This is to certify that the
thesis entitled

HYDROLYSIS OF LIGNOCELLULOSE USING ENZYMES FROM PELLETIZED *TRICHODERMA REESEI* FERMENTATION

presented by

Ying Liu

has been accepted towards fulfillment
of the requirements for the

MS degree in Biosystems Engineering

Major Professor's Signature

05/13/10

Date

HYDROLYSIS OF LIGNOCELLULOSE USING ENZYMES FROM PELLETIZED *TRICHODERMA REESEI* FERMENTATION

By

Ying Liu

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Biosystems Engineering

2010

ABSTRACT

HYDROLYSIS OF LIGNOCELLULOSE USING ENZYMES FROM PELLETIZED *TRICHODERMA REESEI* FERMENTATION

By

Ying Liu

Pelleted *Trichoderma reesei* is a desirable morphology for industrial fungal fermentation to obtain stable cellulase production. Two common cellulosic feedstocks, corn stover and switchgrass were pretreated by acid, alkaline and ammonia fiber expansion (AFEX) methods. The pretreated cellulosic feedstocks were used as fermentation substrates for enzyme production. It has been proved in this study that 15 g/L AFEX corn stover in fermentation medium could stimulate the fungi to yield relatively high cellulase (1.08 U/mL at 93 hours) and xylanase (2.52 U/mL at 24 hours) activities. The enzyme cocktails from pelletized *Trichoderma reesei* fermentation were applied on the pretreated cellulosic feedstocks to produce mono-sugars. A comparison of enzyme cocktails from different pretreated feedstocks and two enzymes sources was conducted to elucidate the effects of pretreated cellulosic materials on enzyme production and enzymatic hydrolysis. The enzyme cocktail (10 U/g dry mass) generated from AFEX corn stover has better enzyme compositions that enhance its hydrolysis especially working on the alkaline pretreated switchgrass which pretreated under 1hour, 121°C, 1% sodium hydrate solution.

TABLE OF CONTENTS

List of Tables

List of Figures

1. Introduction

1.1. Current Transportation Fuels

1.1.1. Fossil Fuels

Since fossil fuels have been commercially produced in the early twentieth century, they have dominated the transportation energy market due to their availability, high energy density, and easy distribution. As the world population has increased in the past several decades, and more developing countries are on the way towards industrialization, the supply of fossil fuels has shrunk. This petroleum shortage could severely hurt the development of the world economy that is powered by the petroleum based fuels. In addition, the consumption of non-renewable petroleum also leads to environmental impacts, such as green house gas emission. The 2010 Copenhagen Conference illustrated that carbon dioxide as one of the major green house gases; it is responsible for at least 50% of the causes to the global climate change (BRDI. 2006). In the United States, around 33% U.S. carbon dioxide emissions is attributed to petroleum-based transportation fuels (Wang et al. 2002). Thus, considering both economic and environmental impacts, renewable energy sources are urgently needed to diversify our energy resources and reduce the negative environmental impacts of fossil fuels.

1.1.2. Starch Ethanol

Ethanol is one of the approved renewable transportation fuels that are currently available in the marketplace. Fuel ethanol, from starch, has been commercially produced since the 1980s. In the past 20 years, starch ethanol production steadily increased. The fuel ethanol

industry in the U.S. is currently producing around 20 billion gallons per year, and it is anticipated that the demands for fuel ethanol would be more than 24 billion gallons/year by 2020 (BRDI 2006). However, starch crops are major food sources. If more starch crops are used for fuel, less starch is available for food production. Following the steady increase of the world population, starch-based crops (such as corn, wheat, cassava, rye and barley) are still not sufficient to satisfy their demands as animal and human foods. Using starch-based crops to increase fuel ethanol production is obviously neither sustainable nor practical (Keshwani et al. 2009).

1.1.3. Lignocellulosic ethanol

Lignocellulose, the most abundant renewable resource on the planet, provides an untapped reserve for bio-fuel production. Agricultural residues, such as crop residues, grasses, sawdust, and wood chips, are the major sources of it (Hofrichter et al. 2002). Due to their high carbohydrate contents, relatively low cost, and worldwide availability, agricultural residues attract significant interest regarding fuel ethanol production. It was estimated that utilizing lignocellulosic materials produce bioethanol could replace up to 40% of the fuel consumption in the United States market (Zheng et al. 2009). Approximate 80-90 billion gallons of fuel ethanol could be annually produced from 1.3 billion tons of available lignocellulosic residues available in the United States. In addition, lignocellulosic ethanol as an alternative fuel also would reduce 86% of the greenhouse gas emission currently generated from fossil fuel consumption (Sun et al. 2002).

1.2. Objectives of This Study

In response to the need of lignocellulosic fuel ethanol production, an environmentally friendly biological process to achieve the highly efficient conversion of carbohydrates in agricultural residues to fermentable sugars for ethanol production needs to be developed. Thus, this study will investigate cellulase and xylanase production using pelletized fungal fermentation, and subsequently apply the enzymes to convert pretreated lignocellulosic biomass into mono-sugars for ethanol production.

The objectives of the study mainly focused on enhancing cellulase and xylanase production on diluted alkaline treated switchgrass and AFEX (Kumar and Wyman) treated fiber using pelletized fungal culture, and improving enzymatic hydrolysis on pretreated cellulosic feedstocks (Esteghlalian, Hashimoto et al.).

1. 3. Pretreatment

1. 3.1. The composition of lignocellulosic material

Fifty to eighty percent of Lignocellulosic materials consist of three polymers of cellulose, hemicellulose and lignin. The three polymers tightly pack together to form a stable and recalcitrant matrix structure. Cellulose is a linear polymer of D-glucose units that linked by beta-1, 4 glycosidic bonds. The polymer has different levels of polymerization degree and crystallinity. Higher polymerization degree and crystallinity, less soluble and degradable cellulose is. Hemicellulose is a complex carbohydrate that links lignin and cellulose fibers. It mainly consists of two polymers of pentoses (D-xylose, D-arabinose) and hexoses (D-mannose, D-glucose, D-galactose). The ratios of pentoses and hexoses in hemicellulose are varied with respect to different sources, for instance, hardwood and

agricultural plants contain more xylan, while softwood has more glucomannan. Hemicellulose is also a linear polymer with short branches. The differences of structure and composition make hemicellulose much easier to be hydrolyzed than cellulose. Lignin is another major component of lignocellulosic materials. It is an amorphous heteropolymer that consists of three units (p-coumaryl, coniferyl and sinapyl alcohol). In a plant, lignin is closely bound to cellulose and hemicellulose makes which make the plant structure stiff, and be able to prevent the plant from microbial attack and enzymatic degradation (Sun et al. 2002).

1. 3.2. Pretreatment

Due to the facts of complicated composition and structure that lignocellulosic materials have (Table 1.), a pretreatment step must be implemented prior to enzymatic conversion of cellulose and hemicellulose components into sugars and further fermentation of ethanol production. Thermal and chemical pretreatment methods were widely used to break down the matrix structure and remove the lignin fraction of lignocellulosic materials. This section presented a brief description of different thermal and chemical pretreatment methods such as steam explosion, ammonia fiber expansion (AFEX), carbon dioxide expansion, acid and alkaline treatments, and ozonolysis etc based on the key factors of selecting effective pretreatment methods for lignocellulosic materials (Table 2.)

1.3.2.1 Steam explosion

High-pressure steam is applied on lignocellulosic materials for a short time period (Sun et al. 2002). At the end of treatment, the pressure will be suddenly released that causes an explosive destruction of the material, which leads to a relatively loose structure. Enzyme

and other catalysts can easily access to attack cellulose and hemicellulose to generate mono-sugars.

1.3.2.2 Ammonia fiber expansion (AFEX)

The ammonia fiber expansion (AFEX) method applies liquid ammonia at mild temperature and high pressure for a short period of time, followed by a sudden release of the pressure (Gao et al. 2009). AFEX process solubilizes lignin and increase surface area that will significantly enhance the following enzymatic conversion of cellulose and hemicellulose into mono-sugars. Even though, the AFEX process was not very effective for the biomass with high lignin content, such as woody biomass, it can effectively improve the enzymatic hydrolysis for most of agricultural lignocellulose such as switchgrass and corn stover.

1.3.2.3 Carbon dioxide expansion

CO2 expansion is another high pressure treatment similar with AFEX and steam explosion (Sun et al. 2002). It has advantages of using non-toxic chemicals and generating non-inhibitory compounds compared to AFEX and steam explosion. The carbonic acid produced during the carbon dioxide expansion, to some degree, helps increase the reaction rate of following enzymatic hydrolysis. However, in terms of hydrolysis performance, CO2 expansion is still not as effective as AFEX and steam explosion.

1.3.2.4 Ozonolysis

Ozonolysis is a chemical method to pretreat lignocellulosic materials (Sun et al. 2002). It can remove lignin under the mild condition like room temperature and pressure. The

reaction is only limited to lignin, and has no negative impacts on cellulose and hemicelluloses (Sun et al. 2002). The ozonolysis reaction has a relatively high efficiency to remove lignin. However, the requirement of large amount of ozone leads to a high cost and makes this technology less feasible.

1.3.4.5 Dilute acid treatment

Dilute acid treatment has been widely used by industry to pretreated lignocellulosic materials. It removes the majority of hemicellulose and break down the recalcitrant structure of lignocellulose in order to make enzyme able to attack cellulose to produce mono-sugars (RJ et al. 1999). The dilute acid treatment is often teamed up with steam expansion to improve performance of the pretreatment. The dilute acid treatment can facilitate the following enzymatic hydrolysis to reach up to 70% conversion rate of cellulose. Due to the fact of use of low concentration of acid, the method has relatively less impact on the environment.

1.3.4.6 Alkaline treatment

Similar with dilute acid treatment, alkaline pretreatment is another chemical treatment method that has been widely used to treat cellulosic materials (Keshwani et al. 2009). The mechanism of this method is that alkaline saponifies intermolecular ester bonds between hemicellulose and other components (lignin and cellulose), and alkaline also extracts the released hemicellulose and lignin so that both hemicellulose and lignin contents are significantly reduced in the treated samples. The dilute alkaline treated lignocellulosic materials can reach a 70% sugar conversion rate (Sun et al. 2002).

1.4. Enzyme production and enzymatic hydrolysis of lignocellulose

Enzymatic conversion of lignocellulosic materials to fermentable sugars is one of the critical steps for lignocellulose utilization of bioethanol production. It has been widely reported that most of lignocellulose enzymes are generated from fungal species such as *T. reesei, A. niger,* and *P. chrysosporium.* etc (Dashtban et al. 2009). A unique enzyme production of pelletized fungal fermentation and enzymatic hydrolysis were discussed in this section.

1.4.1. Enzyme production

1.4.1.1. Stains

Filamentous fungi have been widely used in commercial cellulase production. In the past several decades, numerous studies have been focused on finding different fugal species to improve the efficiency of enzyme production. *Trichoderma reesei* is one of the strains that have been identified and commercially used for cellulase production. T. reesei is used by this study in the same capacity.

1.4.1. 2. Pelletized fungal fermentation technology of enzyme production

Pelletization of filamentous fungi is one of methods that can be applied to improve the oxygen mass transfer and reactor performance. Compared to clump-like fungal morphology, pelletized fungal biomass has several benefits, such as a much larger specific surface area which reduces the mass transfer limitations, better fermentation broth rheology which in return affects momentum, mass, and heat transfer in the reactor; consequently, efficiency of mixing and aeration are enhanced. It has been reported that higher yields and productivity were obtained using pelletized morphology (Liao et al.

2002). Another advantage of fungal pellet fermentation is that the pellet makes it possible to perform high biomass concentration cultures to enhance the productivity (Liao et al. 2002). In addition, pellet morphology not only significantly improves the culture rheology, which results in better mass and oxygen transfer into the biomass, and lower energy consumption for aeration and agitation, but also makes fungal biomass reuse possible. Thus, the pelletized fungal fermentation technology developed by Drs. Liu and Liao was adopted to fulfill the enzyme production for this study.

It has been reported that lignocellulosic materials have often been used as both the substrate and inducers in the fermentation processes for cellulase production (Lee and Fan, 1982). Lower solid concentrations also have been shown to yield higher cellulase production (Szengyel et al, 1997). The highest cellulase yield from *T. reesei* culture was around 150 filter paper units (FPU)/g cellulose using Solka Floc as the cellulose substrate. The productivity of 55 FPU/L-hr was achieved in the same culture (Xia et al. 2006). This study used different agricultural residues (AFEX corn stover, alkaline treated corn stover, acid treated switchgrass, alkaline treated switchgrass, acid treated anaerobically digested manure fiber, and alkaline treated anaerobically digested manure fiber) as the substrates to compare cellulase production from *T. reesei* culture. Recently, due to the considerable increase of feedstock cost (starch and mono-sugars), agricultural residues as the fermentation substrates for cellulase production could have potentials to reduce production cost, and enhances the productivity.

1.4.2. Enzymatic hydrolysis

Main enzymes from fungal fermentation include cellulase system (1, 4-β-D-glucan glucanohydrolase, 1, 4-β-Dglucan cellobiohydrolyase and β-glucosidase) and xylanase system (endo-β-1, 4-xylanase, exoxylanase, and β-xylosidase). The function of the cellulase system is: 1) endoglucanase randomly cleaves cellulose chains to form glucose, cellobiose and cellotriose; 2) exoglucanase attacks the non-reducing end of cellulose to release cellobiose units; and 3) Cellobiase cleaves cellobiose units into fermentable glucose units. However, most fungal cellulase systems are lack of β-glucosidase activity that is critical to convert cellobiose into glucose. β-glucosidase from *A. nigers* is often added into the hydrolysis system to enhance the conversion of cellobiose. The xylanase system has similar function with cellulase system. Endo-β-1, 4-xylanase primarily targets the internal β-1, 4 bonds between xylose units, exoxylanase releases xylobiose units, and β-xylosidase convert xylobiose to xylose (Zheng et al. 2009).

1.5. Problem and possible solution

Current issues of lignocellulosic ethanol production are: 1) strong thermal or chemical methods for feedstock pretreatment; 2) high cost of fungal enzymes production; and 3) relatively low efficiency of enzyme systems. In order to facilitate finding solutions for these issues, this study systematically investigated three components of fungal enzyme production, feedstock pretreatment and enzymatic hydrolysis of sugar production. An integrated solution of pelletized fungal fermentation on AFEX corn stover for enhanced enzyme production, dilute alkaline treatment with less chemical loading, and improved hydrolyzibility with optimized enzyme system was concluded from this study, which

would significantly alleviate the barriers that lignocellulosic biorefineries are encountering.

2. Materials and Methods

2.1. Microorganism

The strain for cellulase and xylanase production was *Trichoderma reesei* ATCC 56765. It was cultured on potato dextrose agar slants at 28 °C for 5-7 days to form spores. The spores were collected by using sterile distilled water to wash the agar. A sterile cheese cloth was used to remove mycelia. The concentration of spore suspension was $1x10^9$ spores /ml. The spore solution was stored at 4 °C.

2.2. Substrate

2.2.1. Substrate for Enzyme Production

Purified cellulose powder was from Sigma-Aldrich (α-D-Glucose, anhydrous, 96 %). AFEX pretreated corn stover was provided by Dr. Bruce Dale at Department of Chemical Engineering of Michigan State University. It contained: 34.4% (dry basis) of glucan, 22.4% (dry basis) of xylan, 4.2% (dry basis) of arabinan, 0.6% (dry basis) of mannan, 1.4% (dry basis) of galactan, 3.8% (dry basis) of uronyl, 11% (dry basis) of lignin and 5.6% (dry basis) of acetyl content.

2.2.2. Substrate for Enzymatic Hydrolysis

Switchgrass was used as the substrate for the enzymatic hydrolysis. It was harvested in the farm at Michigan State and the component is cellulose 36.75 %, hemicellulose 28.76 % and lignin 17.93 %. It was grinded to 4.0 mm prior to biological pretreatment and hydrolysis.

The AFEX corn stover for the enzymatic hydrolysis provided by Dr. Bruce Dale at Department of Chemical Engineering of Michigan State University with the following components : 34.4% (dry basis) of glucan, 22.4% (dry basis) of xylan, 4.2% (dry basis) of arabinan, 0.6% (dry basis) of mannan, 1.4% (dry basis) of galactan, 3.8% (dry basis) of uronyl, 11% (dry basis) of lignin and 5.6% (dry basis) of acetyl content.

2.3. Medium

The medium for spore culture was potato dextrose broth (Sigma-Aldrich, 24 g/L).
Two media for cellulase and xylanase production by *T. reesei* were used. One is the basic chemical defined medium that contained (1 L): glucose 10.0 g, cellulose 10 g, xylan 10g, lactose 10 g, yeast extract 0.3 g, peptone 0.75 g, KH2PO4 15 g, (NH4)2SO4 5 g, MgSO4·7H2O 1.23 g, CaCl2·2H2O 0.8 g, CaCO3 4 g, Tween 80 0.5 g, FeSO4·7H2O 0.00271 g, MnSO4·H2O 0.0016 g, ZnSO4·H2O 0.0014 g, CoCl2·2H2O 0.0036. The other is the modified chemical defined medium that 15g/L AFEX corn stover has been used instead of glucose, cellulose, yeast extract and peptone in the previous basic chemical defined medium, and other salts and trace elements are kept as the same.

2.4. Enzymes

Accellerase® is commercially available through Genencor. The cellulase and xylanase activity are 26 FPU/mL and 20 FPU/mL, respectively. It has been used in this study as a contrast to compare with the cellulases system produced by the laboratory.

