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Fungal growth necessary but not sufficient for effective biopulping of wood for lignocellulosic ethanol applications

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ABSTRACT

A lignocellulosic biomass treatment method utilizing wood rot fungi to improve cellulosic sugar solubilization for ethanol production was modified to provide larger samples for chemical analyses. *Liriodendron tulipifera* wood chips were treated in a novel application of aerobic polypropylene "spawn bags" as experimental bioreactors for biopulping. Treatments were inoculated with culture suspensions of white rot (*Ceriporiopsis subvermispora*) and brown rot (*Postia placenta*) fungi in 40 day incubations. Fungal growth occurred in all treatments, with extensive hyphal coverage of the biomass (ca. >80% of wood chip surfaces). After treatment, physical biodegradation, holocellulose, α-cellulose, and lignin contents of the fungal-biopulped wood chips were comparable to previous results. However, unlike in previous studies, the biopulped wood did not exhibit significant increases in soluble sugars after enzymatic hydrolysis. These results demonstrate that extensive fungal colonization of biomass is not necessarily sufficient for effective biopulping to increase production of free soluble sugars from wood. Environmental controls on hydrolytic activity by wood rot fungi are poorly known, but we hypothesize that treatment inoculation using nitrogen rich medium may have inhibited lignin-specific fungal hydrolysis of the wood. Future development of effective, standardized biopulping techniques for improving soluble sugar production from lignocellulosic biomass will require optimization of methods including characterization of inoculation nutrients and container effects on fungal metabolic activity.
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1. Introduction

Lignocellulosic biomass such as wood has the potential to replace corn and other seed starches as an abundant, sustainable supply of fermentable sugars for fuel ethanol (Perlack et al., 2005; Lin and Tanaka, 2006). However, the abundant sugars contained in wood occur in a complex polymerization of lignin and crystalline cellulose that is difficult to hydrolyze into soluble sugars needed for fermentation (Hahn-Hägerdal et al., 2006). Considerable research has focused on various methods of pretreating wood and other lignocellulosic biomass to improve sugar yield, including mechanical grinding, steam explosion, and acid/base or enzymatic hydrolysis (Sun and Cheng, 2002). These methods generally require high-energy input or hazardous toxic chemicals, the associated costs of which currently impede commercialization of lignocellulosic ethanol (Froese et al., 2008).

Wood decay fungi that naturally biodegrade lignocellulosic polymers could potentially be used as a less energy intensive method of biomass treatment for ethanol production. The process of using whole-organism filamentous wood decay fungi to break down wood or other lignocellulosic biomass is called "biopulping" and has already been used in paper manufacturing (Akhtar et al., 1992; Young and Akhtar, 1997; Akhtar et al., 2000; Guerra et al., 2006). Filamentous wood decay fungi are generically grouped as either white rot or brown rot fungi, based on differences in their effect on the chemical composition of wood. Each type has distinct depolymerization mechanisms that chemically and mechanically degrade lignin and/or depolymerize cellulose. Lignin selective white rot fungi are capable of enzymatically depolymerizing lignin while simultaneously exhibiting a low uptake of cellulose derived sugars (Otjen and Blanchette, 1985; Eriksson et al., 1990; Martinez et al., 2005; Schmidt, 2006). Brown rot fungi on the other hand generally do not break down lignin, but rather depolymerize anamorphous and semi-crystalline cellulose by secreting peroxides that cleave long sugar polymers into shorter chains or monomers, which also has the effect of increasing wood fiber permeability (Kleman-Leyer et al., 1992; Filley et al., 2002; Irbe et al., 2006;

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Howell et al., 2009). Lignocellulosic biomass treatments using a single lignin selective white rot or a single brown rot fungus have previously been examined for their ability to increase downstream ethanol yield (Lee et al., 2008; Shi et al., 2009; Rasmussen et al., 2010; Giles et al., in press). In addition, consecutive treatments using both white rot and brown rot fungi to successively delignify and then hydrolyze wood have also recently been shown to significantly increase soluble glucose after enzymatic hydrolysis (Giles et al., 2011). Further optimization of biomass inoculation and colonization techniques are required for future development of lignocellulosic ethanol pretreatments utilizing multiple fungi.

The practice of fungal biopulping using a white rot fungus to delignify wood substrates for paper production has been previously examined at laboratory and pilot scales (Akhtar et al., 1998; Scott et al., 1998; Masarin et al., 2009). However, these techniques may not be optimal for lignocellulosic ethanol biopulping, where the overall goal is the production of soluble sugars as opposed to delignified cellulose. This different goal conceptually may require application of novel fungi, and the effect of fungal growth parameters on the total fermentable sugars released from woody biomass after enzymatic hydrolysis has not been characterized. Brown rot fungi (e.g. *Postia placenta*) have not been evaluated for use in biopulping for paper manufacture due to this fungal group's lack of observed lignin degradation and relatively rapid cellulose depolymerization (Eriksson et al., 1990; Schmidt, 2006).

