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Enhanced bioprocessing of lignocellulose: Wood-rot fungal saccharification and fermentation of corn fiber to ethanol

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Enhanced bioprocessing of lignocellulose: Wood-rot fungal saccharification and fermentation of corn fiber to ethanol

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ABSTRACT

This research aims at developing a biorefinery platform to convert corn-ethanol co-product, corn fiber, into fermentable sugars at a lower temperature with minimal use of chemicals. White-rot (*Phanerochaete chrysosporium*), brown-rot (*Gloeophyllum trabeum*) and soft-rot (*Trichoderma reesei*) fungi were used in this research to biologically break down cellulosic and hemicellulosic components of corn fiber into fermentable sugars. Laboratory-scale simultaneous saccharification and fermentation (SSF) process proceeded by *in-situ* cellulolytic enzyme induction enhanced overall enzymatic hydrolysis of hemi/cellulose from corn fiber into simple sugars (mono-, di-, tri-saccharides). The yeast fermentation of hydrolyzate yielded 7.1, 8.6 and 4.1 g ethanol per 100 g corn fiber when saccharified with the white-, brown-, and soft-rot fungi, respectively. The highest corn-to-ethanol yield (8.6 g ethanol/ 100 g corn fiber) was equivalent to 42 % of the theoretical ethanol yield from starch and cellulose in corn fiber. Cellulase, xylanase and amylase activities of these fungi were also investigated over a week long solid-substrate fermentation of corn fiber. *G. trabeum* had the highest activities for starch (160 mg glucose/mg protein.min) and on day three of solid-substrate fermentation. *P. chrysosporium* had the highest activity for xylan (119 mg xylose/mg protein.min) on day five and carboxymethyl cellulose (35 mg glucose/mg protein.min) on day three of solid-substrate fermentation. *T. reesei* showed the highest activity for Sigma cell 20 (54.8 mg glucose/mg protein.min) on day 5 of solid-substrate fermentation.

The effect of different pretreatments on SSF of corn fiber by fungal processes was examined. Corn fiber was treated at 30 ^{o}C for 2 h with alkali [2% NaOH (w/w)], alkaline peroxide [2% NaOH (w/w) and 1% H_2O_2 (w/w)], and by steaming at 100 ^{o}C for 2 h. Mild pretreatment resulted in improved ethanol yields for brown- and soft-rot SSF, while white-rot and Spezyme CP SSFs showed no improvement in ethanol yields.

We showed that saccharification of lignocellulosic material with a wood-rot fungal process is quite feasible. Corn fiber from wet milling was best degraded to sugars using aerobic solid state fermentation with the soft-rot fungus *T. reesei*. However, it was shown that both the white-rot fungus *P. chrysosporium* and brown-rot fungus *G. trabeum* had the ability to produce additional consortia of hemi/cellulose degrading enzymes. It is likely that a consortium of enzymes from these fungi would be the best approach in saccharification of lignocellulose. In all cases, a subsequent anaerobic yeast process under submerged conditions is required to ferment the released sugars to ethanol.

To our knowledge, this is the first time report on production of cellulolytic enzymes from wet-milled corn fiber using white- and brown-rot fungi for sequential fermentation of corn fiber hydrolyzate to ethanol.

Keywords: lignocellulose, ethanol, biofuel, bioeconomy, biomass, renewable resources, corn fiber, pretreatment, solid-substrate fermentation, simultaneous

saccharification and fermentation (SSF), white-rot fungus, brown-rot fungus, soft-rot fungus, fermentable sugars, enzyme activities, cellulytic enzymes *Phanerochaete chrysosporium, Gloleophyllum trabeum, Trichoderma reesei, Saccharomyces cerevisiae*.

CHAPTER 1: GENERAL INTRODUCTION

Developing countries demand more energy in the midst of enormous economic development. The increased demand for energy has led to escalating fossil fuel prices. Various alternatives have been sought to manage and stabilize energy security especially in Europe and America. Renewable biofuel generation, application and its research & development have received greater global attention and implication. Corn starch and sugar cane based bio-ethanol production continues to advance and improve in USA and Brazil with current production capacities of 25 and 19 x 10^9 liter per year, respectively (RFA 2008). While this production can be expanded, it is limited by the availability of suitable cropland and climates for the respective crops. Therefore, second generation biofuels, from more ubiquitous and recalcitrant cellulosic crops has recently received increasing attention for its potential to substantially replace fossil fuel demand in coming decades.

Cellulosic ethanol production utilizes primarily lignocellulose (glucose polymer) as feedstock, which requires pretreatment via physical, chemical and/or biological means followed by cellulosic enzymatic hydrolysis to glucose and fermentation to ethanol. Cheap and abundant (native) availability (USA produces ~ 1.3 x10^9 tons of biomass annually, ORNL-USDOE, 2005) of cellulosic biomass is favorable towards sustainable renewable fuel generation. Cellulose occurs mainly in a network with hemicellulose and lignin, thus called lignocellulose, providing recalcitrant properties

to plants. Therefore, pretreatment and enzyme hydrolysis steps are very necessary but costly steps during cellulosic biodegradation and ethanol generation (Mosier et al., 2005). Chemical (alkali and acid) pretreatment and inhibitory compounds, produced during such pretreatment are detrimental to subsequent fermentation and such pretreatment is expensive. It is therefore necessary to reduce chemical cost and environmental footprints, and to explore alternative environment friendly and economically sound processes like direct biological conversion of cellulose to ethanol.

Our ongoing cellulose-ethanol research bio-mimics the natural process of wood biodegradation. Scientists and plant pathologists conduct research to explain physiology, biochemistry and mechanism of various types of fungal wood-rots.. Initial studies explored the cause and prevention of fungal deconstruction of wood. Extracellular enzymatic degradation and non-enzymatic oxidative degradation of lignocellulose were identified as the main wood decay mechanisms. This doctoral research seeks to exploit natural wood-rot degradation mechanism to produce fungal cellulase/hemicellulase enzymes to degrade cellulose for biofuel production. We investigated white- , brown- and soft-rot fungi in biological pretreatment and hydrolysis of wet-milled corn fiber, a collected and abundant lignocellulosic feedstock, mainly corn fiber, to produce fermentable sugar that was bioconverted to ethanol.

1.1 Research objectives

a. Evaluate white-, brown- and soft-rot fungi (*Phanerochaete chrysosporium, Gloeophyllum trabeum and Trichoderma reesei,* respectively) for saccharification of corn fiber via enhanced enzymatic hydrolysis, and the subsequent fermentation of fermentable sugars into ethanol using *Saccharomyces cerevisiae*.

b. Evaluate effectiveness of mild alkali, alkaline peroxide and steam pretreatment of wet-milled corn fiber prior to solid-substrate fermentation by white-, brown- and soft-rot fungi and subsequent fermentation of hydrolyzate to ethanol using *S. cerevisiae*.

c. Evaluate extracellular enzyme profiles during solid-substrate fermentation of corn fiber using three fungi *P. chrysosporium, G. trabeum and T. reesei.*

1.2 Justification

1.2.1 Why corn fiber?

The Renewable Fuels Association (RFA, 2008) reported that the167 ethanol plants, located in 26 states in the United States, have total annual ethanol production capacity over 52 $x10^9$ liter. In addition to ethanol, these industries also produce

excessive quantities of fibrous co-product i.e., corn fiber, which is basically incorporated into animal feed. Depending on the process, dry-grind or wet milling of corn, the co-product is further processed and sold as distiller's dried grain with solubles (DDGS) or corn gluten feed/meal. Over 9 million metric tons of DDGS and 2.4 million metric tons of corn gluten feed were produced in 2006, when the total ethanol production capacity was just about 18 $x10^9$ liter (about 1/3 of present ethanol production capacity). In two years, bioethanol and its co-products production has increased by 300%. Such huge quantities of co-products pose serious management issues. It makes sense to process these collected co-products into more ethanol. Wet-milled corn fiber contains (on dry matter basis [w/w]) comparable amounts of lignin (2%), cellulose (18%), hemicellulose (35%) and some residual starch (18%) (Abbas et al.,2004). Conversion of glucose fraction from cellulose and starch to ethanol would yield an additional 495 $x10^6$ liters of ethanol per year. Xylose bioconversion from hemicelluloses potentially would add $248x10^6$ liters of ethanol. Corn fiber, which is produced in corn wet-milling plants, is comparably a cleaner lignocellulose feedstock compared to other lignocelluloses sources as its generation has been preceded by many cleaning, extraction and operational procedures in the plants. Needless to mention, the lignin content is also very low in corn fiber. Corn fiber therefore serves as a model cellulosic feedstock for cellulosic ethanol production.

1.2.2 Why wood-rot fungi?

Various wood-rot fungi: white- and brown-rot, are reported to degrade lignin, cellulose and hemicellulose. They produce extracellular enzymes like ligninase, cellulase and hemicellulase to degrade these complex polymers. Utilization of their extracellular enzyme consortia for biodegradation of lignocellulose co-products such as corn fiber provides a source of simple sugars which can be fermented to ethanol. *In situ* extracellular enzyme secretion by these wood-rot fungi can eliminate

i. pretreatment cost of lignocellulose degradation, and
ii. enzyme cost by producing on-site enzymes production .

1.2.3 Wood-rot fungi and corn fiber in a consolidated process

We previously reported the two wood-rot fungi: *Phanerochaete chrysosporium* (white-rot fungus) (Shrestha et al., 2008) and *Gloeophyllum trabeum* (brown-rot fungus) (Rasmussen et al., 2008) in bench scale solid-substrate fermentation followed by simultaneous saccharification and fermentation to ethanol. Over a period of 1 to 5 days, solid-substrate fermentation of corn fiber using white- and brown-rot fungi in aerobic conditions at mesophilic temperature (37^{o}C) had outstanding results confirming comparable saccharification of fiber. Enzyme activity assay results confirmed fungal hydrolysis of cellulose, hemicellulose and starch. Lignin degradation activity was also confirmed via Klason lignin assay.

1.2.4 Simultaneous saccharification and fermentation (SSF)

As the fungal saccharification process of corn fiber proceeds, sugar consumption by the fungi occurs. It is necessary to maximize cellulytic enzyme production while minimizing the fungal sugar consumption for maximum net ethanol yield. Shorter aerobic solid-substrate fermentation incubations and subsequent anaerobic simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae* is needed to maximize polysaccharide bioconversion to ethanol. Thus, we minimized the solid-substrate fermentation (which is usually considered to be lengthy process) to 2 days then incorporated SSF incubation for 6 days in anaerobic conditions. This eliminated fungal sugar consumption, retained enzyme active to hydrolyze cellulose to glucose and sequentially fermented the hydrolyzate to ethanol – all in one consolidated process.

1.2.5 Corn fiber to ethanol yield

The fungal saccharification and fermentation of the corn fiber to ethanol result was very exciting. The white-rot saccharification and fermentation led to 3 g ethanol/ 100 gram corn fiber and brown-rot to 4 g ethanol/ 100 g. These fungi were also able to ferment sugar to ethanol without addition of yeast during SSF. The results were 1.7 and 3.3 g ethanol/ 100 g corn fiber for white- and brown-rot fungi, respectively. To our knowledge, this was the first report of this *in situ* bioconversions of corn fiber to

ethanol by these wood-rot fungi (Shrestha et al., 2008a and Rasmussen et al., 2008).

1.2.6 Enhanced enzyme induction

Reported yield of ethanol i.e., ~ 4 g ethanol/ 100 g fiber has potential of producing 50 liters of ethanol per metric ton of corn fiber from a potential of 4 g ethanol/ 100 g fiber (starch and cellulose). Therefore, we continued to conduct research to increase corn fiber bioconversion to ethanol production. The first research manuscript in this dissertation was submitted to *Journal of Agricultural and Food Chemistry (JAFC)* which describes an improved process for enzyme induction and sequential ethanol production via fungal SSF process (Shrestha et al., 2008b). We were able to yield about 120 liters of ethanol per metric ton of corn fiber which was more than twice our previous reported ethanol yields. We continued evaluating parameters to improve hydrolytic enzyme secretion by wood-rot fungi and the sequential SSF process. This thesis dissertation attempts to disseminate the scientific research approaches considered to achieve this improved fungal saccharification and fermentation of corn fiber to ethanol.

1.3 Dissertation organization

This dissertation consists of seven chapters. The first chapter is a general introduction, which also includes research objectives and justification for further research approach. Literature review is contained in the second chapter. It covers global and local energy scenario, introduction to lignocellulose and its application towards second generation biofuel production. It also discusses various pretreatment (physical, chemical and biological) methods for cellulosic biomass. Short discussion on ligninolytic and cellulolytic enzymes are coupled with introductions on wood-rot and soft-rot fungi. Prospects of cellulosic fuel are also discussed in the literature review. Therefore, this chapter may also be considered for publication. Chapters 3, 4 and 5 are presented asjournal papers. The third chapter is focused on enhanced enzymatic induction, using wood and soft-rot fungi, for simultaneous saccharification and fermentation of corn fiber to ethanol. This chapter has been submitted to *Journal of Agricultural and Food Chemistry* and is in process of resubmission with incorporation of edits as per reviewers' comments. The fourth chapter discusses mild pretreatment of corn fiber and its effect on overall fungal saccharification and fermentation of corn fiber to ethanol. The fifth chapter reports on enzyme profiles of wood and soft-rot fungi during solid substrate fermentation. These last two chapters would also be considered for high impact journal publications. Chapter 6 focuses on engineering implications and significance of the outcomes of the dissertation. This chapter discusses practical process validation in existing corn biorefineries and technology transfer towards lignocellulose based bioeconomy. General conclusion is contained in chapter 7. Figures, tables and

equations are embedded within the texts of each chapter and literature citations are added at the end of each chapter.

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CHAPTER 2: LITERATURE REVIEW

(*to be submitted as a journal article*)

2.1 Introduction

Gross domestic product (GDP) is an indicator for a nation's socio-economic development and is correlated with energy consumption (Dincer and Dost, 1997). Most recently, we have experienced the shift of industrial development from developed nations to many developing nations like China and India. The growth in electricity demand in Chinese industries has increased the outputs and henceforth, has improved China's GDP (Shiu and Lam, 2004). These countries increased their share for the world's total energy consumption to 18% in 2005 (EIA-2008). It has been projected that by 2030 the non-OECD (Organization for Economic Co-operation and Development) countries including China and India will increase their energy consumption rate to 25% of the world's energy consumption. Likewise, socio-economic status and purchasing capacities of people (~ 37% of world population) in these developing nations have also improved in recent years. This further demands various forms of energy (coal, natural gas, petroleum and electricity), food and natural resources (to provide raw materials for industries). Nonrenewable resources have dramatically changed our environment by increasing CO_2 a greenhouse gas (GHG). Utilization of energy efficient technologies, reduction in GHG emission and

exploration of renewable energy resources can significantly mitigate the alarming concerns of energy, economy and environment.

Johansson et al. (1992) further addressed that opting energy efficiency strategies could not alone resolve the energy demands of all countries in the world. However, utilization of renewable energy resources (biomass, solar, wind, hydro, geothermal) could resolve a greater portion of the energy demand problem and may replace up to 40% of the fuel demand by the middle of the 21st century. Rosen (2002) suggested that energy efficiency is further required for using sustainable energy resources. By 2030, the United States aims to utilize its plant biomass for replacing 20% of transportation fuel and 25 % of industrial (USDA-DOE, 2005). Plant derived biomass energy has been reported to be 15% of world energy and 38% in developing countries. Biomass has diverse application in producing heat, electricity (e.g. gasification) and liquid fuel (e.g. ethanol). On commercial scale, biomass energy and bio-economy promise sufficient energy supply, rural employment and a closed carbon cycle (Hall et al., 1992).

2.2 Why Biofuel ?

Biomass energy (bioenergy) is basically heat, electricity, and liquid and gas fuels derived from plant materials. Sustainability of biomass energy mainly depends on clean technology, government mandates, wide spread application, minimal impact

on food and feed, alternative but competitive to conventional fossil fuel, and reliable and consistent supply of renewable biomass resources (Hall, 1997). Corn and sugar-cane based ethanol industries in the USA and Brazil, produced over 49 and 38% of the total ethanol production in the world, respectively (RFA, 2008). However, these crops cannot sustainably satisfy the growing demand of liquid transportation fuels. Concerns such as increased planted corn acreage, increased corn into biofuel production versus food production, and increased demand for soil amendments (nitrogen and phosphorus) are some negatives for corn based ethanol production. Thus, non-food carbon sources like lignocelluloses are favorable alternatives.

Lignocellulose (woody) biomass, produced from fixation of carbon dioxide and utilization of solar energy by photosynthetic plants, is abundant in various forms: native forest, dedicated tree crops, forest residues, agricultural residues, industrial residues and so on. It has been estimated that over 1.3 $x10^9$ tons of woody biomass from agricultural, forest and industrial residues are produced annually in the United States (USDA-DOE, 2005). At ca. 2.4 barrels of ethanol per metric ton of biomass (this is equivalent to 100 gallons of ethanol per metric tons of dry biomass), the US can annually produce 3.12 $x10^9$ barrels of ethanol from the aforementioned quantity of biomass in the United States. It has been targeted to replace as much as 30% of total petroleum needs in USA by producing fuels and biobased products from these bioresources. While many research and development projects have been initialized recently, there still exist a lot of barriers in economic and sustainable development of renewable biofuels (Painuly, 2001). The foremost barrier is the hardiness of the raw

materials (plant tissues) towards degradation. Such recalcitrant properties in plants are provided by the structural integrity of lignocellulosic tissues (basically complex carbohydrates and polyaromatic carbons). There are 24 cellulosic ethanol plants that are under development phase in different parts of the USA to produce 200 to 300 MGY of cellulosic ethanol (RFA-2008a). The technical and economical challenges would impede high throughput production of cellulosic ethanol from these industries.

2.3 What is lignocellulose?

Plant and wood cell walls consist of structural carbon polymers: cellulose and hemicellulose. These are basically composed of carbohydrates (c-5 and c-6 sugars) and are often enmeshed by lignin, a complex polymer of methoxylated and hydroxylated polyphenylpropane (Hamelinck et al., 2005). Cellulose, hemicellulose, and lignin together with a little ash form the complex lignocellulose structure (figure 1). The structural complexity of hemicellulose (c-5 and c-6 sugar complex), lignin, crystalline structure of cellulose (β-1,4-glucose polymer) and pectin make lignocellulose highly insoluble and provide plant cell wall resistance to attack (Hamelinck et al., 2005). Therefore, delignification is required to deconstruct the lignin mesh and further liberate cellulose and hemicellulose from lignocellulose. The cellulose and hemicellulose have to be further degraded into pentose and hexose sugars, which could then serve as feedstock for biofuel production (e.g. ethanol) and many other bio-based products. Cellulose is a long-chain polymer of glucose with

β-1,4-glycosidic links that are aggregated, via hydrogen bonding, to form microfibrils (figure 2) and thus impart crystallinity to the structure. Cellulose microfibrils are strengthened and stabilized by linear and branching chains of hemicellulose (Hamelinck et al., 2005), which accounts for about 20 to 40% of the structural integrity in plant cell walls. The holocellulose structure is further hardened by an amorphous and three-dimensional (3D) matrix of a complex polyphenyl propane polymer – lignin, which comprises about 10 to 25% of the woody biomass (Crawford, 1981; Knauf and Moniruzzaman, 2004; Hamelinck et al., 2005).

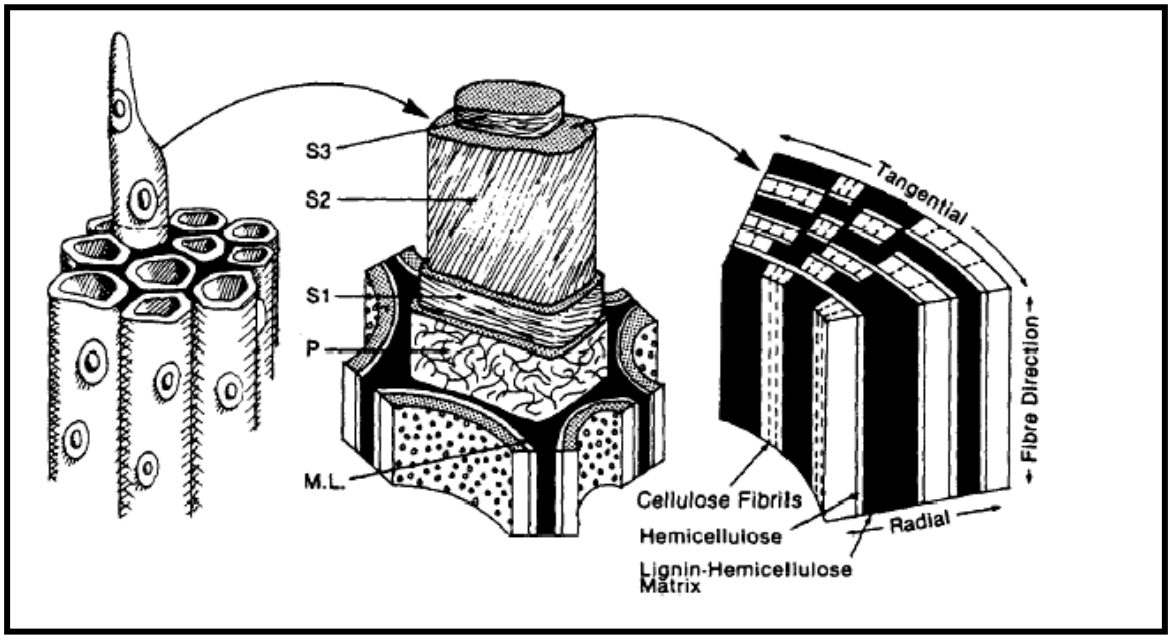


Figure 1: Components of woody tissue. Left: Bundles of woody tissues. Middle: Illustration of cell wall of a single plant cell. Right: Arrangement of hemicellulose and lignin with respect to cellulose microfibrils. Source: *Kirk (1985)*

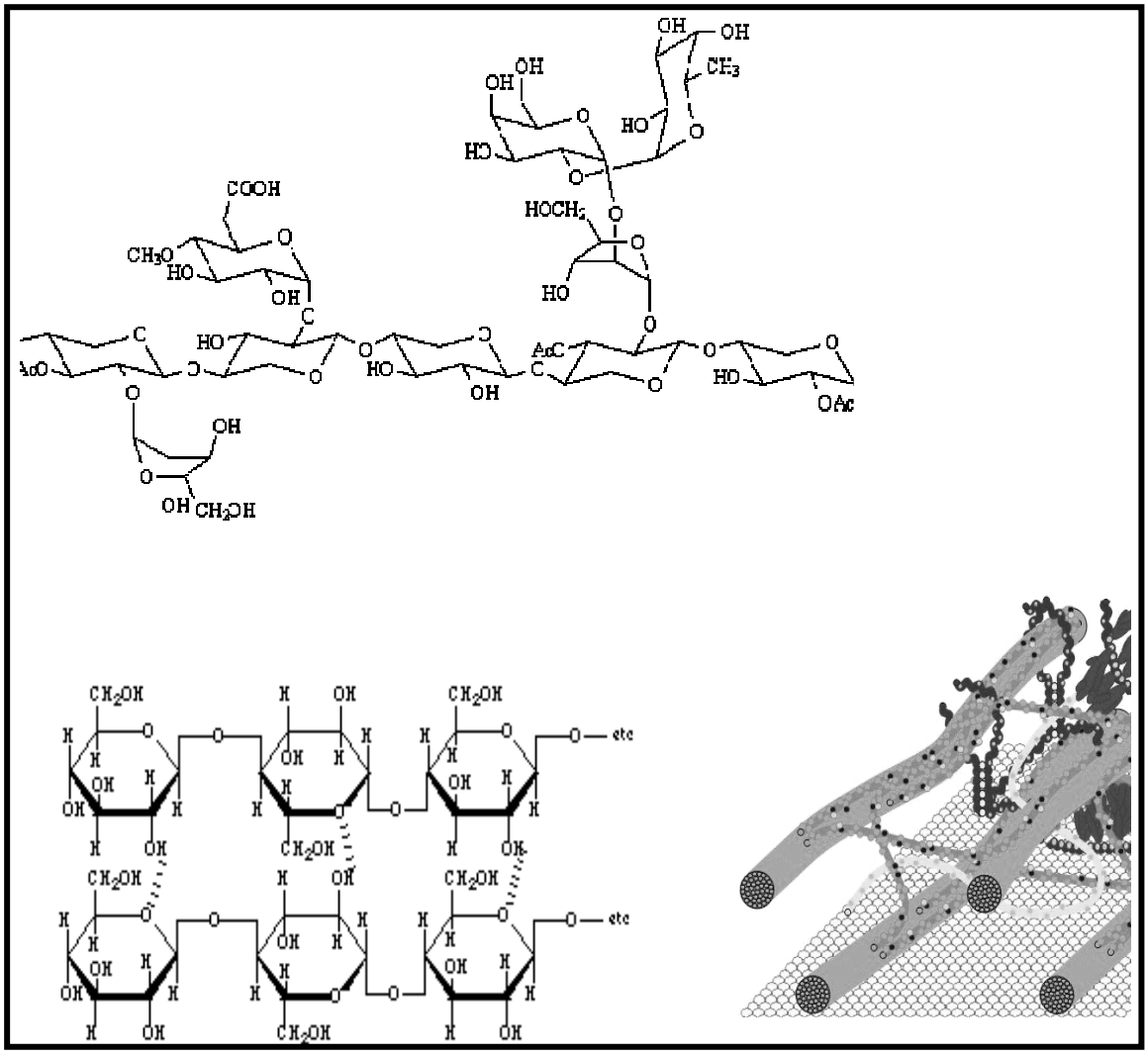


Figure 2: Structure of hemicellulose and cellulose. [Top] Hemicellulose showing glucose, glucuronic acid, mannose, arabinose, and xylose units. [bottom left] Portion on cellulose chains showing the bonds between the glucose molecules. [bottom right] Schematic of cellulose microfibrils enmeshed in hemicellulose.

Sources: Indiana University (2006), Max Planck Institute of Molecular Plant Physiology (2006)

2.4 Lignocellulose as source of sugars

Lignocellulose is abundantly available (10 to 50x10^9 tons annually) and therefore, fractionation (hydrolysis) of its components to lignin, cellulose and hemicellulose may be utilized to produce various commodities (Table 1) listed by Brown (1983) and others. Dale (1987) emphasized the biomass refinery approach – holistic usage of all lignocellulosic components and development of economical biomass pretreatment process to provide cheaper and feasible feedstock. The complexity of lignin and crystallinity of cellulose, both hinders hydrolysis of the lignocellulose via chemical, physical or biological (enzymes and microbes) means (Millett et al., 1976). Enzymatic hydrolysis of lignocellulose is preferable but has been considered to be slow. Physical and chemical pretreatments enhance the enzymatic conversion (Fan et al., 1982; Puri, 1984) but the cost of pretreatment and feedstock may be as much as 50% of the ethanol production cost (Chum et al., 1985). Thus, plant biomass pretreatment represents one of the major hurdles for biofuel production.

