The Transcriptional Response of Phanerochaete Chrysosporium and Trametes Versicolor to Growth on Stems of Helianthus Argophyllus (Silverleaf Sunflower)

Nadh Hamoud Alsubaie

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The Transcriptional Response of Phanerochaete chrysosporium and Trametes versicolor to Growth on Stems of Helianthus argophyllus (Silverleaf sunflower)

by

Nadh Hamoud Alsubaie

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGICAL SCIENCES IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY CHARLESTON, ILLINOIS 2019

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE
Abstract

Traditional physicochemical and physicomechanical pretreatment technologies have improved the deconstruction of lignocellulose, but often require high energy inputs and added chemical reagents. Recent studies have demonstrated the use of white-rot fungi as alternative biological pretreatment agents that can mitigate these concerns. Although, white-rot fungi have been shown to grow on a variety of non-woody substrates, they have not been explored with respect to sunflower, which has the potential to provide raw materials for both biodiesel and bioethanol. In the present study, Silverleaf sunflower stems (*Helianthus argophyllus*) were used as substrates for the white-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor*, and the transcriptome response by these fungi to sunflower stems was explored after six weeks of growth. Those genes that were more highly expressed on sunflower stems compared to malt extract medium were determined at the 25X and 10X thresholds. At the 25X threshold there were 102 transcripts for *P. chrysosporium* and 144 transcripts for *T. versicolor*, while at the 10X threshold there were 412 transcripts for *P. chrysosporium* and 326 transcripts for *T. versicolor*. The majority of these transcripts were, as expected, directly tied to lignocellulose deconstruction, such as peroxidases, oxidases, and glycoside hydrolases. Transporters are not directly involved in lignocellulose breakdown, however they made up 24% and 10% of characterized transcripts at the 25X threshold for *P. chrysosporium* and *T. versicolor*, respectively. In addition, 75% and 37% of transcripts at the 25X threshold for *P. chrysosporium* and *T. versicolor*, respectively, were classified as hypothetical or unknown, which further highlights the need for improved functional characterization of enzymes from these two fungi.
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1. Introduction

Li and Ge (2016) describes biofuel as energy which has been obtained from organic matter, such as plant materials and animal waste. Bioenergy, on the other hand, is energy which has been derived from biofuels, and is the primary source of power in many developing countries. In the past century, fossil fuels were heavily relied on by the global industrial sector. This greatly contributed to issues of global warming as a result of emissions of greenhouse gases (Li and Ge, 2016). In order for warming to be kept below 2°C during the 21st century, 50% of natural gas production together with more than 30% of oil and 80% of coal should remain untapped until 2050 (Li and Ge, 2016). As a result, development of renewable energy is needed urgently in order to cater to the rapidly increasing energy demands by the increasing global population, and to reduce emissions of greenhouse gases.

The earth is expected to be home to 10 billion people by 2050, with many more urban centers having greater than ten million individuals. As a result, 80% of the total available energy in the world will be in demand by 2030 (Guo et al., 2015). For successful reduction of greenhouse gas emissions, there needs to be a maintainable growth of major cities. For example, 20 of the largest cities in the world consume around 80% of the world’s energy, and are responsible for approximately 80% of greenhouse gas emissions (Guo et al., 2015). As a result, there is an urgent need for technologies and innovative approaches that will help reduce or even reverse the effects that are brought about by emissions of greenhouse gases.

Renewable energy is nearly inexhaustible in duration but can be limited in terms of the amount of energy that can be available at a given time. Sustainable energy sources
are highly preferred as they have an advantage over traditional fossil fuels, such as oil and coal. Alternative energy sources tend to pack a much lighter environmental footprint as compared to fossil fuels. There are several major categories of renewable energy. They include solar energy, which is obtained from harnessing the sun's energy, geothermal energy which is harnessed from the earth's energy, wind flow which is harnessed from wind's energy through the use of turbines, hydroelectric energy that relies on water flow, ocean thermal energy that is derived from wind-driven waves, and bioenergy (Boyle, 2004; Guo et al., 2015).