2.5. Cellulase and Xylanase Production

One mL *T. reesei* spore suspension was inoculated into 50 mL of PDB medium in a 125 mL Erlenmeyer flask. After being cultured at 27 °C for 24 h on a rotary shaker at 170 rpm, 10% (v/v) of seeds was inoculated into another 50 mL PDB medium for an extra 24 h. Consequently, the pelletized seeds were inoculated into the production medium with an inoculum size of 10% (v/v). Cellulase and xylanase were produced in 50 mL fermentation medium in a 250 mL flask at 180 rpm, 27°C for 96-120 hours. After that, the culture broth was centrifuged at 3500 rpm for 10 min (AllegaraTM X-12R, Beckman Coulter) and the supernatant was used as cellulose enzyme solution.

2.6. Process for Pretreatment and Enzymatic Hydrolysis

2.6.1. Dilute Alkali Pretreatment of Switchgrass

Switchgrass was treated using an autoclave with various sodium hydroxide concentration (0.1%, 0.5%, 1%, wt), reaction time (1 hour, 2hours) and temperature (105°C, 121°C). Fiber concentration was fixed at 6% dry mass. After the treatment, the reaction solutions were adjusted to pH 4.0-5.0 using 20% sulfuric acid solution. The treated samples were centrifuged, and solid residues were washed 4-5 times using deionized water. The solid residues were alkaline treated switchgrass, and stored in a freezer of -20 °C. The composition of the alkali treated switchgrass was measured to be 61.18% (dry basis) of cellulose, 17.02% (dry basis) of xylan, 9.14% (dry basis) of lignin.

2.6.2. Dilute Acid Pretreatment of Switchgrass

Switchgrass was treated using an autoclave with various sulfuric acid concentration (1%, wt), reaction time (1 hour) and temperature (130 °C). Fiber concentration was fixed at 6% dry mass. After the treatment, the reaction solutions were adjusted to pH 4.0-5.0 using 20% sodium hydroxide solution. The treated samples were centrifuged, and solid residues were washed 4-5 times using deionized water. Solid residues were stored in a freezer of -20 °C. The composition of the acid treated switchgrass was measured to be 60.74% (dry basis) of cellulose, 24.38% (dry basis) of lignin.

2.6.3. AFEX Pretreatment of Corn Stover

The AFEX corn stover was pre-milled (passed through a 10 mm sieve) with 60% moisture (kg water/kg dry biomass) and added into a high-pressure Parr reactor. Heated liquid ammonia (1 kg of ammonia/ kg of dry biomass) was filled up the reactor vessel resulting in immediate rise in temperature to 130°C. The reactor was maintained at 130°C for 15 min by an external heating mantle (within ±10°C). At the end of 15 min, the pressure was reduced to the atmospheric level and the temperature decreased rapidly. The instantaneous pressure drop in the vessel caused the liquid ammonia to vapor, then cooling the biomass down to less than 30°C. The pretreated materials were left under a hood overnight to ensure the residual ammonia volatile completely. The AFEX-treated stover was kept in room temperature until further use. Its composition was measured to be 34.4% (dry basis) of cellulose, 28.60% (dry basis) of xylan, 11% (dry basis) of lignin.

2.6.4. Dilute Acid Pretreatment of Corn Stover

The corn stover was treated using an autoclave with sulfuric acid concentration (1%, wt), reaction time (1 hour) and temperature (120°C). Fiber concentration was fixed at 6% dry mass. After the treatment, the reaction solutions were adjusted to pH 4.0-5.0 using 20% sodium hydroxide solution. The treated samples were centrifuged, and solid residues were washed 4-5 times using deionized water. The solid residues were stored in a freezer of -20 °C. The composition of the acid treated corn stover was 59.31% (dry basis) of cellulose, 0.3% (dry basis) of xylan, 19.74% (dry basis) of lignin.

2.6.5. Enzymatic Hydrolysis of Pretreated Switchgrass

The enzymatic hydrolysis system contains 2 g alkaline pretreated switchgrass (dry basis), 20 FPU cellulase from *T. reesei* produced in the lab or Accellulase® from Genencor, and 0.5 mL 2 M sodium citrate buffer, the total weight of reaction solution was brought to 40 g by adding DI water. The reaction solutions were incubated at 50 °C for 48 hrs at 140 rpm.

2.7. Ethanol fermentation

Saccharomyces cerevisiae D5A, obtained from American Type Culture Collection (ATCC, Manassas, VA), was used in the ethanol production from fermentable sugars. Initial seeds were cultured for 15 h at 30°C in a 250mL flask on ATCC Medium No. 1245 (10 g/L yeast extract, 20 g/L bacto peptone, and 20 g/L glucose). The seed culture solution was centrifuged to collect yeast biomass for next step of ethanol fermentation. The inoculum size was fixed at 10% (v/v) and then the biomass was mixed with an autoclaved nutrition solution (10 g/L of peptone, 5 g/L of yeast extract, and glucose from

hydrolysates) to conduct the fermentation. Samples were taken at the beginning and end of a 26-28h fermentation process for both glucose and ethanol analysis using HPLC.

2.8. Analysis methods

2.8.1. Fiber Content Analysis

Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) of samples were analyzed using the Van Soest Fiber Analysis System (Goering and Van Soest, 1970). NDF, ADF and ADL were used for calculate cellulose, hemicelluloses and lignin contents. Cellulose and hemicelluloses can be determined by the different of %ADF - %ADL and %NDF - %ADF, respectively. Lignin content was expressed by ADL.

2.8.2. HPLC Analysis

2.8.2.1. Enzyme fermentation sample analysis

The supernatants of all the enzyme samples were filtrated through the 0.22 μm filter. Sugar (cellobiose, glucose, xylose and mannose) analysis was performed by high performance liquid chromatography (HPLC) on SHIMADZU LC 20AD series equipped with RI detector using an Aminex HPX-87P column (7.8 ×300 nm; 5 μm) at 65 °C. The mobile phase utilized Millipore pure water at a flow rate of 0.6 mL/min.

2.8.2.2. Enzymatic hydrolysis sample analysis

All of the enzymatic hydrolysis reactions were finished at 48 hours and each reaction solution was diluted to 100 ml in a volumetric flask. The supernatants were filtrated through the 0.22 μm filter, and then analyzed for cellobiose, glucose, xylose and mannose concentrations by HPLC.

2.8.3. Enzyme Activity Analysis

Cellulase activity (measured as filter paper activity), the reaction mixture (4.5 mL) contained 4 mL of 0.05 M citric acid-sodium citrate buffer Solution (pH4.8), 0.5mL enzyme solution and a 1 × 5 cm stripe of Whatman No.1 filter paper. The reaction was incubated at 50 ℃ for 60 min, and stopped the reaction by adding 3 mL DNS Solution and the concentration of the reducing sugar in the reaction mixture was determined by the dinitrosalicylic acid (DNS) method (Ghose, 1987). One unit of cellulase activity was defined as the amount of enzyme needed to produce 1 μmol of glucose per min at 1 hour of reaction time and 50 ℃ of reaction temperature.

Xylanase activity of *Trichoderma reesei* was determined by Mandel's method (Mandel et al.). The reaction mixture (2.5 mL) contained 1 ml citrate-NaOH buffer solution (50 mM, pH4.8), 0.5 mL enzyme solution and 1ml substrate (it was prepared by dissolving xylan in citrate buffer (1%, w/v) and removing any insoluble material by centrifuging for 10 min). The reaction was incubated at 50 ℃ for 30 min, and then the reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS) reagent. The samples were heated in a boiling water bath, and then cooled, diluted with 20 ml water, and the absorbance read at 545 nm. Blank, enzyme and substrate controls were also carried out under the same conditions. One unit of xylanase activity was defined as the amount of enzyme needed to produce 1 μmol of xylose per minute under 1 hour of reaction time and 50 ℃ of reaction temperature.

2.9. Statistical analyses

All experiments with at least two replicates were performed by completely random design (CRD). Analyses of Variance (ANOVA) and Fisher's least significant difference (LSD) test were used to analyze the data.

3. Enzyme Production

3.1. Morphology evaluation

The influence of spore concentration on the pellets was studied using potato dextrose broth (Sigma-Aldrich, 24 g/L). Two different levels of inoculum size were set for 109/ml and 107/ml. Figure 1. shows the pellets after 2 generations of culture, the fungal pellets from higher inoculum size had a smaller diameter of 1.7-1.9 mm. A smaller diameter can facilitate mass transfer and further enhance the cellulase's production.

3.2. Cellulase and xylanase productions on basic chemical defined medium

Cellulase activity was increased following the increase of the culture time, while xylanase activity was dropped in the first 48 hours, and then increased for the rest of culture time (Figure 2.). After the first three days, enzyme production for both cellulase and xylanase reached their peaks. Cellulase activity was 0.9 U/ml, and xylanase activity was 2 U/ml. In the basic chemical defined medium, 10 g/L glucose was the main carbon source for strain growth and produce secondary metabolic products, and 10 g/L lactose was added as an inducer for enzyme production.

3.3. Comparison of basic chemical defined medium and modified medium

According to Figure 3, cellulase activities on both media were increased within first 3 days. There was a significant improvement on cellulase activity on the modified medium at the third day: the activity reached 1.5 U/ml which was twice than the activity in basic

chemical defined medium (0.7 U/ml). Meanwhile, the xylanase activities on both media were also increased in first 2 days. The xylanase activity on modified medium reached the peak value of 1.3 U/ml at 48 hours. After 2 days of culture, the xylanase activities on the modified medium were leveled off, while the xylanase activities on the basic chemical defined medium were still increased.

The enhancement of cellulase and xylanase activity was largely due to the carbon sources of cellulose and xylan in the modified medium. Both substrates also played a role to induce the enzyme production. However, both enzyme activities in the modified medium were leveled off at the 4th day. It was much earlier than the trend of chemical defined medium from previous culture (Figure 2). The main reason was that pH of culture on modified medium was decreased much faster than the chemical defined medium, which means that *T. reesei* on modified medium grew better than chemical defined medium. More metabolic products including various organic acids released into the broth led to a quicker drop of pH on the modified medium. Thus, pH had to be controlled in order to further enhance enzyme production on the modified medium.

3.4. Comparison of CaCO3 and Tris-maleate buffer solution as the neutralizer in cellulase and xylanase production

The impact of neutralizers on enzyme production was presented in Figure 4. The data indicated that both CaCO3 and buffer solution had positive impacts on enzyme production. The figure also elucidated that CaCO3 was a better neutralizer than buffer solution. The maximum enzyme activities for both enzymes were obtained at the fourth day.

Acidity measurements and enzyme activity tests further demonstrated the impacts of pH on enzyme production. The pH values of the medium with CaCO3 as the neutralizer did not change during the entire cultivation, while the pH values of the medium with buffer solution started dropping at the 3rd days.

3.5. Effects of different concentration of CaCO3 on cellulase and xylanase production

Different concentrations (0 g/L, 4 g/L and 10 g/L) of CaCO3 have been used to study the effects of CaCO3 on enzyme production. Figure 5. showed that there were no significant difference on enzyme activities between 4 g/L of CaCO3 and 10 g/L of CaCO3, while enzyme activities on the medium without CaCO3 were significantly lower than other two CaCO3 concentrations.

Besides maintaining the broth system under a stable pH level, the enhanced formation of pellets in the fermentation also indicated that CaCO3 as a neutralizer not only prevented pH from dropping, but was also a favorable factor for the fungal pelletization and biomass accumulation. The calcium ion has been reported as a metal inducer to facilitate mycelial aggregation during fungal growth, which has been verified by this study. The medium with CaCO3 produced smoother and more homogeneous pellets than those without it.

3.6. Comparing different agricultural residues with purified cellulose in cellulose production

Although the relatively high enzyme activity has been achieved using the carbon source modified medium, the purified glucose and cellulose powder were still not the optimal

feedstock for enzyme production at commercial scale. More abundant and less expensive lignocellulosic feedstocks such as agricultural residues should be compared (Table 5., Table 6.).

Seven different lignocellulosic feedstocks (AFEX corn stover, alkaline treated corn stover, acid treated corn stover, alkaline treated switchgrass, acid treated switchgrass, and acid treated anaerobically digested fiber, and alkaline treated anaerobically digested fiber) were compared with the modified medium to evaluate the impacts of them on enzyme production.

The results of cellulase and xylanase production in 250 ml flasks all using 15 g/l lignocellulosic substrates were shown. Compared with dilute alkaline treated substrates, all the dilute acid treated substrates had less enzyme production on both cellulase and xylanase. Alkaline pretreatment did not remove all of hemicellulose in the sample, which might be a reason to enhance both cellulase and xylanase production. Among the alkaline treated samples, AFEX corn stover presented the highest activities on both enzymes, so it was selected as the substrate to produce enzymes for the following study.

3.7. Effects of different concentrations of AFEX corn stover in cellulases production

A further study of effects of concentration of AFEX corn stover on enzyme production was conducted (Figure 12, 13). Cellulase activities were increased for all three concentrations with the increase of culture time, while xylanase acitivities reached the peak at 69 hours. Four different concentrations of AFEX corn stover were tested, and the concentration of 15 g/L showed the highest cellulase activity, while the concentrations of 15 g/L and 30 g/L presented the highest xylanase activities. Considering both cellulase

and xylanase activities, the AFEX corn stover of 15 g/L and cultivation time of three days were chosen as the optimal condition for enzyme production (Figure 14).

3.8. Comparing cellulases production with the optimal condition on both chemical defined medium and AFEX corn stover medium

Enzyme production on the medium with 15 g/L of AFEX treated corn stover was compared with the chemical defined medium (Figure 15). The data showed that 20% and 40% increase were achieved by AFEX medium on xylanase and cellulase, respectively. In addition, enzyme productivity showed even larger improvement by AFEX corn stover (Figure 16). AFEX corn stover obtained 100 % enhancement on cellulase productivity and 79 % enhancement on xylanase fermentation. The increased enzyme activities could be benefits on the enzymatic hydrolysis of lignocellulosic materials (discussed in Section 4).

4. Enzymatic Hydrolysis

4.1. Enzymatic hydrolysis of lignocellulosic materials

4.1.2. Comparing different enzymes and substrates combinations

Nine enzyme cocktails produced from previous fungal fermentation were applied on four pretreated corn stover and switchgrass samples to elucidate the effects of enzyme cocktails from different culture medium on the hydrolysis (Figure 18, 19, 20, and 21). The enzyme cocktail from the culture on the AFEX corn stover had the best hydrolysis performance on all pretreated fibers among all nine cocktails, particularly on AFEX treated corn stover and alkaline treated switchgrass, which the enzyme cocktail had the cellulose conversion rate of 70% and 60%, respectively.

Furthermore, according to the fiber analysis results (Tables 8. through 10.), alkaline treated switchgrass has highest cellulose content of 45% among the treated fiber feedstocks. More cellulose in the sample, more glucose might be produced in the following enzymatic hydrolysis. Thus, alkaline treated switchgrass was chosen as the lignocellulosic material to fulfill the study of enzymatic hydrolysis.

4.1.3. Enzymatic hydrolysis of alkaline pretreated switchgrass using Accellerase®

Ten FPU Accellerase®/g substrate was used to conduct the hydrolysis of 5 % pretreated switchgrass. The switchgrass was pretreated by dilute alkaline using a completely randomized design (CRD). Two pretreatment times (1 and 2 hours), two temperatures (105 and 121°C), and three NaOH concentrations (0.1 %, 0.5 %. 1.0 %) were tested to compare the effects of pretreatment on enzymatic hydrolysis.

The data of reducing sugar (glucose and cellobiose) from twelve treatments (Figure 22, 23, 24 and 25) demonstrated that 0.1% NaOH was too low to break down the matrix structure, while both pretreatment conditions (105°C and 1 hour; 121°C and 1 hour) at the elevated alkaline concentrations of 1% reached the highest sugar concentration of 29 g/L. Longer reaction times had negative impacts on sugar concentrations. In addition, there were no significant differences on sugar concentrations between two pretreatment temperatures (105 and 121°C). Thus, alkaline concentrations (1 and 2%) and pretreatment temperatures (105 and 121°C) were used to conduct the following the experiment of enzyme comparison.

4.1.4. Comparison of different levels of Accellerase® and cellulase on pretreated switchgrass

Commercial cellulase and cellulase cocktail produced from AFEX corn stover were used for the experiment. Two different levels of enzyme activities (5 U and 10 U cellulase/g fiber (all based on dry matter)) were tested. Other experimental conditions were listed in Table 10.

The data indicated that at the same enzyme activity levels the cellulose cocktails performed significantly better than commercial Accellerase (Figure 26, 27, 28 and 29). In addition, relatively high alkaline concentrations and high temperatures showed better sugar conversion. Enzymatic hydrolysis of pretreated switchgrass from the treatment of 121°C, 1.0 % NaOH and 1 hour generated 40 g/L and 30 g/L of sugars for cellulose cocktail and commercial cellulase, respectively.

High substrate concentrations under the optimal pretreatment conditions was applied to further study the effects of different enzyme sources on the hydrolysis (Table 11.), the cellulase cocktail has a better hydrolysis performance than Accellerase. The highest sugar concentration of 60 g/L was obtained on switchgrass using the cellulase cocktail (Figure 30).

The possible reasons of better performance of cellulase cocktail might be: 1) Effects of xylanase supplementation: based on the enzyme activity measurements, the activity ratio (0.65) of xylanase and cellulase in Accellerase® was much lower than that (2.0) from cellulase cocktail. It has been reported that glucose release is improved by hemicellulose removal. 2) The synergistic action among other untested enzymes: a recent published study indicated that there were more than 5 enzymes co-existed in the broth with cellulase and xylanase. They can synergistically work with cellulases and xylanase, and facilitate the conversion of cellulose into glucose.

In addition, another major difference between cellulase cocktail and Accellerase was that cellulase cocktail was production on lignocellulosic materials. The lignocellulosic materials can also induce the strain to release certain secondary metabolic products, which led the enzyme to have better hydrolysis performance.

