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ACIDO-THERMOTOLERANT FUNGI FROM BOILING SPRINGS LAKE, LVNP: POTENTIAL FOR LIGNOCELLULOSIC BIOFUELS

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ABSTRACT

Acidic geothermal environments such as those in Lassen Volcanic National Park (LVNP) may provide novel organisms and enzymes for conversion of plant lignocellulose into ethanol, a process that typically requires hot and acidic pre-treatment conditions to hydrolyze cell wall polysaccharides to fermentable sugars. We evaluated seven Ascomycete fungi associated with LVNP's Boiling Springs Lake (BSL) for utilization of lignocellulose material. We screened the fungi for growth pH and temperature optima, and for growth on purified or natural plant cell wall components. We also examined potential lignin degradation using a (per)oxidase assay, and screened for the presence of potential (hemi)cellulose degradation genes with PCR. Growth analysis showed *Acidomyces* and *Ochroconis* grew best at 35-45 °C and pH <4, and grew up to 48-53 °C. In contrast, *Aspergillus*, *Paecilomyces* and *Penicillium* preferred cooler temperatures for acidic media (25-35 °C), but grew up to 48 °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 and xylan, as well as pine and incense cedar needles, for growth at 40 °C and pH 2. *Acidomyces* and *Ochroconis* showed extracellular (per)oxidase activity at 40 °C and pH 2, and PCR screening showed *Acidomyces*, *Paecilomyces*, and *Ochroconis* contain orthologs to known fungal lignocellulose degradation genes, including glucanases and xylanases. We conclude that the BSL-adapted taxa *Acidomyces*, *Ochroconis* and *Paecilomyces* may be promising sources of enzymes that combine heat- and acid-tolerance, potentially valuable in streamlining the pre-treatment of lignocellulosic biofuels.

Keywords: fungi, ascomycetes, extremophiles, cellulosic biofuels, peroxidases, glucanases, xylanases, biotechnology, extremophiles

INTRODUCTION

Naturally-occurring acidic, geothermal environments such as those in Lassen Volcanic National Park (LVNP) may harbor novel extremophilic organisms and enzymes useful for industrial processing. One important application where tolerance of high temperatures and low pH in particular are needed is production of cellulosic biofuels. Unlike ethanol derived from high sugar-containing crops like sugarcane and corn kernels (Zhang et al. 1995), biofuels based on lignocellulosic biomass use a greater percentage of the whole plant material from non-edible sources like switch grass, sugar cane bagasse, corn stover, wheat straw and rice straw, (Carroll and Somerville 2009; Koçar and Civaş 2013), thus potentially reducing greenhouse gas emissions without interrupting food supply (Stichnothe and Azapagic 2009) and improving regional energy security (Parish et al. 2013). However, converting plant lignocellulose to fermentable sugars requires pre-treatment to fragment polymeric cell wall materials and release sugars, followed by fermentation of the sugars by various microbes (Alvira et al. 2010; Bhatia et al. 2012). Pre-treatment is the most commonly cited limitation to the widespread adoption of lignocellulosic biofuels (Himmel et al. 2007; Howard et al. 2003; Kumar et al. 2009; Parish et al. 2013). Two common pre-treatment schemes are commonly used. *Separate Hydrolysis and Fermentation (SHF)* physically separates fermentation from the hydrolysis of plant biomass (Tao et al. 2011), utilizing a harsh pre-treatment step of acid hydrolysis (pH <1) at 150-200 °C that quickly (10-15 min) degrades the lignocellulosic materials. This step is then 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. However, the heating is energy-intensive, the chemical scrubbing has waste/pollution issues, and the cellulase enzymes are expensive (Chen et al. 2012; Foust et al. 2009; Pienkos and Zhang 2009). In contrast, the *Simultaneous Saccharification and Co-Fermentation (SSCF)* uses co-cultures or genetically-modified single cultures of microbes to hydrolyze and ferment in the same vessel (Amore and Faraco 2012). In SSCF, consolidated bioprocessing is performed at 28-37 °C and at pH conditions more suitable to commonly-used fermenting organisms within the hydrolysis environment (Ali et al. 2012; Carere et al. 2008; Hasunuma et al. 2013; Lynd et al. 2005). However, due to mild temperature and pH ranges, this process is considerably slower (100+ hrs) (Olson et al. 2012). Attempts to reconcile the strengths and weakness of the two approaches by

incorporating microbes and enzymes into the physiochemical pre-treatment stages prior to fermentation have had limited success, as reviewed by Tian et al. (2009).

Genetic and biochemical studies have long confirmed that fungi, which have evolved to degrade almost any plant material (Łaźniewska et al. 2012; Martínez et al. 2005), are a good source of secreted cellulases, xylanases, and peroxidase/laccases (Berka et al. 2011; Cruz Ramírez et al. 2012; Kellner et al. 2009; Kellner and Vandenbol 2010; Shrestha et al. 2011; van den Brink and de Vries 2011). Although novel fungi and their enzymes are of great potential for biofuels and biotechnology (Solomon et al. 2016), many of them cannot survive the hot acid required for a biological/chemical pre-treatment (Krogh et al. 2004; Li et al. 2013). To pair the biological and enzymatic processes with the harsher physical and chemical processes requires enzymes that function under hot and extreme pH conditions. Unsurprisingly, the sources for many of these enzymes come from organisms that themselves are considered extreme-adapted or extremotolerant (Bai et al. 2010; Bhalla et al. 2013; Blumer-Schuette et al. 2013; Kataeva et al. 2013; Luo et al. 2008; Turner et al. 2007; Wijma et al. 2013). Boiling Springs Lake (BSL) is an oligotrophic, pH 2.2, 52 °C, hot spring in Lassen Volcanic National Park, CA, that was a NSF-funded Microbial Observatory from 2009-2015. Although BSL's pH resembles other studied hot springs at Yellowstone National Park or acidic mine drainage sites like the Rio Tinto and Iron Mountain Mine (Amaral Zettler et al. 2003; Baker et al. 2004; Redman et al. 1999), BSL has uniquely low concentrations of dissolved minerals and metals, making it a geochemically unique environment. In addition, this high-elevation site receives a substantial amount of winter snowfall, causing lake surface and shoreline temperatures to frequently drop into the mid 40 °C range during winter storms (Siering et al. 2013), allowing for greater diversity of acid-adapted microeukaryotes. BSL's primary production is extremely low (< 10 μg C L^{-1} d^{-1}), and heterotrophic production dominates, most of which is fueled from allochthonous plant litter from the surrounding coniferous forest (Wolfe et al. 2014). At pH <3 and higher temperatures >40 °C, abiotic thermochemical reactions can begin the plant litter degradation process, but BSL conditions are too mild for significant abiotic glucose release alone (Pedersen et al. 2011), and extreme-adapted prokaryotes and fungi both likely contribute to lignocellulosic breakdown. Evidence for this includes the presence of genes coding known cellulose-, xylan (hemicellulase)- and lignin (aromatic carboxylic acid)-degrading enzymes in BSL microbial community DNA

using GeoChip 3.0 on two separate years (Siering et al. 2013), as well as growth response to inputs of fresh plant materials in *ex-situ* incubation experiments (Wolfe et al. 2014).

Our laboratory previously isolated and characterized seven Ascomycete fungal taxa from BSL water and surrounding thermal features. All grew at low pH (2-5) on easily-utilizable media such as malt agar or potato dextrose agar (PDA) between 28-37 ^{o}C. Fungi were genotyped and were classified into two sets by their growth preferences. The first set, consisting of *Penicillium,* *Aspergillus*, and *Cladosporium,* represents cosmopolitan taxa known to grow over a wide range of conditions and substrates, but typically thrive at mesophilic conditions (Gross and Robbins 2000). These fast-growing fungi were likely isolated from spores that came into the BSL system via wind and/or forest detritus, but they do not appear adapted to growth year-round in the lake's conditions. However, similar taxa produce many characterized lignocellulose-degrading enzymes, include 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*. These share genetic and morphological similarities to published extreme-adapted fungi (Baker et al. 2004; Gross and Robbins 2000; Hujslová et al. 2010; Selbmann et al. 2008; Yamazaki et al. 2010; Yarita et al. 2010). Prior reports have identified and characterized several degradation enzymes from this group, including acid-stable mannase, xylanase, and cellulase from *Phialophora sp*. (Zhang et al. 2011; Zhao et al. 2012) and *Paecilomyces* (Kluczek-Turpeinen et al. 2005; Tribak et al. 2002; Yang et al. 2006). The closely-related extreme-adapted *Acidomyces richmondensis* genome at JGI's MycoCosm database (Grigoriev et al. 2012; Mosier et al. 2016) predicts at least fourteen distinct cellulase enzymes.

Since BSL's environment is a mild version of hot, acid pre-treatment conditions and the BSL fungi are found within that environment, we hypothesized that that BSL-adapted fungi might harbor novel enzymes to degrade woody material under those conditions. To test this idea, we characterized their growth over elevated temperatures and low pH on different lignocellulose substrates, and screened for activity of lignocellulosic enzymes and their potential genes.

MATERIALS & METHODS

Fungal Isolates

Isolation

We previously isolated seven fungi from soil and plant material collected around the western periphery of BSL during the fall and summer months of 2008-2010. The soils and plant materials were agitated with sterile water and then a dilution series was placed onto Petri dishes or into multi-well plates containing neutral or acidic fungal media and incubated at 40 °C. Fungi isolated in this manner were maintained on potato dextrose agar (PDA) at 21 ^{o}C.

