INVESTIGATION OF THE FUNGI FROM BOILING SPRINGS LAKE, LASSEN VOLCANIC NATIONAL PARK, CA FOR POTENTIAL APPLICATIONS TO LIGNOCELLULOSIC BIOFUELS

A Thesis

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Bryan Ervin

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ABSTRACT

INVESTIGATION OF THE FUNGI FROM BOILING SPRINGS LAKE, LASSEN VOLCANIC NATIONAL PARK, CA FOR POTENTIAL APPLICATIONS TO LIGNOCELLULOSIC BIOFUELS

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In the pursuit of producing cleaner fuels, plant lignocellulose is a source of fermentable sugars that don't compete with food production. Conversion of plant components, or lignocellulose, into biofuels typically requires hot and acidic pre-treatment conditions not suitable to most commercial fungal enzymes.

In this work, I evaluated seven Ascomycota fungi associated with Boiling Springs Lake (BSL) in Lassen Volcanic National Park, a hot and acid environment, for utilization of lignocellulose material. Due to the conditions of BSL, I hypothesized these fungal isolates produce thermal/acidic stable enzymes. To test this, the fungi were

screened for: growth on purified or natural components under hot, acid conditions; lignocellulose degradation genes with PCR; and, potential lignin degradation via a (per)oxidase assay.

Growth analysis showed Acidomyces and Ochroconis growing >40 °C and pH <4; Aspergillus, Paecilomyces and Penicillium preferred cooler temperatures for acidic media but still grew above 40 °C; Phialophora only grew up to 27 °C under both acidic and neutral conditions; and Cladosporium showed a preference for cool, neutral conditions. The most promising material utilizers, Acidomyces, Ochroconis and Paecilomyces used cellobiose, xylan, pine and incense cedar needles for growth at 40 °C and pH 2. The remaining fungi used fewer materials. PCR results showed Acidomyces, Paecilomyces, and Ochroconis containing orthologs to known fungal lignocellulose degradation genes. Additionally, Acidomyces and Ochroconis showed extracellular (per)oxidase activity at 40 °C and pH 2.

From these combined results, I concluded that Acidomyces, Ochroconis and Paecilomyces have potential for use in the pretreatment of lignocellulosic biofuels.

CHAPTER I

INTRODUCTION

Lignocellulosic Biofuels

Unlike fossil fuels, which come from deep underground deposits of ancient land plant (coal) or algae (crude oil) materials that return long sequestered CO_2 to the atmosphere when burnt, biofuels are carbon-neutral because they can be harvested from new plant growth. The new growth plants sequester existing CO_2 from the atmosphere as they grow, which is then returned to the atmosphere when processing and using the biofuels products; this cycle repeats with each planting/harvest, effectively balancing the levels of atmospheric CO_2 contributions (Somerville, 2006). Other proposed advantages to using renewable and non-competitive biofuel sources are improve regional energy security and overall reductions in greenhouse emissions (Parish et al., 2013). Finally, the potential for lignocellulosic ethanol to cut greenhouse gas emissions have been estimated around 86% without interrupting the food supply (Stichnothe & Azapagic, 2009).

Currently, the most successful biofuel, ethanol, comes from the fermentation of glucose extracted on an industrial level from high sugar containing crops like sugarcane and corn kernels by naturally occurring organisms, like the brewer's yeast *Saccharomyces cerevisiae* or bacterium *Zymomonas mobilis* (M. Zhang, Eddy, Deanda, Finkelstein, & Picataggio, 1995). Butanol and other small fuel compounds have been generated by *Clostridium beijerinckii* and genetically modified *E. coli* (Ezeji, Qureshi,

&.Blaschek, 2007; Somerville, 2006). Sugar cane, corn kernels and other grains germs are utilized because their starch (polymers of glucose) storage areas are easily accessible. Though these sources are renewable with currently established processes and infrastructure, they have serious limitations: these crops are used by humans and livestock for food and the established processes only utilize a small portion of the plant biomass (low yield per planted area) (Koçar & Civaş, 2013). In contrast, biofuels based on lignocellulosic biomasses use a greater percentage of the whole plant material, sources like switch grass, sugar cane bagasse, corn stover, wheat straw and rice straw, thus increasing yield per planted area; and none of the listed sources are used for food by humans or livestock (Carroll & Somerville, 2009; Koçar & Civaş, 2013). It is within the molecular structure of plant cells walls where we find fermentable sugar polymers (cellulose and hemicelluloses) interwoven with polymeric phenols (lignins) (Figure 1). Cellulose, a large ordered polysaccharide, is the main structural compound in plant cell walls (~50%) and the most abundant source of sugar on the planet (Kumar, Barrett, Delwiche, & Stroeve, 2009). It is comprised of the C6 sugar, glucose, linked together by β-(1,4)-glycoside bonds found in long organized chains, creating strong and flexible plant fibers. The β-(1,4) bond configuration in cellulose sets it apart from the α-(1,4) bonds typically used in starch storage (also glucose chains), requiring a separate sets of enzymes to manipulate (more on these enzymes later).

In addition to the cellulose; hemicelluloses and lignins typically comprise ~30% and ~20%, respectively, of plant biomass (Howard, Abotsi, Jansen van Rensburg & Howard, 2003). Hemicelluloses consist of various C5 sugars attached in a disorganized

manner to the cellulose fibers, acting to cross-stabilize and protect the cellulose. Hemicelluloses, xylose, and arabinose can also be fermented into ethanol by naturally

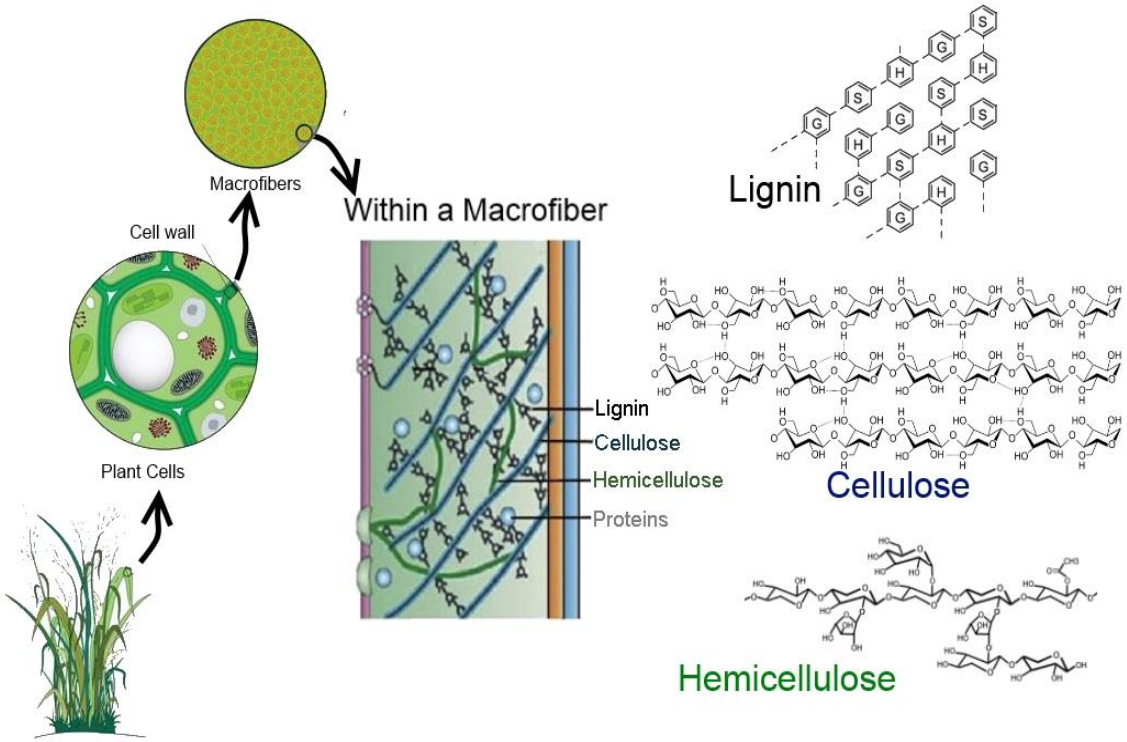

Figure 1. Lignocellulose is composed of three major constituent groups: lignin, cellulose, and hemicellulose giving the plant cell wall strength and structure.

occurring bacteria and fungi (Okamoto, Kanawaku, Masumoto, & Yanase, 2012), but the standard lab organisms *S. cerevisiae* and *E. coli* require genetic modification in order to utilize both glucose and xylose (Avci, Saha, Kennedy, & Cotta, 2013). The most recalcitrant type of lignocellulosic molecules are the oligomers of lignins, typically characterized as the water-proofing and protective molecules containing various phenolic or aromatic rings that require oxygen free radicals for synthesis and degradation (Skyba, Douglas, & Mansfield, 2013). The lignins are not currently being used to produce

biofuels but the lignin subunits (phenols) are useful for industrial or pharmaceutical products (Mendu et al., 2012).

Limitations to Producing Lignocellulosic Biofuels

Producing biofuels from lignocellulose materials requires the efficient conversion of total plant biomass into fermentable sugars. Process designs vary as technologies advance but the current paradigm begins with (1) feedstock collection and transport to facility; then (2) pretreatment to fragment materials and release sugars; and finally (3) fermentation of the sugars by various microbes (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010). Considering the lack of biofuel filling stations, at time of writing, there must be a limiting factor among these three steps. Plant biomass selections (1) for process feedstock are only truly limited by extremes in geography (large deserts or icy tundra) as most human inhabited areas have access to plants, typically grown for food or building materials (Koçar & Civaş, 2013; Perlack & Stokes, 2011). Selecting microorganisms (3) for various fermentation products go back millennia, and more recently (past few decades) designing new functions into existing microbes via genetic modification require little more space than a lab bench (Bhatia, Johri, & Ahmad, 2012; Talebnia, Karakashev, & Angelidaki, 2010) . The remaining step, pretreatment (2), is the most commonly referred limitation to the wide spread adoption of lignocellulosic biofuels. Difficulties in efficiently separating the plant biomass components and conversion of polymeric sugars into simple C6 and C5 sugars, typically via physical and/or chemical methods, significantly contributing to the overall cost (Himmel et al., 2007; Howard et al., 2003; Kumar et al., 2009). Though the three steps are

interdependent, a pretreatment process that can take any biomass feedstock, quickly produce high yields of sugars, and require minimal detoxification before fermentation; would make biofuels economically competitive with fossil fuels (Parish et al., 2013).

Pretreatment: Chance for Combining Chemical and Biological

At the molecular level, the ultimate goal of pretreatment is to separate the lignins from the cellulose and hemicellulose, and further disrupt the crystalline structure of cellulose, so that chemicals or enzymes can easily access and hydrolyze the β-(1,4) glucose linkages. Pretreatment strategies that have been demonstrated on a large scale employ a combination of various techniques: physical destruction of plant biomass (e.g. milling, chopping, and grinding) or physiochemical destruction (steam or ammonia fiber explosion); followed by chemical hydrolysis of components (i.e. acid/base treatment, organic solvent and/or enzymatic treatment) (Alvira et al., 2010; Kumar et al., 2009). From the myriad of combinations, I chose to focus on two common pretreatment schemes, both of which typically use physical or physiochemical destruction to reduce biomass particle size prior to hydrolysis: Separate Hydrolysis and Fermentation (SHF), as implied, fermentation is physically separated from the hydrolysis of plant biomass (Tao et al., 2011); and Simultaneous Sacchrification and Co-Fermentation (SSCF), uses co-cultures or genetically modified single cultures of microbes to hydrolyze and ferment in the same vessel (Amore & Faraco, 2012). Within SHF, a pretreatment step of acid hydrolysis (pH <1) at 150-200 °C quickly (10-15 minutes) degrades the lignocellulosic materials; followed by cooling and scrubbing the sugar liquor of acidic residues and interfering byproducts (acetate and furfural); then adding cellulase enzymes to degrade

the remaining oligomers; and finally pumping the liquor to the fermentation tank. However, the heating is energy-intensive, the chemical scrubbing has waste/pollution issues, and the cellulase enzymes are expensive (Chen et al., 2012; Foust, Aden, Dutta, & Phillips, 2009; Pienkos & Zhang, 2009). In SSCF, consolidated bioprocessing uses 28-37 °C temperatures and pH conditions more suitable to commonly used fermenting organisms within the hydrolysis environment; higher temperatures are also being used with thermotolerant microbes (e.g. *Clostridium thermocellum*) (Ali, Khan, Fagan, Mullins, & Doohan, 2012; Carere, Sparling, Cicek, & Levin, 2008; Hasunuma et al., 2013; Lynd, van Zyl, McBride, & Laser, 2005). The difficulties here are finding or designing organisms that can both break down lignocellulose and ferment the sugars while under heavy solids loading. Additionally, this process is considerably slower (100+ hours) (Olson, McBride, Shaw, & Lynd, 2012).

Attempts to reconcile the strengths and weakness of the two models by incorporating microbe and enzymes into the physiochemical pretreatment stages prior to fermentation have had some success. Storage of beech sapwood chips inoculated with a white rot (lignin-degrading) fungus *Ceriporiopsis subvermispora* for 56 days at 28 °C and neutral pH without addition of any nutrients was calculated to save 15% of the energy of the overall pretreatment process (Itoh, Wada, Honda, Kuwahara, & Watanabe, 2003). Using the same organism in wet storage (~65% moisture content) of corn stover reduced the lignin content by 35% and yielded 64% of the total glucose in 35 days compared to 19% lignin reduction and 47% glucose yield by NaOH treatment [5% w/w] for 90 days (Cui, Shi, Wan, & Li, 2012). Treatment of water hyacinth for 10 days by

Echinodontium taxodii followed by H_2SO_4 [0.25%] treatment at 80 °C for 1 hour resulted in 2-fold higher reducing sugar release than the acid alone (Ma et al., 2010). Many more examples of biological plus physiochemical treatments resulting in improved degradation of various lignocellulose materials are reviewed by Tian, Fang, & Gou, 2012.

Potential of Extreme-Adapted Enzymes and Their Producers

While the promise to lower energy consumption and waste issues is a net positive for biofuels, these treatments still require more time than physiochemical alone (Kumar et al., 2009). Unfortunately, putting the enzymes or microbes from the examples above into the faster, more extreme physiochemical conditions would lead to inactivation or death. Luckily, extreme-adapted organism and their enzymes are the focus of much research, and they may be able to help combine the faster yet harsher physiochemical treatments with the adapted biological and enzymatic processes to decrease time requirements and lower overall costs (Bhalla, Bansal, Kumar, Bischoff, & Sani, 2013; Blumer-Schuette et al., 2013; Kataeva et al., 2013).

To pair the biological and enzymatic processes with the harsher physical and chemical processes, we'll need enzymes that function under hot and extreme pH conditions Extreme-adapted enzymes have evolutionarily driven amino acid configurations that allow function at temperatures up to 90 °C with optimal activities above 55 °C, while also retaining activity in low (<3) or high (>10) pH environments (Bhalla et al., 2013). Tolerance to high salinity or organic solvents (e.g. ethanol) is often considered, though it is usually heat tolerance that gets the most attention. Unsurprisingly, the sources for many of these enzymes come from organisms that

themselves are considered extreme-adapted or extremotolerant (Bai et al., 2010; Luo et al., 2008; Turner, Mamo, & Karlsson, 2007; Wijma, Floor, & Janssen, 2013); though the organisms themselves need not be tolerant to multiple conditions (Zhang et al., 2011). These extreme-adapted organisms can provide 3 major types of lignocellulose degrading enzymes. The major enzyme class required for the hydrolysis step in biofuels is the cellulases that break the beta-1-4glucan bonds (Dashtban et. al., 2010).

1. Endoglucanases, such as 1,4-β-D-glucan-4 glucanohydrolase, target internal cellulose bonds.
2. Exoglucanases, such as 1,4-β-D-glucan cellobiohydrolase, which hydrolyze freed sugar dimers and 1,4-β-D-glucan glucanohydrolase, which cleaves terminal glucose oligomers. Some act from the reducing side and others from the non-reducing side.
3. β-glucosidases or β-glucoside glucohydrolases, which cleave terminal glucose molecules from the non-reducing ends.

Although some cellulase enzymes are suspected to also degrade xylan groups, most organisms have specific hemicellulose-degrading enzymes (xylanases) (Ahmed, Riaz, & Jamil, 2009):

1. Endo-1,4-β-xylanase, such as a-glucuronidase, cleaves hemicellulose to cellulose bonds.
2. Xylan 1,4-β-xylosidase, cleaves terminal/dimer of xylose bonds.

Finally, laccase/peroxidase enzymes catalyze free radical formation from molecular oxygen or hydrogen peroxides and water to disrupt the bonds of lignin phenolic groups (Harald Kellner, Luis, & Buscot, 2007):

1. Laccase and laccase-like multi-copper oxidase (LMCO) enzymes utilize copper ions for redox chemistry and oxide formation.
2. Manganese-peroxidases utilize manganese ions for redox chemistry and peroxide formation.

We look to nature for these extreme-adapted enzymes and organisms, as enzymatic hydrolysis is the pretreatment method of producing fermentable sugars from lignocellulosic biomasses (i.e. decomposition) and the producers of these enzymes inhabit our world in a variety of places. One location that is being searched for potential enzymes is the guts of plant-eating animals, such as ruminants (cows, sheep) or termites, where (hemi)cellulase enzymes are produced by intestinal microbes under comparatively mild conditions (<40 °C and pH 6-7), enabling effective cellulose breakdown without the formation of byproducts as seen in strong acid or alkaline chemical pretreatments (Jahromi & Ho, 2011; Lowe, Theodorou, & Trinci, 1987). Unfortunately, these gut microbes and their enzymes have yet to revolutionize biofuels despite many years of research, partially due to their mild living conditions (Alvira et al., 2010).

Another area of interest in the search for organisms is free-living or community-based water/soil microbes that degrade lignocellulose outside of a host. Though these places are within plain sight of humans we often forgot about the vast number and varieties of microbes involved in recycling nutrients while we go about our

lives: decaying logs, drift wood, beached seaweeds; compost heaps, hay bales, and old wooden structures are all substrates for lignocellulosic microbes (Andrade, Calonego, Severo, & Furtado, 2012; Sharma, Chadha, Kaur, Ghatora, & Saini, 2008). Even though both prokaryote and fungal organisms contribute to the biological decomposition of plant materials in wild and anthropogenic environments, I have chosen to focus on fungal contributions for this work. This decisions was partially based on the opportunities at Boiling Springs Lake (described later) and fungi, like *Trichoderma reesei* and *Aspergillus* spp., are the predominant sources of lignocellulose enzymes used industrial cellulose degradation processes (Hasunuma et al., 2013). Additionally, that typical biofuels bacteria have small zones of enzyme diffusion or use a membrane bound cellulosomes that needs close contact with the substrates (Blumer-Schuette et al., 2013; Hasunuma et al., 2013), whereas fungi rely on secrete enzymes that diffuse further into their environments.

From forest floor duff to frozen tundra to acidic mine drainage, fungi are well known to live almost everywhere and degrade almost any plant materials (Martínez et al., 2005; Łaźniewska, Macioszek, & Kononowicz, 2012). Unsurprisingly, protein and genetic research has long since confirmed fungi as a good source of secreted cellulose degrading enzymes, along with hemicellulose hydrolyzing xylanases, and peroxidase/laccases that oxidize lignin bonds (Berka et al., 2011; Cruz Ramírez et al., 2012; Shrestha, Szaro, Bruns, & Taylor, 2011; Joost van den Brink & de Vries, 2011). The use of community specific metagenomic and metatranscriptomic sequencing of soils for new fungal enzymes turns up more data than can being easily investigated (H.

