Biological abatement of cellulase inhibitors

Guangli Cao a,c, Eduardo Ximenes a, Nancy N. Nichols b, Leyu Zhang c, Michael Ladisch a,*

Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN 47907-2032, United States
Bioenergy Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, USDA, 1815 N. University Street, Peoria, IL 61604, United States
Department of Life Science and Technology, Harbin Institute of Technology, Harbin 150001, China

HIGHLIGHTS

• Bio-abatement removes enzyme inhibitors from biomass liquors.
• Cellulose conversion in bio-abated vs. non-abated liquors increased 1.2 to 1.5-fold.
• Bio-abatement gives similar improvement in cellulose conversion as overliming.
• Bio-abatement is a promising method to detoxify lignocellulosic slurries.

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ABSTRACT

Removal of enzyme inhibitors released during lignocellulose pretreatment is essential for economically feasible biofuel production. We tested bio-abatement to mitigate enzyme inhibitor effects observed in corn stover liquors after pretreatment with either dilute acid or liquid hot water at 10% (w/v) solids. Bio-abatement of liquors was followed by enzymatic hydrolysis of cellulose. To distinguish between inhibitor effects on enzymes and recalcitrance of the substrate, pretreated corn stover solids were removed and replaced with 1% (w/v) Solka Floc. Cellulose conversion in the presence of bio-abated liquors from dilute acid pretreatment was 8.6% (0.1x enzyme) and 16% (1x enzyme) higher than control (non-abated) samples. In the presence of bio-abated liquor from liquid hot water pretreated corn stover, 10% (0.1x enzyme) and 13% (1x enzyme) higher cellulose conversion was obtained compared to control. Bio-abatement yielded improved enzyme hydrolysis in the same range as that obtained using a chemical (overliming) method for mitigating inhibitors.

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1. Introduction

Agricultural crop-based raw materials such as corn stover and sugar cane bagasse are potential commercial feedstocks for production of biofuels. However, due to the recalcitrant nature of lignocellulose, a pretreatment step is required to deconstruct the complex cell wall structure and expose cellulose to cellulolytic enzymes (Hendriks and Zeeman, 2009; Mosier et al., 2005). On the other hand, lignocellulose pretreatment has the detrimental effect of also releasing a wide range of compounds, such as phenolics, acetate, and furan aldehydes, which are inhibitory to fermenting microorganisms and cellulolytic enzymes (Palmqvist et al., 1996; Ximenes et al., 2011, 2010). Hence, the removal of those inhibitors prior to saccharification and fermentation is essential for economically feasible biofuel production from lignocellulosic biomass.

Several physical and chemical methods including dilution, adsorption, and precipitation have been developed to reduce the impact of inhibitory compounds on enzymatic and fermentation processes (Larsson et al., 1999; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000). The drawbacks of these methods are high cost and generation of additional waste streams. Biological abatement (or bio-abatement), using microorganisms to metabolize inhibitors, is a potentially effective and cost-efficient method to eliminate these undesired compounds from lignocellulosic liquors (López et al., 2004; Nichols et al., 2010, 2008, 2005). A fungus, Coniochaeta ligniaria NRR30616, was identified as a promising candidate due to its tolerance of and ability to metabolize these inhibitors (López et al., 2004). This strain of C. ligniaria, which displays yeast-like growth in liquid cultures, utilizes a number of inhibitory compounds such as acetate, phenolic compounds, furfural and hydroxymethylfurfural (HMF) as sources of carbon and energy. When liquors are conditioned (bio-abated) by growth of...
C. ligniaria prior to inoculation with the fermenting microbe, ethanol fermentation efficiency is improved (Nichols et al., 2005). Bio-abatement eliminated extended fermentation lag times associated with the presence of inhibitory compounds, and resulted in improved metabolism of pentoses by a recombinant bacterial strain, *E. coli* FBR5 (Nichols et al., 2008, 2010).

