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DEPARTMENT OF CHEMICAL ENGINEERING

APPLICATION OF TWO DIFFERENT FUNGAL SPECIES FOR BIOLOGICAL PRETREATMENT IN AN INTEGRATED LIGNOCELLULOSIC BIOFUELS PARADIGM

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ABSTRACT

The push for a replacement of fossil fuels with renewable energy sources has stimulated research in the area of liquid biofuels. Lignocellulosic biofuels are considered "second-generation" and would improve upon current liquid biofuel technologies, the majority of which create competition between food and fuel. In order to achieve sufficient production levels, pretreatment of the lignocellulosic material is necessary. Fungal pretreatment by white-rot fungi has been recognized as a possible alternative to expensive, energy intensive pretreatments such as steam hydrolysis. Two white-rot fungi, *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, are examined in this study for their potential use as a fungal pretreatment prior to a consortium mediated bioprocess, which is achieved by a bacteria and yeast co-culture. In this study, preliminary growth observations were made for the two fungi that give relevant insight into the complications that may arise in trying to effectively compare their performance as a pretreatment. Although growth was accomplished for both of these organisms, the growth rate of each one is different depending on the substrate. A method was developed to prepare fungal degraded samples for Sum-frequency Generation (SFG) but it is suggested that this method be further developed for future analyses. Preliminary data is difficult to interpret without acquiring more quantitative information about lignin and cellulose degradation. Also, preliminary efforts were made at obtaining carbon, nitrogen, and energy balance information surrounding the pretreatment by each of these different species of white-rot fungi. Results showed the optimal growth of *P.chrysosporium* led to a shift of 10% of the nitrogen from biomass into chitin, a shift that could potentially make this nitrogen accessible for *C. phytofermentans*, the bacteria of interest in the consortium bioprocess. Based on this work it appears that with improvements in fungal culturing, a large scale biomass conversion process that utilizes fungal pretreatment may be a viable alternative that is compatible with a fully integrated biofuel production paradigm.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Wayne Curtis for allowing me to participate in research studies in his lab over the past two years. His guidance and dedication to creating an outstanding research environment has led to exceptional work in his laboratory. I have always admired his ability to push me and all students that he teaches to think more individually and intellectually.

I would like to give special thanks to Trevor Zuroff and Alex Rajangam for their close guidance during this project and my entire two years in the lab. I started work in 2012 with Trevor, in which he taught me many valuable skills, sometimes ones that were even out of the scope of this project. I continued working closely with both Trevor and Alex throughout my two years in lab, in which both of them were always available for questions and discussions.

I would like to thank Patrick Hillery for his dedication to this project, and for assisting with some analytical methods. Patrick trained me in use of bomb calorimetry, and was responsible for obtaining the majority of bomb calorimetry data used in this project.

I would also like to thank every single CurtisLab member whom which I have had the pleasure to work alongside. Justin Yoo, Sergio Florez, Reed Taylor, Nymul Khan, Stephanie Tran, Mustafa Erbakan, Jun Wang and Tina Lai are all members who I have had the pleasure to get to know, and all of them have at one point helped guide me in my research.

Finally, I would like to thank my friends and family for the support that they have given me for the duration of this project. Their encouragement was much needed for successful completion of this work.

PREFACE

I started work in the CurtisLab during the summer of 2012, in which I was brought in by Trevor Zuroff to work with him on his lab experiments. I assisted Trevor in sample analysis in his experiments that led to a paper titled "Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture", within which I am acknowledged for my work. I learned many valuable skills such as bacterial growth and culturing, yeast growth and culturing, OD sampling, HPLC analysis, and anthrone assay analysis. The skills that I learned during my time with Trevor led to one independent experiment in which I analyzed the effects of salts concentration in growth media on the growth and sporulation of *Clostridium phytofermentans* cultures. In this set of experiments, I discovered that inorganic salts were, in part, responsible for regulating *C.phytofermentans* sporulation – a previously unknown phenomenon. The results of this experiment are presented in Appendix C.

After my initial summer in CurtisLab, I moved on to working with fungal cultures and assessing their potential use as a pretreatment for lignocellulosic biofuels. My initial work with the fungus was influenced by a newly recognized collaborative opportunity between the Curtis and Seong Kim Labs. My preliminary work with the fungal cultures involved efforts in preparing fungal pretreated biomass samples for analysis by Sum-Frequency Generation spectroscopy (SFG). Considerable time and effort was spent simply growing and culturing these fungi in lab, and observing the differences that existed between *Phanerochaete chrysosporium* and *Ceriporiopsis subvermipora*, results of which are presented in Chapter 2 of this thesis. Major efforts were made to develop a successful technique for growing these fungi on biomass substrates and submitting these samples to Kabindra Kafle (Kim Lab), who was responsible for analysis of these samples with SFG. The development of this technique is described in Appendix

A, along with some preliminary data obtained through SFG analysis of these samples. The method was further used in the Curtis Lab by Alex Rajangam, and subsequent results obtained through his sample preparation are presented in Chapter 3 of this thesis.

During the final year of my work in the CurtisLab, a proposal was written for a project that involved analyzing various aspects of a lignocellulosic liquid hydrocarbon fuel paradigm, named FILtH in this thesis. The push for this project recognized a need to obtain preliminary information on mass balances and energy balances for fungal pretreatments. Therefore, I began to work in a collaborative effort with Trevor Zuroff and Patrick Hillery on this project. My primary role was to provide fungal culturing expertise and perform fungal pretreatments of various forms of lignocellulosic biomass. A considerable effort was undertaken to develop pretreatment and sampling methods that would allow for the use of CHNS-O analysis and bomb calorimetry to obtain the preliminary mass and energy balance information. Published scientific literature was used as a basis of my development of these protocols. The methods and data that resulted from this push are presented in Chapter 4 of this thesis. Conclusions and future work that are discussed in the final chapter of the thesis reflect the work that was done and the information that was obtained from the entire body as well as the appendices of this thesi

Chapter 1: Background and Introduction

The Importance of Lignocellulosic Biofuel

Energy is a vital component of the world's economy and is a driving force for productivity. Currently, fossil fuels account for about 80% of the world's total energy usage (IEA, 2011), and it is estimated that three-fourths of the world's energy will still come from fossil fuels by the year 2040 (EIA, 2014). However, greenhouse gas emissions and global warming are strongly linked to fossil fuel combustion (Höök and Tang, 2013). Therefore, a great importance has been recognized for the development of alternative fuel technologies. In fact, renewables are the fastest growing source of energy in the world, with an average annual growth of 2.5% (EIA, 2014).

Technologies currently exist to effectively convert corn to ethanol for high levels of production. However, the use of food for ethanol production creates competition between food and fuel, a problem that would like to be avoided. Government legislation has reflected this concern for negative impacts of using food supplies for fuel, and can be recognized in the amendment placed on the Renewable Fuels Standard by The Energy Independence and Security Act of 2007. This legislation requires that 58.8% of all renewable ethanol comes from "advanced biofuels", such as cellulosic ethanol and biodiesel, that do not require the use of food as feedstocks (Mueller et al., 2011).

Corn ethanol production in the US has also caused price increases for this staple food across the globe, because the United States accounts for the majority of the world's corn exports (Mueller et al., 2011). For instance, a 2007 increase in corn prices from $2.80 to $4.20 per

buschel caused Mexican importers to switch to the cheaper, Mexican-grown white corn for use as animal feed. This caused a price increase for white corn, which is used as the main ingredient in tortilla flour in Mexico. Likewise, increases in cassava-based ethanol production pose a great threat to those who rely on the tropical food to feed their families (Runge et al., 2004).

Lignocellulosic biofuels are classified as second-generation biofuels, and can have a significant positive impact in numbing the competition of fuel versus food. Unlike corn starch, which is used as storage of readily available energy for a plant, lignocellulose is the recalcitrant, structural component of plants. Its recalcitrance is due to a rigid structure comprised of cellulose and lignin, and has been a major obstacle for large scale implementation of lignocellulosic biofuels. Crystalline cellulose contains strong glycosidic hydrogen bonds between glucose units within cellulose polymers, and forms a complex matrix structure. Lignin, an amorphous polymer of aromatic alcohols, fills in spaces between cellulose and makes it far less accessible for modification. This component of lignocellulose is a particular obstacle for most current lignocellulosic biofuel technologies (Limayem and Ricke, 2012). The removal of lignin and alteration of crystalline cellulose to amorphous cellulose, is essential for improving enzymatic hydrolysis during bioconversion of biomass to biofuel (Zhu et al., 2008). Because lignocellulose is the world's most abundant resource available for renewable energy (Limayem and Ricke, 2012), overcoming these obstacles would help to take advantage of this resource for use as a world energy source.

Fungal Pretreatment of Lignocellulosic Biomass

Most lignocellulose pretreatment processes, such as dilute acid, steam explosion, ammonia fiber explosion (AFEX), and ammonia recycle percolation (ARP) require expensive capital and operating costs which outweigh their ability to improve fuel production (Eggeman and

Elander, 2005). These processes remain the most expensive step in lignocellulosic biomass conversion because of necessary requirements such as corrosion resistant reactors, extensive solids washing, and removal of inhibitory compounds from the treated material (Wan and Li, 2012). Also, thermal pretreatments often result in the production of inhibitors which often prevent organism growth that is necessary for downstream processes (Cantarella et al., 2004).

Biological pretreatment, by various types of microorganisms, does not require these expensive capital and operating costs, and provides a very environmentally friendly alternative to other pretreatment technologies. As opposed to brown-rot fungi which mainly consume cellulose, white-rot fungi have the ability to metabolize both lignin and cellulose. For this reason, these fungi have been recognized as an effective pretreatment in lignocellulosic biofuel production (Kumar et al., 2009).

Some white-rot fungi have been recognized for their unique abilities to remove lignin from lignocellulose. *Phanerochaete chrysosporium* produces a plethora of lignin degrading enzymes including lignin peroxidases and manganese-dependent peroxidases (Wymelenberg et al., 2010). Previous work of the Curtis Lab has shown that pretreatment of switchgrass with *P. chrysosporium* improves ethanol production during the downstream fermentation process (Trevor Zuroff, personal communication). Another study has shown that ethanol yield can be increased from 20% to 58% of maximum theoretical yield with proper use of this organism as a form of pretreatment (Bak et al., 2009). However, *P. chrysosporium* has the ability to simultaneously degrade lignin and cellulose, with the potential to lead to significant substrate loss (Fernandez-Fueyo et al., 2012).

Alternatively, the white-rot organism *Ceriporiopsis subvermispora* has been observed to selectively degrade lignin prior to any cellulose removal (Fernandez-Fueyo et al., 2012). Pretreatment with this organism has resulted in as much as 31.33% lignin removal, with only 6% total sugar removal (Wan and Li, 2010). This kind of lignin-specific pretreatment may prove to

be ideal for the application of bioconversion of biomass to biofuel, because it prevents carbon loss and leads to improved fuel production.

***F*ully *I*ntensified (Conversion of) *L*ignocellulose *t*o *H*ydrocarbon**

Previous work in the CurtisLab has showed that consortia mediated bioprocessing with *C. phytofermentans* and *Saccharomyces cerevisiae* cdt-1 significantly improves ethanol production from either of these organisms cultured alone (Zuroff, 2013). This method of bioconversion of lignocellulosic biomass to fuel would like to be taken advantage of by adapting this process for use in a new paradigm, which looks to take advantage of low-cost opportunities that may exist.

The Curtis Lab has recently proposed a vision to demonstrate a new biomass to biofuel paradigm, titled "*F*ully *I*ntensified (Conversion of) *L*ignocellulose *t*o *H*ydrocarbon" (FILtH). This process would take advantage of cost reductions by combining every step of the process into a single reactor system, including pretreatment, enzyme hydrolysis and fermentation.

FILtH envisions a shift away from centralized biofuel production to regionally located, smaller processing facilities (Figure 1a). The vision would provide the necessary low-cost technology for a farm to use its local lignocellulosic crops as feedstocks for the production process. The process itself involves fungal pretreatment of local bioenergy crops, followed by enzymatic hydrolysis and fermentation performed by a symbiotic consortium of bacteria and yeast (Figure 1b), a slight variation of the previously discussed consortium process.

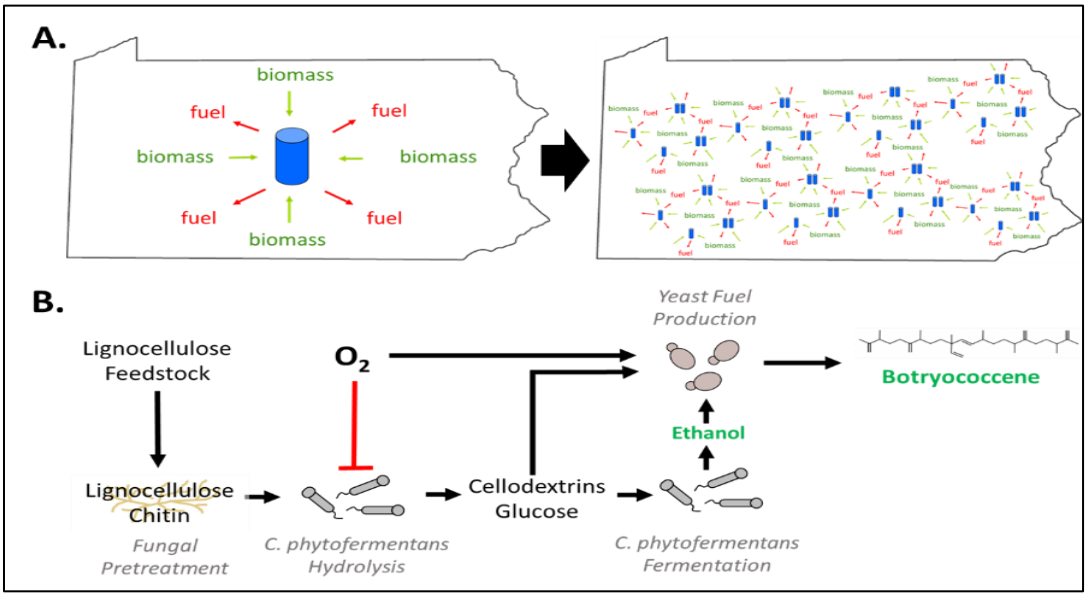

Figure 1. FILtH is designed to shift away from traditional, high-cost centralized biorefining toward regional, low-cost biofuels production (A). Hydrocarbon production in FILtH will be achieved through selective bio-pretreatment followed by fermentation using a symbiotic microbial consortium controlled by oxygen transport (B).

The bacteria that has been chosen as a specific case study for development , *Clostridium phytofermentans*, has the ability to hydrolyze the available carbohydrates (cellulose, hemicelluloses, pectin, and chitin). The hydrolyzed carbohydrates are converted into liquid biofuels through fermentation by yeast. Initially, ethanol was the targeted fuel for because it is a natural electron acceptor during anaerobic digestion. However, CurtisLab seeks to expand its work to advanced biofuels through metabolic engineering and has shifted its interest to a more advanced biofuel, botryococcene. This liquid hydrocarbon has a higher energy density than ethanol, and can be produced in the FILtH process by a genetically engineered strain of *Saccharomyces cerevisiae*.

Comparing Fungal Species for Application in FILtH

The application of a low-cost fungal pretreatment of lignocellulosic substrates has not yet been optimized for this type of bioconversion process, and inevitably different for different feedstocks. Identifying the "best" type of fungal pretreatment requires significant experience growing these white-rot fungi, so that carefully designed experiments can be carried out to compare their effectiveness in improving biofuel production. Also, the ease of culturing these fungi is an important consideration for the use of this pretreatment on a farm, which an ultimate goal of this paradigm. Therefore, significant work has been done to observe the growth of *P. chrysosporium* and *C. subvermispora* on different biomass feedstocks in order to help identify advantages and disadvantages of each species.

Because *P. chrysosporium* and *C. subvermispora* have different methods for attacking lignocellulose, it was of interest to attempt to quantify this difference through analytical means. One optical technique, sum-frequency generation spectroscopy (SFG), has been recognized as a useful tool in observing the orderness of crystalline cellulose in lignocellulosic biomass (Barnette et al., 2012). The CurtisLab wishes to take advantage of this technique to examine the effects of fungal pretreatment on lignocellulose with respect to cellulose decrystallization, in hopes of better understanding how these effects relate to downstream processing. Therefore, preliminary efforts have been made to create successful methods for preparing samples for accurate SFG analysis.

As fungal pretreatment proceeds, the lignocellulosic substrate is converted into fungal biomass, which partly consists of a polymer of N-acetyl-D-glucosamine called chitin ($C_8H_{13}O_5N$). Because *C.phytofermentans* has the ability to convert chitin into downstream fuel products, the

sequestration of nitrogen from the biomass feedstock to the form of chitin may prove to be useful in the downstream fermentation process. This would provide increased savings in nutrient supplementation costs, as well as reduce the possibility of contamination by a wide range of organisms that would compete for supplemented nitrogen. Here, initial experimental efforts have been made to obtain preliminary carbon, nitrogen, and energy balance information of fungal pretreatments.

Chapter 2: Fungal Growth Considerations

Culturing on Agar Media

Fungal culturing begins with growth on media agar, a solidified mixture of nutrients necessary for the organism to grow. The nutrients required by *P. chrysosporium* and *C. subvermispora* are very similar, and it was found that both *P. chrysosporium* and *C. subvermispora* both grow well on either Potato dextrose media or YMPG media. Either media can be used for general growth and culturing.

However, considerable differences in the growth patterns of *C. subvermispora* have been noticed when compared on these two types of media (Figure 2).