There are various systems that are used in the generation of bioenergy, including burning biomass and the use of methane that is produced as a result of decomposition of organic matter. Being one of the largest potential sources of renewable energy, bioenergy accounts for approximately 10% of primary energy supplies around the globe (Somerville, 2007). A variety of energy products such as biomethane, biobutanol, and bioethanol can also be produced from biological feedstocks (Kumar and Gayen, 2011). The four main types of biomass include, manures, woody plants, aquatic plants, and herbaceous plants (McKendry, 2002). Through burning biomass for fuel, the energy stored in these chemical bonds has been exploited by man for ages. Currently, new crops (e.g. miscanthus and switchgrass) are undergoing testing for commercial energy farming. The ideal crops need to possess certain characteristics that include low cost, low energy input, low nutritional requirements, and high yield. For the past decades, biomass has been a topic of interest globally as an energy source although there are still ongoing debates on the benefits of biomass, especially in comparison to other renewable sources of energy (Goldemberg, 2007).
However, there are advantages that biomass has over fossil fuels, such as decreasing the amount of carbon emissions in the atmosphere (Goldemberg, 2018). The global availability of biomass is also an advantage, along with its renewable nature if grown in a sustainable manner. On the other hand, there are disadvantages that have associated with biomass energy. The most common way that this energy has been used for ages, wood burning, leads to the net production of carbon dioxide. Third world countries that lack forest conservation measures have caused deforestation due to the removal of wood for fuel (Goldemberg, 2018). Another issue arises when biofuels are produced from food crops. Many farmers tend to abandon other crops to grow corn, which is sold to various companies that deal in the production of ethanol. This has led to competition between fuel-based crops and food-based crops for fertile land (Graham-Rowe, 2011). In addition, high transportation costs and inefficiency of the biofuels produced are among the challenges associated with biomass (Cherubini et al., 2009).

Furthermore, biofuels that are produced from wood or other high-density plant biomass have an additional challenge, which is the recalcitrance of the material to deconstruction (Zhao et al., 2012). The recalcitrance of this material is due to the properties of the major polymers that make up the woody cell walls (cellulose, hemicellulose, and lignin) and the covalent interconnectivity of these polymers that leads to the overall structure known as lignocellulose (Figure 1). To deconstruct lignocellulose into its major constituents for biofuel purposes, pretreatment of biomass is often necessary. Categories of pretreatment technologies include physical, physiochemical, chemical, and biological (Sanchez, 2009).
Physical pretreatment simply involves the breaking down of the crystallinity and size of biomass, which can be achieved through grinding or milling. The process usually requires a high amount of energy that limits its usefulness at industrial scales. Under chemical pretreatment there is alkaline pretreatment, which involves pretreating lignocellulosic biomass using bases, such as calcium and sodium (Sanchez and Cardona, 2008). Using an alkaline agent causes the glycosidic side chains to be degraded, which causes the cellulose to swell. There is also wet oxidation, whereby oxygen is used as an oxidizer for dissolved substances. During this process, lignin is removed from the lignocellulosic material through fractionation. The hemicellulose is then solubilized. Combining this process with other pretreatment methods can be advantageous as it can help increase the yield for enzymatic sugars. Acid pretreatment, on the other hand, involves using concentrated acids to break down the lignocellulosic material. Dilute sulfuric acid is commonly used as it treats a wide range of biomass. A notable disadvantage of this method is that it produces fermentation inhibitors, such as furfural (Sanchez and Cardona, 2008; Hu et al., 2010; Kim et al., 2015).

Physiochemical pretreatment can involve steam explosion, which is a combination of physical and chemical techniques used to break down the rigid lignocellulosic structure. This process focuses on the disruption of fibrils, which can be attained by subjecting the materials to high temperatures and pressures. There is also ammonia fiber explosion under physiochemical processes. This process involves the subjection of the biomass material to liquid anhydrous ammonia under moderate temperatures and high pressures (Alvira et al., 2010). Temperatures used under this process are lower than those used in the steam explosion process. Hence the process
requires less energy input and is also less costly. However, the use of chemicals creates additional expenses with respect to purchasing and disposal (Sanchez and Cardona, 2008).

Due to the high costs and environmental concerns with physical, chemical, and physiochemical pretreatment, biological pretreatments are a potential alternative with potentially fewer costs and reduced environmental impact (Kumar et al., 2009). Among the more promising biological pretreatment agents available are white-rot fungi, which have developed over hundreds of millions of years the innate ability to degrade lignocellulose through biochemical mechanisms (Floudas et al., 2012). Application of these fungi can be done on a variety of lignocellulosic substrates aside from woody substrates, such as rice and wheat bran (Okamoto et al., 2011), canola straw (Canam et al., 2011), and miscanthus (Alaradi, 2017; Alanazi, 2018). In most cases, the lignin component of lignocellulose will be readily degraded and modified by these organisms while they grow at moderate temperatures through the production of enzymes and chemicals, such as reactive oxygen species.