4.2. Ethanol Fermentation

In order to further evaluate the ethanol production yield from diluted alkaline pretreated switchgrass, an enzymatic hydrolysis at high solid contents (10 % dry basis) followed by ethanol fermentation was conducted. A yeast strain *S. cerevisiae* D5A was used to carry out the ethanol fermentation. A 68% ethanol yield (ethanol yield [%] = ethanol produced [g]/ (0.51* 1.11*cellulose in sample [g]*100)) was obtained on the hydrolysate from

cellulase cocktail treated switchgrass, which was higher than 58% of Accellerase treated switchgrass (Figure 31).

5. Conclusion and Perspectives

This study has concluded that pelletized fungal fermentation on AFEX corn stover significantly improved the enzyme production and enzyme activity, and consequently enzymatic hydrolysis on switchgrass demonstrated high sugar conversion rates.

Fungal morphology is a very important factor in fungal fermentation of cellulose production. Small fungal pellets enhanced enzyme production. Various lignocellulosic materials as carbon sources had different effects on enzyme activities and ratios of cellulase to xylanase. Cellulase and xylanase activities from the cultures on AFEX corn stover were significantly higher than cultures on other lignocellulosic materials. Cellulase production from a culture on 15 g/L AFEX corn stover reached the highest activity of 1.08 U/ml at 93 hours, while xylanase also reached the highest activity of 2.52 U/ml at 72 hours.

The experiments of consequently enzymatic hydrolysis using the enzyme cocktail produced form the pelletized fungal fermentation elucidated that the cocktail from the AFEX corn stover culture had better sugar conversion rates on all 4 pretreated fibers compared to the enzyme from chemical defined culture. Considering both sugar concentration and conversion rate, enzymatic hydrolysis on alkaline treated switchgrass showed better performance than other lignocellulosic materials. A complete randomized design on alkaline treatment concluded that 121°C, 1 % NaOH and 1 hour reaction time is the optimal conditions to treat switchgrass.

The future studies should focus on investigating enzyme compositions of enzyme cocktails from cultures on different lignocellulosic materials, and discovering the interactions of different enzymes during fermentation and following hydrolysis.

Consequently the impacts of various enzymes in the cocktails on hydrolysis can be concluded, which will lead to a mechanistic understanding of synergistic functions of enzymes on hydrolysis, and further make it possible to intentionally design enzyme cocktails with respect to different lignocellulosic materials.

Appendix A: Tables and Figures

Table 1. Correlation between biomass features and enzymatic digestibility of biomass

Biomass features	Relationship between features and digestibility
Cellulose crystallinity	Negative or no correlation
Degree of polymerization	Negative or no correlation
Specific surface area	Positive
Cellulose protection by lignin	Negative
Hemicellulose sheathing	Negative
Degree of hemicellulose acetylation	Negative or no correlation

Table 2. Key factors for an effective pretreatment of lignocellulosic materials

Key properties for low-cost and advanced pretreatment process
• High yields for multiple crops, sites ages, harvesting times;
• Highly digestible pretreated solid;
• No significant sugars degradation;
• Minimum amount of toxic compounds;
• Operation in reasonable size and moderate cost reactors;
• Non-production of solid-waste residues;
• Effectiveness at low moisture content;
• Obtaining high sugar concentration;
• Fermentation compatibility;
• Lignin recovery;
• Minimum heat and power requirements.

Table 3. Literature review of selected diluted acid pretreatment and enzymatic hydrolysis research

Feedstock	T (°C)	T (min)	H_2SO_4 (Zheng, Pan et al. 2007)	Hemicellulose conversion (Ahamed and Vermette)	Enzyme loading (FPU/CBU)	Enzymatic digestibility (Ahamed and Vermette)
Mix wood	230	0.12	1.17	-	-/-	95
Wheat straw	140	60	0.5 (v/v)	80	26/-33/33	>80%
Hard wood	160	10	0.45-0.5 (v/v)	94	42/4.9	90-100
Corn residues	160	10	0.45-0.5 (v/v)	90	42/4.9	85
Douglas fir	200-230	1-5	0.4	90-95	25-60/-	90
Corn stover	121	30-120	2	90	40/8	80
Rye straw	121	90	1.5	55-66	25/75	52-83

Table 4. Literature review of composition of different lignocellulosic materials (% dry wt)

Biomass	Glucan	Xylan	Mannan	Galactan	Arabinan	Lignin
Poplar	43.5	15.5	2.5	2.3	1.5	26.2
Pine	46.4	7.8	10.6	-	2.2	29.4
Spruce	49.9	5.3	12.3	2.3	1.7	28.7
Birch	38.2	18.5	1.2	-		22.8
Corn stover	36.8	22.2	-	2.9	5.5	23.1
Wheat straw	30.2	18.7	-	0.8	2.8	17.0
Barley straw	33.1	20.2	-	0.9	3.8	16.1

Table 5. Comparison of different substrates using in enzyme production

Substrate (Bak, Ko et al.)	CDa	AFEX CSb	Alkaline SGc	Alkaline CSd	Alkaline AD fibere	Acid SGf	Acid CSg	Acid AD fiberh
Cellulase(Szengyel and Zacchi)	0.88	0.92	0.79	0.80	0.47	0.47	0.75	0.60
Xylanase(Chadha and Garcha)	1.78	2.05	1.96	2.01	1.76	1.48	1.50	1.61

a Chemical defined medium
b AFEC pretreated corn stover
c Diluted alkaline pretreated switchgrass
d Diluted alkaline pretreated corn stover
e Diluted alkaline pretreated anaerobic digestion fiber
f Diluted acid pretreated switchgrass
g Diluted acid pretreated corn stover
h Diluted acid pretreated anaerobic digestion fiber

Table 6. Effect of various pretreatment methods on the chemical composition and chemical/physical structure of lignocellulosic biomass

	Increases accessible surface area	Decrystalizes cellulose	Removes hemicellulose	Remove lignin	Alter lignin structure
Dilute Acid	+	-	+	-	+
AFEX	+	+	-	+	+
Dilute Alkaline	+	+	-	+	+

+: Major effect
-: Minor effect

Table 7. Comparison of different substrates using in enzyme production (96hours)

Substrate	CDa	AFEX CSb	Alkaline SGc	Alkaline CSd	Alkaline AD fibere	Acid SGf	Acid CSg	Acid AD fiberh
Cellulase	0.88	0.92	0.79	0.80	0.47	0.47	0.75	0.60
Xylanase	1.78	2.05	1.96	2.01	1.76	1.48	1.50	1.61

a Chemical defined medium
b AFEC pretreated corn stover
c Diluted alkaline pretreated switchgrass
d Diluted alkaline pretreated corn stover
e Diluted alkaline pretreated anaerobic digestion fiber
f Diluted acid pretreated switchgrass
g Diluted acid pretreated corn stover
h Diluted acid pretreated anaerobic digestion fiber

Table 8. Components in different pretreated lignocellulosic materials

Substrate	Cellulose	Hemicellulose	Lignin
AFEX corn stover	34.40%	28.60%	11.00%
Alkaline Switchgrass	61.18%	17.02%	9.14%
Alkaline corn stover	65.33%	22.12%	3.22%
Acid Switchgrass	60.74%	0.00%	24.38%
Acid corn stover	59.31%	0.30%	19.74%
AD fiber	33.07%	9.45%	21.16%

Time: 1 hour
Alkali/Acid Concentration: 1%
Temperature: 121 °C

Table 9. Optimization of enzymatic hydrolysis using commercial cellulase (Accellulase ®)

	Parameters
Substrate	Switchgrass
Pretreatment method	Alkaline treatment
Substrate concentration	5
Alkaline concentrations	0.1/0.5/1.0
Pretreatment time	1/2
Reaction temperature (°C)	105/121
Enzyme hydrolysis	
Cellulase	*Trichoderma Reesei* (10 FPU/g substrate)
Substrate concentration (%)	5
Hydrolysis temperature (°C)	50
Reaction time (hours)	48

Table 10. Optimization of enzymatic hydrolysis using commercial cellulase (Accellulase ®) and cellulase using different enzyme concentration

	Parameters
Substrate	Switchgrass
Pretreatment method	Alkaline treatment
Substrate concentration (%)	5
Alkaline concentrations (%)	0.5/1.0
Pretreatment time (hours)	1
Reaction temperature (°C)	105/121
Enzyme hydrolysis	
Cellulase	*Trichoderma Reesei,* Accellerase® (5 FPU/g or 10 FPU/g substrate)
Substrate concentration (%)	5
Hydrolysis temperature (°C)	50
Reaction time (hours)	48

Table 11. Relatively high activity enzymes working on high solid content pretreated switchgrass and AFEX pretreated corn stover

Pretreatment	121°C, 1% NaOH, 1Hour			
Enzyme	10 U Cellulase/g substrate		10 U Accellulase/g substrate	
Substrate	AFEX CS	Switchgrass	AFEX CS	Switchgrass
Solid Content	10%	10%	10%	10%

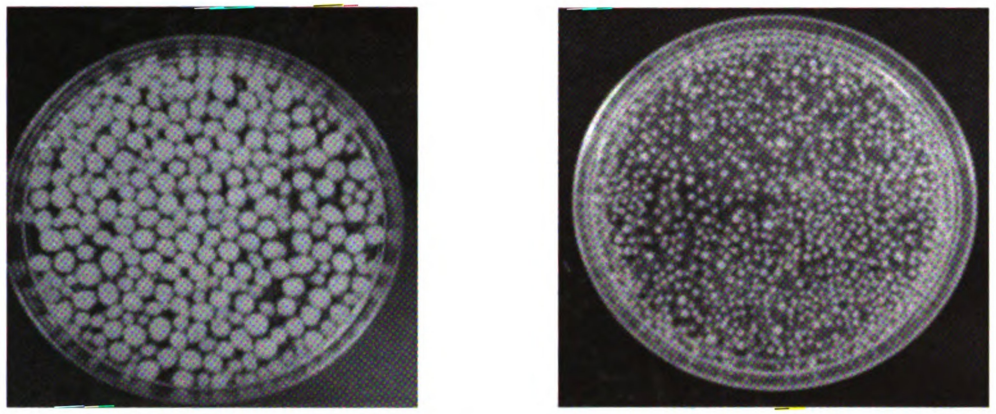

Figure 1. Typical morphologies of T. reesei using the two different inoculum sizes: filamentous mycelium, with inoculum size of 107/ml spores; pelletized with inoculum size of 109/ml spores.

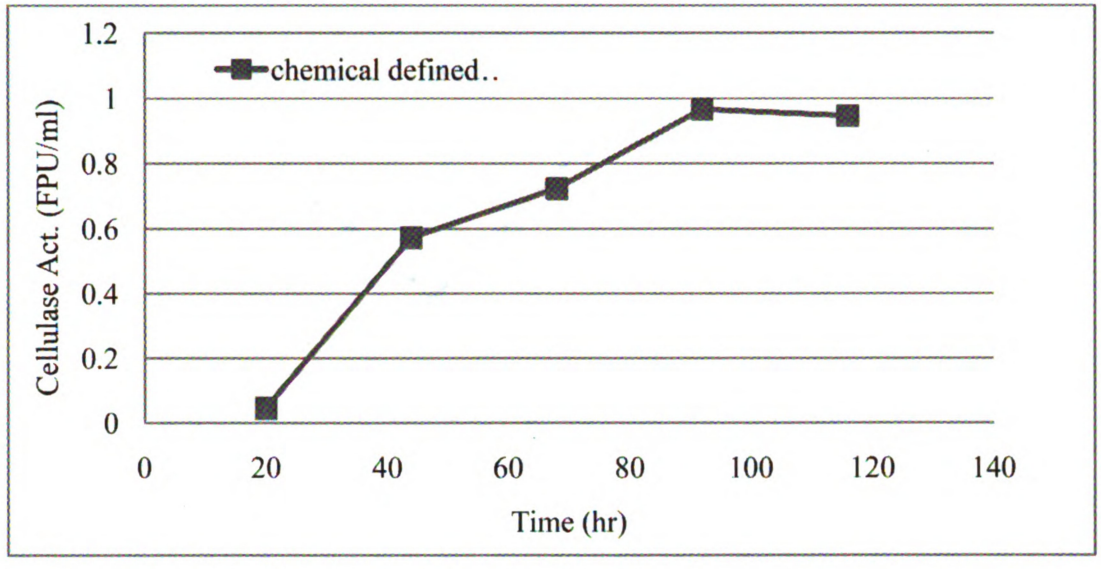

Figure 2. Cellulase and xylanase production time course test for 5 days with the basic chemical defined medium includes: 10g/L of glucose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

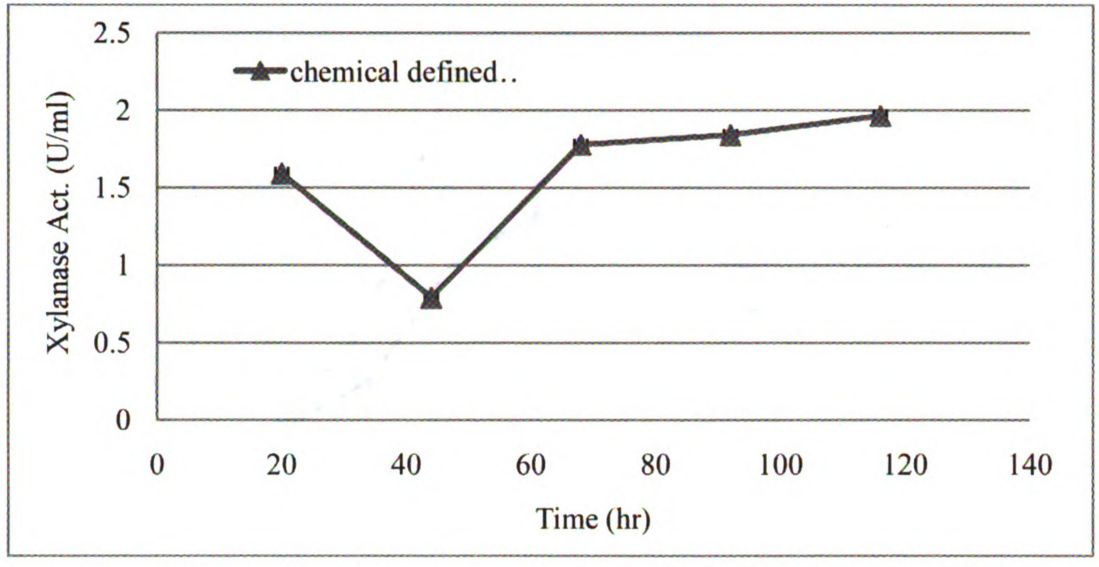

Figure 2. con't Cellulase and xylanase production time course test for 5 days with the basic chemical defined medium includes: 10g/L of glucose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O

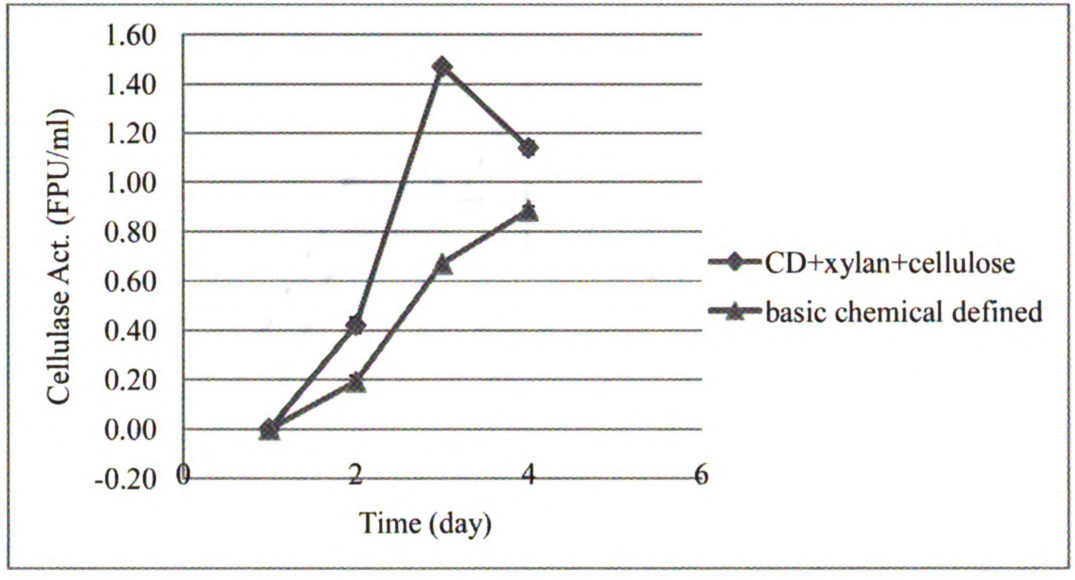

Figure 3. Cellulase and xylanase production with the carbon source modified chemical defined medium includes: 10g/L of glucose, 10g/L of xylan, 10g/L cellulose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

