Saccharification of corn fiber by Phanerochaete chrysosporium in solid-state fermentation and subsequent fermentation of hydrolysate into ethanol

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Saccharification of corn fiber by *Phanerochaete chrysosporium* in solid-state fermentation and subsequent fermentation of hydrolysate into ethanol

by

Prachand Shrestha

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
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This is to certify that the master's thesis of

Prachand Shrestha

has met the thesis requirements of Iowa State University

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The United States imports nearly two-thirds of its petroleum fuels from politically unstable countries to meet its energy needs. Alternative energy sources are being developed and the US produces >4.5 billion gallons of corn-based ethanol currently. The ethanol production is expected to increase to 7.5 billion gallons by 2012. This increase would also result in the generation of vast quantities of low-value, lignocellulosic co-products – corn fiber and distillers dried grains (DDG). One way for value adding to these low-value co-products is production of additional ethanol. Breakdown of lignin followed by saccharification of the cellulose could liberate simple sugars for subsequent yeast fermentation to ethanol.

In this research, laboratory-scale solid state fermentation (SSF) of corn fiber from a wet milling corn plant using the white rot fungus *Phanerochaete chrysosporium* was carried out in 1-L polypropylene bottles. *P. chrysosporium* produced extracellular enzymes *in situ* for lignin degradation and saccharification of cellulose to release sugars. Creating subsequent anaerobic conditions around the fungi by submerging in a buffer solution (pH 4.73 and 37°C), reduced the fungal sugar consumption and enhanced the cellulolytic enzyme activities *in situ* with the subsequent release of additional sugars for yeast fermentation to ethanol. A 5-day *P. chrysosporium* culture under anaerobic conditions converted 2.5 % of corn fiber into reducing sugars; whereas, maximum biomass weight loss was about 34% by day 14.

Investigation of *P. chrysosporium* ligninase inducers (H₂O₂, veratryl alcohol, and MnSO₄) demonstrated significant Klasson lignin reductions for 14 day SSF culture bottles (10 to 41%) with 300 mM MnSO₄ exhibiting the greatest reduction. The production of
extracellular polysaccharides (EPS) by the white-rot fungus during SSF was also investigated by mild acid (0.1N sulfuric acid) hydrolysis, which illustrated a 2-fold increase in acid soluble sugars between the day-9 and day-10 SSF culture bottles. *Saccharomyces cerevisiae* fermentation of the hydrolysates from *P. chrysosporium* SSF and mild acid treatment of SSF produced 4 mg ethanol/g fiber. This suggested low concentrations of fermentable sugars in the hydrolysates. Further studies are needed to optimize SSF protocols in simultaneous saccharification and co-fermentation using wood rot fungi and yeasts for economical ethanol production from the corn fiber and other lignocellulosic biomass.

Keywords: Lignocellulosic, distillers dried grains, corn fiber, solid state fermentation, extracellular enzymes, simultaneous saccharification and fermentation, ethanol, white rot fungi, *Phanerochaete chrysosporium*. 
GENERAL INTRODUCTION

Energy insecurity and global climate changes are the two major concerns with petroleum-based fuels. Corn-based ethanol production has a great potential to abate these issues, particularly for the United States, which imports two-thirds of its liquid fuel, much of it from politically unstable countries. The United States produces about 4.5 billion gallons of ethanol from 97 corn-grain-based ethanol plants (Kansas Ethanol, 2006), and production is expected to increase to 7.5 billion gallons by 2012 (Somerville, 2006). This increase in ethanol production would also result in concomitant increases in residues, such as distillers dried grain (DDG) and corn seed hull from corn milling plants. The production of DDG from ethanol plants is estimated to reach 10 to 14 million metric tons in the near future (University of Minnesota, 2006). Both DDG and corn fiber are currently sold as an animal feed. There is a downward trend in the market price for animal feed. So it is necessary to explore possible alternative uses for these corn-processing coproducts.

The corn residues contain about 80% carbohydrate, and they can potentially serve as a low-cost feedstock to produce renewable biofuels, particularly ethanol. The conversion of DDG to ethanol would increase the net ethanol production by 13% (National Renewable Energy Laboratory [NREL], 2002). The abundant carbohydrate content of these residues is practically inaccessible due to its complex chemical structures and its association with the hardy substance lignin in the plant tissues. Lignin and the complex carbohydrates - hemicellulose and cellulose are together called lignocellulose.

The structural complexity and rigidity of lignin makes it difficult to degrade. The pretreatment of lignocellulosic biomass using physical, chemical, or biological methods
would help to break down the biomass into simple sugars. Due to the higher capital and operating costs of chemical pretreatment methods, biological pretreatment, especially enzymatic hydrolysis, is the current favorite pretreatment option. However, the biomass still needs to be pretreated with a dilute acid to release hemicellulose sugars, and enzyme cost is also an issue with enzymatic hydrolysis.

Wood rot fungi, especially white rot and brown rot fungi, secrete various enzymes in situ that assist in the degradation of lignocellulosic biomass and the release of sugar molecules. These fungi have been studied for various enzymatic production and fermentation processes. White rot fungi secrete extracellular enzymes that help to degrade the lignin in the biomass while leaving the carbohydrate structure (hemicellulose and cellulose) unaltered. The carbohydrates then serve as a carbon source for the fungi. *Phanerochaete chrysosporium* is one of the most widely studied white rot fungi in lignin degradation. It secretes different extracellular lignin degrading enzymes, especially peroxidases. The lignin degradation in the biomass opens up the carbohydrate matrix. The fungus also secretes hemicellulase and cellulase enzymes to hydrolyze the hemicellulose and cellulose, respectively. Thus, the use of wood rot fungi in the fermentation of lignocellulosic biomass for lignin degradation and the production of fermentable sugars for subsequent ethanol fermentation would make a significant contribution to the various biorenewable initiatives being studied in the United States and elsewhere. Based on this premise, *P. chrysosporium* was studied for its potential to produce fermentable sugars in the solid-state fermentation (SSF) of corn fiber from corn wet milling plants.
Thesis Organization

This thesis consists of two chapters. The first chapter is a literature review, which covers pretreatment of lignocellulosic biomass. It also includes review of enzyme system and fermentation studies of the white rot fungus, *Phanerochaete chrysosporium*. The second chapter is a manuscript on a solid-state fermentation study of the fungus. The manuscript will be submitted for publication in the *Journal of Agricultural and Food Chemistry*. Each chapter includes a separate section for references at the end.
1.1 Introduction

The United States produced more than 35% of the world’s ethanol in 2005. Ethanol production in the United States has increased drastically in recent years, as is evident from Figure 1.1 (Renewable Fuels Association [RFA], 2006). In 2005, it produced 4.26 billion gallons of ethanol (all grades). By May 2006, the U.S. production rate reached 4.5 billion gallons, utilizing 1.6 billion bushels of grain, chiefly corn (Kansas Ethanol, 2006). The corn milling plants in the nation, especially the dry milling plants, are increasing in number and

Figure 1.1: Ethanol production in the United States
ethanol production capacity. Iowa leads the nation in total corn and ethanol production (National Corn Growers Association [NCGA], 2006). During ethanol fermentation, large amounts of coproducts such as distillers dried grain (DDG) from dry milling plants and gluten feed from wet milling plants are produced. These coproducts are sold as animal feed or used as fuel in boilers. Dry milling plants in the United States produced about 9 million metric tons of DDG in 2005, and wet milling plants produced about 2.4 million metric tons of gluten feed (RFA, 2006). These coproducts contain mainly complex carbohydrates such as cellulose and hemicellulose bound tightly by lignin molecules. Conversion of the lignocellulosic portion of the DDG into ethanol can produce an additional 13% ethanol in dry milling plants (NREL, 2004). Such conversion not only increases net ethanol production, but also produces less solid residue in the dry milling plants. Production of ethanol from cellulosic biomass satisfies the need for clean renewable fuel. Plant biomass, for example, agricultural and forest residues, is a good source of lignocellulose. Bioethanol and other biobased products produced from such low-value feedstock could potentially be economically more attractive than fossil fuels (Zaldivar et al., 2001; NREL, 2006).

A recent joint report by the United States Department of Agriculture (USDA) and Oak Ridge National Laboratory (ORNL) concluded that the United States could produce about 1.3 billion dry tons of biomass annually (see Figure 1.2) (ORNL and USDA, 2005). The utilization of 1.3 billion dry tons of biomass could produce up to 130 billion gallons of additional fuel ethanol (Somerville, 2006). Thus, the biomass has the potential to make a substantial contribution to reducing the importation of petroleum fuels.

Lee (1997) illustrated the scope of lignocellulosic biomass conversion to ethanol. Iogen Corporation in Ottawa, Canada, annually produces over a million gallons of cellulose
ethanol per year from wheat, oat, and barley straw (RFA, 2006). Various ethanol plants and enzyme companies in the United States are also conducting research in cellulose ethanol production (RFA, 2006). Commercial production of ethanol from cellulose is possible because of innovative pretreatment techniques and fermentation facilities. A cellulosic biomass program has been created under the Energy Policy Act of 2005 for production of 250 million gallons of cellulose ethanol by the year 2013 (RFA, 2006).

Figure 1.2: Biomass resource potential in the United States
Source: ORNL and USDA (2005)

Pretreatment of biomass is an important step in the economical conversion of cellulose material to ethanol (U.S. Department of Energy [DOE], 2006). Physical, chemical, and biological pretreatment techniques facilitate the downstream processing steps in bioethanol and biobased industries (Wyman, 1996). New technologies in the economical production of efficient enzymes have reduced the cost for cellulose conversion to ethanol by 20-fold (NREL, 2006). Therefore, enzymatic hydrolysis is a comparatively favorable pretreatment method over costly and environmentally unfriendly physicochemical methods.
1.2 Lignocellulosic Biomass

Lignocellulosic biomass is the most abundant organic compound on earth (Kirk and Farrell, 1987). Lignocellulosic or woody biomass (Figure 1.3) is composed of carbohydrates and polymers: cellulose and hemicellulose enmeshed by lignin, a complex polymer of methoxylated and hydroxylated polyphenylpropane (Hamelinck et al., 2005). Cellulose, hemicellulose, and lignin form the complex biopolymeric structure of plants. Lignin is the cell bonding agent that holds plant cells together, and thus prevents easy access to these natural polysaccharides. The structural complexity of hemicellulose and lignin and the crystalline structure of cellulose make lignocellulose highly insoluble and resistant to attack (Hamelinck et al., 2005). Therefore, delignification is required to liberate cellulose and hemicellulose from lignocellulose. The cellulose and hemicellulose can be further degraded into to five- and six-carbon sugars, which could serve as a substrate for ethanol fermentation.