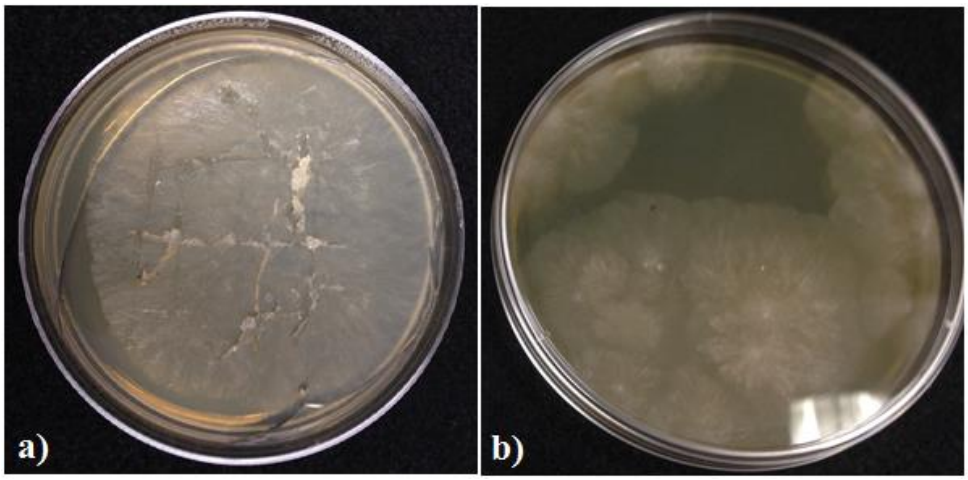

Figure 2. *C. subvermispora* grown for 5 days at 30 °C on Potato dextrose agar (a) and YMPG agar (b).

C. subvermispora grows faster and with a more even spread on the Potato Dextrose media (Figure 2a), and grows slower and with more sporadic growth on YMPG media (Figure 2b).

P. chrysosporium grows in the same fashion on both types of media, with a thick white mycelial mat covering the entire surface of the media (Figure 4). It should be noted that thiamine is a necessary nutrient for growth and lignin metabolism by *P. chrysosporium*, and so it should always be included in the media formulation (Kirk et al., 1978).

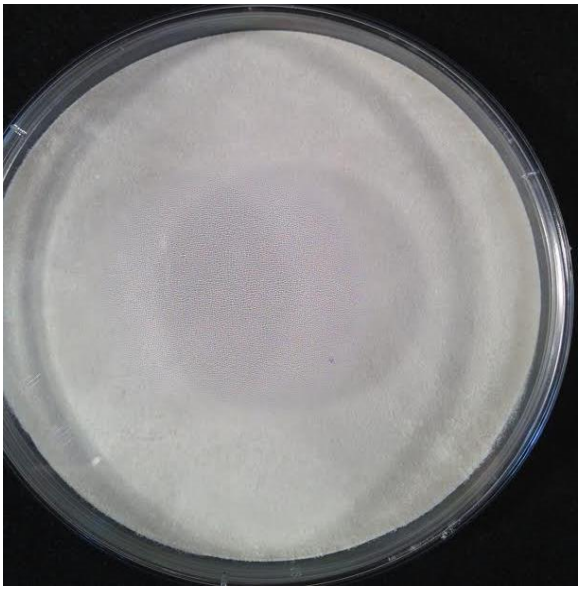

Figure 3. *P. chrysosporium* grown on YMPG agar for 5 days at 30 C.

A more efficient method for storing fungi is to grow them on agar slants instead of on agar plates. An agar slant has a higher surface area to volume ratio than a plate, allowing for the use of less media to grow the same amount of fungus (Figure 3). Slants are also less prone to contamination and drying out, and are useful in the methods for developing spore solutions.

Figure 4. *P. chrysosporium* grown on a YMPG agar slant for 5 days at 30 C.

Homokaryotic Derivatives and Their Use in This Work

P. chrysosporium exists as a dikaryotic (two nuclei) organism in its wild-type form. However, the isolation of a homokaryotic (one nuclei) derivative of this organism has allowed significant strides to be made in genomic sequencing, as well as use of genomic lines in molecular studies (Stewart et al., 2000). This homokaryotic derivative was

acquired for use in the Sum-Frequency Generation analysis part of this work, and used extensively for this purpose. However, there was a major oversight when considering the effects of using this homokaryotic derivative for the biomass pretreatment work in the CurtisLab.

A shift of these preliminary experiments away from SFG work towards solid state cultivation of this organism on biomasss substrates led to an observation of the inability of the homokaryotic strain to groweffectively. The homokaryotic line in solid-state cultivation pretreatments of biomass was severely impaired in growth as compared to the wild type (dikaryotic) strain.

To further substantiate this observation, switchgrass was inoculated by a spore solution of both strains of this fungus. Figure 5 shows the results of a 7 day pretreatment of switchgrass with these two different strains of the fungus.

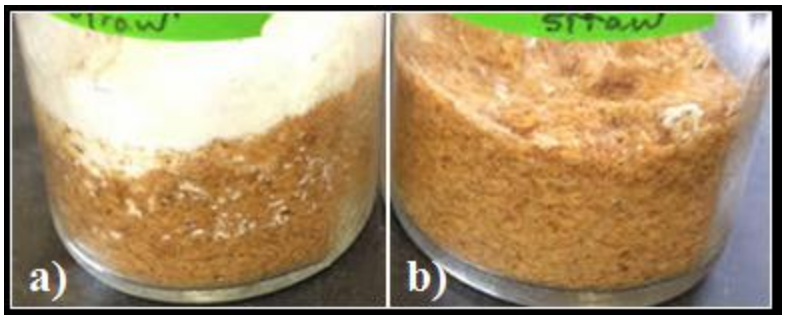

Figure 5. *P. chrysosporium* wild-type strain (a) and homokaryotic derivative (b) at 7 days growth on switchgrass. Inoculation of ~$1.665x10^6$ spores was used for each pretreatment.

Culturing on Lignocellulosic Substrates

Significant differential growth conditions of these organisms on various biomass substrates has been observed while growing them to prepare samples for SFG and fermentation analysis. For example, different extents of growth were observed for each organism on oak wood (Figure 6a and Figure 6b). However, similar amounts of growth were observed for the two different organisms on Poplar wood (Figure 6c and Figure 6d).

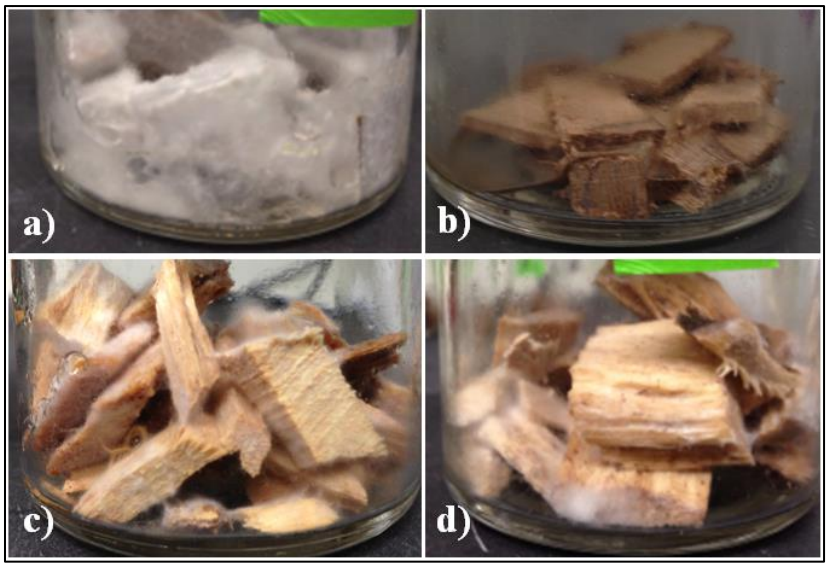

Figure 6. *C. subvermispora* grown on oak (a) and hybrid Poplar (*P. maximowiczii* x *P. trichocarpa*) (c) wood, and *P. chrysosporium* grown on oak (b) and poplar (d) wood.

Clearly, *C. subvermispora* heavily colonizes oak wood chips as evidenced by the coverage of white mycelia. However, no substantial growth is seen for *P. chrysosporium* on oak wood chips, which seems to be of considerable noteworthiness. It is possible that this lack of growth was as a result of the use of a homokaryotic derivative of this organism for initial fungal culturing. It is also possible that the lack of growth resulted from improper preparation of the biomass substrate for fungal inoculation. Previous work of others has involved a thorough washing of this type of substrate to remove brown-colored extractives from the oak chips (Abbas et al., 2005). These seemingly nuances in experimental methods can ultimately have very profound effects on experimental results and interpretation!

Both organisms seem to have similar extents of growth on hybrid poplar wood chips, as evidenced by similar amounts of white mycelia that can be observed growing on the wood. These observations gave the first indication that there may be trouble in developing useful experiments that can compare the performance of these two fungi on the same substrates. Poplar

was chosen as a substrate to move forward with for subsequent SFG experimentation, because of the similar growth patterns initially observed on this substrate.

However, growth of these two organisms on "non-woody" biomass as opposed to these woody biomass substrates yields different results. The progression of growth of these two fungi was monitored on bagasse (Figure 7). *P. chrysosporium* showed initial growth at as early as 4 days of pretreatment with substantial growth observed at 7 days, whereas no growth of *C. subvermispora* could be observed at up to 9 days of pretreatment. *P. chrysosporium* continued to show increased observable growth from 7 days to 9 days of pretreatment. However, results in later experiments in this work will show that no *C. subvermispora* growth is observed even at 16 days of pretreatment.

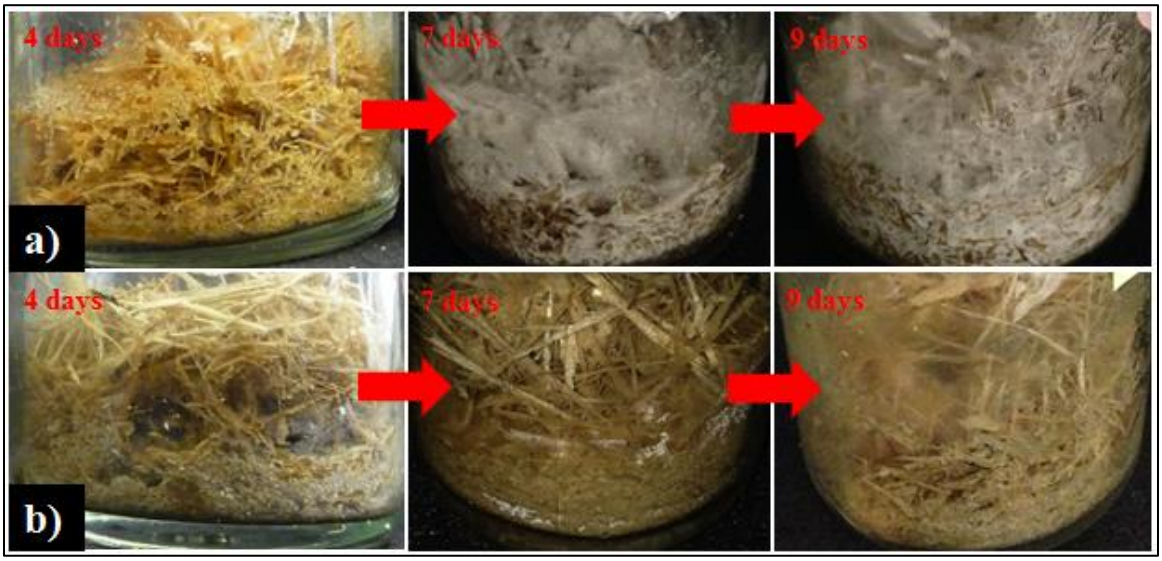

Figure 7. The progression of growth from 4 days to 9 days of *P. chrysosporium* (a) and *C. subvermispora* (b) grown on bagasse.

Growth of *P. chrysosporium* on switchgrass (Figure 8a) yields similar results observed for bagasse. At four days of cultivation, the organism begins to show signs of growth which are limited to the appearance of spotty locations of white fungus on top of the substrate. After 7 days, the substrate is heavily colonized by the fungus, with a slight increase in observable growth after 9 days.

C. subvermispora did not show any observable growth after up to 7 days of pretreatment of switchgrass (Figure 8b). However, white fungus did colonize the biomass at 9 days of incubation. This suggests that *C. subvermispora* can grow better on switchgrass than it can on bagasse, a notable observation that is supported during experiments in Chapter 4.

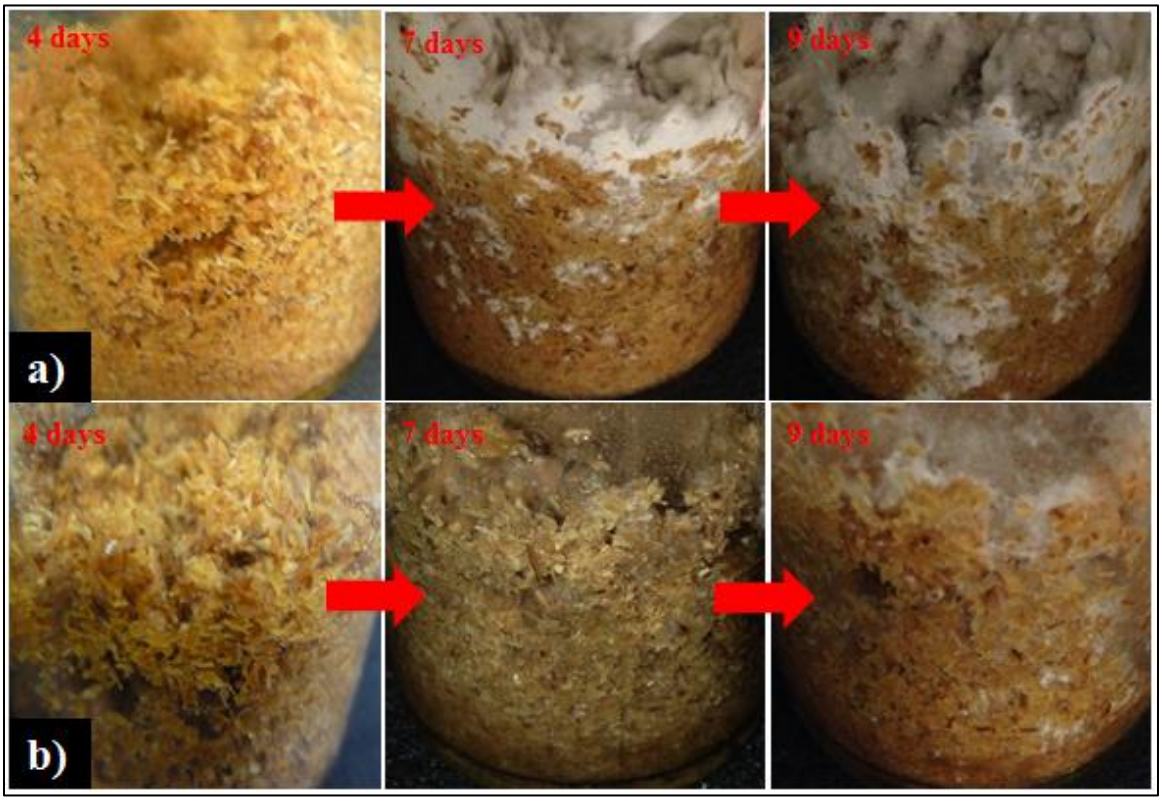

Figure 8. The progression of growth from 4 days to 9 days of *P. chrysosporium* (a) and *C. subvermispora* (b) grown on switchgrass.

Effects of Oxygen Availability on Fungal Growth

The importance of oxygen during fungal growth and lignin degradation by *P. chrysosporium* has been well-known and recognized through experimentation (Liesola et al., 1983). However, during initial fungal culturing, the protocol for *P. chrysosporium* growth used in the CurtisLab (Appendix B) , did not originally stress the importance of allowing for significant

oxygen transfer into fungal cultures. As the importance became understood, an experiment was conducted to examine the effects of oxygen transfer on fungal growth.

P. chrysosporium was grown on agar slants in 15 mL falcon tubes for 7 days by two different methods. In one method, the caps of the slants were tightened and left alone for the entire 7 day during. In the other method, the caps were loosened for a 1-hour time period each day of the duration of the experiment, effectively allowing enhanced diffusion of oxygen into the culture. Clear differences in growth were observed for the two different methods (Figure 9). With no daily opening of the caps, virtually no growth was observable after 7 days (Figure 9a). However, with the daily opening of the caps, high growth was observable after 7 days (Figure 9b).

Figure 9. *P. chrysosporium* grown on YMPG agar slants for 7 days, with (a) and without (b) enhanced oxygen transfer facilitated by periodic opening of the cap.

Ceriporiopsis subvermispora sporulation

For the purpose of the FILtH paradigm, it would be ideal to inoculate biomass with fungal spores. Spores can be kept viable for very long times at cold temperatures, and can be used at the farmer's convenience. Also, quantifying spores is relatively simple as opposed to quantifying a homogenized liquid culture. A technique for developing a spore solution of *P.*

chrysosporium currently exists for use in the CurtisLab, but no such technique exists for *C. subvermispora*. However, *C. subvermispora* has been shown to achieve maximum spore production of 308 x 10^4 spores/mL after 60 hours of treatment in C-GCC stress media (Saxena et al., 2001). For this reason, a preliminary study was conducted for the viability of this method for producing *C. subvermispora* spores. 10 mL of *C. subvermispora* homogenized liquid culture was introduced into D-GCC stress media, and the results were observed after 60 hours and 166 hours of incubation in a rotary shaker at 25°C and 150 rpm.

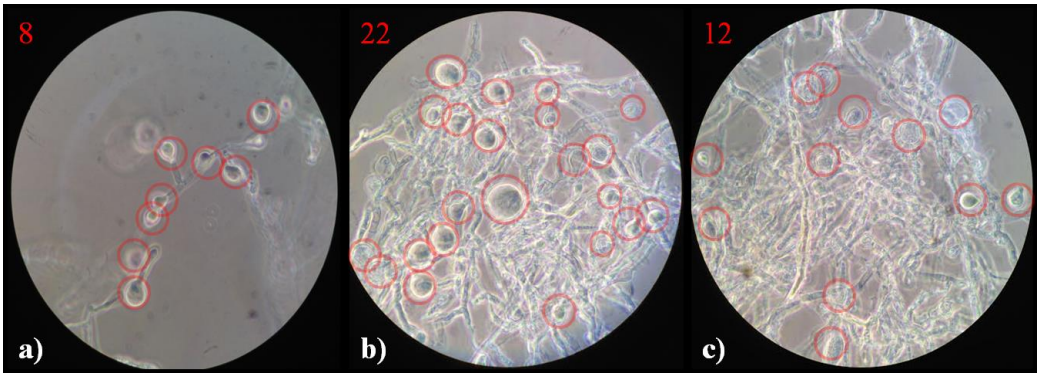

Figure 10. *C. subvermispora* growth in liquid media (3a), and in D-GCC stress media for 60 hrs (3b) and 166 hrs (3c) at 25 °C and 150 rpm.