Identification

Fungal genera were identified on the basis of colony, mycelial, and spore morphology, and color (Gross and Robbins 2000), and by sequencing of the rRNA internal transcribed spacer region (ITS). DNA was extracted from scrapings of mycelial grown on PDA plates into SDS lysis buffer, then separated with 1:1 chloroform, and finished with ethanol precipitation. PCR of the rRNA ITS was done using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). PCR utilized GoTaq Green polymerase master mix (Promega Corp, Madison, WI), ~100 ng of fungal DNA, and 0.5 μM of each primer in a total volume of 20 μL. PCR reactions utilized 30 cycles with 30 sec denaturation (95 °C), 30 sec anneal (54 °C), and 1 min extension (72 °C). The resulting PCR products were sequenced by standard dideoxy sequencing (Univ. Washington High Throughput Genomics, Seattle, WA).

Culturing

Fungi were maintained on PDA (BD, Franklin Lakes, NJ). Two acid-tolerance selection media were developed: 0.4% w/v Malt Extract (ME; BD) and 0.2% w/v Cellobiose/0.1% Yeast Nitrogen Base (CYNB), both made with distilled water, 2% w/v agar, and acidified to pH 2.2 by addition of H_2SO_4 after autoclaving, matching the ~10 mM H_2SO_4 concentration of BSL (Siering et al. 2013). Finally, a basal medium containing 2% w/v agar and 0.1% w/v Yeast Nitrogen Base (YNB; BD) was used to support growth on various sugars, lignocellulose compounds and chopped plant material. Microcrystalline cellulose (Sigma-Aldrich, St. Louis, MO); xylan from Beechwood (Sigma-Aldrich); and cellobiose (BD) provided polysaccharide substrates. For utilization of plant materials, fallen dried leaves were collected from the ground around BSL;

wheat straw and rice straw were gifts from a local feed store, and rice hulls were provided by Dr. L. Hanne, CSU Chico.

Growth Studies

Fungal growth temperature curves

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 and Sawers 2013; Mowat et al. 2007). We 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 measuring rate of fungal mycelial area increase (Reeslev and Kjoller 1995). The fungal plates were imaged and mycelial 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 condition was also used due to very limited *Aspergillus*, *Cladosporium*, *Penicillium*, and *Paecilomyces* mycelial mass (~1 mg, not shown) in hot and acidic liquid ME or CYNB media (pH 2.5) compared to comparable temperatures in neutral pH versions of ME and CYNB (pH 5).

Both liquid and solid media were used to determine the temperature optima of fungal isolates. Growth in liquid media was estimated by measuring mycelial mass after incubation in 10 mL of ME for seven days between 25-52 °C using a custom-built thermal gradient incubator rotating at 75 rpm (Wolfe et al. 2013). Vials of culture/media were filtered onto pre-weighed 5 µm Durapore membrane filters (EMD Millepore, Billerica, MA), 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. For estimates of growth on solid media, vegetative mycelial/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 thirteen days, the fungal plates were imaged and mycelial surface area was calculated to quantify growth ($cm^2\ d^{-1}$).

Thermotolerance of fungi

To combine the growth temperature tolerance data and further define the BSL fungi, mycelial/agar plugs taken from PDA plates grown at 37 °C (27 °C for *Phialophora* sp.) for four days were transferred to new PDA, Myco, or acidic-ME media and incubated at 50 °C for seven

187 days. These were then returned back to 37 °C (27 °C for *Phialophora* sp.) and monitored for
188 growth. A fungus that began re-growing after the return to 37 °C (27 °C) was considered
189 thermotolerant (Maheshwari et al. 2000); conversely, organisms that did not grow were
190 considered mesophilic (Redman et al. 1999).

191 *Oxygen requirements for growth*

192 Mycelial/agar plugs taken from PDA plates grown at 37 °C were transferred to new PDA and
193 ME plates, then incubated at 37 °C for seven days sealed inside either anaerobic (GasPak EZ
194 Anaerobe pouch; BD) or micro-aerobic [5-15% O_2] (GasPak EZ Campy Pouch; BD). Growth
195 rates were calculated as mycelial area (cm^2 d^{-1}) and compared to growth rates on the fully
196 aerobic ME plates at 37 °C.

197 *Initial screen for lignocellulytic activity*

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

203 *Single carbon source growth assay*

204 After wetting a fungal isolate on a ME plate with 1 mL distilled water, 20 µL of plate wash
205 was used to inoculate 10 mL of 0.1% w/v YNB, distilled water/H_2SO_4 at pH 2.2, and 0.1% w/v
206 polysaccharides (microcrystalline cellulose or xylan). Incubation took place for seven days at 37
207 °C. Utilization of a carbon source was determined qualitatively by presence or absence of fungal
208 mycelia.

209 *Plant material utilization growth assay*

210 For growth on cotton, 20 µL of *Acidomyces*, *Ochroconis* and *Paecilomyces* plate washes
211 were introduced into vials containing autoclaved and rinsed 100% cotton fiber balls (Hytop
212 brand) and 10 mL of sterile water, acidified sterile water, sterile water with 0.1% w/v YNB,
213 acidified sterile water with 0.1% w/v YNB, or CYNB media. Each was then incubated at 37 °C
214 with agitation for nine days. Utilization of the cotton was determined qualitatively by presence or
215 absence of fungal mycelia. For growth on whole plant tissues, mycelial/agar plugs taken from
216 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 mycelial area would indicate utilization. Growth rates were calculated to show the mycelial area (cm^2 d^{-1}) 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, we 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 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 (Baker and Adney 1987; Ghose 1987; van den Brink et al. 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 0-0.1 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, we used 2 assays based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to form a green precipitates (Chairattanamanokorn et al. 2006). Control fungi in these assays were white-rot Basidiomycete fungi *Collybia impudica* (syn. *Gymnopus* sp. K254) and *Mutinus caninus*, both a gift from Dr. H. 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 1.3 mM ABTS (Sigma-Aldrich) in 100 mM sodium acetate buffer (pH 5) was applied to one side of inoculated split plates, and then allowed to sit for 10 min before discarding the ABTS/buffer. Plates were allowed to incubate at room temperature for 1 hour to develop precipitate, and then photographed for documentation. A second assay used ABTS (250 mg L^{-1}) with $MnCl_2$ (100 mg L^{-1}) in solid agar medium (acidified to pH 2.5) to also screen for

248 peroxidase enzyme reactions during fungal growth at 40 °C (27 °C for *Phialophora*) over 6 days
249 (Chairattanamanokorn et al. 2006). Fungal cultures that formed green precipitate during growth
250 were considered positive for general peroxidase activity and production of black precipitate
251 specifically indicate manganese-peroxidases.

252 **PCR Screening for Enzyme Genes**

253 To further evaluate the ability of the fungi harboring novel acid- and/or heat-tolerant
254 enzymes, we used PCR primers targeting the conserved domains of fungal genes encoding
255 (hemi)cellulose-degrading enzymes (Kellner and Vandenbol 2010) using degenerate primers
256 (Integrated DNA Technologies; Coralville, IA) (**Table 1**). To screen for potential artefacts of
257 degenerate primers, a matching GH3 primer set with Inosine bases were ordered to compare
258 function. PCR utilized GoTaq Green polymerase master mix (Promega), ~100 ng of fungal
259 DNA, and 0.5 µM of each primer in a total volume of 20 µL. PCR conditions and sequencing
260 were the same as used for ITS-rRNA. PCR bands were excised from the agarose, subjected to
261 freeze/thaw, and re-suspended in TE buffer (pH 8) prior to sequencing. Sequence results were
262 queried via BLASTn and BLASTx at NCBI, or the Joint Genome Institute
263 (www.genome.jgi.doe.gov). Sequences for all detected LVNP fungal (hemi)cellulose-degrading
264 enzyme genes have been deposited in Genbank with accession numbers KX064260-KX062627,
265 except for *Ochroconis* Glc1, which showed evidence of contamination and gave only weak
266 matches.

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RESULTS

Fungal Isolates

Fungal morphological & genetic 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 1).** We confirmed each isolate using rRNA ITS PCR. Results were consistent with previous findings except the suspected *Cladosporium* (**Table 2**). The BSL *Aspergillus* sp. and *Acidomyces* sp. are similar to the annotated genomes (at NCBI and JGI, respectively), 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, Pezizomycotina; followed by Eurotiomycetes (*Aspergillus, Paecilomyces, Phialophora*, and *Penicillium*) or Dothideomycetes [*Ochroconis* and *Acidomyces,* a sister clade to *Tetrosphaeria* (Yamazaki et al. 2010)]. *Talaromyces* is the anamorph of *Penicillium*, and historically the genus names have been used interchangeably (Houbraken and Samson 2011). Mixed sequence fragments from two of the four *Cladosporium* ITS PCR products indicated contamination at time of DNA extraction.

Growth studies

Fungal growth temperature curves

Acidified liquid ME medium supported appreciable growth at warmer temperatures only for *Acidomyces* and *Ochroconis*. 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 due to likely contamination indicated by PCR results.

Acidomyces and *Ochroconis* both showed visible growth in the vials above 40 °C and measureable mycelial mass above 45 °C in pH 2.5 ME media (**Figure 2**). *Acidomyces* growth decreased <27 °C or >40 °C, but grew well within this range. *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 fungi showed a decrease in growth above 30 °C, although though they did produce measureable mycelial mass at the warmer temperatures (**Figure 3**). 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, daily increases in mycelial area were calculated over thirteen days. Similar to liquid media, *Acidomyces* and *Ochroconis* showed an optimum at 37 °C on ME media, or greater on the PDA medium (**Table 3**), while the remaining fungi showed growth on PDA up to 40 °C, but their optima were typically below 37 °C. *Aspergillus* often grew superficially across the maximal area in single day, so we only included the 40 °C rate for later comparisons. For the remaining fungi, only *Paecilomyces* had any growth above 40 °C, and only on acidic ME media. On either medium, *Cladosporium* growth was very limited above 37 °C, while *Phialophora* did not grow above 30 °C. Thus, the largest increases of mycelial density on solid ME medium correlated well with the liquid growth assay optima, further supporting the acid- and heat-tolerance of the adapted fungi. Due to visible differences in growth morphology (mycelial density) between the neutral and acidic media, growth rates calculated from radial measurements could not be extrapolated between media formulations. In later substrate utilization experiments, this issue was remedied by using a standardized basal medium (agar and YNB) and adjusting the pH before pouring the plates.

