pretreated corn stover and three grams of wheat bran after seven days of incubation. The left and middle picture on the top row contained pretreated corn stover while the others did not. Notice the increased fungal growth of the pretreated corn stover samples.



Pictures of samples with eight grams of corn stover and two grams of wheat bran after seven days. The top left photo contains pretreated corn stover, while the others do not.

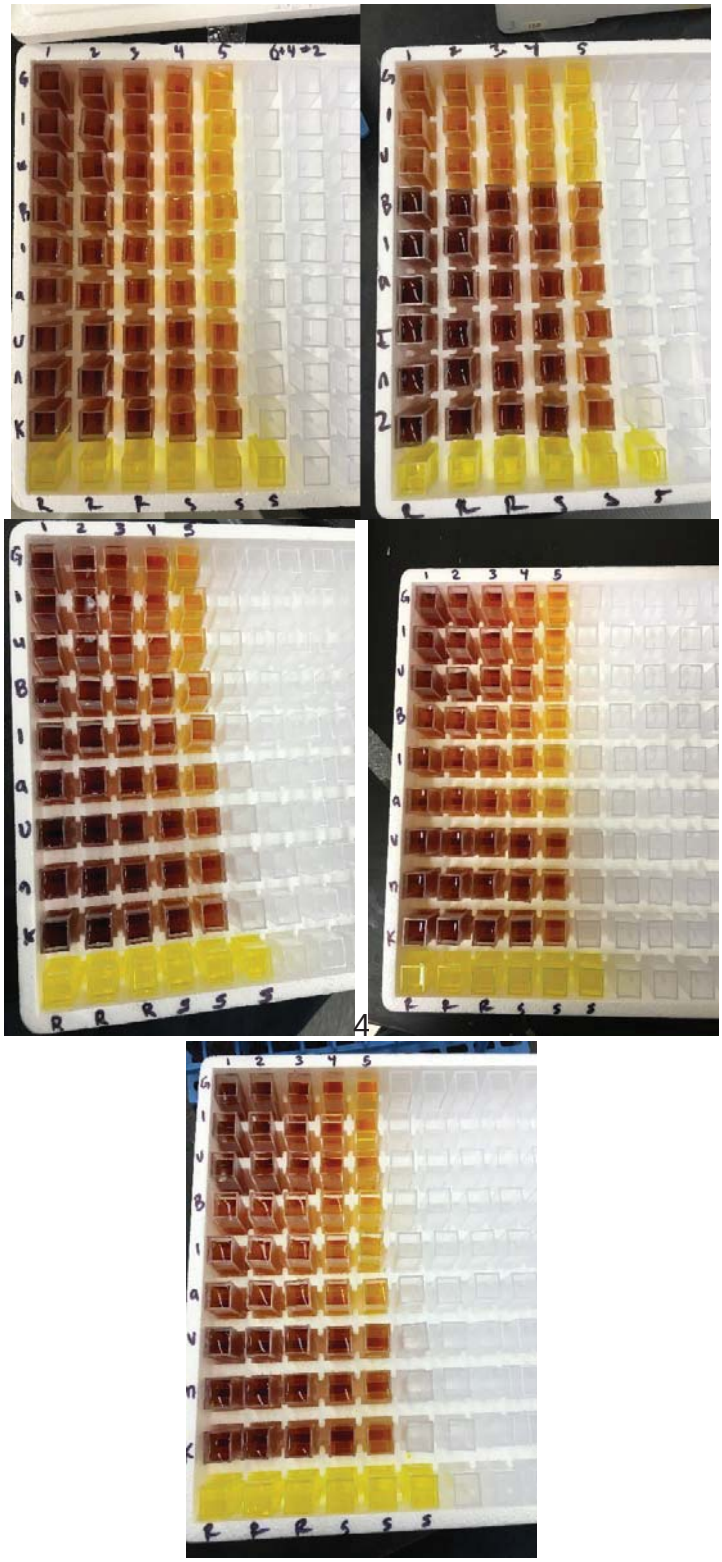
Pictures of samples with nine grams of corn stover and one gram of wheat bran after seven days. The sample on the top left was pretreated while the others were not.



Pictures of samples with ten grams of after seven days. The top left sample was pretreated while the others were not.

Picture of samples after SSC and hydrolysis in Objective II. From samples on the left side of the picture were stored at 50°C for five days after seven days of storage at 30°C. From left to right, the samples contain three grams of corn stover and two grams of wheat bran; four grams of corn stover and one gram of wheat bran; and five grams of corn stover. Samples on the right side of the picture were stored at 30°C for 12 d and from left to right contain three grams of corn stover and two grams of wheat bran; four grams of corn stover and one gram of wheat bran; and five grams of corn stover. All corn stover was pretreated with 0.1 N sodium hydroxide for two hours at room temperature.

Appendix C. Photographs of Cellulase Activity Assays



Representative photos of samples for spectrophotometric assessment. In each case, the top three rows are the same and decrease in glucose concentration from left to right (3.10, 2.25, 1.80, 1.15, and 0.050 mg/0.5mL). The next three rows are also identical and contain the enzyme blanks with decreasing concentration from left to right (100%, 80%, 60%, 40%, and 20%). The next three rows are identical and contain the enzyme unknown samples with decreasing concentration from left to right (100%, 80%, 60%, 40%, and 20%). The final row contains three reagent blank samples on the left and three substrate blank samples on the right.

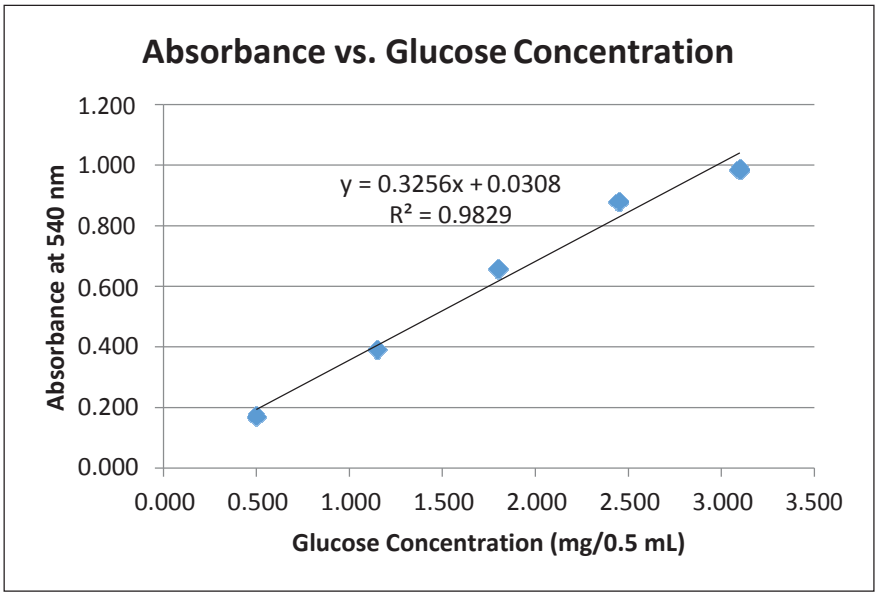
Appendix D. Objective I Cellulase Activity Assay Data Summary.

	Pretreatment	Wheat Bran (g)	Enzyme Activity (IU/mL)	Glucose (g/mL)
1	Y	4.0	0.39	1.89
2	Y	4.0	1.05	4.51
3	Y	4.0	1.29	2.22
1	N	4.0	0.73	2.52
2	N	4.0	1.17	2.54
3	N	4.0	1.29	1.98
1	Y	3.0	0.79	2.56
2	Y	3.0	1.08	4.03
3	Y	3.0	1.93	0.93
1	N	3.0	0.91	2.08
2	N	3.0	0.48	1.63
3	N	3.0	1.13	2.11
1	Y	2.0	0.57	3.53
2	Y	2.0	1.57	3.53
1	N	2.0	0.70	6.32
2	N	2.0	1.03	5.98
3	N	2.0	0.67	2.82
2	Y	1.0	1.04	5.40
3	Y	1.0	0.70	5.55
1	N	1.0	0.39	3.69
2	N	1.0	0.80	5.76
3	N	1.0	0.88	4.86
1	Y	0	0.00	3.75
2	Y	0	0.00	20.08
3	Y	0	0.00	19.08
1	N	0	0.50	18.30
2	N	0	0.28	16.89
3	N	0	0.92	11.82

Six grams pretreated corn stover with four grams wheat bran #1

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams pretreated corn stover with four grams wheat bran for seven days. Sample #1. Diluted with 75 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration						
Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.998	0.983	0.977	0.986	0.011
2	2.450	0.911	0.87	0.855	0.879	0.029
3	1.800	0.742	0.601	0.629	0.657	0.075
4	1.150	0.352	0.432	0.39	0.391	0.040
5	0.500	0.167	0.163	0.184	0.171	0.011

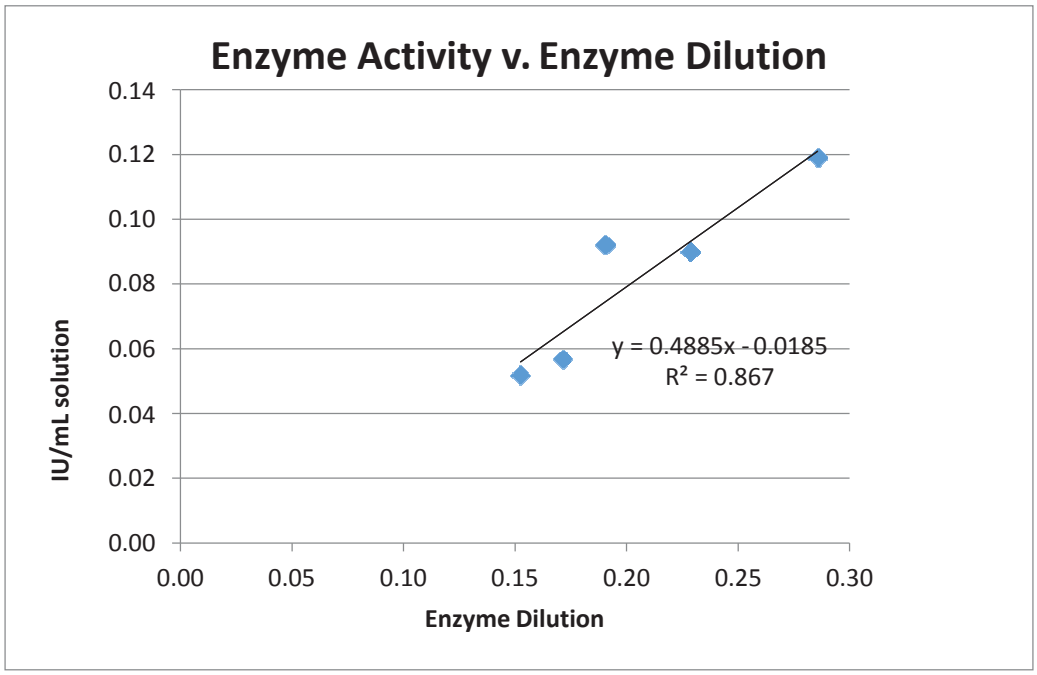


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.29	0.368	0.582	0.615	0.627	0.563	0.664	0.701
0.23	0.321	0.459	0.516	0.556	0.328	0.503	0.626
0.19	0.213	0.334	0.418	0.465	0.277	0.535	0.679
0.17	0.128	0.280	0.253	0.242	0.373	0.290	0.257
0.15	0.039	0.165	0.157	0.162	0.291	0.267	0.282

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.29	0.64	0.12	2.25	0.42
0.23	0.49	0.09	2.13	0.39
0.19	0.50	0.09	2.61	0.48
0.17	0.31	0.06	1.79	0.33
0.15	0.28	0.05	1.84	0.34
		Average:	2.12	0.39

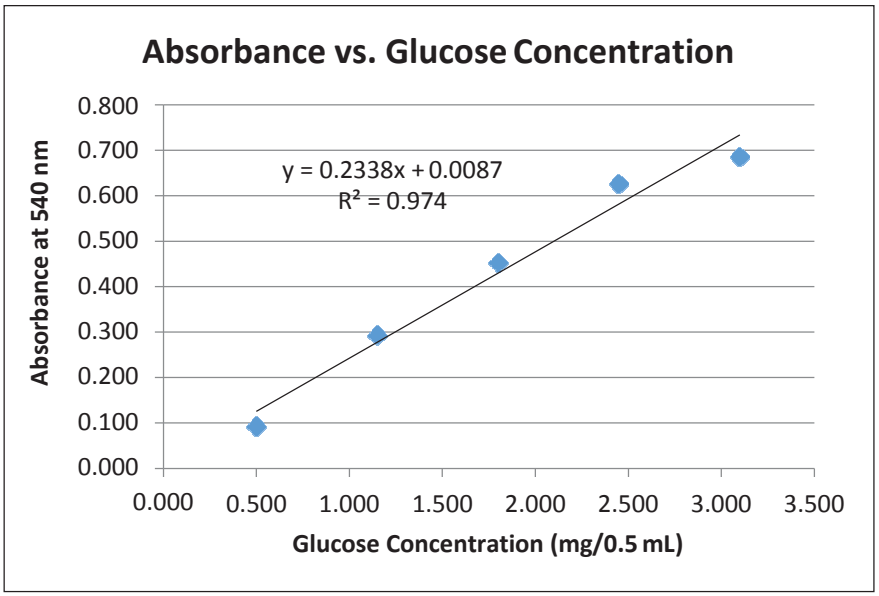


Six grams pretreated corn stover with four grams wheat bran #2

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams pretreated corn stover with four grams wheat bran for seven days. Sample #2. Diluted with 50 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

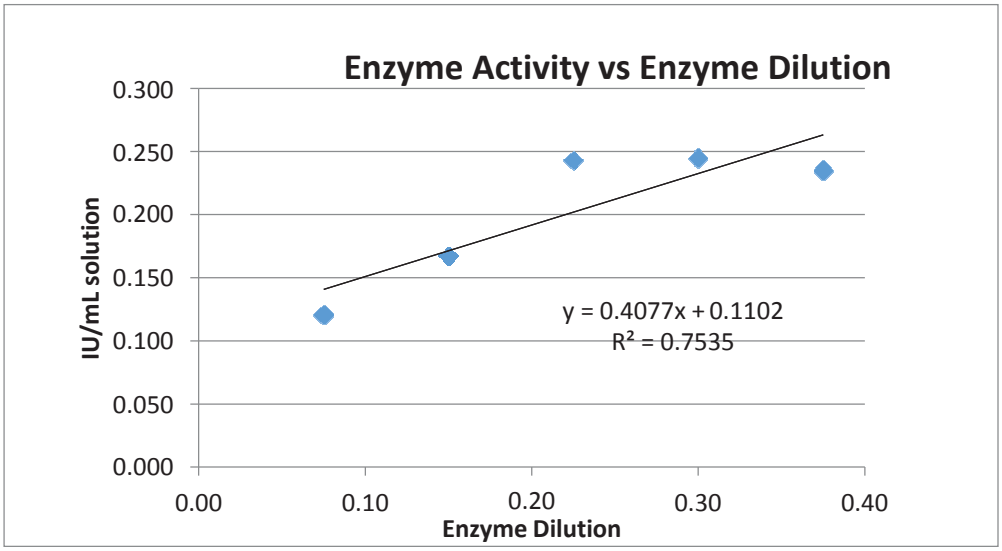
Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.701	0.676	0.679	0.685	0.014
2	2.450	0.624	0.643	0.611	0.626	0.016
3	1.800	0.45	0.447	0.459	0.452	0.006
4	1.150	0.275	0.318	0.285	0.293	0.023
5	0.500	0.104	0.08	0.112	0.092	0.017



Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

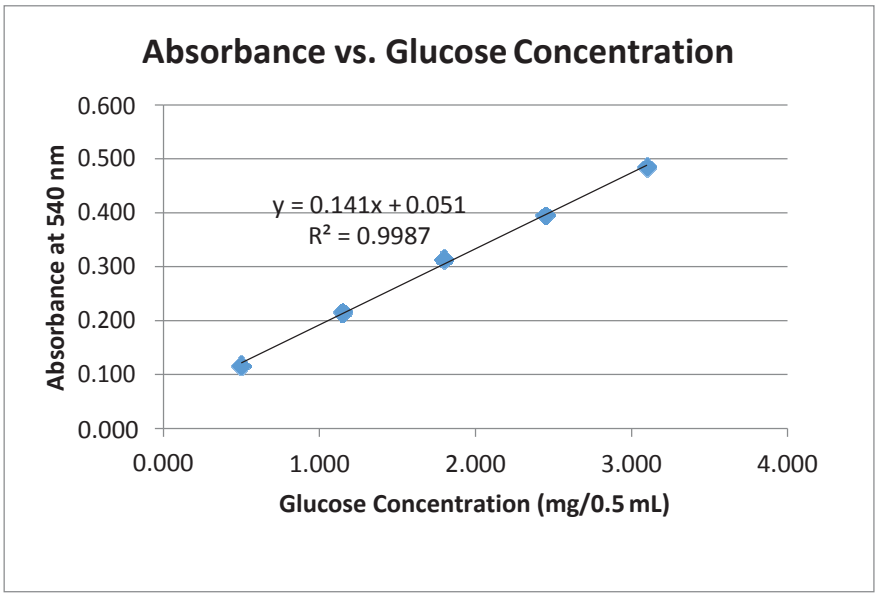
		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.38	0.582	0.826	0.909	0.925	1.008	1.363	1.431
0.30	0.511	0.800	1.007	0.679	1.197	2.083	0.680
0.23	0.375	0.796	0.570	0.704	1.765	0.798	1.371
0.15	0.257	0.484	0.464	0.483	0.934	0.848	0.929
0.075	0.120	0.223	0.316	0.304	0.402	0.800	0.748

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.				
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.38	1.27	0.63	1.27	0.63
0.30	1.65	0.81	1.65	0.81
0.23	2.19	1.08	2.19	1.08
0.15	2.26	1.12	2.26	1.12
0.08	3.25	1.60	3.25	1.60
		Average:	2.12	1.06



Six grams pretreated corn stover with four grams wheat bran #3
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams pretreated corn stover with four grams wheat bran for seven days. Sample #3. Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration						
Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Stud Dev
1	3.100	0.478	0.51	0.466	0.485	0.023
2	2.450	0.397	0.393	-------	0.395	0.003
3	1.800	0.32	0.303	0.316	0.313	0.009
4	1.150	0.209	0.224	0.212	0.215	0.008
5	0.500	0.124	0.112	0.113	0.116	0.007

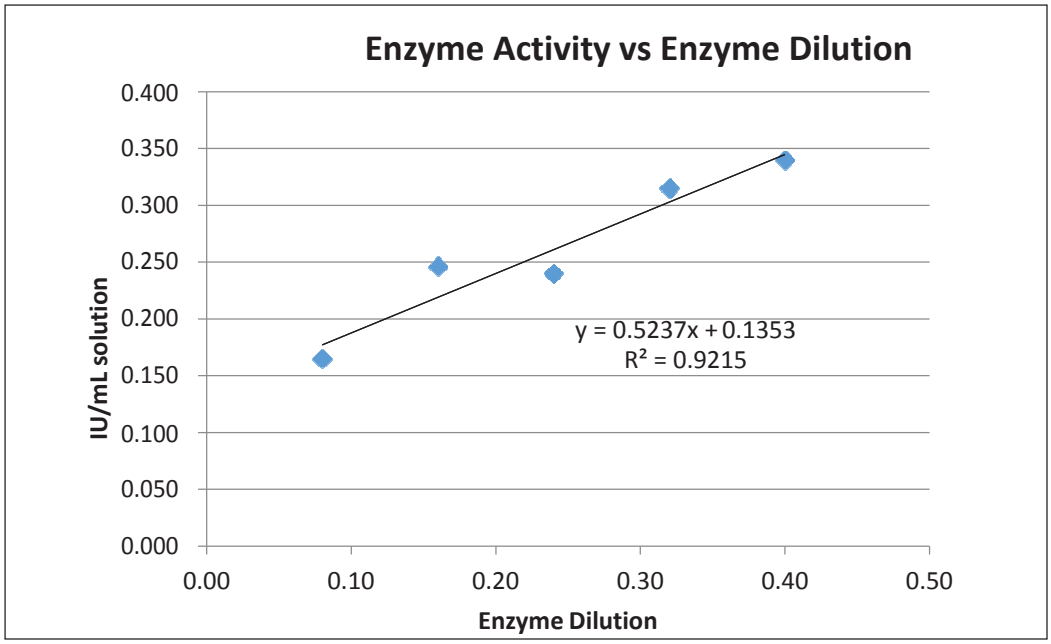


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	0.184	0.485	0.531	0.464	1.775	2.102	1.626
0.32	0.153	0.449	0.428	0.453	1.740	1.591	1.768
0.24	0.122	0.371	0.341	0.356	1.402	1.189	1.296
0.16	0.094	0.342	0.332	0.324	1.395	1.324	1.267
0.08	0.062	0.214	0.256	0.245	0.716	1.014	0.936

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

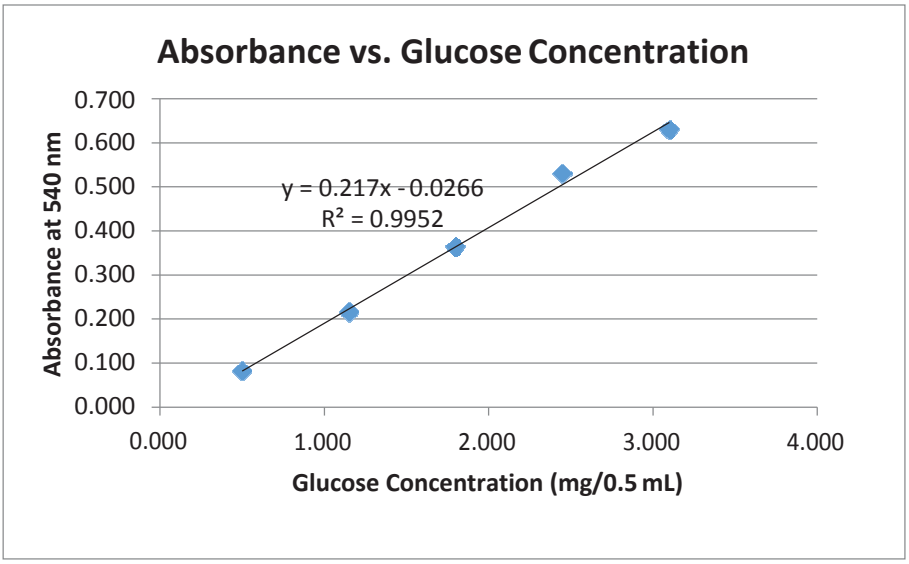
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.40	1.835	0.85	4.59	0.85
0.32	1.700	0.98	5.31	0.98
0.24	1.296	1.00	5.40	1.00
0.16	1.329	1.54	8.30	1.54
0.08	0.889	2.06	11.11	2.06
		Average:	6.94	1.29



