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Fractionation of cellulase and fermentation inhibitors from steam pretreated mixed hardwood

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HIGHLIGHTS

- Steam pretreatment of hardwoods releases lignin-derived phenolic inhibitors.
- Washing of pretreated solids with water removes inhibitors (sugar oligomers, phenolics).
- Hot water (90 °C) recovers more hydrophobic and inhibitory phenolics than cold water (25 °C).
- Phenolic inhibitors act on both enzymes and fermentation microorganisms.
- Inhibition is decreased through fractionation of the inhibitors using XAD-7 or activated carbon.

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ABSTRACT

The purpose of liquid hot water and steam pretreatment of wood is to fractionate hemicelluloses, partially solubilize lignin, and enhance enzyme hydrolysis of cellulose. The pretreatment also solubilizes sugar oligomers, lignin-derived phenolic compounds, acetic acid, and furan derivatives that inhibit cellulase enzymes and/or impede fermentation of hydrolysates by yeasts. This work extends knowledge of the relative contribution of identified inhibitors, and the effect of temperature on their release when pretreated materials are washed and filtered with hot water. Dramatic yield improvements occur when polymeric or activated carbon adsorbs and removes inhibitors. By desorbing, recovering, and characterizing adsorbed molecules we found phenolic compounds were strong inhibitors of enzyme hydrolysis and fermentation of concentrated filtrates by *Saccharomyces cerevisiae* wine yeast NRRL Y-1536 or xylose fermenting yeast 424A (LNH-ST). These data show that separation of inhibitors from pretreatment liquid will be important in achieving maximal enzyme activity and efficient fermentations.

1. Introduction

Hydrothermal pretreatment releases soluble inhibitors that hamper enzymatic and microbial conversion of lignocellulose. The pentose sugars in the filtrates can be converted to fuels and various high-value chemicals, either separately or when fermented in combination with pretreated cellulose. Both inhibitors and sugar oligomers are present after pretreatment. For purposes of this study the pretreated hardwood was washed with water which not only removed inhibitors, but also diluted the soluble components that are recovered in the filtrate. Due to the dilute concentration of oligomers, the filtrate was reconcentrated by evaporation to obtain the higher sugar or ethanol concentrations upon hydrolysis and fermentation. However, compounds that are inhibitory and toxic to enzymes and microorganisms are also concentrated.

The soluble inhibitors include sugars (xylose, xylo-oligomers), furan derivatives (hydroxymethyl furfural, furfural), organic acids (acetic, formic, levulinic acids), and lignin derivatives (poly- and mono-phenolic compounds) (Kim et al., 2011; Palmqvist et al., 1996; Qing et al., 2010; Ximenes et al., 2011, 2010). The washing and filtration of pretreated lignocellulose with hot water improves enzymatic digestibility of the cellulose by removing the inhibitors (Kim et al., 2011, 2009, 2006; Nagle et al., 2002). Nagle et al. (2002) reported that the washing of pretreated poplar above the lignin's glass transition temperature (130–150 °C) improved cellulose digestibility compared to unwashed slurry by decreasing lignin re-deposition to cellulose. Kim et al. (2009) showed that washing pretreated poplar with water at 80–90 °C increases glucose yield

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upon enzyme hydrolysis by 20% compared to solids washed with water at 25 °C.

Recently, studies were directed to identifying the soluble inhibitors in the pre-hydrolysate of aqueous pretreated lignocellulose and understanding the inhibition mechanisms, especially for lignin-derived phenolic compounds (Kim et al., 2011; Ximenes et al., 2011, 2010). Poly- and mono-phenolic acids, such as tannic and gallic acids, and other lignin constituents significantly inhibit cellulases by both reversible and irreversible inhibition (Kim et al., 2011; Ximenes et al., 2011; Vohra et al., 1980).

This work examines the release of cellulase and fermentation inhibitors when pretreated mixed hardwood is stirred in water at either 90 °C or 25 °C and filtered. We identified and compared inhibitors in washates generated at the two different temperatures and measured their inhibitory effects on cellulases and yeasts. Adsorptive removal of these inhibitors using Amberlite XAD resins and activated carbon showed increased hydrolysis of cellulose and fermentation of xylose to ethanol. Methods to separate, recover, and characterize the phenolic compounds from the spent adsorbents are also presented. This study identified inhibitors of enzyme and fermentation processes and gave insights into strategies for reducing soluble inhibitors that affect biochemical conversion of lignocellulose into sugars and ethanol.

2. Methods

2.1. Materials

Steam pretreated mixed hardwood (195 °C/10 min, severity factor = 3.8) was provided by Mascoma Corporation (Lebanon, NH). The material was damp, fibrous, fluffy, and non-flowable. Hot and cold washate concentrates identified as HW1 and CW1, respectively, of the pretreated mixed hardwood were prepared separately in our laboratory as described in the subsequent section. Corresponding filtrates (HW2, CW2) obtained by washing at 85–90 °C and 40 °C and then concentrating by evaporation were also provided by Mascoma Corp.