Thermotolerance of fungi

Attempts to grow the fungi at summertime BSL conditions at temperatures (52 °C) and pH ~2.2 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 50 °C to 37 °C on all three media, while *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 was thermotolerance on acidic (pH 2) ME medium (**Figure 4)**, as this scenario is likely to take place within a biofuel reactor vessel. Due to ongoing discrepancies in defining thermotolerance in

fungi, the remaining fungi could be also considered thermotolerant if they could grow above 40 °C in another assay (Mouchacca 2007). *Phialophora* obviously is not thermotolerant based on the previous growth assay. As noted, only *Aspergillus*, *Ochroconis* and *Paecilomyces* showed growth recovery on the pH 2 ME media.

Oxygen requirements for growth

We observed *Acidomyces* and *Ochroconis* growing up the entire water column of a closed 15 mL conical tube, suggesting a tolerance for low levels of O_2 characteristic of higher temperatures. However, there was no active growth in sealed anaerobic bags on PDA or acidic ME at 37 °C after seven days (not shown). Once the bags were opened to allow gas exchange, the fungi responded with near typical growth rates (**Table 4**) indicating a tolerance for anaerobisis. Further evaluating low-level O_2 tolerance using 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 under micro-aerobic conditions, and resumed typical growth rates once released from the micro-aerobic bags.

Carbon source growth tests

Initial screening all seven BSL fungal for growth on polymeric C source solid media showed strongest growth on acidic 0.1% w/v cellobiose when grown at 37 °C (27 °C for *Phialophora*). Cellobiose, the smallest cellulose dimeric unit, supported growth of all seven fungi under neutral and acidic conditions (not shown) and subsequently became the C source for the acidic control media, and for liquid and solid preparations (CYNB). We observed little difference in fungal growth on the YNB-only (no C source) plates compared to the xylan and cellulose plates (**Figure 5**), 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 into constituents (including glucose; data not shown). To avoid mis-interpretation caused by the unintended growth on Gelrite, we examined fungal growth in liquid media, where growth was determined only qualitatively (presence or absence of fungal mycelia) due to difficulty in separating the fungal mycelia from the remaining cellulose and xylan. The three fungi tested (*Acidomyces, Ochroconis* and *Paecilomyces*) all grew well in the acidic beechwood xylan media, while only *Acidomyces* showed growth in the cellulose media (**Figure 6**). Of the three fungi, only *Acidomyces* grew on cotton ball fibers (**Figure 7**).

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 (**Figure 8**). Additionally, *Acidomyces* was able to utilize chopped wheat straw for growth (**Figure 8**). Under these test conditions *Ochroconis* and *Aspergillus* did not show growth on agricultural byproducts (rice hulls or wheat straw) but were positive for growth on the substrates native to BSL (**Figure 8**). When growth occurred, 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.

Enzyme studies

Cellulase activity based on 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 of 0.03 mg (not shown). In addition, filter paper mass loss was not significantly different from zero. However, both assays, although shown to work in microwell plates (van den Brink et al. 2012), had difficulties, and 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 and Ho 2011).

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. These exhibited limited diffusion compared to the positive control basidiomycete *Collybia impudica* (**Figure 9a).** Neither *Paecilomyces* nor *Phialophora* showed any indication of ABTS oxidation**.** Similar results were displayed by *Acidomyces*, *Collybia impudica* and another control basidiomycete, *Mutinus caninus,* when screened for growth on acidic CYNB medium containing ABTS and $MnCl_2$ (**Figure 9b**), although none of the plates displayed 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 this taxon, but we did not observe it on the acidic CYNB plates; this may indicate another redox reaction happening at higher temperatures compared to the room temperature ABTS assay. Limited description of red pigment production is available (Yarita et al. 2007). *Phialophora* showed increased mycelial spread but did not display oxidation of ABTS 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 growth prohibition by either ABTS or $MnCl_2$ in the medium.

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 (Kellner and Vandenbol 2010) produced fragments within the predicted ranges of 200-500 bp. Of the ten PCR products, seven returned quality reads, with the subsequent translational BLAST queries returning matching or orthologous predications for fungal cellulase, xylanase and cellobiohydrolase enzymes **(Table 7)**.

DISCUSSION

Adaptations of BSL fungi to hot and acidic conditions

To establish their potential usage in the pre-treatment of lignocellulose materials by incorporation into acidic and hot pre-treatment conditions (Barnard et al. 2010), we needed to first show the BSL fungi grew or at least tolerated extreme conditions similar to biofuels pre-treatment. Based on their ability to thrive at various temperatures and pH values, we separated the seven BSL fungi into three groups: 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*). 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. richmondensis*) are found in extremely acid conditions (pH <1) in Iron Mountain Mine in northern California (Baker et al. 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). Similar growth is described in the closely-related acid- and thermal-tolerant taxon *Tetrosphaeria* isolated from Japanese hot springs (Yamazaki et al. 2010). The *Ochroconis* sp. is also similar to a taxon isolated from Japanese hot springs, *Ochroconis calidifluminalis,* that is acid-tolerant with optimal growth at 42 °C, and produces red pigment after multiple plate transfers (Yarita et al. 2010). The *Paecilomyces* genus is quite large at this point, with many isolates showing some thermal and/or acidic tolerance (Gross and 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 pre-treatment. Both *Paecilomyces* and *Ochroconis* returned to typical growth patterns after halting during five-day incubation at 50 °C. This is a useful trait if live fungi are used throughout the pre-treatment process, where temperatures may be higher than optimal. *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, useful attributes during the pre-treatment process where oxygenation can

433 be limited by solids loading and liquid volumes fluctuation. Further, as the typical dilute acid
434 pre-treatment process uses varying water flow regimes to move material through the treatment
435 infrastructure, it is helpful that *Acidomyces* and *Ochroconis* grow equally in static and dynamic
436 liquid environments, often aggregating small particulates. In contrast, use of *Paecilomyces*,
437 which had reduced growth under dynamic liquid conditions, might be limited to static or slow-
438 moving configurations.

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

443 The 'cosmopolitan' taxa *Aspergillus*, *Penicillium* and *Cladosporium* each grew at >40 °C but
444 not with concurrent pH <3. In particular, the *Aspergillus* showed some growth at 50 °C, the
445 highest of all seven fungi, but it was growing on standard PDA medium with pH ~5. It appears
446 that growth of these three fungi species requires a tradeoff of heat tolerance for acid tolerance
447 and vice-versa under our challenging conditions. As the most widely studied fungi of our seven,
448 relatives of these three fungi are found in a wide variety of conditions, but typically grow only at
449 <40 °C in conditions >pH 4 (Gao et al. 2008; Gross and Robbins 2000; Khalid et al. 2006). The
450 use of these fungi in biofuels is less focused on concurrent hot acid pre-treatment processes, and
451 more for free enzyme production that can used after chemical/mechanical steps (Bhalla et al.
452 2013).

453 **Fungal utilization of lignocellulosic material at high temperature and low pH**

454 With the BSL shoreline and side pool environments providing the hot and acidic conditions
455 and the frequent inputs of plant materials, it was not a surprise that several of the fungi could
456 utilize individual lignocellulosic components (cellobiose, cellulose and xylan) or plant materials
457 for growth in laboratory conditions. We selected plant materials found in the BSL environment
458 (incense cedar scales and pine needles), attempting to mimic possible utilization/degradation in
459 the natural system, but which also have higher amount of lignins, pectins, and other compounds
460 that resist microbial degradation (Song et al. 2009). Also tested were agricultural byproducts
461 typical of crops that carry potential for use as biofuel feedstock (e.g. rice hulls, rice straw and
462 wheat straw). These are cheap and have higher ratios of (hemi)celluloses to lignin (Jahromi and
463 Ho 2011; Perlack and 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, we did not screen for growth on isolated lignin compounds (Martínez et al. 2005).

The environmental conditions at BSL (pH 2; 52 °C) likely contribute to chemical hydrolysis of plant materials, resulting in soluble xylose and glucose oligomers diffusing from submerged plant litter. Although we were concerned that the acidified media would chemically hydrolyze cellulose, 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 et al. 2010), it would take about twelve days to chemically hydrolyze half the remaining xylan in the acidic media formulation at 40 °C. To ensure that growth on these substrates resulted from actual fungal degradation of polymeric substrates, not use of abiotically-produced monomers, plant materials were rinsed twice after autoclave sterilization. This minimized solubilized glucose, cellobiose, xylose or arabinose, as well as potential inhibitors such as acetate or furfural.

Using fungal growth as a proxy for potential lignocellulose degradation (Shrestha et al. 2011), we categorize the fungi based on their utilization of diverse lignocellulosic materials under warm and acidic conditions (**Table 8**). The three 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 mycelial 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. *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 versatile *Aspergillus* grew on cellobiose, incense cedar, and pine needles at pH 2 and 40 °C. Thus, the majority of BSL fungi can grow *in vitro* on lignocellulosic materials found in their native environment, and a few also demonstrate the versatility to grow on crop residues typically used for lignocellulosic biofuels.

Extracellular fungal enzymes useful for lignocellulose degradation

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 et al. 2012; Hoegger et al. 2006). The resulting oxidation of ABTS at room temperature on acidic media and growth on the ABTS/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 et al. 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). Our *Paecilomyces*, *Phialophora* and *Aspergillus* isolates did not show ABTS oxidation under either testing condition, although *Aspergillus niger* is known to harbor peroxidase enzymes under non-acidic conditions (Ramos et al. 2011).