Kellner, Luis, Schlitt, & Buscot, 2009; Harald Kellner & Vandenbol, 2010). Additionally, *Pestalotiopsis microspora* and *Engyodontium album* have been shown to degrade synthetic waste materials, such as polyurethane and polypropylenes, respectively, further indicating the potential for broad range of excreted enzymes in bioremediation by yet-uncharacterized fungi (Jeyakumar, Chirsteen, & Doble, 2013; Russell et al., 2011). Although these fungi and their enzymes are starting to revolutionize biofuels and biotechnology, many of them cannot survive the hot acid required for a biological/chemical co-pretreatment (Krogh, Mørkeberg, Jørgensen, Frisvad, & Olsson, 2004; C. Li et al., 2013).

Boiling Springs Lake: an Acidic Hot Spring in the Forest

To help find fungal fungi and their enzymes that can contribute to a concurrent pretreatment system, we need to find additional extreme-adapted fungi that grow at low pH and high temperature while producing enzymes that can degrade lignocellulose. Fortunately for biofuels research and development, there are naturally occurring acidic, hydrothermal environments with lignocellulosic materials that may provide those fungi and enzymes needed to combine the best of both biological and chemical pretreatments (Gross & Robbins, 2000; Maheshwari, Bharadwaj, & Bhat, 2000; Redman, Litvintseva, Sheehan, Henson, & Rodriguez, 1999).

Boiling Springs Lake (BSL), a NSF funded Microbial Observatory, is an oligotrophic, pH 2.2, 52 °C, hot spring in Lassen Volcanic National Park, near Chester, CA (Figure 2). Surrounded by coniferous forest, the relatively geologically stable BSL

stands out as an 'island' of extreme conditions, with H_2S and CO_2 gas bubbling up from a steam vent below (P. L. Siering et al., 2013). The H_2S gas reacting with water to make H_2SO_4 gives the lake its low pH much like other studied hot springs at Yellowstone National Park or acidic mine drainage sites like the Rio Tinto and Iron Mountain Mine (Amaral Zettler, Messerli, Laatsch, Smith, & Sogin, 2003; B. J. Baker, Lutz, Dawson, Bond, & Banfield, 2004; Redman et al., 1999). A major difference from those other sites is that BSL has low concentrations of dissolved minerals and metals making a unique environment to study extremotolerant eukaryotes (Patricia L. Siering, Clarke, & Wilson, 2006). Along with its related hydrothermal siblings in the high elevation Lassen Volcanic Park, the area receives a substantial amount of snow causing the lake surface and shoreline temperatures to frequently drop into the mid 40 °C range during winter storms, opening the window for a more diversity among acid-adapted organisms (P. L. Siering et al., 2013).

Carbon Inputs and Microbial Ecology

Not only is BSL hot and acidic, the estimation for primary production by macroscopic organisms is also quite low; mostly due to kaolinite clay particles limiting light penetrance. Though a few algae can thrive on floating logs, and tethered research equipment, the current hypothesis is that the major source of organic macromolecules fall into the lake as allochthonous plant litter from the surrounding forest (Almasary, 2013) with some lesser input by dead insects; and chemolithoautotrophy driving prokaryote growth in the more extreme locals (Patricia L. Siering et al., 2006). Typically, littoral (shoreline) plant materials in cooler, neutral pH lakes and streams are rapidly broken

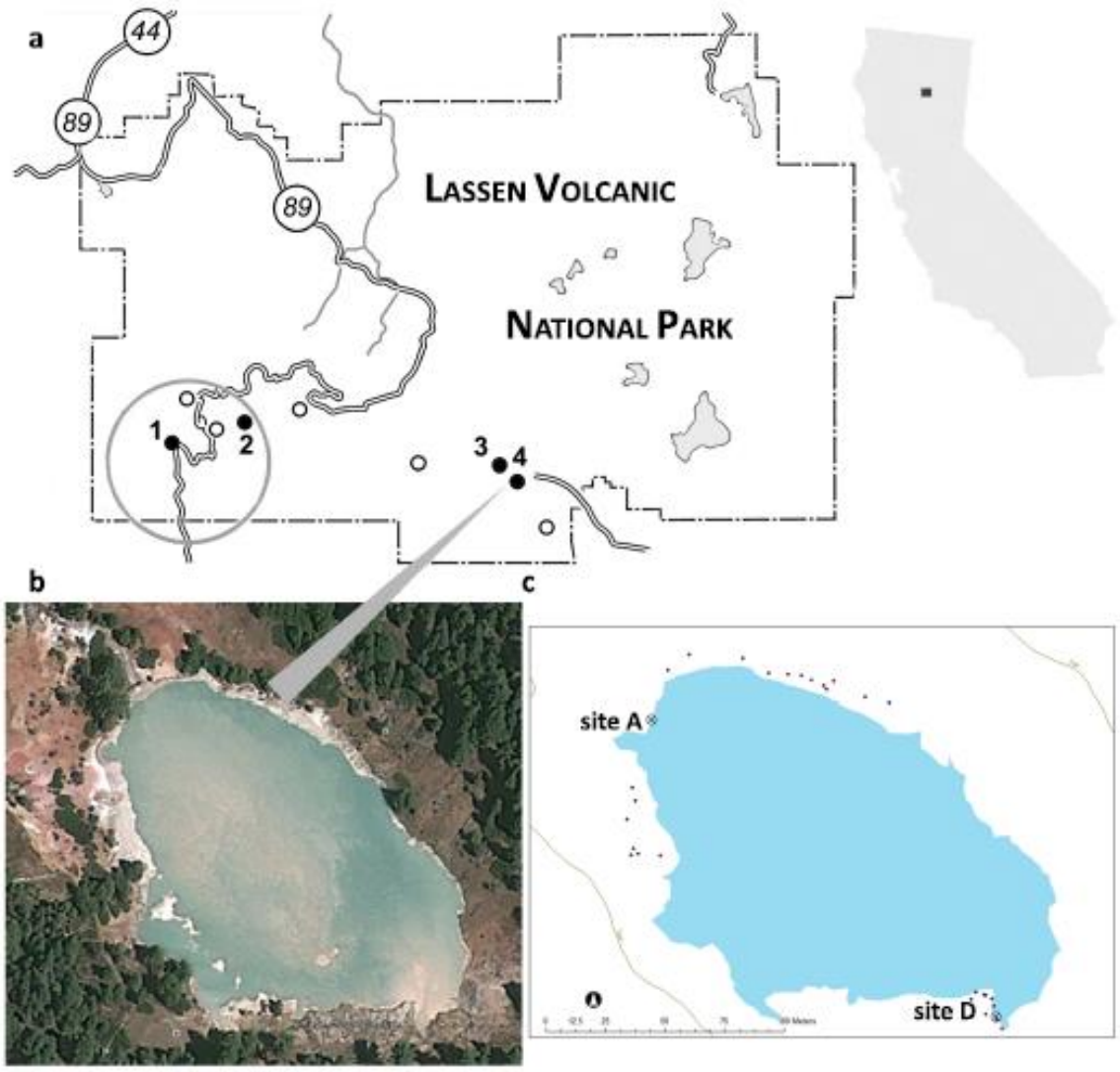

Figure 2. Map of Boiling Springs Lake. (a) Lassen Volcanic National Park and its approximate location in Northern California. Black circles are geothermal sites (1, Sulfur Works; 2, Bumpass Hell; 3, Devil's Kitchen; 4, Boiling Springs Lake). Large gray circle denotes former Brokeoff Mountain caldera. (c) BSL 30-cm resolution GPS map. Water sampling sites A and D are shown, along with major near-shore geothermal features (black diamond). (Credit: G. Wolfe).

down by combinations of shredding invertebrates, fungi, and prokaryotes (Simonis, Raja, & Shearer, 2008; Wurzbacher, Bärlocher, & Grossart, 2010) but when conditions go outside of the ideal (pH: 5-9; temp: 10-30 °C), like in BSL, the shredding invertebrates

and common degradation microorganisms become absent, slowing the decay process (Dangles & Chauvet, 2003; Thompson & Bärlocher, 1989). At pH <3 and higher temperatures >40 °C, abiotic thermochemical reactions can begin the degradation process but the extreme-adapted fungi/prokaryotes are still required to finish the litter decay process.

As part of the current BSL carbon input hypothesis, there appears to be a boom and bust cycle for microbial life hours after the input of fresh plant materials, probably due to initial abiotic thermochemical leaching of hemicellulose and other small soluble molecules (Almasary, 2013). Once the initial materials are utilized by microorganisms, the remaining cellulose fraction requires enzymatic action for degradation and utilization as the lake thermochemical conditions are too mild for significant abiotic glucose release alone (Pedersen, Johansen, & Meyer, 2011).

To date, published studies of BSL microbial ecology have focused on the interactions of the geochemical site with protists, Eubacteria, and Archaea whilst only acknowledging the presence of fungi (Brown & Wolfe, 2006; Patricia L. Siering et al., 2006; Wilson, Siering, White, Hauser, & Bartles, 2007). Former Wolfe lab members included cultivation of fungi in the laboratory from soil samples or incidental identification using eukaryote specific molecular marker approaches (Almasary, 2013; Brown & Wolfe, 2006; Reeder IV, 2011). Analysis of BSL community DNA using a Geo-chip 3.0 hybridization chip on two separate years showed the presence of sequences coding for carbon cycling enzymes that match known cellulose, xylan (hemicellulases)

and lignin (aromatic carboxylic acid) degrading enzymes; of which, some were fungal enzymes (P. L. Siering et al., 2013).

Fungal Candidates from Boiling Springs Lake

As noted above, the Wolfe laboratory previously identified (via rRNA Internal Transcribed Spacer (ITS) genotyping) and partially characterized 7 Ascomycete fungal taxa from the soils surrounding the lake. Preliminary work in the lab showed all 7 fungi grew up to 40 °C (27 °C for *Phialophora* sp.) on standard fungal medium, Potato Dextrose Agar. Each fungus was also shown to utilize glucose, cellobiose (a glucose dimer in the (1,4)-β configuration) and malt extract for growth. Fungi were classified into 2 sets by their growth preferences (Figure 3). The first set represents cosmopolitan taxa known to grow over a wide range of conditions and substrates but typically thrive at mesophilic conditions (Gross & Robbins, 2000): *Penicillium, Aspergillus*, and *Cladosporium*. We suspect these fast-growing fungi were isolated from spores that came into the BSL system via wind and/or on the forest detritus, but do not appear adapted to growth year-round in the lake's conditions. However, these well-studied taxa produce many characterized enzymes, including a few heat- or acid-adapted lignocellulose degrading ones: heat-adapted cellulases from *Penicillium citrinum* (Dutta et al., 2008) and acid-adapted cellulases and xylanases from *Aspergillus terreus* M11 (Gao et al., 2008).

The second set has genera that are more typical of acidic and/or hot habitats, both natural and man-made: *Paecilomyces*, *Phialophora*, *Acidomyces*, and *Ochroconis*.

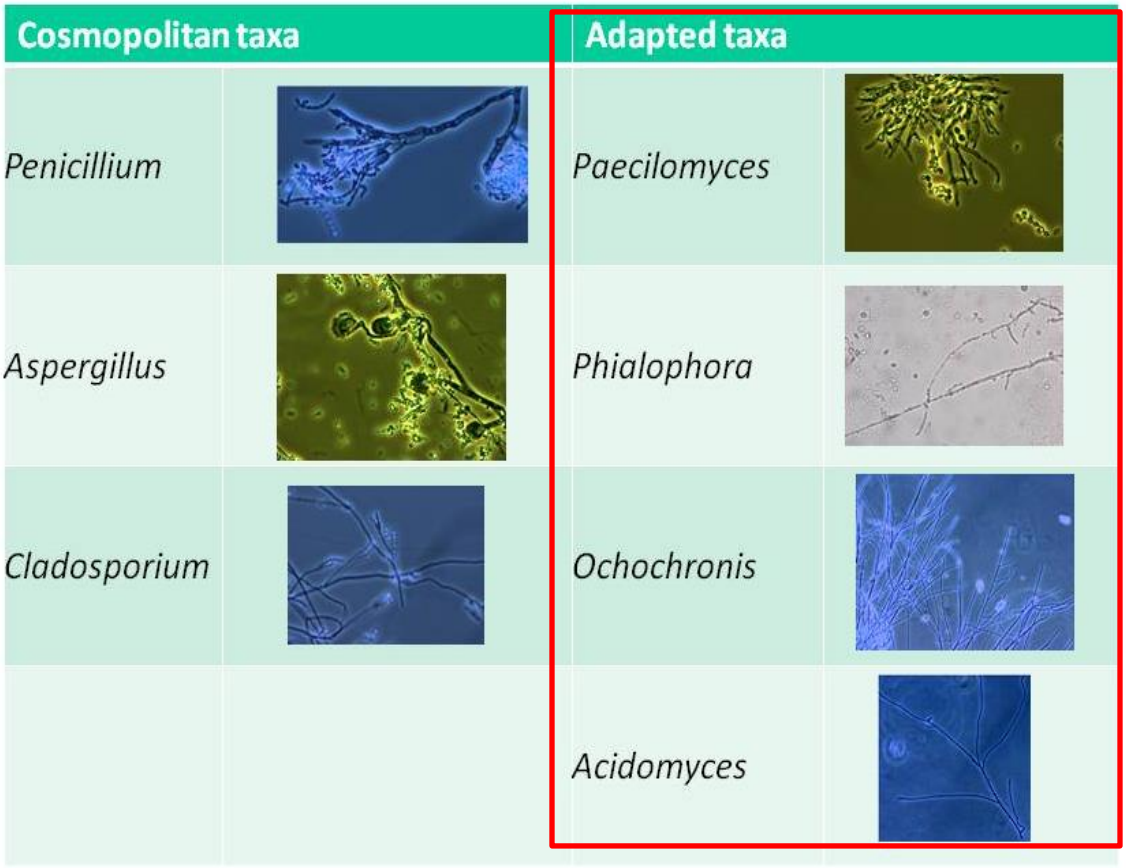

Figure 3. Ascomycete fungi isolated from BSL. Micrographs of hyphae and conidiophores stained with lactophenol blue observed with phase contrast. Taxa characterized by greater tolerance or preference for the BSL niche are at right (red box). Credit: G Wolfe unpublished.

These fungi share ITS sequence and morphological similarities to published extreme-adapted fungi (B. J. Baker et al., 2004; Gross & Robbins, 2000; Hujslová, Kubátová, Chudíčková, & Kolařík, 2010; Selbmann et al., 2008; Yamazaki, Toyama, & Nakagiri, 2010; Kyoko Yarita et al., 2010). The 4 adapted fungi showed growth on media at pH 2, 5 and 7 (unpublished). From this set of BSL fungi, two have previous reports of identified and characterized several degradation enzymes including: acid-stable mannase, xylanase, and cellulase from *Phialophora sp*. (F. Zhang et al., 2011; Zhao et al., 2012); and a thermostable xylanase from *Paecilomyces thermophila* J18 (L. T. Li et al., 2006). A

closely related extreme-adapted fungi *Acidomyces richmodensis* has predicted enzymes coding genes within the genome searchable at JGI (Grigoriev et al., 2012). Similar to our *Ochroconis* sp., Ochroconis *calidifluminalis* is associated with leaf litter in hot spring runoff but it is only evaluated in literature for human disease interactions (Kyoko Yarita et al., 2010).

Objectives

Since the environment imparted by BSL is a mild version of hot, acid pretreatment conditions and the BSL fungi are found within that environment, my objective was to determine whether these fungi might provide a new source for novel enzymes for biofuels pretreatment. To do this, I characterized their growth over elevated temperatures and low pH on different lignocellulose substrates, screened for the genes and activity of lignocellulosic enzymes. These ultimately lead to a determination of which BSL fungi have potential for further use and investigations applicable in biofuels pretreatment.

Questions

1. What are the attributes of fungus capable of surviving in BSL conditions?
2. Can the BSL fungi utilize lignocellulosic material at high temperature and low pH?
3. Can I identify extracellular enzymes useful for lignocellulose degradation?

Hypotheses

1. Active growth and recovery of fungal growth would be observed on, and after, incubation at ≥40 °C and pH ≤3 conditions.
2. Fungal growth would be observed on lignocellulose materials at ≥40 °C, pH <3.
3. Potential heat- and acid-adapted cellulases, xylanases and/or peroxidases from fungi grown on lignocellulosic carbon sources can be confirmed by enzyme assay or identified by PCR.

CHAPTER II

MATERIALS & METHODS

Fungal Isolates

Isolation

The Wolfe lab previously isolated the 7 fungi from soil and plant material collected around the western periphery of BSL during the fall and summer months of 2008, 2009, and 2010. The soils and plant materials were agitated with sterile water and then a series of dilution were placed onto petri dishes or into multi-well plates containing neutral or acidic fugal media and incubated at 40 °C. Fungi isolated in this manner were maintained on potato dextrose agar at room temperature.

Identification

Fungal genera were identified via morphological guides based on colony, mycelia and spore morphology and color (Gross & Robbins, 2000). PCR/sequencing of the rRNA internal transcribed spacer region (ITS) using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White, Bruns, Lee, & Taylor., 1990). DNA was extracted from plated scraping of mycelia grown on PDA plates into SDS lysis buffer, then separated with 1:1 chloroform, and finished with ethanol precipitation. PCR utilized GoTaqGreen polymerase master mix (Promega), approx.100 ng of fungal DNA, and 0.25 mM of each primer in a total volume of 20 μL. PCR reactions utilized 30 cycles with 54 °C annealing and 1 min

extension. The resulting PCR products were sequenced by standard dideoxy sequencing (Univ. Washington High Throughput Genomics, Seattle, WA).

Culturing

Fungi were maintained on Potato Dextrose Agar (denoted: PDA; BD Difco). Two acid-tolerance selection media were developed: 0.4% Malt Extract (denoted: ME; BD Difco) and 0.2% Cellobiose/0.1% Yeast Nitrogen Base (denoted CYNB), both made with distilled water, 2% agar, and acidified to pH 2.2 by addition of H_2SO_4 after autoclaving, matching the approximate 10 mM SO_4 concentration of BSL. Finally a basal medium containing 2% agar and 0.1% YNB was used to support various sugars, lignocellulose compounds and chopped plant material. Sources for adjuvants: Yeast Nitrogen Base (denoted: YNB; BD Difco); microcrystalline cellulose (Sigma-Aldrich); xylan from Beechwood (Sigma-Aldrich); cellobiose (BD Difco). Additional reagents were from Fisher Science or Sigma-Aldrich. Plant materials were collected from the ground around BSL, wheat straw and rice straw were gifts from a local feed store, and rice hulls were gift from Dr. L. Hanne.

Growth Studies

Fungal Growth Temperature Curve

Growth of filamentous fungi in liquid culture tends to be measured by end-point qualifiers due to the inability of removing representative samples throughout the time course; typical procedures include measuring dried fungal mass, dye sorption, or metabolite redox (Fischer & Sawers, 2013; Mowat, Butcher, Lang, Williams, & Ramage, 2007). I chose to use mass as a variable when growing fungi in liquid cultures

with soluble substrates. For insoluble substrates, a plating assay was used and growth quantification was by rate of fungal mycelia area increase: cm^2 per day (Reeslev & Kjoller, 1995). The fungal plates were imaged and mycelia area pixel count was subtracted from the total available pixel area (translating to 56.71 cm^2) to standardize for varying pixel densities/zoom; the initial inoculation plug area (0.1935 cm^2) was also subtracted. This second conditions was also used due to very little amounts of *Aspergillus*, *Cladosporium*, *Penicillium*, and *Paecilomyces* mycelia mass (>1 mg, not shown) in hot and acidic liquid ME or CYNB media (pH 2.5) compared to comparable temperatures in neutral pH conditions versions of ME and CYNB (pH 5). In all, two sets of experimental conditions were used to determine the temperature optima of fungal isolates. As such, a species that grows at 50 °C but not ≤20 °C is considered thermophilic, whereas any growth from >40 °C indicates a thermotolerant fungus (Maheshwari et al., 2000).

