Running title: **Applications of *Trametes versicolor* crude culture filtrates in detoxification of biomass pretreatment hydrolyzates**

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1 Abstract

2 Laccases have wide range of substrate specificity and find applications from pulp industry
3 to waste water remediation. Laccases have also been used in combined pretreatment of
4 biomass hydrolyzates to remove enzymatic and fermentation inhibitors. In this study,
5 laccases production by *Trametes versicolor* strains isolated from different regions of the
6 United States was induced using copper salts. *T. versicolor* crude culture filtrates (CCF),
7 without any purification step, were tested for removal of model inhibitor compounds as
8 well as in poplar and rice straw pretreatment hydrolyzates. Phenolic inhibitors were
9 removed by 76 % and 94 % from the dilute acid hydrolyzates of rice straw and poplar,
10 respectively, when incubated with the CCF for 12 hours, at room temperature. Xylo-
11 oligosaccharide concentrations present in rice straw hydrolyzates were reduced by 64%
12 when incubated with *T. versicolor* CCF. *T. versicolor* CCF could be a low cost technology
13 for decreasing enzymatic and fermentation inhibitors.

14 **Keywords:** *Trametes versicolor* laccase, phenolic fermentation inhibitors, xylo-
15 oligosaccharides, rice straw, poplar

1. Introduction

Cellulosic ethanol is a sustainable alternative to corn ethanol and can be produced from wide range of lignocellulosic feedstock such as, agricultural and forestry residues, energy crops and municipal solid wastes (Perlack and Stokes, 2011). Lignocellulosic biomass represents one of the most abundant and low cost resources and its availability varies from one geographic location to another. In the United States, 1.3 billion dry tons of plant-derived biomass could be used to displace up to 30% of the nation's consumption of liquid transportation fuels (Perlack and Stokes, 2011). Thus, biomass energy system could be designed and implemented, increasing the amount of sustainable energy production.

Cellulose is a major polysaccharide present in lignocellulosic materials and is hydrolyzed to glucose by the synergistic action of endoglucanases, exoglucanases and β -glucosidases (Bras et al., 2011). Pretreatment or preconditioning of biomass is an essential process to enhance the efficiency of enzymatic hydrolysis. Unfortunately, the hydrolysis of hemicellulose and lignin during chemical pretreatments is also accompanied by the production of inhibitors that compromises enzymatic hydrolysis and microbial fermentation (Palmqvist and Hahn-Hägerdal, 2000b). There are at least four main classes of fermentation inhibitors encountered in biomass conversion: 1) furfural and hydroxymethyl furfural, often referred to as furans; 2) weak acids; 3) phenolic compounds; and, 4) oligosaccharides (Palmqvist and Hahn-Hägerdal, 2000a). While furans, weak acids and oligosaccharides are the by-products of cellulose and hemicellulose degradation, phenolic compounds are formed as result of lignin degradation. The nature and concentration of these inhibitors depend on biomass species and the severity of the pretreatment that is used (Kumar and

Wyman, 2009; Palmqvist and Hahn-Hägerdal, 2000a). Acetic acid, formic acid, furfural, 5-hydroxymethylfurfural and phenolic compounds were reported to be inhibitory to fermentation microorganisms (Oliva et al., 2003; Palmqvist and Hahn-Hägerdal, 2000b; Taherzadeh and Karimi, 2011), while xylo-oligosaccharides (XOS) inhibit cellulases, resulting in hydrolysis efficiency losses (Kumar and Wyman, 2009; Zhang and Viikari 2012). For the efficient bioconversion of lignocellulosic material to fermentable sugars and ethanol, inhibitors of cellulases and fermentation microorganisms, such as phenolics, furans and xylo-oligosaccharides, should be mitigated.

Several strategies have been applied to reduce or nullify the effects of inhibitory compounds (Parawira and Tekere, 2011; Palmqvist and Hahn-Hägerdal, 2000a). Laccases (EC 1.10.3.2) have proven to be potentially useful for improving the efficiencies of enzymatic hydrolysis and fermentation of monosaccharides to ethanol by specifically removing the phenolic compounds (Jönsson et al., 1998; Larsson et al., 1999; Chandel et al., 2007). Laccases are known to catalyze the oxidization of substituted phenols and facilitate their subsequent polymerization. In the presence of redox-active compounds, the substrate range, as well as potency of laccase is increased (Fabbrini et al., 2002). Plants, fungi and even some prokaryotes, produce laccases with different properties, according to targeted purposes (Baldrian, 2006). The present study partially characterized and evaluated laccase preparations from *Trametes versicolor*, with and without redox mediators, for the removal of classic phenolic and xylo-oligosaccharide inhibitors. The mitigating effects of *T. versicolor* crude culture filtrates on the inhibitors formed during the dilute acid pretreatments of poplar and rice straw were also evaluated.

