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Sequential saccharification and fermentation of corn stover for the production of fuel ethanol using wood-rot fungi, *Saccharomyces cerevisiae* and *Escherichia coli* K011

by

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All glory, honor and praise to God Almighty, Jesus Christ and the Holy Spirit

God is good all the time and His mercies endure forever

I dedicate this dissertation to my wife, Magdline, my son, Archer and my family in Malaysia
- To my mother, my sisters Garnett and Neela, and their families

This is especially dedicated to my late father
Corporal Vincent Anak Lisa

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ABSTRACT

World oil consumption for energy and transportation applications has increased tremendously over the past decades as the world population grew, and more countries becoming industrialized. Even domestic products like plastics, chemicals, toiletries, clothes, food packaging, automobile parts and building materials are made from petrochemicals. In the United States, world's number one oil consumer, approximately 70% of crude oil goes to the transportation sector. To supplement these fossil based fuels, several ethanol-gasoline blends are currently in the market, and since 2006, a massive increase in the utilization of ethanol is reported in the United States, and this trend is also observed globally. While the present first generation fuel ethanol are produced mainly from sugary and starchy feedstock, numerous efforts are underway in the research, development and production of second generation bioethanol that are derived from lignocellulosic biomass. The latter platform has not fully matured due to the various process and economic challenges in efficiently producing market friendly ethanol from lignocellulosic biomass. Therefore, it is imperative to develop means of bioprocesses that may reduce cost associated with lignocellulosic ethanol production.

In our study, we aim to develop a sequential biological process that converts cellulosic materials into fermentable sugars and ultimately ethanol as a transportation fuel. We performed solid state fermentation at ambient conditions to induce lignocellulolytic activities from three fungal species, namely *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* and *Trichoderma reesei*. We cultivated each of the fungal species on pure cellulose and corn

stover to induce the secretion of cellulases, hemicellulase and lignolytic enzymes via solid state fermentation for several days. Corn stover was chosen as the main material as it is one of the most abundant agricultural residues. The mold mediated processes liberate simple carbohydrates, suitable substrates for downstream microbial utilization. Next, we performed simultaneous saccharification and fermentation (SSF) of the cellulosic materials to produce more sugars that are converted to ethanol.

Prior to the SSF studies on the corn stover, we initially performed enzymatic studies of these fungal species on pure cellulose to evaluate their *in situ* enzyme production and hydrolytic abilities. Filter paper was used in the screening in accordance to the recommendations of several previously reported studies. The efficiency of the fungal species in saccharifying the filter was compared against a low dose (25 FPU/g cellulose) of a commercial cellulase. Fermentation was achieved by using the yeast *Saccharomyces cerevisiae.* Total sugar, cellobiose and glucose concentrations were monitored during the fermentation period, along with three main fermentation products, namely ethanol, acetic acid and lactic acid. Results indicated that the most efficient fungal species in saccharifying the filter paper was *T. reesei* with 5.13 g/100 g filter paper of ethanol being produced at days 5, followed by *P. chrysosporium* at 1.79 g/100 g filter paper. No ethanol was produced from the filter paper treated with *G. trabeum* throughout the five day fermentation stage. Acetic acid was only produced in the sample treated with *T. reesei* and the commercial enzyme, with concentration 0.95 g and 2.57 g/100 g filter paper, respectively at day 5.

Next, we performed enzymatic saccharification of corn stover using *P. chrysosporium* and *G. trabeum*. Subsequent fermentation of the saccharification products to ethanol was achieved via the use of *Saccharomyces cerevisiae* and *Escherichia coli* K011. During the SSF period with *S. cerevisiae* or *E. coli*, ethanol production was highest on day 4 for all samples inoculated with either *P. chrysosporium* or *G. trabeum*. For the corn stover treated with *P. chrysosporium*, the conversion of corn stover to ethanol was 2.29 g/100 g corn stover for the sample inoculated with *S. cerevisiae,* whereas for the sample inoculated with *E. coli* K011, the ethanol concentration was 4.14 g/100 g corn stover. While for the corn stover treated with *G. trabeum*, the conversion of corn stover to ethanol was 1.90 g and 4.79 g/100 g corn stover for the sample inoculated with *S. cerevisiae* and *E. coli* K011, respectively. Other fermentation co-products, such as, acetic acid and lactic acid were also recorded. Acetic acid production ranged between 0.45 g and 0.78 g/100 g corn stover for the samples under different fungal treatments, while no lactic acid production was detected throughout the 5 days of SSF.

In the later stages of our study, we further explore the coupling of mild chemical (dilute NaOH) and biological pretreatment and saccharification on the corn stover. Ethanol production was achieved via the sequential saccharification and fermentation of dilute sodium hydroxide (2% w/w NaOH in corn stover) treated corn stover using *P. chrysosporium* and *G. trabeum.* Ethanol production peaked on day 3 and day 4 for the samples inoculated with either *P. chrysosporium* or *G. trabeum,* slightly plateauing or decreasing thereafter. Ethanol production was highest for the combination of *G. trabeum* and *E. coli* K011 at 6.68 g/100 g corn stover, followed by the combination of *P. chrysosporium*

and *E. coli* K011 at 5.00 g/100 g corn stover. Combination of both the fungi with *S. cerevisiae* generally had lower ethanol yields, ranging between 2.88 g (*P. chrysosporium* treated corn stover) and 3.09 g/100 g corn stover (*G. trabeum* treated corn stover). Acetic acid production ranged between 0.53 g and 2.03 g/100 g corn stover for the samples under different fungal treatments, while lactic acid production was only detected in samples treated with *G. trabeum,* throughout the 5 days of SSF.

The results of our study indicated that mild alkaline pretreatment coupled with fungal saccharification offer a promising bioprocess for ethanol production from corn stover without the addition of commercial enzymes. We believe these sequential procedures are potentially applicable to various other lignocellulosic materials (i.e. switchgrass, poplar, corn cobs) and may assist in environmentally, economical and technological friendlier ethanol production processes.

CHAPTER 1: INTRODUCTION

INTRODUCTION

Over the decades, energy consumption has increased tremendously by 16-fold as the world population quadrupled in the twentieth century, and more countries have become industrialized (Sun and Cheng 2002; Sanchez and Cordona 2008; Zhang 2008). If the current trend persists, the total energy consumption is expected to rise to 27–42 Terawatt (TW) from the current 13 TW by the year 2050 (Whitesides and Crabtree 2007). In the United States, world's number one oil consumer, approximately 70% of crude oil goes to the transportation sector (Sanchez and Cordona 2008). Even plastics products used to make chemicals, toiletries, pharmaceuticals, clothes, food packaging, automobile parts and building materials are made from petrochemicals (National Renewable Energy Laboratory 2002). This is an undesirable situation, both strategically and economically with crude oil and energy prices recently reaching historic highs of over 100 dollars per barrel (Sanchez and Cordona 2008).

Oil is a non-renewable resource that is progressively depleting (Silverstein et al. 2007). According to Sun and Cheng (2002), analysts are predicting that current global oil production would decline by 80% (from 25-30 billion barrels per annum to approximately 5 billion barrels) in 2050. In short, the oil reserve could be depleted within 35-70 years, potentially causing chaos in the economy and transportation sector in the United States and most other nations that depend heavily on oil (Sun and Cheng 2002; Zhang et al. 2007; Zhang 2008).

As the concern about energy security escalates, there is currently a surge of interest both statewide and worldwide in utilizing and producing alternative domestic and renewable energy sources that could reduce needs for fossil based energy supplies (Sheehan et al. 2004; Matsuoka et al. 2009; Wen et al. 2009). Besides energy security issues, other motivations for developing biofuels as a transportation fuel also include needs to keep up with economic growth and environmental health (Perez-Verdin et al. 2009). The adoption of the Kyoto Protocol in 1997 also contributed to this concern as it ignited a global wide concern about climate changes and global warming, which has resulted in unprecedented discussions on the impacts of fossil-based fuel usage to the increase of polluting gases released into the earth's atmosphere (Sanchez and Cordona 2008; Matsuoka et al. 2009). This has led to the research, development and production of biofuels such as bioethanol from plant biomass as the candidate to supplement, substitute and possibly replace fossil fuels (Xuan et al. 2009).

Research, development and production of bioethanol (also called fuel ethanol) have received countless attention not only in the media, but also in the scientific communities, political, and decision-making areas (Perez-Verdin et al. 2009). In his State of Union speech in 2007, President Bush projected that ethanol production in the United States would reach 35 billion gallons per year by 2017 (Zhang 2008). This is equivalent to approximately 20% of biofuels replacement of petroleum usage over the course of next ten years (Wen et al. 2009). Another scenario proposed by the US Department of Energy (US-DOE) indicated that 30% transportation fuels will be from renewable resources such as biomass (approximately 60 billion gallons) by 2030 (Himmel et al. 2007).

According to the National Research Council (NRC) (1999), the motivation for bioethanol in the United States has underwent an interesting evolution that revolves around the tax state and federal gasoline tax exemptions. In the 1980's, the motive was driven by security and domestic economic development, followed by the compliance to meet environmental regulations such as the improvement of air quality, and finally, the reduction of greenhouse gases to the atmosphere as per declared in the Kyoto's Protocol.

Globally, we also notice the similar interest in biofuels in other countries. Brazil made one of the earlier nationwide move soon after 1973, when gasoline was blended with ethanol in 1979, then began manufacturing vehicles that could run on hydrous ethanol (95% ethanol), and by mid-80's, majority of all new cars were manufactured to run exclusively on ethanol (NRC 1999). In the European Union, the European Commission has announced its plan to replace 20% of fossil fuels with renewable fuels in the transportation sector by 2020, and possibly increasing that percentage to 25% (Himmel et al. 2007).

According to a statement by the International Energy Agency (IEA) presented in the 2008 World Energy Outlook report, biofuels (bioethanol and biodiesel) contributed to about 1% of the world's transportation fuel consumption in 2006, and projected to rise to approximately 4% by the year 2030 (Zhou and Thomson 2009). The same report also projected a yearly growth rate of approximately 6.8% from 2006 to 2030 for overall total biofuels consumption. In total, the United States, Brazil, the European Union and China account for approximately 90% of global biofuels production (Sainz 2009).

Fuel ethanol has always been produced via the first generation platform through the conversion of two major groups of feedstocks: (i) sugary feedstocks (sugar cane, sugar beet, sweet sorghum) and (ii) starchy materials (corn, milo, wheat, cassava, sweet potatoes) (Balat and Balat 2009; Perez-Verdin et al. 2009). In particular, corn, however, has created numerous controversies as it is needed as food for human and also feed for animals. In addition, the ethanol prices from sugar or starch feedstocks were too high to compete with gasoline for transportation use (Alkasrawi et al. 2003). Hence, the United States Department of Energy (US-DOE) started promoting the development of fuel ethanol from cheap lignocellulosic feedstocks, such as agriculture residues and energy crops (de La Torre Ugarte et al. 2003; Perez-Verdin et al. 2009). The reasons for these moves are simple - to reduce dependence on foreign oil, decrease trade deficits, rural economics, biodegradability, air pollution and global carbon dioxide reduction (carbon sequestration) (Badger 2002; Balat and Balat 2009).

According to several literatures, lignocellulosic biomass is the most abundant and sustainable bioresources that are still not fully exploited (Chang 2007; Hong et al. 2007; Zhang 2008; Fukuda et al. 2009). Strictly speaking, plant biomass is stored solar energy in forms of complex organic molecules and polymers (Sanchez and Cordona 2008). Plants convert solar energy into various energy-containing organic molecules such as sugars, starch, other carbohydrates, cellulose, hemicellulose and lignin, via photosynthesis, with much of the biomass and bioenergy deposited into the fibrous lignocellulosic parts of the plant (NRC 1999; Ahamed and Vermette 2008; Ling et al. 2009). On average, it is estimated that the amount of carbon fixed by plants during photosynthesis is over 100 billion tons per annum

(Bedford 2001). In a recent report by the US-DOE, the United States alone is reported to have the potential to produce approximately 1.3 billion tonnes of lignocellulosic biomass annually, which could be converted to liquid fuel to supply up to 30% of transportation fuels (~225 billion liters of ethanol) without displacing food crops, such as grains (Chang 2007).

Although very promising, large-scale lignocellulosic ethanol production faces three major logistic obstacles; high processing costs, huge capital investment and narrow margin between feedstock and product prices (Zhang 2008). Process-wise, there are three challenges in efficiently producing cheaper ethanol from lignocellulosic biomass (MacAloon et al. 2000):

1. The need to liberate the cellulose and hemicellulose molecules from their native lignocellulosic state via pretreatment (Zhu et al. 2006). In this respect, different laboratories employ different means of pretreatment, mostly via physical and chemical means, to render the cellulose and hemicellulose more accessible for downstream enzymatic hydrolysis (Yang et al. 2008; Garcia-Cubero et al. 2009). However, other sets of problems accompany this process. When pretreatments are applied, chemical and biochemical compounds are accumulated (i.e. acetic acid, other inorganic acids, phenolics and various salts), that can be inhibitory to microbial fermentation and biocatalysis (Martinez et al. 2000; Talebnia et al. 2004; Keating et al. 2005).

2. The lack of cost-effective enzymes to catalyze the hydrolysis of cellulose to glucose (Alkasrawi et al. 2003). This is a significant factor in fuel ethanol production as

enzymatic hydrolysis still remain a significant portion of the lignocellulosic biomass sugars production cost (Linde et al. 2007).

3. The need for hardier and high performance bio-catalysts (microogansims and enzymes) capable of utilizing all fermentable sugars released from plant biomass hydrolysis and able to adapt to pretreated biomass (MacAloon et al. 2000). Although significant progresses have been achieved in selecting and developing microorganisms capable of effectively producing ethanol from hexoses at high yield, cultures that perform satisfactorily on pentoses still remain elusive (Piskur et al. 2006).

Because of the problems and costs associated with pretreatments and enzymatic hydrolysis of lignocellulosic feedstocks, it is imperative to develop means of pretreatment and enzymatic hydrolysis of lignocellulosics that do not sacrifice ethanol production. Therefore, in attempts to address these issues, we employed several lignocellulolytic microorganisms to perform both pretreatment and enzymatic saccharification on corn stover, our lignocellulosic material of choice. In our study, we performed solid state fermentation at ambient conditions to induce lignocellulolytic activities. The three fungal species used in this study represent three major wood-rot fungi, namely *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* and *Trichoderma reesei*. Corn stover was chosen as it is one of the most abundant agricultural residues in the United States and many other countries (Galbe and Zacchi, 2007; Chen et al. 2009). Prior to performing the main studies on corn stover, we initially perform enzymatic studies of these fungal species on pure cellulose to evaluate their enzyme production and hydrolytic abilities to pure cellulose *in situ*. Filter paper (Whatman No. 1) was used in the

preliminary screening as, according to several studies, this is the material recommended by National Renewable Energy Laboratory (NREL) for measurement of cellulase activities (Decker et al. 2003; Adney and Baker 2008).

We cultivated each of the fungal species on both of the cellulosic material to induce the secretion of cellulases, hemicellulase and lignolytic enzymes via solid state fermentation for several days. The mold mediated processes liberate simple carbohydrates, suitable substrates for downstream microbial utilization. Next, we performed simultaneous saccharification and fermentation (SSF) of the cellulosic materials to convert the fermentable sugar products to ethanol. SSF was chosen as it is reportedly the most logistically and economically favorable process to produce the highest ethanol yield (Tomas-Pejo et al. 2009). In the later stages of our study, we further explore the coupling of mild chemical (dilute NaOH) and biological pretreatment and saccharification on the later part of our study. To perform fermentation, *Saccharomyces cerevisea* and *Escherichia coli* K011 were used to the convert the saccharification products into ethanol. Results are encouraging and we believe these sequential procedures are potentially applicable to various other lignocellulosic materials (i.e. switchgrass, poplar, corn cobs).

RESEARCH IMPORTANCE AND RATIONALE

The realization that oil reserves would someday be extremely expensive and even in short supply spawned the idea of a renewable energy pool, one that could be made from either free

unlimited resources like solar energy or wind, or from replenished bioresources. As of February, 2009, there are 180 bioethanol plants in operations with the total production capacity of 12.2 billion gallons, and the Energy Independence and Security Act of 2007 mandated a gradual increase in the use of renewable biofuels until 2022, until production reaches 36 billion gallons (Stowers 2009). Globally, fuel ethanol production from first generation platforms is estimated to increase to 113.6 billion liters (~30.29 billion gallons) by 2022 (Sainz 2009). However, in the recent years, production of first generation bioethanol based on corn starch has received much criticism in the feed, food, fuel debate (Songstad et al. 2009). There have even been reports that attribute the rising food prices to the production of biofuels such as bioethanol and biodiesel (Armah et al. 2009). In this respect, second generation bioethanol research and production from non-food sources such as lignocellulose biomass are underway as these resources offers great potential to replace conventional fossil fuels without further aggravating the food-fuel debates (Goh et al. 2009).

Research on the production of ethanol from lignocellulosic biomass started as early as the 1970s in response to the same oil crisis that gave birth to the corn-ethanol industry, as new technologies are required if biofuels are to significantly contribute to global energy supplies and to offset the effects of greenhouse gas emissions (Sainz 2009). Since new technologies typically take approximately 25 years to be commercialized, development of alternative, renewable and sustainable transportation fuels is urgently needed in order to meet developmental and environmental needs of both present and future generations (Zhang 2008). Countries like the United States, Canada and China have shown increasing interest and have already committed millions of dollars for the research on lignocellulosic ethanol

research and programs, resulting in the acceleration of research activities in cellulosic ethanol

technologies and funding (Sainz 2009).

The major economic hurdles to viable commercial lignocellulosic ethanol production are

high production costs, associated with feedstocks, processing and enzymes, as the

conventional method for the conversion of lignocellulosics to their monomeric sugars

requires the use of expensive commercial enzymes (Sainz 2009). Therefore in our research,

we address these main issues in the following manners:

1. Utilization of corn stover as main feedstock.

   According to Balat and Balat (2009) and Borjenson et al (2009), to produce "good"

   ethanol, production plants should use cheap and abundant biomass as their feedstocks.

   Corn stover is a substantial source of cheap and abundant lignocellulosic biomass, not

   only in the United States, but also in other countries, such as Europe and China (Galbe

   Zacchi 2007; Chen et al. 2009; Hess et al. 2009). Several recent studies reported that the

   United States produce as much as 75 million tons of this promising feedstock (Perlack et

   al. 2005; Hess et al. 2005). Currently, corn stover is one of the most studied

   lignocellulosic biomass used for bioethanol production, which include harvesting

   technologies and pretreatment procedures (Lu et al. 2008; Aden and Foust 2009; He et al.

   2009; Kumar et al. 2009). Corn stover is recognized as one the most promising feedstock

   by the NREL that a special comprehensive report for a process design and economic

   analysis of the biochemical conversion of corn stover to ethanol was published (Aden,

   2008; Templeton *et al.*, 2009). In fact, by current technological standards, corn stover

based ethanol has the potential to produce approximately 6 billion gallons (85 gal/ton) of liquid fuel (Hess et al., 2009).

2. Utilization of wood-rot fungi as main biological treatment options and enzyme sources.

The three fungal species used in this study are *P. chrysosporium*, *G. trabeum* and *T. reesei*. *P. chrysosporium* has been studied extensively for its efficient lignin degrading enzymes, such as lignin peroxidases, manganese peroxidases and peroxidases, in addition to a variety of cellulases and hemicellulase (Martinez et al. 2009). *P. chrysosporium* has also been used in biotechnological applications, such as biobleaching and pulp-mill effluent treatment (Wymelenberg et al. 2005; Kersten and Cullen 2007; Ravalason et al. 2008). *G. trabeum* is an ecologically important saprophytic fungus contribute significantly to the soil fertility in the ecosystem, that cause the most destructive type of cellulosic decay, attributed largely to several non-enzymatic processes that involve low molecular weight catalysts (Henriksson et al. 1999; Cohen et al. 2005; Schilling et al. 2009). *T. reesei* is a soft-rot mold that is well documented for its highly efficient cellulolytic and hemicellulolytic enzyme systems for the complete hydrolysis of biomass, and is a model fungus for the production of commercial cellulases (Ahamed and Vermette 2008).

The most promising aspects of our research are the possibilities of minimizing or eliminating the most costly part of the lignocellulosic-ethanol process, (i) the pretreatment stage and (ii) the addition of expensive commercial enzymes. By using extracellular enzymes like ligninases, cellulases and hemicellulases from the wood-rot

fungi, *in situ* biodegradation of corn stover will liberate simple sugars that can be fermented to ethanol. Several studies using these fungi on corn fiber showed outstanding results in terms of lignin degradations and cellulolytic activities (Shrestha et al., 2008; Shrestha et al., 2009; Rasmussen et al., 2010).

RESEARCH OBJECTIVE

The overall goal of our study is to develop a sequential biological process that converts cellulosic materials into fermentable sugars and ultimately ethanol as a transportation fuel.

Specifically, the study was performed to meet the following objectives:

1. Investigate the performances of three different species of wood rot, namely white rot (*P. chrysosporium*), brown rot (*G. trabeum*) and soft rot (*T. reesei*) for the production of lignocellulolytic enzymes via solid state fermentation on pure cellulose.
2. Evaluate two different species of wood rot, namely white rot (*P. chrysosporium*) and brown rot (*G. trabeum*) for the production of lignocellulolytic enzymes via solid state fermentation to liberate fermentable sugars that will subsequently be substrates as microbial fermentation feedstock to produce ethanol using *S. cerevisiae* and *E. coli* K011.
3. Examine the effectiveness of mild alkali pretreatment, using sodium hydroxide (NaOH), of corn stover prior to solid state fermentation by selected fungal species, and subsequent fermentation of the hydrolysate to ethanol using *S. cerevisiae* and *E. coli* K011.

DISSERTATION ORGANIZATION

This dissertation is organized into seven chapters. The first chapter of this dissertation is an introduction to the studies, where the research objectives, rationale and justifications are also discussed. Chapter 2 is the Literature Review. In this chapter, subjects such as ethanol, fuel ethanol, history of fuel ethanol and fuel ethanol programs worldwide are covered. This is followed by the discussion of bioethanol production, which is continued by the introduction to the lignocellulosics materials and its contribution towards lignocellulosic ethanol production. The discussion then flows into the current technologies involved in lignocellulosic ethanol production, such as pretreatment (physical, chemical and biological) and SSF processes. Finally this chapter will end with discussions on the organisms used in this study, namely the white rot (*P. chrysosporium*), brown rot (*G. trabeum*), soft rot (*T. reesei*) and the types of lignolytic, cellulolytic and hemicellulolytic enzymes associated with these fungi.

The following three chapters (Chapters 3, 4 and 5) consist of three manuscripts prepared for publication in various international journals:

Chapter 3 - "**Evaluation of Potential Fungal Species for the *in-situ* Simultaneous Saccharification and Fermentation (SSF) of Cellulosic Material**" investigates three different species of wood rot, namely white rot (*P. chrysosporium*), brown rot (*G. trabeum*) and soft rot (*T. reesei*) for the production of lignocellulolytic enzymes via solid state fermentation on pure cellulose. The paper aims to satisfy the first objective of this

dissertation. This paper will be submitted to the **World Journal of Microbiology and Biotechnology.**

Chapter 4 – "**Simultaneous saccharification and fermentation of ground corn stover without pretreatment for the production of fuel ethanol using *Phanerochaete chrysosporium*, *Gloeophyllum trabeum*, *Saccharomyces cerevisiae* and *Escherichia coli* K011**" focuses on the induction of cellulases during the solid state fermentation of the selected fungal species on corn stover to obtain fermentable sugars that will subsequently be substrates as microbial fermentation feedstock to produce ethanol using *S. cerevisiae* and *E. coli* K011. This manuscript is prepared to address the objective 2 of this dissertation, and will be submitted to the **Journal of Microbiology and Biotechnology**.

Chapter 5 – "**Ethanol production via sequential saccharification and fermentation of dilute NaOH treated corn stover using *Phanerochaete chrysosporium* and *Gloeophyllum trabeum***" reports the studies on the mild alkaline pretreatment of corn stover and its effect on overall fungal saccharification and fermentation of corn stover to ethanol by *S. cerevisiae* and *E. coli* K011. This chapter satisfies the third objective, and will be submitted to the **Applied Microbiology and Biotechnology** journal.

Chapter 6 – "**Engineering, economic and environmental implications and significance**" discusses the engineering economic and environmental implications and significance of the production fuel ethanol via the present studies. This chapter will cover the practical processes

and issues in implementing the methodology and technology towards lignocellulose based ethanol production.

Finally, Chapter 7 is the general conclusions for this dissertation. In this dissertation, figures and tables are embedded within the main texts of every chapter, and literature citations are added at the end of each chapter.

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CHAPTER 2: LITERATURE REVIEW

HISTORY OF FUEL ETHANOL

Ethanol production predates even the Industrial Revolution. Written records dated 9,000 years ago found in Northern China indicated that ethanol has been with the human civilizations as early as the Neolithic age (Roach 2005). Prior to 1826, early ethanol productions have always been via the fermentation processes, until Henry Hennel and S. G. Serullas synthetically prepared ethanol. This is followed two years later by Michael Faraday who artificially synthesized ethanol using acid catalysis hydration of ethane, a technology still used today (Wikipedia 2009).

The early application of ethanol has always been for beverages and as lamp fuel. In fact, ethanol was a fuel choice at the beginning of nineteenth century in Europe (Sneller and Durante 2007; Matsouka et al. 2009). During that period, ethanol blended with turpentine was used to replace the more expensive whale oil for lamp fuel (Songstad et al. 2009). Then in 1861, German Engineer and inventor, Nikolas Otto found another use for ethanol, as the fuel for one of his "Otto Cycle" combustion engines (Matsouka et al. 2009). This opened a new chapter for the applications of ethanol that continued to the United States, when Samuel Morey invented the internal combustion engine that ran exclusively on a combination of ethanol and turpentine (Songstad et al. 2009). Simply put, ethanol was already used as fuel years before the discovery of petroleum by Edwin Drake in 1859 and in actuality, the

production of ethanol for fuel spans more than a century (Sneller and Durante 2007; Songstad et al. 2009).

The next important event where fuel ethanol became prominent was linked to the invention of the automobile by Henry Ford, who designed his first car, the Quadricycle, to run on pure ethanol in the 1896 (Goettemoeller and Goettemoeller 2007; Pollok-Newsom 2008). However, during this time, ethanol was heavily taxed, making ethanol more expensive than gasoline, favoring use of gasoline for internal combustion engines (Goettemoeller and Goettemoeller 2007). When the ethanol tax was eventually lifted in 1906, it was an uphill battle for the ethanol to compete with the cheaper gasoline, the then accepted fuel for automobiles (Pollok-Newsom 2008; Songstad et al. 2009). Nonetheless, this did not stop Henry Ford from equipping his "Model T" with engines capable of running on ethanol, gasoline or a combination of the two, in 1908 (Goettemoeller and Goettemoeller 2007; Solomon et al. 2007).

Ford further promoted the usage of ethanol in a movement called Chemurgy during the 1920s and 1930s and fought to have the sales of at least 10% ethanol blend in motor fuel (Songstad et al. 2009). This movement resulted in 2,000 gasoline filling stations with such formulation throughout the Midwest (DiPardo 2003; Solomon et al. 2007). Another indirect impact of this movement was the increase in ethanol demand in the USA as an alternative and additive to gasoline for domestic use. For instance, during World War I, production was at 60 million gallons and this increased to 600 million gallons during the Second World War (Songstad et al. 2009).

Ever since then, the history of ethanol fuel is an epic struggle between ethanol and petroleum, played out in a series of circumstances – taxes, wars, discoveries, prohibition, inventions and champions on each side (Goettemoeller and Goettemoeller 2007). The first major incident that calls for a nationwide search for domestic source of renewable transportation fuel was the oil crisis and the Arab oil embargos of the 1970s that raised concerns about the stability and availability of fossil fuel (DiPardo 2003).

Apart from the energy security concerns, the potential threat of global climate and weather change from the constant consumption of fossil fuels has added new urgency to the development of alternative energy systems (Solomon et al. 2007). This sparked the beginning of programs to develop alternative domestic and renewable energy sources that could reduce needs for imported energy supplies and to counter the unstable and rapid escalation in crude oil prices that has continued until presently (Sheehan et al. 2004). These programs are not only successful but have grown tremendously over the last two decade, and in the last few years, we have seen exponential growth in production, as illustrated in Figure 1 below.
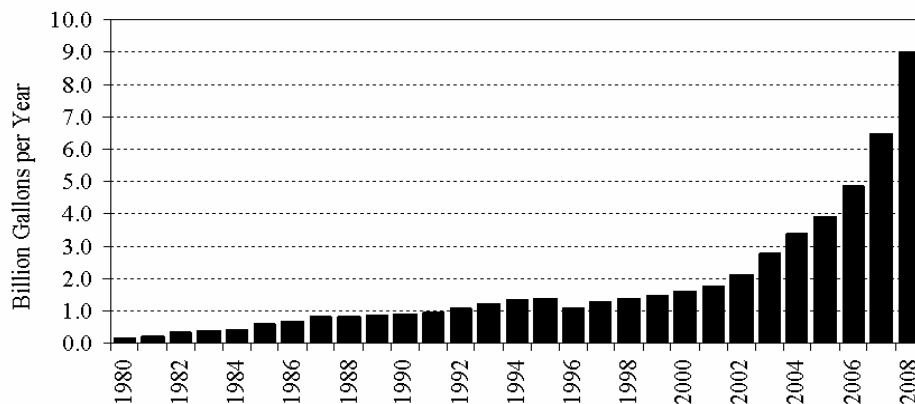
Figure 1. The United States ethanol production from 1980 to 2008 (RFA, 2009).

We are now entering an interesting transition between fossil based fuels and biofuels. As for the United State ethanol industry, *"The [United States ethanol] industry can be proud of the milestones reached in 2008, including record production of 9 billion gallons, implementation of a new Renewable Fuels Standard (RFS), record exports of distillers grains to feed the world's livestock, building new infrastructure, moving to higher blends, and lastly, but perhaps most importantly, technological innovations to improve both starch and cellulose-based ethanol production"* (RFA 2009).

ETHANOL

Ethanol is a clear, colorless, volatile and flammable liquid with a strong distinctive odor (Shakhashiri 2009). It is commonly known as ethyl alcohol, grain alcohol, wine spirit, and cologne spirit. Ethanol belongs to the alcohol family, a group of organic chemical compounds that contain a hydroxyl group, OH, bonded to a carbon atom (Shakhashiri 2009). The term "alcohol" originates from the Arabic word "al-kuhul", which originally referred to any fine powder. It was years later that medieval alchemists applied the term to refined products of distillation (Shakhashiri 2009).

In 1808, Antoine Lavoisier described the chemical composition of ethanol, as a straight chain alcohol compound consisting of carbon, hydrogen, and oxygen. The molecular formula for ethanol is C_2H_5OH (Figure 2), with a molecular weight of 46.07 g/mol (Shakhashiri 2009). It

burns to form CO_2 and H_2O with a non-luminous blue flame, with no soot formation. Table 1

shows the physical and properties of ethanol.
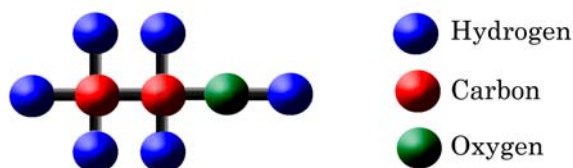
Figure 2. The molecular structure for ethanol.

Table 1. Physical and Chemical Properties of Ethanol (Monick 1968; Shakhashiri 2009).

Properties	Value
Boiling point	78.5°C
Freezing point	-114°C
Heat of combustion of liquid	328 kcal/mole
Heat of vaporization (at boiling pt and 1 atm)	204.3 cal/g
Ignition temperature	371-427°C
Density - relative to water (at 20°C)	0.789
Refractive index, D	1.33614

Traditionally, ethanol has been produced by the anaerobic fermentation of sugars (i.e.

glucose) by yeast. The anaerobic fermentation reaction is represented by the equation below:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Although the equation above seems simple, the reaction is actually very complex. According

to Shakhashiri (2009), impure cultures of yeast and other biological contaminants or foreign

chemical compounds produce varying amounts of other substances, including glycerin and

various organic acids.

Currently, mass production of ethanol is via two routes, biologically via fermentation and synthetically via hydration of ethane (Wikipedia 2009). Overall, approximately 95% of ethanol production are produced via fermentation and the remaining 5% are produced synthetically (Berg and Licht 2004).

Ethanol production globally via the fermentation route generally uses starch (approximately 3% total world production) and sugar crops (approximately 61% total world production) (Berg and Licht 2004), and involves microorganisms (most commonly *Saccharomyces cerevisiae*) that ferments the C6 sugars (usually glucose), into ethanol and other by-products, such as acetic acid. Theoretically, 1 kg of glucose will produce approximately 514 g of ethanol and 488 g of carbon dioxide (Badger 2002).

In Brazil, approximately 79% of the ethanol is produced from sugar cane juice and molasses (Fukuda et al. 2009). In the United States, grains commonly used for ethanol production include sorghum, maize and wheat (Sanchez and Cordona 2008). A bushel of corn (25.3 kg or 56 lb at 15% moisture) can produce from 9.4 to 10.9 L (2.5 to 2.9 gallons) of ethanol (Badger 2002). It is projected that sugar and starchy feedstocks will continue to contribute significantly to the production of ethanol worldwide (Figure 3) (Berg and Licht 2004).
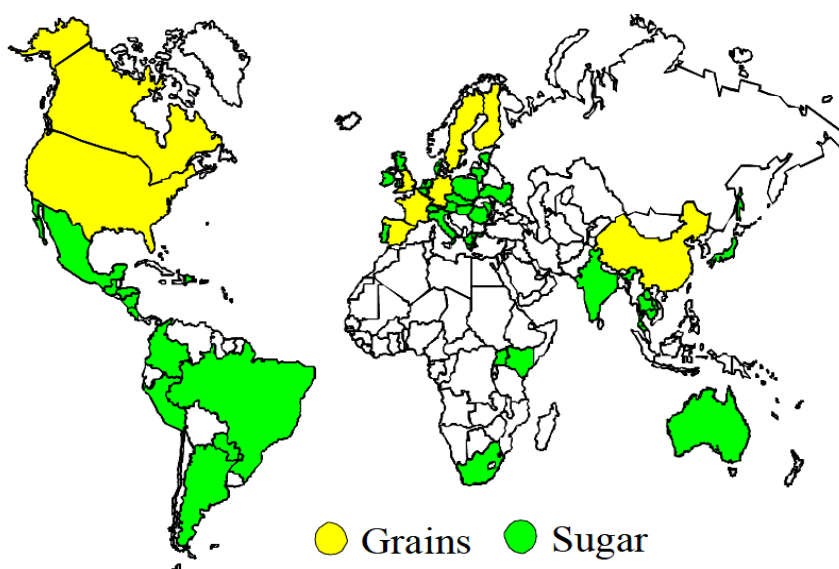
Figure 3. Projected worldwide distribution of sugar and starchy feedstock for ethanol production in 2013 (Berg and Licht 2004).

Other feedstocks for the production of ethanol are lignocellulosic biomass, and currently, lignocellulosic biomass is gaining momentum as potential substrates for bioethanol production (Fukuda et al. 2009). This platform for ethanol production will be discussed further in the later part of this chapter.

Ethanol produced via fermentation ranges in concentration from a few percent (beer) up to about 14% (v/v) (wine). Above 14% (v/v), ethanol stops the fermentation processes as it kills the yeast and destroys the zymase enzyme (Shakhashiri 2009). According to Shakhashiri (2009), all food grade ethanol and more than half of industrial ethanol is still made by this process. To obtain high concentration of up to 95% (v/v), ethanol is usually concentrated by distillation of aqueous solutions (12-15% w/v) (Badger 2002). Further processing, such as the use of dehydrating agents or molecular sieves, is needed to produce pure ethanol.

Ethanol not intended for drinking is now made synthetically. Synthetic production of ethanol involves chemical conversion of ethylene made from petroleum or from acetaldehyde made from acetylene (Badger 2002). Figure 4 shows the world largest synthetic ethanol producers.
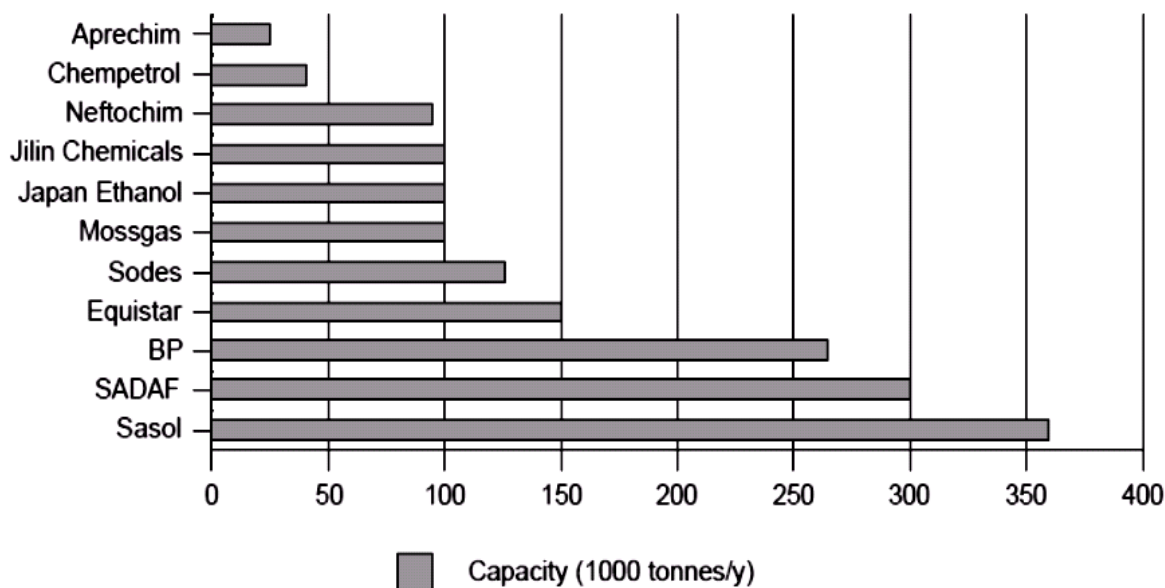
Figure 4. Largest synthetic ethanol producers (Berg and Licht 2004).

Ethanol has been a key industrial and pharmaceutical chemical for many years (Zhu et al. 2006). Because ethanol is fully miscible in water and with most organic solvents, it is the raw material of choice for the production of hundreds of chemicals used in beverages, chemical industries (paints, lacquer), cosmetics (perfumes) and pharmaceuticals (disinfectants) (Dale 1991; Berg and Licht 2004). Most industrial ethanol is denatured by adding small amounts of poisonous or unpleasant substances, to prevent it from being used as a beverage. These chemical denaturants also render ethanol unsuitable for some industrial processes (Goettemoeller and Goettemoeller 2007).

Besides the above applications, ethanol is used as transportation fuel by itself, or it can be mixed with gasoline to form gasohol (Shakhashiri 2009). According to Fukuda et al. (2009), about 73% of produced ethanol worldwide are used as fuel ethanol, while the rest goes to the beverage and industrial sectors (Figure 5).
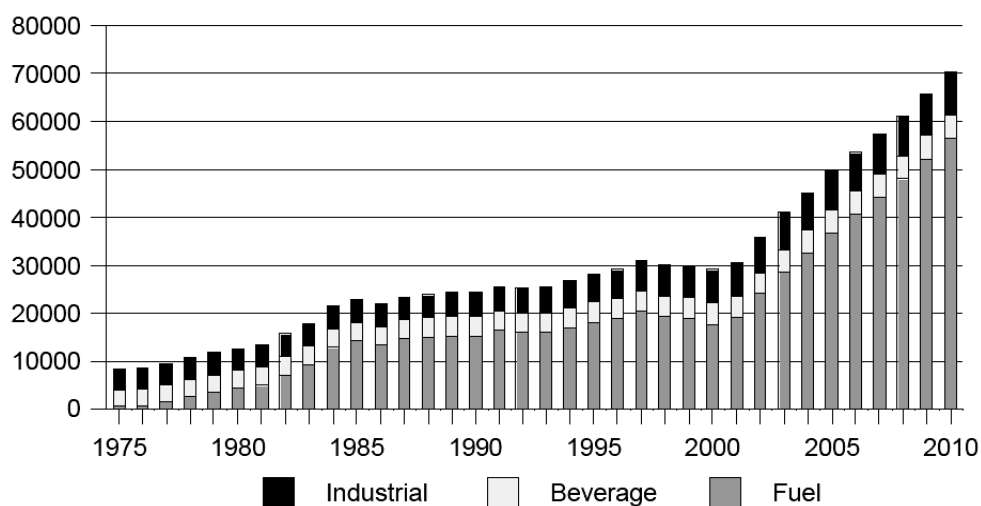
Figure 5. Worldwide ethanol application (million of gallons) (Berg and Licht 2004).

