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FIELD IMPLEMENTATION OF *PHANEROCHAETE CHRYSOSPORIUM* BIOMASS PRETREATMENT: FUNGAL IDENTIFICATION AND INOCULATION TECHNIQUES

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FIELD IMPLEMENTATION OF *PHANEROCHAETE CHRYSOSPORIUM* BIOMASS PRETREATMENT: FUNGAL IDENTIFICATION AND INOCULATION TECHNIQUES

THESIS

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

By

Bobby D. Carey Jr

Lexington, Kentucky

Director: Dr. Sue Nokes, Professor and Chair, Biosystems & Agricultural Engineering Department

Lexington, Kentucky

2014

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ABSTRACT OF THESIS

FIELD IMPLEMENTATION OF *PHANEROCHAETE CHRYSOSPORIUM* BIOMASS PRETREATMENT: FUNGAL IDENTIFICATION AND INOCULATION TECHNIQUES

Scaling biological pretreatment from the bench scale to the production scale may be more economical if unsterilized feedstock are used, however these allow for microbial competition from contaminates. An accurate and rapid method for identifying the desired biological pretreatment organism is necessary to confirm the presence of the desired organism when contaminates are morphologically similar to the target organism. Traditional methods, such as visual identification, sequencing, and selective plating can be time consuming and are sometimes still inconclusive. Based on methods described in the literature, plasmid DNA containing the marker genes *gus* (***β***-glucuronidase), *LacZ*, and *gfp* (green fluorescence protein) incorporated into the lignin-degrading basidiomycete *Phanerochaete chrysosporium* would result in a rapid genetic test for the desired organism. The presence of these genes can be confirmed either through an X-Gluc (cyclohexylammonia salt), X-Gal histochemical assay or observing the *gfp's* fluorescence by a specially equipped confocal microscope. Each reporter systems will allow for rapid, reliable identification of the target species. This study will report on the success of the transformation methods in creating a transformed fungus to be used in the context of a large-scale fermentation operation.

Additionally, a novel in-harvest lignocellulose feedstock biological pretreatment inoculation trial was performed comparing lignolytic performance between fungal inoculum application techniques. Optimization of carbohydrate availability for enhanced saccharification was determined by analyzing glucose release by treated and non-treated unsterilized switchgrass. This study also focused on identifying parameters to enhance saccharification efficacy at the farm-scale.

KEYWORDS: *Phanerochaete chrysosporium*, lignocellulose, genetic transformation, biological pretreatment.

Bobby D. Carey Jr

December 4, 2014

RAPID GENETIC IDENTIFICATION OF PHANEROCHAETE CHRYSOSPORIUM DURING PRETREATMENT OF LIGNOCELLULOSE

By

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December 4, 2014

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CHAPTER 1: INTRODUCTION

1.1 Lignocellulosic conversion

The potential for recovering energy from lignocellulosic sources has long been recognized. However in recent years a new global understanding and a shift from fossil energy has encouraged widespread research in and development of alternative energies. Due to the abundance of lignocellulosic feedstocks in most regions of the world, interest in lignocellulosic-sourced energy is gaining momentum world-wide. In addition to the many forms in which lignocellulosic energy can be made available, clean burning, nearly closed carbon cycles, and opportunity for rural economic development are just a few additional benefits that are associated with this type of energy. However, a variety of challenges hinder optimal conversion efficiency; the primary challenge being the recalcitrant nature of the lignocellulose matrix.

Lignocellulose is predominantly comprised of cellulose, hemicellulose, and lignin. These components account for nearly 90% of most plant material's dry weight, though the ratios of these components vary from feedstock to feedstock. A much smaller fraction of the lignocellulosic weight comes from pectin, protein, ash and trace extractives like free sugars, chlorophyll, waxes, and nitrogen bound molecules (Kumar et al., 2009). Cellulose is composed of D-glucose monomers bound by β-(1,4)-glycosidic bonds that create the long chain cellulose polysaccharide. These chains are bundled together in a microfibril by vander Waals forces and hydrogen bonds. The D-glucose monomers are currently the most valuable component of the lignocellulose as these monomers can be fermented to solvents like acetone, butanol, and ethanol.

Hemicellulose is constructed mainly of C5 sugar monomer with frequent side chains. This configuration enables hemicellulose to be more easily hydrolyzed than cellulose (Kumar et al., 2009). Hemicellulose is found uniformly and non-uniformly intertwined between and around cellulose microfibrils (Cullen, 1998). Hemicellulose is cross-linked with lignin via covalent bonds and surrounds the cellulose microfibrils as a protective and structural reinforcing complex (Kirk and Farrell, 1987).

Lignin is a larger molecule, between 600-1000kd in size, and composed of three different phenyl propionic alcohol monomers that are cross-linked; creating a rigid matrix

space. These three monomers are referred to as coniferyl alcohol, coumaryl alcohol, and sinapyl alcohol respectively called guaiacy, coumaryl and syringyl lignin (Kirk and Farrell, 1987). Specific ratios of the three lignin types vary from species to species. Lignin is found most prevalently in the middle lamella region of the cell wall (Blanchette, 1991).

1.1.1 Pretreatment

Two distinct processing phases occur before fermentation. Initially the lignin polymer needs to be interrupted and cellulose and hemicellulose bundles ruptured to expose adequate surface area of polysaccharides for subsequent enzymatic hydrolysis; this is known as pretreatment. The second phase involves breaking down long chain polysaccharides to their monomeric constituents by enzymatic hydrolysis. The latter phase is known as saccharification, and the effectiveness of saccharification is heavily influenced by the effectiveness of the pretreatment phase.

The primary goal in the pretreatment phase of lignocellulosic conversion is to remove or rearrange the lignin in order to expose the carbohydrates for subsequent hydrolysis. Several lignocellulosic physical properties have been correlated with the degree of difficulty in removing the lignin including;: degree of lignin polymerization, composition of lignin monomers, cellulose crystallinity, available surface area and pore size, and composition of lignocellulosic constituents (Alvira et al., 2010; Kumar et al., 2009).

A variety of pretreatment techniques have been developed. These include physical methods such as comminution to decrease particle size which in-turn increases the overall exposed surface area available for subsequent pretreatment and hydrolysis. Physiochemical pretreatments often employ sudden pressure changes along with steam or strong acids and bases like ammonia fiber explosion or SO_2 steam explosion. Chemical approaches use dilute to strong acid and base (i.e. $NaOH$ or H_2SO_4) reactions, often times combined with elevated temperatures. Biological pretreatments use microbial enzyme systems to metabolize the lignocellulosic fraction. Each of these methods present unique advantages and disadvantages. The optimal choice of pretreatment method depends on the substrate being used and the desired products to be recovered. Pretreatment of biomass is second in cost to the actual biomass itself, therefore cost

effective methods are a priority when determining pretreatment methods (Alvira et al., 2010; Kumar et al., 2009).

Many chemical and physiochemical approaches may leave inhibitory byproducts in the process stream which can drastically reduce fermentation efficiency downstream. Using large volumes of water to wash the residual feedstock to remove inhibitors creates an additional energy-intense handling and purifying step (Isroi et al., 2011). A good review relating feedstock to appropriate pretreatment technique can be found in Alvira et al. (2010) and Kumar et al. (2009).

1.1.2 Biological pretreatment

Research and development in biological pretreatment methods have been overshadowed by all the other pretreatment methods. Rightfully so, biological pretreatments have been associated with long retention times and less than adequate lignin degradation and so the biological pretreatments were deemed inefficient for the time and money-conscious industrial processes. However, lower energy demands, reduced downstream toxicity, lower moisture requirements, and potentially comparable lignin degradation extents have renewed interest in developing efficient biological pretreatment methods for industrial use (Alvira et al., 2010). The structure of lignin and lignocellulose requires biological methods to be extracellular, nonspecific, and oxidative (Kirk and Farrell, 1987). These properties have been observed in a small group of wood-decaying microorganisms.

Both bacteria and fungi have been reported to degrade lignin. Early studies (1980's) observed some potential for using bacterial lignolytic systems for significant lignin degradation. However, many of those reports were not reproducible. The bacterial lignolytic activity that was quantified was insufficient to warrant further research. With the advent of ^{14}C labeled lignin, claims of bacterial lignin degradation were dismissed and attributed to cell adsorption and mineralization of low-molecular weight components (Kirk and Farrell, 1987). A good review of bacterial-related lignin degradation can be found in Kirk and Farrell (1987).

As interest in bacterial lignin degradation fell off, fungi moved into the forefront. Many early ^{14}C confirmed the lignolytic potential from a variety of fungal families (Kirk and Farrell, 1987). Collectively these fungi are considered litter-degrading or wood-

decay fungi. Largely this group of organisms are obligate aerobes and attain the bulk of their nutrient demand from metabolizing lignocellulosic material which are fragmented by extracellular enzymatic activity (Cullen, 1998).

Lignin-degrading fungi can be categorized in three main groups: soft rot, brown rot, and white rot. Soft rot fungi are typically associated with angiosperm wood decomposition and only minor lignin degradation potential. Brown rot fungi have been shown to degrade lignin to beneficial extents however nearly all brown rotting fungi do so while simultaneously exhaustively metabolizing polysaccharides (Blanchette, 1991). The white rot group has received the bulk of scientific attention over the past half century with regard to their potential for biological lignolytic application.

1.1.3 White Rot Fungus – Phanerochaete chrysosporium

Based on a study published in 1991, 94% of all wood degrading fungi were of the white-rot origin (Blanchette, 1991). White-rot fungi are comprised of a collection of Basidiomycota and Ascomycota, however the group is dominated by Basidiomycota and many references do not even mention Ascomycota (Isroi et al., 2011). Often white-rot fungi are white in color but the group name is derived from the color of the cellulose matrix left behind after selective lignin degradation of woody biomass has occurred. A select few white rot fungi have been noted for their high lignin degradation rates and/or preferential lignin degradation; i.e.: *Cyathus stercoreus, Coriolus versicolor* (Keller et al., 2003), *Ceriporia lacerate, Ceriporiopsis subvermispora, Pleurotus ostreaus* (Alvira et al., 2010), *Phlebia subserialis* and *Phanerochaete chrysosporium* (Isroi et al., 2011).

White-rot fungi remove lignin using either selective or non-selective lignin degradation (Blanchette, 1991). Selective degradation results in high lignin depolymerization with little if any of the polysaccharides consumed. Selective degradation leaves the cellulose and the majority of the hemicellulose intact in the biomass which is a favorable characteristic for lignocellulose to biofuel pretreatment applications. Non-selective systems consume both polysaccharides and lignin non-specifically (Blanchette, 1991). Most brown rot fungi have non-selective lignolytic enzyme systems, however non-selectivity has also been observed in many white-rot fungi. Many times the same strain of fungus is noted for expressing both systems without an obvious metabolically motivating factor (Blanchette, 1991). Blanchette

(1991) published electron microscope images of selective and non-selective lignin degradation by a variety of white-rot fungi.

The white-rot basidiomycete *Phanerochaete chrysosporium* stands apart from other organisms for its exceptional selective lignin degradation potential and rate of depolymerization. This organism was officially described as *P. chrysosporium* in 1974 after various cultivars under different names (*Chrysosporium pruinosum, C. lignorum, Sporotrichum pruinosum, and S. pulverulentum*) were consolidated and deemed conspecific (H. H. Burdsall, 1974). An early taxonomic reference was provided by Burdsall and Eslyn (1974). *P. chrysosporium* has been studied more extensively than any other lignolytic microorganism. *P. chrysosporium* has been used in industry as a lignolytic amendment in pulp and paper processes (Cullen, 1998), biobleaching , bioremediation (Kersten and Cullen, 2007), ruminant feed supplementation (Isroi et al., 2011), and in composting applications (Tuomela et al., 2000).

Several studies have concluded that lignin is not consumed as a carbon source for the fungus, and that lignin is always to some degree degraded simultaneously with an amount of sugar metabolized (Kirk and Farrell, 1987) (Blanchette, 1991). The relationship between the extent of lignin, cellulose, and hemicellulose degradation by *P. chrysosporium* is complex, however familiarization with the life cycle of this organism allows a better model to be drawn.

The life cycle of *P. chrysosporium* occurs in four stages: lag phase, primary growth, secondary metabolism, and death phase (autolysis). During primary growth no lignolytic activity is present. The organism consumes available nutrients by employing polysaccharide enzyme degrading systems until a limiting condition is reached. This limitation is often reached fairly quickly in most lignocellulosic biomass as recalcitrant cell walls do not permit access to valuable interior nutrients. Most lignolytic systems in white-rot organisms are induced by nitrogen depletion. A few unique species and cases of transformed species are not dependent on nitrogen limitation to activate secondary metabolism (Blanchette, 1991). A more thorough description of lag, primary growth, and death phases is provided by (Michel et al., 1992). In addition to available nitrogen other factors affect the rate and extent of lignin degradation during secondary metabolism, i.e. oxygen concentration, moisture levels, temperature, pH and other

limiting nutrients like manganese and copper (Isroi et al., 2011) . Some fungi possess pseudosclerotial plates that regulate moisture levels both within the organism and within the immediate vicinity (Blanchette, 1991). Further studies are necessary to determine if *P. chrysosporium* regulates moisture using this physiological mechanism.

Fungi are unique in the eukaryotic domain for the many of the observed physiological states. *P. chrysosporium* can exist in two spore forms – conidiospores and basidiospores. The basidiospores are the sexual spores which maintain two identical haploid nuclei. Basidiospores are produced and ejected by the often less-observed fruiting body of *P. chrysosporium*. Optimal conditions must be achieved before fruiting body formation begins. Refer to Gold and Cheng (1979) for fruiting body inducer conditions and mechanisms. Conidiospores on the other hand are asexual spores which are often referred to as the conidia. The conidia are most commonly observed as white clusters or masses fixed topically to the lignocellulosic biomass. The conidia give rise to the hyphae which grow outwardly in search of pores, cracks, or tears in the biomass. Lignolytic enzymes which promote substrate degradation are secreted from the hyphae, allowing the hyphae to further extend into the interior of the substrate.

1.1.4 Enzyme systems

Two unique extracellular enzymes, ligninase (lignin peroxidase) and manganese peroxidase, work in conjunction with a variety of H_2O_2-forming molecules, and are responsible for the elevated lignolytic activity in *P. chrysosporium.* Around 40 kDa in size, ligninase is a monomeric homoprotien and is considered a strong oxidant (Isroi et al., 2011). In a series of chemical reactions (see equations 1 and 2 provided by Isroi et al., 2011) ligninase will react with H_2O_2 and oxidize the lignin components. During this reaction ligninase changes form twice before completing the reaction and returning to its ground state. Ligninase oxidizes non-phenolic varieties of lignin (Wan and Li, 2012). Ligninase is thought to use selective lignin degradation however it is not likely that ligninase acts directly on the interior of the cell wall due to its large size. Rather it is proposed that ligninase oxidizes lower molecular weight compounds which then enter the pores in the cell wall and these lower molecular weight compounds oxidize lignin from the inside of the cell (Cullen, 1998).

Equation 1: Lignin peroxidase oxidation

$$LiP[Fe(III) + H_2O_2 \rightarrow LiP - I\ [Fe(IV) = O^+] + H_2\ )$$
$$LiP - I + AH \rightarrow LiP - II[Fe(IV)] + A^+$$
$$LiP - II + AH \rightarrow LiP + A^+$$

where LiP is lignin peroxidase; LiP-I is a two electron oxidase intermediate; LiP-II is the second lignin peroxidase intermediate; AH is a donor substrate; and A^+ is a radical cation

Manganese peroxidase (MnP) has been shown to have physical similarities to ligninase (Kersten and Cullen, 2007). MnP activity is dependent on the presence of the nutrient manganese and the activity is influenced by the presence of oxidant H_2O_2. MnP is often referred to as manganese-dependent peroxidase and is known for oxidizing the phenolic portions of lignin. MnP is larger than ligninase but it too has been shown to act in a selective lignin degrading manner suggesting the potential of MnP to oxidize low molecular weight compounds (Cullen, 1998). The oxidation reaction of MnP is shown below (Isroi et al., 2011).

Equation 2: Manganese peroxidase oxidation

$$MnP + H_2O_2 \rightarrow MnP - I + H_2O$$
$$MnP - I + Mn^{2+} \rightarrow MnP - II + Mn^{3+}$$
$$MnP - II + Mn^{2+} \rightarrow MnP + Mn^{3+} + H_2O$$
$$Mn^{3+} + RH \rightarrow Mn^{2+} + R + H^+$$

where MnP is manganese peroxidase; MnP-I and MnP-II are the first and second intermediates respectively; Mn is manganese; and R is the organics substrate

The presence of H_2O_2 is necessary for a variety of oxidase activity. Glyoxal oxidase can utilize many different substrates present within the lignocellulosic media such as a variety of aldehydes produced as metabolites of *P. chrysosporium* (Cullen, 1998) .

Glycolaldehyde, produced by oxidation of the most prominent substructure of lignin, when oxidized by glyoxal oxidase yields H_2O_2. Genetic analysis of the active site glyoxal oxidase has shown it to be a copper radical oxidase (Kersten and Cullen, 2007). Aryl oxidase has also been associated with the *P. chrysosporium* enzyme complex (Isroi et al., 2011). Both extracellular and intracellular enzymes have been confirmed to provide H_2O_2 to other enzyme systems including enzymes associated with carbohydrate degradation (Cullen, 1998).

It is suggested these enzyme systems work in a synergistic manner to effect lignin degradation. Each of these enzymes uses substrates provided by proximal enzymatic reactions and subsequently offers substrates or oxidants for subsequent reactions. The complex and yet fully described nature of these enzymatic pathways has created hypothetical enzymes which have yet been identified in the genome (Kersten and Cullen, 2007). It has also been observed that mineralization of lignin occurs after the initial lignocellulosic disruption (Wan and Li, 2012).

Two enzymes commonly associated with white rot fungi have not been observed in *P. chrysosporium*. Laccase, which is a powerful copper containing oxidase that oxidizes phenolic lignin components (Kirk and Farrell, 1987), and versatile peroxidase though to be a hybrid of ligninase and MnP because of its ability to oxidize both phenolic and non-phenolic portions of lignin (Wan and Li, 2012) have not been found in *P. chrysosporium*.

P. chrysosporium also exhibits a wide range of polysaccharide-degrading enzymes capable of converting cellulose to glucose and hemicellulose to xylose (Cullen, 1998). A more in-depth description of the specific enzyme systems and reactions can be found in Isroi et al. (2011), Kirk and Farrell (1987) and Kirk and Cullen (1998).

1.2 Genetics & genetic marker systems

Successive advancements in genetics have opened our eyes to the potential of understanding while simultaneously providing new curiosities. Early genetic manipulation of *P. chrysosporium* used ultraviolet and X-ray mutation. Different mutants were described by phenotypic expression and those unique characteristics became a marker defining that strain. Often these markers came in the form of auxotrophs (defined by IUPAC as the inability of an organism to synthesize a particular organic compound required for its growth). of specific cofactors or amino acids

(Kirk and Farrell, 1987). Genetic work aimed at up- regulating ligninase production and down- regulating less desirable metabolic processes has been attempted in an effort to increase the industrial viability of using *P. chrysosporium* as a pretreatment. Much of the early understanding of both primary and secondary metabolism came as a result of genetic manipulation studies. Advancements in molecular biology reached a pivotal point for this organism when in 2002 the 30 million base pair haploid genome was sequenced (Martinez et al., 2004). The sequencing of this organism was improved in 2006 and 32.2 M bp were defined (Kersten and Cullen, 2007). This more recent work confirmed much of the physiological and metabolic understanding to date, but also revealed many enzyme systems that had been overlooked or undescribed.