Although the reducing sugar/DNS assay failed to demonstrate evidence of extracellular cellulase activity from *Acidomyces*, *Ochroconis*, and *Paecilomyces* culture supernatants, our PCR-based screening strategy using primers designed to detect conserved regions of known fungal lignocellulose degradation enzymes (Kellner and Vandenbol 2010; Lang and Orgogozo 2011) indicated the presence of potential enzymes. Although we screened genomic DNA, PCR was successful in forming products that represented 3-4 (hemi)cellulose-degrading genes per fungus. Additionally, the positive control fungal cultures also formed products from genomic DNA that agreed with this experimental product (H. Kellner, personal communication). The sequenced PCR products aligned well within the annotated protein sequences of known acid- and thermo-tolerant *Acidomyces richmondensis* (JGI BLASTx) and thermotolerant *Aspergillus fumigatus* (NCBI BLASTx). Even with the lack of matching annotations, our three other sufficiently-performing acid-tolerant fungi (*Ochroconis, Paecilomyces* and *Phialophora*) still maintain potential for biofuels pre-treatment, based on their products typically aligning with orthologous enzymes from thermotolerant fungi (**Table 7**). Although detection of a gene does not imply a functional enzyme, we note that this preliminary genetic screen is likely an underestimate of potential degradation machinery, as it targeted only known, conserved genes. Growth on cellulose requires the cooperative participation of many endo- and exogluconases, and notably only in *Acidomyces*, the sole taxon to grow on cotton, did we detect Glc2, GH6, and

526 GH67 together. We did not try to characterize hemicellulose-degrading xylanases, as the
527 xylose/arabinose oligomers are easily hydrolyzed under the conditions that target the tougher
528 cellulose or lignin components (Hu et al. 2010; Pu et al. 2013; Shen et al. 2011).

IMPLICATIONS

Lignocellulose utilization and ABTS oxidation assays results, combined with PCR-based detection of relevant genes, suggest that 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. These fungi are worthy candidates of enzymes for concurrent hot acid and biological pre-treatment process in the production of lignocellulose biofuels. Enzyme purification and characterization might lead to novel enzymes, or help improve existing enzymes (Gostinčar and Turk 2012; Graham et al. 2011; Wijma et al. 2013). In addition, utilizing the melanization and ABTS 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). Lassen Volcanic National Park's diverse geothermal habitats likely harbor other novel fungal enzymes of use for industry or biotechnology.

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824

825

FIGURE CAPTIONS

Figure 1. Images and micrographs of the seven BSL fungi. (A) Growth on solid media shows pigmentation and mycelial density are greater on PDA (left) than on acidic ME medium (right). Growth for 7 days at 37 °C (27 °C for *Phialophora*). (B) Lactophenol-blue stained fungal mycelia with hyphae connections, conidiophores and conidia numbers (if visible) for each of the seven fungi grown on PDA media (Phase Contrast, 400X).

Figure 2: Growth vs. temperature in liquid medium (ME, pH 2.5) for *Acidomyces* (A) and *Ochroconis* (B). Shown are total mycelial mass produced over 7 days from an inoculum of ca. 800 spores, with shaking at 75 rpm. Data points are averages, and error bars present the range, of replicates. Note slightly different temperature settings.

Figure 3. Growth vs. temperature in liquid medium (CYNB, pH 5) for *Aspergillus*, *Paecilomyces* and *Penicillium*. Other conditions were the same as figure 2, but only single treatments were performed.

Figure 4. Images of fungi on acidic ME plate media during thermotolerance assay. Top rows were photographed after 5 days at 50 °C. Bottom rows 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 after the shift to 37 °C, with mycelia radiating from two separate agar plugs (red arrows).

Figure 5. Images of BSL fungi 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 is evident on all media formulations.

Figure 6. 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, as indicated by black, brown or tan amorphous mycelia (red arrows).

Figure 7. 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 fungal mycelia can be seen on the top portion of the cotton material as dark brown spots (red arrows), most obvious in the cotton+H_2O vial. The CYNB vial is positive for fungal growth with thick dark

857 mycelia (red arrow). (B) Samples of the cotton were removed to verify fungal presence
858 (100X), with three of the four vials showing dark clumped mycelia (red arrows)
859 amongst the thick cotton fibers. The bottom image was from the uninoculated control
860 vial, showing no mycelia, only cotton filaments.
861 Figure 8. Fungal growth on acidified plant material plates. Each plate was inoculated with a
862 mycelia/agar plug and incubated for 13 days at 40 °C (27 °C for *Phialophora*).
863 Figure 9. Peroxidase assay images. (A): Fungi on acidic CYNB plates imaged 60 minutes after
864 challenge with ABTS solution at room temperature. Each plate was previously
865 incubated at 40 °C, 27 °C and RT; as marked for 4 days. BSL specific fungi were
866 challenged on the right half of the split plates, with diffusing green precipitate
867 indicating peroxidase enzyme function (red arrows). Clockwise from top left:
868 *Acidomyces*, *Ochroconis*, *Collybia impudica* (positive control, no split plate),
869 *Paecilomyces*, and *Phialophora*. (B): Fungal growth on ABTS/$MnCl_2$ peroxidase
870 screening plates. Fungi positive for peroxidase enzymes formed diffuse green around
871 fungal mycelia after 6 days at 40 °C or 27 °C (as marked by red arrows). Each plate is
872 representative of duplicate experiments. Clockwise from top left: *Acidomyces*,
873 *Paecilomyces*, *Collybia impudica* and *Mutinus caninus* (positive controls),
874 *Phialophora*, and *Ochroconis.*

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886 **TABLES**

887 Table 1. List of degenerate PCR primers used to screen for glucoside hydrolase (GH) genes
888 within the fungal genomic DNA. Modified from Kellner & Vandanpol (2010).

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

889 * primer sets ordered with and without N base subsitution for Inosine bases.

890 Table 2. Characterisitics of BSL fungi. For morphology, each fugus grown on PDA media for 7 days at 37 °C (27 °C for
891 *Phialophora*). For genetic identity, hits returned from nucleotide (BLASTn) search at blast.ncbi.nih.nlm.gov and *Acidomyces*
892 nucleotide search (BLAST) at genome.jgi.gov. All results returned E-values of 0.0. ITS = internal transcribed spacer.

Taxon	**Pigmentation**	**Condiophores**	**Conidia/Spores**	**Top Match Returned (% identity)**	**Accession**	**Reference**
Acidomyces	Black deep-green	Not observed	Not observed	*Acidomyces richmondensis* v1.0 (from isolate) Aciri1_iso_Assembly Scaffolds_Repeatmasked (99%)	JQ172744.1	Selbmann 2008, Baker 2009
Ochroconis	Pale brown	Modified hyphae	Single, bi-lobed with septate	*Ochroconis gallopava* genes for small subunit rRNA, ITS1 (99%)	AB125284.4	Yarita 2010
Paecilomyces	Light tan	Single	Multiple in chains, eliptical	*Paecilomyces* sp. ALAS-1; ITS 1 (99%)	HM626196.1	Samson et al. 2009
Phialophora	White to pale pink	Not observed	Single, cylinderical	*Phialophora* sp. CGMCC 3329, partial seq; 1 (99%)	GU082377.1	Zhao 2012
Aspergillus	Grey-green	Single vesicule with multiple phialpaes vestigule	Multiples in chains, rounded	*Aspergillus fumigatus* strain SGE57; ITS 1 (99%)	JQ776545.1	Gross 2000
Penicillium	White to off-white	Branching with multiple phialpaes	Multiples in chains, eliptical	*Talaromyces* sp. OUCMBI101202 ITS 1 (96%)	JQ411391.1	Gross 2000
Cladosporium	White	Branching with single phialpaes	Multiples in chains, eliptical	*Candida parapsilosis* strain EN22 18S rRNA gene (98%)	FJ809941.1	Bensch et al. 2012

893 Table 3: Fungal growth rates versus temperature and media. Rates of growth based on increase
894 of fungal mycelia area with bold values representing the optimal temperatures for each fungi.

		Growth (cm^2 d^{-1})			
Taxon	**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

895

Table 4: Fungal growth rates post-anaerobic conditions compared to rates under standard conditions (typical for that medium) at 37 °C (27 °C for *Phialophora*).

		Growth (cm^2 d^{-1})	
Taxon	**Media**	**Post-anaerobic**	**Typical**
Acidomyces	ME	0.88	1.62
	PDA	0.69	1.03
Aspergillus	ME	2.61	0.84
	PDA	8.07	8.03
Cladosporium	ME	0.28	0.19
	PDA	6.97	1.69
Ochroconis	ME	1.70	2.54
	PDA	3.63	3.66
Paecilomyces	ME	2.38	2.66
	PDA	1.61	1.75
Penicillium	ME	0.01	0.78
	PDA	2.73	3.19
Phialophora	ME	0.88	0.86
	PDA	0.61	0.75

901 Table 5: Fungal growth rates under micro-aerobic conditions compared to rates under standard
902 conditions (Typical) both at 37 °C (27 °C for *Phialophora*).

		Growth (cm^2 d^{-1})	
Taxon	**Media**	**Micro-aerobic**	**Typical**
Acidomyces	ME	0.87	1.62
	PDA	0.86	1.03
Aspergillus	ME	0.01	0.84
	PDA	2.08	8.03
Cladosporium	ME	0.08	0.19
	PDA	0.64	1.69
Ochroconis	ME	0.98	2.54
	PDA	1.49	3.66
Paecilomyces	ME	0.80	2.66
	PDA	0.46	1.75
Penicillium	ME	0.47	0.78
	PDA	1.06	3.19
Phialophora	ME	0.77	0.86
	PDA	0.89	0.75

903

904

905 Table 6: Growth rates for fungi at 40 °C (27 °C for *Phialophora*) on each media prepartion based
906 on increased mycelial area incearse over 13 days. Due to non-circular growth patterns
907 *Aspergillus* and *Paecilomyces,* rates were not calculated when growth was present, noted by (+).