Six grams unpretreated corn stover with four grams wheat bran #1
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams unpretreated corn stover with four grams wheat bran for seven days. Sample #1. Diluted with 44 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.632	0.64	0.617	0.630	0.012
2	2.450	0.516	0.518	0.556	0.530	0.023
3	1.800	0.363	0.365	0.362	0.363	0.002
4	1.150	0.207	0.207	0.232	0.215	0.014
5	0.500	0.082	0.079	0.084	0.082	0.003

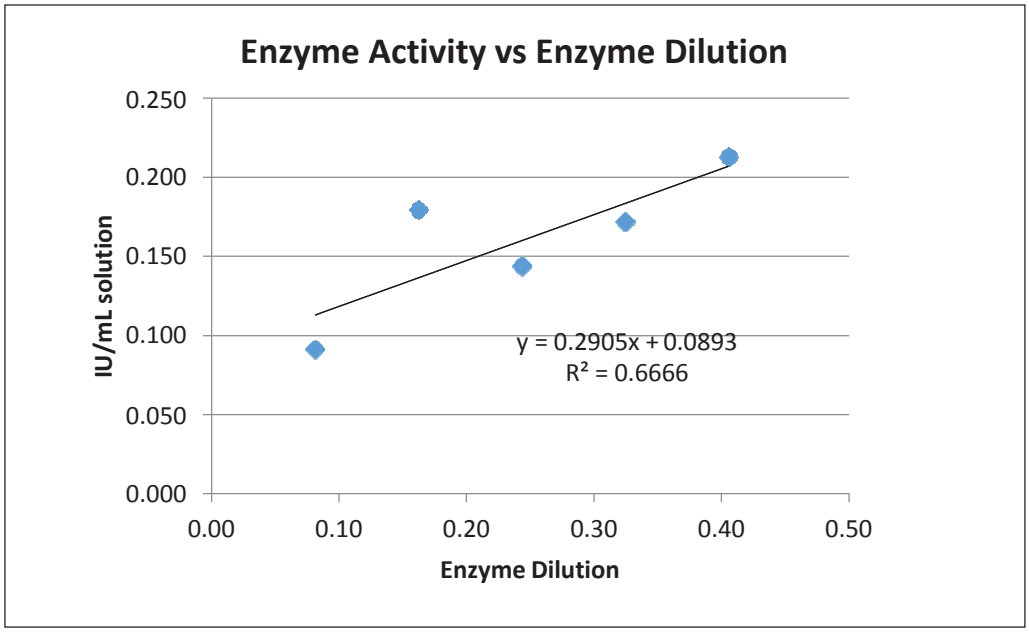


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.41	0.313	0.535	0.55	0.524	1.144	1.213	1.093
0.32	0.283	0.427	0.489	0.457	0.786	1.072	0.924
0.24	0.199	0.397	0.307	0.32	1.033	0.619	0.679
0.16	0.118	0.293	0.302	0.312	0.927	0.969	1.015
0.08	0.058	0.16	0.137	0.117	0.594	0.488	0.396

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

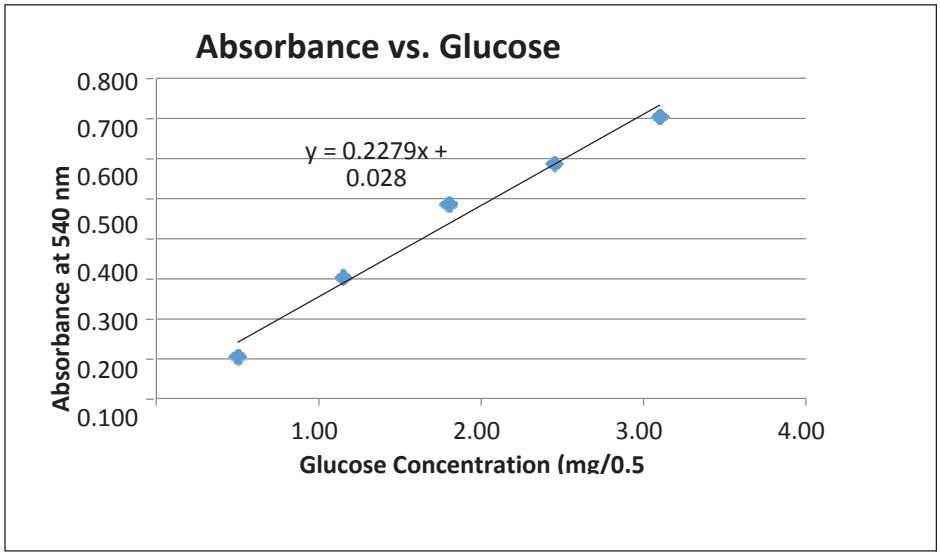
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.41	1.150	0.213	2.84	0.53
0.32	0.927	0.172	2.86	0.53
0.24	0.777	0.144	3.19	0.59
0.16	0.971	0.180	5.98	1.11
0.08	0.493	0.091	6.08	1.13
		Average:	4.19	0.78



Six grams unpretreated corn stover with four grams wheat bran #2
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams unpretreated corn stover with four grams wheat bran for seven days. Sample #2. Diluted with 44 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.682	0.706	0.728	0.705	0.023
2	2.450	0.537	0.581	0.646	0.588	0.055
3	1.800	0.52	0.471	0.471	0.487	0.028
4	1.150	0.296	0.324	0.295	0.305	0.016
5	0.500	0.109	0.092	0.117	0.106	0.013

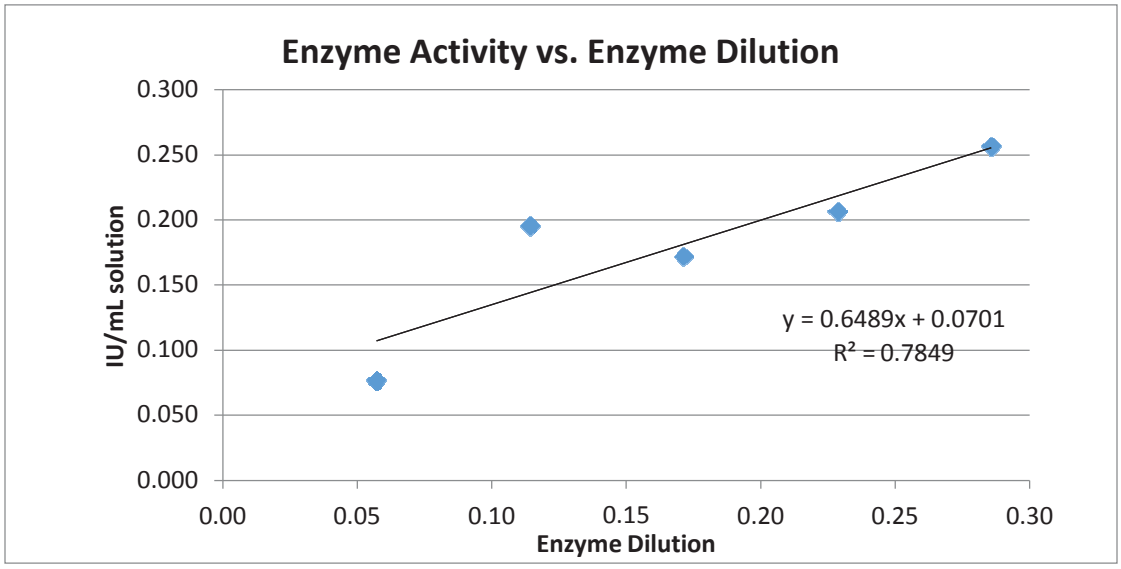


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.29	0.168	0.496	0.52	0.518	1.318	1.423	1.414
0.23	0.130	0.437	0.419	0.381	1.223	1.144	0.977
0.17	0.099	0.337	0.369	0.309	0.923	1.063	0.800
0.11	0.053	0.315	0.321	0.329	1.025	1.052	1.087
0.06	0.021	0.141	0.145	0.144	0.404	0.421	0.417

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

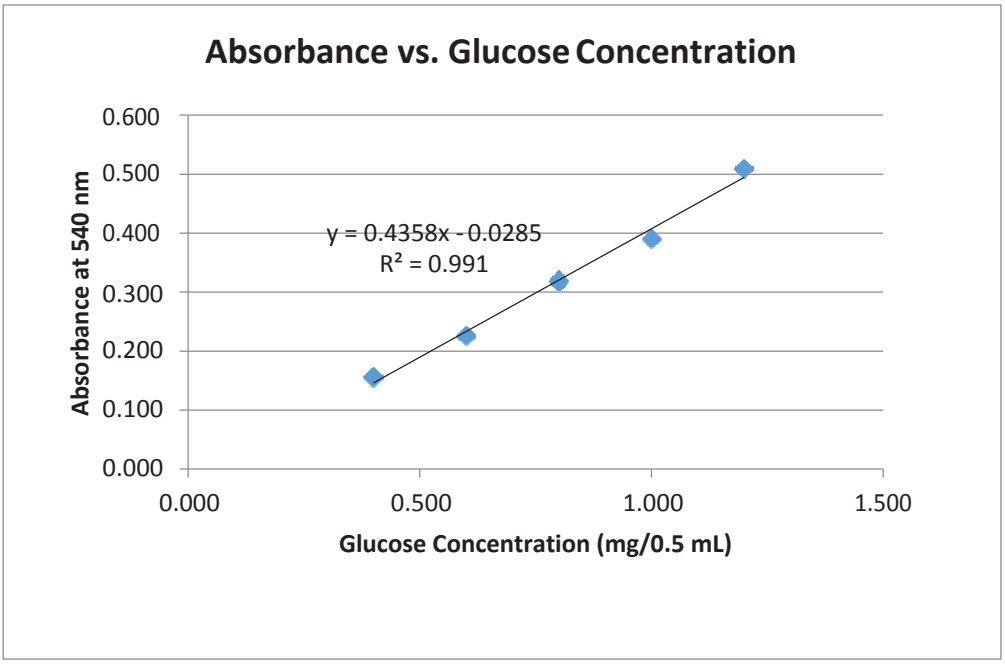
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.29	1.385	0.257	4.85	0.90
0.23	1.115	0.206	4.88	0.90
0.17	0.929	0.172	5.42	1.00
0.11	1.055	0.195	9.23	1.71
0.06	0.414	0.077	7.24	1.34
		Average:	6.32	1.17



Six grams unpretreated corn stover with four grams wheat bran #3
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams unpretreated corn stover with four grams wheat bran for seven days. Sample #3. Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	1.200	0.511	0.549	0.469	0.510	0.040
2	1.000	0.378	0.431	0.361	0.390	0.037
3	0.800	0.32	0.323	0.315	0.319	0.004
4	0.600	0.223	0.243	0.211	0.226	0.016
5	0.400	0.147	0.152	0.169	0.156	0.012

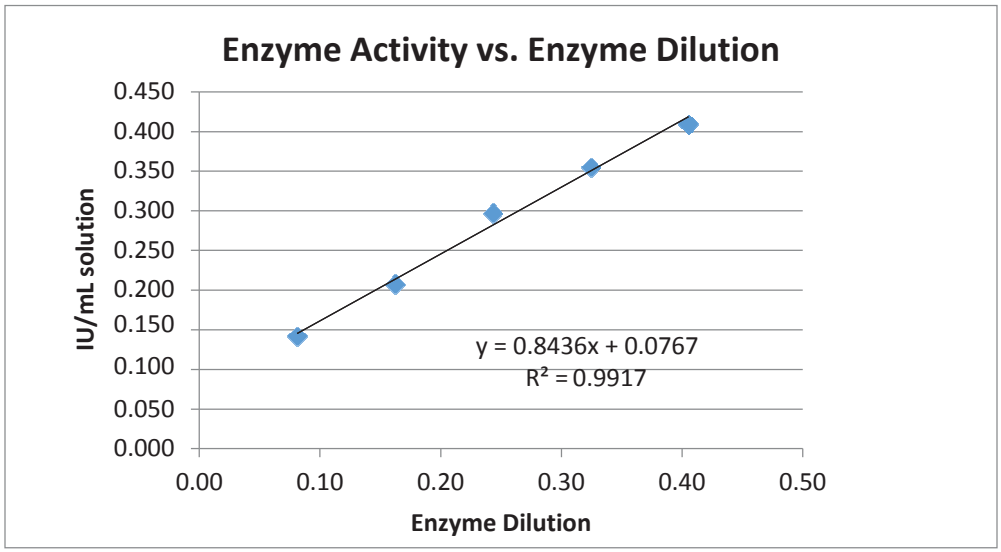


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.41	0.257	0.785	1.282	1.102	1.276	2.417	2.004
0.32	0.211	1.041	1.167	0.847	1.970	2.259	1.525
0.24	0.139	0.919	0.697	0.812	1.854	1.345	1.609
0.16	0.094	0.612	0.449	0.599	1.253	0.879	1.223
0.08	0.032	0.366	0.321	0.32	0.833	0.729	0.727

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

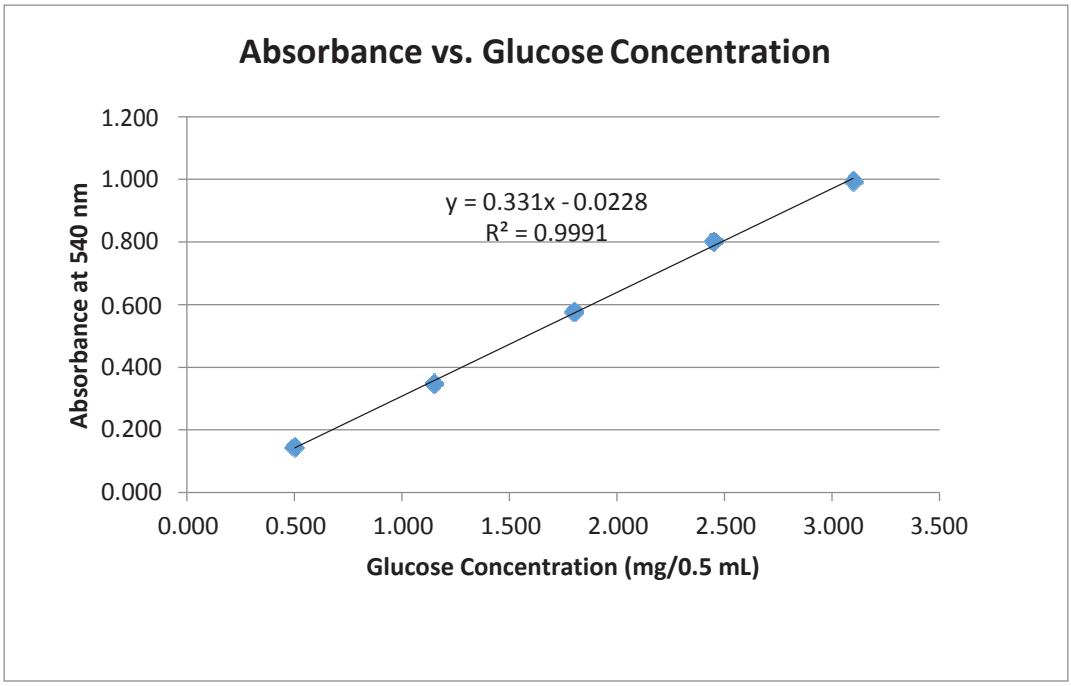
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.41	2.210	0.409	5.45	1.01
0.32	1.918	0.355	5.91	1.10
0.24	1.603	0.297	6.59	1.22
0.16	1.119	0.207	6.90	1.28
0.08	0.763	0.141	9.41	1.74
		Average:	6.85	1.27



Seven grams pretreated corn stover with three grams wheat bran #1
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on seven grams pretreated corn stover with three grams wheat bran for seven days. Sample #1. Diluted with 37 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	0.443	0.453	0.457	0.451	0.007	0.443
2	0.326	0.36	0.372	0.353	0.024	0.326
3	0.269	0.263	0.271	0.268	0.004	0.269
4	0.179	0.175	0.182	0.179	0.004	0.179
5	0.086	0.096	0.095	0.092	0.006	0.086

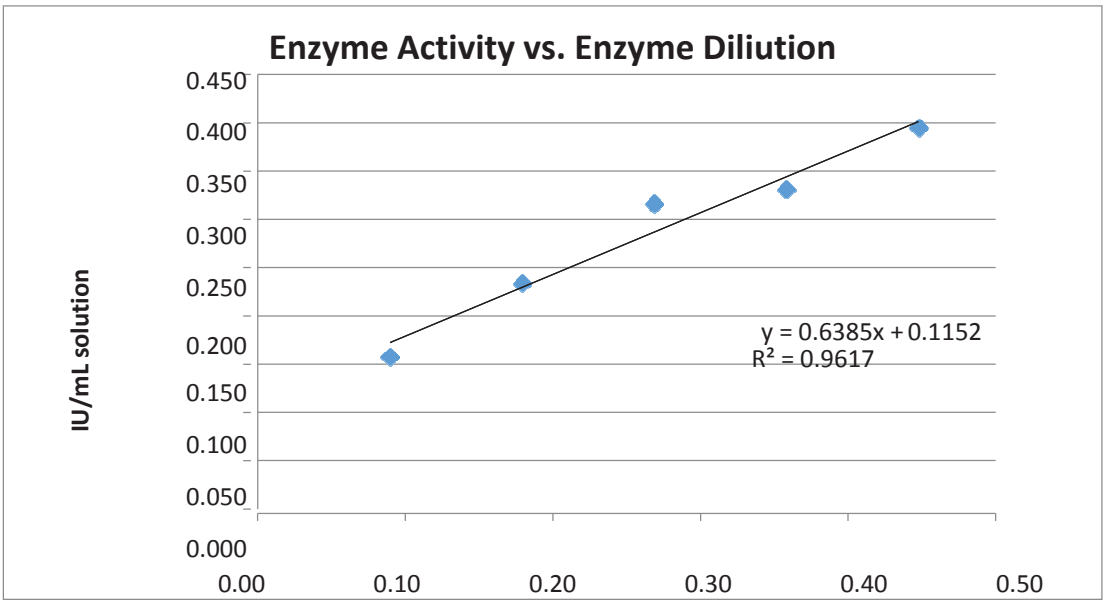


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.45	0.162	0.536	0.418	0.474	2.570	1.710	2.118
0.36	0.139	0.393	0.43	0.394	1.695	1.965	1.702
0.27	0.111	0.374	0.368	0.359	1.758	1.715	1.649
0.18	0.085	0.261	0.29	0.289	1.124	1.335	1.328
0.09	0.056	0.189	0.217	0.176	0.815	1.019	0.720

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

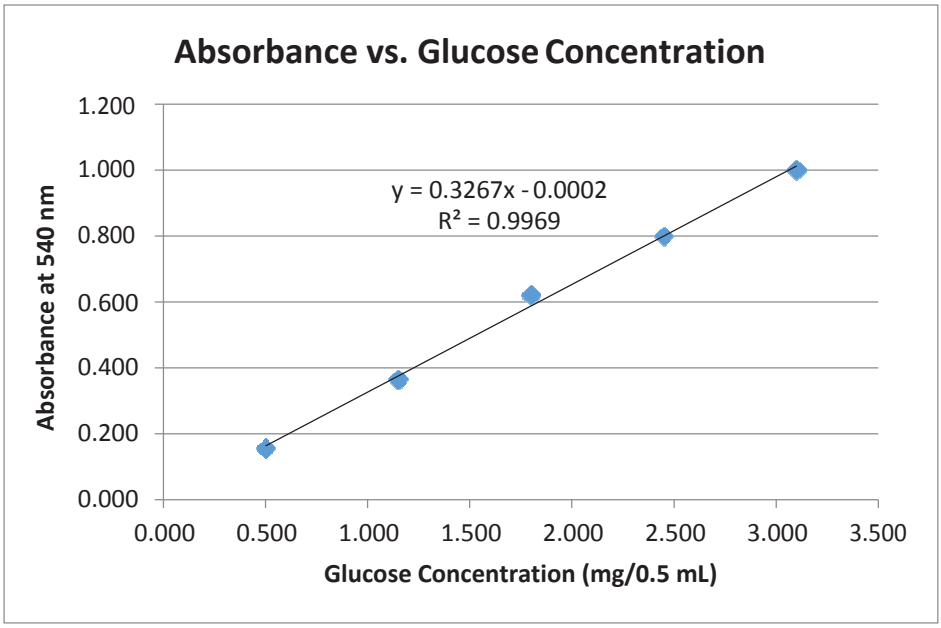
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.45	2.133	0.395	4.76	0.882
0.36	1.788	0.331	4.99	0.924
0.27	1.707	0.316	6.35	1.177
0.18	1.262	0.234	7.05	1.305
0.09	0.851	0.158	9.51	1.761
		Average:	6.53	1.210



Seven grams pretreated corn stover with three grams wheat bran #2
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on seven grams pretreated corn stover with three grams wheat bran for seven days. Sample #2. Diluted with 50 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.962	1.027	1.012	1.000	0.034
2	2.450	0.775	0.822	0.8	0.799	0.024
3	1.800	0.591	0.626	0.644	0.620	0.027
4	1.150	0.362	0.346	0.384	0.364	0.019
5	0.500	0.16	0.154	0.154	0.156	0.003

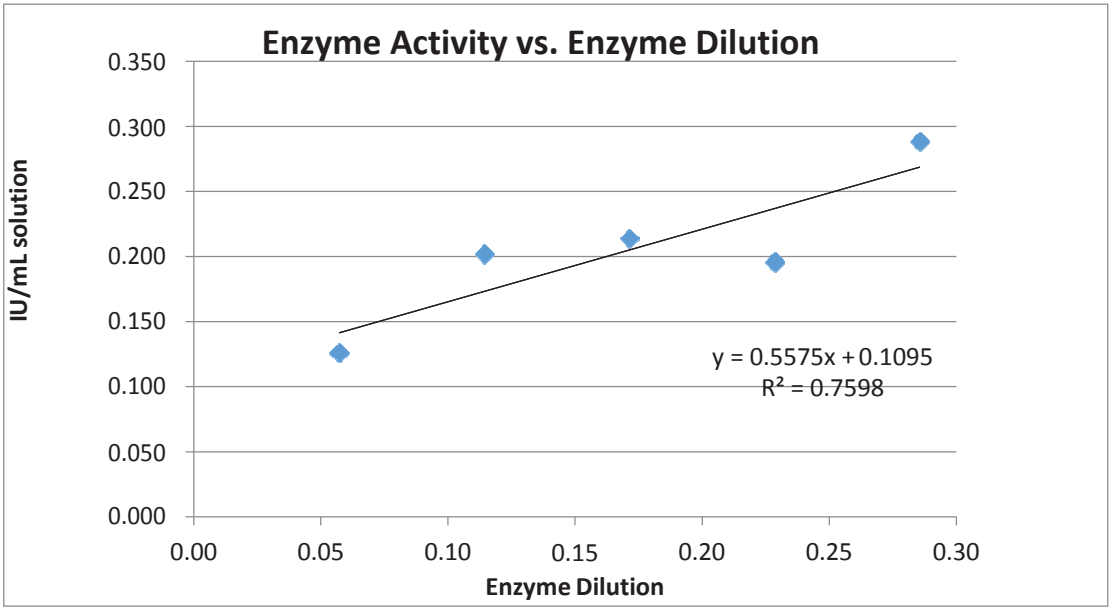


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.38	0.585	1.106	0.995	1.179	1.595	1.256	1.819
0.30	0.440	0.746	0.855	0.753	0.938	1.272	0.960
0.23	0.337	0.73	0.675	0.738	1.203	1.034	1.227
0.15	0.202	0.502	0.639	0.533	0.918	1.337	1.013
0.08	0.096	0.314	0.33	0.309	0.669	0.718	0.654