Figure 1.3: 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)
Cellulose is a long-chain polymer of glucose with β-1,4-glycosidic links that are further aggregated to form microfibril bundles (Figure 1.4) and impart crystalline structure (Linko 1987 in Niamke et al., 2004; Brown, 2003). About 23 to 53% of cell wall materials in plants consists of cellulose. Multilayered and multibundled cellulosic microfibrils are structurally stabilized by straight and branched chains of hemicelluloses: polymers of
hexoses (six-carbon sugars) and pentoses (five-carbon sugars) (Hamelinck et al., 2005). They comprise about 20 to 40% of the structural integrity in plant cell walls. These are further hardened by an amorphous three-dimensional (3D) matrix of a complex polyphenylpropane polymer (the lignin) (Figure 1.5), which comprises of about 10 to 25% of the woody biomass (Crawford, 1981; Knauf and Moniruzzaman, 2004; Hamelinck et al., 2005).

Figure 1.5: Lignin structure. Lignin, polyphenylpropane, basically consists of monomers like coumaric acid, coniferyl alcohol, and sinapyl alcohol.

Sources: University of Hamburg, Department of Biology (2006), Institute of Biotechnology and Drug Research (2006)
1.3 Pretreatment of Lignocellulosic Biomass

Producing sugars from cellulose and hemicellulose is far more difficult than deriving sugars from corn starch or sugar cane (sucrose) (Wyman, 1996). Biotechnology and processing costs for conversion of lignocellulosic biomass to mono- and disaccharides are costly and fail to attract the associated biobased industries (Wyman, 1996). Agricultural and industrial residues and dedicated energy crops like hybrid poplar, switch grass, and others have been widely studied for their prospects in the bioethanol industry. The *Handbook on Bio-ethanol: Production and Utilization* reported that the practical processing cost ceiling for the pretreatment of lignocellulosic materials for bioethanol production has been estimated at about $40/t dry biomass (Wyman, 1996).

The ethanol industries in the United States are flourishing, and lignocellulose has a good niche for fuel ethanol production. 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.

The objective of pretreatment is to alter the structure of the lignocellulosic biomass at micro- and macroscopic levels by physical, chemical, or biological methods and/or a combination of these (Hsu, 1996; Brown, 2003). Pretreatment facilitates the hydrolysis and fermentation processes. Depending on structural complexity and the method employed, pretreatment techniques can render (1) all three components (lignin, hemicellulose, and cellulose) in one product stream (e.g., ball milling), (2) hemicellulose sugars in a liquid phase
and cellulose and lignin in one solid phase (e.g., steam explosion), (3) solubilized lignin and hemicellulose in one liquid phase and cellulose in a solid phase (e.g., alkaline treatment), or (4) all major components in three separate product streams (e.g., biomass fractionation) (Wyman, 1996).

1.3.1 Physical Pretreatment

Physical pretreatment involves processes like communication, irradiation, steam explosion, hydrothermolysis, ultrasonication, and others (Hsu, 1996; Knauf and Moniruzzaman, 2004).

1.3.1.1 Communication

Communication involves mechanical techniques aimed at size reduction of biomass. These include ball milling, compression milling, dry milling/beating, attrition, and wet disk refining (Mosier et al., 2005). The size of the materials is usually 10 to 30 mm after chipping and 0.2 to 3 mm after milling or grinding (Sun et al., 2002). Size reduction helps to increase the surface area of the biomass and thereby enhances the chemical or biological activities in downstream processes. These processes are energy intensive, time consuming, and expensive.

1.3.1.2 Irradiation

Irradiation includes various treatment options such as highly-charged electron beams, gamma rays, and microwaves. Kitchaiya et al. (2003) reported an increase in reducing sugar concentration following enzymatic saccharification when the lignocellulosic waste was
treated with microwave heating at atmospheric pressure. However, many of these methods demonstrated less success and are expensive in full-scale applications.

1.3.1.3 Steaming/Steam Explosion

Applications of high-pressure steam to biomass (e.g., wood chips) for a few minutes, followed by rapid steam venting and flash cooling of the biomass can cause increase in pore volumes (Wyman 1999). Hemicellulose is hydrolyzed by the uncatalyzed steam explosion (Mason 1926; De Long 1981 in Mosier et al., 2005). The removal of hemicellulose facilitates the activity of cellulose degrading enzymes in the downstream saccharification process. The hydrolysis step involves pretreatment of the biomass in the presence of moisture. During this process organic acids such as acetic acid are released from the biomass and catalyze the pretreatment process. This process is sometimes called auto-hydrolysis (Hsu, 1996).

1.3.1.4 Liquid Hot Water Pretreatment (Hydrothermolysis)

This treatment uses pressure to maintain the water in the liquid state at high temperatures (Mosier et al., 2005). High temperature (180 to 220°C) pressurized (350 to 400 psig) water flows through the biomass slurry (16% total solids content) for up to 15 minutes, effectively removing hemicellulose. Hydrothermolysis reduces the need for neutralization and conditioning chemicals because this process does not use acids or alkalis. Removal of hemicellulose exposes sites for cellulose degrading enzymes like cellulbiohydrolases, endoglucanases, and β-glucosidase.
Organic acids such as acetic acid are formed from hemicellulose during liquid hot water treatment with acid. The release of acids helps to catalyze the formation and removal of oligosaccharides. The polysaccharides and especially hemicellulose are hydrolyzed to monomeric sugars. These sugars are partially degraded to furfural and hydroxymethyl furfural by the acid. The furfurals have an inhibitory effect on yeast fermentation (Beery et al., 2004). Modig (2002) reported an inhibitory effect of furfural on the activity of enzymes like alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AIDH), and the pyruvate dehydrogenase (PDH) during yeast fermentation.

1.3.1.5 Ultrasound Treatment

High intensity and high frequency sound waves (ultrasound) have 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, 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 is currently under investigation at Iowa State University (Khanal et al., 2006).
1.3.2 Chemical Pretreatment

Chemicals such as acids, alkalis, and solvents have the ability to increase the accessibility of sites in the biomass by dissolving or altering the lignin fraction and breaking the cellulosic crystals. These pretreatments have received by far the most attention among all pretreatment methods. High chemical costs, equipment, and process complications are the factors impeding its wider application.

1.3.2.1 Dilute Acid Hydrolysis

Addition of small amounts of mineral acids (e.g. sulfuric acid) improves the hydrolysis of hemicellulose at a reduced temperature (Beery et al., 2004; Brown, 2003; Hsu, 1996; Sun et al., 2002). Comminuted biomass is treated with 1% H$_2$SO$_4$ and incubated at 140°C for 30 minutes or at 160°C for as little as 5–10 minutes to achieve complete hemicellulose breakdown. Cellulase enzymes (cellbiohydrolases, endoglucanases, and β-glucosidase) can hydrolyze as much as 90% of the cellulose into glucose molecules (Brown, 2003). Dilute sulfuric acid treatment of cellulosic materials for the commercial production of furfurals for use in the production of pesticides, synthetic resins, and nylon is well-known.

Dilute sulfuric acid treatment requires corrosion-free vessels. The pH adjustment of the acidic sugar solution is also necessary prior to ethanol fermentation. Formation of degradation products such as furfurals from hemicellulose and monomeric sugars was reported to be inhibitory to microbes fermenting the released sugars (Beery et al., 2004).
1.3.2.2 Concentrated Acid Hydrolysis

Both hydrochloric acid (HCl) and sulfuric acid (H$_2$SO$_4$) have been used commercially in concentrated acid hydrolysis (Brown, 2003), the latter being the less expensive. The concentrated acid hydrolysis process is relatively simple and has a high sugar yield (~100% of theoretical hexose yields). However, the solution needs to be neutralized by using an inexpensive base such as lime. For large volumes, the amount of acid required is about equal to the weight of the sugars produced. Brown (2003) addressed the necessity of handling the consequent gypsum (CaSO$_4$) production at a rate of 2 kg gypsum per liter of ethanol produced. This would produce about 380,000 metric tons of gypsum annually from a 50 million gallon/year cellulose-based ethanol industry. Proper disposal or reuse of gypsum is therefore also necessary in a process such as this that involves such a large amount of concentrated acid hydrolysis. Regeneration of acid after acid hydrolysis pretreatment could potentially reduce the cost of such a process.

1.3.2.3 Alkaline Pretreatment

Numerous studies on pretreatment of biomass with various alkalis (e.g., sodium, potassium, calcium, and ammonium hydroxides) have been conducted (Brown, 2003; Mosier et al., 2005). The most commonly used alkalis are sodium hydroxide and its combination with peroxide or others. Sodium hydroxide is easy to handle, but it may be more costly than other feasible agents. Lime can be an alternative. Alkaline pretreatment can also achieve delignification with significant solubilization of hemicellulose. Substrate and treatment conditions may govern the effectiveness of alkaline pretreatment. Regeneration of alkali is also necessary to reduce the overall cost of pretreatment.
1.3.2.4 Organosolv Process

Pan et al. (2005) designed a pre-steam process in an aqueous ethanol “Organosolv” extraction process. The Organosolv process involves use of an organic solvent such as methanol, ethanol, or acetone to solubilize and remove lignin (Hsu, 1996). This is basically a delignification process with varying degrees of simultaneous hemicellulose removal. In the pulp and paper industries, application of several organic solvents resulted in pulp with low papermaking quality because of the carbohydrate degradation that occurred with the delignification.

1.3.2.5 Ammonia

Ammonia fiber explosion (AFEX) is an effective pretreatment process where lignocellulose biomass is treated with an ammonia solution at higher temperatures (60 to 100°C) and pressure (250 to 300 psi) for a period of time (30 minutes), followed by a sudden release of pressure (Dale et al., 1996; Sun et al., 2002; Ramirez, 2005). This simultaneously reduces the lignin content and removes some hemicellulose while decrystallizing the cellulose. Thus, it affects both micro- and macroaccessibility of the cellulases to the exposed cellulose. 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.

1.3.2.6 Sulfur Dioxide

Stenberg et al. (1998) reported pretreatment of mixed softwoods with impregnation of sulfur dioxide (SO_2) steam for production of ethanol. The authors studied the optimal
conditions for both sugar and ethanol yields to assess the effect of inhibitors formed during pretreatment. The parameters investigated were SO$_2$ concentration (1–6% dry matter (w/w)), temperature (190–230°C), and residence time (2–15 minutes). After pretreatment, the material was separated into a solid residue and a filtrate. The solid residue was enzymatically hydrolyzed with 2% dry matter (w/w). Fermentability by *Saccharomyces cerevisiae* on the hydrolyzed filtrate was investigated. The highest sugar yield of 42.1 g/100 g dry matter was obtained at 210°C and 5.5 minutes residence time. However, SO$_2$ is highly toxic and may pose safety and health risks.