Auxotroph marker systems and UV and X-ray shotgun mutation approaches are still frequently used, however more deliberate and specific transformation system have become available. Other systems like polyethylene glycol (PEG), heat shock, electroporation and *Agrobacterium* mediated transformation systems are used to insert a specific gene or set of genes into the target organism. These genes enter the target organism in whole plasmid or partial plasmid form whereupon they will hopefully be expressed by that organism. The particulars of each transformation methods are described later. In addition to the advancement of transformation systems, markers have also improved. Current marker systems allow real time identification. Two marker genes *gfp* and *gus* have demonstrated the appropriate type of expression to be ideal candidate for use in *P. chrysosporium.*

1.2.1 GFP

The green fluorescence protein (GFP) is a protein isolated from the bioluminescent jellyfish *Aquorea victoria*. This protein absorbs longwave UV blue light in the range of 350-480nm and emits green light between 500-550nm (Chalfie et al., 1994). This marker system is unique in that it can be observed in living organisms with no negative effect on the organism. Upon excitation this fluorescence can be observed for up to ten minutes (Chalfie 1994). Detection is performed using a confocal microscope equipped with the appropriate light emitting setting device. In most fungal systems the GFP expression has been limited to the hyphae with relatively little expression in the conidia. Expression is seen intracellularly and the fluorescence is likely emitted from the cytoplasm (Ma et al.,

2001). In addition to GFP an entire spectrum of other fluorescence proteins have been isolated and implemented in white- rot genetic studies such as the red fluorescence protein (RFP) (Schubert et al., 2013). Similarly the *gfp* gene has been superseded by an enhanced green fluorescence (*egfp*) gene whose sequence is a only a few amino acids different than the *gfp* gene yet expresses elevated levels of fluorescence with a slight shift in peak excitation (Ma et al., 2001). However, even with the use of the *egfp* gene, some studies have indicated low to null fluorescence when the gene has been successfully integrated. Two studies have suggested that a unique 5' intron adjoined to the *gfp* or *egfp* gene aids the expression (Ma et al., 2001; Yamagishi et al., 2013). The intron has been described by Ma (2001) as follows: gtcagtacac cacacagcc gaccgcgacg accgcgtgct gacttcgctt tccag. Similarly constructed introns appear to be associated with the majority of genes in *P. chrysosporium* and other fungi apparently confirming its necessity for expression efficiency. This intron was isolated from a region associated with the *P. chrysosporium* native promoters which were used to drive the reporter system in the associated studies. It appears that when a non-native promoter is used (such as CaMV35s) that these introns are not necessary or obsolete as suggested by other successful transformations lacking introns (Sharma et al., 2006; Sharma and Kuhad, 2010).

1.2.2 GUS

β-glucuronidase (GUS) is an enzyeme found in *E. coli* at the *uidA* loci which was first used as a reporter for genetic expression in plants in 1987 by Jefferson and associates. Like the *gfp* gene, the *gus* gene can be fused to promoters that are specific to a particular physiological activity. When the promoters are activated the *gus* gene will encode for the enzyme and a correlation can be made between enzymatic activity and the specific physiological mechanism to which it is bound. This was the first marker system recognized for its ease of use, extreme sensitivity, and budget friendly assay (Jefferson et al., 1987). β-glucuronidase is an enzyme that hydrolyses glucruonidides. Most higher plants and fungi do not produce this enzyme endogenously. Because of this null background, slight enzymatic activity of transformed organisms can be observed via a histochemical assay. The histochemical assay is performed by incorporating a β-glucuronidide substrate with a sample of transformed biomass and incubating for a period

of time. With sufficient incubation time the sample will appear blue as a result of β-glucuronidide cleavage which varies in tint as a function of the enzymatic activity. The activity can be quantified with a fluorometer. A common glucuronidide substrate used in histochemical assays is the 5-bromo-4-chloro-3-indoyl f-D-glucuronide (X-gluc). This assay substrate is relatively inexpensive and simple to use allowing quick and accurate determination of the presence of *gus*.

1.2.3 LacZ

Very similar in function to *gus*, the *LacZ* gene, also from *E. coli*, is a gene that codes for the enzyme β-galactosidase (Wang and Dasilva, 1993). This enzyme cleaves β-glycosidic bonds in D-lactose. When using the reporter substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), the enzyme activity produces a compound that oxidizes to a blue color. Like the X-gluc assay, performing this assay is destructive to the transformed organism. The X-gal assay substrate is nearly half the cost of the X-gluc substrate.

1.2.4 Antibiotic resistance

Antibiotic resistance genes were developed to make selecting for transformed organisms simple and accurate yet avoiding mutation-associated selection (auxotrophic markers). A necessary quality for appropriate antibiotic resistance incorporation is target organism (non-transformed state) sensitivity to the associated antibiotic. With regards to filamentous fungi, and *P. chrysosporium* in particular, Hygromycin B has proven to be a well-suited selectable marker (Magana-Ortiz et al., 2013; Sharma et al., 2006; Sharma and Kuhad, 2010). This antibiotic inhibits growth of *P. chrysosporium* at concentrations as low as 20 μM (see section 2.2.1). The aminoglycosidic antibiotic Hygromycin B interferes with protein synthesis ultimately inhibiting fungal growth. The Hygromcin B antibiotic resistance gene *hptII* was originally isolated from *Streptomyces hygroscopicus* and *Escherichia coli*. *hptII* codes for a Hygromyin B phosphotransferace which phosphorylates the antibiotic (Punt and Vandenhondel, 1992). Hygromycin resistance successfully transformed into *P. chrysosporium* has been shown to remain stable after successive generations without selection pressure (Sharma and Kuhad, 2010).

1.2.5 Promoter selection

The driving force behind gene expression exists within the promoter region of DNA for each gene or set of genes. Transcription of a gene will take place only when DNA polymerase identifies and securely attaches to these essential regions of a gene known as the promoter. Promoter activation is most frequently dependent on environmental, metabolic, or stress-related conditions. Some promoters are activated for transcription nearly all the time, while others may only call for transcription once during the life of an organism. Identifying the most suitable promoter to drive gene expression is an essential step in transformation. For this study, promoters are desired that will drive transcription at all times during the life of the organism.

The Cauliflower Mosaic Virus (CaMV) 35S promoter is responsible for the majority of gene expression in commodity GMO crops. The CaMV 35S promoter is noted for its continual activation of genes associated with the sequence. This promoter is ideal for a marking system when the target organisms will need to be identified at all stages of life. As the name suggests this promoter was isolated from a virus associated with cabbages and cauliflower and therefore is not a native gene construct in fungi.

The glyceraldehyde-3-phosphate dehydrogenase (*gdp*) gene has also been implemented as a viable promoter for trans gene expression (Sharma and Kuhad, 2010). Additionally the *gdp* gene was isolated from wild type *P. chrysosporium* cultures and therefore may be a more reliable promoter for marker gene expression in *P. chrysosporium* (Punt and Vandenhondel, 1992).

The ToxA promoter was isolated from the fungi *Pyrenophora triticirepe* which induces necrosis -causing protein in wheat (Ciuffetti et al., 1997). The ToxA promoter has been used to drive the *sgfp* reporter in the pCT74 plasmid successfully in Ascomycota (Andrie et al., 2005). Such work has allowed for a range of fluorescent colors to be expressed using the ToxA promoter.

Plasmid construction

1.2.6 Agrobacterium mediated transformation

Agrobacterium mediated transformation (AMT) in plant species has been employed for more than half a century. In 2006, Sharma et al. reported that 80% of all transgenic plants were transformed using AMT (Sharma et al., 2006). This technique uses a living

transformation mediator, namely a gram-negative soil bacteria known as *Agrobacterium tumefaciens*. In nature this organism is responsible for crown gall disease that frequently affects dicotyledonous plants (de Groot et al., 1998). Tumorigenesis occurs when an *Agrobacterium* cell recognizes a wound site on a plant. This recognition occurs when membrane-bound virulence receptors are excited by wound-related phenols. This virulence response induces a set of virulence genes that are incorporated in the tumor-inducing DNA (Ti-plasmid) (Hooykaas and Beijersbergen, 1994). This phenomenon is deemed induction. Upon induction, a segment of the Ti-plasmid known as the T-DNA is transferred to the host organism and there upon integrated at a random location in the host genome (de Groot et al., 1998). The virulence pathway is quite complex and a more detailed explanation is described Hooykaas et al. (1994).

AMT was the standard technique for plant transformation through most of the twentieth century; however fungal transformations were carried out by other means. Electroporation or heat shock to protoplast spores, often in conjunction with PEG and calcium chloride treatments, were proven techniques on a variety of fungal strains (Randall et al., 1991). However an associated low transformation frequency accompanied this method. Additionally a loss of expression was commonly reported after only a few generations. In an attempt to improve transformation frequencies of filamentous fungi, de Groot and associates (1998) used a modified AMT to successfully transform seven well-studied fungal strains. de Groot reported remarkably high transformation frequencies; and due to genomic incorporation, expression was not lost after many generation even in the absence of selection.

Transformation of *P. chrysosporium* was performed using non-AMT techniques both before and after de Groot's work (Ma et al., 2001; Randall et al., 1989; Randall et al., 1991). It was not until 2006 that Sharma et al. transformed a strain of *P. chrysosporium* using AMT techniques (Sharma et al., 2006). They refined their modified AMT protocol in 2010 demonstrating another successful AMT of *P. chrysosporium* (Sharma and Kuhad, 2010).

The phenols associated with wound sites in plants account for the virulence activity. In fungi these phenols are not associated with a wound site (wound sites found in plants are not transferable to fungi). To counter the absence of inducing phenols, a modification

is made in the AMT protocol. Acetosyringone (AS) (3', 5'-dimethoxy-4-hydroxyacetophenone) has been commonly used as a place holder (Sigma-Aldrich product D134406). Transformation methods are assisted by an inducing media containing 100-400μM AS (Bundock et al., 1995; Sharma et al., 2006). Elevated concentrations of AS can become inhibitory to *Agrobacterium's* tumorigenesis activity. Evidence from Sharma et. al (2006) suggests that AMT can occur successfully without addition of AS. This result is only valid for co-culture in the presence of lignin. Lignin degradation by *P. chrysosporium* produces phenol by-products including AS (Sharma and Kuhad, 2010).

1.2.7 Alternative transformation techniques

Prior to AMT of filamentous fungi, protoplast formation followed by cell wall lysing and plasmid adsorption had proved to be satisfactory for fungal genomic research. Decades of work at the Oregon Graduate Center on *P. chrysosporium* has optimized transformation protocols using protoplast techniques (Alic et al., 1989). The first protoplast transformation technique to be widely used was performed using $CaCl_2$ to encourage plasmid uptake by the protoplast cell (Mandel and Higa, 1970). This method has been greatly improved in the years after its initial development with exclusion and inclusion of different chemicals. In particular Polyethylene glycol (PEG) incorporation in the late stages of the transformation significantly increased transformants per μl of plasmid DNA (Klebe et al., 1983). PEG mediated transformation is suitable for a wide range of species including *P. chrysosporium*.

Electroporation-mediated transformation also proved successful for filamentous fungi in 1990 (Chakraborty and Kapoor, 1990). Later *P. chrysosporium* was transformed using this technique (Dombrowski et al., 2011). The later study improved upon optimizing electroporation parameters to increase transformation frequency.

1.2.8 Spore formation

Whether to enhance transformation frequencies or for obtaining the appropriate cell type for large scale application the basidiospore formation phenomenon must be understood. Many of the transformation protocols including AMT and PEG are capable of successful transformant production using all cell types associated with *P. chrysosporium*. However, basidiospores appear to be the most susceptible and robust for

each of these methods. Culturing *P. chrysosporium* at normal growth incubation temperatures, light levels, and media composition will rarely give rise to the basidia (fruiting body) responsible for genetically pure basidiospore ejection. Precise media recipes**,** optimum temperatures, moisture, and light are mandatory to isolate basidiospores from conidia and hyphae (Dhawale and Kessler, 1993; Gold and Cheng, 1979). The carbon source must be limited and in the form of a sugar alcohol (i.e. glycerol) or cellulose for best results. Additionally nitrogen must be limited as it is correlated with the inorganic salt concentrations which have a direct effect on basidiospore production. Light and a small list of trace molecules were also found to be directly related to basidiospore production (Gold and Cheng, 1979). Besides the use of basidiospores for genetic study, basidiospores may prove to be more tolerant to environmental extremes and time in storage. These characteristics will be beneficial for inoculating unsterilized bales in the field that may be stored for an undetermined amount of time before pretreatment begins.

1.3 *P. chrysosporium* Inoculation for Pretreatment

1.3.1 In-Harvest P. chrysosporium Inoculation

Few studies are available on experimental attempts to inoculate lignocellulosic feedstocks in-field to pretreat the substrate for subsequent fuel conversion. Commercial applicator designs are available to apply preservative to hay. An experiment that used this commercial applicator to assess ammonia pretreatment applied during the baling process (Horn et al., 1983b) found a significant increase in in-vitro dry matter ruminant digestibly at a specific rate of ammonia application, however issues arose with retention of $NH_3 - N$ in the biomass.

A report by Keller et al. (2003) describes methods of blending pelleted fungal mycelia into a slurry. Disruption of the cell matrix in this fashion was reported to have no significant effect on growth compared to whole pellet cultures (Keller et al., 2003). These findings are significant because a slurry would be more easily used in the commercial applicators than whole pellet cultures.

1.3.2 Moisture content & water activity

The majority of microorganisms thrive within a particular range of moisture levels and perform primary and secondary metabolic functions at a peak rate relative to an optimum moisture content. The measurement referred to as the water activity level describes the physical relationship of water in vapor phase and the surrounding heterogeneous substrate (Gervais and Molin, 2003). Moisture content and water activity are related; however there is not a direct one to one correspondence. The effect of substrate moisture content on *P. chrysosporium* performance has been well studied however with mixed results. In addition, the literature does not report a specific value for optimal water activity for *P. chrysosporium* growth or secondary metabolism; however Gervais et al. (2003) report values for similar filamentous fungi which range between 0.96-0.99 for optimal growth, sporulation, and product recovery.

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CHAPTER 2: FUNGAL TRANSFORMATION

2.1 Summary

The objective of this study was to genetically transform *P. chrysosporium* with a selectable marker which could be used to quantify and hence optimize fungal growth for a biological pretreatment process. Agrobacterium-Mediated Transformation (AMT) and electroporation transformation techniques were performed in attempt to incorporate Hygromycin B resistance, *gus, LacZ*, or *gfp* genes into *P. chrysosporium*. This experiment demonstrated the first attempt at using the marker gene *LacZ* as a reporter for *P. chrysosporium.* Though the experiments did not produce a viable positive transformant culture, valuable knowledge was gained towards improving conditions for future attempts. The following study is presented as a tool to expedite future attempts to genetically transform *P. chrysosporium*.

2.2 Introduction

Lab-scale experiments focused on optimizing lignin degradation rates and extent have been performed on a variety of lignocellulosic feedstocks using the lignin-degrading fungi *P. chrysosporium*. Many studies suggest scaling-up these pretreatment techniques and state that biological pretreatment could reduce processing costs, energy demands, and wastewater streams.

The practical aspects of scaling up from the bench top to the farm-scale present several challenges. One such challenge is the impracticality of sterilizing the volume of material produced on-farm. Without sterilization *P. chrysosporium* is in direct competition with many native organisms present on the feedstock. *P. chrysosporium* and several other non-lignolytic fungi appear phenotypically similar. Though *P. chrysosporium* is robust in nature, the inoculated organism can be quickly outpaced by contaminant organisms such as *Aspergillus sp., Fusarium sp*., etc.

Sterilizing the large amount of lignocellulosic biomass to be processed on farm is highly energy intensive. An alternative may be to optimize growth conditions for *P. chrysosporium* for peak performance in the presence of other organisms, so that *P. chrysosporium* would out-compete the other organisms. These studies could be greatly

aided by a quick and reliable identification and quantification method for the target organism, *P. chrysosporium*.

A few studies are available in the literature that has successfully transformed *P. chrysosporium* with a genetic marker. Sharma et al. (2006 and 2010) used an *Agrobacterium* mediated transformation to incorporate a Hygromycin B resistance gene (*hpt*) and a green fluorescence protein coding gene (*gfp*) into *P. chrysosporium* using the pCAMBIA 1304 plasmid. This was the first AMT of *P. chrysosporium* and the only one to date of which we are aware. Schubert et al. (2013) successfully transformed another white-rot fungal species, *Physisporinus vitreus* via AMT. Polyethylene glycol, electroporation, and shockwave transformation techniques have also been successful in incorporating marker genes such as auxotrphic phenotypes, *gus,* and a spectrum of fluorescence proteins to *P. chrysosporium* (Alic et al., 1989; Ma et al., 2001; Magana-Ortiz et al., 2013; Randall et al., 1989; Randall et al., 1991).

2.1 Materials and Methods

2.1.1 Transformation preparation

2.1.1.1 Organism

Phanerochaete chrysosporium strain MYA-4764 was used as a target species during preliminary attempts at transformation. This strain was replaced with the *P. chrysosporium* strain ATCC 24725 because strain ATCC 24725 was found to have elevated levels of lignolytic activity and more aggressive growth than strain MYA-4764. For plasmid subcloning, the *Escherichia coli* strain TOP10 (provided by Dr. DeBolt's lab) will be used. *Agrobacterium* mediated transformation (AMT) implemented the gram negative soil bacteria *Agrobacterium tumefaciens* strain GV3101 (provided by Dr. DeBolt's lab).

2.1.1.2 Transformation vectors

For AMT the pCAMBIA 1304 plasmid (provide by Dr. Debolts lab) was used which contains an *hptII* (Hygromycin B antibiotic resistance) fungal selection and a *kan* (Kanamycin antibiotic resistance) bacterial selection (Figure 1). The reporter genes on the Ti region are a fused *gfp:gusA* gene. This region is driven by a CaMV35s promoter.

For electroporation- mediated transformation the plasmid pCT74 (Figure 2) and pBSII (Figure 3) was used for transforming *P. chrysosporium*

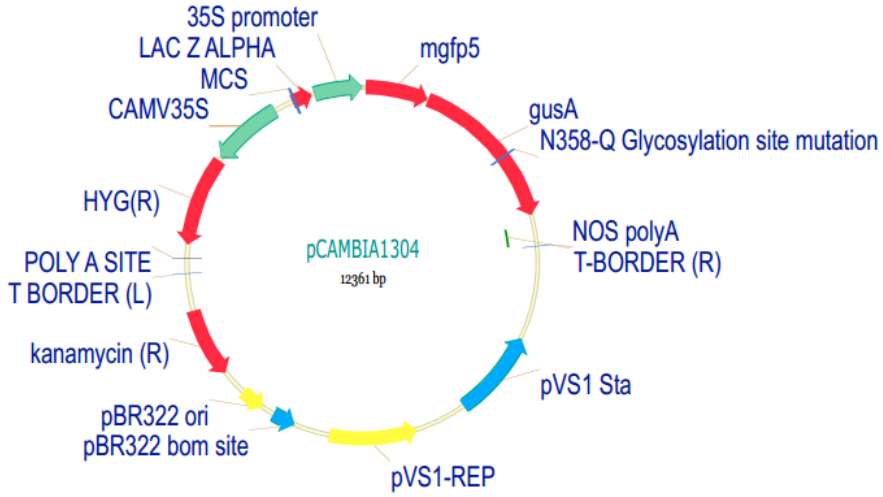

Figure 1: pCambia 1304 with *gfp* and *gus* marker (figure from Cambia).

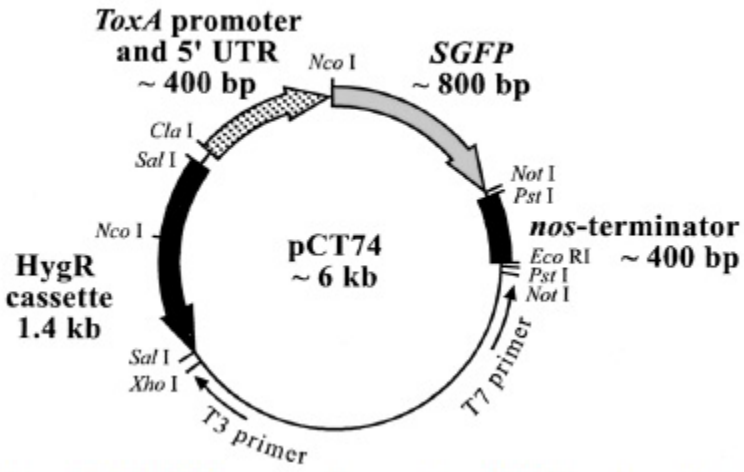

Figure 2: pCT74 with *gfp* marker (Lorang et al., 2001)

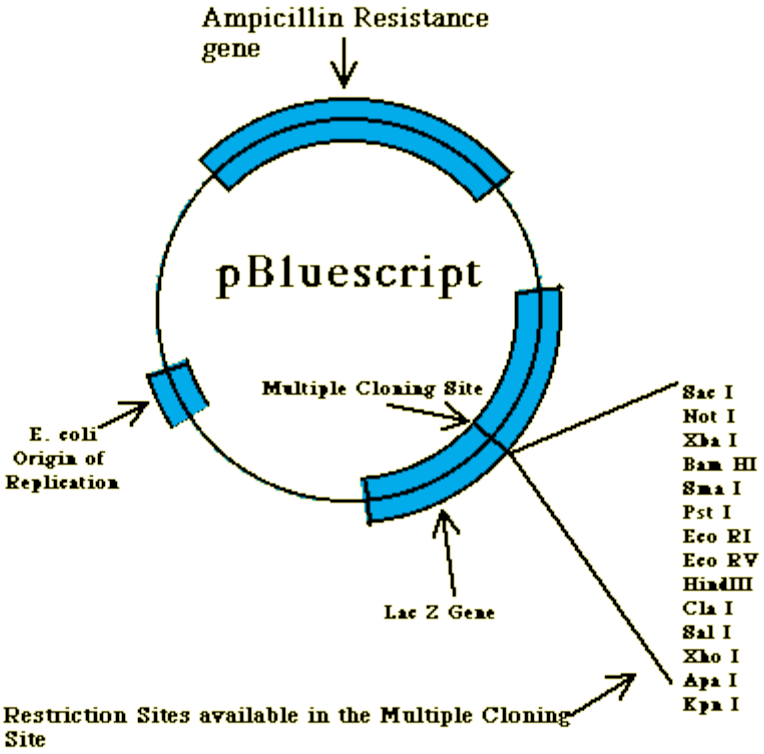

Figure 3: pBluescript with *LacZ* marker (figure from Davidson College).