After 60 hours of incubation, spores were observable and identified (Figure 10b). However, the concentration of spores in the stress media did not seem to be significantly greater than the concentration of spores in a basic liquid culture (Figure 10a), suggesting that the stress media used was not able to effectively enhance the sporulation of *C. subvermispora*. After 166 hrs of incubation time, even less spores were observed in the stress media (Figure 10c). The separation of the spores from mycelia requires expensive chitinase treatment (Saxene et al., 2001), and was outside the scope of this project. Therefore, the alternate biomass inoculation technique, homogenized culture biomass, was utilizied. for the rest of the work of this project.

Chapter 3: Characterizing Fungal Activity with Sum Frequency Generation Spectroscopy

Sum frequency generation spectroscopy is a new technique that has been used to detect the orderness of crystalline cellulose in plant cell walls (Barnette et al., 2011). An advantage of this optical detection over others is that there is no contribution to the signal from amorphous cellulose in lignocellulose, such as hemicelluloses and lignin. Wood samples give similar SFG spectra as Avicel crystalline cellulose, which indicates that all other components in wood are not detected (Barnette et al., 2012). This technique has demonstrated the ability to give more accurate results than IR and Raman spectroscopy, because of the ability to give fewer and sharper vibrational peaks (Barnette et al., 2011).

However, SFG intensity varies nonlinearly with amount of crystalline cellulose. Also, there are many uncertainties about the effect of other lignocellulosic components on SFG signal (Barnette et al., 2012). Therefore, a limitation has been recognized for SFG's inability to provide substantial information about the quantity of crystalline cellulose present. If this technique can be adapted to analyze cellulose crystallinity in pretreated lignocellulosic substrates accurately, then it could give great insight into the specificity of white-rot fungi for lignin degradation and the alterations made to crystalline cellulose.

There are two important SFG signal peaks for the application of this tool to analysis of biological pretreatments. The peak at 2945 cm^{-1} represents the ***inter***chain CH_2 stretch that occurs as a result of interaction between cellulose polymers. The shrinking of this peak represents the breaking of attractions between individual cellulose polymer chains. The peak at 3325 cm^{-1} represents the ***intra***chain OH bonding that occurs between glucose units in an individual cellulose polymer (Figure 11) (Barnette et al., 2011).

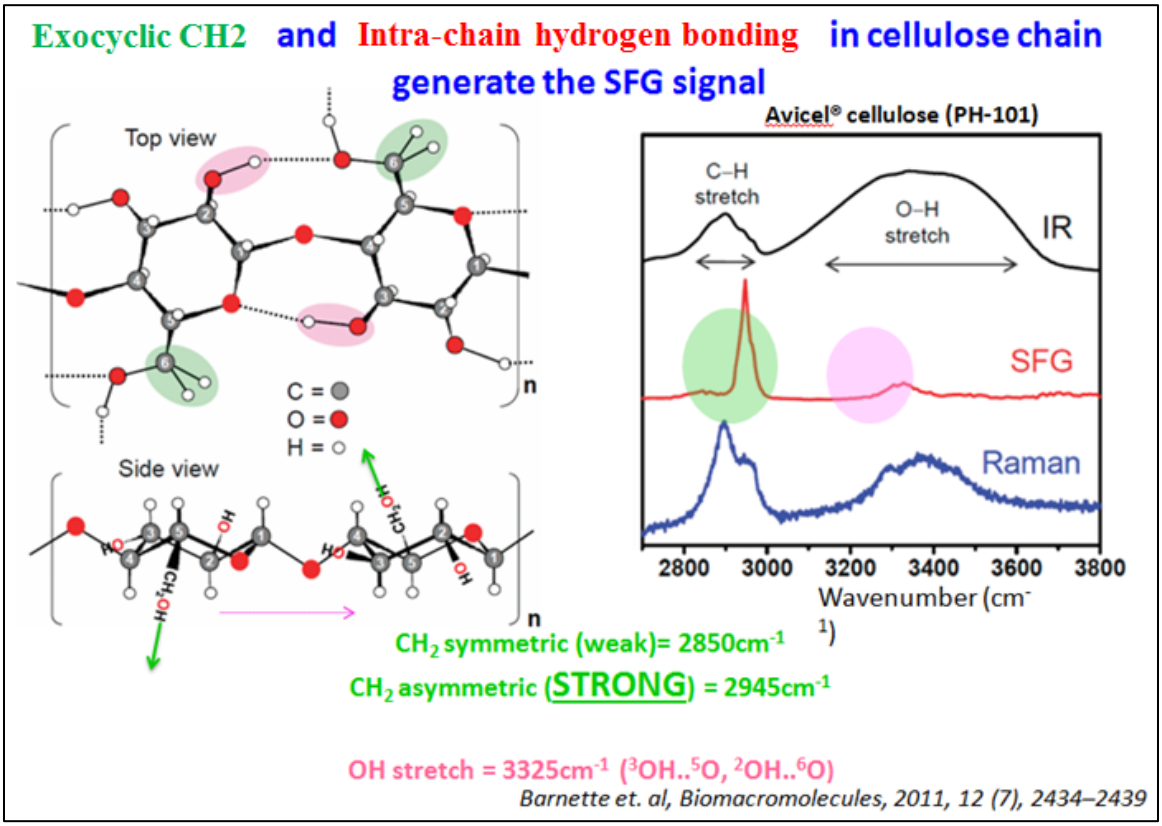

Figure 11. A top and side view of two glucopyranose units in a cellulose chain (a). The linkages observed with SFG and their respective signals (b) are highlighted in green and pink. (Barnette et al., 2011)

Methods

A significant amount of time and thought was dedicated to developing a method to achieve accurate SFG results for fungal pretreated biomass samples. The development of the methods for SFG sample preparation is described in Appendix A, with the optimal method described in this section.

Fungal cultures were grown on agar plates for about 3-5 days, until sufficient fungal biomass was observed (Figure 12).

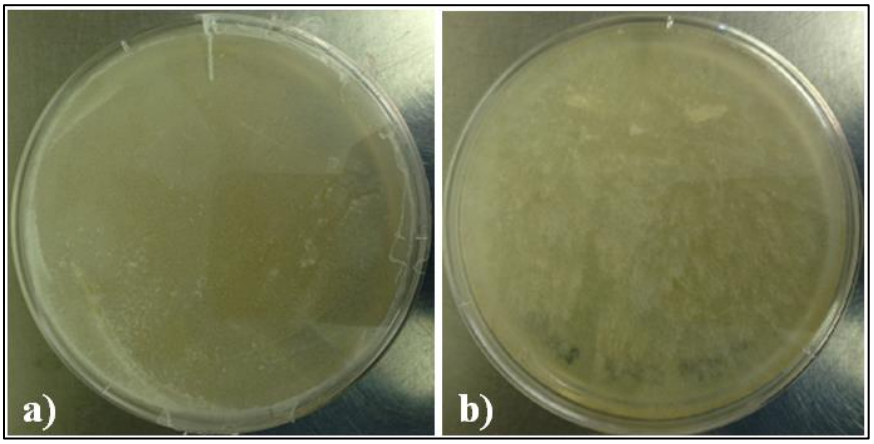

Figure 12. *P. chrysosporium* (a) and *C. subvermispora* (b) grown for 5 days at 30 C on Potato dextrose agar.

Samples are cut to a 2 mm thickness and introduced onto the fungal plate cultures. In effect the biomass sample rests on a mycelial mat of the fungus (Figure 13). Therefore, the fungus has access to the media as well as the biomass substrate. This technique allows for sufficiently even growth of fungus on the biomass substrates and smooth enough surfaces for SFG analysis, and limits the interference of the media with results.

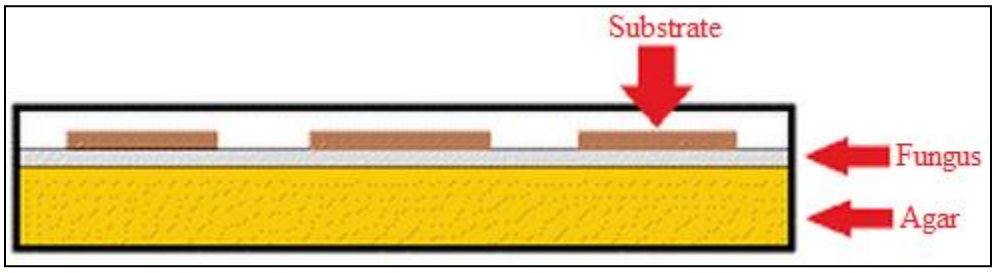

Figure 13. Substrate placement technique for SFG sample preparation.

Placing the biomass substrates in a spiral-like fashion (Figure 14) allows for incorporation of multiple replicates for each time point, necessary to gain insight on how degradation changes over time. To obtain the most recent results of this study, three replicates of each sample were removed from the plate at each time point.

Figure 14. 8 days of growth of *P. chrysosporium* on poplar wood chips for the purpose of SFG sample preparation.

Results and Discussion

Early results obtained during the development of the use of this technique, results from analysis of samples that were provided by me, are discussed in Appendix A. For the purpose of a meaningful discussion, only the most recent results are presented in this section. Alex Rajangam and Kabindra Kafle used these methods from Appendix A to prepare fungal degraded samples that were analyzed to obtain the following results.

SFG results for *P. chrysosporium* and *C. subvermispora* growth on poplar are presented in Figure 15a, along with presentation of the calculated percentage of cellulose present based on an Avicel calibration curve in Figure 15b.

After 8 days of fungal growth, both *P. chrysosporium* and *C. subvermispora* caused a substantial decrease in SFG intensity corresponding to the interchain. *P. chrysosporium* caused a decrease from 40% to about 23% of cellulose present, whereas *C. subvermispora* caused a

decrease from 40% to about 32% of cellulose present. After another week of fungal growth, the two fungi caused opposite effects. *P. chrysosporium* continued to decrease the SFG intensity, while *C. subvermispora* increased the SFG intensity. This trend continued until day 30, when the SFG signals converged for both fungi to about 57%.

During cellulolytic activity by these two fungi, the beta 1-4 glycosidic bond between glucose units in the cellulose chain is broken. The adjacent O-H region that is detected by SFG analysis (highlighted in pink in Figure 11) will be substantially affected and vary the SFG signal. It should be noted that the SFG signal only corresponds to the crystalline cellulose part of the lignocellulose.

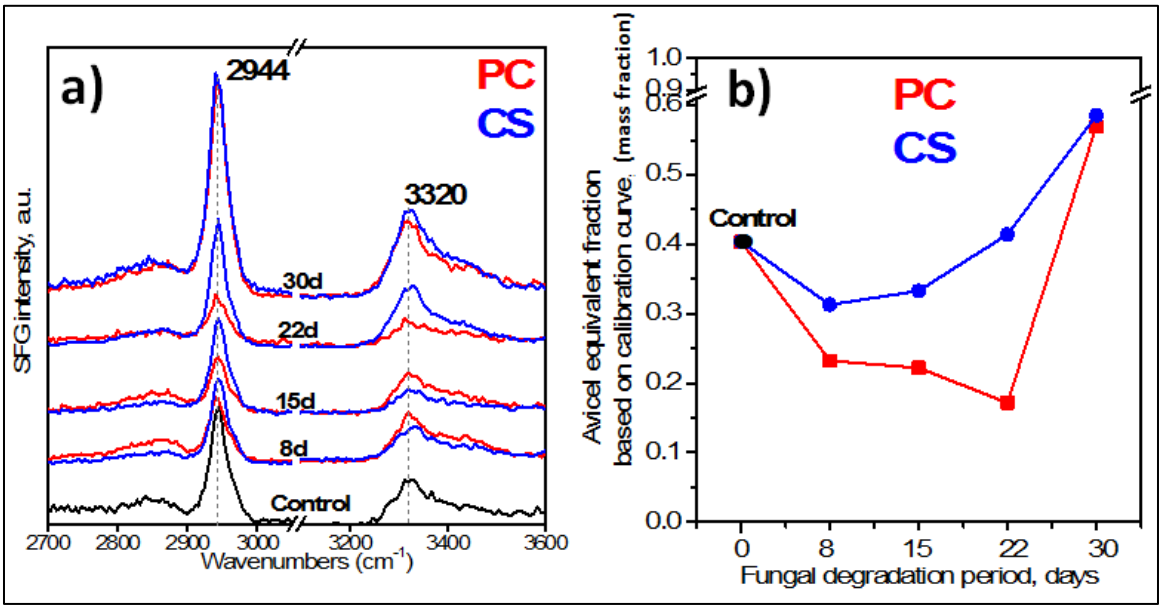

Figure 15. SFG Intensity signals (a) for P. chrysosporium and C. subvermispora pretreated samples. The SFG signals for different time lengths of pretreatment (8, 15, 22, and 20 days) are all represented on one chart. The peak intensity at each time point was used to calculate the Avicel equivalent mass fraction of crystalline cellulose based on a calibration curve. (Data provided by Kabindra Kafle)

The larger initial decrease in intensity for *P. chrysosporium* and the smaller decrease in intensity for *C. subvermispora* supports the differences in substrate selectivity of these two fungi. Because *P. chryosporium* simultaneously degrades lignin and cellulose, this fungus results in the removal of a higher fraction of crystalline cellulose from the biomass substrate. On the other hand, *C. subvermispora* is more selective toward lignin degradation and therefore removes a smaller fraction of crystalline cellulose.

Cellulose Degradation

After presentation of the methods for preparing SFG samples, there was some question as to whether or not the fungi were even growing on the biomass substrates. Therefore, quantification of cellulose in the pretreated samples were determined by Alex Rajangam using the Updegraff method (Appendix B) to prove that cellulose was being degraded in the samples. This method detects only the long-chain polymer cellulose, as the smaller cellodextrins and sugars are washed away in the process (Alex Rajangam, personal communication). The results are presented in Figure 16.

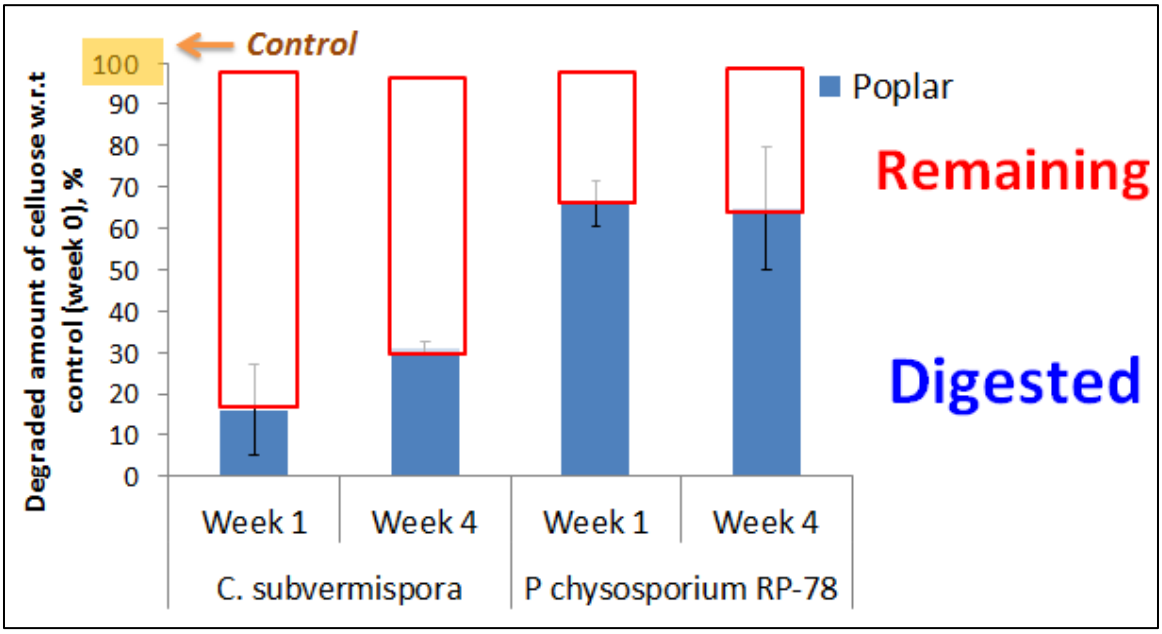

Figure 16. Cellulose removal during pretreatment of poplar by C. subvermispora and P. chrysosporium. (Data provided by Alex Rajangam)

This data shows that cellulose is in fact being degraded by both fungi during the method presented for SFG sample preparation, though it does not necessarily confirm growth on the biomass – only release of cellulose degrading enzymes.

The data substantiates that *P. chrysosporium* treatment resulted in higher cellulose digestion than *C. subvermispora* treatment. *P. chrysosporium* resulted in the removal of about 65% of the cellulose in the poplar wood after four weeks of pretreatment, whereas *C. subvermispora* resulted in about 30% removal of cellulose after four weeks of pretreatment. This supports the idea that *P. chrysosporium* degrades more cellulose because of its more active cellulolytic enzymes. Support for this data comes from a recent study that shows that *C. subvermispora* removed considerable less cellulose than *P. chrysosporium* after 4 weeks of pretreatment on wheat straw (Cianchetta et al., 2014).

The data also shows that removal of cellulose by *P. chryosporium* halted after one week of pretreatment, as virtually no change in cellulose content was observed between one week and

four weeks of pretreatment. On the other hand, cellulose degradation nearly doubled from one week to four weeks during *C. subvermispora* treatment. This suggests that the time frame for cellulose degradation differs between these two fungi. This may simply be a result of the difference in the timeframe for fungal growth, or it may have a more substantial implication on the result of the difference between enzymatic activity of the two species. Because *C. subvermispora* is thought to release its lignin degrading enzymes prior to its cellulolytic enzymes, cellulose degradation may be slower during the earlier part of its growth.