FUEL ETHANOL PRODUCTION AND PROGRAMS

The current global interest in bioethanol technology and industry goes back to the oil embargo in the 1970s, and it has truly experienced a dynamic emergence from the 1980s to the present. Worldwide biofuels production and applications has increased tremendously in recent years, from a little over 18.2 billion liters (~4.85 billion gallons) in 2000 to approximately 60.6 billion liters (~16.4 billion gallons) in 2007 (Sainz, 2009). In 2008, this

figure stands at least 17.335 billion gallons (Table 2) (RFA 2009), with bioethanol contributes to about 85% of the overall total, and it is projected that this volume will surpass 113.6 billion liters (~30.29 billion gallons) by 2022 (Goldenberg and Guardabassi 2009). The United States alone is expected to produce 135 billion liters of renewable fuels, of which about 60% is cellulosic ethanol (Kim et al. 2009)

Table 2. World ethanol production in the year 2008 (RFA 2009).

Country	Millions of Gallons
USA	9000.0
Brazil	6472.2
European Union	733.6
China	501.9
Canada	237.7
Other	128.4
Thailand	89.8
Colombia	79.29
India	66.0
Australia	26.4
Total	17,335.2

Currently, the United States and Brazil are the top starch/sugar-ethanol producers (Maki et al. 2009; Sainz 2009). In 2008, the United States led the production with 9.0 billion gallons, and Brazil was close behind at about 6.5 billion gallons (RFA 2009). The top ten ethanol producing states in the United States is shown in Table 3.

Table 3. Top ten ethanol producer by state in the United States (RFA 2009).

	Nameplate	Operating	Under Construction/ Expansion	Million of gallons
Iowa	3,076.0	2,856.0	690	3,766.0
Nebraska	1,444.0	1,164.0	319	1,763.0
Illinois	1,190.0	1,190.0	293	1,483.0
Minnesota	1,081.6	837.6	50	1,131.6
South Dakota	1,016.0	799.0	33	1,049.0
Indiana	899.0	697.0	88	987.0
Ohio	470.0	246.0	65	535.0
Kansas	491.5	436.5	20	511.5
Wisconsin	498.0	498.0	-	498.0
Texas	250.0	140.0	115	365.0

In the United States, national energy security concerns, lack of reliable energy sources, new federal gasoline standards, and government incentives have been the primary stimuli for the production of fuel ethanol (Kim et al. 2009). According to Urbanchuk (2009), as at the end of 2008, the ethanol industry in the United States comprised of 172 operating plants in 25 states with the total production capacity of 10.6 billion gallons, although the official figure is 9 billion gallons distributed among 170 plants according to the RFA (2009). Public policies aimed at encouraging ethanol development are largely motivated by the desire to improve air quality and enhance energy security. This need has led to not only nationwide gasoline station selling the minimum blend of E10 (gasoline blend with 10% ethanol), but also the increase in stations dispensing the E85 blend (Figure 6) (RFA 2009).
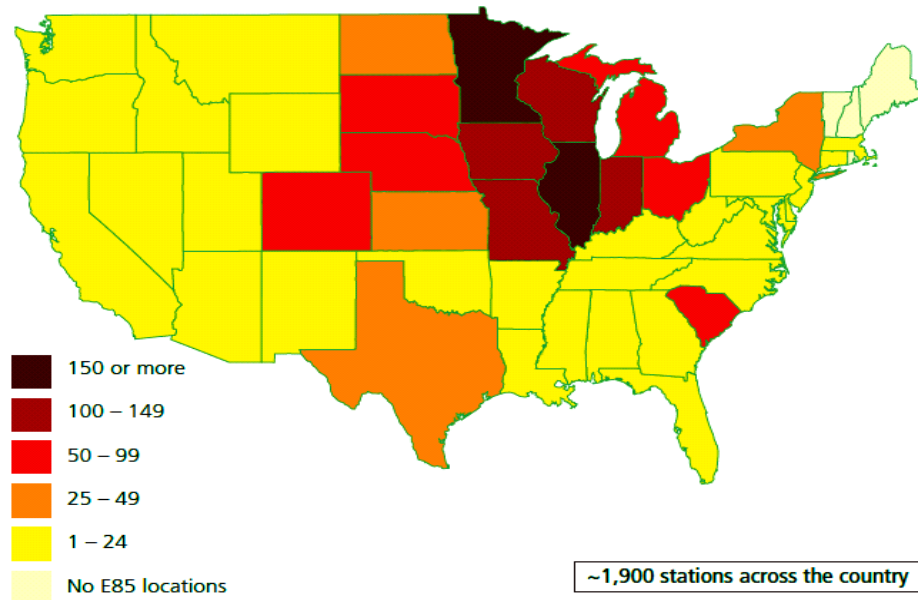
Figure 6. E85 Refueling locations by state (RFA 2009).

A long term objective for biofuel applications in the United States aims to displace 30% of the 2004 gasoline use (3.4 billion gallons) with biofuels (60 billion gallons) by 2030 (DOE-EERE 2009). Of that projected amount, 45 billions are estimated to come from lignocellulosic resources, while grains contribute the other 15 billion (Hess et al. 2009). In addition, agricultural policymakers see the expansion of the ethanol industry as a means of stabilizing farm income and reducing farm subsidies. Increasing ethanol production induces a higher demand for corn and raises the average corn price that will result in reduced farm program payments (Shapouri et al. 2002).

In the United States, fuel ethanol is typically produced from starch, primarily from corn (Silverstein et al. 2007). The type of corn *(Zea mays)* commonly used for fuel ethanol are the yellow dent corn *(Zea mays* var. *indentata)*, also known as commodity corn. Two methods

are used to process corn into ethanol – wet milling (~20%) and dry milling (~80%) (Kim et al. 2008). One reason for more dry grind corn ethanol plants is because of low capital costs required to build and operate these type of plants. According to the RFA (2009), in total, there are currently 170 ethanol plants nationwide (Figure7) in the United States and 20 more are under construction (RFA 2009).

Biorefineries in production (170) ● ○ Biorefineries under construction (20)

Figure 7. Location of biorefineries in the United States (RFA, 2009).

In recent years, government and privately sponsored research has resulted in new technologies that have lowered the cost of production of ethanol made from corn (Hohmann 1993). As a result, corn and ethanol production are now so efficient that it takes less energy to grow the crop and process it than the amount of energy in the ethanol itself. According to Aden (2008), based on the 2007 dollar, the minimum ethanol selling price was at $2.43 per

gallon and this is projected to decrease at $1.33 by the year 2012 with the advancement of

technologies (Figure 8). Table 4 shows the ethanol production targets up to the year 2020.

Table 4. The United States renewable fuels, advanced and cellulosic ethanol targets (2009-2022) (Haigwood and Durante 2009).

Year	Total Volume of Renewable Fuels (millions of gallon)	Cellulosic Ethanol (millions of gallon)	Advanced Biofuel (Cellulosic ethanol and biodiesel) (millions of gallon)
2009	11.10	-	0.60
2010	12.95	0.10	0.95
2011	13.95	0.25	1.35
2012	15.20	0.50	2.00
2013	16.55	1.00	2.75
2014	18.15	1.75	3.75
2015	20.50	3.00	5.50
2016	22.25	4.25	7.25
2017	24.00	5.50	9.00
2018	26.00	7.00	11.00
2019	28.00	8.50	13.00
2020	30.00	10.50	15.00
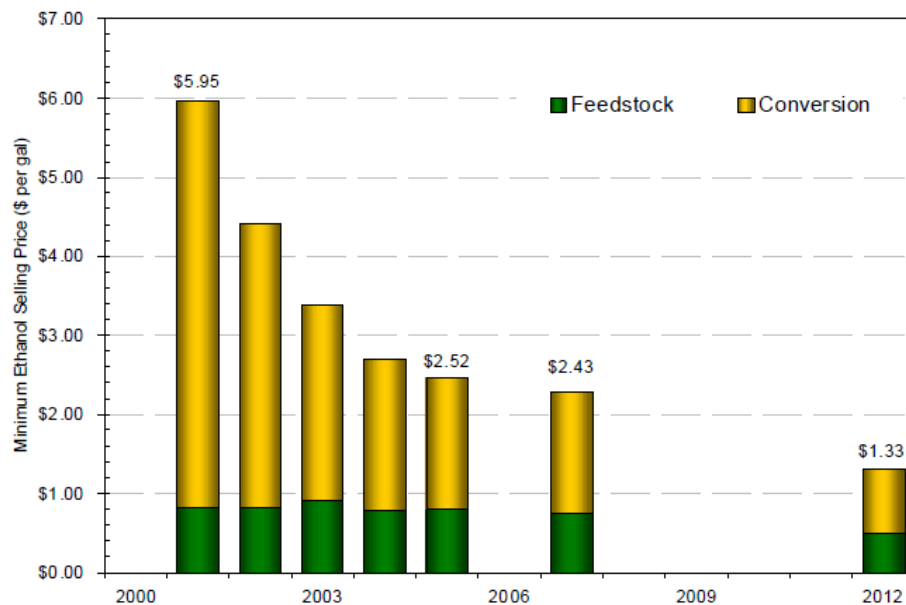
Figure 8. State of technology progress toward the 2012 goal (Aden, 2008).

Brazil has developed one of the most successful fuel ethanol programs among the fuel ethanol producing countries (Badger 2002). Even before the worldwide fuel crisis that escalated in the mid 1970s, Brazil has provided a major shift in the industry by mandating ethanol as a motor fuel as early as the 1960s. Unlike other leading countries with fuel ethanol programs, Brazil retains a blending requirement that all gasoline used in the country contains a minimum of 20-25% ethanol (Janssen et al. 2009). Diesel-powered personal vehicles are also banned in Brazil to boost the demand for ethanol powered vehicles, and government bodies are required to use 100% alcohol fueled vehicles (Janssen et al. 2009). Brazil's tax regime favors ethanol over gasoline, and other programs have been implemented to support the domestic ethanol industry. Brazil is currently running a sugarcane ethanol program called ProAlcohol for the last 30 years, and with further innovations and improvements, it is expected that sugarcane based fuels could supply over 30% of Brazil's energy needs by 2020 (Matsuoka et al. 2009).

The European Union (EU) has a specific objective as stated in the European White Paper ''European transport policy for 2010: time to decide", published in 2001 (later endorsed by Directive 2003/30/CE), to promote the use of renewable biofuels (Garcia–Cubero et al. 2009; Janssen et al, 2009). According to this directive, EU members must have minimum blends of renewable automotive fuels for the public at 2% in 2005, which should be 5.75% by 2010, and by the end of 2020, biofuels ratio should be increase to 10% (Galbe and Zacchi 2007; Janssen et al. 2009). Other members such as Germany imposed fuel tax exemption and in 2004, motor fuel blends containing up to 5% biofuel (bioethanol and biodiesel) also became

exempted from the fuel tax (RFA 2009). In the United Kingdom, subsidies on biofuels include both biodiesel and bioethanol until 2007 (Flach et al. 2009).

In Canada, similar developments are also observed with the Canadian renewable fuel standard warranting that motor fuel should contain 5% ethanol by 2010 (Maki et al. 2009). In another review by Sanchez and Cardona (2008), Canada is reported to blend 7.5-10.0% ethanol, produced mainly from corn, wheat and barley, into their current gasoline supplies. The review added that Canada is also offering tax incentives to promote the use of fuel ethanol, in an effort to comply with the Kyoto Protocol.

Although all these are encouraging developments worldwide, the problem with corn ethanol is that the high demand for corn has caused the increase in price of corn kernels for human food and animal feed (Songstad et al. 2009). Therefore, attentions are moved towards the utilization of the less costly and most abundant feedstock, lignocellulose materials such as corn stover, baggase (sugar cane waste), rice straw, wood chips or "energy crops" (fast-growing trees and grasses) as the primary starting material (Goettemoeller and Goettemoeller 2007). Concerted efforts are currently underway to make mass production of lignocellulosic ethanol available. These are discussed in a later section of this chapter.

LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass are complex biological materials that include agricultural residues (corn stover, wheat straw, sugar baggase, rice straw, rice hull, corn cob, corn fiber, cotton stalks), office waste, industrial cardboard and forestry products (Lim 2004; Kumar et al. 2009). These resources are abundant and widespread, with a yearly supply of approximately 200 billion metric tons (Table 5) (Zhang 2008; Fukuda et al. 2009). However, only 3% of the available lignocellulosic sources are exploited, often in non-food manufacturing, such as the paper and pulp industries (Zhang 2008). Because these materials are outside the human food chain, lignocellulosics are relatively low cost feedstocks that is an ideal source of sugars for sustainable fuel ethanol and value added commodities production via the development of lignocellulose-based biorefineries (Kumar et al. 2009; Xuan et al. 2009; Yu et al. 2009).

Lignocellulose consists primarily of plant cell wall materials that are composed of cellulose (insoluble fibers bundles of β-1,4-glucan), hemicellulose (polysaccharides that includes xylan, glucan, arabinan, mannan) and lignin (recalcitrant poly-phenol-propane) (Fukuda et al. 2009; Hendriks and Zeeman 2009). Lignocellulose biomass compositions and structures vary greatly according to the plant parts, species and growth conditions (Zhang et al. 2007). Depending on the biomass, lignocellulosic materials comprise of 10-25% cellulose, 20-35% hemicellulose and 35-50% lignin (Table 6), and the variations in these materials also correlate to the amount of fermentable components (Kerstetter 2001). A schematic representation of a typical plant secondary wall showing cellulose, hemicelluloses chains and lignin matrix is shown in Figure 9.

Table 5. Lignocellulosic residues from different agricultural sources (Sanchez 2009).

Lignocellulosic residues	Ton×10^6/year
Sugar cane bagasse	317–380
Maize straw	159–191
Rice shell	157–188
Wheat straw	154–185
Soja straw	54–65
Yuca straw	40–48
Barley straw	35–42
Cotton fiber	17–20
Sorgoum straw	15–18
Banana waste	13–15
Mani shell	9.2–11.1
Sunflower straw	7.5–9.0
Bean straw	4.9–5.9
Rye straw	4.3–5 2
Pine waste	3.8– 4.6
Coffee straw	1.6–1.9
Almond straw	0.4–0.49
Sisal a henequen straw	0.077–0.093

Table 6. The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes (Saha 2003; Mosier et al. 2005; Lee et al. 2007; Yu et al. 2009).

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Corn stover	38	26	16
Soybean hull	33	14	-
Paper	85–99	0	0–15
Wheat straw	30	50	15
Rice straw	35	25	12
Rice hull	35-40	15-20	20-25
Cotton seed hairs	80-95	5-20	0
Coastal Bermuda grass	25	36	6
Switch grass	45	31	12.0
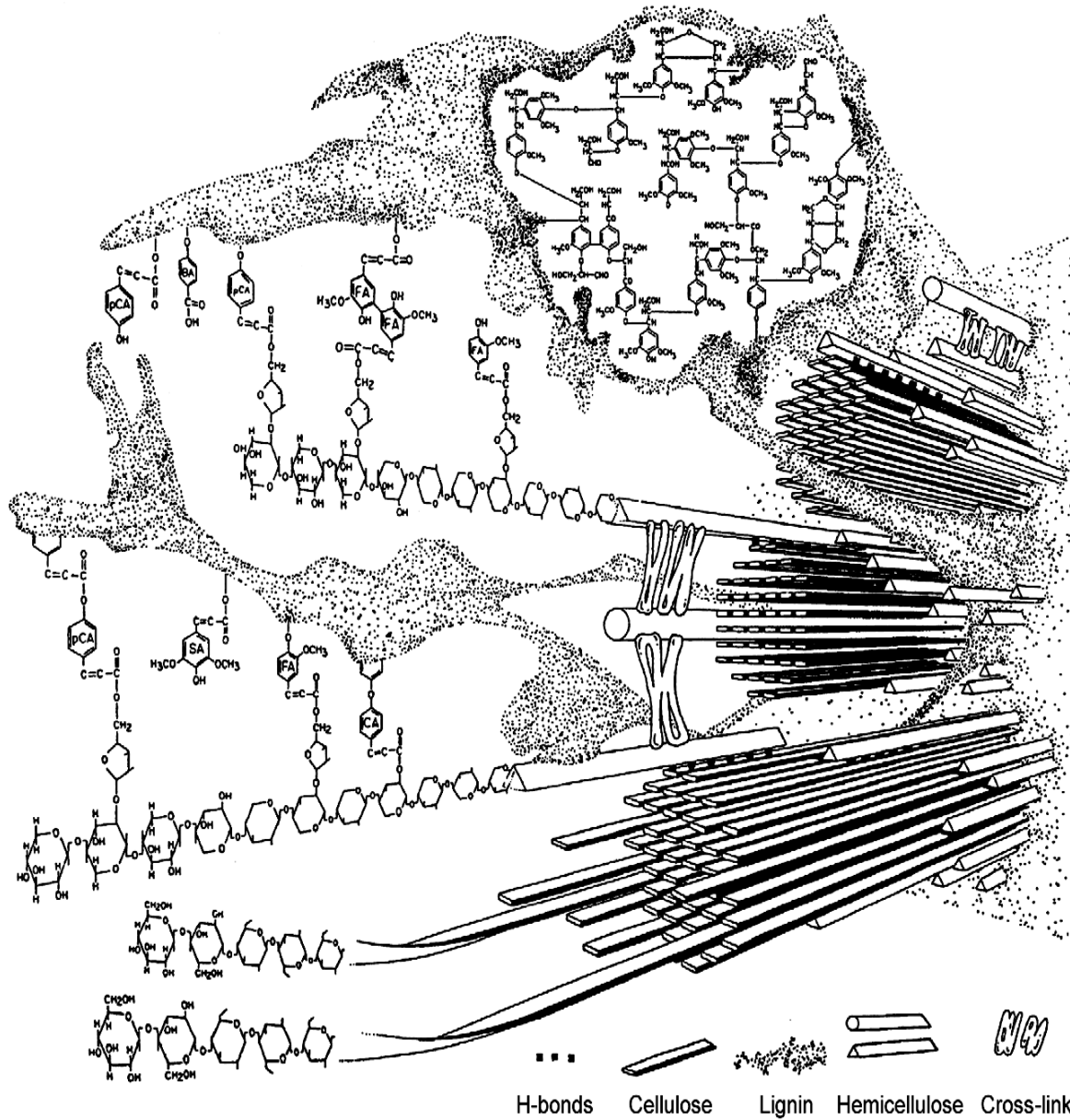
Figure 9. Schematic representation of plant wall showing linear cellulose and branched hemicelluloses chains surrounded by a lignin matrix (Martinez *et al*., 2009).

CELLULOSE

Cellulose is the most predominant component in plant cell walls. It is the primary product of photosynthesis in terrestrial environments and is the most abundant renewable polymer produced in the biosphere, reported to be approximately 100 billion dry tons synthesized annually (Zhang et al. 2006).

Cellulose in plant cell walls is a polymer consisting of D-anhydroglucopyranose (glucose monomers) joined together by β-1,4-glucosidic bonds that form long chains of linear cellulose micro fibril, with a degree of polymerization (DP) from 100 to 20,000 (Figure 10) (Zhang et al. 2006; Chang 2007; Hendriks and Zeeman 2009). Neighboring glucose molecules in the chain rotated 180° with respect to the adjacent molecules. The coupling of these molecules by extensive intrastrand hydrogen bonds and van der Waals forces results in a crystalline structure, termed cellulose fibrils or cellulose bundles, that is chemically and structurally stable and highly resistant to depolymerization (Heck et al. 2002; Lim 2004; Hendriks and Zeeman 2009). These structural characteristics, plus the encapsulation by lignin and hemicellulose makes cellulose extremely recalcitrant and inaccessible to microbial and enzymatic degradation (Heck et al. 2002; Zhang et al. 2006). However, in nature, cellulose is degraded much faster by fungal and bacterial cellulases and this is an important biological process to return carbon to the atmosphere (Zhang et al. 2006)
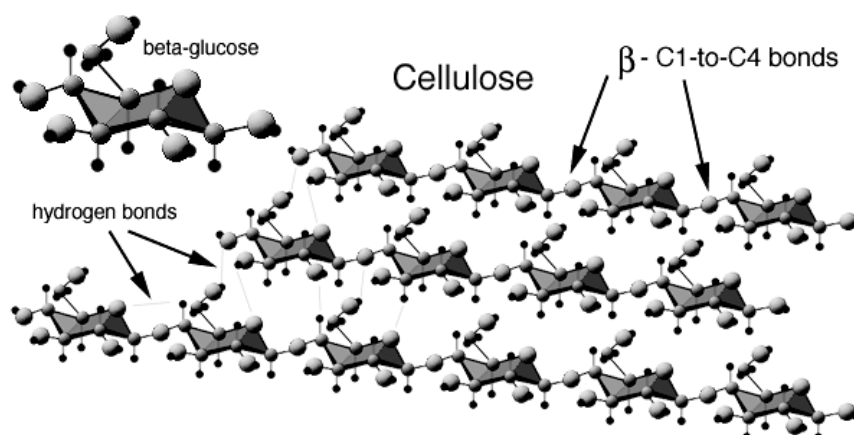
Figure 10. Schematics of cellulose molecules.
(http://www.generalbiomass.com/fig_cellulose.gif)

The long chain of glucose molecules are unlike starch molecules, both structurally and in configuration. Celluloses, either in its crystalline (organized) or amorphous forms, are highly stable and resistant to physio-chemical attack, making it difficult to hydrolyze (Badger 2002; Hendriks and Zeeman 2009). However, because of the abundance of cellulosic biomass and the fact that this feedstock can be hydrolyzed into fermentable sugar, cellulose has been the major focus for the development of next-generation biofuel production (Chang 2007). The key is to hydrolyze these complex structures efficiently and economically for the production of cellulosic ethanol (Brekke 2005). The good news is that present advanced bioethanol technology allows fuel ethanol production from these cellulose and hemicellulose, greatly expanding the renewable and sustainable resources available for fuel ethanol production.

HEMICELLULOSE

Hemicelluloses are the second most abundant biopolymer found in plant biomass (Chang 2007). Like celluloses, hemicelluloses are also comprised of long chains of sugar molecules. However, hemicellulose differs from cellulose as it is an amorphous heterogeneous branched polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and minor sugar acids (4-O-Methyl-D-glucuronic acid, L-fucose, L-galacturonic acid) derived from pectin or pectic acids (Saha 2003; Jovanovic et al. 2009).

β-1,4 Xylan, the major component of hemicellulose, is a complex polysaccharide structure made of a backbone of β -1,4 linked xylopyranoside (xylose) that is extensively branched and linked with acetyl, glucuronosyl, and arabinosyl side chains to other sugar molecules (Sluiter et al. 2005; Zhu et al. 2008; Fukuda et al. 2009). The frequency and composition of the branches vary greatly according to the plant cells. Approximately 80% of the xylan backbone is linked in this manner, and also by oligomeric side chains containing arabinose, and galactose residues (Figure 11). Because of these structures, xylans are also categorized as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan (Saha 2003).

A: Arabinose
X: Xylose
G: Galactose
GlcA: Glucuronic Acid
FeA: Ferulic Acid
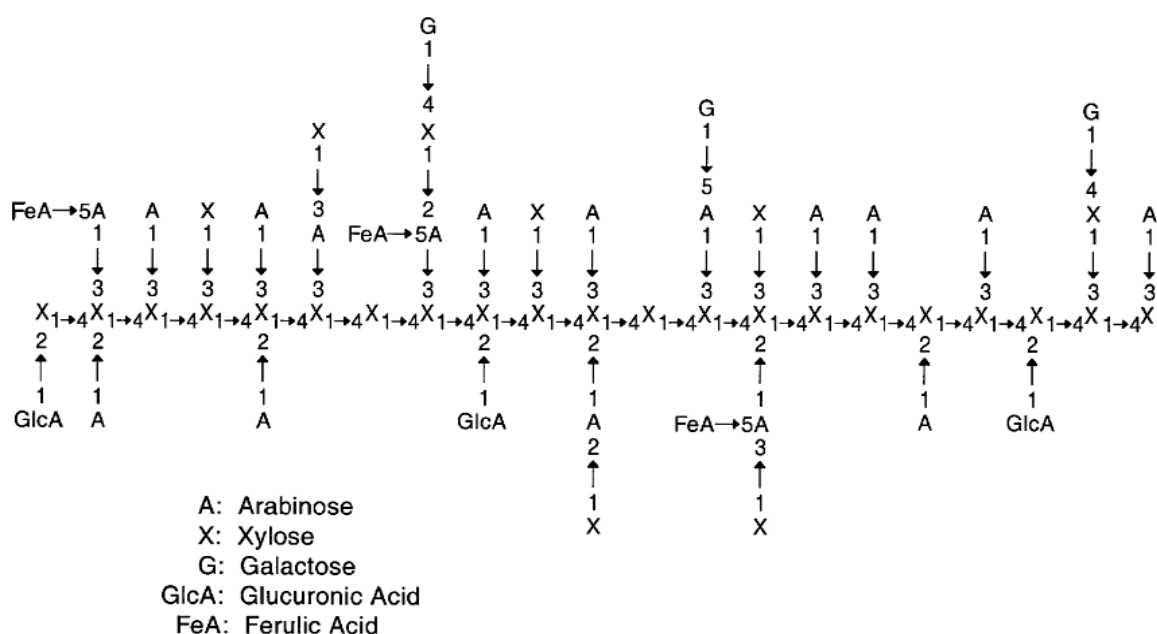
Figure 11. Schematic structure of corn fiber heteroxylan (Saha 2003).

The exact sugar composition of hemicellulose can vary depending on the plant species (Table 7). In general, hardwood hemicelluloses contain mostly xylans with the degree of polymerization in the range of 150–200, whereas softwood hemicelluloses contain mostly glucomannans with the DP in the range of 70–130 (Saha 2003).

Table 7. The composition of sugars in the hemicellulose of several biomass (Saha 2003).

	Xylose (%)	Glucose (%)	Arabinose (%)	Galactose (%)	Others[a] (%)
Birch wood	89.3	1.4	1.0	-	8.3
Rice bran	46.0	1.9	44.9	6.1	1.1
Wheat	65.8	0.3	33.5	0.1	0.1
Corn fiber	48.0-54.0	-	33.0-35.0	5.0-11.0	3.0-6.0

[a] mannose, glucuronic acid and/or anhydrouronic acid

Hemicellulose functions as an intermediate between cellulose and lignin, conferring the whole cellulose–hemicellulose–lignin biocomplex more rigidity and structural integrity (Hendriks and Zeeman 2009). In typical lignocellulosic biomass, cellulose fibrils are interlaced and embedded within a matrix of hemicelluloses, which are highly connected via diferulic linkages and isodityrosine bridges to form feruloylated heteroxylans, forming an insoluble network (Figure 12) (Saha 2003; Chang 2007). These interactions often time block the physical access to the cellulose surface to the activities of the cellulases (Yoon 1998). According to Zhu et al. (2008), removal of these acetyl groups from xylan greatly enhanced biomass availability and digestibility thereby increasing the enzymatic hydrolysis rate.
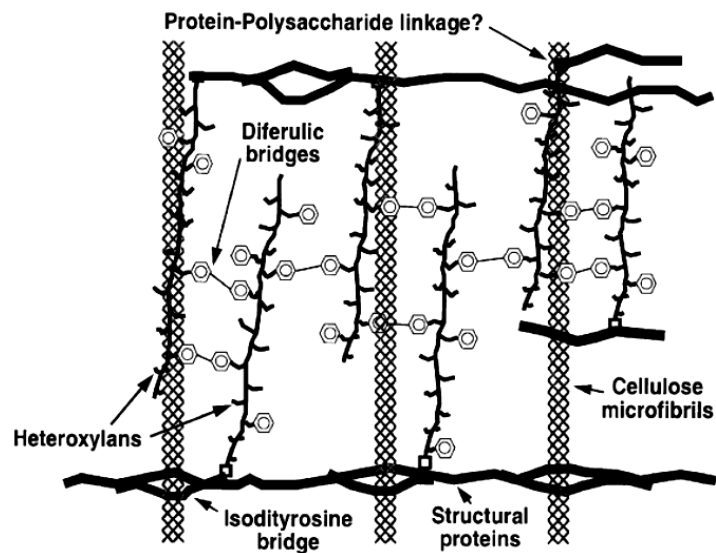
Figure 12. Typical hemicellulose-cellulose structures showing networks of diferulic linkages (Saha 2003).

Traditionally, just like cellulose, hemicellulose serve as a major source of food and nutrients for herbivores and as substrates for the production of food, textiles, paper and pulp industries. Recently, technology has found another use for hemicellulose - as feedstocks for the

production of ethanol. To produce fuel ethanol from these biopolymers, hemicellulose are first hydrolyzed into sugars such as glucose, xylose and other sugars by enzymes (Heck et al. 2002). However, the wide variety of sugar monomers, and other by-products, creates a challenge to fermentation because these five carbon sugars are not universally metabolized by most microbes, especially the common industrial yeast. One approach is to genetically engineering the heterologous ethanol biosynthesis pathways into xylose-consuming microbes such as *Escherichia coli* (Chang 2007). Nonetheless, these sugars or other by-product of hemicellulose degradations (i.e furfural) may also be used for other applications (Table 8).

Table 8. Hemicellulose products and applications (Saha 2003).

Hemicellulose components	Products and applications
Oligosaccharides	Oxygen-barrier film, thickeners, adhesives, emulsifiers, protective colloids, stabilizers, animal feed and nutrients
Xylose	Xylitol, ethanol, organic acids
Furfural	Lubricants, coatings, adhesives, plastics, furan resins, polytetramethylene ether, nylon-6, nylon-6,6

LIGNIN

Lignin is the third most abundant component of the plant cell wall, and the most abundant renewable aromatic composed of phenylpropane moieties (80-90%) (Dashtban et al. 2009). It is a complex biopolymer that is synthesized from the combination three types of phenolic monomers, namely *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 13) (Hendriks and Zeeman 2009). These monomers then form larger hydroxyphenylpropanoid

units (Figure 14) that aggregate into very complex heterogeneous polymer that are interlinked by a variety of non-hydrolysable C-C and C-O-C bonds, such as phenylcoumarans, β-aryl ethers, resinols, biphenyls and biphenyl ethers (Figure 15) (Lim 2004; Chang 2007; Hammel and Cullen 2008). These organic molecules interlink with polysaccharide polymers, like cellulose and hemicellulose, to form a complete lignocellulosic biomass. Of the three major cell wall components, lignin is probably the most recalcitrant, mainly due to its biochemical qualities such as aromaticity, structural heterogeneity, and extensive carbon–carbon crosslinks (Chang 2007). The three-dimensional surface of lignin is complex and non-repeating with no clear chemical composition, and, like hemicellulose, varies greatly according to the plant species (Lim 2004; Hammel and Cullen 2008).
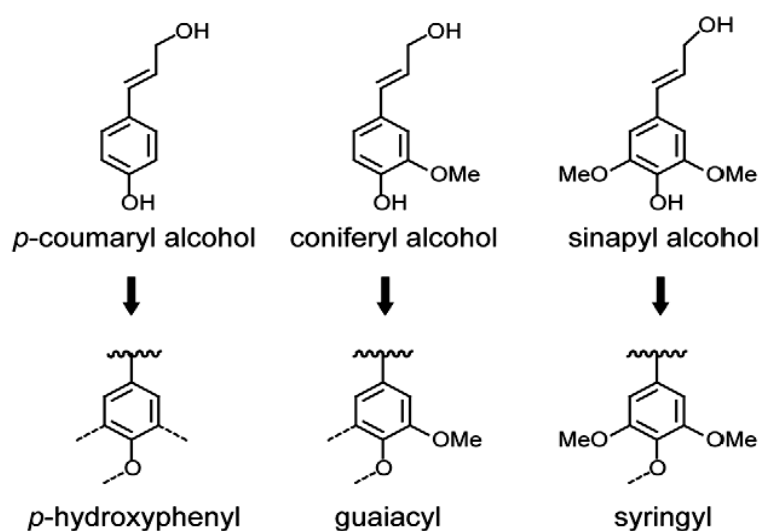
Figure 13. Lignin monomers (top) and lignin units (bottom) (Chang, 2007).
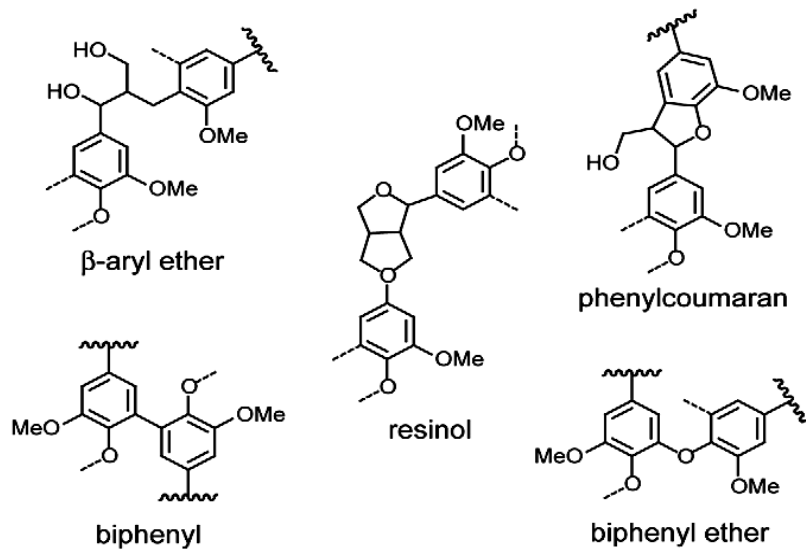
Figure 14. Lignin units crosslinks (Chang, 2007).
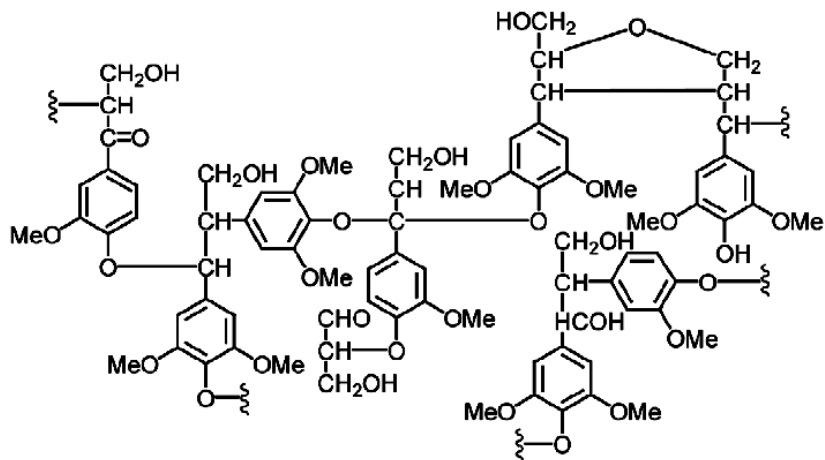
Figure 15. Multiple modes of bonding in heterogeneous polymer of lignin (Chang, 2007).

Lignin contains no sugars. It is optically inactive and non-water soluble, making it difficult to degrade (Hendriks and Zeeman 2009).The primary function of lignin in plant cell wall is for structural support, impermeability, and defense against oxidative stress and microbial attack (Hendriks and Zeeman 2009). In addition, this complex material also binds cellulose and

hemicellulose together. Lignin is a major deterrent to enzymatic degradation on cellulose and hemicellulose, because its close association with the microfibrils prevents enzyme accessibility (Badger 2002). Lignin removal or delignification of biomass greatly increases digestibility, as this causes the swelling of biomass, and disruption of lignin structure, that leads to an increase of exposed surface area and pore volume, subsequently increasing enzyme accessibility to cellulose and hemicellulose (Draude et al. 2001; Zhu et al. 2008).

Several chemical, physical and biological pulping methods are currently being used to remove lignin from plant biomass. Usually these processes generate much modified-lignin residues that are problematic to dispose of (Zhang 2008). Although many applications are available for these residues, most pulping residues are burned to generate electricity to provide steam and power for running refineries, as conventionally done in Brazil, to produce high-energy returns from ethanol (Chang 2007). This is possible because lignin has high energy content. There are, however, various other applications for lignin that further add value to lignocellulosic biomass. These are shown in Figure 16.
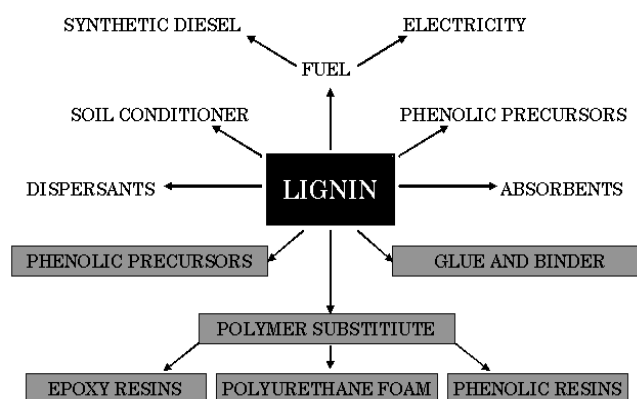
Figure 16. Possible lignin applications. The shaded boxes represent high selling-price products (Zhang 2008).

ETHANOL FROM LIGNOCELLULOSIC BIOMASS

Currently, most ethanol production in the United States and Brazil comes from starch and sugar-based crops, using so-called first-generation technologies (Galbe and Zacchi 2007). There are several issues with this platform, as both crops are in the human food chain (Badger 2002; Perez–Verdin et al. 2009). Furthermore, increasing demand for fuel ethanol has resulted in higher price for these crops, their production and all downstream applications involved in the production chain (Perez–Verdin et al. 2009). Starches and sugars from which ethanol are made are just a very small portion of available biomass that includes the full range of plant materials that, in fact can be converted to ethanol themselves (Figure 17) (Zhu et al. 2008). Therefore, common sense would dictate that these underused plant materials be used for the production of ethanol for liquid fuels (Hendriks and Zeeman 2009).

Generally, in lignocellulosic ethanol production, feedstocks are typically dedicated feedstocks (such as mischantus, switchgrass, willow and hybrid poplar), agricultural residues (corn stover or cobs) and forest residues (woody residues, mill residues and urban waste) (Perlack et al. 2005; Ruark et al. 2006; Perez–Verdin et al. 2009). However, the process of extracting the sugars becomes from lignocellulosic material becomes more difficult because these biomass are composed of recalcitrant complex of cellulose, hemicellulose, lignin, ash and other insoluble substances.
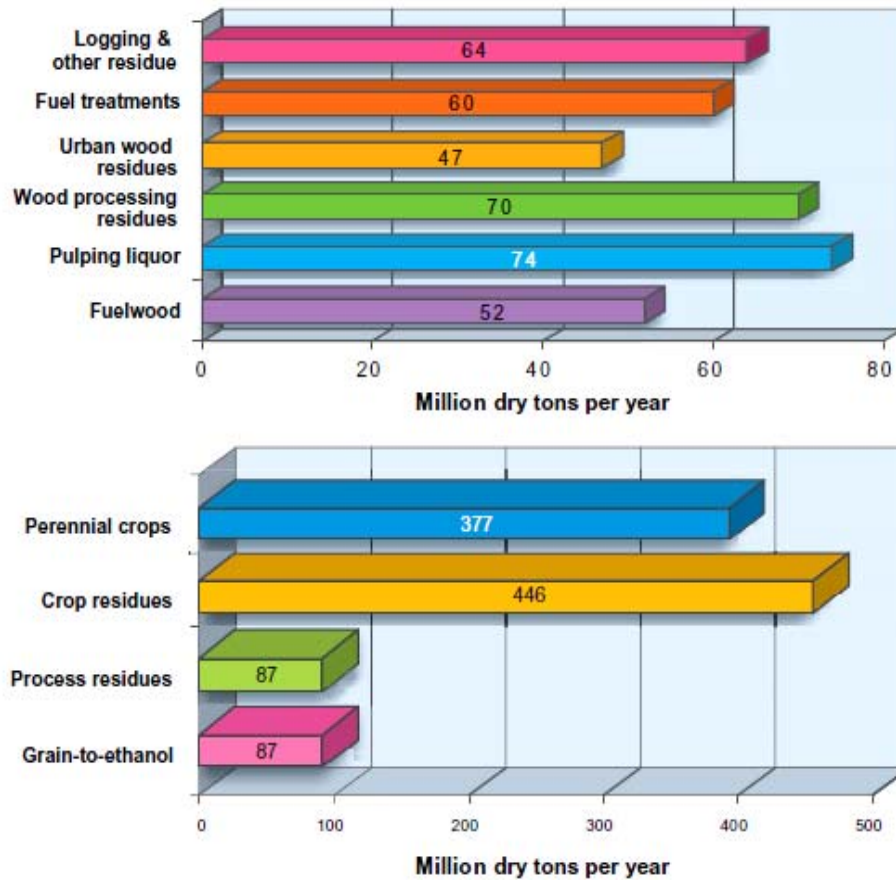
Figure 17. Summary of potential forest and agriculture residue (Perlack et al. 2005).

