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Single versus simultaneous species treatment of wood with *Ceriporiopsis subvermispora* and *Postia placenta* for ethanol applications, with observations on interspecific growth inhibition

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ABSTRACT

In order to examine the potential efficacy of simultaneous dual-species fungal treatment of wood for lignocellulosic ethanol production, whole organism fungal biopulping methods utilizing white rot (*Ceriporiopsis subvermispora*) and brown rot (*Postia placenta*) fungi alone or in co-culture were compared for effect on wood microstructure, chemical composition, and enzymatic sugar solubilization. *Liriodendron tulipifera* wood chips exposed for 30 days to *C. subvermispora* and/or *P. placenta* fungi alone or in co-culture exhibited qualitative differences in wood microstructure, but did not significantly differ in final percent composition of holocellulose, α-cellulose, or lignin content compared to controls. All fungal treatments increased the soluble reducing sugar yield of enzymatic hydrolysis by ca. 28–30% over sterile controls. The co-culture fungal treatment did not significantly differ in reducing sugar yield compared to monoculture treatments, suggesting an unexpected lack of additive or other synergistic species effects on wood degradation using these fungi in co-culture. Paired interaction agar plate assays demonstrated that *C. subvermispora* and *P. placenta* exhibited mutual distance-mediated growth inhibition that was independent of substrate type or availability, suggesting an explanation for the observed lack of degradative synergy between these taxa. This study is the first to report the effects of simultaneous co-treatment with white and brown rot fungi, highlights the need for further optimization of methods to account for specialized fungal degradative mechanisms, and examines the potential influence of competitive interactions in whole-organism biopulping treatments utilizing different taxa.

Introduction

Lignocellulosic biomass potentially represents a significant source of low-cost, renewable carbohydrates for production of biofuels such as ethanol or biobutanol (Hahn-Hägerdal et al., 2006; Dürre, 2007). However, the highly polymerized lignin and cellulose in plant cell walls requires intensive pretreatment to release fermentable sugars or other useful substrates for biofuel production, and the current cost of these pretreatments is the primary impediment to commercialization of cellulosic biofuels (Hahn-Hägerdal et al., 2006; Gupta et al., 2009). Recently, searches for low cost methods of biomass pretreatment have sparked interest in natural woody biomass degradation processes. One such process, generally referred to as "biopulping", utilizes living whole-organism fungi as a low-energy pretreatment to degrade lignocellulosic biomass for industrial paper and/or potential biofuel production (Pérez et al., 2002; Tortella et al., 2008; Vimala and Nilanjana, 2009; Giles et al., 2011, 2012a). Of particular interest are filamentous wood decay fungi belonging to the order Polyporales (within the phylum Basidiomycota), which are ecologically central in the breakdown and nutrient recycling of forest residues (i.e. woody plant lignin and carbohydrate polymers) (Alexopoulos et al., 1996). Forest wood decay fungi of the order Polyporales are grouped polyphyletically as either white rot or brown rot fungi,

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according to the physical characteristics they impart to wood in the process of degrading it. White rot fungi refers to species that cause a rapid depolymerization of lignin and subsequent exposure of cellulose microfibrils within the wood, giving the substrate a white, cottony appearance (Eriksson et al., 1990; Martinez et al., 2005). Within this group, some taxa are distinguished as "lignin selective white rot fungi" due to their high affinity for the polymer lignin and low cellulose degradation activity. Lignin selective white rot fungi are capable of enzymatically depolymerizing lignin while maintaining a relatively low uptake of cellulose sugars, thus potentially increasing the surface area of embedded cellulose microfibrils within S1, S2, and S3 cell wall layers (Otjen and Blanchette, 1985; Eriksson et al., 1990; Martinez et al., 2005; Schmidt, 2006). Brown rot fungi are primarily known for degradation of wood in service, such as structural lumber (Martinez et al., 2005). Brown rot fungi tend to selectively degrade cellulose while leaving a modified lignin behind, giving the wood a brown cubical appearance (Eriksson et al., 1990; Martinez et al., 2005). Brown rot fungi rapidly depolymerize anmorphous and semi-crystalline cellulose by secreting peroxides that cleave long carbohydrates into shorter chains, reducing cell wall tensile strength and increasing microfibril permeability (Kleman-Leyer et al., 1998; Filley et al., 2002; Irbe et al., 2006).