Due to the powerful deconstruction mechanisms of white-rot fungi, they have generated increased attention as agents for biomass pretreatment (Canam et al., 2013b; Sharma et al., 2019). Although the enzymes produced by white-rot fungi can be extracted or expressed in host organisms (e.g. E. coli), pretreatment of biomass by growing the fungi directly on the biomass may be the most cost-efficient strategy (Canam et al., 2013a). Additionally, there is preference for fungal species and strains that are highly selective for lignin degradation so that the cellulose, which is rich in glucose, is available for fermentation. Fungal selectivity, however, shows variance among species and also
with the time of pretreatment (Canam et al., 2013a). Additionally, different biomass feedstocks show variance in fungal growth and degradation, and resulting digestibility.

Among plants with high potential to contribute significantly to biofuel production, sunflowers have been of particular interest due to their ability to yield seed oil for biodiesel production while also producing considerable above-ground, lignocellulose biomass that can be used for traditional fermentation of ethanol (Ziebell et al., 2013). The Silverleaf sunflower (*Helianthus argophyllus*) is an extremely drought tolerant desert-dwelling species that produces a dense stem that resembles wood (Sanchez, 2009). In a single year, *H. argophyllus* can grow up to a height of 5 m and 9 cm in diameter, and the stem material is similar in quality to quaking polar and aspen, and is obviously much denser than silage and forage cellulosic biomass feedstock (Sanchez, 2009).

The present study investigated the suitability of *H. argophyllus* stems as a substrate for the growth of *Phanerochate chrysosporium* and *Trametes versicolor*. These white-rot fungi species have been studied extensively in the context of their metabolism of woody material, but have not been reported in the scientific literature as being able to utilize *H. argophyllus* as a growth medium. The transcriptome of both *P. chrysosporium* and *T. versicolor* after growth on stem material from *H. argophyllus* for six weeks was analyzed to determine the most highly expressed genes from these fungi.

2. Methods

2.1 Biomass Sources

*Helianthus argophyllus* was grown in the Thut Greenhouse courtyard at Eastern Illinois University. After one season of growth, the stems of *Helianthus argophyllus* were harvested and chopped by using a DEK CH1 wood chipper. The chip size of stems in
length and width ranged from approximately 0.5 cm to 3 cm, respectively. Prior to inoculation the stem chips were placed into a biohazard bag and sterilized in an autoclave for 15 minutes at 121°C.

2.2 Strains of Fungi, Growth and Inoculation

Trametes versicolor (52J; #20869; Alanazi, 2018) and Phanerochaete chrysosporium (VKM F-1767; #24725; Alaradi, 2017) were acquired from the American Type Culture Collection (ATCC; Manassas, VA). Both types of fungi were grown separately at room temperature on malt extract agar with subculturing to fresh media every week. For biomass pretreatment, liquid cultures of each fungi were prepared by scraping the surface of approximately 50 petri plates into a 600 mL beaker containing malt extract broth. The fungi were then blended into a fine slurry using a DeLonghi DHB716 hand mixer and five short bursts for 30 seconds. This concentrated slurry was mixed with additional malt extract broth for a total volume of 3 L. Then the liquid cultures were incubated at room temperature while shaking at 100 rpm for 3 days. After incubation, the fungal biomass was filtered from malt extract broth through a plastic filter (75 µm mesh screen) and rinsed with 1500 mL of deionized water to remove extra malt extract broth. The rinsed slurry was then mixed with 500 mL deionized water for biomass inoculation. Approximately 250 mL of sterilized Helianthus argophyllus chips were added to eight ethanol-sterilized plastic containers (Glad Food Storage Containers 25 Ounce) with both sides of the containers having 0.5 cm holes covered by 3M micropore tape for gas exchange. Four of the containers were inoculated with 50 mL each of the rinsed P. chrysosporium slurry and four were inoculated with 50 mL each of T. versicolor slurry. The containers were covered and mixed by shaking for 5-10 s, and the cultures were
incubated at room temperature for 6 weeks.