The production of fuel ethanol from lignocellulosic biomass via biological conversion comprises of six main steps. The basic process steps in producing ethanol from biomass are as follow (Tifanny and Eidman 2003; Zhang et al. 2007; Hendriks and Zeeman 2009):

i.   Size reduction and pretreatment

ii. Hydrolysis

iii. Fermentation

iv. Distillation and dewatering of the ethanol

vi. Denaturing of the ethanol

Lignocellulosic biomass fermentation is complimentary to, and in some cases, better than conventional grain fermentation (Erickson 2004). Currently, several efforts are underway in to mass produce ethanol from lignocellulosic materials as a primary feedstock product, as shown in Table 9. Zhang (2008) reported that the US-DOE has proposed a scenario to produce 30% of transportation fuels (60 billion gallons) each year from biomass by 2030. According the April 2009 industry assessment by the US-EPA, eleven lignocellulosic ethanol plants are currently at various advanced stages of planning and these facilities are likely to go online soon. It is projected that these facilities will enable the US to fulfill the 100 million gallon cellulosic ethanol target in 2010.

Table 9.   Projected Cellulosic Ethanol Production Capacity (Top ten producers) (Haigwood and Durante 2009).

Operational	Location	Feedstock	Size (Gal/Yr)
Greenfield Ethanol	Edmonton, Canada	Municipal Solid Waste (MSW)	36,000,000
POET (Project Liberty)	Scotland, SD	Corn cobs, fiber	20,000,000
Abengoa Bioenergy (York)	York, NE	Wheat Straw	11,600,000
Verenium (Celunol/Diversa/BP)	Jennings, LA	Sugar Cane/Bagasse	1,500,000
Western Biomass Energy (KL Process Design)	Upton, WY	Wood	1,500,000
Abengoa Bioenergy	Babilafuente, Spain	Ag Waste	1,500,000
Gulf Coast Energy	Livingston, AL	Wood waste, sorted MSW	200,000
Mascoma Corp. (NY)	Rome, NY	Wood chips	200,000
AE Biofuels	Butte, MT	Crop Residue	150,000
BRI Energy	Fayetteville, AR	MSW waste, Wood, coal	40,000

PRETREATMENT

One very important step in lignocellulosic ethanol production is the initial pretreatment of the feedstocks (Mosier et al. 2005; Galbe and Zacchi 2007). Although they are the most costly steps in cellulosic ethanol production, pretreatments are crucial as they greatly improve the enzymatic digestibility of the lignocellulosic materials via the alterations of both the chemical and structural properties of biomass (Silverstein et al. 2007; Zhu et al. 2006). These alterations include the removal of lignin, reduction of cellulose crystallinity, increasing the surface area and increasing the porosity of the biomass (Wyman et al. 2005; Hendriks and Zeeman 2009). Some pretreatment may also liberate the sugar monomers that can be used directly for fermentation (Sorensen et al. 2008, Shrestha et al. 2008).

The schematic of typical pretreatment processes is shown in Figure 18, as illustrated by Mosier et al. (2005). Whatever the pretreatment procedures may involve, effective pretreatment of lignocellulosic biomass should meet the following requirements (Sun and Cheng 2002; Galbe and Zacchi 2007; Mosier et al. 2005; Yu et al. 2009):

i.   High recovery of all sugars, especially the fermentable glucose and xylose using enzymes (requires over 240 g/l of fermentable sugars to be economical).

ii. Improves accessibility to the hemicellulose and cellulose for enzymatic hydrolysis (swelling, hydration of the polysaccharide or delignification).

iii. Pretreatment end-products should be usable with minimal post detoxification or conditioning and simplifies downstream processes (environmentally friendly).

iv. Removes saccharification and/or fermentation inhibitors from solids.

v. Requires low energy inputs and operational costs (minimal water use and waste; pretreatment chemical should be inexpensive and/or easy to recover).

vi. Requires low capital and generate extra revenues from by-products (i.e. lignin by-products).
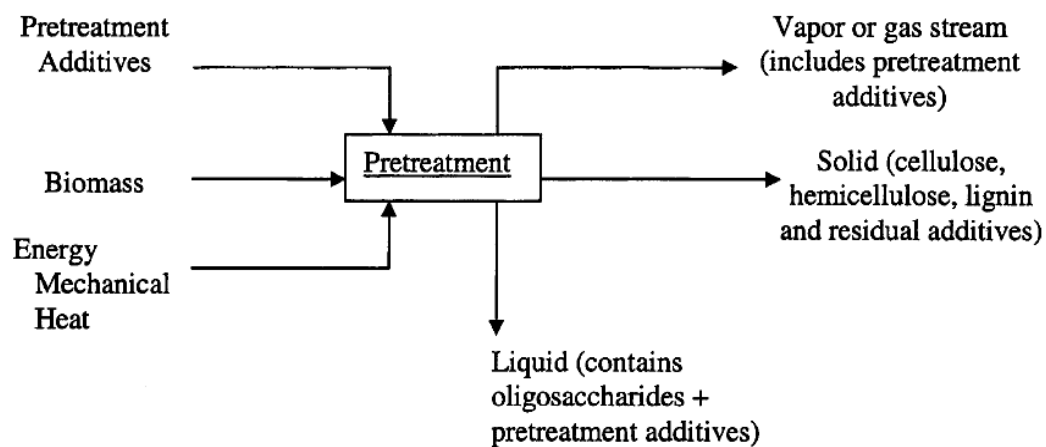
Figure 18. Schematic of Pretreatment Process (Mosier et al. 2005).

Numerous pretreatment methods have been suggested and developed in the last decade, and can be either simple or more technologically and logistically intensive (Lim 2004). These pretreatment protocols can be loosely divided into several groups, such as physical, chemical, physicochemical, biological and the combinations of these (Sorensen et al. 2008; Yang et al. 2008; Garcia-Cubero et al. 2009). In generally, different types on pretreatments work differently on the cellulose, hemicellulose and lignin components of biomass (Mosier et al. 2005). Table 10 details these mechanisms.

Table 10. Effects of various pretreatments on the composition and structure of lignocellulosic biomass (Mosier et al. 2005; Hendrikks and Zeeman 2009).

	Increases surface area	Cellulose Decrystalization	Hemicellulose removal	Lignin removal	Furfural/ HMF formation
Steam explosion	■		■		□
CO2 explosion	■		■		
Acid	■		■	□	■
Alkaline	■		□	■	■
pH controlled hot water	■	ND	■		ND
Liquid hot water	■	ND	■		□
Flow-through liquid hot water	■	ND	■	□	□
Flow-through acid	■		■		■
Oxidative (H2O2)	■	ND		■	□
Ammonia explosion	■	■	□	■	■
ARP	■	■	□	■	■
Lime	■	ND	□	■	■

■ : Major effect   □ : Minor effect   ND: Not determined

The effectiveness of the individual pretreatments is very much dependent on both operating conditions and the biomass composition (Hendriks and Zeeman 2009; Olofsson et al. 2008). Pretreatment is still one of the most expensive and environmentally controversial processing steps in lignocellulosics conversion, with costs as high as 30¢/gallon of ethanol produced (Mosier et al. 2005; Wyman et al. 2005). Therefore, more studies are needed to optimize their practicality in mass production of lignocellulosic ethanol (Hendriks and Zeeman 2009).

PHYSICAL PRETREATMENT

Physical pretreatment of lignocellulosic feedstocks usually involves particle size reduction or degradation of molecular structure (Hendriks and Zeeman 2009). This will increase the effective surface area of the cellulose and reduce cellulose crystallinity (DP) (Mani et al. 2004; Galbe and Zacchi 2007). Several simple methods such as grinding or the more technically intensive procedures, such as irradiation and ultrasonification are currently under laboratory and pilot studies.

1. *Milling and grinding.* This form of physical pretreatment can be a combination of milling, grinding and chipping to smaller aggregates or even fine powder. Upon size reduction, the size of the biomass is usually 0.2–2 mm after milling or grinding, and 10–30 mm after chipping (Keshwani and Cheng 2009). The particulate size reduction is necessary to maximize mass and heat transfer during downstream hydrolysis (Mani et al. 2004). According to Hendriks and Zeeman (2009), the reduction of crystallinity and the shearing and increase in overall surface area greatly increases the total hydrolysis yield. Logistically, size reduction also makes feedstocks handling easier in the subsequent processing steps, and reduces bulk density (Mosier et al. 2005).

2. *Irradiation.* Irradiation, usually using Cobalt-60 isotope to generate gamma ray, has been used on several lignocellulosic materials such as rice straw, bagasse, corn stover and oil palm empty (Yang et al. 2008). Although promising in lab studies, in reality, this method is too expensive to be used in a mass-scale production (Galbe and Zacchi 2007).

3. *Ultrasonics.* Ultrasonification as a mean for physical pretreatment has emerged rather recently. For the purpose of biomass pretreatment, "high power" ultrasound, that usually involves lower frequencies (20-100 kHz) and high amplitudes (12-320 μm) are used with biomass in a water slurry of 35% suspended solids (Mason and Lorimer 2002). At these settings, higher acoustic energy are generated, resulting in surface erosion due to the cavitational collapse in the surrounding liquid, and size reduction due to particle fission during antiparticle collision (Yu et al. 2009).

CHEMICAL PRETREATMENT

Chemical pretreatment of lignocellulosic feedstock economics is impacted by time, concentration, temperatures and environment. Two main chemical pretreatments of lignocellulosic feedstock are acid and alkaline pretreatment (Garcia-Cubero et al. 2009; Hendriks and Zeeman 2009). Other chemical pretreatment methods utilize organolvants, hydrogen peroxide (H_2O_2) and ozone (Keshwani and Cheng 2009; Yu et al. 2009).

1. *Acid pretreatment.* Dilute acid pretreatment of lignocellulosic materials is probably the most widely studied of all the chemical pretreatment methods because this process is effective and inexpensive (Sun and Cheng 2005; Gupta et al. 2009). During acid based pretreatment, the biomass is soaked in dilute acid solution, usually sulfuric acid (H_2SO_4) at concentrations between 0.5-3% (v/v) (Wyman et al. 2005; Garcia-Cubero et al. 2009). The mixtures are then heated to temperatures between 130∘C and 200∘C, at 3-15 atm

from several minutes up to a few hours (Wyman et al. 2005). H_2SO_4 is normally used as it is effective and inexpensive compared to other inorganic industrial acid such as hydrochloric acid (HCl) and nitric acid (HNO_3) (Lim 2004; Silverstein et al. 2007). Upon pretreatment, the hemicellulose component of the biomass is hydrolyzed to its monomeric sugars (xylose, glucose, arabinose, galactose and mannose) and other soluble oligomers, leaving cellulose intact or partially hydrolyzed to glucose (Sun and Cheng 2005; Galbe and Zacchi 2007). Pretreatment using dilute acid pretreatment is reported to achieve high yields and greatly improve cellulose hydrolysis, especially on agricultural wastes such as corn stovers and cobs (Sun and Cheng 2002; Silverstein et al. 2007).

According to Keshwni and Cheng (2009), acid pretreatment can also be done using strong acids to further solubilize lignin. However, harsher treatments with strong acids and extreme temperature may result in the generation of toxic by-products, such as furfural from pentoses and 5-hydroxymethylfurfural from hexoses, warranting post-treatment such as overliming and activated charcoal adsorption (Gupta et al. 2009; Keshwani and Cheng 2009; Tasic et al. 2009). Furthermore, strong acids are not only highly toxic, they are also corrosive and hazardous, thus requiring reactors that are expensive and resistant to corrosion (Sun and Cheng 2002; Lim 2004).

2. *Alkaline pretreatment.* Alkaline pretreatment involves the use of alkaline solution such as sodium hydroxide (NaOH) and ammonium hydroxide combined with high temperature. This procedure is best used on biomass such as agricultural residues and herbaceous crops, where it basically dissolves the lignin and various hemicellulose uronic

acid portions of the lignocellulosic materials (Galbe and Zacchi 2007; Silverstein et al. 2007; Garcia-Cubero et al. 2009). This form of pretreatment also hydrolyzes some portion of the hemicellulose, leaving behind mostly cellulose residues (Lim 2004). A significant fraction of the solubilized hemicellulose sugars can be recovered as oligosaccharides (Galbe and Zacchi 2007).

During alkaline hydrolysis, initial solvation and saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and lignin (Figure 14) occur extensively, causing increase in the porosity of the lignocellulosic materials (Hendriks and Zeeman 2009; Keshwani and Cheng 2009) Furthermore, alkaline solution also results in the swelling of the lignocellulosic materials (Galbe and Zacchi 2007). This subsequently decreases the degree of polymerization and crystallinity of the lignocellulosic structure (Sun and Cheng 2002). In addition, there is also increase in the surface area disruption of the hemicellulose and lignin molecules (Hendriks and Zeeman 2009).

3. *Oxidative pretreatment*. Another pretreatment method that causes lignin degradation is via oxidative pretreatment using oxidizing chemicals, such as hydrogen peroxide (H_2O_2) and peracetic acid (Teixeira et al. 1999; Yanez et al. 2006; Yu et al. 2009). According to Hendriks and Zeeman (2009), during oxidative pretreatment, several chemical reactions that occur include displacement of side chains, electrophilic substitution, cleavage of organic linkages (aryls, esters) or the cleavage of aromatic rings of the lignin subunits. Sun and Cheng (2002) reported that this procedure has been proven successful on sugar cane bagasse, with approximately 50% lignin removal by 2% (v/v) H_2O_2 at $30^{\circ}C$ within 8

hours. In another study done by Teixeira and colleagues (1999), peracetic acid at ambient temperatures pretreatment significantly increased the enzymatic hydrolysis of the cellulose from hybrid poplar and sugar cane bagasse. The same study further reported that minimal carbohydrate were loss, as peracetic acid is highly lignin selective.

4. ***Organosolvant pretreatment.*** One relatively novel method for the pretreatment of lignocellulosic materials is the organosolvant pretreatment (Geddes et al. 2009; Li et al. 2009). Termed COSLIF (Cellulose solvent- and organic solvent-based lignocellulose fractionation), this process combines an organic or aqueous volatile organic cellulose solvents (methanol, ethanol, acetone, ethylene, glycol, oxalic, acetylsalicylic, salicylic acid and phosphoric acid) and another inorganic nonvolatile acid catalysts (usually HCl or H_2SO_4) to hydrolyze the lignin and hemicellulose bonds (Sun and Cheng 2002; Geddes et al. 2009; Li et al. 2009; Sathitsuksanoh et al. 2009).

This form of pretreatment offers several advantages as it requires only moderate reaction conditions, such as 50^oC and 1 atm (Li et al. 2009). In addition, according to Zhang et al. (2007), this procedure easily separates the lignocellulose compounds from both solvents, making recycling of the solvents easy. Furthermore, this pretreatment produce not only high-value lignocellulosic end-products, but also causes minimal sugar degradation which mean greater yields upon enzymatic hydrolysis (Li et al. 2009). Recent studies using this procedure have shown success with perennial plants, hard wood and cotton-based waste textiles (Kim and Mazza 2008; Li et al. 2009).

5. ***Ozonolysis.*** The final chemical pretreatment is Ozonolysis, that utilizes ozone gas (O_3). Ozone is a powerful water soluble oxidant "*that is highly reactive towards compounds incorporating conjugated double bonds and functional groups with high electron densities*" (Garcia-cubero et al. 2009). Ozonolysis or ozone pretreatment is mainly for lignin degradation that subsequently releases soluble compounds with lower molecular weight, usually organic acids such as formic and acetic acid (Garcia-cubero et al. 2009) Hemicellulose is slightly affected, while the cellulose portion remains intact (Silverstein et al. 2007).

Among the advantages of ozonolysis pretreatment are as follows; no production of inhibitory residues that can interfere with the downstream processes, effective removal of lignin and reactions can be performed in ambient conditions (Sun and Cheng 2002; Silverstein et al. 2007). The only negative aspect of this pretreatment is the large amount of ozone that is required for effective result which makes the overall process very expensive (Sun and Cheng 2002).

The application of ozonolysis has been reported on both agricultural (wheat straw, bagasse, hay, peanut, pine, cotton straw) and forestry wastes (poplar sawdust) (Sun and Cheng 2002; Silverstein et al. 2007).

PHYSICOCHEMICAL PRETREATMENT

Physicochemical pretreatment of lignocellulosic feedstocks is basically a combination of both physical and chemical pretreatment (Galbe and Zacchi 2007). Among the more popular procedures are steam/steam explosion pretreatment, hydrothermolysis and ammonia fiber explosion (AFEX) (Gupta et al. 2009).

1. *Steaming/steam explosion*. To perform steam pretreatment, ground biomass is heated (160-260°C) with high-pressure (0.69-4.83 MPa) saturated steam for a few seconds to several minutes, usually in a retort (Keshwani and Cheng 2009). After treatment, parts of the hemicellulose is solubilized while, lignin and cellulose remain intact (Lim 2004). Factors that significantly affect steam pretreatment are temperature, residence time, moisture content and particle size (Sun and Cheng 2005). Adding acid catalysts, such as sulfuric acid, oxalic acid, sulfur dioxide or carbon dioxide has been reported to improve the procedure (Sassner et al. 2008; Viola et al. 2008; Jurado et al. 2009).

   Steam explosion is one of the most commonly used and perhaps the most successful physicochemical pretreatment of lignocellulosic biomass, especially hardwoods and agricultural residues (Gou et al. 2008; Sassner et al. 2008). The difference between steam pretreatment and steam explosion pretreatment is the sudden decompression of the materials that causes the water molecules to expand rapidly or 'explode' (Hendriks and Zeeman 2009). According to Lim (2004), this procedure is among a few that has advanced to pilot scale.

2. ***Hydrothermolysis/Liquid hot water (LHW).*** Another form of thermal pretreatment is hydrothermolysis or 'liquid hot water' pretreatment (Kim et al. 2008). This process is also known as hot aqueous fractionation and involves the 'cooking' of the lignocellulosic biomass in hot water, at high temperature (Lim 2004). The overall process is to hydrate lignocellulose and to solubilize some hemicellulose component to render better access to cellulose (Hendriks and Zeeman 2009). When performing LHW, it is recommended that the pH of the mixture should be kept between 4 and 7 to minimize the formation of toxic residues (Hendriks and Zeeman 2009).

3. ***Ammonia fiber explosion (AFEX) pretreatment.*** AFEX is another physico-chemical pretreatment method (Kim et al. 2008). In contrast to the previous alkaline procedure such as dilute NaOH, AFEX is accomplished at high pressure (250–300 psi), using an extruders, that is quickly released by the end of the treatment (Teymouri et al. 2005).

AFEX is best done on agricultural waste and grassy feedstocks (Kim et al. 2008). AFEX pretreatment does little degradation of cellulose and hemicelluloses but alters the structure of the biomass significantly, resulting in higher digestibility and water retention capacity (Kim et al. 2008; Keshwani and Cheng 2009).

One advantage to using AFEX pretreatment is that literally high portion of the ammonia can be recovered to be reused, while any residual left behind in the biomass serves as nitrogen source for yeast or other microbes during fermentation (Teymouri et al. 2005; Kim et al. 2008).

BIOLOGICAL PRETREATMENT

Physical, chemical and physicochemical pretreatments require highly specialized instrument and consume a lot of resources such as chemical and energy, often needing higher initial capital investment, larger processing costs and bigger investment risks (Zhang et al. 2007; Yu et al. 2009). Furthermore, these processes suffer from low sugar yield, loss of sugars and generation of toxic inhibitors to downstream processes such as enzymatic hydrolysis and microbial fermentation (Zhang et al. 2007; Yu et al. 2009). However, there is an alternative which utilizes less severe procedures, such as biological pretreatments.

For the biological pretreatment of lignocellulosic biomass, microorganisms, especially wood degrading fungi such as white-, brown- and soft-rot fungi are used to degrade lignin and hemicellulose (Sun and Cheng 2002; Galbe and Zacchi 2007; Shrestha et al. 2008; Shrestha et al. 2009; Rasmussen et al. 2010). Each of these fungi has their respective mechanisms as discussed further in the following sections. Among some of the fungal species that have been investigated are listed in Table 11.

The ability of these fungi to breakdown lignocellulosic biomass is due to their highly synergistic enzymatic complexes (Sanchez 2009). Basically, these fungi selectively degrade lignin and hemicellulose over a longer time period, making it less attractive for mass application (Galbe and Zacchi 2007; Keshwani and Cheng 2009). However, the mild reaction conditions and low energy input associated with this form of pretreatment as compared to physical, chemical and physico-chemical pretreatments demands a closer look (Keshwani

and Cheng 2009; Sanchez 2009).   Furthermore, this form of pretreatment require no

chemicals, making it also environmentally friendly (Saqib and Whitney 2006; Galbe and

Zacchi 2007).

Table 11. List of fungal species used in biological pretreatment of lignocellulosic biomass
(Sun and Cheng, 2002; Cho et al. 2008; Shrestha et al. 2008; Dashtban et al.
2009; Sanchez 2009; Shrestha et al. 2009; Rasmussen et al. 2010).

Type of rot fungus	Fungal species
White rot	*Strobilurus ohshimae*
	Phanerochaete chrysosporium
	Trametes versicolor
	Pleurotus ostreatus
	Pleurotus florida
	Clonostachys rosea
	Penicillium sp.
	Pycnoporus cinnabarinus
	Sporotrichum pulverulentum
	Xylaria hypoxylon
	Ceriporiopsis subvermispora
	Cyathus stercoreus
	Xylaria polymorpha
Brown rot	*Aspergillus niger*
	Fusarium oxysporus
	Fusarium merismoides
	Fomitopsis palustris
	Gloeophyllum trabeum
Soft rot	*Trichoderma reesei*

There are reports of biological pretreatment applications on wood chips, wheat straw,

Bermuda grass, softwood *Pinus densiflora*, corn stalks, corn fiber, Japanese beech and

Japanese cedarwood (Lee et al. 2007; Shrestha et al. 2008; Keshwani and Cheng 2009;

Sanchez 2009; Shi et al. 2009; Shrestha et al. 2009; Tanaka et al. 2009). According to

Wyman and colleagues (2005), biological pretreatment offers low costs alternative procedure

and with the advancement in biotechnology, this process can further be improved. Another suggestion is to use this pretreatment as a first step to be followed by some of the other types of pretreatment methods (Galbe and Zacchi 2007).

Phanerochaete chrysosporium

P. chrysosporium is a wood-decay white-rot fungus that has been studied extensively due to its abilities to completely and efficiently degrade, depolymerization and mineralize all major components of plant cell walls including cellulose, hemicellulose, and the more recalcitrant lignin (Wymelenberg et al. 2005; Kersten and Cullen 2007; Hamid and Rehman 2009). Because of these properties, *P. chrysosporium* has been deemed as a model organism in the study of lignin biodegradation and other biotechnological applications, such as biopulping, biobleaching and pulp mill effluents treatment (Ravalason et al. 2008). This fungus effectively performs all these processes, because it secretes various cellulases and hemicellulase such as endoglucanases, exocellobiohydrolase, cellobiose dehydrogenase, β–glucosidases, endoxylanases, β-xylosidase and α-galactosidase, among others (Abbas et al. 2005; Wymelenberg et al. 2005; Suzuki et al. 2008).

Molecular analysis of this species using restriction-fragment length polymorphism (RFLP) mapping and pulsed field gels electrophoresis suggest that *P. chrysosporium* genome consists of up to nine chromosomes. In 2004, Martinez and colleagues documented the complete profile of the *P. chrysosporium* genome. Table 12 shows a general feature of *P.*

chrysosporium genome. A dedicated genome database for this species is now hosted by the
United States Department of Energy Joint Genome Institute (Martinez et al. 2004).

Table 12. General features of the *P. chrysosporium* genome (Martinez et al. 2009).

Assembly size	34.5 Mbp
GC content overall	57%
Protein coding genes	11,777
Intron size (average)	117 bp
Intron size (mode)	54 bp
Exon size (average)	232 bp
Exon size (mode)	89 bp

Analysis of the *P. chryso*sporium genome reveals impressive diversity among genes that
encode carbohydrate-active enzymes (Martinez et al. 2004). Several studies reported that this
particular fungus harbors the genetic information of proteins from at least 69 distinct families
that encode 180 glycoside hydrolases (GH) and 282 putative carbohydrate hydrolyzing
cellulases and hemicellulases (Martinez et al. 2004; Abbas et al. 2005; Wymelenberg et al.
2005; Suzuki et al. 2008; Martinez et al. 2009). Martinez updated their database in 2009 and
a more comprehensive annotation of the *P. chrysosporium* lignocellulolytic gene complexes
are shown in Table 13.

Many of the cellulolytic, hydrolytic and oxidative enzymes of *P. chrysosporium* have been
purified and their cDNAs isolated and characterized (Abbas et al. 2005). These cellulases
and hemicellulases are produced, not only in large amounts, but also in different variety that
act synergistically, making it a choice microorganism for their production (Wymelenberg et
al. 2005; Suzuki et al. 2008).

Table 13. List of lignocellulolytic genes in *P. chrysosporium* (Martinez et al. 2009).

Types of protein	Specific protein/enzymes	Total
Lignin degradation proteins	Lignin peroxidases (LiP)	10
	Manganese peroxidases (MnP)	5
	Low redox-potential peroxidases	1
Iron reduction	Multicopper oxidases	4
	Quinone reductases	4
	Cellobiose dehydrogenases	1
Peroxide generation	Copper-radical oxidases	7
	Pyranose-2 oxidases (GMC)	1
	Glucose oxidases (GMC)	1
	Aryl-alcohol oxidases (GMC)	4
	Methanol oxidases (GMC)	1
	Total GMC oxidoreductases	35
Carbohydrate active proteins	GH with cellulose-binding domain	30
	Exocellobiohydrolases	7
	Endoglucanases	>40
	β-Glycosidases	9-10
	Esterases and transferase	87
	Expansins	11
Miscellaneous heme-protein reactions	Cytochrome P450-type enzymes	148
	Chloroperoxidase-peroxygenases	1-3
Total proteins predicted		10,048

Gloeophyllum trabeum

G. trabeum is a brown-rot basidiomycete from the order *Gloeophyllales*, family *Gloeophyllaceae* and genus *Gloeophyllum* (www.ncbi.nlm.nih.gov). A bracket fungus that forms spongy basidiocarps measuring up to 5 x 8 cm wide and 0.2-0.8 cm thick, this cinnamon-brown colored fungus possesses radial banding patterns (Overholts 1967) (Figure 19). According to Overholts (1967), the underside is ochre to tan-colored and is lined with a

network of 1-3 mm wide spore tubes that houses cylindric-elliptic shaped brown spores (measuring approximately to 4 x 10 µm).

Figure 19. Morphology of *G. trabeum.*
(http://micologia.net/g3/Gloeophyllum-trabeum/Gloeophyllum_trabeum_001)

G. trabeum and other saprophytic brown-rot fungi are major contributors to the plant biomass biodegradation, recycling, humus formation and soil fertility in the ecosystem (Kerem et al. 1999; Cohen et al. 2005). Brown-rot fungi also cause the most destructive type of decay in wooden built structures, making them highly studied in wood durability related studies (Kerem et al. 1999; Schilling et al. 2009). These basidiomycetes are characterized by the rapid and extensive depolymerization of cellulose, incurring significant strength loss, in the early stages of wood decay (Cho et al. 2008; Schilling et al. 2009). Cho and colleagues (2008) further commented that this unique form of wood depolymerization can easily be observed under microscopy such as "*loss of birefringence, absence of erosion troughs, and near-normal morphological appearance of the degraded wood cells*".

G. trabeum is unique in its wood-rotting mechanism because they rapidly hydrolyze the cellulose while leaving most of the encasing brown pigmented lignin (Kerem et al. 2009). *G. trabeum* modifies the lignin molecular structure, mainly via partial depolymerization, oxidation, demethoxylation and demethylation processes (Xu and Goodell 2001; Schilling et al. 2009). The remaining residues after degradation are sugar-free brown mass as shown in Figure 20 (Schilling et al. 2009).

Figure 20. Brown rot patterns on a tree trunk cause by *G. trabeum.*
(http://www.wolman.de/imagepool/Braunfaeule_in_NH_1.jpg)

G. trabeum degrades lignocellulose via a two-part mechanism (Schilling et al. 2009). Firstly, it modifies the plant cell wall non-enzymatically via a Fenton reaction with hydroxyl radical generation within the plant cell wall (Varela et al. 2003). Secondly, *G. trabeum* secretes cellulases and hemicellulases that further degrade the cellulose and hemicellulose. *G. trabeum* possesses a very efficient cellulolytic system that include a variety of cellulases and hemicellulases including endoglucanases, exoglucanases, β-glucosidases, xylanases and other hemicellulases, that rapidly degrades cellulose and hemicellulose, making it an ideal

biocatalyst for the hydrolysis of lignocellulosic materials (Kerem et al. 1999; Cohen et al. 2005). However, the typical brown-rot cellulases systems are unlike those of *P. chrysosporium* and *T. reesei*, as the former are not influenced by free glucose concentrations, and most often the mechanisms lack exo-acting cellobiohydrolases (Martinez et al. 2009; Schilling et al. 2009).

Trichoderma reesei

T. reesei is a filamentous mesophilic soft-rot fungus that was first documented during World War II (Martinez et al. 2008). This ascomycete is known for its efficient polysaccharide degradation system (Jovanovic et al. 2009). Presently, *T. reesei* serves as an important model organism for lignocellulose degradation studies and has been widely used for the mass production of cellulases and hemicellulases for various applications (Jovanovic et al. 2009*)*. The full *T. reesei* genome was successfully sequenced from high-quality draft assemblies using the Department of Energy Joint Genome Institute (JGI) shotgun assembler and reported in 2008 by Martinez and colleagues (Martinez et al. 2008). The general features of the genome are listed in Table 14.

Table 14. General features of the *T. reesei* genome (Martinez et al. 2008).

Assembly size	33.9 Mbp
GC content overall	52.0%
Coding genes overall	40.4%
No. of genes	9,129

T. reesei is a well-studied cellulolytic organism that is known to secrete various types of cellulolytic, hemicellulolytic and other carbohydrate active enzymes (Fukuda et al. 2009; Jovanovic et al. 2009). In total, 200 glycoside hydrolases (GH) have been found in the *T. reesei* genome (Martinez et al. 2009; Jovanovic et al. 2009). A listing of these carbohydrate active enzymes is shown in Table 15.

Table 15. Number of carbohydrate active enzymes in *T. reesei* (Martinez et al. 2008; Donohoe et al. 2009; Martinez et al. 2009).

Cellulase/Hemicellulase	Cellulase/Hemicellulase type	Total
Cellulase	CBH1, Exocellobiohydrolase I (GH7)	1
	CBH2, Exocellobiohydrolase II (GH6)	1
	EG1, Endoglucanase I (GH7)	1
	EG2, Endoglucanase II (GH5)	2
	EG3, Endoglucanase III (GH12)	1
	EG4, Cel61 (GH61)	3
	EG5, endoglucanase V, Cel45	1
Hemicellulase	GH43	2
	GH10	1
	GH11	4
	GH74	1
	GH62	1
	GH54	2
	GH67	1
	GH95	4
Other carbohydrate active enzyme	Carbohydrate Binding Module (CBM)	35
	Carbohydrate Binding Module I (CBMI)	14
	Glycosyl Transferease Modules	87
	Carbohydrate Esterases CE)	15
	Polysaccharide Lyases (PL)	3
	Expansins (EXPN)	7

In recent years, advancement in molecular biology has improved this strain to produce more cellulases, and the result is the mutant hypercellulolytic *T. reesei* Rut C30 (Ahamed and Vermette 2008). However, these strains still lack the abilities to secrete significant amount of

extracellular β-glucosidase, thus requiring supplementation of β-glucosidases from other sources (Kovacs et al. 2009). Nonetheless, the most commonly available commercial enzyme mixtures for lignocellulosic degradation are mainly obtained from this fungus (Olofsson et al. 2008; Tomas-Pejo et al. 2009).

CORN STOVER AS A LIGNOCELLULOSIC ETHANOL FEEDSTOCK

Corn stovers (Figure 21), the agricultural residues (the stalks and leaves) that remain after corn is harvested, are a substantial source of inexpensive and abundant lignocellulosic biomass (Hess et al. 2009). It is also one of the most abundant agricultural residues in other countries, such as Europe and China (Galbe Zacchi 2007; Chen et al. 2009). According to one of the most current studies on biomass, it is estimated that the United States produce as much as 1.3 billion tons biomass per year with corn stover leading the total volume at 75 million tons (Perlack et al. 2005; Hess et al. 2005; Templeton et al. 2009). Table 16 below shows a detailed analysis of a typical corn stover.

Figure 21. Shredded corn stover

Table 16. Detailed corn stover composition analysis (Aden and Foust 2009).

Component	Dry basis (%)
Glucan	37.4
Xylan	21.1
Lignin	18.0
Ash	5.2
Acetate	2.9
Protein	3.1
Extractives	4.7
Arabinan	2.9
Galactan	2.0
Mannan	1.6
Unknown soluble solids	1.1
Moisture	15.0

Corn stover is one of the most studied lignocellulosic biomass used for bioethanol production (Sokhansanj et al. 2002). These studies include harvesting techniques developments (Figure 24) and pretreatment procedures. Among some of the pretreatment studies that have been conducted on corn stovers are AFEX, ARP, dilute acid (both Sunds and Parr systems), flow through, SO_2 alkaline pretreatments, steam explosion and LHW (Galbe and Zacchi 2007; Lu et al. 2008; Aden and Foust 2009; He et al. 2009; Kumar et al. 2009).

Figure 22. (a) Corn stover a typical corn field, (b) Harvesting and baling of corn stovers, (c) A corn stover bale in the square format, (d) The loading of bales onto a flat-bed semi-tractor trailer, (e) Loaded bales being transport (Hess et al. 2009).

The DOE-funded research from 1978 to 2002 on woody plants and other "*perennial energy crops such as switchgrass was largely discontinued in 2002 and the focus shifted to the use of crop residues*" (i.e. corn stover) for bioethanol production (Varvel et al. 2008). Of all the

crop residues, corn stover is deemed one of the model and most promising feedstock that the National Renewable Energy Laboratory (NREL) published a comprehensive report for a process design and economic analysis of the biochemical conversion of corn stover to ethanol (Aden 2008; Templeton et al. 2009).

LIGNIN, CELLULOSE AND HEMICELLULOSE DEGRADING ENZYMES

The most crucial steps in lignocellulosic ethanol production are the hydrolysis of the cellulose and hemicellulose polymers to their respective monomeric sugars. In contrast to starch, cellulose and hemicellulose degradation requires more enzymes for complete hydrolysis to fermentable units (Keshwani and Cheng 2009). In nature, the enzymatic hydrolysis of cellulose and hemicellulose are performed by microorganisms that can be found either free in nature (i.e. fungi) or in the rumin of higher animals (i.e. *Archaebacteria*) (Galbe and Zacchi 2007; Maki et al. 2009). These processes are extremely slow and challenging because of cell wall insoluble rigid nanostructures and the minute amount of efficient cellulolytic and hemicellulolytic release by most microorganisms (Shallom and Shoham 2003; Chang 2007).

Enzymatic hydrolysis occurs outside the plant cell using either a free or complexed cellulolytic/hemicellulolytic system. Aerobic microorganisms such as *T. reesei* usually use the free cellulases mechanism where they secrete a set of single cellulases, most of which contain a carbohydrate binding module (CBM) joined by a peptide linker to the catalytic

domain (CD) (Chang 2007; Wilson 2009). Anaerobic cellulolytic microorganism, such as

Clostridium thermocelluum uses a large cellulase complex known as cellulosomes (Figure

23), that are attached to the outer surface of the bacterial cell wall via a structural protein

called scaffoldin (Maki et al. 2009; Wilson 2009). According to Chang (2007), cellulosomes

are more effective cellulase systems than the freely secreted enzymes.
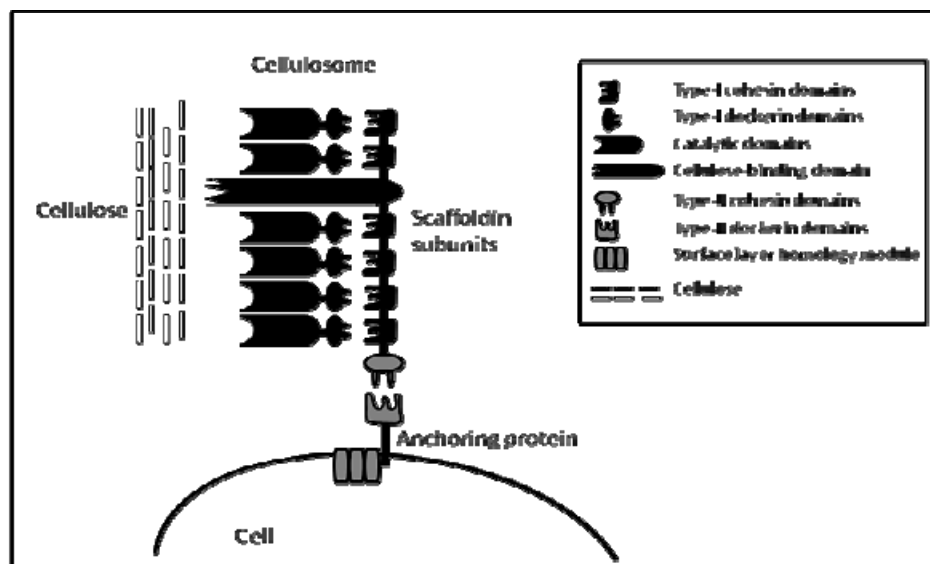
Figure 23. A schematic of a typical cellulosome complex connected to the cell surface *C. thermocelluum* (Maki et al. 2009).

According to the Carbohydrate-Active enZymes (CAZy) database, cellulases and most

hemicellulases are members of the GH group of enzymes (Dashtban et al. 2009). Multiple

isozymes of the different classes of cellulases, hemicellulases and ligninases work in unison

to achieve the complete hydrolysis of cellulose, hemicellulose and lignin. Currently more

than 2500 GH have been identified, with 148 recognized in the enzyme classification system

(EC 3.2.1.X) and classified into 115 families (Dashtban et al. 2009; Jovanovic et al. 2009).

Cellulases and hemicellulases have attracted much interest in the recent years because of the diversity and importance of their applications, especially in the production of lignocellulosic ethanol for transportation purposes (Das et al. 2008). Since the cost of producing these enzymes is a huge factor in cost effective biofuel production, accounting for approximately 40-50% of the total cost, attentions are drawn on the possibilities of using available low cost carbon source and on effective fermentation systems (Hao et al. 2006; Liu and Yang 2007; Muthuvelayudham and Viruthagiri 2007; Das et al. 2008; Zhang et al. 2006). Recently, in partnering with Genencor International and Novozymes Biotech, NREL announced the development of technologies that has reduced the cellulase cost 20-30 fold for the cellulosic ethanol to about 10-25 cents per gallon of ethanol (Zhang et al. 2006; Chang 2007). In these technologies, the improvements were focused on the economical aspects of cellulase production by using less expensive medium and a development of higher potency in the enzyme activities to reduce the enzyme loadings (Zhang et al. 2006).

In this section, we will also discuss the enzymes that perform lignin degradation, as part of the big lignocellulolytic theme.

LIGNIN DEGRADING ENZYMES

In a typical plant cell wall, lignin forms a binding matrix that encases and impedes the breakdown of cellulose and hemicellulose (Aro et al. 2005). Therefore, solubilization of lignin is a prerequisite for liberation of cellulose and hemicelluloses and to achieve an optimal biological conversion of lignocellulosic biomass to ethanol.

Lignin is difficult to breakdown because of its hydrophobicity, free radical coupling mechanism and its four stereoisomers (Hamid and Rehman 2009). However, many white rot fungi, such *P. chrysosporium, C. versicolor* and *T. versicolor*, are capable of producing efficient lignin degrading enzymes (Aro et al. 2005). Most of these enzymes are non-specific, oxidative and act via non-protein mediators (Aro et al. 2005). There are currently four main fungal ligninolytic enzymes (Martinez et al. 2005; Hammel and Cullen 2008):

1. ***Lignin Peroxidases (EC 1.11.1.14)***. Lignin peroxidase (LiP) was first reported in 1983 as part of the extracellular enzyme systems of *P. chrysosporium*, under nitrogen limitation (Hammel and Cullen 2008; Hamid and Rheman 2009). They are monomeric proteins with molecular weights of approximately 40 kDa with an optimal pH of above 4 with addition of H_2O_2 (Hammel and Cullen 2008; Hamid and Rheman 2009). In addition, these enzymes also degrade a variety of complex aromatic compounds and oxidize a number of recalcitrant polycyclic aromatic and phenolic compounds, making them highly important for the biodegradation of industrial effluents (Hamid and Rehman 2009). To

date, 10 LiP genes designated *lip*A through *lip*J have been discovered, although the reason why there are so many LiPs are still unclear (Hammel and Cullen 2008).

2. ***Manganese peroxidases (EC 1.11.1.13)***. Manganese peroxidases (MnP) are produced by *P. chrysosporium* under nutrient limitation and also by the presence of Mn^{2+} (Hammel and Cullen 2008). MnPs are strong oxidizing enzymes but they do not oxidize non-phenolic lignin-related structures (Hammel and Cullen 2008). These group of enzymes have also been reported to catalyze the oxidation of several phenols and aromatic dyes compounds via lipid peroxidation reactions, with reactions greatly stimulated by the presence of manganese and certain types of buffer solutions (Hamid and Rehman 2009; Sanchez 2009).