	Growth rate (cm^2 d^{-1})			
Taxon	**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

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910 Table 7: Top matches of known genes (using NCBI BLASTx) for PCR products of each functional primer set.

Taxon	Primer target: Top Hit returned	E-value	Max Ident.	Accession
Acidomyces	Glc1 (GH3): glycoside hydrolase family 3 protein [*Acidomyces richmondensis*]	1E-34	100%	KXL41872.1
	GH6: glycoside hydrolase family 6 protein [*Acidomyces richmondensis*]	8E-74	94%	KXL46768.1
	GH67: glycoside hydrolase family 67 protein [*Acidomyces richmondensis*]	9E-109	99%	KXL45919.1
Ochroconis	Glc1 (GH3): β-glucosidase [*Diaporthe ampelina*]	1E-15	90%	KKY29838.1
Paecilomyces	Glc1 (GH3): β-glucosidase [*Byssochlamys spectabilis* No. 5]	5E-37	96%	GAD99276.1
	GH67: glycoside hydrolase family 67 [uncultured fungus]	2E-97	83%	ADJ38273.1
Phialophora	Glc1 (GH3): β-glucosidase [*Ophiostoma piceae* UAMH 11346]	1E-09	46%	EPE10815.1
	GH67: glycoside hydrolase family 67 [uncultured fungus]	6E-40	48%	ADJ38275.1
Aspergillus	GH67: α-glucuronidase [*Aspergillus fumigatus* var. RP-2014]	5E-108	99%	KEY93400.1
Collybia impudica	Glc1 (GH3): β-glucosidase [*Penicillium occitanis*]	4E-20	76%	ABS71124.1
	GH5: endo-1,4-β-mannosidase, putative [*Talaromyces stipitatus* ATCC 10500]	9E-52	89%	XP_002480621.1
	GH6: cellobiohydrolase II [*Talaromyces cellulolyticus* CF-2612]	2E-64	77%	BAA74458.1
	GH67: glycoside hydrolase family 67 protein [*Acidomyces richmondensis*]	5E-88	78%	KXL45919.1
Mutinus canidus	Glc1 (GH3): glycoside hydrolase family 3 [*Spherobolus stellatus* SS14]	1E-21	74%	KIJ48071.1

911 Table 8. Summary of lignocellulose material utilization by the BSL fungi at pH 2 and 40 °C or
912 37 °C (except *Phialophora* at 27 °C). C sources: CB = cellobiose; C = cellulose; X = xylan; PN =
913 pine needles; IC = incense cedear; RH = rice hulls RS = rice straw; WS = wheat straw. Symbols:
914 confirmed growth (+), lack of growth (-); blank = not tested.

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

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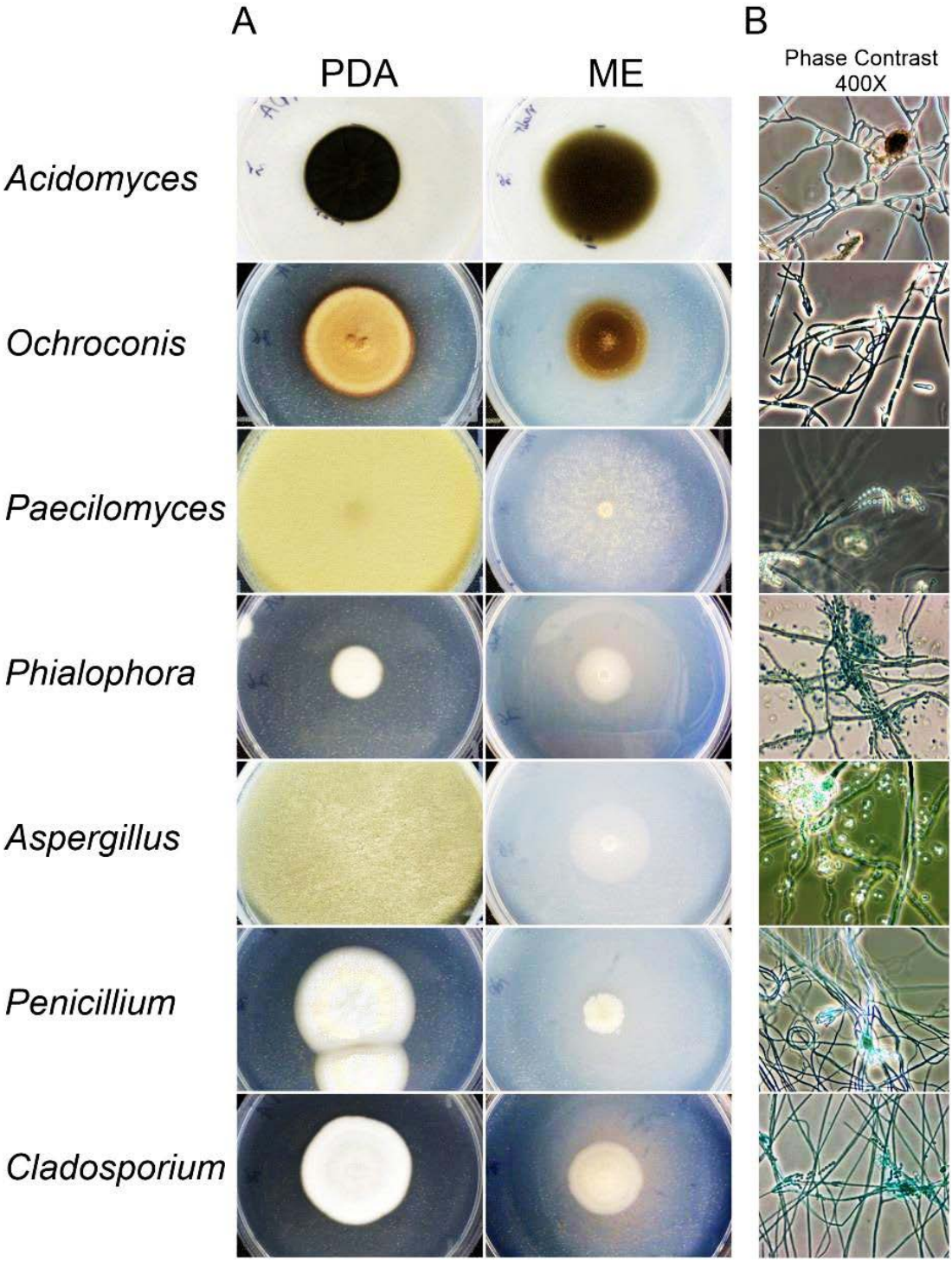
916 **FIGURES**

917



918

919 Figure 1

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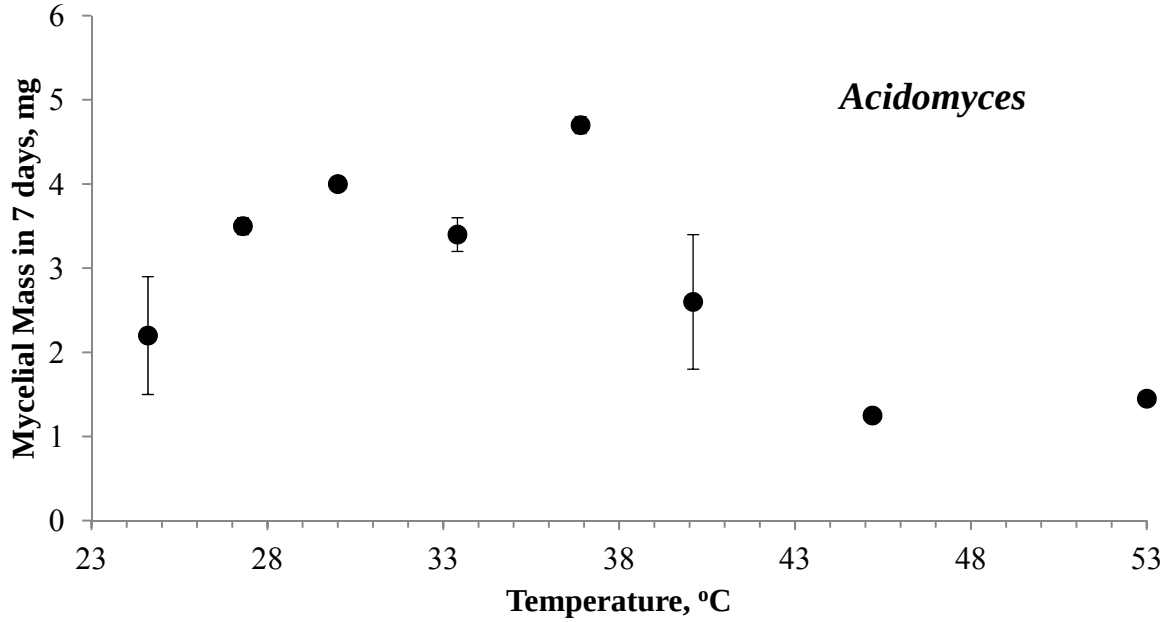
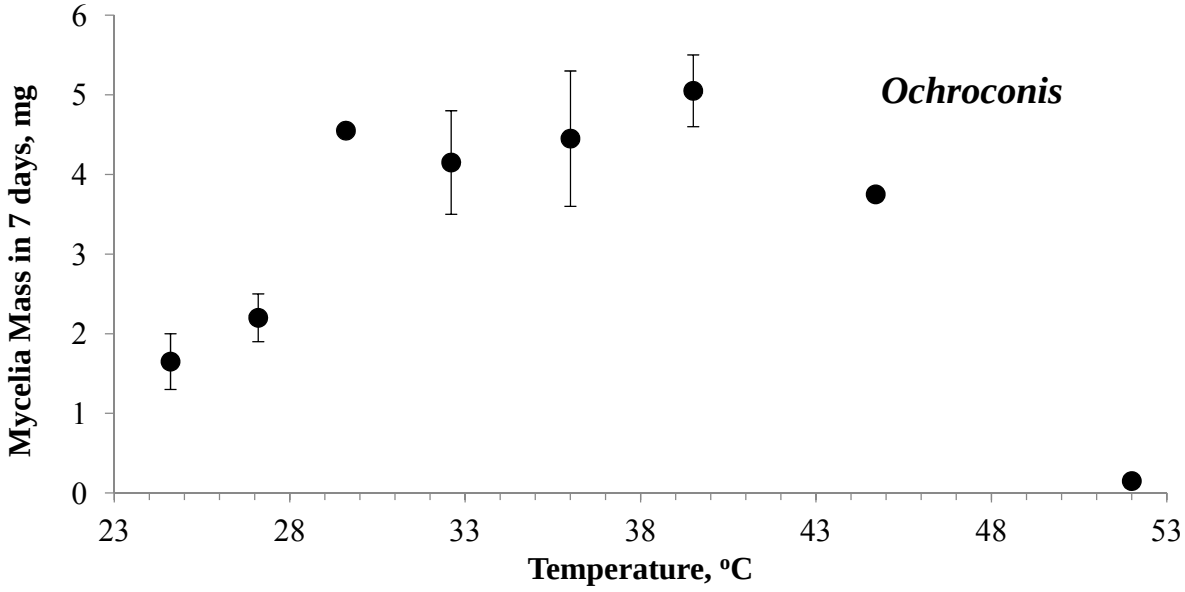
920 **A**