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

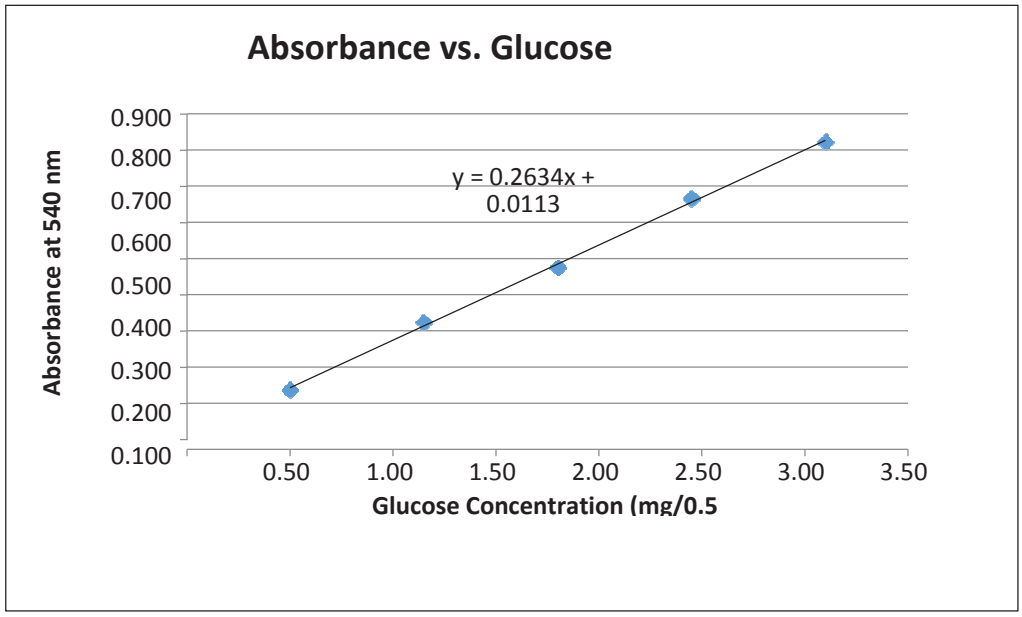
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.29	1.557	0.288	5.45	1.01
0.23	1.057	0.196	4.62	0.86
0.17	1.155	0.214	6.74	1.25
0.11	1.089	0.202	9.53	1.77
0.06	0.680	0.126	11.90	2.20
		Average:	7.65	1.42



Seven grams pretreated corn stover with three grams wheat bran #3
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on seven grams pretreated corn stover with three grams wheat bran for seven days. Sample #3. Diluted with 44 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.795	0.817	0.856	0.823	0.031
2	2.450	0.614	0.64	0.745	0.666	0.069
3	1.800	0.447	0.495	0.486	0.476	0.026
4	1.150	0.325	0.333	0.315	0.324	0.009
5	0.500	0.137	0.145	0.131	0.138	0.007

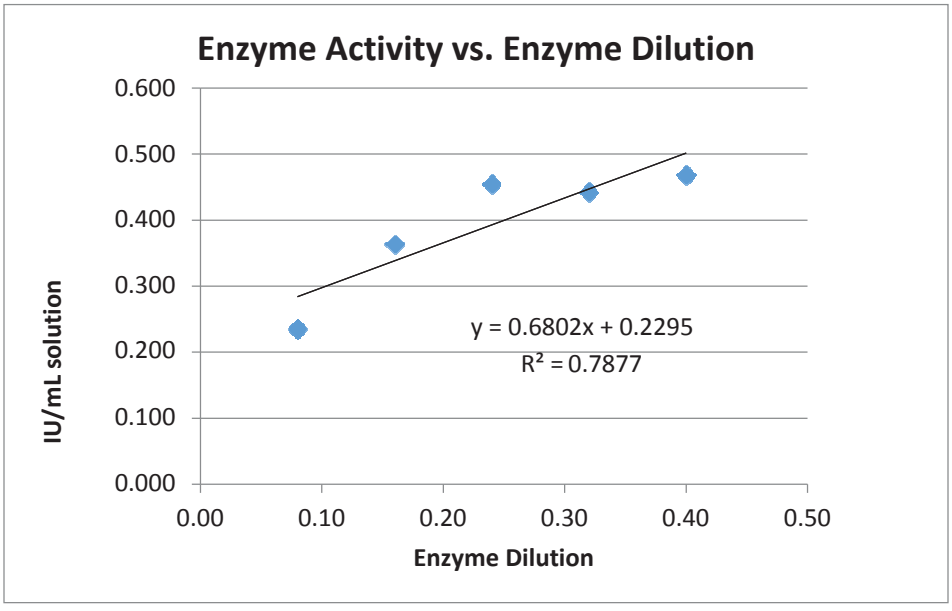


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	0.181	0.786	0.930	-----	2.254	2.801	-----
0.32	0.132	0.794	0.766	0.757	2.470	2.364	2.330
0.24	0.098	0.714	0.712	0.843	2.294	2.287	2.784
0.16	0.062	0.564	0.679	0.529	1.862	2.298	1.729
0.08	0.021	0.351	0.352	0.398	1.210	1.214	1.388

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

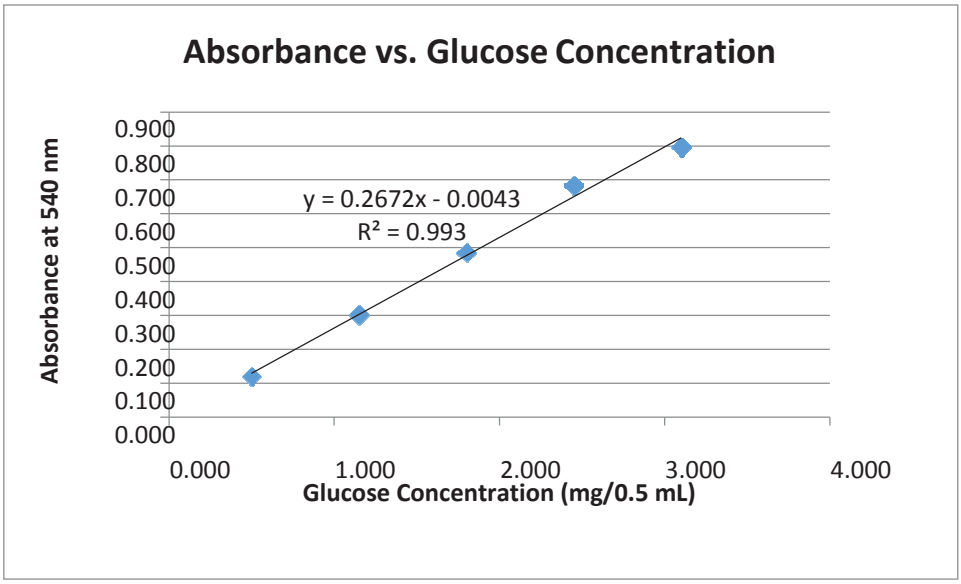
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.40	2.527	0.468	6.32	1.17
0.32	2.388	0.442	7.46	1.38
0.24	2.455	0.455	10.23	1.89
0.16	1.963	0.364	12.27	2.27
0.08	1.271	0.235	15.88	2.94
		Average:	10.43	1.93



Seven grams unpretreated corn stover with three grams wheat bran #1
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on seven grams unpretreated corn stover with three grams wheat bran for seven days. Sample #1. Diluted with 40 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.772	0.813	0.801	0.795	0.021
2	2.450	0.648	0.706	0.697	0.684	0.031
3	1.800	0.485	0.497	0.474	0.485	0.012
4	1.150	0.313	0.286	0.303	0.301	0.014
u	0.500	0.119	0.108	0.128	0.118	0.010

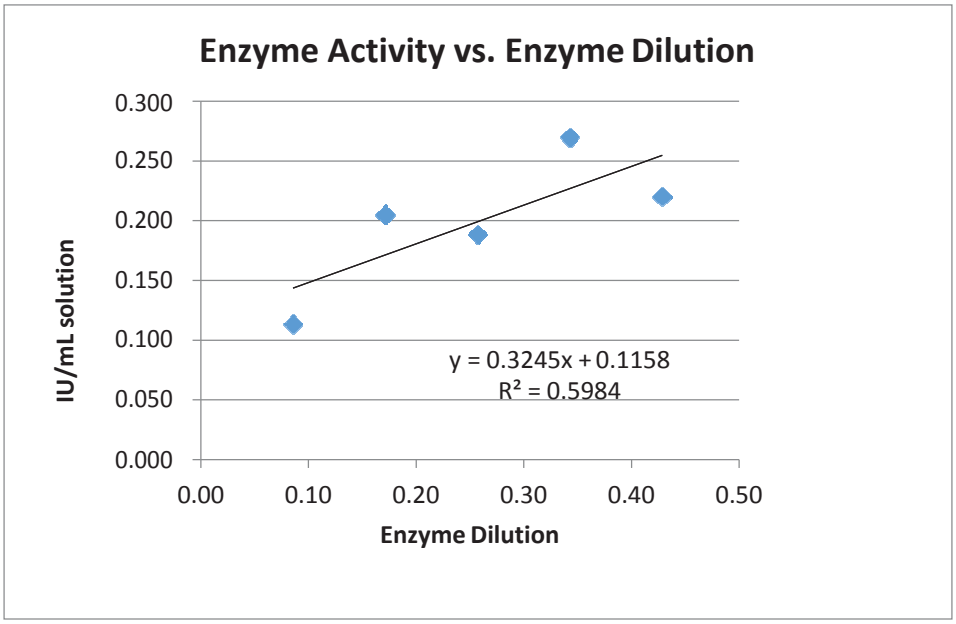


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.43	0.397	0.690	0.730	-----	1.113	1.262	-----
0.34	0.318	0.750	0.689	0.670	1.632	1.403	1.332
0.26	0.237	0.501	0.496	0.517	1.004	0.985	1.064
0.17	0.150	0.455	0.431	0.439	1.156	1.066	1.096
0.09	0.064	0.230	0.220	0.220	0.636	0.599	0.599

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the

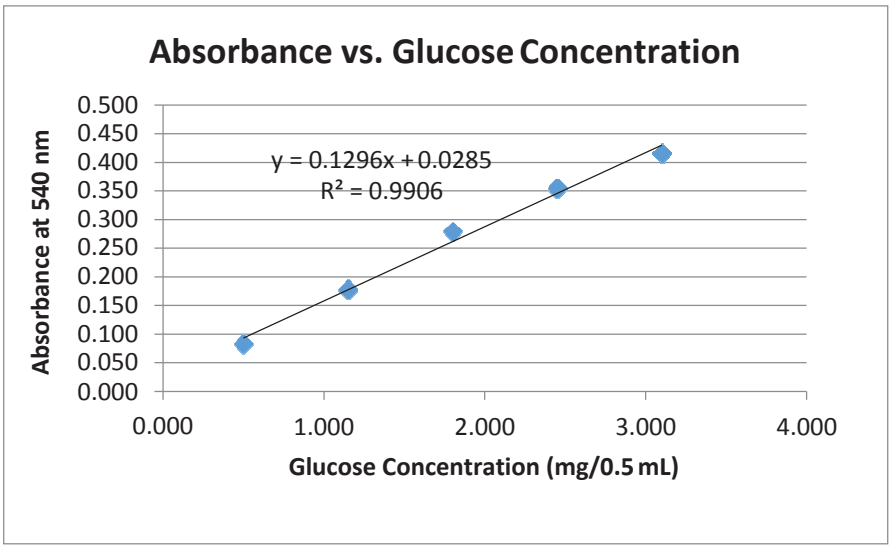
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	1.188	0.220	2.77	0.51
0.34	1.456	0.270	4.25	0.79
0.26	1.018	0.188	3.96	0.73
0.17	1.106	0.205	6.45	1.20
0.09	0.611	0.113	7.13	1.32
		Average:	4.91	0.91



Seven grams unpretreated corn stover with three grams wheat bran #2
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on six grams unpretreated corn stover with four grams wheat bran for seven days. Sample #2. Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.386	0.440	0.421	0.416	0.027
2	2.450	0.341	0.357	0.364	0.354	0.012
3	1.800	0.279	0.275	0.282	0.279	0.004
4	1.150	0.183	0.172	0.178	0.178	0.006
5	0.500	0.082	0.084	0.082	0.083	0.001

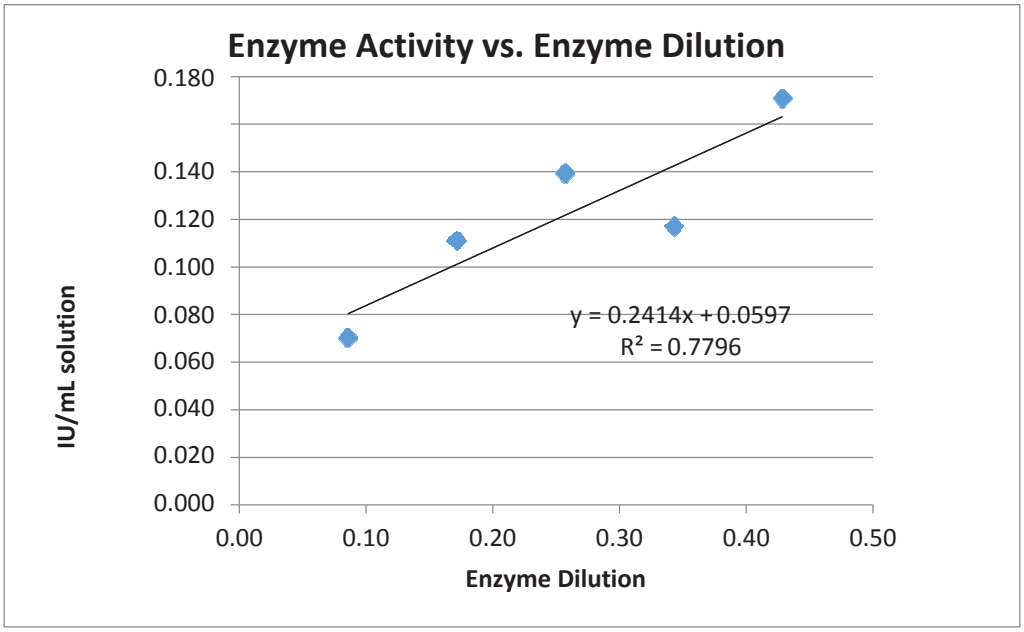


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	0.184	0.303	0.328	0.365	0.698	0.891	1.177
0.32	0.150	0.248	0.277	0.255	0.540	0.764	0.594
0.24	0.128	0.27	0.268	0.225	0.873	0.858	0.526
0.16	0.093	0.183	0.207	0.208	0.475	0.660	0.667
0.08	0.061	0.137	0.137	0.143	0.364	0.364	0.410

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

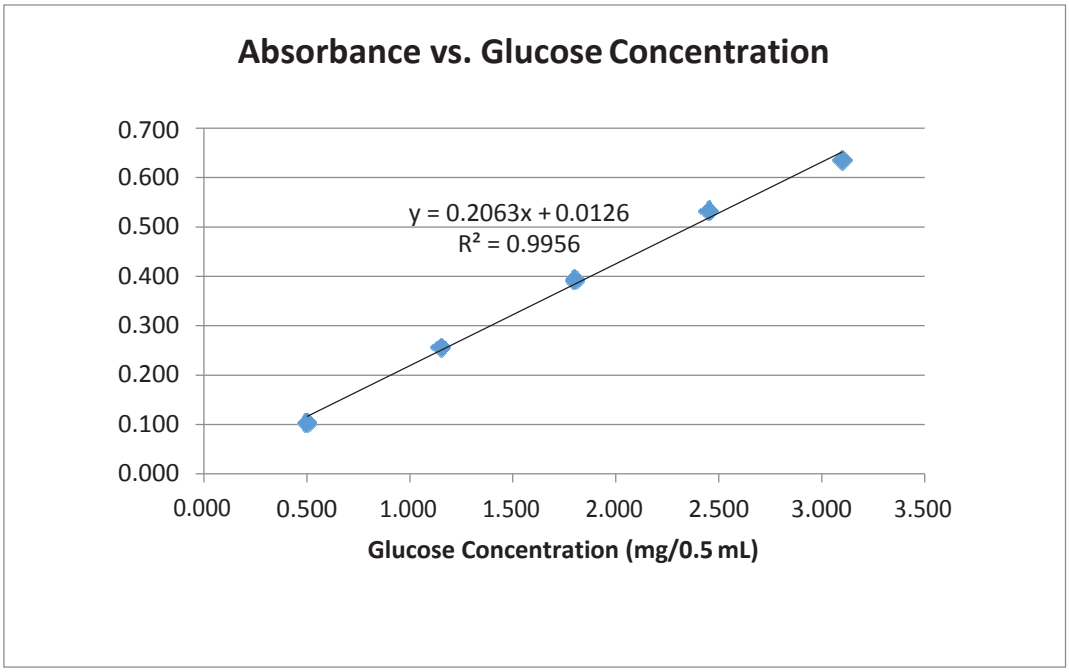
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	0.427	0.079	1.00	0.18
0.34	0.384	0.071	1.12	0.21
0.26	0.388	0.072	1.51	0.28
0.17	0.250	0.046	1.46	0.27
0.09	0.113	0.021	1.31	0.24
		Average:	1.28	0.24



Seven grams unpretreated corn stover with three grams wheat bran #3
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on seven grams unpretreated corn stover with three grams wheat bran for seven days. Sample #3. Diluted with 36 mL for extraction.

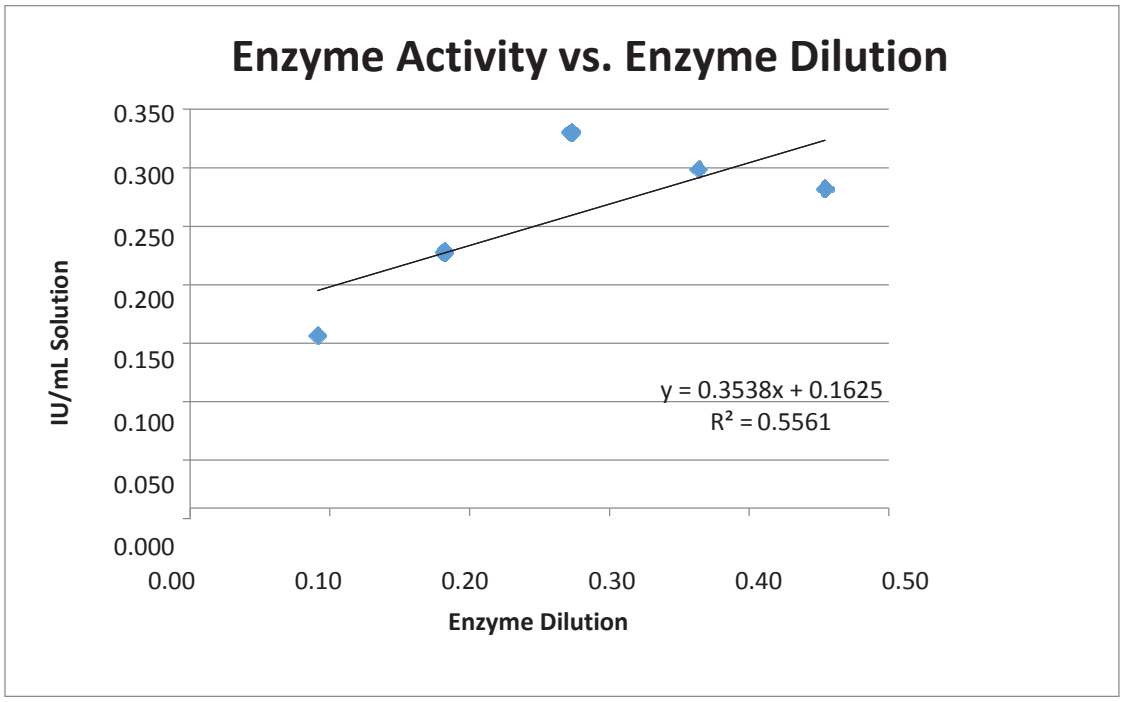
Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.654	0.635	0.616	0.635	0.019
2	2.450	0.519	0.551	0.529	0.533	0.016
3	1.800	0.396	0.39	0.393	0.393	0.003
4	1.150	0.249	0.26	0.259	0.256	0.006
5	0.500	0.103	0.105	0.101	0.103	0.002



Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

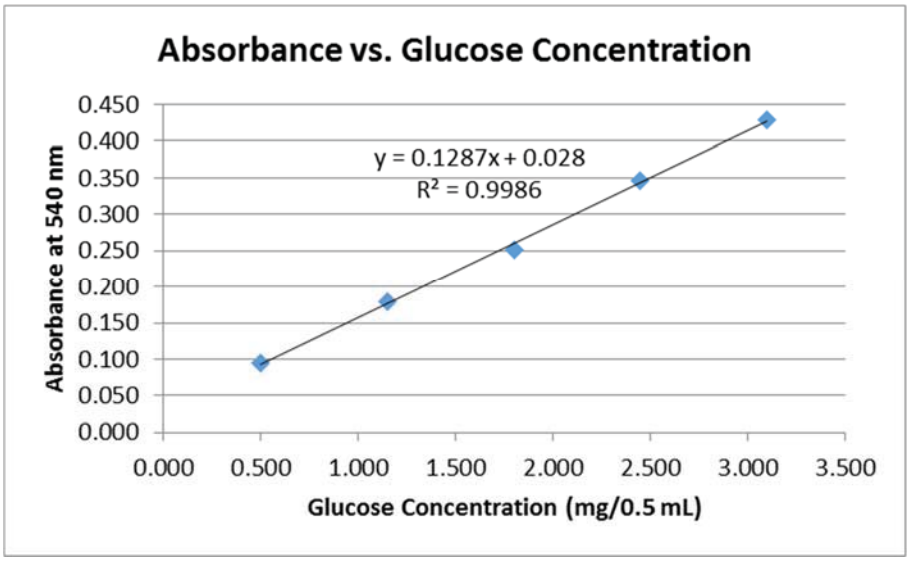
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.45	1.522	0.282	3.35	0.62
0.36	1.613	0.299	4.44	0.82
0.27	1.782	0.330	6.53	1.21
0.18	1.230	0.228	6.76	1.25
0.09	0.845	0.157	9.30	1.72
		Average	6.08	1.13



Eight grams pretreated corn stover with two grams wheat bran #1 Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on eight grams pretreated corn stover with two grams wheat bran for seven days. Sample #1. Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.441	0.415	0.428	0.428	0.013
2	2.450	0.352	0.339	0.35	0.347	0.007
3	1.800	0.254	0.24	0.259	0.251	0.010
4	1.150	0.177	0.181	0.177	0.178	0.002
5	0.500	0.096	0.091	0.095	0.094	0.003