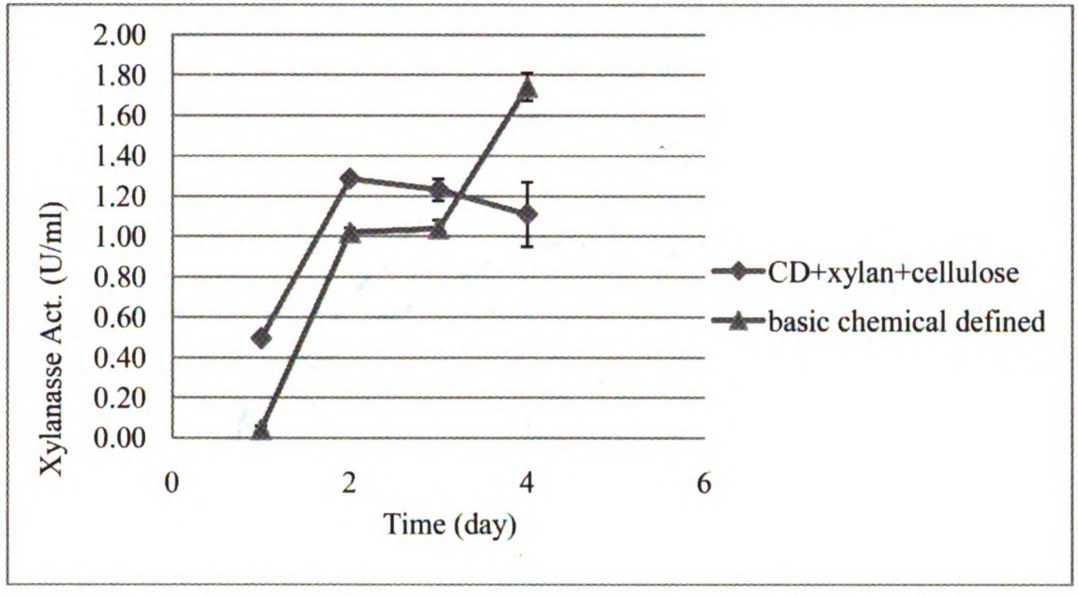

Figure 3. con't Cellulase and xylanase production with the carbon source modified chemical defined medium includes: 10g/L of glucose, 10g/L of xylan, 10g/L cellulose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

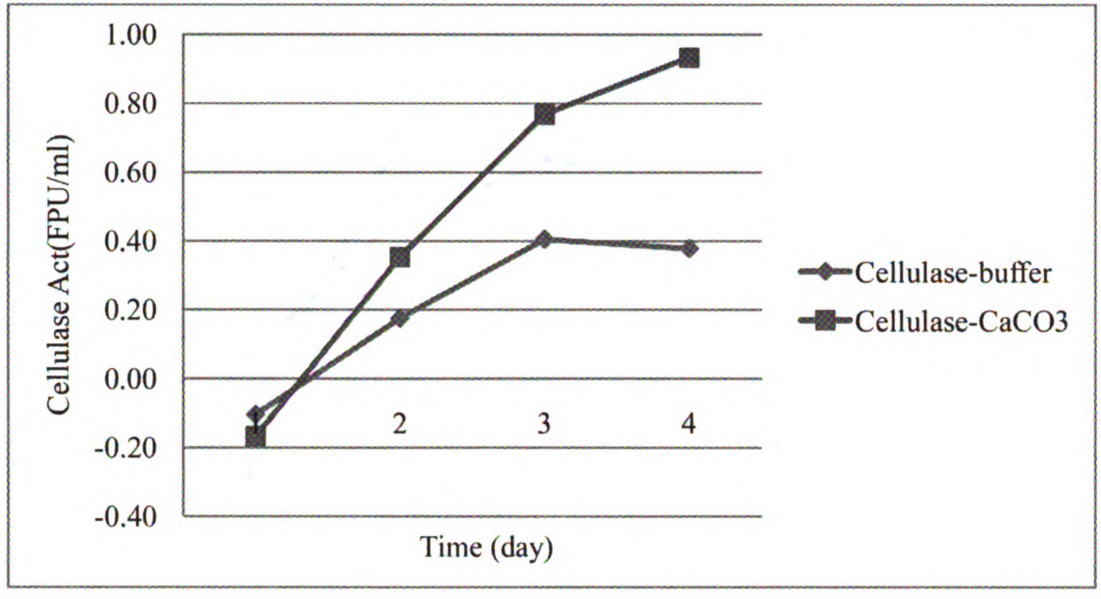

Figure 4. Enzyme production with both neutralizers added in the basic medium which includes: 10g/L of glucose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

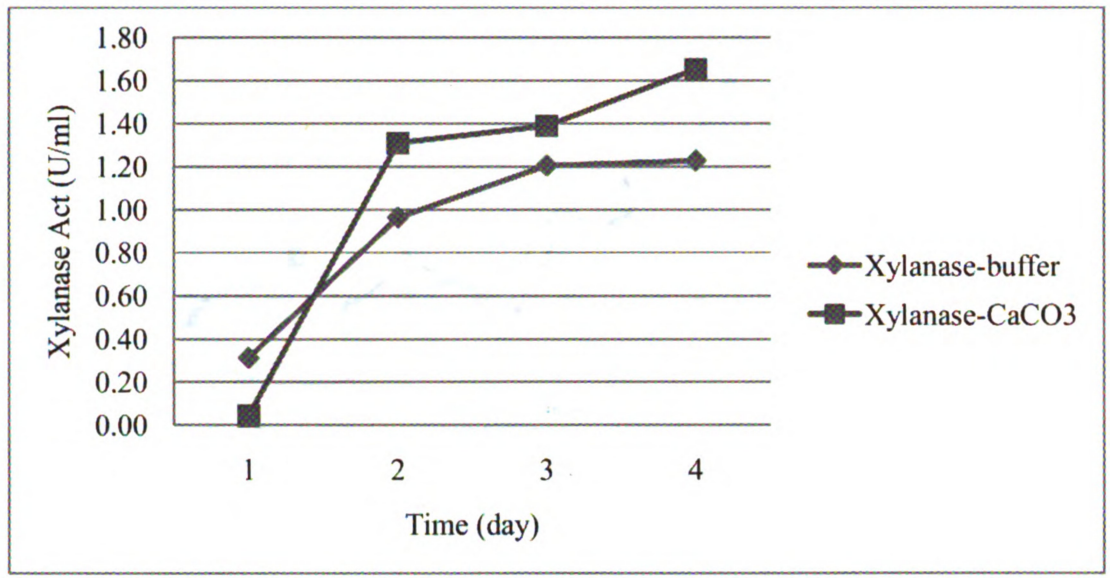

Figure 4. con't. Enzyme production with both neutralizers added in the basic medium which includes: 10g/L of glucose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

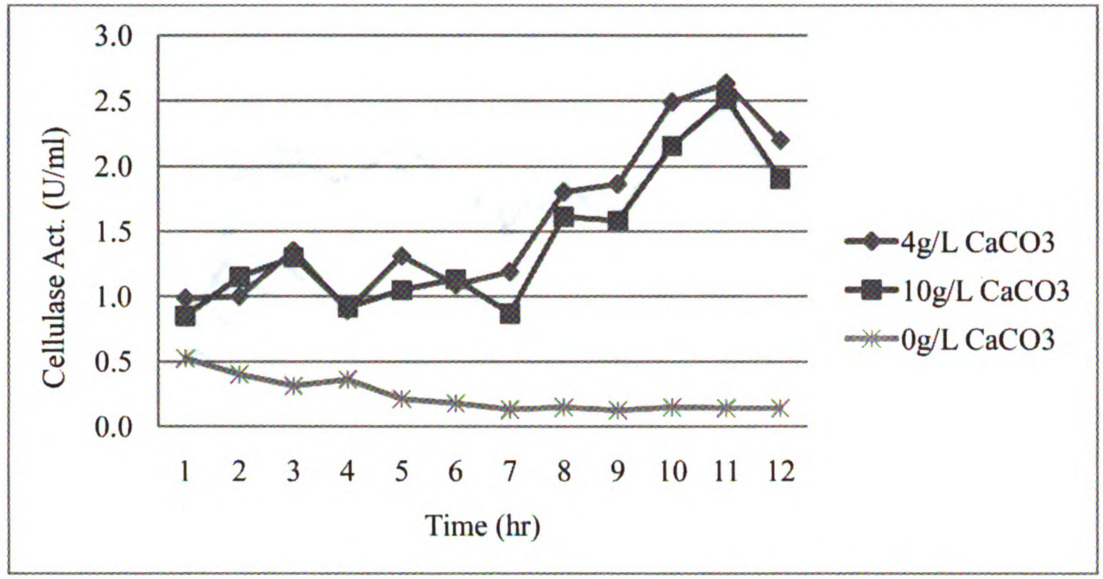

Figure 5. The time course of cellulase and xylanase production in flasks with 4g/L of CaCO3, 10g/L of CaCO3 or without CaCO3 added in the carbon source modified chemical defined medium which includes: 10g/L of glucose, 10g/L of xylan, 10g/L cellulose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

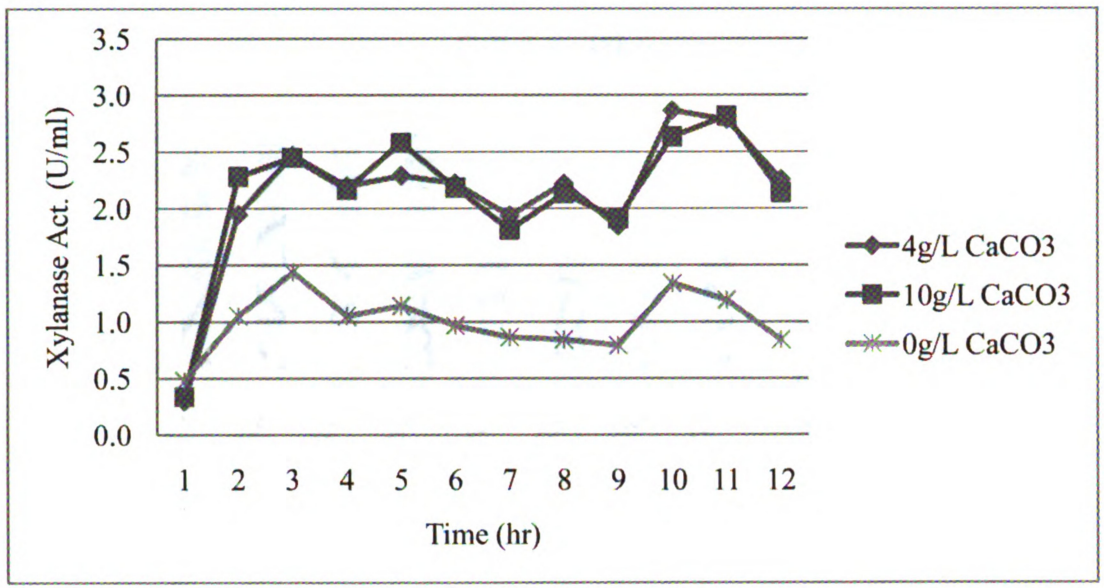

Figure 5. con't The time course of cellulase and xylanase production in flasks with 4g/L of CaCO3, 10g/L of CaCO3 or without CaCO3 added in the carbon source modified chemical defined medium which includes: 10g/L of glucose, 10g/L of xylan, 10g/L cellulose, 0.3 g/L of yeast extract, 0.75 g/L of peptone, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 10 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

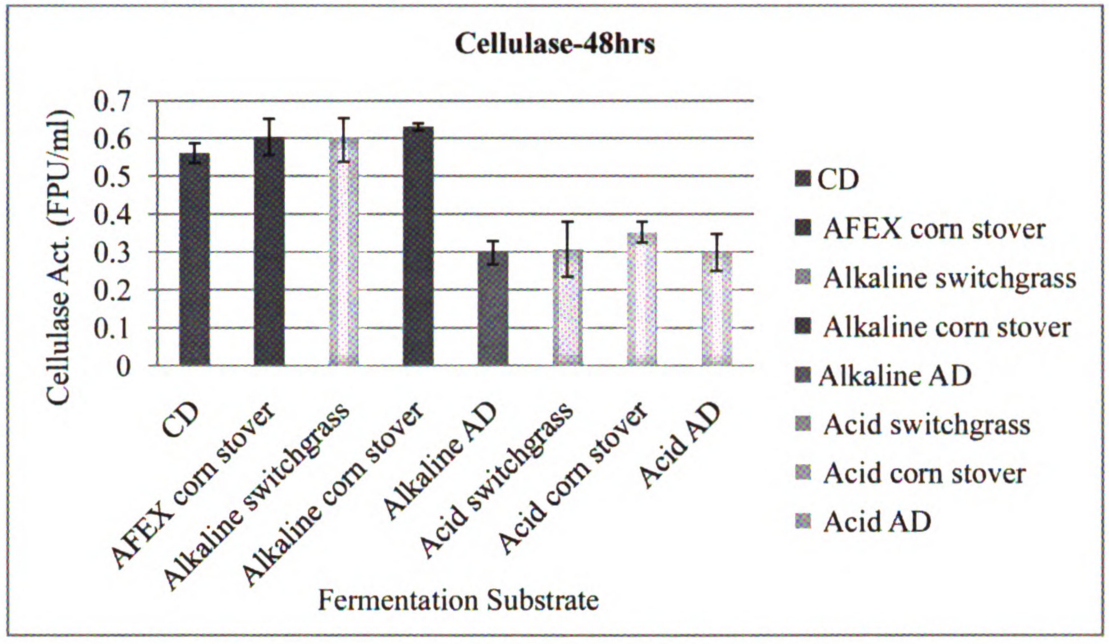

Figure 6. Comparing different agricultural residues using as the substrate in the enzyme production. The modified medium includes: 15 g/L Agricultural residues, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

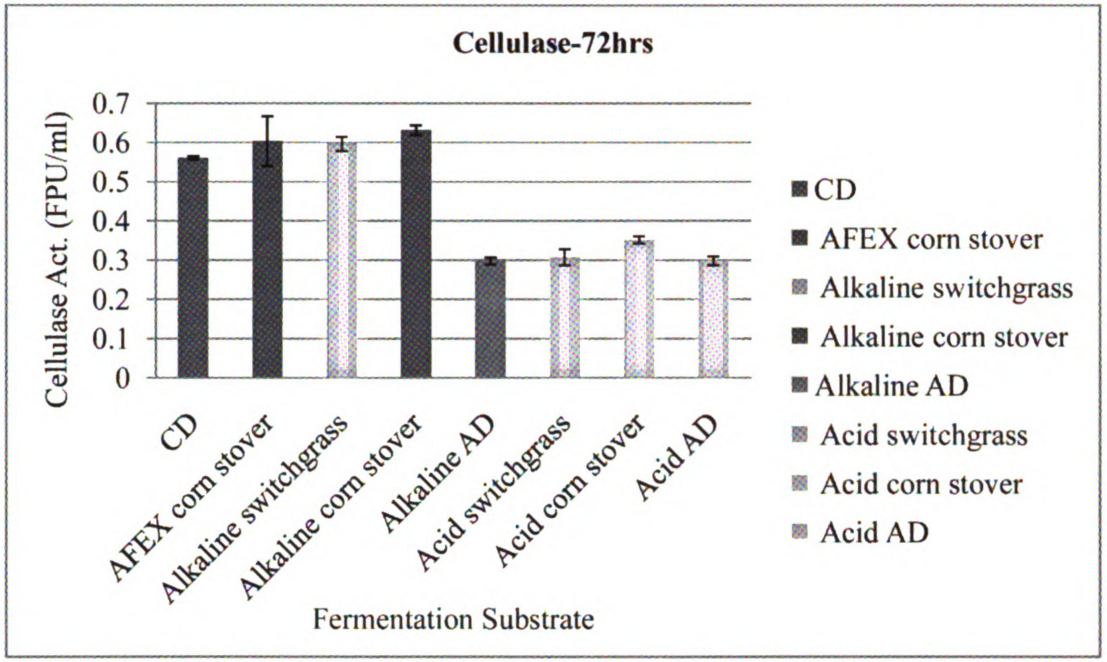

Figure 7. Comparing different agricultural residues using as the substrate in the enzyme production. The modified medium includes: 15 g/L Agricultural residues, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

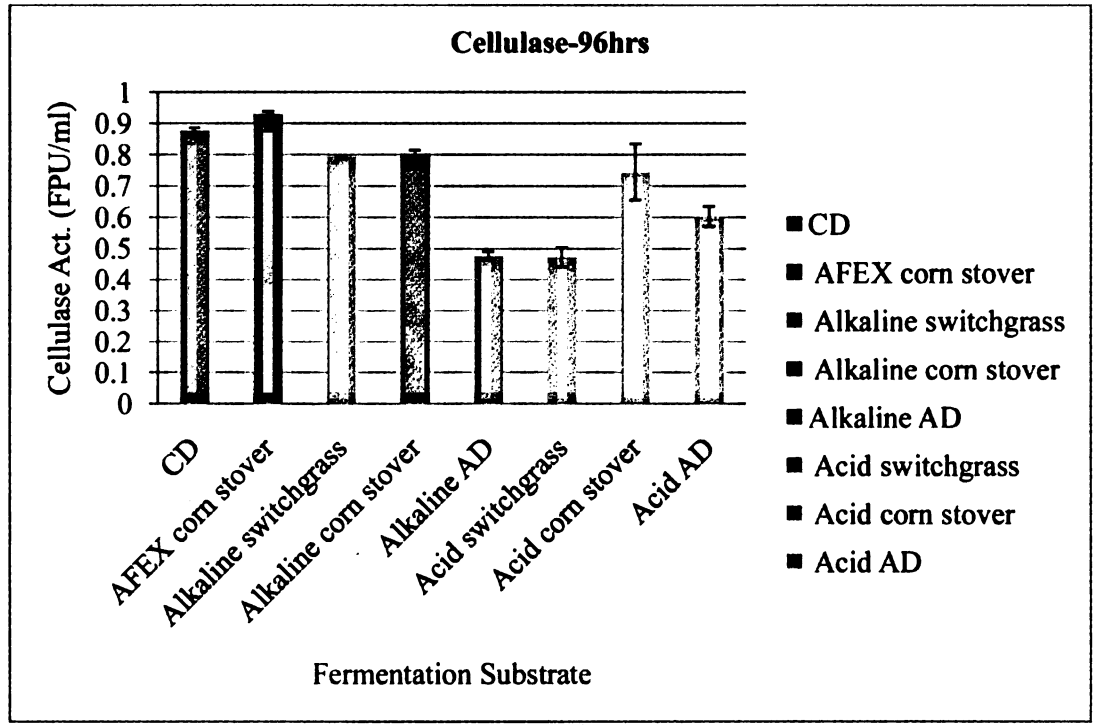

Figure 8. Comparing different agricultural residues using as the substrate in the enzyme production. The modified medium includes: 15 g/L Agricultural residues, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