3. ***Laccases (EC 1.10.3.1).*** Laccase, or also known as phenol oxidase, are blue copper oxidases that catalyze the one-electron oxidation of phenolic compounds (Dashtban et al. 2009). As their name describe, phenol oxidases oxidize phenolic compounds and reduce molecular oxygen to water (Sanchez 2009).

4. ***Glyoxal oxidase***. Glyoxal oxidases are also called GLOX. These enzymes generate peroxides that are essential for peroxidase function (Martinez et al. 2005).

CELLULOSE DEGRADING ENZYMES

Enzymatic hydrolysis of crystalline and amorphous cellulose is a complicated process that is performed by a group of enzymes called cellulases (Hong et al. 2007). Although the current lignocellulosic ethanol interest has sparked great interest in cellulose degrading enzymes, the application of cellulases actually has long been established. Among some of the applications of cellulases as mentioned by Zhang et al. (2006) are "*in the textile industry for cotton softening and denim finishing; in the detergent market for color care, cleaning, and anti-deposition; in the food industry for mashing; and in the pulp and paper industries for deinking, drainage improvement, and fiber modification.*"

Cellulases are constructed of independently folding and functionally specialized units called domains (Maki et al. 2009). As shown in Figure 24, a typical free cellulase is composed of a CBD joined by a flexible peptide linker to the CD (Maki et al. 2009).
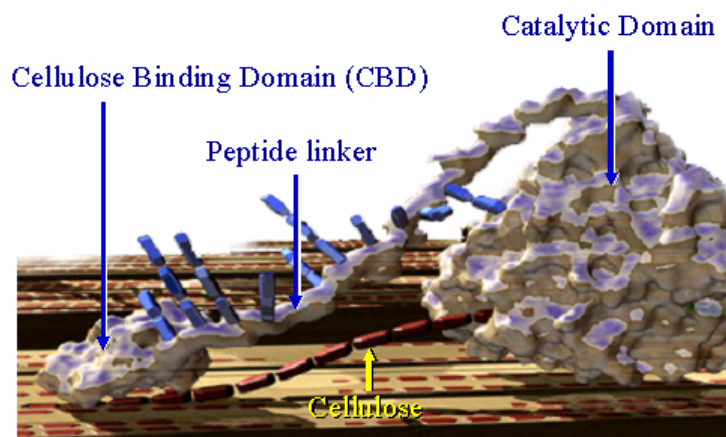
Figure 24. A typical structure of free cellulase.
(http://genome.gsc.riken.go.jp/hgmis/graphics/slides/images/01-0618R3cellulase.jpg)

Cellulases are classified into three main groups according to the mode of action and biochemical structure of the protein (Figure 25). These major groups are generically termed endoglucanases, exoglucanases and β-glucosidases (Zhang and Lynd 2004; Mussatto et al. 2008; Maki et al. 2009). Enzymatic hydrolysis by these three enzyme groups occur simultaneously (Zhang et al. 2006). Endoglucanase and exoglucanases perform the synergistic primary hydrolysis on the surface of solid substrates to release soluble sugars with a DP of up to 6 into the liquid substrate (Maki et al. 2009). Processive cellulases, both exo- and endo-, are major components of the free cellulase concoction, and often constitute more than 60% of the total cellulase mixture (Wilson 2009). β-Glucosidases then perform the secondary hydrolysis the liquid substrate to hydrolyze the free cellobiose or longer cellodextrins to glucose (Zhang et al. 2006).
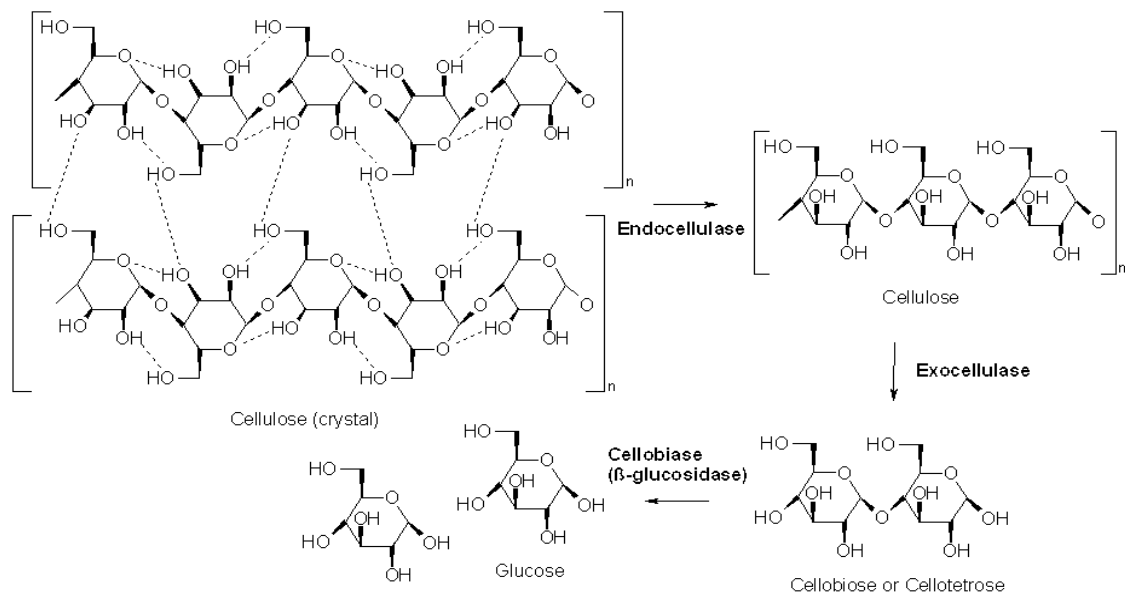
Figure 25. The mode of mechanisms of cellulolytic enzymes. (Adapted from http://www.enzymeindia.com/enzymes/images2/Cellulase_map.jpg)

The degradation of cellulose by cellulases is affected by several factors such as types of enzymes, quantity of enzymes, quality of substrate (accessibility, crystallinity, DP, particle size and pore volume) and environmental factors (temperature, pH, nutrient, etc.) (Gregg and Saddler 1996; Hong et al. 2007).

1. ***β-1-4-Endoglucanase (Endogluccanases - EC 3.2.1.4)***. β-1-4-Endoglucanases are relatively small enzymes with molecular weights between 22 and 45 kDa (Dashtban et al. 2009). While many endoglucanases consist of the typical CD-linker-CBD structures, some smaller endoglucanases lack the CBD (Baldrian and Valaskova 2008; Dashtban et al. 2009). These group of endoglucanases degrade the amorphous regions of cellulose works best at 50-70°C and at pH of 4-5 (Sun and Cheng 2002; Dashtban et al. 2009).

Endoglucanases break the internal bonds that form the crystalline structure of cellulose and cut the long cellulose chains at random positions to create free chain-ends (Zhang et al. 2006; Saqib and Whitney 2006; Sanchez 2009). Thus, endoglucanases activities can be tested using soluble cellulose substrates, such as carboxymethylcellulose (CMC) (Maki et al. 2009). It is because of this assay that endoglucanases are also termed carboxymethylcellulases (CMCase).

According to Sanchez (2009), many fungi produce multiple EGs. For instance, *T. reesei* produces at least 5 EGs (EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A, EGIV/Cel61A and EGV/Cel45A) whereas *P. chrysosporium* secretes three EGs (EG28, EG34 and EG44) (Dashtban et al. 2009).

2. ***β-1-4-Exoglucanase (Exoglucanases - EC 3.2.1.74) and Cellobiohydrolase (CBH) - EC 3.2.1.91)***. Exoglucanases are also known as CBH. These monomeric enzymes have molecular weights of between 50 and 65 kDa (Baldrian and Valaskova 2008). Exoglucannases degrade the crystalline regions of cellulose and the optimal conditions are at 37-60°C and at pH of 4-5 (Dashtban et al. 2009).

    These isoenzymes cleave 2-4 units from the ends of the exposed chain-ends produced by endocellulases, resulting in the disaccharide such as cellobiose or tetrasaccharides such as cellotetraose (Sun and Cheng 2002; Dashtban et al. 2009). According to Cohen and colleagues (2005), there are two main types of exo-cellulases, one type working processively from the reducing end and one type working processively from the non-reducing end of cellulose.

    Exoglucanases are also found in multiple copies, at times accounting for 40–70% of the total cellulase proteins (Sanchez 2009). For example, *T. reesei* has two exoglucanases acting from both reducing ends (CBHI/Cel7A) and non-reducing (CBHII/Cel6A), making it a very efficient cellulolytic fungi (Dashtban et al. 2009)

3. ***β-Glucosidase (EC 3.2.1.21)***. β-Glucosidases are a mixture of monomeric, dimeric and trimeric enzymes that has a larger range of molecular weights of between 35 and 450 kDa (Baldrian and Valaskova 2008). Exoglucanases hydrolyses the cellobiose into glucose monomers under a wide pH range and in a temperature range of 45-75°C (Sun and Cheng 2002; Dashtban et al. 2009).

β–Glucosidases are sometimes not categorized as a cellulase, because it does not act on the cellulose itself. However, due to its specificity to β-1,4-glucosidic bonds and its essential role in the cellulolytic processes, it is often mentioned as a cellulase. β-Glucosidase hydrolysis of cellobiose to glucose is highly important, as without this mechanism, degradation activities of the other cellulases will be inhibited (Keshwani and Cheng 2009; Sanchez 2009).

Because cellobiose is a common carbohydrate, β-Glucosidases are produced by the majority of cellulolytic microorganisms (Baldrian and Valaskova 2008). In *T. reesei*, two β-glucosidases (BGLI/Cel3A and BGLII/Cel1A) are found but the expression is very low when compared to other cellulolytic fungi such as *A. niger* (Dashtban et al. 2009).

HEMICELLULOSE DEGRADING ENZYMES

Hemicellulose degradation is an important process for the optimal utilization of biomass, as this liberates fermentable pentoses like xylose (Fukuda et al. 2009). More importantly, hemicellulose needs to be hydrolyzed first before cellulose is exposed for the action of cellulases. In comparison to cellulose, hemicellulose is a very heterogeneous polymer both in structures and organization, thus it requires a more extensive repertoire of enzymes to be completely hydrolyzed to its forms of soluble sugars (Heck et al. 2002; Dashtban et al. 2009). Collectively, these enzymes are known as hemicellulases, and are usually classified under cellulase in general.

According to Dashtban et al. (2009), hemicellulases and cellulases share several similarities. Firstly, hemicellulases are mostly modular proteins and have other functional domains, like the CBD of cellulase, attached to their catalytic domains. These CBDs are responsible for the attachment of the enzymes to the insoluble polysaccharides. In addition, there are also dockerin modules that assist in the binding of the catalytic domains via cohesin–dockerin interactions (Shallom and Shoham 2003). Secondly, most of these synergistic hemicellulases belong to the GHs family of enzymes (Dashtban et al. 2009; Keshwani and Cheng 2009). However, in addition to the GHs, hemicellulase also contain another enzyme group, that hydrolyzes the ester linkages from the acetate or ferulic acid side group that are known as carbohydrate esterases (CEs) (Shallom and Shoham 2003). And, thirdly, hemicellulases from aerobic fungi, such as *Trichoderma* and *Aspergillus,* are expressed in high amount in several varieties, believed to enable the efficient hydrolysis of their substrates (Aro et al. 2005; Chang 2007, Dashtban et al. 2009).

One of the major hemicellulase enzyme groups is the xylanases. This is believed to be partly an evolutionary respond the fact that xylan is the largest component of hemicellulose (70%) (Dashtban et al. 2009). Endo-xylanases (EC 3.2.1.8) hydrolyze the β-1,4 linkages in xylan to release xylooligosaccharides that are finally hydrolyzed into xylose by β-Xylosidases (EC 3.2.1.37) (Dashtban et al. 2009; Fukuda et al. 2009). Xylanases have an optimum working pH of 4 and a very high temperature optimum of $80^{\circ}C$. It is a relatively large protein with a molecular mass of 39–42 kDa, while other hemicellulases are smaller (about 20 kDa) monomeric proteins (Saha 2003).

In addition to the xylanases, hemicellulose degradation requires a whole consortium of additional enzymes. A comprehensive list of the specific substrates and chemical bonds hydrolyzed by the hemicellulolytic enzymes are shown in Table 17, and a schematic view on degradation of the different hemicellulose components is shown Figure 26 (Shallom and Shoham 2003).

Table 17. The hemicellulolytic enzymes, their substrates and optimum working conditions (Saha 2003: Shallom and Shoham, 2003; Sanchez 2009).

Enzyme	Substrates	Optimal pH	Optimal temp
Endo-β-1,4-Xylanase	β-1,4-xylan	5.0	45
Exo-β-1,4-Xylosidase	β-1,4-xylooligomers xylobiose	5.0	50
α-L-Arabinofuranosidase	α-Arabinofuranosyl xylooligomers α-1,5-arabinan	3.4-4.5	50-60
Endo-α-1,5-Arabinanase	α-1,5-arabinan	4.5-5.0	50-55
α-Glucuronidase	4-O-methyl-a-glucuronic acid (1→2) xylooligomers	3.5	50
Endo-β-1,4-Mannanase	β-1,4-mannan	2.9-3.3	72.74
Exo-β-1,4-Mannosidase	β-1,4-mannooligomers mannobiose	3.3	72-74
α-Galactosidase	α-galactopyranose mannooligomers	4.0	60
β-Glucosidase	β-glucopyranose (1→4) mannopyranose	5.0	50
Endo-Galactanase	β-1,4-galactan	3.5	50-55
Acetyl xylan esterase	2- or 3-O-acetyl xylan	7.7	30
Ferulic acid esterases	feruloylester bonds	5.0	55
p-coumaric acid esterases	*p*-coumaric ester bonds	-	-
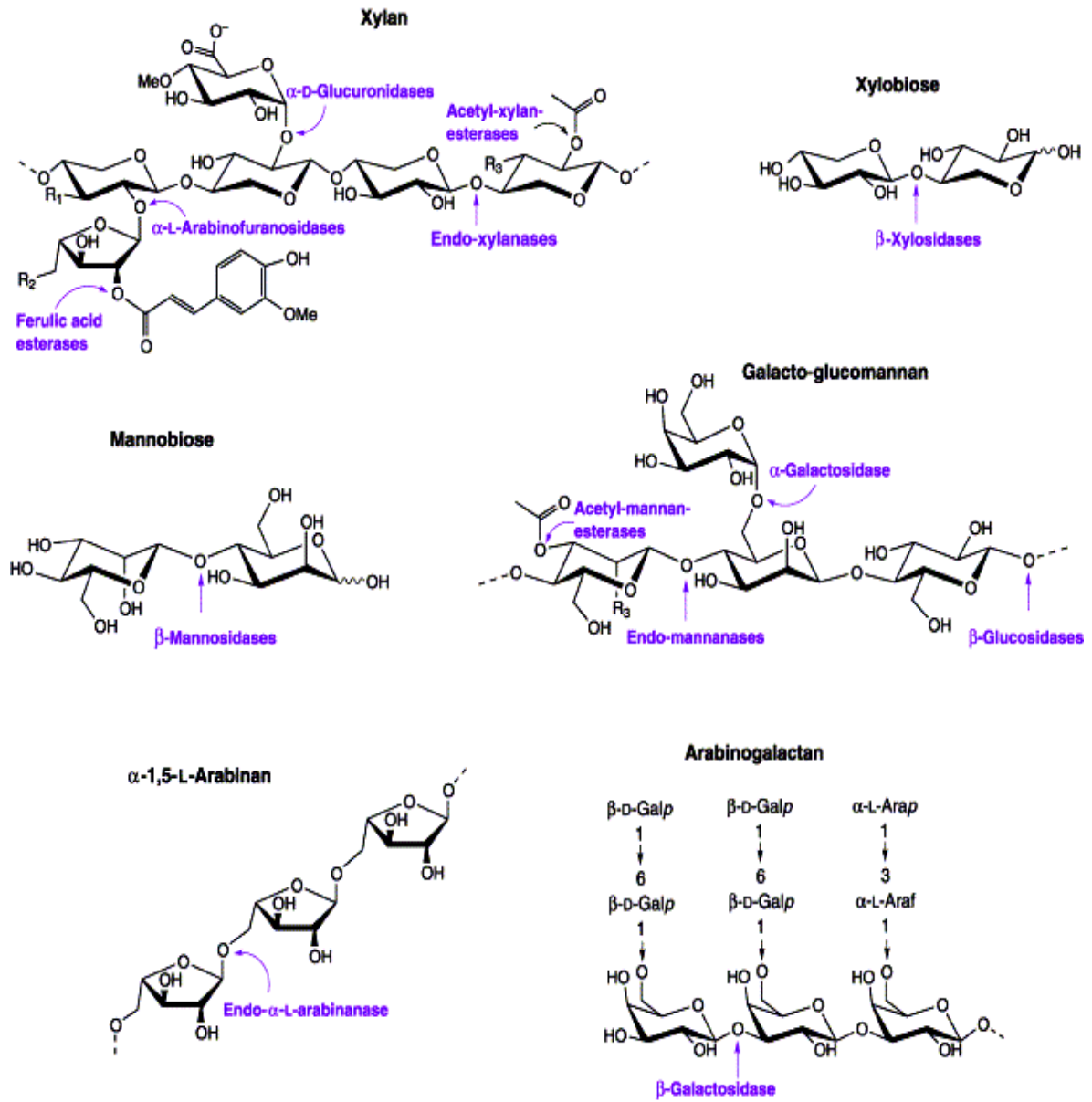
Figure 26. A schematic view on degradation of the different hemicellulose components (Shallom and Shoham 2003)

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

Simultaneous saccharification and fermentation or SSF is a process whereby both enzymatic saccharification of lignocellulosic feedstocks and fermentation of the resultant sugar to ethanol, are performed in the same vessel, at the same time (Olofsson et al. 2008). During the saccharification step, the supernatant from the enzymatic hydrolysis of the pretreated lignocellulosic biomass contain both hexoses and pentoses, mostly glucose and xylose (Keshwani and Cheng 2009). Simultaneously, *S. cerevisiae* or other fermenting species in the broth then convert the free sugars to ethanol (Keshwani and Cheng 2009). The initial concept of performing the enzymatic saccharification and fermentation simultaneously was first suggested in 1976 by Gauss and colleagues (Olofsson et al. 2008). According to Tomás-Pejó et al. (2009), SSF results in higher ethanol yields compared to another related process that is called separate hydrolysis and fermentation (SHF) that separates the saccharification and fermentation steps.

In study by Olofsson et al. (2008), they reported that SSF offers the following advantages:

i. End-product (glucose and cellobiose) inhibition of the enzymatic saccharification is greatly reduced (Shapouri 2007).

ii. The potential loss of fermentable sugars (glucose and xylose) can be avoided as the supernatant constituents do not need to be separated or transferred.

iii. Lower capital and maintenance cost as the number of vessels for processing are fewer. The decrease in capital investment is estimated to be more than 20%.

Efficient SSF is dependent on several factors:

1. ***Substrate loading.*** Optimal substrate loading is crucial in SSF to achieve a high final ethanol concentration, and has to be optimized empirically as high solid content in the SSF reactor decreases ethanol yield (Olofsson et al. 2008).

2. ***Enzyme loading.*** Enzyme dosing is also one of the key factors that affect hydrolysis rate and efficiencies. Linde and colleagues (2007) documented that there is a strong positive correlation between enzyme loading and the overall ethanol yield.

3. ***Temperature.*** In performing SSF, a compromise between the optimal temperatures for the hydrolytic enzymes activities and the microbial fermentation is needed. Previous SSF experiments recommends a temperature of 32-37°C to facilitate both *S. cerevisiae* (optimal temperature ~30°C) and saccharifying enzymes (optimal temperature ~55°C) (Sassner et al. 2006; Olofsson et al. 2008).

One problem with an SSF process is the optimum temperature for both the saccharification and fermentation stages. For enzymatic hydrolysis using cellulases and hemicellulases, the best temperature about $50^{\circ}C$, whereas most fermenting microorganisms of choice, such *S. cereviseae*, *Zymomonas mobilitis* and *E. coli* K011, have an optimum temperature ranging between $30^{\circ}C$ and $37^{\circ}C$ (Tomás-Pejó et al. 2009). One possible solution is to use thermo tolerant yeasts such as *Kluveromyces marxianus*, as suggested by Fonseca et al. (2007).

CONCLUSION

Through a thorough research and analysis of the literature, we conclude that the goals to introduce lignocellulosic ethanol into the fuel ethanol supply are attainable with the concerted efforts in improving and maturing the current technologies involved. Undoubtedly, cellulosic ethanol has the potential to comply with President Bush's goals, but there are still huge rooms for improvements and breakthroughs to be made, and any attempts to displace gasoline consumption by supplementing the supply with ethanol would require an enormous change in agricultural and industrial practices.

With nationwide and worldwide production and application of fuel ethanol being the universal target, the technologies should be efficient, cost effective and environmentally friendly in every aspect. In recent years, we have seen many Government and privately sponsored research which has resulted in new technologies that lowered the cost of production of ethanol made from corn starch. But for the production of ethanol should go beyond the current first generation technologies. In our discussion, we strongly believe that that efforts should be made in transitioning the feedstock from corn and sugar based to lignocellulosic biomass, as the low cost and abundance of a wide range of lignocellulosic materials offer many possibilities for the development and implementation of biobased industries that supply the world energy needs for the international biofuel market. For cellulosic fuel ethanol production to be competitive at the commercial level, processing expenditures, especially for pretreatment and enzymatic hydrolysis should be improved.

Currently, concerted efforts are underway to improve pretreatment technologies and the enzymes used for lignocellulosics saccharification to improve production, and reduce the overall cost of lignocellulosic ethanol. The biological pretreatment and enzymatic saccharification mechanisms of wood-rot fungi such as *P. chrysosporium*, *G. trabeum* and *T. reesei* on corn stover is a very promising area for research as there are numerous advantages in the application of these processes. Advancement in molecular biology and genetic engineering may also assist in strains improvement of these wood-rot fungi for biological pretreatment and simultaneous enzymatic hydrolysis of lignocellulosic materials. In the field of fuel ethanol production from corn stovers, the optimization of these biological processes can lead to the following advantages:

i.   Inexpensive ethanol production - manufacturers can produce their own enzymes without the need to buy expensive commercially available ones.

ii. More effective ethanol production - using adaptive and living fungus, such as *P. chrysosporium*, *G. trabeum* and *T. reesei* will reduce the inhibitory effects of by-products during saccharification and fermentation.

iii. More environmentally friendly processing – lignocellulosic ethanol producers can skip the environmentally detrimental pretreatments process.

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CHAPTER 3: EVALUATION OF POTENTIAL FUNGAL SPECIES FOR THE *IN SITU* SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) OF CELLULOSIC MATERIAL

*(to be submitted to the **World Journal of Microbiology and Biotechnology**)*

ABSTRACT

Three fungal species were evaluated for their abilities to saccharify pure cellulose from Whatman No.1 filter paper. The three species chosen represented the three major wood-rot molds; brown rot (*Gloeophyllum trabeum*), white rot (*Phanerochaete chrysosporium*) and soft rot (*Trichoderma reesei*). After solid state fermentation of the fungi on the filter paper for four days, the hydrolysis products released from the saccharified filter paper was then subsequently fermented to ethanol by using *Saccharomyces cerevisiae* for a period of five days. The efficiency of the fungal species in saccharifying the filter was compared against a low dose (25 FPU/g cellulose) of a commercial cellulase. Total sugar, cellobiose and glucose were monitored during the fermentation period, along with three main fermentation products, namely ethanol, acetic acid and lactic acid. Results indicated that the most efficient fungal species in saccharifying the filter paper was *T. reesei* with 5.13 g/100 g filter paper of ethanol being produced at days 5, followed by *P. chrysosporium* at 1.79 g/100 g filter paper. No ethanol was detected for the filter paper treated with *G. trabeum* throughout the five day fermentation stage. Acetic acid was only produced in the sample treated with *T. reesei* and

the commercial enzyme, with concentration 0.95 g/ 100 g filter paper and 2.57 g/100 g filter paper, respectively at day 5. Lactic acid production was not detected for all the fungal treated filter paper after day 5. Our study indicated that there is potential in utilizing *in situ* enzymatic saccharification of biomass by using *T. reesei* and *P. chrysosporium* that may lead to a more economical simultaneous saccharification and fermentation of biomass for downstream applications such as production of fuel ethanol.

Keywords *Phanerochaete chrysosporium*, *Trichoderma reesei*, *Gloeophyllum trabeum*, *Saccharomyces cerevisiae*, Simultaneous Saccharification and Fermentation (SSF), Cellulase

INTRODUCTION

Lignocellulosic materials from biomass such as agricultural crop residues and other energy crops is the most abundant and renewable biopolymer on Earth (Bedford 2001; de La Torre Ugarte et al. 2003; Zhang 2008; Fukuda et al. 2009). Made of 75–80% cellulose and hemicellulose, they are low cost feedstocks for various industrial purposes that can be used in the production of chemicals and fuel ethanol, which is a good substitute for gasoline in internal combustion engines (Adsul et al. 2005; Ahamed and Vermette 2008; Ling et al. 2009). However, the production of fuel grade ethanol from lignocellulosic materials as an alternative or additives for fossil fuels is still expensive. According to Alkasrawi et al. (2003), recent economical calculations showed that the production cost of fuel ethanol from

lignocellulosic biomass would be higher than the price of gasoline. Thus, additional cost reductions are necessary to achieve economic competitiveness against the existing conventional fuels.

Currently, the most promising platform for the bioconversion of lignocellulosics to ethanol is based on the enzymatic hydrolysis of biomass using cellulase and hemicellulase enzymes via simultaneous saccharification and fermentation (SSF) process, first reported in 1976 by Gauss and colleagues (Ahamed and Vermette 2008; Olofsson et al. 2008). SSF is a technology that has gained a lot of interest, as it is both logistically and economically favorable in terms of higher final ethanol yield (Ohgren et al. 2007; Tomas-Pejo et al. 2009). Furthermore, this type of process has lower energy consumption when compared to the closely related separate hydrolysis and fermentation (SHF) (Olofsson et al. 2008). However, the drawback of SSF is the high enzyme concentrations that are required for significant hydrolysis of cellulose and hemicellulose (Alkasrawi et al. 2003; Linde et al. 2007). According to Ahamed and Vermette (2008), cellulase production is the most expensive step during ethanol production from cellulosic biomass, accounting for approximately 40% of the total cost. Therefore, because the high cost of cellulase enzyme production and enzyme loading is a major economical factor in the overall ethanol production cost, it is imperative to find methods of reducing the enzyme loading and increasing the hydrolysis of cellulose to fermentable sugars (Gregg et al. 1998; Adsul et al. 2005).

Another challenge in making the bioconversion of lignocellulosics to ethanol more feasible is the pretreatments that are needed to be performed on the feedstocks prior to enzymatic

hydrolysis (Silverstein et al. 2007; Zhu et al. 2009). The problems with many current pretreatments technologies are the generations of toxic by-products that can hinder the bio-mechanisms of the cellulolytic and hemicellulolytic enzymes, and may also inhibit downstream alcoholic fermentation (Ortega et al. 2001; Keating et al. 2005). Furthermore, these practices are environmentally detrimental and energy intensive (Chundawat et al. 2006). Therefore, it is imperative to develop means of direct enzymatic hydrolysis of lignocellulosics that do not sacrifice ethanol production. One possible solution is to use lignolytic, cellulolytic and hemicellulolytic organisms, such as fungi, to perform enzymatic saccharifications that will liberate fermentable sugars from the biomass.

Many fungal groups have been known to be able to degrade the main components of lignocellulosics, such as cellulose, hemicellulose and lignin (Arantes and Milagres 2006; Sanchez 2009; Shrestha et al. 2009; Rasmussen et al. 2010). The first of this group, the filamentous molds are well documented for their highly efficient cellulolytic and hemicellulolytic enzyme systems for the complete hydrolysis of biomass into its monomeric sugar components. The extracellular cellulolytic system of this fungus group composed of 60–80% cellobiohydrolases or exoglucanases, 20–36% of endoglucanases and 1% of β-glucosidases that act synergistically (Ahamed and Vermette 2008).

The next fungal group, the white-rots, have been studied extensively for their abilities to efficiently degrade and depolymerize major plant cell wall components, especially the more recalcitrant lignin, making it extensively used in the study of lignin biodegradation and other biotechnological applications, such as biobleaching and pulp mill effluents treatment

(Wymelenberg et al. 2005; Kersten and Cullen 2007; Ravalason et al. 2008). White rots effectively perform all these processes because they secrete several varieties of lignin degrading proteins, such as lignin peroxidases (LiPs), manganese peroxidases (MnPs) and other low redox-potential peroxidases, in addition to expressing multiple cellulases and hemicellulase (Suzuki et al. 2008; Martinez et al. 2009).

The third fungal group consists of the brown-rots. These saprophytic fungi are major forest biomass degraders that contribute significantly to the soil fertility in the ecosystem (Kerem et al. 1999; Cohen et al. 2005). Brown-rot fungi also cause the most destructive type of decay in wooden structures, although their biodegradation mechanisms are still relatively unknown (Kerem et al. 1999; Schilling et al. 2009). Fungi from this group appear to produce some cellulases, but a larger part of the cellulose degradation seems to be non-enzymatic, involving low molecular weight catalysts such as chelating peptides and radicals (Henriksson et al. 1999; Cohen et al. 2005).

In this study, we evaluated three fungal species that represent the three major wood-rot; brown-rot (*Gloeophyllum trabeum*), white-rot (*Phanerochaete chrysosporium*) and soft-rot (*Trichoderma reesei*), for their abilities to enzymatically saccharify filter paper via *in situ*. The efficiencies of their enzyme activities are measure via the release of cellobiose, glucose and the end fermentation products in the form of ethanol and organic acids. To perform fermentation, *Saccharomyces cerevisea* was used to maximize the conversion of the saccharification products.

MATERIALS AND METHODS

Microorganisms stocks and culture preparation

All fungal cultures used in this study were obtained from American Type Culture Collection (Rockville, MD). The *Gloeophyllum trabeum* (ATCC 11539), *Phanerochaete chrysosporium* (ATCC 24725), *Trichoderma reesei* (ATCC 13631) and *Saccharomyces cerevisiae* (ATCC 24859) cultures were revived onto potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD) at 24°C with shaking at 120 rpm overnight (Shrestha et al. 2009). For long term storages, the stock cultures were aliquoted in Yeast Malt (YM) extract broth (glucose, 10.0 g/l; peptone, 5.0 g/l; yeast extract, 3.0 g/l; and malt extract, 3.0 g/l) (Difco) supplemented with 20% (v/v) glycerol, at -80°C in an ultralow-temperature freezer (So-Low Environmental Equipment Co., Inc., Cincinnati, OH) (Shrestha et al. 2008; Shrestha et al. 2009).

Seed cultures from spore suspension of *G. trabeum*, *P. chrysosporium* and *T. reesei* were prepared in 1 liter YM broth and incubated at 30°C, agitated at 150 rpm. After a 7-day of incubation period, the mycelial pellets were separated from the broth via centrifugation (Sorvall-RC3B Plus centrifuge, Thermo Fisher Scientific, Wilmington, DE) at 7,277g for 20 min in a sterilized 1 L polypropylene centrifuge bottle (Nalgene, Nalge Nunc, Rochester, NY) (Shrestha et al. 2008). Next, the mycelial pellets were rinsed with a solution containing 50 mM Phosphate buffer (pH 4.5-4.8), 0.5% $(NH_4)_2SO_4$ and basal salt solution (0.25 g KH_2PO_4, 0.063 g $MgSO_4 \cdot 7H_2O$, 0.013 g $CaCl_2 \cdot 2H_2O$, in 1 L water) and 1.25 mL of premix

trace element solution (3.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.181 g $CoSO_4 \cdot 7H_2O$, 0.082 g $CaCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $Al_2(SO_4)_3 \cdot 2H_2O$, 0.01 g H_3BO_3, and 0.01 g $NaMoO_4$) in 1 L of deionized water (Shrestha et al. 2009). The mycelial pellets were once more separated from the broth via centrifugation at 7,277g for 20 min in a sterilized 1 L polypropylene centrifuge bottle. The final mycellial mat collected was mixed with an equal volume of the same solution mixture.

S. cerevisiae culture inoculum for the fermentation stage was prepared by growing the stock culture in sterile 50 ml YM broth, in 250-ml Erlenmeyer flasks at 32°C (120 rpm). After harvesting the yeast cells in 50 ml conical centrifuge tubes (BD Falcon, BD, Franklin Lakes, NJ) at 2,852 g for 10 min (Beckman J2-21centrifuge, Beckman Coulter Inc., Brea, CA), the cell concentration was adjusted with sterile YM broth to 10^7-10^8 CFU/ml as determined turbidometrically at 600 nm (Nguyen et al. 2009).

Filter Paper Compositional Analysis

The compositional analysis of the filter paper used in this study was performed in triplicate via complete enzymatic analysis as described by Selig et al. (2008), with minor modifications. Filter paper strips (0.1 g) were soaked in 50.0 mL 0.1 M citrate buffer (pH 4.8) and 1.38 ml (60 FPU/mL) of Spezyme CP (Genencor, Rochester, NY) in a 250 mL Flask. Distilled water and 1.0 ml of a 2% sodium azide solution, as microbial inhibitor, was added to bring the total volume in each flask to 100.0 mL. The flask was incubated in an incubator shaker at 50°C for 5 days for complete hydrolysis of the filter paper.

Solid State Fermentation for Enzyme Induction

Prior to the addition of fungal inoculum for enzyme induction, 2.0 g of shredded filter paper with 5 ml buffered basal salt solution was sterilized at $121^{\circ}C$ for 1 hour in loosely mouth covered polypropylene bottles. Then, 2 ml of fungal mycelia (1.5% w/v *P. chrysosporium,* 1.0% w/v *G. trabeum* and 0.8% w/v *T. reesei,* based on dry weight) in 100 mM phosphate buffer (pH 4.5-4.8), 0.5% $(NH_4)_2SO_4$ and basal salt solution was added. Solid state fermentation was then performed for 4 days at $37^{\circ}C$, in a humidified incubator, for the production of cellulases and hemicellulases.

Determination of total protein concentration and enzyme activities

Sample aliquots of 1.5 ml were taken from the medium washed fungal grown filter paper (Whatman No. 1, Whatman Inc., Clifton, NJ) at day 4 of solid substrate fermentation for each of the three fungal species treated filter paper. The supernatant was centrifuged at 1,118 g for 5 min (MiniSpin Plus, Eppendorf, Hauppauge, NY) and filtered through a 0.2 μm nylon syringe filter (VWR International, Batavia, IL), and was used to perform total protein analysis and enzyme activities assay.

Protein production by *P. chrysosporium* and *G. trabeum* grown on the filter paper was measured via the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). This system measures a loading of 2 ul sample size and calculates the protein concentration (mg/ml) from the protein's absorbance at 280 nm (A280). A separate

fermentation broth from the filter paper control bottle with no fungal culture was used as the blank reading.

The commercial cellulase enzyme (Spezyme CP) was kindly provided by Genencor International (Palo Alto, CA). The cellulase activity was measured using the filter paper activity (FPase) assay, expressed in filter paper units (FPU/ml) according to the standard procedure of the National Renewable Energy Laboratory (NREL) (Adney and Baker 2008). This procedure measures the release of reducing sugar produced in 60 min from a mixture of enzyme solution (0.5 mL) and of citrate buffer (0.05 M, pH 4.8, 1 mL) in the presence of 50 mg Whatman No. 1 filter paper (1 x 6 cm strip) and incubated at $50^{\circ}C$. The released sugars were analyzed by the dinitrosalicylic (DNS) acid reducing sugar assay. One unit of enzyme activity was defined as the amount of enzyme releasing 2.0 mg reducing sugar from 50 mg of filter paper in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). All samples were analyzed in triplicate and mean values were calculated.

Simultaneous Saccharification and Fermentation (SSF)

SSF reactions were carried out in 250 ml polypropylene bottles with batch cultures of 100 ml final volume, consisting of 25 ml 4X Yeast Extract Broth (1.8 g yeast extract (Difco), 0.07 g $CaCl_2 \cdot 2H_2O$ (Thermo Fisher Scientific), 0.45 g of KH_2PO_4 (Thermo Fisher Scientific), 1.2 g $(NH_4)_2SO_4$ (Thermo Fisher Scientific) and 0.3 g $MgSO_4 \cdot 7H_2O$ (Thermo Fisher Scientific) per liter of deionized water) (Shrestha et al. 2009) buffered basal medium (pH 4.5-4.8) (50 mM

Phosphate Buffer + Basal Salt Solution) (Shrestha et al. 2009). For the sample set that was treated with the commercial cellulase enzyme, 25 FPU of Spezyme CP/g cellulose was added. The flasks were then aseptically inoculated with *S. cerevisiae* suspension. Batch culture SSF was performed under static condition for 5 days at 37°C. All experiments were performed in triplicates.

Total Sugars Assays

Sample aliquots of 1.8 ml were collected aseptically from each bottle every 24 hours. The sample mixtures were centrifuged and filtered through a 0.2 μm nylon syringe filter. The filtered supernatants were tested for total sugars via the phenol-sulfuric (Crawford and Pometto 1988) method. The total sugar determination was determined via the phenol sulfuric carbohydrate test at 490 nm (SpectraMax Plus384, Molecular Devices, Inc., Sunnyvale, CA, U.S.A) with glucose standards. The equivalent sugar concentration (g/l) was determined based on a standard glucose solution curve that was generated prior to the assays.

High Pressure Liquid Chromatography (HPLC) Analyses

Filtered sample aliquots were tested for cellulose, glucose and fermentation products (ethanol, acetic acid, lactic acid) were analyzed by using a Waters High Pressure Liquid Chromatography (Millipore Corp., Milford, MA) equipped with a Waters Model 401 refractive index (RI) detector, column heater, autosampler and computer controller. The separation and analysis of ethanol and other fermentation constituents was done on a Bio-Rad Aminex HPX-87H column (300.0 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N H_2SO_4 as a mobile phase with a flow rate of 0.6 ml/min, a 20 µl injection volume and a column temperature of 65.0°C (Ramos 2003, Liu et al. 2008, Shrestha et al. 2009). Percentages of theoretical maximum ethanol yields (TEY) were calculated based on a theoretical ethanol yield of 56.8 g per 100 g of cellulose (Doran and Ingram 1993).

Statistical Analyses

The experimental data were analyzed statistically using the statistical software, JMP 8.0 (SAS Institute Inc., Cary, NC). The data (n=3) on ethanol production were fitted to non-linear polynomial (2nd degree) models. Error bars were determined based on the standard deviation from the mean values. Student's t-test for significant differences were also performed for all final data set to determine multiple comparisons of the ethanol production based on the different fungal treatments. A p-value of less than 0.05 was considered significantly different.

RESULTS AND DISCUSSION

Cellulose degrading microorganisms hydrolyze cellulose using complicated consortia of different enzymes that work individually, but synergistically on the cellulose, converting it to cellobiose and glucose (Henrikkson et al. 1999). This group of enzymes is produced by a wide variety of bacteria and fungi, aerobes and anaerobes, mesophiles and thermophiles (Bhat and Bhat 1997). However, only few of these microorganisms produce a complete cellulase complex and significant levels of extracellular cellulase capable of efficient depolymerization and solubilizing lignocellulosic biomass (Ahamed and Vermette 2008).

These cellulolytic enzymes are inducible enzyme systems (Iyayi et al. 1989; Suto and Tomita 2001; Ling et al. 2009). The induction process hypothesizes that basal levels of cellulase that is constitutively produced by fungi first hydrolyses cellulose to soluble oligosaccharides or their derivative sugars that is then absorbed into the cells, ultimately becoming the actual inducers (Lynd et al. 2002; Ling et al. 2009). In the case of *Trichoderma*, the conidial bound cellobiohydrolase hydrolyses the cellulose chains, liberating cellobiose and cellobiono-1,5-lactone (CBL) that are then taken up by the mycelia and promote further cellulase expressions (Szakmary et al. 1991; Bhat and Bhat 1997; Suto and Tomita 2001).

We chose filter paper as the cellulosic starting material because of its high cellulose and low impurities content (www.whatman.com). From the results of the total enzymatic analysis done on the filter paper, the content of the filter paper was approximately 98.0% cellulose. Because of its high cellulose purity, filter paper contains no lignin or other inhibitory

compound that may inhibit the fermentation of the glucose released into ethanol, or interfere with other analyses. It was also used in previous fungal enzyme induction studies (van Wyk 1999), largely due to its crystallinity index (CrI) of 0.45, that is within the range of susceptible cellulosic substrates of 0.4-0.7 like other pretreated biomass, and its degree of polymerization (DP) of 750-2800 that is also very close to conventional pretreated cellulosic substrates of 400-1000 (Zhang et al. 2006). In fact, it is the material that is recommended by NREL for standardized method of cellulase activities measurement (Decker et al. 2003; Adney and Baker 2008).