2.1.1.3 Non-Transformed organism response to Hygromycin B

Determining the appropriate selection agent for the organism being transformed is necessary to avoid false positive transformations. Preliminary experiments were conducted to determine the level of effectiveness of Hygromycin B on the target organism and competing organisms. The target organism was brought out of storage at -40℃ in 80% glycerol and grown on PDA plates for one week. Contaminant organisms were isolated from a variety of sources in the lab and cultured on PDA plates for one week. Isolates from each plate were individually placed in 10ml of sterile DI water and vortexed to suspend cells. Three treatments were prepared for each organism containing varying concentrations of Hygromycin B (0µg/ml, 100µg/ml, and 200µg/ml) in 250 ml flasks containing 100ml of PDB (potato dextrose broth) media. One hundred microliters (µl) of each cell suspension was added individually into each treatment flask. Flasks were agitated at 135rpm at 35℃ for two weeks. Visual observation of growth was made

every 24 hours. The experiment was subsequently repeated to test Hygromycin B concentrations between 0 and 100µg/ml.

2.1.1.4 E.coli transformation

Previously prepared chemically competent cells of the *E. coli* strain TOP10 were allowed to thaw on ice. A 0.5μL volume of the plasmid pCAMBIA 1304 (gifted from Dr. DeBolt's lab), pCT74, and pBSII (gifted from Dr. Farman's lab) were individually combined with approximately 50μl chemically competent *E. coli* and gently tapped to induce mixing. The mixtures were allowed to sit for 15 minutes in an ice bath. The vial was submitted to heat shock at 42°C for 30 sec in water bath. Two hundred μl of Loria broth (LB) media was added to the solution (4x competent cell volume) and incubated for 1 hour at 37°C in a stationary incubator. Contents of the vials were dispersed on LB agar plates amended with the appropriate selection (pCAMBIA 1304: 200µg/ml Kanamycin; pCT74 and pBSII: 100µg/ml Ampicillin).

The plates were then incubated overnight at 37°C. One colony from the plate was isolated and cultured in liquid LB-Kanamycin (200$\mu g/ml$) and allowed to again grow overnight. Cells were then spun down and washed in preparation for plasmid extraction using a mini-prep technique (Axygen Biosciences Mini-Prep Plasmid Extraction Kit; #AP-MN-P-4; Tewksbury MA). The extraction was carried out following the provided AxyPrep Plasmid Mini-Prep Spin Protocol.

2.1.1.5 Agrobacterium plasmid uptake

Freezer stock *A. tumefaciens* was grown up in LB media liquid culture overnight. A 0.5 mL volume of *A. tumefaciens* was pipetted into a uniquely designed pre-chilled electroporation cuvette. One and a half μl volume of the plasmid pCAMBIA 1304 was added to the cuvette and mixed gently by drawing in and releasing the mixture using a pipette. The cuvette was tapped to remove air bubbles and moisture was removed from the sides of the cuvette to avoid arcing during electroporation. Using an Eppendorf brand Electroporator 2510 (Hauppauge, NY; S/N: EC1530) set on 1900 V and a 5ms pulse length the plasmid was incorporated into the *A. tumefaciens* cell. Immediately upon completion of electroporation, 1 ml of LB media was added to contents of the cuvette and incubated for three hours at 28 °C and 125rpm and then plated onto LB triple selection

media containing Rifampicin and Gentimycin at 50$\mu g/ml$ and Kanamycin at 100μg/ml to select for *A. tumefaciens* transformants. This culture was incubated for 2-3 days at 28°C until isolated colonies formed. Once colonies were large enough to sample, LB triple selection media liquid cultures were prepared by inoculating from a single colony. Cultures were allowed to grow for 2-3 days at 28°C to achieve a large volume of cells.

2.1.2 AMT: Following Sharma et al. (2010) protocol

Procedures for transforming organisms are far from an exact science. Protocols exist as guidelines or suggestions based on previous success and often need modification to incorporate favorable conditions for the particular organism being transformed. In the following sections successive attempts at AMT of *P. chrysosporium* are illustrated. Significant changes from one attempt to the next are described. Procedures that remained consistent from one attempt to the next are not reiterated.

With relatively few AMT protocols available for *P. chrysosporium* the Sharma et al. (2010) procedures was chosen due to their simplicity. The original *P. chrysosporium* strain MYA-4764 was co-cultured with transformed *Agrobacterium* strain GV 3101 containing the pCAMBIA 1304 vector.

The protocol provided by Sharma was followed exactly with the exception that lignin was not amended to the transformation agar in the co-culturing plates (otherwise known as double plates, Figure 4). The protocol suggested an optimum range of temperatures between 20-29℃ with higher transformation success at the lower end of that range. Due to previous success with AMT on other organisms in our lab, we set the co-culturing temperature at 28℃ in an incubator in the absence of light. After 48 hours of co-cultivation, transformation media disks were harvested using a sterile cork-borer. Mycelial disks were washed in augmentin (100μg/ml) then rinsed with DI water and plated on Hygromycin B-amended plates at concentrations of 50 or 100μg/ml.

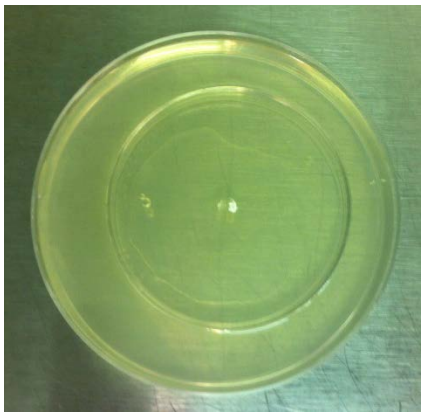

Figure 4: Double plate inoculated with *P. chrysosporium* in the center

2.1.3 AMT: Modified with light, temperature, and bacterial selection

The next transformation was performed following the procedures explained in 2.1.2 with three modifications. Sharma et al (2006) suggests light plays an important role during the insertion and expression of vector genes. The co-culture took place in an incubator with a glass door and interior light (3500K) that was illuminated during the entire 48 hour co-culture period. The second modification made in this transformation was the elimination of the augmentin and DI water rinsing step. Rather, disks were taken from the co-culture plate and plated directly on Hygromycin (50 or 100 μg/ml) amended PDA plates. The third modification was the reduction in temperature from 28℃ to 22℃ for the duration of the co-cultivation.

2.1.4 AMT: Fungal strain replacement

This transformation was performed as described in Section 2.1.3 however the original MYA-4764 target species strain was replaced by the *P. chrysosporium* strain ATCC 24725. This strain is noted for being more robust and effective at preferential lignin degradation.

2.1.5 AMT: Induced virulence response with lignin in co-culture media

This transformation attempt was performed following the procedures outlined by the Sharma et al. (2010) AMT protocol. AS was not available at the time of this attempt therefore lignin (details below) was incorporated into the co-culture media to induce a virulence response in the *Agrobacterium* as described by Sharma et al. (2010). This experiment was the most extensive and tested the widest range of conditions in an

attempt to identify the most conducive conditions for successful transformation. Variables tested included type of lignin, percent of lignin in the co-culture media, and light response. Two types of lignin were gifted by Ravinder Garlapalli which was originally purchased from BOC Sciences: Alkaline lignin (AL) (CAS#8068-08-1) and Organosolv lignin (OL) (CAS#8068-03-9). Sharma suggested 0.1% lignin amendment to the center dish of the co-culture plate. This experiment tested 0.1% and 1.0% lignin in the center dish and varied the outer dish between 0 or 0.1% lignin. Each variation was also replicated for both light and dark conditions for the co-culture period. Table 1 details the treatments tested. Note all treatments were performed in triplicate.

Table 1: AMT co-culture conditions

Replications	Lignin Type	% Lignin Center	% Lignin Outer	Light
3	AL	0.1	0	Yes
3	AL	0.1	0.1	Yes
3	AL	1	0	Yes
3	AL	1	0.1	Yes
3	AL	0.1	0	No
3	AL	0.1	0.1	No
3	AL	1	0	No
3	AL	1	0.1	No
3	OL	0.1	0	Yes
3	OL	0.1	0.1	Yes
3	OL	1	0	Yes
3	OL	1	0.1	Yes
3	OL	0.1	0	No
3	OL	0.1	0.1	No
3	OL	1	0	No
3	OL	1	0.1	No

AL - Alkaline Lignin; OL - Organosolv Lignin

Co-culture plates subjected to dark conditions were wrapped in aluminum foil and placed inside the incubator set at 22℃. Those subjected to light were placed inside the same chamber, face up, and exposed to light for the entire 48 hour period. One plate from each treatment was removed from the incubator after 24 hours and cultures were immediately plated on selection plates. Both 24 and 48 hour potential transformants were grown on PDA plates amended with Hygromycin B (200µg/ml) for selection; and no cultures were washed in any reagent before plating to selection plates. Unlike previous attempts all potential transformants cultured to selection plates were inoculated by loop rather than cork-borer method. This adjustment in procedure was intended to save time and reduce potential contamination during handling with the cork-borer.

2.1.6 AMT: Transformation Enhancers

The two transformation enhancers AS and 5-Azacytidine were the focus of this experiment. AS, an *Agrobacterium* virulence inducer, is considered necessary for non-plant AMT (Sharma et al., 2006) (de Groot et al., 1998). In this attempt AS was used to pre-charge the *Agrobacterium* before co-culturing with *P. chrysosporium* and was provided in the co-culture plates to be available during the length of the incubation period. The optimal concentration of AS is not precisely known. De Groot, Sharma, and a list of other transformation protocols suggest a range between 150-200µM. Higher concentrations are known to be inhibitory. 5-Azacytidine is a demethylating agent used to reverse transgene silencing or to prevent transgene silencing from occurring (Palmgren et al., 1993). Carlo Alberto Petti (post-doc in Horticulture at UK) suggested using a concentration of 15µM based upon previous success during master's work.

Two known transformation enhancers AS (Sigma-Aldrich) and 5-Azacytidine (Sigma-Aldrich) served as the primary variable for this transformation attempt. As in previous attempts, transformed *Agrobacterium* was grown up from a single colony in 25ml of triple selection media for 48 hours at 27℃. A 1ml aliquot was transferred to liquid LB media and grown up for an additional 24 hours. In this attempt AS was added (200µM) to the 24 hour old culture and allowed to incubate at 25℃ for 2 hours to charge the *Agrobacterium's* virulence system. For this attempt double plates were not used; rather a single plate composed of PDA amended with a combination of 200µM AS, 100 μg/ml Kanamycin, and 15µM 5-Azacytidine was used. Conidia from a seven day

old *P. chrysosporium* PDA plate was scraped and washed with DI water into a sterile 50ml centrifuge tube and vortexed. Five ml of the spore suspension was added to the *Agrobacterium* after the 2 hours of *Agrobacterium* and AS incubation. This co-culture was allowed to incubate at room temperature for 3 hours before pouring onto co-culture plates. The co-culture plates were allowed to dry and was then covered and sealed with parafilm. Ten plates were covered with aluminum foil and another ten were left exposed to the light in the same incubator as in previous attempts, with the incubator held at 25℃. These plates were allowed to incubate for three days before cultures were transferred to selection plates. For this attempt selection plates were composed of PDA amended with a combination of 200μg/ml Hygromycin B, 100μg/ml Augmentin, 200µM AS, and 15µM 5-Azacytidine.

2.1.7 AMT: Farman Lab protocol

The AMT "Farman Lab Protocol" provided by Dr. Melanie Crawford (Appendix F) was used for this AMT experiment. This protocol was designed for transforming the Ascomycete *Magnaporthe oryzae* and has been performed with high transformation frequencies in Dr. Farman's lab in UK's Plant Pathology department. In general the protocol utilizes the same mechanisms as in previous experiments, except rather than using the double plate co-culturing technique, this protocol uses filter paper disks as the staging substrate for the transformation. Both organisms are placed onto the sterile filter paper disks and allowed to interact. The disk is located on the surface of an induction media agar plate. After two days of co-culturing the disk is moved to a selection plate. If a transformation occurs the fungal growth will no longer be restricted to the filter paper and hyphal growth can be observed on the selection agar. The Farman Lab has had success with this protocol, however their experience suggests that transformation frequencies are dependent on the presence of a variety of nutrients and trace minerals. The composition of the induction medium (IM), co-cultivation medium (CC), and the selection medium or complete medium (CM) includes an array of nutrients/minerals that had not been used in previous experiments. It is not known whether all the nutrients/minerals are necessary, but increased transformation frequencies have been observed (with different organisms) when incorporated. The protocol did not explicitly mention the lighting conditions for the transformation therefore the plates were left at

room temperature in an area receiving natural sunlight and lab lighting during the day and dark conditions at night.

2.1.8 Fungal electroporation

The principles utilized for incorporating plasmid DNA into *E. coli* and *A. tumefaciens* have been applied to incorporate plasmid DNA into fungi (Chakraborty and Kapoor, 1990; Dombrowski et al., 2011). Using the plasmids pCT74 and pBSII an electroporation transformation was performed as an alternative to AMT. For this experiment *P. chrysosporium* hyphal protoplasts were used to increase transformation frequency.

2.1.8.1 Electroporation plasmid composition

The electroporation transformation separately attempted to incorporate the vectors pBS and pCT74 into *P. chrysosporium*. The pBS vector (Figure 3) includes the markers *LacZ* and a Hygromycin B resistant gene. The pCT74 vector (Figure 2) is a pBS vector modified to contain the *gfp* reporter under control of the *ToxA* promoter (Lorang et al., 2001).

2.1.8.2 Protoplast preparation

The *Magnaporthe* protoplasting protocol (Appendix A. provided by Dr. Jinrong Xu's lab,Purdue University) was adapted to prepare *P. chrysosporium* hyphal protoplasts. *P. chrysosporium* was inoculated into three PDB and three CM (suggested by protocol) 250ml flasks containing 50ml of media and incubated at 35℃ at 125rpm for two days. Each day cultures were blended (Waring commercial laboratory blender for 30 sec on high speed) and any contaminated flasks were discarded. The most robust culture from each media type was used for protoplasting. The cell wall lysing enzyme Lysozyme (Fisher Bioreagents BP535-1) was added to each flask at a 10mg/ml dose. After beginning day 3, protocol step 3 the protoplasts were observed at the one hour mark. Cells appeared fragmented, shriveled, and pitted. The lysing step was allowed to run for one hour and forty-five minutes total before proceeding. After completion of protoplasting, cells from each (PDB and CM) cultures were frozen with and without glycerol (1:1). Protoplasts were stored at -40℃ for 4 days, at which time samples were taken from frozen sample and plated to test for vigor.

2.1.8.3 Electroporation transformation procedures

The procedure was adapted from two publications describing electroporation transformation of filamentous fungi (Chakraborty and Kapoor, 1990) and specifically of *P. chrysosporium* (Dombrowski et al., 2011). *P. chrysosporium* protoplasts were thawed and 800ml of media with protoplasts was added to four pre-chilled and sterilized electroporation cuvettes (Eppendorf 4mm-800µl). Five µl of the plasmid pCT74 (two uniquely prepared stocks) were added to two cuvettes, and to the remaining two cuvettes 5µl of the plasmid pBSII (two uniquely prepared stocks) was added. The content of each cuvette (Eppendorf 4mm/800µl) was subjected to electroporation using the Eppendorf Electroporator 2510 for 5ms at a 1.5kV. The electroporator operated at a capacitance of 10µF and a resistance of 600Ω. Immediately upon completion of electroporation, 1 ml of CM media was added to the cuvette. The cuvette contents were then pipette mixed, and transferred to an eppendorf tube which was incubated for three hours at 26 °C and 65rpm. After incubation the contents of each potential transformant sample were spread onto PDA Hygromycin B (100µg/ml) selection media and allowed to dry before incubating at 35℃ until transformants appeared.

2.1.9 Gene expression confirmation

After transformations occurred and transformant cultures are selected on Hygromycin B antibiotic-amended plates, transformant *P. chrysosporium* will be further confirmed by *gfp*, *LacZ* and *gus* detection as described below.

2.1.9.1 Antibiotic selection

Transformed and non-transformed (wild type) fungi were grown on PDA supplemented with varied amounts (25-200 $\mu g/ml$) of the antibiotic Hygromycin B, and the bacterial specific antibiotic Cefotaxime to kill any residual *A. tumefaciens*. Colonies formed on selection plates that grew at comparable rates to the non-transformed organism on non-selection plates were isolated. Isolates were allowed to grow on selection media again. Colonies forming on the second round of selection were used for further transformation confirmation.

2.1.9.2 GFP

Gfp transgene presence was confirmed by plating transformed and non-transformed cultures side by side and allowing for 1-2 days of growth on PDA plate. Plates were allowed to air dry in a bio-hood to evaporate any residual moisture that may affect fluorescence detection. Plates were observed in a dark room under a dissecting microscope equipped with a GFP filter at the appropriate light emitting wave length. A difference in detection should be observable between the transformed and non-transformed cultures. However, a fair percentage of previous studies report a lack of GFP detection in successfully transformed species. In which case, a fluorometer with the appropriate filters could be used to detect slight differences in excitation between the two cultures.

2.1.10 Fruiting body and basidiospore production

Many early and more recent transformations of *P. chrysosporium* and other white rot fungi have been performed using the basidiospores or protoplasted basidiospores (Alic et al., 1989; Ma et al., 2001). Both PEG and AMT style transformations have implemented basidiospores due to their robust nature and benefits of being a sexual spore. However, special conditions must be met in order for *P. chrysosporium* to produce a fruiting body. Further unique conditions are necessary for the fruiting body to produce basidiospores. In this section an experiment is described which aimed to produce an observable amount of basidiospores to be used in subsequent transformation attempts.

The attempt generally followed the protocol outlined by Dhawale and Kessler (1993). A heterothallic state was first attained by encouraging mycelial interaction. On malt extract media agar plates the fungus was inoculated on opposite sides and allowed to grow at 35℃ for 3 days. After that time the heterokaryotic zones were clearly distinguishable as a linear cluster of conidia in the center of the plate. Four loops were taken from this region and plated to the fruiting plates separately in the four quadrants of the plate. Fruiting plate media is detailed in Appendix D. For the first attempt sixteen fruiting plates were prepared. After plates were inoculated they were placed in a twenty count plate bag, sealed, and placed upright in an incubator at 35℃ for 6 days in dark conditions. Plates were then removed from the plastic bag, inverted, and left in ambient lab conditions between 24-26℃ for 3 weeks. After 5 days out of the bag plates were

checked frequently for visual accumulation of basidiospores that would have fallen onto the lid of the plate.

2.2 Results and Discussion

2.2.1 Non-transformed organism response to Hygromycin B

The treatment containing 0µg/ml Hygromycin B resulted in detectable levels of growth observed in all organisms between day 2 and day 5. At Hygromycin B levels of 100µg/ml and 200µg/ml, no growth was observed in any organism until three weeks after the experiment began. Inhibition of growth was observed at antibiotic concentrations as low as 20µg/ml Hygromycin B.