In the application of a fungal pretreatment for downstream conversion of biomass to biofuel, this evidence is unclear as to which fungus would provide a better pretreatment. In one way, *C. subvermispora* could provide a better pretreatment because it has the ability to selectively degrade lignin. However, the fact that *P. chrysosporium* changes the crystallinity of cellulose may actually prove to be beneficial to further downstream processing. Although delignification has proven to be more beneficial to enzymatic hydrolysis than decrystallization (Zhu et al., 2008), a proper mixture of both may prove to be the ideal pretreatment. If the decrystallization of cellulose that is being accomplished by *P. chrysosporium* does not result in significant carbon loss, this pretreatment may be more beneficial than a more lignin-specific pretreatment.

Chapter 4: Pretreatment Effects on Composition and Energy Density

For the purpose of the FILtH paradigm, it is important to investigate the movement of carbon and nitrogen during the process of fungal pretreatment. Since *C.phytofermentans* has the ability to consume chitin, it would be useful to monitor the amount of lignin that is consumed by the fungus and converted to chitin. The energy content of biomass before and after pretreatment could also give insight into how this shift of carbon and nitrogen usage affects the availability of energy. Closing a mass balance on pretreatment will help to optimize this process for use in the FILtH paradigm. Various analytical techniques such as elemental analysis and bomb calorimetry were used in order to obtain the necessary data for this analysis.

Methods

Fungal Growth

P. chrysosporium was grown on YMPG agar slants for 6 days at 30 °C. A spore solution was obtained by adding 2 mL of distilled water to each slant and vortexing. This solution was filtered through a cotton plug into a 50 mL falcon tube. The resulting solution contains only *P. chrysosporium* spores in water. An optical density measurement (OD) of the resulting spore solution was measured to be 0.977. Distilled water was added to this solution until the resulting OD was 0.525, which corresponds to about $2.5x10^6$ spores/mL (Kirk et al., 1978).

C. subvermispora was grown on YMPG agar plates for 6 days at 30 °C. Three discs of 5 mm diameter of this culture were inoculated into YMPG liquid media, and this culture was grown in a rotary shaker at 28 °C and 150 rpm for 12 days. The resulting culture was homogenized by bead beating, and re-suspended in distilled water. This solution was used as the *C.*

subvermispora inoculums for all pretreatments. One mL of this inoculum corresponds to 10 mg of dry weight of fungus.

Pretreatment

There are several variables that are important to consider when preparing for an effective fungal pretreatment, one of which is substrate moisture content (MC). Previous studies have revealed that 75% moisture content of cotton stalks resulted in the greatest lignin degradation (Shi. et al.., 2008), and that a 75% moisture content of corn stover led to maximum lignin degradation and glucose yields (Wan and Li, 2010). Therefore, this moisture content was used for all of the different biomass substrates in this study. Moisture content was calculated using the following equation:

$$MC = \frac{mass\ of\ water}{mass\ of\ substrate + mass\ of\ water} \qquad \textit{Eq. 1}$$

Another important experimental variable when optimizing fungal pretreatment is the length of pretreatment. In general, studies have shown that a longer pretreatment time leads to greater lignin degradation as well as improved downstream bioprocessing. Lignin degradation continues after ten days of pretreatment, suggesting that this is too short to be an effective pretreatment. A pretreatment time of 18 days has been shown to effectively degrade lignin and prepare the substrate for subsequent fermentation (Wan and Li, 2011).
Shaken cultures do not degrade lignin as effectively as stationary ones (Kirk, 1978).

Oxygen transfer is essential for effective lignin degradation by white-rot fungi. Research has shown that diffusion of oxygen into *P. chrysosporium* cultures is the major rate-limiting factor in efficient lignin degradation (Leisola et al., 1983). For this reason, it is important to keep

the surface area to volume ratio constant for pretreatments, minimize gradients by providing a small but significant forced convection of air within the treatment bed, so as not to cause variations in oxygen levels in the different pretreatment atmospheres. Because of differences in density of the substrates used in this study, different starting masses were used for each substrate. Keeping surface area to volume ratio constant resulted in starting masses of 6.5 g, 2.5 g, and 4.5 g, for switchgrass, bagasse, and steam treated bagasse, respectively. In previous studies conducted by Salvador Barri in Wayne Curtis' laboratory, 2.5 g of biomass was treated with about 2,500 *P. chrysosporium* spores. In the current experiment, this ratio of 1000 spores/g of substrate was kept constant.

Prior to pretreatment, the biomass substrates were dried at 70°C for 3 days in order to remove all water content. The biomass substrates were then inoculated with either *P. chrysosporium* (1000 spores/g of biomass) or *C. subvermispora* (10 mg dry weight/g of biomass). Distilled water was added to the jars in order to bring the final moisture content of the biomass to 75%. These pretreatments were carried out in baby food jars at 30 °C for 16 days. After 16 days of pretreatment, the treated material was dried at 105 °C for 24 hours to remove all water. The treated material was split into a top half (~25% of the total mass) and a bottom half (~75% of the total mass), and these samples were frozen at -20 °C for future analysis.

Analysis

Elemental analysis sample preparation and the analysis itself was performed in Dr. Ephiram Govere's Lab at Penn State University. For elemental analysis of lignocellulose, it is important to grind samples to fine particles on the scale of hundreds of microns. In this experiment, samples were milled down to this particle size using an SPEX Sample Prep

Mixer/Mill 8000D (Figure 17a). Samples are placed into a capsule with a ball, topped with a ring seal and capsule lid, and placed into the capsule holder with capsule holder lid (Figure 17c). It is important to balance the weight in the ball mill by placing the same number of full capsules in each capsule holder. Both capsule holders are placed into the mill, and securely tightened (Figure 17b).

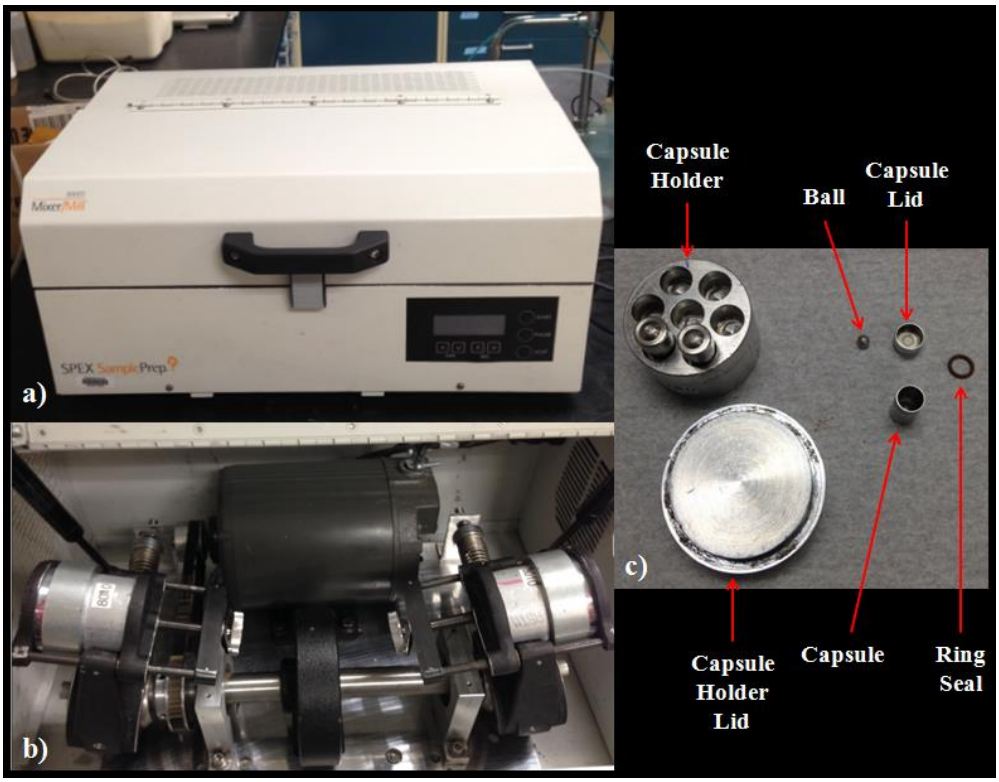

Figure 17. An SPEX Sample Prep Mixer/Mill 8000D (a) was loaded with two capsule holders full of samples (b) and grinded down to a homogeneous sample powder. Various parts are necessary for the effective use of this technique (c).

. The samples were ball milled to a fine particle size, and placed in a dessicator for 16 hrs prior to being weighed and analyzed. Different types of substrates required different lengths of milling time, because of their difference in robustness. 5 minutes of milling was sufficient for switchgrass and steam treated bagasse, whereas bagasse required at least 15 minutes of milling

time. Therefore, it is recommended to mill samples of the same substrate type simultaneously for most efficient use of time.

2.5 – 3.0 mg of each ground sample was carefully weighed into a tin boat and was introduced into a CE Instruments EA 1110 CHNS-O elemental analyzer.

Heat combustion properties were determined by Patrick Hillery, by using a Parr model 1710 adiabatic bomb calorimeter, a device owned by the Penn State Energy Institute. Samples ranging in size from 25-100 mg were introduced into the bomb calorimeter, along with a benzoic acid tablet of known size.

Results and Discussion

Growth Observations and Total Mass Loss

Post-treatment observations showed varied growth patterns across different substrates, and also varied growth patterns throughout the depth profile of each individual pretreatment. Both fungi seemed to grow differently on the top section of the biomass substrate than it did on the bottom section of the biomass substrate. These observations illustrate a need for forced convection that would be required in a larger scale pretreatment process. After 16 days, a significant amount of *P. chrysosporium* growth was observed on the top section of the pretreatment, with less growth observed at greater depths (Figure 18a). Opposite observations were made of *C. subvermispora's* growth on switchgrass, in which considerably higher growth was observed in the bottom section of the pretreatment (Figure 18b).

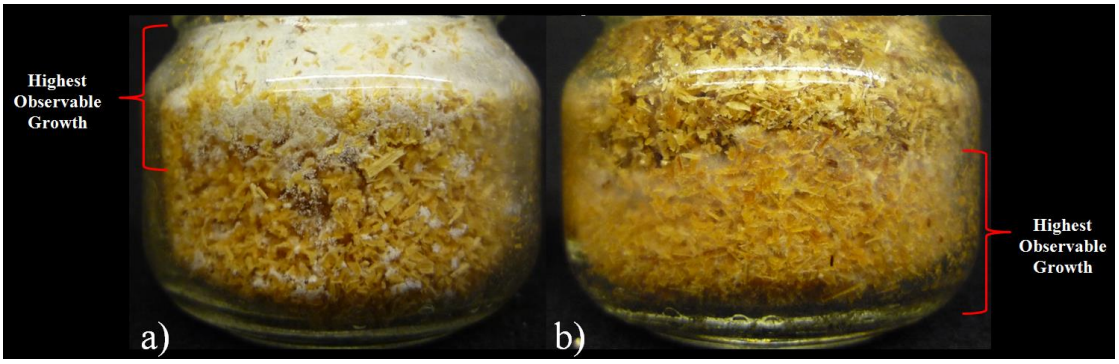

Figure 18. Switchgrass pretreated with P. chrysosporium (a) and C. subvermispora (b) for 16 days at 30 C.

Although this same phenomenon was seen for *P. chrysosporium* growth on bagasse (Figure 19a), very minimal total growth was observed for *C. subvermispora* on bagasse (Figure 19b). However, there was a noticeable color change between the top section and bottom section of the *C. subvermispora* pretreated bagasse, suggesting that fungal growth may be present but not readily observable.

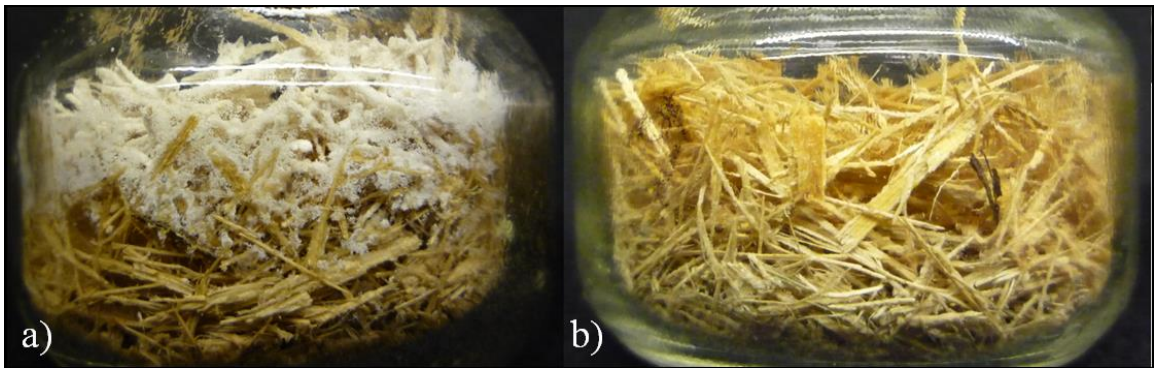

Figure 19. Bagasse pretreated with P. chrysosporium (a) and C. subvermispora (b) for 16 days at 30 °C.

Various ideas have been presented to explain this phenomenon, with the most plausible being differential oxygen requirements. It has been demonstrated that in 4% oxygen concentration atmospheres, many fungal species can only grow to about 40% of their full potential when 21% oxygen is available (Wells and Uota, 1970).

The total mass of each system was determined before and after fungal pretreatment, and the results are presented below (Figure 20). Switchgrass pretreated samples had the highest total mass loss, with the *P. chrysosporium* treatment resulting in 21.08% mass loss and *C. subvermispora* treatment resulting in 8.77% mass loss, when compared to the control sample. The higher mass loss due to *P. chrysosporium* pretreatment when compared to *C. subvermispora* can be explained by growth observations. As stated before, much higher growth was observed for *P. chrysosporium* than was observed for *C. subvermispora*, which would result in a higher mass loss in the pretreatment due to CO_2 production.

These results remain consistent for the bagasse treated samples, in which there was a 4.46% mass loss for *P. chrysosporium* treatment and a 1.23% mass loss for *C. subvermispora* treatment. It is hard to say if the mass loss of *C. subvermispora* treated bagasse samples was actually as a result of fungal pretreatment activity. The methods involved in this experiment often resulted in minor mass losses (maximum 5%), due to drying and sampling.

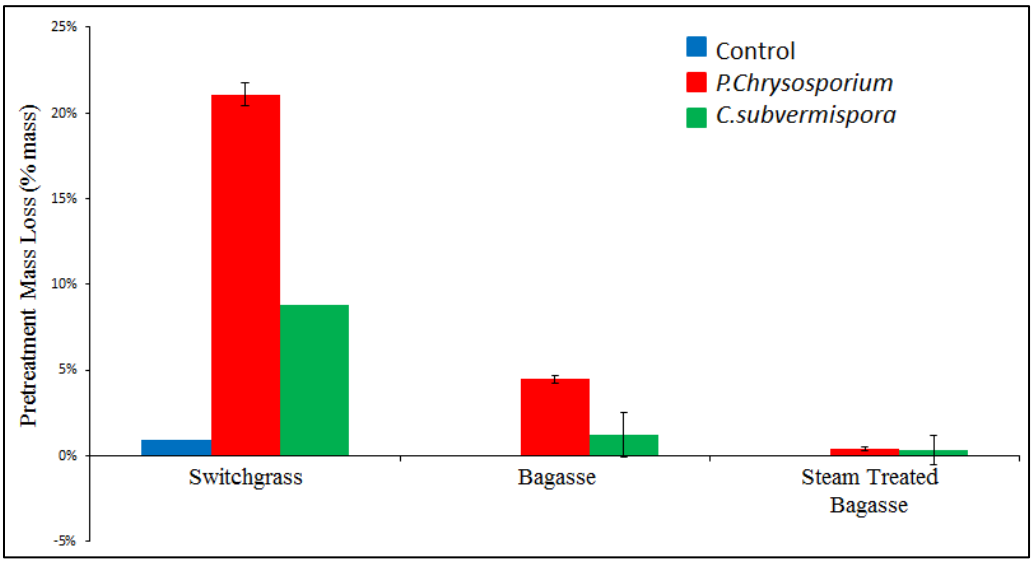

Figure 20. Total pretreatment mass loss after 16 days. An obvious trend is recognized that P. chrysosporium results in greater pretreatment mass loss than does *C. subvermispora*. Higher observable growth corresponded well with higher pretreatment mass loss.

Virtually no mass loss was recorded for steam treated bagasse, which corresponds well with the observations of no growth on this substrate. The lack of growth and activity on steam treated bagasse was most likely due to inhibitors that are introduced during the steam treatment process, including formic acid, acetic acid, levulinic acid, furfural, 5-hydroxymethyl furfural, syringaldehyde, 4-hydroxy benzaldehyde, and vanillin (Cantarella et al., 2004).

Elemental Composition Alterations

Fungal pretreatment effects on carbon and nitrogen content were determined by performing elemental analysis on samples with and without pretreatment, with varying results for each type of substrate analyzed. The "top" section of the pretreated material, which is defined as the top 25% of the total mass, was analyzed separately from the "bottom" section of the pretreated material, in order to examine the effects of varying levels of observable growth.

P. chrysosporium treated switchgrass showed almost a two-fold increase in nitrogen content from the control (0.337%) to the pretreated "top" section (0.636%). This large increase in nitrogen content was expected because of the shift of mass from the forms of lignin and cellulose into fungal biomass, mainly chitin. Although no nitrogen was added to the system, carbon, hydrogen, and oxygen were lost from the system in the form of CO_2 and H_2O, effectively increasing the mass fraction of nitrogen in relation to the total mass. The shift of carbon and nitrogen from the biomass substrate to the fungal biomass is examined in the next section, in which the amount of fungal biomass present is estimated.