The general outline of our study is shown in Figure 1. In our study, the induction of enzyme production from the three fungal species was performed at pH 4.5-4.8, a condition suitable for both the growth of the fungi but also cellulolytic enzyme reactions (Bhat and Bhat 1997; Xia and Shen 2004; Shrestha et al. 2009; Rasmussen et al. 2010).

While many studies have been done on *P. chrysosporium, G. trabeum* and *T. reesei* to produce various cellulases, hemicellulases and lignolytic enzymes, and their direct cellulose hydrolysis activities, only few have reported their coupled applications in SSF (van Wyk 1999; Decker et al. 2003; Howard et al. 2003; Cohen et al. 2005; Shrestha et al. 2008; Shrestha et al. 2009; Rasmussen et al. 2010). Therefore, our study was extended to further examine the efficiencies of the respective fungal species and their enzymatic mechanisms on high cellulose feedstock, such as filter paper, in the presence of *S. cerevisiae*, as the fermenting organism. To achieve this, we performed SSF on the filter paper and measured the final fermentation products via HPLC. This technology combines continous enzymatic

hydrolysis of cellulose with the simultaneous fermentation of the sugars released to ethanol via a chosen fermenting microorganisms (i.e. the yeast *S. cerevisiae*), in a single reactor (Ballesteros et al. 2004).
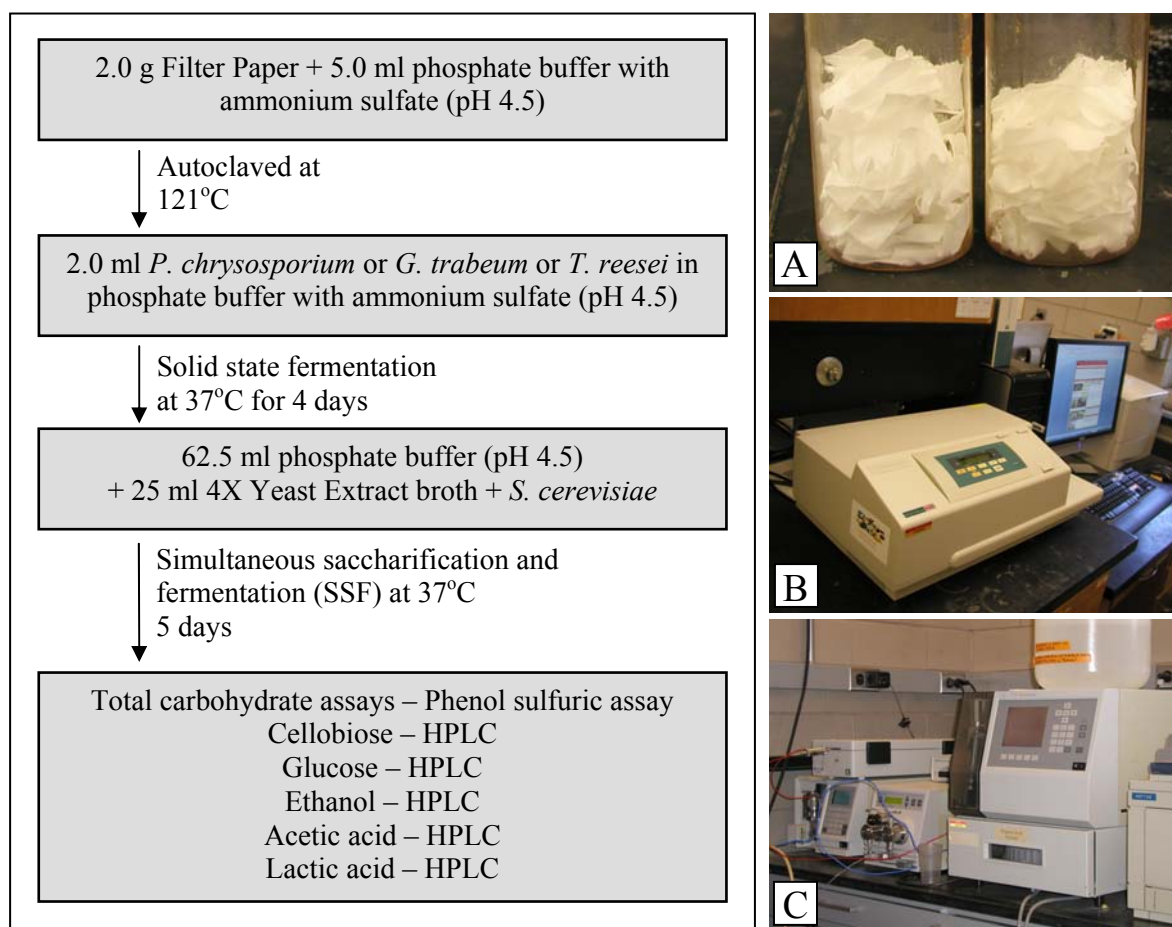
Figure 1. Flow-chart of process outlining the steps for solid state fermentation of *P. chrysosporium* or *G. trabeum* or *T. reesei* on filter paper, followed by SSF using *S. cerevisiae* as the fermenting organisms. (A) Whatman No.1 filter paper strips before treatment. (B) The SpectraMax Plus384 system used for the phenol-sulfuric total sugar assay. (C) The Waters HPLC system used for sample analysis.

During SSF, the presence of the fermenting organism reduces the accumulation of glucose within the vessel, thereby increasing saccharification rate and ethanol production (Figure 2).

We also prepared a separate sample set that was added with the commercial cellulase enzyme, Spezyme CP, at a low dose of 25 FPU/g cellulose, as a comparison of enzymatic activities. The combination of Spezyme CP and *S. cerevisiae* yielded 47.91 g/100 g filter paper of ethanol (86.06% theoretical).
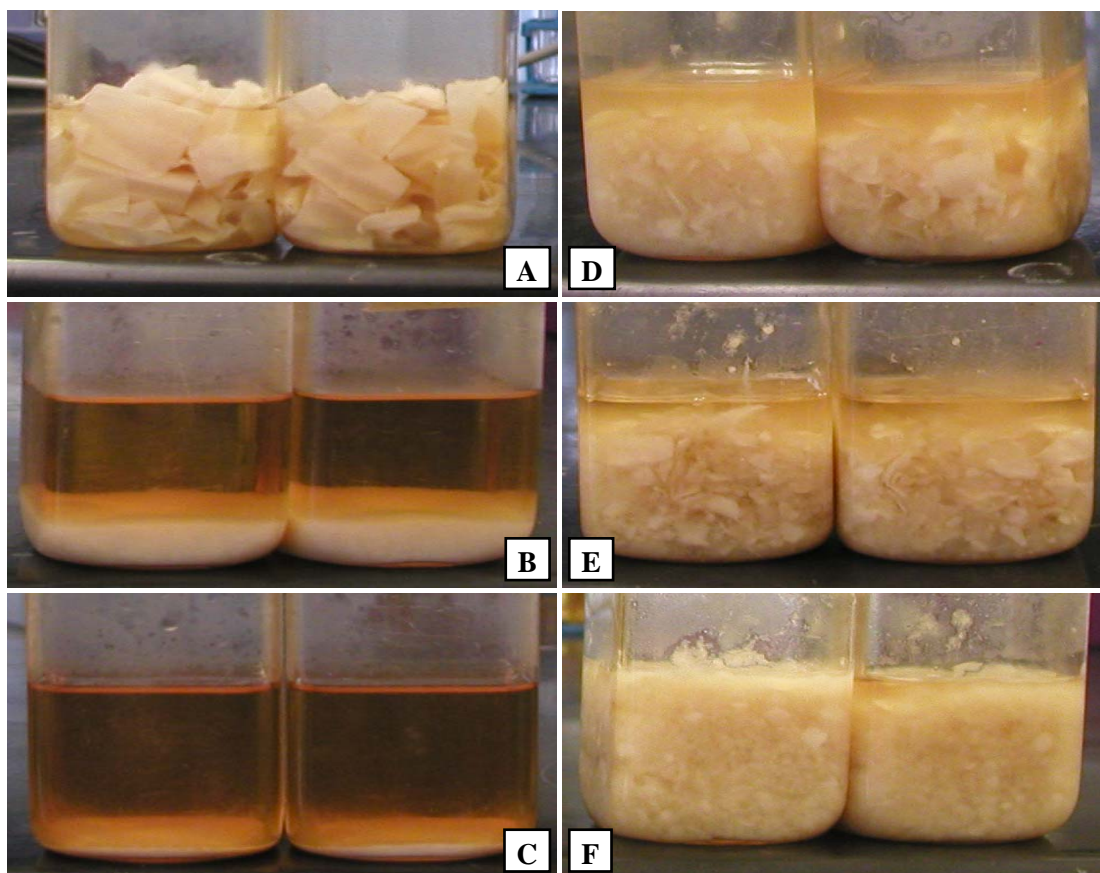
Figure 2. Simultaneous saccharification and fermentation (SSF) batches of Whatman No. 1 Filter paper at day 3. (A) *S. cerevisiae* only (B) 25 FPU/ g cellulose Spezyme only (C) 25 FPU/ g cellulose Spezyme CP + *S. cerevisiae* (D) *P. chrysosporium + S. cerevisiae* (E) *T. reesei + S. cerevisiae* (F) *G. trabeum + S. cerevisiae.*

From Table 1, the results of the total protein assay using the NanoDrop[TM] 1000 spectrophotometer showed that the highest protein concentration of 10.67 mg/ml was produced in the sample treated with *T. reesei*, followed by 10.52 mg/ml in the sample treated

with *P. chrysosporium* while in the sample treated with *G. trabeum*, the concentration was at

10.04 mg/ml. We then determined the enzyme activities based on the filter paper units

(FPU), as described previously (Ghose 1987; Adney and Baker 2008). This assay do not

report the enzyme activities in the conventional I.U. units, as Ghose (1987) pointed out that

"*because the FPU assay is non-linear, the use of the International Unit per se is incorrect as*

this unit is based on initial velocities, i.e., linear reactions in which the product is produced

at the same rate during each and every minute of the reaction". Ghose (1987) concluded that

the FPU values for a given cellulase solution be given simply as "FP units/ml".

The result from FPase assays (Table 1) from the induction experiments indicated that

cellulase activities were highest in the sample treated with *T. reesei*, at 1.76 FPU/ml. The

sample treated with *G. trabeum* had a lower protein activities value of 1.52 FPU/ml and the

sample treated with *P. chrysosporium* had the lowest FPase activities of 0.76 FPU/ml.

Table 1. Enzyme activity and total protein assays (n=3).

	Protein Assay (mg/ml)[a]	Enzyme Assay (FPU/ml)[b]
P. chrsysosporium	10.52	0.76
T. reesei	10.67	1.76
G. trabeum	10.04	1.52

[a] Protein was determined by NanoDrop™ 1000 Spectrophotometer.
[b] Filter paper unit activities (FPase) based on the value of 2.0 mg of reducing sugar as
   glucose from 50 mg of filter paper, at 4% conversion, in 1 hour (units FPU/ml)

This trend is expected as *T. reesei* have been known to produce high concentration of potent

cellulases (Jovanovic et al. 2009), and in fact, this fungus serves as a reference organism for

cellulose degradation studies and for the mass production of cellulases and hemicellulases for

various applications (Martinez et al. 2008). During the five-day SSF period, total sugar production was recorded. From Figure 3, residual total sugar remained at a very steady level for all the samples treated with the three different fungi. The concentration ranged from 2.60 - 2.82 g of total sugar per 100 g of filter paper at day 0 and by day 5, the concentration ranged from 3.58 - 2.55 g. The total sugar profile for the Spezyme control showed a sharp increase in day 1, followed by a sharp dive in day 2.
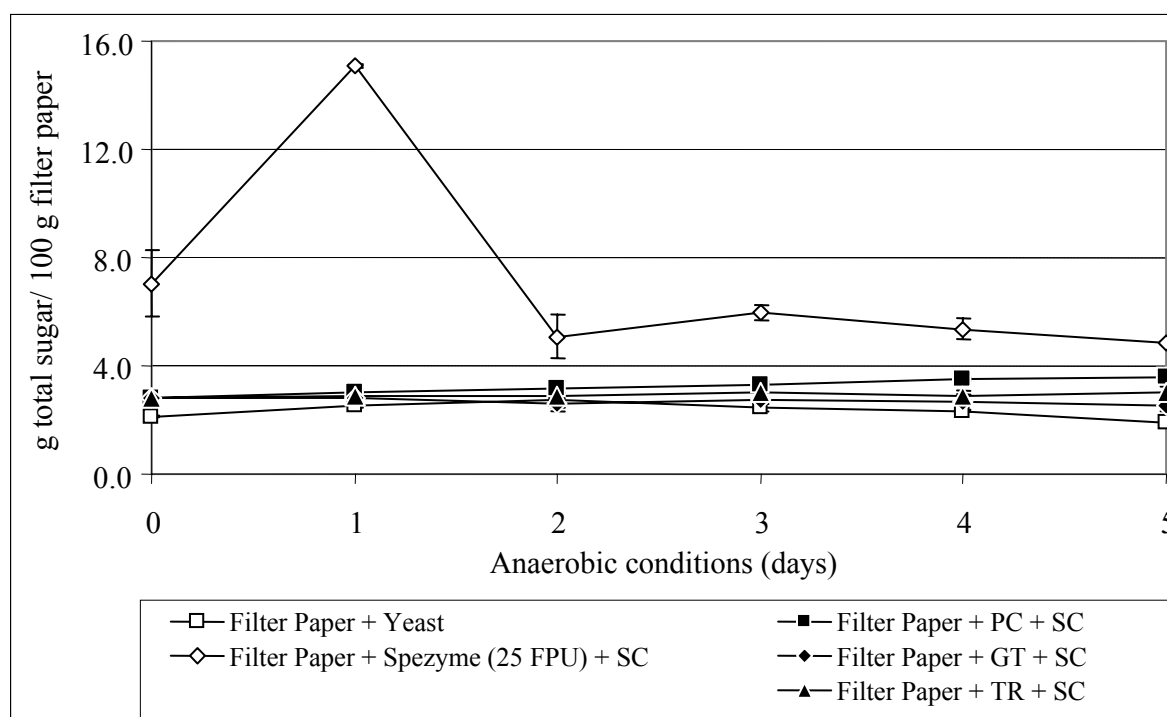
Figure 3. Time course of total sugar production, as determined via the phenol-sulfuric method. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisea*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium, G. trabeum* or *T. reesei*).

Close examination via HPLC (Figure 4) showed that much of this is from the release of cellobiose at day 1, that was then hydrolyzed at day 2. Another observation from the HPLC

readings indicated that cellobiose was detected all throughout the five day SSF period in the sample treated with *P. chrysosporium,* indicating the possible partial hydrolysis of the cellulose.
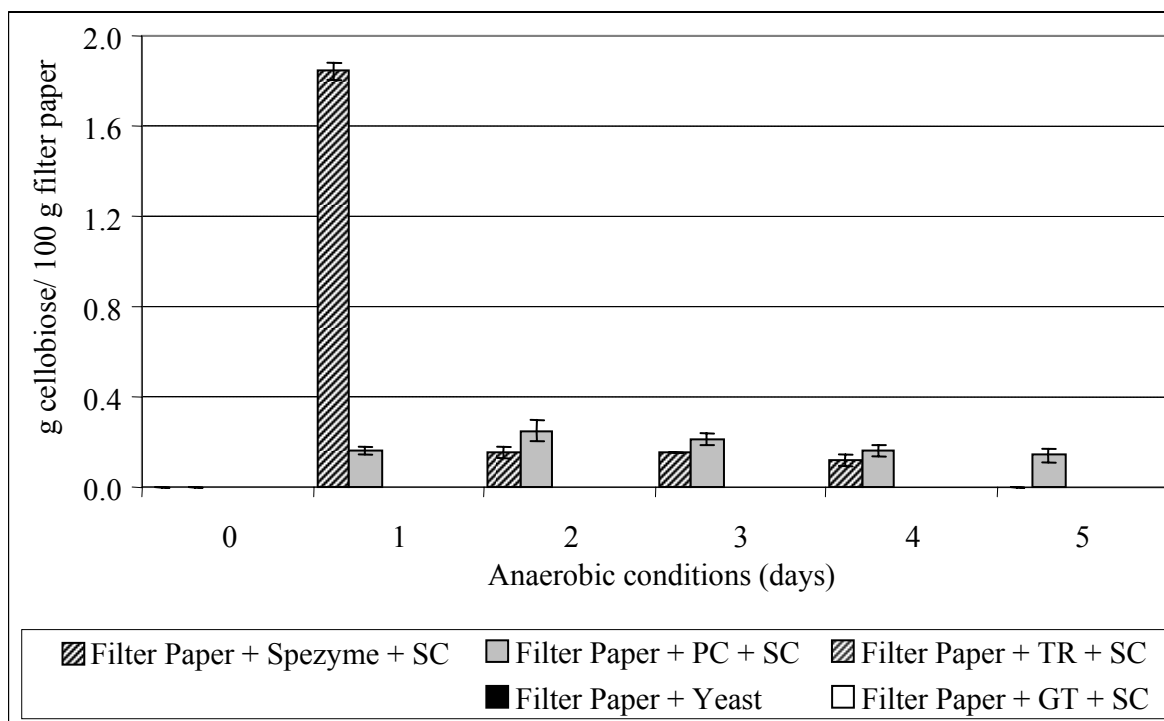
Figure 4. Time course of cellobiose production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisea.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium, G. trabeum* or *T. reesei*).

From Figure 5, ethanol production was highest for the filter paper inoculated with *T. reesei*. Ethanol production was in steady increments even during the final day of experiment at day 5, with the concentration values of 5.13 g/ 100 g filter paper, corresponding to 9.33% TEY (Table 2). A longer SSF period may provide the necessary information on the day further ethanol production will stop. The filter paper inoculated with *P. chrysosporium* was at 1.79

g/ 100 g filter paper (3.25 TEY). In comparing the results of the ethanol production at day 5, the FPU values between *T. reesei* and *P. chrysosporium* treated filter paper reflects the final ethanol concentration. Higher enzymatic activities in *T. reesei* resulted in more ethanol production, and in fact the difference of approximately 286%. Another explanation to the lower ethanol yield in the sample treated with *P. chrysosporium* is the possibility incomplete hydrolysis of the cellulose to glucose, as seen in Figure 4. Statistic analyses validated the significance of these results (Table 3, Table 4 and Figure 6).
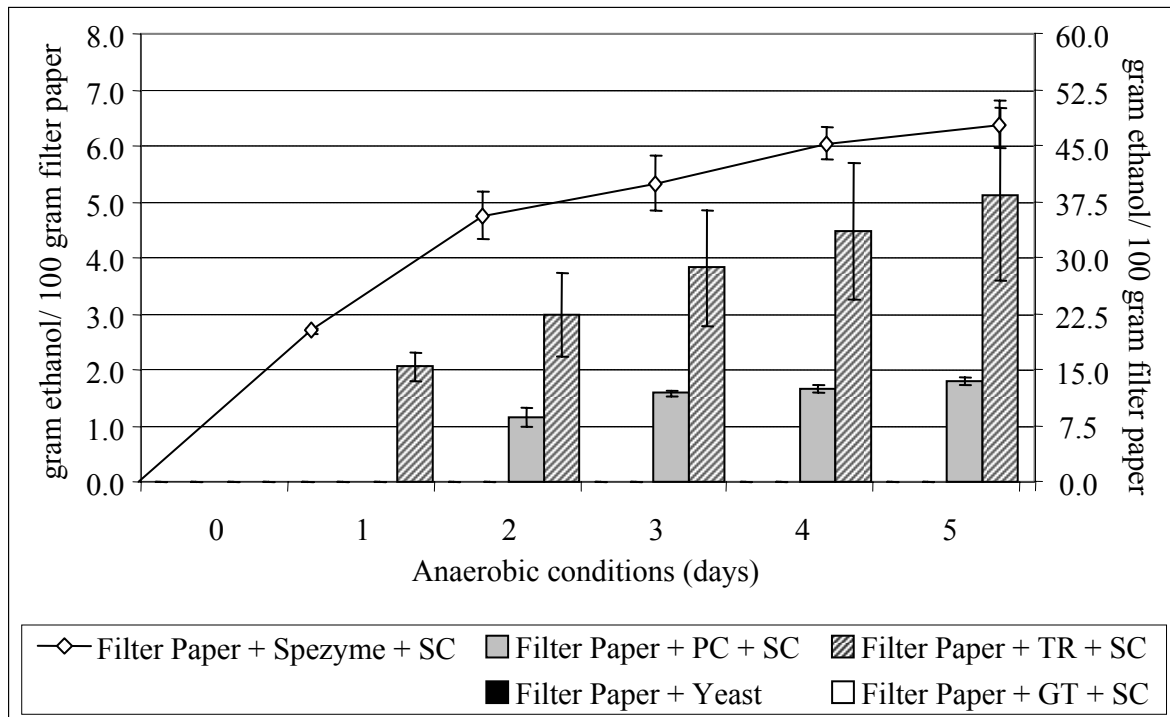
Figure 5. Time course of ethanol production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisea*. Left y-axis represents the bar charts, Right y-axis represents the line regression. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium, G. trabeum* or *T. reesei*).

Table 2. Cellulose conversion and theoretical ethanol yield at day 5 (n=3).

	Cellulose Conversion (g / 100 g filter paper)	Theoretical ethanol yield (%)
P. chrysosporium	1.79	3.25
T. reesei	5.13	9.33
G. trabeum	n.d.	n.d.
Spezyme (25 FPU/g cellulose)	47.91	87.11

Table 3. Statistical analysis of the significant differences in ethanol production (g ethanol /100 g filter paper) between *P. chrysosporium, T. reesei* and *G. trabeum* treated filter paper as determined via the Student's t test.

	p - value
P. chrysosporium + *S. cerevisiae* vs. *T. reesei* + *S. cerevisiae*	< 0.0007
P. chrysosporium + *S. cerevisiae* vs. *G. trabeum* + *S. cerevisiae*	< 0.0219
T. reesei + *S. cerevisiae* vs. *G. trabeum* + *S. cerevisiae*	< 0.0001

Table 4. Summary of non-linear (polynomial, 2nd degree) model fits of ethanol production.

	R^2	Prob > F
P. chrysosporium + *S. cerevisiae*	0.990	0.0010
G. trabeum + *S. cerevisiae*	0.909	0.0275

Unlike the previous two fungi, the data (Table 2, Figure 5 and Figure 6) showed that none of the samples inoculated with *G. trabeum* produced cellobiose, glucose, and other fermentation products (ethanol, acetic acid and lactic acid), suggesting that *G. trabeum* may not be an effective fungus for the use in the hydrolysis of pure cellulose, albeit to its highly documented potent cellulolytic enzyme systems on other substrates (Cohen et al. 2005; Daniel et al. 2007). There are several possible explanations to these observations.
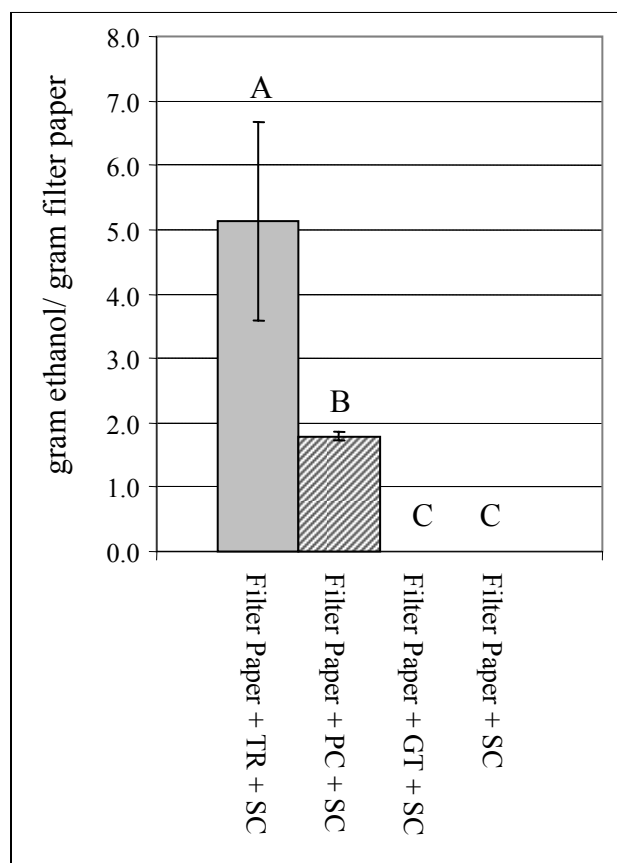
Figure 6. Maximum ethanol yields of different fungal treatments conditions. Letters on top of the columns indicate significant differences (Student's t test, α=0.05)

Firstly, *G. trabeum* is reported to lack the complete combination of the enzymes needed for efficient cellulose hydrolysis on pure cellulose (Mansfields et al. 1998; Cohen et al. 2002). Unlike the cellulases of *T. reesei*, *G. trabeum* lacks cellobiohydrolases, although endoglucanases were detected (Henriksson et al. 1999). This is an important finding as, in many cases, CBHs are also more efficient on cellulose than EGs (Henriksson et al. 1999). However, brown-rots compensate the lack of processive cellulases by degrading biomass largely through non-enzymatic mechanisms, via a hydroquinone-driven system for the production of extracellular reactive oxygen species (ROS) in an 'enhanced' Fenton system (Paszczynski et al. 1999; Cohen et al. 2002).   The Fenton system plays an extremely

important role in the early stages of cellulose degradation by brown-rot fungi. However, this reaction only occurs under favorable conditions, catalyzed by a low-molecular-weight peptide, termed Gt factor (Wang and Gao 2003). According to Xu and Goodell (2001), these conditions must include the presence of iron, hydrogen peroxide, biochelators, oxalate and light. Iron is present in woody biomass as bound iron and ferric hydroxide complexes. However, in our experiment, the absence on iron on the highly cellulose-pure filter paper may have adverse effect on the natural iron dependent hydrolytic processes. Lighting condition was also not conducive as during the aerobic enzyme induction period, it was performed in the darkness of an incubator that may have negatively impacted the Fenton reactions. Further experimentation in the future may have to take these conditions into consideration in order to fully evaluate the effectiveness of the cellulolytic system of *G. trabeum* in performing SSF on filter paper.

Secondly, another study done by Cohen and colleagues (2005) added that the cellulolytic system of *G. trabeum* may hydrolyze amorphous cellulose but not crystalline cellulose. However, in the degradation of amorphous cellulose, hydrolysis is partial with the end product being cellotriose instead of glucose, a phenomenon also reported in other microorganims (Hash and King 1958; Reese et al. 1959; Lejuene et al. 1988). This same observation is supported by our result with the negative glucose reading in all samples inoculated with *G. trabeum*. This is further supported by another related work done by Schilling and colleagues (2009) that observed the difficulties of brown-rot fungi in metabolizing lignin-free microcrystalline cellulose. The same study further hypothesized that

brown-rot fungi make initial tissue modifications that facilitate in hydrolytic efficiencies that is specific for the specific cellulases they secrete.

The production of other fermentation co-products, such as, acetic acid and lactic acid were also recorded. No lactic acid was produced by any of the samples at the end of the five-day experiments. Acetic acid was only detected in the samples inoculated with *T. reesei* at 0.95 g/l00 g filter paper (Figure 7). This trend is supported by other studies that documented high production of acetic acid by *T. reesei* (Chambergo et al. 2002; Shrestha et al. 2009). This is due to the enzymatic actions of the two paralogous genes for aldehyde dehydrogenase (*ALD*1 and *ALD*2) capable of converting acetaldehyde to acetate, present in the *T. reesei* genome (Chambergo et al. 2002; Shrestha et al. 2009). Furthermore, other genes such as acetyl esterases are also reported to function in the same manner and interestingly, these genes interact with other cellulases for the production of acetates from other biomass (Poutanen and Sundberg 1998; Harrison et al. 2002). Acetic acid (2.57 g/100 g filter paper) was also detected in the sample treated with Spezyme CP (Figure 7).
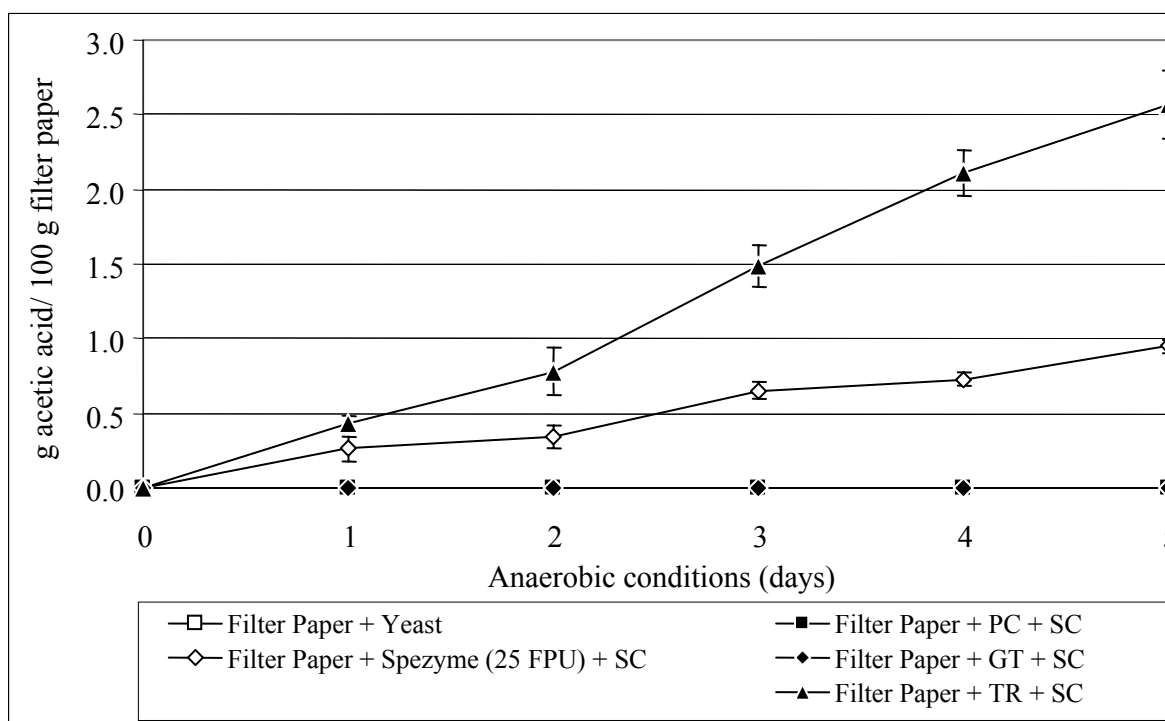
Figure 7. Time course of acetic acid production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisea*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium, G. trabeum* or *T. reesei*).

By comparing these three fungal species, our study suggests that the most efficient fungal species in saccharifying pure cellulose was *T. reesei,* followed by *P. chrysosporium*, while *G. trabeum* failed to effectively liberate fermentable product. Of the three fungal species evaluated in this study, *P. chrysosporium* is worth noted as not only it is the cellulolytic enzymes system efficient, but it offers greater flexibilities when lignocellulosic biomass is the feedstock for ethanol production. This is because *P. chrysosporium* also harbors lignolytic enzyme that may be advantageous in eliminating a major inhibitor in conventional SSF, which is lignin (Ballesteros et al. 2004).

In conclusion, the results from our study of the solid state fermentation of cellulose-rich filter paper for the production of ethanol indicated that the fungal species *P. chrysosporium* and *T. reesei* are potentially useful for this form of application. Further experimentation may be done by inoculating these two species onto more complex feedstocks that are lignin rich, such as switchgrass, corn stover and other perennial grasses, to evaluate their enzymatic efficiencies against more recalcitrant feedstocks and the presence of potential inhibitors (Sokhansanj et al. 2002; Varga et al. 2004; Wyman et al. 2005). Direct fungal enzymatic saccharification mechanisms for SSF are indeed very promising and can lead to a more environmentally friendlier processing, whereby ethanol producers can skip or minimize the environmentally detrimental pretreatment steps. This will ultimately lead to a more economically sound ethanol production when manufacturers can produce their own enzymes *in situ* to supplement the use of expensive commercial preparations.

Acknowledgment

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CHAPTER 4: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF GROUND CORN STOVER FOR THE PRODUCTION OF FUEL ETHANOL USING *PHANEROCHAETE CHRYSOSPORIUM, GLOEOPHYLLUM TRABEUM, SACCHAROMYCES CEREVISIAE* AND *ESCHERICHIA COLI* K011

*(to be submitted to the **Journal of Microbiology and Biotechnology**)*

ABSTRACT

Enzymatic saccharification of corn stover using the white rot, *Phanerochaete chrysosporium*, and the brown rot, *Gloeophyllum trabeum* and subsequent fermentation of the saccharification products to ethanol was achieved via the use of *Saccharomyces cerevisiae* and *Escherichia coli* K011. Prior to the simultaneous saccharification and fermentation (SSF) for ethanol production, *P. chrysosporium* or *G. trabeum* solid-state-fermentation for four days with ground corn stover was performed for 4 days to induce *in situ* cellulase production. During the SSF period with *S. cerevisiae* or *E. coli*, ethanol production was highest on day 4 for all samples inoculated with either *P. chrysosporium* or *G. trabeum*. For the corn stover treated with *P. chrysosporium*, the conversion of corn stover to ethanol was 2.29 g/100 g corn stover for the sample inoculated with *S. cerevisiae,* whereas for the sample inoculated with *E. coli* K011, the ethanol concentration was 4.14 g/100 g corn stover. While for the corn stover treated with *G. trabeum*, the conversion of corn stover to ethanol was 1.90 g/100 g and 4.79 g/100 g corn stover for the sample inoculated with *S. cerevisiae* and *E. coli* K011,

respectively. Other fermentation co-products, such as, acetic acid and lactic acid were also reported. Acetic acid production ranged between 0.45 to 0.78 g/100 g corn stover for the samples under different fungal treatments, while no lactic acid production was detected throughout the 5 days of SSF. The results of our experiment suggest that it is possible to perform SSF of corn stover by using *P. chrysosporium*, *G. trabeum, S. cerevisiae* and *E. coli* K011 for the production of fuel ethanol without pretreatments and without the use of commercial enzymes.

INTRODUCTION

The present use of ethanol for transportation purposes is conventionally produced in large quantities from corn grain and sugarcane juice. However, this is only a temporary solution as this practice conflicts with the food and feed industry (Chundawat et al. 2007). Thus, there is great interests in the development of fuel ethanol from agricultural residues and other lignocellulosic feedstocks, which are inexpensive feedstocks, and are the most abundant bio-resources available in the biosphere (de La Torre Ugarte et al. 2003). Currently, corn stover is considered one of the first candidates for lignocellulosic biomass use for cellulosic bioethanol production, because this it is an abundant agricultural by-product in many

European countries and in the USA, and it can be collected during harvest (Sokhansanj et al. 2002; Varga et al. 2004). Although promising, using corn stover as raw material to produce ethanol faces many challenges, as unlike starch from corn, the polysaccharides in corn stovers are cellulose and hemicellulose, which are difficult to degrade (Hendriks and Zeeman 2009; Keshwani and Cheng 2009; Nguyen et al. 2009). Thus hydrolyzing these components into fermentable sugars is essential to the efficient and economical production of cellulosic ethanol (Brekke 2005).

Biohydrolysis of cellulose and hemicellulose is an enzymatic process carried out by a family of cellulolytic and hemicellulolytic enzymes that are highly specific (Keshwani and Cheng 2009). These enzyme consortia are usually a mixture of several enzymes, that may include endoglucanases, exoglucanases or cellobiohydrolases, glucosidases or cellobiases, endoxylanases, xylosidases and galactosidases, among others (Abbas et al. 2005; Musatto et al. 2008; Suzuki et al. 2008; Wymelenberg et al. 2005). The conventional method for the breakdown of lignocellulosics to fermentable sugars requires the use of expensive commercial enzymes (Donohoe et al. 2009; Kumar et al. 2009; Weiss et al. 2010). However, these enzymes are not only substrate specific, and they are largely susceptible to inhibition from compounds usually associated with lignin. Thus, prior to enzymatic hydrolysis, pretreatment of the ground lignocelluloses is required (Keating et al. 2006).

Pretreatments of plant biomass are crucial for the production of cellulosic ethanol as they greatly improve the enzymatic accessibility of the feedstocks (Saqib et al. 2006; Duguid et al. 2009; He et al. 2010; Kim et al. 2009; Seliq et al. 2009). In recent years, several

pretreatments have been tested on corn stover, either via physical, chemical, physicochemical or the combinations of these procedures (Sorensen et al. 2008; Yang et al. 2008; Garcia-Cubero et al. 2009). However, these current pretreatment technologies are energy intensive, environmentally unfriendly and may produce many toxic by-products such as weak acids, phenolic derivatives and furans which inhibit alcoholic fermentation (Cantarella et al. 2001; Keating et al. 2006; Chundawat et al. 2007). Therefore, it is imperative to develop alternative means of lignocellulosics saccharification that can overcome these obstacles.

One potential form of pretreatment and hydrolysis of lignocellulosic materials is by using biological means (Sun and Cheng 2002; Galbe and Zacchi 2007). This type of procedure usually involves lignocellulolytic fungal species such as *Phanerochaete chrysosporium* and *Gloeophyllum trabeum* (Sanchez 2009; Shrestha et al. 2008; Shrestha et al. 2009; Rasmussen et al. 2010).

P. chrysosporium is a white-rot fungus that has been studied extensively in the degradation of plant cell walls components which includes cellulose, hemicellulose and lignin (Kersten and Cullen 2007; Wymelenberg et al. 2005). *P. chrysosporium* performs efficient lignocellulolytic processes using the various ligninolytic peroxidases or laccases, cellulases and hemicellulases it is known to secrete (Martinez et al. 2004; Wymelenberg et al. 2005; Suzuki et al. 2008).

G. trabeum is a brown-rot basidiomycete. Like a typical brown rot, *G. trabeum* primarily attack the polysaccharide while leaving the brown pigmented lignin behind (Cohen et al.

2005). These degradative processes culminate in the rapid loss of wood strength and the darkening of the affected substrate (Daniel et al. 2007). *G. trabeum* is known to secrete a potent cellulolytic enzyme family, consisting of endoglucanases, exoglucanases, β-glucosidases and other hemicellulases (Cohen et al. 2005; Kerem et al. 1999). In contrast to white rots, *G. trabeum* rapidly degrades cellulose and hemicellulose, while leaving the undigested lignin modified mainly through demethoxylation and demethylation mechanisms (Arantes et al. 2006).

In this paper, we report the use of *in situ* cellulases and hemicellulases from *P. chrysosporium* and *G. trabeum* for the saccharification of corn stover cellulose that is subsequently fermented to ethanol by using *Saccharomyces cerevisiae* and *Escherichia coli* K011. We performed our work at conditions and with equipment that would generate commercially relevant results.

MATERIALS AND METHODS

Corn stover analysis

Corn stover was obtained from the Department of Agronomy, Iowa State University. The field dried corn leaf and corn stalk were ground in a Wiley mill to pass through a 2 mm screen and then screened using a 20 mesh sieve and further oven dried at $80^{o}C$ for four days prior to compositional analysis. The composition of cellulose and hemicellulose was determined by Iowa State University, Department of Agronomy, using the ANKOM method (ANKOM Technol. Corp., Fairport, NY) as described previously (Vogel et al. 1999). The Klason lignin content was determined by using a modified Klason lignin assay, which measures lignin as the acid-insoluble fraction of lignocellulosic material after hydrolysis by strong acid (H_2SO_4) and heat. Klason lignin analysis was performed following the method by Crawford and Pometto (1988) with a slight modification, where by glass fiber filters (1.6 μm) (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were used for capturing the lignin residues. The residue on the filter paper was thoroughly rinsed with deionized water and dried in the oven at $105^{o}C$ for four days. The Klason lignin was determined as the weight of dry residue collected on the filter paper.

Microorganisms

Phanerochaete chrysosporium (ATCC 24725)*, Gloeophyllum trabeum* (ATCC 11539), *Saccharomyces cerevisiae* (ATCC 24859) and *Escherichia coli* K011 (ATCC 55124) were

used in this study. All cultures were obtained from American Type Culture Collection (Rockville, MD). The fungal cultures were revived by inoculating onto potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD), bacterial culture on LB broth (Becton Dickinson and Co. , Sparks, MD), and was incubated with shaking at 24°C (Shrestha et al. 2008; Shrestha et al. 2009). Stock cultures were stored in stored in Yeast Malt extract (YM) broth (Becton Dickinson and Co.) supplemented with 20% (v/v) glycerol at -80°C in an ultralow-temperature freezer (So-Low Environmental Equipment Co., Inc., Cincinnati, OH) for long term storage.