At the mesophilic incubation temperature used in this experiment, Hygromycin B activity drastically decreases with time, which is likely why growth was observed after three weeks in the treatment flasks. Additionally the growth after three weeks was phenotypically inconsistent with the inoculated organism in some cases. These observations suggest that the antibiotic successfully inhibited and terminated the organism and a secondary contamination ensued after the antibiotics activity was reduced.

2.2.2 AMT: Following Sharma et al. (2010) protocol

Growth appeared on all selection plates between about 48 and 72 hours of incubation at 35℃. Cultures however were not phenotypically representative of *P. chrysosporium*. Organisms produced green spore-forming units with a leading periphery of growth that was white in color. Growth was consistent across all plates regardless of the concentration of Hygromycin. After one week of incubation from plating on selection media, organisms produced black crystalline structures on the surface of the fungal sheet. In attempt to determine the presence of multiple organisms, isolations were plated on selection media. All isolations produced growth consistent with that of the original transformant (Figure 5: left). When the organism was plated on PDA without Hygromycin B the resulting growth appeared structurally similar with the exception that all growth was yellow (Figure 5: right).

Based on physiological characterization alone the organism was determined to be a

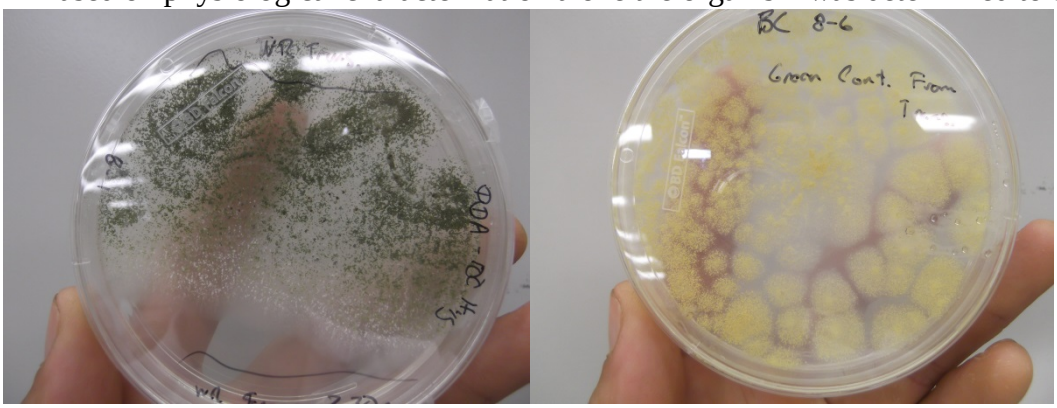

Figure 5: Transformed organism cultured on Hygromycin B amended plate (left); transformed organism cultured on non-Hygromycin B amended plate (right)

contaminant (non-target) organism and was destroyed. The contaminant growth was not observed on any co-culture plates suggesting one of two possible explanations. Either the contaminant organism was present in small (unobservable by the naked eye) concentrations on the co-culture plates, and conditions were favorable for successful transformation of the contaminant organism and not the target organism and therefore a successful transformation occurred in a non-target organism. Or the contaminant could have been contracted during the original isolation step where disks were removed from the co-culture plate and rinsed and plated on selection media. A likely source for contamination could be attributed to the augmentin rinsing step. The augmentin stock was gifted to the lab for the purpose of this transformation and may not have been prepared in a sterile environment. A question that still remains is why the contaminating organism was unaffected by the antibiotic Hygromycin B. This antibiotic resistance could be a wild type characteristic for the organism or the organism could have been transformed with a Hygromycin B resistance from a previous experiment. Hygromycin B antibiotic resistance has become a popular means for selecting transformed organisms in recent years.

2.2.3 AMT: Modified with light, temperature, and bacterial selection

No growth was observed at any time on either the 50 or the 100 μg/ml Hygromycin B amended PDA selection plates. Selection plates were allowed to incubate for three weeks at 35℃ in their respective light or dark conditions. Control plates (containing no

Hygromycin B) were inoculated with potential transformants and growth was observed as expected beginning 48 hours after inoculation in both the light and dark scenarios. Based on visual observation of the selection plates it was determined that no transformation occurred. Because no transformation occurred, this experiment was inconclusive as to whether or not light plays a significant role in transformation success.

2.2.4 AMT: Fungal strain replacement

No growth was observed at any time point on both the 50 and 100 μg/ml Hygromycin B amended PDA selection plates. Selection plates were allowed to incubate for three weeks at 35℃ in their respective light or dark conditions. Control plates (containing no Hygromycin B) were inoculated with potential transformants and growth was observed as expected beginning 24 hours after inoculation in both light and dark treatments. Based on visual observation of selection plates it was determined that no transformation occurred. This experiment was also inconclusive at determining whether or not light plays a significant role in transformation success.

Consistent with the description of the strain, growth on PDA plates was accelerated and was observable about 24 hours before the MYA-4764 strain under identical conditions. The ATCC 24725 also produced a full sheet of conidia in about 3 days where the MYA-4764 strain took up to one week. The more aggressive growth from ATCC 24725 may be more successful at establishing a competitive culture of *P. chrysosporium* on unsterilized feedstock in the presence of other microorganisms.

2.2.5 AMT: Induced virulence response with lignin in co-culture media

Table 2 lists the treatments remaining after removing plates that contained any degree of contamination. Only plates that appeared to contain pure target species cultures were used for inoculating selection plates. After allowing selection plates to incubate at 35℃ for two weeks, two plates showed some degree of growth. Asterisks on Table 2 indicate the two plates and their respective transformation conditions. Growth rates of the potentially transformed species were slower than that of stock *P. chrysosporium* species. After two weeks of incubation the two potential transformants were re-isolated onto PDA plates amended with Hygromycin B 0, 50, and 100 µg/ml. Growth on non-selective plates was observed after five days. Hyphal growth was significantly slower but continued for seven additional days. Growth on Hygromycin B 50 µg/ml was observed

after 12 days for both potential transformants. Growth on Hygromycin B 100 µg/ml selective plates was not observed until three weeks after re-isolation. This delayed growth on both concentrations of Hygromycin B plates is not indicative of a positive transformation. It is likely the antibiotic lost effectiveness over time and we were observing contaminants growing on the plates.

Table 2: AMT inventory and potential transformants

Transf.	Replications	Lignin Type	% Lignin Center	% Lignin Outer	Light
	2	AL	0.1	0	Yes
	3	AL	0.1	0.1	Yes
	2	AL	1	0	Yes
*	2	AL	1	0.1	Yes
	3	AL	0.1	0	No
	3	AL	0.1	0.1	No
	2	AL	1	0	No
	2	AL	1	0.1	No
	2	OL	0.1	0	Yes
	1	OL	0.1	0.1	Yes
	3	OL	1	0	Yes
	2	OL	1	0.1	Yes
*	3	OL	0.1	0	No
	3	OL	0.1	0.1	No
	3	OL	1	0	No
	2	OL	1	0.1	No

* - Potential Transformant; AL - Alkaline Lignin; OL - Organosolv Lignin

To date no other AMT attempt yielded cultures that showed any sign of growth on selection media therefore the two potential transformants were subjected to GFP microscopy. After comparing both non-transformed stock *P. chrysosporium* cultures with the two potential transformants it was determined there was no significant visual

difference of fluorescence between the cultures (Figure 6). High background fluorescence made GFP screening difficult. Eventually it was determined that the agar composition was the source of the autofluorescence and further GFP observations were performed on minimal media water agar plates. A positive control (a *gfp* positive fungal sp.) would have been highly beneficial to ensure potential transformants were truly non-transformed. Based on observation through selective plating and GFP screening it was determined that no transformations were successful under any conditions tested in this experiment. This test was also inconclusive in determining if lignin of either source was able to induce a virulence response in the *Agrobacterium*.

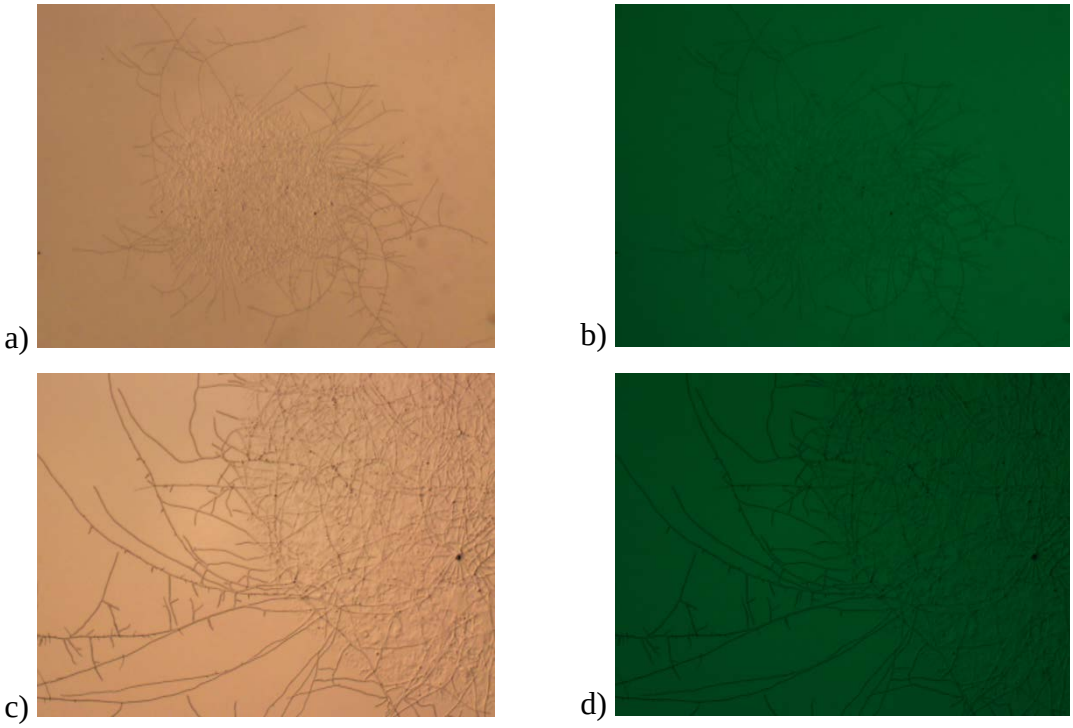

Figure 6: *gfp* negative (Figure a and b are the light field and GFP filtered non-transformed cultures respectively; figure c and d are the light field and GFP filtered potential transformants from this experiment)

2.2.6 AMT: Transformation Enhancers

After plating potential transformants onto selection plates, cultures were allowed to incubate for three weeks at 35℃. Growth was not observed at any time during the selection period. Plates were held for an additional two weeks of monitoring. Three plates showed some hyphal growth during that period. Isolations were made of each of

the cultures on PDA with and without Hygromycin B (200µg/ml). Cultures transferred to non-selection plates were delayed in growth and showed reduced vigor. Cultures plated to selection plates showed no signs of growth over an additional three week incubation period. Due to visual observation of selection plating it was determined that no successful transformants were produced. The false positives were likely a result of loss of effectiveness of the antibiotic near the end of the first three weeks of monitoring. This experiment was inconclusive at determining if the transformation enhancers, AS and 5-Azacytidine, were beneficial.

2.2.7 AMT: Following the Farman Lab protocol

Twenty co-culture plates were prepared for this attempt. After 10 days of incubation, only one plate showed signs of growth. The growth was not physiologically representative of *P. chrysosporium*, but rather a suspected *Penicillium sp.* Plates were allowed to incubate for an additional 20 days, after which time bacterial growth was observed abundantly on the agar suggesting the deterioration of antibiotic activity.

2.2.8 Fungal Electroporation

Protoplasts from PDB and CM cultures frozen with and without glycerol and plated on malt extract agar (MEA) plates all showed vigorous hyphal growth after 24 hours, indicating successful protoplasting. Twenty hours after plating to selection plates, growth on the control (0µg/ml Hygromycin B) was apparent through visually observable hyphae clusters. Hygromycin B amended plate cultures showed no growth at that time. Visual observations for growth continued for two weeks however no fungal growth persisted. This attempt was unsuccessful at producing a positively transformed fungal culture. Hyphal protoplasts may be unnecessary or even less beneficial than using whole (non-protoplasted) hyphae or conidia. Future attempts should aim to transform whole conidia with the same vectors using an electroporator with variable voltage, capacitance, and resistance to achieve the conditions suggested by Dombrowski et al. (2011).

2.2.9 Fruiting body and basidiospore production

This experiment did not yield any positively identified basidiospores. By visual observation no spores were evident on the lid of the plate after 3 weeks of inverted ambient incubation. The lids of a few selected plates were rinsed with DI water and

observed under a microscope. Some spores were apparent but due to the low concentration and relative size comparison to known conidiospores these observed spores were determined to be conidia. It was also not confirmed that a fruiting body was produced. However, the fungal growth observed on the fruiting plates after 5 days was characteristic of a fruiting body determined by comparison to published figures (Gold and Cheng, 1979). Fungal formations on fruiting plates produced after 9 days can be seen in.

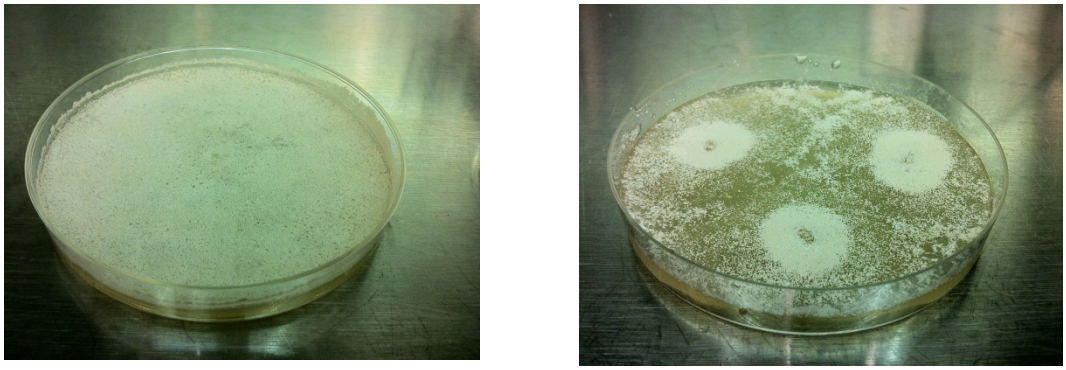

Figure 7: Typical *P. chrysosporium* growth on PDA (left); fruiting body formations on fruiting media agar.

Many transformations using *P. chrysosporium* basidiospores reference Gold and Cheng (1979) or Alec et. al (1987) for fruiting protocols. Both suggest specific criteria which are necessary for basidiospore production. The carbon source in the fruiting media appears to play a significant role. Results from experiments by Gold and Cheng (1979) show little to no basidiospores produced when glucose is used as the sole carbon source. The largest basidiospore yields were attained when using cellulose or xylose. Other essential reagents and conditions suggested as significantly necessary were incorporated in the attempt made here. Likely removing glucose and amending with cellulose would produce observable amounts of basidiospores.

2.3 Conclusion

The objective of this study was to transform *P. chrysosporium* with a selectable marker which could be used as a means to quantify and hence optimize fungal growth for a biological pretreatment process. Two types of transformation methods were attempted: *Agrobacterium* mediated and electroporation. No confirmed positive transformant culture was produced however a great deal of knowledge was compiled on the topic.

Transforming *P. chrysosporium* is still an emerging process. Though less stable PEG transformations of the organism occurred as early as 1989 (Alic et al., 1989) it was not until 2006 when *P. chrysosporium* was transformed using *Agrobacterium* (Sharma et al., 2006) just eight years after filamentous fungi were first transformed with AMT (de Groot et al., 1998). Furthermore electroporation of *P. chrysosporium* did not occur until 2011(Dombrowski et al., 2011).

The primary selection agent for this experiment, Hygromycin B, was determined to be an appropriate selection antibiotic for use with *P. chrysosporium* transformations. Concentrations as low as 50µg/ml were sufficient at completely inhibit growth for up to three weeks at mesophilic conditions. Special consideration should be taken to avoid false positive identification due to loss of antifungal efficacy after the three week period. The experiments described above were often inconclusive at determining whether light, temperature, media composition, target cell type, or transformation enhancers played a significant role in transformation success. However, based upon review of published protocols these afor mentioned parameters are likely to have profound effects on transformation frequencies and should be thoroughly incorporated into future work in this area.

It has become evident that transformation protocols are not an exact science but rather they are guidelines and suggestions and may require small adjustments to be successful. Re-attempting any of the experiments detailed in the section could result in a positive transformant using developed and refined skills acquired over the course of these trials. The field of biological pretreatment with white-rot fungi could be greatly benefited through the development and implementation of marker systems within transformed organisms. Future work in light of the results of these experiments should implement PEG style transformations using *gus*, *LacZ*, and *sgfp* incorporated vectors driven by fungal promoters.

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CHAPTER 3: INOCULATION OPTIMIZATION

3.1 Summary

The following study compares fungal activity in lignocellulosic biomass that has 1) been uniformly inoculated and allowed to sit dormant before being exposed to favorable fungal growth conditions to biomass that has 2) been uniformly inoculated and immediately exposed to favorable fungal growth conditions. These treatments were compared to a control which consisted of biomass that was not inoculated but was exposed to favorable fungal growth conditions. The objectives of the following experiment was to simulate in-field fungal applications of *P. chrysosporium* to recently harvested switchgrass and determine if such a biological pretreatment application would produce equivalent cellulose availability as other more conventional methods (i.e. treatment 2). Additionally this study investigated parameters known to affect saccharification efficacy under conditions which arise in commercial farm-scale lignocellulosic biological pretreatments.

The composition of total cellulose and lignin do not drastically change between treatments. One treatment did show statistically greater cellulose availability as determined by enzymatic saccharification glucose yield. Many potential factors thought to affect saccharification were ruled out as saccharification inhibitors in this experiment. Sterilization procedures were indicated as having a large effect on the extent of cellulose hydrolyzed during saccharification. The findings do not support in-field fungal application due to a low percent cellulose hydrolysis, however the fungal application did show a treatment effect in treatment 2.

3.2 Introduction

Large scale lignocellulosic to solvent conversion currently takes place in a bunker similar to that of a silage bunker. One challenge encountered when opting for a biological pretreatment (i.e. white rot fungi delignification) is the difficulty of achieving uniform inoculum dispersal throughout the entirety of the biomass within the bunker. Applying the inoculum directly to the top of the biomass may limit the microorganism's ability to effectively delignify biomass at depths below the surface. Frequent and heavy flushing with water could move the *P. chrysosporium* cells down through the biomass

however the density of biomass and poor space may hinder the extent to which the fungi may travel. An alternative approach to uniformly inoculating the biomass is to apply inoculum during harvest between flakes in the baler with a pressurized spraying device. Such devices have been affixed to balers in order to apply chemical treatments to increase storage life of feed hay (Horn et al., 1983a). By applying the inoculum in this fashion a greater amount of biomass is exposed to the organism. Currently there are no published data suggesting how to apply fungi in this way and if the fungi will be viable over a period of time in conditions that have a low water activity.

3.3 Materials and Methods

3.3.1 Reactor design

The three treatments mentioned above were carried out in triplicates by preparing three identical 100L bins. Bins and lids were cleaned and sterilized with bleach, rinsed, and dried. The bins contained 1.5kg of switchgrass harvested and baled at the University of Kentucky's North Farm. The raw bails were stored in ambient building conditions. The switchgrass flakes in each bin were separated by chicken wire partitions in three equal mass (0.5kg) layers. This partitioning allowed for stratified sampling while minimizing void space between layers. Each bin was fitted with a lid that allowed for some air exchange.

3.3.2 Inoculum preparation

The white rot fungus *Phanerochaete chrysosporium* (Parent strain: ATCC 24725) was thawed from the -40 ℃ freezer stock, plated on PDA, and allowed to grow for one week at 37℃. After the one week a segment of the PDA was cut with sterile razor and placed into 30ml of DI water in a 50ml centrifuge tube and vortexed for 30 sec to dislodge conidia from the agar. A 6L flask was prepared with 1L of DI water, 10g Dextrose, and 4g potato extract, covered with aluminum foil and autoclaved. After the media cooled the flask was inoculated with the 30ml of DI water containing conidia. The flask was allowed to incubate for 72 hours in a shaking incubator at 125 rpm and 37℃. After 72 hours small uniform pellets were observed. The content of the flask was used to inoculate a 100L New Brunswick Scientific reactor that was sterilized in place and prepared with the same media recipe with the addition of 10 ml of antifoam (Antifoam

204, Sigma A6426). This reactor ran for 72 hours at 250 rpm and 37 ℃. The fungus was harvested after the allotted time and emptied onto metal screen trays in ambient conditions. The fungus was washed with tap water for 5 min to remove any residual media. After washing the fungus was allowed to sit and drain over the screen for 10 min before measuring pellet volume. The fungal dry matter was approximately 0.03g/ml determined by oven drying the pellets at 105℃until a constant weight was achieved. For each treatment requiring inoculum, 600 ml of pellets were blended with 1200 ml of DI water in a Waring commercial laboratory blender for 30 sec on high speed. The inoculum for each bale was placed into a graduated spray bottle.