Table 1: Products from lignocellulose. Source: *Brown* (1983)

product	application
mixed sugars liquor	fermentation processes; single cell protein (SCP), ethanol, butanol, organic acids, antibiotics, enzymes, etc.
glucose	fermentation processes
	fructose syrups
	ethylene, butadiene, hydroxymethyl furfural (HMF), laevulinic acid,
xylose	fermentation processes with selective organisms
	furfural, adiponitrile, xylitol sweetner
other sugars	fermentation processes with selected organisms
	animal feed carbohydrates
lignin	fuel, carbon black
	sulfonates as dispersants and emulsifiers in drilling muds, dyes, etc.
	chelating agents, humectants, resin extenders
	phenol, benzene, phenolic resins, vanillin, dimethylsulphoxide, methylmercaptan

2.5 Pretreatment of Lignocellulosic Biomass

Agricultural and industrial residues and dedicated energy crops have been widely studied for their prospects in the bioethanol industry. Producing sugars from cellulose and hemicellulose is far more difficult than deriving sugars from corn starch or sugar cane (Wyman, 1996). The processing of lignocellulosic materials to ethanol consists of four major unit operations: pretreatment, hydrolysis, fermentation, and product separation/purification (Mosier et al., 2005). The authors stated that pretreatment is one of the most expensive steps, with costs as high as $0.30/gallon of ethanol produced. With associated chemical, equipment, and/or time factors, this may account for more than 20% of the total ethanol production cost.

Biomass pretreatment processes alter the structure of the lignocellulosic biomass at the micro- and macroscopic levels by physical, chemical, or biological methods and/or a combination of these (Hsu, 1996; Brown, 2003). Pretreatment facilitates the enzymatic hydrolysis and hence the fermentation processes as well. The structural complexity and compositional variability of lignocellulosic biomass would direct pretreatment options to (i) have lignin, hemicellulose, and cellulose - all in one product stream, (ii) separate hemicellulose but keep cellulose and lignin together, (iii) separate cellulose via solubilizing lignin and hemicellulose, and (iv) separate lignin, hemicellulose, and cellulose. The cost for such pretreatment will also increase with the complexity and superior quality of the lignocellulosic fraction (Wyman, 1996).

2.5.1 Physical Pretreatment

Physical pretreatment involves processes such as grinding,- irradiation, steam explosion, ultrasonication, and others (Hsu, 1996; Knauf and Moniruzzaman, 2004).

a. Grinding

Grinding involves mechanical techniques to reduce the size of biomass by application of ball milling, compression milling, attrition, wet disk refining etc. (Mosier et al., 2005). The size of the materials can vary: 10 to 30 mm after chipping and 0.2 to 3 mm after milling or grinding (Sun et al., 2002). Size reduction increases the surface area of the biomass and therefore, enhances the chemical or biological reactions. Specific energy requirement during grinding of biomass is inversely proportional to size of the finished product (Mani et al., 2004). Moisture content, particle size and bulk densities of feedstock influence the energy requirement. These processes are energy intensive, slow, and expensive.

b. Irradiation

Irradiation includes various treatment options such as highly-penetrating electron beams, gamma rays, and microwaves. Increase in reducing or total sugar yields

has been reported when the substrate was treated with microwave at atmospheric condition (Kitchaiya et al., 2003) or when alkali soaked switch grass, pretreated in microwave, was enzymatically hydrolyzed by cellulase (Hu and Wen, 2008). However, many of these methods demonstrated less success and are expensive in full-scale applications.

c. Steaming/Steam Explosion

In a review paper by Sun and Cheng (2002), steam explosion process is defined as treating biomass at 160 to 260^{o}C at high pressure (0.7 to 5 MPa) for several minutes and then rapid exposure of the hot and pressurized biomass to atmospheric pressure causes hemicellulose and lignin transformation. Such process also helps to increase the pore volumes of residual biomass (Wyman 1999). Hemicellulose hydrolysis is reported for the uncatalyzed steam explosion of biomass (Mason 1926; De Long 1981). Though steam explosion can considerably reduce the energy requirement cost compared to mechanical milling process, this process also has some limitations like incomplete lignin disruption and chances of forming inhibitory compounds that may further affect the downstream fermentation process. Due to release of acetic acid from hemicellulose fraction during this process and possible enhancement of biomass pretreatment, this process is sometimes also referred to as auto-hydrolysis (Hsu, 1996). Steam-explosion pretreatment (3.53 MPa for 2 min) of rice straw followed by enzymatic hydrolysis was reported for increased glucose yield (Moniruzzaman, 1996).

d. Ultrasound Treatment

High frequency sound waves in the range of 20 KHz (ultrasound) have many applications in biotechnology fields (Shoh, 1975). Exposure of material to sound energy of 1.5 kW at a frequency of 20 kHz for a period of time helps to produce cavitation in the slurry phase. The sound energy, frequency, and exposure time required to produce effective cavitation are governed by the type of ultrasonic system used and the nature of the material to be treated. Ultrasound can be applied in pretreatment of lignocellulosic biomass (reference ?), where the cavitation can help to reduce the size of the biomass particles and thereby improve the accessibility of sites for enzymatic saccharification and fermentation. Application of ultrasound in a dry-corn milling ethanol plant to enhance enzymatic saccharification and fermentation has been studied (Khanal et al., 2007). Similar work has also been done for sonication of cassava chips for enhanced sugar yield following enzymatic hydrolysis (Nitayavardhana et al., 2008;).

2.5.2 Chemical Pretreatment

Chemical pretreatment methods utilize concentrated or dilute acids, alkalis, peroxides and other solvents that improve and increase accessibility sites in the biomass for sequential enzymatic hydrolysis process. Dissolution or alteration of

lignin structure and degradation of cellulose crystallinity are possible. The effectiveness and less time consuming factors favor chemical pretreatment methods but in the mean time, high chemical, equipment, and processing cost may impede wide application of chemical pretreatment of biomass for biofuel generation.

a. Dilute Acid Hydrolysis

Acid hydrolysis using mineral acids (e.g. sulfuric acid) can improve hemicellulose hydrolysis (Beery et al., 2004; Brown, 2003; Hsu, 1996; Sun et al., 2002). Treatment of ground biomass with 1% H_2SO_4 at 140^{o}C for 30 min or at 160^{o}C for 5–10 min can achieve complete hemicellulose breakdown. This would further improve the activity of cellulose degrading enzymes (cellobiohydrolases, endoglucanases, and β-glucosidase) and thus may hydrolyze as much as 90% of the cellulose into glucose molecules (Brown, 2003).

Acid pretreatment would require corrosion proof containers to hold acid and biomass. Neutralization of hydrolyzate would also be necessary prior to ethanol fermentation. Formation of Hydroxymethylfurfurals (HMFs) and phenolic compound inhibit downstream process of sugar fermentation (Beery et al., 2004).

b. Concentrated Acid Hydrolysis

Concentrated hydrochloric and sulfuric acids have been commercially used in biomass pretreatment (Brown, 2003). High sugar yield (~ 100% of theoretical hexose

yields) following acid hydrolysis of biomass is possible. Following pretreatment, neutralization of hydrolyzate is usually done by the addition of lime. The consequence would be a production of gypsum ($CaSO_4$) at a rate of 2 kg gypsum per liter of ethanol produced. This would produce about 200,000 metric tons of gypsum annually from a $100x10^6$ L capacity cellulosic ethanol plant. Proper disposal or reuse of gypsum and as well as regeneration of acid for consecutive acid hydrolysis of biomass are both highly desirable options.

c. Alkaline Pretreatment

Alkaline pretreatment of biomass is basically carried out for delignification process. Subsequently significant solubilization of hemicellulose may be possible during alkali pretreatment. Various alkalis (e.g., sodium, potassium, calcium, and ammonium hydroxides) have been used at various concentrations for pretreatment of biomass (Brown, 2003; Mosier et al., 2005). Many times, sodium hydroxides alone or in combination with different concentrations of hydrogen peroxide have been effectively used for biomass pretreatment. Sodium hydroxide is costly but is easy to handle. Lime can be an alternative. Reduction in pretreatment cost can be possible by using alternative option like lime treatment or regeneration of spent alkali after the pretreatment.

d. Ammonia

In ammonia fiber explosion (AFEX) lignocellulose biomass is treated with gaseous ammonia at higher temperatures (60 to 100^{o}C) and pressure (1.7 to 2.1 MPa) for a period of time (30 min), followed by a sudden release of pressure (Dale et al., 1996; Sun et al., 2002; Ramirez, 2005). This simultaneously reduces the lignin content, removes some hemicellulose and break crystallinity of cellulose. Aqueous ammonia pretreatment of corn stover via ammonia recycle percolation (ARP) at higher temperature was effective in digestibility of the pretreated stover (Kim and Lee, 2005) Other processes include application of supercritical ammonia pretreatment and ammonia soaking pretreatment at ambient and slightly higher temperatures . The cost of ammonia and especially of ammonia recovery drives the cost of this pretreatment.

2.5.3 Biological Pretreatment

It involves (i) direct application of commercial cellulose and hemicellulose hydrolyzing enzymes like cellulase and hemicellulase or (ii) *in situ* secretion of these enzymes from microorganisms (especially bacteria and fungi) by growing them on the biomass (as substrate) for its degradation into sugars via enzymatic hydrolysis. Many times the enzymatic hydrolysis process utilizes consortia of enzymes (hemicellulase and cellulase) from the same or multiple microorganisms. Over the decades, many fungal and bacterial species have been identified for their potential to produce extracellular enzymes (Cloete and Malherbe, 2002) to obtain sugars:

hexoses and pentoses, from complex cellulose substrates. Needless to say, the annual carbon recycling from huge volumes of organic matter on forest floors have been possible due to the degradation of organics via enzymes produced by the surface and subsurface microorganisms (Perej et al., 2002). Many of these microorganisms have been identified, isolated, studied, and modified according to the need in biotechnological experiments and therefore, their application in efficient enzymatic hydrolysis of cellulosic biomass for ethanol fermentation is not an exception. Lee (1997) emphasized that biological conversion of lignocellulose to ethanol requires a delignification process to release holocellulose, hydrolysis of cellulose and hemicellulose, and fermentation of not only hexose (glucose) but also pentose sugars. A few bacteria and soft- and wood-rot fungal species are capable of degrading or modifying the lignin structures and hydrolyzing the hemicellulose and cellulose. These bacteria and fungi out-compete other organisms.

Wood-rot fungi such as white- and brown-rot fungi have been studied for their cellulolytic abilities (Highley and Dashek, 1998). *Trichoderma reesei* has been studied frequently for its cellulase activities (Schulein, 1988). Martinez et al. (2005) reviewed various aspects of lignin degradation by white-, brown- and soft-rot fungi. Many research papers have reported cellulose and hemicellulose degrading properties of brown- rot and white-rot fungi, however, the importance of these organisms and their saccharification and fermentation ability of lignocellulose have been overshadowed by the exploration and implication of enzyme systems from the fungus: *Trichoderma reesei* (soft-rot fungus). In the advent of favoring biological

pretreatment and saccharification of lignocellulose as a whole, wood-rot fungi are of greater importance in research and development. White-rot fungi are able to produce lignin-degrading enzymes that can completely mineralize lignin to carbon dioxide and water, exposing the hemicellulose and cellulose in the wood matrix (Cowling, 1961). Further, consortia of hemicellulase and cellulase hydrolyze the holocellulose and help fungi to absorb sugars, as carbon source, into the fungal cells. Interestingly, in contrast to the white-rot delignification mechanism, brown-rot fungi modify the lignin structure in the wood matrix (Highley and Dashek, 1998) facilitating the access of enzymes for holocellulose degradation.

Biological processes pose no environmental hazards as they do not require the use of any chemical. Enzymatic hydrolysis is one of the most widely employed pretreatment methods, which may or may not be preceded by chemical pretreatments, for releasing cellulosic sugars. The cost of producing cellulases for cellulose hydrolysis has dropped by more than 20-fold due to efficient pretreatment technologies and production of effective enzymes at low cost (NREL, 2006) and yet annual cost associated with enzymes is still very high.

2.6 Enzyme systems for lignocellulose degradation

2.6.1 Enzymes for lignin degradation

Much of the information on the degradation of lignin comes from biodegradation of lignin by the white-rot fungi like *Phanerochaete chrysosporium, Trametes versicolor, Phlebia radiata, Pleurotus ostreatus* (Penttila and Saloheimo, 1999). These fungi produce peroxidases (EC 1.1.1.7) like lignin peroxidase and manganese peroxidase along with laccase (EC 1.10.3.2) and not necessarily that all these enzymes have to be produced by every white-rot fungus. *P. chrysosporium* produces lignin peroxidase and manganese peroxidase (Hatakka, 1994).

a. Manganese Peroxidase (MnP)

The catalytic reaction of heme containing manganese peroxidase (MnP), in presence of hydrogen peroxide, during lignin degradation involves the oxidation of Mn^{2+} ions to Mn^{3+} ions that are further stabilized by chelator (for example organic acids). The MnP - Mn^{3+} complex (MnP-compound II + Mn^{3+}) oxidizes phenolic compounds (AH_2) to phenolic radicals (AH •). The stepwise reactions of phenol oxidation by MnP enzyme has been described by Hatakka (1994) as:

$$\text{Ferri-MnP} + H_2O_2 \rightarrow \text{MnP-compound I} + H_2O$$

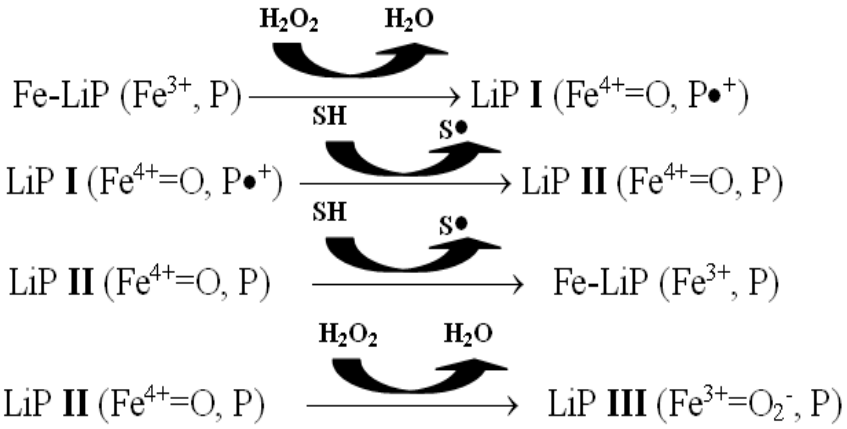
$$\text{MnP-compound I} + Mn^{2+} \rightarrow \text{MnP-compound II} + Mn^{3+}$$

$$\rightarrow \text{Ferri-MnP} + Mn^{3+} + H_2O$$

$$Mn^{3+} + AH_2 \rightarrow Mn^{2+} + AH\bullet$$

b. Lignin Peroxidase (LiP)

Similar to manganese peroxidase, the ferric enzyme (LiP) is oxidized in the presence of H_2O_2 to LiP compound I (LiP I). Radical cations (**S•**) are formed during one-electron oxidation of the reducing substrate (**S**) and Lip I is converted to LiP II. Another one-electron oxidation of reducing substrate further yields reactive radical cation (**S•**) and LiP II is converted back to ferric enzyme (Fe-LiP) as reviewed by Ward et al (2004).



Source: Ward et al., 2004

2.6.2 Enzymes for cellulose degradation

The enzyme mechanisms involved in cellulose degradation have also been investigated extensively (Eriksson, 1978; Highley and Dashek, 1998). Much of the research work on enzymatic fractionation of cellulosic substrate to sugars have been studied on many bacteria (*Clostridium sp, Bacillus sp and Cellulomonas sp*) and fungi (*Trichoderma sp, Penicillium sp, Aspergillus sp, Phanerochaete chrysosporium*) but only one fungal species, *Trichoderma reesei,* and its cellulase enzyme system has been compared with cellulolytic activity of many other fungi and bacteria, though wild strains of *Trichoderma reesei* lack optimal β-gluocidase activity compared to other fungi like *Aspergillus sp .*(Kadam, 1996). Lutzen et al. (1983) furnished a model presented by Klyosov et al. (1980) for synergistic enzymatic hydrolysis of cellulose to glucose (Figure 3).The Klyosov model also depicted that enzymatic degradation of cellulose involves consortia of different enzymes like: endoglucanase (or endocellulase), exoglucosidase (or exocellulase), cellobiohydrolase, and cellobiase (or β-glucosidase). Ramos and Fontana (2004) grouped exoglucosidase and cellobiohyrolase of *T. reesei* as exocellobiohydrolase. The mechanism of cellulase synergism for cellulose hydrolysis is depicted in figure 4. Endoglucanase acts randomly on the cellulose microfibrils (especially on amorphous regions) to liberate cello-oligosaccharides, which are then acted upon by exoglucanase to liberate cellobiose or glucose. Cellobiase (β-glucosidase) acts on cellobiose molecules to glucose.

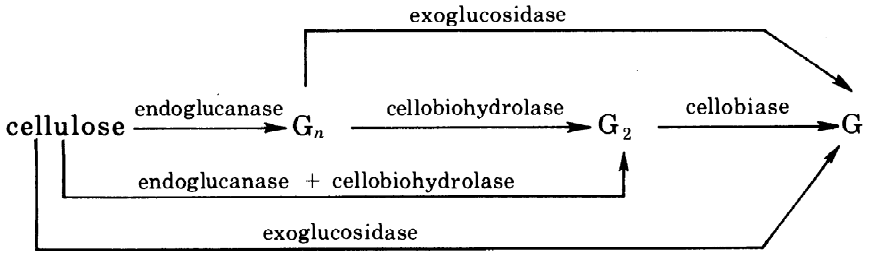


Figure 3: Model represented by Klyosov et al (1980) for enzymatic breakdown of cellulose to glucose. G_n = oligosaccharide (of glucose monomers), G_2 = Cellobiose, G = Glucose. Source: *Lutzen et al., 1983*

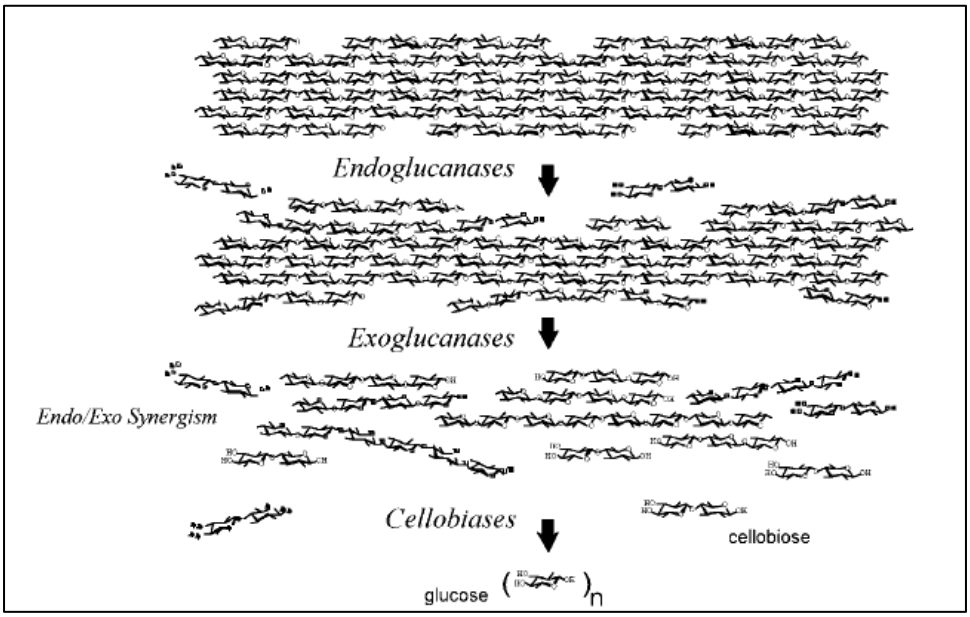


Figure 4: Cellulase synergism for hydrolysis of cellulose.

Source: *Ramos and Fontana (2004)*

A similar mechanism of cellulose degradation by cellulase enzyme systems has been described by Ubhayasekara (2005) as shown in figure 5, where two types of cellobiohydrolases attack cello-oligosaccharides from both reducing and non-reducing ends to liberate cellobiose.

Several research works have been conducted for the production of cellulase enzymes. Various carbon sources have been experimented with for cellulase (and hemicellulase) enzyme induction either in solid-substrate fermentation or submerged fermentation using cellulose, cellobiose, lactose, sophorose or lignocellulose residues (Kadam, 1996). Many researchers still debate on mechanism of cellulase induction from insoluble substrate (Zhang et al., 2006). Expression of cellulase involves 3 steps: expression at basal level, where small levels of cellulase hydrolyze cellulose, in vicinity, to cello-oligosaccharides or cellobiose that act as an inducer. The inducer incorporated within the cytoplasm helps in overall transcription of cellulase under favorable condition. The synergism of cellulase cleaves cellulose to cellobiose or glucose, an accumulation of which might incur feedback inhibition (Suto and Tomita, 2001). Therefore, an adequate amount of glucose utilization and presence of β-glucosidase is very necessary.

Kadam (1996) reported that a volumetric production rate of 200 FPU $l^{-1}h^{-1}$ for cellulase is considered as economical. It has also been argued that cost of enzyme protein production via optimizing enzyme secretion and screening of hyper-enzyme

secreting microbial species would still be very costly, at least by a factor of 100, compared to enzymes required for saccharification of starch.

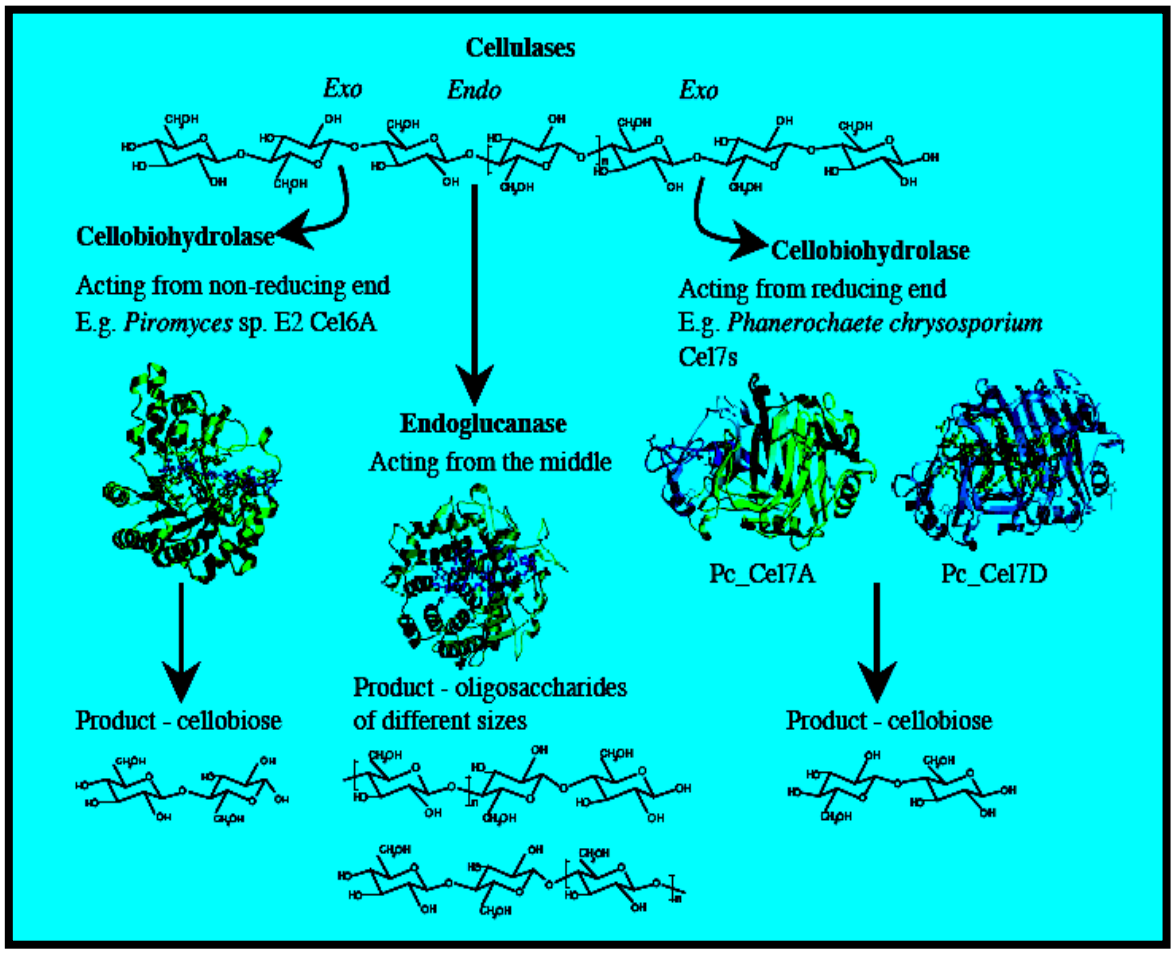


Figure 5: Cellulose degrading enzymes secreted by *P. chrysosporium*.

Source: Ubhayasekera (2005)

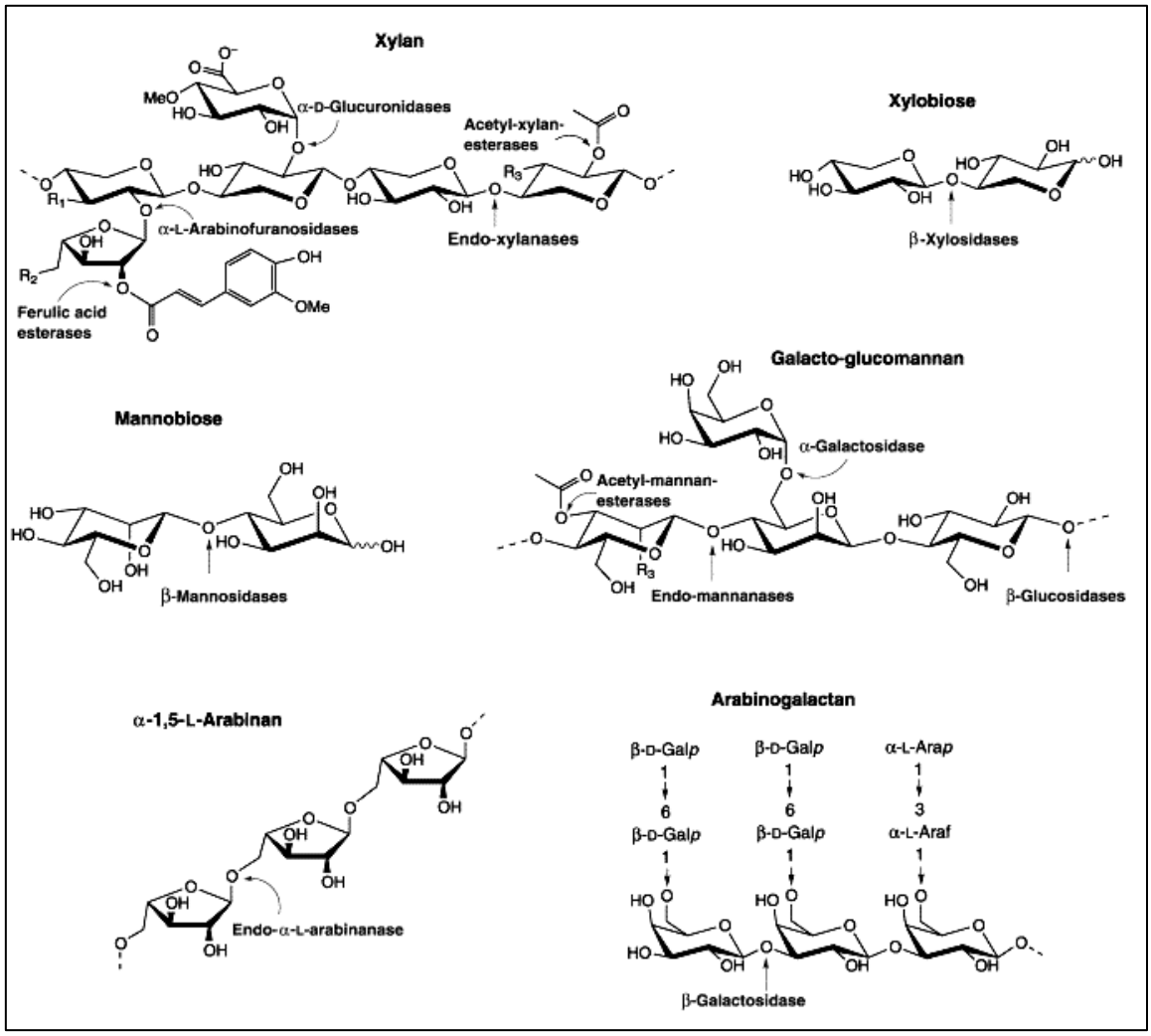


Figure 6: Different types of hemicellulases to hydrolyze various structural components of hemicellulose. Source: *Shallom and Shoham (2003)*

2.6.3 Hemicellulose Degradation

The structure, bonds, and subunits for lignin and hemicelluloses is different from plant to plant. Hemicellulose is a very complicated polysaccharide and thus has high degree of substitution (figure 6). Hemicellulose degrading enzymes (hemicellulases) are comprised of various enzymes that cleave different sugar and substituted groups off the parent polysaccharide (Shallom and Shoham, 2003 ; Highley and Dashek, 1998; Sinnott et al., 1999).