921

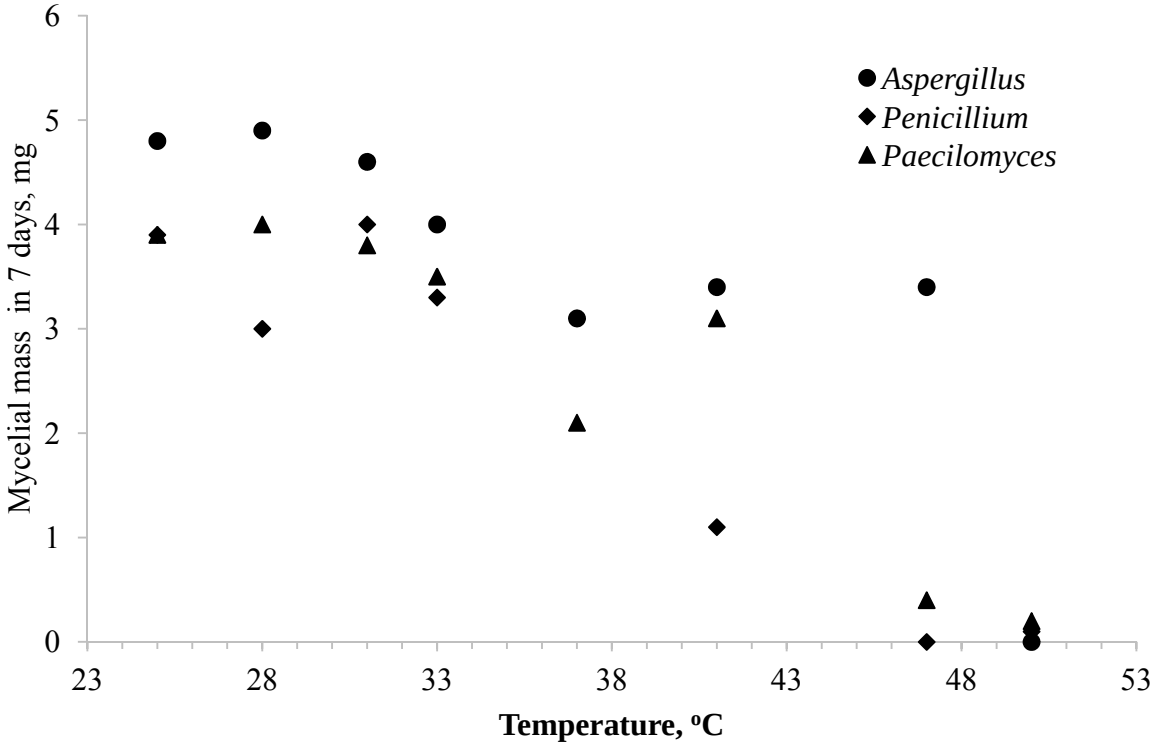
922 **B**



923

924 Figure 2

925



926

927 Figure 3

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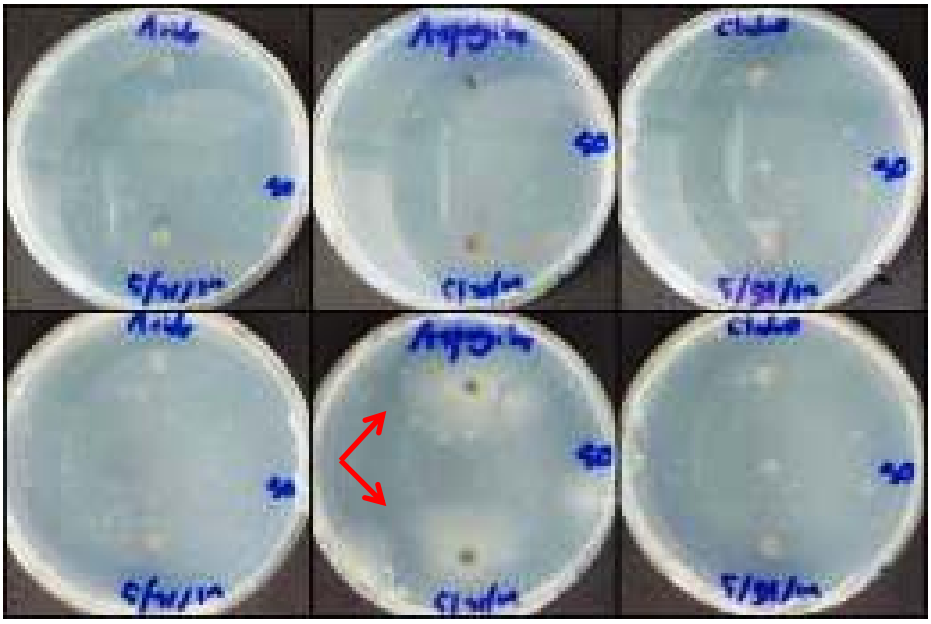
Acidomyces *Aspergillus* *Cladosporium*

50 °C
(no growth)

move to
37 °C



Paecilomyces *Penicillium* *Ochroconis* *Phialophora*

50 °C
(no growth)

move to
37 °C

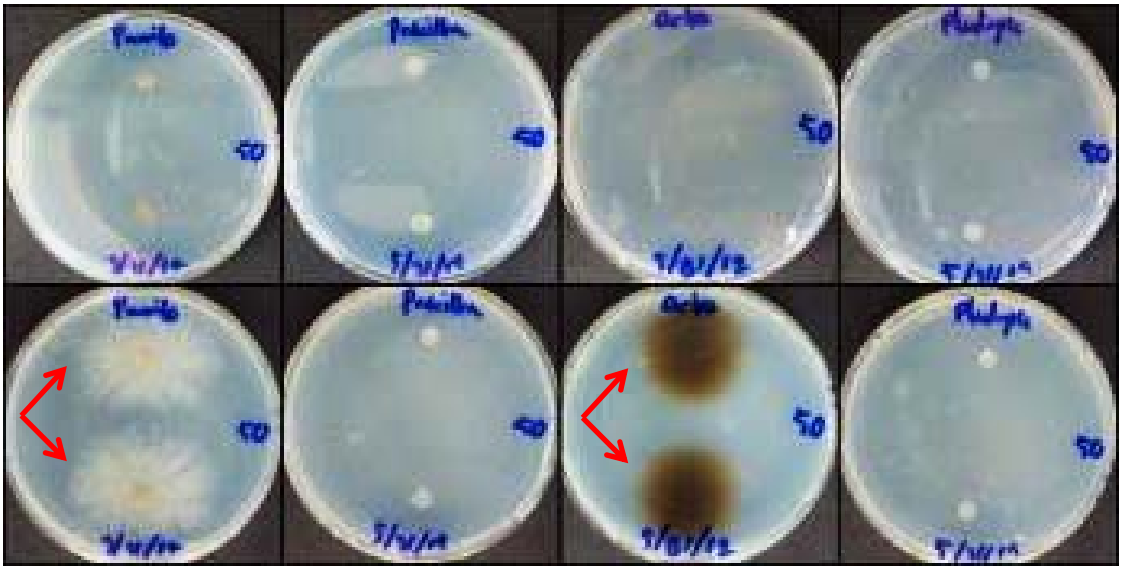
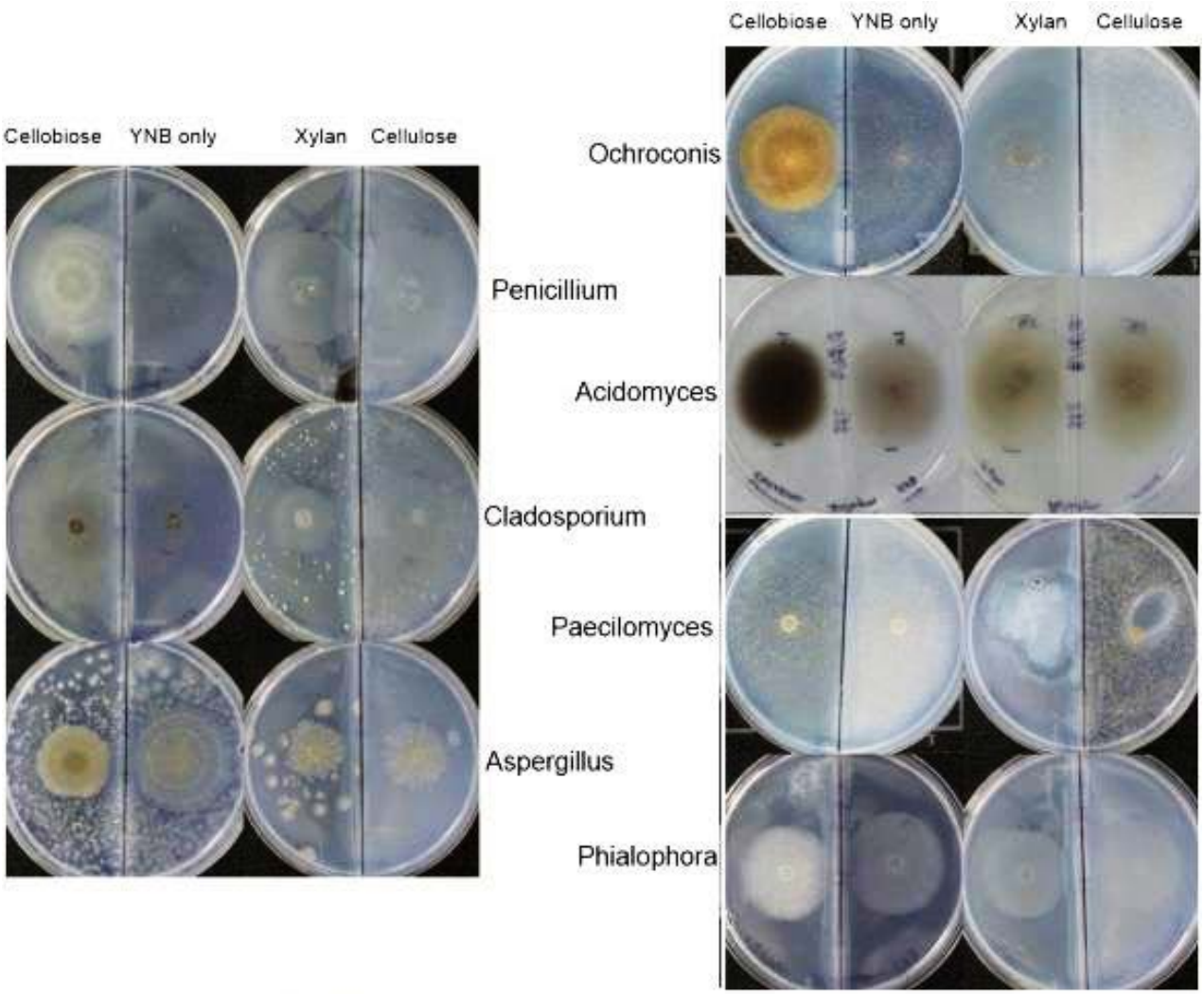


