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Biological pretreatment with a cellobiose dehydrogenase-deficient strain of *Trametes versicolor* enhances the biofuel potential of canola straw

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ABSTRACT

The use of *Trametes versicolor* as a biological pretreatment for canola straw was explored in the context of biofuel production. Specifically, the effects on the straw of a wild-type strain (52 J) and a cellobiose dehydrogenase (CDH)-deficient strain (m4D) were investigated. The xylose and glucose contents of the straw treated with 52 J were significantly reduced, while only the xylose content was reduced with m4D treatment. Lignin extractability was greatly improved with fungal treatments compared to untreated straw. Saccharification of the residue of the m4D-treated straw led to a significant increase in proportional glucose yield, which was partially attributed to the lack of cellulose catabolism by m4D. Overall, the results of this study indicate that CDH facilitates cellulose access by *T. versicolor*. Furthermore, treatment of lignocellulosic material with m4D offers improvements in lignin extractability and saccharification efficacy compared to untreated biomass without loss of substrate due to fungal catabolism.

1. Introduction

The cost associated with chemical, thermal and mechanical pretreatment of plant biomass destined for biofuel and biomaterial production impinges on the overall energy balance of these processes, thereby decreasing the market competitiveness of the products. These energy intensive pretreatments typically focus on the fractionation of one or more of the major components of plant biomass to enhance the efficiency of downstream processing (Chandra et al., 2007; Hendriks and Zeeman, 2009). As a result, alternative strategies for more cost-effective fractionation of lignocellulose are key for sustainable biofuel and biomaterial production.

Biological pretreatments have the potential to mitigate some of the costs associated with current biomass pretreatment strategies by taking advantage of the innate ability of certain microbial species to selectively deconstruct the various components of lignocellulose (Anderson and Akin, 2008). As a consequence, identifying organisms capable of processing recalcitrant biomass has been the focus of recent research initiatives. For example, an investigation of the metatranscriptome of the muskoxen rumen illuminated the breadth of both the genomic and transcriptomic diversity associated with lignocellulosic decomposition (Qi et al., in press). Similarly, exploration of cow rumen digestion of switchgrass revealed a plethora of microbiota associated with the expression of over 27,000 putative lignocellulose-modifying enzymes (Hess et al., 2011). The results of these studies and others of this nature will undoubtedly identify organisms and enzymes that will enhance future industrial biomass conversion strategies.

In addition to recent microbial community analyses, there has been renewed interest in several well-known microbial species due to their ability to deconstruct recalcitrant biomass. Of particular interest are white-rot and brown-rot fungi, which specialize in lignocellulose depolymerization. This unique ability has provided the impetus for genomic, secretomic and transcriptomic analyses of notable lignocellulose-degrading fungi, such as *Postia placenta* (Martinez et al., 2009), *Phanerochaete chrysosporium* (Sato et al., 2009; Vanden Wymelenberg et al., 2009), and *Phanerochaete carnosa* (MacDonald et al., 2011). Due to our increasing understanding of the mechanisms underpinning their unique ability to decompose lignocellulose, these organisms have the potential to be effective low-cost biomass pretreatment agents. To this end, a number of studies have investigated the efficacy of white rot fungi with respect to biological pretreatment of lignocellulosic material including wheat and rice straw, switchgrass and corn stover (Anderson and Akin, 2008; Patel et al., 2007; Talebnia et al., 2010; Wan and Li, 2010). The high residue production of canola makes it an attractive substrate for biofuel production, although its composition limits the amount of fermentable glucose available using standard alkali or acid pretreatments (George et al., 2010). Although

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pretreatment with white-rot fungi has generally provided significant increases in the extractability of lignin as well the amount of fermentable glucose in lignocellulosic substrates (Anderson and Akin, 2008; Patel et al., 2007; Talebnia et al., 2010; Wan and Li, 2010), the consumption of cellulose by the fungus itself remains a limitation. In this context, there exists a delicate balance between enhancement of lignocellulosic fractionation of plant biomass and loss of substrate due to fungal catabolism.

In the present study, the efficacy of white-rot fungus, *Trametes versicolor*, as a biological pretreatment agent for canola straw was explored. Specifically, the pretreatment effects on canola straw after treatment with a wild-type *T. versicolor* (52 J) were compared with those from a cellobiose dehydrogenase (CDH)-deficient strain, m4D (Dumonceaux et al., 2001). The biological role of CDH and the efficacy of the pretreatments in the context of sustainable bioenergy production are discussed.

2. Methods

2.1. Fungal strains

T. versicolor monokaryotic strain 52 J (ATCC 96186) (Addleman and Archibald, 1993) and a mutant strain that was shown to be deficient in producing functional cellobiose dehydrogenase (Dumonceaux et al., 2001) were used in this study. Strains were maintained as glycerol stocks at −80 °C and grown on potato dextrose agar (EMD chemicals, Gibbstown NJ) for fewer than 30 serial passages.