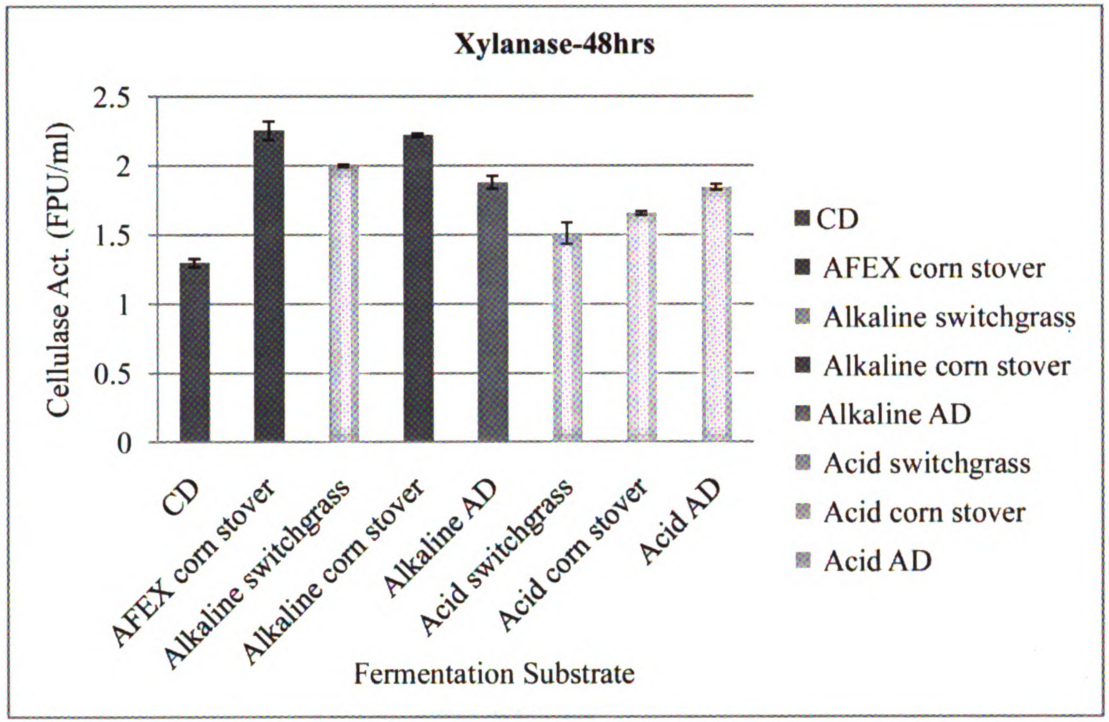

Figure 9. Comparing different agricultural residues using as the substrate in the enzyme production. The modified medium includes: 15 g/L Agricultural residues, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

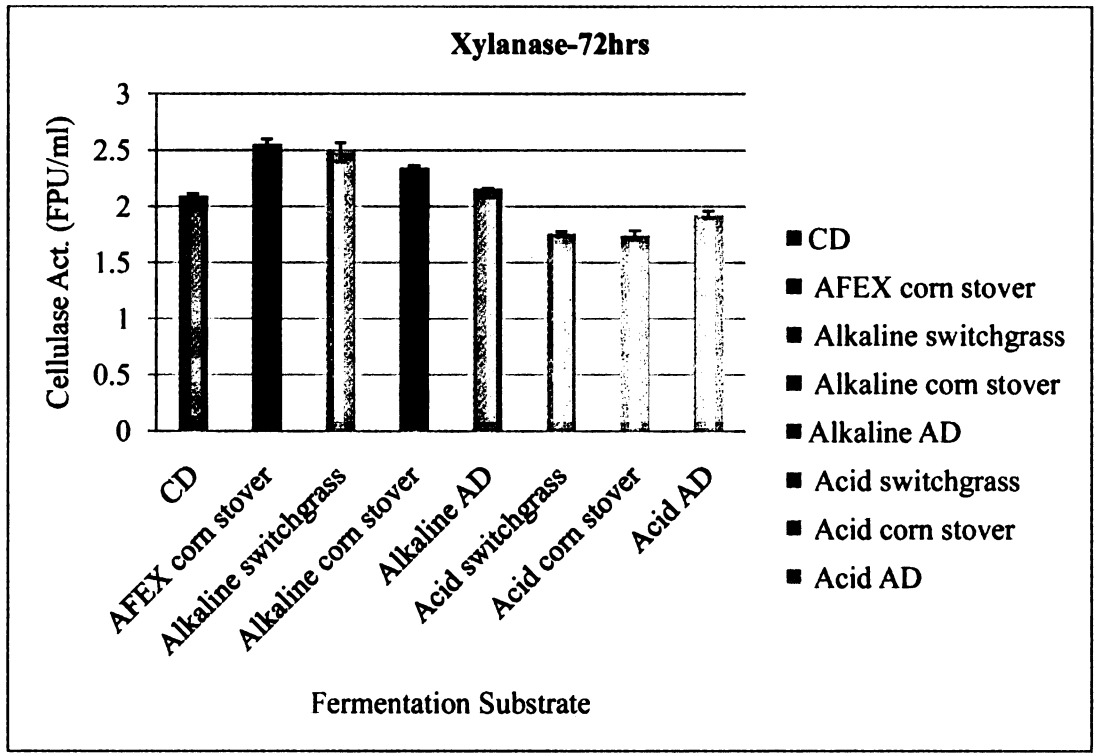

Figure 10. Comparing different agricultural residues using as the substrate in the enzyme production. The modified medium includes: 15 g/L Agricultural residues, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

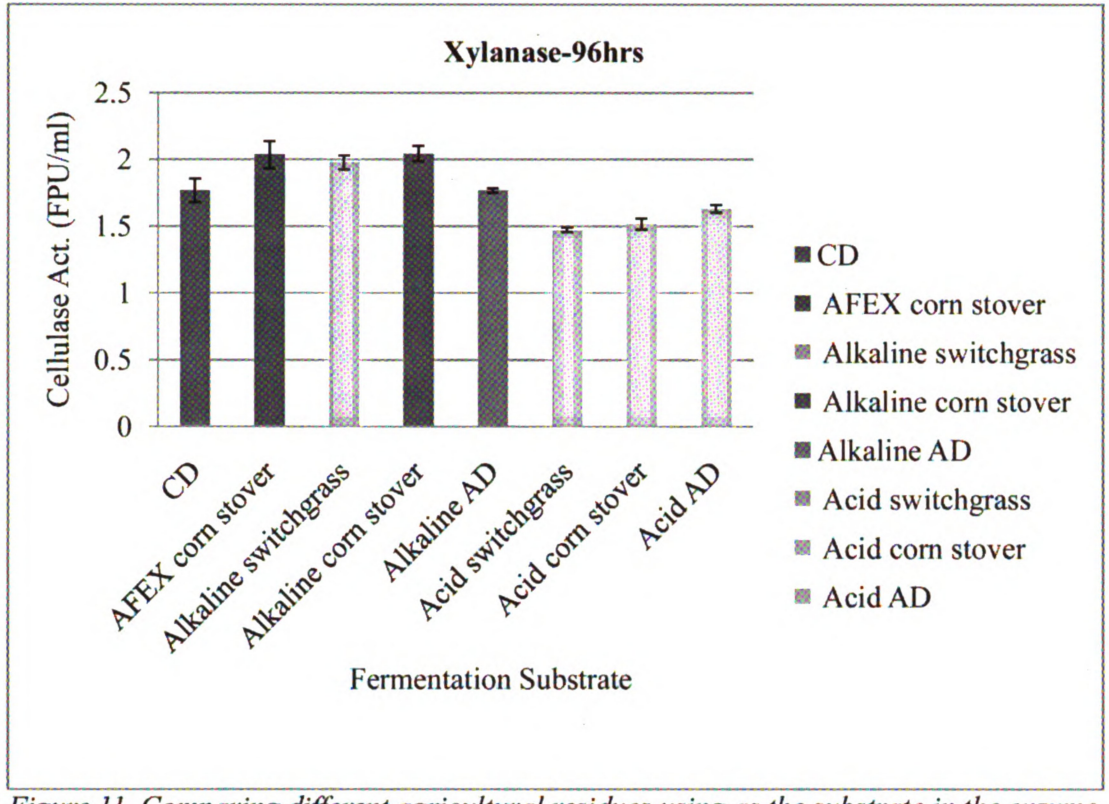

Figure 11. Comparing different agricultural residues using as the substrate in the enzyme production. The modified medium includes: 15 g/L Agricultural residues, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

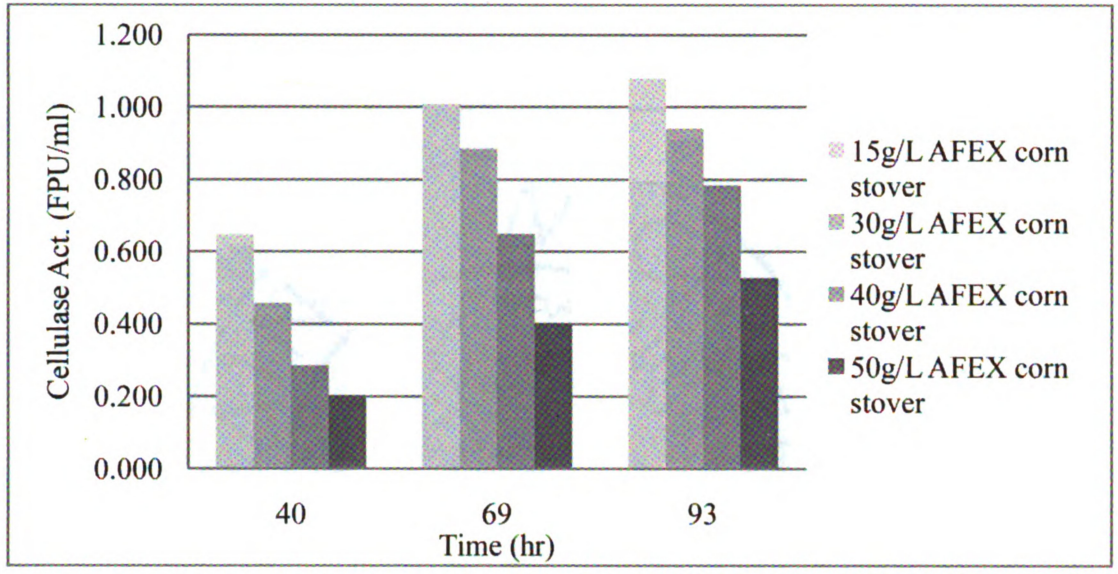

Figure 12. Comparing different concentrations of AFEX treated corn stover as the substrates working on cellulases fermentation for 40hours, 69hours and 93hours, respectively. The modified medium includes: 15/30/40 g/L AFEX corn stover, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

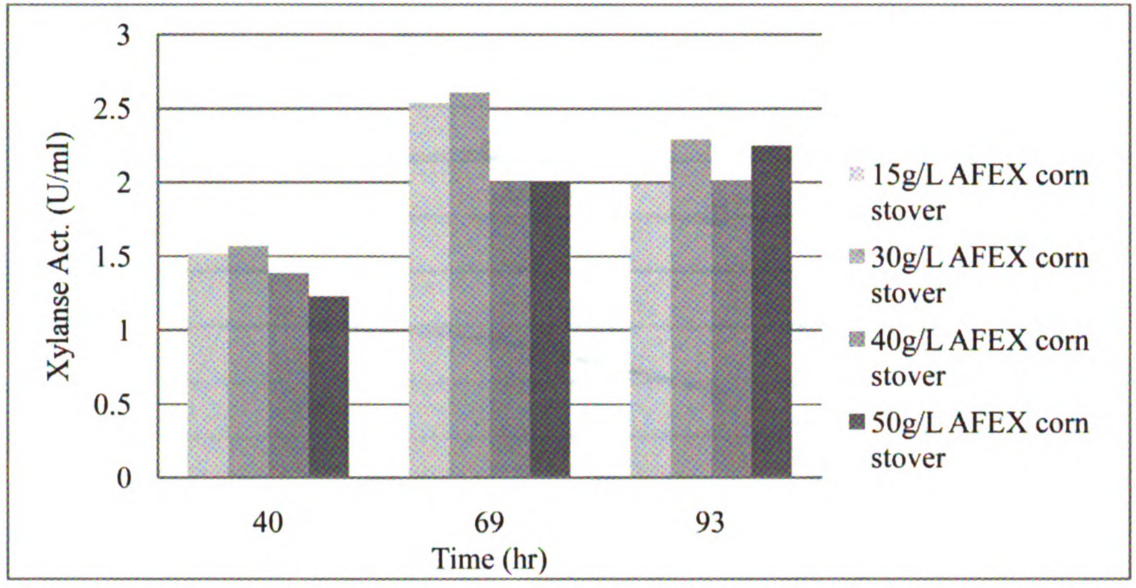

Figure 13. Comparing different concentrations of AFEX treated corn stover as the substrates working on cellulases fermentation for 40hours, 69hours and 93hours, respectively. The modified medium includes: 15/30/40 g/L AFEX corn stover, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

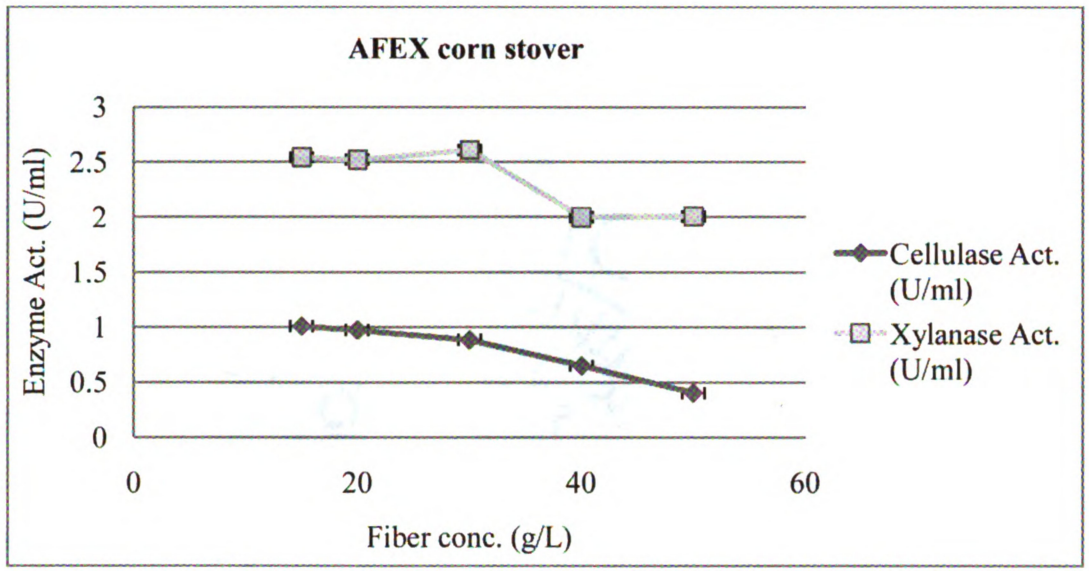

Figure 14. The modified medium includes: 15 g/L AFEX corn stover, 15 g/L of KH2PO4, 5 g/L of NH4Cl, 0.5 g/L of Tween 80, 5 g/L of lactose, 1.23 g/L of MgSO4.7H2O, 0.00271 g/L of FeSO4.7H2O, 0.0016 g/L of MnSO4.H2O, 0.0014 g/L of ZnSO4.H2O, 0.0036 g/L of CoCl2.6H2O, 0.8 g/L of CaCl.2H2O.