Figure 4



952

953 Figure 5

954

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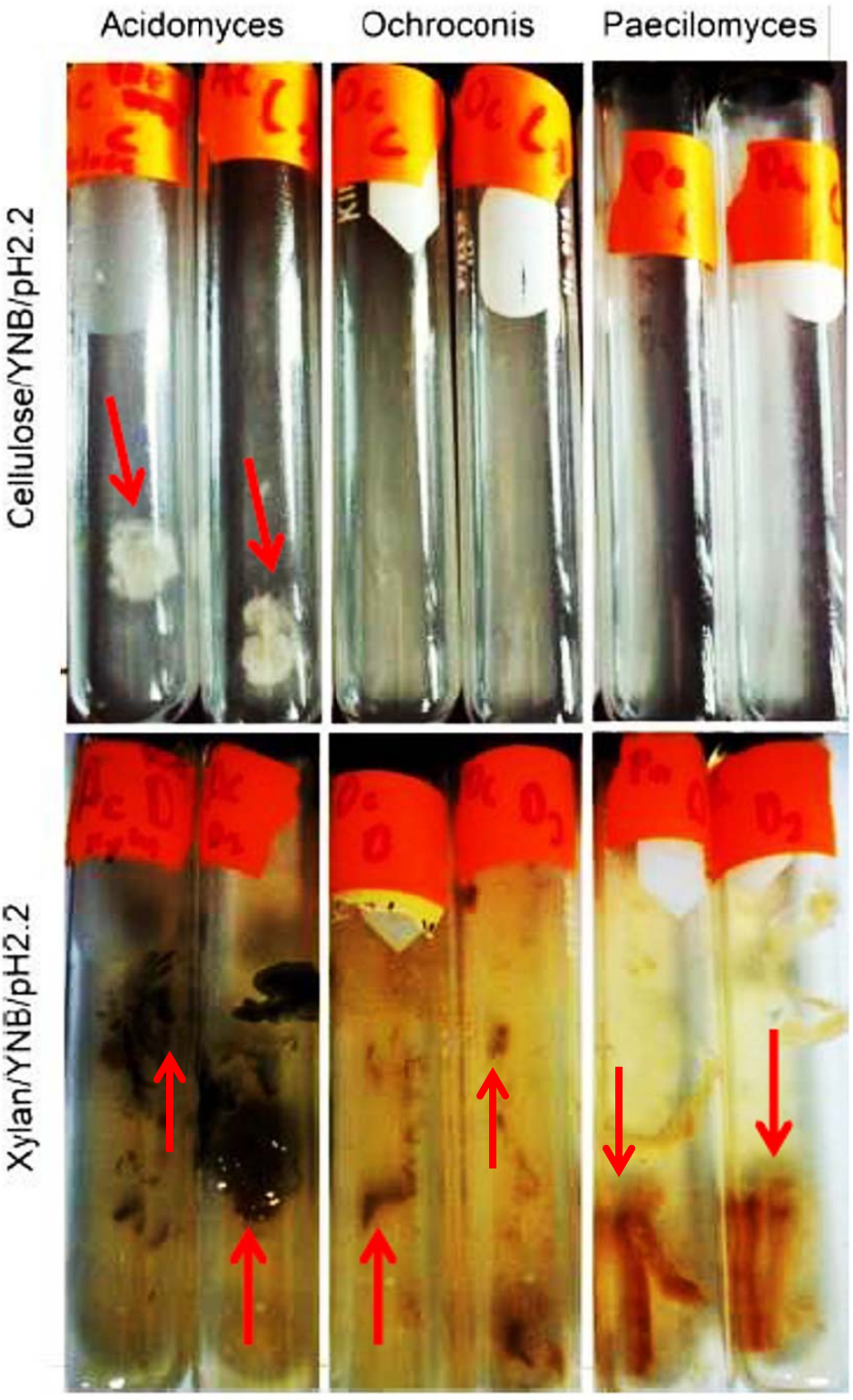

Figure 6

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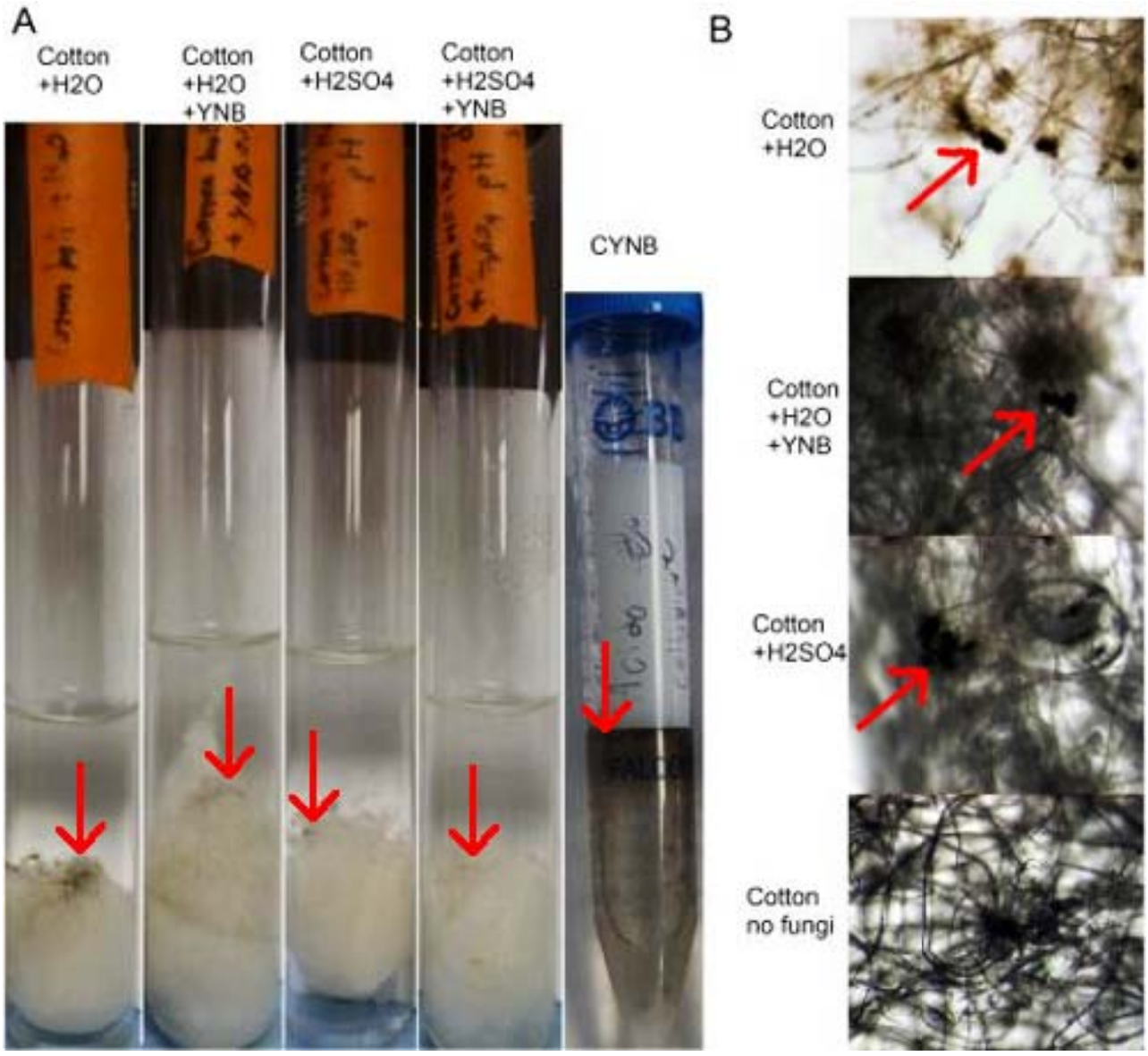