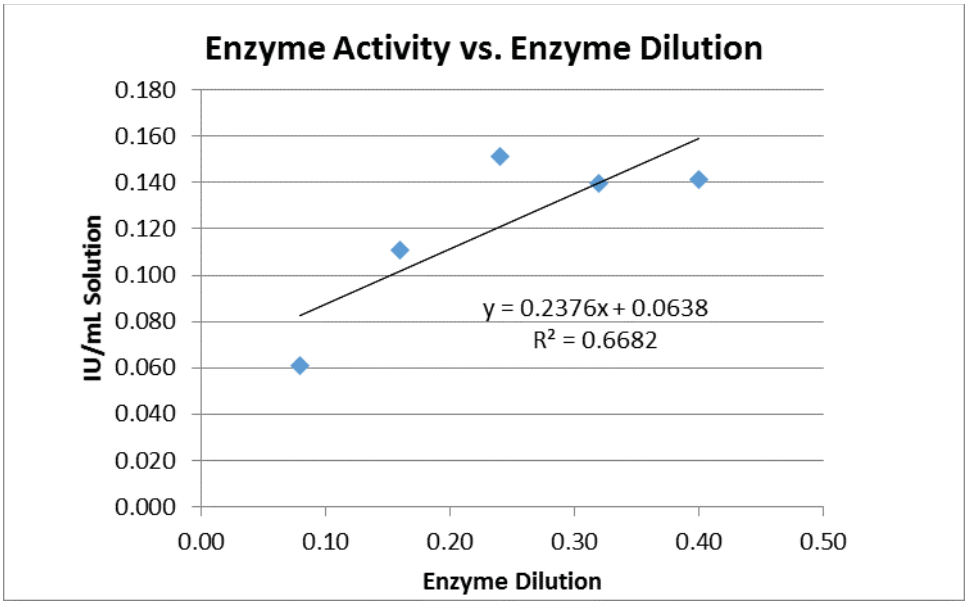


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	0.255	0.348	0.343	0.349	0.774	0.734	0.782
0.32	0.224	0.297	0.315	0.332	0.615	0.758	0.893
0.24	0.179	0.243	0.303	0.286	0.543	1.020	0.885
0.16	0.125	0.167	0.218	0.203	0.368	0.774	0.654
0.08	0.075	0.103	0.115	0.118	0.257	0.352	0.376

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase

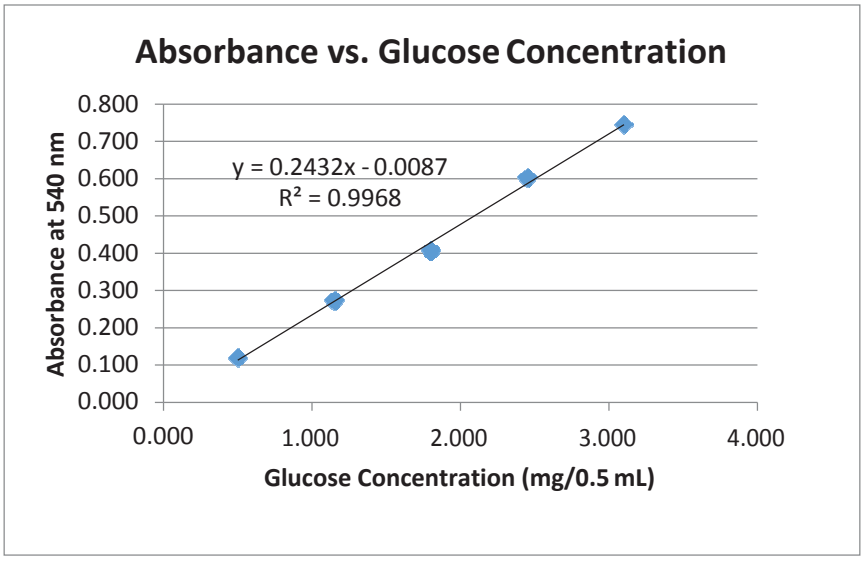
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.40	0.763	0.141	1.91	0.35
0.32	0.755	0.140	2.36	0.44
0.24	0.816	0.151	3.40	0.63
0.16	0.599	0.111	3.74	0.69
0.08	0.328	0.061	4.10	0.76
		Average:	3.10	0.57



Eight grams pretreated corn stover with two grams wheat bran #2 Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on eight grams pretreated corn stover with two grams wheat bran for seven days. Sample #2. Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.751	0.739	0.744	0.745	0.006
2	2.450	0.625	0.579	0.604	0.603	0.023
3	1.800	0.417	0.401	0.401	0.406	0.009
4	1.150	0.282	0.26	0.275	0.272	0.011
5	0.500	0.109	0.119	0.13	0.119	0.011

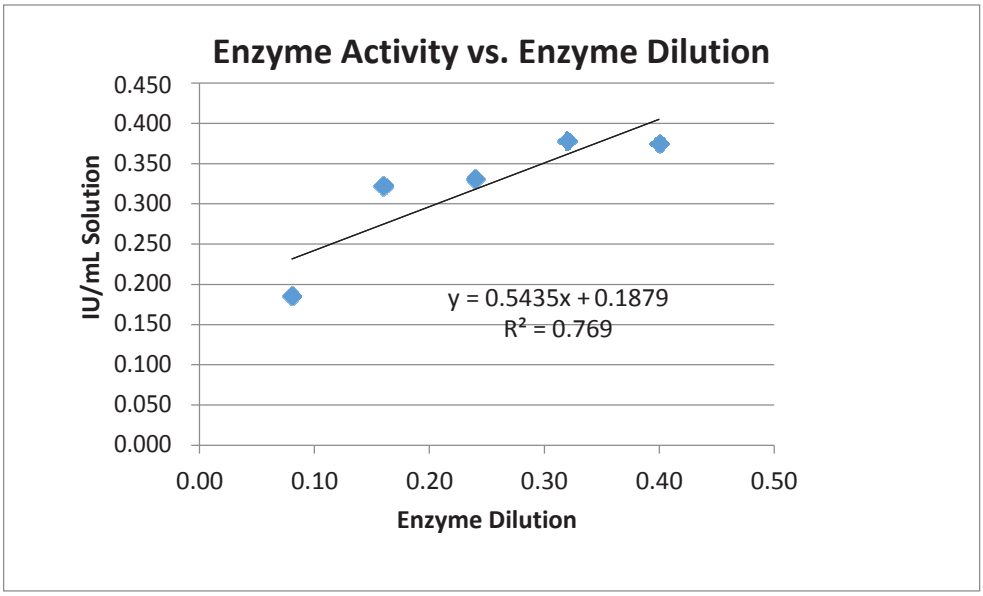


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	0.398	0.878	0.891	0.876	2.009	2.063	2.001
0.32	0.299	0.769	0.771	0.822	1.967	1.975	2.185
0.24	0.235	0.638	0.675	0.67	1.691	1.844	1.823
0.16	0.149	0.507	0.619	0.563	1.509	1.970	1.739
0.08	0.060	0.268	0.301	0.315	0.892	1.028	1.086

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

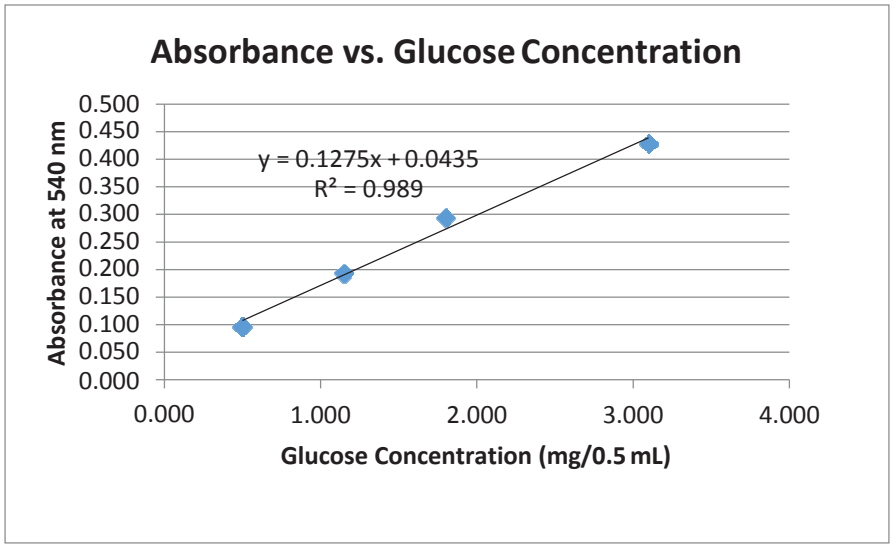
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.40	2.025	0.375	5.06	0.94
0.32	2.042	0.378	6.38	1.18
0.24	1.786	0.331	7.44	1.38
0.16	1.739	0.322	10.87	2.01
0.08	1.002	0.186	12.53	2.32
		Average:	8.46	1.57



Eight grams pretreated corn stover with two grams wheat bran #3 Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on eight grams pretreated corn stover with two grams wheat bran for seven days. Sample #3. Diluted with 37 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.442	0.407	0.435	0.428	0.019
3	1.800	0.281	0.281	0.318	0.293	0.021
4	1.150	0.189	0.197	0.190	0.192	0.004
5	0.500	0.09	0.101	0.096	0.096	0.006

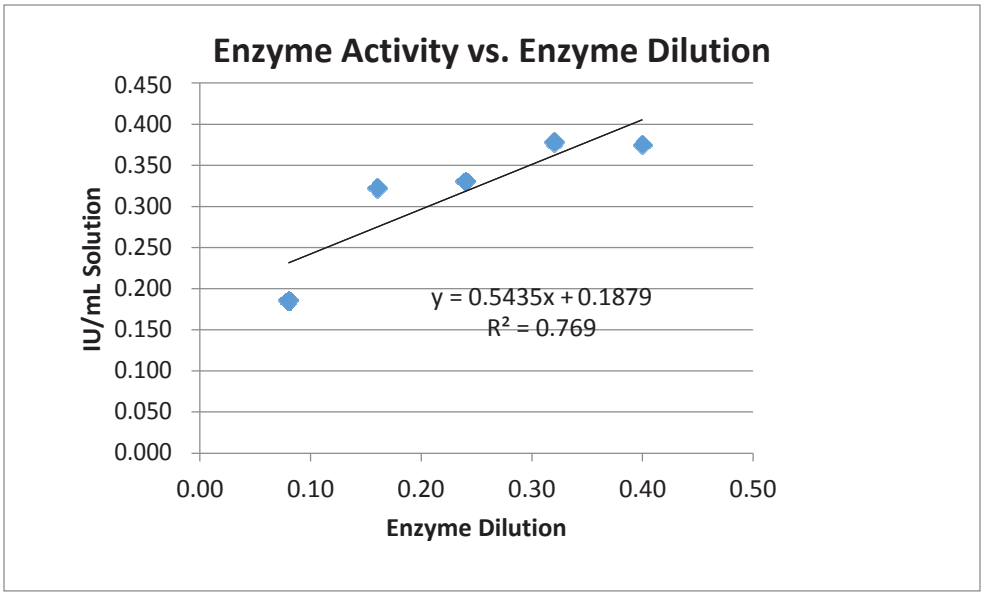


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.45	0.327	0.573	0.585	0.602	1.586	1.680	1.813
0.36	0.273	0.453	0.518	0.503	1.068	1.578	1.460
0.27	0.220	0.444	0.447	0.481	1.418	1.442	1.708
0.18	0.158	0.365	0.361	0.269	1.282	1.251	0.529
0.09	0.088	0.190	0.233	0.252	0.459	0.796	0.945

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

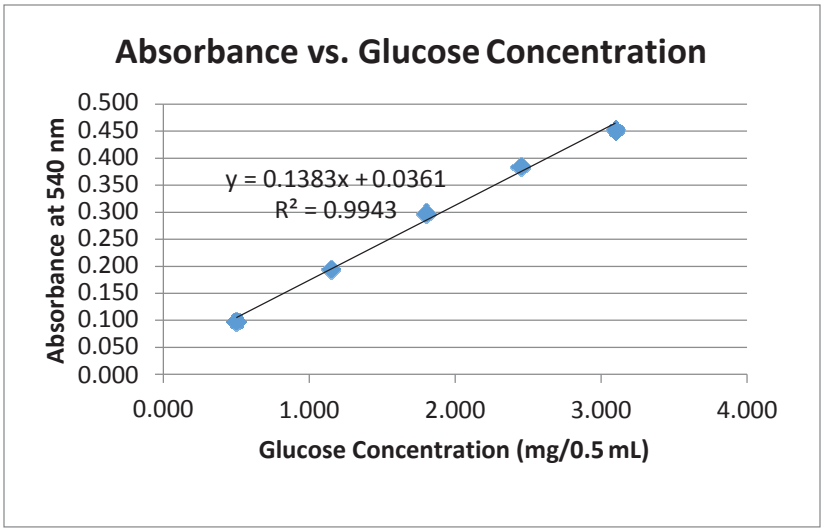
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average
0.40	2.025	0.375	5.06	0.94
0.32	2.042	0.378	6.38	1.18
0.24	1.786	0.331	7.44	1.38
0.16	1.739	0.322	10.87	2.01
0.08	1.002	0.186	12.53	2.32
		Average:	8.46	1.57



Eight grams unpretreated corn stover with two grams wheat bran #1
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on eight grams unpretreated corn stover with two grams wheat bran for seven days. Sample #1. Diluted with 40 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.459	0.458	0.439	0.452	0.011
2	2.450	0.38	0.381	0.39	0.384	0.006
3	1.800	0.282	0.307	0.304	0.298	0.014
4	1.150	0.189	0.201	0.191	0.194	0.006
5	0.500	0.096	0.1	0.097	0.098	0.002

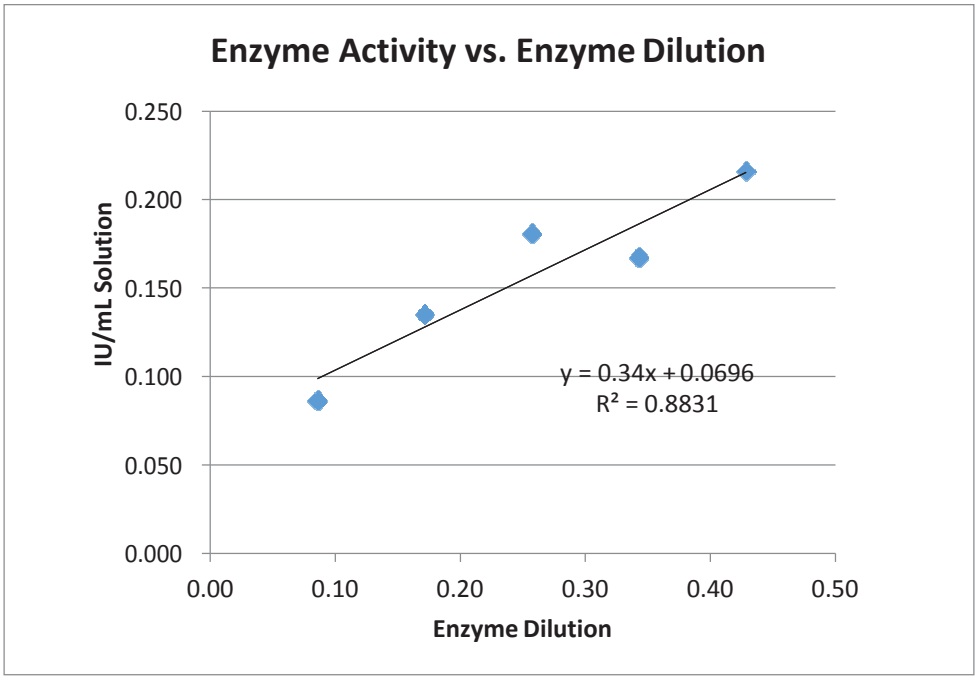


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.43	0.305	0.509	0.487	0.512	1.212	1.053	1.233
0.34	0.258	0.436	0.374	0.447	1.026	0.578	1.106
0.26	0.187	0.374	0.328	0.373	1.089	0.756	1.081
0.17	0.146	0.266	0.299	0.285	0.604	0.843	0.742
0.09	0.085	0.171	0.2	-----	0.361	0.570	-----

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

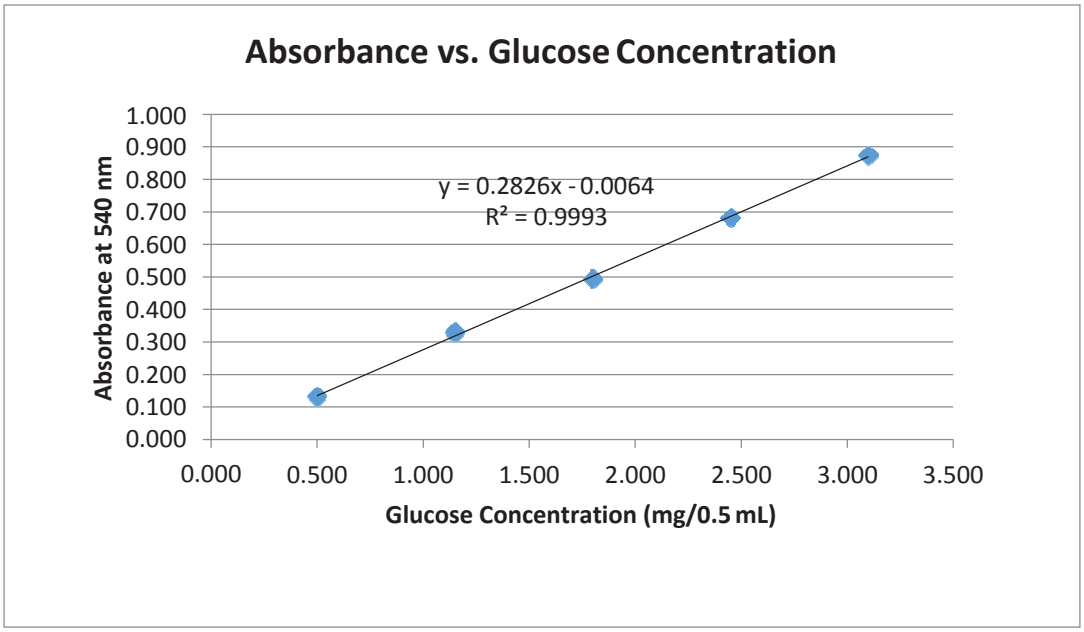
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	1.166	0.216	2.72	0.50
0.34	0.903	0.167	2.63	0.49
0.26	0.975	0.181	3.79	0.70
0.17	0.730	0.135	4.26	0.79
0.09	0.466	0.086	5.43	1.01
		Average:	3.77	0.70



Eight grams unpretreated corn stover with two grams wheat bran #2 Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on eight grams unpretreated corn stover with two grams wheat bran for seven days. Sample #2. Diluted with 35 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.849	0.897	0.876	0.874	0.024
2	2.450	0.696	0.69	0.661	0.682	0.019
3	1.800	0.477	0.514	0.490	0.494	0.019
4	1.150	0.327	0.325	0.338	0.330	0.007
5	0.500	0.126	0.133	0.136	0.132	0.005

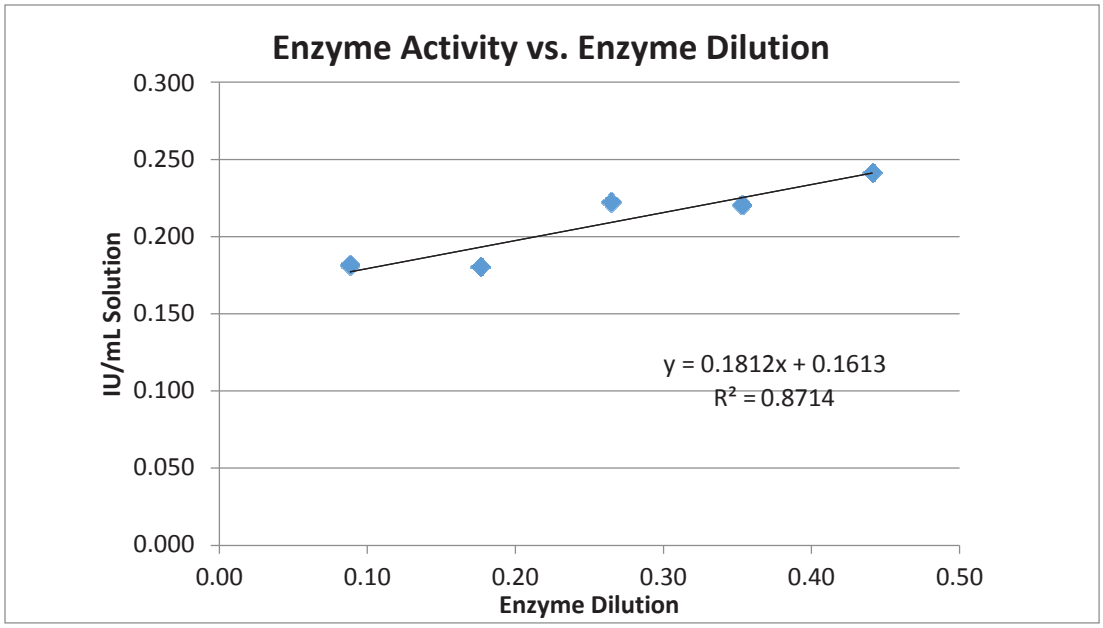


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.46	0.885	1.296	1.213	1.233	1.476	1.182	1.253
0.37	0.807	1.088	1.155	1.168	1.017	1.254	1.300
0.28	0.577	0.918	0.917	0.996	1.228	1.225	1.504
0.18	0.401	0.652	0.671	0.686	0.912	0.979	1.032
0.09	0.187	0.452	0.456	0.463	0.962	0.976	1.001

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase

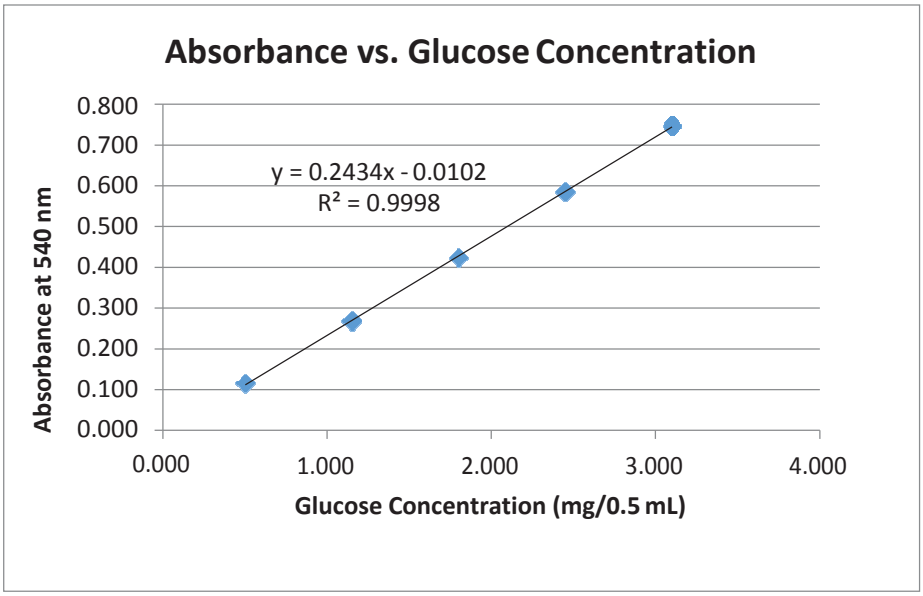
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.44	1.304	0.241	2.95	0.55
0.35	1.190	0.220	3.37	0.62
0.26	1.201	0.222	4.54	0.84
0.18	0.975	0.180	5.52	1.02
0.09	0.980	0.181	11.10	2.06
		Average:	5.50	1.02