3.3.3 Treatments

Treatment 1: After each bin was prepared, a standard amount of inoculum was applied over the entirety of the biomass at each of the layers. An equal volume (250ml per layer) of inoculum was applied to each of the three layers using a spray bottle. Layers were stacked carefully to ensure that void space was eliminated and the lids were secured on each bin. The water activity was measured at each layer using a water activity probe (Rotronic Hydro Palm). The three replicate bins were stored for 14 days at room temperature. During this time the fungus should not have been active due to the low water activity and less than optimal temperature. On the 14^{th} day samples of approximately 25g were taken from each layer of the three replicate bins and the water activity was measured at each layer. The bins were then filled with tap water and the biomass was submerged for 10 minutes before draining completely. Bins were filled and drained gently to avoid dislodging hyphae from the biomass. Conidial mats remained intact and affixed to the biomass after draining. The lids were secured to the bins and placed in a Parameter Generation and Control (Precise Humidity Control) environmental chamber at 35 ℃ and 50% relative humidity for an additional 14 days to encourage delignification of the biomass by the fungus. On the 28^{th} day of the trial 3 samples of approximately 50g were taken at each layer in all three replicate bins. Additionally the water activity was measured at each layer. The remaining biomass was then sterilized and discarded.

Treatment 2: After the bins were prepared each was filled with tap water and the lignocellulosic biomass was submerged for 10 minutes before completely draining. A

standard amount of inoculum was applied over the entirety of the biomass at each of the layers. An equal volume (250ml per layer) of inoculum was applied to each of the three layers using a spray bottle. The water activity was measured at each layer by a water activity probe (Rotronic Hydro Palm). Layers were restacked carefully to ensure that void space was eliminated and then the lids were secured to each bin. Bins were then placed in an environmental chamber at 35 ℃ and 50% relative humidity for 14 days to encourage delignification of the biomass by the fungus. On the 14th day of the trial, 3 samples of approximately 50g were taken at each layer in all three bins for a total of nine samples per bin resulting in 27 samples per treatment. Additionally the water activity was measured at each layer. After the required samples were obtained, the remaining biomass was then sterilized and discarded.

Control Treatment: After the bins were prepared each was filled with tap water and the biomass was submerged for 10 minutes before completely draining. Water activity was measured at each layer by a water activity probe and then approximately 25g from each layer were collected to test for dry matter content. Lids were then secured on the bins and the bins placed in the environmental chamber under the same conditions as for Treatment 1 and 2 for a 14 day period. No inoculum was applied to the control treatment; however it is worth noting that the biomass for all bins was unsterilized. On the 14th day of the trial, three samples of approximately 50g were taken at each layer in all three bins. Additionally the water activity was measured at each layer. After sampling was complete, the remaining biomass was sterilized and discarded.

3.3.4 Sampling protocol

Before sampling, each layer was divided into 6 equal quadrants and each quadrant assigned a number. Using a random number generator, sampling quadrants were selected from which to take each 50g of wet sample. No quadrants in any layer were ever sampled twice. Using the "quick" setting on a Rotronic Hydro Palm water activity meter a measurement was provided within 3-8 minutes for each layer at the time of sampling. Water activity measurements were within a range of accuracy of + or – 0.005 aw (1.000aw = 100% RH). Samples were also taken of the raw switchgrass bales that were used to prepare each of the bins before the trials began. All samples taken were immediately weighed and placed a pre-weighed tin or paper bag and incubated at 44℃.

Sample weights were measured each day until samples maintained the same weight for 3 days. After samples were thoroughly dried using the 45℃ drying method a dry matter weight was determined and samples were ground to 2 mm, placed in plastic bags, labeled, and refrigerated until ready for saccharification and analysis.

3.3.5 Analyses

In order to determine the effectiveness of the pretreatment techniques samples were uniformly hydrolyzed for 72 hours at 50℃ to convert exposed carbohydrates to glucose. The hydrolyzate was then analyzed by a YSI analyzer (YSI 2900D; YSI, Inc.; Yellow Springs, Ohio) to quantify glucose. The enzymatic saccharification was performed following the NREL Laboratory Analytical Procedures (LAP) for Enzymatic Saccharification of Lignocellulosic Biomass protocol (Selig et al., 2008). The cellulase enzyme was purchased from American Laboratories Inc. (lot number ALI 14175-04). Protein concentration was determined using a modified Bradford method provided in Appendix D. Cellulase activity was determined by following NREL's Measurement of Cellulase Activities LAP (Adney and Baker, 1996). Cellobiase activity was determined using a pNPG method for B-Glucosidase provided in Appendix F.

3.3.6 Saccharification

Due to the volume of samples to be hydrolyzed and analyzed the first saccharification attempt was broken down into three different runs over the course of two weeks. Conveniently each treatment was performed in triplicate (i.e. three individual bins labeled A, B, and C) therefore each run analyzed one bin's samples from each treatment. Additionally two sets of raw samples were included with each run. Distributing samples in this way ensured that any variability in the saccharification treatment or YSI analysis would be equally distributed across all samples (blocked on replication).

The NREL LAP for Enzymatic Saccharification of Lignocellulosic Biomass suggests using 0.1g of cellulose or 1.5g total biomass on a 105℃ dry weigth basis. The compositional analysis of switchgrass from UK's North Farm previously performed in the lab provided a consensus of 30% (w/w) cellulose. Dry matter was determined using a Ohaus MB35 Halogen moisture analyzer provided by Dr. Mike Montross' lab. Moisture content was determined for each sample immediately prior to preparing saccharification

vials. Cellulose (0.1g) from each sample was placed into a scintillation vile. Equation 3 was used to determine the amount of wet biomass needed to equal 0.1g of cellulose.

Equation 3: Wet biomass adjustment for cellulose

$$Sample\ x\ contains\ 10\%\ moisture\ therefore:$$

$$0.1g\ cellulose * \frac{1g\ dry\ biomass}{0.3g\ cellulose} * \frac{1g\ wet\ biomass}{(1.0 - .10)g\ dry\ biomass} = 0.37g\ wet\ biomass$$

For each layer (consisting of three samples; one from each replication) a blank was prepared without enzyme by duplicating the first sample and adding an equal volume of DI water in place of the enzyme aliquot. After the appropriate amount of biomass was added to each scintillation vial 5ml of 0.1M Na-citrate buffer (pH = 4.89), 100µl of 20% Na-azide, 1ml of enzyme dilution, and an amount of DI water were added sufficient to bring the total volume to 10.00mland the specific gravity of solution to 1.000g/ml. Using Sample x (at 10% moisture) from Equation 3 the remaining volume needed can be calculated as follows using Equation 4.

Equation 4: DI water adjustment

$$10.00ml\ total\ volume - 5ml\ buffer - 0.1ml\ Na\ azide - 1.0ml\ enzyme\ dillution - 0.37ml\ (biomass\ assumed\ to\ occupy\ equal\ g - ml) = 3.53ml\ DI$$

Note that the blanks with no enzyme replaced the 1.0ml of enzyme dilution with 1.0ml of DI water. To achieve the LAP specified 60FPU/g cellulose, an enzyme dilution of the American Labs Cellulase (American Laboratories, Inc.; Omaha, Nebraska) was prepared using the previously determined activities and protein content of the enzyme (Equation 5).

Equation 5: Enzyme concentration

$$0.1g\ cellulose * \frac{60FPU}{g\ cellulose} * \frac{1.0mg\ protein}{3.0\ FPU} * \frac{1.0mg\ enzyme}{0.1157\ mg\ protein} * \frac{1g}{1000mg}$$
$$= 0.01729g\ enzyme$$

A stock enzyme dilution was prepared by combining 1.729g enzyme with 100ml DI water and stirring continuously (note continuous stirring is necessary to suspend contents of enzyme powder). The LAP also suggests using 64pNPGU/g cellulose. Based on determination from Appendix F. the accompanying the cellulase enzyme in the enzyme powder will exceed this value. Excess β-Glucosidase activity will ensure cellobiose will not accumulate and become inhibitory.

Enzyme blanks were prepared in triplicate by combining 5ml of 0.1M Na-citrate buffer, 100µl of 20% Na-azide, 1ml of enzyme dilution, and 3.9ml DI water. The enzyme aliquot was added last to each vial before tightly fixing the screw cap to each vial and vortexing for 10s to suspend biomass particles. Samples prepared for saccharification had a final pH of 4.80 with all components added. All vials were placed in a rack and into an incubator set at 50℃ at 150rpm's for 72 hours. After the allotted time, the vials were placed into a 93℃ water bath for exactly 15 minutes to denature the enzyme thereby stopping enzyme activity. Vials were vortexed for 10s to mix contents and 1.5ml was poured into pre-labeled eppendorf tubes. Samples in the eppendorfs were then centrifuged at 6000rpm for 10 minutes. During the boiling and centrifuging time the YSI machine was calibrated and a standard run at 9.00g/l glucose. Samples were not analyzed until the standard analysis read within a range of ± 0.5g/l glucose. After samples were centrifuged the caps were opened and the sample contents were placed into a 24 well container which was placed inside the YSI analyzing chamber.

3.3.7 Saccharification efficacy

3.3.7.1 Comparison of Extracted and non-extracted raw biomass

A series of ranging experiments were performed aimed at identifying the expected efficiency of saccharification. Our first objective was to determine if inhibitors were present in the raw switchgrass that might interfere with enzymatic activity. Raw

switchgrass was extracted using (what piece of equipment?) using both hot water and ethanol to remove proteins, ash, soluble sugars, and other debris from the switchgrass. An amount (approximately 360g) of each of the six raw samples was packed tightly into individual small extraction cells. The cells were placed into the Dionex ASE 350 extractor and run at 1500psi and 100℃ with a preheat time of 5min with three static cycles of 7min each, a flush volume of 150%, and a purge time of a 120sec to remove extractables such as proteins, and soluble sugars. After samples were extracted they were allowed to sit in open plastic bags for three days to dry and evaporate off any residual ethanol. After reaching equilibrium the moisture content was determined for each sample using the moisture analyzer. As in the previous section samples were weighed such that 0.1g of cellulose was added to each scintillation vial. For this saccharification, each raw sample was run in triplicate with a substrate blank that was not treated with enzyme. Additionally an enzyme blank was analyzed in triplicate. All other conditions were identical to the experiment described in section 3.3.6.

3.3.7.2 Effect of enzyme concentration on saccharification

The cellulase activity experiment was designed to determine if higher concentrations of the American Labs Cellulase became inhibitory. Enzyme concentrations of 15, 30, 60, and 120 FPU/g cellulose were tested in triplicate following the same conditions as described in Section 3.3.6. An enzyme and substrate blank was prepared for each cellulase concentration.

The second experiment compared the Sigma Cellulase from *Trichoderm reesei* (ATCC 26921; aqueous) to the American Labs Cellulase . The Sigma Cellulase was tested at three different activity concentrations (30, 60, and 120 FPU/g cellulose). The Sigma Cellulase activity was assayed by Dr. Alicia Modenbach and found to be 39.4FPU/ml of enzyme. Each activity was tested in triplicate and an enzyme and substrate blank was prepared as previously described. Untreated switchgrass ground to 2mm was used as the saccharification substrate.

3.3.7.3 Inhibition and substrate effect

Experiments were performed to quantify the effects of Na-azide, microbial competition, and xylose on the enzymatic activity. For these three sets of experiments, the saccharification conditions were performed as outlined in Section 3.3.6 with an

enzyme activity of 60FPU/g cellulose. Each variable tested was performed in triplicate with an enzyme blank and a substrate blank. The standard for comparison was raw (unsterilized) switchgrass from the same lot as in the previous experiment. Sufficient switchgrass to provide 0.10g of cellulose was added to each sample. The necessary weight of switchgrass was calculated with consideration of the moisture content and substrate composition.

To quantify the inhibitory effects of the antimicrobial agent Na-azide a set of samples were run with and without Na-azide (Na-azide substituted with equal volume of water).

To determine the viability of the American Labs Cellulase enzyme a set of samples were prepared with Avicel as the cellulose substrate. 0.1g of Avicel was used in place of the biomass and water was used to adjust the final volume to 10.0ml. This test allowed us to benchmark saccharification using a substrate with no structural or chemical inhibitors.

To determine the effect of microbial competition on the saccharification results, a set of samples were prepared using biomass that had been autoclaved for 30 minutes at 121℃ and ambient moisture content. This pretreatment is termed “dry” sterilization. Another set of samples was prepared using biomass that was autoclaved for 30 minutes at 121℃ that was pre-moistened to 75% moisture content with DI. This pretreatment is termed “wet” sterilization. The results of these saccharification trials were compared to raw, unsterilized biomass subjected to the same treatment.

To determine the effect of xylose concentration on enzymatic activity, three sets of samples were prepared same as the standards but were amended with varying xylose concentrations (1X = 0.131g, 0.5X=0.0655g, 0.1X=0.0131g xylose amended). The liquid volume was adjusted for the addition of xylose and brought to a final volume of 10.0ml

3.3.7.4 Compositional analysis

Select samples from each trial were analyzed for total biomass composition using the NREL Biomass Compositional Analysis LAP (Sluiter et al., 2008). Hydrolyzate samples were analyzed for glucose and xylose with HPLC (HPLC conditions: Dionex Ultimate 3000; 5mM Sulfuric Acid solution mobile phase at 400µl/min; Biorad Aminex 87-H column; Shodex RI 101 detector; Chromeleon 7.1 analyzing software); glucose was also determined with a YSI glucose analyzer (YSI 2900D; YSI, Inc.; Yellow Springs, Ohio)

as a confirmation of the HPLC data. The top layer subsamples of one replicate from the control and treatment 1; the top layer subsamples of all three replicates of treatment 2; and three raw samples were analyzed. The objective for performing this analysis was to compare total biomass glucose and total lignin composition between treatments. Other data including ash, xylose, approximate cellulose, acid insoluble lignin (AIL) and acid soluble lignin (ASL) is provided however not all components of hemicellulose were determined.

3.4 Statistics

A split plot experimental design was used. The treatments were applied to the whole plot (bins) and the main effect of depth was evaluated as the split plot subunit. A PROC GLM model was used in SAS (version 9.3) with "treatment" analyzed as the whole unit and significance was evaluated with an error term of "bin" x "treatment". The response variable tested was glucose concentration after enzymatic hydrolysis. The subunit (bin) was analyzed for the main effect of depth and the interaction of depth x treatment. Initially a repeated measure model (as suggested by the statisticians) was applied to the same data to confirm significance (not shown) before selecting the split plot analysis.

Comparisons were made between treatments and between depth x treatment combinations using t-tests (in EXCEL using the "=T.TEST(array1,array2,trials,type)" command) .Mean and standard deviation values for the compositional analysis data were computed in Excel using the "=AVERAGE(array1)" and "STDEV(array1)" functions.

3.5 Results and Discussion

3.5.1 Pretreatment observations

These water activity and percent moisture data are provided in Appendix G. Pictures were taken immediately prior to sampling to provide evidence of growth and a record for *P. chrysosporium* physiology on biomass with the given conditions. Those images can be found in Appendix G. Growth appeared as expected on all bins that were treated with *P. chrysosporium*. The control bin biomass appeared to harbor heavy microbial activity observed by the loss of rigidity of stem pieces when handled. Varieties of fungi were

observed on the control samples however never in concentrations or in mats to the extent *P. chrysosporium* appeared on treated samples.

3.5.2 Saccharification

The raw data containing YSI values for every sample analyzed are provided in Appendix H. The glucose released from each sample was determined by subtracting the glucose from the enzyme blanks from glucose values determined by the YSI for each sample. The substrate blanks are also provided for reference however they were not used to adjust final glucose concentrations. Substrate blanks were not subtracted as these values are reflective of the effectiveness of pretreatment methods. The analysis of variance, other pertinent data and the SAS codes used are provided in Appendix I.

Results from the analysis show a difference between treatments ($p < 0.05$). The saccharification of treatment two released nearly twice as much glucose (avg. 0.77g/l) as either treatment one (avg. 0.44g/l) or the control (avg. 0.46g/l). Though a statistical significance was determined, all three of these values fall short of the theoretical yield. Theoretical yield for each sample was approximately 11.1g glucose/l. All treated and control samples analyzed released between 1.5-13.2% of theoretical glucose. Raw untreated biomass released between 5.0-7.1% of theoretical glucose. Previous trials (using *sterilized* switchgrass and a similar organism) claim as much as 10% for control and non-treated samples and up to 37% of theoretical for treated samples (Wan and Li, 2011). Another set of trials tested glucose released after a 14 day incubation with *P. chrysosporium* using non-sterile switchgrass. After saccharification under similar conditions as described here the glucose yield from treated switchgrass was approximately 11.7% of theoretical (Tanjore, 2009). Results from these two sources provide some insight into the influence sterility has on effective pretreatment. Biomass that has undergone a sterilization procedure (i.e. moist autoclave cycle) has drastically reduced active microbial activity and autoclaving destroys the majority of contaminant spores. Most “sterilization” procedures for large volumes of large-particle material like switchgrass likely do not remove all contaminant organisms. However, contaminant microbial populations are suppressed at the time of target organism inoculation. This suppression provides a window for the target species (in this case *P. chrysosporium*) to grow, mature, and sporulate to a point that is competitive against vigorous contaminant

species. When the target species becomes dominant, metabolic activities, including lignolytic systems, can work efficiently. Additionally, the autoclave sterilization procedure is a form of pretreatment as suggested by results provided in section 3.5.3.3 of this study. This disruption of the lignin matrix may expose greater portions of the biomass thereby allowing the hyphae of the inoculated organism to increase its biological pretreatment effectiveness. The elevated glucose yields measured by Wan and Li (2011) are likely a result of optimum conditions for lignolytic activity due to the sterile nature of the biomass. The resultant glucose yields from the experiment presented in this manuscript and Tanjore (2009) suggest reduced lignolytic activity as a result of competition with contaminant species. Though growth was apparent on the unsterilized biomass, the extent of growth and hyphal biomass interaction is unknown.

The substrate blanks (no enzyme applied during saccharification) glucose values provide insight into additional enzymatic characteristics relative to treatment type. Treatment two substrate blank values were significantly higher than either treatment one, control, or raw biomass ($p < 0.0001$). Though the compositional analysis did not show a difference in lignolytic degradation between treatments, more soluble sugars were produced during treatment two. This could be due to enhanced conditions for the cellulolytic activity of *P. chrysosporium* (which is present in small amounts even though typically only its lignolytic activity is discussed) or by release of free sugars by disruption of the lignin matrix.

Table 3: ANOVA of bin-scale saccharification

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	1.28161235	0.09154374	8.15	0.0004
Error	12	0.13470617	0.01122551		
Corrected Total	26	1.41631852			

R-Square	Coeff Var	Root MSE	sugar Mean
0.904890	19.13488	0.105951	0.553704

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	0.60915802	0.30457901	27.13	<.0001
Bin(Trt)	6	0.28496049	0.04749342	4.23	0.0161
Depth	2	0.17567654	0.08783827	7.82	0.0067
Trt*Depth	4	0.21181728	0.05295432	4.72	0.0161

Tests of Hypotheses Using the Type I MS for Bin(Trt) as an Error Term					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Trt	2	0.60915802	0.30457901	6.41	0.0324

The analysis of the bin-scale experiment also revealed a layer main effect (Table 3). The interaction effect of treatment by layer was also significant. We see in the treatment 2 bin-scale saccharified samples that the top and middle layers produced higher yields of glucose than did the bottom layer ($p > 0.05$ and $p > 0.001$ respectively). In neither the control nor treatment 1 did we observe a difference in glucose released between any layers. This finding further suggests that the experimental conditions of treatment 2 were more favorable for lignocellulosic biological pretreatment by *P. chrysosporium*.