2.7 White-, Brown- and Soft-rot Fungi

There have been a lot of studies in fungal degradation of wood in natural forest systems. Huge quantities of live and dead trees are under constant microbial attacks. Intensive scientific and ecological studies have reported many fungi and bacteria responsible for wood and organic matter decay. The following texts incorporate short discussion on white-, brown- and soft-rot fungi (table 2):

2.7.1 White-rot fungi

White-rot fungi, name given as such for the white crystalline cellulose deposits during wood degradation, are considered to be efficient lignin degraders (Kirk and Farrell, 1987) as they can completely mineralize lignin into carbon dioxide and water. These fungi represent the advanced fungal subdivision– basidiomycota. They produce laccases and peroxidases like lignin peroxidase and manganese peroxidase for delignification processes (Tuor et al., 1995). These ligninolytic enzymes are further complemented by consortia of cellulose and hemicellulose degrading enzymes. The synergistic enzyme degradation mechanism helps these fungi to hydrolyze woody material and absorb simple sugars for their metabolism and growth.

Non-selective degradation of lignocellulose by white-rot fungi which included *Phanerochaete chrysosporium* was reported (Eriksson et al., 1990). The fungi degrade not only lignin but also the holocellulose (hemicellulose and cellulose) so that they can release sugars by the virtue of their efficient enzyme consortia. In addition to their natural habitat, these fungi have also been successfully grown on agricultural and industrial residue for their extensive application in decontamination and removal of aromatic contaminants (Reddy, 1995). These fungi primarily colonize their hyphal cells in cell lumen. As non-selective degradation of secondary cell wall tissues proceeds towards middle lamellaand ultimately coalesce the adjusting damaged cells (Blanchette., 1991)

Table 2: Generalized characteristic of white-, brown- and soft-rot fungi (*Copied from Ward et al., 2004*)

Organism	Sub division	Examples	Actions	distribution
White-rot fungi	Basidiomycetes	*Phanerochaete chrysosporium, Trametes versicolor, Phlebia radiata etc*	Mineralize lignin to CO_2 and H_2O; some species selectively degrade lignin; others degrade lignin and cellulose simultaneously	Predominantly degrade wood from deciduous trees (angiosperms), containing hardwood
Brown-rot fungi	Basidiomycetes	*Gloeophyllum trabeum, Serpula lacrymans, Neolentinus lepidus etc*	Modify lignin by demethylation, limited aromatic hydroxylation, and ring cleavage	Prefer coniferous substrates (gymnosperms), which are softwoods
Soft-rot fungi	Ascomycetes, Deuteromycetes	*Chaetomium sp, Ceratocystis sp etc*	Some lignin degradation	Active generally in wet environments and plant litter; attack hardwood and softwood

2.7.2 Brown-rot fungi

Contrary to white-rot delignification mechanisms, brown-rot fungi (also from basidiomycota) have different mechanism for the degradation of polysaccharides in plant cell walls. Brown-rot fungus like *Serpula lacrymans* is an efficient degrader of wood in service (building lumber). Unlike white-rot, brown-rot wood decay mechanism is propelled via modification of lignin structure by demethylation and oxidation, degradation and utilization of hemicellulose and cellulose leaving modified lignin, which imparts brown color in advent of wood-decay (Green and Highley, 1997). Colonization of brown-rot fungal mycelia also starts in cell lumens and wood decay proceeds towards middle lamella, without major deconstruction of secondary cell wall layer # 3 (S-3) that has higher proportion of lignin. It has therefore been suggested by many researchers that brown-rot degradation of wood involves oxidation of cellulosic tissues via diffusible oxidative agents much smaller than the hydrolytic enzymes like xylanase, endocellulase and exocellulase, whose productions are also reported (Higley and Illman, 1991). Extracellular fenton mechanisms, oxalic acid and hydroxyl radicals have been reported for demethylation of lignin and oxidative degradation of cellulose and hemicellulose (Green and Highley, 1997).

2.7.3 Soft-rot fungi

Soft-rot fungi (subdivision ascomycota and deuteromycota) like *Trichoderma reesei, Chaetomium sp, Ceratocystis sp etc* preferably attack high moisture wood and plant litter with lower lignin content (Goodwell et al., 2008). Savory (1954) also described the degradation pattern of wood decay by soft-rot fungi. The fungal hyphae were extended especially in the central part of secondary cell walls to degrade cellulose in zones with less lignin content. The wood decay was advanced with the supplementation of inorganic salts. In a study of 2700 years old archaeological wood, Nelson et al. (1995) reported two types of soft-rot decay of woods: Type-I has longitudinal cavity formation within secondary cell walls and Type-II has erosive degradation resulting from secondary cell wall erosion. Blanchette (2000) further explained that such decay process ultimately results to higher lignin concentrations in the wood residue. Worrall et al. (1997), in their investigation of 78 fungal species for wood decay, contrasted that the soft-rot decay of wood was different from white-rot in terms of lesser Klason lignin degradation and lower alkali solubility in comparison to brown-rot decay. *Trichoderma reesei*, is a soft-rot fungus, thas is studied for production, characterization and application of cellulose (and hemicellulose) degrading enzymes. The cellulolytic enzymes production and characterization have been studied under various conditions like solid-substrate fermentation or submerged fermentation using standard cellulose or cellulosic agricultural or industrial residues. Chahal (1985) reported on the cellulose and hemicellulose degrading enzymes (e.g. cellulase, β-glucosidase and xylanase) production by *T. reesei QMY -1* during solid-state fermentation of wheat straw. Li et al. (2005) also reported on cellulolytic enzyme profiles of two strains of *T. reesei* (*T.*

reesei QM9414 and *T. reesei Rut C-30*) when grown on crude or fractionated corn fiber.

2.8 Cellulosic Feedstock for Ethanol

Utilization of structural plant tissues such as cellulose fibers have been sought for feedstock to produce renewable fuel such as ethanol. Wiegel (1982) reported the two steps process of converting cellulose to ethanol (1) hydrolysis of the polysaccharide and (2) fermentation of glucose to ethanol using yeast, *Saccharomyces cerevisiae*. The author also suggested the possibility of directly converting the cellulose to ethanol using bacteria like *Clostridium thermocellum.* There has also been a school of thought regarding converting not only hydrolyzate from cellulose but also the ones from hemicellulose to ethanol. The overall conversion of hollocellulose (hemicellulose and cellulose) to ethanol would be a favorable option. Singh et al. (1982) discussed the potential application of filamentous fungal species of genera *Fusarium, Monilla and Neurospora* for production of extracellular enzymes for hemi/cellulose hydrolysis and fermentation of glucose and xylose to ethanol. However, ethanol and sugar tolerance by these fungi is lower, which demonstrates slower sugar to ethanol conversion compared to yeast. South et al. (1993) conducted simultaneous saccharification and fermentation (SSF) of acid pretreated hardwood flour via supplementation of cellulase from*T. reesei* and β-glucosidase and *Saccharomyces cerevisiae* to convert glucose to ethanol. The

authors also reported direct microbial conversion of acid pretreated hardwood flour to ethanol using *C. thermocellum.* So and Brown (1999) compared the Waterloo fast pyrolysis process followed by fermentation process with SSF and dilute acid hydrolysis and fermentation in terms of ethanol production cost for a hypothesized 25 MGY cellulosic ethanol industry. The unit production cost of ethanol via fast pyrolysis and fermentation was reported to be slightly higher than SSF and dilute acid hydrolysis and fermentation process. The authors suggested further feasibility research on pyrolysis coupled with ethanol production and the recovery of lignin as a value product to minimize the unit ethanol production cost. The fascinating research on lignocellulosic ethanol still has big challenges to overcome, especially the high cost of converting lignocellulose to fermentable sugar mixture. For the optimization of (ligno-) cellulosic ethanol process, it is also very necessary to first understand the morphological, anatomical and physiological characteristics of plant cell wall tissues that pose recalcitrant and robust structural assembly against degradation (Himmel et al., 2007)

2.9 Conclusion

The outlook on cellulosic biofuel development is progressive. In recent years, a lot of efforts have been put to optimize the overall cellulose-to-ethanol production cost. In the United States, emphasis has been given to utilize an annual production of over a billion tons of cellulosic biomass from forest, agricultural and industrial residues to

produce biofuel and other biobased products, so as to substitute as much as 30% of the demand on petroleum fuels. A cellulosic biomass program, created under the Energy Policy Act of 2005 for production of 250 million gallons of cellulose ethanol by the year 2013 (RFA, 2006) is very promising in the present context when 24 cellulosic ethanol plants have already been under development phase in different parts of USA to produce 200 to 300 MGY of cellulosic ethanol (RFA-2008). Under feasible legislative, economical and technical scenarios the targeted 250 MGY cellulose ethanol is achievable before 2013.

While one school of experts are under constant quests of improving the cellulosic ethanol yield, there are other schools of scientists who are focused on research to understand the anatomical and physiological aspects of various fuel crops. The structural recalcitrance of plant cell wall materials imposes the greatest barrier in economically converting cellulosic feedstock to fermentable sugar. Physico-chemical pretreatment of biomass helps in enzymatic hydrolysis of feedstock to produce sugars. These promising technologies are still costly and impart greater cost in overall feedstock to ethanol production cost. Direct enzymatic conversion of lignocellulose is desirable, but not foreseeable, due to high enzyme dosage and variety of requirements. The cost of producing cellulolytic enzyme is still high to date.

It is well understood that many research laboratories and enzyme companies are continuously conducting research to improve the enzyme systems and reduce the unit cost. Fungal and bacterial enzyme systems have to be explored constantly.

Genetic manipulation and improvement process for enzyme development is as important as conducting intensive research on fungal and bacterial species selection for even efficient enzyme systems. As such, wood-rot fungi can be among several candidates that need detailed research and experiments to explore their wood-degradation mechanisms and exploit their enzyme systems for a better cause – cellulose degradation and henceforth for biofuel production.

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CHAPTER 3: CORN FIBER INDUCED EXTRACELLULAR ENZYMES PRODUCTION BY WOOD-ROT AND SOFT-ROT FUNGI FOR SUBSEQUENT FERMENTATION OF HYDROLYZATE TO ETHANOL

(submitted to the Journal of Agricultural and Food Chemistry)

3.1 Abstract

The use of bio-based feedstock to support an economy based on renewable resources is becoming extremely important for generating renewable clean energy and reducing the developed nations' dependency on imported fossil fuels. This research aims at developing a biorefinery platform to convert lignocellulosic biomass to fermentable sugars at a low temperature with minimal use of chemicals. White-rot (*Phanerochaete chrysosporium*), brown-rot (*Gloeophyllum trabeum*) and soft-rot (*Trichoderma reesei*) fungi were used in this research to biologically break down cellulosic and hemicellulosic components of corn fiber into fermentable sugars. Previous studies (genesis of present study) on solid-substrate fermentation of corn fiber by either white-rot or the brown-rot fungi followed by simultaneous saccharification and fermentation (SSF) using the yeast *Saccharomyces cerevisiae* showed possibilities of enhancing wood-rot saccharification of corn fiber for significant ethanol fermentation. Laboratory-scale SSF process proceeded by *in-situ* cellulolytic enzyme induction enhanced overall enzymatic hydrolysis of

hemi/cellulose from corn fiber into simple sugars (mono-, di-, tri-saccharides). The yeast fermentation of hydrolyzate yielded 7.8, 8.6 and 4.9 g ethanol per 100 g corn fiber when saccharified with the white-, brown-, and soft-rot fungi, respectively. The highest corn to ethanol yield (8.6 g ethanol/ 100 g corn fiber) is equivalent to 35% of the theoretical ethanol yield from starch and cellulose in corn fiber. This research has significant commercial potential to increase net ethanol production per bushel of corn.

Keywords. Lignocellulosic biomass, corn fiber, solid-state fermentation, simultaneous saccharification and fermentation, enzymatic hydrolysis, ethanol, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum, Trichoderma reesei, Saccharomyces cerevisiae*.

3.2 Introduction

The annual corn ethanol production capacity exceeded 8.5 billion gallon per year in early 2008 from 147 biorefineries in the United States. Over 55 new plants, currently under construction, will add additional 5.1 billion gallons ethanol per year (RFA, 2008). Needless to say, these industries also produce millions of tons of low-value feed-grade co-products like distiller's dried grains with soluble (DDGS) and gluten feed from corn dry-grind and wet-milling plants, respectively. Excess coproducts will soon saturate the feed sector and their bulk management may pose a serious issue.

Approximate analyses of DDG or corn fiber (which is supplemented with condensate from steep water evaporation to make gluten feed) showed that these co-products contain largely cellulose, hemicellulose, and residual starch (Abbas et al., 2004). The National Renewable Energy Laboratory (NREL, 2004) estimated an increase in the net ethanol yield per bushel of corn by 13% (from 2.7 to 3.1 gallon ethanol/bushel corn) via utilization of the cellulosic fraction and enhanced starch saccharification. Cellulosic conversion to ethanol also reduces overall bulk production of co-products.

The recalcitrance and structural complexity of cellulose and hemicelluloses (hemi/cellulose) matrix requires a certain degree of pretreatment involving physical, chemical, and biological techniques. Mosier et al. (2005) reported various pretreatments such as mechanical milling, pressurized steam, acids, ammonia, or enzymes in a separate or combined process. Such pretreatments break down the heterogenic and crystalline lignocellulosic fiber matrix thereby improving downstream enzymatic saccharification of hemi/cellulose to sugars and their subsequent fermentation to ethanol. High energy and chemical costs associated with these pretreatments and downstream waste management are the major drawbacks. Hydrolysis with commercial enzymes is the more favorable pretreatment method compared to costly and environmentally unfriendly chemical methods. The costs of biomass pretreatment and enzymes are still the major limiting factors for the overall cost of cellulosic ethanol production.

Studies showed potential application of indigenous fungi to break down lignocellulosic biomass. Shrestha et al. (2008a) reported application of the white-rot fungus *Phanerochaete chrysosporium* in solid-substrate fermentation of corn fiber (co-product from wet milling plant) and subsequent simultaneous saccharification and fermentation to ethanol. Similar work was also examined by Rasmussen et al. (2008) using the brown-rot fungus *Gloeophyllum trabeum.* These studies on wood-rot fungi opened a new frontier for biological saccharification and fermentation of lignocellulosic biomass to ethanol. These fungi were also reported to produce ethanol without yeast co-culture. Wood-rot fungi, otherwise, had been studied mainly for degradation of lignocelluosic substrates (Cowling, 1961; Highley and Dashek, 1998) while cellulase activities have been extensively studied for *Trichoderma reesei* (Shulein, 1988)).

Solid-substrate fermentation, which involves developing selected culture and enzymatic activities of microbes on selected substrate, was reported as a promising fermentation technique for *in-situ* production of ligninolytic and cellulolytic enzymes (Pandey et al., 2000). Previous studies examined solid-substrate fermentation using *P. chrysosporium* and *G. trabeum* for saccharification of corn fiber and conversion of hydrolyzate into ethanol using *Saccharomyces cerevisiae* in the subsequent submerged fermentation. Net ethanol yields were low (18%) in terms of theoretical maximum yield of corn fiber cellulose and starch. Based on these findings, the objective of this research was to evaluate *P. chrysosporium* and *G. trabeum* saccharification of corn fiber via enhanced enzymatic hydrolysis, and the

subsequent fermentation of fermentable sugars into ethanol using *S. cerevisiae*. The performance of these wood-rot fungi was also compared with *T. reesei* in terms of net ethanol yield from corn fiber.

3.3 Materials and Methods

3.3.1 Fungal Culture

Fungal cultures were obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Phanerochaete chrysosporium* (ATCC # 24725), *Gloeophyllum trabeum* (ATCC # 11539), *Trichoderma reesei* (ATCC #13631) and *Saccharomyces cerevisiae* (ATCC #24859) were separately revived by inoculating each culture in potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD) and were incubated with shaking (150 rpm) at 24^{o}C. Stock cultures in 10% sterile glycerol were stored in sterile 2 ml-cryogenic vials and preserved in an ultra-low temperature freezer (-75^{o}C, So-Low, Cincinnati, OH).

Fungal inocula for the saccharification and fermentation studies were prepared from the stock culture. The culture vials were thawed and poured aseptically, 1 vial into 1 L of sterilized yeast mold (YM) broth (Difco). The seed culture was incubated with

shaking at 150 rpm and 37^{o}C for rejuvenation. The mycelia grew into pellets of 2 to 3 mm size in 7 days.

3.3.2 Substrate

Corn fiber, obtained from a corn wet milling plant (Archer Daniel and Midland, Decatur, IL), had been processed through hot water steeping and sulfur dioxide (SO_2) treatment at the beginning of the wet milling process. The wet-milled corn fiber (Table 1) was oven dried at 80 ^{o}C for 4 days followed by desiccation prior to use. Sterilization of the fiber was done by autoclaving at 121^{o}C for 75 min.

Table 1: Constituent analysis of corn fiber using Anknom Technology (2005)

Cellulose	**Hemicellulose**	**Lignin**	**Cell solubles**	**Ash**
%, (w/w)	%, (w/w)	%, (w/w)	%, (w/w)	%, (w/w)
16.4	45.2	1.3	37	0.1

3.3.3 Experimental Setup

a. Fungal culture preparation

White-rot (*P. chrysosporium*), brown-rot (*G. trabeum*) and soft-rot (*T. reesei*) fungi were grown separately in 1L YM broth at 37^{o}C in shake flasks (at 150 rpm) for 7 days and the mycelia pellets of uniform diameter (~ 3 to 4 mm) were formed. The media with fungal pellets were aseptically transferred into sterile 1-L polypropylene centrifuge bottles. The bottles were centrifuged at 7277 *g* for 20 min. The supernatant was decanted and the centrifuge bottle was filled aseptically to the top with basal medium (Kirk et al., 1972), which contained 0.25 g of KH_2PO_4, 0.063 g of $MgSO_4{\cdot}7H_2O$, 0.013 g of $CaCl_2{\cdot}2H_2O$, and 1.25 ml of trace element solutions in 1L of deionized water. The trace element solution (in 1L deionized water) contained 3.0 g of $MgSO_4{\cdot}7H_2O$, 0.5 g of $MnSO_4{\cdot}H_2O$, 1.0 g of NaCl, 0.1 g of $FeSO_4{\cdot}7H_2O$, 0.181 g of $CoSO_4{\cdot}7H_2O$, 0.082 g of $CaCl_2{\cdot}2H_2O$, 0.1 g of $ZnSO_4$, 0.01 g of $CuSO_4{\cdot}5H_2O$, 0.01 g of $Al_2(SO_4)_3{\cdot}2H_2O$, 0.01 of H_3BO_3 and 0.01 g of $NaMoO_4$.

The pellets were resuspended in the basal medium; the centrifugation and supernatant decantation procedure was repeated to reduce the possibility of introduction of organic nutrients into the suspension. There were duplicate sets of 2-L Erlenmeyer flasks for each of the three fungal species and controls, which had no fungal cultures. Each flask contained 600 ml of dense resuspended pellets of specific fungal species. Approximately 7.8, 5.5 and 4.4 g (dry weight)/L of white-, brown- and soft-rot fungus was used, respectively, for enzyme induction and SSF.

b. Enzyme induction and simultaneous saccharification and fermentation

Sterile corn fiber (10 g) was added to each flask containing respective fungal pellets. The control flasks had 600 ml of basal medium but no fungal cultures. These flasks, covered with sterile autoclave cloth, were placed in a shaker at 150 rpm and 37^{o}C for 48h. Samples, 10 ml, from each flask were collected on the second day for enzyme activity assay. The content (~600 ml) of each flask was emptied into individual sterile 1-L polypropylene bottles, which contained 15 g of sterile corn fiber, 200 ml of yeast media, and 1 ml of *S. cerevisiae* culture (cell count = 2.9 x10^{9} cells/ml). All bottles including controls contained 600 ml of basal media, 200 ml of yeast media, 1-ml yeast culture and 25 g of sterile corn fiber. The bottles were then loosely capped and incubated standing in an incubator at 37^{o}C. The caps were placed in such a way that it would create an anaerobic environment inside the bottle, yet allow the excess CO_2 to escape from the bottles.

The overall experimental procedure is schematically presented in Figure 1.

3.3.4 Analytical Methods

Every alternate day, 5 ml samples were collected aseptically from each bottle. The samples were centrifuged and syringe filtered (0.45 µm) for the following assays:

a. Sugar Assays

Total and reducing sugar analyses were conducted via phenol sulfuric and modified Somogyi-Nelson methods, respectively (Crawford and Pometto, 1988). The samples were analyzed for total and reducing sugars using spectrophotometer (Spectronic™ 20 Genesys™, Thermo Electron, Cambridge, UK) at 490 and 500 nm, respectively. The absorbance readings were then converted into equivalent sugar concentration (g/L) using a standard glucose solution curve.

b. Ethanol and organic acids assays

Ethanol, and lactic and acetic acid production were measured by using a Bio Rad Aminex 87-H (78x300) organic acid column in Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford, MA, USA) as described by Shrestha et al (2008)).

c. Specific enzyme activity assay

Specific enzyme activity assays for α- and glucoamylase, xylanase, endocellulase and exocellulase were performed using protocol described by Lee et al. (1998). Specific enzyme activity for each enzyme was expressed as mg product/mg protein/min.

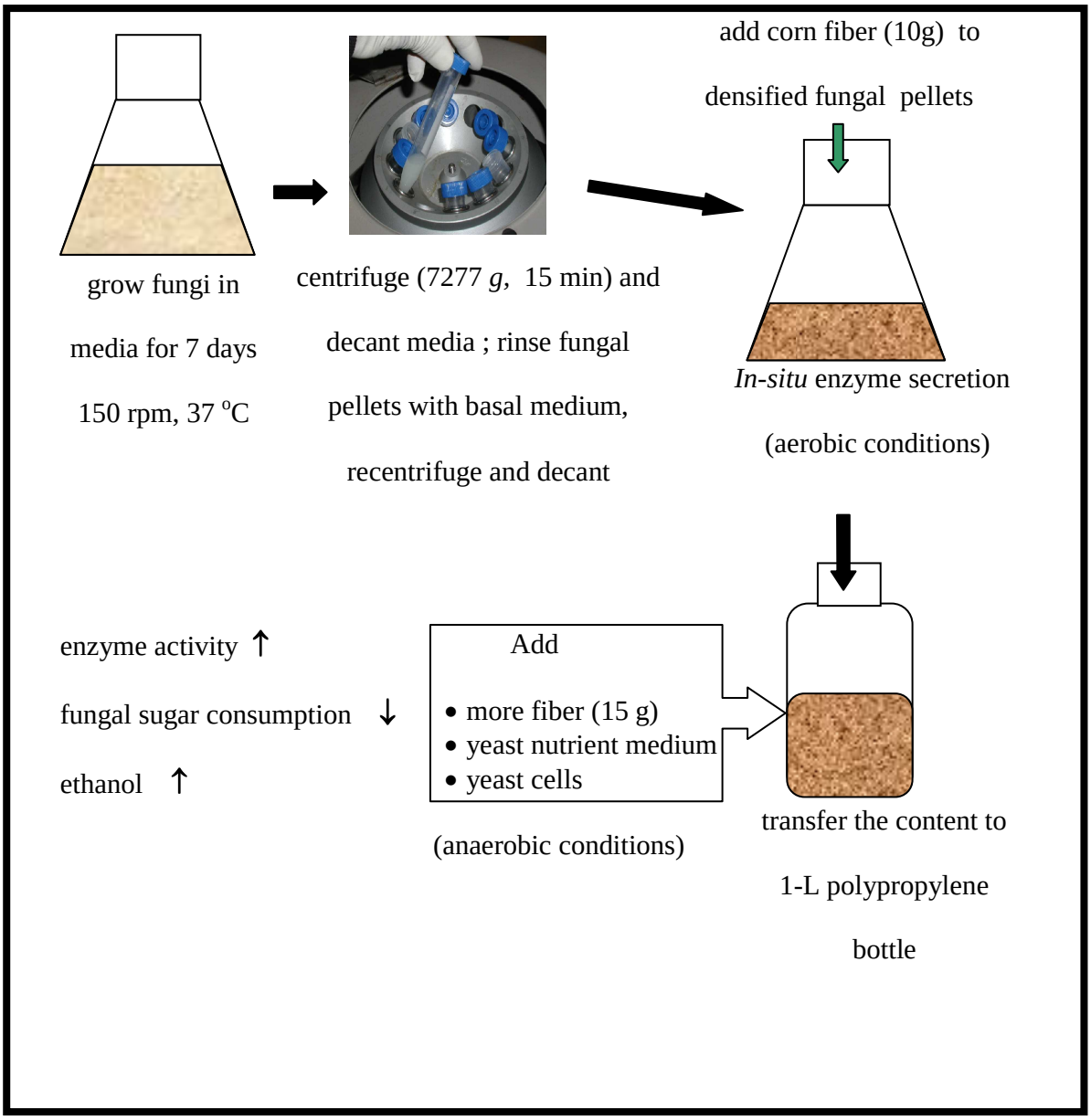

Figure 1: Schematic of bench-scale in-situ fungal enzyme induction and simultaneous saccharification and fermentation of corn fiber to ethanol.

3.3.5 Statistical Analyses

The experimental data were validated by statistical analyses using a statistical tool, SAS. The SSF results on sugar, ethanol and organic assays were fitted to two-factor fixed effects model. All assays and fermentations were performed in replicates of two (n=2), and significant difference of p value of 0.05 was employed. Student's t-test analyses were performed for data obtained from specific enzyme activity assays.

3.4 Results and Discussion

3.4.1 Sugar release in simultaneous saccharification and fermentation (SSF)

During aerobic enzyme induction phase, extracellular enzyme production resulted in the production of water soluble simple sugars from the residual starch, cellulose and hemicellulose fractions of corn fiber and also their consumption by the fungi. To minimize fungal sugar consumption and maximize enzymatic hydrolysis after two-day aerobic incubation, the fungi were placed in an anaerobic condition with added fiber and yeast cultures for simultaneous saccharification and fermentation (SSF) to ethanol. The saccharification of corn fiber polysaccharides was monitored via reducing and total sugars assays. The sugar values were interpreted in terms gram

sugar produced per 100 g corn fiber. Higher sugar values compared to control (without fungi) confirmed active enzyme activities during SSF process. The released fermentable sugars (especially glucose) would be fermented by yeast during anaerobic conditions to ethanol. The non-fermentable sugars such as cellobiose, pentoses, tri- and oligosaccharides accumulated during SSF as reported in Figures 2 and 3.