Amberlite™ XAD-4 and XAD-7 resins, activated carbon, and all the other reagents and chemicals, unless otherwise noted, were purchased from Sigma–Aldrich (St. Louis, MO). Enzymes were from Novozyme. The *Saccharomyces cerevisiae* wine yeast NRRL Y-1536 strain was provided by USDA-ARS (Peoria, IL) through Dr. Eduardo Ximenes and the xylose-cofermenting yeast, 424A (LNH-ST) (Sedlak and Ho, 2004) was provided by Dr. Nancy Ho from Purdue University.

2.2. Post-pretreatment washing (laboratory procedure)

The steam pretreated mixed hardwood as received was well mixed in 25 °C (cold) or 90 °C (hot) de-ionized water at 5% w/w dry solids. After 10 min of mixing, the slurry was filtered through a filter cloth and then Whatman No. 1 filter paper to remove any particulates. The resulting filtrate was concentrated tenfold using a rotary evaporator (Rotavapor® R-210/R-215, BÜCHI Labortechnik AG, Switzerland) at 40 °C under vacuum. The cold and hot filtrate concentrates prepared in the lab were denoted as CW1 and HW1, respectively, throughout this paper. Corresponding washed and filtered solids were denoted as PCS and PHS. The hot and cold filtrate concentrates from Mascoma were denoted as HW2 and CW2.

2.3. Removal of phenolic compounds from the washate concentrates

Activated carbon or Amberlite™ XAD-7 resin was used to remove phenolic compounds from the washate concentrates. Prior to use, the activated carbon and resins were washed with an excess amount of de-ionized water and isopropyl alcohol, and then air-dried. The washed adsorbents were added to filtrate concentrates at 10% w/v and mixed for 4 h at 250 rpm in a shaking incubator at room temperature. The washate concentrates before and after the resin treatment were analyzed for compositions using NREL LAP-014 procedure (Sluiter et al., NREL, LAP014). The total phenolics concentration was measured using Folin–Ciocalteu method and expressed as the equivalent gallic acid amount (Makkar, 2004). All measurements were made in triplicate.

The spent XAD-7 resin was filtered through Whatman No.1 filter paper. The resin was then washed with 3 v of de-ionized water. After washing with water, a sequence of three different solvents, in order of increasing hydrophobicity (decreasing polarity), was used to desorb phenolics from the resin for purposes of further study. First, the spent XAD-7 resin was mixed in 50% v/v methanol/water mixture at 10% w/v (resin dry wt/solvent volume), incubated for 4 h at 250 rpm at room temperature, and filtered through Whatman No.1 filter paper. The spent XAD-7 resin, desorbed with 50% v/v methanol as described above, was then mixed in 100% acetone at 10% w/v for the second round of desorption and filtration, followed by desorption with 100% chloroform at 10% w/v to desorb any residual phenolics. The flasks containing phenolics recovered in each solvent were covered in aluminum foil and stored in a freezer until use.

Different volumes (0.25–8.0 mL) of phenolics recovered in 50% methanol were dried using a Speedvac® concentrator (Thermo scientific, Waltham, MA) at 40 °C and re-dissolved in pH 4.8 citrate buffer to give different levels of phenolics. The same volumes (0.25–8.0 mL) of phenolics recovered in 100% acetone were also dried following the same procedure. The dried phenolics recovered in acetone were re-dissolved in 6.25% aqueous acetone instead of pH 4.8 buffer because they were only partially soluble in the buffer.

2.4. Enzymatic hydrolysis

Enzymes: Cellic™ Ctec (94 FPU/mL, 154 mg protein/mL) and Cellic ™ Ctec 2 (120 FPU/mL, 514 mg protein/mL) were donated from Novozyme, North America Inc. (Franklinton, NC). Protein concentration of enzyme was determined by total nitrogen analysis after trichloroacetic acid (TCA) precipitation as described by Hames (1981). All hydrolyses were performed in triplicate at 50 °C, 800 rpm using a shaking incubator (Vortemp™ 56 shaking incubator, Labnet International, Inc., Edison, NJ) for 72 h.

Avicel® PH101 in presence of washate concentrates: the effect of inhibitors in the cold and hot wash liquids (HW1, CW1) was examined using Avicel® PH101 as a substrate. Avicel® PH101 mixed in pH 4.8 citrate buffer at 1% w/w loading was hydrolyzed using Cellic™ Ctec at 30 FPU/g cellulose (=50 mg protein/g cellulose) in the presence of various volume ratios of the washate concentrates. The pH was adjusted to 4.8 using ammonium hydroxide for all hydrolysis runs.

Avicel® PH101 in presence of fractionated phenolics: the phenolics were recovered in 50% methanol and 100% acetone from the spent XAD-7 resin as described in Section 2.3. These fractions were tested for their inhibitory effects on cellulase using Avicel® PH101 as a substrate. Avicel was added to the buffer solutions containing the re-dissolved phenolics at 1% w/v cellulose loading. Cellic™ Ctec was added to the mixture at 30 FPU/g cellulose (=50 mg protein/g cellulose). Control runs used the same buffer or solvents without phenolics.

Pretreated, washed solids: the hot or cold washed solids were mixed in pH 4.8 citrate buffer and enzymatically hydrolyzed at 1% dry solids using Cellic™ Ctec2 for 72 h at various cellulase loadings (10–130 mg protein/g cellulose).