3.5.3 Saccharification efficacy

3.5.3.1 Comparison of Extracted and non-extracted raw biomass

Post-extraction, the glucose in substrate blanks for the extracted samples (below instrument detection limit) was lower than in substrate blanks for raw non-pretreated switchgrass (0.14-0.29 g/l) saccharification, likely due to the removal of soluble sugars

from the raw biomass by the extraction procedure. When comparing the total glucose released by the enzyme, the values were statistically equivalent for raw samples and those samples which were extracted ($p > 0.05$). If extractables were inhibiting the enzyme, then the removal of extractables would increase enzymatic activity yet this was not observed.

3.5.3.2 Effect of enzyme concentration on saccharification

Figure 8 below shows the glucose recovered from hydrolysis as a function of enzyme activity. The glucose released for each both the American Labs and the Sigma Cellulase was equivalent at the suggested optimal 60FPU/g cellulose activity ($p > 0.05$). This suggests that the cellulase concentration (between the ranges of 15 FPU/g cellulose and 120 FPU/g cellulose) did not inhibit the work of the enzymes. Doubling the activity from optimum to 120FPU/g cellulose only increased glucose released by roughly 25%.

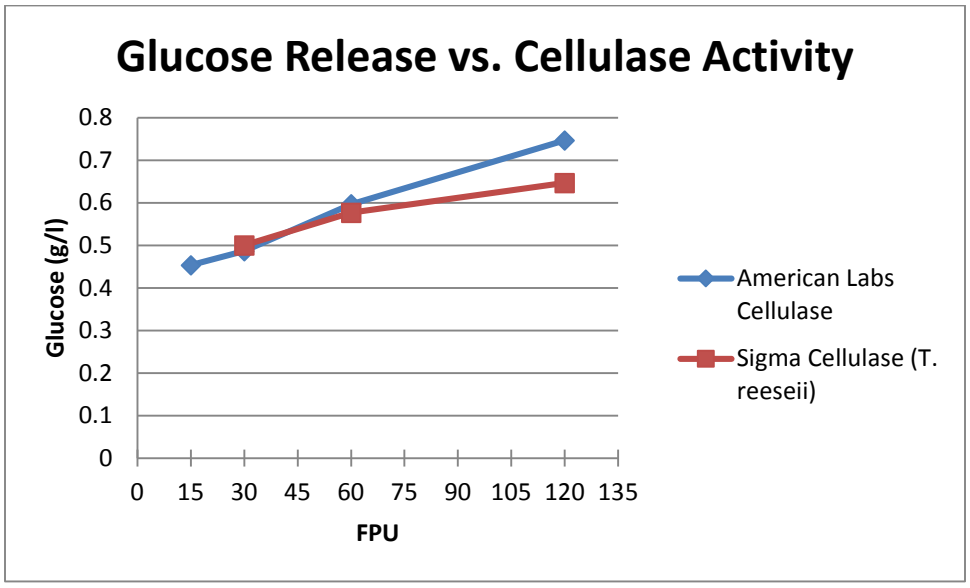

Figure 8: Glucose Release vs. Cellulase Activity

3.5.3.3 Inhibition and substrate effect

The data for the investigative experiments quantifying the effects of Na-azide, lignin, microbial competition, and xylose concentration can be found in Appendix H. Figure 9 below summarizes the results.

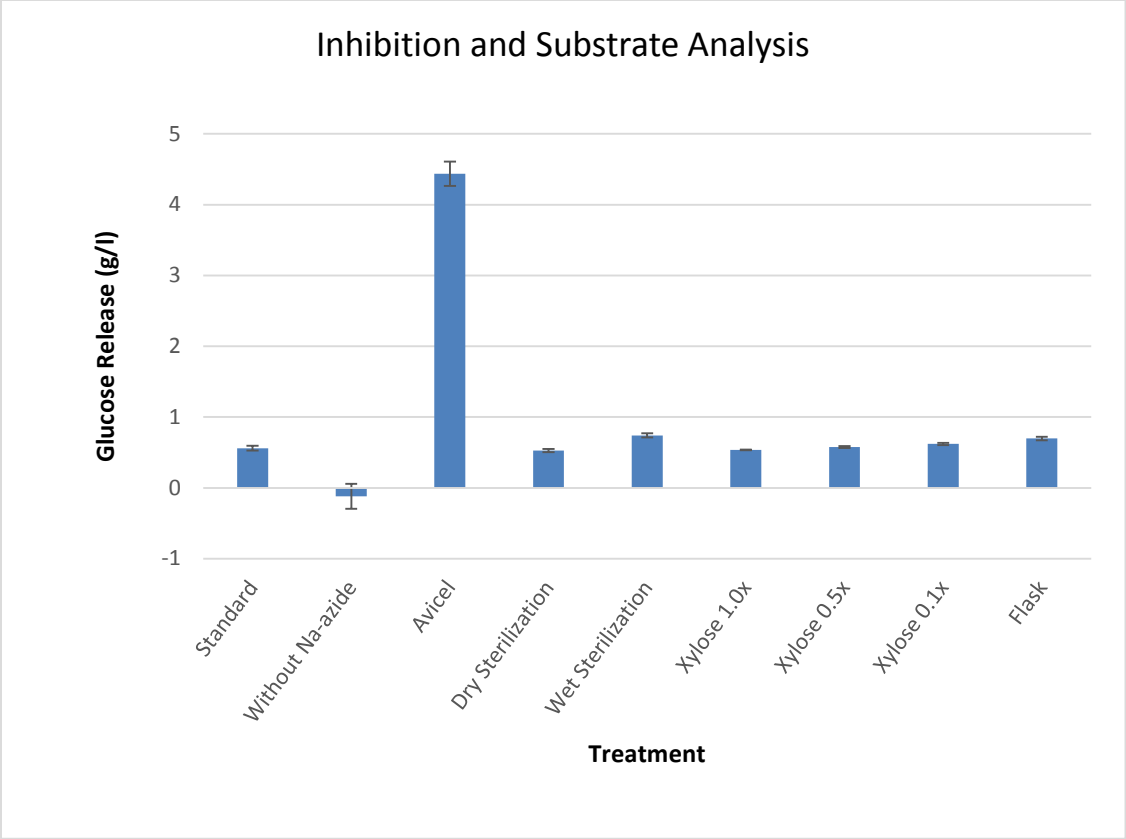

Figure 9: Inhibition and substrate analysis

The glucose recovered from the comparison standard matched previous trials with raw untreated switchgrass. The negative values reported for samples without a Na-azide amendment reflect microbial activity during the saccharification which consumed all soluble biomass sugars, any sugars resulting from hydrolysis, and a portion of the sugars present in the enzyme powder. These results suggest the necessity of including the antimicrobial Na-azide for the saccharification. Additionally these results do not suggest Na-azide -related enzymatic inhibition.

In the samples using Avicel as the carbon source an average of 4.44g/l glucose was released. This concentration is 40% of the theoretical potential conversion. At the end of the saccharification the Avicel within the samples had settled to form a pellet at the bottom of the vial. This result is consistent with other similar reports in our lab suggesting the enzyme and saccharification conditions are not the source of low glucose release. A more plausible reason is inability of the enzymes to access the cellulose.

Cellulose was more available in Avicel (no lignin present) and more glucose was released.

The dry and wet sterilization samples, when saccharified, produced glucose concentrations of 0.53 and 0.74g/l respectively. The dry sterilization values were not significantly different from the standard ($p > 0.05$) however the wet sterilization values were significantly different ($p < 0.005$). In comparison to the theoretical glucose release these differences are only marginal. The "wet" sterilization technique provides a mild pretreatment to the biomass that dry sterilization and untreated switchgrass do not experience, which may account for the increased glucose released from the sample prepared using the wet sterilization technique.

Amending the switchgrass with xylose at 1.0X, 0.5X, and 0.1X the concentration of theoretical xylose produced 0.54, 0.58, 0.62g/l glucose respectively. These values are not significantly different from the standard for comparison samples ($p > 0.05$). However a trend can be seen suggesting slight enzymatic inhibition associated with xylose. An increase in xylose concentration correlated to a decrease in glucose released ($p < 0.05$). Xylanase activity was determined for the American Labs Cellulase at 1.08IU/mg protein. The Xylanase activity is relatively low compared to the cellulase and β-Glucosidase activities within the enzyme pool.

3.5.3.4 Compositional analysis

Results for samples analyzed in the compositional analysis experiment can be found in Table 4 below. A slight significance was determined between concentrations of lignin where the lignin content of treatment 2 was slightly less than that of either the control or treatment 1 ($p < 0.05$). However, so little material was analyzed compared to the amount of material which was taken from the bin-scale experiment that this lignin reduction in treatment 2 may not be representative. More interestingly, the results show that there was no difference in cellulose composition between any treatments including the raw ($p > 0.05$). This result suggests that lignin does not necessarily need to be removed in order to improve cellulose availability for enzymatic saccharification. Because the experiment showed a significant difference in glucose yield between treatments, and there was not a large amount of difference in lignin concentration between treatments, either a very small

amount of lignin removal or lignin disruption is sufficient to produce a significant difference in glucose yield.

Table 4: Compositional analysis data

Treatment	Bin	Layer	Cellulose	Glucose	Xylose	AIL	ASL	Total Lignin	Ash
Raw	n/a	n/a	38.34% (0.0162)	42.55% (0.0162)	27.10% (0.0089)	22.29% (0.0027)	0.85% (0.0001)	23.11% (0.0033)	0.52% (0.0008)
Control	A	Top	39.56% (0.0605)	43.90% (0.0605)	27.58% (0.0272)	24.99% (0.0054)	0.71% (0.0009)	25.70% (0.0052)	1.13% (0.0094)
Trx 1	A	Top	40.07% (0.0226)	44.48% (0.0226)	27.63% (0.0029)	25.28% (0.0093)	0.85% (0.0087)	26.13% (0.0087)	0.41% (0.0011)
Trx 2	A	Top	38.18% (0.0173)	42.38% (0.0173)	26.96% (0.0128)	22.32% (0.0143)	1.16% (0.00008)	23.48% (0.0144)	0.30% (0.0011)
Trx 2	B	Top	37.13% (0.0373)	41.22% (0.0373)	26.08% (0.0233)	21.48% (0.0133)	1.08% (0.0010)	22.20% (0.0187)	0.37% (0.0017)
Trx 2	C	Top	37.33% (0.0164)	41.44% (0.0164)	27.23% (0.0109)	22.54% (0.0092)	1.06% (0.0002)	23.60% (0.0091)	0.38% (0.0006)

AIL: Acid Insoluble Lignin; ASL: Acid Soluble Lignin. Note: Values given in percent are means of subsamples and values in parenthesis are standard deviations of subsamples.

3.6 Conclusion

The objective of this experiment was to simulate in field fungal applications (treatment 1) to switchgrass and determine if such a biological pretreatment application would produce equivalent cellulose availability as other more conventional method (treatment 2). These findings do not support the implementation of in-field inoculation because of low cellulose conversion upon enzymatic saccharification, however the results do provide some insight into effective biological pretreatment techniques.

In general glucose yields from saccharification analysis were lower than anticipated. However these results were consistent with findings from a similar experiment employing *P. chrysosporium* on unsterilized switchgrass (Tanjore, 2009). A leading theory for the sterilized vs un-sterilized saccharification glucose yield difference is supported by findings in our compositional analysis experiment. Though total lignin and cellulose composition did not change measurably between treatments, an enhanced pretreatment effect was observed after saccharification. Further work should be done to assess the impacts of sterility with regards to contaminant microbial activity and the

pretreatment effect of the sterilization procedure. Such experiments should more thoroughly compare treated biomass composition to saccharification yields.

The low glucose yield from treated samples should not prompt the end of research and development of lignocellulosic biological pretreatments. Rather work should continue to optimize conditions for both sterile and unsterile feedstock and to determine if repeated inoculation and saccharification could result in increased sugar yield of a particular amount of starting biomass.

This experiment did provide some insight regarding how fungal pretreatment is affected by depth of biomass. A significance in treatment two showed an increased pretreatment effect of the top two layers compared with that of the bottom layer. It is not clear whether this would be the case if the biomass was not uniformly inoculated. This study should be compared to currently ongoing experiments in our lab employing topical fungal inoculation to determine if more in-depth trials should be conducted this regard.

Lastly some of the most practical knowledge gained through the trials described in this chapter related to beneficial and inhibitory factors affecting saccharification efficiency. Extractables, Na-azide being present at a concentration of (0.02%), and biologically-relevant xylose concentrations did not inhibit saccharification. Additionally the enzyme concentration did not appear to inhibit saccharification rather the NREL reconfirmed the enzyme concentration used as optimal for the given conditions. One factor, autoclave sterilization, did proved grounds for discussion. The glucose release from moist autoclave sterilization was improved compared to dry autoclaved and non-autoclaved biomass. And though this experiment resulted in low overall glucose yields, the potential lignin disruption or slight removal may in turn result in significantly enhanced biological pretreatment effects if inoculated after sterilization. This may explain other published white-rot pretreated lignocellulosic biomass trials reporting glucose yields after saccharification nearing 40% of theoretical (Wan and Li, 2011).

3.7 References

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FUTURE WORK

3.7.1 PEG transformation

An alternative method of transformation could be attempted for production of a positive transformant. Different transformation techniques integrate plasmid DNA into the target organism differently and at different frequencies. Comparing successful transformants from multiple techniques may be beneficial in choosing the most fit cultivar. The PEG mediated *Magnaporthe oryzae* transformation protocol provided by the Farman Lab can be modified for *P. chrysosporium* hyphal protoplasts. Two separate transformations could be performed to integrate *gus* containing pCT74 and *LacZ* containing pBS vectors into the target organism separately.

3.7.1.1 PEG transformation procedures

The PEG transformation would employ protoplasts of the *P. chrysosporium* strain ATCC 24725 prepared by adapting the *Magnaporthe* Protoplasting Protocol (Dr. Jinrong Xu's lab at Purdue University) found in Appendix B. These protoplasts could be transformed by performing the PEG Transformation of MagnaporthePprotocol (Appendix C.) modified for our target organism. The transformation could be performed separately with the vectors pCT74 and pBSII.

Linearization of the plasmid DNA has been found to be beneficial for transformation success (Dombrowski et al., 2011). The Eco R1 restriction site was determined to be a single cutter (using the nebcutter application from the neb website). An overnight digestion at 37℃ using the enzyme EcoR1 (Thermo Scientific #ERO271) at a concentration of 1µl in 33µl 10x buffer (provided with enzyme) to 300µl plasmid suspension could be used to linearize the plasmid. Plasmids can be then be purified in 1:1 phenol chloroform. The top precipitate DNA- containing layer can be re-suspended in two volumes of absolute ethanol and 1:10 Na-acetate (3M pH = 5.2). This mixture can be incubated at -20℃ for 20 minutes where then a pellet is formed by centrifugation in a cold chamber. The DNA pellet can be washed in 70% ethanol and stored at -40℃ until ready for use. A pre and post linearized sample of both vectors can be characterized by gel electrophoresis for linearization confirmation.

3.7.1.2 GUS

GusA transgene presence can be confirmed via X-Gluc histochemical assay. X-Gluc compounds are purchased through Sigma-Aldrich Co. Transformed and non-transformed cultures can be cultured for a standard time and prepared for assay following the same procedures. Assays performed on non-transformed organisms provide a negative control and a null background to observe any color difference between the transformed samples. Any detectable color difference indicates successful transformation in the transformed culture.

3.7.1.3 LacZ

LacZ transgene presence can be confirmed via X-gal histochemical assay. X-gal compounds are purchased through Sigma-Aldrich Co. Transformed and non-transformed cultures can be cultured for a standard time and prepared for assay following the same procedures. Assays performed on non-transformed organisms provide a negative control and a null background to observe any color difference between the transformed samples. *LacZ* expression in the pBSii plasmid is typically used to determine if a successful insertion into the multiple cloning sites (MCS) occurred. If the insertion were successful, the colonies would appear white. In this application the MCS was undisturbed and therefore the color of a successful transformant will report blue.

3.7.2 Enzymatic competence trials

Agrobacterium mediated transformation effectively inserts vector DNA into host genome, however the particular placement of the insertion is non-specific. The insertion could cause incomplete coding for other metabolizing functions that are essential for fungal implementation. To ensure vector DNA does not interfere with such activity an experiment could be conducted to compare enzymatic activity between the transformed species and the wild-type parent strain.

Cultures of each species will be inoculated into a volume of liquid media containing a specific amount of lignin. Conditions will be optimized for lignin degradation based upon cited examples. Lignin peroxidase, manganese peroxidase, peroxide forming enzymes, and other ligninase activities can be quantified via lignin degradation by the NREL protocol and sugar availability using HPLC analysis. Assay results will be

compared side by side to determine whether enzymatic activity between transformed and non-transformed cultures are statistically different.

3.7.3 Plasmid DNA viability

Agrobacterium mediated transformation effectively relocates a segment of DNA from the plasmid vector to the fungal host genome. Using this method to transform fungi is a relatively novel practice (within the past 16 years (de Groot et al., 1998)). Other forms of transformation only place the plasmid within the cell wall of the host; replication and expression of genetic information is extra-chromosomal. Because the gene will exist in the fungal genome via insertion mutation, it is likely the most robust means to ensure consistent reporter gene replication. However it is necessary to confirm the viability of the gene post-transformation.

A simple yes/no series of trials could be performed starting once a successful transformation has been verified. Duplicate PDA plates will be inoculated with transformant species and incubated at 35℃ for seven days in the absence of light. Upon the seventh day samples from each plate will be taken and plated onto X-GLUC agar plate. Additionally, a sample will be taken from each plate and placed on its own fresh PDA plate to begin the next week's trial. Samples taken for X-GLUC assay will be analyzed the following day. Trials will continue every week until the either genetic expression of *gus* gene is absent or until viability is confirmed. If a positive transformant fungal strain is produced with a vector not containing the *gus* gene then this experiment can be tailored to the reporter available.

3.7.4 Growth curve comparison

There are some inherent concerns when implementing transformed organisms. When adding genetic information energy is used by the organism to express the gene. The particular gene, *gusA*, will code for production of an enzyme which in the presence of a salt (5-bromo-4-chloro-3-indoyl f-D-glucuronide) will react to precipitate a substance blue in color. It is our understanding that this enzyme is only produced in the presence of the very unique salt and therefore does not demand metabolic energy to produce this enzyme at any other time. It is necessary however to confirm this theory. In order to confirm this, a series of side by side trials will be performed with two organisms (transformed sp. and wild-type sp.). Growth curves will be monitored (as described in

the next paragraph) over the course of a period of time (likely 1 to 2 weeks). Replicates of each scenario will be prepared for each trial. Trials will be varied in working volume (250ml and 5L).

In general: equivalent concentrations of inoculum for each type of organism (transformed sp. and wild-type) will be placed in liquid culture of PD broth media. Cultures will incubate in agitated, low light, growth temperature conditions (35℃) for a period of time. Each day upon the first day equivalent volumes will be taken from each sample (10ml). Such samples will be subject to serial dilution methods of cell counting. For trials at the 250ml flask scale X-Gluc assays will be performed randomly to ensure contamination or cross-culturing does not persist. It is likely there will not be an issue at this scale as observed from previous experiments. When trials increase in size to the 5L stage, it may become necessary to perform simultaneous X-Gluc assays on additional aliquot to determine level of contaminant presence.

A growth curve will be established for each scenario to determine whether or not transformant maximum growth rate is influenced by presence of gene.

3.7.5 Influence of sterility on effective pretreatment

To further support the claims made in chapter 3 regarding low glucose release in pretreated unsterile biomass a side by side single variable experiment can be performed. On a scale suitable for sterilization of biomass sterile and non-sterile switchgrass can be pretreated for 14 days with *P. chrysosporium* at the conditions specified in section 3.3. Sterile switchgrass can be prepared via 60 minute autoclave cycle at 121℃. Samples from each layer can be analyzed through the saccharification technique previously described in section 3.3.6. Results could determine if a significant difference in pretreatment effectiveness is due to biomass sterility.

3.7.6 Re-inoculation

Based upon the findings in the previously described experiment and other preliminary trials a single biological pretreatment application followed by saccharification yields sugars in concentrations much lower than desirable. Improving the biological pretreatment activity and saccharification conditions is one option for increasing glucose yield. An alternative would be to work with the productivity of the technology at hand

and attempt to repeat the process on the same biomass. Multiple pretreatment and saccharification loops may allow for extraction of sugars nearing the theoretical potential.