Eight grams unpretreated corn stover with two grams wheat bran #3
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on eight grams unpretreated corn stover with two grams wheat bran for seven days. Sample #3. Diluted with 36 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.739	0.756	0.000	0.748	0.012
2	2.450	0.579	0.602	0.575	0.585	0.015
3	1.800	0.433	0.427	0.411	0.424	0.011
4	1.150	0.267	0.259	0.277	0.268	0.009
5	0.500	0.130	0.109	0.107	0.115	0.013

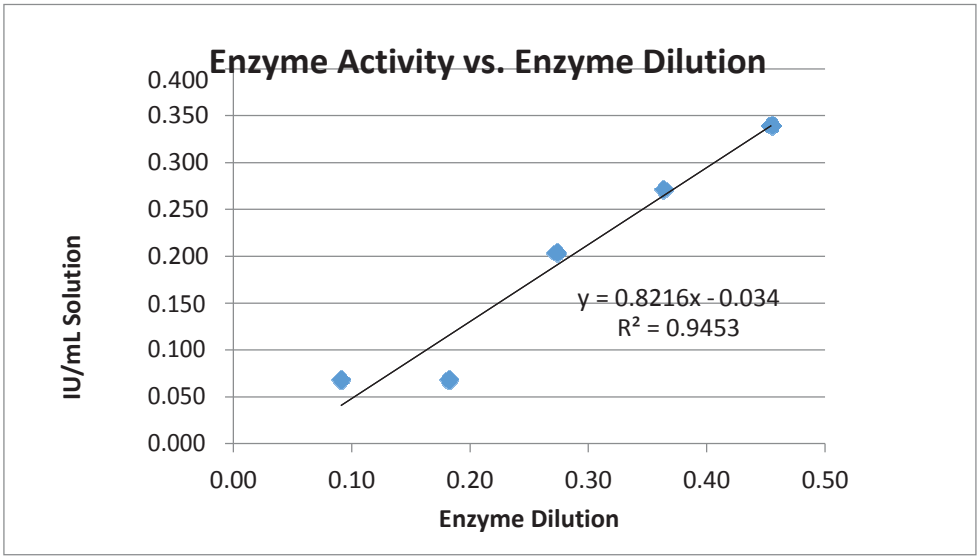


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.45	0.512	0.896	0.837	0.918	1.621	1.379	1.712
0.36	0.411	0.705	0.812	0.707	1.250	1.690	1.258
0.27	0.290	0.570	0.597	0.605	1.194	1.305	1.338
0.18	0.195	0.471	0.519	0.486	1.176	1.373	1.238
0.09	0.083	0.278	0.294	0.264	0.844	0.910	0.787

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.45	1.83	0.340	4.03	0.75
0.36	1.47	0.272	4.03	0.75
0.27	1.10	0.204	4.03	0.75
0.18	0.37	0.068	2.02	0.37
0.09	0.37	0.068	4.03	0.75
		Average:	3.63	0.67



Nine grams pretreated corn stover with one grams wheat bran #1

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on nine grams pretreated corn stover with one gram wheat bran for seven days. Sample #1. Diluted with 36 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.454	0.426	0.425	0.435	0.016
2	2.450	0.373	0.363	0.364	0.367	0.006
3	1.800	0.268	0.281	0.258	0.269	0.012
4	1.150	0.182	0.187	0.183	0.184	0.003
5	0.500	0.101	0.103	0.101	0.102	0.001

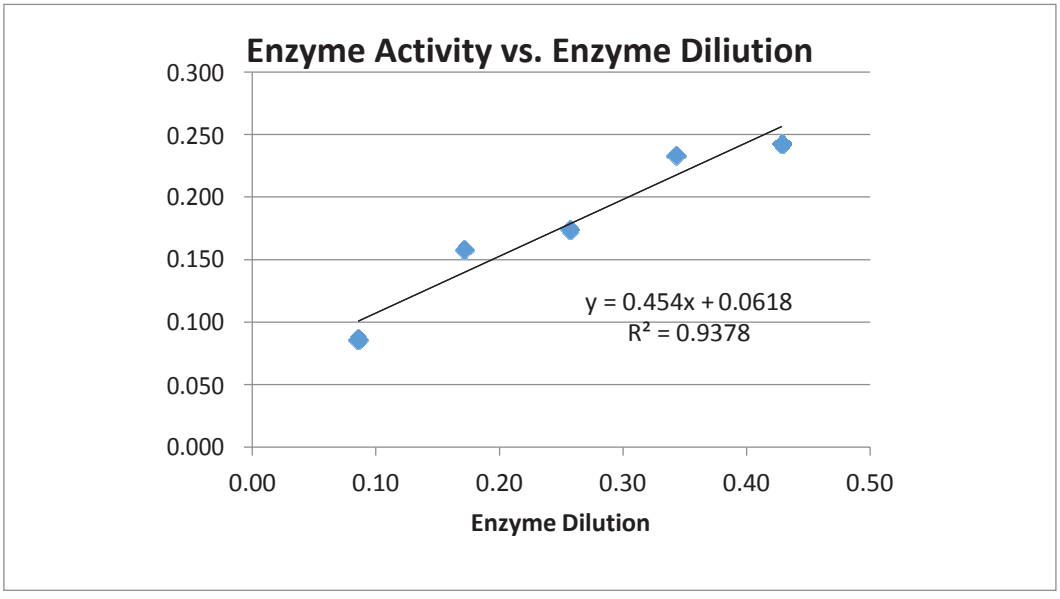
Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

Enzyme Dilution	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.45	0.182	0.344	0.413	0.41	0.966	1.494	1.471
0.36	0.151	0.318	0.369	0.368	1.002	1.392	1.384
0.27	0.126	0.28	0.290	-----	0.900	0.976	-----
0.18	0.092	0.225	0.244	0.248	0.744	0.889	0.920
0.09	0.059	0.155	0.16	0.152	0.458	0.497	0.435

IU/mL solution

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase

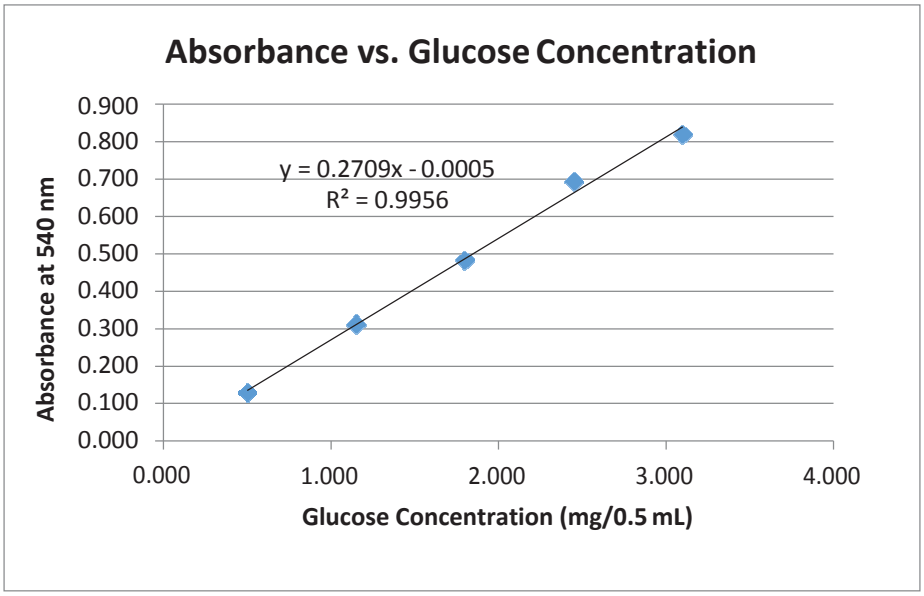
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	1.310	0.243	3.06	0.566
0.34	1.259	0.233	3.67	0.680
0.26	0.938	0.174	3.65	0.675
0.17	0.851	0.158	4.96	0.919
0.09	0.463	0.086	5.41	1.001
		Average:	4.51	0.768



Nine grams pretreated corn stover with one grams wheat bran #2 Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on nine grams pretreated corn stover with one gram wheat bran for seven days. Sample #2. Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.826	0.861	0.770	0.819	0.046
2	2.450	0.687	0.687	0.706	0.693	0.011
3	1.800	0.450	0.473	0.524	0.482	0.038
4	1.150	0.307	0.314	0.314	0.312	0.004
5	0.500	0.124	0.128	0.136	0.129	0.006

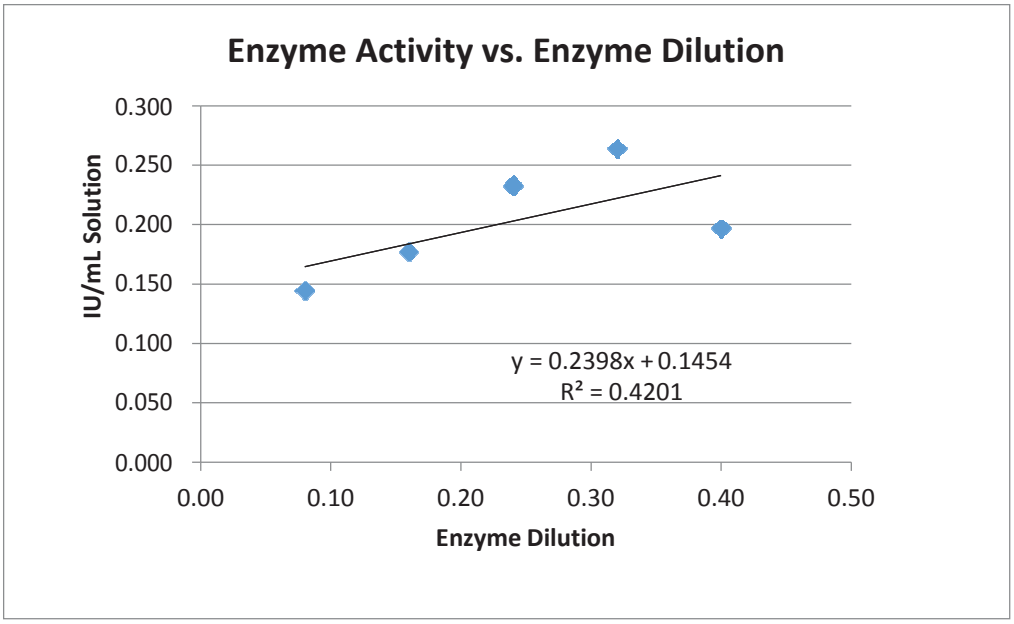


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	1.019	1.470	1.088	1.361	1.667	0.257	1.264
0.32	0.820	1.100	1.214	1.304	1.034	1.455	1.787
0.24	0.631	0.895	1.071	0.948	0.975	1.625	1.171
0.16	0.381	0.610	0.678	0.629	0.848	1.099	0.919
0.08	0.174	0.358	0.395	0.400	0.682	0.819	0.837

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

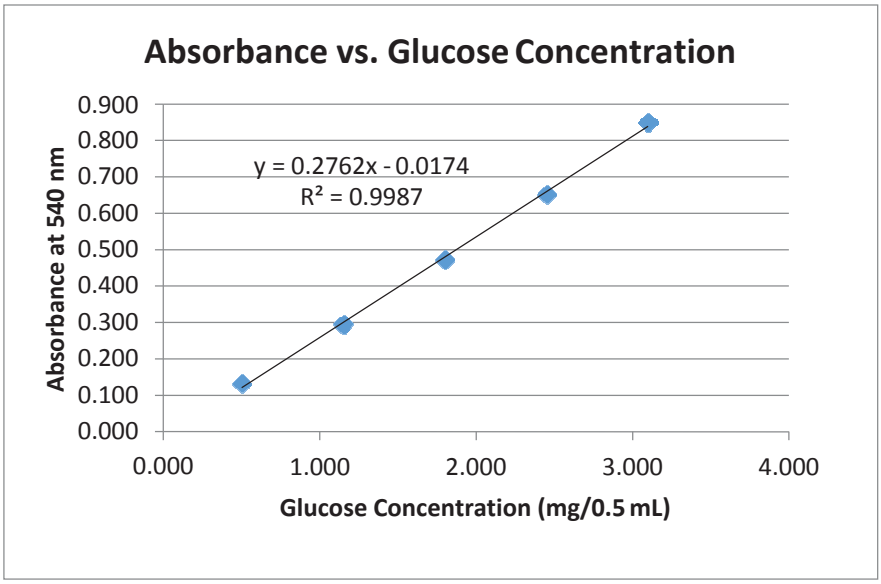
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.40	1.063	0.197	2.66	0.492
0.32	1.425	0.264	4.45	0.825
0.24	1.257	0.233	5.24	0.970
0.16	0.955	0.177	5.97	1.106
0.08	0.780	0.144	9.74	1.804
		Average:	5.61	1.039



Nine grams pretreated corn stover with one grams wheat bran #3 Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on nine grams pretreated corn stover with one gram wheat bran for seven days. Sample #3. Diluted with 30 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.754	0.864	0.932	0.850	0.090
2	2.450	0.614	0.713	0.627	0.651	0.054
3	1.800	0.482	0.451	0.483	0.472	0.018
4	1.150	0.293	0.31	0.278	0.294	0.016
5	0.500	0.127	0.125	0.142	0.131	0.009

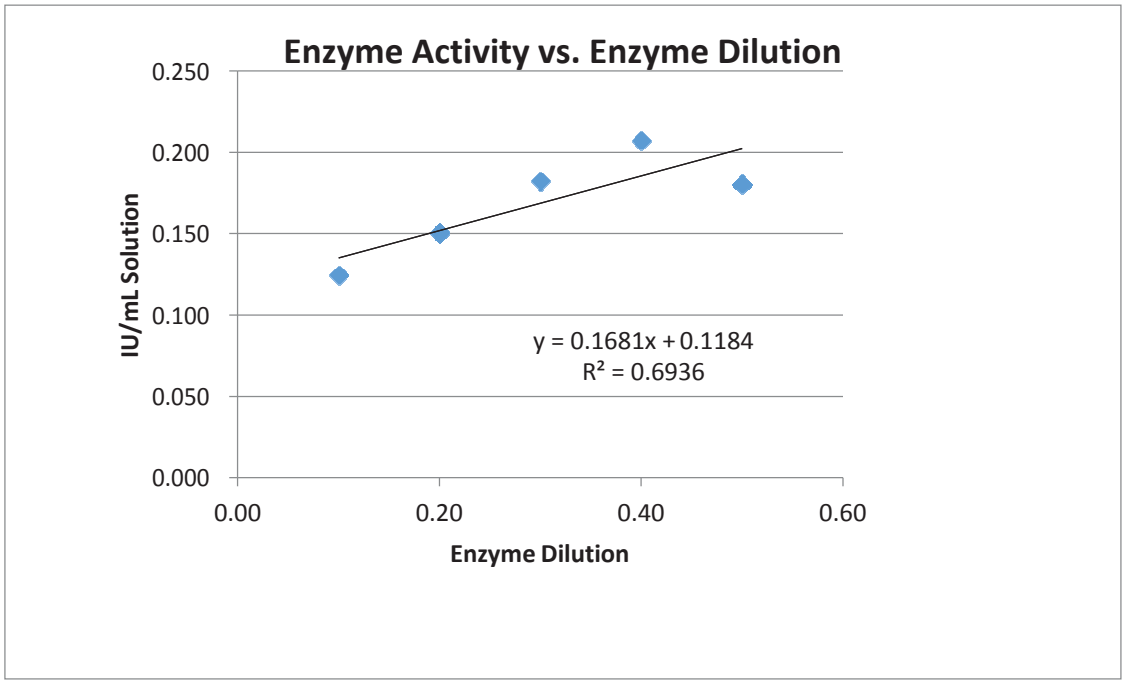


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.50	0.977	1.218	1.232	1.234	0.937	0.987	0.995
0.40	0.792	1.079	1.077	1.095	1.101	1.094	1.159
0.30	0.618	0.934	0.847	0.837	1.206	0.891	0.855
0.20	0.428	0.68	0.627	0.596	0.977	0.785	0.672
0.10	0.203	0.309	0.394	0.410	0.448	0.756	0.814

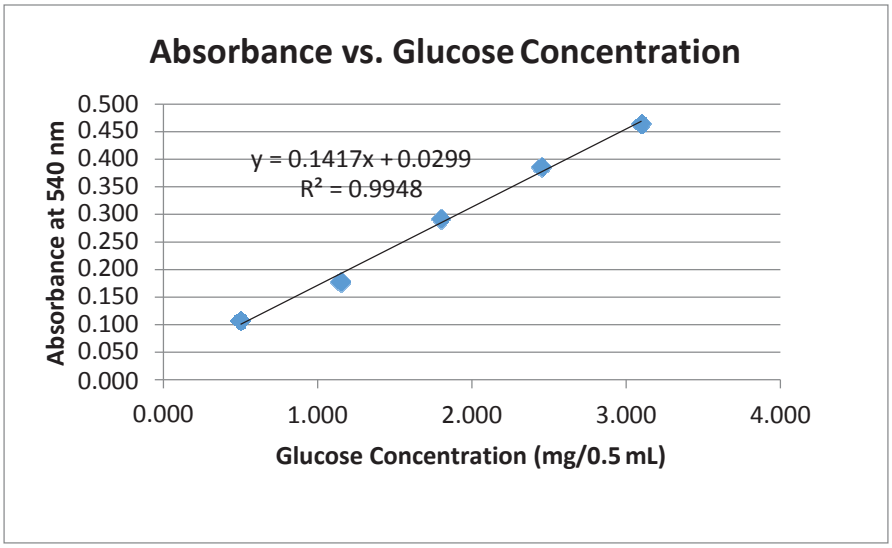
Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.50	0.973	0.180	1.95	0.36
0.40	1.118	0.207	2.79	0.52
0.30	0.984	0.182	3.28	0.61
0.20	0.811	0.150	4.06	0.75
0.10	0.672	0.125	6.72	1.25
		Average:	3.76	0.70



Nine grams unpretreated corn stover with one grams wheat bran #1
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on nine grams pretreated corn stover with one gram wheat bran for seven days. Sample #1. Diluted with 40 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration						
Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.458	0.474	0.459	0.464	0.009
2	2.450	0.389	0.399	0.369	0.386	0.015
3	1.800	0.278	0.321	0.275	0.291	0.026
4	1.150	0.174	0.173	0.184	0.177	0.006
5	0.500	0.111	0.103	0.108	0.107	0.004

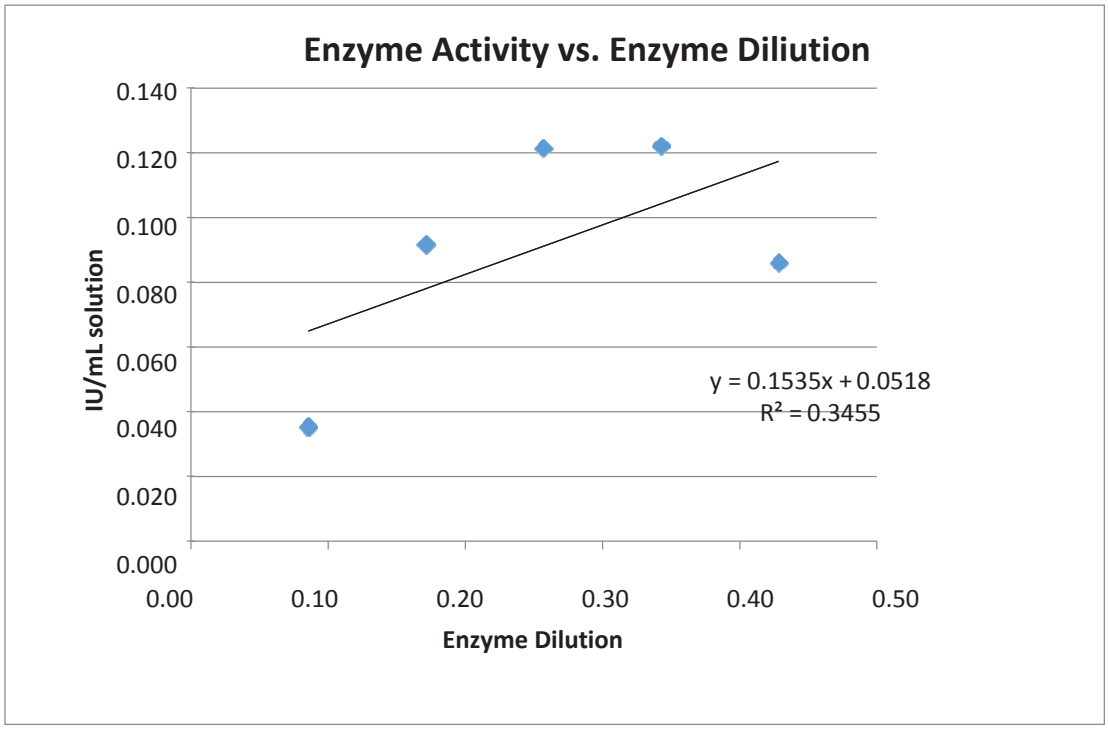


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.43	0.249	0.334	0.356	0.344	0.389	0.544	0.459
0.34	0.216	0.325	0.343	0.349	0.561	0.688	0.730
0.26	0.166	0.291	0.277	0.297	0.675	0.576	0.717
0.17	0.117	0.200	0.209	0.241	0.377	0.441	0.666
0.09	0.078	0.120	0.134	0.151	0.085	0.184	0.304

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

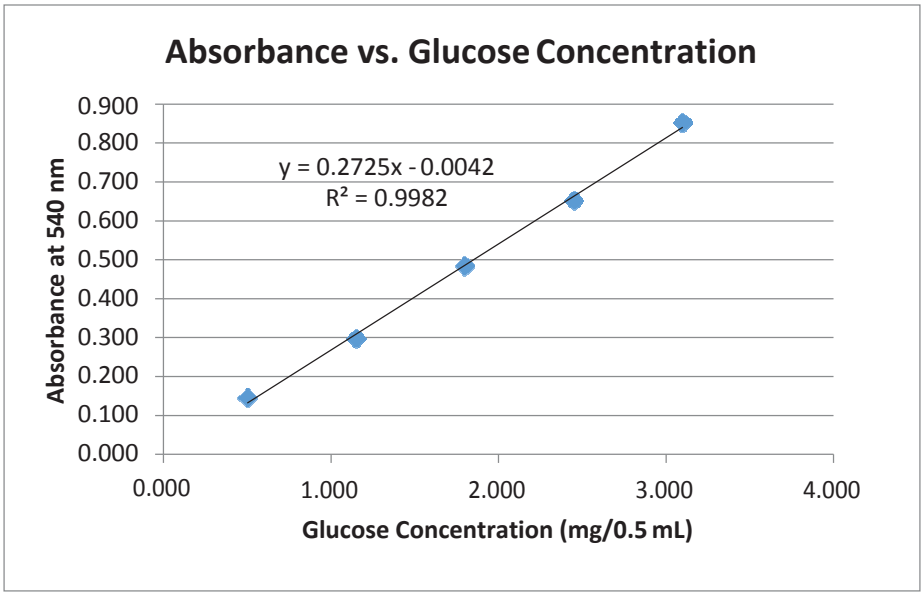
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	0.464	0.086	1.08	0.20
0.34	0.659	0.122	1.92	0.36
0.26	0.656	0.121	2.55	0.47
0.17	0.495	0.092	2.89	0.53
0.09	0.191	0.035	2.23	0.41
		Average:	2.11	0.39