2.3 RNA Extraction from Sunflower (H. argophyllus) Stems

The RNA extraction method for the mixture of fungi and sunflower stems followed that for woody specimens (Kolosova et al., 2018). Pliers were used to break the fungal-treated biomass (Figure 2) into smaller pieces to fit the grinding tubes until the vial was about half full. The grinding vials had metal hammer cylinders that crushed the material into a fine powder using a SamplePrep 6870 (SPEX) system (Figure 3) that was in pre-chilled with liquid nitrogen. The grinding vials were shaken at 15 cycles for 4 min.

Approximately, 5 mL of frozen milled material was quickly added to 50 centrifuge tubes, and 15 mL of freshly prepared extraction buffer [200 mM Tris pH 8.5, 1.5% lithium dodecylsulfate, 300 mM LiCl, 10 mM disodium EDTA, 1% (w/v) sodium deoxycholate, 1% (w/v) Tergitol NP40, 5 mM thiourea, 1 mM aurintricarboxylic acid, 10 mM dithiothreitol, and 2% (w/v) polyvinylpyrrolidone] was added.

The tubes were then flash frozen for 20-25 seconds in liquid nitrogen and then placed in a 37°C water bath until just thawed. The tubes were placed in a centrifuge and spun at 3000 rpm for 10 minutes at 4°C to pellet the biomass. Two layers of Kimwipes were placed on the top of new 50 mL centrifuge tubes and the top liquid layer (approximately 12 mL) after centrifugation was poured through. To each filtered solution, 1/30th volume of 3.3 sodium acetate (pH 6.1) and 1/10th volume of 100% ethanol was added. After thoroughly mixing, the tubes were centrifuged for 45 minutes at 4°C and 3000 rpm to precipitate soluble carbohydrates. The resulting solution was poured into new 50 mL centrifuge tubes while avoiding the carbohydrate pellets. Then 1/9th volume of 3.3 M sodium acetate (pH 6.1) and 3/5th volume of ice-cold isopropanol was
added, followed by mixing by inversion several times, and the tubes were placed at -80°C overnight.

The frozen mixture was thawed by placing in a water bath set to 40°C. The tubes were then centrifuged for 45 minutes at 4°C to pellet the RNA. The supernatants were removed, and the pellets resuspended in 3 mL of TE (pH 8.0) and 3 mL of 5 M NaCl. The tubes were placed between ice and vortexing every five minutes for 30 minutes to dissolve the RNA. A total of 1.3 mL of 10% CTAB was added to the tubes to further capture polysaccharides, then the tubes were placed in a water bath at 65°C for 5 minutes. After the resulting solution cooled to room temperature, 7.5 mL of 24:1 chloroform:isoamyl alcohol was added, the sample was mixed using inversion, and the solution was centrifuged for 5 minutes at 3000 rpm and 4°C. The top phase was carefully transferred to a new 50 mL tube and 7.5 mL of 24:1 chloroform:isoamyl alcohol was added, followed by mixing by inversion, and then centrifugation at 3000 rpm and 4°C for 5 minutes. The resulting top phase (containing RNA) was transferred to a new 15 mL tube and 1 mL of 8 M LiCl was added before mixing by inversion. The solution was then stored overnight at -20°C.

After the tubes were thawed, they were centrifuged for 30 minutes at 3000 rpm and 4°C to precipitate the RNA. After adding 0.7 mL of TE, the tubes were vortexed and iced periodically over 30 minutes to resuspend the RNA. The solution was then transferred to 1.5 mL microcentrifuge tubes. An aliquot of 0.63 mL of ice-cold isopropanol (0.9 volume) and 70 µL (0.1 volume) of 3.3 M sodium acetate (pH 6.1) were added to the solution followed by mixing by inversion. The tubes were placed for 1 hour at -20°C, then the solution was thawed and centrifuged for 30 minutes at 4°C at 14,000
rpm. After removing the supernatant, the pellets were rinsed with 1 mL of 70% ethanol and then centrifuged at 14,000 rpm and 4°C for 4 minutes. After 10 minutes to evaporate the ethanol, the pellet was resuspended in 50 µl of DEPC-treated water.

2.4 RNA Extraction (Plate Culture)

Week-old fungal mycelia was collected using a clean lab spatula from malt extract plates and placed in chilled microtubes containing plastic beads (Lysing Matrix A; MP Bio). The tubes were immediately frozen in liquid nitrogen and 1 mL of TRizol solution (Invitrogen) was added. The samples were then ground using a Mini-Beadbeater-24 (BioSpec Products). The rest of the procedures followed the TRizol reagent instructions for RNA extraction. The RNA pellets were suspended in 50 µL of DEPC-treated water.