Initially the experiment should be performed on a bench-top scale with a biomass samples size between 1g to 250g. Furthermore biomass should be initially sterilized via wet autoclave sterilization. These conditions allow for a near controlled microbial population. If preliminary findings look promising the experiment should be performed with larger amounts of biomass and in unsterile conditions. Biological pretreatment conditions for the initial experiment should model the conditions described in the previously described experiments. The saccharification step will be a point of discussion. This is because commercial enzyme saccharification requires a microbial inhibitor (i.e. Sodium-azide) to reduce sugar losses through bacterial metabolism at the elevated temperatures. If the saccharification is followed by a subsequent fungal inoculation will the microbial inhibitor also inhibit the fungi? The microbial inhibitor may denature by the time of a re-inoculation. These parameters will need to be investigated to ensure an effective subsequent pretreatment. The saccharification may also be performed by active bacteria such as *Clostridium thermocellum*. An antimicrobial may be unnecessary depending upon the co-habitation characteristics of the fungal and bacterial species. If a simple fluctuation in temperature could turn on one process and off the other the pretreatment/saccharification could be performed rapidly without externally re-inoculating. Preliminary trials are currently being performed to determine the viability of this possibility.

The pretreatment/saccharification should be repeated at a minimum of three times to reveal any yield rate change between loops. If the process continuous to yield between 10% and 20% of theoretical sugar each round the process could be performed eight to ten times. If such a process can remove as much as 50% of theoretical it could become a viable alternative to chemical technologies.

APPENDICES

Appendix A. **Farman Lab: *Magnaporthe oryzae* transformation using *Agrobacterium tumefaciens* mediated method (modified for *P. chrysosporium*)**

Fungal strain and culture conditions:
Activate from stock on oatmeal agar (25 g/l). Grow for 7-14 days under continuous illumination.

Use sterile water and a plastic spreader to harvest conidia. Quantify spore number using a hemacytometer. Centrifuge at 4000 rpm for 10 minutes, then re-suspend in Induction Medium (IM) to the desired concentrations. (We typically dilute the spores to a concentration of 10^6/ml).

Transformation:
Activate the Agrobacterium from storage to solid LB+50 µg/ml Kanamycin at least 72 hours prior to the experiment. Incubate at 29℃ for 40 h at 250 rpm. Poor suspension onto IM plate and allow to grow overnight or until individual colonies form.
(**IM requires the addition of Thiamin and Acetosyringone).

Use a blue tip (1000µl) to pipette 100 µl of the spore suspension. Drop the suspension on the center of the nitrocellulose membrane placed on the co-cultivation (CC) medium (**nitrocellulose membrane is also known as #1 filter paper that has been cut into small blocks and sterilized by autoclave).

Eject the blue tip from the pipette and use it to pick a single Agrobacterium colony. Then use the same tip to spread the spore suspension to cover the whole surface of the membrane. This allows Agro cells and fungal spores to be mixed on the membrane. Allow the membrane to dry in the hood for approximately 10 minutes. Incubate the plate for 2 days at room temperature.

Transfer the membrane onto selection medium (CM) containing Hygromycin B (200µg/ml). Incubate at room temperature for 5-7 days before isolating Hygromycin B-resistant colonies.

Notes

This procedure generates more than five colonies on a single membrane.

To set up multiple transformations (e.g., two or three membranes per constructs), repeat the steps 2 and 3 with different Agro colonies.

Induction Medium – Solid (IM) [100 ml]

Minimal Salts	4 ml
1 M MES (pH 5.3)	4 ml
1 M glucose	0.5 ml
100% glycerol	0.5 ml
Agar	1.5 g

Autoclave

Immediately after cooling medium drops to 50℃ add:

AS (100mM)	0.2 ml
Thiamin (1 g/l)	100 µl (to 1 µg/l)

Minimal Salts

(25X Strength Stock)

Dissolve successively:

$Na_3citrate$ (5.5 H_2O) in 750 ml water	150 g
[or $Na_3citrate$ (2 H_2O) in 775 ml water]	125 g
KH_2PO_4, anhydrous	250 g
NH_4NO_3, anhydrous	100 g
$MgSO_4$ (7 H_2O)	10 g
$CaCl_2$ (2 H_2O)	5 g

**dissolve separately in 20 ml water and ad the solution slowly

Biotin stock solution	2.5 m l
Trace element solution (i.e. Wolf's)	5 ml

Store at 4℃

Complete Medium – solid (SELECTION) [300 ml]

Sucrose	3 g
Yeast extract	1.8 g
Casamino acids	1.8 g
Agar	4.5 g

Autoclave

Immediately after cooling medium drops to 50℃ add:

Thiamin (1 g/l)	300 µl (to 1 µg/l)
Hygromycin B (100 mg/ml)	600 µl (to 200 µg/l)
Tecarcillin (150 µg/ml)	

Appendix B. *Magnaporthe* Protoplasting Protocol

(from Dr. Jinrong Xu's lab at Purdue University)

Day 1

Slice 1 square inch of 2 week old oatmeal culture and blend it for 30 seconds with 50 ml complete medium (CM) containing 100 µg/ml ampicillin. Transfer mixture to a 250 ml flask and shake O/N at room temperature.

Day 2

Blend the culture for 30 seconds and pour mixture back to the 250 mixture flask. Add 50 ml CM containing 100µg/ml ampicillin. Shake overnight at room temperature.

Day 3

1. Collect mycelia by filtering through a sterile Miracloth. Wash mycelia with 1M sorbitol twice.
2. Re-suspend the mycelia well in 50 ml 1M sorbitol containing 1-2 mg NOVOZYME lysing enzyme per ml.

   Alternative: 10 mg/ml Sigma lysing enzyme
3. Incubate at 30-32℃ for 2 hours or less with shaking at 90 rpm. Check the stage of protoplasting with a light microscope after 1 hour.
4. Collect protoplasts by filtering through Miracloth.
5. Rinse with 50 ml 1M sorbitol to recover protoplasts from Miracloth.
6. Spin down protoplasts at 5000 rpm for 15 minutes at room temperature. Dump supernatant.
7. Re-suspend the pellet with 20-40 ml 1x SuTC.
8. Spin down protoplasts and re-suspend in 1x SuTC to a concentration of $5x10^7 - 1x10^8$ protoplasts/ml.
9. You can stop here and store the protoplasts at -80℃ with or without 7% DMSO.

Reagents and Media

1. Complete Medium

0.6% Yeast Extract

0.6% Casamino Acids

1% Sucrose

2. 1M Sorbitol
3. 500 ml 1x SuTC

    20% Sucrose

    50 mM Tris-HCl, pH 8.0

    50 mM $CaCl_2$

Autoclave Tools

1. Blenders
2. Funnels lined with Miracloth

Appendix C. **PEG Transformation of *Magnaporthe***

(from Dr. Farman's Lab at the University of Kentucky)

Day 1

1. In a 50 ml centrifuge tube combine DNA and 100µL of protoplasts.
2. Let sit for 20 minutes at room temperature. During this time combine 9 ml 66% PEG, 0.5 ml (50mM) 1M Tris-HCl pH 7.5 and 0.5 ml (50mM) $CaCl_2$ (Total volume 10 ml PTC; 60% PEG). Mix on a tube rotator.
3. Remove ejector from a p1000 pipette. Sterile barrel using 70% ethanol. Add 1 ml of PTC to DNA and protoplasts dropwise and mix well by stirring with tip after addition of each drop. Continue until entire 1 ml has been added. Add another 1 ml in a straight shot and mix well. Let sit for 20 minutes at room temperature.
4. Add SuTC to a total volume of 20 ml.
5. Centrifuge for 5 minutes at 5000 rpm. Pour off supernatant.
6. Add 5 ml Regeneration Medium (RM) and incubate O/N on bench.

Day 2

1. Check germination of protoplasts under the microscope.
2. Prepare Complete Medium (CM) – 100 ml in 250 ml flask/sample, autoclave, then cool in water bath to 50℃. Cool further until flask can be held comfortably in your hand. Flame outside of flask to sterilize. Allow to cool for a couple of minutes.
3. Pour cooled CM into 50 ml centrifuge tube containing DNA, protoplasts, and RM to temper. Then pour back into flask and mix well.
4. Pour plates- just until covers the bottom of the plate (25 ml). (100 ml flask of medium = 4plates)
5. Allow to solidify, then place on lighted shelf overnight.

Day 3

1. Prepare 1.5% Water Agar (WA)- 15 ml/plate, autoclave, then cool in water bath to 50℃. Cool further until flask can be held comfortably in your hand. Flame outside of flask to sterilize. Allow to cool for a couple of minutes.
2. Add Hygromycin B to a total plate concentration of 200 µg/ml (actual concentration going into 1.5% WA will be 2.66 x 200 µg/ml). Use 15 ml centrifuge tube to pour into plates.
3. Allow to solidify, then place on lighted shelf until transformants appear.
4. Pick onto Oatmeal Agar (OA) + Hyg plates.

Solutions and Media for PEG Transformation

Regeneration Medium (RM)

0.6% Yeast Extract

0.6% Casamino Acids

20% Sucrose

Autoclave

Complete Medium (CM)

0.6% Yeast Extract

0.6% Casamino Acids

20% Sucrose

1.5% Agar

Autoclave

1.5% Water Agar (WA)

15 g Agar

1000 ml Water

Autoclave

SuTC

20% Sucrose

50 mM Tris-HCl, pH 8.0

50 mM $CaCl_2$

Filter sterilize into autoclaved bottle.

Appendix D. **Fruiting media**

In 1L

1 ml 5x Wolf's trace minerals

Ingredient	1 L
Milli-Q H_2O	500 ml
Nitrilotriacetic Acid	1.5 g
$MgSO_4$ * 7 H_2O	3.0 g
$MnSO_4$ * H_2O	0.5 g
NaCl	1.0 g
Fe SO_4 * 7 H_2O	0.1 g
$CoCl_2$ * 6 H_2O	0.1 g
$CaCl_2$	0.1 g
$ZnSO_4$ * 7 H_2O	0.1 g
$CuSO_4$ * 5 H_2O	0.01 g
$AlK(SO_4)_2$ * 12 H_2O	0.01 g
Na_2MnO_4 * 2 H_2O	0.01 g
Complete volume with Milli-Q H_2O to	1000 ml

Use iron-free beaker, stir bar, canulas, and disposable pipettes

1. Put 500 ml milli-Q water into a beaker with a stir bar
2. Add nitrilotriacetic acid
3. Wait for the NTA to dissolve fully.
4. Adjust pH of the solution to 6.5 with KOH
    a. When the pH approaches 5.0 use a lower normality base such as 5N or even 2N.
5. Once the solution is at a sufficient pH add the rest of the ingredients one at a time.
6. Complete volume with milli-Q water in a graduated cylinder

10 ml Basal III medium

Ingredients	1 L
KH2PO4	20 g
MgSO4	5 g
CaCl2	1 g
Complete volume with Milli-Q H_2O to	1000 ml

0.12 g	Na acetate
10 g	glucose
0.8 g	saponin (Sigma, catalog no. S1252)
3 g	malt extract
18 g	agar

pH 4.5 acetic acid

Autoclave

Let cool to 65 C before addition of thiamin – HCl to final concentration of 1.2 mg/L

Appendix E. **Bradford Method for Protein Determination**

Assay prepared by Pedro Ricardo (University of Kentucky)

Enzyme stock:

3g/l enzyme stock- 0.30 g enzyme powder
100 ml DI

Assay:

1.5 ml Bradford Reagent (Sigma-Aldrich)

50 µl sample

Blank:

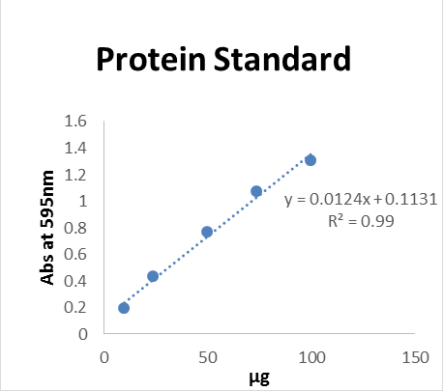
1.5 ml Bradford Reagent (Sigma-Aldrich)

50 µl DI

1. Prepare triplicates of Assay mixtures and a single blank in test tubes. Vortex for 15 seconds or pipet mix.
2. Pipet 1.5 ml of contents of each tube to individual cuvette.
3. Read absorbance at 595 nm. Subtract out blank from each assay sample.
4. Determine mass of protein using the standard curve equation below.
5. Multiply by 20 to determine µg protein/ml of stock.

Note: Curve is only suitable for protein contents up to and not exceeding 2mg/ml. Original stock may need to be adjusted to fit within the range of the curve.

Following standards prepared by Pedro Ricardo using bovine albumin serum:



Results for American Laboratories Inc. enzyme:

Stock Enzyme Dilution:	3.0 g/l
Protein:	0.318 mg/ml

Appendix F. **B-Glucosidase Enzymatic Activity Assay**

Dr. Nokes Lab Protocol (University of Kentucky – Biosystems & Agricultural Engineering)

Prepared by Bobby Carey and Pedro Vieira Hamann (August 2014)

Principal:

Cellobiose is a disaccharide composed of two glucose units connected by a β (1-4) glycosidic bond. A handful of enzymes can cleave this bond to produce two separate glucose units however most of this activity is attributed to B-Glucosidase. In this Assay a substrate known as 4-Nitrophenyl β-D-glucopyranoside (PNPG) containing the same linkage is hydrolyzed by B-Glucosidase. When the PNPG molecule is cleaved it results in two separate molecules – Glucose and a Nitrophenol (PNP). When the assay is terminated by addition of NaCO_3 the Nitrophenol and the NaCO_3 bond resulting in the intense color change from clear to yellow. The level of absorbance can be correlated to the abundance of free Nitrophenol which is related to the activity of the enzyme. Using a standard curve produced using known concentrations of PNP a measurement of B-Glucosidase activity can be made.

Reagents:

1. 0.05M Na-Citrate Buffer – Begin by preparing a stock of 1M Na-Citrate
    1. 84g Citric Acid Monohydrate C_6 H_8 O_7·H_2 O
    2. 300ml DI H_2 O
    3. Add NaOH until pH = 4.5 (Should be around 15-18g)
        4. Dilute to 400ml and check pH (if necessary add NaOH until pH = 4.5); you now have a 1M stock of Na-Citrate
        5. To make 100 ml of 0.05M Na-Citrate add 5ml 1M Na-Citrate to 95ml DI H_2 O. When diluted to 0.05M, pH should equal 4.8
2. 4mM 4-Nitrophenyl β-D-glucopyranoside (Sigma-Aldrich/500mg) PNPG- add 0.120mg of PNPG to 100ml of 0.05M Na-Citrate Buffer.
3. 4-Nitrophenol solution (Sigma-Aldrich/100ml) PNP

4. Enzyme stock-combine desired amount (g) of enzyme with 100ml DI water. *A good place to start when using lyophilized powder enzyme is 0.5g/l. The goal is to use a stock that will place the assay readings within the range of the standard curve. If the value does not fall in the range, the assay should be reattempted adjusting the enzyme stock or adjusting the volume of enzyme aliquot used for the assay.
5. 1M NaCO_3- combine 10.599 g with 100ml DI. Or if you plant to do the assay again in the near future make a larger volume and store on the bench top.

Assay Procedures:

1. Prepare water bath at 50℃.
2. Prepare standards by combining the following in individual test tubes.

PNP (µL)	DI H_2O (µL)
0	200
5	195
10	190
15	185
20	180
25	175
30	170

*Total volume in each tube should be 200µL

3. Prepare enzyme assay tubes by pipetting 100µL of 4mM PNPG into empty test tube. Prepare in triplicate. Do not add enzyme yet.
4. Prepare control by adding 100µL of 4mM PNPG into empty test tube. If you are testing a range of enzyme dilutions – prepare controls for each dilution. Prepare all controls in triplicate.
5. Place all tubes in rack and into the water bath at 50℃ for 10 minutes to acclimate. *The standard temperature is 37℃ for this assay however if you want to know your enzyme activity at the temperature at which you plan to perform your hydrolysis then perform the assay at that temperature.

6. After the 10 minute acclimation time. Quickly add 100µL of enzyme stock to each assay tube. Do not add enzyme to standards or control.

   *You can use one dilution of enzyme stock or multiple in attempt to ensure readings fall within the standard curve range. But prepare each in triplicate.

7. Incubate in water bath for exactly 30 minutes.
8. After the incubation period is up remove from water bath and quickly add 1ml of 1M NaCO_3 to all tubes. This will stop the reaction.
9. Add 100µL of enzyme dilution to the control. If testing multiple dilutions, ensure the appropriate enzyme dilution is mixed with the corresponding control.

   *No color change should be visually observed in controls after addition of enzyme solution.

10. Poor contents of each tube into cuvette and read absorbance at 400nm.

Analysis:

1. Plot the standard absorbance readings on a graph. Y-axis: Abs at 400nm, and X-axis: µmol of PNP. Fit a line and equation for the standard curve.
2. Determine the average absorbance for the enzyme assay samples then subtract out the average control for the corresponding sample. Using equation from standard curve line determine µmol of PNP for the enzyme assay samples.
3. Divide this number by the reaction time (30min) to determine the international units (IU)in µmol/min. IU are sometimes wrote as pNPGU or PNPGU.
4. Divide that number by the volume of enzyme in the reaction (0.1ml) to determine Units per ml of original stock.
5. Divide that number by mg of protein in the enzyme stock to retain the most useful unit which is in IU/mg protein. Use the Bradford method of protein determination to find mg of protein in the original stock.

Appendix G. **Bin Sampling Observations**

Treatment	Bin	Layer	Sample MC (%)	Water Activity (aw)	Sample Initial Temp (℃)
S-1	NA	NA	5.3	NA	NA
S-2	NA	NA	4.4	NA	NA
S-3	NA	NA	4.4	NA	NA
S-4	NA	NA	5.8	NA	NA
S-5	NA	NA	5.4	NA	NA
S-6	NA	NA	5.7	NA	NA
C	A	Top	46.4	0.987	46.4
C	A	Mid	57.5	0.983	36.1
C	A	Bot	70.7	0.993	31.2
C	B	Top	53.0	0.991	33.7
C	B	Mid	61.9	0.99	31.5
C	B	Bot	66.8	0.997	30.8
C	C	Top	54.9	1.001	34.1
C	C	Mid	65.9	1	35.0
C	C	Bot	76.0	1.001	28.1
1	A	Top	55.2	1.004	32.0
1	A	Mid	67.2	0.91	35.9
1	A	Bot	77.6	0.997	31.8
1	B	Top	66.8	0.997	27.6
1	B	Mid	66.2	0.996	31.1
1	B	Bot	73.9	0.997	32.5
1	C	Top	60.4	0.997	29.9
1	C	Mid	64.0	1	32.7
1	C	Bot	71.3	0.999	30.9

1*	A	Top	51.5	0.888	23.9
1*	A	Mid	40.7	0.968	24.8
1*	A	Bot	34.3	0.987	24.4
1*	B	Top	63.2	0.954	23.3
1*	B	Mid	29.9	0.994	25.3
1*	B	Bot	22.3	0.98	24.8
1*	C	Top	34.5	1.005	25.6
1*	C	Mid	36.1	0.969	24.7
1*	C	Bot	30.0	0.974	26.3
2	A	Top	73.6	0.988	36.6
2	A	Mid	68.0	0.989	28.8
2	A	Bot	77.1	0.994	36.5
2	B	Top	68.6	1.007	25.8
2	B	Mid	60.1	0.991	24.2
2	B	Bot	76.1	0.999	21.4
2	C	Top	73.6	1	30.6
2	C	Mid	69.8	1	26.9
2	C	Bot	79.0	0.999	31.7

Notes: Treatments: S-x are raw samples; C is control samples; 1 and 2 are Treatment 1 and Treatments 2; and 1* are the samples taken from treatment 1 two weeks after initial inoculation and immediately prior to placing in environmental chamber.

Treatment 1 Bin A: Large areas consumed with contaminant organisms. Patches of P. chrysosporium growth.	
Treatment 1 Bin B: Observable hyphal-biomass interaction. Biomass partly consumed in contamination presenting black hyphae.	
Treatment 1 Bin C: Picture shows the top of the middle layer. Similar to all Treatment 1 bins reduced conidial growth observed below top layer.	