### 1.3.2.7 Carbon Dioxide

Hsu (1996) reported explosion pretreatment of lignocellulosic biomass using carbon dioxide (CO$_2$). CO$_2$ dissolution before the expansion to produce an explosion produces carbonic acid that would enhance the hydrolysis process (Sun et al., 2002). Supercritical carbon dioxide (SC-CO$_2$) has been widely used as an extraction solvent in conjunction with the enzymatic hydrolysis step to release sugar from the lignocellulose in moist substrates like aspen and southern yellow pine (Kim and Hong, 2001). The authors reported that the SC-CO$_2$ pretreatment of both aspen and southern yellow pine with moisture contents of 40, 57, and 73% (w/w), followed by cellulase activity, produced a significantly higher final sugar yield than thermal pretreatment without SC-CO$_2$. Carbon dioxide is inexpensive, clean, and easy to recover after use. The CO$_2$ produced during fermentation of sugar into ethanol can be used up-front in the industry for pretreatment of lignocellulose.
1.3.3 Biological Pretreatment

Enzymatic hydrolysis employs the application of enzyme extracts from various microorganisms cultured on complex substrates. There are numerous bacteria and fungi in the wild that contribute to the degradation of cellulose and hemicellulose to their monomers: hexoses and pentoses, respectively (Cloete and Malherbe, 2002). These monomers would then be further utilized in their metabolic processes for growth. There are a few fungal species, especially wood rot fungi that are capable of degrading or modifying the lignin structures while hydrolyzing the hemicellulose and cellulose. These bacteria and fungi out-compete other organisms. Some of them have been identified, isolated, studied, and modified according to the need in biotechnological experiments. NREL has been conducting numerous experimental studies in enzymatic hydrolysis using genetically modified bacteria and fungi (NREL, 2006).

Wood rot fungi such as white and brown rot fungi have been studied for their cellulolytic abilities. *Trichoderma reesei* has been studied frequently for its cellulase activities (Schulein, 1988). Cellulose and hemicellulose degrading properties have been reported for brown rot and white rot fungi. These organisms are of great importance in saccharification and fermentation of lignocellulosic biomass. White rot fungi produce lignin-degrading enzymes that degrade the lignin to carbon dioxide and water, exposing the hemicellulose and cellulose in the wood matrix (Cowling, 1961 in Crawford, 1981). The hemicellulase and cellulase enzymes work systematically to restore the carbon energy in the fungal body. Likewise, brown rot fungi have the ability to modify the lignin structure in the wood matrix (Highley and Dashek, 1998).
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 for releasing cellulosic sugars. The cost of cellulose hydrolysis has dropped by more than 20-fold due to efficient pretreatment technologies and production of effective enzymes at low cost (NREL, 2006).

1.3.4 Combination of Pretreatment Technologies

Many of these pretreatment techniques can be combined for efficient lignocellulosic biomass breakdown. Various combinations of pretreatments have been studied (Hsu, 1996).

Two chemical pretreatments applied in sequence:

a) a chemical pretreatment followed by a biological pretreatment,
b) two physical pretreatments in sequence,
c) a physical pretreatment followed by a chemical pretreatment, and
d) a chemical pretreatment followed by a physical pretreatment

have been employed for effective breakdown.

The development of environmentally friendly and cost-effective pretreatment methods governs the success of the biofuel industries. Therefore, biofuel processing designs should employ a pretreatment method with (a) lower chemical use, (b) higher sugar recovery, (c) easy separation of released sugars, and (d) overall lower capital and operation costs.
1.4 Microbial Scissoring of Lignocellulose

Cellulose and hemicellulose together comprise a vast resource of carbon polymers on Earth. They comprise about 50% and 30% of the plant biomass, respectively (Niamke and Wang, 2004). Lignin and minor amounts of inorganic substances (ash) make up the remainder of the biomass. Lignin imparts strength and protection for plant biomass. Cellulose is a long linear polymer ranging from 1,000 to 1,000,000 D-glucose units, which are linked together by β-1,4-glycosidic bonds to form highly stable chains. The chains of glucose units are aggregated together by hydrogen bondings at places and form the crystalline structure of cellulose. Hemicellulose is a relatively short-chain branched copolymer of pentoses (xylose, arabinose, mannose, etc.) and hexoses (glucose, galactose, etc.) that form amorphous structures that vary depending on the source of the biomass (Niamke and Wang, 2004). Compared with cellulose, hemicelluloses are more susceptible to hydrolysis. They are solubilized under milder conditions than cellulose during acid hydrolysis (Hamelinck et al., 2005). However, the hydrolyzed products of hemicellulose include both hexoses and pentoses, thereby limiting the efficient microbial fermentation of these products to a great extent. On the other side, degradation of lignin and cellulose is confined to the enzymatic activities of some microorganisms (Kirk 1985). Furthermore, lignin is not subject to enzymatic hydrolysis, and the initial attack must be oxidative, nonspecific, nonhydrolytic, and extracellular (Kirk and Farrell, 1987; Hatakka, 1994).

White rot fungi are the most efficient lignin degraders, and they are able to completely break down lignin to carbon dioxide and water (Cowling, 1961 in Crawford, 1981). Lignin is not utilized as a true carbon source. The enzymatic activity of the white rot fungi depends on polymeric complexities, the environment, and the available
polysaccharides. Lignin degradation provides access to underlying cellulose and hemicellulose, which serve as a source of carbon and energy. The white rot fungi are a heterogeneous group that may degrade greater or lesser parts of specific cell wall components (Ward et al., 2004). Some preferentially degrade lignin, leaving the white crystalline cellulose undisturbed (hence the name, white rot fungi), while others degrade lignin and cellulose simultaneously. The former process is a selective delignification and leaves cellulose as crystalline pockets; the latter is a nonselective delignification. *Phlebia radiate*, *Phanerochaete chrysosporium*, and other white rot fungi degrade lignin selectively, while *Trametes versicolor* degrades lignin nonselectively (Eriksson et al., 1990). *P. chrysosporium* is the most studied white rot fungus for delignification and is therefore best known in the areas of bioremediation, environmental conservation, and renewable bioenergy. Mechanisms of various extracellular enzymes in lignin degradation have been reported as very complex reactions both because of the complexity of real lignin and the variability in enzymatic activities influenced by different factors.

### 1.5 *Phanerochaete chrysosporium*: Taxonomy and Morphology

*Phanerochaete chrysosporium* is one of the most studied white rot fungi especially for lignin degradation. The name ‘white rot’ is given to the fungi for the crystalline cellulose left behind during the delignification process in wood (Fungal Genomics Project, 2005). This fungus belongs to family: Phanerochaetaceae, order: Polyporales, subclass: Agaricomycetidae, class: Basidiomycetes, and phylum: Basidiomycota.

Microscopic examination of two-week mycelial mats on potato dextrose agar typically reveals simple septate hyphae ranging from 3–9 µm in diameter with sparse to
moderate branching as well as the presence of thick-walled terminal or intercalary chlamydospores, 50–60 µm in diameter (Figure 1.6). The blastoconidia are round to ellipsoid in appearance, 6–9 µm in diameter, and borne by poorly differentiated branched conidiophores.

Figure 1.6: Culture pictures of *P. chrysosporium* (a) Mycelia mat of *P. chrysosporium* on corn fiber during solid-state fermentation. (b) Mycelia and chlamydospores (400X). (c) Chlamydospore (1000X). (d) terminal chlamydospores (1000X). Microscope photos using a bright field microscope (Olympus BH-2, Leeds Precision Instruments, Inc. Minneapolis, MN, USA) by Prachand Shrestha, 2006.

White rot fungi degrade lignin at the onset of the secondary growth phase, when utilisable nutrients are depleted and primary fungal growth ceases (Zacchi et al., 2000). Carbon, nitrogen, and manganese are all critical nutritional variables in triggering secondary metabolism and production of ligninolytic enzymes, including lignin peroxidase (LiP) and manganese peroxidase (MnP), by *P. chrysosporium* and other white rot fungi. The ligninolytic activity, delignification mechanism in *P. chrysosporium* is also triggered in cultures where carbon becomes limiting. Jeffries et al. (1981) studied the effect of limiting carbohydrate, sulfur, and phosphorus. Carbon and sulfur were reported to be limiting factors to trigger the ligninolytic activity.
1.6 Ligninolytic and Other Enzyme Systems in *P. chrysosporium*

Most of the available information concerning fungal biodegradation of lignin has come from studies on the white rot fungus *P. chrysosporium*. This fungus is able to degrade lignin by secreting peroxidases that are able to catalyze the initial oxidation involved in lignin degradation (Glenn et al., 1983). Two types of peroxidases, lignin peroxidase and manganese peroxidase, were discovered in *P. chrysosporium*.

1.6.1 Manganese Peroxidase (MnP)

Manganese peroxidase (MnP) oxidizes manganese ion from the +II to the +III state (Mn$^{2+}$ to Mn$^{3+}$), and cannot be replaced by any other metal at physiological concentrations. Some organic acids produced by the white rot fungus are able to chelate the Mn$^{3+}$, resulting in a relatively stable enzyme-substrate complex. This complex can then oxidize phenolic lignin model compounds and various phenols through phenoxy radical (AH•) formation.

In many fungi, MnP is thought to play a crucial role in the primary attack on lignin. It generates Mn$^{3+}$, a strong diffusible oxidant that is able to penetrate the small “molecular pores” between cellulose microfibrils. The primary reducing substrate in the MnP catalytic cycle is Mn$^{2+}$, which efficiently reduces compound I and compound II, generating Mn$^{3+}$, which then serves to oxidize phenols to phenoxy radicals (AH•) (Hatakka, 1994).

\[
\begin{align*}
\text{Ferri-MnP} + \text{H}_2\text{O}_2 & \rightarrow \text{MnP-compound I} + \text{H}_2\text{O} \\
\text{MnP-compound I} + \text{Mn}^{2+} & \rightarrow \text{MnP-compound II} + \text{Mn}^{2+} \\
& \rightarrow \text{Ferri-MnP} + \text{Mn}^{3+} + \text{H}_2\text{O} \\
\text{Mn}^{3+} + \text{AH}_2 & \rightarrow \text{Mn}^{2+} + \text{AH•}
\end{align*}
\]
1.6.2 Lignin Peroxidase (LiP)

Oxidation reactions catalyzed by LiP are cleavage of the Cα-Cβ and aryl Cα bond, aromatic ring opening, and demethylation. Because of the bonding and reaction complexity of ligninolytic enzyme systems, different dimeric model compounds representing different proportions of linkages in the lignin polymer have been studied for the activities of lignin peroxidases. Hammel et al. (1993) and Kirk and Farrel (1987) reported that LiP cleaves the lignin between Cα-Cβ in the dimeric model compounds. Ward et al. (2004) stated furthermore that dimeric lignin model compounds have the disadvantage of low molecular weight. Unlike lignin, they can be taken up and metabolized intracellularly by microorganisms, thereby limiting the real understanding of ligninolytic activity.