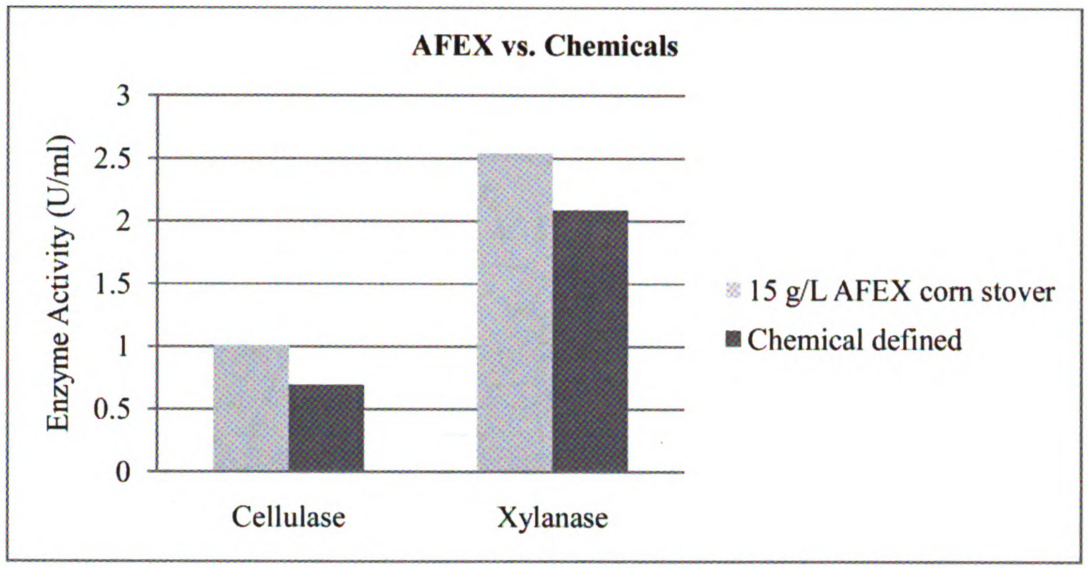

Figure 15. Comparing the enzymes activity produced on 15 g/L AFEX corn stover and chemical defined medium

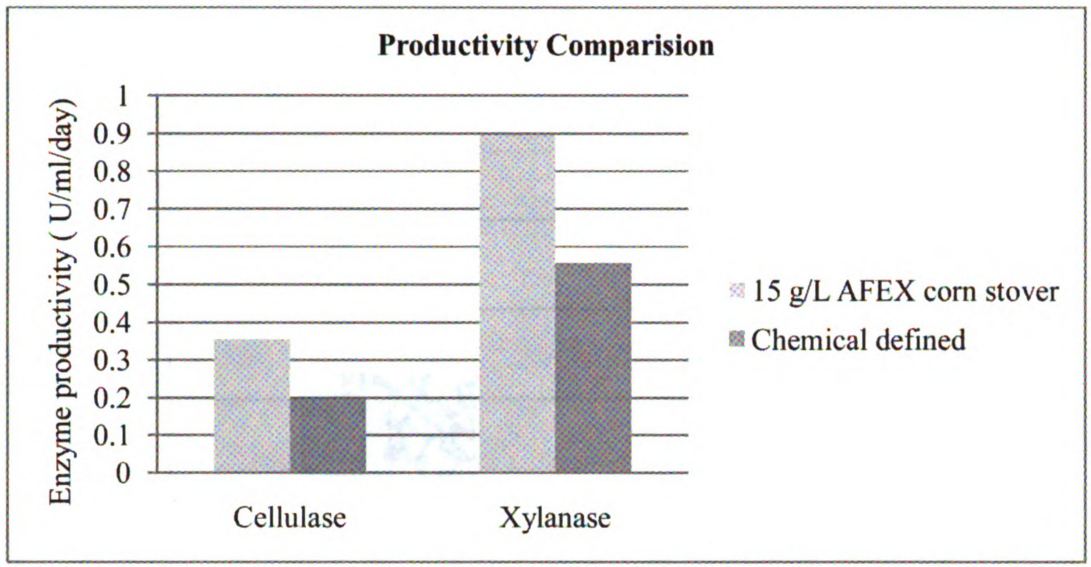

Figure 16. Comparing the enzyme productivity based on 15 g/L AFEX corn stover and chemical defined medium

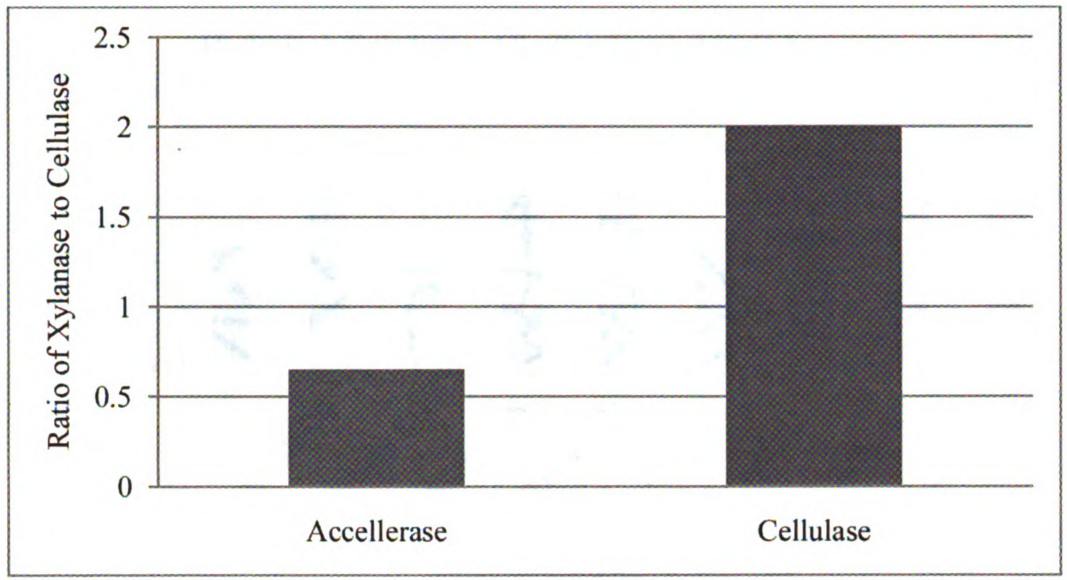

Figure 17. Different ratio of xylanase over cellulase in both Accellerase and cellulase enzyme cocktails

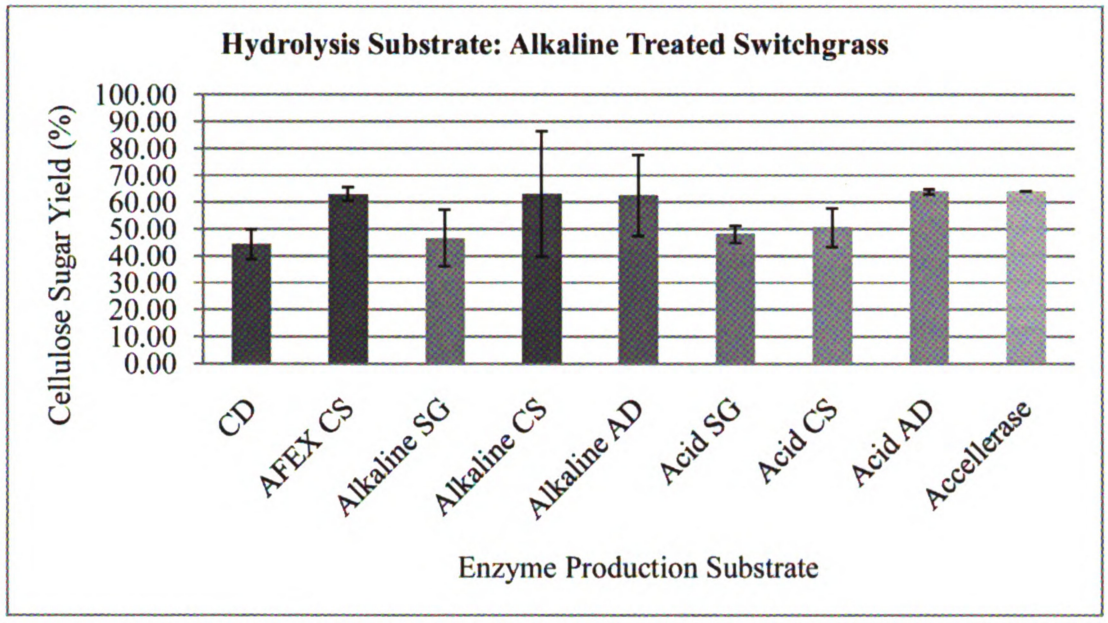

Figure 18. Hydrolysis of alkaline treated switchgrass, acid treated switchgrass, AFEX treated corn stover and acid treated corn stover with different enzyme cocktails

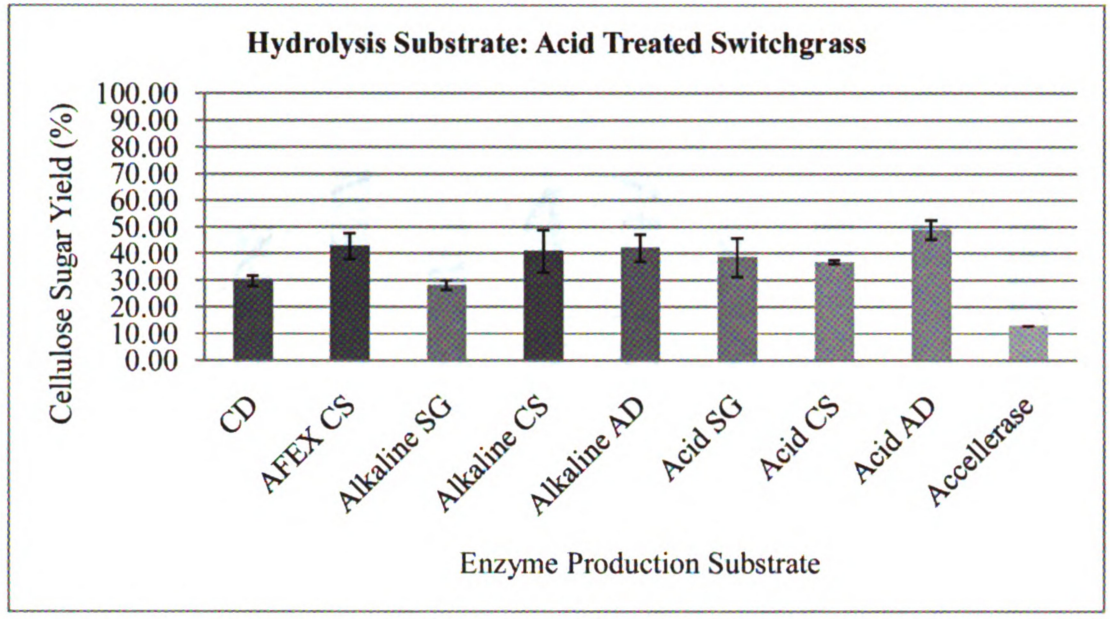

Figure 19. Hydrolysis of alkaline treated switchgrass, acid treated switchgrass, AFEX treated corn stover and acid treated corn stover with different enzyme cocktails

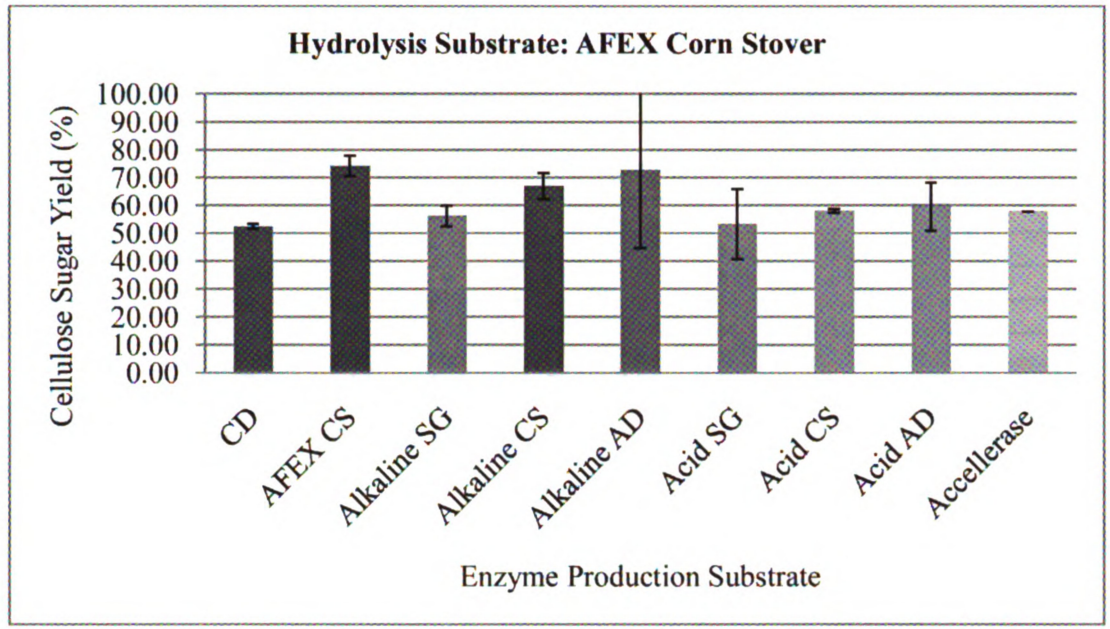

Figure 20. Hydrolysis of alkaline treated switchgrass, acid treated switchgrass, AFEX treated corn stover and acid treated corn stover with different enzyme cocktails

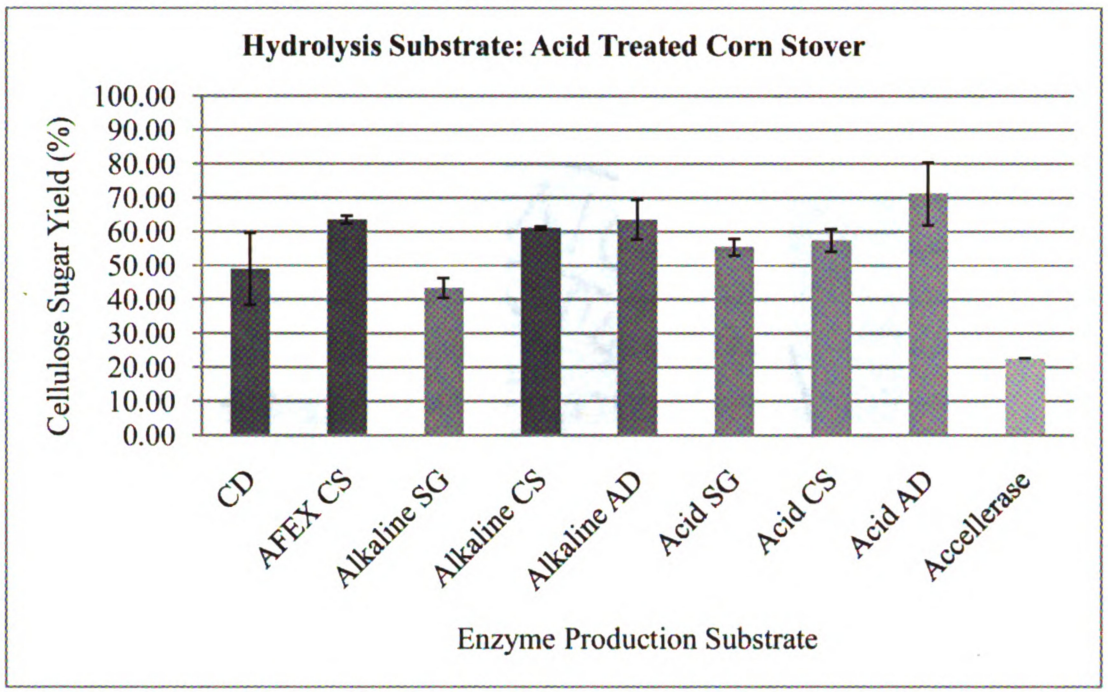

Figure 21. Hydrolysis of alkaline treated switchgrass, acid treated switchgrass, AFEX treated corn stover and acid treated corn stover with different enzyme cocktails

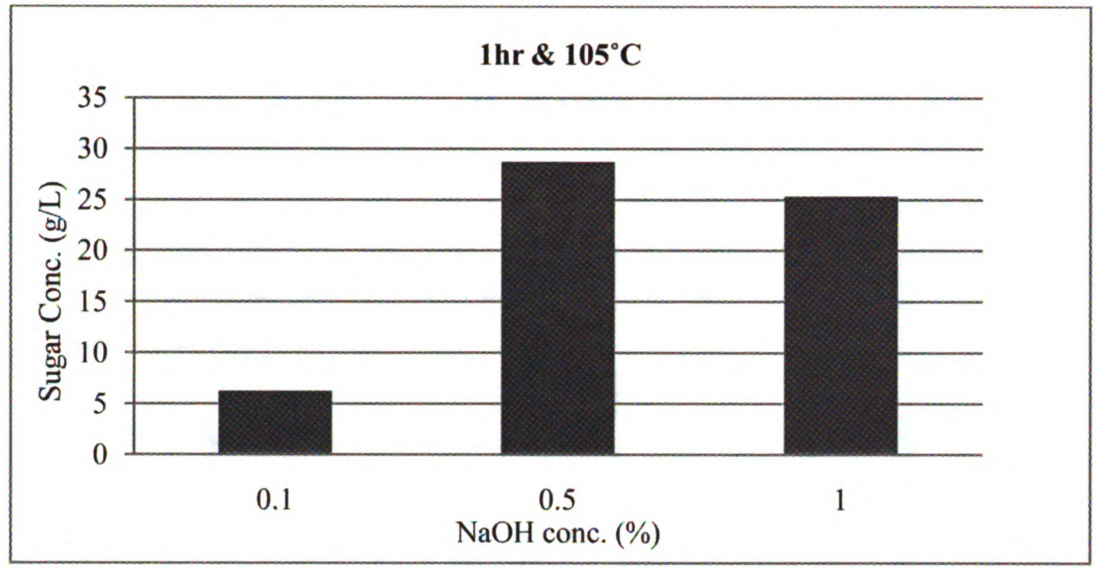

Figure 22. Effects of Accellulase working on 1hour, 105C, 0.1 /0.5/1% NaOH solution pretreated switchgrass.

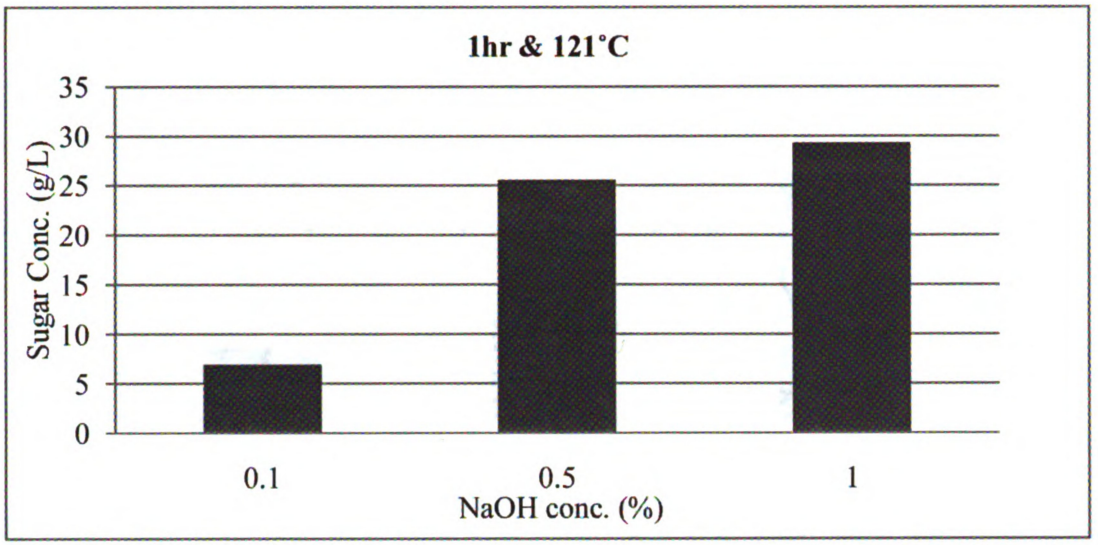

Figure 23. Effects of Accellulase working on 1hour, 105C, 0.1 /0.5/1% NaOH solution pretreated switchgrass.