2. Materials and methods

2.1 Chemicals

Commercial standards of *p*-coumaric acid, *trans* ferulic acid, furfural, 5-hydroxymethylfurfural (HMF) (Sigma-Aldrich, St. Louis, MO), vanillic acid, syringaldehyde, 4-hydroxybenzoic acid, vanillin, salicylic acid, gallic acid (TCI chemicals, Montgomeryville, PA) and *trans*-cinnamic acid (Alfa-Aesar, Ward Hill, MA), were used to test the selective degradation potential of the fungal laccases. Xylo-oligosaccharides such as xylobiose (DP 2), xylotriose (DP 3), xylo-tetraose (DP 4) and xylopentaose (DP5), having > 95 % purity were purchased from Megazyme International (Wicklow, Ireland). Chemicals, such as malt extract (bacterial grade), potassium dihydrogen phosphate (Amresco, Solon, OH), magnesium sulfate, calcium nitrate, copper sulfate, remazol brilliant blue-R (RBBR) (Acros Organics, NJ) and agar, bacterial grade (BD Biosciences, Franklin Lakes, NJ) were used for culturing and testing *Trametes versicolor*. Guaiacol was obtained from Alfa Aesar (Ward Hill, MA). The compound 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma, St. Louis, MO), was used as a substrate to determine the laccase activity. *T. versicolor* laccase, purchased from Sigma (St. Louis, MO), was used for comparison with in-house crude culture filtrates. The laccase mediator, 1-hydroxybenzotriazole hydrate (HOBt), was also purchased from Sigma (St. Louis, MO). Chemicals of molecular biology grade, such as acrylamide, *bis*-acrylamide, tetramethylethylenediamine (TEMED), Tris base, glycerol (Amresco, Solon, OH) and ammonium persulfate (IBI Scientific, Peosta, IA) were used in the polyacrylamide gel electrophoresis (PAGE) analysis.

2.2. Microorganisms

Five different strains of *T. versicolor* isolated from different regions of the United States were supplied by the United States Department of Agriculture, Madison (WI, USA). The various strains of *T. versicolor* had the following origins: Aho 23 (Portland, OR), ERT 201 (Stoneville, MS), FP-103969 (Red wood forest, CA), HBB-2740 (Nantahala National Forest, NC), and Shigo61-7-3-1 (White Mountains, NH).

2.3 Culture and selection of fungal strains

The fungal cultures were grown on sterile malt extract agar (MEA) media composed of malt extract 20 g/L, agar 20 g/L, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 0.5 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L and KH_2PO_4 0.5 g/L, with the pH adjusted to 5.2. After 7 days of incubation, in a forced-air incubator (VWR, Chicago, IL), at 28 °C, all five strains of *T. versicolor* were tested for their lignolytic ability using a RBBR dye-based assay. Petri dishes containing 2.0 % (w/v) MEA media supplemented with 1 g/L of RBBR were inoculated with one 8.0 mm diameter mycelial discs, cut from the growing edge of the fungal hyphae and were incubated for 15 days, at 28 °C, in the forced-air incubator.

2.4 Production of Crude culture filtrate (CCF) laccase

Strains of *T. versicolor* selected for high laccase production, were grown in 1 L Erlenmeyer flasks. Sterilized malt extract broth was prepared by mixing malt extract 20 g/L, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 0.5 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L and KH_2PO_4 0.5 g/L, at pH 5.4, as reported by Vasdev and Kuhad (1994). The malt extract broth was supplemented with 0.1

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6 87 mmol/L copper sulfate on the third day of the culture, in order to induce higher laccase
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8 88 production (Cordi et al., 2007). Fresh mycelial disks of 8.0 mm diameter were added to 150
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10 89 mL of the sterilized malt extract broth and incubated at 28 °C, in a forced air incubator,
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12 90 under static conditions. Culture broth samples of 1 mL were collected every 24 h and were
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14 91 used to determine the laccase activity. Thereafter, contents of the flasks were vacuum
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16 92 filtered using Whatman filter paper No.1 (GE Healthcare Bio-Sciences, PA) and the crude
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18 93 culture filtrates (CCF) were subsequently used for characterization.
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24 94 **2.5 Characterization of *T. versicolor* CCF laccase**

25 26 27 95 **2.5.1. Enzymatic activity determination (laccase, lignin peroxidase, manganese** 28 29 30 96 **peroxidase and xylanase)** 31 32