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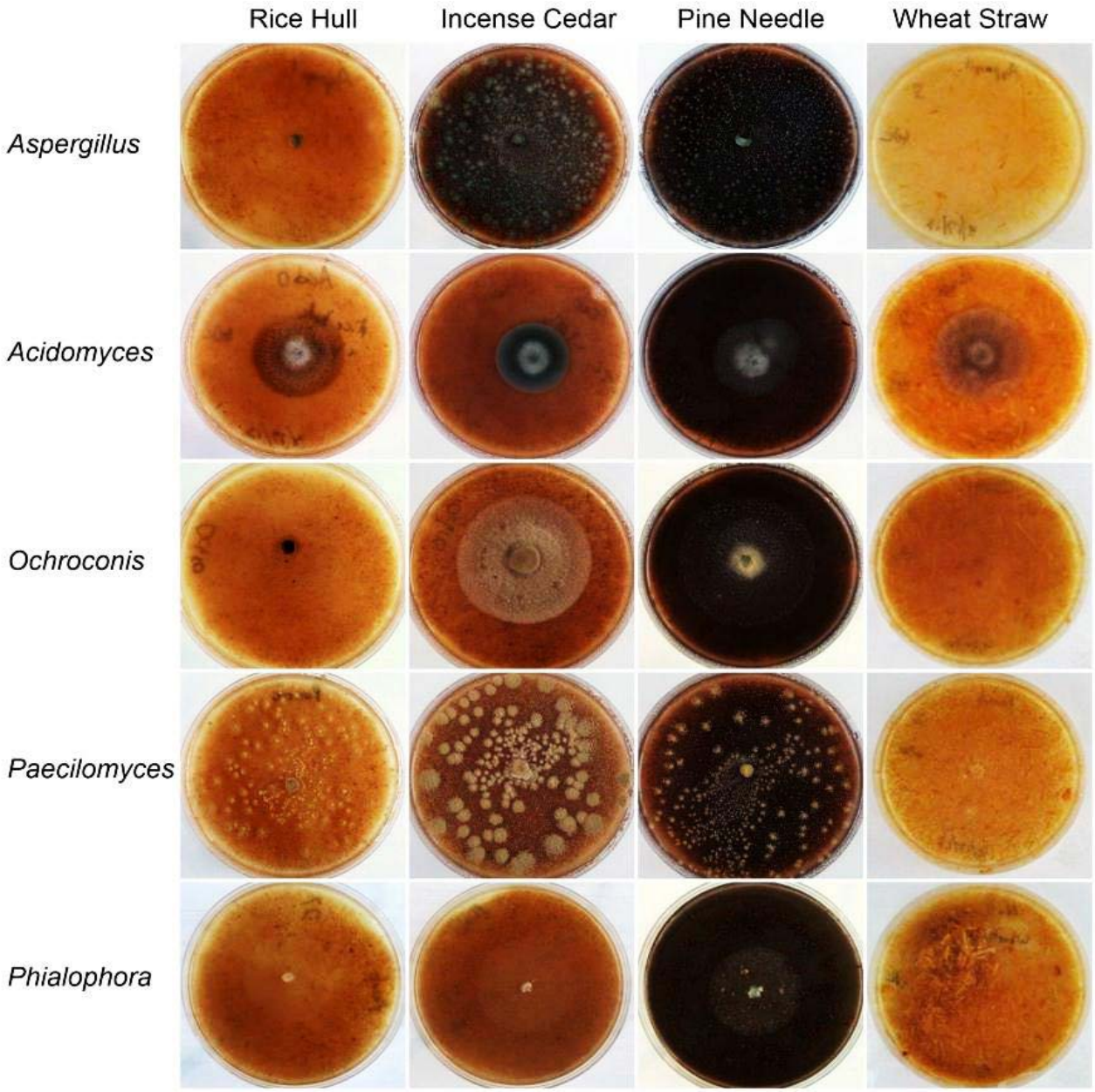
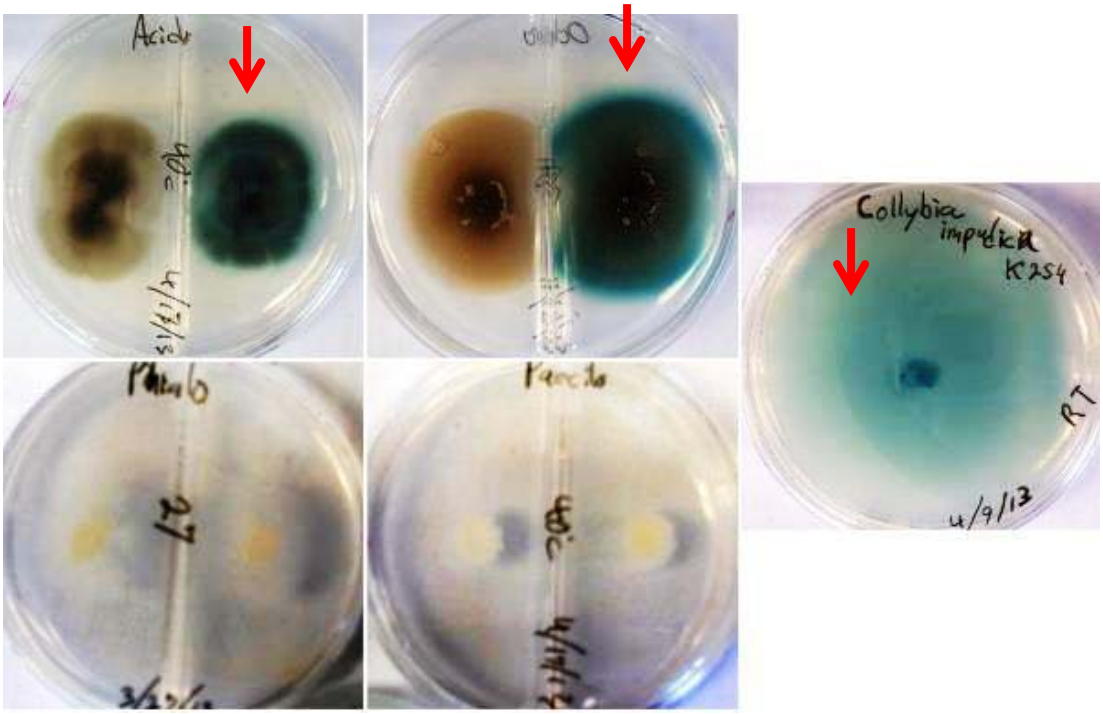
958 Figure 7



959
960
961 Figure 8
962

963 A



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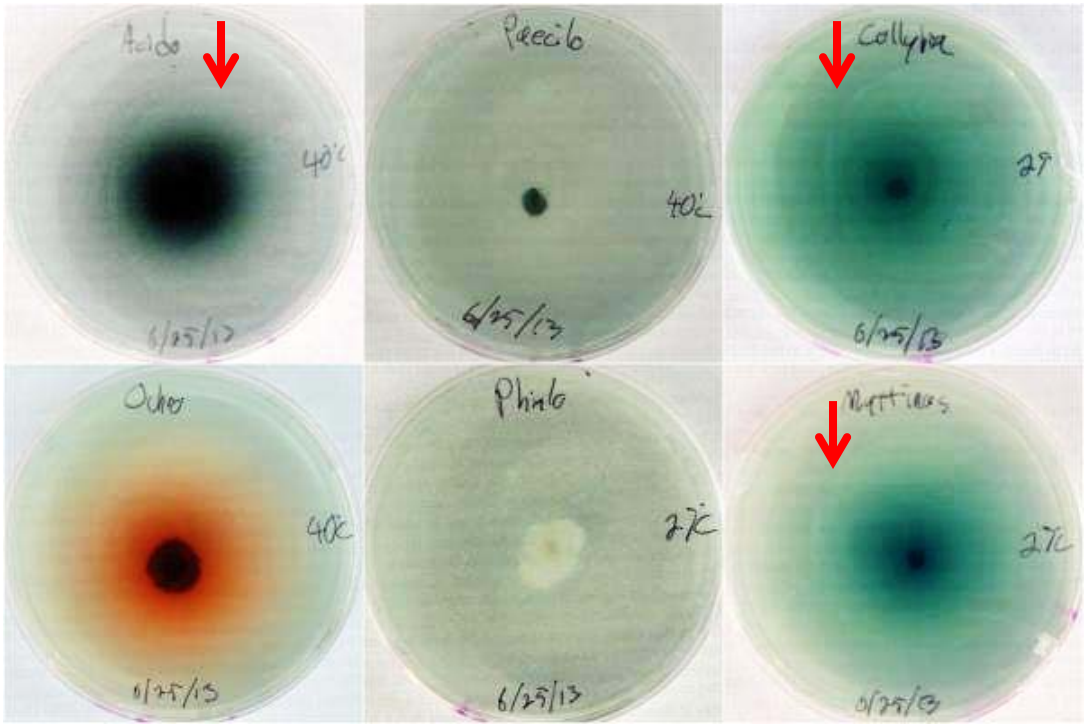
965 B



966

967 Figure 9