It is well known that the enzymes used in the hydrolysis step are a major contributor to the total cost of producing ethanol from biomass (Nguyen and Saddler, 1991; Tengborg et al., 2001). Therefore, it is critical to minimize enzyme usage, while maintaining the efficiency of enzymatic hydrolysis. Some of the fermentation inhibitors, especially phenolic compounds, which are metabolized by *C. ligniaria*, have also been shown to be strong enzyme inhibitors and/or deactivators (Kim et al., 2011; Ximenes et al., 2010, 2011), hence motivating the study of the possible beneficial effect of bio-abatement on improving enzyme performance by alleviating inhibitor and/or deactivator effects. A clear elucidation of the relationship between enzymatic hydrolysis and bio-abatement may contribute to a rational design of economic biomass-to-biofuels processes with reduced usage of cellulosic enzymes.

The aim of the present study was to investigate the effect of bio-abatement by *C. ligniaria* NRRL30616 on enzymatic conversion of cellulose in dilute acid- (DA) and liquid hot water- (LHW) pretreated corn stover liquors (Kim et al., 2009; Ladisch et al., 1998; Zeng et al., 2012a,b). Metabolism and removal of potential enzyme and fermentation inhibitors including acetate, furan aldehydes, and phenolic compounds from those liquors by bio-abatement was characterized. In addition to the well-known effect of end products (e.g., glucose and xylose for cellulose and xylan, respectively), hemicellulose products (mainly xylo-oligosacharides, especially for LHW pre-treatment) and phenols are major inhibitors generated during pretreatment processes (Ximenes et al., 2010; Qing et al., 2010). Enzymatic hydrolysis of cellulose in corn stover liquors was evaluated at two enzyme loading rates, and bio-abated samples were compared to those treated by overliming, a chemical method commonly used to detoxify microbial fermentation inhibitors (Purwadi et al., 2004; Saha et al., 2005; Stoutenburg et al., 2011).

### 2. Methods

#### 2.1. Materials

Corn stover was harvested in central Illinois, air-dried to 5–8% moisture, and milled (Model 4 Wiley, Thomas Scientific, Swedesboro, NJ) to pass through a 2 mm screen. The stover comprised (dry basis) 36.8% glucan, 23.6% xylan, 1.9% galactan, 3.8% arabinan, 18.9% total lignin, and 5.0% ash. Solka Floc® 300 FCC was purchased from International Fiber Corporation (Urbana, Ohio). Spezyme CP (cellulase) was provided by Genencor, a Danisco Division (Palo Alto, CA). Novozyme 188 (β-Glucosidase, Novo Nordisk, Novo Allé, Denmark) was purchased from Sigma (Cat. No. C6150). Spezyme CP and Novozyme 188 contain 82 mg protein/ml (50 FPU/ml) and 152 mg protein/ml (515 CBU/ml), respectively (Ximenes et al., 2011). All other reagents and chemicals were purchased from Sigma Aldrich (St. Louis, MO).

#### 2.2. Preparation of corn stover prehydrolysates

DA-pretreated slurries of ground corn stover were prepared in a rotating stainless steel reactor system using infrared heating (da Cruz et al., 2012) (Labomat BFA-12 V200, Werner Mathis, Concord, NC). Samples (10 g biomass in 100 ml 0.7% (v/v) H2SO4) were incubated at 150 °C for 15 min with heating and cooling times of approximately 35 and 20 min, respectively. LHW-pretreated slurries of ground corn stover were prepared using 10 g biomass in 100 ml water. Samples were heated to 190 °C and incubated for 15 min, with heating and cooling times of approximately 45 and 30 min., respectively. For both methods, samples were rotated at 50 rpm during pretreatment to ensure adequate mixing. Solids were removed by centrifugation (20 min, 25 °C, 15,000g) and washed with a 10% volume of sterile water. After the supernatant was combined with the wash liquid, the pH was adjusted to 6.5 with Ca(OH)2 and filter-sterilized (0.2 μm pore size) prior to use.

#### 2.3. Microorganism and growth conditions

*C. ligniaria* NRRL30616 was isolated from furfural-contaminated soil (López et al., 2004). It was maintained in defined mineral medium (25 mM each KH2PO4 and Na2HPO4, 0.1% (NH4)2SO4, 0.1% Hutner mineral base) (Gerhardt et al., 1981) (final pH 6.8) containing 10 mM furfural as sole source of carbon and energy. Inocula for bio-abatement were grown in YPD medium (10 g/l yeast extract, 20 g/L peptone, and 20 g/L glucose) overnight at 30 °C, with aeration by shaking.