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34 97 Laccase activities of the CCF were determined by monitoring the oxidation of the
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36 98 substrate 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), to its cation radical, at
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38 99 420 nm ($\epsilon_{420} = 0.36 \text{ L/mol/m}$) (Palmieri et al., 1997) using a Synergy HT micro-well plate
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41 100 reader (Bio-Tek Instruments Inc., Winooski, VT). The assay mixture contained 0.5 mmol/L
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43 101 ABTS and 100 mmol/L citrate-phosphate buffer adjusted to pH 5.2, at ambient
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47 103 is the amount of laccase that formed 1 μmol of ABTS cation radical per min. For further
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49 104 characterization, the relative laccase activities were tested at a range of pH (2.0 to 8.0) and
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51 105 temperatures (10 to 80 °C), using 1.0 mmol guaiacol as substrate. Thermo-stabilities at 30
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53 106 °C, 45 °C and 60 °C were determined by incubating the CCF in 100 mmol citrate phosphate
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buffer at pH 5.2, for 10 h. Laccase stability, at various pH (3.0 to 8.0), was observed by incubating the CCF at 28 °C for 50 h.

Lignin peroxidase activity was determined based on the oxidation of veratryl alcohol to veratraldehyde in the presence of H₂O₂; the increase in absorbance was monitored at 310 nm ($\epsilon_{420} = 9,300 \text{ mol}^{-1} \text{ cm}^{-1}$), using the micro-well plate reader (Kirk et al., 1990). Manganese peroxidase activity was determined by monitoring the formation of manganese (III) malonate complex, at 270 nm, induced by the addition of 0.1 mM H₂O₂; Mn (III) complexes were formed by reacting 50 mM sodium malonate at pH 4.5, with 0.5 mM MnSO₄ (Wariishi et al., 1992). Filtered CCF was used as sample blanks in all the assays. Xylanase activity was determined using oat spelt xylan (1.5% w/v) as substrate, following the protocol of Konig et al., (2002), where the end point was analyzed using DNS (3,5- dinitrosalicylic acid) reagent and the absorbance was measured at 530 nm. In house xylose calibration curves were used to determine the rate of product formation.

2.5.2 Gel electrophoresis

Native-PAGE (polyacrylamide gel electrophoresis) was performed at an alkaline pH under non-denaturing conditions (VWR® Mini Vertical PAGE System, Sugarland, TX). The separating and stacking gels were made of 9% acrylamide, with 50 mmol/L Tris-HCl, at pH 9.5 and 4% acrylamide, with 18 mmol/L Tris-HCl, at pH 7.5, respectively. The gel-loading buffer was made of 25 mmol/L Tris and 190 mmol/L glycine, at pH 8.4. Zymography and visualization of the protein (laccase) bands was achieved by adding 5 mmol/L ABTS to the separating gel, which was submerged in the citrate phosphate buffer (100 mmol/L, pH 5.2) after electrophoresis.

2.6 Effect of CCF on model cellulase and fermentation inhibitors

Stock solutions of 100 mmol/L were prepared by first dissolving the various phenolic compounds, namely, *p*-coumaric acid, *trans*-ferulic acid, gallic acid, 4-hydroxybenzoic acid, salicylic acid, syringaldehyde, *trans*-cinnamic acid, vanillic acid and vanillin, in 10 % (v/v) methanol and then making up the volume with 100 mmol/L citrate phosphate buffer, at pH 5.2. Stock solutions of 50 mmol/L HOBt and ABTS and 2 mmol/L xylo-oligosaccharides, xylobiose (DP2), xylotriose (DP3), xylotetraose (DP4), and xylopentaose (DP5), were also prepared in the citrate phosphate buffer. Sample blanks were prepared by diluting the phenolic stocks to 5 mmol/L and the xylo-oligosaccharide stocks to 0.5 mmol/L. Treatments with CCF were carried out both in the presence and absence of mediators. For each experiment, 10 mmol/L of HOBt was used with the phenolic samples and a mixture of 5 mmol/L ABTS and 5 mmol/L HOBt was used for the xylo-oligosaccharide samples. Commercial laccase and CCF were added to the reaction mixtures such that they all had a uniform laccase activity of 1.2 U/mL. The treatments were carried out in 1.5 mL Eppendorf tubes (Hauppauge, NY) incubated in a forced air incubator (VWR, Chicago, IL) at 28 °C, for 12 h. An orbital shaker (VWR, Chicago, IL) was used to continuously agitate the samples at 100 rpm.