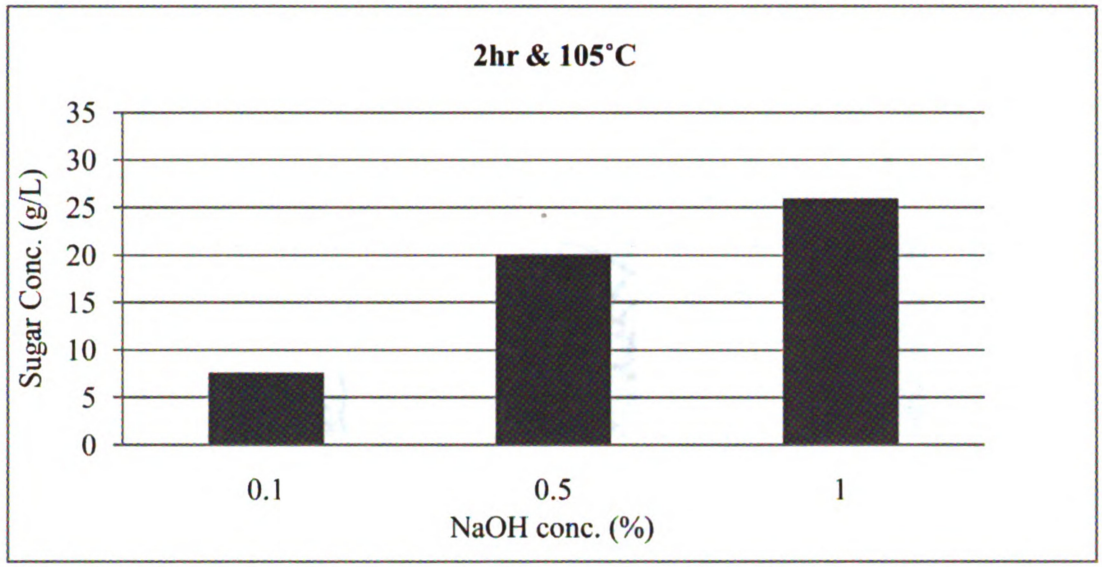

Figure 24. Effects of Accellulase working on 1hour, 105C, 0.1 /0.5/1% NaOH solution pretreated switchgrass.

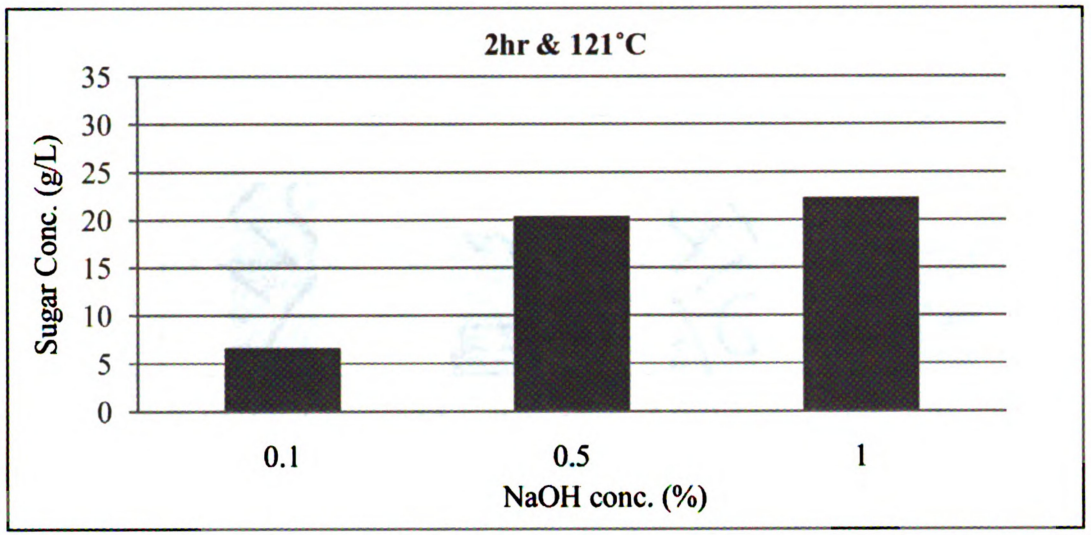

Figure 25. Effects of Accellulase working on 1hour, 105C, 0.1 /0.5/1% NaOH solution pretreated switchgrass.

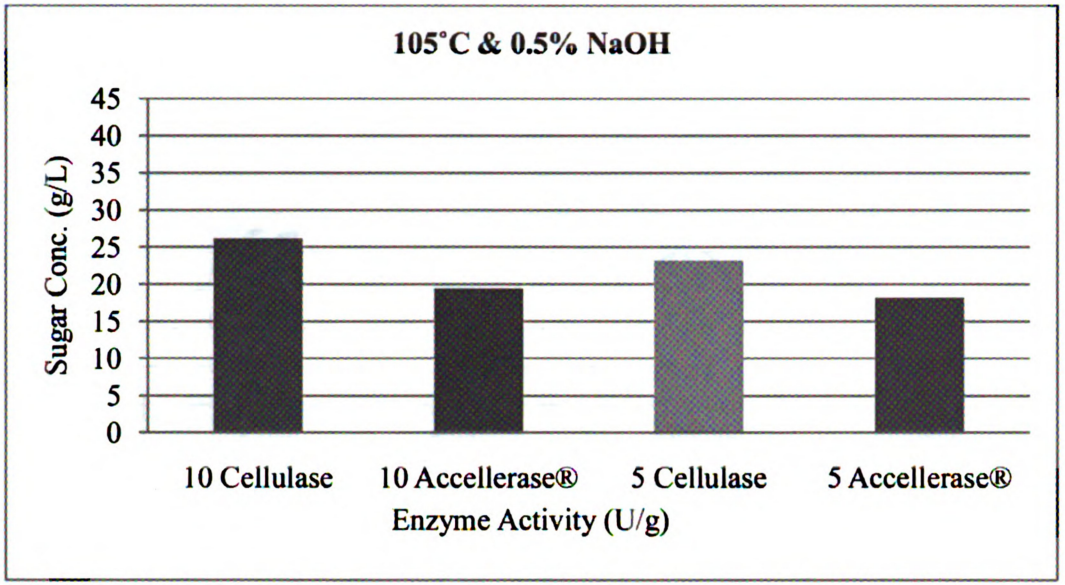

Figure 26. Comparing the effects of 10 FPU/g substrate and 5 FPU/g substrate Accellulase working on 1hour, 105C, 0.5 % NaOH solution pretreated switchgrass.

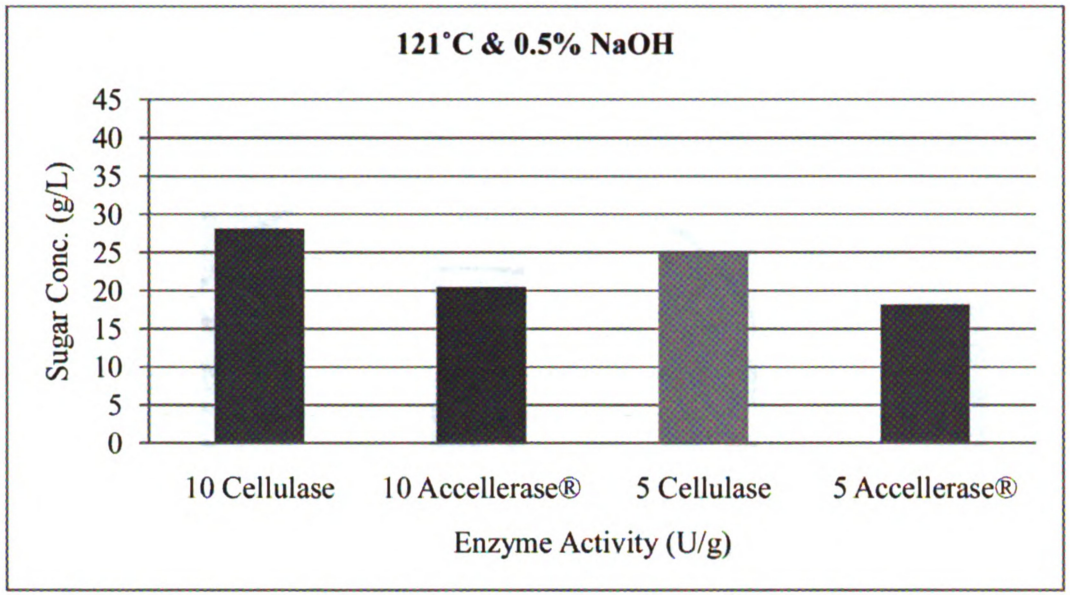

Figure 27. Comparing the effects of 10 FPU/g substrate and 5 FPU/g substrate Accellulase working on 1hour, 105C, 0.5 % NaOH solution pretreated switchgrass.

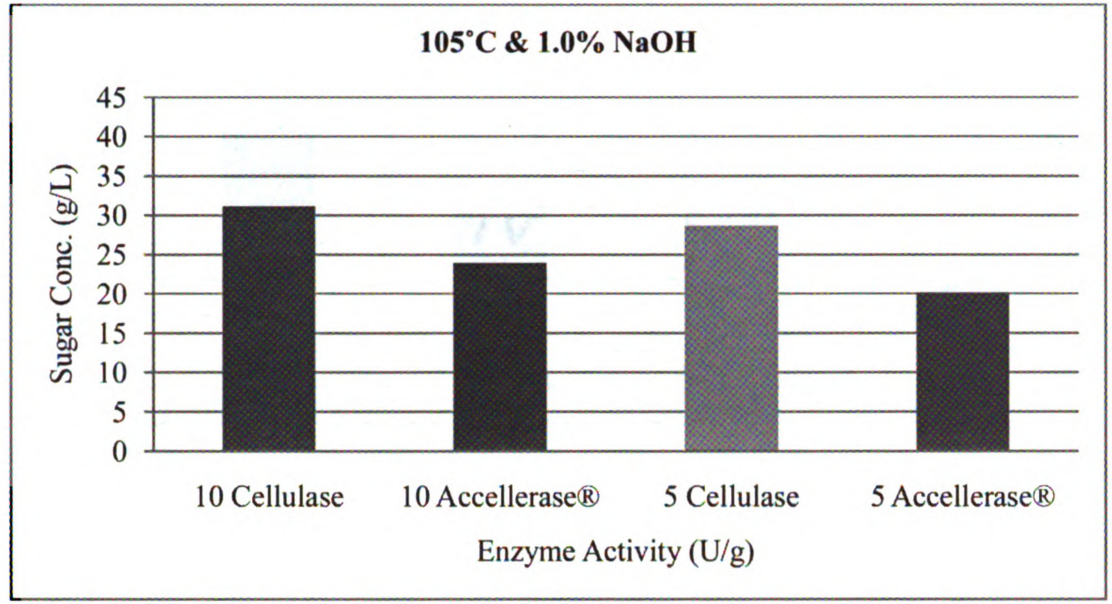

Figure 28. Comparing the effects of 10 FPU/g substrate and 5 FPU/g substrate Accellulase working on 1hour, 105C, 0.5 % NaOH solution pretreated switchgrass.

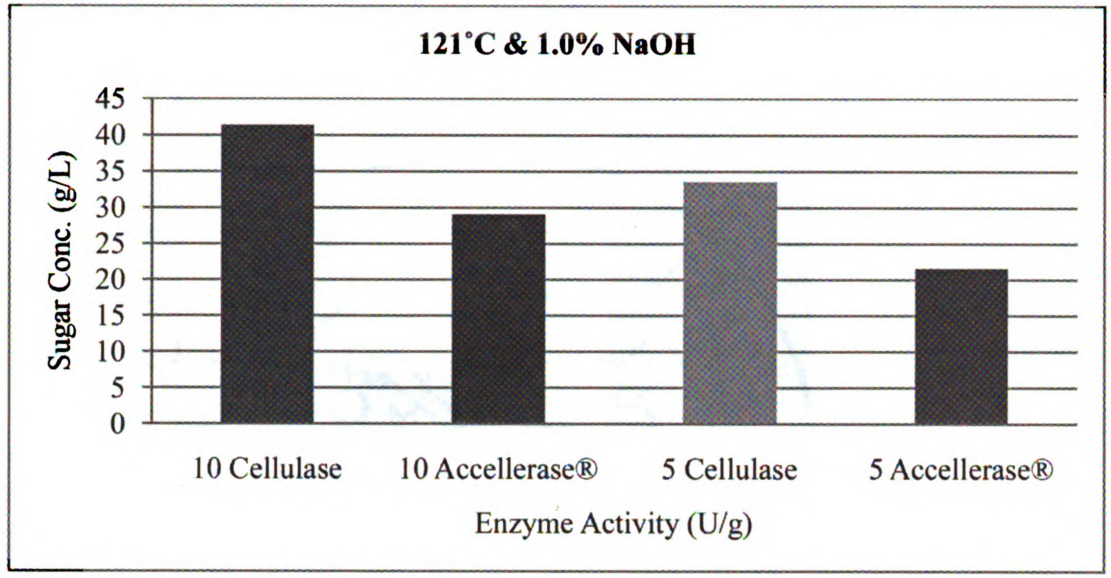

Figure 29. Comparing the effects of 10 FPU/g substrate and 5 FPU/g substrate Accellulase working on 1hour, 105C, 0.5 % NaOH solution pretreated switchgrass.

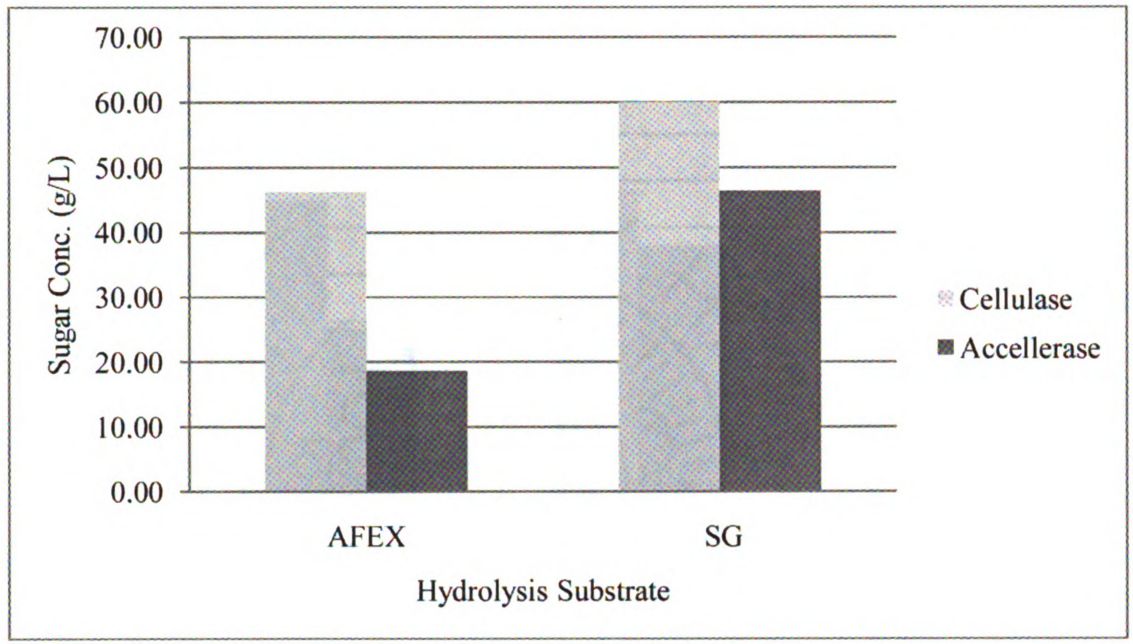

Figure 30. 10 FPU/g substrate Accellulase and cellulase working on 1hour, 121C, 1% pretreated switchgrass and AFEX pretreated corn stover, respectively.