First conditions, by measuring mycelia mass after incubation in 10 mL of liquid ME for 7 days between 25-52 °C using a custom-built thermal gradient; were rotating at 75 rpm (Wolfe, Reeder IV, & Ervin, 2013). Vials of culture/media were filtered onto pre-weighed 5 µm Durapore membrane filters (Millipore), and then dried overnight to constant weight at 82 °C. On the following day, the filters were re-weighed and fungal mass was plotted vs. temperature to produce a temperature tolerance growth curve.

The second condition, vegetative mycelia/agar plugs (~5 mm), from PDA plates grown at 40 °C were transferred to PDA and ME plates in incubation ovens at 27,

37, 40, 45, and 50 °C. Over multiple days, the fungal plates were imaged and mycelia surface area was calculated to quantify growth [cm^2d^{-1}].

Thermotolerance of Fungi

Though the distinctions between thermophilic and thermotolerance are not routinely followed when defining fungi, the terms are common buzzwords in biotechnological setting helping to quickly discern the potentiality of new taxa to harbor enzymes of interest (Mouchacca, 2007). To combine the growth temperature tolerance data and further define the BSL fungi, mycelia/agar plugs taken from PDA plates grown at 37 °C (27 °C for *Phialophora* sp.) for 4 days were transferred to new PDA, Myco, or acidic-ME media and incubated at 50 °C for 7 days. These were then returned back to 37 °C (27 °C for *Phialophora* sp.) and monitored for growth. A fungus that began re-growing after the return to 37 °C (27 °C) was considered highly thermotolerant; conversely, organisms that did not grow were considered mesophilic organism (Redman et al., 1999).

Oxygen Requirements for Growth

I was prompted to evaluate oxygen requirements for growth after noticing some of the fungi growing in liquid media near the bottom of static flasks and tubes at elevated temperatures (not shown). The heating also lowers dissolved oxygen and could indicate micro-aerobic or anaerobic tolerance. Mycelia/agar plugs taken from PDA plates grown at 37 °C were transferred to new PDA and ME plates then incubated at 37 °C for 7 days; sealed inside of anaerobic (GasPak EZ Anaerobe pouch; BD Difco) or micro-aerobic [5-15% O_2] (GasPak EZ Campy Pouch; BD Difco). Growth rates were

calculated as mycelia area (cm^2 d^{-1}) and compared against growth rates on the fully aerobic ME plates at 37 °C.

Initial Screen for Lignocellulytic Activity

Fungal isolates grown in liquid ME for 96 h at 37 °C were vortexed and spotted (20 µL) onto a basal media of 0.1% YNB, 0.8% Gelrite (Research Products International) and distilled water adjusted to pH 2.2; containing: 0.1% cellobiose, 0.1% microcrystalline cellulose, 0.1% xylan, or no-carbon control. Appearance of fungal mycelia after 13 days on a medium was considered positive for compound utilization.

Single Carbon Source Growth Assay

After wetting a fungal isolate on a ME plate with 1 mL distilled water, 20 µL of plate wash was used to inoculated 10 mL of 0.1% w/v YNB, distilled water/H_2SO_4 at pH 2.2, and 0.1% w/v of polysaccharides (microcrystalline cellulose or xylan). Incubation took place for 7 days at 37 °C. Utilization of a carbon source was determined qualitatively by presence or absence of fungal mycelia.

Cotton Material Utilization
for Growth Assay

Twenty µL of *Acidomyces*, *Ochroconis* and *Paecilomyces* plate wash were introduced into vials containing autoclaved and rinsed 100% cotton fiber balls (Hytop brand) and 10 mL of sterile water, acidified sterile water, sterile water with 0.1% YNB, acidified sterile water with 0.1% YNB, or CYNB media. Each was then incubated at 37 °C with agitation for 9 days. Utilization of the cotton was determined qualitatively by presence or absence of fungal mycelia.

Plant Material Utilization for Growth Assay

Mycelia/agar plugs taken from PDA plates grown at 37 °C were transferred to acidic basal medium containing chopped, autoclaved and two-times sterile washed plant material (pine needles, incense cedar scales, wheat straw, rice straw and rice hulls, all approx. 1% w/v). The presence of actively increasing mycelia area would indicate utilization. Growth rates were calculated to show the mycelia area per day and were compared against growth rates on CYNB plates at 37 °C (27 °C for *Phialophora*).

Enzyme Studies

Cellulase Activity via Freed Reducing Sugar

Based on the substrate utilization versatility, I tested 50 µL of (i) filtered culture supernatant from *Acidomyces* or *Ochroconis*, (ii) a boiled supernatant control to denature enzymes (95 °C for 5 min), (iii) a purified cellulase (5U, from *Aspergillus niger*; Sigma-Aldrich) in a acetate buffer (pH 5), or (iv) a fungal free media. All were mixed with 150 µL of 1% w/v carboxymethyl cellulose (CMC, ~1.5 mg total; Sigma-Aldrich) in 50 mM citrate buffer (pH 2.5), or 0.5 mL of the above sources were also mixed with cellulose filter paper (Whatman #1, approx. 50 mg) in 1.5 mL of citrate buffer (pH 2.5). Both assays were incubated at 40 °C for 0, 30, 60, 90 and 180 min, then 100 µL of each were mixed with 300 µL of 3,5-dinitrosalicylic acid (DNS) and heated at 95 °C for 5 min (Adney & Baker, 1996; Ghose, 1987; J. van den Brink, van Muiswinkel, Theelen, Hinz, & de Vries, 2012). Once cooled to room temperature, 200 µL was then used to establish absorbance at 540 nm in a 96-well plate and compared to a reacted glucose standard

curve (range 0 mg to 0.1 mg; detection limit of ~0.03 mg) to determine the concentration of freed reducing sugars.

Laccase/Peroxidase Activity

Screening Assays

To evaluate the production of laccases and peroxidase enzymes, I used 2 assays based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) (ABST) to form a green precipitates (Chairattanamanokorn, Imai, Kondo, Ukita, & Prasertsan, 2006). Control fungi in these assays were white-rot Basidiomycetes fungi *Collybia impudica* (syn. *Gymnopus* sp. K254) and *Mutinus caninus*, both a gift from Dr. Harold Kellner. For the first assay, fungi were grown on acidic CYNB media for 4 days at 40 °C (27 °C for *Phialophora* and RT for *Collybia impudica*). Once removed to room temperature, 5 mL of a 1.3 mM ABST solution (in 100 mM sodium acetate pH 5 buffer) was apply to one side of inoculated pre-cultured split petri plates, and then allowed to sit for 10 min before discarding the ABST/buffer. Plates were allowed to incubate at room temperature for 1 hour to develop precipitate, and then photographed for documentation.

A second assay used ABST (250 mg L^{-1}) with $MnCl_2$ (100 mg L^{-1}) mixed into the solid agar medium (acidified to pH 2.5) to also screen for laccase/peroxidase enzyme reactions during active fungal growth at 40 °C (27 °C for *Phialophora*) over 6 days (Chairattanamanokorn et al., 2006). Fungal cultures that formed green precipitate (oxidized ABST) during growth were considered positive for general laccase/peroxidase activity and production of black precipitate (MnO_2) specifically indicate manganese-peroxidases

PCR Screening for Enzyme Genes

To further evaluate the ability of the fungi harboring novel acid- and/or heat-tolerant enzymes, I used PCR primers targeting the conserved domains of fungal genes encoding (hemi)cellulose degrading enzymes (Harald Kellner & Vandenbol, 2010). These mixed base primers (Table 1), typically known as degenerate primers, were used in PCR with fungal DNA. I introduced modifications into the primers by ordering

Table 1. List of degenerate PCR primers used to screen for glucoside hydrolase (GH) genes within the fungal genomic DNA. Modified from Kellner & Vandanpol (2010). The * indicates primers ordered with and without N base subsitution for Inosine bases.

Primer set	Sequences (5'→3')	Potential Target
Glc1 (GH3)*	GGNMGNAAYTGGGARGGNTT AYNGCRTCNGCRAANGGCCA	β-glucosidase (EC 3.2.1.21) xylan 1,4-β-xylosidase (EC 3.2.1.37)
GH5	GNGTHTGGGGNTTYAAYGA GGYTCRTTNSGNARYTCCCA	β-mannosidase (EC 3.2.1.25)
GH6	GAYGCNGGNCAYGCNGGNTGG TCNCCNCCNGGYTTDACCCA	Cellobiohydrolase II (EC 3.2.1.91)
GH67	GGNCCNATHGAYTTYCARGT GCNCKNGTCCAYTGNCCCCA	α-glucuronidase (EC 3.2.1.139)

"N" bases instead of Inosine nucleotides (Integrated DNA Technologies; Coralville, IA). To screen for issues with the increased the degeneracy of more "N" bases; a matching GH3 primer set with Inosine bases were ordered to compare function. PCR utilized GoTaqGreen polymerase master mix (Promega), ~100 ng of fungal DNA, and 1 mM of each primer in a total volume of 20 µL. PCR conditions and sequencing were the same as used for ITS-rRNA. Sequence results were queried via BLASTx to reveal translated sequence matches from the NCBI database (blast.ncbi.nim.nih.gov) or the Joint Genome Institute (www.genome.jgi.doe.gov).

Fermentation Screening

To further evaluate the versatility of BSL fungi for biofuel production - in particular SSCF configurations - the isolates were screened for ethanol production. This was spurred by observations of growth at the bottom of static vials filled with various media at elevated temperatures (not shown). Fungi were incubated in 15 mL conical vials with acidic ME media, filled to the lid to limit O_2 penetration, at 37 °C for 7, 17, and 20 days. At each time point, a 500 µL sample of cell-free media was mixed with 500 µL of tributyl phosphate and mixed vigorously using a 96-well plate mixer (setting 6 on Jitterbug; Boekel Scientific) for 10 min. Tubes were centrifuged at 1100xg for 5 min, and then 300 µL of upper phase was mixed vigorously with 300 µL of potassium dichromate reagent for 10 min [(Okamoto, Nitta, Maekawa, & Yanase, 2011; Seo et al., 2008)]. Two hundred microliters of the reaction was transferred into a 96-well plate and absorbance measured at 420 nm using path-length correction (BioTek Synergy). Absorbance values were compared against uninoculated ethanol standards prepared in the same acidic ME medium (0-2% v/v; detection limit of 0.1 % v/v).

CHAPTER III

RESULTS

Fungal Isolates

Fungal Morphological Identification

Fungal morphology on standard PDA medium and micrographs of hyphae and conidia/conidiophores were matched to related genera described in the literature (Table 2). When grown on the acidic ME plates, only *Acidomyces* and *Ochroconis*, displayed secondary metabolic characteristics like pigmentation and aerial hypha (conidiophore) formation (Figure 4).

Fungal Genetic Identification

Previous students in the Wolfe lab isolated the BSL fungi via acidic media and identified them via PCR with ITS primers (unpublished). I re-investigated each isolate using the same PCR primers and resulting nucleotide sequence data in the BLASTn program on the NCBI website. Results were congruent with the previous findings except the suspected *Cladosporium* (Table 3). The BSL *Aspergillus* sp. and *Acidomyces* sp. are similar to the annotated genomes (at NCBI and JGI, respectively), a which allowed for high values of genus/species identities, but the remaining BSL taxa are only known by partial sequences and phenotypes. Current taxonomic classifications shared by all BSL fungi: Fungi, Dikarya, Ascomycota, Pezizomcotina; followed by Eurotiomycetes (Aspergillus, Paecilomyces, Phialophora, and Penicillium) or

Table 2. Morpholigical characterisitics of BSL fungi. Each fugus grown on standard PDA media for 7 days at 37 °C (27 °C for *Phialophora*). Pigmentation was determined by odserving the plates under florescent and naturally lighting; while conidiophore and conidia/spore determiniantion used a 100X power light microscopy with lacto phenol blue staining.

Fungi	Pigmentation	Condiophores	Conidia/Spores	Reference
Acidomyces	Black deep-green	Not observed	Not observed	Selbmann 2008, Baker 2009
Ochroconis	Pale brown	Modified hyphae	Single, bi-lobed with septate	Yarita 2010
Paecilomyces	Light tan	Single	Multiple in chains, eliptical	Samson 2009
Phialophora	White to pale pink	Not observed	Single, cylinderical	Zhao 2012
Aspergillus	Grey-green	Single vesicule with multiple phialpaes vestigule	Multiples in chains, rounded	Gross 2000
Penicillium	White to off-white	Branching with Multiple phialpaes	Multiples in chains, eliptical	Gross 2000
Cladosporium	White	Branching with single phialpaes	Multiples in chains, eliptical	Bensch 2012

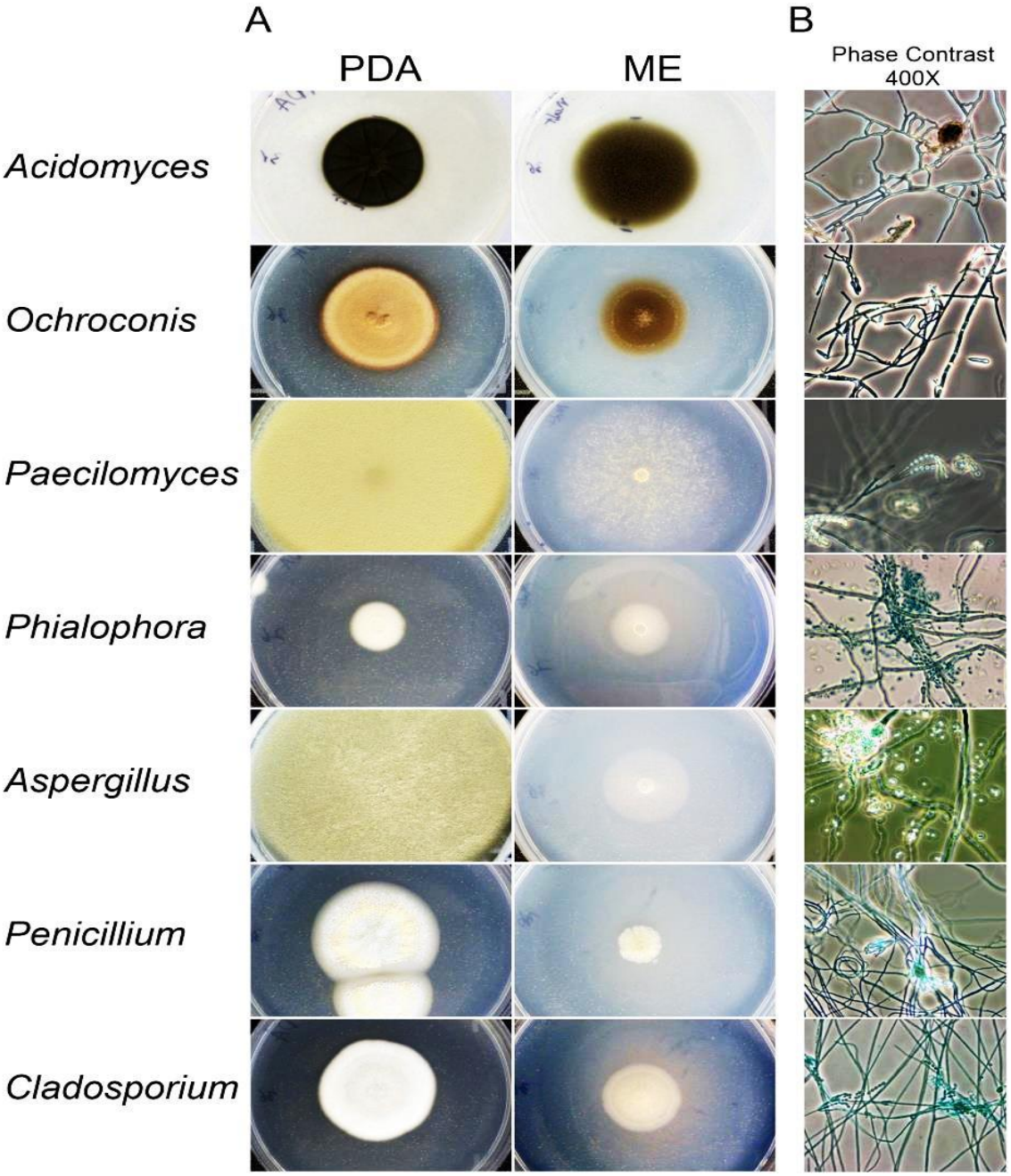

Figure 4. Images and micrographs of the seven BSL fungi. (A) Growth on solid medium shows pigmentation and mycelia density are greater on PDA (left) than on acidic ME (right) medium. Growth for 7 days at 37 °C (27 °C for *Phialophora*). (B) Lacto phenol-blue stained fungal mycelia with hyphae connections, conidiophores and conidia numbers (if visible) for each of the seven fungi grown on PDA media, Bright field with phase contrast at 400X.

Table 3. Sequence identities for ITS1 products. Hits returned from nucleotide (BLASTn) searchs at blast.ncbi.nih.nlm.gov and *Acidomyces* nucleotide search (BLAST) at genome.jgi.gov. All results returned E-values of 0.0.

Taxon	Top Match Returned	Accession	%Ident
Acidomyces sp.	Acidomyces richmondensis v1.0 (from isolate) Aciri1_iso_Assembly Scaffolds Repeatmasked	JQ172744.1	99%
Ochroconis sp.	Ochroconis gallopava genes for small subunit rRNA, ITS1	AB125284.4	99%
Paecilomyces sp.	Paecilomyces sp. ALAS-1; internal transcribed spacer 1	HM626196.1	99%
Phialophora sp.	Phialophora sp. CGMCC 3329, partial seq; internal transcribed spacer 1	GU082377.1	99%
Aspergillus sp.	Aspergillus fumigatus strain SGE57; internal transcribed spacer 1	JQ776545.1	99%
Penicillium sp.	Talaromyces sp. OUCMBI101202 internal transcribed spacer 1	JQ411391.1	96%
Cladosporium sp.	Candida parapsilosis strain EN22 18S ribosomal RNA gene	FJ809941.1	98%

Dothideomycetes (Acidomyces and Ochroconis). The resulting Talaromyces is the anamorphic form of Penicillium, and historically these genus names have been used interchangeably (Houbraken & Samson, 2011). Mixed sequence fragments from 2 of the 4 *Cladosporium* ITS PCR products indicated contamination at time of DNA extraction; the other two matched *Candida parapsilosis*; a yeast commonly found on human hands (Clark et al., 2004). I was unable to re-sequence the hyphae-forming, non-yeast fungi suspected to be *Cladosporium*, so I cannot be sure of its true identity; as such its inclusion on subsequent assays are highly preliminary.

Growth Studies

Fungal Growth Temperature Curves

In describing the temperature range for fungal growth in liquid media, I quickly found that the acidified liquid ME medium only supported appreciable growth of *Acidomyces* and *Ochroconis* at warmer temperatures. Thus, a non-acidified liquid medium was used for the less acid-tolerant fungi. *Phialophora* would grow on acidic (pH 2) ME and CYNB media, but not above 30 °C on any tested media, neutral or acidic. *Cladosporium* was not included in these tests. *Acidomyces* growth decreased <27 °C or >40 °C, but grew well within this range. *Acidomyces* and *Ochroconis* both showed visible growth in the vials above 40 °C and measureable mycelia mass above 45 °C in pH 2.5 ME media (Figure 5). *Ochroconis* showed more sharply reduced growth above or below its 40 °C optimum. Both of these fungi can be considered acid- and heat-adapted. The remaining taxa (Paecilomyces, Penicillium, and Aspergillus) were tested in a weakly-acidic (pH 5) formulation of CYNB media over a similar temperature range. All showed a decrease in growth beyond 30 °C; though they did produce measureable mycelia mass at the warmer temperatures (Figure 6). These fungi may not be adapted for acidic liquid environments like the other BSL fungi as they are typically found on dry substrates, damp soils, and decomposing vegetation.