2.7 Laccase treatment of biomass hydrolyzates

Biomass hydrolyzates were prepared by the hot water treatment of rice straw at 220 °C, 54 min and dilute acid treatment of poplar at 160 °C, 30 min, using 1.0 % (v/v) sulfuric acid. Rice straw and poplar wood hydrolyzates have been previously characterized

by Rajan and Carrier (2014) and Frederick et al., (2014), respectively. The liquid hydrolyzates were separated from the pretreated solids, using a Buchner filtration apparatus, fitted with Whatman #1 filter paper and their pH was adjusted to 5.2, using 5 N sodium hydroxide. The neutralized hydrolyzates were centrifuged at $14,000 \times g$ in a MiniSpin® plus centrifuge (Eppendorf, Hamburg, Germany) for 5 min to remove solids. Enzyme treatments of the biomass hydrolyzates were carried out by adding 1.2 U/mL of the *T.versicolor* CCFs and commercial laccase, either in the presence or absence of mediator. A mixture of 5 mmol/L HOBt and 5 mmol/L ABTS in 1:1 volumetric ratio, was used as the mediator. Sample blanks were prepared without any enzyme preparations or mediators. The treatments were carried out in 1.5 mL Eppendorf tubes incubated in a forced air incubator, at 28°C, for 12 h and agitated at 100 rpm, on an orbital shaker.

2.8 Liquid chromatography analysis of various inhibitors

After incubation with the CCF, the samples containing phenolic compounds were analyzed using an Acquity Ultra Performance Liquid Chromatography (UPLC) system equipped with a BEH C₁₈ (1.7 $\mu\text{m} \times 2.1 \text{ mm} \times 50 \text{ mm}$) analytical column and an Acquity VanGuard precolumn (Waters, Milford, MA, U.S.), modified from the method reported by Frederick et al., (2014). The mobile phase consisted of 0.1 % formic acid and methanol, mixed in a gradient of 88.5:11.5 to 30:70, during a run time of 3.5 min. The column was heated to 50 °C and the samples were eluted at a flow rate of 0.4 mL/min and detected using a photodiode array detector. The λ_{max} values varied for different phenolic compounds; 215 nm for HMF, furfural, syringaldehyde and 4-hydroxybenzoic acid, 230 nm for vanillin, vanillic acid and salicylic acid, 270 nm for gallic acid and *trans*-cinnamic acid, 280 nm for

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6 173 *trans*-ferulic acid, and 300 nm for *p*-coumaric acid. Xylo-oligosaccharides were quantified
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8 174 using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA),
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10 175 fitted with a Bio-Rad Aminex-HPX 42A analytical column (Bio-Rad, Hercules, CA) and a
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14 177 Carrier, 2014). The sugars and phenolic compounds were quantified using in-house
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16 178 calibration curves.
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22 179 All experiments were carried out in duplicate. The results were analyzed using
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24 180 Analysis of Variance (ANOVA) and Dunnett's test, in order to determine the significant
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26 181 difference between the control and treatments. In case of laccase treatment of the biomass
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28 182 hydrolyzates, Tukey's test was used to compare all the means. Data analysis was performed
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30 183 using JMP Pro v.11.0 software (SAS, Cary, NC).
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35 184 **3. Results and Discussion**

36 37 38 185 **3.1 Selection and characterization of crude fungal culture filtrates**

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42 186 The five strains of *Trametes versicolor* viz. Aho-23, ERT 201, FP-103969, HBB-
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44 187 2740 and Shigo61-7-3-1 used in this work were isolated from different regions of the
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46 188 United States, and showed diverse levels of expression of laccase. All five strains were
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48 189 prescreened for their lignolytic potential using the RBBR dye-based qualitative assay.
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50 190 Complete decolorization of the plates was observed within 15 days for *T. versicolor* strains
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52 191 FP-103969 and Aho-23, whereas no decolorization was observed for the strains ERT 201
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54 192 and Shigo61-7-3-1. A partial but not complete, decolorization was obtained with *T.*
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56 193 *versicolor* strain HBB-2740. *T. versicolor* strains degraded RBBR dye most likely due to
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the production of extra-cellular lignin-modifying enzymes such as laccase (Glenn and Gold, 1983). To evaluate the strains in the context of laccase production, *T. versicolor* strains Aho 23, FP-103969 and HBB-2740 were selected for culturing in 2% malt extract broth supplemented with 0.1 mmol/L copper sulfate. Addition of copper salts to the culture media has been reported to induce laccase activity in *T. versicolor* (Cordi et al., 2007; Tavares et al., 2005). The culture broth samples were tested for laccase activity, using ABTS as the substrate. The highest laccase activity, of 18 U/mL, was produced by FP-103969 on day 11, followed by Aho-23 and HBB-2740, which were 15 and 12 U/mL on days 14 and 15, respectively, (Fig. 1). Tavares et al., (2005) have reported that supplementation of culture broth with 0.075 mmol/L of copper sulfate resulted in four-fold increase of *T. versicolor* laccase production. The culture broths of Aho 23, FP-103969 and HBB-2740, were also tested for the presence of lignolytic enzymes such as, lignin peroxidase and manganese peroxidase; however, no significant activities were detected (data not shown). It is likely that laccase constituted a major share of the copper induced CCFs. Previous studies have also shown that laccase can single-handedly decolorize the RBBR dye (Wong and Yu, 1999).