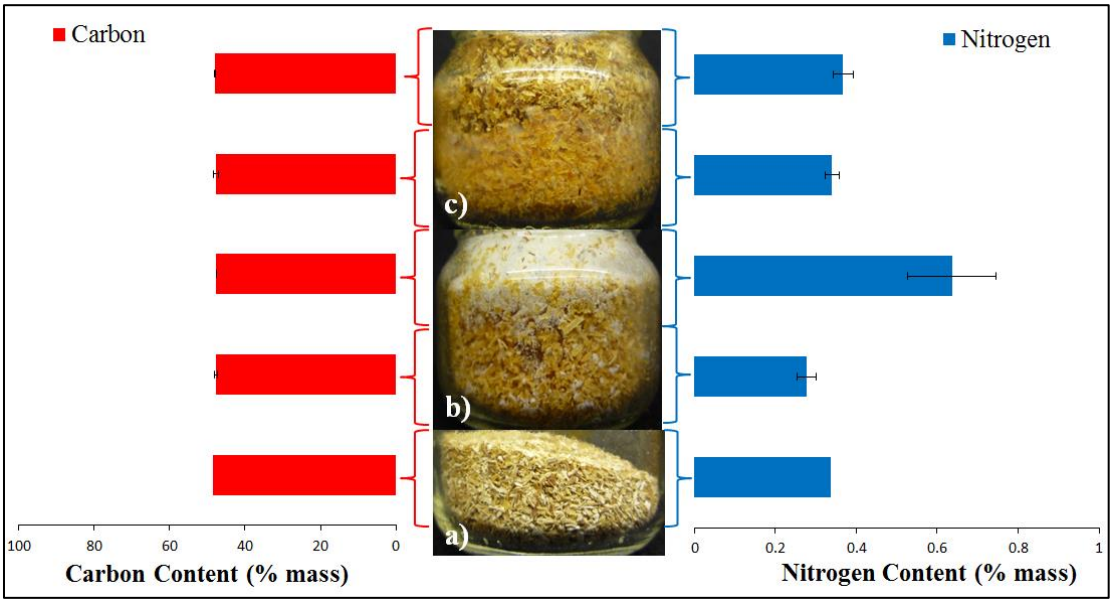

Figure 21. The nitrogen and carbon content are presented on a % of total mass basis for 16 day pretreatment of non-treated (a), *P. chrysosporium* treated (b), and *C. subvermispora* treated (c) switchgrass. The most significant change occurs in the "top" section of the *P. chrysosporium* pretreatment, which corresponds with the highest observed growth of any pretreatment.

Virtually no difference in nitrogen content for the *C. subvermispora* pretreatment was recognized from the data, most likely resulting from lack of growth and very little total mass of chitin. Carbon content decreased slightly in both fungal pretreatments, with a slightly higher decrease for *P. chrysosporium* pretreatment than for *C. subvermispora*. This higher decrease in carbon content suggests higher activity for the *P. chrysosporium* pretreatment, resulting in higher losses of carbon from the system.

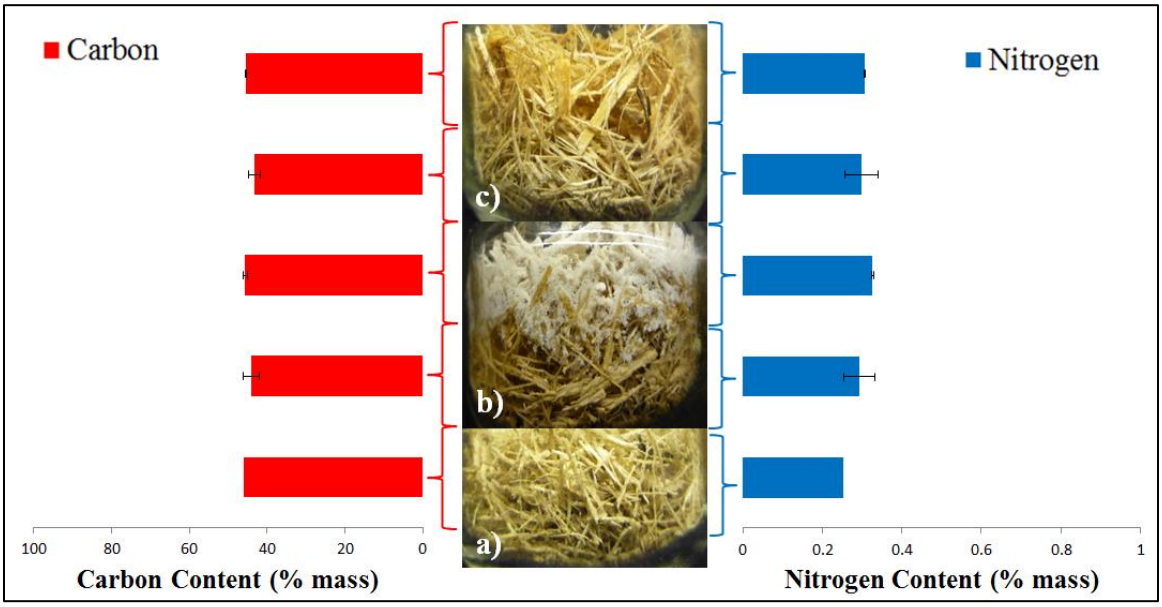

Figure 22. The nitrogen and carbon content are presented on a % of total mass basis for 16 day pretreatment of non-treated (a), *P. chrysosporium* treated (b), and *C. subvermispora* treated (c) bagasse. Nitrogen content increased in both fungal pretreatments, with a slightly higher increase for *P. chrysosporium*.

The bagasse samples showed similar results as the switchgrass samples, with a less substantial increase in the nitrogen content of pretreated samples. The "top" section of the *P. chrysosporium* treated bagasse resulted in an increase in nitrogen content from 0.252% to 0.325%, a smaller increase in content than was determined for the switchgrass treated samples. Bagasse treated samples differed from switchgrass, in that *C. subvermispora* treatment had a slight effect on the nitrogen content in the system. Both the top and bottom sections of the pretreatment contained about 0.300% nitrogen, an increase of 0.05% from the control sample. This suggests that *C. subvermispora* growth may have been present, but was not readily observable. There was virtually no change in nitrogen content for the steam treated material, supporting the hypothesis that there was no fungal growth on this substrate.

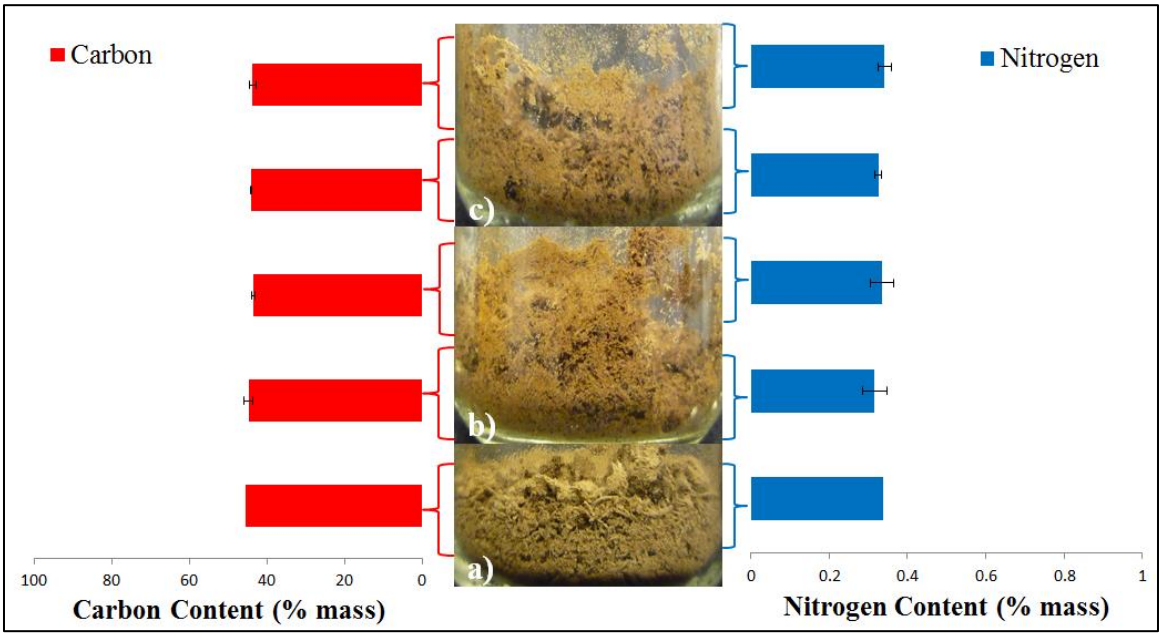

Figure 23. The nitrogen and carbon content are presented on a % of total mass basis for 16 day pretreatment of non-treated (a), P. chrysosporium treated (b), and C. subvermispora treated (c) steam treated bagasse. Nitrogen and carbon content remained virtually unchanged as a result of fungal pretreatment, supporting the observation that little to no growth occurred.

Estimation of Fungal Biomass

For the simplicity of estimating the shift of biomass to fungal biomass, the content of oxygen and hydrogen were neglected. Therefore, all mass balance equations include only carbon and nitrogen, the two most important elements to be monitored for the purpose of the FILtH paradigm.

The main component of fungal biomass is chitin, which has a composition of $C_8H_{13}O_5N$. For the estimation of fungal biomass, it was assumed that 100% of the fungal biomass was present in the form of chitin. This assumption allowed the analysis to be performed on the following simple mass balance.

$$Biomass \rightarrow Biomass + Chitin + CO2$$

The estimation of fungal biomass was performed on the top portion of the *P. chrysosporium* treated switchgrass, which was depicted in Figure 18a. The mass of the "top" portion of the pretreatment is defined to be the top 25% of the mass of the entire system. The following table presents the necessary information needed to perform all calculations (Table 1).

Table 1. Switchgrass Pretreatment Mass and Elemental Composition Information

	Total Mass	Nitrogen %	Carbon %	Nitrogen (g)	Carbon (g)
Switchgrass Control	1.625	0.3374	48.4350	0.005482719	0.7870684
Switchgrass - P.chrysosporium - Top	1.31004	0.6362	47.6405	0.008290476	0.62412065

Using basic stoichiometry, the molar composition of the switchgrass control was determined to be $C_{167}N_1$, and the pretreated top was determined to be $C_{88}N_1$. This pretreated top can be assumed to be a mixture of biomass and chitin. Therefore, the simple mass balance is represented by the following sequence of equations:

$$Biomass \rightarrow (Biomass + Chitin) + CO2$$

$$C_{167}N \rightarrow (C_{88}N) + CO_2 \qquad \textit{Eq. 2}$$

$$Biomass \rightarrow Biomass + Chitin + CO2$$

$$C_{167}N \rightarrow C_8N + C_{167}N + CO2 \qquad \textit{Eq. 3}$$

It was assumed that all carbon loss from the system was lost as CO_2, which can be calculated by knowing the amount of total mass lost from the system. In the case of the pretreated top of switchgrass, it was measured that there was a total carbon loss of 0.163 g, or 0.0136 moles of carbon. Therefore, the amount of carbon left in biomass and chitin after pretreatment is 0.0655 mol – 0.0136 mol = 0.0519 mol carbon. That means that 0.0519 moles of carbon is split by some ratio into biomass and chitin. Using the known final elemental composition of the system ($C_{88}N$), this ratio can be determined by using basic stoichiometry to set

up the following set of equations (Eq. 3-7) with two unknowns, moles of carbon in biomass (X_b) and moles of carbon in chitin (X_c):

$$88 = \frac{moles\ of\ carbon}{moles\ of\ nitrogen} \quad \textit{Eq. 4}$$

$$moles\ of\ carbon = (C_c + C_b)/(MM_c) \quad \textit{Eq. 5}$$

$$moles\ of\ nitrogen = (N_c + N_b)/(MM_N) \quad \textit{Eq. 6}$$

$$C_c + C_b = X_c(EC_{Cc})(MM_c) + X_b(EC_{Cb})(MM_c) \quad \textit{Eq. 7}$$

$$N_c + N_b = X_c(EC_{Nc})(MM_N) + X_b(EC_{Nb})(MM_N) \quad \textit{Eq. 8}$$

where C_C is mass of carbon in chitin, C_b is mass of carbon in biomass, N_C is mass of nitrogen in chitin, N_b is mass of nitrogen in biomass, MM_C is the molar mass of carbon, MM_N is the molar mass of nitrogen, EC_{Cc} is the elemental composition of carbon in chitin, EC_{Cb} is the elemental composition of carbon in biomass, EC_{Nc} is the elemental composition of nitrogen in chitin, and EC_{Nb} is the elemental composition of nitrogen in biomass.

Excel's solver was used to simultaneously solve these equations to determine the distribution of carbon in biomass and chitin. It was determined that X_b=0.4995 and X_c=0.0571, or that there was 0.4995 moles of biomass ($C_{167}N$) after pretreatment, and 0.0571 moles of chitin (C_8N) after pretreatment. This means that about 90% of the moles of nitrogen in the system remained is in the form of biomass, and 10% of the moles of nitrogen in the system was present in chitin.

Ideally, it is desired to be able to pretreat an entire system of biomass to the extent that the "top" portion. If this would be able to be achieved, one could assume that the entire pretreatment mass (in this case, 6.5) was pretreated uniformally and experienced the same change in elemental composition throughout the system. If this were the case, 0.0021931 g of the

nitrogen in the biomass could be converted into chitin and made available to *C.phytofermentans*. This calculation suggests that even under these highly un-optimized conditions, up to 10% of the nitrogen available in biomass can be sequestered for future use be *C.phytofermentans*. This could mean a potential cost reduction and points to the possibility of a future system in which nitrogen addition is very minimal based on optimized transfer of nitrogen through fungal biomass.

Energy Content Analysis

The energy content of all three substrates utilizied in this experiment were determined via bomb calorimetry. The heat of combustion for switchgrass, bagasse, and steam treated bagasse were calculated to be 18.3, 15.6, and 16.3 kJ/g, respectively (Figure 24).

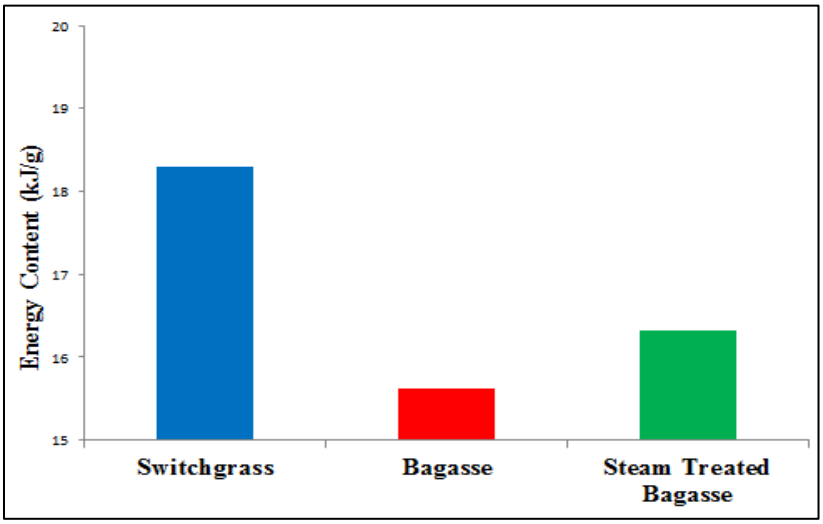

Figure 24. Experimental values for the heat of combustion of three different biomass substrates (switchgrass, bagasse, and steam treated bagasse), determined by bomb calorimetry.

After fungal pretreatment of bagasse, increases in energy content were observed for both *P. chrysosporium* and *C. subvermispora* treated samples (Figure 25). The "top" section of *P. chrysosporium* treated bagasse (Figure Xb) resulted in a 14.7% increase in energy content relative to no fungal pretreatment, whereas the "bottom" section of the same fungal pretreatment resulted

in no substantial change in energy content. This means that the shift of mass from the biomass substrate to the fungal biomass causes an increase in energy density in the system.

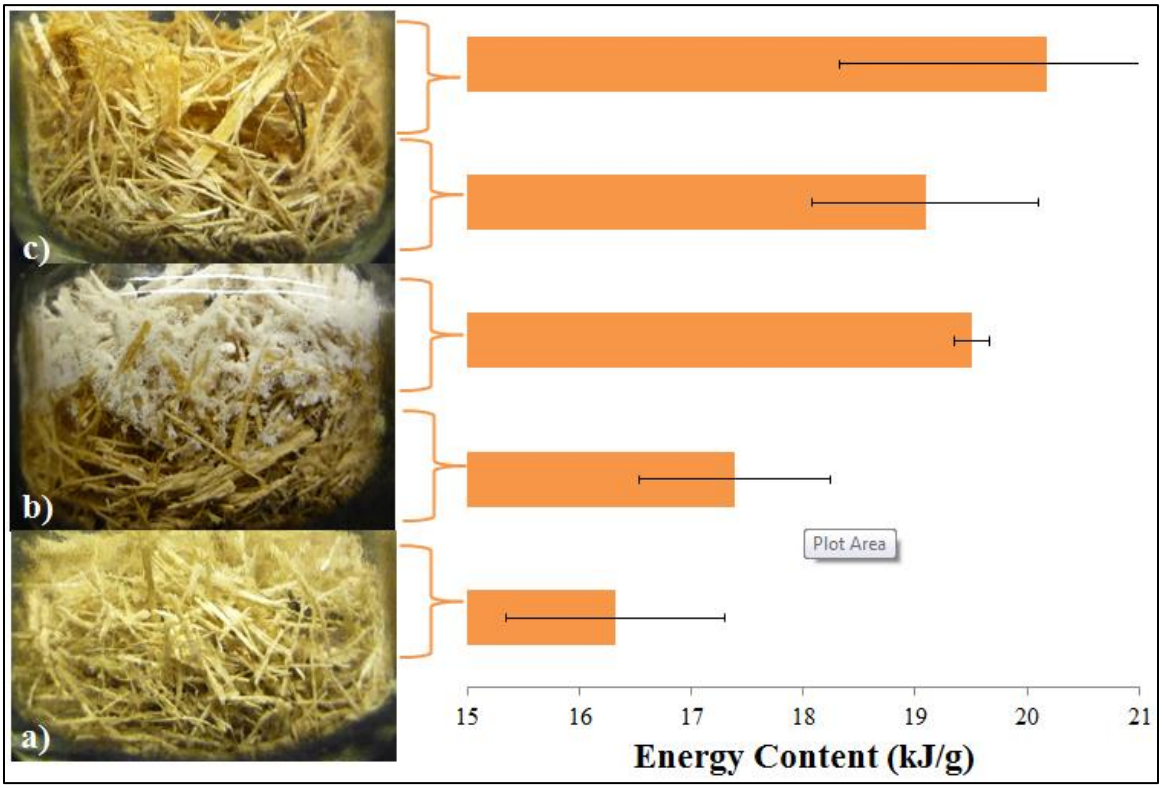

Figure 25. The heat of combustion of non-treated (a), P. chrysosporium treated (b), and C. subvermispora treated (c) bagasse after 16 days of pretreatment.