To quantify the effects of temperature on growth using solid media, increases in mycelia area were calculated per day. Resulting in similar data to the liquid media, *Acidomyces* and *Ochroconis* showed an optimum at 37 °C on ME media, or greater on the PDA medium (Table 4), while the remaining fungi showed the growth on PDA up to 40 °C, but their optima were typically below 37 °C. The *Aspergillus* often grew

superficially across the maximal area in single day, so I only included the 40 °C rate for later comparisons. The acidic ME media was still difficult for the remaining fungi; only

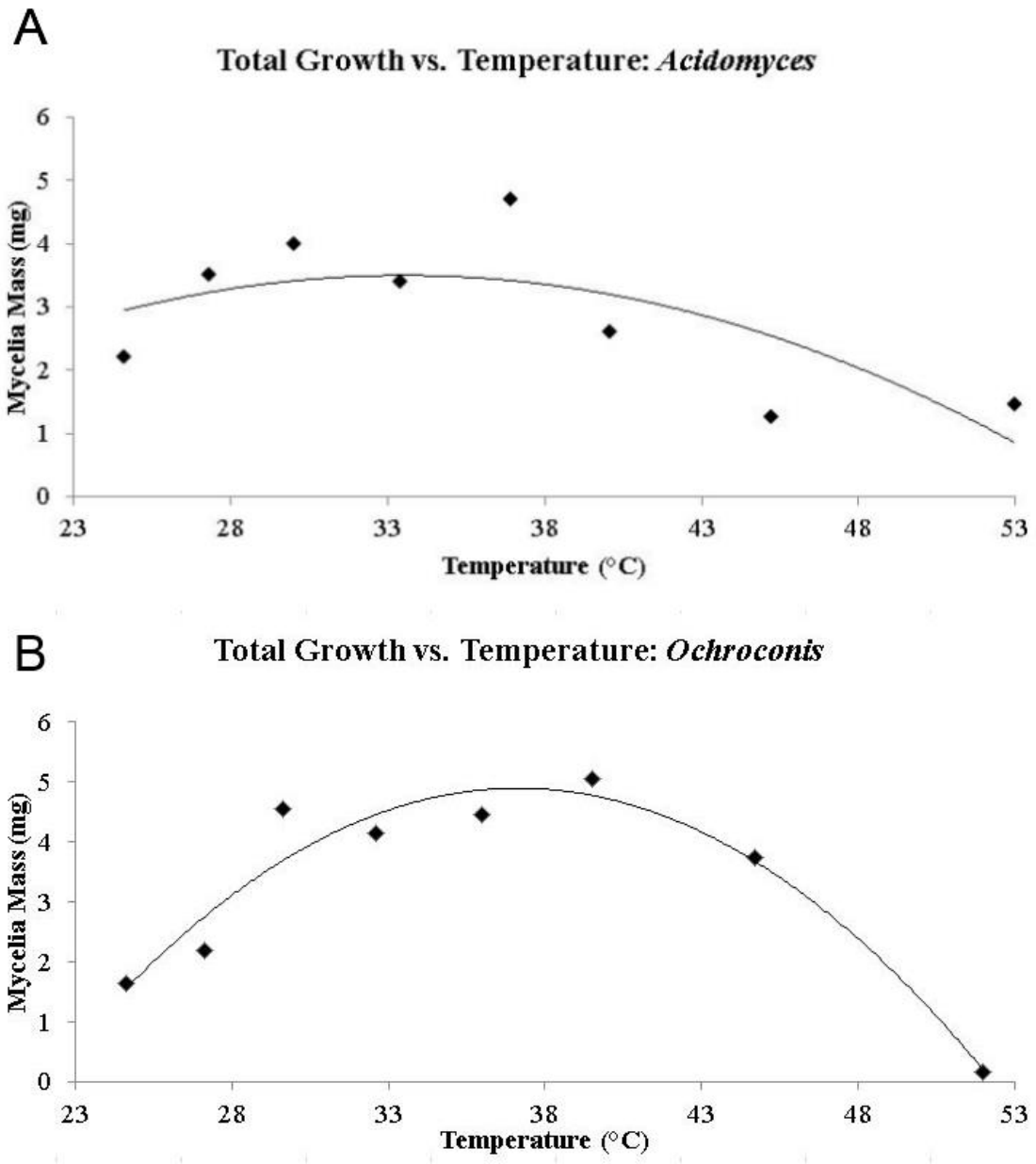

Figure 5. Temperature range of growth via mycelia mass. The average masses of Acidomyces (A) showed a broad range of temperatures, while Ochroconis (B) showed a preference near 38 °C; both shared an affinity for the upper 30 °C range.

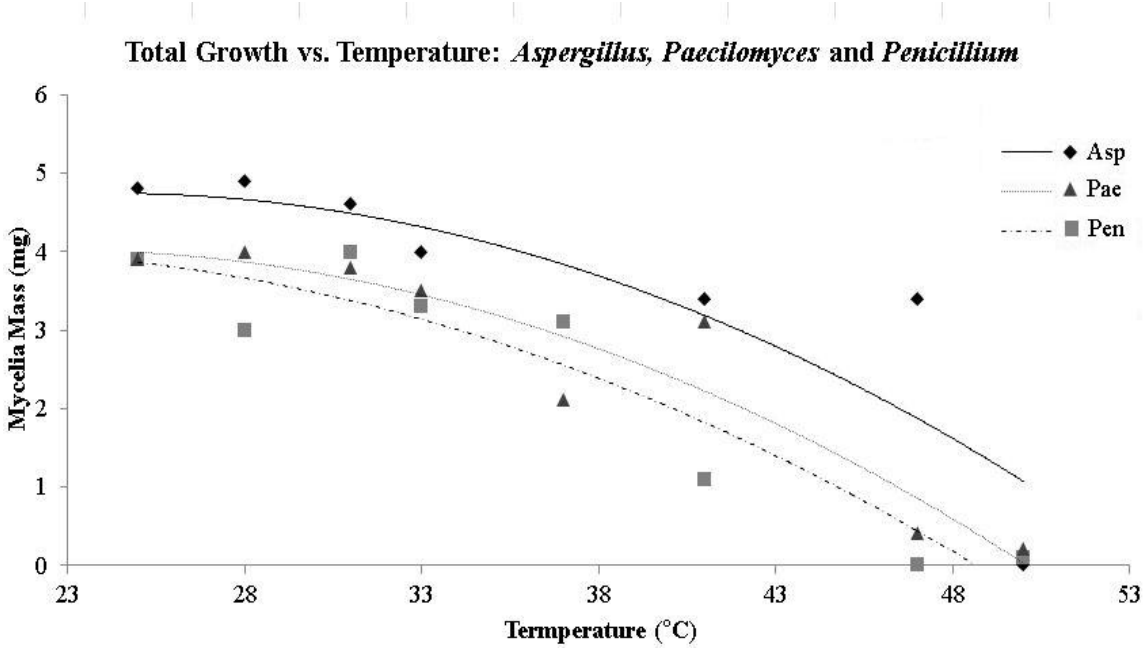

Figure 6. Temperature range for growth via mycelia mass. Mycelia mass measurements (mg) of *Aspergillus*, *Paecilomyces* and *Penicillium* as a function of temperature. Growth of fungi in less-acidic liquid medium show negative trends as the temperature increases. Thought the highest masses for all three below 33 °C, each did show some growth above 38 °C.

Paecilomyces had any growth above 40 °C. Again, *Phialophora* did not grow above 30 °C on either media, and *Cladosporium* was very limited past 37 °C. For the most part, the largest increases of mycelia density on solid ME medium correlated well with the liquid growth assay optima, further supporting the acid and heat tolerance of the adapted fungi over the cosmopolitan. Due to the visible differences in growth morphology (mycelia density) between the neutral and acidic media, growth rates calculated from radial measurements of could not be extrapolated between media formulations. An example with *Acidomyces* is shown in Figure 7. In later substrate utilization experiments, this issue was remedied by using a standardized basal medium (agar and YNB) and adjusting the pH soon before pouring the testing petri plates made the different lignocellulosic materials.

Table 4. Fungal growth rates versus temperature and media. Rates of growth based on increase of fungal mycelia area with bold values representing the optimal temperatures for each fungi. These calculation include subrtacting off the initial inoculum area (0.1963 cm^2). *Aspergillus* did not grow in an increaseing circlular are pattern under multipple trials, as such only positive growth was noted by a +.

		Growth (cm2 d-1)			
Fungi	Medium	27 °C	37 °C	40 °C	45 °C
Acidomyces	ME	1.01	**1.62**	0.91	0.25
	PDA	0.57	**1.03**	0.57	0.25
Ochroconis	ME	0.61	**2.54**	2.42	1.31
	PDA	1.31	3.66	**4.17**	3.66
Paecilomyces	ME	**4.17**	2.66	0.57	0.13
	PDA	**4.17**	1.75	1.14	1.43
Phialophora	ME	**0.34**	0.00	0.00	0.00
	PDA	**0.84**	0.00	0.00	0.00
Aspergillus	ME	+	0.84	+	+
	PDA	+	8.03	+	+
Penicillium	ME	**1.62**	0.78	0.09	0.00
	PDA	2.75	**3.19**	0.33	0.00
Cladosporium	ME	0.05	0.07	0.01	0.00
	PDA	**3.25**	2.43	0.08	0.00

Thermotolerance of Fungi

Attempts to grow the fungi at summertime BSL conditions at temperatures (48-52 °C) and pH ~2.2 (P. L. Siering et al., 2013) were unsuccessful. Aspergillus grew slowly at 50 °C on Myco agar (pH 7) and PDA (pH 5) media, but not on the acidic ME (pH 2) media and none of the adapted taxa grew at 50 °C on any medium. However, they were tolerant of this temperature and began re-growing after being returned to 37 °C. Specifically, Ochroconis and Paecilomyces began growing after the shift from a week at

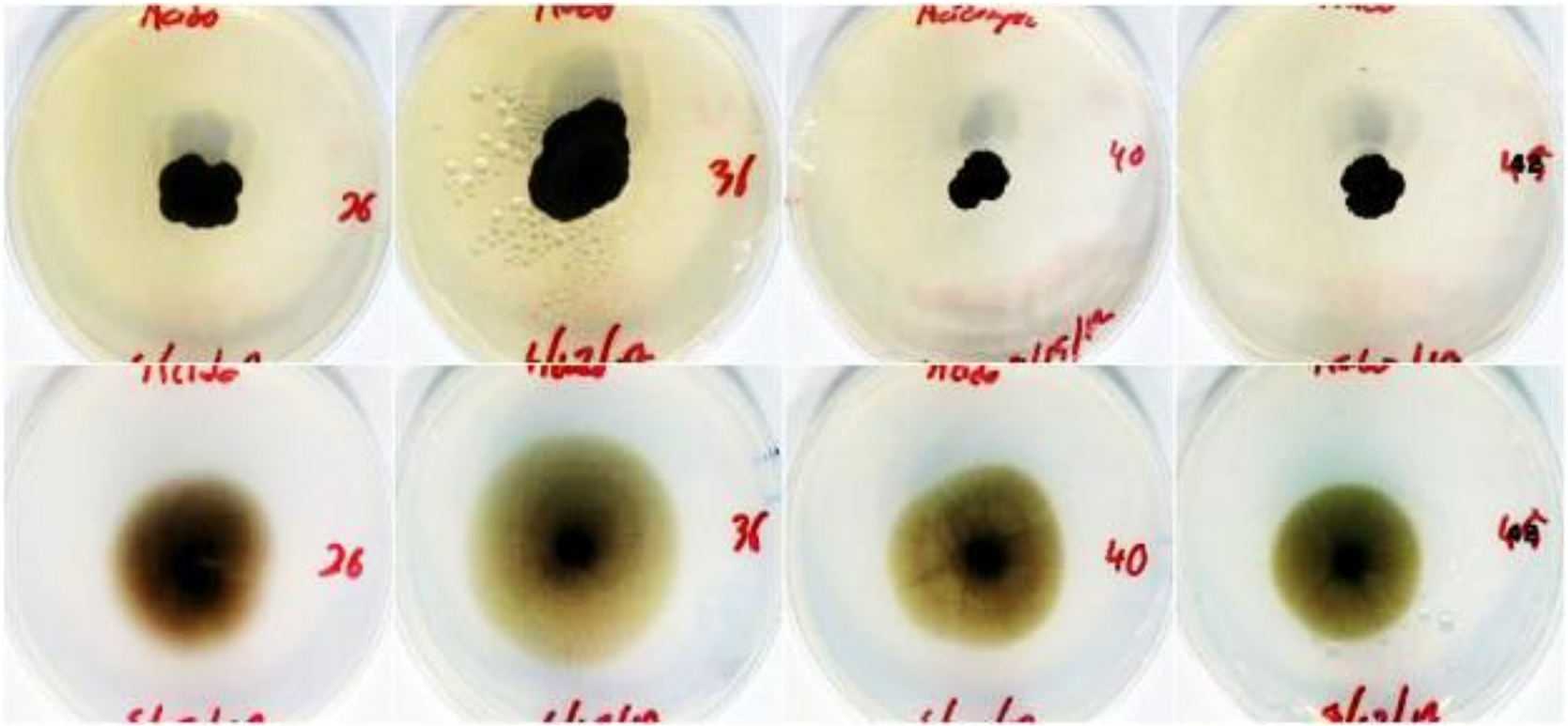

Figure 7. Images of *Acidomyces* growth at increasing temperatures. Visible differences in hyphae density emphasize the incompatibility of comparing growth rates based on fungal growth diameter across different media composition. Fungal growth on PDA (top row) and acidic ME (bottom row) media; temperatures left to right: 26, 36, 40 and 42 °C.

50 °C to 37 °C on all 3 media, meanwhile Acidomyces only showed recovery of growth on the PDA medium. The remaining fungi did not grow after the shift down to 37 °C (27 °C for Phialophora). Of greatest interest for any proposed usage in biofuel production were growth outcomes on acidic (pH 2) ME medium (Figure 8), as a heat spike scenario is likely to take place within a biofuels reactor vessel.

There are some ongoing discrepancies on the definition of thermotolerance in fungi so, confusingly, the remaining fungi could be also considered thermotolerant if they could grow above 40 °C in another assay (Mouchacca, 2007). This discrepancy has left me with no choice but to use a more general phrasing instead: heat- and/or acid-adapted. Consistency aside, the *Phialophora* is obviously not thermotolerant based on the previous growth assay. As noted, only *Aspergillus*, *Ochroconis* and *Paecilomyces* showed growth recovery on the pH 2 ME media (Figure 8).

Oxygen Requirements for Growth

I observed *Acidomyces* and *Ochroconis* growing up the entire water column of a closed 15 mL conical tube, suggesting a tolerance for low levels of oxygen. In testing with standard sealed anaerobic bags, there was not active growth of fungi on PDA or acidic ME at 37 °C after 7 days (not shown). Once the bags were opened to allow gas exchange, the fungi responded with near typical growth rates (Table 5) indicating a tolerance for anaerobisis. Further evaluating low-level O_2 tolerance using standard sealed micro-aerobic testing bags showed a considerably slower growth rate for all of the fungi compared to the aerobic plates (Table 5). Fungi maintained typical growth morphologies

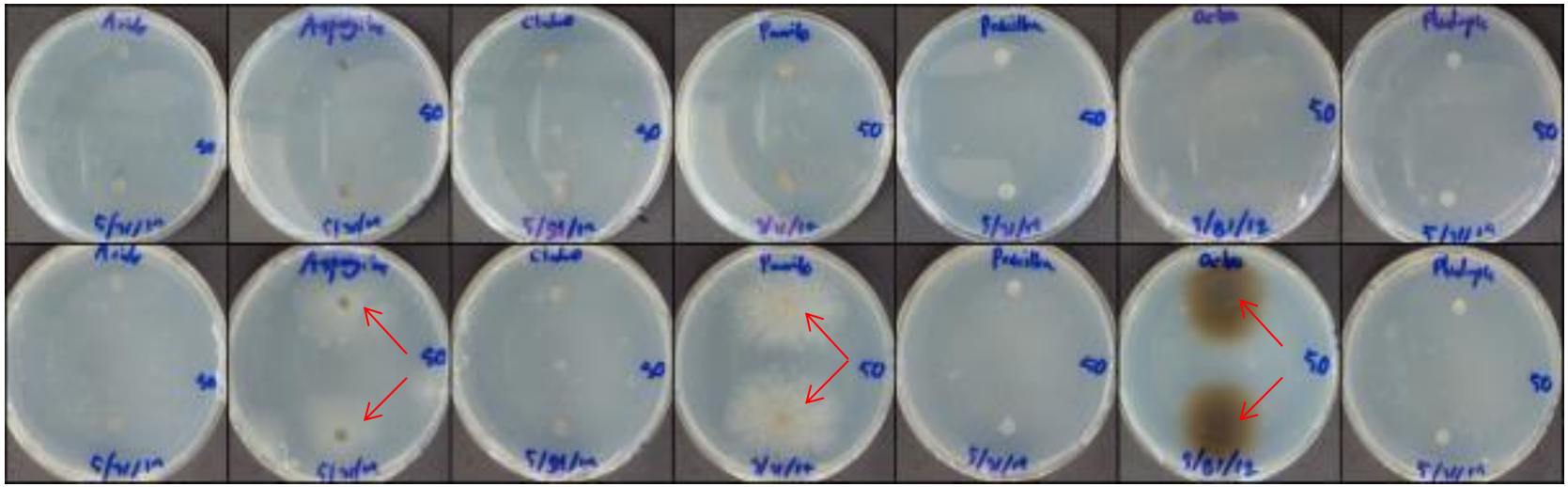

Figure 8. Images of fungi on acidic ME media during thermotolerance assay. The presence of mycelia radiating from the two initial agar plugs (red arrows) indicated growth. Top row were taken after 5 days at 50 °C, with no visible growth. Bottom row were the same plates after moving to 37 °C (27 °C for *Phialophora*) for 5 more days. Only *Aspergillus*, *Paecilomyces* and *Ochroconis* began re-growing on the acidic media after the shift to 37 °C.

under micro-aerobic conditions, and resumed typical growth rates once released from the micro-aerobic bags.

Table 5. Fungal growth rates post-anaerobic and under micro-aerobic conditions compared to rates under standard conditions (typical for that medium) at 37 °C (27 °C for *Phialophora*).