Nine grams unpretreated corn stover with one grams wheat bran #2
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on nine grams unpretreated corn stover with one gram wheat bran for seven days. Sample #2. Diluted with 35 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.794	0.861	0.903	0.853	0.055
2	2.450	0.609	0.674	0.675	0.653	0.038
3	1.800	0.475	0.506	0.47	0.484	0.020
4	1.150	0.284	0.304	0.305	0.298	0.012
5	0.500	0.154	0.135	0.145	0.145	0.010

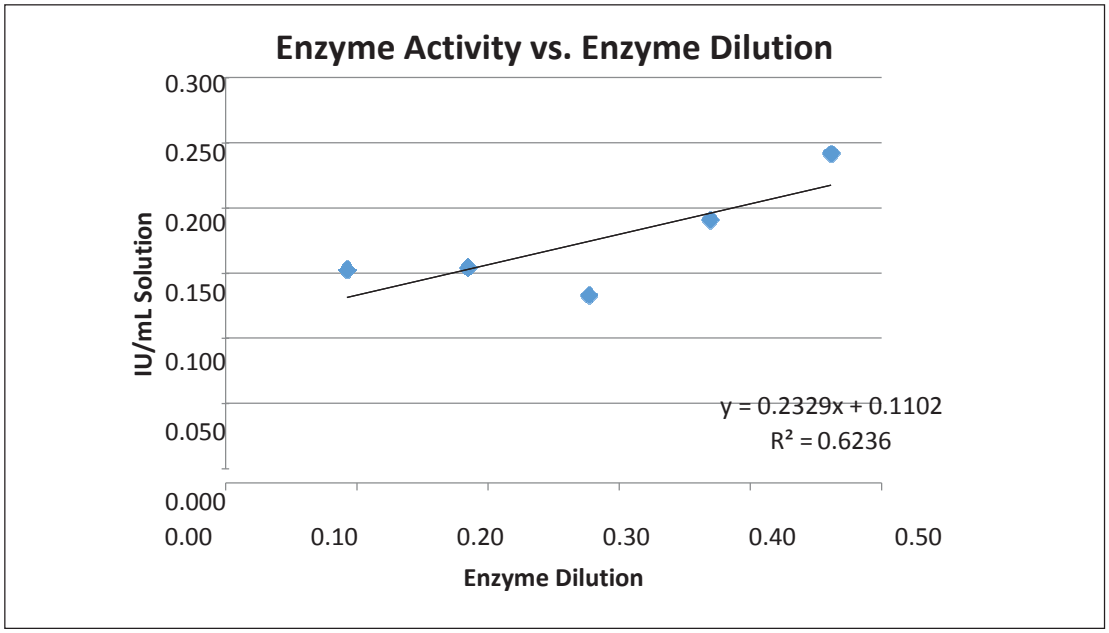


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.46	1.018	1.404	1.416	1.288	1.434	1.478	1.008
0.37	0.898	1.179	1.18	1.167	1.045	1.049	1.001
0.28	0.750	0.902	0.956	0.967	0.573	0.771	0.812
0.18	0.452	0.669	0.653	0.702	0.813	0.754	0.934
0.09	0.220	0.462	0.426	0.433	0.905	0.772	0.798

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase

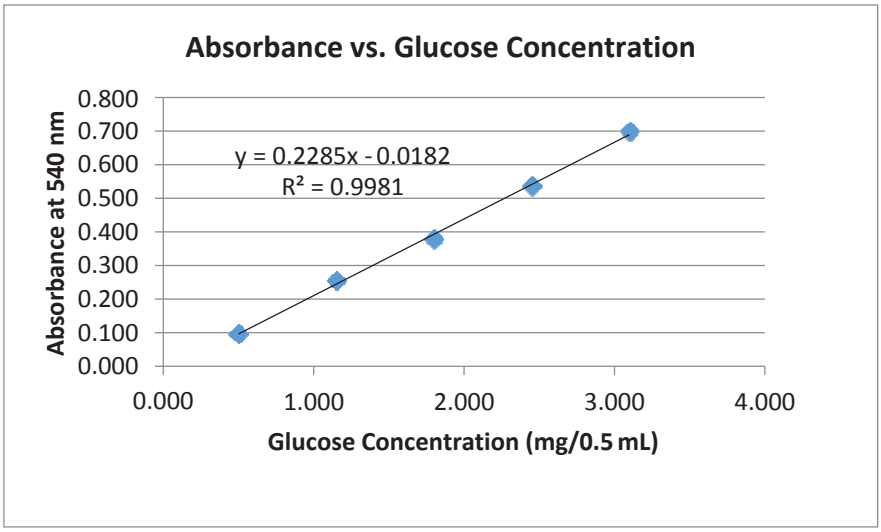
Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.46	1.307	0.242	0.52	2.83
0.37	1.032	0.191	0.52	2.79
0.28	0.719	0.133	0.48	2.60
0.18	0.834	0.154	0.84	4.52
0.09	0.825	0.153	1.66	8.94
		Average:	4.34	0.80



Nine grams unpretreated corn stover with one grams wheat bran #3
Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on nine grams unpretreated corn stover with one gram wheat bran for seven days. Sample #3. Diluted with 35 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.692	0.700	0.703	0.698	0.006
2	2.450	0.561	0.529	0.521	0.537	0.021
3	1.800	0.371	0.381	0.383	0.378	0.006
4	1.150	0.259	0.252	0.256	0.256	0.004
5	0.500	0.104	0.103	0.082	0.096	0.012

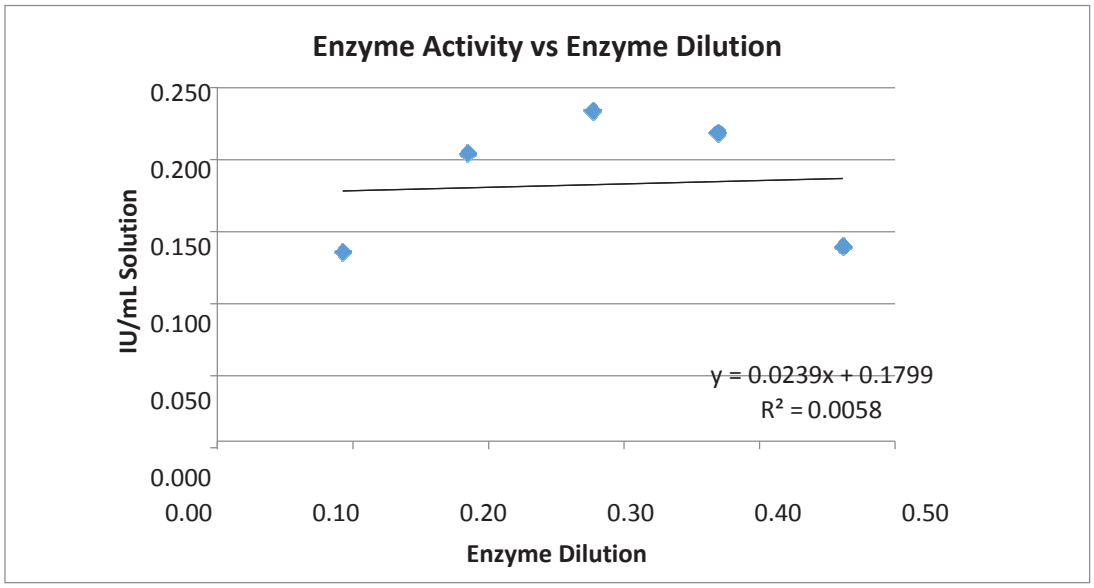


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.46	0.665	0.828	0.810	0.891	0.794	0.716	1.070
0.37	0.564	0.688	0.761	0.870	0.622	0.942	1.419
0.28	0.437	0.607	0.691	0.724	0.822	1.190	1.334
0.18	0.277	0.433	0.518	0.503	0.765	1.137	1.071
0.09	0.124	0.270	0.277	0.249	0.719	0.749	0.627

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.46	0.755	0.140	1.64	0.30
0.37	1.180	0.219	3.20	0.59
0.28	1.262	0.234	4.56	0.84
0.18	1.104	0.204	5.98	1.11
0.09	0.734	0.136	7.95	1.47
		Average:	4.66	0.86

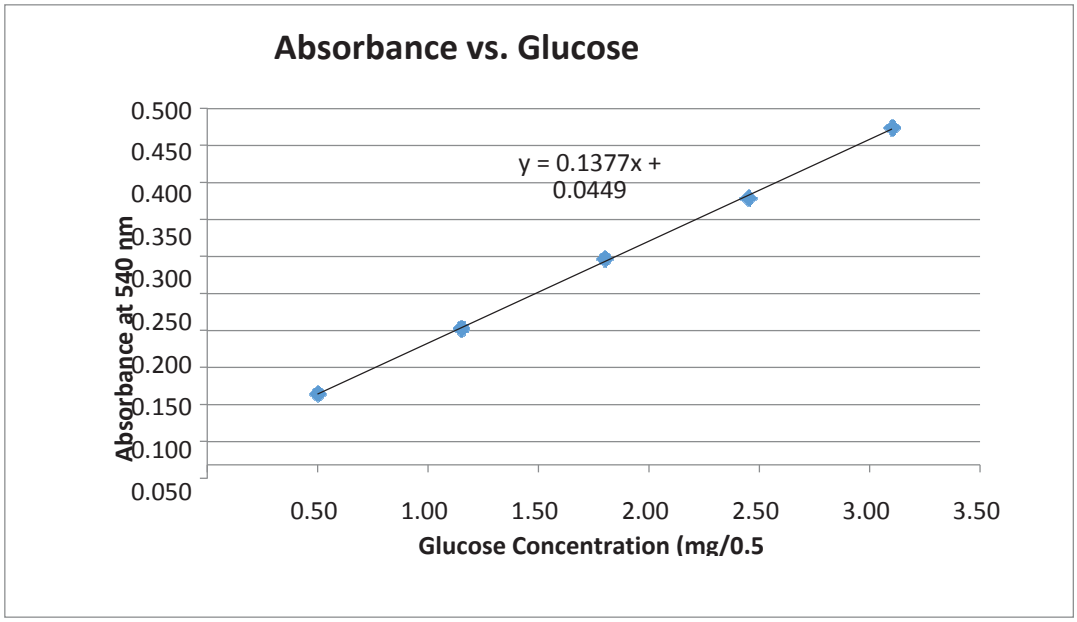


Ten grams pretreated corn stover #1

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on ten grams pretreated corn stover without wheat bran supplementation after seven days. Sample #1. Diluted with 75 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.465	0.485	0.470	0.473	0.010
2	2.450	0.364	0.3777	0.393	0.378	0.015
3	1.800	0.290	0.300	0.299	0.296	0.006
4	1.150	0.198	0.209	0.200	0.202	0.006
5	0.500	0.117	0.117	0.107	0.114	0.006

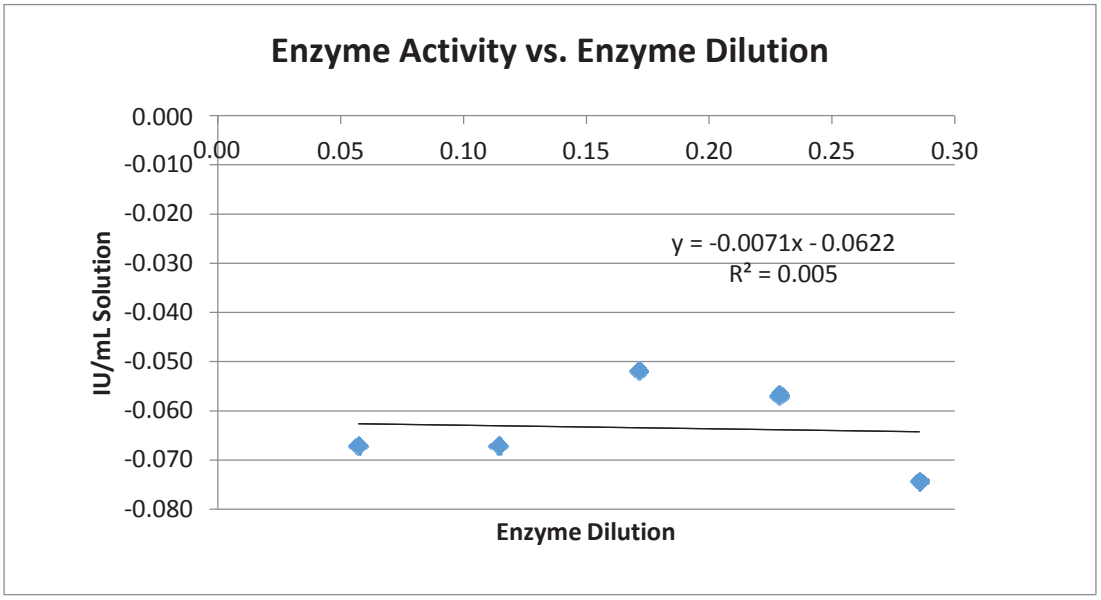


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.29	0.267	0.277	0.258	0.25	-0.290	-0.428	-0.486
0.23	0.216	0.207	0.225	0.24	-0.430	-0.299	-0.191
0.17	0.171	0.163	0.185	0.198	-0.418	-0.258	-0.164
0.11	0.133	0.133	0.127	0.138	-0.360	-0.404	-0.324
0.06	0.079	0.072	0.082	0.082	-0.411	-0.338	-0.338

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.29	-0.401	-0.074	0.00	0.00
0.23	-0.307	-0.057	-0.25	-1.34
0.17	-0.280	-0.052	-0.30	-1.63
0.11	-0.362	-0.067	-0.59	-3.17
0.06	-0.362	-0.067	-1.17	-6.34
		Average:	-0.46	-2.50



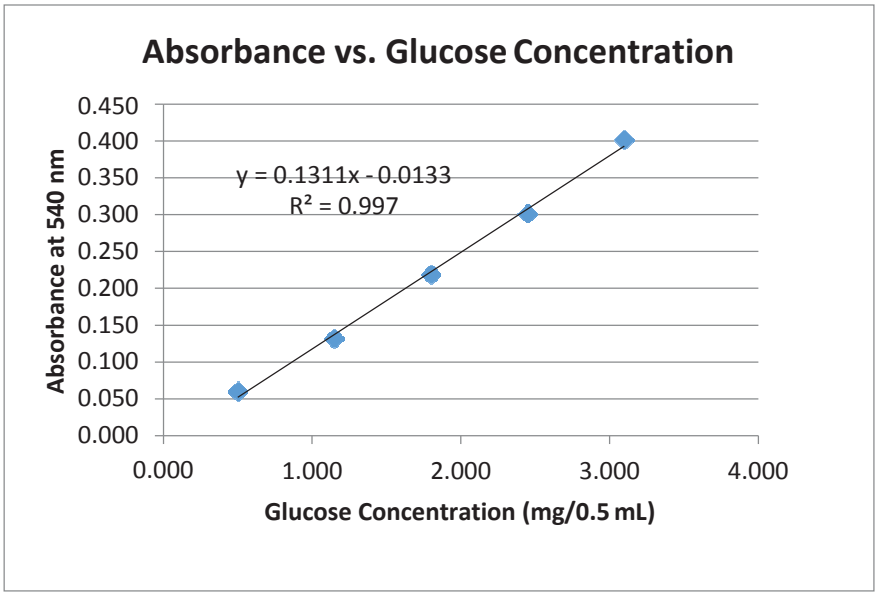
Ten grams pretreated corn stover #2

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on ten grams pretreated corn stover without supplementation for seven days. Sample #4.

Diluted with 45 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.377	0.392	0.435	0.401	0.030
2	2.450	0.283	0.274	0.347	0.301	0.040
3	1.800	0.206	0.246	0.204	0.219	0.024
4	1.150	0.106	0.145	0.145	0.132	0.023
5	0.500	0.057	0.064	0.059	0.060	0.004

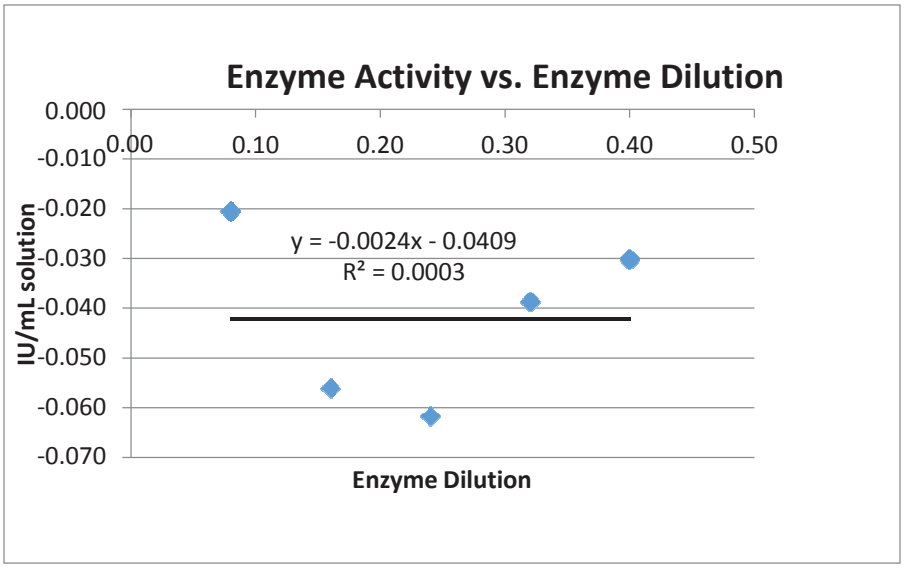


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.40	1.162	1.003	1.209	1.171	-1.114	0.457	0.168
0.32	1.058	0.959	0.991	1.103	-0.656	-0.412	0.442
0.24	0.796	0.684	0.751	0.783	-0.755	-0.244	0.000
0.16	0.574	0.463	0.581	0.518	-0.743	0.157	-0.323
0.08	0.266	0.225	0.26	0.228	-0.207	0.059	-0.185

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.40	-0.163	-0.030	-0.41	-0.08
0.32	-0.209	-0.039	-0.42	-0.12
0.24	-0.333	-0.062	-0.67	-0.26
0.16	-0.303	-0.056	-0.61	-0.35
0.08	-0.111	-0.021	-0.22	-0.26
		Average:	-0.46	-0.21

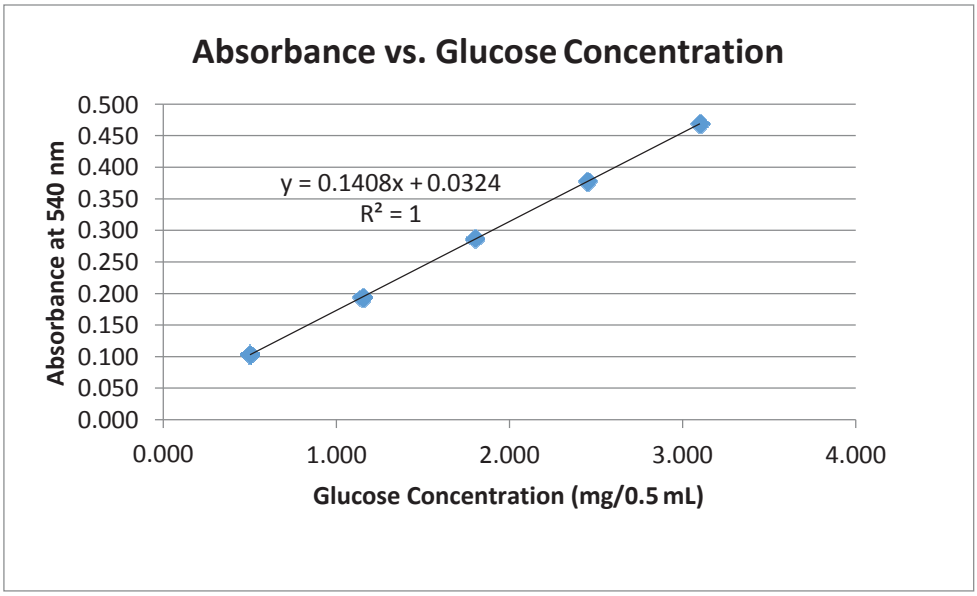


Ten grams pretreated corn stover #3

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on ten grams pretreated corn stover without wheat bran supplementation for seven days. Sample #3. Diluted with 40 mL for extraction.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.473	0.463	0.470	0.469	0.005
2	2.450	0.366	0.385	0.381	0.377	0.010
3	1.800	0.263	0.316	0.281	0.287	0.027
4	1.150	0.191	0.195	0.193	0.193	0.002
5	0.500	0.112	0.101	0.097	0.103	0.008

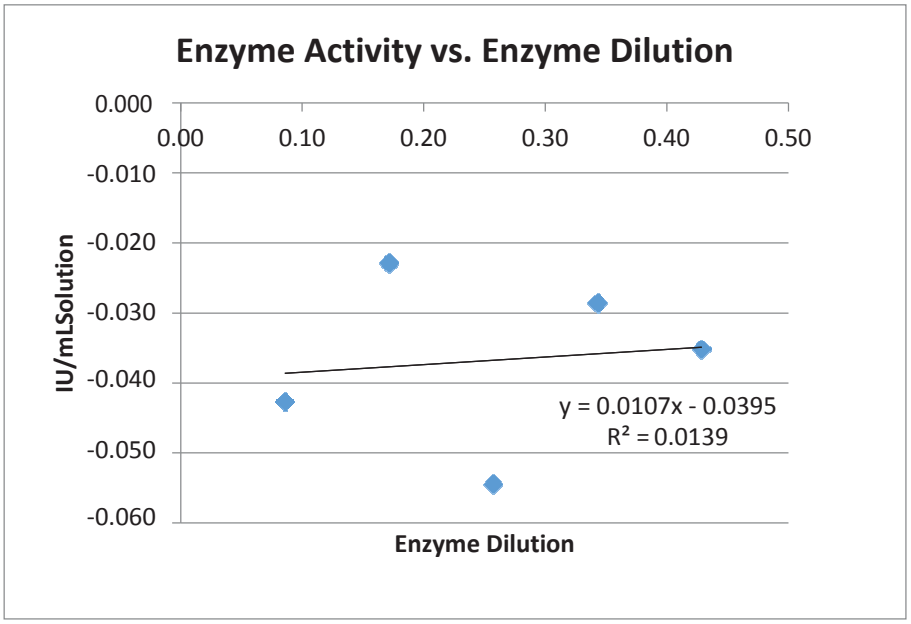


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.43	1.219	1.241	1.273	1.159	-0.071	0.156	-0.654
0.34	1.013	1.010	0.952	1.108	-0.249	-0.661	0.447
0.26	0.853	0.853	0.889	0.791	-0.232	0.023	-0.673
0.17	0.595	0.604	0.638	0.589	-0.169	0.073	-0.275
0.09	0.325	0.350	0.293	0.332	-0.053	-0.457	-0.180