2.5 Quantification of RNA

All fungal RNA extracted from biomass or the agar plates was quantified using a NanoDrop Lite (Thermo Scientific). The NanoDrop concentrations were used to dilute the RNA to approximately 100 ng/µL in DEPC water. The samples were then analyzed using an Experion Automated Electrophoresis Station (Bio-Rad) and a Standard Sensitivity RNA chip (Bio-Rad) for final RNA quantification and purity analysis. The three replicates per fungus species and substrate type (sunflower or agar plate) with the highest quality scores were submitted for sequence analysis (12 samples total).

2.6 RNA Sequencing

The total RNA submitted for sequencing for each sample was 1 µg in DEPC-treated water. The samples were prepared for sequencing using a TruSeq Stranded mRNAseq Sample Prep Kit (Illumina) at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Using a HiSeq 4000 system (Illumina), the samples
were multiplexed to a single lane that generated reads of 150 nucleotides in length.

2.7 Sequence Analysis and Annotation

The ArrayStar program of DNASTAR software was used to process the fastq sequence files returned from the sequencing facility. The sequences were sorted using either the *T. versicolor* transcript identification file (*Travel1_GeneCatalog_transcripts_20101111.nt.fasta.gz;* Floudas et al., 2012) or the *P. chrysosporium* RP-78 v2.2 transcript identification file (*Phchr2_GeneCatalog_transcripts_20131210.nt.fasta.gz;* Ohm et al., 2014) provided from the Joint Genome Institute (JGI). The data were represented as reads per kilobase of transcript per million mapped reads (RPKM) values and then annotated using GO (Gene Ontology) files available from JGI for each fungus: *Travel1_GeneCatalog_proteins_20101111_GO.tab.gz* and *Phchr2_GeneCatalog_proteins_20131210_GO.tab.gz*. Transcripts of interest were further annotated using nucleotide BLAST (NCBI). The data were transferred to Excel for computational analysis and graphing, and the program XLSTAT (Excel add-on) was used for generating heatmaps.

3. Results and Discussion

3.1 RNA Extraction and Sequencing

High quality RNA from *P. chrysosporium* and *T. versicolor* that was grown on *Helianthus argophyllus* stem material for six weeks was extracted using a custom lithium chloride-based extraction procedure designed for woody species (Kolosova et al., 2018). The RNA from each fungus grown on agar plates were extracted using the TRIzol method because the strong extraction method used for *H. argophyllus* samples resulted in poor quality RNA (Alaradi, 2017; Alanazi, 2018). RNA extractions from *H. argophyllus* stem material without fungal growth had undetectable RNA concentrations, and were
therefore not subjected to sequence analysis. The undetectable RNA levels from sunflower stems without fungal treatment was expected because the *H. argophyllus* stems had been harvested several months prior to these experiments.

The twelve RNA samples were estimated for quality and concentration using an Experion Automated Electrophoresis System. The digital gel pictures of these twelve RNA samples are shown in Figures 4 and 5. Two dark bands representing 28S/26S and 18S rRNA were clearly visible for all samples, suggesting RNA samples with high integrity and limited degradation. The total pattern of RNA banding from malt agar plate samples was similar to previous studies (Alaradi, 2017; Alanazi, 2018), where several low molecular mass bands may represent residual macromolecules from the rich media. The Experion system provides RQI scores as a measure of RNA quality, with 10 being the highest score. The RQI scores ranged from 8.4-9.9 for *P. chrysosporium*, and from 7.4-9.9 for *T. versicolor*, which were considered to be suitable for sequence analysis. The highest score of RNA extracted was 9.9 from *P. chrysosporium* and *T. versicolor* grown on *H. argophyllus*, while the lowest score of RNA extracted was 7.4 from *T. versicolor* grown on malt agar plates. RNA-Seq analysis generated a total of 299,065,173 million reads for *P. chrysosporium* across six samples, and 188,129,372 million reads across the six *T. versicolor* samples. This led to an average per sample of 49,844,196 million reads for *P. chrysosporium*, and 31,354,895.3 million reads per sample for *T. versicolor* (Tables 1 and 2).