Treatment 2 Bin A: Observable bleaching effect and abundant conidial growth.	
Treatment 2 Bin B: Most significant bleaching effect.	
Treatment 2 Bin C:	

Appendix H. **Saccharification Raw Data**

Treatment	Layer	Type	Glucose (g/l)		Treatment	Layer	Type	Glucose (g/l)		Treatment	Layer	Type	Glucose (g/l)
Control A	Top	Blank	0.11		Control B	Top	Blank	0.27		Control C	Top	Blank	0.14
Control A		a	0.96		Control B		a	1.23		Control C		a	1.11
Control A		b	0.93		Control B		b	1.11		Control C		b	1.22
Control A		c	0.94		Control B		c	1.29		Control C		c	1.22
Control A	Mid	Blank	0.1		Control B	Mid	Blank	0.26		Control C	Mid	Blank	0.22
Control A		a	0.97		Control B		a	1.24		Control C		a	1.5
Control A		b	0.94		Control B		b	1.11		Control C		b	1.31
Control A		c	0.96		Control B		c	1.33		Control C		c	1.18
Control A	Bot	Blank	0.13		Control B	Bot	Blank	0.26		Control C	Bot	Blank	0.12
Control A		a	1.1		Control B		a	1.18		Control C		a	1.25
Control A		b	0.97		Control B		b	1.19		Control C		b	1.24
Control A		c	0.9		Control B		c	1.21		Control C		c	1.58
Trx 1 A	Top	Blank	0.14		Trx 1 B	Top	Blank	0.13		Trx 1 C	Top	Blank	0.25
Trx 1 A		a	0.9		Trx 1 B		a	1.03		Trx 1 C		a	1.42
Trx 1 A		b	0.92		Trx 1 B		b	1.06		Trx 1 C		b	1.36
Trx 1 A		c	0.95		Trx 1 B		c	1.3		Trx 1 C		c	1.17
Trx 1 A	Mid	Blank	0.16		Trx 1 B	Mid	Blank	0.16		Trx 1 C	Mid	Blank	0.15
Trx 1 A		a	1.1		Trx 1 B		a	1.02		Trx 1 C		a	1.21
Trx 1 A		b	1.44		Trx 1 B		b	1.08		Trx 1 C		b	1.48
Trx 1 A		c	0.9		Trx 1 B		c	1.01		Trx 1 C		c	1.61
Trx 1 A	Bot	Blank	0.12		Trx 1 B	Bot	Blank	0.11		Trx 1 C	Bot	Blank	0.15
Trx 1 A		a	0.94		Trx 1 B		a	1.17		Trx 1 C		a	1
Trx 1 A		b	1.02		Trx 1 B		b	1.01		Trx 1 C		b	1.24
Trx 1 A		c	1.32		Trx 1 B		c	1.06		Trx 1 C		c	1.02
Trx 2 A	Top	Blank	0.32		Trx 2 B	Top	Blank	0.53		Trx 2 C	Top	Blank	0.36
Trx 2 A		a	1.25		Trx 2 B		a	2.19		Trx 2 C		a	1.4
Trx 2 A		b	1.27		Trx 2 B		b	1.64		Trx 2 C		b	1.44
Trx 2 A		c	1.17		Trx 2 B		c	1.74		Trx 2 C		c	1.47
Trx 2 A	Mid	Blank	0.46		Trx 2 B	Mid	Blank	0.52		Trx 2 C	Mid	Blank	0.48
Trx 2 A		a	1.47		Trx 2 B		a	1.74		Trx 2 C		a	2
Trx 2 A		b	1.71		Trx 2 B		b	2		Trx 2 C		b	1.27
Trx 2 A		c	1.55		Trx 2 B		c	1.67		Trx 2 C		c	1.75
Trx 2 A	Bot	Blank	0.28		Trx 2 B	Bot	Blank	0.43		Trx 2 C	Bot	Blank	0.14
Trx 2 A		a	1.16		Trx 2 B		a	1.47		Trx 2 C		a	0.9
Trx 2 A		b	0.96		Trx 2 B		b	1.35		Trx 2 C		b	1.48
Trx 2 A		c	0.85		Trx 2 B		c	1.27		Trx 2 C		c	1.38
Raw S-1		Blank	0.16		RawS-3		Blank	0.16		Raw S-5		Blank	0.29
Raw S-1		a	1.32		Raw S-3		a	1.46		Raw S-5		a	1.55
Raw S-1		b	1.28		Raw S-3		b	1.48		Raw S-5		b	1.47
Raw S-1		c	1.3		Raw S-3		c	1.46		Raw S-5		c	1.5
Raw S-2		Blank	0.14		Raw S-4		Blank	0.29		Raw S-6		Blank	0.17
Raw S-2		a	1.32		Raw S-4		a	1.53		Raw S-6		a	1.48
Raw S-2		b	1.35		Raw S-4		b	1.52		Raw S-6		b	1.44
Raw S-2		c	1.35		Raw S-4		c	1.5		Raw S-6		c	1.5
Enzyme A	Blank	a	0.63		Enzyme B	Blank	a	0.73		Enzyme C	Blank	a	0.74
Enzyme A	Blank	b	0.62		Enzyme B	Blank	b	0.74		Enzyme C	Blank	b	0.74
Enzyme A	Blank	c	0.64		Enzyme B	Blank	c	0.74		Enzyme C	Blank	c	0.73
					Enzyme B	Blank	d	0.72		Enzyme C	Blank	d	0.73
					Enzyme B	Blank	e	0.74		Enzyme C	Blank	e	0.73
					Enzyme B	Blank	f	0.73		Enzyme C	Blank	f	0.73

Appendix I. **Saccharification analysis**

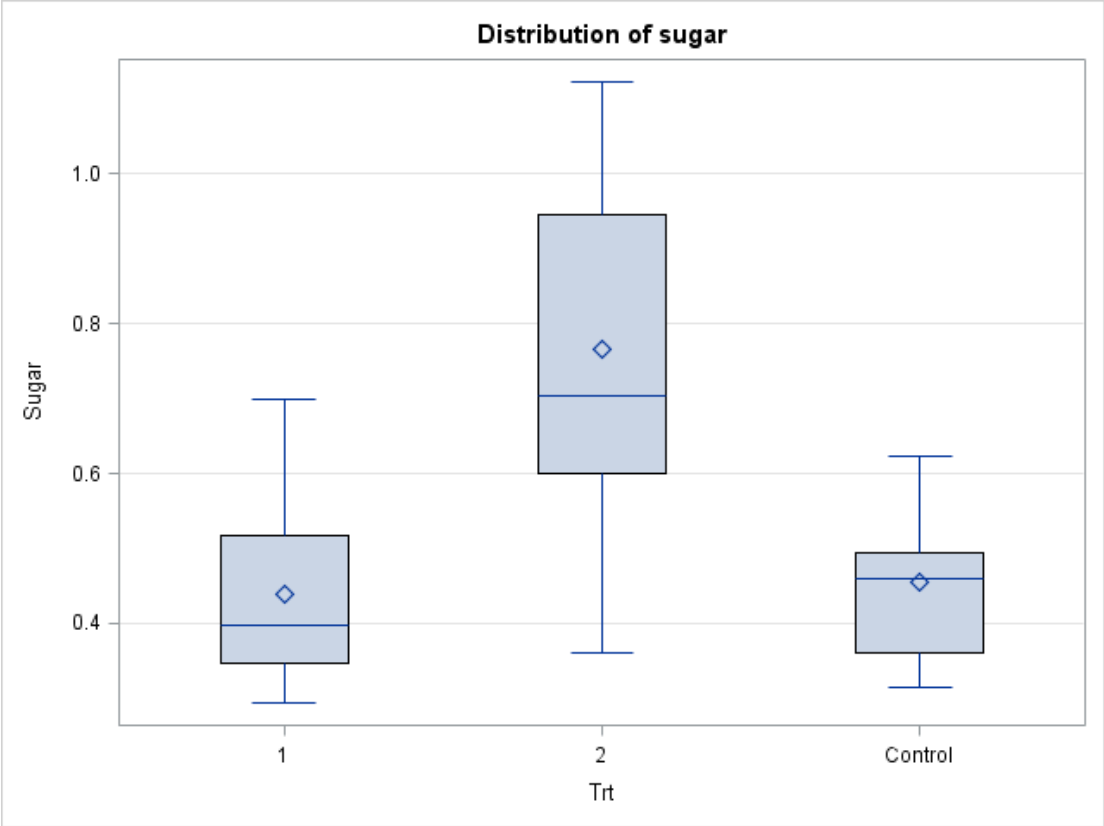
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	1.28161235	0.09154374	8.15	0.0004
Error	12	0.13470617	0.01122551		
Corrected Total	26	1.41631852			

R-Square	Coeff Var	Root MSE	sugar Mean
0.904890	19.13488	0.105951	0.553704

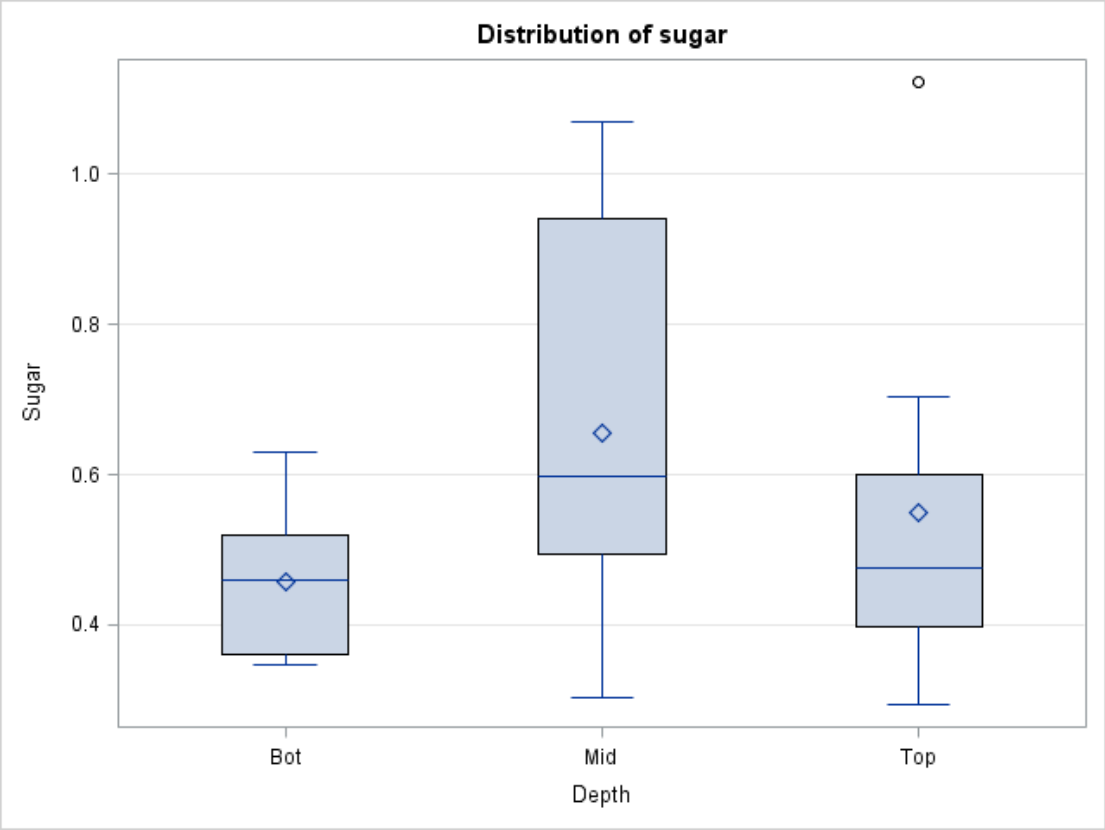
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	0.60915802	0.30457901	27.13	<.0001
Bin(Trt)	6	0.28496049	0.04749342	4.23	0.0161
Depth	2	0.17567654	0.08783827	7.82	0.0067
Trt*Depth	4	0.21181728	0.05295432	4.72	0.0161

Tests of Hypotheses Using the Type I MS for Bin(Trt) as an Error Term					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Trt	2	0.60915802	0.30457901	6.41	0.0324

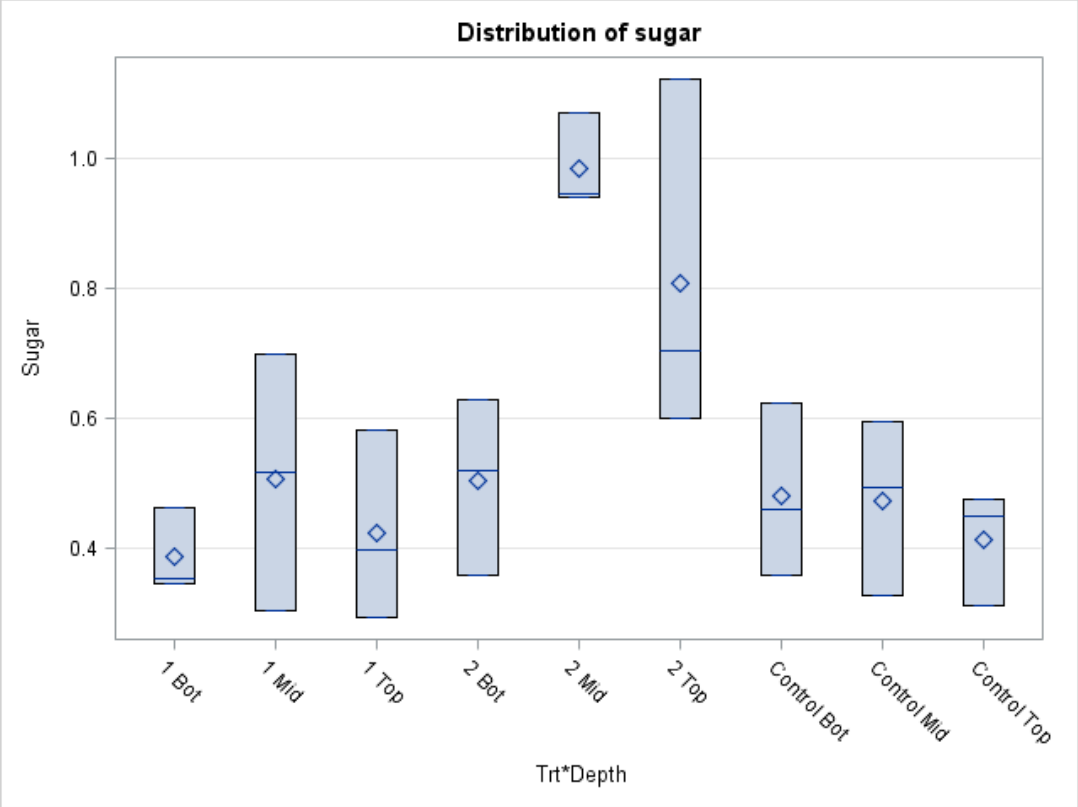
The GLM Procedure



Level of Trt	N	sugar	
		Mean	Std Dev
1	9	0.43962963	0.13822731
2	9	0.76592593	0.26418521
Control	9	0.45555556	0.10951915



Level of Depth	N	sugar	
		Mean	Std Dev
Bot	**9**	0.45740741	0.11366102
Mid	**9**	0.65481482	0.27854339
Top	**9**	0.54888889	0.25411612



Level of Trt	Level of Depth	N	sugar	
			Mean	Std Dev
1	**Bot**	**3**	0.38777778	0.06551788
1	**Mid**	**3**	0.50666667	0.19852232
1	**Top**	**3**	0.42444444	0.14698198
2	**Bot**	**3**	0.50333333	0.13576941
2	**Mid**	**3**	0.98555556	0.07320696
2	**Top**	**3**	0.80888889	0.27717490
Control	**Bot**	**3**	0.48111111	0.13292995
Control	**Mid**	**3**	0.47222222	0.13623237
Control	**Top**	**3**	0.41333333	0.08762293

SAS code:

```
PROC IMPORT OUT=sugar
DATAFILE="C:\Users\bdca227\Desktop\Sugar.xlsx"
DBMS=xlsx REPLACE;
SHEET="Sheet4";
GETNAMES=yes;
RUN;
proc sort data=Sugar;
     by trt bin depth;
run;
proc means data=Sugar mean noprint;
     by trt bin depth;
     var sugar;
     output out=data mean=sugar;
run;

proc glm data=data plots(unpack)=all;
     class trt bin depth;
    model sugar=trt bin(trt) depth depth*trt/ss1;
    test h=trt e=bin(trt);
     means trt depth depth*trt;
   run;
```

Appendix J. **Inhibition and substrate effect data**

Treatment	Sample Type	Glucose Recovered (g/l)	Adjusted Average (g/l)
Standard	Enzyme Blank	0.73	0.56
	Substrate Blank	0.17	
	a	1.5	
	b	1.44	
	c	1.44	
Without Na-azide	Enzyme Blank	0.75	-0.12
	Substrate Blank	0.14	
	a	0.64	
	b	0.97	
	c	0.7	
Avicel	Enzyme Blank	0.74	4.44
	Substrate Blank	0.01	
	a	5.29	
	b	5.28	
	c	4.99	
Dry Sterilization	Enzyme Blank	0.72	0.53
	Substrate Blank	0.14	
	a	1.37	
	b	1.41	
	c	1.38	
Wet Sterilization	Enzyme Blank	0.74	0.74
	Substrate Blank	0.16	
	a	1.61	
	b	1.67	
	c	1.64	
Xylose 1.0x	Enzyme Blank	0.75	0.54

	Substrate Blank	0.26	
	a	1.55	
	b	1.54	
	c	1.55	
Xylose 0.5x	Enzyme Blank	0.73	0.58
	Substrate Blank	0.22	
	a	1.54	
	b	1.53	
	c	1.51	
Xylose 0.1x	Enzyme Blank	0.71	0.62
	Substrate Blank	0.17	
	a	1.52	
	b	1.49	
	c	1.49	
Flask	Enzyme Blank	0.81	0.70
	Substrate Blank	0.17	
	a	1.68	
	b	1.65	
	c	1.7	

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VITA

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Education

University of Kentucky, Lexington KY, 2012-present

Masters of Science - Biosystems and Agricultural Engineering, (3.64/4.0)

Eastern Kentucky University, Richmond KY, 2007-2011

Bachelor of Science – Biology (3.35/4.0)

Certification

Engineer in Training State of Kentucky (Passed Fundamentals of Engineering Exam: Fall 2013)

Awards and Honors

Education Abroad at UK Scholarship

Alpha Epsilon, 2014

Research Assistantship (USDA), 2012-present

Regents Scholarship, 2007-20011

U.S. Marine Corps Junior ROTC Scholarship

Research Experience

Masters Research: Biological Pretreatment Applications Department of Biosystems and Agricultural Engineering, University of Kentucky, 2012- present, (research adviser: Dr. Sue Nokes).

- Design and perform biological pretreatment applications to effectively and efficiently degrade lignin and expose carbohydrates in biomass for lignocellulosic fuel conversion processes.
- Protocol design for up-scaling microorganism growth and enzyme activity.
- Transform fungi and bacteria for swift and accurate target species identification.
- Participate in field research on lignocellulosic biomass variety trials and harvest operations.

- Study Abroad Brazil (2014): A look in to modern and traditional agriculture practice and value added products.

Undergraduate Research: Copperhead Environmental Consulting, Paint Lick, KY, 2011, (supervisor: Mark Gumbert).

- Monitor bat populations across the Central and Eastern U.S. through field survey mist netting.
- Identify hibernacula and range of individual bats through tagging and radio telemetry tracking.
- Survey bat species through ultrasonic monitoring.

Teaching and Leadership Experience

Research Coordinator: University of Kentucky, 2012-Present.

- Managed and advised undergraduate researchers (5).

Trip Leader: Adventure Programs, Eastern Kentucky University, 20010-2011.

- Led student participants on a variety of outdoor recreation trips including hiking, climbing, mountain biking, orienteering, paddling, etc. over a range of durations up to a week in length.
- Designed and led workshops and demonstrations to groups of students on topics including wilderness survival, expedition nutrition, kayak roll clinics, and climbing and biking technical skills.

Adventure Guide: American Canadian Expeditions, Oak Hill WV, 2009-2010.

- Led participants in navigating class IV-V whitewater on the New and Gauley rivers while building hard skills, teamwork, and informing them on the cultural and natural history of the area.
- Led participants on mountain bike and zip line trips in the New River Gorge area.

Presentations

- Institute of Biological Engineering (IBE) International Conference, Lexington KY, 2014. *Rapid Genetic Identification of Bioprocess Organisms*
- American Society of Agricultural and Biological Engineers (ASABE) International Meeting, Montreal Canada, July 2014. *Rapid Genetic Identification of Phanerochaete chrysosporium during Pretreatment of Lignocellulose*