The crude culture filtrates (CCFs) of the strains Aho 23, FP-103969 and HBB-2740, were further characterized using gel electrophoresis (Native-PAGE) followed by zymography, using ABTS as the oxidizing agent. The CCF displayed the presence of laccases, but with banding patterns slightly different from those of commercial *T. versicolor* laccase. Dissimilarity in banding patterns indicated that the laccases produced by the three *T. versicolor* strains were composed of possibly different isozymes; variability in

their expression could be due to the presence of unique genes that are acquired by horizontal gene transfer or mutagenesis (Andersen et al., 2011). Based on the laccase production, two strains of *T. versicolor* viz Aho 23, FP-103969 were selected for further studies. The temperature and pH optima of FP-103969 and Aho-23, with regards to laccase activity, was determined to be 60 °C and between 5.0 and 5.5, respectively, as shown in Fig. 2a,b. It was determined that after incubation at 30 °C for 10 h, the loss in laccase activity was 11% and 12% for Aho-23 and FP-103969, respectively (Fig. 2c). It was also determined that the loss of laccase activity was higher at higher temperatures. At 60 °C, at least 97% of relative laccase activity was lost for Aho-23 and FP-103969 (Fig. 2c). The pH stability of various laccase preparations were also studied; at a pH of 5.2 there was only 5% decrease in relative laccase activity for CCF after 50 h of incubation at 28 °C. Therefore, future enzyme treatments were performed at 28 °C, pH of 5.2 and incubation time of 12 h in order to minimize energy input while maintaining CCF laccase activity.

3.2 Effect of *T.versicolor* CCF on model phenolic inhibitors

CCF of FP-103969 and Aho-23, as well as commercial *T. versicolor* laccase, were individually tested for their potential to mitigate model phenolic fermentation inhibitors, such as *p*-coumaric acid, *trans*-ferulic acid, gallic acid, 4-hydroxybenzoic acid, salicylic acid, syringaldehyde, *trans*-cinnamic acid, vanillic acid and vanillin. Ximenes et al., (2011) used tannic, gallic, *trans*-cinnamic, ferulic, *p*-courmaric and sinapic acids, ranging in concentrations between 2 and 15 mg/mL and reported that tannic, gallic and *p*-courmaric acids, inhibited the activity of *Trichoderma reesei* β -glucosidase by at least 65 %. Initial screening conducted with various laccase concentrations showed that 1.2 U/mL of laccase

was optimal for the removal of different phenolic inhibitors. At laccase loadings above 1.2 U/mL, no further decrease was observed in the concentrations of the phenolic compounds. Kolb et al., (2012), working with *T. versicolor* laccase, also observed that lower laccase dosages, such as 0.2 to 0.5 U/mL, were more effective than higher dosages. Therefore, in subsequent studies, all laccase loadings were uniformly maintained at 1.2 U/mL. The use of a mediator (10 mmol/mL HOBt) was tested in order to determine the ideal mitigating conditions for the phenolic inhibitors. Even in the absence of HOBt, the treatment groups exhibited 100% reduction in the concentrations of *p*-coumaric acid, *trans*-ferulic acid, gallic acid, syringaldehyde, vanillic acid and vanillin, as shown in Fig. 3a. Kolb et al., (2012) studied the effect of commercial *T. versicolor* laccase on synthetic phenolic compounds, and showed that *p*-coumaric acid and vanillin were readily degraded by laccase, supporting results presented in Fig. 3a. On the other hand, 4-hydroxybenzoic acid, *trans*-cinnamic acid and salicylic acid, were still detected in the test systems (Fig. 3a). In the presence of HOBt, concentrations of 4-hydroxybenzoic acid and salicylic acid were, on average, reduced by 55 % and 22 %, respectively (Fig. 3b). The phenolic inhibitor, *trans*-cinnamic acid was recalcitrant to all three treatments and there was no significant reduction in its concentrations, with or without HOBt. No significant differences were observed between the use of commercial laccase preparation and CCF from FP-103969 and Aho-23, even with HOBt supplementations (Fig. 3b). Action of laccase is enhanced in the presence of mediators, such as HOBt, because the latter acts as an electron carrier between enzyme and substrate (Fabbrini et al., 2002); however, as they are expensive and can possibly inhibit downstream processing, their use could be omitted. Overall, transformation of phenolics by laccase is dependent on their chemical features; number of hydroxyl groups,

presence of side groups and their position on the aromatic ring (Canfora et al., 2008).