3.2 General Expression Patterns

The *P. chrysosporium* and *T. versicolor* data sets sorted by transcript abundance, with those transcripts with less than 6.0 RPKM values across all six samples from each species
(i.e. average of 1.0 RPKM per sample) removed. To visualize overall trends, log plots were applied to examine overall gene expression patterns between *P. chrysosporium* grown on *H. argophyllus* and on agar plates, and *T. versicolor* grown on *H. argophyllus* and on agar plates (Figures 6 and 7). With log plots, transcripts with identical expression values between two samples are located on the central diagonal. The results from the present experiment indicate substantial differences in gene expression between any of the agar plate samples and the sunflower samples because of the scattering of many data points away from the central diagonal (Figures 6 and 7). Specifically, the *P. chrysosporium* genes that were expressed while growing on *H. argophyllus* stems were generally expressed at different levels (if at all) when *P. chrysosporium* was grown on malt extract agar. These findings were not surprising because of the recalcitrance of lignocellulose as material in contrast to the easily attainable carbohydrates of the agar plate. These trends were similar to *P. chrysosporium* and *T. versicolor* grown on maple and miscanthus compared to malt extract agar (Alaradi, 2017; Alanazi, 2018). In those studies, gene expression comparisons between maple and miscanthus (both lignocellulose feedstocks) using log plots showed much greater similarity, which further supports these conclusions.

To further examine broad gene expression patterns between the biomass types, heatmaps were prepared with all six samples for *P. chrysosporium* (three replicates for sunflower and malt extract agar) and all six samples for *T. versicolor* (Figures 8 and 9). In both cases dendrograms (from hierarchical clustering using XLSTAT) show that the samples within a replicate set are more similar to each other than samples from the other replicate set. Specifically, *P. chrysosporium* (Figure 8) and *T. versicolor* (Figure 9) that
grew on the sunflower stem samples were located in the left cluster and the agar plate samples were clustered on the right. This indicates a reasonable level of consistency between the separate biological replicates, which provided confidence in further data analysis.

3.3 Differential Gene Expression Analysis

Examining the differences in average gene expression of each transcript by the fungi on the different substrates allowed for finer levels of detail with respect to potential differences in metabolism by each fungus in response to each substrate. To achieve this, average RPKM values for one substrate type were divided by the average RPKM values of the other substrate type within each species. The sorting feature of Excel was then used to arrange transcripts by the biggest differences in gene expression between sample types. To further limit the analysis, only transcripts with higher expression on sunflower than malt extract agar were analyzed in this study due to having a scope that focused on those genes most important to lignocellulose degradation by the fungi. Accordingly, a threshold of 25X or greater expression on sunflower compared to malt extract agar was used for initial comparisons (Figures 10-13), which allowed for identification of the most abundantly expressed genes by the fungi on sunflower stems. At the 25X or greater expression threshold, there were 102 transcripts from *P. chrysosporium*, while 144 transcripts from *T. versicolor* that met the filtering criteria (Figure 13). The transcripts at the 10X or greater threshold were also examined (Figures 14-17),

During the analysis, some of the transcripts that were identified using BLAST searches were found to be ‘hypothetical proteins,’ which are predicted proteins from known transcripts but without identification. There were also several transcripts labeled
as 'unknown function,' which are transcripts that have not been well characterized. For
this study, these two categories were combined. The combined category of unknown
transcripts resulted in 77 transcripts for *P. chrysosporium* and 53 for *T. versicolor* at the
25X or greater level, which represent 75% and 37% of all transcripts above that
threshold, respectively (Figure 13). For comparison, at the 10X or greater expression
threshold there were 335 transcripts from *P. chrysosporium* and 140 transcripts from *T.
versicolor*. Of those, 81% and 43% were from the combined unknown category in *P.
chrysosporium* and *T. versicolor*, respectively (Figure 17). In all cases, some of the
hypothetical/unknown proteins are perhaps participants in lignocellulosic biomass
deconstruction. Of particular interest are the three highest transcripts on sunflower of *T.
versicolor* (transcript numbers 136701, 136699, and 48241) and the three highest
transcripts on sunflower of *P. chrysosporium* (transcript numbers 2701563, 3034584, and
2976663). Further studies of these high-level transcripts and their proteins (if they are
coding transcripts) will be necessary to elucidate their function and purpose.