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	-0.190	-0.035	-0.44	-0.08
0.34	-0.154	-0.029	-0.45	-0.08
0.26	-0.294	-0.054	-1.14	-0.21
0.17	-0.124	-0.023	-0.72	-0.13
0.09	-0.230	-0.043	-2.68	-0.50
		Average:	-1.09	-0.20

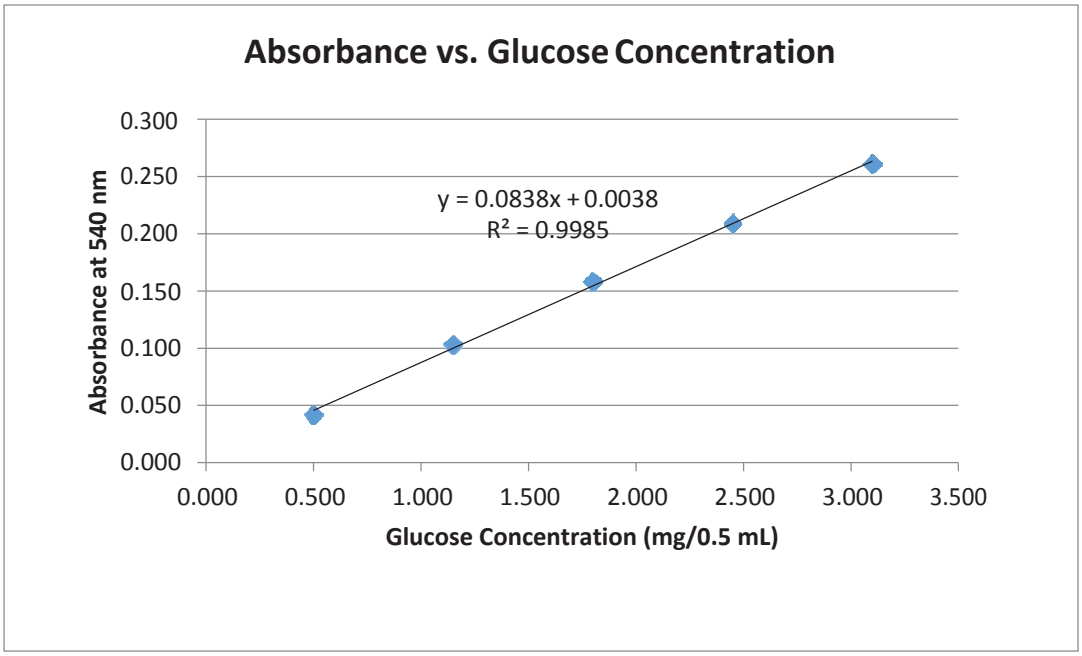


Ten grams unpretreated corn stover #1

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on ten grams unpretreated corn stover without supplementation with wheat bran for seven days. Sample #1. Diluted with 31 mL for extraction. Spectrophotometer samples were diluted 100µL samples to 2.600 mL RO water due to high absorbance of samples.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.262	0.259	0.262	0.262	0.055
2	2.450	0.208	0.21	0.209	0.208	0.038
3	1.800	0.155	0.159	0.161	0.155	0.020
4	1.150	0.101	0.100	0.108	0.101	0.012
5	0.500	0.041	0.041	0.043	0.041	0.010

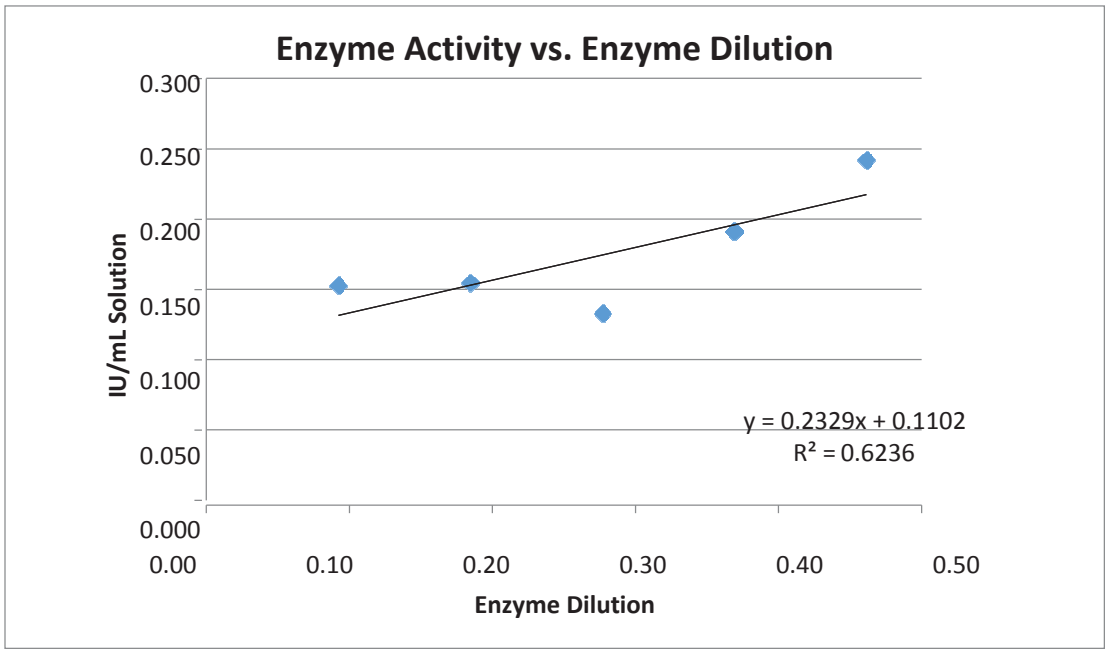


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.46	1.018	1.404	1.416	1.288	1.434	1.478	1.008
0.37	0.898	1.179	1.18	1.167	1.045	1.049	1.001
0.28	0.750	0.902	0.956	0.967	0.573	0.771	0.812
0.18	0.452	0.669	0.653	0.702	0.813	0.754	0.934
0.09	0.220	0.462	0.426	0.433	0.905	0.772	0.798

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.46	1.307	0.242	0.52	2.83
0.37	1.032	0.191	0.52	2.79
0.28	0.719	0.133	0.48	2.60
0.18	0.834	0.154	0.84	4.52
0.09	0.825	0.153	1.66	8.94
		Average:	4.34	0.80

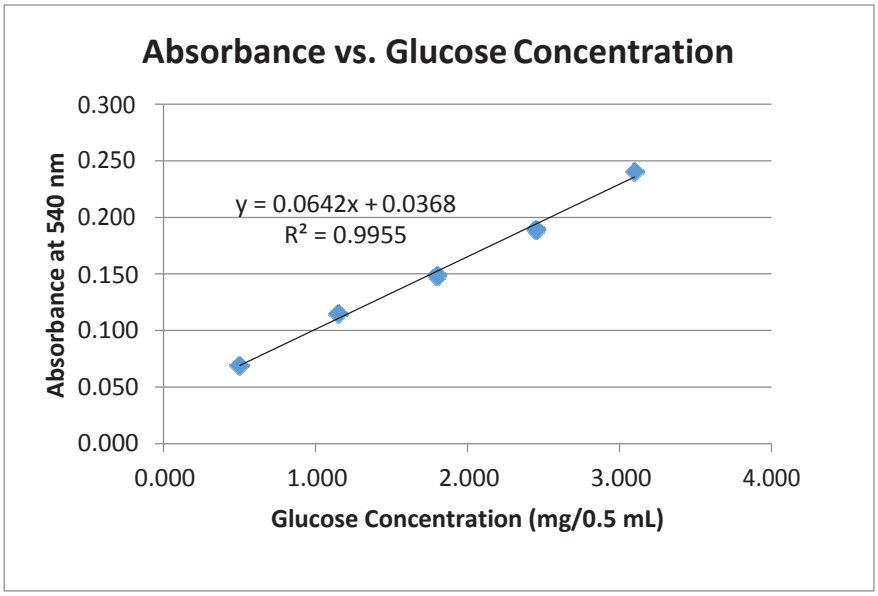


Ten grams unpretreated corn stover #2

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on ten grams unpretreated corn stover for seven days. Sample #2. Diluted with 40 mL for extraction. Spectrophotometer samples were diluted 100µL samples to 2.600 mL RO water due to high absorbance of samples.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.229	0.241	0.251	0.240	0.011
2	2.450	0.167	0.198	0.202	0.189	0.019
3	1.800	0.153	0.139	0.153	0.148	0.008
4	1.150	0.114	0.115	0.115	0.115	0.001
5	0.500	0.072	0.066	0.069	0.069	0.003

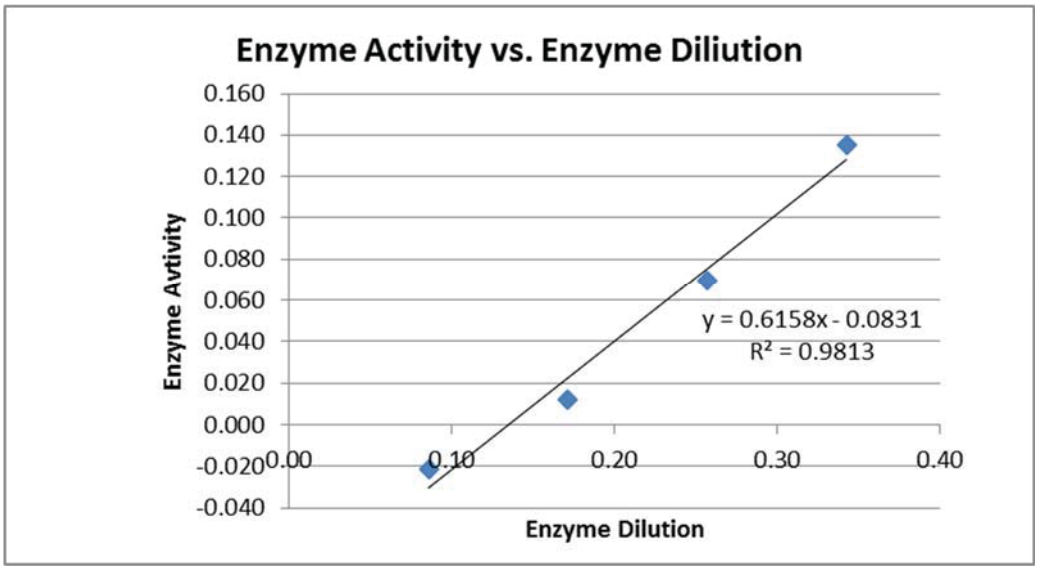


Unknown absorbance data. Enzyme dilutions, average absorbance of blanks at 540 nm, and absorbance of unknown samples are given. The glucose released was calculated using the trend line produced in the previous graph and the difference between absorbance of the unknown samples and the corresponding blank.

	Absorbance at 540 nm				Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.43	0.539	0.625	0.645	0.625	0.761	1.073	0.761
0.34	0.477	0.527	0.577	0.577	0.211	0.990	0.990
0.26	0.403	0.467	0.430	0.495	0.424	-0.153	0.860
0.17	0.305	0.353	0.366	0.318	0.180	0.382	-0.366
0.09	0.176	0.208	0.203	0.205	-0.075	-0.153	-0.121

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.43	0.865	0.160	2.02	0.37
0.34	0.730	0.135	2.13	0.39
0.26	0.377	0.070	1.47	0.27
0.17	0.065	0.012	0.38	0.07
0.09	-0.116	-0.022	-1.36	-0.25
		Average:	1.50	0.28

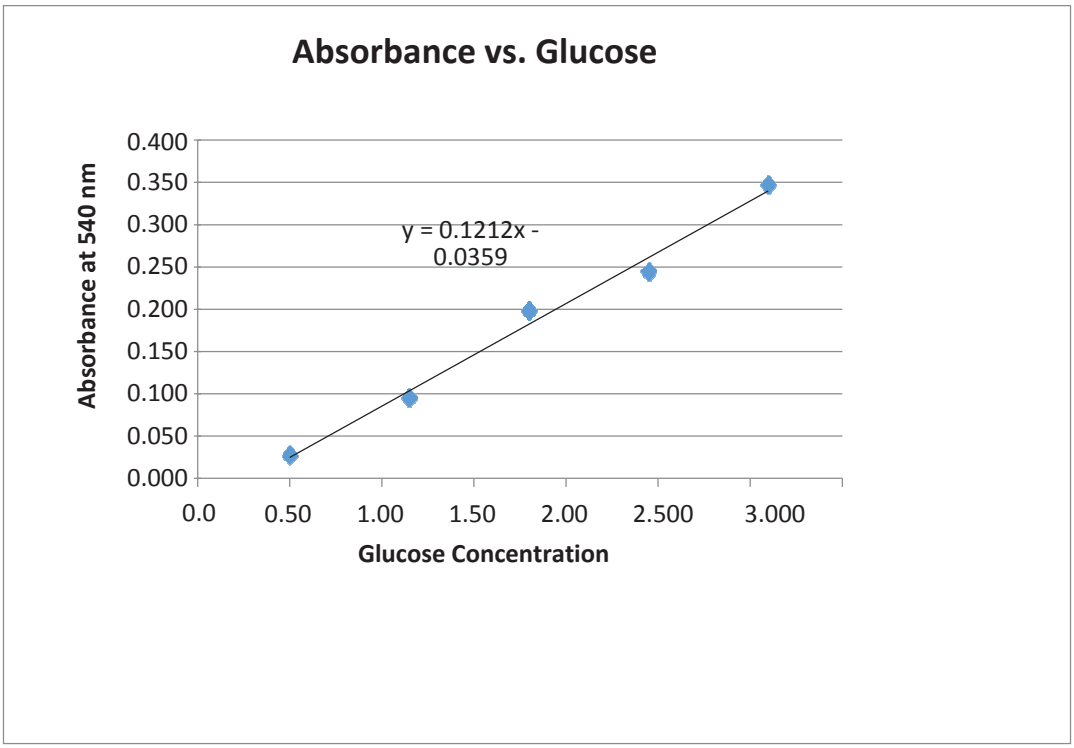


Ten grams pretreated corn stover #3

Cellulase activity assay data from *T. reesei* RUT-C30 cultivated on ten grams unpretreated corn stover without wheat bran supplementation for seven days. Sample #3. Diluted with 32 mL for extraction. Spectrophotometer samples were diluted 100µL samples to 2.600 mL RO water due to high absorbance of samples.

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration

Tube #	[Glucose] (mg/0.5mL)	Rep 1	Rep 2	Rep 3	Average	Std Dev
1	3.100	0.310	0.384	0.617	0.347	0.052
2	2.450	0.234	0.254	0.556	0.244	0.014
3	1.800	0.181	0.215	0.362	0.198	0.024
4	1.150	0.106	0.084	0.232	0.095	0.016
5	0.500	0.027	0.028	0.084	0.028	0.001

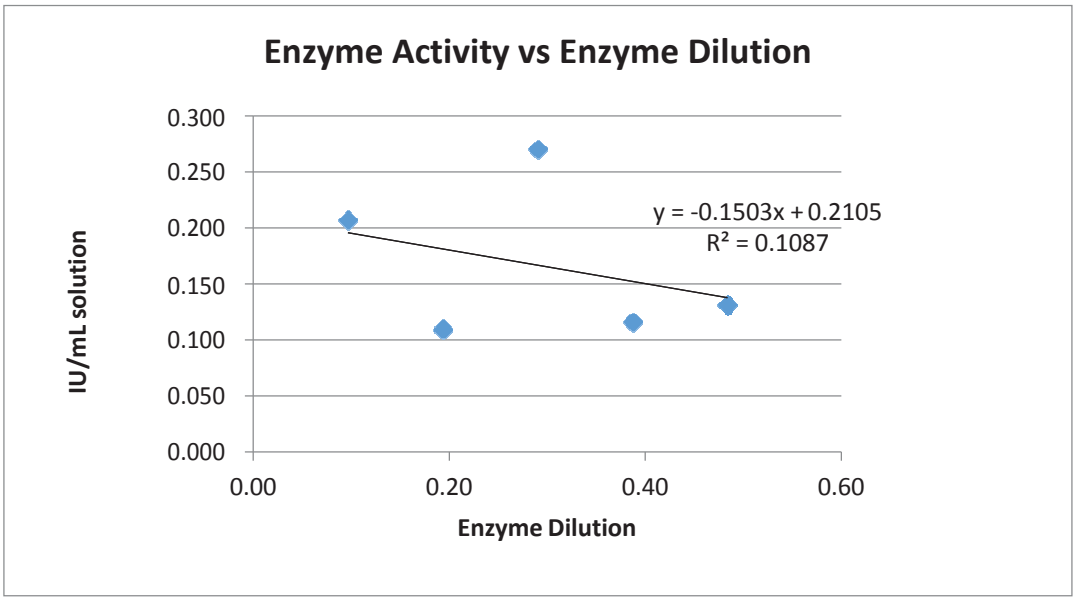


Absorbance data. Enzyme dilution, average absorbance of blanks at 540 nm, and absorbance at 540 of unknown samples are given. Glucose was calculated using the trend line produced in the previous graph.

		Absorbance at 540 nm			Glucose Released (mg/0.5 mL)		
Enzyme Dilution	Blank	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.48	0.956	0.984	1.017	1.018	0.524	0.797	0.805
0.39	0.858	0.89	0.904	0.9	0.560	0.676	0.643
0.29	0.601	0.748	0.765	0.712	1.512	1.652	1.215
0.19	0.468	0.488	0.513	0.509	0.465	0.672	0.639
0.10	0.219	0.331	0.293	0.331	1.223	0.910	1.223

Enzyme activity data. Corrected glucose released and corrected enzyme activity correct for the dilution during extraction and during the cellulase activity assay.

Enzyme Dilution	Average glucose released (mg/0.5 mL)	Average IU/mL	Corrected average glucose released (mg/0.5 mL)	Corrected average IU/mL
0.48	0.709	0.131	1.46	0.27
0.39	0.626	0.116	1.62	0.30
0.29	1.460	0.270	5.03	0.93
0.19	0.592	0.110	3.06	0.57
0.10	1.119	0.207	11.56	2.14
		Average:	4.55	0.84



Appendix E. Sample Cellulase Assay Calculations

The average light absorbance for each glucose standard was plotted against the known glucose concentrations in milligrams per half milliliter. A linear function was created. The R^2 was calculated to ensure accuracy of pipetting. The average absorbance of enzyme blank for each sample solution was calculated and subtracted from each corresponding enzyme solution. The difference between the absorbance at 540 nm was then used to calculate the glucose released by the enzyme. For example, if the average absorbance of the enzyme blanks prepared using an 80% sample solution with an overall dilution of 0.20 was 0.247 nm, an enzyme unknown absorbance from an 80% sample solution was 0.602, and the standard curve was y=0.1189x+0.0504, then the glucose released would be calculated as follows:

y=0.1189x+0.0504 mg/0.5 mL

y=0.602 nm – 0.247 nm

y=0.355 nm

x=(y-0.0504 mg/0.5 mL)/0.1189 nm

x=(0.355 nm-0.0504 mg/0.5 mL)/0.1189 nm

x=2.56 mg/0.5 mL

The glucose released was then used to calculate the International Units present in each milliliter of enzyme sample for each concentration by multiplying by two to find milligrams to milliliters, converting to micromoles, dividing by the hydrolysis time (sixty minutes), and converting to international units. For example:

(2.56 mg/0.5 mL glucose)*(2 0.5 mL/mL) = 5.12 mg/mL glucose

(5.12 mg/mL glucose)*(1000µg/mg)*(1 µmol glucose/180 µg glucose) = 28.4 µmol glucose

(28.4 µmol/60 min glucose)*(1 IU/(µmol/min glucose)) = 0.477 IU/mL

(0.477 IU/mL)/0.2 =2.38 IU/mL

Appendix F. Objective II Glucose and Preliminary Cellulase Activity Data

Rep	Dry weight corn stover (g)	Dry weight wheat bran (g)	Temperature during days 8-12 (°C)	Final Glucose Concentration (g/L)
1	3.0	2.0	50	8.06
2	3.0	2.0	50	10.5
3	3.0	2.0	50	11.8
1	3.0	2.0	30	1.01
2	3.0	2.0	30	1.28
3	3.0	2.0	30	0.35
1	4.0	1.0	50	13.0
2	4.0	1.0	50	13.8
3	4.0	1.0	50	15.3
1	4.0	1.0	30	0.54
2	4.0	1.0	30	0.30
3	4.0	1.0	30	0.92
1	5.0	0.0	50	29.0
2	5.0	0.0	50	28.2
3	5.0	0.0	50	41.0
1	5.0	0.0	30	1.86
2	5.0	0.0	30	2.20
3	5.0	0.0	30	6.14

Glucose Standard Data. Measured absorbance at 540 nm, averages, and standard deviations for glucose samples with given concentration						
1	3.100	0.174	0.1676	0.173	0.172	0.003
2	2.500	0.14	0.131	0.124	0.132	0.008
3	1.800	0.117	0.113	----	0.115	0.003
4	1.110	0.084	0.087	----	0.086	0.002
5	0.500	0.058	0.056	----	0.057	0.001
1	3.100	0.174	0.1676	0.173	0.172	0.003

Sample	Enzyme Dilution	Absorbance at 540 nm				Enzyme Activity (IU mL^{-1})
		Blank	Rep 1	Rep 2	Rep 3	
5-1-1	0.28	0.448	0.472	0.423	0.487	-0.39
5-2-1	0.50	0.556	0.659	0.635	0.664	0.53
5-2-2	0.39	0.553	0.537	0.600	0.552	-0.31
5-3-1	0.50	0.858	0.817	0.868	0.837	-0.48
5-3-2	0.32	0.769	0.680	0.802	0.750	-0.86
3-1-1	0.44	0.482	0.464	0.457	0.462	-0.58
3-1-2	0.40	0.360	0.370	0.377	0.432	-0.04
3-1-3	0.39	0.437	0.435	0.429	0.442	-0.44
3-2-1	0.41	0.510	0.527	0.520	0.581	-0.05
3-2-2	0.39	0.551	0.554	0.577	0.591	-0.16
3-2-3	0.29	0.900	0.959	0.935	0.877	-0.22
3-3-1	0.41	0.692	0.693	0.629	0.641	-0.81
3-3-2	0.32	0.458	0.489	0.491	0.494	-0.04

Samples are labeled A-B-C where A is the number of grams of 0.1N sodium hydroxide pretreated corn stover in the five gram sample; B is the replicate number in Objective II; and C is the replicate number in the cellulase measurement (i.e., sample 5-3-1 and 5-3-2 were taken from the same flask containing five grams of pretreated corn stover).