The activity of cellulase enzymes depends on the microbial source, the types of substrate, and the operating conditions (i.e., pH and temperature). Meyer et al. (2006) reported that the pH of 5.0 and the temperature of 50^{o}C were optimal for maximum yield of glucose from steam-pretreated barley straw using cellulase enzymes from cultures of five thermophilic fungi: *Chaetomium thermophilum, Thielavia terrestris, Thermoascus aurantiacus, Corynascus thermophilus and Myceliophthora thermophila, and* from the mesophilic *Penicilium funiculosum.* The starting pH for SSF in this study was at 4.7 to 5.2 and the temperature was maintained at 37^{o}C. The pH gradually decreased to 4.2 and then remained nearly constant as the SSF progressed. The moderate temperature was required for anaerobic yeast fermentation.

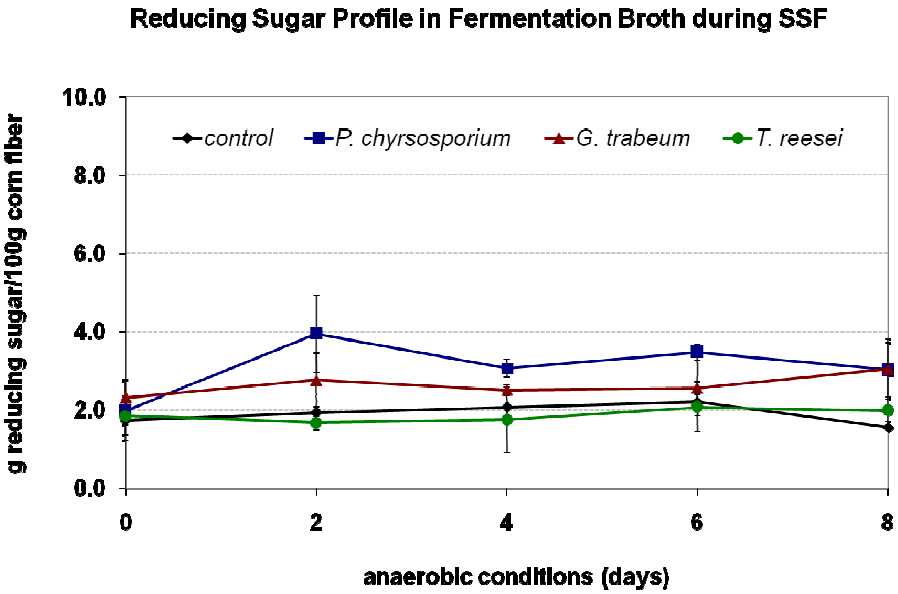

Figure 2: Residual reducing sugars present in culture broth from anaerobic simultaneous saccharification and fermentation for ethanol production in white-, brown-, and soft-rot (*P. chrysosporium, G. trabeum, and T. reesei* fungi co-cultured with *S. cerevisiae* (n=2).

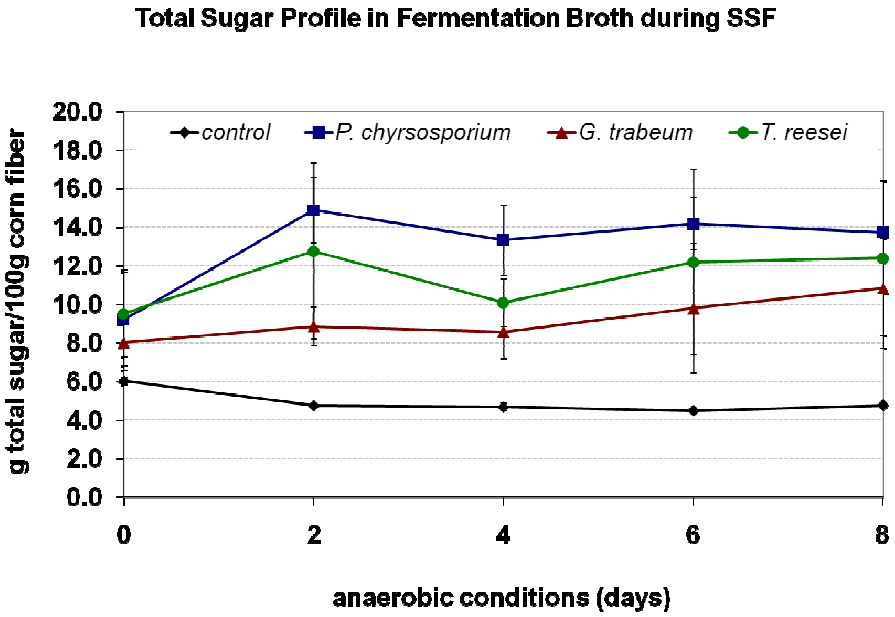

Figure 3: Total sugars present in culture broth from anaerobic simultaneous saccharification and fermentation for ethanol production in white-, brown-, and soft-rot (P. *chrysosporium, G. trabeum*, and *T. reesei*) fungal co-cultured with *S. cerevisiae* (n=2). Control is with yeast cells but no fungal culture

The accumulation of end-products (e.g., glucose and cellobiose) suppresses enzyme activity (Sarkar and Etters, 2004). Thus, SSF helps to overcome the product inhibition by converting fermentable end-products into ethanol as soon as they are produced (Manzanares et al., 2004; Reczey et al., 2004), and facilitates continuous cellulase activity. The hemicellulase activity of *P. chrysosporium was* studied by Highley and Dashek (1998). The hydrolysis of hemicellulose releases both hexoses and pentoses. The reducing sugar measurements depend on the availability of aldose or ketose reducing end, and mono-, di-, tri- and short-chained carbohydrates have one reducing end each. The fermentable portion of the reducing sugar can be determined by quantifying ethanol produced by yeast fermentation.

The increase in total sugars in culture SSF bottles, compared to controls, confirmed the enzyme activities of the fungi (Figure 3). The total sugar decreased from 6 to 4.8 g per 100 g corn fiber, which then remained constant throughout the experimental period. Similarly, the maximum and minimum (in parentheses) total sugar production for *P. chrysosporium* and *T. reesei* were, respectively, 14.9 (13.7) and 12.8 (12.4) g total sugar per 100 g corn fiber. The total sugar, however, had an increasing trend for *G. trabeum* from 8 to 10.8 g total sugar per 100 g corn fiber. The overall total sugar data was not statistically different (p-value = 0.5) between three fungal species. The difference between the total and the reducing sugars also indicates that soluble sugars were not completely hydrolyzed to monosaccharides. The difference in total sugar levels was basically due to the differences in the enzyme activities between these fungal species. There was also no statistical difference for

reducing sugar data (Figure 2) between fungal species and control samples (*p*-value = 0.29)

3.4.2 Ethanol fermentation in simultaneous-saccharification and fermentation (SSF)

One mole of glucose (C-6) is converted into 2 moles of ethanol and 2 moles of carbon dioxide during yeast fermentation. Thus, stoichiometrically, 1 g of glucose would yield 0.51 g of ethanol and 0.49 g of carbon dioxide. Fungal SSF yielded higher ethanol production compared to control. The net fiber to ethanol conversion (based on initial corn fiber weight of 25 g) was as high as 8.6 g ethanol per 100 g corn fiber in case of brown-rot fungus (*G. trabeum*), followed by 7.1 and 4.6 g ethanol per 100 g corn fiber, respectively, for *P. chrysosporium* and *T. reesei* (Figure 4). The brown-rot saccharification and SSF of corn fiber yielded about 42 % of the theoretical maximum yield (theoretical maximum ethanol yield is 20.4 g ethanol per 100 g fiber, if glucose from starch and cellulose is utilized) and this would also mean that the current ethanol yield can produce 29 gallons (~110 L) of ethanol per ton of corn fiber. There was significant difference in ethanol data between the different fungal treatments (*p*-value = 0.0557), however, the white- and brown-rot ethanol production data were not significantly different for the number of experiments (*p*-value = 0.8491). As seen from the contrasts output, ethanol yield following *T. reesei* treatment was significantly different when compared with the *P.*

chrysosporium (*p*-value = 0.0336) and *G. trabeum* (*p*-value = 0.0388). The ethanol profile would be expected to increase slightly for *G. trabeum* if the anaerobic incubation period was prolonged. However, it would not be economically sound to extend the fermentation process for such an extended time. The decreasing profile of ethanol and sugar values might indicate the low activity of saccharification and fermentation processes at a later phase. Decreasing pH trend (not reported here) was also observed in the SSF bottles with fungal biomass.

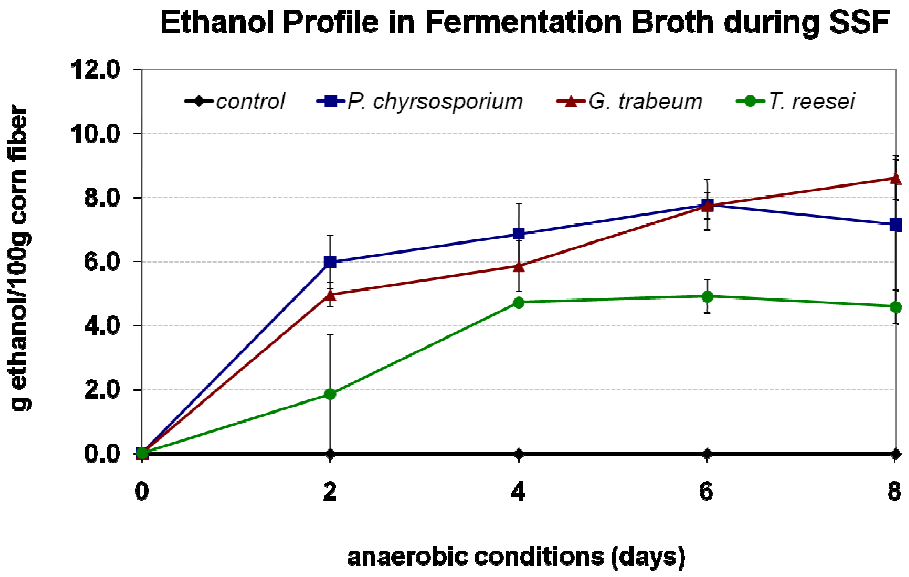

Figure 4: Ethanol profile in culture broth from anaerobic simultaneous saccharification and fermentation for ethanol production in white-, brown-, and soft-rot (*P. chrysosporium, G. trabeum,* and *T. reesei*) fungal co-cultured with *S. cerevisiae* (n=2). Control is with yeast cells but no fungal culture.

3.4.3 Acetic acid production in simultaneous-saccharification and fermentation (SSF)

The white- and brown-rot SSF had 1.7 and 1 g acetic acid per 100 g corn fiber (*p*-value = 0.6121), respectively. In case of soft-rot SSF, the acetic acid profile was as high as 11.3 g acetic acid per 100 g corn fiber (Figure 5). Chambergo et al. (2002) discovered the paralogous gene for enzyme aldehyde dehydrogenase (ALD1 and ALD2) responsible for converting acetaldehyde to acetate. The increasing acetic acid profile in *T. reesei* SSF may have affected the activity of the co-culture: *S. cerevisiae*, in converting glucose to ethanol and therefore, leading to lower yield of ethanol. Graves et al. (2006) reported inhibition of ethanol production by *S. cerevisiae* at various acetate concentrations.

3.4.4 Lactic acid production in simultaneous saccharification and fermentation (SSF)

Lactic acid profile also showed valid differences between three fungal SSF (*p*-value=0.043). By the end of the experiment, 0.9, 1.4, 1.4, and 0.5 g lactic acid per 100 g corn fiber had accumulated in white-, brown-, soft-rot fungi and control samples, respectively (Fig. 6).

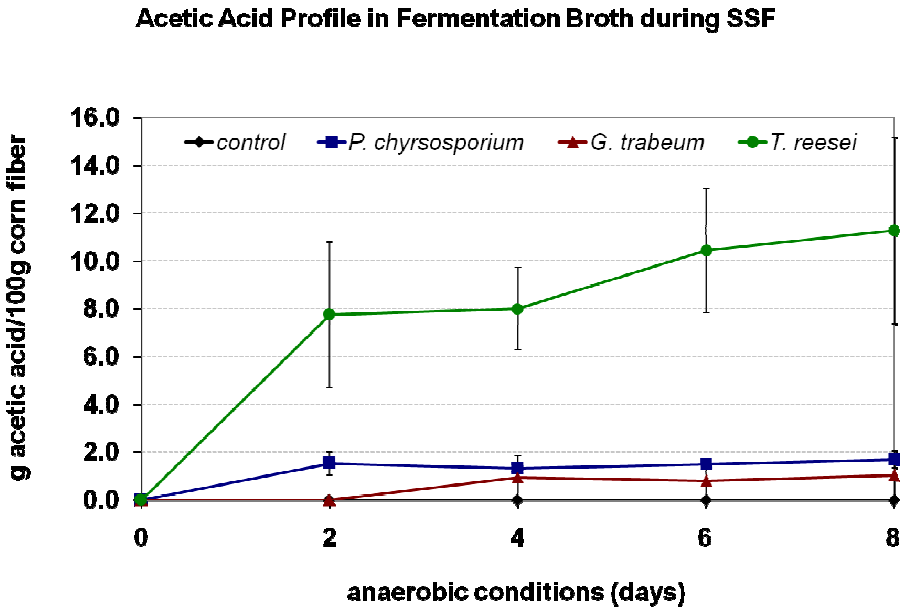

Figure 5: Acetic acid profile in culture broth from anaerobic simultaneous saccharification and fermentation for ethanol production in white-, brown-, and soft-rot (*P. chrysosporium, G. trabeum, and T. reesei*) fungal co-cultured with *S. cerevisiae* (n=2). Control is with yeast cells but no fungal culture.

From the contrasts output, it was found that lactic acid production in SSF involving *T. reesei* was significantly different when averaged over time than the SSF involving *P. chrysosporium* (p-value=0.019); but *P. chrysosporium* was not significantly different from *G. trabeum* when averaged over time (*p*-value=0.08). Similar findings were observed between *G. trabeum* and *T. reesei* when averaged over time (*p*-value=0.137). Various conditions, like broth composition and conditions of yeast cells during fermentation affect lactic acid formation (Stenber et al., 2000). There could also be the possibility of lactic acid bacteria contamination during SSF.

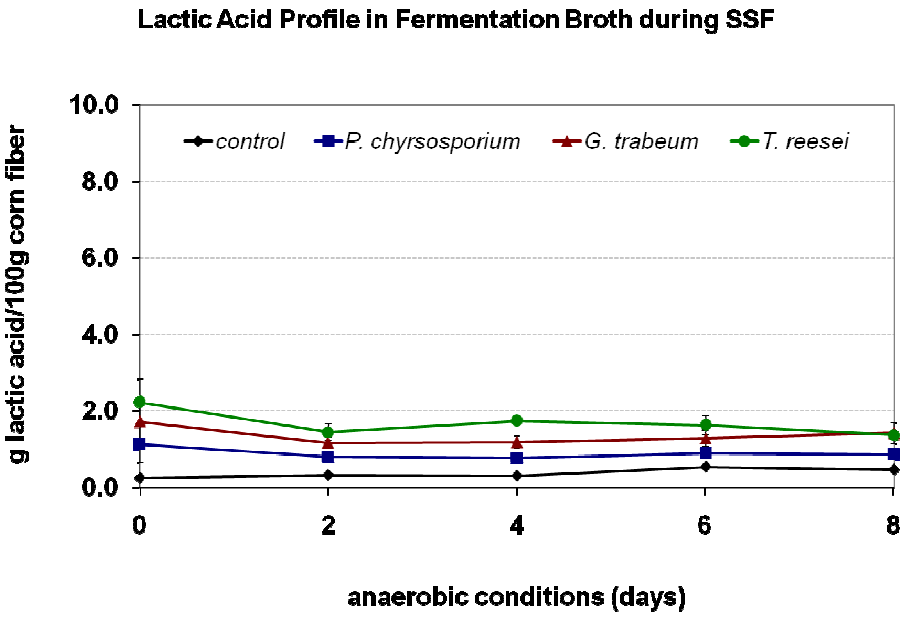

Figure 6: Lactic acid profile in culture broth from anaerobic simultaneous saccharification and fermentation for ethanol production in white-, brown-, and soft-rot (*P. chrysosporium, G. trabeum, and T. reesei*) fungal co-cultured with *S. cerevisiae* (n=2). Control is with yeast cells but no fungal culture.

3.4.5 Specific enzyme activity assays

Table 2 (a) shows the specific enzyme activity assay result on five different enzymes (α-amylase, glucoamylase, xylanase, endocellulase and exocellulase). It is evident that all three fungal species had shown activities for starch, xylan and cellulose. The corn fiber induced enzyme secretion in all three fungal cultures during aerobic submerged culture for 2 days. The residual starch and hemi/cellulose fractions had higher enzyme induction for white-rot fungus as compared to brown- and soft-rot fungi. Therefore, it is evident that both starch and hemi/cellulose fractions contributed significantly to enzyme induction and thereby saccharification and fermentation of corn fiber to ethanol. Similar results were also observed by Shrestha et al. (2008) and Rasmussen et al. (2008*)* for white- and brown-rot saccharification studies, respectively. There were no statistical differences between α-amylase and glucoamylase activities for all three fungal cultures (Table 2(b)). Xylanase, endo-, and exo-cellulase activities were significantly different between the fungal species. Higher biomass inventory (7.8 g/L, dry weight, DW) for white-rot fungus compared to brown (5.5 g/L, DW) and soft (4.4g/L, DW) fungi introduced to a fixed amount of corn fiber (10g) during the enzyme induction step may have resulted in higher enzyme activities of the white-rot fungus compared to brown- and soft-rot fungi.

Table 2a: Specific enzyme activities of different enzymes expressed as milligrams of product per minute per milligram of protein in 2-day-old submerged corn fiber fermentation with three fungal cultures (n=2)

Specific Enzyme Activity	P. chrysosporium	G. trabeum	T. reesei
α-amylase (mg maltose/mg protein.min)	0.230 ±0.06	0.160 ± 0.04	0.330 ± 0.08
glucoamylase (mg glucose/mg protein.min)	0.380 ± 0.15	0.180 ±0.01	0.375 ±0.175
xylanase (mg xylose/mg protein.min)	0.740 ± 0.17	0.060 ±0.00	0.060 ±0.00
endocellulase (mg glucose/mg protein.min)	0.505 ±0.05	0.215 ±0.04	0.050 ±0.00
exocellulase (mg glucose/mg protein.h)	1.030 ±0.05	0.090 ±0.05	0.265 ±0.05

All chemicals were purchased from Sigma Chemical Inc. (St. Louis, MO).

Table 2(b): Comparison of specific enzyme activities of three fungal species using p values obtained from student t-test

Specific Enzyme Activity	P. chrysosporium vs G. trabeum	P. chrysosporium vs T. reesei	G. trabeum vs T. reesei
α-amylase	0.4341	0.4266	0.1977
glucoamylase	0.3148	0.9847	0.3817
xylanase	0.0572	0.0572	not determined
endocellulase	0.0365	0.0096	0.0422
exocellulase	0.0056	0.0101	0.1615

3.5 Conclusion

Corn fiber from a wet milling plant represents cleaner lignocellulosic substrate for fungal SSF with no further pretreatment requirements. This study envisaged the concept of enzyme induction and subsequent simultaneous saccharification and fermentation processes to further enhance the enzymatic hydrolysis in conjunction with reduced mold-sugar-consumption during saccharification, and facilitate improved ethanol fermentation via co-culture of yeast. All three (white-, brown- and soft-rot) fungi illustrated extracellular enzyme production for the hydrolysis of corn fiber. SSF of *P. chrysosporium* and *G. trabeum,* with *S. cerevisiae had* higher saccharification and ethanol fermentation yield, (i.e., 35% of the theoretical maximum yield) whereas *T. reesei* had lower fermentation yields. This might be due to excess acetic acid formation compared to ethanol. Enzyme activities and yeast ethanol fermentation might have been affected by variable initial fungal biomass and sugar consumption by fungi during the enzyme induction phase, acidic pH, organic acid production and prolonged anaerobic conditions. Mild physical-chemical treatment of fiber prior to SSF is expected to enhance the net ethanol yield.

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CHAPTER 4: STUDY OF WOOD-ROT AND SOFT-ROT FUNGI FOR SACCHARIFICATION AND FERMENTATION OF MILD ALKALI AND STEAM PRETREATED CORN FIBER TO ETHANOL

(to be submitted to Bioresource Technology)

4.1 Abstract

The effect of pretreatment on simultaneous saccharification and fermentation of corn fiber by fungal processes was evaluated. Corn fiber was treated at 30^{o}C for 2 h with alkali [2% NaOH (w/w)], alkaline peroxide [2% NaOH (w/w) and 1% H_2O_2 (w/w)], and by steaming at 100^{o}C for 2 h. Solid-substrate fermentation of pretreated and untreated corn fiber separately with *Phanerochaete chrysosporium, Gloeophyllum trabeum and Trichoderma reesei* followed by simultaneous saccharification and fermentation (SSF) in anaerobic conditions for six days resulted in 2.55 g ethanol per 100 g of untreated corn fiber for white-rot SSF and 3.27 and 5.79 g ethanol per 100 g of alkaline peroxide pretreated corn fiber for brown-rot and soft-rot SSF, respectively. SSF of untreated and alkalipretreated corn fiber with Spezyme CP respectively resulted in 7.65 g and 7.74g ethanol per 100 g corn fiber. Mild pretreatment resulted in improved ethanol yields for brown- and soft-rot SSF, while white-rot and Spezyme CP SSFs showed no improvement in ethanol yield as a result of pretreatment. Steam pretreatment resulted in partial hydrolysis (~ 28%) of starch present in corn fiber. Fungal consumption of sugar during 4 days of solid-

substrate fermentation also reduced the availability of fermentable sugars during SSFs.

Keywords. Lignocellulosic biomass, corn fiber, solid-substrate fermentation, simultaneous saccharification and fermentation, enzymatic hydrolysis, ethanol, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum, Trichoderma reesei, Spezyme CP, Saccharomyces cerevisiae*.

4.2 Introduction

The United States ethanol industries have an annual production capacity of 10.7 billion gallons (~ 40.4×10^9 L) ethanol . There has been an almost four fold increase in ethanol production between 2000 and 2007 (RFA, 2008). Along with ethanol, these industries also produce huge quantities of fibrous co-products and are used for animal feed as corn gluten feed, corn gluten meal, and distiller's dried grains. In 2007, over 14.6 million metric tons of distillers' dried grains (DDG) was produced on corn dry-grind ethanol plants. Over 2.4 million metric tons of corn gluten feed (20% protein, dry mass) and 0.9 million metric tons of corn gluten meal (60% protein, dry mass) were also produced in corn wet-milling plants in 2006. Excessive production of these co-products may soon saturate animal feed demand and pose material handling problems. Utilization of corn-fiber and DDG, other than animal feed, has

recently been studied for producing monosaccharides like xylose and arabinose via pretreatments and enzymatic hydrolysis (Dien et al., 2006). Corn fiber arabinoxylan can also be potential source of gum (Yadav et al., 2007). Hicks et al. (2002) listed several potential application of corn fiber like industrial biobased products, corn fiber gum (CFG), corn fiber oil, hemi/cellulose and ethanol. Conversion of corn fiber into value added product like ethanol has potential to increase net ethanol production per bushel of corn both in corn dry-grind and corn wet-milling industries. Saha and Bothast (1999) reported the various chemical pretreatments of corn fiber and subsequent enzymatic saccharification to yield high monomeric sugars. Shrestha et al. (2008) reported conversion of corn fiber hydrolyzate, followed by white-rot fungal saccharification, into ethanol. Corn fiber as cellulosic feedstock represents (residual) starch (17%), cellulose (18%) and hemicellulose (35 %) (Abbas et al., 2004). Therefore, an 80% bioconversion efficiency of hexoses in corn fiber by SSF can yield as much as 200 liters (~ 53 gallons) of ethanol per metric tons of corn fiber, which would substantially improve net corn to ethanol conversion efficiency per acre.

Because of the recalcitrant nature and the presence of enzymatic and microbial inhibitors, the hemi/cellulosic component of any cellulosic feedstock including corn fiber needs to undergo physico-chemical pretreatment. This is an important and costly step in overall hydrolysis and fermentation of cellulosic feedstock into ethanol. Mosier et al. (2005) evaluated various pretreatment techniques for cellulosic feedstock. Chemical (acid and alkali) pretreatments are costly; require expensive and chemical resistant reactors; produce hydrolysis byproducts that inhibit

fermentation process and impose environmental problems. Therefore, it is necessary to develop environment-friendly, cost-effective and highly efficient enzymatic hydrolysis process for economic cellulosic ethanol production (Saha et al., 1998). Steam pretreatment followed by enzymatic hydrolysis of starch-free wheat fiber achieved ~ 75% of theoretical sugar yield (Palmarola-Adrados et al., 2004). Boussaid et al. (2000) reported proportionate hydrolysis of hemicellulose and cellulose when SO_2 impregnated Douglas-fir wood were steam exploded at different severity levels. Alkali (1%) pretreatment at 55^{o}C for 2 h followed by hydrogen peroxide pretreatment at various concentrations resulted in proportionate hemicellulose hydrolysis in rice straw (Sun et al., 2000). Alkali pretreatment at 2% (w/w) NaOH has been considered optimal for effective hemicellulose degradation and subsequent enzymatic hydrolysis (Singh et al., 1989). Saha and Cotta (2005) also discussed alkaline peroxide (2.15 % [v/v]) pretreatment of wheat straw at 35^{o}C for 24 h followed by 5 days of enzymatic hydrolysis and 2 days of fermentation, which resulted into ~ 29% bioconversion of straw into ethanol at conversion efficiency of 0.46 g ethanol/ g of available sugar. In addition to chemical pretreatments, reports of biological pretreatments of lignocellulosic biomass have been reported regularly. Lee et al. (2007) conducted biological pretreatment of Japanese red pine chips over 8 weeks using three white-rot fungal species. Over 20% increase in sugar yield was reported following enzymatic hydrolysis of red pine chips pretreated with the white-rot fungus, *Stereum hirsutum*. Biodegradation of eucalyptus wood chips by white- and brown-rot fungi have been studied in solid substrate fermentation by Machuca and Ferraz (2001). White-rot fungi, including

Phanerochaete chrysosporium, have been studied for pretreatment of various agricultural residues like sugarcane bagasse (Rolz et al., 1987), wheat straw (Muller et al., 1986), cotton stalk (Shi et al., 2008) and corn fiber (Shrestha et al., 2008). Our research group has been conducting research on white-rot (*Phanerochaete chrysosporium*) and brown-rot (*Gloeophyllum trabeum*) fungi in saccharification and fermentation of cellulosic feed stocks like corn fiber and corn stover (Shrestha et al., 2008a,b). Restriction of commercial enzyme usage for saccharification of cellulose feedstock is one of the research achievements. In our earlier research work, we had been partially successful to convert corn fiber into ethanol via wood-rot fungal pretreatment of corn fiber followed by simultaneous saccharification and fermentation of hydrolyzate to ethanol. A maximum yield potential of 110 L ethanol per metric ton of corn fiber has been reported (Shrestha et al., 2008b).