Previous research examining biopulping for biofuel applications have utilized relatively small amounts of lignocellulosic biomass (<5 g) to evaluate (both white and brown rot) fungal wood degradation in standardized laboratory-scale treatments conducted in small batch "bioreactor" containers such as test tubes, screw-capped culture bottles, or Petri dishes (Otjen and Blanchette, 1985; ASTM, 1996; Chi et al., 2007; Giles, 2008). The relatively small amounts of biomass treated in such experiments permits only limited chemical analysis of critical wood decay parameters such as lignin, cellulose, and soluble sugar composition, which are required to determine the suitability of biopulping for lignocellulosic ethanol production or other processes. Future comparative studies using different biomass types and/or novel fungal species would benefit from a standardized method for examining larger amounts of biomass to allow for repeated destructive testing of chemical composition during the biopulping process. Furthermore, previous biopulping for paper research (using single applications of white rot fungi) suggests fungal degradation is affected by treatment parameters such as biomass quantity, homogeneity, gas exchange, moisture content, and temperature (Akhtar, 1997; Young and Akhtar, 1997; Ferraz et al., 2008). In the laboratory, these parameters are influenced by treatment container effects, which have varied considerably in previous research. Biopulping using brown rot fungi for lignocellulosic ethanol applications has not been evaluated using larger amounts of biomass (>5 g), and thus the effects of scale-up and bioreactor conditions on this process are unknown.

Future comparative studies of biopulping for lignocellulosic ethanol (or other novel applications) would benefit from demonstration of a more standardized laboratory bioreactor design that is large enough to provide sufficient biomass for repeated chemical testing, while at the same time supporting active fungal growth, mimicking theoretical industrial scale-up conditions as closely as is experimentally feasible, and remaining relatively inexpensive and easy to use. A goal of this study was to test the suitability of polypropylene "spawn bags" used in commercial mushroom cultivation as bioreactor containers for laboratory-scale, sequential fungal biopulping of wood as a treatment process for increasing soluble sugar yield for ethanol production. These bags are inexpensive, hold sufficient biomass to allow repeated chemical testing (>500 g), and have aerobic gas/moisture exchange and contaminant control characteristics suitable for aseptic growth of a variety of fungal species (Upadhyay and Singh, 2010). Our hypothesis was that this simple bioreactor design would yield biopulping efficiency at or better than previous studies using similar biomass and staged fungal applications, while providing larger quantities of biomass for chemical testing compared to previous smaller treatments. The overall objective was to develop and demonstrate a useful, more standardized method for future comparative biopulping studies.

2. Materials and methods

2.1. Wood, fungal isolates, and culture conditions

Fresh cut *Liriodendron tulipifera* (Tulip Poplar) wood chips ($\sim 3 \times 3 \times 0.5$ cm) were collected from a 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. *Ceriporiopsis 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, WI, USA). The fungi were initially cultured on autoclave-sterilized malt extract agar medium (20 g malt extract L^{-1} in deionized water with 1.5% agar) and incubated at 28 °C for 20 days. Fungi were then transferred into autoclave-sterilized modified malt extract liquid medium (20 g malt extract, 1.0 g yeast extract L^{-1} in deionized water) and incubated at 28 °C for 20 days prior to use.

All treatments were conducted in triplicate. For each, approximately 100 g (oven dry weight) of wood chips were placed in commercial spawn bags (model FDB1000, Field and Forest Products, Peshtigo, WI, USA). These autoclavable gusseted polypropylene bags are transparent and are equipped with a 10 cm^2 fiber gas exchange filter. Distilled water was added to increase wood moisture content to 70% for optimal fungal growth (Young and Akhtar, 1997). The bags were then steam sterilized at 121 °C for 1 h, allowed to cool, and aseptically inoculated with 10 mL of well-mixed liquid culture suspension of either *C. subvermispora* or *P. placenta*. The bags were then incubated in darkness for 40 days at 28 °C, considered optimal for delignification and depolymerization (Clausen and Kartal, 2003; Ferraz et al., 2003; Giles et al., 2011), then steam sterilized at 121 °C for 1 h. *C. subvermispora* treatment bags designated for two-stage biopulping were inoculated with 10 mL of liquid culture of the second fungal species, *P. placenta*. These two-stage treatment bags (and controls) were incubated for an additional 40 days at 28 °C then steam sterilized at 121 °C for 1 h. It was determined in previous research that a second sterilization cycle does not increase significant differences in chemical composition and enzymatic hydrolysis between sterile controls (Giles et al., 2011). The optimal order of staged fungal colonization was previously determined to be *C. subvermispora* followed by *P. placenta* (Giles et al., 2011).

2.2. Chemical analyses

Gravimetric determination of wood extractives, holocellulose, alpha cellulose, and Klason lignin content of the sterile controls and decayed samples was performed using previously described microanalytical techniques (Yokoyama et al., 2002; Yeh et al., 2004; Sluiter et al., 2008; Giles et al., 2011). Briefly, ground wood meal was oven dried at 104 °C before weighing, then extracted for 24 h using a Soxhlet apparatus to wash 95% EtOH over the samples. The extracted material was then oven dried at 104 °C before weighing to determine extractive content which was recorded as a percentage of original sample mass.

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