When comparing differential expression at the 25X or greater expression
threshold without hypothetical/unknown proteins, there were 25 transcripts from *P.
chrysosporium* and 91 from *T. versicolor* (Figure 12). Many of these identified transcripts
are known to be involved in the breakdown of lignocellulose, which is not surprising
given that the malt extract agar plates did not contain lignocellulose. For example, the
category 'glycoside hydrolase' represented 20% and 16% of *P. chrysosporium* and *T.
versicolor* transcripts, respectively, at the 25X or greater threshold (excluding
hypothetical/unknown transcripts). Glycoside hydrolase is an enzyme responsible for
catalyzing the hydrolysis of glycosidic bonds that are found between sugars, especially in
polysaccharides, such as cellulose and hemicellulose. As a result, they are common enzymes in fungi that lead to lignocellulose degradation, such as *P. chrysosporium* (Igarashi et al., 2008; Wymelenberg et al., 2005).

Similarly, the category of 'oxidase/reductase' was 8% of transcripts for *P. chrysosporium* and 16% for *T. versicolor*. Both oxidases and reductases are known to be involved with lignocellulose breakdown, and are common enzymatic categories for white-rot fungi that specialize in lignin deconstruction. A related category of enzymes is 'dehydrogenase /NADH oxidase' (4% for *P. chrysosporium* and 9% for *T. versicolor*), which are involved in transferring electrons typically to and from NAD+/NADH. This group of enzymes is expected to be involved in general disassembly of lignocellulose polymers.

Among the most abundant transcripts at the 25X level that did not translate into enzymes for directly deconstructing lignocellulose was the category of 'transporter' (24% for *P. chrysosporium* and 10% for *T. versicolor*). Transporters are membrane-bound proteins that shuttles ions and molecules across membranes into cells or out of cells. White-rot fungi are known to secrete a variety of proteins during lignocellulose degradation, and must also import materials for general metabolism, which makes this category unsurprising. However, some of these transporters may be more pertinent to molecules associated with breakdown of lignocellulose given the relatively low transcript numbers while growing on malt extract agar, which would also require transporters for fungal survival.