2.2. RNA extraction and reverse transcription (RT)-PCR

Fungal liquid pre-cultures were prepared by removing 10 agar plugs (0.5 cm diameter) from the edge of a colony grown on a potato dextrose agar plate and added to 100 mL of *Trametes* defined medium (TDM) (Addleman and Archibald, 1993) with glucose (2% w/v) as a carbon source. This mixture was blended in an Eberbach blender cup on a Waring blender base for three pulses of 10 s each, and then the mycelial macerate was transferred to a sterile 250 mL Erlenmeyer flask and incubated at room temperature (20–22 °C) with shaking (150 rpm) for 4 d. Cultures were centrifuged at 1000*g* for 5 min and washed twice in 100 mL TDM with no carbon source. The washed cultures were then resuspended in 50 mL of TDM with no carbon source and added to flasks containing 50 mL of autoclaved TDM containing 2 g of cellulose (SigmaCell, Sigma, St. Louis, MO), spring wheat straw (milled with a Retsch homogenizer fitted with a 1 mm screen), or glucose (Sigma) in a 250 mL Erlenmeyer flask. Duplicate cultures of each substrate were prepared and incubated as above for 6 d. Alternatively, to generate a time course of *cdh* expression, duplicate fungal cultures were prepared in *cdh* induction medium (Dumonceaux et al., 2001) from TDM-glucose plates using a Waring blender as described above. Aliquots of 10 mL were removed at 6 h, 24 h, 3 d, 4 d, and 7 d post-inoculation.

At each time point, an aliquot of 10 mL of each fungal culture was removed and the liquids were separated by filtration on Whatman #1 filter paper. The solid residue (fungal biomass with, in certain cases, insoluble substrate) was scraped off of the filter paper into a pre-cooled mortar containing liquid nitrogen and was pulverized into a fine powder using a pestle. The powdered biomass (approximately 0.5 g including a variable amount of fungal biomass) was immediately used for RNA extraction with Trizol reagent (Invitrogen, Burlington ON) or with an RNeasy Plant Kit (Qiagen, Toronto ON) according to the manufacturers' recommendations. RNA samples that were extracted with Trizol were post-treated with DNaseI (Ambion Turbo DNA-free kit, Applied Biosystems Inc., Streetsville, ON), while RNA samples that were extracted with an RNeasy Plant Kit were subjected to on-column DNaseI treatment as recommended by the manufacturer. RNA was quantified using a Quant-IT RNA assay kit and Qubit instrument (Invitrogen).

DNaseI-treated RNA samples (1 μg) were reverse-transcribed using SuperScript III (Invitrogen) or iScript (BioRad Laboratories, Mississauga, ON) reverse transcriptase (RT). Controls were included without RT to rule out amplification from contaminating genomic DNA. PCR primers were designed using Beacon Designer (BD) v7.0 software (Premier Biosoft, Palo Alto, CA) for *cdh* from the reported sequence of the *T. versicolor* mRNA (GenBank accession No. AF029668): 5′-CCACGTCCGTTCCTAGCG-3′ and 5′-TGTCCTTGCACCACCAGAATG-3′. These primers were designed to generate a product of 300 base pairs (bp) from mRNA and spanned introns such that DNA-derived bands could be distinguished from RNA-derived bands. Amplification conditions for the *cdh* primer set were: 95 °C, 3 min (1×); 95 °C 30 s, 55 °C 30 s, 72 °C 30 s (40×). PCR primers and amplification conditions for 18S rRNA were from Matityahu et al. (2008); these primers generated a product of 200 bp from either genomic DNA or RNA. To normalize gene expression using another mRNA species, the sequence of the chaperonin-60 (*cpn60*) universal target was retrieved (GenBank accession No. FJ973632) and PCR primers were designed with BD 7.0 to generate a product of 75 bp from mRNA or gDNA: 5′- TGAGGACGAGATTGAGATCAC-3′ and 5′- ACGTCGGTCACGAAGTAGG-3′. Amplification conditions for the *cpn60* RT-PCR were: 95 °C, 3 min (1×); 95 °C 30 s; 55 °C 30 s; 72 °C 30 s (40×). All PCRs used 2 μL of RT product as template and had primer concentrations of 400 nM. Conventional PCRs had a $MgCl_2$ concentration of 2.5 mM, 0.5 mM each dNTP, 1 U *Taq* polymerase (Invitrogen), and used a C1000 thermocycler base with a dual 48-well alpha unit (Bio-Rad). Quantitative PCRs used SsoFast EvaGreen Supermix (Bio-Rad) and were run using a CFX96 quantitative PCR module on a C1000 thermocycler base (Bio-Rad).

2.3. Solid-state fermentation

The straw (stems) of senesced canola (*Brassica napus* L.) was collected after seed harvest in the fall of 2009 from a field in Swift Current, Saskatchewan (50° 17′ 17″ N, 107° 47′ 38″W). The straw was subsequently chopped into ~5 cm segments and 20 g was placed in a 2.25 L plastic bucket with a tight-fitting lid. To suppress the outgrowth of endogenous moulds, 15 mL of water was added to the straw and the bucket was microwaved (Kenmore, 700 W) for 2 min. Inocula for solid state fermentation cultures were prepared in 100 mL mycological broth (10 g soytone, 40 g D-glucose, and 1 mL of *Trametes* trace metal solution (Addleman and Archibald, 1993) per L) as described in Section 2.2. The liquid culture was then blended using a sterile probe homogenizer (Heidolph DIAX 900; generator probe attachment with 1 cm diameter sawtooth blade) prior to inoculating solid-state fermentation cultures (see Section 2.3). Homogenized liquid *T. versicolor* culture (22 mL) was inoculated over the surface of the cut, microwaved straw, using a pipette to distribute the inocula evenly over the entire substrate. The moisture content for the culture was 62.4%. A 1 mL aerosol-barrier pipette tip was fitted into the top of the lid to provide aeration while maintaining an axenic, humid environment. After 84 d of growth the straw was freeze-dried prior to milling with a ZM 200 Retsch Mill (1 mm screen) for subsequent analyses.

2.4. Ergosterol extraction

Ergosterol was extracted from approximately 15 mg of milled biomass using a saponification method described by Hobbie et al. (2009). After passing through a nylon filter (4 mm × 0.2 μm), a