		Growth (cm^2 d^{-1})		
Fungi	Media	Post-anaerobic	Micro-aerobic	Typical
Acidomyces	ME	0.88	0.87	1.62
	PDA	0.69	0.86	1.03
Aspergillus	ME	2.61	0.01	0.84
	PDA	8.07	2.08	8.03
Cladosporium	ME	0.28	0.08	0.19
	PDA	6.97	0.64	1.69
Ochroconis	ME	1.70	0.98	2.54
	PDA	3.63	1.49	3.66
Paecilomyces	ME	2.38	0.80	2.66
	PDA	1.61	0.46	1.75
Penicillium	ME	0.01	0.47	0.78
	PDA	2.73	1.06	3.19
Phialophora	ME	0.88	0.77	0.86
	PDA	0.61	0.89	0.75

Carbon Source Growth Tests

Initial screening all 7 BSL fungal for growth on polymeric C source solid media showed strongest growth on acidic 0.1% cellobiose when grown at 37 °C (27 °C for *Phialophora*). Cellobiose, the smallest cellulose polymeric unit, supported growth of all 7 fungi under neutral and acidic conditions (not shown) and subsequently became the C source for my acidic control media, and for liquid and solid preparations (CYNB; this work, multiple figures). I observed little difference in fungal growth on the YNB-only (no C source) plates compared to the xylan and cellulose plates (Figure 9), and later tests confirmed that Gelrite with added YNB at acidic pH could support growth of the fungi,

possibly due to chemical/thermal decomposition of the Gelrite gellum gum into constitutes (including glucose) (data not shown). To avoid mis-interpretation caused by the unintended growth on Gelrite, I examined fungal growth in liquid media (see following sections). In using liquid cultures for fungal utilization of cellulose and xylan was determined qualitatively by presence or absence of fungal mycelia due to difficulties in separating the fungal growth from the remaining cellulose and xylan. The 3 fungi

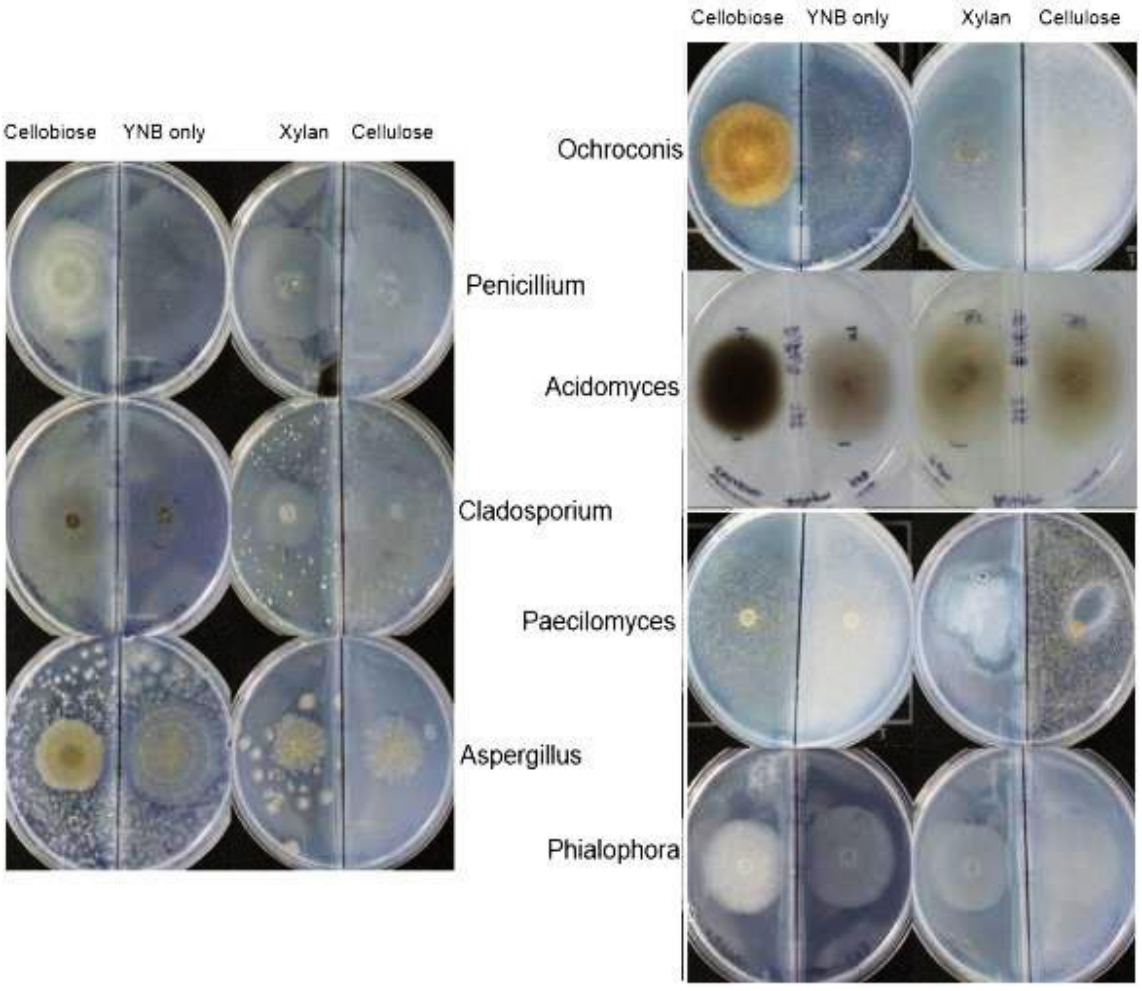

Figure 9. Images of BSL fungal growth from initial lignocellulose degradation screening. Acidic basal medium with YNB and lignocellulose component split plates (as labeled) for all seven fungi after 13 days at 37 °C (27 °C for *Phialophora*). Growth seen on all media formulations lead to further investigation of medium solidification choice.

tested (*Acidomyces*, *Ochroconis* and *Paecilomyces*) all grew well in the acidic beechwood xylan media, while only *Acidomyces* showed growth in the cellulose media (Figure 10).

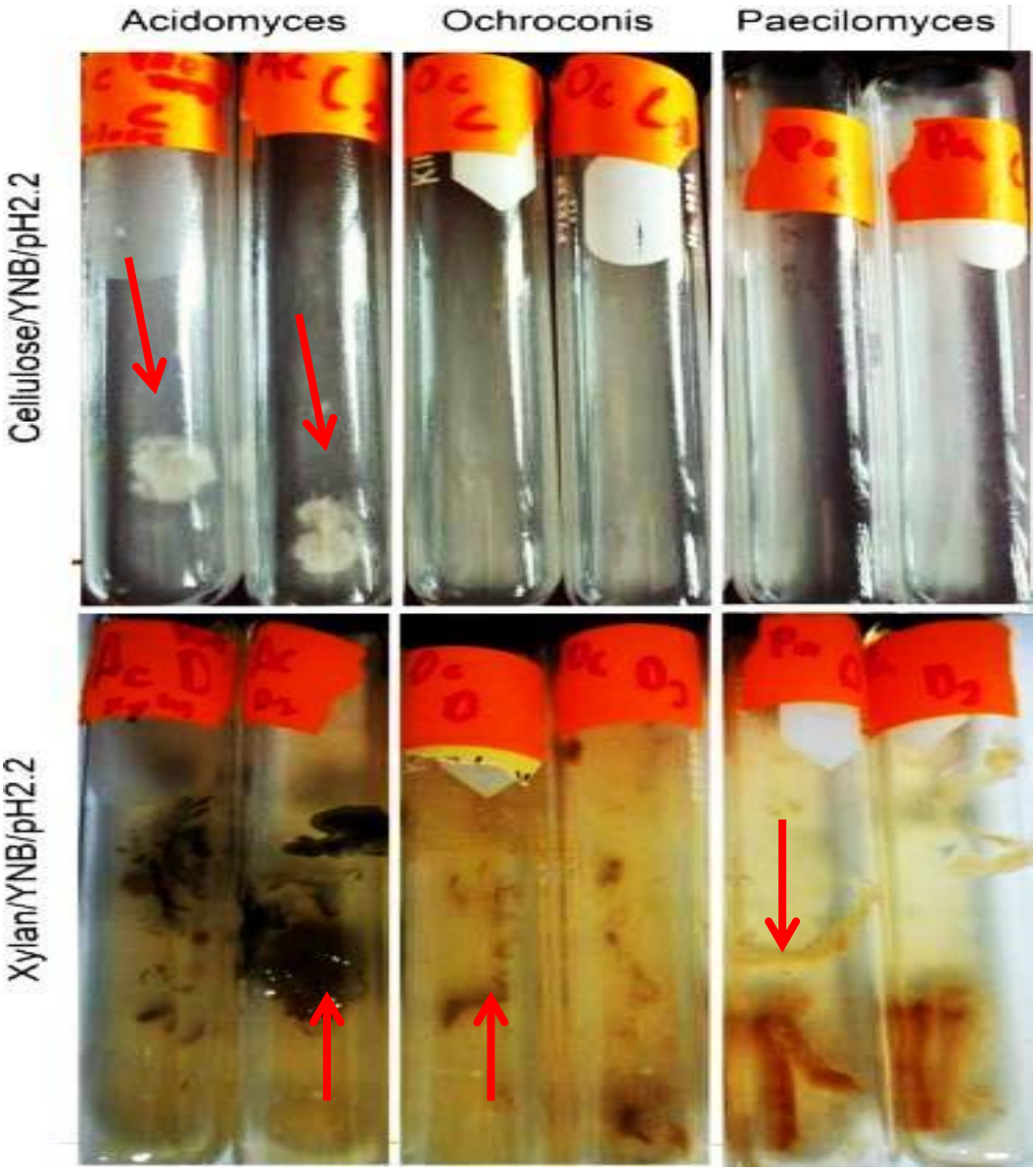

Figure 10. Images of duplicate cellulose (top) and xylan (bottom) test vials, showing the presences or absence of fungal growth. Cellulose only supported growth of Acidomyces (red arrows), while all three fungi utilized xylan (red arrows indicating representative black, brown and tan colored amorphous tissues, respective of each fungi, in the vials)

Following up on the cellulose, only *Acidomyces* showed any growth on cotton ball fibers; of the various conditions tested, 40 C without added YNB, produced considerable fungal growth for a suspected low nutrient environment (Figure 11).

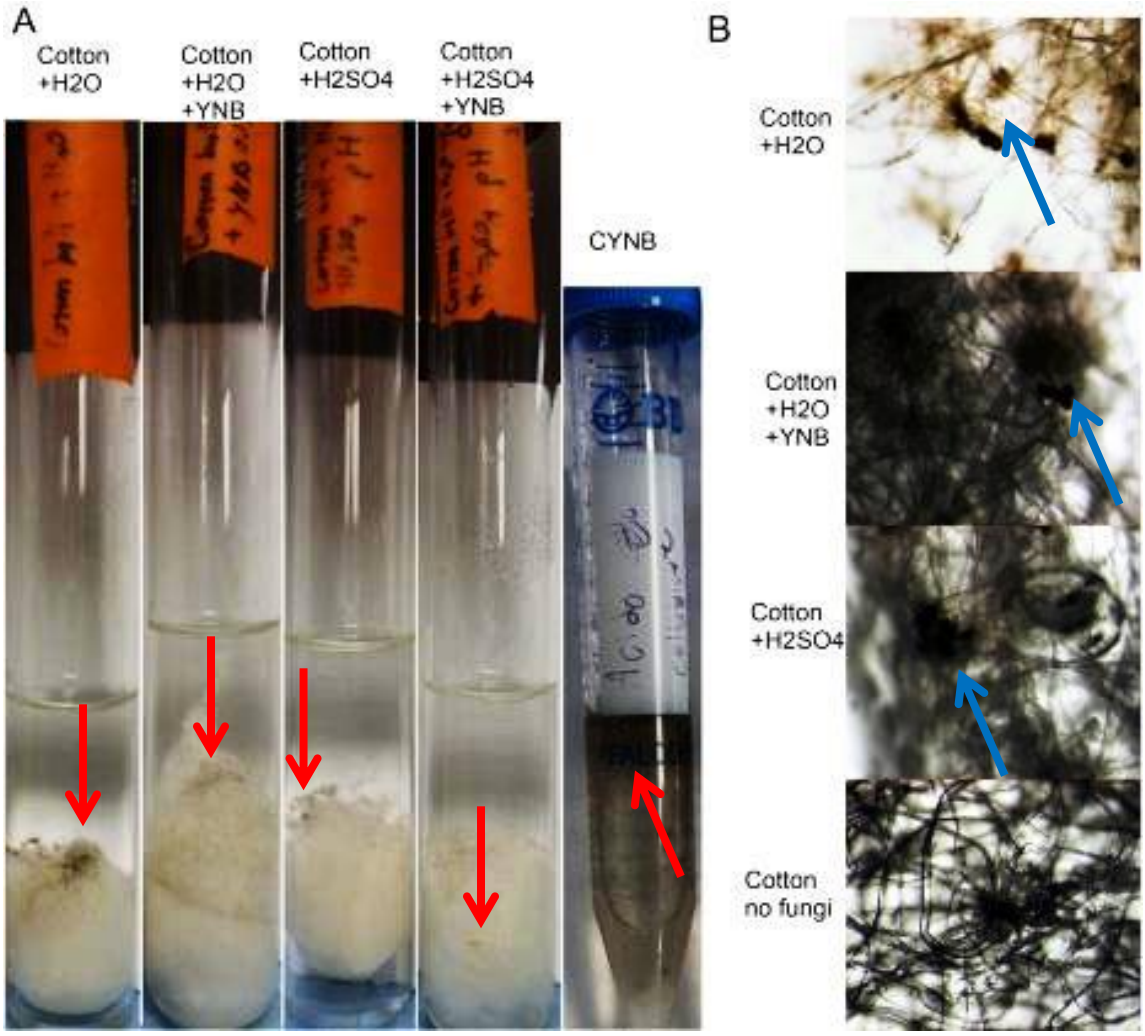

Figure 11. Images of the *Acidomyces* cotton assay vials and cotton fiber. (A) The white cotton material is at the bottom of the vials and some visible dark brown fungal mycelia (red arrow) can be seen on the top portion of the cotton material, particularly in the cotton+H_2O vial. The CYNB vial is positive for fungal growth with thick dark mycelia (red arrow). (B) Samples of the cotton were removed to verify fungal presence (100x), with three of the four vials showing dark clumping filamentous mycelia (blue arrows) amongst the thick cotton fibers. The bottom cotton image was from the un-inoculated control vial there were no thin filaments.

Plant Material Utilization for Growth

Solid acidic media containing YNB and cellobiose, ground rice hulls, chopped incense cedar scales, or chopped pine needles supported growth of *Acidomyces*, *Paecilomyces* and *Phialophora* at permissive temperatures. Additionally, *Acidomyces* was able to utilize chopped wheat straw for growth. Under these test conditions *Ochroconis* and *Aspergillus* did not show growth on agricultural by-products (rice hulls or wheat straw) but were positive for growth on the substrates native to Boiling Springs Lake (Figure 12).Overall, when the fungi grew, the rates of growth were similar between each substrate used in the media; the only confounding factor in making calculations was the lack of uniform growth by a few of the fungi (Table 6). Interestingly, *Ochroconis* did grow in liquid acidified media prepared with rice hulls and YNB (data not shown); these opposite results hint at the inherent differences of using solid versus liquid media for growth testing experiments.

Table 6. Growth rates for fungi at 40 °C (27 °C for *Phialophora*) on each substrate media prepartion. Calcualtions were based on increasing mycelial area over the 13 day inucbation period. Due to non-circular growth patterns *Aspergillus* and *Paecilomyces* growth rates were not calculated but postive growth is noted by a (+).

	Growth rate (cm^2 d^{-1})			
Fungi	Rice Hull	Incense Cedar	Pine Needle	Wheat Straw
Aspergillus	0.00	+	+	0.00
Acidomyces	0.81	0.54	0.65	0.64
Ochroconis	0.00	1.77	2.09	0.00
Paecilomyces	+	+	+	0.00
Phialophora	0.82	0.7	0.7	0.00

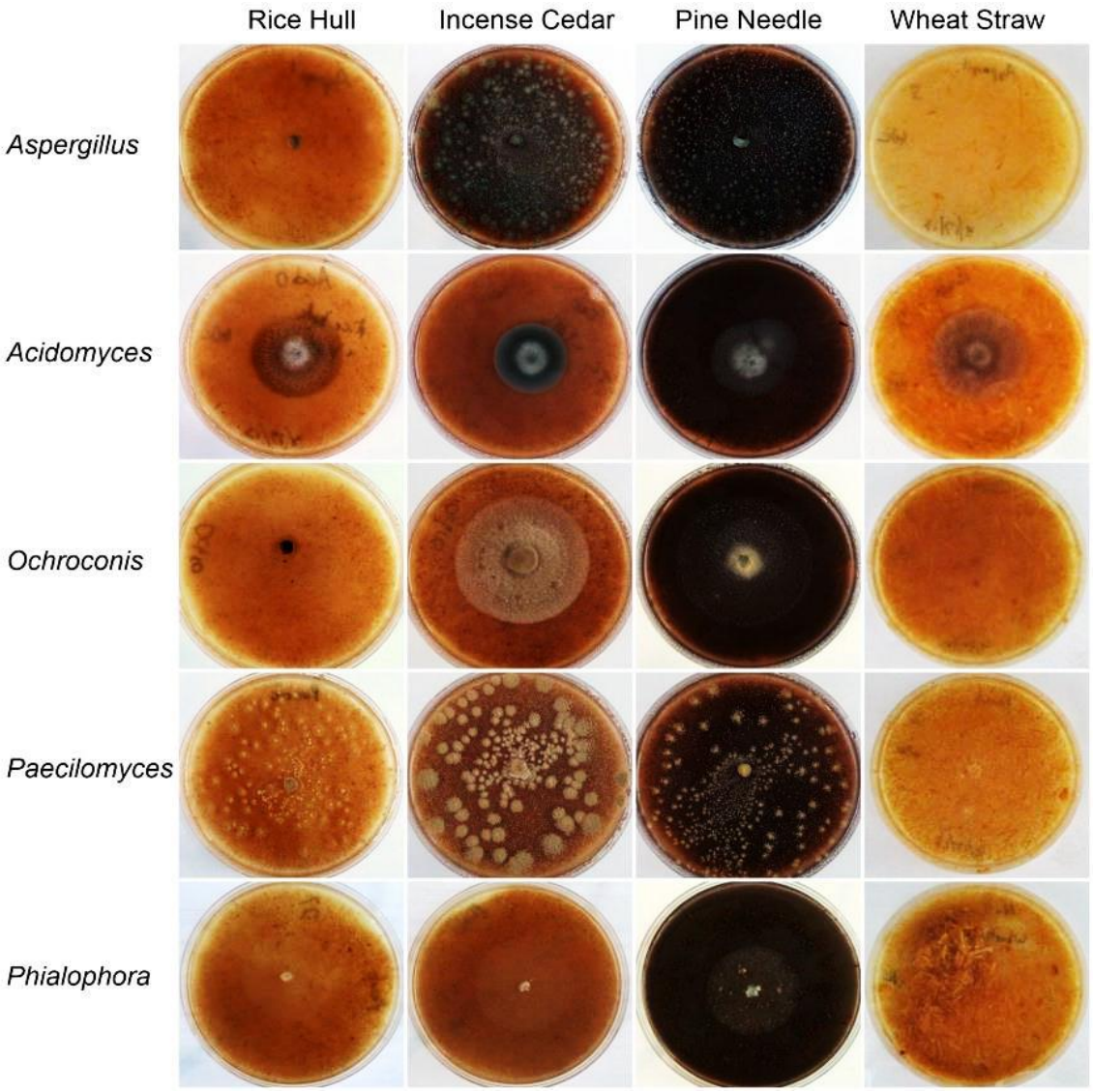

Figure 12. Resulting images fungal growth on acidified plant material plates. Each plate was inoculated with a mycelia/agar plug and incubated for 13 days at 40 °C (27 °C for *Phialophora*).

Enzyme Studies

Cellulase Activity via Freed Reducing Sugar

Though the reacted glucose standard produced linear curves that were reproducible across the trials, the amount of freed reducing sugars from both the CMC

and filter paper assays were no greater than the boiled or no-fungi controls, which themselves were below the detection limit (0.03 mg) (Table 7).