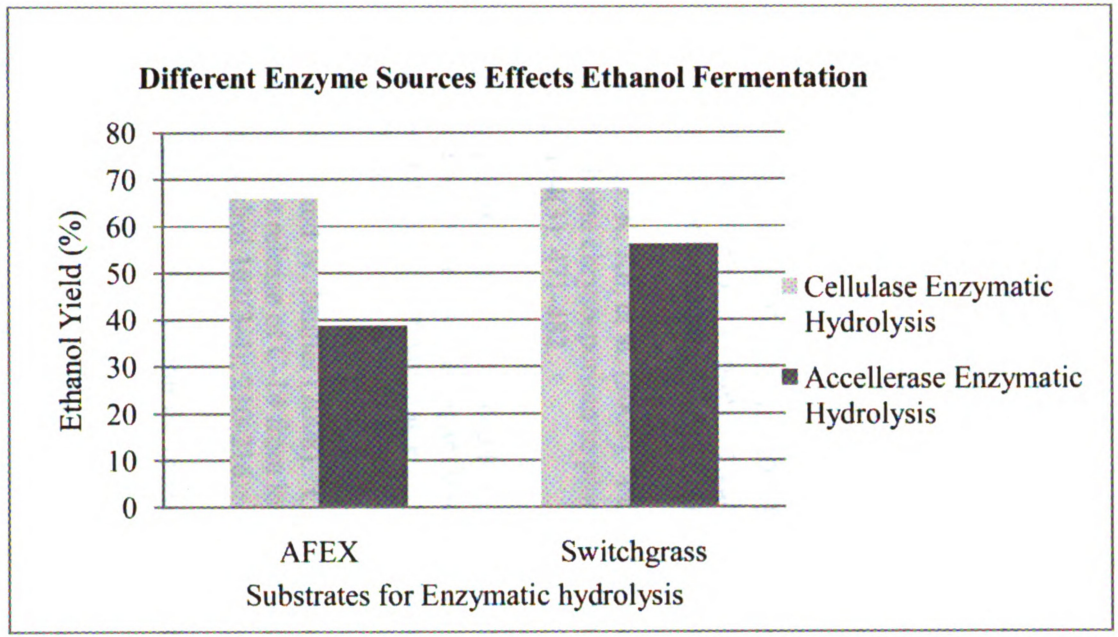

Figure 31. Compared the effects of different hydrolysates based on 2 different enzyme sources working on ethanol fermentation.

Reference

1 A., W., T. J., et al. (1996). "Biomass feedstock resources and composition."

2 Ahamed, A. and P. Vermette (2009). "Effect of culture medium composition on Trichoderma reesei's morphology and cellulase production." Bioresour Technol **100**(23): 5979-5987

3 Alvira, P., E. Tomas-Pejo, et al. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review." Bioresour Technol **101**(13): 4851-4861.

4 Azzam, A. M. (1989). "Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation." Environ.Sci.Health **B. 24(4)**: 421-433.

5 Bailey, M. J. and J. Tahtiharju (2003). "Efficient cellulase production by Trichoderma reesei in continuous cultivation on lactose medium with a computer-controlled feeding strategy." Appl Microbiol Biotechnol **62**(2-3): 156-162.

6 Bak, J. S., J. K. Ko, et al. (2009). "Fungal pretreatment of lignocellulose by Phanerochaete chrysosporium to produce ethanol from rice straw." Biotechnol Bioeng **104**(3): 471-482.

7 Boominathan, K. and C. A. Reddy (1992). "cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete Phanerochaete chrysosporium." Proc Natl Acad Sci U S A **89**(12): 5586-5590.

8 Bracke, G. and W. Muller (2008). "Contribution to a more realistic approach in assessing the release of C-14 from low-level radioactive waste repositories." J Contam Hydrol **102**(3-4): 210-216.

9 BRDI (2006). "2006 Vision for bioenergy and biobased products in the United States."

10 Cambell, C. J. and J. H. Laherrere (1998). "The end of cheap oil." Sci Am **3**: 78-83.

11 Chadha, B. S. and H. S. Garcha (1992). "Mixed cultivation of Trichoderma reesei and Aspergillus ochraceus for improved cellulase production." Acta Microbiol Hung **39**(1): 61-67.

12 Chen, F. and R. A. Dixon (2007). "Lignin modification improves fermentable sugar yields for biofuel production." Nature Biotechnology **25**(7): 759-761.

13 Colina, A., B. Sulbaran-De-Ferrer, et al. (2003). "Xylanase production by Trichoderma reesei rut C-30 on rice straw." Appl Biochem Biotechnol **105 -108**: 715-724.

14 Coughlan Michael P. (1989). Enzyme systems for lignocellulose degradation. London ; New York, Elsevier.

15 Dashtban, M., H. Schraft, et al. (2009). "Fungal bioconversion of lignocellulosic residues; opportunities & perspectives." Int J Biol Sci **5**(6): 578-595.

16 Depardo, J. (2000). "Outlook for biomass ethanol production." Energy information administration.

17 Deshpande, P., S. Nair, et al. (2009). "Water hyacinth as carbon source for the production of cellulase by Trichoderma reesei." Appl Biochem Biotechnol **158**(3): 552-560.

18 Domingues, F. C., J. A. Queiroz, et al. (2000). "The influence of culture conditions on mycelial structure and cellulase production by Trichoderma reesei Rut C-30." Enzyme Microb Technol **26**(5-6): 394-401.

19 Duff, S. J., D. G. Cooper, et al. (1985). "Effect of Colloidal Materials on Cellulase Production by Trichoderma reesei Rut-C30." Appl Environ Microbiol **49**(4): 934-938.

20 Duff, S. J. and W. D. Murray (1996). "Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review." Bioresour Technol **55**(1-33).

21 Eriksson, K. E. and S. C. Johnsrud (1986). "Microbial Delignification of Lignocellulosic Materials." Papier **40**(10A): V33-V37.

22 Esteghlalian, A. R., A. G. Hashimoto, et al. (1997). "Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass." Bioresour Technol **59**: 129-136.

23 Fan, L. T., M. M. Gharpurary, et al. (1987). "Cellulose hydrolysis biotechnology monographs." Springer, Berlin: 57.

24 Farrell, A. E., R. J. Plevin, et al. (2006). "Ethanol can contribute to energy and environmental goals." Science **311**(1): 506-508.

25 Ghose, T. K. (1978). Bioconversion of cellulosic substances into energy, chemicals, and microbial protein : symposium proceedings, held at New Delhi, February 21-23, 1977 under the auspices of the Biochemical Engineering Research Centre, Indian Institute of Technology, Delhi and the Institute of Microbiology, Swiss Federal Institute of Technology, Zurich. New Delhi

26 Zurich, Indian Institute of Technology ;

27 Swiss Federal Institute of Technology.

28 Ghose, T. K., T. Panda, et al. (1985). "Effect of culture phasing and mannanase on production of cellulase and hemicellulase by mixed culture of Trichoderma reesei D 1-6 and Aspergillus wentii pt 2804." Biotechnol Bioeng **27**(9): 1353-1361.

29 Hadar, Y., Z. Kerem, et al. (1993). "Biodegradation of lignocellulosic agrucutrural waste by Pleurotus ostreatus." J.Biotechnol **30133-139**.

30 Hendriks, A. T. W. M. and Z. G. (2009). "Pretreatment to enhance the digestibility of lignocellulosic biomass." Bioresour Technology **100**(1): 10-18.

31 Himmel, M. E. and J. O. Baker "Enzymatic conversion of biomass for fuels production." American chemical society: 363-371.

32 Himmel, M. E., M. F. Ruth, et al. (1999). "Cellulase for commodity products from cellulosic biomass." Curr Opin Biotechnol **10**(4): 358-364.

33 Hofrichter, M. (2002). "Review: lignin conversion by manganese peroxidase (MnP)." Enzyme and Microbial Technology **30**(1): 454-466.

34 Huang, X. L. and M. H. Penner (1991). "Apparent Substrate-Inhibition of the Trichoderma-Reesei Cellulase System." Journal of Agricultural and Food Chemistry **39**(11): 2096-2100.

35 Jager, A., S. Croan, et al. (1985). "Production of ligninases and degradation of lignin in agitated submerged cultures of phanerochaete chrysosporium." Environ.Microbiol **50**: 1274-1278.

36 Janick, J. "Perspectives on new crops and new uses." ASHS press: 282-299.

37 Jeffries, T. W. (1990). "Biodegradation of lignin-carbohydrate complexes." Biodegradation **1**: 163-176.

38 Keller, F. A., J. E. Hamilton, et al. (2003). "Microbial pretreatment of biomass: potential for reducing severity of thermochemical biomass pretreatment." Appl Biochem Biotechnol **105 -108**: 27-41.

39 Keshwani, D. R. and J. J. Cheng (2009). "Switchgrass for bioethanol and other value-added applications: a review." Bioresour Technol **100**(4): 1515-1523.

40 Kim, T. H., Y. Y. Lee, et al. (2006). "Pretreatment of corn stover by low-liquid ammonia recycle percolation process." Applied Biochemistry and Biotechnology **133**(1): 41-57.

41 Kirk, T. K., E. Schulze, et al. (1978). "Influence of culture parameters on lignin metabolism by phanerochaete chrysosporium." Arch.Microbiol **117**: 277-285.

42 Koullas, D. P., P. Christakopoulos, et al. (1992). "Correlating the Effect of Pretreatment on the Enzymatic-Hydrolysis of Straw." Biotechnology and Bioengineering **39**(1): 113-116.

43 Kovacs, K., S. George, et al. (2009). "Comparative enzymatic hydrolysis of pretreated spruce by supernatants, whole fermentation broths and washed mycelia of Trichoderma reesei and Trichoderma atroviride." Bioresour Technology **100**(1): 1350-1357.

44 Kubicek, C. P., R. Messner, et al. (1993). "The Trichoderma cellulase regulatory puzzle: from the interior life of a secretory fungus." Enzyme Microb Technol **15**(2): 90-99.

45 Kumar, R. and R. P. Singh (2001). "Semi-solid-state fermentation of Eicchornia crassipes biomass as lignocellulosic biopolymer for cellulase and 3-glucosidase production by cocultivation of Aspergillus niger RK3 and Trichoderma reesei MTCC164." Appl Biochem Biotechnol **96**(1-3): 71-82.

46 Kumar, R. and C. E. Wyman (2009). "Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies." Bioresour Technol **100**(18): 4203-4213.

47 Latifian, M., Z. Hamidi-Esfahani, et al. (2007). "Evaluation of culture conditions for cellulase production by two Trichoderma reesei mutants under solid-state fermentation conditions." Bioresour Technol **98**(18): 3634-3637.

48 Leonowicz, A., A. Matuszewska, et al. (1999). "Biodegradation of lignin by white rot fungi." Fungal Genet Biol **27**(2-3): 175-185.

49 Lewandowski, I., J. M. O. Scurlock, et al. (2003). "The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe." Biomass & Bioenergy **25**(4): 335-361.

50 Liao, W., Y. Liu, et al. (2007). "A new approach of pellet formation of a filamentous fungus -Rhizopus oryzae." Bioresour Technol **98**(18): 3415-3423.

51 Liming, X. and S. Xueliang (2004). "High-yield cellulase production by Trichoderma reesei ZU-02 on corn cob residue." Bioresour Technol **91**(3): 259-262.

52 Linko, M., K. Poutanen, et al. (1989). "New developments in the application of enzymes for biomass processing." Elsevier Applied Science: 331-345.

53 Lynd, L. R., J. H. Cushman, et al. (1991). "Fuel Ethanol from Cellulosic Biomass." Science **251**(4999): 1318-1323.

54 Mandels, M. (1976). "Production and Applications of Trichoderma Cellulase." Abstracts of Papers of the American Chemical Society: 82-82.

55 Mandels, M., L. Hontz, et al. (2010). "Enzymatic hydrolysis of waste cellulose. Biotechnol Bioeng. Vol. XVI, pages 1471-93 (1974)." Biotechnol Bioeng **105**(1): 3-25; discussion 21-22.

56 McMillan, J. D. (1994). "Pretreatment of lignocellulosic biomass."

57 Miettinen-Oinonen, A. and P. Suominen (2002). "Enhanced production of Trichoderma reesei endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric." Appl Environ Microbiol **68**(8): 3956-3964.

58 Montenecourt, B. S. and D. E. Eveleigh (1977). "Preparation of mutants of Trichoderma reesei with enhanced cellulase production." Appl Environ Microbiol **34**(6): 777-782.

59 Moser, L. E., L. E. Sollenberger, et al. "American society of agronomy." 561-588.

60 Mosier, N., C. Wyman, et al. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass." Bioresour Technol **96**(6): 673-686.

61 Orpin, C. G. (1988). "genetic approaches to the improvement of lignocellulose degradation in the rumen." Academic press: 171-179.

62 Parker, A. A. (2005). "The end of suburbia: what happens when all the cheap oil is gone and what are the health implications?" Health Promot J Austr **16**(1): 61-68.

63 Rahman, Z., Y. Shida, et al. (2009). "Application of Trichoderma reesei cellulase and xylanase promoters through homologous recombination for enhanced production of extracellular beta-glucosidase I." Biosci Biotechnol Biochem **73**(5): 1083-1089.

64 Reddy, C. A. and T. M. D'Souza (1994). "Physiology and molecular biology of the lignin peroxidases of Phanerochaete chrysosporium." FEMS Microbiol Rev **13**(2-3): 137-152.

65 Reese, E. T., American Chemical Society., et al. (1963). Advances in enzymic hydrolysis of cellulose and related materials. New York,, Pergamon Press;.

66 RJ, B., N. Nichols, et al. (1999). "Fermentation with new recombinant organisms." Biotechnol Prog **15**: 875.

67 Sardar, S., A. K. Paul, et al. (2008). "A quantum-classical approach to the molecular dynamics of butatriene cation with a realistic model Hamiltonian." Phys Chem Chem Phys **10**(42): 6388-6398.

68 Sawada, T., Y. Nakamura, et al. (1995). "Effects of fungal pretreatment and steam explosion pretreatment on enzymatic saccharification of plant biomass." Biotechnol Bioeng **48**(6): 719-724.

69 Schaffner, D. W. and R. T. Toledo (1991). "Cellulase production by Trichoderma reesei when cultured on xylose-based media supplemented with sorbose." Biotechnol Bioeng **37**(1): 12-16.

70 Schafner, D. W. and R. T. Toledo (1992). "Cellulase production in continuous culture by Trichoderma reesei on xylose-based media." Biotechnol Bioeng **39**(8): 865-869.

71 Sendich, E. N., M. Laser, et al. (2008). "Recent process improvements for the ammonia fiber expansion (AFEX) process and resulting reductions in minimum ethanol selling price." Bioresour Technol **99**(17): 8429-8435.

72 Shi, J., R. R. Sharma-Shivappa, et al. (2009). "Microbial pretreatment of cotton stalks by submerged cultivation of Phanerochaete chrysosporium." Bioresour Technol **100**(19): 4388-4395.

73 Singhania, R. R., R. K. Sukumaran, et al. (2007). "Improved cellulase production by Trichoderma reesei RUT C30 under SSF through process optimization." Appl Biochem Biotechnol **142**(1): 60-70.

74 Sivers, M. V. and G. Zacchi (1995). "A techno-economical comparison of three processes for the production of ethanol from pine." Bioresour Technol **51**: 43-52.

75 Sternberg, D. and S. Dorval (1979). "Cellulase production and ammonia metabolism in Trichoderma reesei on high levels of cellulose." Biotechnol Bioeng **21**(2): 181-191.

76 Sun, Y. and J. Cheng (2002). "Hydrolysis of lignocellulosic materials for ethanol production: a review." Bioresour Technol **83**(1): 1-11.

77 Sun, Y. and J. Cheng (2002). "Hydrolysis of lignocellulosic materials for ethanol production: a review." Bioresour Technol **83**(1): 1-11.

78 Szengyel, Z. and G. Zacchi (2000). "Effect of acetic acid and furfural on cellulase production of Trichoderma reesei RUT C30." Appl Biochem Biotechnol **89**(1): 31-42.

79 Szengyel, Z., G. Zacchi, et al. (2000). "Cellulase production of Trichoderma reesei Rut C 30 using steam-pretreated spruce. Hydrolytic potential of cellulases on different substrates." Appl Biochem Biotechnol **84-86**: 679-691.

80 Szijarto, N., Z. Szengyel, et al. (2004). "Dynamics of cellulase production by glucose grown cultures of Trichoderma reesei Rut-C30 as a response to addition of cellulose." Appl Biochem Biotechnol **113-116**: 115-124.

81 Tholudur, A., W. F. Ramirez, et al. (1999). "Mathematical modeling and optimization of cellulase protein production using Trichoderma reesei RL-P37." Biotechnol Bioeng **66**(1): 1-16.

82 Tomas-Pejo, E., O. J. M, et al. (2008). "Realistic approach for full-scale bioethanol production from lignocellulose: a review." Journal of Scientific & Industrial Research **67**(1): 874-884.

83 Varga, E., Z. Szengyel, et al. (2002). "Chemical pretreatment of corn stover for enhancing enzymatic digestibility." Appl Biochem Biotechnol **98-100**(1): 0272-0289.

84 Velkovska, S., M. R. Marten, et al. (1997). "Kinetic model for batch cellulase production by Trichoderma reesei RUT c30." J Biotechnol **54**(2): 83-94.

85 Vidal, P. F. and J. Molinier (1988). "Ozonolysis of Lignin - Improvement of Invitro Digestibility of Poplar Sawdust." Biomass **16**(1): 1-17.

86 Wang, M., C. Saricks, et al. (1999). "Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions." Argonne National Laboratory.

87 Wen, Z., W. Liao, et al. (2005). "Production of cellulase/beta-glucosidase by the mixed fungi culture of Trichoderma reesei and Aspergillus phoenicis on dairy manure." Appl Biochem Biotechnol **121-124**: 93-104.

88 Wu, J. and L. K. Ju (1998). "Enhancing enzymatic saccharification of waste newsprint by surfactant addition." Biotechnology Progress **14**(4): 649-652.

89 Yang, B. and W. C. E. (2007). "Pretreatment: the key to unlocking low-cost cellulosic ethanol." Biofuels Bioproducts & Biorefining **2**(1): 26-40.

90 Zaldivar, J., J. Nielsen, et al. (2001). "Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration." Appl Microbiol Biotechnol **56**(1-2): 17-34.

91 Zheng, Y., Z. Pan, et al. (2007). "Evaluation of different biomass materials as feedstock for fermentable sugar production." Appl Biochem Biotechnol **137-140**(1-12): 423-435.

92 Zheng, Y., P. Zhongli, et al. (2009). "Overview of biomass pretreatment for cellulosic ethanol production." Int J Biol Sci **2**: 51.