Literature values for the energy density of different components of this pretreatment system were examined in order to help explain this phenomenon (Table 2).

Table 2. Energy Densities of Various Compounds

	Energy Density (kJ/g)	Reference
Cellulose	17.3	(Kaltschmitt 2009)
Hemi-cellulose	16.2	(Kaltschmitt 2009)
Chitin	21.2	(Whitaker Jr. et al., 2004)
Fungus (Mucor)	22.4	(Prochazka et al., 1970)
Lignin	27	(Kaltschmitt 2009)

Because lignin has higher energy density than chitin and the other components of fungus, removal of only lignin and addition of only fungal biomass would result in a decrease in energy density of the system. Because cellulose has lower energy density than chitin and the other components of fungus, removal of only cellulose and addition of only fungal biomass would result in an increase in energy density of the system. Therefore, in order for the energy content of the system to increase, as in the case for the *P. chrysosporium* treated bagasse samples, it is likely that a higher mass of cellulose than lignin was removed.

Because *C. subvermispora* is suggested to have more specificity toward lignin degradation, this fungus would essentially be removing more higher energy content material (lignin) and less lower energy content material (cellulose). Assuming that optimal *C. subvermispora* growth occurred, one would expect it's pretreatment to result in less energy content than a *P. chrysosporium* pretreatment. At the current time, it is puzzling as to why a *C. subvermispora* pretreatment may result in higher energy density than a *P. chrysosporium* pretreatment.

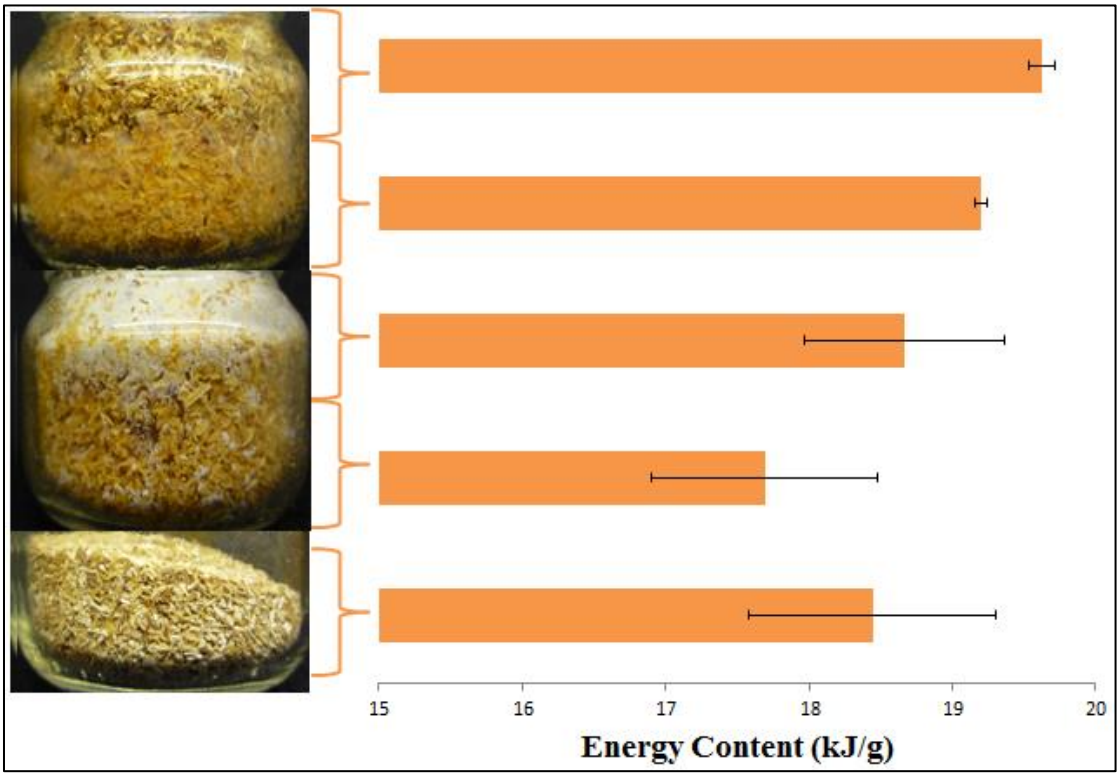

Figure 26. The heat of combustion of non-treated (a), *P. chrysosporium* treated (b), and *C. subvermispora* treated (c) switchgrass after 16 days of pretreatment.

In contrast, *P. chrysosporium* pretreatment of switchgrass did not show any significant change in energy content (Figure 26). This may suggest that there is a difference in the way that *P. chrysosporium* attacks different substrates with varying chemical compositions. It is possible that more lignin is removed from switchgrass than is removed from bagasse, resulting in a lower overall energy content in the switchgrass pretreatment than in the bagasse pretreatment.

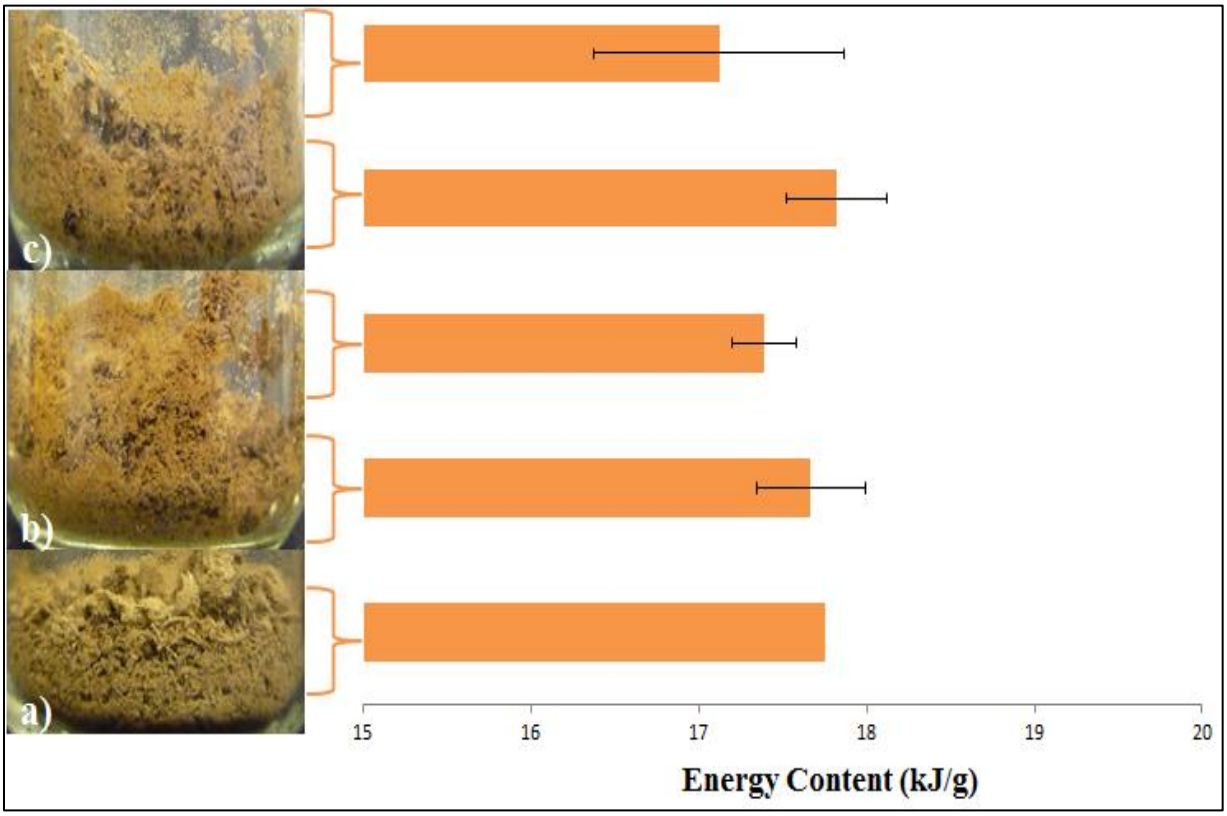

Figure 27. The heat of combustion of non-treated (a), *P. chrysosporium* treated (b), and *C. subvermispora* treated (c) steam-treated bagasse after 16 days of pretreatment.

No significant change in energy density was observed for either of the fungal pretreatments of steam treated bagasse (Figure 27), which supports the theory that not fungal growth occurred on this substrate.

Chapter 5: Conclusions and Future Work

Implications of Varying Fungal Growth

This work has succeeded in recognizing the fact that substantial differences exist between *P. chrysosporium* and *C. subvermispora* and their ability to grow on different lignocellulosic feedstocks. Generally, *C. subvermispora* has shown the ability to effectively colonize woody biomass (such as oak and hybrid poplar) and has shown a relative inability to effectively colonize non-woody material (such as switchgrass and bagasse). On the other hand, growth of *P. chrysosporium* on woody biomass did display poor growth as compared to growth on non-woody biomass. This presents the idea that it may not be possible to identify "one" fungus that is best for every case scenario in the FILtH paradigm. In fact, it is likely that it may not be possible to develop a single biofuel production process that would work for every different type of substrate. All different distributed processing locations in this paradigm will likely have access to different biomass feedstocks, and therefore will require the fungus that can most effectively grow on that specific substrate. It would be particularly interesting as well, to conduct studies of mixed white rot fungal growth, on mixtures of substrates to see if there is synergy or even inhibition beyond simple competition.

Future experiments would also benefit from achieving more comparable inoculation methods for these two fungi. Throughout the pretreatment experiments, *P. chrysosporium* was inoculated as a spore solution, and *C. subvermispora* was inoculated as a homogenized liquid culture. These two forms of inoculation are difficult to compare quantitatively, and ideally we could compare the growth rates on substrates based on the same inoculation method. Although the method presented in this thesis for attempting to collect *C. subvermispora* spores did not yield

significant spore production, it was based on a single publication that apparently had never been replicated. Since spores were observed in *C. subvermispora* cultures in this work, it is likely that with trial and error it will be possible to achieve mass sporulation of this fungus. The reality is that the methods for sporulation techniques presented in the referenced paper were not well documented, hard to follow and could not be reproduced in our hands.

Quantification and Observation of Differing Fungal Activity

Considerable differences have been recognized for the effect of pretreatment by two different fungal species on lignocellulosic biomass substrates. *P. chrysosporium* and *C. subvermispora* treatment on poplar wood results in differences in Sum-frequency Generation signal intensity, supporting recent discoveries describing the differences in the genomic sequences of these two fungi.

Solid-state cultivation of these fungi have resulted in differing observation of extent of growth on lignocellulosic substrates. Neither fungal species had the ability to grow on steam-treated bagasse, most likely resulting from the presence of thermally produced inhibitors. This is somewhat surprising since white-rot fungi have been used to degrade toxic environmental compounds such as polyaerobic hydrocabarbons and polychloriniated biphenyls. It is possible that cultures can adapt to these inhibitors upon gradual exposure.

There was considerably higher observed growth for *P. chrysosporium* than for *C. subvermispora* on switchgrass and bagasse, with differences in location of growth for the two fungi. After 16 days of pretreatment, the majority of *P. chrysosporium* fungal biomass was present in the "top" portion of the system, whereas *C. subvermispora* showed higher growth in the "bottom" portion of the system. This observation of the potential important role of oxygen availability suggests that an important aspect of pretreatment is a more defined availability of

oxygen, CO_2 or other atmospheric gasses such as ethylene which is known to be produced by some fungi, and is a stress metabolite in plants. An examination of pretreatment performance under very low forced convection conditions seems to be a critically important assessment that should be made to better understand the role of the gas phase in the scaleup of such a biological pretreatment process.

A significant increase in nitrogen content in a heavily cultivated portion of *P. chrysosporium* pretreated switchgrass was quantified, suggesting that nitrogen has been retained in the system with a loss of other elemental components. The retention of nitrogen within the system may prove to be beneficial for *C. phytofermentans* growth during downstream processing in the FILtH paradigm.

For more concrete evidence of these quantifications and observations, all of these studies could be repeated with aims to achieve the same results. Because of the time constraints of this project, only duplicates of each pretreatment variation was examined in the analyses. It is recommended that these experiments be repeated in triplicate, to further validate the data that was obtained.

Fully Encompassed Quantification of Pretreatment Activity

In order to better determine whether *P. chrysosporium* or *C. subvermispora* would provide a better fungal pretreatment for use in low energy in put FILtH paradigm, it would be important to quantify additional aspects of the pretreatment process. The accurate quantification of lignin, cellodextrins, and sugars during pretreatment would help to explain some of the phenomena examined in this work. Knowing the ratio of lignin to cellulose removal of the two fungi during pretreatment is absolutely necessary to gain better insight into the results of SFG.

Also, sugar determination would help to explain the positive and negative aspects of *P. chrysosporium*'s ability to degrade cellulose during delignification.

Quantification of chitin through a chitin assay or another accurate analytical technique would be helpful when considering the application of the fungal pretreatment in the FILtH paradigm. Because there is an interest in *C .phytofermentans*' ability to consume chitin as a nitrogen source, it would be important to quantify the availability of this nitrogen supply. Significant literature research has determined that a colorimetric assay performed by acid hydrolysis of pretreated biomass, a technique discussed in "Estimation of mycelial growth of basidiomycetes by means of chitin determination" (Plassard et al., 1982), would result in accurate chitin and fungal quantification. A tentative procedure for this chitin assay was produced, but is continuing to be modified (Appendix B). It would be interesting to see how this analytical quantification compares with the estimation provided in this work.

These quantifications would also help to better explain the phenomenon of changing energy content due to fungal pretreatment. It would be interesting to see a correlation between lignin and cellulose removal with the change in energy density of the pretreatment.

Effect of Fungal Pretreatment on Downstream Processing

It must not be forgotten that the main goal in optimizing a fungal pretreatment is to increase overall productivity of biofuel. Therefore, there should be ongoing demonstrations that fungal pretreatment does in fact increase productivity of biofuels during subsequent processing to biofuels. Work is currently in progress by Trevor Zuroff to assess fungal pretreatment effects on downstream processing. Many analyses can be envisioned; the effect of the presence of chitin and fungal biomass on growth of *C. phytofermentans*and *Saccharomyces cerevisiae*, the variation

of change in productivity of biofuel as a result of fungal pretreatment on different substrates, effects of length of downstream processing of pretreated biomass, and many more.

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Appendix A

Development of SFG Sample Preparation

Because this was the first time that this type of SFG work had been performed in Curtis Lab, significant methods had to be developed and optimized to obtain useful results. First, various methods of fungal growth were tested for their ability to produce good samples for SFG analysis. The first round of samples were prepared by inoculating wood chips with mycelia from a plate culture. Three inoculation loopfulls of plate culture were introduced into jars that contained the biomass substrates.

The fungi grew considerably well on biomass, with varying degrees of growth for the two different fungal species. *C. subvermispora* seemed to grow very well on Oak wood chips (Figure 6a), while *P. chrysosporium* seemed to have trouble growing on this substrate (Figure 6b). Both of the fungi seemed to grow to relatively the same level on poplar wood chips (Figure 6c and Figure 6d).

These samples were analyzed with Sum Frequency Generation by Kabindra Kafle to produce SFG signal results. Different trends in the results were seen when comparing the poplar treated samples and the oak treated samples. Both fungi caused a substantial decrease in SFG intensity for the treated poplar samples (Figure 28a). *P. chrysosporium* treatment resulted in about a two-fold decrease in the SFG intensity, whereas *C. subvermispora* treatment resulted in about a four-fold decrease in the SFG intensity. For treated oak samples, *P. chrysosporium* treatment resulted in about a three-fold decrease in SFG intensity, whereas *C. subvermispora* resulted in about a two-fold increase in intensity.

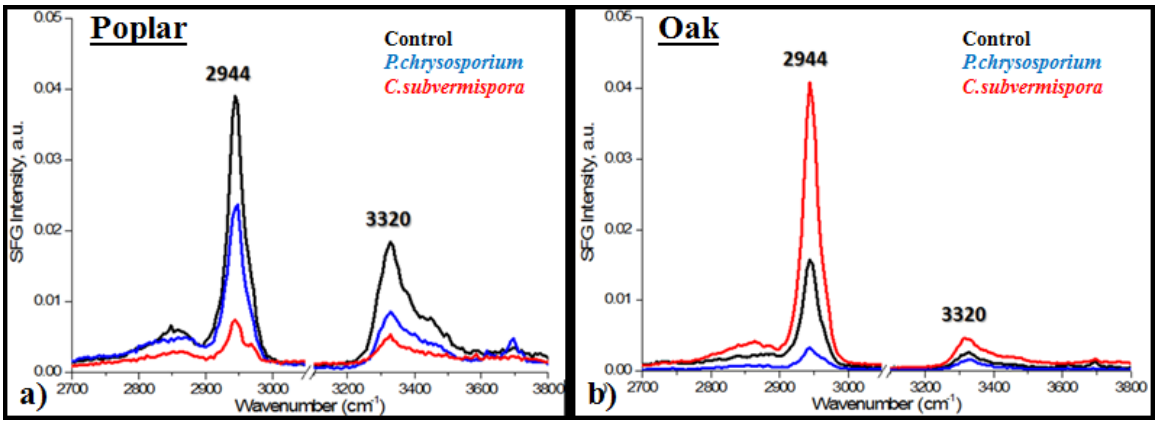


Figure 28. SFG signals of both P. chrysosporium and C. subvermispora pretreated poplar (a) and oak (b).