P. chrysosporium and *G. trabeum* culture preparation

P. chrysosporium and *G. trabeum* seed culture was prepared from spores in 1 liter of YM Broth (Difco, Becton Dickinson and Co.) and incubated at 30°C, agitated at 150 rpm. After 7 days of growth, the fungal mycelia (approximately 2-3 mm in diameter) was harvested via centrifugation in a sterilized 1-L polypropylene centrifuge bottle (Nalgene, Nalge Nunc, Rochester, NY), at 7,277 g for 20 min using a Sorvall-RC3B Plus centrifuge (Thermo Fisher Scientific, Wilmington, DE) (Rasmussen et al. 2010). The fungal cell pellets were rinsed with fungal mineral salt solution (pH 4.5-4.8) (50 mM Phosphate Buffer + 0.5% $(NH_4)_2SO_4$ + Basal Medium). Basal medium was prepared according to the formulation of Shrestha *et al.* (2009), consisting of 0.25 g of KH_2PO_4 (Fisher Scientific, Pittsburgh, PA), 0.063 g of $MgSO_4.7H_2O$ (Fisher Scientific), 0.013 g of $CaCl_2.2H_2O$ (Fisher Scientific), and 1.25 mL of trace element solutions in 1 L of deionized water (Shrestha et al. 2008).

Solid State Fermentation for Enzyme Induction

All ground corn stover used in this study received no pretreatment except what weathering occurred in the field prior to harvest. Prior to the addition of fungal inoculum for enzyme induction, 2 g of ground corn stover and glass marbles with 5 ml fungal mineral salt solution was sterilized in 250-ml polypropylene bottles (Nalgene) at 121°C for 1 hr followed by rapid exhaust. Then, 2 ml of fungal (1.5% w/v *P. chrysosporium* and 1.0% w/v *G. trabeum*) biomass in mineral salt solution was added. The bottles were rolled on their sides and the marbles assisted to uniformly disperse and coat the corn stover and fungi mixture along the inner surface (Shrestha et al. 2009; Rasmussen et al. 2010). Solid state fermentation was then performed for 4 days at 37°C in a humidified incubator for *in situ* production of cellulases and hemicellulases prior to the addition of ethanolic microorganism.

Protein Assay

Total protein was analyzed by using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The NanoDrop 1000 module measures the protein's absorbance at 280 nm (A280) and calculates the concentration (mg/ml) from a loading of 2 ul sample size. Sample aliquots of 1.5 ml were taken from the minimal salt media washed fungal grown corn stover at day 4. The supernatant was centrifuged using a MiniSpin Plus centrifuge (Eppendorf, Hauppauge, NY) at 1,118 *g* for 5 min and filtered through a 0.2 μm nylon syringe filter (VWR International, Batavia, IL). Parts of the filtered solution were also used to perform the enzyme activities assay.

Enzyme Activities Assay

Specific enzyme activity assay was performed using the protocol described by the official National Renewable Energy Laboratory (NREL) procedure [2]. This method is based on the International Union of Pure and Applied Chemistry (IUPAC) guidelines to determine cellulase activity in terms of "filter-paper units" (FPU) per milliliter (FPU/ml) of original enzyme preparation (Ghose 1987).

***S. cerevisiae* and *E. coli* K011 culture preparation**

Culture inoculum of *S. cerevisiae* and *E. coli* K011 were prepared by growing cultures in sterile 50 ml YM Broth at 32°C with constant agitation (120 rpm). Cells were harvested via centrifugation in a 50 ml conical centrifuge tubes (BD Falcon, BD, Franklin Lakes, NJ) at 2852 *g* for 10 min using a Beckman J2-21centrifuge (Beckman Coulter Inc., Brea, CA). Prior to use in SSF, cell counts were set at 10^7-10^8 CFU/ml as determined turbidometrically at 600 nm via a standard curve (Nguyen et al. 2009).

Simultaneous Saccharification and Fermentation (SSF)

SSF reactions were carried out in 250-ml polypropylene bottles with batch cultures of 100 ml final volume, consisting of 25 ml 4X Yeast Extract Broth (1.8 g yeast extract (Difco), 0.07 g $CaCl_2 \cdot 2H_2O$ (Fisher Scientific), 0.45 g KH_2PO_4 (Fisher Scientific), 1.2 g $(NH_4)_2SO_4$ (Fisher Scientific), and 0.3 g $MgSO_4 \cdot 7H_2O$ (Fisher Scientific) per liter of water) (Shrestha et al.

2009) and basal medium (pH 4.5-4.8) (50 mM Phosphate Buffer + 0.5% $(NH_4)_2SO_4$ + Basal Medium). The flasks were then aseptically inoculated with *S. cerevisiae* and *E. coli* K011 suspension. Batch culture fermentations were incubated at 37^oC under static condition. These bottles were then subjected to SSF in anaerobic conditions for 5 days. The SSF experiments were performed in triplicates (n=3).

High Pressure Liquid Chromatography (HPLC) Analyses

Sample aliquots of 1.8 ml were taken daily, centrifuged at 1,118 g for 5 min and filtered through a 0.2 μm nylon syringe filter. Glucose, xylose and the fermentation products (ethanol, acetic acid, and lactic acid) were analyzed by using a Waters High Pressure Liquid Chromatography (Millipore Corp., Milford, MA) equipped with a Waters Model 401 refractive index (RI) detector, column heater, autosampler and computer controller. The separation and analysis of ethanol and other fermentation constituents was done on a Bio-Rad Aminex HPX-87H column (300.0 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N H_2SO_4 as a mobile phase with a flow rate of 0.6 ml/min, a 20 μl injection volume and a column temperature of 65°C (Ramos 2003, Liu et al. 2008; Shrestha et al. 2009).

Total and Reducing Sugars Assays

The filtered supernatants from the fermentation broth were tested for free reducing sugar and total reducing sugars, via the Somogyi-Nelson (Antai and Crawford 1981) and phenol-

sulfuric (Crawford and Pometto 1988) methods, respectively. The Somogyi-Nelson carbohydrate assay was performed at 500 nm with a glucose standard, whereas total sugar determination was determined via the phenol sulfuric carbohydrate test at 490 nm with a glucose standard. The absorbance readings of samples were achieved using a spectrophotometer (SpectraMax Plus384, Molecular Devices, Inc., Sunnyvale, CA, U.S.A). The absorbance readings were then converted into equivalent sugar concentration (g/L) based on a standard glucose solution curve. All sugar analyses were performed in triplicate (n=3).

Statistical Analyses

The SSF results were statistically analyzed using the statistical software, JMP 8.0 (SAS Institute Inc., Cary, NC). The data on ethanol production were fitted to exponential fit models, and a significant difference of p value of 0.05 was employed. Student's t-test analyses were also performed for all final data set to determine multiple comparisons of the ethanol production. A p-value of less than 0.05 was considered significantly different.

RESULTS AND DISCUSSION

Enzyme Induction on Untreated Corn Stover

In this study, we performed SSF on ground corn stovers that received no pretreatment. The main components of the corn stover used are hemicellulose, cellulose, lignin, and ash (Table 1). An interesting observation from the compositional analysis is the high content of ash in the corn stover. This is in agreement with another analysis done previously (Pordesimo et al. 2005; Su et al. 2006) that reported the ash contents of corn leaf and corn stalk to be considerably higher than that of other biomasses. A flow-chart of our experimental design is shown in Figure 1. Unlike other previous works, our SSF process do not use pretreated corn stover samples (Duguid et al. 2009; Kim et al. 2009; Selig et al. 2009; He et al. 2010) or the addition of expensive commercial enzymes (Donohoe et al. 2009; Kim et al. 2009; Weiss et al. 2010). Instead, cellulases and hemicellulases produced by *G. trabeum* and *P. chrysosporium in situ* upon corn stover enzyme induction were performed in a pH range of 4.5-4.8 at 37°C for 4 days via solid state fermentation (Figure 2), a condition suitable not only for the growth of the fungus but also the ideal pH for production of cellulolytic enzymes (Shrestha et al. 2008; Shrestha et al. 2009; Rasmussen et al. 2010). As seen in Table 2, our protein assay using the NanoDrop^{TM} 1000 spectrophotometer indicated that protein was produced during the induction stage, and production is higher in quantities in the corn stover and *P. chrysosporium* combination, compared the corn stover and *G. trabeum* combination, at 14.06 mg/ml and 11.61 mg/ml, respectively.

Table 1. Composition of corn stover (as percentage based on dry weight; n=3).

Main components	Composition based on cell mass (%, w/w)
Cellulose	38.08
Hemicellulose	30.72
Klasson lignin	20.70
Ash	8.77
Others	0.31

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┌─────────────────────────────────────────────┐
│   2.0 g Corn stover + 5.0 ml phosphate buffer with   │
│          ammonium sulfate (pH 4.5)            │
└─────────────────────────────────────────────┘
                    │  Autoclaved at 120°C
                    ▼  1 hour
┌─────────────────────────────────────────────┐
│  2.0 ml G. trabeum/P. chrysosporium in phosphate │
│       buffer with ammonium sulfate (pH 4.5)   │
└─────────────────────────────────────────────┘
                    │  Solid state fermentation at 37°C
                    ▼  4 days
┌─────────────────────────────────────────────┐
│   66.0 ml phosphate buffer (pH 4.5)           │
│         + 25 ml 4X Yeast Extract broth         │
└─────────────────────────────────────────────┘
        │                           │
        ▼                           ▼
┌──────────────────┐      ┌──────────────────┐
│  + S. cerevisiae  │      │  + E. coli K011    │
└──────────────────┘      └──────────────────┘
        │   Simultaneous saccharification   │
        │   and fermentation at 37°C          │
        ▼   5 days                            ▼
┌─────────────────────────────────────────────┐
│       Total sugar (Phenol-sulfuric)           │
│     Reducing sugar (Somogyi-Nelson)           │
│              Xylose (HPLC)                     │
│             Glucose (HPLC)                     │
│             Ethanol (HPLC)                     │
│            Acetic acid (HPLC)                  │
│             Lactic acid (HPLC)                 │
└─────────────────────────────────────────────┘
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Figure 1. Flow-chart of process outlining the steps for solid state fermentation of *G. trabeum* and *P. chrysosporium* on corn stover without pretreatment, followed by SSF using *S. cerevisiae* and *E. coli* K011.

Figure 2. Solid state fermentation of corn stover with *P. chrysosporium* (left) and *G. trabeum* (right) at day 4.

Table 2. Enzyme activity and total protein assays (n=3).

	Corn stover + *P. chrysosporium*	Corn stover + *G. trabeum*
Enzyme Assay (FPU/ml)[a]	0.65	1.72
Protein Assay (mg/ml)[b]	14.06	11.61

[a] Filter paper unit activities (FPase) based on the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper, at 4% conversion, in 1 hour (units FPU/ml)
[b] Protein was determined by NanoDrop™ 1000 Spectrophotometer.

Enzyme assays done to determine the FPase showed that more cellulase was being secreted by the brown rot, at 1.72 FPU/ml, as compared to white rot at 0.65 FPU/ml (Table 2). This concentration, however, does not correlate to the amount of total protein being produced extra-cellularly as mentined earlier. White-rot fungi such as *P. chrysosporium*, produce additional extracellular enzymes such as laccases and peroxidases, when grown in lignin

impregnated biomass such as corn stover and other lignocellulosic material (Wymelenberg et al. 2005; Suzuki et al. 2008).

In situ **Enzymatic Hydrolysis**

The efficiencies of the cellulolytic enzymes hydrolysis of lignocelluloses were further evaluated and validated via the assays of the saccharification and fermentation products. Saccharification of the stover to its free reducing and total sugars was measured via the Somogyi-Nelson and phenol-sulfuric methods. After four days solid state fermentation for enzyme induction (day 0 of SSF), between 2.42 - 2.91 g of reducing sugar per 100 g of stover, was detected for *G. trabeum* treated stover and 0.23 - 0.29 g reducing sugar per 100 g of stover, was detected for *P. chrysosporium* treated stover. Although there was a significant difference in the amount of reducing sugar, it is quite different for the total sugar. Total water soluble sugar profile was almost similar for both fungi and ranged from 5.57 – 5.94 g sugar per 100 g of stover. Both of these assays support the ability of both fungal strains to perform *in situ* saccharification and these trends were observed throughout the five day SSF period (Figure 3 and Figure 4), especially for total sugar.
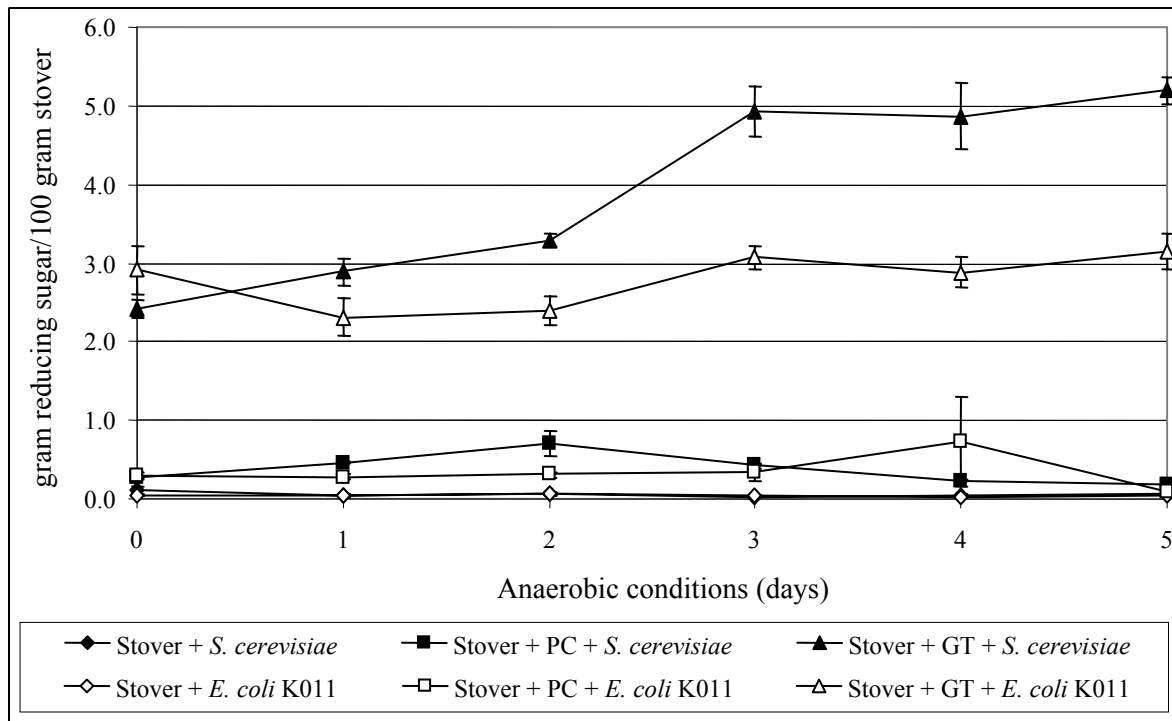
Figure 3. Time course of reducing sugar production, as determined via the Somogyi-Nelson method. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).

To supplement the reducing sugar assay, the concentration of glucose and xylose were determined using the HPLC, as these sugars are the main monomeric end products from the cellulosic and hemicellusic polymers hydrolysis (Hendriks and Zeeman 2009; Lim 2004). During the anaerobic ethanolic fermentation, no glucose was detected. Xylose was detected in all fungi treated samples that were inoculated with *S. cerevisiae*, (Figure 5). This is expected since *S. cerevisiae* cannot utilized pentoses such as xylose (Eliasson et al. 2000; Liu et al. 2010).
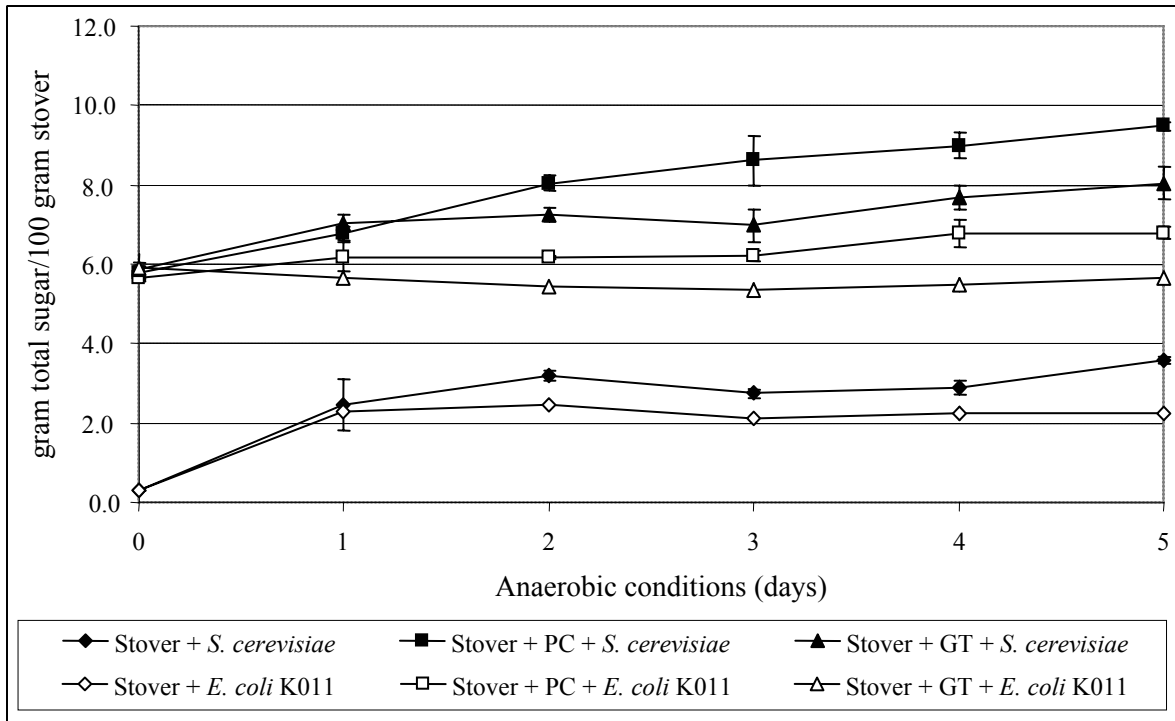
Figure 4. Time course of total sugar production, as determined via the phenol-sulfuric method. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).
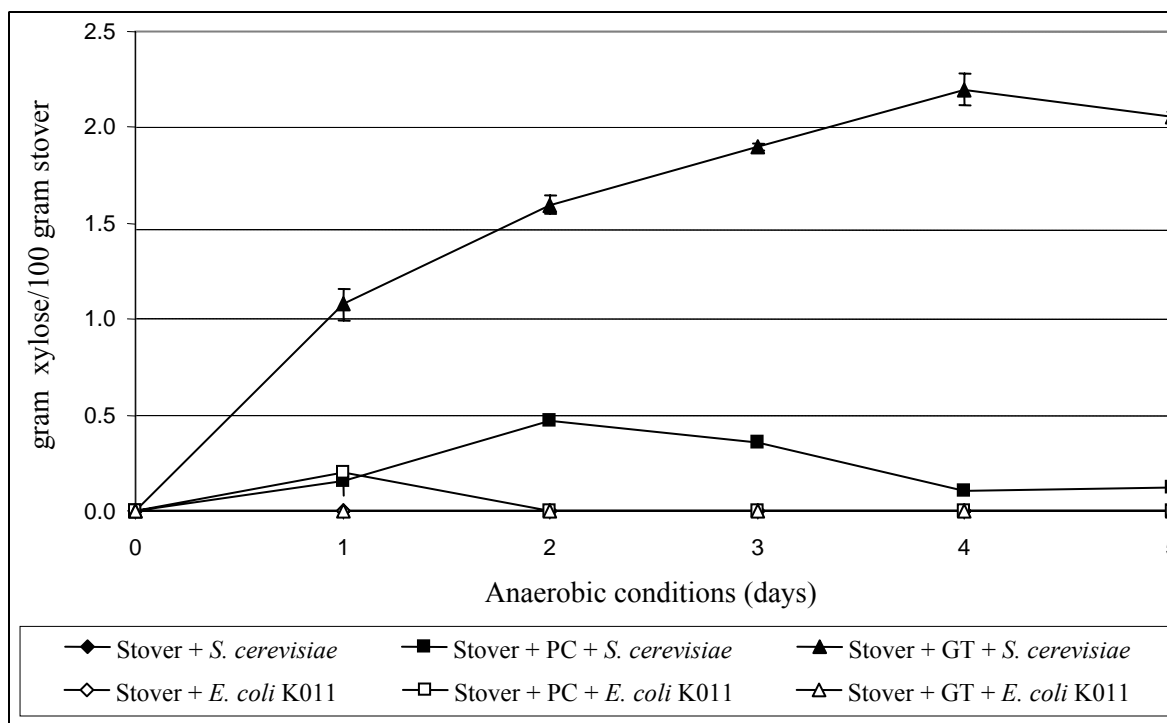
Figure 5. Time course of xylose production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).

Simultaneous Saccharification and Fermentation of Fungal Treated Corn Stover

The fermentability of the saccharification products was further evaluated by using *S. cerevisiae* and *E. coli* K011 as the fermenting organisms. *S. cerevisiae* and *E. coli* K011 was chosen as both of these microorganisms are efficient ethanolic fermenters, with the former capable of fermenting glucose from the breakdown of cellulose, and the latter capable of fermenting both glucose and other fermentable sugars such as xylose, arabinose and galactose from the enzymatic hydrolysis of hemicelluloses (Liu et al. 2010).

From the graph in Figure 6, ethanol production started from day 1 and increased steadily for all corn stover samples, indicating that the sugars released during the saccharification were readily converted to ethanol. Our results showed that ethanol production was highest on day 4 for all samples inoculated with either *P. chrysosporium* or *G. trabeum*. For the corn stover treated with *P. chrysosporium*, the conversion of corn stover to ethanol was 2.29 g/100 g corn stover for the sample inoculated with *S. cerevisiae,* whereas for the sample inoculated with *E. coli* K011, the ethanol concentration was 4.14 g/100 g corn stover. For the corn stover treated with *G. trabeum*, the conversion of corn stover to ethanol was 1.90 g and 4.79 g/100 g corn stover for the sample inoculated with *S. cerevisiae* and *E. coli* K011, respectively. A general trend in ethanol production among the fungal treatments is that samples inoculated with *E. coli* K011 has greater ethanol yield. This is due to the ability of *E. coli* K011 to ferment both hexoses (C6 sugars) (i.e. glucose) and pentoses (C5 sugars) (i.e. xylose) (Liu et al. 2010). The result as seen in Figure 5 further supports this observation, with corn stover not inoculated with *E. coli* K011 still containing xylose even after day 5 of SSF.

One interesting observation in the ethanol production profile is the production of trace amount of ethanol (1.45 g/100 g corn stover at day 4) for the sample inoculated only with *E. coli* K011. This, however, is not a new finding as *E. coli* have been documented to secrete cellulases and several hemicellulases, such as xylanases, mannosidase and galactases that may have librated xylose from the hemicellulose polymers (Gebler et al. 1992; Park and Yun 1999; Sampaio et al. 2004; Okuyama et al. 2005).
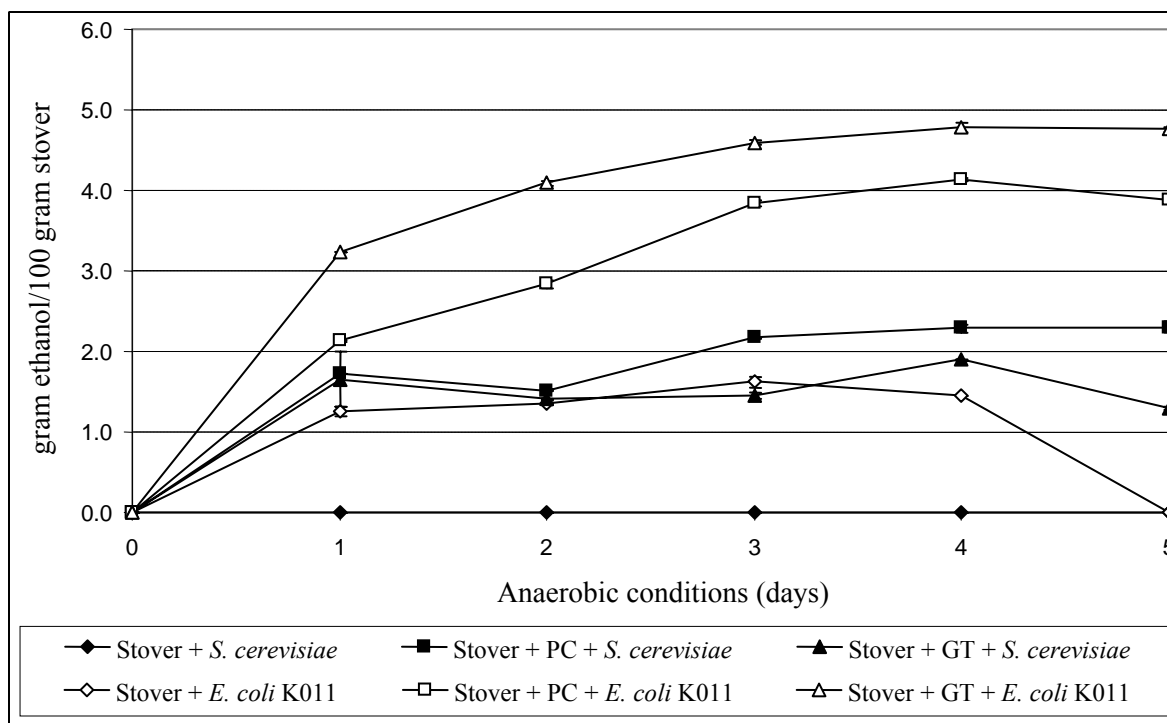
Figure 6. Time course of ethanol production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).

Throughout the experiment, other fermentation co-products, such as, acetic acid and lactic acid were also monitored (Figure 7). Acetic acid production ranged between 0.45 g and 0.78 g/100 g corn stover for the samples under different fungal treatments, while no lactic acid production was detected throughout the 5 days of SSF.

Statistical analyses of the ethanol production via non-linear regression using exponential model fits, as summarized in Table 3, strongly endorsed the reliability of the ethanol production, with all p values being < 0.05. Further analyses performed using the Student's t

test showed statistically significant ethanol yield (Figure 8) among the different treatments. This reinforces the interrelationship between fungal species treatments and fermenters used.
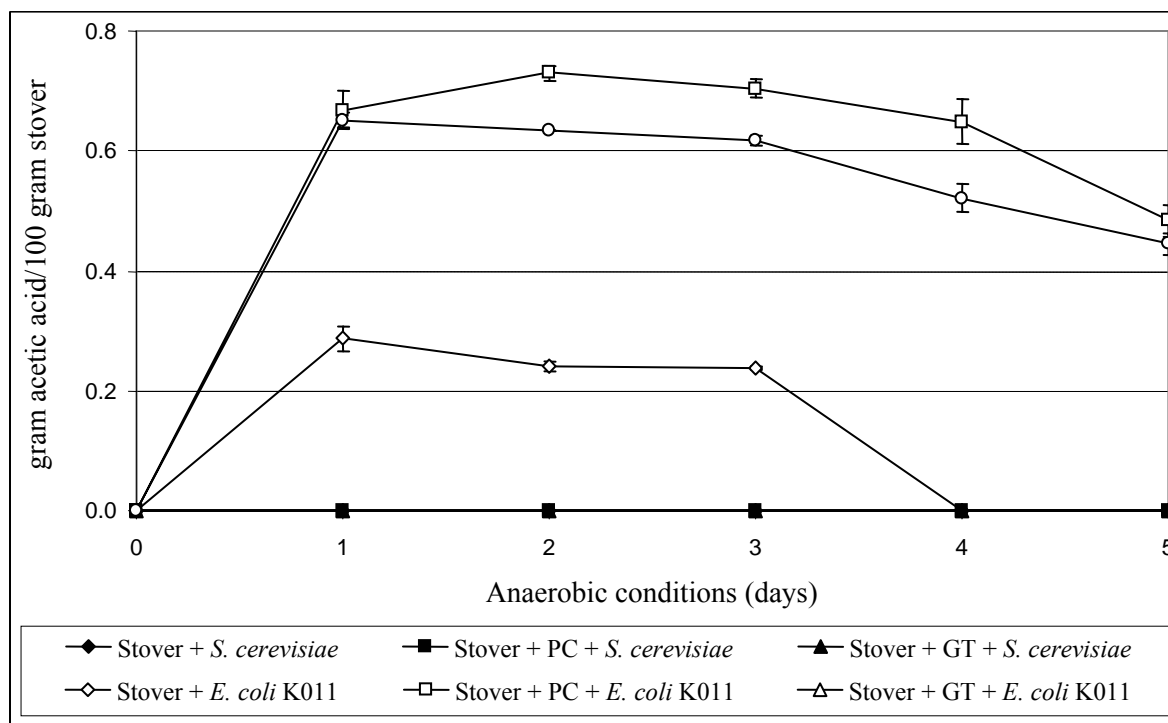
Figure 7. The course of acetic acid production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).

Table 3. Summary of non-linear (exponential) fit models of ethanol production (n=3).

	R^2	F - value
Corn stover + *E. coli* K011	0.911	0.0265
Corn stover + *P. chrysosporium* + *S. cerevisiae*	0.925	0.0022
Corn stover + *P. chrysosporium* + *E. coli* K011	0.839	0.0103
Corn stover + *G. trabeum* + *S. cerevisiae*	0.893	0.0044
Corn stover + *G. trabeum* + *E.coli* K011	0.937	0.0015
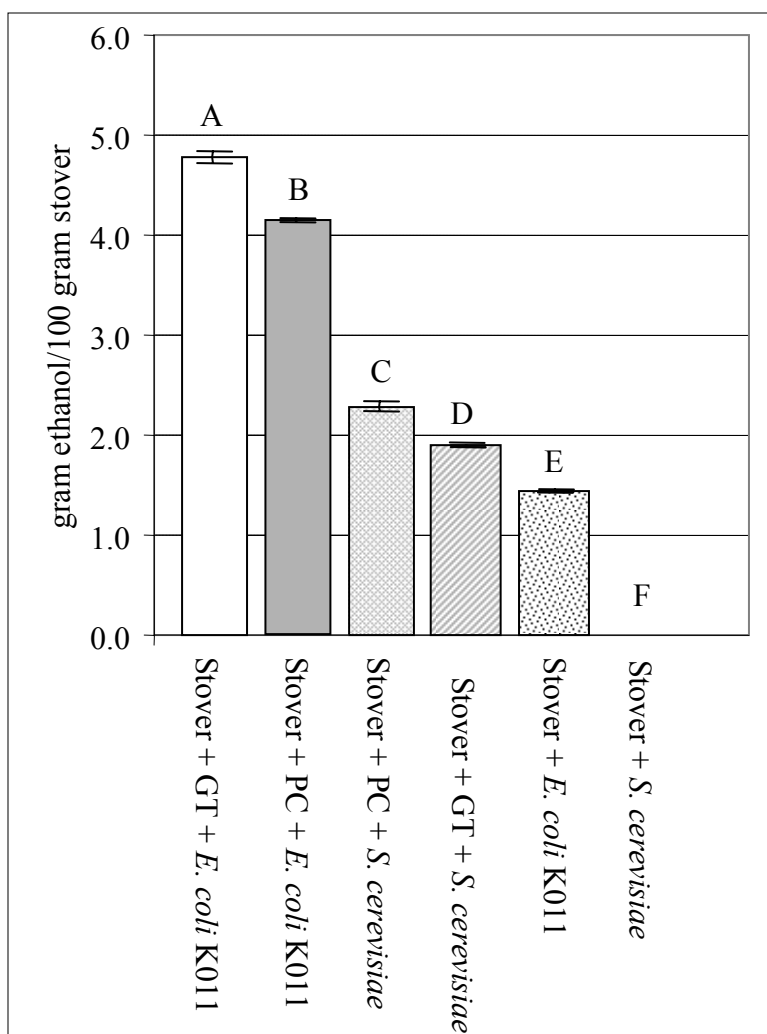
Figure 8. Maximum ethanol productions of different fungal treatments and fermentation conditions. Letters on top of the columns indicate significant differences (Student's t test, $\alpha=0.05$).

To realize large scale applications for cellulosic feedstocks such as corn stovers, low conversion costs are essential. The usage of commercial enzymes makes the production of fuel ethanol not economically feasible or profitable. Furthermore, these enzymes are highly susceptible to inhibitions and are very substrate specific. An ideal lignocellulolytic biocatalyst should degrade the three main components of corn stovers, namely, the cellulose,

hemicellulose and lignin. Thus, *P. chrysosporium* and *G. trabeum* as the organism to provide *in situ* enzymes for the degradation of the lignocellulosic components of corn stovers offers a promising solution. In the production of fuel ethanol production from corn stovers, the optimization of this mechanism can lead to reduced ethanol production costs, as ethanol plants can produce their own enzymes to supplement the usage of commercial enzymes. Another advantage in using this process is an environmentally friendlier approach that eliminates the needs to perform potentially environmentally detrimental pretreatments.

Acknowledgment

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CHAPTER 5: ETHANOL PRODUCTION VIA SEQUENTIAL SACCHARIFICATION AND FERMENTATION OF DILUTE NAOH PRETREATED CORN STOVER USING *PHANEROCHAETE CHRYSOSPORIUM* AND *GLOEOPHYLLUM TRABEUM*

*(to be submitted to the **Applied Microbiology and Biotechnology** journal)*

ABSTRACT

Ethanol production was achieved via the sequential saccharification and fermentation of dilute sodium hydroxide (2% w/w NaOH in corn stover) treated corn stover using *Phanerochaete chrysosporium* and *Gloeophyllum trabeum.* Fermentation was performed by using *Saccharomyces cerevisiae* and *Escherichia coli* K011 after a four-day solid state fermentation of the wood rots to induce cellulolytic and hemicellulolytic enzymes production on the alkaline treated stover which was followed by an ethanolic simultaneous saccharifiation and fermentation (SSF). Ethanol production peaked on day 3 and day 4 for the samples inoculated with either *P. chrysosporium* or *G. trabeum,* slightly plateauing or decreasing thereafter. Ethanol production was highest for the combination of *G. trabeum* and *E. coli* K011 at 6.68 g/100 g stover, followed by the combination of *P. chrysosporium* and *E. coli* K011 at 5.00 g/100 g stover. SSF with *S. cerevisiae* generally had lower ethanol yields, ranging between 2.88 g/100 g stover (*P. chrysosporium* treated stover) and 3.09 g/100 g stover (*G. trabeum* treated stover). The production of total sugar, reducing sugar, acetic acid

and lactic acid were also recorded. Acetic acid production ranged between 0.53 g and 2.03 g/100 g corn stover for the samples under different fungal treatments, while lactic acid production was only detected in samples treated with *G. trabeum,* throughout the 5 days of SSF. The results of our study indicated that mild alkaline pretreatment coupled with fungal saccharification offer a promising bioprocess for ethanol production from corn stover without the addition of commercial enzymes.

Keywords: *Phanerochaete chrysosporium, Gloeophyllum trabeum*, *Saccharomyces cerevisiae*, *Escherichia coli* K011, solid state fermentation, simultaneous saccharification and fermentation (SSF).

INTRODUCTION

Ethanol has been an important industrial chemicals for decades (Zhu et al. 2006). It's application is broad due to its total miscibility in water and many organic solvents, making it the choice material for use in beverages, chemical industries, cosmetics and pharmaceuticals (Dale 1991; Berg and Licht 2004). Another more recent and significant application of ethanol is as transportation fuel, either by itself, or as additives to regular gasoline (Shakhashiri 2009). According to a study by Fukuda et al. (2009), it is estimated that about 73% of worldwide ethanol productions goes into the transportation sectors. In fact, in the United States alone, it is projected that by 2017, fuel ethanol will replace approximately 20% fossil fuel (35 billion gallons), a practice that is envisioned to reduce the amount of gasoline

used, and also the dependence on foreign oils (Wen *et al*. 2009). This interest is also shared globally and can be traced back to the oil embargo in the 1970s. According to Sainz (2009), worldwide biofuels production and applications has increased tremendously in recent years, from a little over 18.2 billion liters (~4.85 billion gallons) in 2000 to approximately 60.6 billion liters (~16.4 billion gallons) in 2007, with bioethanol contributing to about 85% of the amount. In 2008, this figure stands at least 17.335 billion gallons (RFA 2009).

Presently, one major controversy with bioethanol production is the high demand for corn that it has caused, leading to the increase in price of corn kernels for human food and animal feed, which questions the sustainability of this technology (Bommarius et al. 2008; Alvira et al. 2009). Therefore, attentions are now moving towards the utilization of non-food lignocellulosic materials such as corn stover, baggase (sugar cane waste), rice straw, wood chips or other "energy crops" (fast-growing trees and grasses) as the primary feedstock (Mosier et al. 2005; Dwivedi et al. 2009). Together with other lignocellulosic biomass in world forests, these biopolymers are the most abundant renewable resources with a yearly supply of approximately 200 billion metric tons (Zhang 2008; Fukuda et al. 2009).

Although available in great abundance, utilization of lignocellulosics, such as corn stover, for ethanol production faces many obstacles. Firstly, the individual lignocellulosic components such as cellulose and hemicellulose have to be liberated from the encasing lignin barriers. Secondly, the exposed cellulosic and hemicellulosic has to be hydrolyzed into fermentable sugar. To tackle these two hurdles, pretreatments, usually via physical and chemical means are deployed and, commercial enzymes are used. With corn stover, many pretreaments that

have been tested are chemical based which include Ammonia Fiber Explosion (AFEX), Ammonia Recycle Perlocation (ARP), dilute acid, lime and SO_2 alkaline pretreatments (Galbe and Zacchi 2007; Aden and Foust 2009; He et al. 2009; Kim et al. 2009; Kumar et al. 2009). These pretreatments, however, are highly unfavorable, especially in the mass production of fuel ethanol as they result in downstream post treatments and in environmental problems, usually associated with disposal. Furthermore, according to several other reports, pretreatments can be expensive with costs as high as 30¢/gallon of ethanol produced (Mosier et al. 2005; Wyman et al. 2005). Thus, more studies are needed to optimize their use in commercial production of lignocellulosic ethanol (Hendriks and Zeeman 2009).

One possible solution to reduce the cost of these expensive chemical based pretreatment is to couple the process with biological pretreatment and the sequential saccharification of the feedstock, using biological means. In this study, we perform initial pretreatment of corn stover with mild sodium hydroxide (NaOH) at high temperature. Next, we hydrolze the treated corn stover using two wood rots, *Phanerocheate chrysosporium* and *Gloeophyllum trabeum*, which have proven to be effective in this form of application (Shreshta et al. 2008, Shrestha et al. 2009, Rasmussen et al. 2010). *P. chrysosporium* and *G. trabeum* are choosen for their abilities to efficiently degrade cellulose and hemicellulose, with their repertoire of cellulases and hemicellulase such as endoglucanases, exocellobiohydrolase, cellobiose dehydrogenase, β–glucosidases (cellobiases), endoxylanases, β-xylosidase and α-galactosidase, among others (Kerem et al. 1999; Abbas et al. 2005; Cohen et al. 2005; Wymelenberg et al. 2005; Suzuki et al. 2008). We further subject the saccharification products to be fermented to ethanol by using *Saccharomyces cerevisiae* and *Escherichia coli*

K011. This bacterium utilizes hexose and pentose sugars generated from cellulose and hemicelluloses.

MATERIALS AND METHODS

Experimental setup

A flow-chart of the overall experimental setup is shown in Figure 1. All experiments were done in triplicates (n=3). Carbohydrate analyses, total protein and enzymatic activities assays were also performed in triplicates.
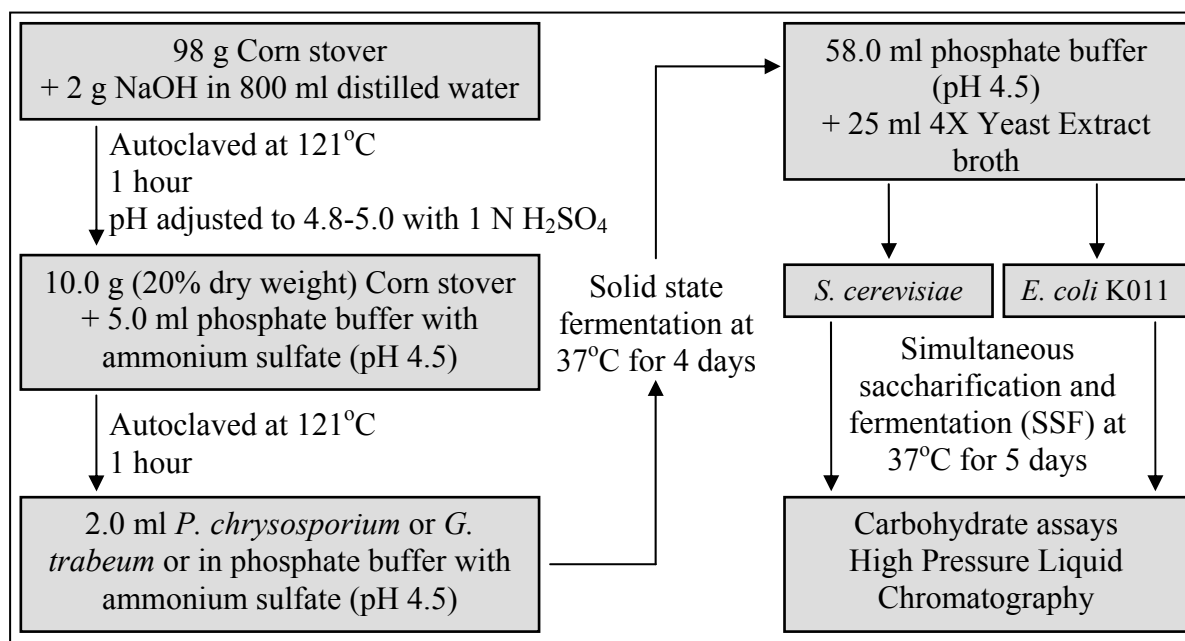
Figure 1. Flow-chart of process outlining the steps for dilute NaOH treatment of corn stover, followed by solid state fermentation of *P. chrysosporium and G. trabeum* on corn stover, and simultaneous saccharification and fermentation (SSF) using *S. cerevisiae* and *E. coli* K011.