Table 7. Reducing sugars (mg) detected from degardation of the intial 15mg of carboxy-methyl cellulose. Reaction volume of freed sugars and DNS was 200 uL.

Time (minutes)	No Enzyme	Heat Killed	Acidomyces Trial 1	Acidomyces Trial 2
30	0.09	0.07	0.07	0.08
60	0.01	0.03	0.03	0.04
90	0.00	0.01	0.07	0.01
180	0.02	0.10	0.01	0.06

In addition, filter paper mass loss was not significantly different from zero (Table 8). The CMC and filter paper assays had a few issues which I did not spend more time to overcome, such as the purified cellulase reactions changing color even without substrates present, indicating a possible cross-reaction between the DNS reactant, the cellulase in a phosphate buffer (pH 5), and the citrate buffer (pH 2).

Table 8. Mass loss (mg) from filter paper measured at each time point for the different treatments

Time (minutes)	No Enzyme	Heat Killed	Acidomyces Trial 1	Acidomyces Trial 2	Cellulse (5U)
30	1.6	1.9	1.8	2.1	2.1
60	2.0	n/t	2.0	2.4	n/t
90	2.1	n/t	2.2	2.6	n/t
180	2.1	2.0	2.2	2.4	2.6

Another issue was clumping of the remaining CMC, which required an extra transfer and centrifugation step, resulting in 25-30 μL losses of reactant. Though assays have been

shown to work in microwell plates (J. van den Brink et al., 2012), they may require larger volumes of supernatant to capture more enzymes and more DNS for greater sensitivity; typical assays use total volumes of 1 mL with 3 mL of added DNS (Jahromi & Ho, 2011). Although these assays did not show active fungal cellulase enzymes in *Acidomyces* or *Ochroconis,* other metrics (PCR) indicated enzyme potential.

Laccase/Peroxidase Activity
Screening Assays

Production of green precipitate around *Acidomyces* and *Ochroconis* mycelia after growth on acidic CYNB media (pH 2) indicated high production of peroxidase enzymes. The putative (per)oxidase) activity of these two fungi showed a limited diffusion range indicated by the smaller zones of diffusion compared to the positive control Basidiomycetes *Collybia impudica***.** Neither of the other two acid-tolerant fungi, *Paecilomyces* and *Phialophora*, showed any indication of ABST oxidation at any distance from the fungal growth (Figure 13**).**

When screening for growth on acidic CYNB medium containing ABST and $MnCl_2$; *Acidomyces*, *Collybia impudica,* and another control Basidiomycetes, *Mutinus caninus,* displayed similar results as above (Figure 14). Although none of the fungal plates displayed the black MnO_2 precipitate that is typically used to indicate Mn-peroxidase specific function. The diffusion of a red pigment by *Ochroconis* on this media is a typical phenotype for the taxon, but I did not observe it on the acidic CYNB plates; this may indicate another redox reaction happening at higher temperatures compared to the room temperature ABST assay. Limited description of red pigment production is available (K. Yarita et al., 2007). Again, the *Phialophora* showed increased mycelia

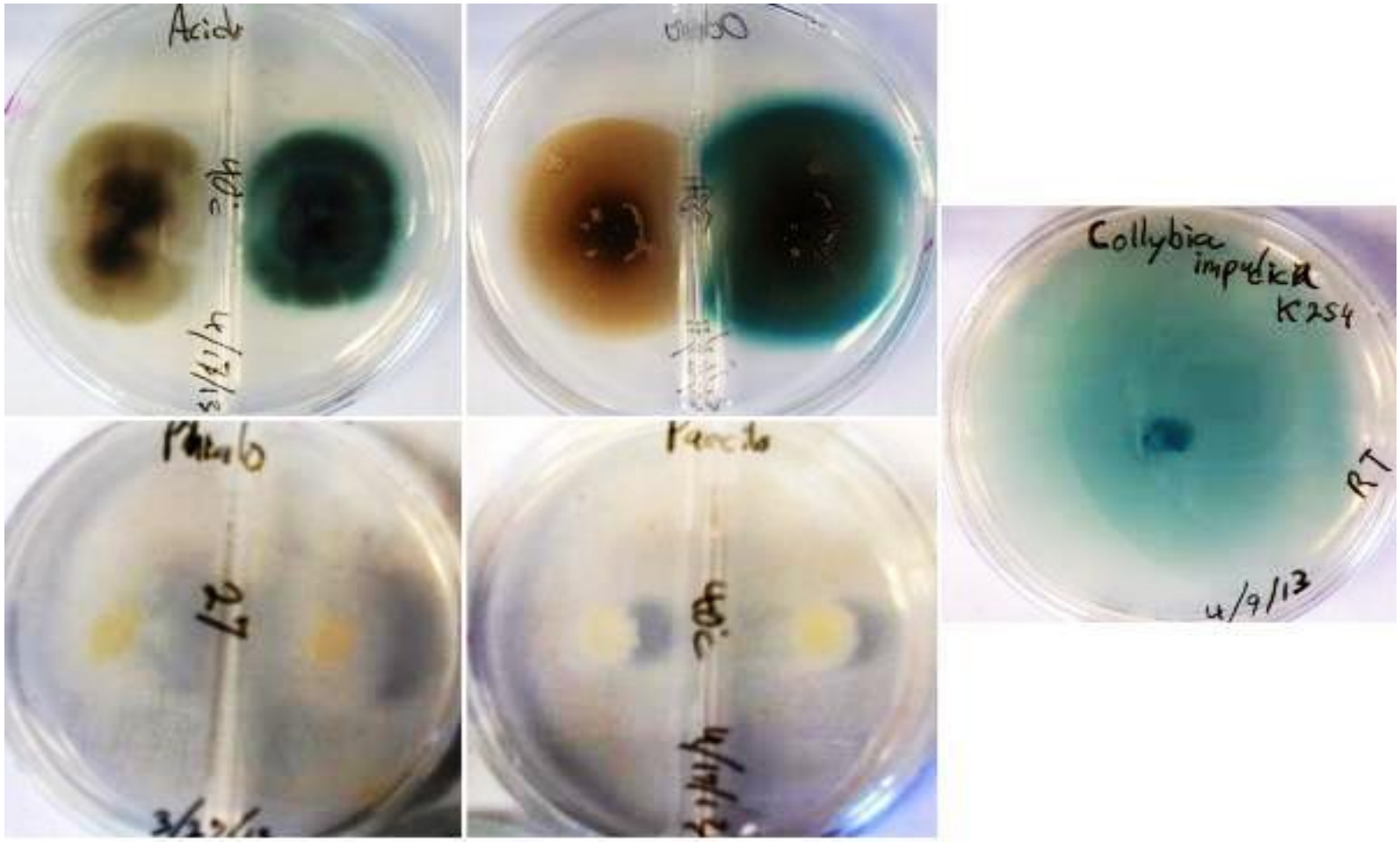

Figure 13. Fungi on acidic CYNB plates imaged 60 minutes after challenge with ABST solution at room temperature. Each plate was previously incubated at 40 °C, 27 °C and RT; as marked for 4 days. BSL specific fungi were challenged on the right half of the split plates, with diffusing green precipitate indicating peroxidase enzyme function. Clockwise from top left: *Acidomyces*, *Ochroconis*, *Collybia impudica*, *Paecilomyces*, and *Phialophora.*

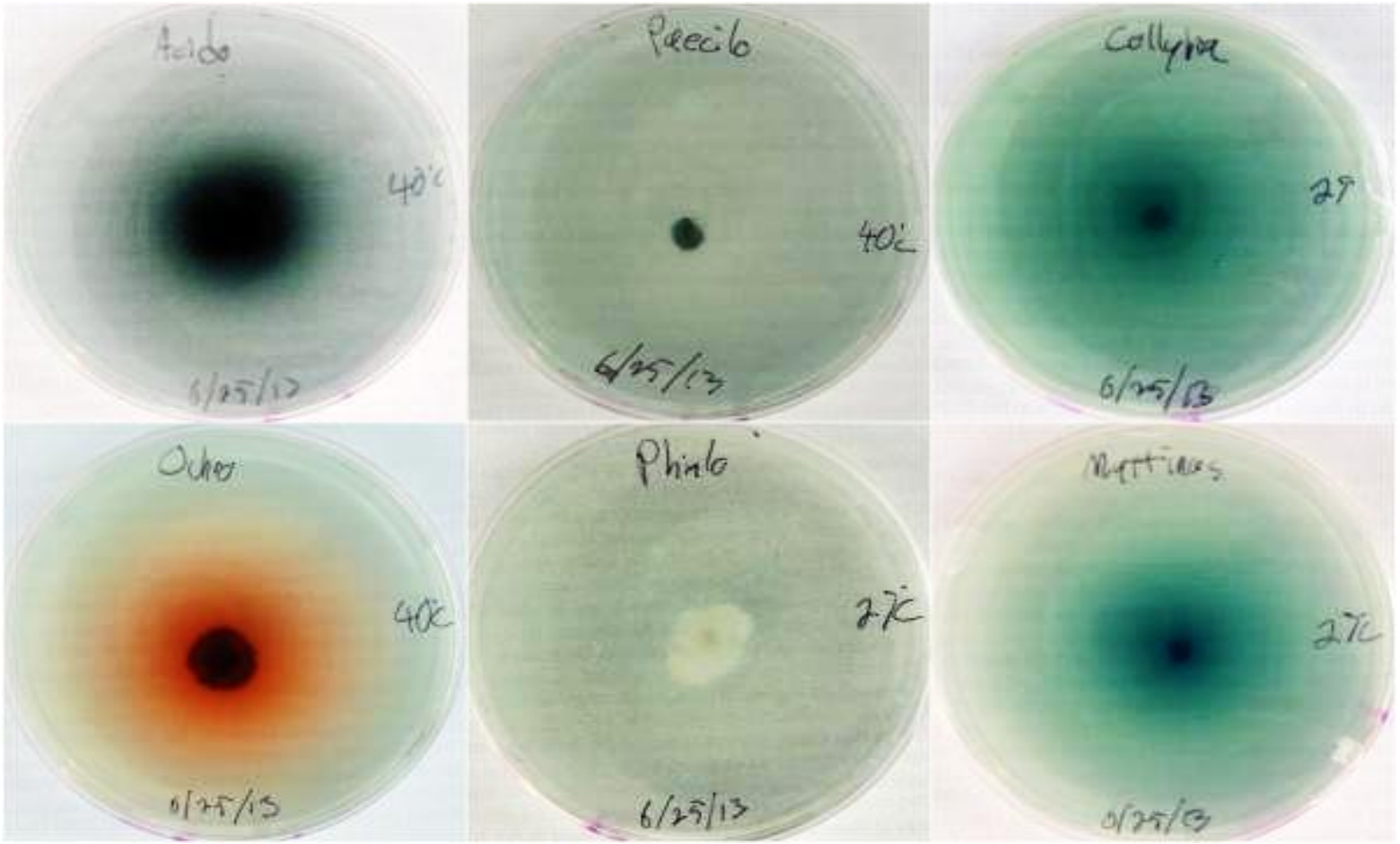

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Figure 14. Fungal growth on ABST/$MnCl_2$ peroxidase screening plates. Fungi positive for peroxidase enzymes formed diffuse green around fungal mycelia after 6 days at 40 °C or 27 °C (as marked). The red around *Ochroconis* is not directly indicated as a peroxidase reaction. Each plate is representative of duplicate plating; clockwise from top left: *Acidomyces*, *Paecilomyces*, *Collybia impudica*, *Mutinus caninus*, *Phialophora*, and *Ochroconis.*

spread but did not display oxidation of ABST nor $MnCl_2$. The *Paecilomyces* did not show growth on the test media, though it is regularly grown on the acidic CYNB media at 40 °C, possible indicating prohibition of growth by either ABST or $MnCl_2$ in the medium during the entire incubation period.

PCR Screening for Enzyme Genes

To complement the growth and enzymatic results, the screening for glycoside hydrolase (GH) genes from genomic DNA using the degenerate PCR primers (Harald Kellner & Vandenbol, 2010) produced fragments within the predicted ranges of 200-500 bp (Figure 15). PCR bands were excised from the agarose, subjected to freeze/thaw, and re-suspended in TE buffer (pH 8) prior to sequencing.

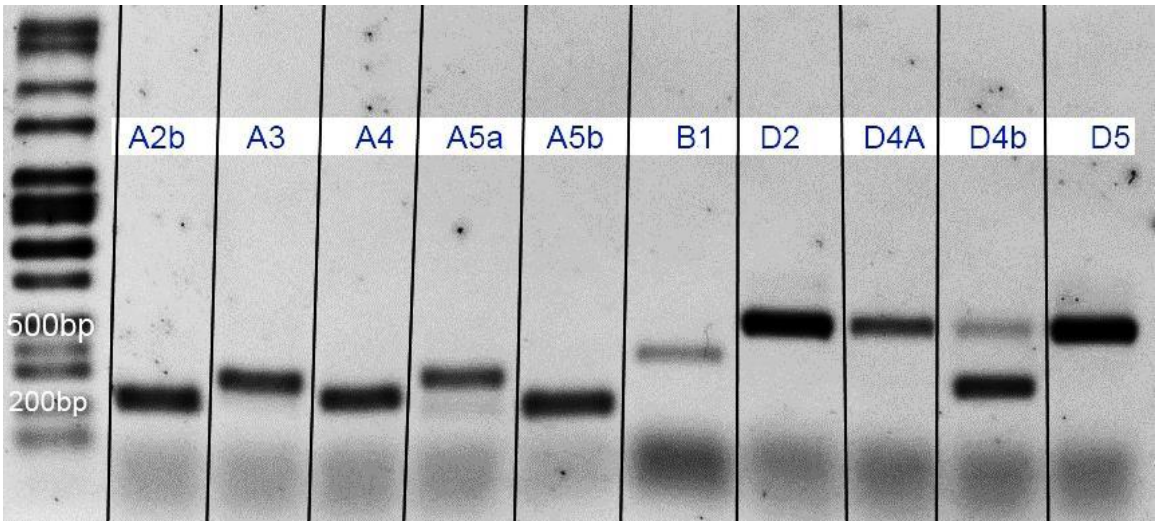

Figure 15. 2% agarose gel of PCR results of degenerate primers reactions. Lanes labeled A were GH3 products from *Paecilomyces*, *Ochroconis,* and two bands (separated after initial electrophoresis) from *Phialophora*; lane B1 was GH6 in *Acidomyces*; and lanes labeled D were GH67 products from *Paecilomyces*, two from *Phialophora* and *Aspergillus.*

Of the 10 PCR products, 7 returned quality reads, with the subsequent translational BLAST queries returning matching or orthologous predications for fungal cellulase, xylanase and cellobiohydrolase enzymes (Table 9). The top matches for BSL fungi did share either thermal or acidic tolerance characteristics. *Mutinus caninus* and *Collybia impudica* are not typically associated with thermotolerance and served as controls for the primer sets (H. Kellner, personal communication).

Fermentation Screening

The promise of lignocellulose utilization and micro-aerobic growth did not culminate in the detectable amount of ethanol from fungi in submerged cultures. Even though the ethanol standard curve was reproducible across the attempts, the calculated ethanol values for the reacted culture samples were less than the 0% v/v ethanol standard at 7, 17, and 20 days (resulting fungal data ranged -0.001 to 0.001 ethanol % v/v). A possible limitation to the assay was using ME medium that may provide too rich of an environment for the acid-adapted fungi, allowing them to slowly maintain growth without resorting to fermentation (if they can).

Table 9. Top matches for PCR products of each primer set for the fungi. Sequence identities where queried using translated BLASTx at blast.ncbi.nlm.nih.gov. Accession numbers correspond to the database entry for each returned match.

Fungi	Primer target: Top Hit returned	E-value	Max Ident	Accession
Acidomyces	Glc1 (GH3):glycoside hydrolase family 3 protein [*Pseudocercospora fijiensis* CIRAD86]	2.00E-26	77%	EME89291.1
	GH6:glycoside hydrolase family 6 protein [*Baudoinia compniacensis* UAMH 10762]	4.00E-56	74%	EMD00967.1
	GH67:glycoside hydrolase family 67 [uncultured fungus]	4.00E-84	70%	ADJ38269.1
Ochroconis	Glc1 (GH3): *Aspergillus terreus* β-glucosidase	5.00E-16	86%	D0VKF5.1
Paecilomyces	Glc1 (GH3):β-glucosidase [*Thermoascus aurantiacus*]	6.00E-34	91%	AFU51372.1
	GH67: glycoside hydrolase family 67 [uncultured fungus]	7.00E-99	83%	ADJ38273.1
Phialophora	Glc1 (GH3):hypothetical protein GGTG_05800 [*Gaeumannomyces graminis* var. *tritici* R3-111a-1]	3.00E-11	47%	EJT75871.1
	GH67:glycoside hydrolase family 67 [uncultured fungus]	2.00E-41	48%	ADJ38275.1
Aspergillus	GH67: α-glucuronidase [*Aspergillus fumigatus*]	3E-108	99%	AFN44902.1
Collybia impudica	Glc1 (GH3):β-glucosidase [*Penicillium occitanis*]	2.00E-24	76%	ABS71124.1
	GH5:endo-1,4-β-mannosidase, putative [*Talaromyces stipitatus* ATCC 10500]	4.00E-56	89%	XP_002480621.1
	GH6:cellobiohydrolase II [*Acremonium cellulolyticus* Y-94]	7.00E-69	77%	BAA74458.1
	GH67:α-glucuronidase precursor, putative [*Talaromyces marneffei* ATCC 18224]	1.00E-112	92%	XP_002149572.1
Mutinus canidus	Glc1 (GH3): glycoside hydrolase family 3 [uncultured fungus]	3.00E-24	67%	ADJ38174.1

CHAPTER IV

DISCUSSION

For my thesis project, I set out to investigate the potential of BSL fungi for use in a biofuels pretreatment based on growth abilities, utilization lignocellulosic materials and extracellular enzyme activity. Here I present my major findings with implications for lignocellulosic biofuels.

Adaptations of BSL Fungi to Hot and Acidic Conditions

In order to establish their potential usage in the pretreatment of lignocellulose materials by incorporation into acidic and hot pretreatment conditions (Barnard, Casanueva, Tuffin, & Cowan, 2010), I needed to first show the BSL fungi grew or at least tolerated extreme conditions similar to biofuels pretreatment. Based on their ability to thrive at various temperatures and pH values, I separated the seven BSL fungi into three groups while keeping the scheme currently adopted by our lab: the 'adapted' fungi which showed acid and heat preference and/or tolerance (*Acidomyces*, *Ochroconis* and *Paecilomyces*); an acid-tolerant but not heat tolerant fungi (*Phialophora*); and the 'Cosmopolitan' fungi that proved to be moderately heat-tolerant but less tolerant of acidic conditions (*Aspergillus*, *Penicillium* and *Cladosporium*). This was a slight change from the lab's previous categorization, as under my testing conditions I did not observe *Phialophora* growing above 27 °C on any solid or liquid media.

The three ‘adapted’ taxa *Acidomyces, Ochroconis,* and *Paecilomyces* were capable of growth at >40 °C and pH 2.5; these results are congruent with published works on the genera. Relatives of the BSL *Acidomyces sp*. (*A. richmondesis*) are found in extremely acid conditions (pH <1) in Iron Mountain Mine in northern California (Brett J. Baker, Tyson, Goosherst, & Banfield, 2009), acidic peat bogs in Czech Republic (*A. acidophilus*) (Hujslová et al., 2010) and a sulfur stock pile in Alberta, Canada (*syn. Scytalidium acidophilum)* (Selbmann et al., 2008); additional description as an acid thermal tolerant genera came from a Japanese hot spring stream (Yamazaki et al., 2010). The *Ochroconis* sp. also has a relative in Japan, *Ochroconis calidifluminalis,* an acid-tolerant isolate from a hot spring with optimal growth at 42 °C, and producing red pigment after multiple plate transfers (Kyoko Yarita et al., 2010). The *Paecilomyces* genus is quite large at this point, with many isolates showing some thermal and/or acidic tolerance (Gross & Robbins, 2000; Hujslová et al., 2010).