The results of the oak treated samples were expected. An increase in SFG signal for *C. subvermispora* treated supports that fact that the fungus is selective in degrading lignin over cellulose. *C. subvermispora* removes lignin from the substrates without making any major changes in the crystalline cellulose, resulting in a higher concentration of crystalline cellulose relative to the entire biomass sample. On the other hand, *P. chrysosporium* simultaneously degrades cellulose as it removes lignin. For the SFG intensity to actually decrease as it did with the oak treated samples, the mass fraction of crystalline cellulose relative to the entire substrate must have decreased. This suggests that *P. chrysosporium* is substantially altering the crystallinity of the cellulose in the substrate.

This preliminary round of results demonstrates the need for a better sample preparation technique for SFG analysis. Substantially different results were obtained for the oak treated samples versus the poplar treated samples.

The preliminary sample preparation resulted in substrates with rough surfaces and uneven fungal growth, problems that must be resolved to achieve accurate results. To achieve a smoother substrate surface, the substrates were cut to a 2 mm thickness.

To achieve even fungal growth, a new inoculation technique was developed. In this method, the finely cut substrate was introduced onto fungal plate cultures with 7 days of growth. As pretreatment progressed, the substrates are overtaken by the fungal culture, allowing it to effectively pretreat the samples. Samples can be easily removed from the plate at any time, allowing analysis to be performed at various time points during the pretreatment. Figure 29 shows this new technique in action at 0 days of pretreatment (Figure 29a and Figure 29c) and at 8 days of pretreatment (Figure 29b and Figure 29d).

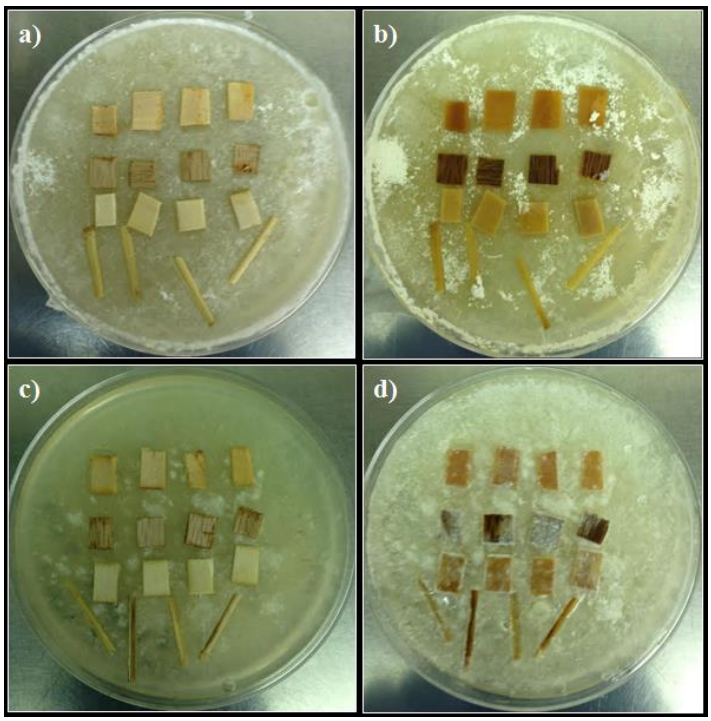


Figure 29. Demonstration of the pretreatment of biomass samples for application in SFG analysis. Poplar, oak, pine, and switchgrass were laid on plate cultures of fungus. Growth of P. chrysosporium at 0 and 8 days(a and b) and C. subvermispora at 0 and 8 days(c and d) are presented here.

Higher fungal growth on top of the substrates was observed for *C. subvermispora* than was observed for *P. chrysosporium*, most likely due to the faster growth rate and sporulation of *P. chrysosporium*.

Possible Fungus Synergy

It was observed that co-culturing P. chyrsosporium and C. subvermispora together on swtichgrass resulted in faster growth of fungus than did either one of these organisms cultured individually. Figure 30 shows pretreatment of *P.chrysosporium* (Figure 30a), *C. subvermispora* (Figure 30c), and a co-culture of the two (Figure 30b), after four days of growth on switchgrass. One can see here that only the co-culture shows any observable fungal growth. Based on the color of the fungal growth, it is most likely that this growth is mainly P.chrysosporium. This means that the addition of C.subvermispora into the pretreatment may for some reason give P. chrysosporium an enhanced growth rate. It is possible that this is merely a result of minimal amount of media that was introduced along with the homogenized liquid culture of C. subvermispora. Although this homogenized liquid culture was suspended in DI water, there may have been some media left behind from the liquid culture that it was prepared from. This addition of media would allow for faster growth of P.chrysosporium. However, it is possible, and should be tested that these two organisms have some type of synergy when cultures together.

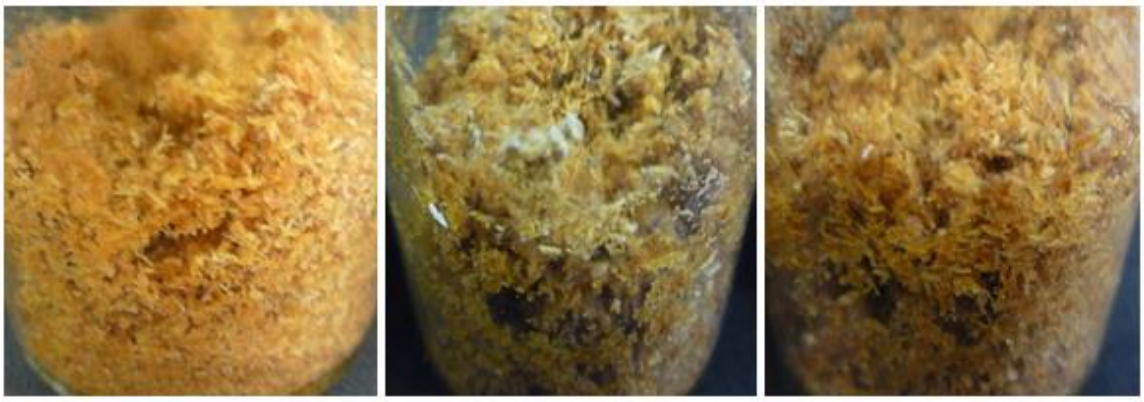


Figure 30. P.chrysosporium (a), C. subvermispora (b), and a co-culture of the two (c), grown on switchgrass for 4 days at 30 C.

Appenix B: Standard Operating Procedures and Media Recipes

Chitin Assay

Chitin Assay - Fungal Cell Wall Quantification
Version: Taylor Maher Feb 2014

Principle of the Assay:
Chitin can be hydrolysed completely into acetyl and glucosamine residues using HCl. De-amination of glucosamine occurs by addition of HNO_2 to solution, to yield 2,5-anhydromannose. Reaction of this compound with MBTH yields a strong blue color that can be measured at 653 nm against a reagent blank.

Calculations:
The total hydrolysis of P weight of chitin produces N molecules of glucosamine and N molecules of acetic acid. The formation of P weight polymer of acetylglucosamines causes the loss of N-1 water molecules.

Therefore:

P=221*N - 18*(N-1)
221 = MW of acetylglucosamine
18 = MW H20
X= mass of glucosamine present
N= molecules of glucosamine present
X=179*N

The materials needed are:

- 6 M HCl
- Nitrous Acid (HNO_2)
- 0.5% 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) **
- NaOAc
- Chitin (for calibration curve)
- 5% $NaNO_2$ solution
- 5% $KHSO_4$ solution
- 12.5% $NH_4SO_3NH_2$
- 0.5% $FeCl_3$ (0.83 g $FeCl_3*6H_2O$ in 100 mL) **
- 96 well plate

**Need to be prepared 3 days fresh and stored in a refrigerator

Tentative Procedure:

1. Collect representative sample of pretreated biomass
2. Measure out a known mass of this representative sample for three separate eppi-tubes
3. Add 1 mL of 6 M HCl to each eppi-tube, and place in 80 degree celsius incubator for 16 hr.
*At this point, all chitin has been hydrolyzed into acetyl and glucosamine residues, and we now follow methods laid out in this paper.
4. To 1 mL of sample solution containing glucosamines, add 1.25 M NaOAc to pH the solution to ~ 3.0 (should be around 5 mL).
5. Remove 1 mL of the pHed solution (if 5 mL of NaOAc was added, then the concentration of glucosamine is now ⅙ of what is was before)
6. To 1 mL of sample, add 1 mL of 5% KHSO4, and 1 mL of 5% NaNO2
7. Leave the solution standing for 15 min, shaking occasionally, to complete deamination.
8. Remove excess nitrous acid by adding 1 mL of 12.5% NH4SO3NH2.
9. Add 1 mL of 0.5% MBTH, and allow to stand for 60 min.
10. Add 1 mL of 0.5% FeCl3, let sit for 30 min, and read the absorbance at 653 nm against the reagent blank.

The absorbance of the solution has a direct relationship with the mass of glucosamine present. Therefore, we can determine the number of molecules present, and then the total mass of our polymer, chitin

YMPG Media

Note: The following recipe is for 1-L of media; however it can be made in any volume by adjusting the mass of ingredients accordingly.

Procedure:

1. To a 2-L autoclavable glass flask, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

D – Glucose, Anhydrous (Dextrose; CAS# 492-62-6)	**10g**
Malt extract (VWR Part# 90001-014)	**10g**
Peptone (VWR Part# 61001-506)	**2g**
Yeast extract (Sigma-Aldrich Part# 70161-100G)	**2g**
L – Asparagine, Anhydrous (CAS# 70-47-3)	**1g**
KH_2PO_4 (CAS# 7778-77-0)	**2g**
$MgSO_4 \cdot 7H_2O$ (CAS# 10034-99-8)	**1g**

3. **If making slants, add 20g of agar** (CAS# 9002-18-0)**. (Omit agar if making liquid media.)**
4. Remove the stir bar, and take the final volume to 1 L with DDI water.
5. Wrap aluminum foil over the opening of the flask and add a piece of autoclave tape.
6. Place flask in an autoclavable bin and fill with several inches of tap water to aid in equal heating.
7. Autoclave for 30 minutes.
8. Remove from autoclave and allow media to cool until you can place your hands on the sides of the flask without being burnt.

D-GCC Stress Media

Note: This media recipe was taken from an experiment done in "Induction and mass sporulation in lignin degrading fungus *C. subvermispora* for its potential usage in pulp and paper industry." (Saxena et al., 2001).

1. To a 2-L autoclavable glass flask, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

D – Glucose, Anhydrous	**10g**
$CaCl_2$ * $2H_2O$	**7g**

*It has been calculated that adding 5.28 g of anhydrous $CaCl_2$ would be equivalent to adding the 7g of $CaCl_2$*$2H_2O$

3. Remove the stir bar, and take the final volume to 1 L with DDI water.
4. Wrap aluminum foil over the opening of the flask and add a piece of autoclave tape.
5. Place flask in an autoclavable bin and fill with several inches of tap water to aid in equal heating.
6. Autoclave for 30 minutes.
7. Remove from autoclave and allow media to cool until you can place your hands on the sides of the flask without being burnt.

CurtisLab *P. chrysosporium* Protocol

The following Standard Operating Procedures, written by Salvador Barri, were used in this study for the culturing and inoculums preparation of *P. chrysosporium*:

Making agar plates

1. After adding the filter-sterilized thiamine hydrochloride in the laminar flow hood in room 232 as stated in step 9 in the YMPG media recipe, pour a small amount fresh YMPG media into a petri dish. *You can pour it directly from the bottle without using a serological pipet. Pour enough so that it covers the entire surface area with no air bubbles.*
2. Cap the petri dish and set to a side of the hood
3. Repeat step 1 and 2 for each petri dish or until you have used about 750 ml of the YMPG media (the rest of the volume will be used in Slants).
4. Make a small pile of 3 or 4 plates on top of each other to make space and let it solidify.
5. After the plates have solidified, Store them in on a stack, like they were packaged, inside the bag and label the bag properly with label tape (initial, date, media)
6. Store the agar plates in the cabinet in 225 until you need them again

I. Making Agar Slants

1. Place the test tube rack in vertically against the laminar flow hood.
2. Take 5 ml of YMPG media from the leftover media from Making Agar Plates using a 10 ml sterile serological pipet with the help of a Pipet-aid. *There should be 250 ml left for making the slants. When extracting the media make sure not to touch the bottle with the pipet.*
3. Carefully open the cap of the 15 ml conical tube with your non-dominant hand. *Hold the cap close to the conical tube.*
4. Cautiously transfer the 5 ml YMPG to conical tube. *Do not touch the inside of the tube with the pipet since it is a possible source of contamination. Also try not make any bubbles.*
5. Seal the conical tube.
6. Place the 15 ml conical tube in the test tube rack on a 10º angle with the slant on the non-label side. *Make sure the agar does not touch the cap.*
7. Let the slants solidify in the laminar flow hood.
8. Store the slants in the cabinet next to the YMPG plates.

II. Inoculation

1. Take one of the agar plates made in Making Agar Plates section and label it with the name of the Fungus (*P. chrysosporium*), initials, date, media type, and initial quantity of spore solution.
2. Take the *P. chrysos* spore solution into the laminar flow hood.
3. Carefully open the spore solution bottle
4. With a sterile 200 ul pipette, extract 100 ul of spores.

5. Decap the agar plate and carefully introduce 100 ul of spore solution in the center. *Be sure not to touch the agar with the pipet tip.*

7. Cap the agar and cover the space between the cover and the base with parafilm.
8. Place the plate in the incubator in the molecular biology room in 232. *The incubator MUST always be at 37C^{o}. Be sure not to tilt the agar plate when moving it into the incubator.*
9. The spores are ready for suspension after 5-6 days.

III. Heavy Suspension of P. Chryso

1. Make new YMPG as detailed in YMPG, but do not add agar to make liquid YMPG media.
2. In the flow hood located in room 225, place the vortex genie and plug it into the outlet in the hood
3. Uncover the slants and add YMPG medium up to the 15 ml mark. *Two slants should be enough to make +20 cryotubes.*
4. Cover it, shake it well with the vortex genie. *Try not to shred or cut the agar with the vortex genie.*
5. After most of the spores have dissolved in the liquid, filter it with an autoclaved thin wool cloth pour the liquid in a 50 ml conical tube. *Use the cloth in conjunction with the strainer to remove any mycelia that is in the liquid. As with the slan ts, label the 50 ml conical tubes.*
   Repeat steps 3 - 5 for each slant. *You can add the liquid from multiple slant into the same 50 ml conical tube.*

IV. OD Sampling

1. With a sterile 1000 ul pipette tip, extract 1 ml of the spore suspension from the 50 ml conical tube.
2. Place it a clean cuvette. *Gently Pipette up and down 3 times to have a homogeneous solution*
3. Make a "blank" sample by placing 1 ml of liquid YMPG made in Heavy Suspension of *P.chrysos* in another cuvette.
4. Place the "blank" cuvette in the Spectrophotometer located in room 222.
5. Change the wavelength, button with lamda, to 650 nm. *Make sure it is in Absorbance mode. You can change the mode by clicking the T% / A button.*
6. Close the lid and press the Ref button. *The LED screen should show 0.000 under the cuvette label*
7. Take out the "blank" sample and introduce the cuvette with the actual sample in its place.
8. Close the lid, again, and now press the Read cuvette button. *The OD for refrigeration should be around 0.5 and for cryopreservation is 1.0.*

a. *If the OD is higher than desired, add more YMPG to the solution in the 50 ml conical tube to dilute it until you get the desired OD.*

If the OD is too low, suspend more spores into the solution by ret urning some liquid in the 50 ml conical tube back to the slant and shake well as stated in step 4 of Heavy Suspension of P. chrysos. Put the liquid again in the same conical tube and revise its OD Repeat until you have the desired OD.

V. Getting the Pretreatment Ready

1. Adding 2.5g of milled biomass in an empty serum bottle. *This gives a final concentration of 50 g / L of biomass during fermentation. Using a funnel is highly recommended*
2. Cutting a sponge plug in half. Use one half to plug the serum bottle. *Make sure there are no air spaces*
3. Covering the entire bottle mouth with aluminium foil.
4. Autoclave the serum bottles for 30 min in dry/gravity.
5. Let it cool in the hood in room 232.