Methoxy groups, via their electron donating capacities, increase the rate of oxidation, while carboxyl moieties, with their electron-withdrawing action, have the opposing effect. The mechanisms proposed by Canfora et al., (2008) could possibly explain the results reported in this work where concentrations of *trans*-ferulic acid, syringaldehyde, vanillic acid and vanillin were reduced, but *trans*-cinnamic acid and salicylic acid were not.

3.3 Effect of *T. versicolor* laccases on lignocellulosic biomass hydrolyzates

The effect of CCFs was also studied on hydrolyzates from poplar wood, produced at 160 °C, 30 min, 1.0 % sulfuric acid, and rice straw, generated in 220 °C water for 54 min. The following phenolic fermentation inhibitors were detected during UPLC analyses in both hydrolyzates: gallic acid, HMF, furfural, 4-hydroxybenzoic acid, vanillic acid, vanillin, syringaldehyde, *p*-coumaric acid, *trans*-ferulic acid, *trans*-cinnamic acid and salicylic acid. During treatment with CCF, concentrations of *trans*-cinnamic acid and 4-hydroxybenzoic acid, which showed only sparse reductions in model systems, were completely removed from biomass hydrolyzates. Although not completely depleted, vanillic acid concentrations in the laccase-mediator treated poplar and rice straw hydrolyzates were reduced by 60 % and 75 %, respectively, as shown in Tables 1 and 2. Similarly, vanillin was totally depleted in poplar hydrolyzates and minimized by 70 % in the rice hydrolyzates. Canfora et al., (2008) reported that laccase transformation on phenolics, when present as mixtures, can be different than that with individual compounds. Highest reduction in the concentrations of phenolic fermentation inhibitors was obtained in the presence of mediators and for FP-103969 *T. versicolor* CCF.

Differential effect of laccases on susceptible substrates, with respect to different biomass has been previously observed in several reports. *T. versicolor* laccase treatments of steam-exploded wheat straw and dilute acid-treated sugarcane bagasse were previously reported to result in 50% and 92% reductions of their respective total phenolic concentration (Chandel et al., 2007; Moreno et al., 2013). Jönsson et al., (1998) reported a 94% decrease of phenolic compounds in softwood hydrolyzates treated with *T. versicolor* laccase. Lee et al., (2012) described a 92% reduction of phenolic cellulase inhibitors, in rice straw treated with *Yarrowia lipolytica* laccase- HOBt mediator system, while Guo et al., (2013) reported a 45% reduction in total phenolic inhibitors when spruce hydrolyzates were treated with 5 g/L of *T. versicolor* laccase. In this study, treatment with CCF supplemented with mediators removed 83 % and 95 % of the target phenolic compounds in rice straw and poplar hydrolyzates, respectively. The use of laccase for removal of phenolics or detoxification has been reported after lyophilization of purified enzymes (Cordi et al., 2007). Commercially available laccases from *Pycnoporus cinnabarinus* (Moreno et al., 2013) were reported to pretreat hydrolyzates; while detoxification work have been described with laccases from *T. versicolor* (Guo et al., 2013; Jönsson et al., 1998), or with partially purified *Cyathus stercoreus* laccase (Chandel et al., 2007). Laccase from *Y. lipolytica* was concentrated by ultrafiltration and used for the pretreatment of rice straw by Lee et al., (2012). None of the reports have used the CCF directly for detoxification.

Previous reports on the treatment of biomass hydrolyzates with fungal laccases showed the removal of phenolic compounds, without affecting the concentration of weak acids and furan derivative (Chandel et al., 2007; Larsson et al., 1999; Moreno et al., 2012).

When treated with the *T. versicolor* CCF, HMF concentrations in poplar hydrolyzates were reduced by 17 % and 25 %, respectively, in the presence and absence of mediators (Table 1). Furfural concentrations of poplar wood hydrolyzates were markedly reduced by 40 % and 27 %, respectively, in the presence and absence of mediators (Table 1). For rice straw, no significant differences were observed in HMF and furfural concentrations of for all treatments (Table 2). Overall, furan modification by fungal laccases has not been reported before. However, it is possible that furan oxidizing enzymes, such as, aryl-alcohol oxidase and unspecific peroxygenases (Carro et al., 2015), were present in the *T. versicolor* CCF; the presence of these enzymes was not tested in this study.