The reaction of the ferric enzyme with H₂O₂ yields the LiP compound I (LiP I).

One-electron oxidation of the reducing substrate yields the radical cation (S•) and a one-electron oxidized intermediate, LiP compound [LiP II (Fe⁴⁺=O, P)]. A single one-electron oxidation of a second substrate molecule returns the enzyme to a Fe-LiP, completing the enzyme cycle. However, in the absence of a suitable reducing substrate or at high H₂O₂
concentrations, LiP II is further oxidized to LiP III, which does not revert to the native state.

Veratryl alcohol (VA): 3,4-dimethoxybenzyl alcohol is normally produced in ligninolytic cultures of P. chrysosporium. VA is believed to protect LiP from H$_2$O$_2$-dependent inactivation by reverting LiP III to the native state. VA has also been shown to act as a charge transfer mediator in LiP-catalyzed reactions. During the catalytic cycle of LiP, VA is oxidized to the VA cation radical (VA$^{+*}$), which in the presence of a suitable reducing substrate is reduced back to VA and is ready for another LiP-catalyzed charge transfer reaction (Ward et al., 2004).

\[
\text{VA, } \text{H}_2\text{O}_2 \xrightarrow{\text{Unknown}} \text{LiP III (Fe}^{3+}=\text{O}_2, \text{P)} \rightarrow \text{Fe-LiP (Fe}^{3+}, \text{P)}
\]


1.6.3 Cellulose Degradation

The enzyme mechanisms involved in cellulose degradation have been extensively investigated (Eriksson 1978 in Highley and Dashek, 1998). The degradation of crystalline cellulose by white rot fungi, similar to that of other fungal cellulases, is carried out by a multiple enzyme complex in which the individual component interacts synergistically to degrade cellulose to glucose (Figure 1.7). Endoglucanases (EGs) act randomly over the exposed surfaces of cellulose microfibrils, exposing nonreducing termini that are hydrolyzed by cellobiohydrolases (CBHs), and producing cellobiose. Cellobiose may be cleaved by β-glucosidase, yielding glucose.
1.6.4 Hemicellulose Degradation

Hemicellulose is structurally far more complex than cellulose. Hemicelluloses are a group of homo- and heteropolymers consisting largely of anhydro-β-(1→4)-D-xylopyranose, mannopyranose, glucopyranose, and/or galactopyranose main chains with a number of substituents (Highley and Dashek, 1998). Degradation of hemicellulose by white rot fungi proceeds in a manner roughly analogous to that of cellulose. The hemicellulose chains are attacked first by endoenzymes (mannanases and xylanases) that produce progressively shorter chains, which are hydrolyzed to simple sugars by glycosidases (mannosidases, xylosidases, and glucosidases). The enzymes involved in the removal of side-chain
substituents (arabinose, uronic acids, and acetylcs) have received little attention (Sinnott et al., 1999).

1.6.5 Cellobiose Dehydrogenase (CDH)

This extracellular enzyme is produced by many white rot fungi. \textit{P. chrysosporium} produces relatively high levels of the enzyme, approximately 0.5\% of secreted protein on a mass basis (Ward et al., 2004). Some of the functions suggested for CDH include cellulose degradation. In the context of lignin degradation, it is suggested that CDH reduces the aromatic radicals formed by ligninolytic enzymes, prevents their repolymerization and thus supports the lignin degradation.

1.7 Extracellular Polysaccharide

\textit{P. chrysosporium} produces an extracellular glucan type polysaccharide when grown in a chemostat under nitrogen limitation (Leisola et al., 1982). When cells were transferred to a standing mode of cultivation in the presence of excess glucose (6 g/L), the amount of nonglucose total carbohydrate in the culture increased from 0.58 to 1.76 g/L in 15 days. The change in total carbohydrate was due to an increase in extracellular and cell-bound glucan-type polysaccharides. This increase occurred simultaneously with the formation of mycelial mats. Excess polysaccharide formation is the result of excess glucose in situ. Such a condition also reduces lignin degradation activity.

Experiments with \textit{P. chrysosporium} regarding the ligninolytic enzymatic systems revealed the production of extracellular glucan by the fungus. The slimy polysaccharide
around the fungal hypha assists the fungus in creating a suitable environment (pH and H$_2$O$_2$) for lignin degradation. Bes et al. (1987) reported the alternative system facilitated by the fungus to control the higher glucose levels by production of extracellular glucan. Higher glucose levels repress the endoglucanases, sugar oxidizing enzymes. H$_2$O$_2$ is essential for lignin degradation due to extracellular peroxidases and is mostly derived from oxidation of sugars. When endoglucanases are repressed, H$_2$O$_2$ production is also affected. When glucose concentrations fall below the level necessary for adequate hydrogen peroxide production, secretion of enzyme (1→3)-linked β-D-glucanase is induced. This enzyme degrades the poly-β-D-glucan back to glucose.

Acid hydrolysis of the polysaccharide material isolated from the culture medium by precipitation with 50% ethanol yielded glucose, mannose, galactose, fructose, and xylose in molar ratios of 1.0:0.03:0.01:0.01:0.02 (Buchala et al., 1987). Structurally, glucan consists of a backbone of (1,3)-linked residues with a branch in 6-position on approximately every second and some internal (1,6)-linked D-glucose (Bes et al., 1987). Experiments on wood samples of the aspen tree (Populus tremula) inoculated with the P. chrysosporium showed that the hyphae were encapsulated by a sheath that had the β-1,3-1,6-D-glucan nature (Katia et al., 1991). The sheath provides a transient junction between the hyphae and the wood, thus establishing a point of attachment to the site of wood degradation.

The metabolic response study on P. chrysosporium in conditions of oxygen and nitrogen limitation produced small amounts of ethanol from substrate (glucose, mannose, cellobiose, maltose, and sucrose) in a sealed serum bottle (Kenealy et al., 2004). The authors also reported production of ethanol by the white rot fungus in a nitrogen-purged culture that was grown aerobically on aspen wood chips at the beginning. The ethanol production was as
high as 1.61 g/L in 800 hours under anaerobic conditions. Formation of a slimy glucan matrix both in wood and in liquid cultures would limit the oxygen transfer to deep-seated fungal mycelia. Genome sequence analysis revealed the multiple alcohol dehydrogenase genes in *P. chrysosporium*. Such a finding supports the fermentative capacity of the fungus under anaerobic conditions (Kenealy et al. 2004).

### 1.8 Solid-State Fermentation of *P. chrysosporium*

Yu et al. (2005) quoted various sources regarding the enzymatic capability of *P. chrysosporium* in the degradation of lignin and various environmental pollutants. Of four fungi tested, *P. chrysosporium* had the best ligninolytic activity in solid-state fermentation (SSF) on wheat straw. During 60 days of incubation up to 45% biomass weight loss was observed (Dorado et al., 1999).

SSF of *P. chrysosporium* was studied for production of biopulp. The wheat straw was pretreated with steam explosion followed by autoclaving prior to inoculation with the fungus spores (Chen et al., 2002). Steam explosion of wheat straw facilitated the hemicellulose hydrolysates as a carbon source for the fungus. Fungal lignin degradation improved by five times compared with normal substrate.

Immobilization of the white rot fungus for biofilm development in membrane systems (Sheldon and Small, 2005) and on carriers like polyurethane foam (Yu et al., 2005) has been tried for the study of different enzyme systems under various conditions. Khiyami et al. (2006) reported the necessity of immobilizing *P. chrysosporium* for production of manganese peroxidase (MnP) and lignin peroxidase (LiP). The fungus produced these enzymes after
they had been immobilized in plastic composite supports (PCSs). The enzyme activity was enhanced with addition of veratryl alcohol and manganese sulfate.

1.9 Conclusion

The United States and other countries focus on developing a huge infrastructure for sustainable biobased industries, which reduces their dependency on foreign imports of petroleum and oil products. In the long run, the United States aims to mitigate the environmental and energy crises by utilizing local resources to produce substantial quantities of clean transportation fuel (bioethanol) and biobased products from renewable lignocellulosic biomass. The annual production of over a billion tons of biomass per year from agricultural and forest resources potentially provides enough feedstock for bioethanol production to meet the demand for transportation fuel, in particular. The structural hardiness of biomass tissue imposes difficulties in obtaining simple sugars. However, various pretreatment options are available to break down the complexity of the woody biomass. Promising technological advances can help improve the enzymatic saccharification of cellulosic biomass to monomeric sugars. Many of the pretreatment methods are costly and some or polluting. Direct enzymatic conversion of lignocellulosic material without the use of chemicals, high pressure or high temperatures seems to be more effective and less polluting. Research laboratories and enzyme companies are collaborating on various research and developmental work for the commercial production of efficient enzymes. Furthermore, the microbial system of delignification and biodegradation of lignocellulose materials in nature can be mimicked in laboratory- or bench-scale research. Understanding the complex biochemistry of lignin degradation is very challenging, but utilization of surplus residues like
DDG and corn hull from corn milling plants in biological cellulose degradation research has
drawn the attention of research scientists, industrial economists, and environmental analysts.
The future of biomass energy is focused in cellulose ethanol.

The efficient wood rot fungi can be grown on these substrates for conversion of
cellulose into sugar and then to ethanol. This appears to be the most efficient approach as
this eliminates the need to produce enzymes externally. Direct production of ethanol from
lignocellulosic material using nothing but fungi seems like an economical way to go,
provided the operational conditions are simple and the process is fast enough.