Beyond their temperature and pH tolerances, a few other attributes increases the utility of these three BSL fungi for pretreatment. Both *Paecilomyces* and *Ochroconis* returned to typical growth patterns after halting due to temporary 5 day incubation at 50 °C. This is a useful trait if live fungi are used throughout the pretreatment process where temperatures can fluctuate up and down. *Acidomyces* was not as tolerant to the 50 °C shift; even if it just required a few more days to recover, the slow response would still be a disadvantage. All three of the adapted fungi grew under micro-aerobic conditions and quickly recovered to typical growth after transfer from anaerobic conditions; again these attributes are useful during the pretreatment process where oxygenation can be limited by

solids loading and liquid volumes fluctuation. Further, as the typical dilute acid pretreatment process uses water to move through the treatment infrastructure, it is helpful that our *Acidomyces* and *Ochroconis* grow equally in static and dynamic liquid environments, often aggregating small particulates. Our *Paecilomyces* had reduced growth under dynamic liquid conditions so, use of whole organisms in pretreatment may be limited to shallow configurations.

The *Phialophora* sp. isolate was successful at pH 2.5, showing acid tolerance similar to descriptions of related genera (Gross & Robbins, 2000; F. Zhang et al., 2011), but only displayed mycelia growth up to 27 °C. However, *Phialophora* may be better suited for acidic processes that take place closer to ambient temperatures.

The 'cosmopolitan' taxa *Aspergillus*, *Penicillium* and *Cladosporium* each grew at >40 °C but not with concurrent pH <3. In particular, the *Aspergillus* showed some growth at 50 °C, the highest of all seven fungi, but it was growing on standard PDA medium with pH ~5. It appears that growth of these three fungi species requires a trade of heat tolerance for acid tolerance and vice-versa under my challenge conditions. As the most widely studied fungi of our seven, relatives of these three fungi are found in a wide variety of conditions, but typically grow only at <40 °C in conditions >pH 4 (Gao et al., 2008; Gross & Robbins, 2000; Khalid, Yang, Kishwar, Rajput, & Arijo, 2006). The use of these fungi in biofuels is less focused on concurrent hot acid pretreatment processes and more for free enzymes production that can used in after chemical/mechanical steps (Bhalla et al., 2013).

Though I made the decision to focus more on the adapted fungi in some of the later assays due to their ideal tolerances, much of the further testing included the cosmopolitan fungi and *Phialophora* as part of the big picture for microbiology at BSL. As such, the 3 adapted fungi showed an obvious preference for the warm and acidic environment while the rest can at least tolerate a component. I felt this established a basis to further investigate the fungi on lignocellulose materials.

Fungal Utilization of Lignocellulosic Material at High Temperature and Low pH

With the BSL shoreline and side pool environments providing the hot and acidic conditions and the frequent inputs of plant materials, it was not a surprise that several of the fungi could utilize individual lignocellulosic components (cellobiose, cellulose and xylan) or plant materials for growth in laboratory conditions. I selected plant materials found in the BSL environment (incense cedar scales and pine needles) attempting to mimic possible utilization/degradation in the natural system but later realized that the results were more interesting because these materials have higher amount of lignins, pectins, and other compounds that resist microbial degradation (Song, Tian, Fan, & He, 2009). Also tested were agricultural byproducts indicative of crops grown around the world that carry potential for use as biofuel feedstock (e.g. rice hulls, rice straw and wheat straw); these are cheap, have higher ratios of (hemi)celluloses than lignins, and concurrently use agricultural land with food crops (Jahromi & Ho, 2011; Perlack & Stokes, 2011). Because lignin components are often not utilized by brown-rot and soft-rot fungi, or modified by white-rot fungi to allow the fungus access to more

(hemi)cellulose, I did not screen for growth on isolated lignin compounds (Martínez et al., 2005).

Using fungal growth as a proxy for potential lignocellulose degradation (Shrestha et al., 2011), I categorized the fungi based on their utilization of diverse lignocellulosic materials under warm and acidic conditions (Table 11). The 3 fungi showing the strongest growth in warm and acidic conditions also showed the most potential for biofuels with their flexibility of substrates. *Acidomyces* showed the most versatility at pH ~2.5 and 37-40 °C, with qualitative growth on major lignocellulose components: cellobiose, cellulose, xylan, and cotton fiber; and quantifiable growth rates of mycelia increase on incense cedar and pine needles, rice hulls, rice straw, and wheat straw. The second most versatile BSL fungus, *Paecilomyces*, had growth verified on cellobiose and xylan; and comparable growth rates on rice hulls, incense cedar, and pine needles to control plates, all at pH ~2.5 from 37-40 °C. Third, *Ochroconis* sustained growth on cellobiose, xylan, incense cedar, or pine needle media at pH ~2.5 from 37-40 °C. *Phialophora*, though not thermotolerant, could utilize cellobiose, rice hulls, incense cedar, and pine needles at pH ~2.5 at 27 °C. The well-studied and already versatile *Aspergillus* did show growth on cellobiose, incense cedar and pine needles at pH 2 and 40 °C. The remaining two fungi, *Penicillium* and *Cladosporium* were slow-growing on the pH 2 cellobiose media at 37 °C and due to limited growth on acidic cellobiose media, the *Cladosporium* was not tested on acidified plant substrates.

To minimize false positive growth from the small amounts of solubilized glucose, cellobiose, xylose or arabinose, or false negatives from inhibitors such as acetate

Table 11. Summary of lignocellulose material utilization by the BSL fungi at pH 2 and 40 °C or 37 °C (except *Phialophora* at 27 °C). C sources: CB = cellobiose; C = cellulose; X = xylan; PN = pine needles; IC = incense cedear; RH = rice hulls RS = rice straw; WS = wheat straw. Symbols: confirmed growth (+), lack of growth (-); blank = not tested.

Fungi	CB	C	X	PN	IC	RH	RS	WS
Acidomyces	+	+	+	+	+	+	+	+
Paecilomyces	+	-	+	+	+	+	-	-
Ochroconis	+	-	+	+	+	-	-	-
Phialophora	+			+	+	+		-
Aspergillus	+			+	+	-	-	-
Penicllium	+			-	-	-	-	-
Cladosporium	+							-

or furfural the plant materials were rinsed twice after autoclave sterilization (DI water at 121 °C for 15 minutes) (Mittal, Scott, Amidon, Kiemle, & Stipanovic, 2009). I also had concerns that the acidified media would chemically hydrolyze cellulose, but the H_2SO_4 concentration (~0.1% w/v) and incubation temperature (40 °C) are well below typical dilute-acid treatments that employ H_2SO_4 at 0.5% w/v and 140 °C (Avci et al., 2013). Hemicelluloses are easily degraded abiotically, but based on extrapolations from hot water treated sugar maple chips (Hu, Lin, Liu, & Liu, 2010), it would take ~12 days to chemically hydrolyze half the remaining xylan (after autoclaving and twice rinsing) in my acidic media formulation at 40 °C. I bring up this distinction because the environmental conditions at BSL (pH 2; 50 °C) are likely contributing to chemical hydrolysis of plant materials, resulting in soluble xylose and glucose oligomers diffusing from submerged plant litter -of recent input- as an easy source of nutrients (Almasary, 2013). Rinsing twice was intended to remove any soluble compounds, leaving the fungi as the driving force for degradation in the laboratory condition.

Thus, the majority of BSL fungi can utilize lignocellulosic materials found in their "native" environment *in vitro,* and a few of them have increased versatility to grow on plant materials beyond BSL's environment.

Extracellular Fungal Enzymes Useful for Lignocellulose Degradation

But growth is more than a proxy for lignocellulose utilization; it can also indicate fungal enzymes that are actively degrading the lignocellulose within the hot and acid conditions of BSL One of my original goals was to isolate and characterize extremophilic secreted cellulase and laccase enzymes. While I was not able to accomplish this ambitious goal, I tried to show indirect evidence by detecting enzyme activity in situ, or detecting genes of known enzymes.

Lignin degradation is usually thought to be a specialty of the white-rot Basidiomycetes (e.g. *Phanerochaete chrysosporium* and *Trametes versicolor*), but ascomycetes associated with plant degradation also have various suites of laccases or Mn-peroxidases (Baldrian, 2006; Chang, Fan, & Wen, 2012; Hoegger, Kilaru, James, Thacker, & Kues, 2006). The resulting oxidation of ABST at room temperature on acidic media and growth on the ABST/manganese plates by the BSL *Acidomyces* and *Ochroconis* at 40 °C and pH 2 indicates laccase/peroxidase enzymes that function under acidic and hot conditions, though the lack of dark precipitate may indicate a lack of Mn-peroxidase activity (Steffen, Hofrichter, & Hatakka, 2000). These conditions are comparable to *Pycnoporus coccinues*, which also functions at elevated temperatures, but was only tested at pH 4.5 (Chairattanamanokorn et al., 2006). It is unfortunate that our

Paecilomyces, *Phialophora* and *Aspergillus* did not show ABST oxidation under either testing conditions, as this may suggest a limit to their utility. I note here that *Aspergillus niger* is known to harbor peroxidase enzymes under non-acidic conditions (Ramos, Barends, Verhaert, & de Graaff, 2011). Fungi that can produce functioning lignin and cellulose degrading enzymes under hot and acidic conditions are a more valuable asset to the pretreatment processes

In evaluating potential extracellular cellulase enzymes from in *Acidomyces*, *Ochroconis*, and *Paecilomyces* culture supernatant, I was unfortunately not able to troubleshoot issues with the reducing sugar/DNS assay for any of these fungi. Though it is possible these fungi lack cellulytic enzymes, I suspect the small volumes and high dilutions were below detection limits (Ghose, 1987). Short of the physical evidence, the related *Acidomyces richmondosis* genome has annotations predicating at least 14 distinct cellulase enzymes within its entry on MycoCosm database at the Joint Genome Institute website (Grigoriev et al., 2012). I was unable to find reports about the potential for cellulases within the *Ochroconis* genus, whose literature is predominantly concerned with human pathogenicity (Kyoko Yarita et al., 2010). I chose not to focus on hemicellulose degrading xylanases, as the xylose/arabinose oligomers are easily hydrolyzed under the conditions that target the tougher cellulose or lignin components (Hu et al., 2010; Mittal et al., 2009; Pu, Hu, Huang, Davison, & Ragauskas, 2013; Shen, Kumar, Hu, & Saddler, 2011).

With lack of success in direct cellulase enzyme assays, but armed with a few annotated fungal genomes, I turned to a PCR-based screening strategy. Using primers

designed to complement conserved regions of fungal degradation enzyme cDNAs (Harald Kellner & Vandenbol, 2010; Lang & Orgogozo, 2011), I took a chance on using BSL fungi genomic DNA instead. Even though fungi are known to harbor introns, PCR was successful in forming products that represented 3-4 (hemi)cellulose-degrading genes per fungus. Additionally, the positive control fungal cultures sent to us by Dr. Harald Kellner also formed products from genomic DNA that agreed with his experimental products (personal communication). The sequenced PCR products aligned well within the annotated protein sequences of known acid and thermotolerant *Acidomyces richmondosis* (JGI BLASTx) and thermotolerant *Aspergillus fumigatus* (NCBI BLASTx). Even with the lack of matching annotations, our 3 other sufficiently performing acid tolerant fungi (*Ochroconis*, *Paecilomyces* and *Phialophora*) still maintain potential for biofuels pretreatment, based on their products typically aligning with orthologous enzymes from thermotolerant fungi (**Table 7**). Further, previously published studies have characterized cellulases and xylanases enzymes in *Phialophora* (F. Zhang et al., 2011; Zhao et al., 2012) and *Paecilomyces* (Kluczek-Turpeinen, Maijala, Tuomela, Hofrichter, & Hatakka, 2005; L. T. Li et al., 2006; Tribak, Ocampo, & García-Romera, 2002).

In conclusion, by coupling the PCR results to lignocellulose utilization data and the ABST oxidation assays results suggests the BSL fungi, in particular *Acidomyces* and *Ochroconis*, and to a lesser extent *Paecilomyces*, contain viable sets of (hemi)cellulose and lignin degrading enzymes that likely function in warm and acidic conditions. As such, these fungi are worthy candidates for a concurrent hot acid and biological pretreatment process in the production of lignocellulose biofuels.

Suggestions for Future Work

Although I was able to answer a few questions about the fungi of BSL, I was not able to achieve all my goals, and much work remains. Focusing on lignocellulytic assays, protein purification and mass spectrometry to characterize individual enzymes from the 3 adapted fungi could lead to new amino acid configuration for improved biotechnological designs (Gostinčar & Turk, 2012; Graham et al., 2011; Wijma et al., 2013). Also, using genomics to compare the BSL fungi to relations found around the world could shed more light on the gene/protein functions needed to maintain life in hot acid with applications beyond biofuels, like the changes from global warming and continued pollution or possibilities for astrobiology.

Utilizing the melanization and ABST oxidation capabilities of *Acidomyces* and *Ochroconis* might replace or complement current chemical treatments for bioremediation or decolorizing wastewater (Chairattanamanokorn et al., 2006; Kalpana et al., 2012). Since *Ochroconis* also produces a water soluble red pigment after prolonged growth on nutrient media, this may have application as a renewable dye source; or since it is related to a human pathogen, may provide non-infectious model to for genomic/transcriptomic comparisons in hopes of identifying pathogenicity pathways.

LITERATURE CITED

LITERATURE CITED

Adney, B. & Baker, J. (1996). Measurement of cellulase activities. NREL Analytical Procedure, National Renewable Energy Laboratory, Technical, Report NREL/TP-510-42628.

Ahmed, S., Riaz, S., & Jamil, A. (2009). Molecular cloning of fungal xylanases: an overview. *Applied Microbiology and Biotechnology, 84*(1), 19-35-35.

Ali, S. S., Khan, M., Fagan, B., Mullins, E., & Doohan, F. M. (2012). Exploiting the inter-strain divergence of *Fusarium oxysporum* for microbial bioprocessing of lignocellulose to bioethanol. *AMB Express, 2*(1), 1.

Almasary, A. (2013). *Contribution of allochthonous carbon to the microbial community in an acidic geothermal lake.* (M.S.), California State University, Chico, Chico.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology, 101*(13), 4851-4861.

Amaral Zettler, L. A., Messerli, M. A., Laatsch, A. D., Smith, P. J. S., & Sogin, M. L. (2003). From Genes to Genomes: Beyond Biodiversity in Spain's Rio Tinto. *The Biological Bulletin, 204*(2), 205-209.

Amore, A., & Faraco, V. (2012). Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production. *Renewable Sustainable Energy Review, 16*(5), 3286-3301.

Andrade, F. A. d., Calonego, F. W., Severo, E. T. D., & Furtado, E. L. (2012). Selection of fungi for accelerated decay in stumps of *Eucalyptus* spp. *Bioresource Technology*. 110, 456-461.

Avci, A., Saha, B. C., Kennedy, G. J., & Cotta, M. A. (2013). Dilute sulfuric acid pretreatment of corn stover for enzymatic hydrolysis and efficient ethanol production by recombinant Escherichia coli FBR5 without detoxification. *Bioresource Technology*, *142*, 312-319.

Bai, Y., Wang, J., Zhang, Z., Shi, P., Luo, H., Huang, H., Yao, B. (2010). A novel family 9 β-1,3(4)-glucanase from thermoacidophilic *Alicyclobacillus* sp. A4 with potential applications in the brewing industry. *Applied Microbiology and Biotechnology, 87*, 251-259.

Baker, B. J., Lutz, M. A., Dawson, S. C., Bond, P. L., & Banfield, J. F. (2004). Metabolically active eukaryotic communities in extremely acidic mine drainage. *Applied and Environmental Microbiology, 70*(10), 6264-6271.

Baker, B. J., Tyson, G. W., Goosherst, L., & Banfield, J. F. (2009). Insights into the Diversity of Eukaryotes in Acid Mine Drainage Biofilm Communities. *Applied and Environmental Microbiology, 75*(7), 2192-2199.

Baldrian, P. (2006). Fungal laccases occurrence and properties. *FEMS Microbiology Reviews, 30*(2), 215-242.

Barnard, D., Casanueva, A., Tuffin, M., & Cowan, D. (2010). Extremophiles in biofuel synthesis. *Environmental Technology, 31*, 871-888.

Berka, R. M., Grigoriev, I. V., Otillar, R., Salamov, A., Grimwood, J., Reid, I., Tsang, A. (2011). Comparative genomic analysis of the thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. *Nature Biotechnology, 29*(10), 922-927.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., & Sani, R. K. (2013). Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology, 128*, 751-759.

Bhatia, L., Johri, S., & Ahmad, R. (2012). An economic and ecological perspective of ethanol production from renewable agro waste: a review. *AMB Express, 2*(1), 65.

Blumer-Schuette, S. E., Brown, S. D., Sander, K. B., Bayer, E. A., Kataeva, I., Zurawski, J. V., Kelly, R. M. (2013). Thermophilic lignocellulose deconstruction. *FEMS Microbiology Reviews*. 1574-6976

Brown, P. B., & Wolfe, G. V. (2006). Protist Genetic Diversity in the Acidic Hydrothermal Environments of Lassen Volcanic National Park, USA. *The Journal of Eukaryotic Microbiology, 53*, 420-431.

Carere, C. R., Sparling, R., Cicek, N., & Levin, D. B. (2008). Third generation biofuels via direct cellulose fermentation. *International Journal of Molecular Sciences, 9*(7), 1342-1360.

Carroll, A., & Somerville, C. (2009). Cellulosic Biofuels. *Annual Review of Plant Biology, 60*(1), 165-182.

Chairattanamanokorn, P., Imai, T., Kondo, R., Ukita, M., & Prasertsan, P. (2006). Screening thermotolerant white-rot fungi for decolorization of wastewaters. *Applied Biochemistry and Biotechnology*, *128*(3), 195-204.

Chang, A. J., Fan, J., & Wen, X. (2012). Screening of fungi capable of highly selective degradation of lignin in rice straw. *International Biodeterioration & Biodegradation*, *72*, 26-30.

Chen, X., Tao, L., Shekiro, J., Mohaghaghi, A., Decker, S., Wang, W., Tucker, M. (2012). Improved ethanol yield and reduced Minimum Ethanol Selling Price (MESP) by modifying low severity dilute acid pretreatment with deacetylation and mechanical refining: 1) Experimental. *Biotechnology for Biofuels, 5*(1), 69.

Clark, T. A., Slavinski, S. A., Morgan, J., Lott, T., Arthington-Skaggs, B. A., Brandt, M. E., . . . Hajjeh, R. A. (2004). Epidemiologic and molecular characterization of an outbreak of *Candida parapsilosis* bloodstream infections in a community hospital. *Journal of Clinical Microbiology*, *42*(10), 4468-4472.

Cruz Ramírez, M. G., Rivera-Ríos, J. M., Téllez-Jurado, A., Maqueda Gálvez, A. P., Mercado-Flores, Y., & Arana-Cuenca, A. (2012). Screening for thermotolerant

ligninolytic fungi with laccase, lipase, and protease activity isolated in Mexico. *Journal of Environmental Management, 95, Supplement*, S256.

Cui, Z., Shi, J., Wan, C., & Li, Y. (2012). Comparison of alkaline- and fungi-assisted wet-storage of corn stover. *Bioresource Technology, 109*, 98-104.