3.4 Effect of CCF on xylo-oligosaccharide model system

Effect of laccase on pure xylo-oligosaccharides was evaluated. Commercially available DP2, DP3, DP4 and DP5 were incubated with FP-103969 or Aho-23 CCF in the presence and absence of mediators. Controls were composed of identical solutions, but without any enzyme or mediator. For the CCF treatments without mediators the concentrations of DP4 and DP5 showed 100 % reduction, DP2 showed 40% reduction and DP3 remained unaffected, as presented in Fig. 4a. There were significant differences between the CCFs and commercial *T.versicolor* laccase in the mitigation of DP4 and DP5. CCF of FP-103969 and Aho-23 were tested for xylanase activity and results showed less than 0.01 U/mL activity, which was deemed unlikely to produce any significant interferences. It was interesting to note that there were no increases in concentrations of lower DPs or monomers, possibly indicating a different underlying mechanism than hydrolysis of β - (1 $\rightarrow$ 4) glycosidic linkages. The performance CCFs did not improve

significantly in the presence of mediator (Fig. 4b), especially for DP2, where the activity of CCFs was reduced in the presence of mediator compared to that of the control. Although laccases have been studied with respect to lignin and its degradation products, few reports provide insights on its role in oxidizing polysaccharides. Laccase-mediated polymerization of wood galacto- and glucomannan, via cross-linking of the aromatic moieties bound to hemicelluloses, has been reported (Oinonen, et al., 2013). Formation of xylo-oligosaccharide polymerization products was not tested in this study.

3.5 Effect of *T.versicolor* laccases on rice straw xylo-oligosaccharides

Hot water hydrolyzates of rice straw also xylo-oligosaccharides. Previous experiments with model xylo-oligosaccharide compounds indicated that CCF of FP-103969 and Aho-23 reduced DP4 and DP5 concentrations. In rice straw hydrolyzates, concentration of DP5 was reduced by 67 % when treated with Aho-23 laccase (Fig. 5). Rice straw hydrolyzate concentrations of DP2 were on an average reduced by 60 % in the presence of both commercial laccase and Aho-23 laccase. There were no significant decreases in DP3 and DP4 concentrations, between the control and treatments. Moreover, there were no significant differences between control and laccase-mediator treatments for all rice straw xylo-oligosaccharides, indicating that the addition of mediators was ineffective against xylo-oligosaccharides.

Xylo-oligosaccharides have been reported to be detrimental to the cellulolytic enzymes viz. endoglucanases, exoglucanases and β -glucosidases. Xylo-oligosaccharides from birchwood xylan were reported to decrease the activity of exoglucanases by 24 to

50 % (Zhang and Viikari, 2012); specifically, 1 mmol/mL of DP2 and DP3 were demonstrated to competitively inhibit cellobiohydrolase-1 from *Thermoascus aurantiacus*. Rajan and Carrier, (2014) also reported 60% and 92% inhibition of cellobiohydrolase-1 and endoglucanase-1, respectively, by rice prehydrolyzates containing mainly xylo-oligosaccharides and phenolics. Supplementation with xylanases has been demonstrated to provide synergistic interactions with cellulases and improve the glucose yields from corn stover hydrolyzates (Kumar and Wyman, 2009). However, there have been no reports on mitigation of xylo-oligosaccharides in biomass hydrolyzates; this work was an attempt to study the effect of laccase on xylo-oligosaccharides abatement.

4. Conclusion

Thus the crude filtrates of *T. versicolor* FP-103969 and Aho-23 cultures were as effective as the commercially purified laccase in achieving significant reduction in the concentrations of model phenolic and cellulolytic inhibitors. Moreover, treatment of poplar and rice straw hydrolyzates with CCF alone, showed 85 % and 46 % reduction in phenolic and xylo-oligosaccharide inhibitors, respectively. Dilute acid hydrolysis of biomass releases inhibitors that inhibit cellulose hydrolyzing enzymes and delay microbial fermentation, thereby decreasing final yields and productivities. Incubation with crude *T. versicolor* culture filtrates can provide a novel strategy for detoxification of biomass hydrolyzates and overall improvement of bioconversion efficiency.

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Figure captions

Fig. 1. Specific activity of laccases in the crude culture filtrates of *T. versicolor* ● HBB-2740, ○ Aho-23 and ▼ FP-103969 grown in 2% malt extract broth in the presence of 0.1 mmol/L CuSO₄ as inducer. Mean and standard deviations were calculated for $n=2$.

Fig. 2. Relative laccase activities of commercial enzyme and crude culture filtrates (CCF) of FP-103969 and Aho-23 when tested at (a) different pH and (b) different

incubation temperatures (°C), using 1.0 mmol guaiacol as substrate. (c) Residual laccase activities of Aho-23 and FP-103969 CCF, at pH 5.2 and at temperatures 30°C, 45°C and 60°C, calculated using 1.0 mmol guaiacol as substrate. For all experiments, mean and standard deviations were calculated for $n=2$.