In previous research, individual lignin selective white rot or brown rot fungal species have typically been examined for utility as single-species biopulping pretreatments to increase downstream paper fiber or sugar yield from wood or other lignocellulosic biomass (Blanchette, 1984; Otjen and Blanchette, 1985; Akhtar et al., 1992; Scott et al., 1998; Giles, 2008; Shi et al., 2009; Fissore et al., 2010; Rasmussen et al., 2010; Wan and Li, 2012; Giles et al., 2012b). Depending upon biomass type and desired breakdown products, exposure to the respective lignolytic and cellulolytic activities of both white rot and brown rot fungi may also be useful as a biopulping strategy. Staged biopulping using successive exposures to white rot and/or brown rot fungi have recently been explored as methods for increasing carbohydrate availability for subsequent enzymatic hydrolysis into glucose (Lee et al., 2008; Giles et al., 2011, 2012a). While staged successive applications of both white and brown rot fungi have been found to significantly increase the sugar yield of enzymatic hydrolysis, the process requirements of staged fungal exposures have disadvantages that may impede commercial adoption. In particular, previous staged treatments required that the biomass be sterilized between fungal exposures, and each fungus required a minimum 30 day colonization period for effective biopulping (Giles et al., 2011). A potential but untested solution to minimize these energy (biomass sterilization) and time (~60 day) costs is simultaneous co-exposure of the biomass to both fungi at the same time. However, it is unknown how the two taxa most commonly used in biopulping experiments (white rot *Ceriporiopsis subvermispora* and brown rot *Postia placenta)* might interact in terms of growth and decay mechanisms when exposed to the same substrate at the same time.

In general, wood decay fungal mycelia interactions are poorly understood but appear to be highly species specific, and range from combative to mutualistic (Rayner and Boddy, 1988). To our knowledge, the effects of simultaneous co-culture applications of white and brown rot fungi for biopulping purposes have not been previously examined. Increased wood lignin and holocellulose degradation has been observed in simultaneous co-culture using two different white rot fungi, however brown rot species have not been so examined (Chi et al., 2007; Carabajal et al., 2012). In the present research, we hypothesized that combining the lignin selective white rot fungus *C. subvermispora* with the cellulose-depolymerizing brown rot fungus *P. placenta* in a simultaneous co-culture biopulping treatment would significantly affect wood degradation and chemical composition, compared to respective monospecific fungal treatments of the same duration. The overall goal was to examine the effects of this novel simultaneous fungal treatment on wood biomass in order to explore its potential as a lower-cost, single stage biopulping process. In addition, paired interaction agar plate assays were conducted to examine the gross potential for close growth interactions between *C. subvermispora* and *P. placenta* in a fashion not possible to observe *in situ* within woody biomass.

Materials and methods

Fungal isolates, culture conditions, and biomass treatments

C. subvermispora FP-90031-sp (a lignin-selective white rot fungus), and *P. placenta* Mad-698-R (a brown rot fungus) were obtained from USDA Forest Products Laboratory, Madison, Wisconsin, USA. The fungi were maintained in the laboratory at 28 °C in darkness on modified malt extract agar (20 g malt extract, 1.0 g yeast extract, and 20 g agar L^{-1} in deionized water). Fresh cut *Tulip Poplar* (*Liriodendron tulipifera*) wood chips were collected from a local hardwood lumber mill and stored at 4 °C until use. The wood was a mixture of earlywood and latewood chipped from duramen without orientation. The chips were visually inspected to remove bark pieces or defect wood and to verify the homogeneity of all samples before treatment. Chips were flat in shape and approximately 2–3 cm in diameter.

Single stage biopulping was performed using modification of previously described techniques (Giles et al., 2011, Giles et al. 2012a, Giles et al., 2012b). Prior to inoculation onto wood, *C. subvermispora* and *P. placenta* isolates were subcultured in low nitrogen malt extract agar (20 g malt extract, 20 g agar L^{-1} in deionized water) at 28 °C for 7–10 d. A fungal plug from the malt extract agar plate of each species was then placed in 500 mL of low nitrogen malt extract liquid medium (20 g malt extract L^{-1} in deionized water) and incubated at 28 °C for 7–10 d. For each treatment, 1 g (oven dry weight) of wood chips was placed into 20 mL glass scintillation vials and distilled water was added to increase moisture content to 70%. The loosely capped vials were then steam sterilized for 30 min. The uninoculated controls were also sterilized. The liquid fungal cultures were vigorously shaken for one min before use and 0.2 mL was used to aseptically inoculate each wood chip monoculture treatment vial. Co-culture treatment vials were inoculated with 0.2 mL of both *C. subvermispora* and *P. placenta.* All treatments and controls were conducted in triplicate. All vials were incubated for 30 d at 28 °C for optimal delignification and depolymerization (Clausen and Kartal, 2003; Ferraz et al., 2003; Giles et al., 2011). After the 30 d treatment period, all vials (including uninoculated controls) were steam sterilized for 30 min to halt biological activity.

Microscopy

Random wood specimens were selected from each treatment and control for observation of fungal growth and cell wall degradation. Samples were oven dried at 104 °C, mounted on aluminum stubs with carbon tape, and coated with 30 nm Au–Pd in a plasma chamber. Samples were imaged with a JEOL JSM-6460 low vacuum scanning electron microscope (SEM) at an accelerating voltage of 5 kV to reduce sample charging.

Chemical analyses of wood

Gravimetric determination of wood holocellulose, α-cellulose, and Klason lignin content of the sterile controls and biopulped samples was performed using previously described microanalytical