This research is aimed at evaluating the effectiveness of mild alkali, alkaline peroxide and steam pretreatment of wet-milled corn fiber prior to solid-substrate fermentation by white-, brown- and soft-rot fungi and subsequent fermentation of hydrolyzate to ethanol.

4.3 Materials and Methods

4.3.1 Fungal Culture

Fungal stock cultures: white-rot fungus: *Phanerochaete chrysosporium* (ATCC 24725), brown-rot fungus: *Gloeophyllum trabeum* (ATCC 11539), soft-fort fungus: *Trichoderma reesei* (ATCC # 13631), and yeast: *Saccharomyces cereivisiae* (ATCC # 24859) were prepared as described earlier (Shrestha et al., 2008b).

4.3.2 Substrate

Archer Daniel and Midland – ADM, Decatur, Illinois provided the wet-milled corn fiber with moisture content about 65%. The corn fiber was dried at 80^{o}C for 4 days followed by overnight desiccation. The dried corn fiber was milled to pass a 20-mesh screen via a Wiley Mill (Thomas Scientific, Swedesboro, NJ)

4.3.3 Pretreatment

Various pretreatment methods of 100 g ground corn fiber in 500 ml deionized water were performed in three 1-L polypropylene centrifuge bottles. The treatments were:

(a) Alkali pretreatment – 2 g of NaOH per 100 g of ground corn fiber in 500 ml of deionized water shaking at 150 rpm and 30 ^{o}C for 2 h (i.e., 2% NaOH, w/w of corn fiber),

(b) Alkaline peroxide pretreatment – 2 g of NaOH and 2.94 ml of 33% hydrogen peroxide per 100 g of ground corn fiber in 500 ml of deionzied water shaking at 150 rpm and 30^{o}C for 2 h (i.e., 2 % NaOH and 1% H2O2, w/w of corn fiber), and

(c) Steam pretreatment – 100 g of corn fiber in 500 ml deionized water in a steaming cabinet at 100 ^{o}C, standing for 2 h with occasional shaking

Following pretreatment, the bottles were centrifuged at *7277 g* for 20 minutes to collect the pretreated biomass. The residue in each bottle was remixed with sterile deionized water and the liquid was decanted. The process was repeated three times and finally pH was adjusted to 4.5 by sodium hydroxide addition before final decantation. The residues were then autoclaved at 121 ^{o}C for 15 minutes to reduce contamination.

4.3.4 Experimental Setup

4.3.4.1 Fungal culture preparation

White-rot (*P. chrysosporium*), brown-rot (*G. trabeum*) and soft-rot (*T. reesei*) fungi were cultured in shake flasks, collected aseptically by centrifugation and densified fungal pellets were prepared as described earlier (Shrestha et al., 2008b).

4.3.4.2 Solid-substrate fermentation

Solid-substrate fermentation experiment consisted of sterile 100-ml polycarbonate bottles, in replicates of two, each with 20 g (wet weight) pretreated or untreated (5 g of ground corn fiber and 15 ml of water autoclaved at 121^{o}C for 15 minutes) corn fiber and 20 g (wet weight) of densified fungal pellets. Altogether, there were four sets of duplicate polycarbonate bottles: three for pretreated and one for untreated corn fiber for solid-substrate fermentation using each of the three fungal species. The mixing and spreading of the fungal pellets and corn fiber was performed as described by Shrestha et al. (2008a). The polycarbonate bottles with uniformly spread mix of fungal pellets and corn fiber were capped with sterile autoclave cloth. The culture bottles were then kept static for four days in an incubator (37^{o}C) equipped with humidified air supply through a water trough at the base.

4.3.4.3 Simultaneous saccharification and fermentation (SSF)

After 4 days of solid-substrate fermentation, 70-ml basal medium (*21*), 25 ml of yeast peptone (YP) medium and 5 ml of 24 hr yeast culture (~ $7x10^7$ cells/ml) were added to each bottle. In another set of experiments, the aforementioned pretreated or untreated corn fiber after fungal treatment was also used for the SSF process using commercial cellulosic enzyme: Spezyme-CP (Genencor) (50 filter paper units(FPU)/ g of cellulose; 25 FPU/ml). For enzyme treatment of each fungal treated corn fiber. 20 g of corn fiber was mixed with 68 ml of basal medium, 25 ml of yeast peptone medium, 2 ml of Spezyme CP and 5 ml of 24 hr yeast culture (~ $7x10^7$ cells/ml). For each treatment the bottles were loosely capped and kept static in an incubator at 37^oC for 6 days.

The overall experimental procedure is schematically presented in Figure 1.

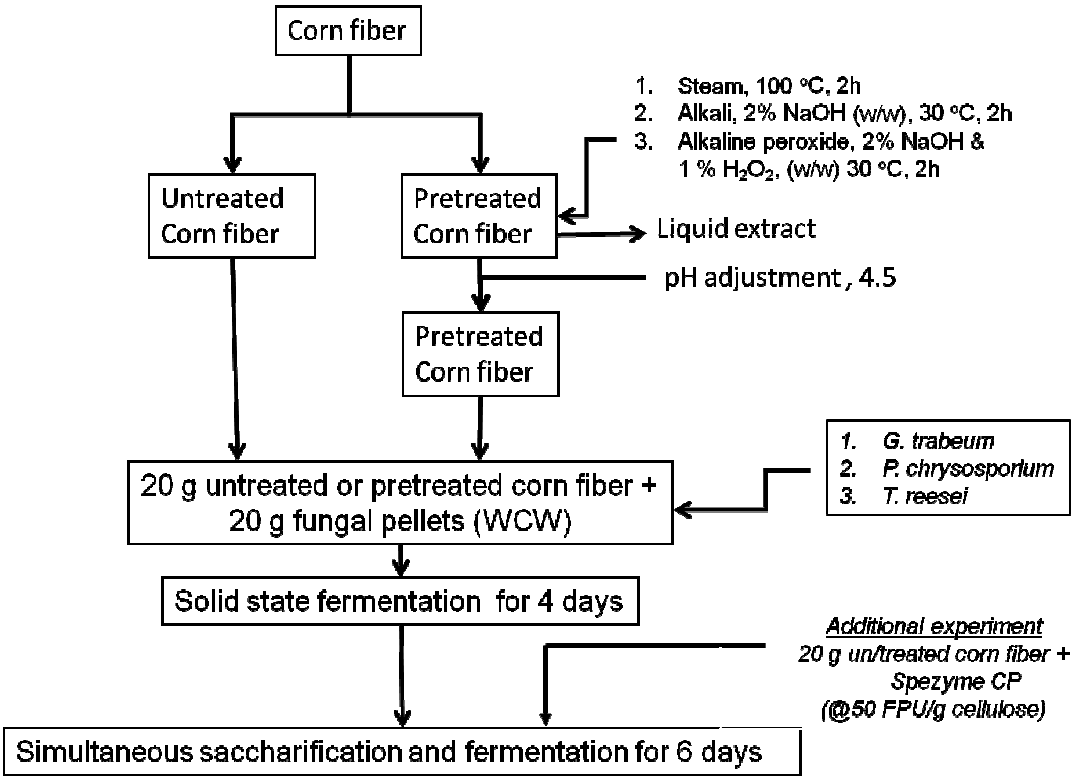


Figure 1: Schematic of overall experiment on solid substrate fermentation and simultaneous saccharification and fermentation of corn fiber (untreated and pretreatments like (1) Steaming, (2) 2% sodium hydroxide or (3) 2% sodium hydroxide and 1% hydrogen peroxide)

4.3.5 Analytical Methods

Every alternate day, 4 ml samples were collected aseptically from each bottle. The samples were steamed in sealed tubes for 15 minutes, cooled and centrifuged at 14000 *g* for 3 min. The supernatants were syringe filtered through 0.22 µm syringe filters (Acrodisc Premium 25 mm Syringe Filter with glass fiber pre filter and hydrophilic polypropylene, pore size: 0.22 µm , Pall Life Sciences) for the HPLC assays to determine glucose, xylose, ethanol, acetic and lactic acids measured by using a Bio Rad Aminex 87-H (78x300) organic acid column in Waters High Pressure Liquid Chromatography (Millipore Corporation, Milford, MA, USA). Kim and Lee (1996) reported that xylose, mannose and galactose are detected as a single peak, and therefore referred as xylose-mannose-galactose (XMG), in Bio Rad Aminex 87-H column. Xylose (20%, DM) and arabinose (11%, DM) are major components of hemicellulose compared to mannose (1.4%, DM) and galactose (4.2%, DM) in corn fiber. Therefore, for our convenience, XMG concentrations was considered as xylose in interpretation of result.

4.3.6 Statistical Analyses

The SSF results on ethanol, acetic acid and xylose were analyzed for null model likelihood ratio test; solution for fixed effects; estimates and contrasts using the statistical tool –SAS. All assays and fermentations were performed in replicates of

two (n=2) and significant difference of p value of 0.10 was employed. Glucose and biomass weight loss data were verified for type 3 tests of fixed effects and contrasts.

4.4 Results and Discussion

4.4.1 Pretreatment and fungal solid-substrate fermentation effect on corn-fiber monosaccharide release

4.4.1.1 Glucose profile

Extractable glucose concentrations were the highest for the soft-rot solid-substrate fermentations at day 0. Corn fiber to glucose conversion rate, in grams glucose per 100 grams of corn fiber, were 12.17, 11.50, 7.32 and 10.07 for pretreatment with alkaline peroxide, alkali, steam and no pretreatment (control), respectively (Figure 2).

Comparatively, the glucose released from steam treated corn fiber was consistently the lowest for all of the pretreated samples, and white-rot solid-substrate fermentation released the least glucose for the fungal solid-substrate fermentations. In case of Spezyme CP, the enzyme was added at day 0 of anaerobic fermentation. The representative lower glucose concentration could be from residual sugar in enzyme and soluble sugar from corn fiber. Steaming hydrolyzed ~ 28% of the residual starch in corn fiber, which would be consumed by the fungus (table 1).

Thus, during fungal solid-substrate fermentation, a reduction in released glucose concentration would be expected.

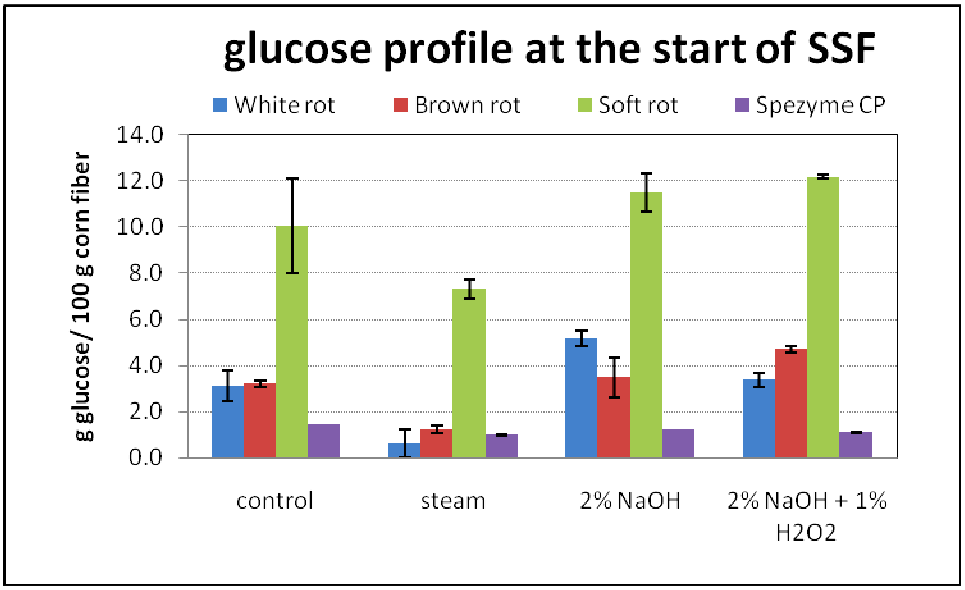


Figure 2: Glucose concentration at the start of simultaneous saccharification and fermentation of untreated and pretreated corn fiber (n=2)

Alkali and alkaline-peroxide pretreatment have significant effects on fungal saccharification of corn fiber. Glucose concentration was higher for white-rot saccharification for alkali pretreated fiber compared to untreated fiber but statistically not different ($p = 0.1153$). Alkaline peroxide pretreatment has higher and significantly different ($p=0.0192$) glucose yield in case of brown-rot saccharification when compared to untreated corn fiber. On the other hand, glucose concentration was the highest for soft-rot saccharification of corn fiber when compared to white- ($p = 0.0845$) and brown-rot ($p=0.0794$) saccharification. There was no statistical

difference in glucose concentration from brown-rot saccharification between either alkali or alkaline-peroxide pretreated corn-fiber (*p=0.2889*). The same was true for soft-rot saccharification of alkali or alkaline-peroxide pretreated corn fiber (*p=0.5000*). White-rot saccharification had a significantly (*p = 0.0602*) higher glucose concentration for alkali pretreated corn fiber compared to alkaline-peroxide pretreatment. Shrestha et al. (2008) reported enzymatic action of white-rot fungus over starch and hemi/cellulose in corn fiber. Fungal consumption of sugars during 4 days of solid-substrate fermentation also resulted in lower glucose yields. In the advent of anaerobic conditions, glucose was converted to ethanol by *Saccharomyces cerevisiae* and therefore, glucose profile was not recorded in successive fermentation samples.

Table 1: Starch analyses result for untreated and pretreated corn fiber using total starch assay procedure (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) with modifications.

The values in parentheses are the standard deviation

%RS: % Residual starch; %SS: % Soluble sugar and %TSD: % Total starch derivatives = %RS + %SS.

Sample	**% RS**	**% SS**	**% TSD**
1. Control	18.83 (0.40)	2.75 (0.19)	21.58
2. Steam (100 ^{o}C, 2 h	13.02 (0.28)	2.58 (0.03)	15.60
3. 2% NaOH and 1% H_2O_2 (30°C, 2 h)	19.24 (0.33)	2.56 (0.07)	21.80
4. 2% NaOH (30°C, 2 h)	18.22 (0.39)	2.62 (0.10)	20.84

4.4.1.2 Xylose profile

Xylose release were statistically different for pretreatments, fungal SSF and sampling days – individually or two-way and three-way interactions ($p<0.0001$) (Figure 3 a,b). Since xylose is not utilized by *S. cerevisiae,* it will accumulate during SSF. Day 0 brown-rot SSF of untreated corn fiber had the maximum xylose released (1.51 g xylose per 100 g corn fiber). Similarly, Spezyme CP SSF, white-rot SSF and soft-rot SSF had 1.445, 1.325 and 1.16 g xylose per 100 g untreated corn fiber on sampling days 6, 0 and 6, respectively. By the end of the SSFs, on day 6, the xylose profiles were the highest for Spezyme-CP SSFs of untreated and treated corn-fiber compared to respective fungal SSFs of untreated, steam, alkali and alkaline-peroxide pretreated corn-fiber. Spezyme CP SSFs of steam pretreated corn-fiber had significantly more xylose released (1.385 g xylose per 100 g corn fiber) when compared the results between alkali (1.165 g xylose per 100 g corn fiber, $p = 0.0707$) and alkaline peroxide (1.16 g xylose per 100 g corn fiber, $p = 0.0649$) pretreatments. Similar results were obtained for brown-rot SSFs of steam pretreated corn-fiber (1.045 g xylose per 100 g corn fiber) when compared with alkali (0.655 g xylose per 100 g corn fiber, $p = 0.0025$) and alkaline peroxide (0.61 g xylose per 100 g corn fiber, $p = 0.0009$) pretreatments.

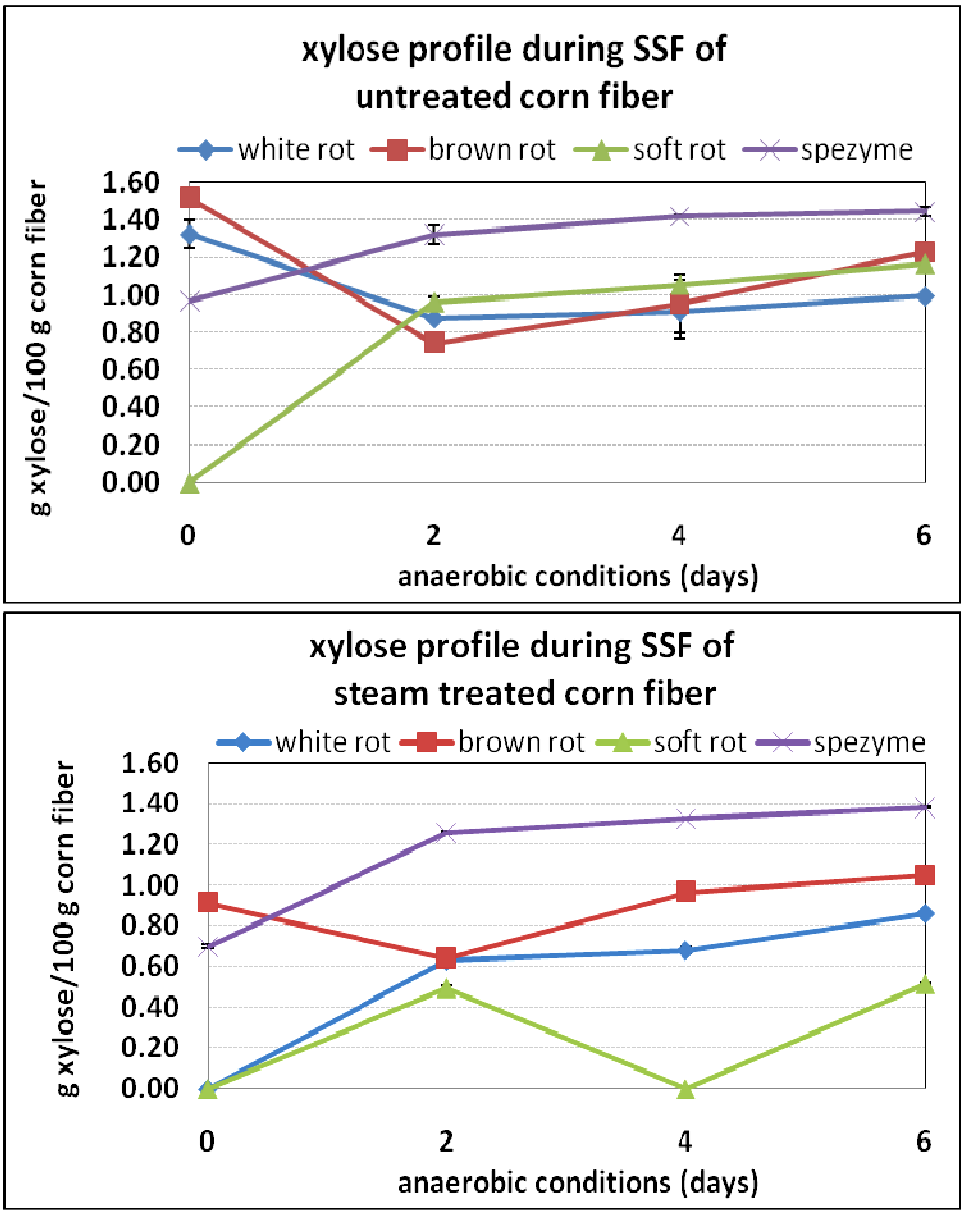


Figure 3a: Xylose profile during simultaneous saccharification and fermentation of untreated and steam pretreated corn fiber (n=2)

Among the pretreatments, Spezyme CP SSF of steam pretreated corn-fiber had the highest xylose yield of 1.385 g xylose per 100 g corn fiber. This was significantly different among brown-rot (1.045 g xylose per 100 g corn fiber, $p = 0.0084$), white-rot (0.86 g xylose per 100 g corn fiber, $p = 0.0002$) and soft-rot (0.51 g xylose per 100 g corn fiber, $p<0.0001$) SSFs of steam pretreated corn fiber. For white-rot SSFs, significant difference in xylose profiles were between alkaline-peroxide and untreated corn-fiber ($p= 0.0457$). Soft-rot SSFs also had the highest xylose of 1.16 g xylose per 100 g untreated corn fiber and was statistically different from SSFs for steam (0.51 g xylose per 100 g corn fiber, $p <0.0001$), alkali (0.615 g xylose per 100 g corn fiber, $p<0.0001$) and alkaline-peroxide (0.78 g xylose per 100 g corn fiber, $p = 0.0031$) pretreated corn-fiber.

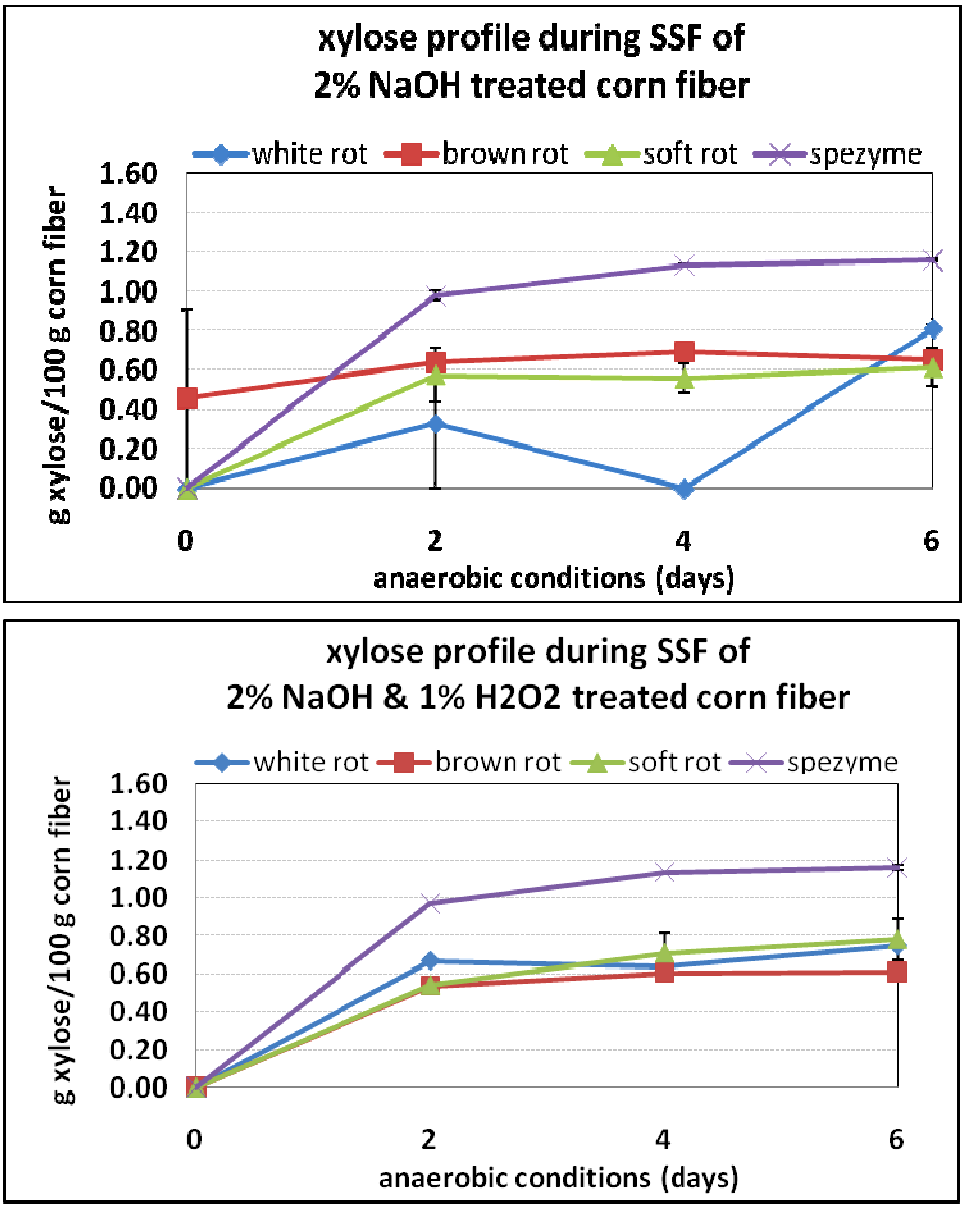


Figure 3b: Xylose profile during simultaneous saccharification and fermentation of alkali and alkaline peroxide pretreated corn fiber (n=2)

4.4.2 Ethanol production in simultaneous saccharification and fermentation (SSF)

There was a strong significance in differences for pretreatments (*p =0.0034*), fungal SSF (*p <0.0001*) and sampling days (*p <0.0001*), on ethanol yield from corn fiber (Figure 4 a,b). Interactions between pretreatment and fungal SSF (pretreatment*fungi, *p <0.0001*), fungal SSF and sampling days (fungi*day, *p <0.0001*) and pretreatment, fungal SSF and sampling days (pretreatment*fungi*day, *p <0.0001*) were all statistically significant. The ethanol yield was determined as g ethanol per 100 g of corn fiber. The highest ethanol yield, at day 6, was 7.74 g of ethanol per 100 g corn fiber when Spezyme CP was used for alkali pretreated corn fiber. This is however, not significantly different with the ethanol yield from untreated corn fiber (7.65 g ethanol per 100 g corn fiber, *p =0 .7019*) and alkaline peroxide corn fiber (7.46 g ethanol per 100 g corn fiber, *p = 0.2560*).

Soft-rot SSF

When compared with soft-rot SSF ethanol yield result from similar corn fiber (pretreated versus pretreated, control vs control etc.), Spezyme CP SSF ethanol yields were significantly different (*p<0.0001*). Alkaline-peroxide pretreatment enhanced ethanol yield for soft-rot SSF (5.79 g ethanol per 100 g corn fiber, *p = 0.0105*) when compared with soft-rot SSF of untreated corn fiber (5.11 g corn fiber

per 100 g corn fiber). Alkaline peroxide and alkali pretreatments of corn fiber did not have differences in ethanol yields ($p = 0.2991$) for soft-rot SSF.