#### 2.4. Bio-abatement and overliming of corn stover liquors

For bio-abatement of enzyme and fermentation inhibitors, a 10% (v/v) inoculum of *C. ligniaria* NRRL30616 cells was washed and added to filter-sterilized liquors and incubated for 24 or 48 h with agitation (225 rpm) at 30 °C in an Innova 4230 incubator shaker. For each experiment, a non-inoculated (non-abated) control was incubated at 30 °C under the same conditions. Before biomass liquors were used for enzymatic hydrolysis, they were subjected to centrifugation (10 min, 25 °C, 4000g) to remove *C. ligniaria* cells from bio-abated samples, and the pH was adjusted to 4.8 using 2 M HCl.

For chemical mitigation of inhibitors, samples of DA and LHW-pretreated liquors were “overlimed” by adjusting the pH to 10.5 using Ca(OH)2 (Purwadi et al., 2004; Saha et al., 2005; Stoutenburg et al., 2011). Samples were heated for 30 min at 90 °C in rotating stainless steel reactors as described above, then cooled to room temperature. The pH of samples was reduced to 6.5 with H2SO4 prior to filtration, then to 4.8 with 2 M HCl prior to enzymatic hydrolysis.

#### 2.5. Enzymatic hydrolysis

Solka Floc, containing 80% glucan and 20% xylan, was used as the cellulose substrate. Enzymatic hydrolysis experiments were performed at cellulose (Solka Floc) concentration of 1% (w/w) suspended in DA- or LHW-pretreated liquors, with Spezyme CP (1.5 FPU/g glucan, corresponding to 2.6 mg protein/g glucan, or 15 FPU/g glucan, corresponding to 26 mg protein/g glucan) and Novozyme 188 (3.0 CBU/g glucan, corresponding to 0.64 mg protein/g glucan, or 30 CBU/g glucan, corresponding to 6.4 mg protein/g glucan). Prior to the enzymatic hydrolysis assay, liquors were placed in boiling water for 5 min. to avoid the possible influence on cellulose conversion of enzymes produced by *C. ligniaria* NRRL30616 during bio-abatement. As a positive control, enzymatic hydrolysis of Solka Floc cellulose was also performed in 50 mM sodium acetate buffer, pH 4.8. In all cases, enzymatic hydrolysis was carried out at pH 4.8–5.0, 50 °C, with a final reaction volume of 1 ml incubated in a shaker at 250 RPM. Each experiment was performed in triplicate. For 3.2 (0.1x) and 32 (1x) mg protein/g glucan loadings, the hydrolysis time was 168 and 72 h, respectively. Samples were taken at timed intervals for analysis by HPLC.
2.6. Analytical methods

Pretreated samples were analyzed for sugar and acetate content using a Bio-Rad Aminex HPX-87H ion exchange column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA) connected to a Milton Roy mini pump (Milton Roy Co., Ilyland, PA), Waters™ 717 plus autosampler, and Waters™ 2414 refractive index detector (Waters Corp., Milford, MA). The data were stored and processed using Empower™ 2 Chromatography Data Software (Waters Corp., Milford, MA). The mobile phase was 5 mM sulfuric acid in distilled, de-ionized sterile water. The mobile phase flow rate was 0.6 mL/min. The column temperature was maintained at 60 °C (Eppendorf CH-30 Column Heater controlled by an Eppendorf TC-50 (Eppendorf, Westbury, NY)). Oligomeric sugars were calculated by subtracting free sugars from sugars liberated by trifluoroacetic acid treatment (Dien et al., 1997). Furfural and HMF concentrations were measured using reverse-phase HPLC with ultraviolet detection at 277 nm as described by López et al. (2004). Total concentration of phenolics in samples was determined by spectrophotometric analysis at 280 nm (Somers and Ziemelis, 1985).