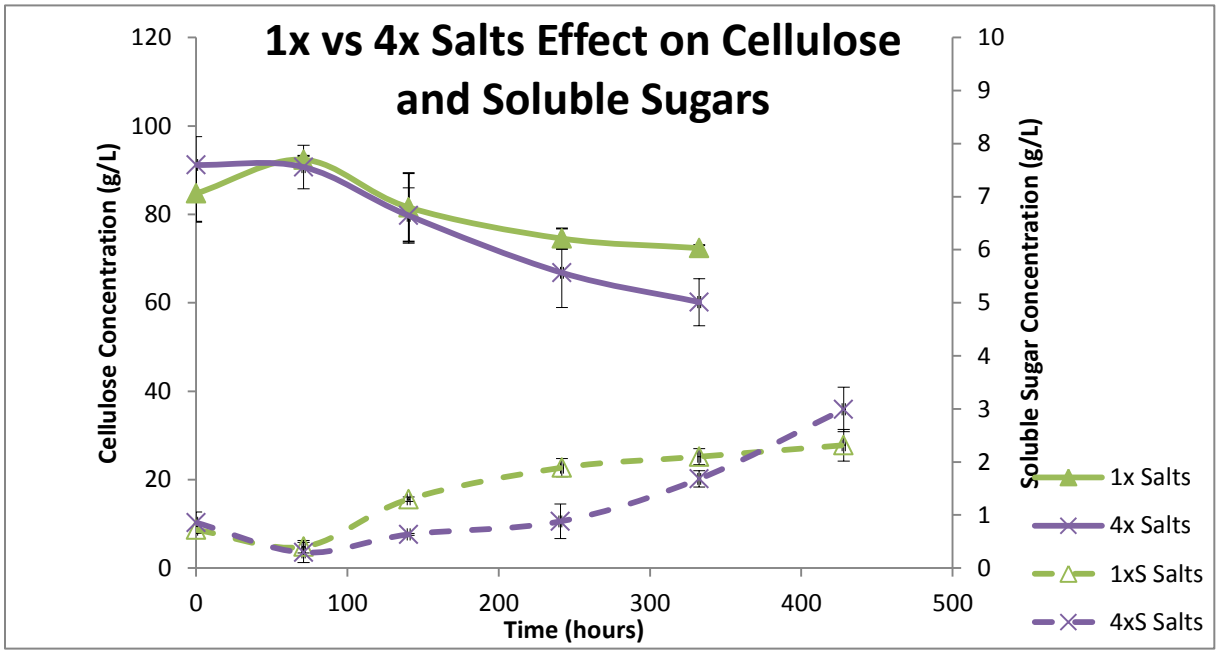
VI. Pretreatment

1. Take the serum bottles to the hood in room 225
2. Sterilize the environment and a pair of tweezers by using a bunsen burner. *To sterilize the tweezers we recommend spraying a little bit of ethanol on the tips and then use the bunsen burner to heat it.*
3. With the tweezers, carefully remove the aluminium foil and the sponge plug.
4. Fill a 15ml conical tube with 5 ml of sterile DI water
5. With a 10 ul pipette, extract 1 ul of *P.chysos* from the liquid culture and add it to the 5 ml of DI water in the conical tube. *The 10 pipette is located in microbial lab room in 232. As before the pipette should be sterile.*
6. Mix well and add the solution into the serum bottle.
7. Place the sponge plug and the aluminium just as they were before. *Make sure the sponge plug covers the entire surface area of the opening.*
8. Place the serum bottle in the shaker. *The pretreatment will be done in 10 days*

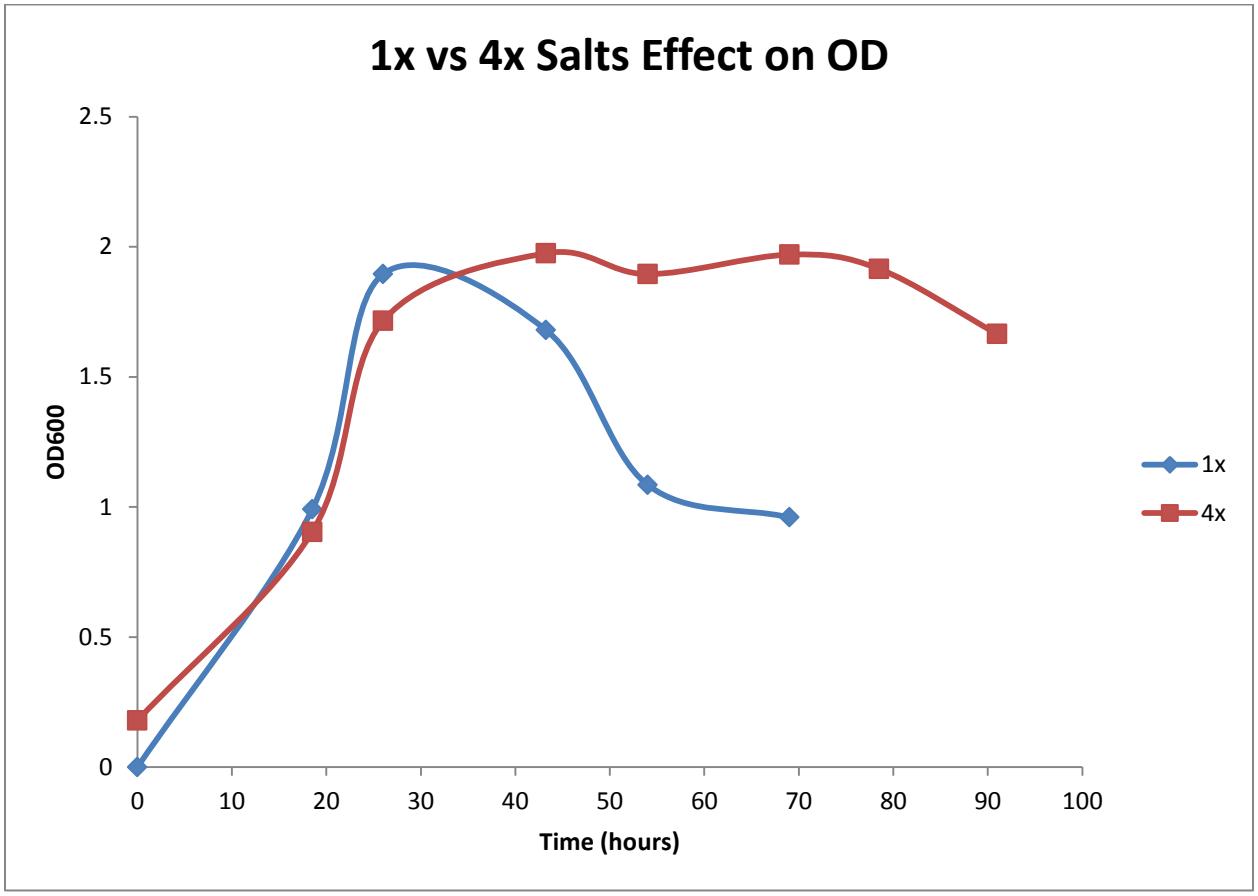
Appendix C: Effects of Increased Salts on *C.phytofermentans* Growth and Sporulation

Work done by me, alongside Trevor Zuroff, produced the following results:

It was observed that quadrupling the salts concentration (containing magnesium, calcium, and iron) in the media formulation for *C.phytofermentans* resulted in increased cellulose hydrolysis and increased ethanol production. An interesting profile for the accumulation of soluble sugars was also noticed. The system with 4 times (4x) the amount of salts seemed to keep its soluble sugar concentration lower than the system with normal (1x) concentration of salts for a longer period of time, as can be seen by the following chart:

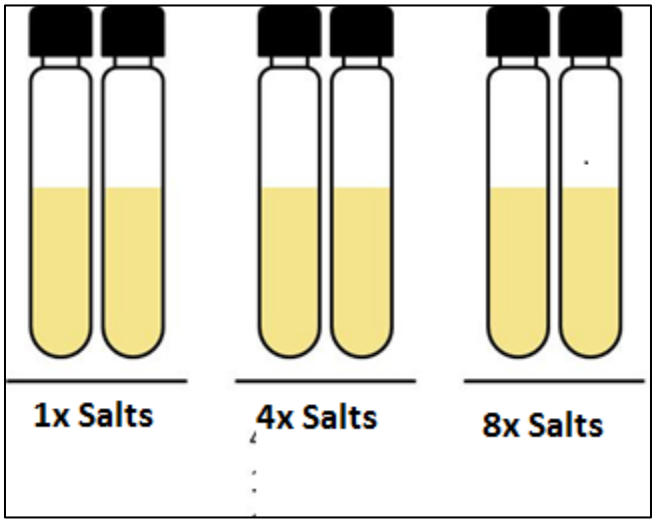


It was observed that quadrupling the concentration of all three salts when growing C.phytofermentans on 50 g/L glucose led to increased OD stability. As can be seen by the figure below, both the 1x and 4x systems resulted in a very similar maximum OD. However, the 4x salts system was able to retain this maximum OD for a longer period of time.

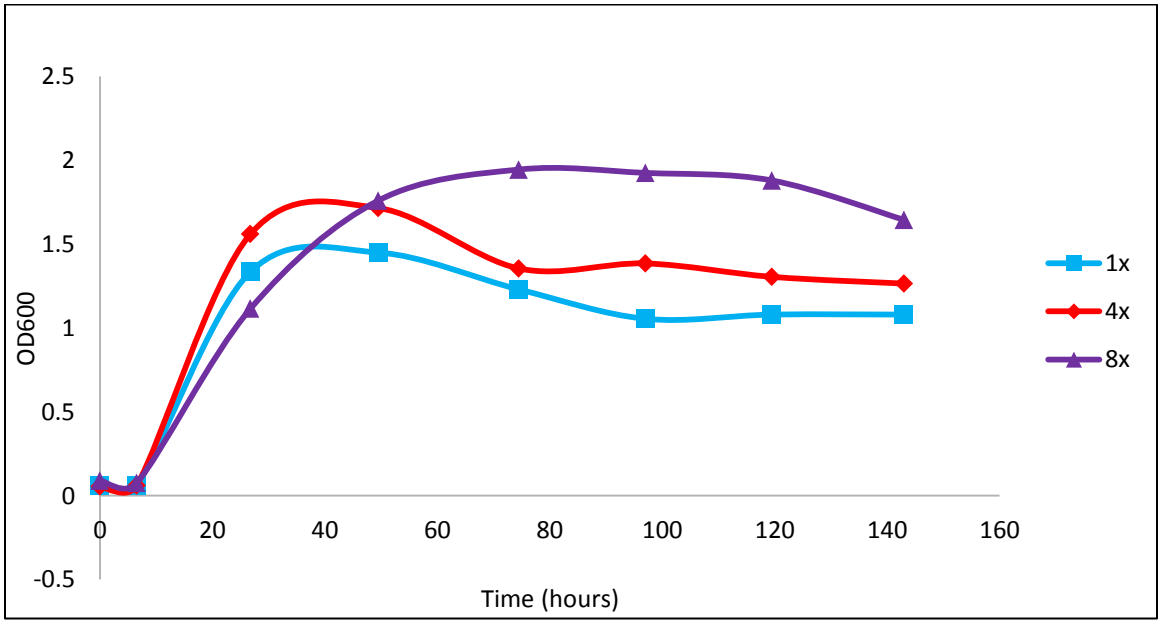


This OD stability was thought to possiblly be related to the ability of *C.phytofermentans* to continue to ferment soluble sugars to ethanol and therefore not accumulate as much. It was hypothesized that sporulation may be what results in the decrease in OD in the normal media formulation with only 1x concentration of salts. Therefore, it is possible that the increase in salts to a 4x concentration delayed sporulation of *C.phytofermentans*, leading to increased ethanol production.

A final experiment was set up to provide insight as to what was physically happening to the *C.phytofermentans* population and cells during the bacteria's growth and fermentation. The experiment had a total of six tubes. There was three different concentrations of salts tested (1x, 4x, and 8x). *C. phytofermentans* was grown on 50 g/L glucose in each of the tubes.

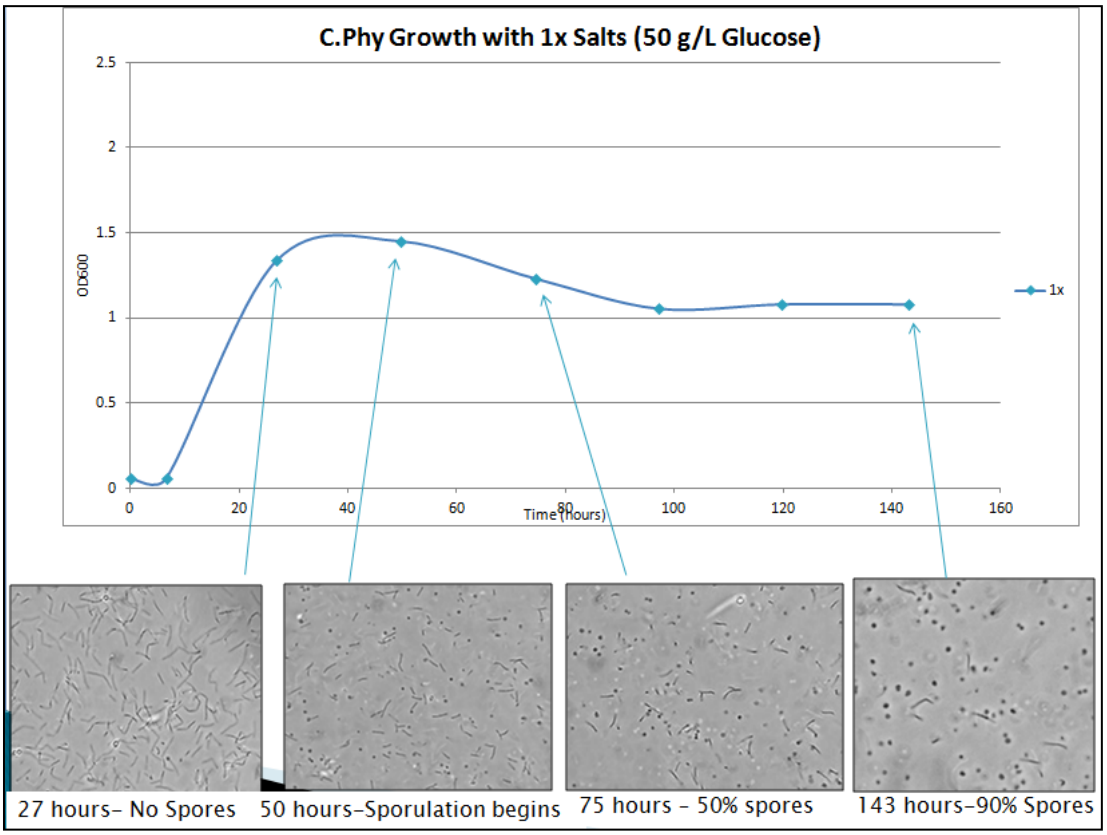


Every day, and sometimes twice a day, a 350 uL sample was taken. This sample was used to take an OD reading, run HPLC, and to look at the sample under the microscope. This experiment obtained very interesting results. The following OD data was obtained from this experiment:



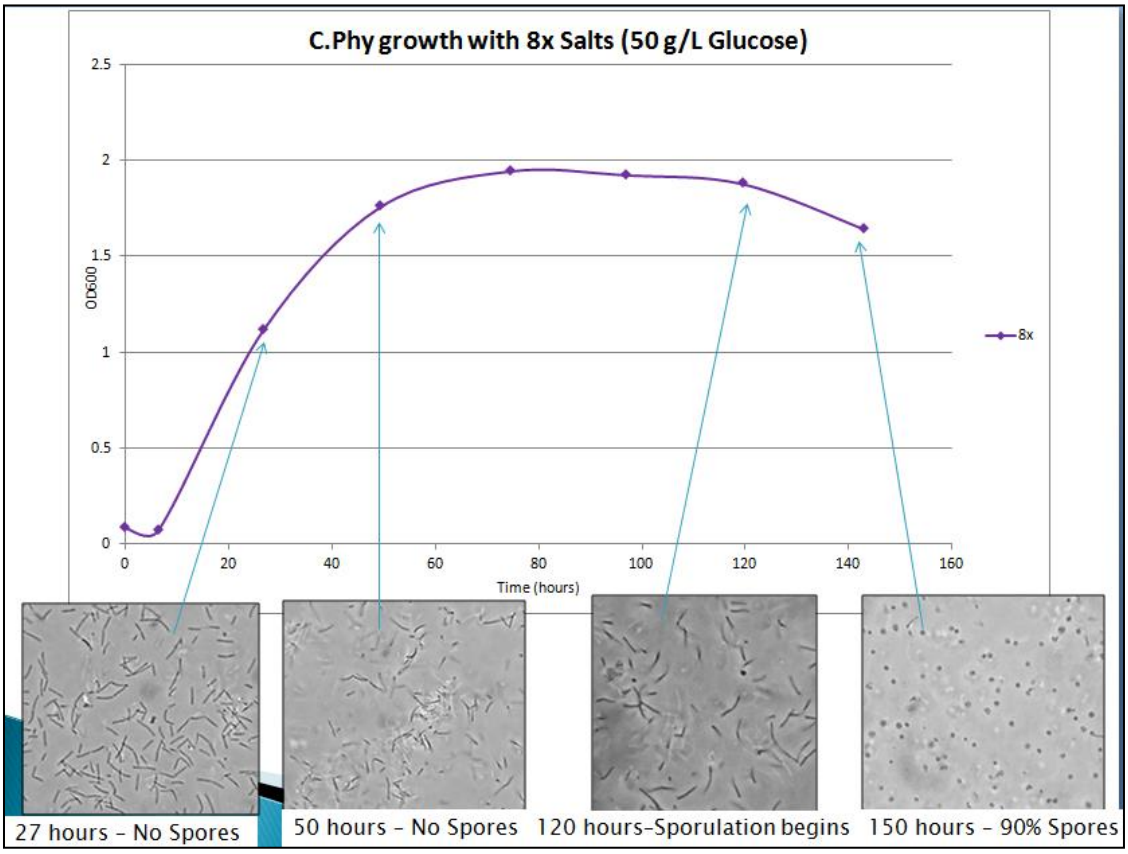
As can be seen from the chart, the system with 8x concentration of salts had a maximum OD for a longer time period. This result is different from what was seen in the past, when we had seen a longer maximum OD time for 4x concentration of salts. This difference may have resulted from different experimental conditions, such as using larger tubes, using a higher volume of media, and using gas relief valves. Although results were different, valuable information was still obtained about sporulation.

The following pictures were taken during the growth of *C. phytofermentans* in media with normal concentration of salts:



As can be seen from this chart, sporulation begins at the point when OD begins to drop. At about 50 hours, this culture began to sporulate.

The following pictures were taken during the growth of *C. phytofermentans* in media with 8x concentration of salts:



This culture did not begin to sporulate until about 120 hours, which suggests that an increase in salts leads to delayed sporulation in cultures of *C. phytofermentans*. This experiment was a strong step in investigating the effects of salts on *C. phytofermentans'* sporulation and ability to degrade cellulose effectively.

There are many things that can be done to move this work forward in the future. To more accurately determine the effects of salts on sporulation, there would need to be accurate spore counts during cellulose hydrolysis by *C. phytofermentans*. In order to do this, a reliable heat shock procedure or some other procedure should be developed. Monitoring *C. phytofermentans* sporulation during growth on cellulose would be a large step forward in understanding the bacteria's ineffective cellulose degradation.

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Member, **Penn State Ski Club**
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Professional Experience

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- Performed process analysis and developed material and energy balances
- Prepared project cost analysis

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