Appendix G. Statistical Analysis

The code used to perform statistical analysis using ANOVA and Tukey's range test in SAS Version 9.4 was:

```
data cas;
input rep pt wb iu glu;
cards;
1 1 4 0.39 1.89
2 1 4 1.05 4.51
3 1 4 1.29 2.22
1 0 4 0.73 2.52
2 0 4 1.17 2.54
3 0 4 1.29 1.98
1 1 3 1.21 1.56
2 1 3 1.08 4.03
3 1 3 1.93 0.91
1 0 3 0.91 2.08
2 0 3 0.48 1.63
3 0 3 1.13 2.11
1 1 2 0.57 3.53
2 1 2 1.57 3.03
3 1 2 1.05 3.35
1 0 2 0.70 6.32
2 0 2 1.03 5.72
3 0 2 0.67 2.77
1 1 1 0.77 1.87
2 1 1 1.04 5.40
3 1 1 0.70 4.76
1 0 1 0.39 3.69
2 0 1 0.80 5.76
3 0 1 0.86 4.86
1 1 0 0.00 21.04
2 1 0 0.00 19.08
3 1 0 0.00 17.57
1 0 0 0.50 19.13
2 0 0 0.28 16.89
3 0 0 0.92 11.82
;
run;
title "cas";
proc print data=cas;
run;
proc anova data= cas;
        class Rep Pt wb;
            model IU = pt wb pt*wb;
            means pt wb /tukey;
```

```
                means wb*pt/tukey;
run;
proc anova data = cas;
        class rep pt wb;
                model glu = pt wb pt*wb;
                means pt wb /tukey;
                means wb*pt/tukey;
run;
```

The effects of pretreatment, supplementation of each level of wheat bran (zero to four grams); and the interaction of pretreatment and wheat bran supplementation were examined. The code and tables produced use “rep” to refer to repetition number; “pt” to refer to pretreatment status of corn stover where “1” represents pretreated corn stover and “0” refers to unpretreated corn stover; “wb” refers to the mass of wheat bran used in the sample; “iu” refers to the enzyme activity of the sample before dilution in international units per milliliter; and “glu” refers glucose grams per milliliter in the sample before dilution. The term “pt*wb” was used to examine whether there was an interaction between pretreatment status of corn stover and the amount of wheat bran used for supplementation.

The code used in SAS 9.4 to analyze the data in Objective II was:

```
data hydrolysis;
input rep temp wb glu;
cards;
1    30    40    1.01
2    30    40    1.28
3    30    40    0.35
1    30    20    0.54
2    30    20    0.30
3    30    20    0.92
1    30    0     1.86
2    30    0     2.20
3    30    0     6.14
1    50    40    8.06
2    50    40    10.5
3    50    40    11.8
1    50    20    13.0
2    50    20    13.8
3    50    20    15.3
1    50    0     29.0
2    50    0     28.2
```

```
3       50      0       41.0
;
run;
title "hydrolysis"
proc print data=hydrolysis;
run;
proc glm data=hydrolysis;
        class rep temp wb;
                model glu= temp wb temp*wb;
                means temp*wb temp wb/tukey;
run;
proc anova data= obj2;
              class Rep temp wbpt;
                   model glu = temp wbpt temp*wbpt;
                   means temp wbpt temp*wbpt /tukey;
run;
proc boxplot data= hydrolysis;
        plot glu*temp;
        inset min mean max stddev;
        insetgroup Q1 Q3;
run;
```

The abbreviation “rep” referred to repetition number; “temp” referred to the temperature during the hydrolysis phase, “wb” referred to the percentage of wheat bran in the sample, and “glu” referred to the measured and calculated glucose concentration of the sample in grams per liter. “temp*wb” was used to analyze the interaction between the temperature during the hydrolysis phase and the percentage of wheat bran.

The code used in SAS 9.4 to compare the final glucose concentration of samples in Objective I with those which completed a hydrolysis phase at 50°C in Objective II was:

```
data compare;
input rep time wb glu;
cards;
1       7       40      1.89
2       7       40      4.51
3       7       40      2.22
1       7       20      3.53
2       7       20      3.03
3       7       20      3.35
1       7       0       21.0
2       7       0       19.1
```

```
3       7       0       17.6
1       12      40      8.06
2       12      40      10.5
3       12      40      11.8
1       12      20      13.1
2       12      20      13.8
3       12      20      15.3
1       12      0       29.0
2       12      0       28.2
3       12      0       41.0
;
run;
title "compare";
proc print data=compare;
```

The abbreviation “rep” referred to the repetition number of the sample, “time” referred to the number of total days passed since the original inoculation, “wb” referred to the percentage of wheat bran in the sample, and “glu” referred to the final glucose concentration in grams per liter.

Appendix H. Summary of Objective II results.

Rep	Time (d)	Temperature after 7 d (°C)	Wheat bran (%)	Glucose (g L^{-1})
1	12	30	40.0	1.01
2	12	30	40.0	1.28
3	12	30	40.0	0.35
1	12	30	20.0	0.54
2	12	30	20.0	0.30
3	12	30	20.0	0.92
1	12	30	0.0	1.86
2	12	30	0.0	2.20
3	12	30	0.0	6.14
1	12	50	40.0	8.06
2	12	50	40.0	10.5
3	12	50	40.0	11.8
1	12	50	20.0	12.98
2	12	50	20.0	13.82
3	12	50	20.0	15.26
1	12	50	0.0	29.0
2	12	50	0.0	28.2
3	12	50	0.0	41.0

References

Adney, B. and J. Baker (2008). Measurement of Cellulase Activities Laboratory Analytical Procedure (LAP). U. S. D. o. Energy. Golden, CO.

Awafo, V. A., et al. (1996). "Production of cellulase systems by selected mutants of Trichoderma reesei in solid-state fermentation and their hydrolytic potentials." Applied Biochemistry and Biotechnology **57**(1): 461-470.

Bailey, M. J. (1981). "The effect of β-glucosidase on some assays for cellulolytic enzymes." Biotechnology Letters **3**(12): 695-700.

Bailey, M. J., et al. (1992). "Interlaboratory testing of methods for assay of xylanase activity." Journal of biotechnology **23**(3): 257-270.

Bansal, N., et al. (2011). "A novel strain of Aspergillus niger producing a cocktail of hydrolytic depolymerising enzymes for the production of second generation biofuels." BioResources **6**(1): 552-569.

Baral, N. R. and A. Shah (2014). "Microbial inhibitors: formation and effects on acetone-butanol-ethanol fermentation of lignocellulosic biomass." Applied Microbiology and Biotechnology **98**(22): 9151-9172.

Binder, J. B. and R. T. Raines (2010). "Fermentable sugars by chemical hydrolysis of biomass." Proceedings of the National Academy of Sciences **107**(10): 4516-4521.

Bolado-Rodríguez, S., et al. (2016). "Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse." Bioresource technology **201**: 182-190.

Brijwani, K., et al. (2010). "Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran." Process Biochemistry **45**(1): 120-128.

Brown, R. C. and T. R. Brown (2003). Biorenewable resources: engineering new products from agriculture, John Wiley & Sons.

Brown, R. C. and T. R. Brown (2012). Why are We Producing Biofuels?: Shifting to the Ultimate Source of Energy, Brownia LLC.

Bull, S. R. (1991). "The US Department of Energy biofuels research program." Energy sources **13**(4): 433-442.

Camassola, M. and A. Dillon (2007). "Production of cellulases and hemicellulases by Penicillium echinulatum grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation." Journal of Applied Microbiology **103**(6): 2196-2204.

Capehart, T. (2016). Feed Grains Data: Yearbook Tables. USDA Economic Research Service.

Chahal, D. (1985). "Solid-state fermentation with Trichoderma reesei for cellulase production." Applied and Environmental Microbiology **49**(1): 205-210.

Considine, P., et al. (1988). "Hydrolysis of beet pulp polysaccharides by extracts of solid-state cultures of Pencicillium capsulatum." Biotechnology and bioengineering **31**(5): 433-438.

Coward-Kelly, G., et al. (2003). "Suggested improvements to the standard filter paper assay used to measure cellulase activity." Biotechnology and bioengineering **82**(6): 745-749.

Dashtban, M., et al. (2011). "Effect of different carbon sources on cellulase production by Hypocrea jecorina (Trichoderma reesei) strains." International journal of biochemistry and molecular biology **2**(3): 274.

Dashtban, M., et al. (2010). "Cellulase activities in biomass conversion: measurement methods and comparison." Critical reviews in biotechnology **30**(4): 302-309.

Deswal, D., et al. (2011). "Optimization of cellulase production by a brown rot fungus Fomitopsis sp. RCK2010 under solid state fermentation." Bioresource technology **102**(10): 6065-6072.

Dhillon, G. S., et al. (2012). "Lactoserum as a moistening medium and crude inducer for fungal cellulase and hemicellulase induction through solid-state fermentation of apple pomace." Biomass and bioenergy **41**: 165-174.

Dhillon, G. S., et al. (2011). "Apple pomace ultrafiltration sludge–A novel substrate for fungal bioproduction of citric acid: Optimisation studies." Food Chemistry **128**(4): 864-871.

Dhillon, G. S., et al. (2012). "Improved xylanase production using apple pomace waste by Aspergillus niger in Koji fermentation." Engineering in Life Sciences **12**(2): 198-208.

Dhillon, G. S., et al. (2012). "Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation." Industrial Crops and Products **38**: 6-13.

Dhillon, G. S., et al. (2011). Value-addition of agricultural wastes for augmented cellulase and xylanase production through solid-state tray fermentation employing mixed-culture of fungi.

Dionisi, D., et al. (2014). "The potential of microbial processes for lignocellulosic biomass conversion to ethanol: a review." Journal of Chemical Technology and Biotechnology **90**(3): 366-383.

Domingues, F., et al. (2000). "The influence of culture conditions on mycelial structure and cellulase production by Trichoderma reesei Rut C-30." Enzyme and Microbial Technology **26**(5): 394-401.

dos Santos, T. C., et al. (2013). "Application of response surface methodology for producing cellulolytic enzymes by solid-state fermentation from the puple mombin (Spondias purpurea L.) residue." Food Science and Biotechnology **22**(1): 1-7.

EIA (2016, March 11, 2016). "International Energy Outlook 2016." Retrieved May 31, 2016.

Elshafei, A. M., et al. (1991). "The saccharification of corn stover by cellulase from Penicillium funiculosum." Bioresource technology **35**(1): 73-80.

Ezeji, T., et al. (2003). "Production of acetone, butanol and ethanol by Clostridium beijerinckii BA101 and in situ recovery by gas stripping." World Journal of Microbiology and Biotechnology **19**(6): 595-603.

Ghose, T. (1987). "Measurement of cellulase activities." Pure and applied Chemistry **59**(2): 257-268.

Guo, T., et al. (2012). "Clostridium beijerinckii mutant with high inhibitor tolerance obtained by low-energy ion implantation." Journal of industrial microbiology & biotechnology **39**(3): 401-407.

Gutierrez-Correa, M. and R. P. Tengerdy (1997). "Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation." Biotechnology Letters **19**(7): 665-667.

Hendriks, A. and G. Zeeman (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass." Bioresource technology **100**(1): 10-18.

Hill, J., et al. (2006). "Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels." Proceedings of the National Academy of Sciences **103**(30): 11206-11210.

Holker, U., et al. (2004). "Biotechnological advantages of laboratory-scale solid-state fermentation with fungi." Applied Microbiology and Biotechnology **64**(2): 175-186.

Holtzapple, M., et al. (1990). "Inhibition of Trichoderma reesei cellulase by sugars and solvents." Biotechnology and bioengineering **36**(3): 275-287.

Jessup, R. W. (2009). "Development and status of dedicated energy crops in the United States." In Vitro Cellular & Developmental Biology-Plant **45**(3): 282-290.

Juhasz, T., et al. (2005). "Characterization of cellulases and hemicellulases produced by Trichodermareesei on various carbon sources." Process Biochemistry **40**(11): 3519-3525.

Kehail, A. and C. Brigham (2015). "Biofuels Production Process: Microorganisms Utilizing Carbon to Produce Butanol and Ethanol." Int. J. of Thermal & Environmental Engineering **10**(1): 57-62.

Kilikian, B., et al. (2014). "Filamentous fungi and media for cellulase production in solid state cultures." Brazilian Journal of Microbiology **45**(1): 279-286.

Krishna, S. H., et al. (2001). "Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast." Bioresource technology **77**(2): 193-196.

Kubicek, C. P. (1992). The cellulase proteins of Trichoderma reesei: structure, multiplicity, mode of action and regulation of formation. Enzymes and Products from Bacteria Fungi and Plant Cells, Springer**:** 1-27.

Kumar, P., et al. (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production." Industrial & Engineering Chemistry Research **48**(8): 3713-3729.

Leisola, M. and M. Linko (1976). "Determination of the solubilizing activity of a cellulase complex with dyed substrates." Analytical biochemistry **70**(2): 592-599.

Lever, M., et al. (2010). "Ethanol from lignocellulose using crude unprocessed cellulase from solid-state fermentation." Bioresource technology **101**(18): 7083-7087.

Li, Y., et al. (2004). "Enzymatic hydrolysis of corn stover pretreated by combined dilute alkaline treatment and homogenization." TRANSACTIONS-AMERICAN SOCIETY OF AGRICULTURAL ENGINEERS **47**(3): 821-826.

Ma, K. and Z. Ruan (2015). "Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi." Bioresource technology **175**: 586-593.

Maurya, D. P., et al. (2012). "Optimization of solid state fermentation conditions for the production of cellulase by Trichoderma reesei." 5-8.

Miao, Z., et al. (2011). "Energy requirement for comminution of biomass in relation to particle physical properties." Industrial Crops and Products **33**(2): 504-513.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar." Analytical chemistry **31**(3): 426-428.

Modenbach, A. (2013). "Sodium hydroxide pretreatment of corn stover and subsequent enzymatic hydrolysis: An investigation of yields, kinetic modeling and glucose recovery."

Montgomery, D. C. (2013). Design and analysis of experiments, John Wiley & Sons.

Moosavi-Nasab, M. and M. Majdi-Nasab (2008). "Cellulase Production by Trichoderma reesei using Sugar Beet Pulp." Iran Agricultural Research **25**(1.2): 107-116.

Moreno, A. D., et al. (2015). "A review of biological delignification and detoxification methods for lignocellulosic bioethanol production." Critical reviews in biotechnology **3**(35): 342-354.

Mosier, N., et al. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresource technology **96**(6): 673-686.

Nelson, D. L., et al. (2008). Lehninger principles of biochemistry, Macmillan.

Nogawa, M., et al. (2001). "L-Sorbose induces cellulase gene transcription in the cellulolytic fungus Trichoderma reesei." Current genetics **38**(6): 329-334.

Pachauri, R. K., et al. (2014). "Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change."

Pandey, A., et al. (2000). "New developments in solid state fermentation: I-bioprocesses and products." Process Biochemistry **35**(10): 1153-1169.

Qureshi, N., et al. (2010). "Production of butanol (a biofuel) from agricultural residues: Part II - Use of corn stover and switchgrass hydrolysates." Biomass and bioenergy **34**(4): 566-571.

Rana, V., et al. (2014). "Comparison of SHF and SSF of wet exploded corn stover and loblolly pine using in-house enzymes produced from T. reesei RUT C30 and A. saccharolyticus." SpringerPlus **3**(1): 516.

Rathmann, R. g., et al. (2010). "Land use competition for production of food and liquid biofuels: An analysis of the arguments in the current debate." Renewable Energy **35**(1): 14-22.

Reese, E. and A. Maguire (1969). "Surfactants as stimulants of enzyme production by microorganisms." Applied microbiology **17**(2): 242-245.

Saqib, A. A. N. and P. J. Whitney (2011). "Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono-and di-saccharide sugars." Biomass and bioenergy **35**(11): 4748-4750.

Selig, M. J., et al. (2009). "The effect of lignin removal by alkaline peroxide pretreatment on the susceptibility of corn stover to purified cellulolytic and xylanolytic enzymes." Applied Biochemistry and Biotechnology **155**(1-3): 94-103.

Shrestha, P., et al. (2015). "Fungi isolated from Miscanthus and sugarcane: biomass conversion, fungal enzymes, and hydrolysis of plant cell wall polymers." Biotechnology for biofuels **8**(1): 38.

Shrestha, P., et al. (2010). "Ethanol production via in situ fungal saccharification and fermentation of mild alkali and steam pretreated corn fiber." Bioresource technology **101**(22): 8698-8705.

Shrestha, P., et al. (2009). "Enzyme production by wood-rot and soft-rot fungi cultivated on corn fiber followed by simultaneous saccharification and fermentation." Journal of agricultural and food chemistry **57**(10): 4156-4161.

Singhania, R. R., et al. (2007). "Improved cellulase production by Trichoderma reesei RUT C30 under SSF through process optimization." Applied Biochemistry and Biotechnology **142**(1): 60-70.

Singhania, R. R., et al. (2010). "Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases." Enzyme and Microbial Technology **46**(7): 541-549.

Singhania, R. R., et al. (2006). "Solid-state fermentation of lignocellulosic substrates for cellulase production by Trichoderma reesei NRRL 11460." Indian journal of Biotechnology **5**(3): 332-336.

Sukumaran, R. K., et al. (2009). "Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production." Renewable Energy **34**(2): 421-424.

Taha, M., et al. (2015). "Enhanced Biological Straw Saccharification Through Coculturing of Lignocellulose-Degrading Microorganisms." Applied Biochemistry and Biotechnology **175**(8): 3709-3728.

Teixeira, R. S. S., et al. (2012). "Amino acids interference on the quantification of reducing sugars by the 3, 5-dinitrosalicylic acid assay mislead carbohydrase activity measurements." Carbohydrate research **363**: 33-37.

Thirmal, C. and Y. Dahman (2012). "Comparisons of existing pretreatment, saccharification, and fermentation processes for butanol production from agricultural residues." The Canadian Journal of Chemical Engineering **90**(3): 745-761.

Tian, F., et al. (2015). "Comparative secretome analysis of Fusarium sp. Q7-31T during liquid fermentation using oat straw as a carbon source." Annals of Microbiology: 1-10.

Tyson, K. S. (1993). Fuel cycle evaluations of biomass-ethanol and reformulated gasoline. , National Renewable Energy Lab., Golden, CO (United States); Oak Ridge National Lab., TN (United States); Pacific Northwest Lab., Richland, WA (United States). **I**.

van Tilbeurgh, H., et al. (1982). "The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes." FEBS letters **149**(1): 152-156.

Varga, E., et al. (2002). "Chemical pretreatments of corn stover for enhancing enzymatic digestibility." Applied Biochemistry and Biotechnology **98**(1-9): 73-87.

Vintila, D., et al. (2014). "Sorghum bagasse as substrate for cellulase production by submerged and solid-state cultures of Trichoderma." Scientific Papers Animal Science and Biotechnologies **47**(1): 121-125.

Vinzant, T., et al. (2001). "Fingerprinting Trichoderma reesei hydrolases in a commercial cellulase preparation." Applied Biochemistry and Biotechnology **91**(1-9): 99-107.

Von Sivers, M. and G. Zacchi (1995). "A techno-economical comparison of three processes for the production of ethanol from pine." Bioresource technology **51**(1): 43-52.

Wahid, M. Z. A., et al. (2011). "Factors affecting endoglucanase production by Trichoderma reesei RUT C-30 from solid state fermentation of oil palm empty fruit bunches using Plackett-Burman design." African Journal of Biotechnology **10**(46): 9402-9409.

Wallace, J. (1991). "Phenol." Kirk-Othmer encyclopedia of chemical technology.

Wang, Z., et al. (2013). "Butanol production from wheat straw by combining crude enzymatic hydrolysis and anaerobic fermentation using Clostridium acetobutylicum ATCC824." Energy & Fuels **27**(10): 5900-5906.

Wen, Z., et al. (2014). "A novel strategy for sequential co-culture of Clostridium thermocellum and Clostridium beijerinckii to produce solvents from alkali extracted corn cobs." Process Biochemistry **49**(11): 1941-1949.

Wooley, R., et al. (1999). "Process design and costing of bioethanol technology: a tool for determining the status and direction of research and development." Biotechnology Progress **15**(5): 794-803.

Xie, L., et al. (2015). "Efficient hydrolysis of corncob residue through cellulolytic enzymes from Trichoderma strain G26 and l-lactic acid preparation with the hydrolysate." Bioresource technology **193**: 331-336.

Yang, L., et al. (2015). "Challenges and strategies for solid-state anaerobic digestion of lignocellulosic biomass." Renewable and Sustainable Energy Reviews **44**: 824-834.

Yoon, L. W., et al. (2014). "Fungal solid-state fermentation and various methods of enhancement in cellulase production." Biomass and bioenergy **67**: 319-338.

Zhang, Y. H. P. and L. R. Lynd (2004). "Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems." Biotechnology and bioengineering **88**(7): 797-824.

Vita

Danielle I. Empson

Place of Birth: Chloe, West Virginia

Education

University of Kentucky Master of Science in Biosystems and Agricultural Engineering
Lexington, KY Anticipated Graduation August 2016, GPA: 3.92

West Virginia University Bachelor of Science in Animal and Nutritional Sciences
Morgantown, WV Graduated December 2012
Dean's List Spring 2010 and Spring 2011

Research Experience

Graduate Research Assistant, University of Kentucky January 2014 - present
Biochemical engineering research in and out of laboratory setting; design of experiments, analysis, and procedures

Undergraduate Research Assistant, West Virginia University May 2012 - August 2012
Nutritional biochemistry research in laboratory setting; presented scientific findings to other SURE researchers at end of summer

Leadership Activities

President's Sustainability Advisory Council August 2015 – May 2016
- Appointed to represent student body on council with administrators, faculty, and staff
- Worked in Subcommittee with UK Greenthumb to write Greenhouse Gas Emissions Reduction Commitment for UK
- Upheld the university's commitment to create policies and programs to advance economic vitality, ecological integrity and social equity

Greenthumb Student Environmental Group August 2014 – May 2016
- Presented and gained approval for A Resolution Relating To: Environmental Climate at the University of Kentucky to Student Government Association in favor of UK adopting a climate action plan
- Selected to represent group in meeting with UK president and other top administrators
- Elected as Student Sustainability Council Representative

Co-chair, Kentucky Student Environmental Coalition December 2014 – June 2016
- Co-wrote successful Patagonia Environmental Grant ($8,000)
- Planned and facilitated bi-monthly conference calls with 3-8 participants
- Worked with coalition partners to strategize and organize Clean Energy Lobby Day with 60 citizen participants in 2015 and 20 student participants in 2016

Student Sustainability Council August 2015 – May 2016
- Served as Director of Operations by facilitating general and directors' meetings, organizing and distributing proposals, and enforcing attendance policy
- Worked with Sustainability Director and council to allocate $150,000 from the Student Sustainability Fee through voting process

Vice President, Alpha Epsilon Honor Society April 2015 – May 2016

Volunteered at local school for E-day; volunteered for science and engineering fairs; writing department newsletter; adopting a butterfly garden

Information technologist, Graduate Student Congress January 2014 – May 2014
Co-planned Graduate Student Appreciation Week, maintained listserv and website

Member, American Society of Agricultural and Biological Engineers

Scholarships
UK Education Abroad Scholarship, $1500 Summer 2014
ASABE Student Branch Education Abroad Scholarship, $2300 Summer 2014
PROMISE Scholarship, $4750/semester Spring 2010 - Spring 2011