Corn stover pretreatment and analysis

Corn stover used in this study was obtained from the Department of Agronomy, Iowa State University. The field dried corn stovers were ground in a Wiley mill (Model 4 Wiley Laboratory Mill, Thomas Scientific Inc., Swedesboro, NJ) and then screened using a 20 mesh sieve. To perform the dilute NaOH pretreatment of the corn stover, 98 g of stover was soaked in a NaOH solution that was prepared by diluting 2 g NaOH pellets in 800 ml of distilled water (0.25% w/v NaOH solution or 2% w/w NaOH per stover). The mixture was heated at $121^{\circ}C$ for 1 hour. Heat treated corn stover was initially rinsed with distilled water, and the pH was adjusted to a final pH of 4.8-5.0 with 1 N (0.5 M) H_2SO_4 at room temperature. The acidified corn stover is then dried to a 20% w/w solid content.

For compositional determination, the oven dried corn stover and NaOH treated corn stover were subjected to cellulose and hemicellulose analysis using the ANKOM method (ANKOM Technol. Corp., Fairport, NY) as described previously (Vogel et al. 1999). The lignin content for both untreated and NaOH treated corn stover were determined by using a modified Klason lignin assay, following the method by Crawford and Pometto (1998) which measures lignin as the acid-insoluble material after hydrolysis by strong acid (H_2SO_4) and heat. The lignin analysis was performed with a slight modification, where by glass fiber filters (1.6 μm) (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were used instead of Whatman No.1 filter papers for capturing the solid lignin residues. The Klason lignin was determined as the weight of dry residue collected on the filter paper after the remaining solids were thoroughly rinsed with deionized water and dried in the oven at $105^{\circ}C$ for four days.

Microorganisms

Stock cultures of *Phanerochaete chrysosporium* (ATCC 24725)*, Gloeophyllum trabeum* (ATCC 11539), *Saccharomyces cerevisiae* (ATCC 24859) and *Escherichia coli* K011 (ATCC 55124) used in this study were obtained from the American Type Culture Collection (Rockville, MD). The fungal cultures were revived by inoculating onto potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD), while the bacterial culture was grown on LB broth (Difco). All cultures were incubated at 24°C with shaking at 120 rpm overnight (Shrestha et al. 2009). Long term storage of the stock cultures in Yeast Malt extract (YM) broth (Difco) supplemented with 20% (v/v) glycerol, were kept at -80°C in an ultralow-temperature freezer (So-Low Environmental Equipment Co., Inc., Cincinnati, OH).

Microorganism culture preparation

P. chrysosporium and *G. trabeum* cultures were grown in 1 liter of YM Broth and incubated at 30°C, agitated at 150 rpm. After 7 days of growth, the fungal mycelia was harvested via centrifugation at 7,277 g for 20 min in a sterilized 1-L polypropylene centrifuge bottle (Nalgene, Nalge Nunc, Rochester, NY), using a Sorvall-RC3B Plus centrifuge (Thermo Fisher Scientific, Wilmington, DE) (Shrestha et al. 2008; Shrestha et al. 2009). The collected fungal mycellia was rinsed with a mineral salt solution (pH 4.5-4.8) containing 50 mM Phosphate Buffer, 0.5% $(NH_4)_2SO_4$ and Basal Medium (0.25 g KH_2PO_4, 0.063 g $MgSO_4 \cdot 7H_2O$, 0.013 g $CaCl_2 \cdot 2H_2O$, and 1.25 mL of trace element solutions in 1 L of deionized water) (Shrestha et al. 2009).

S. cerevisiae and *E. coli* K011 culture inocula were prepared in sterile 50 ml of YM Broth at 32°C with constant agitation (120 rpm). The respective yeast and bacterial cells were harvested via centrifugation in a 50 ml conical centrifuge tubes (BD Falcon, BD, Franklin Lakes, NJ) at 2852 g for 10 min using a Beckman J2-21centrifuge (Beckman Coulter Inc., Brea, CA) and cell density was adjusted at 10^7-10^8 CFU/ml as determined at 600 nm via a standard curve (Nguyen et al. 2009).

Solid State Fermentation

Solid state fermentation was performed on the autoclave sterilized corn stover (10 g, 20% dry weight) that was mixed with 5 ml mineral salt medium and three marble balls (Shrestha et al. 2008; Shrestha et al. 2009). Next, rinsed *P. chrsosporium* or *G. trabeum* inocula were mixed with fungal mineral salt solution, and 2 ml of the mixture was added to the sterilized corn stover. The bottles were rolled on their sides to uniformly disperse and coat the corn stover and fungi mixture along the inner surface with the assistance of glass marbles (Shrestha et al. 2008; Shrestha et al. 2009). The four days incubation for enzyme induction production was kept at 37°C in a humidified incubator.

Simultaneous Saccharification and Fermentation (SSF)

SSF experiments were carried out in batch cultures of 100 ml, consisting of 25 ml 4X Yeast Extract Broth (1.8 g yeast extract (Difco), 0.07 g $CaCl_2 \cdot 2H_2O$ (Fisher Scientific, 0.45 g of KH_2PO_4 (Fisher Scientific), 1.2 g of $(NH_4)_2SO_4$ (Fisher Scientific), and 0.3 g of $MgSO_4 \cdot 7H_2O$ (Fisher Scientific) per liter of deionized water) (Shrestha et al. 2009) and buffered basal medium (pH 4.5-4.8) (50 mM Phosphate Buffer + Basal Medium). The flasks were then aseptically inoculated with *S. cerevisiae* or *E. coli* K011 cultures. These SSF culture bottles were incubated for 5 days at 37^oC under static condition (Figure 2).

Figure 2. Simultaneous saccharification and fermentation (SSF) of corn stover with *P. chrysosporium* and *E. coli* K011 (day 3), clearly showing the formation of carbon dioxide gas bubbles.

Protein and Enzyme Activities Assays

On day 4 of the solid state fermentation a 1.5 ml samples was aspectially collected. The sample was centrifuged at 1,118 g for 5 min (MiniSpin Plus, Eppendorf, Hauppauge, NY) and the supernatant was filtered through a 0.2 μm nylon syringe filter (VWR International, Batavia, IL). The filtered solutions were used to perform total protein analysis and enzyme activities assay.

Protein production by *P. chrysosporium* or *G. trabeum* cultured on corn stover was measured via the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). This system measures a loading of 2 μl sample size and calculates the protein concentration (mg/ml) from the protein's absorbance at 280 nm (A280). A separate fermentation broth from uninoculated corn stovers was used as the blank reading.

Enzyme activity assay in terms of "filter-paper units" (FPU) per milliliter of enzyme preparation was performed using the protocol described by the official National Renewable Energy Laboratory (NREL) procedure (Adney and Baker 2008). This method was developed based on the International Union of Pure and Applied Chemistry (IUPAC) guidelines to determine cellulase activities (Ghose 1987).

Total and Reducing Sugars Assays

Sample aliquots of 1.8 ml were collected aseptically from each SSF bottle every 24 hours. The sample mixtures were centrifuged (MiniSpin Plus) at 1,118 g for 5 min and supernatant filtered through a 0.2 μm nylon syringe filter. The filtered supernatants were tested for total sugars and reducing sugar, via the phenol-sulfuric (Crawford and Pometto 1988) and Somogyi-Nelson (Antai and Crawford 1981) methods, respectively. The total sugar determination was determined via the phenol sulfuric carbohydrate test at 490 nm (SpectraMax Plus384, Molecular Devices, Inc., Sunnyvale, CA, U.S.A) while the Somogyi-Nelson carbohydrate assay was performed at 500 nm (SpectraMax Plus384) with glucose standards. The equivalent sugar concentration (g/l) was determined based on a standard glucose concentration curve that was generated prior to the assays.

High Pressure Liquid Chromatography (HPLC) Analyses

Fermentation products (ethanol, acetic acid, and lactic acid) and glucose were measured by using a Waters High Pressure Liquid Chromatography (Millipore Corp., Milford, MA) system. The separation and analysis the fermentation products was done on a Bio-Rad Aminex HPX-87H column (300.0 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N H_2SO_4 as a mobile phase with a flow rate of 0.6 ml/min (Ramos 2003; Liu et al. 2008; Shrestha et al. 2009).

Statistical Analyses

The experimental data were validated statistically using the statistical software, JMP 8.0 (SAS Institute Inc., Cary, NC). The data (n=3) on ethanol production were fitted to polynomial (2nd degree) fit models. Error bars were determined for triplicate samples based on the standard deviation from the mean values. Tukey-Kramer's HSD (Honestly Significantly Different) test analyses were also performed for all final data set to determine multiple comparisons of the ethanol production. A *p*-value of less than 0.05 was considered significantly different.

RESULTS

The main components of the oven dried corn stover used are cellulose, hemicellulose, lignin, and ash (Table 1). Following 2% (w/w) NaOH pretreatment, overall weight loss was recorded at 14.1% with Klason lignin reduction by 4%. The Klason lignin reduction corresponds to approximately 19.3% of initial lignin content of untreated corn stover.

Table 1. Composition of dried biomass for initial and dilute NaOH treated corn stover using the ANKOM method and Klason Lignin method (as percentage based on dry weight; n=3).

Main components	Initial Biomass (%)	NaOH Treated Biomass (%)
Cellulose	38.0	44.1
Hemicellulose	30.7	31.6
Klasson lignin	20.7	16.7
Others	10.6	7.6

The sample from the day 4 solid-state-fermentation was used to determin the total protein and FPase concentrations (Table 2). Results showed that protein production was higher with the *P. chrysosporium* inoculated corn stover (12.14 mg/ml), compared to the *G. trabeum* treated (10.98 mg/ml). Following the protein assays, enzyme activities were determined based on the Filter paper unit (FPase) (Ghose 1987; Adney and Baker 2008). From Table 2, the assay indicated that more cellulase activities were observed from the brown rot *G. trabeum*, at 2.54 FPU/ml, as compared to white rot *P. chrysosporium* at 1.48 FPU/ml.

Table 2. Enzyme activity and total protein assays after 4 day SSF (n=3).

	Corn stover + *P. chrysosporium*	Corn stover + *G. trabeum*
Enzyme Assay (FPU/ml)[a]	1.48	2.54
Protein Assay (mg/ml)[b]	12.14	10.98

[a] Filter paper unit activities (FPase) based on the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper, at 4% conversion, in 1 hour (units FPU/ml)
[b] Protein was determined by NanoDrop™ 1000 Spectrophotometer

During the five day SSF period, the samples were analyzed for solubilized saccharification products (both free reducing and total sugars) via the Somogyi-Nelson and phenol-sulfuric methods. At day 4 of the enzyme induction stage (day 0 of SSF), between 5.42 – 5.58 g of reducing sugar per 100 g of corn stover, was detected in the broth of the *G. trabeum* treated corn stover and 3.78 – 4.34 g reducing sugar per 100 g of corn stover, was detected in the broth of the *P. chrysosporium* treated corn stover. From Figure 3, the concentration of the reducing sugars decreased steadily with all the samples, including the control samples inoculated with a single ethanolic fermenters. Sequential fermentations with *P. chrysosporium* and *E. coli* K011 showed a very dramatic drop from 3.78 g reducing sugar per

100 g of corn stover to 0.767 g reducing sugar per 100 g of corn stover. At day 4 of the solid state fermentation, solubilized total-sugar ranged from 8.62 – 10.04 g per 100 g of corn stover for the corn stover treated with *G. trabeum,* and 6.03 – 6.54 g of total sugar per 100 g of corn stover was detected in the *P. chrysosporium* treated corn stover (Figure 4). The corn stovers that was not treated with either the white- or brown-rot (control) showed the presence of some total sugar after 4 days incubation (day 0 of SSF) (4.33 – 4.39 g of total sugar per 100 g of corn stover), indicating that some sugar was liberated via the dilute NaOH pretreatment on the ground corn stover.
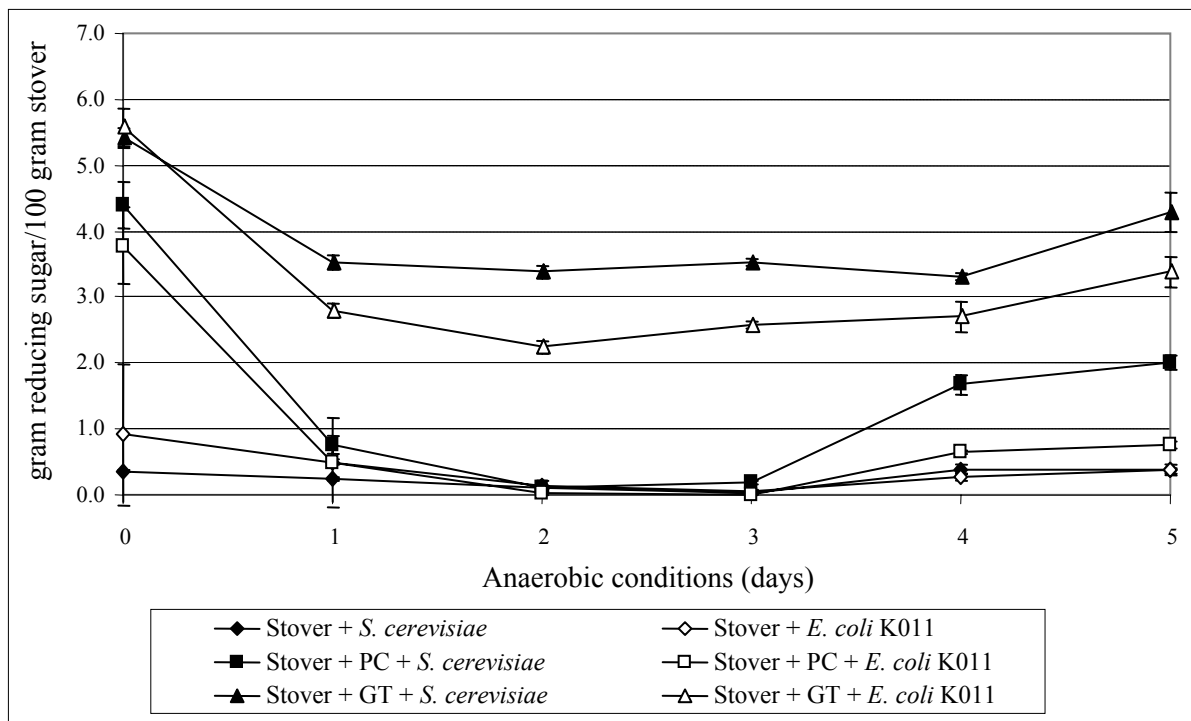
Figure 3. Time course of reducing sugar production, as determined via the Somogyi-Nelson method. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum)*.
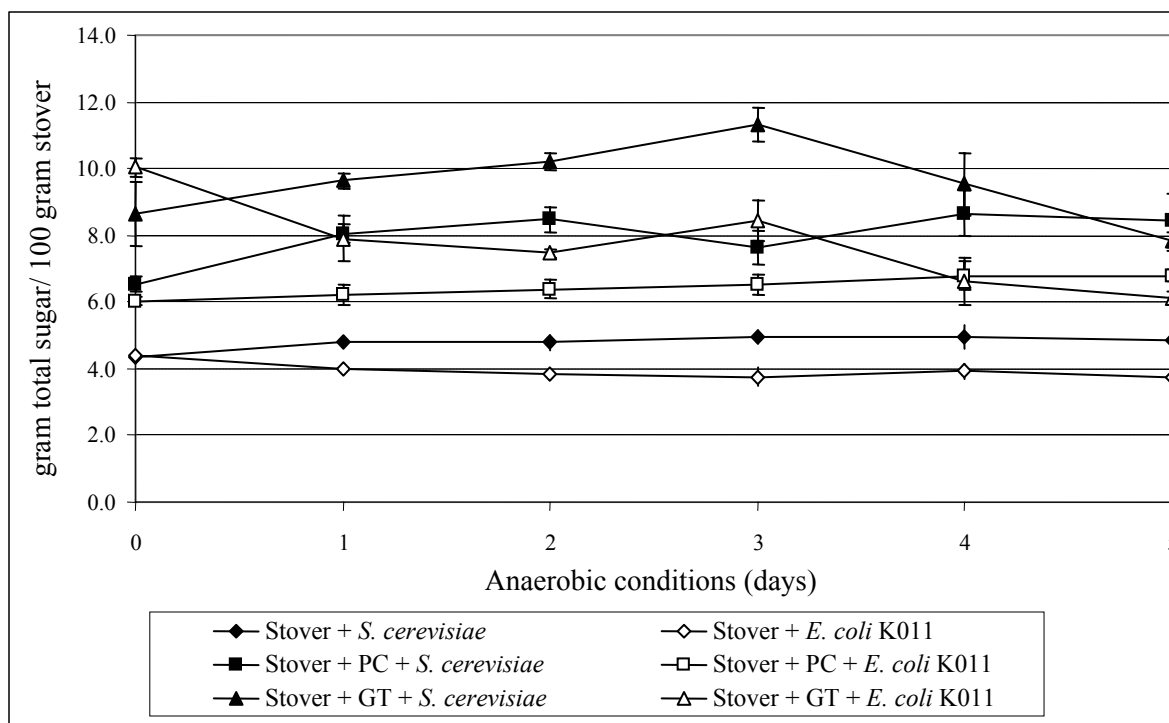
Figure 4. Time course of total sugar production, as determined via the phenol-sulfuric method. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.* Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum)*.

From Figure 5, ethanol production started from day 1 of SSF and increased steadily for all fungal treated samples, indicating the significant liberation of fermentable sugars during saccharification. As expected ethanol production was typically greater for fungal solid-state-fermented corn stovers sequential fermentation with *E. coli* K011 SSF due to its ability to utilize both C6 and C5 sugars. The highest ethanol yield was for the corn stover inoculated with *G. trabeum* and *E. coli* K011 at 6.68 g/100 g corn stover followed by the combination of *P. chrysosporium* and *E. coli* K011 at 5.00 g/100 g corn stover which represents 15.42% and 11.55% of theoretical yields, respectively. Both combination of *G. trabeum* and *S. cerevisiae*

and the combination of *P. chrysosporium* and *S. cerevisiae* generally illustrated lower ethanol

yields, at 3.09 g and 2.88 g/100 g corn stover, respectively.
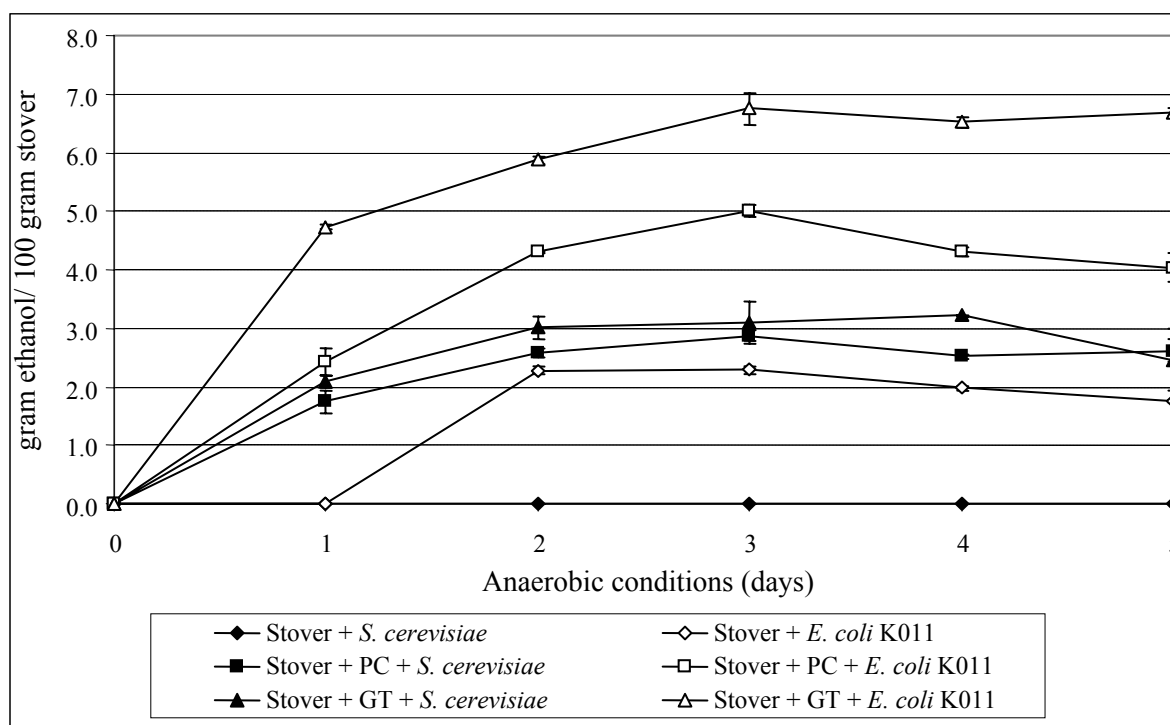
Figure 5. Time course of ethanol production. The data points represent the averages of three
independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum.*
Time zero is after 4 days of solid state fermentation with a specific fungus (*P.
chrysosporium* or *G. trabeum*).

The production of other fermentation co-products, such as, acetic acid and lactic acid were

also recorded. Acetic acid production ranged between 0.53 g and 2.03 g/100 g corn stover

for the samples under different fungal treatments (Figure 6). Lactic acid production was only

detected in samples treated with *G. trabeum,* throughout the 5 days of SSF (Figure 7).
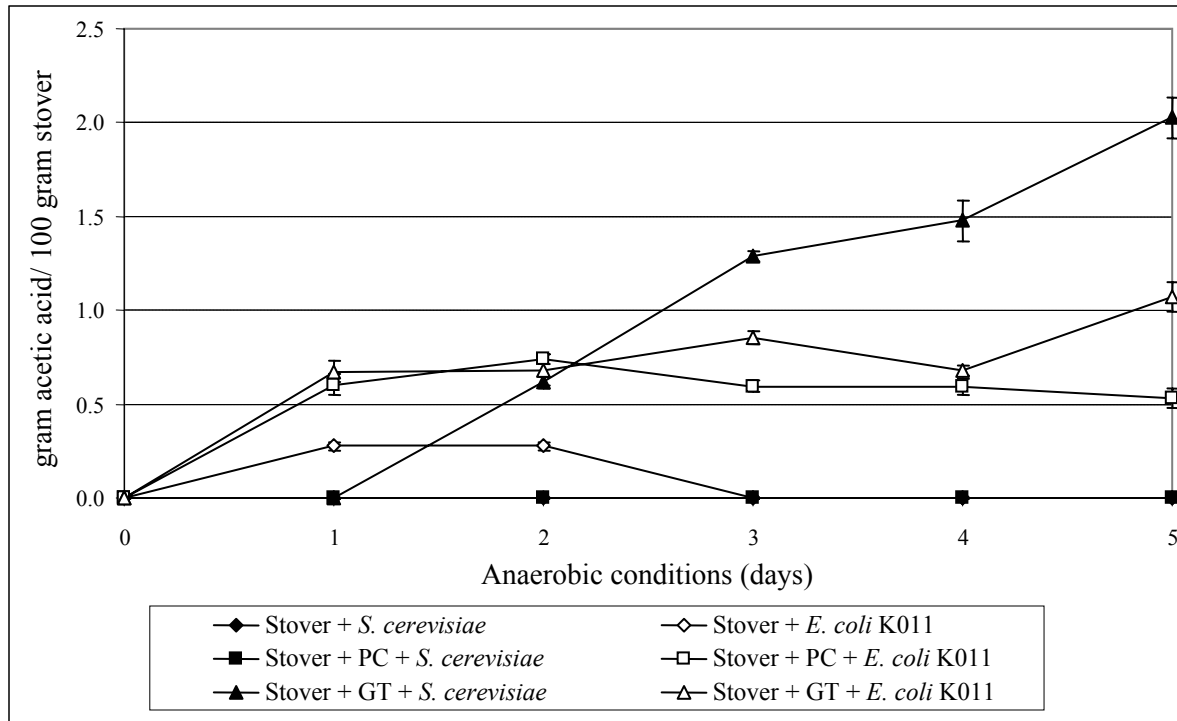
Figure 6. Time course of acetic acid production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).
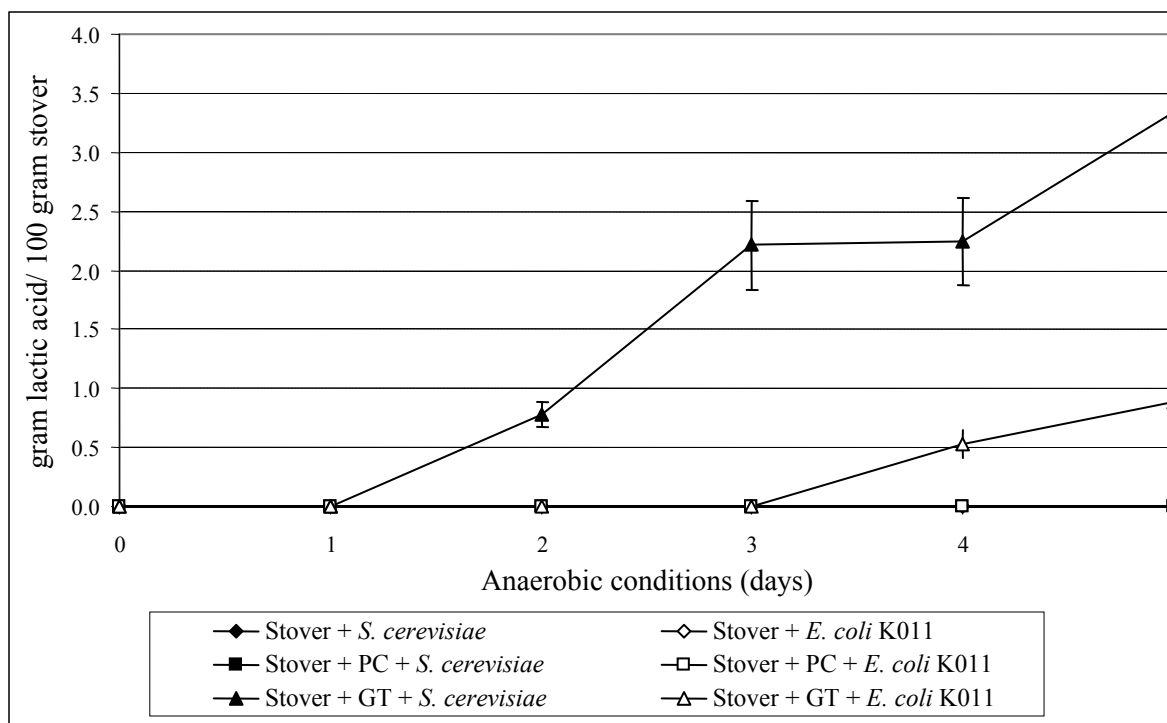
Figure 7. Time course of lactic acid production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, GT – *G. trabeum*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium* or *G. trabeum*).

DISCUSSION

In this study, we performed SSF on dilute alkaline (NaOH) treated corn stovers. Mild alkaline have been proven to be very effective on biomass with low lignin content, such as herbaceous crops and agricultural residues, such as corn stover (Galbe and Zacchi 2007; Garcia-Cubero et al. 2009). Dilute NaOH (0.25% w/v) was chosen as a pretreatment method as it offers several advantages, such as the swelling of stover for better enzymatic saccharification and the partial elimination of lignin and hemicellulose, while leaving the

cellulose untouched (Lim 2004; Silverstein et al. 2007). The removal of lignin and/or partial reduction of hemicellulose can substantially reduce the recalcitrance of biomass to enzymatic hydrolysis (Wyman et al. 2005). In addition, Varga et al. (2003) stated that alkaline pH was previously found to be very efficient for increasing the cellulose convertibility to glucose, consequently leading to high sugar recoveries.

Furthermore, dilute NaOH pretreatment of lignocellulosics produces minimal inhibitory by-products (i.e. complex mixtures of acetate compounds from the deacetylation of xylan, furan dehydration products [furfural and hydroxymethylfurfural] and aliphatic acids [formic and levulinic acid] from sugars, and cocktails of phenolic compounds from lignin when using dilute acid) (Martinez et al. 2000). The presence of furfural and hydroxymethylfurfural is highly undesirable as these two compounds are known to be two of the strongest inhibitory compounds present in acid pretreated hydrolyzates (Talebnia et al. 2004). Previous research has demonstrated that even low concentrations of furfural at 2 g/l reduced the rate of glucose, galactose, glucose, and mannose consumption in *S. cerevisiae*-catalyzed fermentations, an effect that can be attributed to the inhibition of glycolytic enzymes (Keating et al. 2004; Chundawat et al. 2006).

To achieve saccharification of the corn stover, we employed the lignocellulolytic abilities of *P. chrysosporium* and *G. trabeum*. Both these fungal species have been studied extensively due to their abilities to degrade, depolymerize and modify all major components of plant cell walls including cellulose, hemicellulose, and the more recalcitrant lignin (Kerem et al. 1999; Cohen et al. 2005; Wymelenberg et al. 2005; Kersten and Cullen 2007; Hamid and Rehman

2009). Like most other wood-rotters, *P. chrysosporium* and *G. trabeum* effectively performs these biomass degradations via the secretion of various cellulases and hemicellulases (Cohen et al. 2005; Wymelenberg et al. 2005; Suzuki et al. 2008).

Visual observations of solid-state fermentation products showed that *P. chrysosporium* grew more aggresively visually when compared to *G. trabeum*. Total mycelial coverage of the corn stover was seen even at day 2 for *P. chrysosporium* (Figure 8a) , while visible mycelia from *G. trabeum* was only observed after day 3 (Figure 8b). For *G. trabeum*, slower visible growth is in agreement with Rasmussen et al. (2010), whereby substantial growth of *G. trabeum* was only seen on day 5 when the same strain was grown on corn fiber. The slower growth rate could also explain the lower amount of overall protein being secreted (Table 2). Unlike other previous works, our SSF experiments do not use additional commercial enzymes (Kumar and Wyman 2009; Weiss et al. 2010). Instead, the cellulases and hemicellulases produced by *G. trabeum* and *P. chrysosporium in situ* upon corn stover induction was performed in a pH range of 4.5-4.8 at 37°C for 4 days, conditions suitable for the induction of the lignocellulotic enzymes enzymes (Shrestha et al. 2008; Shrestha et al. 2009; Rasmussen et al. 2010).
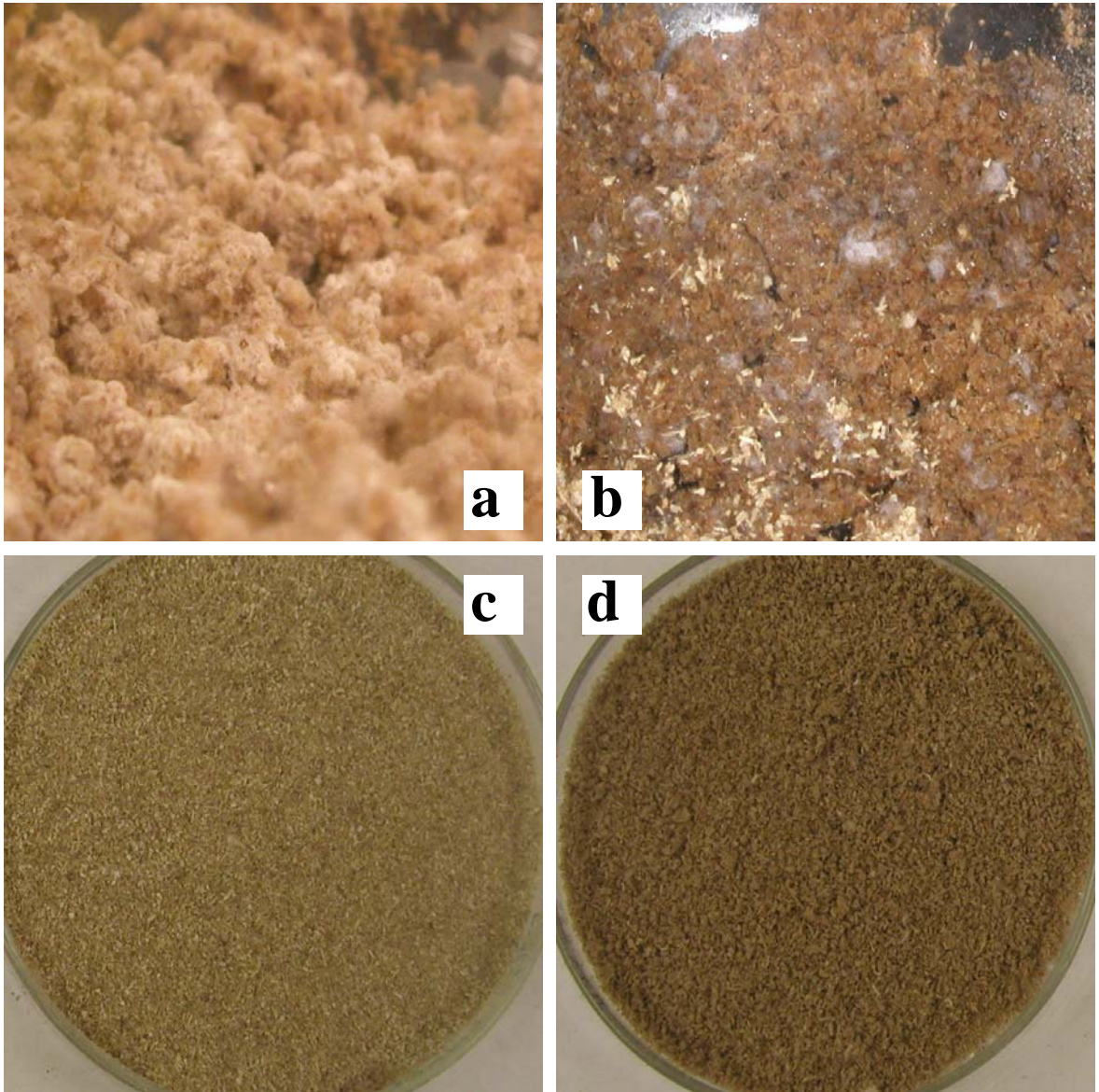
Figure 8. (a) NaOH treated corn stover inoculated with *P. chrysosporium* at day 2 of enzyme induction stage. (b) NaOH treated corn stover inoculated with *G. trabeum* at day 3 of enzyme induction stage. (c) Oven dried NaOH treated corn stover inoculated with *P. chrysosporium* at day 4. (d) Oven dried NaOH treated corn stover inoculated with *G. trabeum* at day 4.

Although the protein concentration is lower in the sample treated with *G. trabeum*, FPase actitivies are higher at 2.54 FPU/ml as compared to 1.48 FPU/ml in the sample treated with *P. chrysosporium*. More proteins are detected in the *P. chrysosporium* treated corn stover

could be due to the secretion of other lignocellulosic active protein such as laccases and peroxidases, when this particular fungal species is grown in lignin rich biomass such as corn stover and other lignocellulosic material (Wymelenberg et al. 2005; Suzuki et al. 2008). Although no specific lignolytic enzyme assays were performed to verify this, visual inspection of the corn stover indicated the discoloration of the feedstock due to lignin reduction. This can be seen in Figure 8c. Corn stover treated with *G. trabeum* do not share this physical manifestation as brown-rot basidiomycetes only slightly modify the encasing brown pigmented lignin, leaving behind brown residues (Figure 8d) (Xu and Goodell 2001; Kerem et al. 2009; Schilling et al. 2009).

Throughout the course of the five day SSF, reducing sugar concentration continued to decrease (Figure 3), while the ethanol production continued to increased (Figure 5) until day 3, for most of the samples. To supplement the reducing and total carbohydrate assay, HPLC analysis was also performed to monitor the glucose level. However, during the anaerobic SSF period, no glucose was detected, a good indication that efficient conversion to ethanol was achieved.

Fermentation of the enzymatic saccharification products was further achieved by using *S. cerevisiae* or *E. coli* K011 as ethanolic fermenters, chosen as both of these microorganisms are efficient fermenters (Garrote et al. 1999a; Garrote et al. 1999b; Liu et al. 2010). From the result shown in Figure 5, samples inoculated with *E. coli* K011 generally has higher ethanol yield due to the ability of this particular strain to ferment both hexose and pentose sugars (Liu et al. 2010). When comparing efficiencies of saccharification among the *P.*

chrysosporium and *G. trabeum,* the results shows that the latter has more effective cellulolytic and hemicellulolytic systems. These are supported by the amount of initial reducing sugar and total sugar release at day 4 of the enzyme induction stage, and also the significant differences in the amount of ethanol production at day 3 (Table 3). The highly efficient cellulolytic and hemicellulolytic systems of *G. trabeum* are well documented. According to Kerem et al. (1999) and Schilling et al. (2009), brown-rot fungi like *G. trabeum* cause the most destructive type of decay in wooden structures, as they rapidly and extensively depolymerize cellulose in the early stages of wood decay (Cho et al. 2008; Schilling et al. 2009).

Table 3. Statistical analysis of the significant differences in ethanol production (g ethanol /100 g corn stover) between *P. chrsysporium* and *G. trabeum* treated corn stover as determined via the Tukey-Kramer HSD test.

Ethanol production (g ethanol /100 g corn stover)	*p* - value
PC + *S. cerevisiae* (2.88 g ethanol /100 g corn stover) vs. GT + *S. cerevisiae* (3.09 g ethanol /100 g corn stover)	< 0.0001
PC + *E. coli* K011 (5.00 ethanol /100 g corn stover) vs. GT + *E.coli* K011 (6.68 g ethanol /100 g corn stover)	< 0.0001

We also observe the production of ethanol (2.28 g/100 g corn stover at day 2) for the sample inoculated only with *E. coli* K011 (Figure 5). However, this is quite expectable as *E. coli* have been reported to secrete cellulases and several hemicellulases, such as xylanases, mannosidase and galactases (Gebler et al. 1992; Park et al. 1999; Sampaio et al. 2004; Okuyama et al. 2005) that may have librated fermentable sugars such glucose, xylose, arabinose, mannose and galactose from the cellulose and hemicellulose fibers.

Statistical analyses of the ethanol production via non-linear regression using 2nd degree polynomial model fits, as summarized in Table 4 validated the reliability of the ethanol production, with all p-values < 0.005. Further analyses performed using Tukey-Kramer's HSD test showed statistically significant ethanol yield (Figure 9) among the different fungal treatments, further reinforcing the interrelationship between fungal species treatments and fermenters used.

Table 4 Summary of polynomial (2nd degree) fit models of ethanol production (g ethanol /100 g Switchgrass vs. Day).