It is noteworthy that the transcript for cellobiose dehydrogenase (*P. chrysosporium* transcript 3030639; *T. versicolor* transcript 73866) was expressed at
12.5X greater levels in *P. chrysosporium* 4.7X greater levels in *T. versicolor* when grown on sunflower compared to malt extract agar. This enzyme is known to enhance the access to carbohydrate polymers within lignocellulose by white-rot fungi (Canam et al., 2013a). For example, a mutant strain of *T. versicolor* (known as m4D) was generated that had no detectable cellobiose dehydrogenase activity (Dumonceaux et al., 2001), which was later demonstrated to significantly decrease this mutant’s ability to catabolize cellulose while not affecting the lignin-degrading characteristics (Canam et al., 2011). The specificity of this enzyme for cellulose, the most valuable polymer for lignocellulosic ethanol production, continues to make it a target for customized strains of white-rot fungi, such as *T. versicolor* m4D.
Figure 1. A schematic structure of lignocellulose showing representations of cellulose, hemicellulose, and lignin polymers (image from Streffer, 2014).
Figure 2. *Helianthus argophyllus* (Silverleaf sunflower) after six weeks of incubation with *Trametes versicolor*.
Figure 3. The 6870 SamplePrep Freezer Mill (SPEX) system utilized for RNA extraction of fungal-treated *Helianthus argophyllus* (Silverleaf sunflower). The inserted image is the sample vial used for powdering the material.
**Figure 4.** Experion Automated Electrophoresis System (Bio-Rad) image of RNA samples from *P. chrysosporium* after growth on malt extract agar plates (Plate 1-3) and *Helianthus argophyllus* (Silverleaf sunflower; Sun 1-3). The ladder was composed of RNA fragments measured in base pairs. The dark band at approximately 3000 bp represents the 28S/26S rRNA subunit, while the other dark band at approximately 1800 bp represents the 18S rRNA subunit.
Figure 5. Experion Automated Electrophoresis System (Bio-Rad) of RNA samples from *T. versicolor* after growth on malt extract agar plates (Plate 1-3) and *Helianthus argophyllus* (Silverleaf sunflower; Sun 1-3). The ladder was composed of RNA fragments measured in base pairs. The dark band at approximately 3000 bp represents the 28S/26S rRNA subunit, while the other dark band at approximately 1800 bp represents the 18S rRNA subunit.
Table 1. The number of sequencing reads of the *P. chrysosporium* RNA samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequencing Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate 1</td>
<td>44,300,949</td>
</tr>
<tr>
<td>Plate 2</td>
<td>42,030,046</td>
</tr>
<tr>
<td>Plate 3</td>
<td>36,999,519</td>
</tr>
<tr>
<td>Sunflower 1</td>
<td>54,556,991</td>
</tr>
<tr>
<td>Sunflower 2</td>
<td>58,947,064</td>
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<tr>
<td>Sunflower 3</td>
<td>62,230,604</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>299,065,173</strong></td>
</tr>
</tbody>
</table>
Table 2. The number of sequencing reads of the *T. versicolor* RNA samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequencing Reads</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>30,347,631</td>
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<tr>
<td>Plate 3</td>
<td>22,292,628</td>
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<tr>
<td>Sunflower 2</td>
<td>32,426,392</td>
</tr>
<tr>
<td>Sunflower 3</td>
<td>37,486,777</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>188,129,372</strong></td>
</tr>
</tbody>
</table>
Figure 6. Average RPKM values (Log2 transformed) for all *P. chrysosporium* transcripts after six weeks of growth on malt extract agar (plate) and sunflower (*Helianthus argophyllus*). The data points on the diagonal line represent transcripts with the same expression values with both sample types.
Figure 7. Average RPKM values (Log2 transformed) for all *T. versicolor* transcripts after six weeks of growth on malt extract agar (plate) and sunflower (*Helianthus argophyllus*). The data points on the diagonal line represent transcripts with the same expression values with both sample types.
Figure 8. Heatmap of all transcripts of *P. chrysosporium* after six weeks of growth on malt extract media (three replicates on the right) and sunflower (three replicates on the left). Hierarchical cluster analyses generated using XLSTAT. Colors are relative in each row (transcript) with bright green indicating high expression and bright red indicating low expression (while passing through black).
Figure 9. Heatmap of all transcripts of *T. versicolor* after six weeks of growth on malt extract media (three replicates on the right) and sunflower (three replicates on the left). Hierarchical cluster analyses generated using XLSTAT. Colors are relative in each row (transcript) with bright green indicating high expression and bright red indicating low expression (while passing through black).
Figure 10. Heatmap of 102 transcripts from *P. chrysosporium* with 25X or greater expression on sunflower compared to malt extract plates. Hierarchical cluster analyses generated using XLSTAT. Colors are relative in each row (transcript) with bright green indicating high expression and bright red indicating low expression (while passing through black).
**Figure 11.** Heatmap of 144 transcripts from *T. versicolor* with 25X or greater expression on sunflower compared to malt extract plates. Hierarchical cluster analyses generated using XLSTAT. Colors are relative in each row (transcript) with bright green indicating high expression and bright red indicating low expression (while passing through black).
Figure 12. The 25 transcripts from *P. chrysosporium* (top) and 91 transcripts from *T. versicolor* (bottom) with 25X greater expression when growing on *Helianthus argophyllus* (sunflower) compared to malt extract plate, organized by predicted function. Hypothetical and unknown transcripts are not shown.
Figure 13. The 102 transcripts from *P. chrysosporium* (top) and 144 transcripts from *T. versicolor* (bottom) with 25X greater expression when growing on *Helianthus argophyllus* (sunflower) compared to malt extract plate, organized by predicted function. Hypothetical and unknown transcripts are included.
Figure 14. Heatmap of 412 transcripts from *P. chrysosporium* with 10X or greater expression on sunflower compared to malt extract plates. Hierarchical cluster analyses generated using XLSTAT. Colors are relative in each row (transcript) with bright green indicating high expression and bright red indicating low expression (while passing through black).
Figure 15. Heatmap of 326 transcripts from *T. versicolor* with 10X or greater expression on sunflower compared to malt extract plates. Hierarchical cluster analyses generated using XLSTAT. Colors are relative in each row (transcript) with bright green indicating high expression and bright red indicating low expression (while passing through black).
Figure 16. The 77 transcripts from P. chrysosporium (top) and 186 transcripts from T. versicolor (bottom) with 10X greater expression when growing on Helianthus annuus (sunflower) compared to malt extract plate, organized by predicted function. Hypothetical and unknown transcripts are not shown.
Figure 17. The 412 transcripts from *P. chrysosporium* (top) and 326 transcripts from *T. versicolor* (bottom) with 10X greater expression when growing on *Helianthus argophyllus* (sunflower) compared to malt extract plate, organized by predicted function. Hypothetical and unknown transcripts are included.
4. References