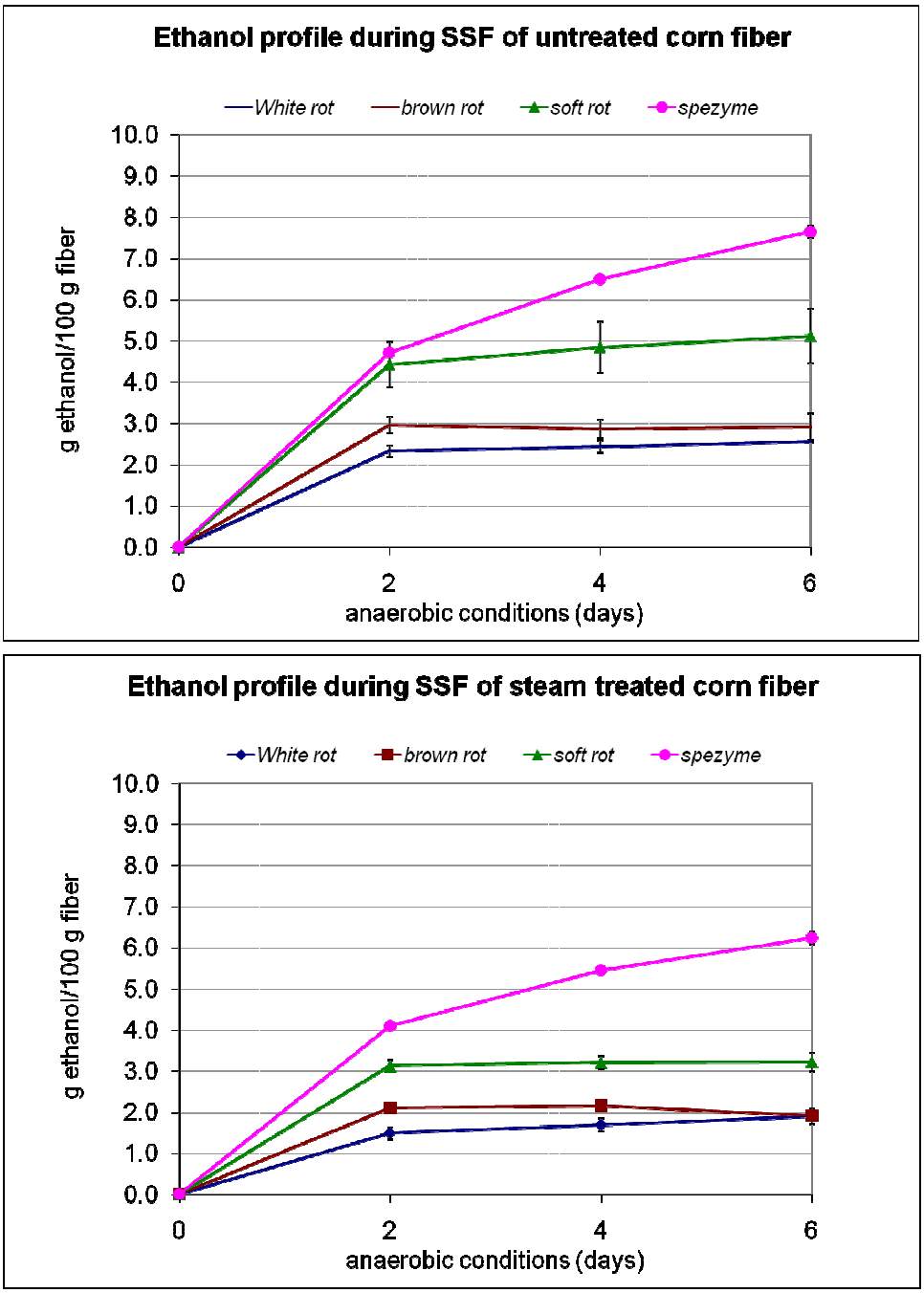


Figure 4a: Ethanol profile during simultaneous saccharification and fermentation of untreated and steam pretreated fiber (n=2)

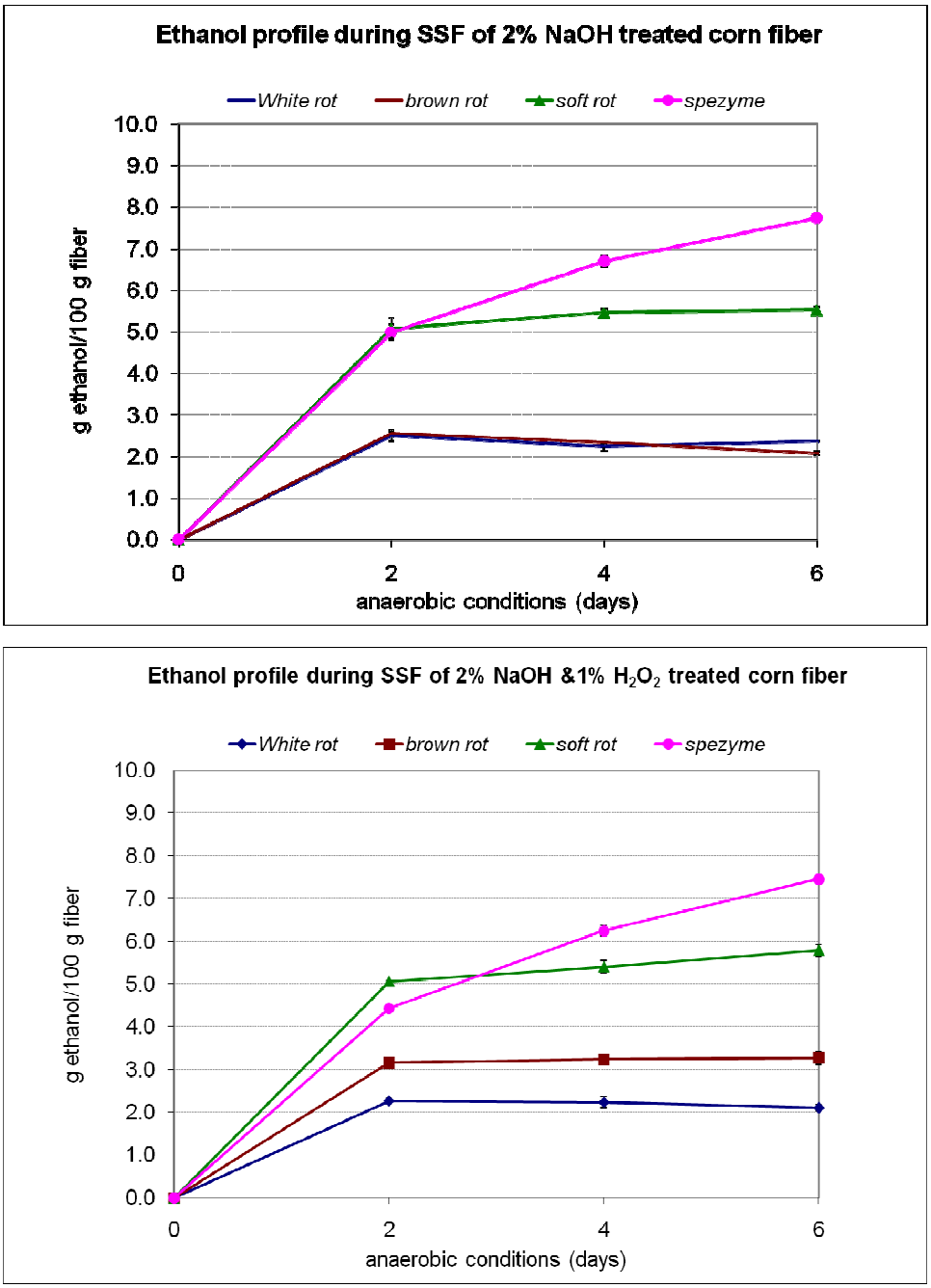


Figure 4b: Ethanol profile during simultaneous saccharification and fermentation of alkali and alkaline peroxide pretreated fiber (n=2)

Wood-rot SSF

The white-rot and brown-rot SSF of corn fiber resulted in lower ethanol yields for alkaline peroxide, alkali and steam pretreatments when compared to soft-rot SSF (*p<0.0001*). For brown-rot SSF, alkaline peroxide pretreatment had significant (*p<0.0001*) ethanol yield (3.27 g ethanol per 100 g corn fiber) compared to alkali pretreated corn fiber (2.08 g ethanol per 100 g corn fiber) and steam pretreated corn fiber (1.91 g ethanol per 100 g corn fiber) but not significantly different (*p = 0.*1598) with untreated corn fiber (2.91 g ethanol per 100 g corn fiber).

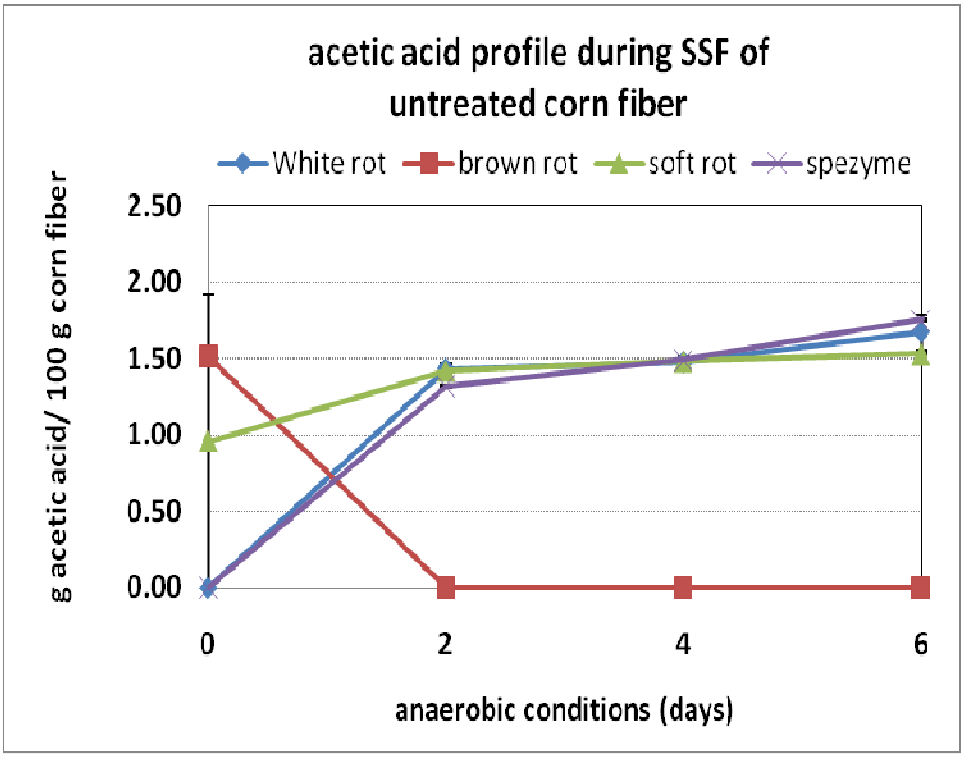
White-rot SSF resulted in the highest ethanol yield for untreated corn fiber (2.55 g ethanol per 100 g corn fiber) but was insignificantly different for ethanol yield from alkaline-pretreated corn fiber (2.375 g ethanol per 100 g corn fiber, *p = 0.4699*) and significantly different with alkaline peroxide (2.105 g ethanol per 100 g corn fiber, *p = 0.0779*) and steam (1.9 g ethanol per 100 g corn fiber, *p = 0.0128*) pretreatments of corn fiber.

Alkaline peroxide pretreatments had enhanced ethanol yield for soft- and brown-rot SSF of corn fiber. However, white-rot SSF did not result into higher ethanol yield when compared to untreated or steam treated corn-fiber. Steam pretreated corn fiber resulted into lower ethanol yields for all SSF studies. This could be attributed to partial hydrolysis of residual starch and separation of hydrolyzed starch in liquid fraction after steam pretreatment. Steam pretreatment had hydrolyzed ~ 28% of the

residual corn fiber starch (Table 1). Lower ethanol yield for white- and brown-rot SSF could also be attributed to consumption of sugar during solid-substrate fermentation of untreated and pretreated corn fiber. The concentration of glucose at 0 day of anaerobic fermentation, preceded by 4 days of solid-substrate fermentation, was the highest for soft-rot saccharification compared to white- and brown-rot saccharification.

4.4.3 Acetic acid production in simultaneous-saccharification and fermentation (SSF)

Overall acetic acid production in different fungal SSF ranged between 1 to 2 g acetic acid per 100 g of treated or untreated fiber. Contrary to ethanol yields, pretreatment of corn fiber did not significantly affect acetic acid profiles ($p = 0.8415$), whereas fungal saccharification of corn-fiber has significant effect ($p<0.0001$) (Figure 5). Soft-rot SSF consistently demonstrated high levels of acetic acid even on day 0, whereas Spezyme CP SSF demonstrated continuous acetic acid production over the 6 day SSF incubation with final concentrations of 1.75 g acetic acid per 100 g untreated corn fiber. Interestingly, for brown-rot SSF, acetic acid was degraded or it was not produced at all. Acetic acid might be produced due to degradation of hemicelluloses and/or as by-product of ethanol fermentation during SSF.



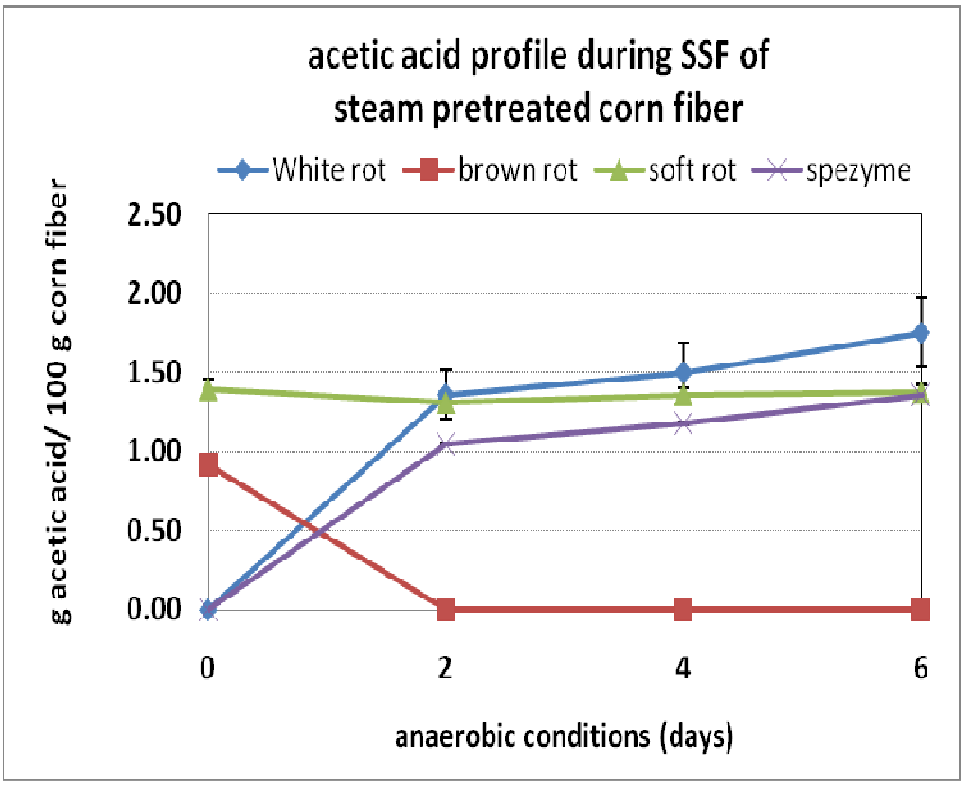


Figure 5a : Acetic acid profile during simultaneous saccharification and fermentation of untreated and steam pretreated fiber (n=2)

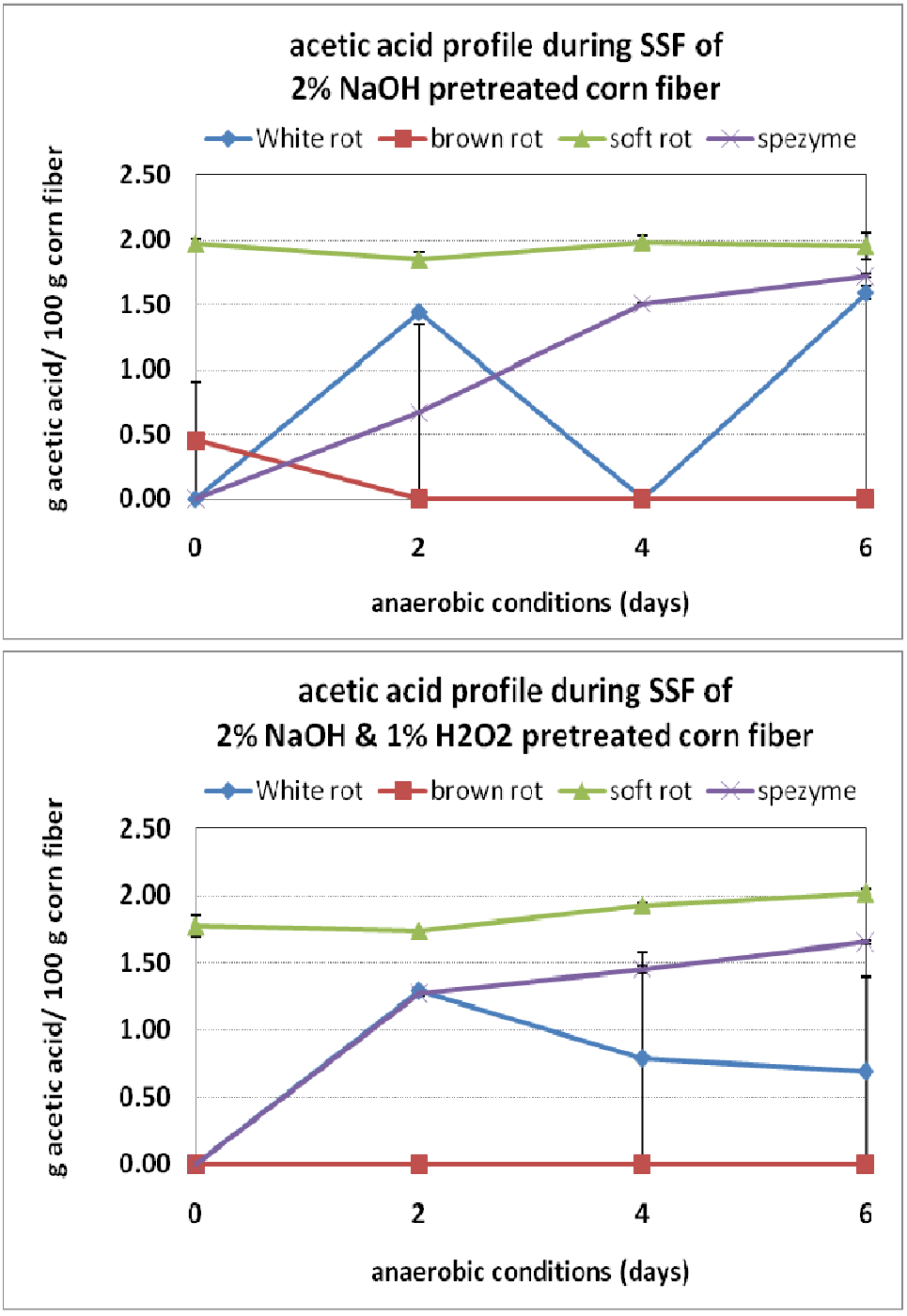


Figure 5b: Acetic acid profile during simultaneous saccharification and fermentation of alkali and alkaline peroxide pretreated fiber (n=2)

As SSF incubation continued, sugar (e.g. glucose) became scarce and this could have also have resulted in scarcity of NADH. Incorporation of ethanol back into yeast cells could be possible (Piskur et al., 2006) for production, by alcohol dehydrogenase, of NADH and acetaldehyde. The later could further convert to acetic acid, by aldehyde dehydrogenase, with production of NADPH.

4.4.4 Lactic acid production in simultaneous saccharification and fermentation (SSF)

The lactic acid profile was very inconsistent among fungal and Spezyme SSF of untreated and pretreated corn fiber as determined by HPLC. A range of 0 to 1.88 g of lactic acid per 100 g of corn fiber was observed.

4.4.5 Biomass weight loss

Fungal and Spezyme CP SSFs also resulted in weight loss of untreated and pretreated corn fibers respectively (Figure 6). The highest biomass weight loss (55±4.61 %, w/w) was for SSF of Spezyme CP untreated corn fiber and this was significantly different among the biomass weight losses from fungal-SSFs of untreated corn fiber ($p = 0.005$). Similarly, biomass weight loss for SSF of Spezyme

CP for steam ($p = 0.1000$), alkali ($p = 0.086$) and alkaline peroxide pretreated ($p = 0.008$) corn fiber were also significantly different (90% confidence interval) when compared with white-, brown- and soft-rot SSFs. However, no significant difference in biomass weight losses was observed between SSFs within each untreated and pretreated corn fiber.

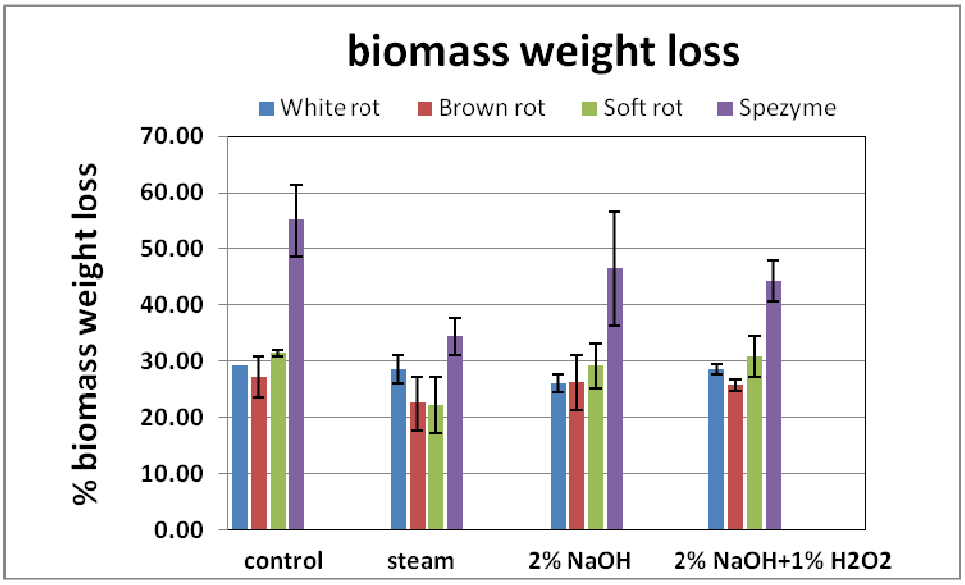


Figure 6: Biomass weight loss following simultaneous saccharification and fermentation of untreated and pretreated fiber (n=2)

4.5 Conclusion

Saccharification and fermentation of untreated and pretreated corn fiber by white-, brown- and soft-rot fungi and Spezyme CP had varied results in terms of ethanol, acetic acid, and monosaccharide released (e.g., glucose and xylose) and biomass weight loss. SSF with Spezyme CP had higher ethanol yield compared to the yields from wood- and soft-rot SSFs. Among the fungal SSFs, soft-rot SSFs results in terms of ethanol yields were comparable to SSFs with Spezyme CP. Though pretreatment (alkaline peroxide) had positive impact on brown-rot SSF, the ethanol yield was very low (over 50%) compared to soft-rot SSFs. SSFs yields from steam pretreated corn fiber were the lowest. Separation of partially hydrolyzed reisdual starch (~ 28%) during steam pretreatment of corn fiber could have been utilized during SSF process to yield extra ethanol. Therefore, steam pretreatment could be simple and possible pretreatment prior to simultaneous saccharification and fermentation of corn fiber to ethanol to increase net ethanol production per bushel of corn in corn wet-milling industries. Prolonged, 4 days, SSF might have helped brown-rot fungus to consume sugars. Acetic acid production was enhanced by alkali/alkaline peroxide pretreatments only in case of soft-rot SSFs.

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CHAPTER 5: STUDY OF ENZYME ACTIVITIES DURING SOLID-SUBSTRATE FERMENTATION OF CORN FIBER BY WOOD-ROT AND SOFT-ROT FUNGI

(to be submitted to Journal of Agricultural and Food Chemistry)

5.1 Abstract

Cellulase, xylanase and amylase activities of three fungi: *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* and *Trichoderma reesei,* were investigated over a week long solid-substrate fermentation of corn fiber. *P. chrysosporium* had the highest activities for starch (13.53 mg glucose/l.min) and for xylan (12.10 mg xylose/l.min) on day five of solid-substrate fermentation. *G. trabeum* had the highest activity for carboxymethyl cellulose (6.24 mg glucose/l.min) on day seven and *T. reesei* had the highest activity for Sigma cell 20 (3.46 mg glucose/l.min) on day 5 of solid-substrate fermentation. To our knowledge, this is the first reported production of celluloytic enzyme from wet-milled corn fiber solid-substrate fermentations for these fungi. Optimization of physical (temperature, moisture, pH and oxygen diffusibility) and chemical (nutrients and metals) parameters are still necessary to improve the induction of cellulase, hemicellulase and amylase for these fungi.

Keywords. Lignocellulose, corn fiber, solid-substrate fermentation, simultaneous saccharification and fermentation, enzymatic hydrolysis, ethanol, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum, Trichoderma reesei.*

5.2 Introduction

Biological cellulosic ethanol production from plant biomass generally requires pretreatment via physical, chemical and/or biological followed by enzymatic hydrolysis and fermentation of released sugar (i.e., glucose) to ethanol (Wyman, 1996). Abundant (native) availability (USA produces ~1.3 x10^9 tons of plant biomass annually) of cellulosic biomass is favorable towards sustainable renewable fuel generation (ORNL-USDA, 2005). Lignocellulose is a network of cellulose, hemicellulose and lignin (Hamelinck et al., 2005). Chemical (alkali and acid) lignocellulose pretreatment removes inhibitory compounds which are detrimental to subsequent enzymatic hydrolysis of polysaccharides and microbial fermentation to biofuels; such pretreatment is expensive (Mosier et al., 2005). It is therefore necessary to reduce chemical cost and environmental footprints and explore alternative environment friendly and economically sound processes like direct biological conversion of cellulose to ethanol .

In situ hydrolytic enzymes production for biological conversion of cellulosic biomass to biofuel will significantly reduce biofuel production costs. Hydrolysis of cellulose

and hemicelluloses (holocellulose) requires group of enzymes like hemicellulase (xylanase, arabinase, mannase etc) and cellulase complexes (endocellulase, exocellulase, cellobiase etc) (Cloete and Malherbe, 2002). Many bacterial and fungal species have been studied in recent decades for their ability to produce aforementioned enzyme complexes. Recently, agricultural and industrial lignocellulosic residues have also recieved a lot of attention for their application as cheap substrates in cellulolytic enzyme production studies using different fungi. Li et al (2005) reported on induced hemi/cellulolytic enzyme activities of *Trichoderma reseei* Rut C-30 grown on crude and fractionated corn fiber. Though, *T. ressei* has been extensively studied for cellulase enzymes synthesis (Kadam , 2006) over varieties of cellulosic feedstock, the overall enzymatic hydrolysis of cellulose is highly affected by limited β-glucosidase (cellobiase) activity leading to the accumulation of cellobiose, which further inhibits the activity of other endocellulase and exocellulase (Zhiyou Wen, Wei Liao and Shulin Chen – 2005). Co-culture of *T. reesei* with *Aspergillus phoenicis* in animal manure supplemented with basal salts boosted enzyme consortia and had effective results in overall cellulose hydrolysis. Many other fungal species especially white- and brown-rot fungi have also been explored for their efficacy in cellulase production. Highley (1973) studied white- and brown-rot fungi for induction of cellulase enzymes using hard and soft woods. Likewise, Hatakka (1983) conducted white-rot treatment of wheat straw and reported the improvement in enzymatic hydrolysis. Fungal treatment of biomass had however, been reported to be time consuming and necessitated optimization of the technique. Our research group has also been conducting solid-substrate

fermentation of corn fiber (using white-rot fungus: *Phanerochaete chrysosporium* and brown-rot fungus: *Gloeophyllum trabeum*) for few days followed by simultaneous saccharification and fermentation (SSF) to ethanol (Shrestha et al., 2008a; Rasmussen et al., 2008). We successfully conducted fermentation of corn fiber using these fungi and compared that with performance of *T. reesei* (wild type) (Shrestha et al 2008 b) in terms of ethanol yield. This research is, however, focused on comparing enzyme profiles during 7 days of solid-substrate fermentation of corn fiber using three fungi *P. chrysosporium, G. trabeum and T. reesei.*

5.3 Materials and Methods

5.3.1 Fungal Culture

Fungal stock cultures: white-rot fungus: *Phanerochaete chrysosporium* (ATCC 24725), brown-rot fungus: *Gloeophyllum trabeum* (ATCC 11539), and soft-fort fungus: *Trichoderma reesei* (ATCC # 13631) were prepared as described earlier (Shrestha et al., 2008b).