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Chapter 2: Saccharification of Corn Fiber by *Phanerochaete chrysosporium* in Solid-State Fermentation and Subsequent Fermentation of Hydrolysate into Ethanol

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Abstract

Ethanol production in the United States is expected to increase to 7.5 billion gallons by 2012 from its current production of 4.5 billion gallons. This increase would also result in generation of huge quantities of low-value, lignocellulosic coproducts—corn fiber and distillers dried grain (DDG). Conversion of large amounts of the low-value lignocellulosic biomass into high-value products like ethanol is needed for sustainability of corn processing industries. In this research, laboratory-scale solid-state fermentation (SSF) of corn fiber from a wet milling corn plant using white rot fungus *Phanerochaete chrysosporium* was carried out in 1-L polypropylene bottles. *P. chrysosporium* produced extracellular enzymes in situ for lignin degradation and saccharification of cellulose to release sugars. Anaerobic incubation of SSF in buffer solution (at pH 4.73 and 37°C) reduced the fungal sugar consumption and enhanced the cellulolytic enzyme activity in situ, with the release of additional sugars for subsequent yeast fermentation to ethanol. *P. chrysosporium* converted 2.5% of the corn fiber into reducing sugars. The corresponding biomass weight loss was
about 34% and Klason lignin reduced by up to 41%. The storage of extracellular polysaccharides (EPS) by the white-rot fungus during SSF was also investigated by mild acid (0.1N sulfuric acid) hydrolysis. *Saccharomyces cerevisiae* fermentation of the hydrolysates from SSF and mild acid treatment produced 4 mg ethanol/g fiber. This apparently suggests that lower concentrations of fermentable sugar were present in the hydrolysates. Further studies are needed to optimize SSF protocols in simultaneous saccharification and co-fermentation using wood rot fungi and yeasts for economical ethanol production from the corn fiber and other lignocellulosic biomass.

Keywords: Lignocellulosic biomass, distillers dried grains, corn fiber, solid-state fermentation, extracellular enzymes, ligninase, simultaneous saccharification and fermentation, ethanol, white rot fungi, *Phanerochaete chrysosporium*.

### 2.1 Introduction

The United States produces over 4.5 billion gallons of corn-based ethanol (Kansas Ethanol, 2006) and imports nearly two-thirds of its petroleum fuels from politically unstable countries to meet its energy needs. The annual ethanol production is expected to reach 7.5 billion gallons by 2012. The ethanol industry also produces a huge amount of low-value coproducts like distillers dried grains (DDGs) and corn fiber. These contain ample amounts of cellulose, hemicellulose, and leftover starch associated in a complex matrix with lignin (Beery et al., 2004). Conversion of these cellulosic coproducts into ethanol has the potential to add an extra 13% in net ethanol production (National Renewable Energy Laboratory [NREL], 2004). The leftover residues can still be used as a low-carbohydrate animal feed.
In addition, technological advances make it possible to use a billion metric tons per year of lignocellulosic materials—varying from industrial residues to agricultural and forest resources—for over billion gallons of ethanol production to meet the nation’s entire transportation fuel demand (NREL, 2004; U.S. Department of Agriculture and U.S. Department of Energy, 2005). Lee (1997) illustrated the scope of lignocellulosic biomass conversion to ethanol. Iogen Corporation in Ottawa, Canada, successfully produces over a million gallons of cellulose ethanol annually from wheat, oat, and barley straw (Renewable Fuels Association [RFA], 2006). Conversion of lignocellulosic material to ethanol involves several steps such as pretreatment, hydrolysis, fermentation, and separation/purification (Mosier et al., 2005).

Pretreatment of biomass is an important step in the economical conversion of cellulosic material into ethanol (USDOE, 2006). Physical, chemical, and biological pretreatments involve the use of mechanical milling, pressurized steam, acids, ammonia, or enzymes in a separate or combined process to break down the heterogenic and crystalline lignocellulosic fiber matrix and facilitate the conversion of the fiber components, that is, cellulose and hemicellulose, into sugars for ethanol and bioproducts production (Wyman, 1996; Mosier et al., 2005). New technologies in the economical production of efficient enzymes have reduced the cost for cellulose conversion to ethanol by nearly 20-fold (NREL, 2006). Therefore, enzymatic hydrolysis is a comparatively favorable pretreatment method over costly and environmentally unfriendly physicochemical methods.

Numerous indigenous bacteria fungi are reported capable of degrading cellulose and hemicellulose to their monomers, that is, hexoses and pentoses (Cloete and Malherbe, 2002). Wood rot fungi such as white and brown rot fungi have been examined for their cellulolytic
abilities. *Trichoderma reesei* has been studied frequently for its cellulase activity (Schulein, 1988). White rot fungi produce lignin-degrading enzymes that degrade the lignin to carbon dioxide and water, exposing the hemicellulose and cellulose in the wood matrix (Cowling, 1961 in Crawford, 1981). Likewise, brown rot fungi have the ability to modify the lignin structure in the wood matrix (Highley and Dashek, 1998). Most of the information available on fungal biodegradation of lignin has been derived from studies on the white rot fungus *P. chrysosporium*, which is capable of degrading lignin by secreting peroxidases (Glenn et al., 1983).

The overall cost of the enzymatic hydrolysis can further be reduced by coupling the enzyme production with hydrolysis of lignocellulose materials into a single step such as solid-state fermentation (SSF). Pandey et al. (2000) reviewed SSF as a promising fermentation technique for *in situ* production of ligninolytic and cellulolytic enzymes. Several studies explored ligninolytic and cellulolytic enzyme activities in SSF using *P. chrysosporium* under various substrates and operating conditions (Hongzhang et al., 2001; Rodriguez-Vazquez et al., 2003). Bes et al. (1987) reported the storage of extra glucose as extracellular polysaccharides (EPS) in situ by *P. chrysosporium*. EPS formed by the fungus primarily consisted of glucose, mannose, galactose, fructose, xylose, and similar sugars (Buchala et al., 1987). These sugars can potentially serve as an ideal feedstock for ethanol fermentation.

SSF has both technological and economic advantages over liquid fermentation techniques in the enzymatic hydrolysis of lignocellulosic materials to produce the fermentable sugars (Couto and Sanroman, 2005). Based on these rationales, the objective of this research was to evaluate the performance of *P. chrysosporium* in saccharification of corn
fiber in a laboratory-scale solid-state fermentation and in the subsequent fermentation of hydrolysates into ethanol using *Saccharomyces cerevisiae*.

## 2.2 Materials and Methods

### 2.2.1 Fungus Culture

The culture of *Phanerochaete chrysosporium* was obtained from American Type Culture Collection (ATCC # 24725, Rockville, MD, USA). The fungus culture was revived by inoculating the culture in potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD, USA) and was incubated with shaking at 24°C (ATCC, 2004). Stock cultures were preserved in an ultra-low temperature freezer (-80°C).

Mycelia pellets of 5 to 10 mm diameter were visible in 1 week on culture grown in shaker flasks containing PDB incubated at 24°C and 150 rpm. The whole fungal content was aseptically poured into a sterilized laboratory blender and homogenized at low speed for 1 min at room temperature to produce smaller mycelia. The disintegrated mycelia were allowed to rejuvenate for a day or two by pouring the blended mycelia into the flask for incubation with shaking as described earlier. Then 10 mL of mycelia suspension was aseptically mixed into 10 mL of 20% (v/v) sterilized glycerol in water such that the final glycerol concentration was 10% (v/v). After mixing, 2 mL culture suspension was dispensed into a sterile cryogenic vial (Nalgene, Nalge Nunc, International, Rochester, NY, USA). A number of stock cultures were prepared in vials in the same manner. The vials were then stored in an ultra freezer (So-Low, Cincinnati, OH, USA) at -80°C.

Fungal inocula for solid-state fermentation (SSF) were prepared for each experiment from the cryogenic vials stored in the ultra freezer. The culture was thawed and poured
aseptically into 1 L of sterilized yeast mold (YM) broth (Difco). The seed culture was incubated with shaking at 150 rpm and 37°C for rejuvenation. The mycelia grew into pellets of 2 to 3 mm size in 3 to 5 days.

2.2.2 Substrate for Solid-State Fermentation

The substrate for the SSF experiments was corn fiber from corn wet milling plants, (Archer Daniels Midland (ADM), Decatur, IL, USA). The fiber had been processed through hot water steeping and sulfur dioxide (SO₂) treatment at the beginning of the wet milling process. Sterilization of the fiber was done during the preparation of culture bottles.

2.2.3 Experimental Set-up

Batch SSF experiments were conducted on corn fiber using *P. chrysosporium*. Culture bottles with fungal mycelia growing on substrate at 37°C incubation temperature (Kenealy and Dietrich, 2004) served as microcosms. The SSF was carried out in a number of

![Flow chart of a solid-state fermentation (SSF) batch experiment](image-url)
1-L polypropylene bottles (Nalgene, Nalge Nunc., International, Rochester, NY, USA). Control bottles had fiber, marbles, and inoculation media but no fungi. Culture and control bottles were taken out of the incubators to determine sugar content and biomass weight loss. Anaerobic incubation of the culture bottles in buffer solution was studied for enzyme activity and saccharification of the fiber. Lignin degradation in the corn fiber was studied under the influence of various inducers like hydrogen peroxide, manganese sulfate, and veratryl alcohol. The experimental set-up is illustrated in Figure 2.1.

### 2.2.3.1 Substrate Quantification and Pretreatment

The SSF process used was modified from that of Crawford and Pometto (1988). The authors developed empirical formulae to quantify the fiber and volume of microbial inocula for uniform spread of substrate and fungal biomass along the inner wall of the culture bottles, as illustrated below:

Inoculum volume, \[ V = 1.5(D) \]

Weight of lignocellulosic material, \[ W = 2dh \text{ (0.1cm)/ (2.5/D)} \]

Where, \( D \) = density of the lignocellulosic material (mL fiber/g), \( d \) = diameter of vessel (or bottle) in cm, and \( h \) = height of the vessel in cm.

The moisture content of the fiber (Figure 2.2) was about 7%. The fiber was oven dried at 80°C for 4 days. The dry fiber was cooled in a desiccator. Loosely capped culture bottles (each containing 25 g dried and cooled fiber, five marbles, and 5 ml of deionized water) were autoclaved at 121°C for 1 h. The autoclave was rapidly exhausted. The bottle caps were replaced with blue autoclave cloth (Propper Steri-Wrap III, Propper manufactu-
ring Co. Inc., Long Island City, NY, USA). The bottles were then resterilized for 15 min at 121°C.

Figure 2.2: Corn hull from ADM wet milling plant in Decatur, IL, was used as substrate for fungal solid-state fermentation

2.2.3.2 Fungal Inoculum Preparation and Incubation

The fungal mycelia grown for 3 to 5 days in YM broth were aseptically transferred to a sterilized 1-L polypropylene centrifuge bottle. The bottle was centrifuged at 7,277 g for 20 min. The supernatant was decanted, and the centrifuge bottle was aseptically filled to the top with fungal inoculum medium (Kirk et al., 1972). After the pellets were mixed into suspension, the centrifuge and supernatant decantation procedure was repeated to reduce the chances of transferring complex nutrients to the sterile corn fiber SSF culture bottle during fungal inoculation. Once the complex nutrient (from YM broth) was washed and rinsed out, the washed fungal suspension was aseptically transferred into a sterile container (e.g., a 2-L Erlenmeyer flask), and sterile fungal inoculum medium was poured to make approximately 1 L of dense mycelia slurry. The SSF bottles were inoculated with 75 ml of dense fungal slurry. Control bottles contained the same inoculum medium, but without fungal mycelia.
The bottles were rolled on their sides soon after the fungal inoculation. The marbles in the bottles helped to mix and disperse the corn fiber and fungi uniformly over the inner surface of the bottles. The bottles were placed in a humidified incubator set at 37°C. Moist air in the incubator was maintained by placing water troughs at the bottom and passing air into the water.