	R^2	Prob > F
Corn stover + *E. coli* K011	0.785	0.0998
Corn stover + *P. chrysosporium* + *S. cerevisiae*	0.895	0.0341
Corn stover + *P. chrysosporium* + *E. coli* K011	0.981	0.0026
Corn stover + *G. trabeum* + *S. cerevisiae*	0.977	0.0036
Corn stover + *G. trabeum* + *E.coli* K011	0.943	0.0138

In order to realize mass production of lignocellulosic ethanol, low conversion costs are essential. The optimization of both feedstock pretreatment and hydrolysis will lead to the reduction of production costs. The results of our study indicate that mild alkaline pretreatment coupled with fungal saccharification offer a promising alternative to produce ethanol from agricultural wstes such as corn stover. While our study may not show that the addition of commercial cellulases can be eliminated altogether, the results of do offer promising potentials signs that there could be savings in enzyme costs.
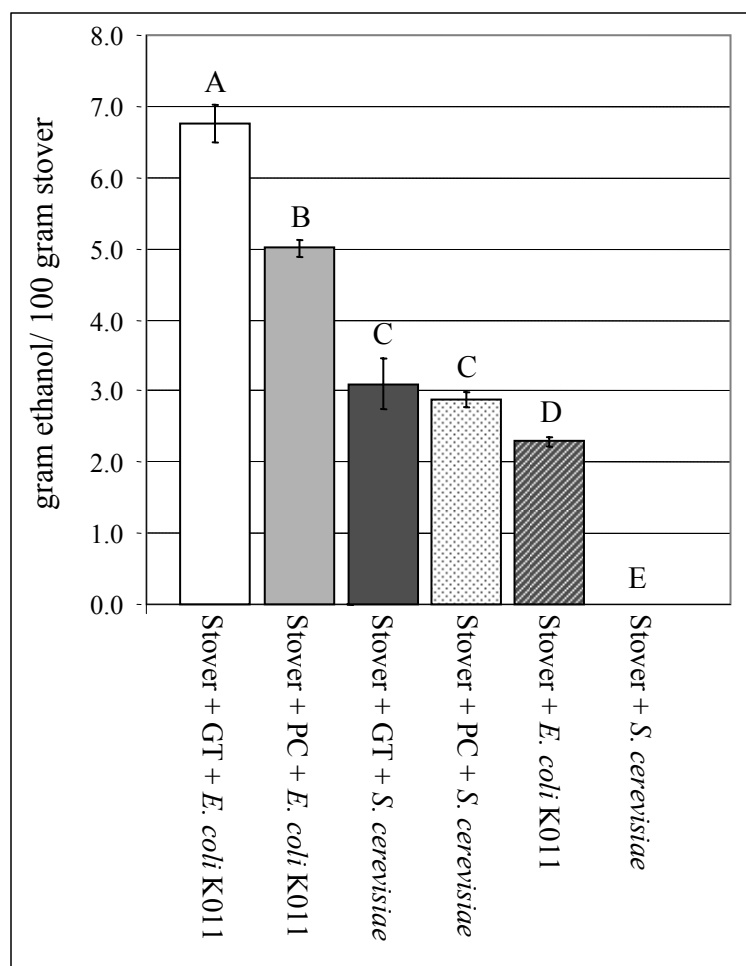
Figure 9. Maximum ethanol yields of different fungal treatments and fermentation conditions. Letters on top of the columns indicate significant differences (Tukey-Kramer HSD, α=0.05).

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CHAPTER 6: ENGINEERING, ECONOMIC AND ENVIRONMENTAL IMPLICATIONS AND SIGNIFICANCE

INTRODUCTION

Currently, in the United States, ethanol-gasoline blends range from 10 to 85%. In fact, the combined production of present ethanol industries is more than 10 billion gallons, representing about 7% of the gasoline supply (Sainz 2009). Sainz (2009) further commented that approximately 70% of all gasoline sold nationwide contains some ethanol. It is expected that if the current trend persists, fuel ethanol will replace approximately 20% fossil fuel (35 billion gallons) by 2017 (Wen et al. 2009). According Stowers (2009), in a recent report by the U.S. Department of Commerce International Trade Administration Study, the United States will generate sufficient cellulosic feedstock by 2020 to generate approximately 50 billion gallons of lignocellulosic ethanol. These statements clearly indicate the bright prospect for the research and production of second generation bioethanol.

This chapter aims to discuss the engineering, economic and environmental implications, and significance of fuel ethanol in two aspects, the general outlook of ethanol productions and the specific significances and implications of our present research. The purpose of this chapter is to discuss the following:

i.   The importance of bioethanol as transportation fuel

ii. The importance of bioethanol for the environment

iii. The importance of bioethanol for the economy

iv. Engineering and processing implications

v.   Research recommendations

THE IMPORTANCE OF BIOETHANOL AS TRANSPORTATION FUEL

Ethanol is an oxygenated liquid fuel that contains 35% oxygen (Badger 2004). It is a perfect replacement for lead additives to gasoline used to boost the octane level and performances because of its high octane content (ethanol octane number is 113) (Durante et al. 2009; Wen et al. 2009). In fact, ethanol will soon establish itself as an octane enhancer (Shapouri et al. 2002). Higher oxygen content is good for the engine as this result in motor fuel that burns cleaner, reduces emissions of carbon monoxide and ozone-forming compounds, and reduces the build-up of gummy deposits in the engine (Wen et al. 2009).

Another feature unique to ethanol blended fuel is the ability to absorb water and prevent automotive gas-line freeze-up during cold weather (Goettemoeller and Goettemoeller 2007). Even the lowest ethanol-gasoline blend of E10 is able to absorb more moisture than a regular dosage of methyl or isopropyl alcohol, eliminating the need for adding additional gas line anti-freeze formula (Wen et al. 2009). Furthermore, ethanol burns cooler that gasoline, thus preventing engine valves from overheating and burning (Wen et al. 2009). Logistically,

ethanol is also more favorable as it is much less flammable than gasoline, making it safer than gasoline to store, transport and refuel (Goettemoeller and Goettemoeller 2007).

Routine operation for ethanol fueled vehicles are very similar to those conventionally fueled (Goettemoeller and Goettemoeller 2007). Special lubricants, to prevent pipe corrosion, for ethanol fueled vehicles are sometimes needed at a slightly higher cost than normal motor oils, but not all vehicles require these lubricants (US-DOE 1999). In addition, oil changes are less frequent, defraying some incremental costs (Wooley et al. 1999; Sheehan et al. 2004).

Fuel ethanol (E10) is attractive as a gasoline extender and, a mean of increasing the gasoline supply. When blended with gasoline, ethanol will increase the quantity of motor fuel available for consumers, while not compromising the quality of the vehicle performances (Goettemoeller and Goettemoeller 2007). According to the Sainz (2009), ethanol (113 to 115 octane rating) fueled vehicles exhibit the same power, acceleration, payload and cruise speed on high-compression engines, as their gasoline (87 octane) fueled counterparts.

THE IMPORTANCE OF BIOETHANOL FOR THE ENVIRONMENT

In general, ethanol is one of the safest and most environmentally friendly components in reformulated gasoline. Ethanol is nontoxic, water-soluble, and rapidly biodegrades in essentially all environments (Goettemoeller and Goettemoeller 2007). When blended with gasoline, ethanol proportionally reduces other toxic components in normal gasoline, such as sulfur and benzene (Durante et al. 2009; Wen et al. 2009). Ethanol can be used as a fuel oxygenate substitute for methyl tertiary butyl ether (MTBE), while achieving equal combustion performance (Wen et al. 2009). However, unlike MTBE, ethanol poses no threat to surface or ground water (Wen et al. 2009). In fact, in the United States, blending ethanol with gasoline is a common practice to meet the new oxygen requirements mandated by the Clean Air Act Amendments (CAA) of 1990 as ethanol is not a toxic pollutant when used as a motor fuel (Durante et al. 2009).

The U.S. Environmental Protection Agency (US-EPA 2002) credits ethanol blended gasolines with the reduction of hazardous emissions, which threaten air quality. When blended with motor fuel, ethanol reduces the use of cancer-causing gasoline compounds such as benzene, toluene, xylene, and ethyl benzene (Durante et al. 2009). Because of this, ethanol blended gasoline programs are currently being introduced in cities that exceed public health standards for carbon monoxide and ozone pollution (Sainz 2009). As a result, more than one-third of the gasoline for United States motor fuels contains some level of ethanol oxygenates to reduce harmful emissions and improve air quality (Jennings 2005).

The data that support the contributions of ethanol blended gasoline towards environmental friendliness are numerous. According to the US-EPA (2002), ethanol blended with gasoline produces fewer total toxic substances (Durante et al. 2009). Further studies made by the US-EPA (2002) also reported reductions in particulate emissions (20%), nitrogen oxide (10%) and sulfate emissions (80%). According to another study by the Argonne National Laboratory, vehicles that use ethanol actually help offset fossil fuels' greenhouse gas emissions, which contribute to global warming, by 35 to 46% (Shapuori et al. 2002; Durante et al. 2009; Sainz 2009). Corn-based ethanol alone shows between 20 to 30% reduction in emissions according to the same model (Brekke 2005). These data are summarized in Figure 1 below.
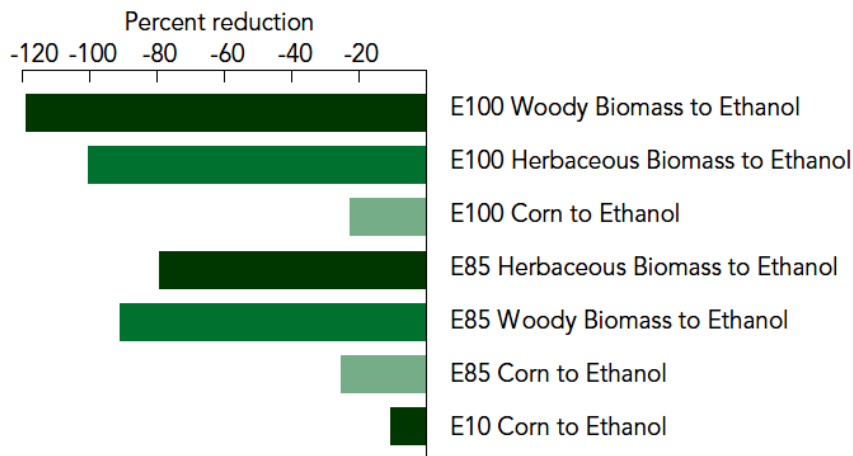
Figure 1. Greenhouse gas reductions compared to standard gasoline (Durante et al. 2009).

Ethanol programs, especially bioethanol from lignocellulosic biomass and agricultural feedstocks are perhaps one of the best means humankind has to fight air pollution and create a more sustainable carbon-neutral energy (Zhang 2008).

THE IMPORTANCE OF BIOETHANOL FOR THE ECONOMY

Ethanol and ethanol related industries have a tremendously positive impact on the US economy. According to Jenkins (2008), the rapid growth and maturing of the ethanol industries is an important business and economic success story of the past several years, spanning more than twenty states. These amounts covers three major areas: production operations (annual operations and transportation), capital spending for construction of new production facilities and research and development, adding $65.6 billion to the nation's Gross Domestic Product (GDP) in 2008 (Urbanchuck 2009). Along with this, fuel ethanol has also contributed to the lowering of transportation fuel prices, approximately $12 billions to the consumers as reported by Sainz (2009).

Demand for fuel ethanol will increase between 2002 and 2016, resulting in the increment of farm-level corn prices by 11.8% (Urbanchuck 2009). Currently, ethanol production has helped boost U.S. farm income by $5.5 billion, and over the next 15 years, an additional $6.6 billion of net cash income will be available annually for America's farmers (Vaughann 2000; Sneller and Durante 2007).

In 2008, ethanol production provided more than 494,000 jobs in all sectors, from ongoing production, constructions and R&D spurring growth in many rural areas (Urbanchuck 2009). From the agriculture sectors alone, about 54,000 jobs are created in the United States by the year 2020 (Stowers 2009). Another study estimated that increased production and use of renewable fuels will also create as many as 300,000 new jobs by 2016 (Vaughan 2000).

Production of fuel ethanol also helps curb the massive transfer of energy dollars to other countries, presently amounting more than $700 billion annually (Jenkins 2008). According to Urbanchuck (2009), crude oil imports account for more than 65% oil supplies and the imports of this commodity are the largest contributor to US trade deficit. Therefore, production of nine billion gallons of fuel ethanol reduces crude oil import by 321.4 million barrels in 2008, a value of approximately $32 billion.

The ethanol industry has a tremendously positive impact on the local economies around the plants themselves. For example, in a study focused on 50 mgpy wood-to-ethanol plant reported by Sneller and Durante (2007), the construction of this type of infrastructure will generate up to $200 million in income, and create about 6,000 jobs, with 540 to 830 permanent jobs that result in up to $48 million of annual income.

In a case study on Iowa's 12 farmer-owned ethanol facilities (with the capacity to produce 493 million gallons of ethanol, from 182 million bushels of corn annually), a total of $2.8 billion in sales went back to local communities (www.iowacorn.org 2009). Iowa's ethanol industry also has contributed more than 80,000 new job opportunities statewide, benefiting all employment sectors, especially rural communities (www.iowacorn.org 2009). Figure 2 shows the location of all biofuels (bioethanol and biodiesel) plants in Iowa. This is just one of the more than 100 communities that have benefit from similar infrastructures (Sneller and Durante 2007).
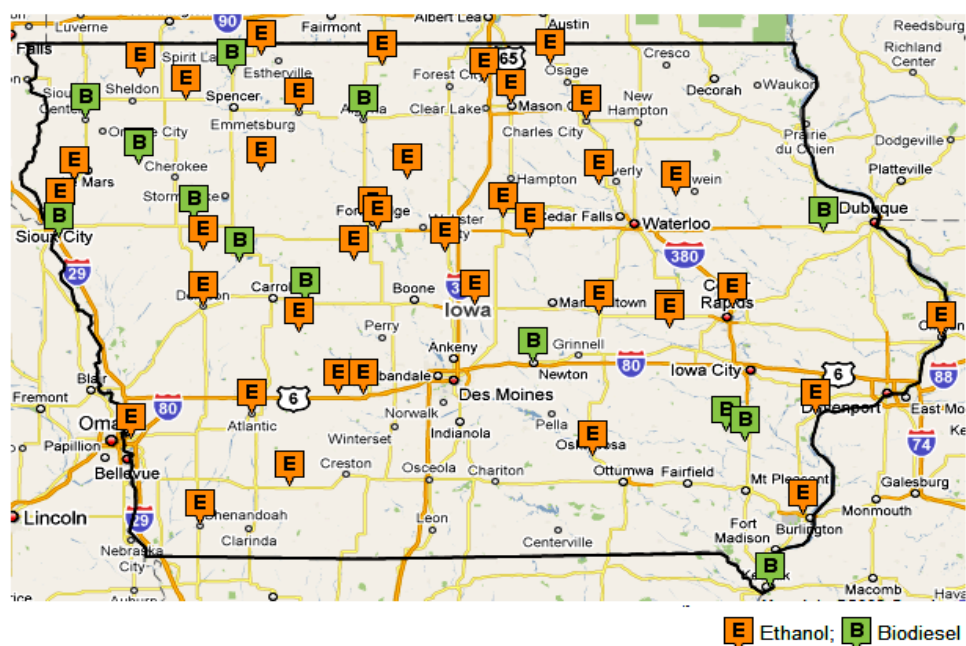
Figure 2. Biofuel plants in Iowa (http://data.desmoinesregister.com/ethanol2/index.php).

ENGINEERING AND PROCESSING IMPLICATIONS

Our present study was designed to address the issues with pretreatment and enzymatic saccharification of lignocellulosic biomass, namely corn stover. We aimed to eliminate, if not, minimize the harsh and detrimental pretreatment process by using biological means and a coupling of biological and mild chemical pretreatment. Just as important, we did not use expensive commercial enzymes, but, by inducing the production of the necessary enzymes in situ from the wood-rot fungi (white-rot fungus: *P. chrysosporium* and brown-rot fungus: *G. trabeum*). This dissertation therefore significantly reduces the costs associated with the

pretreatment and enzymatic processes, which reportedly are the most expensive steps in the production of cellulosic ethanol.

We determined the ethanol yields based on two calculations – theoretical yield (Doran and Ingram 1993) and practical yield based on the fermentation efficiencies assumption set by Balat and Balat (2009) and Goh et al (2009). This was done for both glucose (from cellulose) and xylose (from hemicellulose). The first calculation is shown in Table 1 and the second is shown in Table 2. We calculated the conversion values based on the compositional analysis of our corn stover (untreated corn stover with 38% cellulose, 31% hemicellulose; NaOH treated corn stover with 44% cellulose, 32% hemicellulose).

The yield values may seem low, probably due to the reason that part of the sugars liberated are consumed by the fungi as they proliferate during the enzyme induction phase (Shrestha et al 2008; Shrestha et al 2009).The enzymatic saccharification of untreated corn stover using *P. chrysosporium* and *G. trabeum* followed by SSF had ethanol yields of 10.55 and 8.76% of the theoretical maximum yield, which corresponds to approximately of practical 14.14 and 11.73% yields, respectively, with *S. cerevisea* as the fermenting organism (Table 3). When *E. coli* K011 is used as the fermenting organism, the theoretical yield was 10.53% (*P. chrysosporium*) and 12.19% (*G. trabeum*), while practical yield was 16.56% (*P. chrysosporium*) and 19.04% (*G. trabeum*) (Table 3).

Ethanol yields are generally higher for the alkaline and fungal treated corn stover samples. Ethanol yields of 11.47% (*P. chrysosporium*) and 12.31% (*G. trabeum*) of the theoretical

maximum yield was recorded, which corresponds to approximately of practical 15.32% (*P. chrysosporium*) and 16.44% (*G. trabeum*) yields, respectively, with *S. cerevisea* as the fermenting organism. When *E. coli* K011 is used as the fermenting organism, the theoretical yield was 11.55% (*P. chrysosporium*) and 15.42% (*G. trabeum*), while practical yield was 17.92% (*P. chrysosporium*) and 23.94% (*G. trabeum*) (Table 4). Thus, in our study, based on the cellulose and hemicellulose compositional analysis and taking into consideration the practical values and assumptions, the best ethanol yield is when the corn stover is pretreated with dilute NaOH followed by *G. trabeum* enzymatic saccharification with the percentage of yield at 23.94% (23.94 gram of ethanol/100 gram of corn stover) with *E. coli* K011.

For a larger scale study, our research conceptualizes four production platforms:

i. Biological (fungal) pretreatment and saccharification of corn stover with *S. cerevisea* as fermenting organism (Figure 3)

ii. Biological (fungal) pretreatment and saccharification of corn stover with *E. coli* K011 as fermenting organism (Figure 4)

iii. Coupled biological (fungal) and mild alkaline (NaOH) pretreatment, and saccharification of corn stover with *S. cerevisea* as fermenting organism (Figure 5)

iv. Coupled biological (fungal) and mild alkaline (NaOH) pretreatment, and saccharification of corn stover with *E. coli* K011 as fermenting organism (Figure 6)

Table 1. Theoretical ethanol yield from cellulose and hemicellulose using calculations from Doran and Ingram (1993).

Dry corn stover (1000 kg)	Untreated corn stover	NaOH pretreated
Cellulose		
Cellulose content	× 0.38	× 0.44
Ethanol stoichiometric yield	× 0.57	× 0.57
Final yield	217 g	251 g
Hemicellulose		
Hemicellulose content	× 0.31	× 0.32
Ethanol stoichiometric yield	× 0.57	× 0.57
Final yield	176 g	182 g

Note : untreated corn stover consist of 38% cellulose and 31% hemicellulose; NaOH treated consist of 44% cellulose, 32% hemicellulose.

Table 2. Practical ethanol yield from lignocellulose using the calculation from Balat and Balat (2009).

| Dry corn stover (1000 kg)	Untreated corn stover	NaOH pretreated
Cellulose		
Cellulose content	× 0.38	× 0.44
Ethanol stoichiometric yield	× 0.57	× 0.57
Glucose fermentation efficiency	× 0.75	× 0.75
Final yield	162 g	188 g
Hemicellulose		
Hemicellulose content	× 0.31	× 0.32
Ethanol stoichiometric yield	× 0.57	× 0.57
Xylose fermentation efficiency	× 0.50	× 0.50
Final yield	88 g	91 g

Note : untreated corn stover consist of 38% cellulose and 31% hemicellulose; NaOH treated consist of 44% cellulose, 32% hemicellulose.

Table 3. Ethanol yield from untreated corn stover.

	P. chrysosporium		*G. trabeum*	
	S. cerevisea	*E. coli* K011	*S. cerevisea*	*E. coli* K011
Ethanol per 100 g corn stover	2.29	4.14	1.90	4.79
Theoretical yield (%)	10.55	10.53	8.76	12.19
Practical yield (%)	14.14	16.56	11.73	19.04

Table 4. Ethanol yield from untreated NaOH treated corn stover.

	P. chrysosporium		*G. trabeum*	
	S. cerevisea	*E. coli* K011	*S. cerevisea*	*E. coli* K011
Ethanol per 100 g corn stover	2.88	5.00	3.09	6.68
Theoretical yield (%)	11.47	11.55	12.31	15.42
Practical yield (%)	15.32	17.92	16.44	23.94
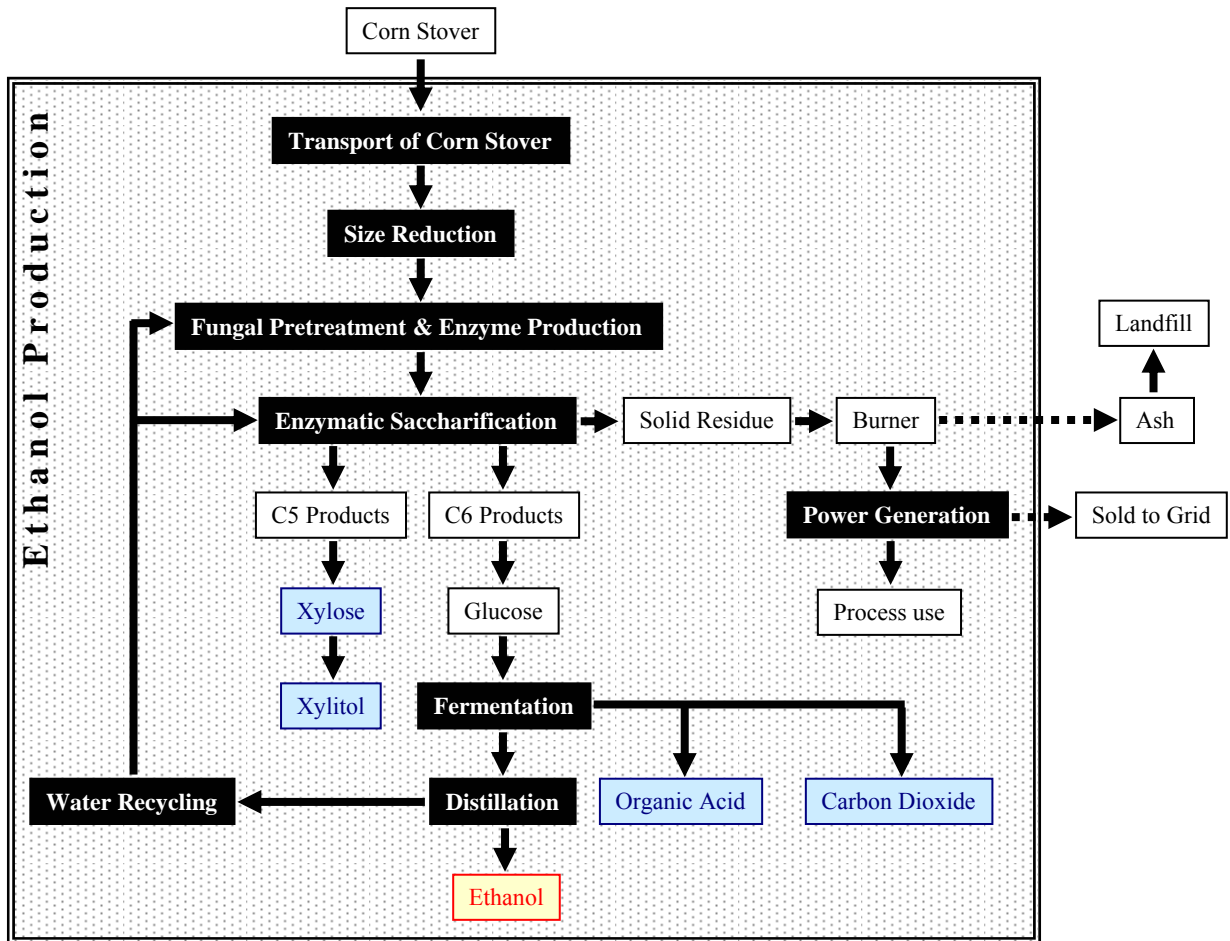
Figure 3. Biological (fungal) pretreatment and saccharification of corn stover with *S. cerevisea* as fermenting organism.

Main product – Ethanol

By products – Carbon dioxide, organic acid (mainly acetic acid), xylose, xylitol, yeast extract

Residue/waste – Water (to be recycled), solid residues (for power generation)
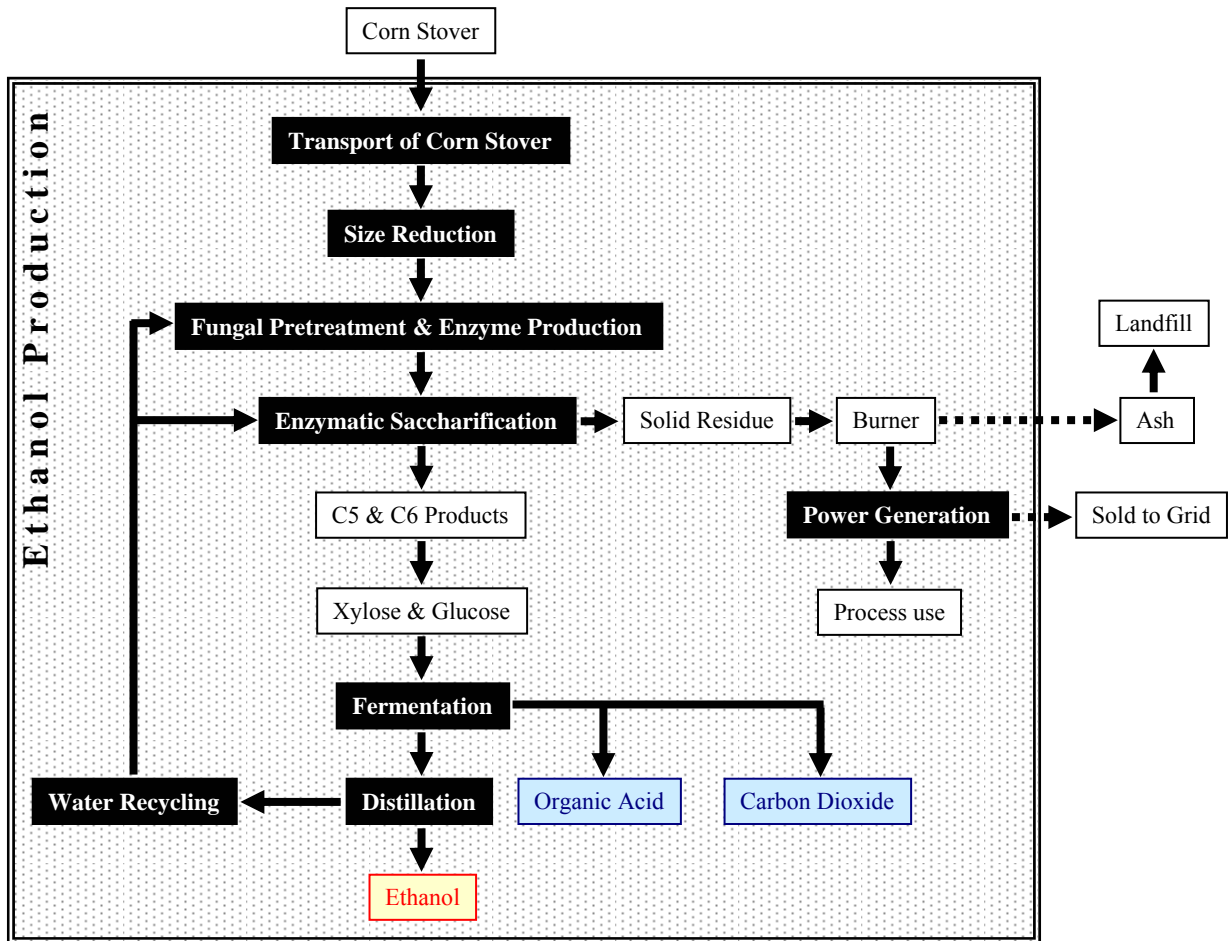
Figure 4. Biological (fungal) pretreatment and saccharification of corn stover with *E. coli* K011 as fermenting organism.

Main production – Ethanol

By products – Carbon dioxide, organic acid (mainly acetic acid)

Residue/waste – Water (to be recycled), solid residues (for power generation)
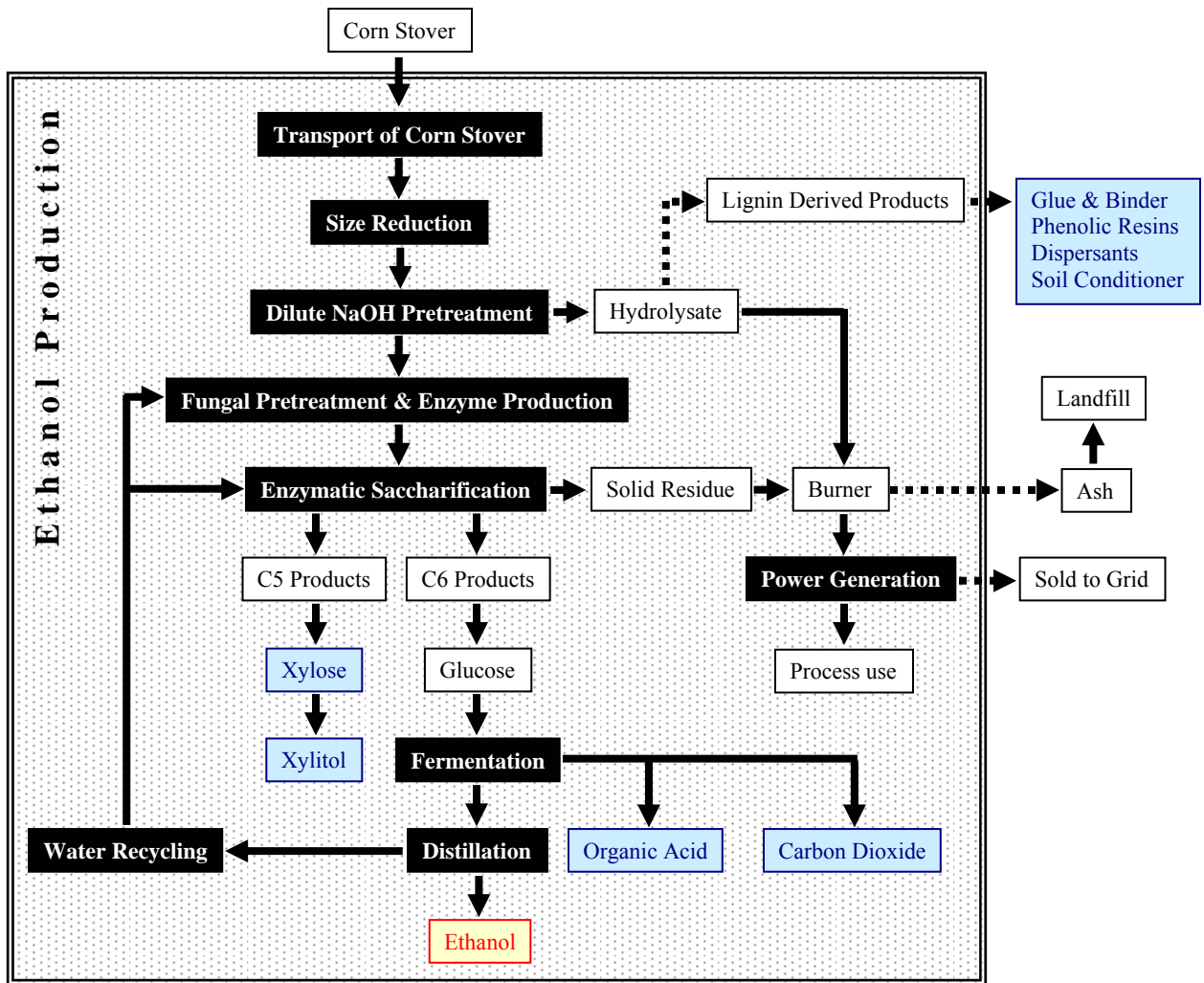
Figure 5. Coupled biological (fungal) and mild alkaline (NaOH) pretreatment, and saccharification of corn stover with *S. cerevisea* as fermenting organism.

Main production	– Ethanol
By products	– Lignin related/derived products, carbon dioxide, organic acid (mainly acetic acid), xylose, xylitol, yeast extract
Residue/waste	– Water (to be recycled), solid residues (for power generation)

Figure 6. Coupled biological (fungal) and mild alkaline (NaOH) pretreatment, and saccharification of corn stover with *E. coli* K011 as fermenting organism

Main product   − Ethanol

By products   − Lignin related/derived products, carbon dioxide, organic acid (mainly acetic acid), yeast extracts

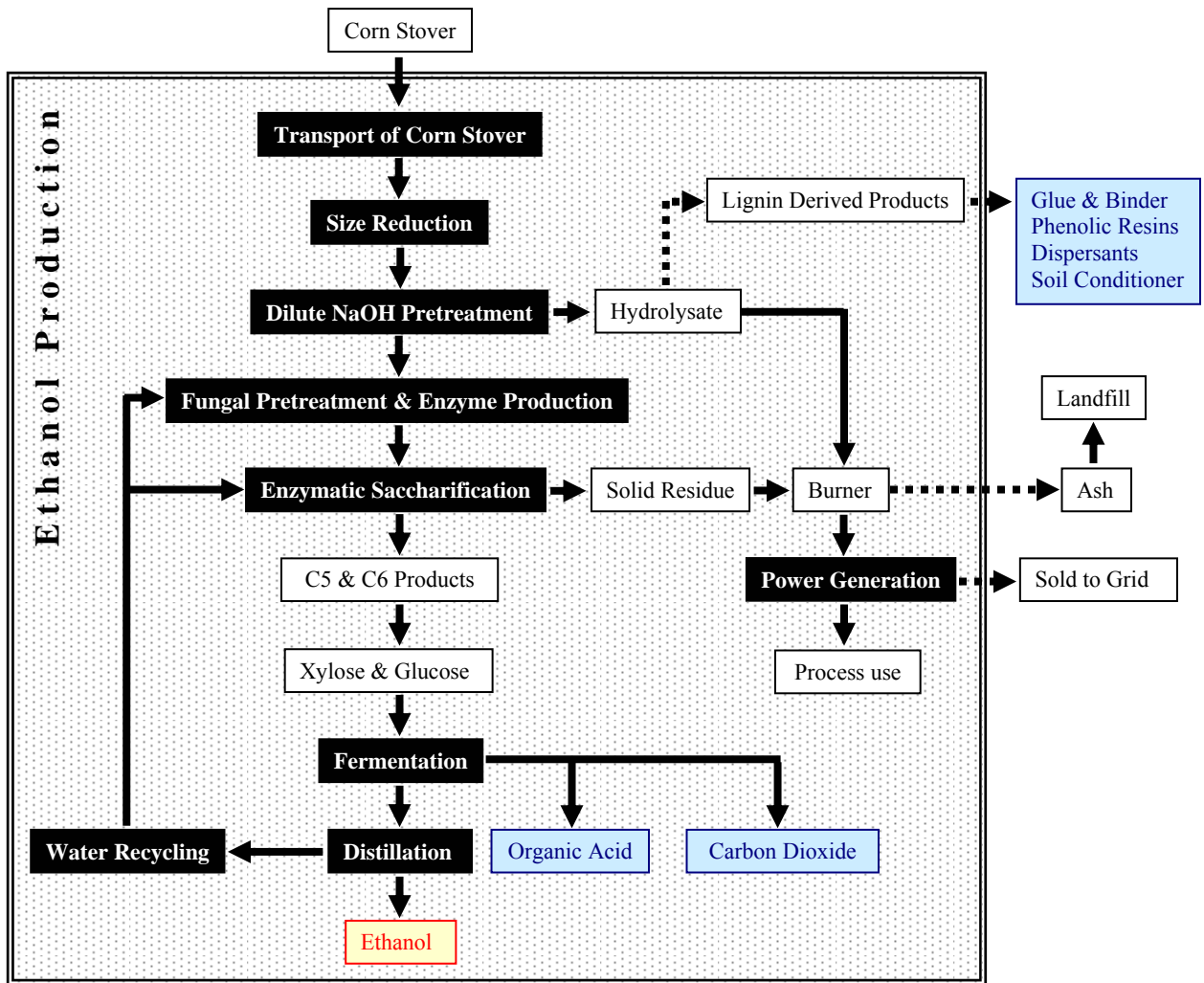**Residue/waste**   − Water (to be recycled), solid residues (for power generation)

CONCLUSION

The ethanol industries are indeed making significant contributions to the economy, both locally and nation-wide, by creating demand for local goods and services, stimulating investment, generating tax revenues, invigorating grain markets, creating employment opportunities with salaries exceeding regional averages, and displacement of imported oil. As for the case of lignocellulosic based bioethanol, engineering aspects, process optimization and production economics are important considerations that may render cost to be competitive with fossil fuels. This will ensure marketability and create the incentive for the mass consumers to adopt renewable sources of fuel.

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CHAPTER 7: GENERAL CONCLUSION AND RESEARCH RECOMMENDATIONS

Since 2006, there has been a significant increase in the utilization of ethanol in the United States, and this trend is also observed globally. Research and production of fuel ethanol are currently being promoted in many sectors, especially in the transportation sector. In the hope of increasing the production and use of biofuels for transportation and energy purposes, biorenewable resources and environmentally friendly processes are of particular interest because they offer energy security, economics possibilities and mitigation of greenhouse gases. Efforts being made to transition the feedstock from corn starch and sugar based to lignocellulosic biomass are indeed noble, and lignocellulosic ethanol is projected to make a significant contribution to future energy needs that will be even more environmentally friendly than first-generation biofuels.

Lignocellulosic ethanol platform offers greater flexibilities as the technologies may be adapted across various types of plant biomass materials. These abundant feedstocks are low cost and offer many possibilities for the development and implementation of biobased industries that supply the world energy needs for the international biofuel market. Of the various feedstocks being studied, corn stover is a very attractive candidate because of its close proximity to existing ethanol plants. Corn stover and corn kernel can be collected at the same time, thus lowering labor, harvesting and transportation costs.

In the past decade, there are worldwide efforts being explored to improve production and reduce the overall cost of plant biomass ethanol, as the success of lignocellulosic ethanol is

directly related to the profitability and environmental sustainability of the overall production process. One method to boost competitiveness is by introducing cost effective pretreatment and enzymatic processes. An ideal lignocellulolytic biocatalyst should degrade the three main components of corn stovers, namely, the cellulose, hemicellulose and lignin. In this dissertation, we have successfully applied inexpensive fungal and mild alkaline pretreatment and saccharification of corn stover into sugars, both C5 and C6, that are readily fermentable by *S. cerevisiae* and *E. coli* K011.

The biological pretreatment and lignocellulolytic activities of *P. chrysosporium*, *G. trabeum* and *T. reesei* on other lignocellulosic biomass is a very promising area for research as there are numerous applications warranting the application of these processes. We believe that through advancement in biotechnology, molecular biology and genetic engineering, these wood-rot fungi could be further manipulated to perform maximized biological pretreatment and simultaneous enzymatic hydrolysis of lignocellulosic materials for ethanol production. While the addition of commercial cellulases may not be eliminated altogether, the results of our studies do offer promising potentials signs that there could be savings in enzyme costs.

Thus, the outcome of our dissertation may lead to the following advantages:

i. ***Environmentally friendlier processing*** – ethanol producers can minimize the environmentally detrimental pretreatments processes by coupling the current chemical and physical based technologies with biological means.

ii. ***Economical and technological viable ethanol production*** – ethanol plants can produce their own enzymes *in situ* to supplement expensive commercial preparations.

iii. ***More effective ethanol production*** - using adaptive and living fungi, such as *P. chrysosporium* or *G. trabeum*, will reduce the inhibiting by-products during saccharification and fermentation, as these fungi have the abilities to convert and degrade some of these chemicals.

In conclusion, lignocellulosic ethanol has the potential to fulfill the President's goals. With emerging technologies that are more efficient and less expensive, the problem of biomass recalcitrance, that hinders the commercial production of ethanol from various lignocellulosic biomasses, may be resolved and the future of biofuels from lignocellulosic sources is promising. These biotechnological advances will address the issues such as biomass feedstock yield improvement and processing steps.

Future studies recommendations:

While the results of our studies show promising applications of our procedures, we recommend the following strategies to further improve on the existing processes:

1. **Screening of other lignocellulosic enzyme inducers**

   We see that there are significant differences of activities when pure cellulose and corn stover are used to induce enzyme productions, especially in *G. trabeum*. While no fermentable sugar was detected in pure cellulose induced *G. trabeum*, corn stover (untreated and treated) clearly indicates that this fungus has the best enzymatic performance in liberating fermentable sugars. Therefore, we suggest the screening of several other lignocellulosic biomass, such as poplar, switchgrass and distillers dried grains (DGGs) to determine the best inducers.

2. **Optimize solid state parameters**

   We also recommend the manipulation of other parameters such as the duration of solid state fermentation, aeration level, pH and carbon:nitrogen ratios.

3. **Concentration of enzymes**

   The saccharification of the raw material and ethanol yield during SSF are highly dependent on the enzymatic activities of the fungal species. Therefore, to possibly improve the performances of the enzymes from the *P. chrysosporium* and *G. trabeum* innocula, we suggest to concentrate of the enzyme preparation, and perhaps to include

protease inhibitor to prevent enzyme degradations. Purified and concentrated enzyme consortia would definitely improve the liberation of sugar. The potency of these enzymes preparations can be compared against the popular commercial blends (Spezyme CP and Accellearase 1000).

4. Mixed culture

Another possible process improvisation is to perform solid state fermentation using a mixed culture of the three fungi. The logic behind this suggestion is that while *P. chrysosporium* effectively degrade the lignin components, co-culturing of *G. trabeum* and *T. reesei* may assist in further hydrolysis of the cellulose-hemicellulose components.

ACKNOWLEDGEMENT

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