5.3.2 Substrate

Corn fiber from a corn wet-milling industry (Archer Daniel and Midland – ADM, Decatur, Illinois) was used as substrate for solid-substrate fermentation as described earlier (Shrestha et al., 2008a; 6). Oven dried corn fiber was milled to 20-mesh size in a Wiley Mill (Thomas Scientific, Swedesboro, NJ).

5.3.3 Pretreatment

Prior to fungal inoculation in the corn fiber, 5 g of corn fiber was added with 5 ml of deionized water in a 50-ml centrifuge tube. The mixture of fiber and water was vortexed and the tube-mouth was wrapped with autoclave cloth. Numbers of tubes were prepared as described above and were then autoclaved at 121^{o}C for 15 min and allowed to cool for 30 min.

5.3.4 Experimental Setup

5.3.4.1 Fungal culture preparation

White-rot (*P. chrysosporium*), brown-rot (*G. trabeum*) and soft-rot (*T. reesei*) fungi were grown and densified fungal pellets were prepared as described previously (*20*).

5.3.4.2 Solid-substrate fermentation

The autoclaved 50-ml centrifuged tubes (5 g corn fiber each) were inoculated aspectically with 5 g of densified (wet weight) white-, brown- or soft-rot fungal pellets. With the tubes' mouths covered with sterile autoclave cloth, the fungal and fiber mixture was vortexed. There were five sets of tubes in duplicate for each fungal solid-substrate fermentation experiment. The tubes were then kept static in a humidified incubator at 37^{o}C equipped with humid air via air supply through water trough at the base. Starting at day 0, of solid-substrate fermentation, two tubes for each of fungi were processed for enzyme activity assays. Separate tubes (replicate of two) were also successively processed on day 1, 3, 5 and 7 of solid-substrate fermentation.

The overall experimental procedure is schematically presented in Figure 1.

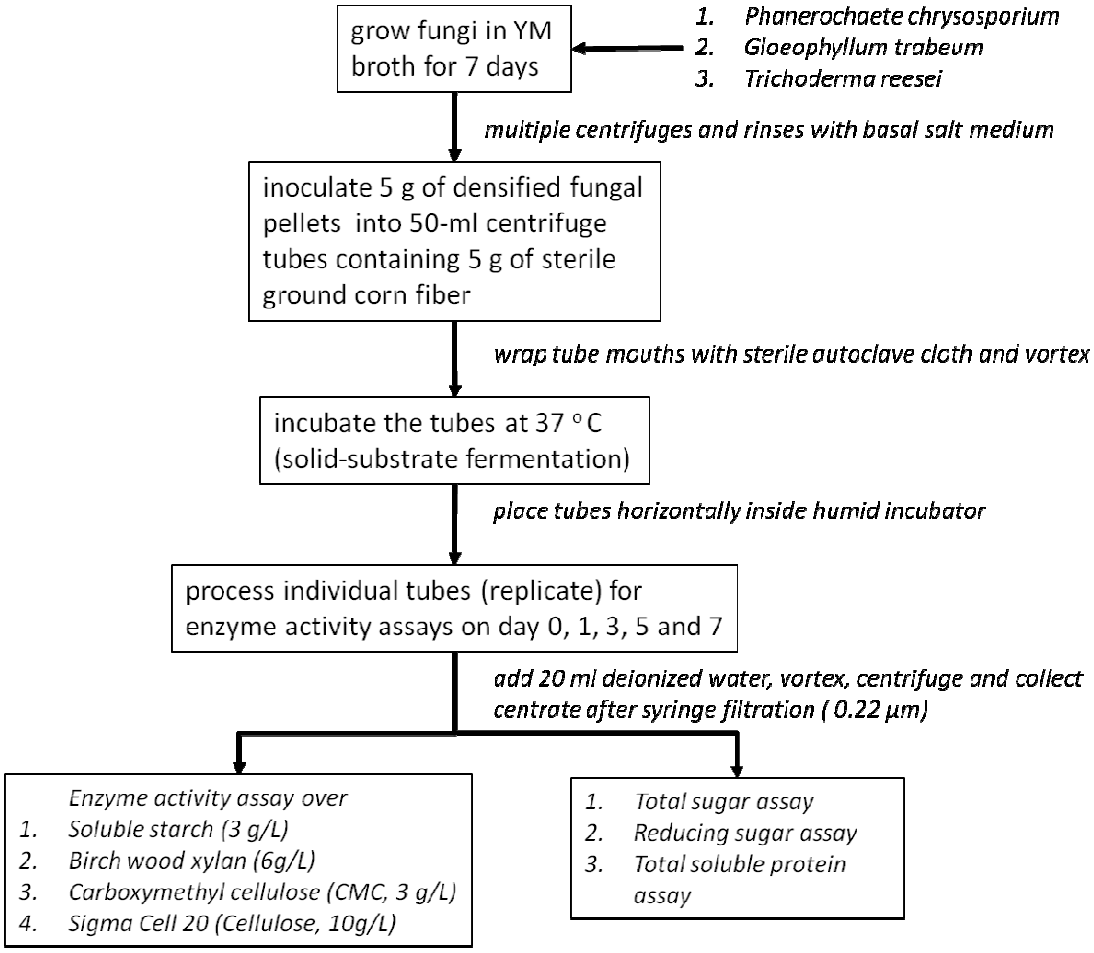

Figure 1: Schematic of overall experiment on solid substrate fermentation of corn fiber and subsequent enzyme activity assays

5.3.4.3 Analytical Methods

Starting on day 0, then day 1, 3, 5 and 7, two culture tubes of solid-substrate fermentation by white-, brown- and soft-rot fungi were taken out from the incubator. To each culture tube 20 ml sterile deionized water was added, mixed by vortexing, then centrifuged for 10 minutes. Centrates from these tubes were filtered through 0.22 µm syringe filters (Acrodisc Premium 25 mm Syringe Filter with glass fiber pre filter and hydrophilic polypropylene, pore size0.22 µm, Pall Life Sciences). The samples were then stored at -21^{o}C till enzyme activity assays were performed.

5.3.4.4 Enzyme activity tests

Enzyme activity tests were performed over various substrates like soluble corn starch, birch wood xylan, carboxymethyl cellulose (CMC) and Sigma Cell 20 as described below (Abd El-Nasser et al., 1997; Lee et al., 1998).

a. Amylase activity

Soluble corn starch (3 g/L) was used as standard substrate for amylase activity. In four 10-ml glass tubes, 0.2 ml of sodium acetate buffer (pH 4.5), 0.2 ml substrate (3 g/L soluble corn starch) and 0.2 ml of syringe filtered centrate, from respective solid-substrate fermentation, were added. Two of the four tubes were used as time 0

(zero) samples and two other tubes were placed in a water bath at 37^{o}C for 60 min. Time 0 tubes were further mixed with 1.8 ml Somogyi reagent mixture for reducing sugar measurements (Crawford and Pometto, 1988) to restrict the enzyme reaction. After the 60 min water bath incubation at 37^{o}C, the two tubes were also mixed with 1.8 ml Somogyi reagent mixture to restrict further enzyme reaction. All tubes mouths were capped with marbles, placed in steaming cabinet for 60 min followed by cooling at cold room for 30 minutes. To each tube1.8 ml of Nelson's reagent was added then vortexed, followed by 6 ml of deionized water and revortexed. From each of the representative samples, 200 µL, were then transferred into three microtiter wells in a 96-well spectrophotometer plate. Absorbance reading was measured at 500 nm (Spectra Max Plus 384, Molecular Devices Corporation, CA) and the average values were converted to glucose equivalents by using standard glucose curve. Amylase activity was calculated as difference in glucose concentration per unit time for the 60 min sample minus the zero time sample (i.e., (glucose concentration in g/L at time 60 min minus glucose concentration at time 0 minute)/60 minutes).

b. Xylanase activity (hemicellulase activity)

Birchwood xylan (6 g/L) was used as standard substrate. In four 10-ml glass tubes, 0.2 ml of sodium acetate buffer (pH 4.5), 0.2 ml substrate (6 g/L birchwood xylan) and 0.2 ml of syringe filtered centrate, from respective solid-substrate fermentation, were added. Two of the four tubes were used as time 0 (zero) samples and two other tubes were incubated in water bath at 37^{o}C for 60 min. To stop enzyme activity

the time 0 tubes were mixed with 1.8 ml Somogyi reagent mixture (Crawford and Pometto, 1988). Following 60 min at 37^{o}C, the two tubes were mixed with 1.8 ml Somogyi reagent mixture to restrict further enzyme reaction. All these tubes were treated as above with Nelson's reagent, placed in a steam chamber, diluted, added to microtiter well spectrophotometer plate, and absorbance determined at 500 nm. Absorbance readings were converted to xylose concentrations using previously prepared standard xylose curve. Enzyme activity over xylan is calculated as difference in xylose concentration at 60 min minus the xylose concentration at time zero divided by 60 min.

c. Endocellulase activity (carboxymethyl cellulose)

Carboxymethyl cellulose (CMC) (3 g/L) was used as standard substrate. In four 10-ml glass tubes, 0.2 ml of sodium acetate buffer (pH 4.5), 0.2 ml CMC substrate (3 g/L) and 0.2 ml of syringe filtered centrate, from respective solid-substrate fermentation, were added. Two of the four tubes were used as time 0 (zero) samples and two other tubes were incubated in water bath at 37^{o}C for 60 min. All these tubes were treated as above with Nelson's reagent, placed in a steam chamber, diluted, added to microtiter well spectrophotometer plate, and absorbance determined at 500 nm. Absorbance readings at 500 nm were converted to glucose concentrations using previously prepared standard glucose curve. Enzyme activity over CMC is calculated as difference in glucose concentration at 60 min minus glucose concentrations at time zero divided by 60 min.

d. Exocellulase activity (Sigma cell 20 Cellulase activity)

Sigma cell 20 (10 g/L) was used as standard substrate. In four 10-ml glass tubes, 0.2 ml of sodium acetate buffer (pH 4.5), 0.2 ml Sigma cell substrate (3 g/L) and 0.2 ml of syringe filtered centrate, from respective solid-substrate fermentation, were added. Two of the four tubes were used as time 0 (zero) samples and two other tubes were incubated in water bath at 37^{o}C for 60 min. All these tubes treated as above with Nelson's reagent, placed in a steam chamber, diluted, added to microtiter well spectrophotometer plate, and absorbance determined at 500 nm. Absorbance readings at 500 nm were converted to glucose concentrations using previously prepared standard glucose curve. Enzyme activity over insoluble cellulose (Sigma cell 20) was calculated as difference in glucose concentration at 60 min minus glucose concentration at time zero divided by 60 min.

5.3.4.5 Total and Reducing Sugar Assays

The centrate samples were also analyzed for total and reducing sugar concentrations via phenol sulfuric and modified Somogyi-Nelson Carbohydrate assays respectively (Crawford and Pometto, 1988).

5.3.4.6 Total Protein Assay

The total soluble protein concentrations in the centrate samples, from solid-substrate fermentation, were analyzed via modified Lowry's Protein Assay (Shrestha et al., 2008a).

5.3.5 Statistical Analyses

The experimental data were analyzed for statistical validation via one-way analysis of variance (ANOVA). All assays and fermentations were performed in replicates of two (n=2) and significant difference of p value of 0.1 was employed.

5.4 Results and Discussion

5.4.1 Enzyme activity over starch (amylase)

Successive higher yields of glucose (mg glucose/L.min) were observed for the extracts collected from white-, brown-, and soft-rot fungal solid-substrate fermentation of corn fiber (Figure 2). White- and brown-rot fungi had their highest amylase activity over starch at day 5 with glucose yield potential of 13.53 mg

glucose/L.min and 10.77 mg glucose/L.min, respectively. The soft-rot fungal fermentation had glucose yield potential of 6.14 mg glucose/L.min on day 5. These results were significantly different with each other ($p = 0.039$). The soft-rot fungal fermentation of corn fiber had 10.43 mg glucose/L.min yield potential at day 7. The glucose yield rates increased up to day 5 of solid-substrate fermentation for white- and brown-rot fungi and then it decreased on day 7. Whereas soft-rot fungal fermentation, demonstrated glucose yield rates demonstrated a three day lag with increased rates on day 5 and 7. Significant differences in maximum amylase activity were demonstrated for *P. chrysosporium ($p = 0.002$), G. trabeum ($p<0.001$) and T. reesei ($p = 0.002$).*

5.4.2 Enzyme activity over xylan (hemicellulase)

Xylanase activities were confirmed for the extract samples collected from solid-substrate fermentation of corn fiber by white-, brown- and soft-rot fungi (Figure 3). Xylose yield rate (mg xylose/L.min) from standard substrate (birch wood xylan, 6g/L) was the highest (12.10 mg xylose/L.min) for extract from white-rot solid-substrate fermentation of corn fiber at day 5. The brown-rot solid-substrate fermentation also had the highest xylase activity of 7.55 mg xylose/L.min at day 5. Between fungal species the solid-substrate fermentation of corn fiber also had significantly different for maximum xylanase activities for *P. chrysosporium ($p = 0.021$), G. trabeum ($p = 0.02$) and T. reesei ($p = 0.007$).*

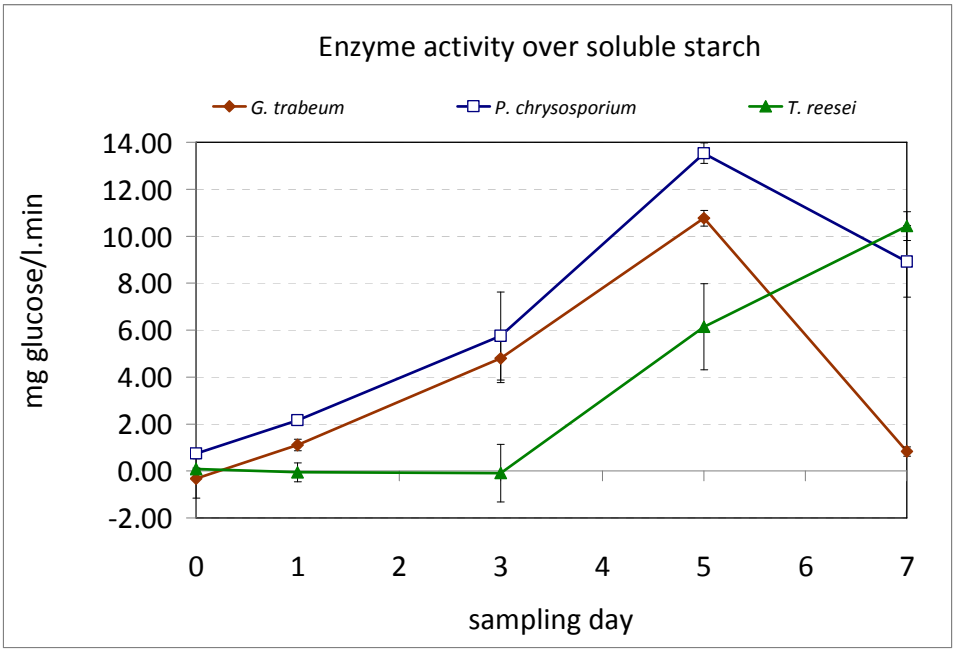

Figure 2: Amylase activity over starch determined as mg glucose/L.min released from soluble corn starch (3 g/l) sample from fungal solid-substrate fermentation extracts (n=2)

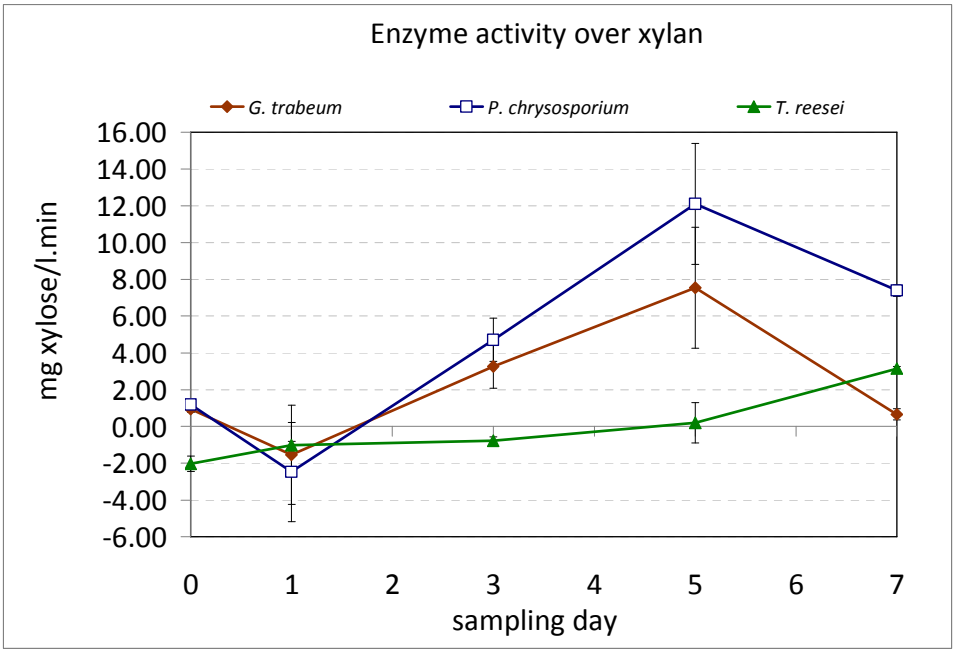

Figure 3: enzyme activity over xylan determined as mg xylose/L.min released from birch wood xylan (6 g/l) sample (n=2)

5.4.3 Enzyme activity over CMC (endocellulase)

Endocellulase activities in extracts from solid-substrate fermentation were confirmed (Figure 4). The highest glucose yield rate from CMC was 6.24 g glucose/L.min for brown-rot solid-substrate fermentation on day 7, whereas white-rot solid-substrate fermentation had 2.77 g glucose/L.min and soft-rot solid-substrate fermentation had 0.97 g glucose/L.min. The glucose yield rates from CMC were not significantly different on day 7 among white-, brown- and soft-rot solid-substrate fermentation of corn fiber ($p = 0.128$).

5.4.4 Enzyme activity over sigma cellulose (exocellulase)

The highest exocellulase activities during solid-substrate fermentation of corn fiber with *P. chrysosporium* (day 7), *G. trabeum* (day 5) and *T. reesei* (day 5) were respectively calculated as 3.15, 2.8 and 3.46 mg glucose/L.min, when representative samples extracts were reacted with standard cellulose sample for 60 minutes (figure 5). The exocellulase activities were however, not significantly different between corn fiber fermentation with different fungi on day 5 ($p = 0.539$) and day 7 ($p = 0.177$),

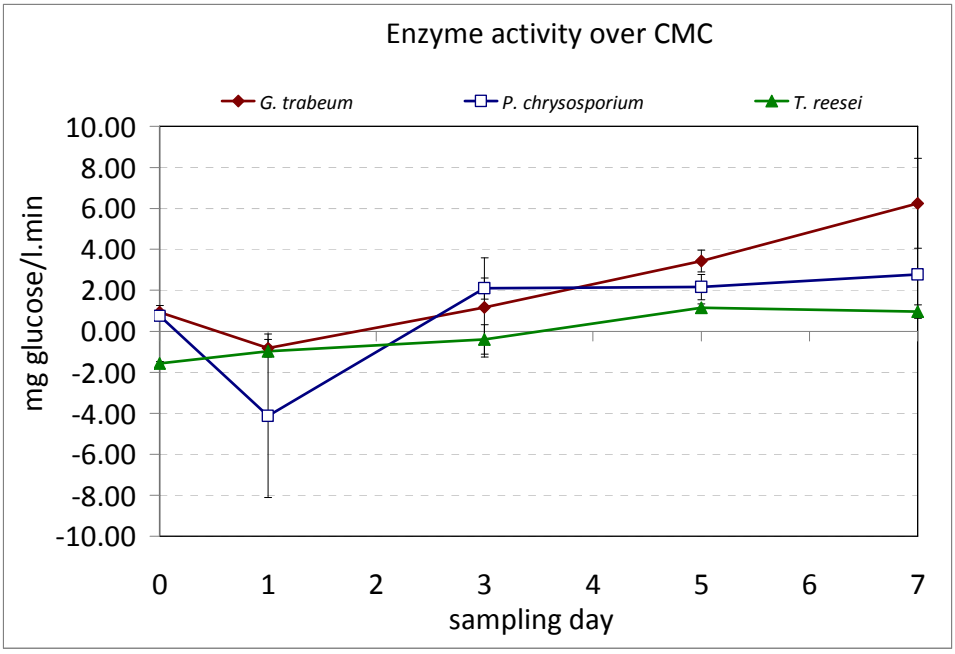

Figure 4: enzyme activity over carboxymethyl cellulose (CMC) determined as mg glucose/L.min released from CMC (3 g/l) sample (n=2)

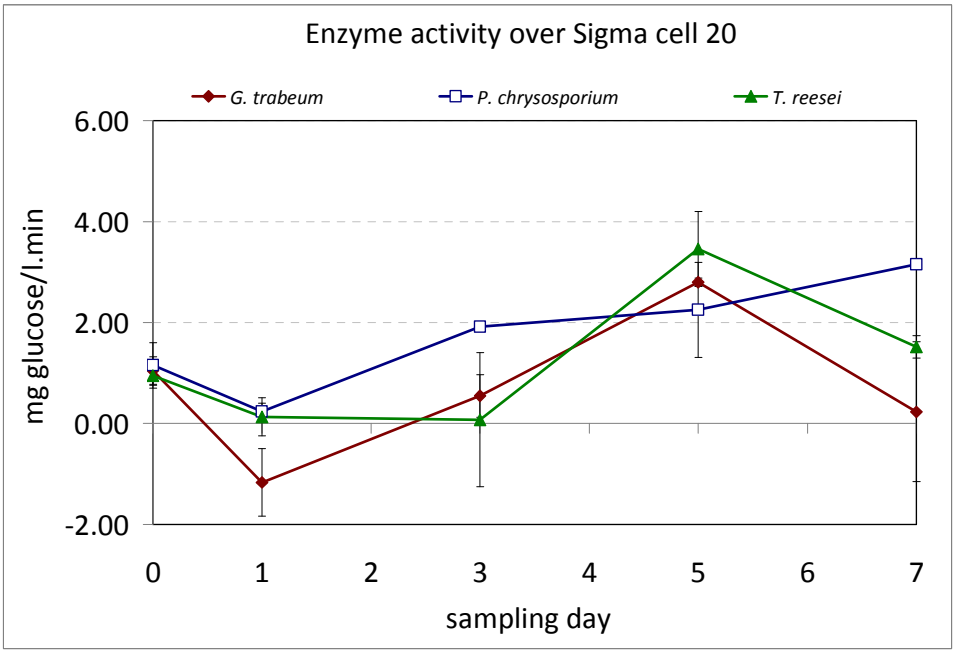

Figure 5: enzyme activity over Sigma cell 20 (cellulose) determined as mg glucose/L.min released from Sigma cell 20 (10 g/l) sample (n=2)

5.4.5 Total and Reducing Sugar

The total sugar concentrations represents mono-, di-, tri- and oligo-sacchardes in the water extracts due to extracellular enzyme activity. The day 1 concentrations were 10.6, 9.2 and 7.9 g total sugar/L, respectively, for the samples extracted from solid-substrate fermentation of corn fiber using soft-, white- and brown-rot fungi (figure 6). The total sugar concentrations decreased as the fermentation proceeded for days 3, 5 and 7. This clearly indicates fungal consumption of sugars during solid-substrate fermentation. The total sugar concentrations increased to 15 g/L on day 7 of solid-substrate fermentation with brown-rot fungus. This could be attributed to enhanced endocellulase activity of brown-rot fungus.

Reducing sugar concentrations profiles illustrate the potential fermentable sugar concentrations released demonstrated a similar patterns to that of total sugar (Figure 7). The sugar concentrations increased after 24 h of solid-substrate fermentation for all fungi. The highest reducing sugar concentration, 5.2 g/L, was calculated for 24 h white-rot fermentation. Both brown- and soft-rot fermentation had 4.3 g reducing sugar/L which was similar to total sugar (4.7 g/L on day 7) of solid-substrate fermentation with brown-rot fungus.

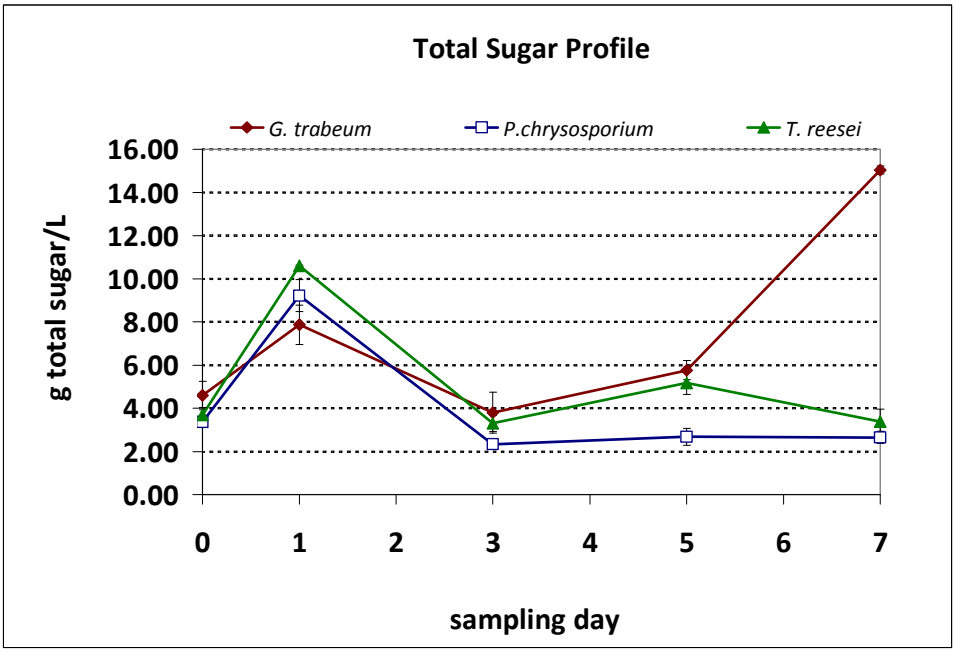

Figure 6: total sugar concentrations (in g/l) determined for solid-substrate fermentation samples (n=2)

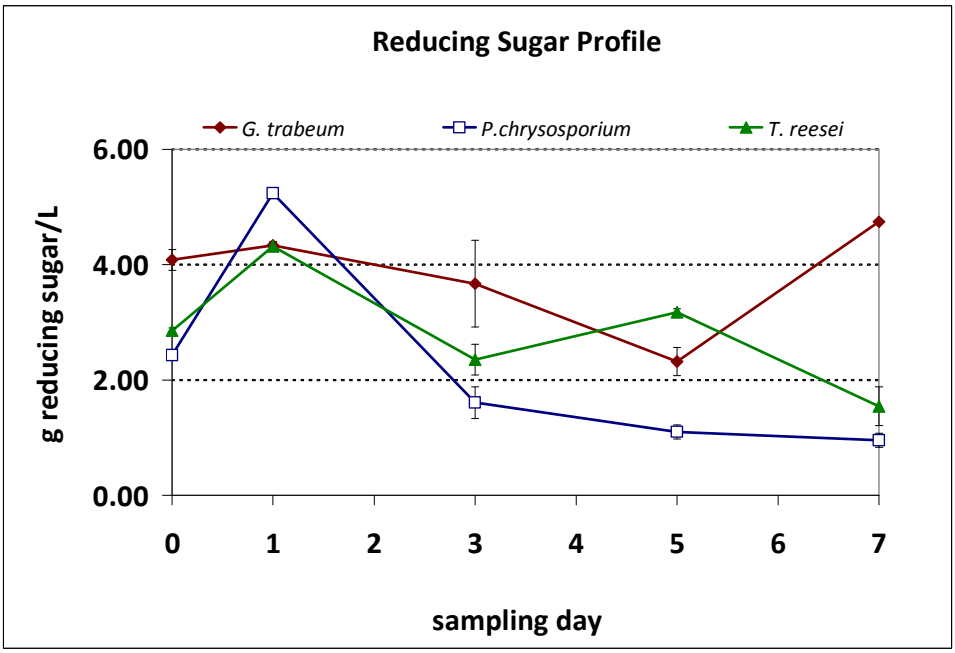

Figure 7: reducing sugar concentrations (in g/l) determined for solid-substrate fermentation samples (n=2)

5.4.6 Total protein

The total soluble protein concentrations increased as the solid-substrate fermentation proceeded. The highest protein concentration: 16 g/L was calculated for samples extracted on day 7 of solid-substrate fermentation of corn fiber with brown-rot fungus (Figure 8). The protein concentration profile were significantly different on 1 ($p = 0.034$), 5 ($p = 0.011$) and 7 day ($p < 0.001$) solid-substrate fermentation of corn fiber. The enzymatic activities improved as the protein concentrations increased with succession of solid-substrate fermentation.