**Fungal Inoculum Media and Trace Element Solution Composition**

The fungal inoculum medium (Kirk et al., 1972) contained: 0.25 g KH₂PO₄, 0.063 g MgSO₄·7H₂O, 0.013 g CaCl₂·2H₂O, 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 MgSO₄·7H₂O, 0.5 g MnSO₄·H₂O, 1.0 g NaCl, 0.1 g FeSO₄·7H₂O, 0.181 g CoSO₄·7H₂O, 0.082 g CaCl₂·2H₂O, 0.1 g ZnSO₄, 0.01 g CuSO₄·5H₂O, 0.01 g Al₂(SO₄)₃·2H₂O, 0.01 of H₃BO₃ and 0.01 g NaMoO₄.

2.2.4 Analytical Methods

Individual control and culture bottles were taken out of the incubators after completion of required incubation time according to the experimental design. The content of the bottles were washed down to the bottom by adding 300 ml of deionized water to each bottle. Then the bottles were kept inside a steam cabinet for 1 h. Steam treatment would release the sugar in the solution. Analyses of total and reducing sugars, biomass weight loss, lignin degradation, and ethanol fermentation were conducted for harvested SSF bottles. Effects of inducers like hydrogen peroxide (Sun et al., 1999), manganese sulphate, and veratryl alcohol: 3, 4 – dimethoxybenzyl alcohol (Pometto et al. 2006) were also studied for delignification. Different concentrations of these inducers were added aseptically to the
culture bottles as one concentration per bottle. The concentrations and volume added were:
10 ml of 0.4 mM, 4 mM and 40mM hydrogen peroxide; 3 mM, 30 mM and 300mM veratryl alcohol and manganese sulfate. The biomass inside these bottles was analyzed for Klason lignin degradation one week after the addition of inducers.

2.2.4.1 Klason Lignin Assay

The lignin content was determined by a modified Klason procedure (Crawford and Pometto, 1988), which measures lignin as the acid-insoluble fraction of lignocellulose samples (Klason lignin) subjected to hydrolysis by strong acid. First, 1 ml of concentrated sulfuric acid (36N) was added to 50 mg of dried, ground substrate. This mixture, in a 50-ml Erlenmeyer flask, was occasionally mixed with a glass rod for 1 h. Then 28 ml of deionized water was added to the flask. The flask mouth was covered, and then the flask was autoclaved for 1 h at 121°C. When the solution was cooled, it was filtered through Whatman #54 filter paper. The residue on the filter paper was thoroughly rinsed with deionized water and dried in the oven at 80°C for 2 days. The Klason lignin was estimated as the weight of dry residue collected on the filter paper.

The filtrate was then collected in a 50 ml volumetric flask. For neutralization, 18 ml of 2N sodium hydroxide was added, and a few milliliters of deionized water were also added to make a final volume of 50 ml. The reducing sugar concentration was then analyzed via a modified Somogyi Nelson Carbohydrate Assay (Crawford and Pometto, 1988), as described in the following section.
2.2.4.2 Reducing Sugar Assay

The Somogyi-Nelson reducing sugar assay was selected because it measures hexoses, pentoses, and other sugars with reducing ends (Crawford and Pometto, 1988). For sugar harvesting, 300 ml of deionized water was carefully poured into each of the bottles so that it would wash down the fiber and fungal mycelia at the bottom of the bottle while being poured. The bottles were placed in a steam cabinet and steam-treated for 1 h. The contents were then poured into 500-ml polypropylene centrifuge bottles (Nalgene, Nalge Nunc, International, Rochester, NY, USA). The marbles were trapped out while pouring the contents into the centrifuge bottles. The bottles were centrifuged at 17,696 g for 20 min, and the supernatants were filtered through predried and preweighed Whatman #54 filter paper. The filtrates were then analyzed for reducing sugar by the modified Somogyi-Nelson Carbohydrate Assay. The filtrates were neutralized or diluted as needed for accurate results.

One milliliter of filtrate, Klason filtrate, or sample was added to a test tube, followed by addition of 3 ml of Somogyi Reagent prepared by mixing four parts of Somogyi Reagent A with one part of Somogyi Reagent B (Figure 2.3). The tube was capped by placing a marble over the mouth and placed in a steam chamber for 1 h. It was then cooled at 4°C for 30 min, after which 3 ml of Nelson reagent was added. The solution was vortexed, and then 10 ml of deionized water was added. The solution was vortexed again before being transferred into spectrophotometer tubes. All tests were carried out in triplicate to reduce errors. The samples were diluted so that the sugar concentration in the diluted sample could be accurately detected. The absorbance reading was taken in a spectrophotometer (Spectronic™ 20 Genesys™, Thermo Electron, Cambridge, UK) at 500 nm. The absorbance
readings were then converted into equivalent concentration using standard glucose solution curve.

The aldehyde groups of carbohydrates are oxidized to carboxylic acid by the copper sulfate (Cu +II), which itself is reduced to cuprous oxide (Cu +I). This mechanism is carried out in the Nelson-Somogyi Carbohydrate Assay for reducing sugars. The presence of reducing sugars was detected visually by the brick colored precipitate in heated (steamed 1 h in the experiment) sample solutions with Somogyi reagents (Figure 2.4). Higher concentration of sugar solution was diluted for accurate spectrophotometer readings.

Reagent Ingredients for the Somogyi-Nelson Carbohydrate Assay

The reagents for Somogyi-Nelson Carbohydrate assay were Somogyi reagent C and Nelson reagent. Somogyi reagent C was prepared by mixing 1 part of Somogyi Reagent A (180 g Na₂SO₄, 15 g Rochelle salt, 30 g Na₂CO₃ and 20 g NaHCO₃ in 1 L deionized water)
and 4 parts of Somogyi Reagent B (180 g Na₂SO₄, 20 g CuSO₄·5H₂O in 1L of deionized water).

Nelson reagent contained 50 g (NH₄)₆Mo₇O₂₄·4H₂O) added to 42 ml of concentrated H₂SO₄, 900 ml deionized water and 6 g Na₂HAsO₄·7H₂O in 50 ml of deionized water and additional deionized water to bring the mixture volume to 1 L. This reagent was incubated at 37°C for 24–48 h before its use.

### 2.2.4.3 Total Carbohydrate Assay

The samples were also tested for total sugar via acid hydrolysis using the phenol sulfuric carbohydrate test (Crawford and Pometto, 1988). One milliliter of sample was mixed with 1 ml of 5% (w/v) phenol and 5 ml of concentrated sulfuric acid in a spectrophotometer tube. After being vortexed and cooled, the solution was observed for absorbance at 490 nm. The absorbance readings were then used to calculate the concentration using a standard glucose solution curve.

The higher concentrations of sugar sample developed an intense golden color with a reddish tinge. Light yellow and orange colors indicated low or moderate sugar concentrations, respectively (Figure 2.5). However, this should be confirmed via colorimetric testing in a spectrophotometer at 490 nm. Higher concentration of sugar solution was diluted for accurate spectrophotometer readings.
2.2.4.4 Biomass Weight Loss

Following the filtration and sugar harvesting steps, the centrifuge bottles contained wet fibers. These bottles were dried in an oven at 80°C for 4 days. After cooling in a desiccator, they were weighed in an analytical balance (Mettler PM 6100, Hightstown, NJ, USA; accuracy ±10 mg). The difference between the weights of dried and cooled (in desiccator) bottles with fibers and the initial weights of dried centrifuge bottles would give the weights of biomass remained after SSF. The fraction biomass weight loss was then calculated as the ratio of the difference in the biomass weight after SSF to the weight of initial weight of the dry corn fiber.

2.2.4.5 Incubation under Anaerobic Condition

Anaerobic incubation of the SSF bottles was conducted to evaluate the cellulase activity of *P. chrysosporium*. The anaerobic condition minimizes the consumption of sugar
by the fungi. Meyer et al. (2006) performed studies on cellulase enzymes at pH 3.5 to 5.0 and temperature ranging from 35° to 60°C. Anaerobic incubation of SSF bottles was performed by adding 300 ml of sterile 0.2M acetate buffer (sodium acetate and acetic acid, pH 4.73) solution and keeping the tightly capped bottles in a water bath at 37°C for 2 days. Control and culture bottles that were incubated for 5, 7, 9 and 11 days were treated anaerobically in buffer solution as described above. Sugar and biomass weight loss analyses were done following the sugar harvesting step, as described earlier.

2.2.4.6 Extracellular Polysaccharides (EPS)

The filtration step in the sugar harvesting process was very tedious. The filter papers clogged frequently during the filtration of sugar solution from the P. chrysosporium SSF bottle as compared with the control SSF bottle. Filamentous microorganisms (bacteria and fungi) produce extracellular polysaccharides (EPS). Encapsulations of the microbial cells or hyphae by EPS provide protection, anchorage, and a medium for nutrient transfer to the organisms. Studies showed the production of extracellular glucan by P. chrysosporium (Leisola et al. 1982; Bes et al. 1987). EPS formation by P. chrysosporium during SSF was suspected as the cause of the filter paper clogging.

The EPS produced by P. chrysosporium was determined by mild acid treatment of the SSF fiber. The SSF fiber and fungal biomass (in culture bottles) was washed down to the bottom of the bottles by adding 200 ml of sterile deionized water and incubated for 3 and 10 days. Once the biomass was submerged, 0.55 ml of concentrated sulfuric acid (36N) was added to the bottles, the bottles were capped loosely and steam treated in a steam chamber for 1 h. The EPS produced by P. chrysosporium was hydrolyzed by mild acid treatment to
release higher sugar concentration. The fermentability of the released sugars was determined via yeast fermentation. Aseptically, 100 ml of sterile triple strength, yeast culture media (Veale et al., 2003) without glucose was added to the bottles, and pH was adjusted to 5.5. Freeze-dried yeast cells (5.6 x 10^8 cell counts) were aseptically inoculated as one vial per bottle. Some acid-free bottles were also inoculated with yeast. The bottles were loosely capped and kept in the incubator at 37°C. Samples were analyzed for total and reducing sugars and ethanol every 48 h.