Fig. 3. Effect of commercial *T. versicolor* laccase & copper-induced crude culture filtrate of *T. versicolor* FP-103969 and Aho-23, on different model phenolic compounds: (a) without mediators and (b) in the presence of 10 mmol/L HOBt. All preparations had a laccase activity of 1.2 U/mL. Controls had no enzyme preparations or mediators. Bars not connected by the same letters were significantly different.

Fig. 4. Effect of commercial *T. versicolor* laccase & copper-induced crude culture filtrate of *T. versicolor* FP-103969 and Aho-23, on different xylo-oligosaccharides: (a) without mediators and (b) in the presence of 5 mmol/L ABTS + 5 mmol/L HOBt. All preparations had a laccase activity of 1.2 U/mL. Controls had no enzyme preparations or mediators. Bars not connected by the same letters were significantly different.

Fig. 5. Effect of commercial *T. versicolor* laccase and copper-induced crude culture filtrate of *T. versicolor* Aho-23 on hot water hydrolyzates of rice straw, quantified using HPLC. Control was the original rice straw hydrolyzate. Bars not connected by the same letters were significantly different.

Fig. 1

Fig. 2a

Fig. 2b

Fig. 2c

Fig. 3a

3b

Fig. 4a

4b

Fig. 5

Table 1. Effect of commercial *T. versicolor* laccase (T1) and crude culture filtrates of *T. versicolor* FP-103969 (T2) and Aho-23 (T3) on poplar hydrolyzates (160°C, 30 min, 1.0% sulfuric acid) in the presence and absence of mediators (5mmol/L ABTS + 5mmol/L HOBt), analyzed using a UPLC

Compounds [#]	Control	No mediator			With Mediator		
		T1	T2	T3	T1	T2	T3
Gallic acid	0.3±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
HMF	1.2±0.1	1.0±0.0 ^a	1.0±0.0 ^a	1.0±0.0 ^a	0.9±0.0 ^b	0.9±0.0 ^b	0.9±0.0 ^b
Furfural	8.3±0.2	5.9±0.8	6.0±0.9	6.1±0.7	4.9±0.7	5.2±0.9	5.0±0.6
4-hydroxybenzoic acid	2.0±0.7	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Vanillic acid	0.9±0.1	0.4±0.0 ^a	0.4±0.0 ^a	0.4±0.0 ^a	0.3±0.0 ^b	0.3±0.0 ^b	0.3±0.0 ^b
Vanillin	1.4±0.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Syringaldehyde	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
<i>trans</i> -Ferulic acid	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Salicylic acid	2.2±0.0	0.0±0.1 ^a	0.0±0.1 ^a	0.1±0.0 ^{ab}	0.0±0.0 ^a	0.0±0.0 ^a	0.1±0.0 ^{ab}

[#] Average concentrations and standard deviations were calculated for *n*=4

^{a,b,ab} The control and treatment groups were significantly different, *p* <0.05. Values denoted by different alphabets were significantly different within the group, *p* <0.05

Table 2. Effect of commercial *T. versicolor* laccase (T1) and crude culture filtrates of *T. versicolor* FP-103969 (T2) and Aho-23 (T3) on rice straw hydrolyzates (220°C, 54 min) in the presence and absence of mediators (5mmol/L ABTS + 5mmol/L HOBt), analyzed using a UPLC

Compounds [#]	Control	No mediator			With Mediator		
		T1	T2	T3	T1	T2	T3
Gallic acid	0.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
HMF	1.1±0.2	1.0±0.0	1.0±0.1	1.0±0.0	0.9±0.1	0.9±0.1	0.9±0.1
Furfural	0.5±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0	0.5±0.0
Vanillic acid	0.4±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.1±0.1	0.1±0.1
Vanillin	1.0±0.7	0.3±0.2	0.3±0.2	0.3±0.1	0.3±0.2	0.3±0.2	0.3±0.2
Syringaldehyde	n.d	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
<i>p</i> -Coumaric acid	0.4±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
<i>trans</i> -Ferulic acid	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Salicylic acid	1.0±0.5	0.2±0.1	0.2±0.1	0.2±0.1	0.0±0.0 ^a	0.1±0.0 ^b	0.2±0.1
<i>trans</i> -Cinnamic acid	n.d.	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[#] Average concentrations and standard deviations were calculated for *n*=4

^{a,b,c} The control and treatments were significantly different, *p* <0.05

n.d.- Below reportable limits