Dangles, O., & Chauvet, E. (2003). Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. *Water Research, 37*(3), 533-538.

Dashtban, M., Maki, M., Leung, K. T., Mao, C., & Qin, W. (2010). Cellulase activities in biomass conversion: measurement methods and comparison. *Critical Reviews in Biotechnology,* 30, 302-309.

Dutta, T., Sahoo, R., Sengupta, R., Ray, S. S., Bhattacharjee, A., & Ghosh, S. (2008). Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. *Journal of Industrial Microbiology & Biotechnology, 35*, 275-282.

Ezeji, T., Qureshi, N., & Blaschek, H. P. (2007). Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. *Biotechnology and Bioengineering, 97*(6), 1460-1469.

Fischer, M., & Sawers, R. G. (2013). A universally applicable and rapid method for measuring the growth of streptomyces and other filamentous microorganisms by methylene blue adsorption-desorption. *Applied and Environmental Microbiology, 79*(14), 4499-4502.

Foust, T. D., Aden, A., Dutta, A., & Phillips, S. (2009). An economic and environmental comparison of a biochemical and a thermochemical lignocellulosic ethanol conversion processes. *Cellulose, 16*(4), 547-565.

Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., & Xi, Y. (2008). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technology, 99*(16), 7623-7629.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry, 59*(2), 257-268.

Gostinčar, C., & Turk, M. (2012). Extremotolerant fungi as genetic resources for biotechnology. *Bioengineered, 3*(5), 293-297.

Graham, J. E., Clark, M. E., Nadler, D. C., Huffer, S., Chokhawala, H. A., Rowland, S. E., Robb, F. T. (2011). Identification and characterization of a multidomain hyperthermophilic cellulase from an archaeal enrichment. *Nature Communications, 2*, 375.

Grigoriev, I. V., Nordberg, H., Shabalov, I., Aerts, A., Cantor, M., Goodstein, D., Dubchak, I. (2012). The genome portal of the Department of Energy Joint Genome Institute. *Nucleic Acids Research, 40* (Database issue), D26-32.

Gross, S., & Robbins, E. I. (2000). Acidophillic and acid-tolerant fungi and yeasts. *Hydrobiolgia, 433*, 91-109.

Hasunuma, T., Okazaki, F., Okai, N., Hara, K. Y., Ishii, J., & Kondo, A. (2013). A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. *Bioresource Technology, 135*, 513-522.

Himmel, M. E., Ding, S.-Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., & Foust, T. D. (2007). Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science, 315*(5813), 804-807.

Hoegger, P. J., Kilaru, S., James, T. Y., Thacker, J. R., & Kues, U. (2006). Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS Journal, 273*(10), 2308-2326.

Houbraken, J., & Samson, R. A. (2011). Phylogeny of *Penicillium* and the segregation of Trichocomaceae into three families. *Studies in Mycology, 70*(1), 1-51.

Howard, R. L., Abotsi, E., Jansen van Rensburg, E. L., & Howard, S. (2003). Review-Lignocellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of Biotechnology, 2(12), 602-619.*

Hu, R., Lin, L., Liu, T., & Liu, S. (2010). Dilute sulfuric acid hydrolysis of sugar maple wood extract at atmospheric pressure. *Bioresource Technology, 101*(10), 3586-3594.

Hujslová, M., Kubátová, A., Chudíčková, M., & Kolařík, M. (2010). Diversity of fungal communities in saline and acidic soils in the Soos National Natural Reserve, Czech Republic. *Mycological Progress, 9*(1), 1-15.

Itoh, H., Wada, M., Honda, Y., Kuwahara, M., & Watanabe, T. (2003). Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. *Journal of biotechnology, 103*(3), 273-280.

Jahromi, M. F., & Ho, Y. W. (2011). Efficiency of rice straw lignocelluloses degradability by *Aspergillus terreus* ATCC 74135 in solid state fermentation. *African Journal of Biotechnology, 10*(21), 4428-4435.

Jeyakumar, D., Chirsteen, J., & Doble, M. (2013). Synergistic effects of pretreatment and blending on fungi mediated biodegradation of polypropylenes. *Bioresource Technology, 148,* 78-85.

Kalpana, D., Velmurugan, N., Shim, J. H., Oh, B.-T., Senthil, K., & Lee, Y. S. (2012). Biodecolorization and biodegradation of reactive Levafix Blue E-RA granulate dye by the white rot fungus *Irpex lacteus*. *Journal of Environmental Management, 111*, 142-149.

Kataeva, I., Foston, M. B., Yang, S.-J., Pattathil, S., Biswal, A. K., Poole Ii, F. L., Adams, M. W. W. (2013). Carbohydrate and lignin are simultaneously solubilized from unpretreated switchgrass by microbial action at high temperature. *Energy & Environmental Science, 6*(7), 2186-2195.

Kellner, H., Luis, P., & Buscot, F. o. (2007). Diversity of laccase-like multicopper oxidase genes in Morchellaceae: identification of genes potentially involved in extracellular activities related to plant litter decay: Laccase-like multicopper oxidases in Morchellaceae. *FEMS Microbiology Ecology, 61*(1), 153-163.

Kellner, H., Luis, P., Schlitt, B., & Buscot, F. (2009). Temporal changes in diversity and expression patterns of fungal laccase genes within the organic horizon of a brown forest soil. *Soil Biology and Biochemistry, 41*(7), 1380-1389.

Kellner, H., Zak, DR, & Vandenbol, M. (2010). Fungi Unearthed: Transcripts Encoding Lignocellulolytic and Chitinolytic Enzymes in Forest Soil. *PLoS ONE, 5*(6).

Khalid, M., Yang, W., Kishwar, N., Rajput, Z. I., & Arijo, A. G. (2006). Study of cellulolytic soil fungi and two nova species and new medium. *Journal of Zhejiang University-Science B, 7*(6), 459-466.

Kluczek-Turpeinen, B., Maijala, P., Tuomela, M., Hofrichter, M., & Hatakka, A. (2005). Endoglucanase activity of compost-dwelling fungus *Paecilomyces inflatusis* stimulated by humic acids and other low molecular mass aromatics. *World Journal of Microbiology & Biotechnology, 21*, 1603-1609.

Koçar, G., & Civaş, N. (2013). An overview of biofuels from energy crops: Current status and future prospects. *Renewable and Sustainable Energy Reviews, 28*, 900-916.

Krogh, K., Mørkeberg, A., Jørgensen, H., Frisvad, J., & Olsson, L. (2004). Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. *Applied Biochemistry and Biotechnology, 114*(1), 389-401.

Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial & Engineering Chemistry Research, 48*, 3713-3729.

Lang, M., & Orgogozo, V. (2011). Identification of homologous gene sequences by PCR with degenerate primers. *Methods in Molecular Biology, 772*, 245-256.

Li, C., Yang, Z., He, R., Zhang, D., Chen, S., & Ma, L. (2013). Effect of pH on cellulase production and morphology of *Trichoderma reesei* and the application in cellulosic material hydrolysis. *Journal of Biotechnology*. 168(4), 470–477.

Li, L. T., Yang, S. Q., Yan, Q. J., Jiang, Z. Q., Tian, H. M., & Wang, Y. Z. (2006). High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. *Bioresource Technology, 97*(15), 1794-1800.

Lowe, S. E., Theodorou, M. K., & Trinci, A. P. (1987). Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan. *Applied and Environmental Microbiology, 53*(6), 1216–1223.

Luo, H., Wang, Y., Wang, H., Yang, J., Yang, Y., Huang, H., Yao, B. (2008). A novel highly acidic β-mannanase from the acidophilic fungus *Bispora* sp. MEY-1: gene cloning and overexpression in *Pichia pastoris*. *Applied Microbiology and Biotechnology, 82,* 453-461.

Lynd, L. R., van Zyl, W. H., McBride, J. E., & Laser, M. (2005). Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology, 16*(5), 577-583.

Ma, F., Yang, N., Xu, C., Yu, H., Wu, J., & Zhang, X. (2010). Combination of biological pretreatment with mild acid pretreatment for enzymatic hydrolysis and ethanol production from water hyacinth. *Bioresource Technology, 101*(24), 9600-9604.

Maheshwari, R., Bharadwaj, G., & Bhat, M. K. (2000). Thermophilic fungi: their physiology and enzymes. *Microbiology and Molecular Biology Reviews, 64*(3) 461–488.

Martínez, A. T., Speranza, M., Ruiz-Dueñas, F. J., Ferreira, P., Camarero, S., Guillén, F., del Río, J. C. (2005). Biodegradation of lignocellulosics: microbial, chemical, and

enzymatic aspects of the fungal attack of lignin. *International Microbiology 8*(3), 195-204.

Mendu, V., Shearin, T., Campbell, J. E., Stork, J., Jae, J., Crocker, M., DeBolt, S. (2012). Global bioenergy potential from high-lignin agricultural residue. *Proceedings of the National Academy of Sciences, 109*(10), 4014-4019.

Mittal, A., Scott, G. M., Amidon, T. E., Kiemle, D. J., & Stipanovic, A. J. (2009). Quantitative analysis of sugars in wood hydrolyzates with 1H NMR during the autohydrolysis of hardwoods. *Bioresource Technology, 100*(24), 6398-6406.

Mouchacca, J. (2007). Heat tolerant fungi and applied research: Addition to the previously treated group of strictly thermotolerant species. *World Journal of Microbiology and Biotechnology, 23*, 1755-1770.

Mowat, E., Butcher, J., Lang, S., Williams, C., & Ramage, G. (2007). Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *Journal of Medical Microbiology, 56*(9), 1205-1212.

Okamoto, K., Kanawaku, R., Masumoto, M., & Yanase, H. (2012). Efficient xylose fermentation by the brown rot fungus *Neolentinus lepideus*. *Enzyme and Microbial Technology, 50*(2), 96-100.

Okamoto, K., Nitta, Y., Maekawa, N., & Yanase, H. (2011). Direct ethanol production from starch, wheat bran and rice straw by the white rot fungus *Trametes hirsuta*. *Enzyme and Microbial Technology, 48*(3), 273-277.

Olson, D. G., McBride, J. E., Shaw, A. J., & Lynd, L. R. (2012). Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology, 23*(3), 396-405.

Parish, E. S., Kline, K. L., Dale, V. H., Efroymson, R. A., McBride, A. C., Johnson, T. L., Bielicki, J. M. (2013). Comparing scales of environmental effects from gasoline and ethanol production. *Environmental Management, 51*(2), 307-338.

Pedersen, M., Johansen, K. S., & Meyer, A. S. (2011). Low temperature lignocellulose pretreatment: effects and interactions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw. *Biotechnology for Biofuels, 4*, 11.

Perlack, R. D., & Stokes, B. J. (2011). *U.S. Billion-Ton Update: Biomass Supply for a Bioenergy and Bioproducts Industry.* Oak Ridge National Laboratory, Oak Ridge, TN.

Pienkos, P. T., & Zhang, M. (2009). Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. *Cellulose*, *16*(4), 743-762.

Pu, Y., Hu, F., Huang, F., Davison, B. H., & Ragauskas, A. J. (2013). Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. *Biotechnology for Biofuels, 6*(1), 15.

Ramos, J. T., Barends, S., Verhaert, R., & de Graaff, L. H. (2011). The *Aspergillus niger* multicopper oxidase family: analysis and overexpression of laccase-like encoding genes. *Microbial Cell Factories, 10*, 78.

Redman, R. S., Litvintseva, A., Sheehan, K. B., Henson, J. M., & Rodriguez, R. J. (1999). Fungi from geothermal soils in Yellowstone National Park. *Applied and Environmental Microbiology*, *65*(12), 5193–5197.

Reeder IV, W. H. H. (2011). *Diversity and activities of phagotrophic micro-eukaryotes in Boiling Springs Lake, Lassen Volcanic National Park.* (M.S.), California State University, Chico, Chico.

Reeslev, M., & Kjoller, A. (1995). Comparison of biomass dry weights and radial growth rates of fungal colonies on media solidified with different gelling compounds. *Applied and Environmental Microbiology, 61*(12), 4236-4239.

Russell, J. R., Huang, J., Anand, P., Kucera, K., Sandoval, A. G., Dantzler, K. W., Strobel, S. A. (2011). Biodegradation of Polyester Polyurethane by Endophytic Fungi. *Applied and Environmental Microbiology, 77*, 6076-6084.

Selbmann, L., De Hoog, G. S., Zucconi, L., Isola, D., Ruisi, S., van den Ende, A. H. G., Onofri, S. (2008). Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. *Studies in Mycology, 61*(1), 1-20.

Seo, H.-B., Kim, H.-J., Lee, O.-K., Ha, J.-H., Lee, H.-Y., & Jung, K.-H. (2008). Measurement of ethanol concentration using solvent extraction and dichromate oxidation and its application to bioethanol production process. *Journal of Industrial Microbiology & Biotechnology, 36*(2), 285-292.

Sharma, M., Chadha, B. S., Kaur, M., Ghatora, S. K., & Saini, H. S. (2008). Molecular characterization of multiple xylanase producing thermophilic/thermotolerant fungi isolated from composting materials. *Letters in Applied Microbiology, 46*(5), 526-535.

Shen, F., Kumar, L., Hu, J., & Saddler, J. N. (2011). Evaluation of hemicellulose removal by xylanase and delignification on SHD and SSF for bioethanol production with steam-pretreated substrates. *Bioresource Technology*, 102(19), 8945-8951.

Shrestha, P., Szaro, T. M., Bruns, T. D., & Taylor, J. W. (2011). Systematic Search for Cultivatable Fungi That Best Deconstruct Cell Walls of Miscanthus and Sugarcane in the Field. *Applied and Environmental Microbiology, 77*, 5490-5504.

Siering, P. L., Clarke, J. M., & Wilson, M. S. (2006). Geochemical and Biological Diversity of Acidic, Hot Springs in Lassen Volcanic National Park. *Geomicrobiology Journal, 23*, 129-141.

Siering, P. L., Wolfe, G. V., Wilson, M. S., Yip, A. N., Carey, C. M., Wardman, C. D., Zhou, J. (2013). Microbial biogeochemistry of Boiling Springs Lake: a physically dynamic, oligotrophic low pH geothermal ecosystem *Geobiology, 11*(4), 356-376.

Simonis, J. L., Raja, H. A., & Shearer, C. A. (2008). Extracellular enzymes and soft rot decay: Are ascomycetes important degraders in fresh water. *Fungal Diversity, 31*(1), 135-146.

Skyba, O., Douglas, C. J., & Mansfield, S. D. (2013). Syringyl-Rich Lignin Renders Poplars More Resistant to Degradation by Wood Decay Fungi. *Applied and Environmental Microbiology, 79*(8), 2560-2571.

Somerville, C. (2006). The Billion-Ton Biofuels Vision. *Science, 312*(5778), 1277.

Song, F., Tian, X., Fan, X., & He, X. (2009). Decomposing ability of filamentous fungi on litter is involved in a subtropical mixed forest. *Mycologia, 102*(1), 20-26.

Steffen, K. T., Hofrichter, M., & Hatakka, A. (2000). Mineralisation of 14C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi. *Applied Microbiology and Biotechnology, 54*(6), 819-825.

Stichnothe, H., & Azapagic, A. (2009). Bioethanol from waste: Life cycle estimation of the greenhouse gas saving potential. *Resources, Conservation and Recycling, 53*(11), 624-630.

Talebnia, F., Karakashev, D., & Angelidaki, I. (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technology, 101*(13), 4744-4753.

Tao, L., Aden, A., Elander, R. T., Pallapolu, V. R., Lee, Y. Y., Garlock, R. J., Warner, R. E. (2011). Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. *Bioresource Technology, 102*(24), 11105-11114.

Thompson, P. L., & Bärlocher, F. (1989). Effect of pH on leaf breakdown in streams and in the laboratory. *Journal of the North American Benthological Society*, 8, 203-210.

Tian, X.-f., Fang, Z., & Gou, F. (2012). Impact and prospective of fungal pre-treatment of lignocellulosic biomass for enzymatic hydrolysis. *Biofuels, Bioproducts and Biorefining, 6*(3), 335-350.

Tribak, M., Ocampo, J. A., & García-Romera, I. (2002). Production of xyloglucanolytic enzymes by *Trichoderma viride*, *Paecilomyces farinosus*, *Wardomyces inflatus*, and *Pleurotus ostreatus*. *Mycologia, 94*(3), 404-410.

Turner, P., Mamo, G., & Karlsson, E. N. (2007). Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbial Cell Factories, 6*, 9.

van den Brink, J., & de Vries, R. P. (2011). Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and Biotechnology, 91*(6), 1477-1492.

van den Brink, J., van Muiswinkel, G. C. J., Theelen, B., Hinz, S. W. A., & de Vries, R. P. (2012). Efficient Plant Biomass Degradation by Thermophilic Fungus *Myceliophthora heterothallica*. *Applied and Environmental Microbiology, 79*(4), 1316-1324.

White, T. J., Bruns, T., Lee, S., & Taylor., J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White (Eds.), *PCR Protocols: A Guide to Methods and Applications* (pp. 315-322). New York: Academic Press, Inc.

Wijma, H. J., Floor, R. J., & Janssen, D. B. (2013). Structure- and sequence-analysis inspired engineering of proteins for enhanced thermostability. *Current Opinion in Structural Biology, 23*(4), 588-594.

Wilson, M. S., Siering, P. L., White, C. L., Hauser, M. E., & Bartles, A. N. (2007). Novel Archaea and Bacteria Dominate Stable Microbial Communities in North America's Largest Hot Spring. *Microbial Ecology, 56*, 292-305.

Wolfe, G. V., Reeder IV, W. H. H., & Ervin, B. (2013). Novel materials enable a low-cost termpature-light gradient incubator for microbial studies. *Journal of Microbiological Methods, 97, 29-33*.

Wurzbacher, C., Bärlocher, F., & Grossart, H. (2010). Fungi in lake ecosystems. *Aquatic Microbial Ecology, 59*, 125-149.

Yamazaki, A., Toyama, K., & Nakagiri, A. (2010). A new acidophilic fungus *Teratosphaeria acidotherma* (Capnodiales, Ascomycota) from a hot spring. *Mycoscience, 51*, 443-455.

Yarita, K., Sano, A., Murata, Y., Takayama, A., Takahashi, Y., Takahashi, H., Nishimura, K. (2007). Pathogenicity of *Ochroconis gallopava* isolated from hot springs in Japan and a review of published reports. *Mycopathologia, 164*(3), 135-147.

Yarita, K., Sano, A., Samerpitak, K., Kamei, K., Hoog, G. S., & Nishimura, K. (2010). *Ochroconis calidifluminalis*, a Sibling of the Neurotropic Pathogen *O. gallopava*, Isolated from Hot Spring. *Mycopathologia, 170*, 21-30.

Zhang, F., Shi, P., Bai, Y., Luo, H., Yuan, T., Huang, H., Yao, B. (2011). An acid and highly thermostable xylanase from *Phialophora* sp. G5. *Applied Microbiology and Biotechnology, 89*(6), 1851-1858.

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., & Picataggio, S. (1995). Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic *Zymomonas mobilis*. *Science, 267*(5195), 240-243.

Zhao, J., Shi, P., Bai, Y., Huang, H., Luo, H., Zhang, H., Yao, B. (2012). A Thermophilic Cellulase Complex from *Phialophora* sp. G5 Showing High Capacity in Cellulose Hydrolysis. *Applied Biochemistry and Biotechnology, 166*(4), 952-60.

Łaźniewska, J., Macioszek, V. K., & Kononowicz, A. K. (2012). Plant-fungus interface: The role of surface structures in plant resistance and susceptibility to pathogenic fungi. *Physiological and Molecular Plant Pathology, 78*, 24-30.