2.2.4.7 Ethanol Analysis

*P. chrysosporium* has been studied for its ability to produce EPS and ethanol under oxygen-limiting conditions (Kenealy and Dietrich, 2004). Ethanol fermentation by yeast in acid-free and mild acid treated bottles was determined at intervals of 2 days. Percentages of ethanol were measured by using a Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford, MA, USA) equipped with a Waters Model 401 refractive index detector, column heater, auto-sampler and computer controller. The separation of ethanol, glucose, and other broth ingredients was done on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA, USA) using 0.012N sulfuric acid as a mobile phase at a flow rate of 0.8 mL/min with a 20 µl injection volume and a column temperature of 65°C.
2.3 Results and Discussion

2.3.1 Substrate Composition

The lignocellulosic composition of corn fiber obtained from an ADM wet milling plant was analyzed by the neutral detergent fiber (NDF) and acid detergent fiber (ADF) procedures (Ankom, 2006). The fiber was found to be high in hemicellulose and cell-solubles, which include soluble carbohydrates, organic acids, and proteins, among others (Table 2.1).

<table>
<thead>
<tr>
<th>% cell-soluble</th>
<th>% hemicellulose</th>
<th>% cellulose</th>
<th>% lignin</th>
<th>% ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>36.98</td>
<td>45.30</td>
<td>16.42</td>
<td>1.27</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 2.1: Corn fiber composition as determined by the NDF and ADF methods (n = 2).

Beery et al. (2004) reported the following composition for unprocessed corn fiber: 35% hemicellulose, 18% cellulose, 17% starch, 11% protein, 6% ash, 6% galactan, 3% oil, 1% manna, and 3% other materials. These cellulose and cell-soluble (starch, protein, galactan, oil, etc.) contents are comparable to the values given in Table 2.1. The modified Klason lignin method (Crawford and Pometto, 1988) measured 17% Klason lignin in the corn fiber. This lignin content is significantly different from the value obtained by the ADF method. Hatfield et al. (2005) reported that the acid detergent lignin method had the lowest lignin value of all the methods. They also suspected the inclusion of protein contaminant in the Klason lignin determination. The possibility of error due to protein should be scrutinized further, as it is unlikely that proteins would be present in residue from the concentrated acid treatment in the Klason lignin assay.
2.3.2 Reducing Sugar Harvested from SSF

Extracellular enzymes secreted by *P. chrysosporium* are capable of hydrolyzing the carbohydrate polymers into smaller components, such as monosaccharides, disaccharides, trisaccharides, and oligosaccharides. The reducing sugar assay quantified the carbohydrates with reducing ends. The reducing sugar values in control bottles varied between 9 and 12 mg/g initial fiber. In *P. chrysosporium* culture bottles, the reducing sugar values decreased gradually from 13 to 9 mg/g initial fiber during 3 weeks of SSF (Figure 2.6). The increase in reducing sugars from week 0 to week 1 confirmed that the enzyme system was active during SSF. The decreasing values of sugar in culture bottles suggest sugar consumption by the fungi during SSF.

![Figure 2.6: Reducing sugar released in steamed SSF of *P. chrysosporium* culture and control (n = 4)](image-url)
*P. chrysosporium* has been studied for cellulose degradation (Ubayasekera, 2005). The activity of cellulase enzymes depends on the types of substrate and operating conditions such as pH and temperature (Meyer et al., 2006). They reported pH 5.0 and 50°C as optimal for maximum yield of glucose from steam-pretreated barley straw using a blend of cellulase enzymes. The cellulase enzyme activity of *P. chrysosporium* in this study was performed at a pH 4.73 (which was close to pH 5.0), and the temperature was set at 37°C, the same temperature as in SSF. Anaerobic conditions in buffer solution for SSF bottles released, or at least preserved, higher concentrations of reducing sugar in culture bottles (Figure 2.7). Thus, simultaneous extracellular cellulase/hemicellulase activities and reduced sugar consumption by the fungi under anaerobic conditions was observed. The control SSF bottles were deprived of fungi and hence also lacked a cellulase enzyme system. The increase in reducing
sugar yield was as high as 140% for the 7-day-old culture bottle (24 mg reducing sugar/g initial fiber) with anaerobic incubation compared to the 7-day-old culture bottle (10 mg reducing sugar/ g initial fiber) without anaerobic incubation (Figure 2.7). The 9- and 11-day culture bottles had less enzyme activity and hence poor release of extra reducing sugars. The cellulolytic system is sensitive to end-product (glucose and cellobiose) inhibition (Sarkar and Etters, 2004). Thus, accumulation of glucose and cellobiose suppresses cellulase enzyme activity. Simultaneous saccharification and fermentation is employed to overcome this inhibition by converting these end-products into ethanol as they are produced (Manzanares et al., 2004; Reczey et al., 2004), and thus facilitating continuous cellulase activity.

The hemicellulase activity of P. chrysosporium has been studied by Highley and Dashek (1998). Pentoses are released in addition to hexoses in hydrolysis of hemicellulose. Reducing sugar measurements depend on an available aldose or ketose reducing end; mono-, di-, tri-, and short-chained carbohydrates have one reducing end each. The fermentable portion of the reducing sugar can be determined by yeast fermentation (bioassay) in the hydrolysate collected during sugar harvesting. Saccharomyces cerevisiae ferments most hexoses to ethanol. Genetically modified organisms are available that can ferment both five- and six-carbon sugars and increase the net ethanol production from biomass-derived sugars (NREL, 2004).
2.3.3 Total Sugar Harvested from SSF

Total sugar assays determined the total sugar present in the saccharification products. This test quantified polysaccharides, oligosaccharides, and other carbohydrates that were not detected by the reducing sugar test. Total sugar values were as high as 77 mg/g initial fiber in 3-day-old SSF with *P. chrysosporium* (Figure 2.8). The increase in total and reducing sugars in culture SSF bottles, compared with controls, confirmed the active cellulolytic activities of the fungus. However, the decreasing pattern also suggested the fungal consumption of sugar as the time of incubation was extended (Figure 2.8). The difference in the quantities of sugar values between the total and reducing sugars revealed the availability of soluble sugars that had not been hydrolyzed. Consistently, the sugar data were highly

![Figure 2.8: Released total and reducing sugar in steamed SSF of *P. chrysosporium* culture and control (n = 1)](image-url)
variable for the control samples. It appears that there might be various physicochemical/thermal factors influencing the sugar data.

These sugar values were only from the solution extracted after the steaming process of the SSF bottles. Roughly, normal SSF resulted in 7.7% (and maximum of 10%) conversion of biomass to total sugar and 1.5% to reducing sugar. Enhanced cellulolytic/hemicellulolytic activities during anaerobic incubation of SSF yielded 2.5% conversion of biomass to reducing sugars. Based on weight loss data (Figure 2.9), prolonged SSF incubations converted a greater proportion of fiber to sugar. These experiments showed an active enzyme system within a few days of *P. chrysosporium* SSF. Thus, simultaneous saccharification and fermentation appears to be the method of choice. This process is envisaged for future work in cellulose fermentation using *P. chrysosporium*.

### 2.3.4 Increase in Biomass Weight Loss

In this study, the term *biomass* referred to both fungal biomass and residual corn fiber in SSF culture. This eased in overall calculation of biomass weight loss during SSF. The difference between total dry biomass weight following sugar harvesting and the initial fiber weight (~25 g) was regarded as biomass weight loss.

In control bottles, percent biomass weight loss varied between 13 and 15% (Figure 2.9). The biomass weight loss in SSF of *P. chrysosporium* gradually increased from 12 to 34% in 3 weeks of SSF. Significant biomass weight loss was observed in *P. chrysosporium* SSF by the first week of SSF. Similar results were also obtained when the SSF bottles were analyzed for biomass weight loss (and reducing sugar) after 0, 5, 7, 9, and 11 days of incubation. The control samples (without fungi) varied between 10 and 12% in biomass.
weight loss. This value was close to the weight loss values in earlier experiments. The loss of cell-soluble fraction (Table 2.1) from the corn fiber during steam treatment could be one reason for biomass weight loss in control bottles. Cell-soluble fraction was analyzed, and was found to contain carbohydrates, proteins, organic acids, and other soluble materials.

Carbohydrate fraction was detected by the reducing and total sugar assays. Control samples contained as high as 7.5% total sugar, and this would be about half of the biomass weight loss for the controls. The other portion of the biomass weight loss could be due to organic acids and proteins in the solution harvested from steam treatment. The total Kjeldahl nitrogen (TKN) test results (data not shown here) suggested that the protein content in the liquid sample collected from the control SSF bottles was only about 1.5% of the total biomass.
2.3.5 Inducer Enhanced Lignin Degradation in SSF

The study on the application of inducers like hydrogen peroxide, veratryl alcohol (VA), and manganese sulfate showed improvement in lignin degradation. In two different experimental trials, 10 ml of various concentrations of inducers (0.4 mM, 4 mM and 40 mM of hydrogen peroxide; 3 mM, 30 mM and 300 mM of veratryl alcohol; 3 mM, 30 mM and 300 mM of manganese sulfate) were added separately to 9 SSF culture bottles. In one batch, inducers injected into 7-week-old SSF bottles and tested for Klason lignin produced no change in percentage Klason lignin after 1 week (Figure 2.10).

![Lignin Degradation](image)

Figure 2.10: Effect of inducers (hydrogen peroxide, veratryl alcohol [VA], and manganese sulfate) in lignin degradation for 7-week *P. chrysosporium* SSF culture bottles (n = 1)

When the same experiment was performed on 2-week SSF culture bottles (Klason lignin test done after 1 week of incubation), there was a difference in Klason lignin percentages between the bottles with different inducers (Figure 2.11). Manganese sulfate had a marked effect on Klason lignin. The Klason lignin reduction was >30% with all three
concentrations, whereas 10 ml of 3 mM veratryl alcohol reduced the Klason lignin by 37%. The effect of hydrogen peroxide on lignin degradation was less than that of the other two inducers. Table 2.2 summarizes the effects of various concentrations of inducers on lignin degradation. *P. chrysosporium* produces both manganese peroxidase (MnP) and lignin peroxidase (LiP) enzymes that catalyze lignin degradation (Kirk, 1985; Hatakka, 1994). The addition of inducers like H₂O₂, VA, and MnSO₄ enhanced the activities of lignin-degrading enzymes (Sun et al., 1999; Pometto et al., 2006). These inducers provided enzyme substrates to catalyze the ligninolytic activities. VA is believed to protect the lignin peroxidase (LiP) from excess hydrogen peroxide (H₂O₂) *in situ*. Excess H₂O₂ oxidizes the LiP enzyme system II to LiP compound III. Ward et al. (2004) reported that LiP compound III would not revert back to the native LiP. The exogenous H₂O₂ addition during SSF could have oxidized the LiP II to LiP III. This would reduce the enzyme catalyzed lignin degradation reaction. VA helps to bring LiP III back to its native state, as illustrated in the following equation.