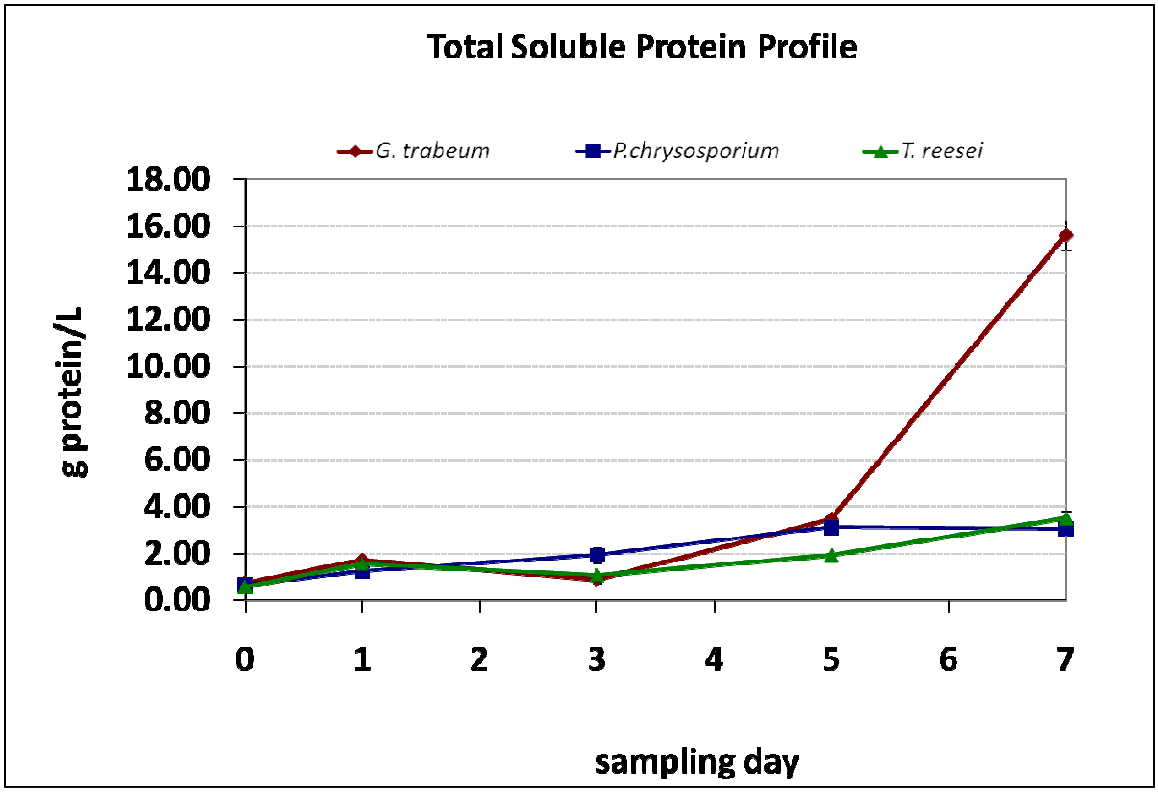

Figure 8: total soluble protein concentrations (in g/l) determined for solid-substrate fermentation samples (n=2)

5.5 Conclusion

Solid-substrate fermentation of corn fiber using *P. chrysosporium* (white-rot fungus), *G. trabeum* (brown-rot fungus) and *T. reesei* (soft-rot fungus) were studied for extracellular hydrolytic enzyme productions by these fungi. Hydrolyase activities for starch, xylan, CMC and cellulose were observed. There were no consistency in enzyme activities as the activities were decreased on day 1 of solid-substrate fermentation then increased to maximum on day 5 and again decreased on day 7 of solid-substrate fermentation. *P. chrysosporium* had highest enzyme activity for starch (13.53 mg glucose/l.min) and xylan (12.10 mg xylose/l.min) on day 5 of solid-substrate fermentation, whereas *G. trabeum* had highest activity on CMC (6.24 mg glucose/l.min) on day 7. *T. reesei* had the highest activity for cellulose (3.46 mg glucose/l.min) on day 5 of solid substrate fermentation. Various other factors like temperature, pH, oxygen diffusibility, moisture and limited nutrients influence overall performance in solid-substrate fermentation of cellulosic feedstock like corn fiber. Induction of hemi/cellulose and starch degrading enzymes in fungal fermentation can be greatly influenced by the concentration of end products like glucose, which may repress the enzyme induction but the sugar profiles clearly showed sugar consumption during solid-substrate fermentation. Hence, end product inhibition towards enzyme activities cannot be justified. Further research work would require optimization of these parameters, comparison of cellulosic feedstock with standard substrate and determination of individual enzyme concentrations.

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CHAPTER 6: ENGINEERING IMPLICATIONS AND SIGNIFICANCE

The primary products in corn wet-milling biorefineries are ethanol, starch, high fructose corn syrup (HFCS), organic acids, corn oil and animal feed. The latter is comprised of basically fibrous co-products, i.e. corn fiber (representing bran coat, germ fiber and tip cap of the corn kernel) that are further blended with gluten (protein) and sold as corn gluten feed and corn gluten meal. Similarly, the dry-grind corn ethanol plants also produce a fibrous co-product: distillers dried grains (DDG) usually combined with solubles and sold as DDGS. In recent years, the corn ethanol production (especially from corn-dry grind industries) and planted corn acreage in the United States has increased significantly. The amount of co-products generated as corn gluten feed/meal or DDGS has increased in proportion with ethanol production. A large amount of energy is spent to produce these co-products but the supply exceeds demand for this animal feed. This has a serious effect on the profitability of ethanol plants.

On dry-mass basis, over 70 % of corn fiber is complex carbohydrate (residual starch integrated with hemicellulose and cellulose). Starch and cellulose fractions of the fiber occur in equal proportions, 18 % dry mass each (with some variations). Hemicellulose fraction (~ 35 %, dry mass) is higher than starch and cellulose fractions. The lignin fraction is constitutively very low in corn fiber (~ 1.3 %, dry mass). Therefore, corn fiber has been primarily sought as a cleaner and valuable

feedstock for producing high value products like arabinoxylan gum (from hemicellulose), corn fiber oil, enzymes and ethanol from cellulose and starch fractions. However, the structural complexity and recalcitrance properties of the constituent polysaccharides currently require physico-chemical pretreatment to facilitate enzymatic saccharification. Pretreatment methods are costly and may produce inhibitory compounds. Effective enzyme production for cellulose degradation is still very costly.

6.1 Fungal conversion of corn fiber to ethanol

This research focused on no/minimal pretreatment of cellulosic biomass, mainly corn fiber, and further hydrolysis of the polysaccharides at low temperature to produce sugars for ethanol production. Wood-rot fungi (white-rot fungus: *Phanerochaete chrysosporium* and brown-rot fungus: *Gloeophyllum trabeum*) have been extensively studied for their hemi/cellulose degrading enzymes. The *in-situ* production of these enzymes in solid-substrate or submerged fermentation (under aerobic conditions) was followed by anaerobic simultaneous saccharification and fermentation (SSF) with co-culture of *Saccharomyces cerevisiae* to convert hydrolyzate from corn fiber to ethanol. This dissertation therefore,

(i) conceptualizes on-site production of enzymes (from wood-rot fungi),

(ii) develops saccharification and fermentation of corn fiber to ethanol and

(iii) improves the residue quality as animal feed or development of value-added products.

6.2 Enzyme activities: solid-substrate fermentation vs. submerged fermentation

Studies of amylase, hemicellulase (represented by xylanase) and cellulase (represented by endocellulase and exocellulase) enzyme activities of wood-rot fungi and *T. reesei* showed that the latter fungus has comparable activities on starch, xylan and cellulose. The specific enzyme activities of all three fungi are very high in solid-substrate fermentation compared to submerged fermentation. It is therefore envisaged that starch and hemi/cellulose degrading enzymes production from these fungi can be improved (and concentrated) via solid-substrate fermentation. Solid-substrate fermentation at optimal moisture, temperature and nutrient supply mimics the natural systems of the wood-rot and soft-rot fungi. Therefore, their enzymatic activities are expected to enhance as thefungal growth and metabolism progresses.

Submerged fermentation simplifies controlling pH, temperature and nutrient levels while the fungi grow in presence of cellulosic feedstock. Operation of fermentors, adequate oxygen supply, intermittent sampling and downstream processing for enzyme harvesting would add extra cost and equipment footprints. Fungal enzyme activities in submerged fermentation may be lower compared to solid-substrate

fermentation as observed from the reported experiments. It may also enhance other products like acetic acid rather than ethanol (as in case of *T. reesei*) during SSF process.

It is therefore envisaged that sizable solid-substrate fermentation of corn fiber would be more appropriate to produce fungal enzymes using a portion of corn fiber as substrate. The white- and brown-rot fungi had higher enzymatic activities over starch, xylan and carboxymethyl cellulose. This confirms higher amylase, xylanase and endocellulase activities of these fungi. *T. reesei* had higher exocellulase activity compared to wood-rot fungi. All these enzyme activities are necessary to hydrolyze starch and hemi/cellulose fractions of corn fiber. The exocellulase activities of *T. reesei* complement the consortia of enzymes secreted by wood-rot fungi. Therefore, a mixed culture of the fungi (white-rot and *T. reesei*, brown-rot and *T. reesei,* white- and brown-rot fungi, or all three) can be used for solid-substrate fermentation. Fungal proliferation would enhance enzymatic activities and may further degrade substrate to provide sugars for fungi. Fungal sugar consumption should be restricted or kept minimal. Periodic extraction of enzyme complex via water or buffer rinse of the fungi-substrate bed followed by purification and concentration would ensure the enzyme supply and quality. This also helps to mitigate the problem of fungal sugar consumption.

The ethanol yield in SSF process primarily depends on the enzymatic activities of the fungal species. The higher the enzymatic activities, the faster and higher would

be the ethanol production in SSF process. The results strongly support hydrolytic fungal enzyme production in solid-substrate fermentation. Purified and concentrated enzyme consortia would definitely improve the corn fiber to ethanol yield in the sugar to ethanol fermentation process.

6.3 Ethanol yield

The SSF of corn fiber preceded by enzyme induction in submerged fermentation using brown- and white-rot fungi respectively had ethanol yields of 42 and 34 % of the theoretical maximum yield (ca. 20.4 g ethanol per 100 g of corn fiber) from an estimated 18 % starch and 18 % cellulose fraction in corn fiber. These ethanol yields were higher when compared to the ethanol yield from a similar SSF study with *T. reesei,* which had 20 % of theoretical maximum ethanol yield. However, solid-substrate fermentation of corn fiber using these fungi and the following SSF process had a higher ethanol yield for *T. reesei* (25 % of the theoretical maximum yield). It must also be understood that the fungi would also consume sugar as they proliferate during the enzyme induction phase. This would then result in lower ethanol yields like the reported yield of 25 to 42 % of the theoretical maximum.

6.4 Process Economics

The corn fiber to ethanol production trends in the fungal SSF process leveled off after 2 days of fermentation. Extension of anaerobic conditions to 6 or 8 days increased but did not significantly improve the ethanol yield. Therefore, an extended period for SSF fermentation would not be necessary.

Ethanol yield had not significantly improved after mild alkali and alkaline peroxide pretreatment of corn fiber. Therefore, pretreatment of corn fiber prior to fungal saccharification and fermentation would not be necessary as well.

Separate SSF process for corn fiber would require more reactors, higher retention time for fermentation and additional processing steps. In addition, a separate SSF process for converting corn fiber to ethanol would not be economical in terms of the highest corn fiber to ethanol conversion and concentration of ethanol in the fermentors. At ca. 30% solid loading (corn fiber) the theoretical maximum ethanol concentration would be around 61 g/L (i.e., 6.1 % w/v or 7.7 % v/v). Such a low ethanol concentration might not be economical for separation by distillation.

It would be more practical to add the germ fiber and fiber separated in the upstream process together with separated corn-starch into the fermentors. The fungal enzymes, collected from solid-substrate fermentation, and commercial amylase

enzymes (lesser quantity than required as fungi also have amylase activities) will saccharify the free and residual starch, hemicellulose and cellulose. The fermentable sugar (glucose) is converted into ethanol by *Saccharomyces cerevisiae.* Conversion of xylose to ethanol is also a possibility. Usually it takes 48 to 72 hours to maximize conversion of sugar to ethanol in ethanol biorefineries. Integration of corn fiber and starch hydrolysis in the same fermentor and conversion of sugar to ethanol would, therefore, anticipated to yield more ethanol within 48 to 72 hours.

6.5 Utilization of waste

The corn to ethanol conversion rate is expected to increase. Utilization of starch and cellulose fraction would reduce bulk generation of solid residue. The residue is also expected to be higher in hemicellulose content (depending on enzyme supplementation) and therefore, be more a favorable feedstock for hemicellulose applications such as the production of arabinoxylan gum. The liquid fraction may be further explored to separate valuable products like antioxidants, enzymes, nutrients and proteins. Recycle of enzymes and nutrients back to upstream processes are possibilities.

The solid residue can otherwise be supplemented with fungal enzyme complex and the residue is not mixed with fungal cells (hyphae). The enzyme supplemented residue could be considered as safe animal feed. Their inclusion in animal rations

will enhance the hydrolysis and improve the digestibility of the substrate in the animal gut. The holistic process will improve corn to ethanol yield with ancillary benefits from solid residue and liquid streams as summarized in the schematic diagram (figure 1).

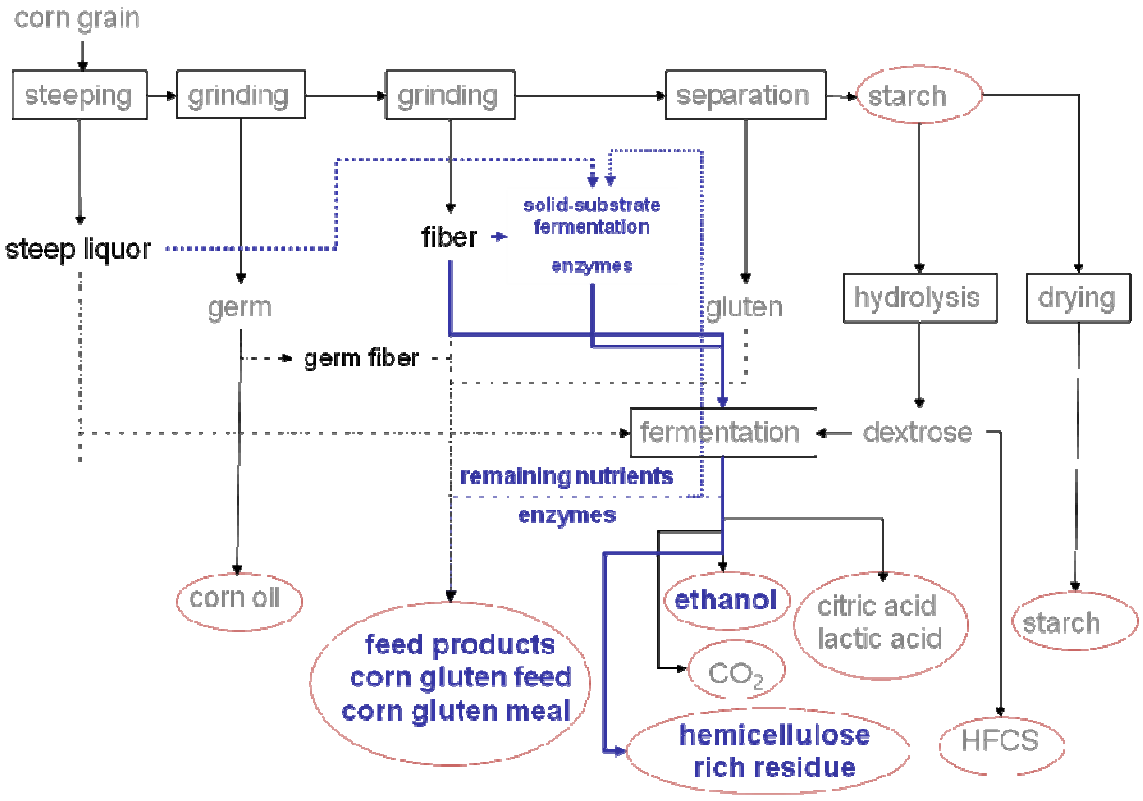

Figure 1: Addition of processing steps (bold letters and arrows) for improvement of the existing corn wet milling process for improved corn to ethanol yield, animal feed products and ancillary high-value products like hemicellulose-rich residue.

6.6 Biological conversion of lignocellulose to fuel and biobased products

This dissertation also has significant implications in the broader perspective of biological conversion of lignocellulose biomass into ethanol and biobased products. Availability and compositional variability of lignin, hemicellulose and cellulose in various lignocellulose feedstocks like corn stover, bagasse, switchgrass, etc. greatly determine the desirable end product(s). Higher cellulosic content would favor cellulose ethanol production along with separate end usage of hemicellulose and lignin. Higher lignin content may route the processing towards economical usage of lignin. Unlike corn fiber, lignocellulosic biomass may require mild to severe pretreatment prior to biological conversion of feedstock to sugars and other derivatives. It is also desirable that both physical-chemical and biological pretreatments keep the substrates (and polymers) in their natural states and yet enhance the enzymatic hydrolysis process.

Wood-rot and soft-rot fungal treatment of lignocellulosic feedstock can be applicable to untreated or pretreated substrate. Since each and every microbial species has different types and strength of enzymatic activities, in most cases a mixture of microbial population would benefit in holistic degradation of complex polymer to simple sugars, which can further be fermented to ethanol. For example, white-rot and soft-rot fungi (*P. chrysosporium* and *T. reesei*) can be co-cultured in solid

substrate fermentation to provide a complete consortium of lignin and hemi/cellulose degrading enzymes such that the hydrolyzate can be completely fermented to ethanol using genetically modified yeasts or bacteria. In many cases, it is also desirable to have multiple products in addition to ethanol. Conversion of glucose and xylose to ethanol and xylitol respectively, may be profitable. Brown-rot fungus (*G. trabeum*) can be used to solely convert lignin and hemicellulose rich feedstock into modified lignin, hemicellulose and cellulose hydrolyzate. The lignin and hemicellulose fraction may be used separately for other purposes. There are many possible products other than ethanol from lignocellulose. Contrary to ethanol production, depending on market demand and product values, biomass feedstock can also be utilized for production of organic acids, anti-oxidants, enzyme assisted improved animal feed as conceptualized in figure 2.

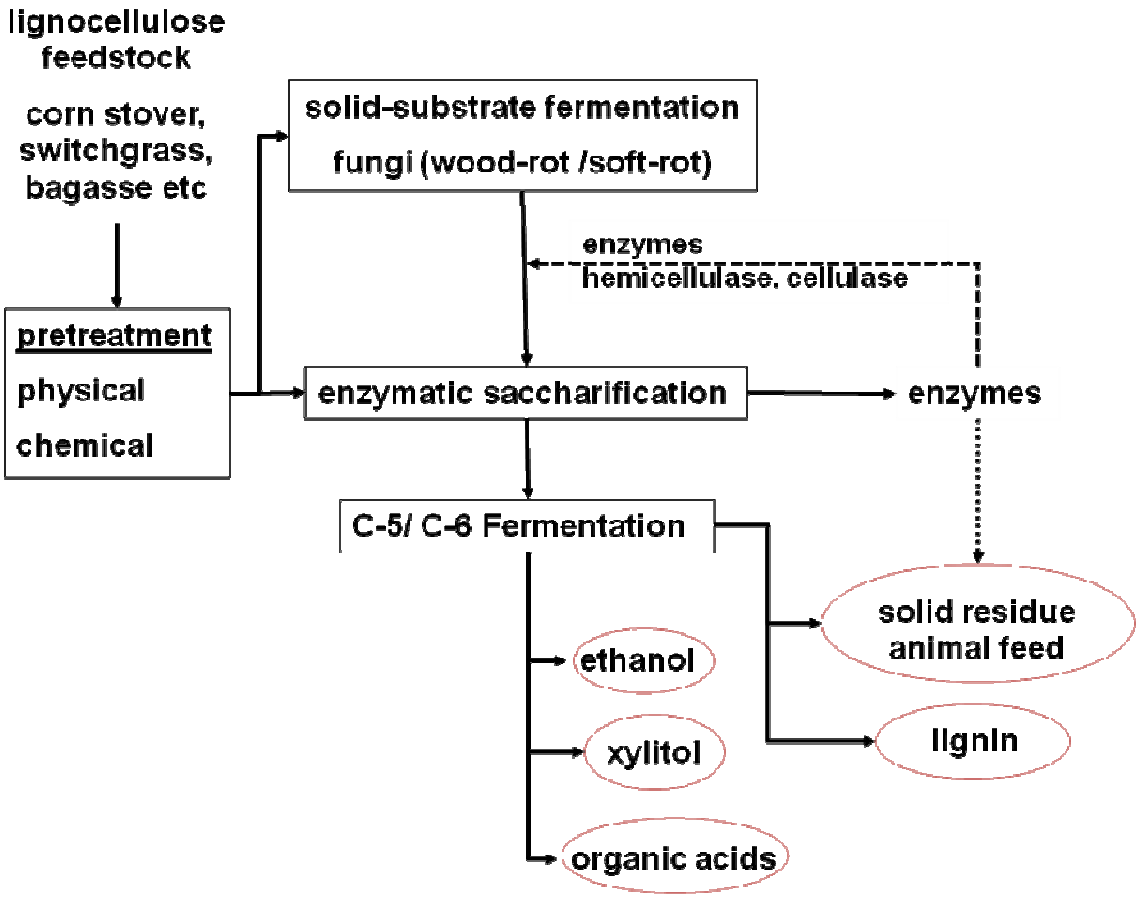

Figure 2: Integrated biorefinery concept to utilize lignocellulosic biomass for various products like fungal enzymes, alcohols, organic acids, lignin etc.

6.7 Conclusion

The corn-based ethanol industries in the US can be expected to be the main producers of ethanol well into the future. The utilization and management of co-products will be challenging issues in the near future. The main implications of the research findings in this dissertation are three-fold as below.

Wet corn milling. Utilization of corn fiber into extra ethanol production helps to increase the net ethanol yield from corn. It is calculated that at a reported yield rate of ~ 9 g ethanol per 100 g of corn fiber, 56 million gallons of ethanol can be produced from the wet-milling corn fiber produced in 2006. The corn fiber to ethanol conversion rate and production could still be improved and made more economical by integrating on-site fungal enzyme production into the process as described earlier. The residue, on the other hand, will be rich in protein and fetch higher prices and a greater market.

Dry-grind corn milling. Strategic processing can also be employed in increasing the net ethanol yield in dry-grind corn ethanol plants and reduction in production of DDGS. Conversion of fibrous co-product to extra ethanol and high-value (protein enriched) animal feed not only helps to minimize corn acreage for fuel but also to minimize bulk generation of residues.

Ethanol from lignocellulosic biomass. The practicality and process validation of such processes would also be very beneficial to implement such technological concepts into long-term and sustainable transition to utilize abundant lignocellulosic biomass to fuel and other biobased products. Progressive exploration for wood-rot fungal enzymes should lead to techno-economic development of biological conversion of lignocellulosic feedstock to renewable biofuels and biobased products. This could well be the key to sustainable biofuel production to meet the growing need for transportation fuels.

CHAPTER 7: GENERAL CONCLUSION

This research successfully evaluated wood-rot fungi for biological conversion of cellulose feedstock, especially corn fiber, into fermentable sugar. The corn fiber to ethanol yields was as high as 8.6 and 7.1 g ethanol per 100 g of corn fiber respectively for *Gloeophyllum trabeum* and *Phanerochaete chrysosporium* in SSF process preceded by enzyme induction in submerged fermentation. In similar experiment with *Trichoderma reesei* resulted in 4.1 g ethanol per 100 g of corn fiber. Higher amount of acetic acid (11.3 g acetic acid per 100 g of corn fiber) production was also observed. Submerged fermentation of cellulosic feedstock would not truly represent natural environment of fungi. Limited fungal enzyme activities and metabolism favoring acetic acid production (in case of *T. reesei*) was observed in submerged fermentation followed by SSF process. Contrary to that wood-rot fungi and *T. reesei* had very good enzymatic activities in solid-substrate fermentation of corn fiber. Ethanol yield was also higher for *T. reesei,* compared to wood-rot fungi, in SSF process that followed solid-substrate fermentation of corn fiber. Organic acids like acetic and lactic acid profiles were also very low. Fungal consumption of released sugar during solid-substrate fermentation has been highly suspected.

Starch content in corn fiber and comparable enzyme activities over starch could have significant impact on hemi/cellulolytic enzyme activities and overall ethanol yield from corn fiber. Corn fiber can be first destarched via enzyme and hot water

treatment. Separated starch and sugar fractions can be added back during fermentation. Effectiveness of wood-rot fungi and *T. reesei* can then be evaluated for their hemi/cellulolytic enzyme activities. Mild alkali pretreatment of corn fiber also had positive impact on ethanol yield for *G. trabeum* and *T. reesei.*

It would take 2 to 3 days to produce effective enzyme consortia from wood-rot fungi in solid-substrate fermentation. SSF process, that follows enzyme induction phase, further requires 2 to 4 days for effective corn fiber to ethanol conversion. Overall, the whole process would require 4 to 7 days to convert fermentable sugars in corn fiber to ethanol. At present ethanol yield from corn fiber, it would not be advantageous to build a whole new fungal process to convert corn fiber to ethanol. However, with further improvement of fungal enzymatic activities (possibly with mixed fungal culture), relatively small unit of solid-substrate fermentation can be integrated to existing corn biorefinery system. Such system will provide enzyme cocktail to saccharify hemi/cellulose and starch fractions from corn fiber in SSF process. Separate fungal enzyme productions via solid-substrate fermentation also ease in extracting, purifying and concentrating enzymes. The solid residue may be of better quality in terms of its hemicellulose content and therefore, can be utilized as feedstock for hemicellulose derived products like arabinoxylan gum. Therefore, many products in addition to ethanol can be produced. Addition of hemi/cellulase enzymes to fibrous animal feed may also improve rumen digestibility.

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