![Diagram](image)

*Adapted from Ward et al. 2004*

The easily diffusible manganese ion (Mn³⁺) formed by oxidation of Mn²⁺ catalyzed the oxidative lignin degradation reaction. Manganese serves as substrate for the manganese peroxidase. The presence of manganese in situ is important for expression of extracellular manganese peroxidase (Gold and Peric, 1991).
Figure 2.11: Effect of inducers (hydrogen peroxide, veratryl alcohol [VA], and manganese sulfate) in lignin degradation for 2-week *P. chrysosporium* SSF culture bottles (n = 1)

<table>
<thead>
<tr>
<th>Inducers</th>
<th>% Klason lignin reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>0.4 mM H₂O₂</td>
<td>-6</td>
</tr>
<tr>
<td>4.0 mM H₂O₂</td>
<td>10</td>
</tr>
<tr>
<td>40 mM H₂O₂</td>
<td>11</td>
</tr>
<tr>
<td>3 mm VA</td>
<td>37</td>
</tr>
<tr>
<td>30 mM VA</td>
<td>11</td>
</tr>
<tr>
<td>300 mM VA</td>
<td>16</td>
</tr>
<tr>
<td>3 mM MnSO₄</td>
<td>34</td>
</tr>
<tr>
<td>30 mM MnSO₄</td>
<td>37</td>
</tr>
<tr>
<td>300 mM MnSO₄</td>
<td>41</td>
</tr>
</tbody>
</table>

Table 2.2: Klason lignin reduction with addition of ligninase inducers at various concentrations (n=1)
2.3.6 Extracellular Polysaccharide (EPS) Deposition by *P. chrysosporium*

Filtration of hydrolysate after the steam treatment of SSF bottles proved to be difficult. The SSF bottles had to be centrifuged to lower the burden on the filtration process. The filter papers clogged quickly, and filtration became progressively slower for SSF cultures. Longer SSF incubation periods retarded the filtration even more. However, the filtration was fast when the hydrolysate was filtered hot, soon after steam treatment. There were some compounds in hydrolysate that became viscous when cooled and clogged the filter paper rapidly. These viscous compounds were suspected to be extracellular polysaccharides (EPS) deposited by *P. chrysosporium* during SSF of corn fiber. Mild acid (0.1N sulfuric acid) steam treatment for 1 h of the filtered and nonfiltered hydrolysate cleaved the polysaccharides into monomeric sugars. The reducing sugar values increased with mild acid treatment (Figure 2.12). Mild acid and heat will hydrolyze the polysaccharides and oligosaccharides present in the harvested sugar solution into monosaccharides. There was no significant difference in reducing sugar between nonfiltered and filtered samples. The EPS could have passed through the filter paper when the harvested solution was filtered hot. It is also possible that the EPS formed on the substrate and did not dissolve during steam treatment.

Mild acid treatment of the whole SSF bottle (biomass plus the harvested solution) using 0.1N H₂SO₄ and steam treatment for 1 h resulted in higher reducing sugar values in 10-day SSF cultures compared with 3-day cultures. EPS is suspected to be present in 10-day SSF cultures (Figure 2.13). Comparison between the reducing sugars in 9-day SSF hydrolysate (Figure 2.12) and 10-day SSF hydrolysate (Figure 2.13) suggested additional
sugar was released after mild acid treatment. The difference in reducing sugar between these two hydrolysates was 2-fold (27 and 54 mg reducing sugar/g initial fiber, respectively).

![Extracellular Polysaccharide](image)

**Figure 2.12:** Additional reducing sugar released after mild acid treatment of sugar solution harvested from *P. chrysosporium* SSF bottles incubated for 0, 3, 5, 7, and 9 days (n=1).

Production of EPS by *P. chrysosporium* in a glucose-rich environment has been reported (Leisola et al., 1982; Bes et al., 1987; Buchala et al., 1987). Acid hydrolysis of the polysaccharide material isolated from the culture medium by precipitation with 50% ethanol yielded glucose, mannose, galactose, fructose, and xylose in molar ratios of 1.0:0.03:0.01:0.01:0.02 (Buchala et al., 1987). Sutherland (1990) reported carbohydrates (e.g., D-glucose, D-galactose, D-mannose, etc.) as the main components of EPS. The possibility of excess glucose in situ during SSF and its conversion to EPS by the white rot fungus is questionable. The breakdown of cellulose to glucose by enzymatic hydrolysis is a slow process. When glucose is not easily available in SSF of lignocellulose, EPS formation
by the fungus is very doubtful. The reducing sugar after acid hydrolysis of EPS, if present, might release comparably higher concentrations of fermentable sugars. Fermentation of the

![Graph: Extracellular Polysaccharide](image)

Figure 2.13: Higher reducing sugar released after mild acid treatment of entire control and culture SSF bottles which included corn fiber residue (n=1)

hydrolysate into ethanol using *S. cerevisiae* would quantify the fermentable sugar present in the acid-hydrolyzed product.

### 2.3.7 Ethanol Fermentation of Hydrolysate from SSF

The reducing sugar harvested after steam treatment of SSF bottles was analyzed for fermentable sugar content. One way of quantifying the fermentability of the reducing sugar is yeast fermentation (bioassay). The mild acid (0.1N H₂SO₄) treated and untreated SSF culture bottles (control and *P. chrysosporium*) were fermented with the yeast *S. cerevisiae* (ATCC 24859). There was no ethanol production except in the 3-day *P. chrysosporium* SSF bottle without acid treatment (Figure 2.14). Under anaerobic conditions, the ethanol
Figure 2.14: Ethanol fermentation from 3-day SSF hydrolysate using S. cerevisiae (n=1)

Figure 2.15: Mild acid treatment of hydrolysate spiked with 2% glucose and additional yeast, Saccharomyces cerevisiae after 12 hr incubation at 37°C (n=1)
production was completed by day 2 at about 4 mg ethanol/g initial fiber with no significant change in pH.

However, the mild acid hydrolysis step can release some toxic chemicals from the fiber and create an inhibitory effect on ethanol fermentation by yeasts. The other possibility was the presence of low concentrations of fermentable sugar in the hydrolysate (with or without acid treatment). An acid hydrolysis step might also have released higher concentrations of non-fermentable sugars. The higher reducing sugar concentration was observed following the acid treatment.

The confirmation test on the possibility of toxic compounds produced by the mild acid treatment and their inhibitory effect on yeast fermentation showed no toxic components (Figure 2.15). The ethanol value was as high as for the positive control sample, which had 2% glucose, yeast (5.6x10^8 cells), and the glucose-free yeast growth media. Thus, no compounds with toxic or inhibitory effects on yeast fermentation were released.

Formation of degradation products such as furfurals from hemicellulose and monomeric sugars was reported to be inhibitory for microbial fermentation (Beery et al., 2004). The furfurals formed by the acid hydrolysis of monosaccharides inhibit enzymes like alcohol dehydrogenases and pyruvate dehydrogenase during yeast fermentation (Modig, 2002). Conversion of sugars to furfurals after mild acid treatment in laboratory trials possibly reduced the concentration of fermentable sugar. Simultaneous saccharification and fermentation of corn fiber after SSF could boost the release of fermentable sugars via cellulolytic and/or hemicellulytic enzyme action and their subsequent fermentation (cofermentation) to ethanol (Manzanares et al., 2004; Reczey et al., 2004; NREL, 2006). This
objective is envisaged as future work in continuation of the research on fungal lignocellulose conversion to ethanol fermentation.

2.4 Conclusion

Corn fiber from a wet milling plant provided a good lignocellulose substrate for the study of solid-state fermentation (SSF) by *P. chrysosporium*. Increase in reducing sugar, total biomass weight loss (up to 35%), and reduction of lignin content (>40%) of the biomass were good indications for the success of the SSF study with white rot fungi. Mild acid hydrolysis of SSF fibers released more reducing sugars in the hydrolysate. This could be due to either hydrolysis of EPS formed by *P. chrysosporium* or the cleavage of polysaccharides present in the hydrolysate. Lower concentrations (4 mg/g of fiber) of ethanol were fermented in the hydrolysate from SSF. Anaerobic incubation of the SSF bottles enhanced the cellulolytic and/or hemicellulolytic enzyme activity to produce additional sugars. Such treatment can be an important intermediate step during cellulose saccharification and fermentation of sugars to ethanol. This study envisaged the concept of simultaneous saccharification and fermentation processes to reduce the time in the enzymatic hydrolysis step, reduce fungal consumption of sugar during saccharification of substrate, and facilitate improved ethanol fermentation from fungal bioconversion of lignocellulose to ethanol.

Acknowledgement

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GENERAL CONCLUSIONS

The solid state fermentation (SSF) of *P. chrysosporium* on corn fiber was successfully carried out in laboratory-scale batch experiments. The cellulolytic and ligninolytic activities of the fungus during the SSF were indirectly evaluated by the analyses of total and reducing sugars, biomass weight loss and Klason lignin degradation. Based on this research, the following conclusions were drawn:

- *P. chrysosporium* was able to convert about 2.5% of corn fiber into reducing sugars with reduction in the biomass weight loss and Klason lignin content by over 35% and 40%, respectively. This apparently suggests an excellent growth and lignocellulose biodegradation.

- Increase in reducing sugar content revealed that cellulolytic activity was eminent by day 3 of SSF. An anaerobic treatment in buffer solution (acetate buffer pH 4.7 and 37°C) helped to enhance the cellulase activity, thereby releasing more sugar. Reducing sugar increased by as much as 140% following the anaerobic treatment.

- The formation of extracellular polysaccharides by the white rot fungus during SSF could not be accurately assessed. The release of additional sugar by mild acid hydrolysis of EPS might have been attributed to the hydrolysis of polysaccharides and oligosaccharides in the hydrolysates.

- Lower ethanol yield (about 4 mg/ g corn fiber) confirmed a higher concentration of non-fermentable sugar in the SSF hydrolysates. Glucose (2% w/v) spiking in the hydrolysates followed by the yeast ethanol fermentation confirmed that toxic compounds were not released from the sugars following mild acid treatment in the SSF bottles.
Further studies are needed to optimize SSF protocols in simultaneous saccharification and co-fermentation using wood rot fungi and yeasts for economical ethanol production from the corn fiber and other lignocellulosic biomass.

Protocols developed for laboratory-scale SSF using wood rot fungi (e.g. *P. chrysosporium*) can be evaluated using other lignocellulosic substrates, such as soy hull, switchgrass, corn stover, forest residues etc. Pretreatment like size reduction, hot water extraction, ultrasound, acid/alkali hydrolysis etc., may enhance their breakdown.
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