3. Results and discussion

3.1. Detoxification by bio-abatement

C. ligniaria NRRL30616 efficiently detoxifies dilute acid-pretreated biomass liquors prior to fermentation. Previous studies showed effective removal, via metabolism, of numerous inhibitory compounds including acetate, furfural, hydroxymethyl furfural (HMF), and aromatic compounds, which were converted to cell mass or reduced to less-toxic intermediates (Nichols et al., 2005, 2008, 2010). Bio-abatement effectively detoxified liquors of corn stover and other bioenergy feedstocks, enabling more efficient fermentation using either conventional or engineered S. cerevisiae or ethanologenic E. coli. However, the utility of bio-abatement to relieve inhibition of biomass-degrading enzymes had not been examined.

The amount and type of inhibitory compounds varies in liquors with the pretreatment method. Therefore, in this work, bio-abatement was performed on liquors prepared from both DA and LHW pretreatments of 10% (w/v) corn stover to evaluate the application of C. ligniaria NRRL30616 for conditioning liquors prior to enzyme hydrolysis. Inhibitors generated during these pretreatments included furan aldehydes (furfural and HMF) and aromatic compounds, which were converted to cell mass or reduced to less-toxic intermediates (Nichols et al., 2005, 2008, 2010). Bio-abatement effectively detoxified liquors of corn stover and other bioenergy feedstocks, enabling more efficient fermentation using either conventional or engineered S. cerevisiae or ethanologenic E. coli. However, the utility of bio-abatement to relieve inhibition of biomass-degrading enzymes had not been examined.

As shown in Table 1, the inhibitory compounds were substantially or completely removed in bio-abated (for 48 h) DA liquor compared to the control (non-bio-abated samples). In these samples, inhibitor concentrations decreased a little or were unchanged in liquor bio-abated for 24 h. By 48 h, nearly all acetate was removed, and the concentrations of HMF, furfural, and phenolic compounds were decreased by more than 50%. For bio-abated LHW liquors, bio-abatement also removed a substantial amount of acetate (>95%), as well as HMF, furfural, and phenolic compounds (>65%) from liquor bio-abated for 48 h. Acetate was removed more quickly and completely from DA liquors compared to LHW liquors. Our results clearly indicate the potential use of bio-abatement as a detoxification method, as illustrated by major reduction of well-known enzyme and fermentation inhibitors from DA and LHW liquors (Table 1). Similar results regarding bio-abatement have been published previously for DA-pretreated biomass (Nichols et al., 2005, 2008, 2010), but this had not previously been examined for LHW-pretreated biomass. Compared to our results using bio-abatement, less removal of acetic acid (unchanged), furans and phenolic compounds (45.7%) were obtained when using overliming (Table 1), a widely used method for detoxification in which inhibitors are precipitated and/or converted to less toxic compounds as a result of alkali treatment (Jönsson et al., 2013; Musiatto and Roberto, 2004; Persson et al., 2002).

Although the bio-abatement procedure efficiently removed large quantities of inhibitory compounds from DA and LHW liquors, loss of sugar monomers generated during pretreatment was also observed, as is the case for other methods of detoxification such as overliming and ion exchange (Nichols et al., 2010; Sárvári Horváth et al., 2005). In bio-abatement, monitoring and control of inhibitor and sugar concentrations during growth of the bio-abatement microbe may lead to an efficient balance between mitigation of inhibitory substances and conservation of fermentable sugars in the liquors.

3.2. Effect of bio-abatement on enzymatic hydrolysis of cellulose

The influence of bio-abated liquor on cellulose conversion during enzymatic hydrolysis with a dosage of 15 FPU/g glucan (26 mg protein/g glucan) cellulase (Spezyme CP) and 30 CBU/g glucan (6.4 mg protein/g glucan) β-Glucosidase (Novozyme 188) is shown in Fig. 1. In these experiments, biomass solids were removed from liquor and replaced with a defined substrate (Solka Floc, 1% w/v) to compare cellulase inhibition in liquor that had been bio-abated to mitigate inhibitors, to inhibition in non-abated liquor. This approach was selected to give a clear reading of the effect of inhibitors, isolated from the residual but inherent recalcitrance of...
pretreated lignocellulosic biomass. The conversion of cellulose into glucose in buffer (a control to determine enzymatic activity on Solka Floc in the absence of inhibitors) was 80.5% after 72 h hydrolysis. When the pretreatment liquid prepared from DA-treated corn stover without inhibitor abatement replaced the buffer, glucose conversion was reduced to 54.2% and 58.1% after 72 h hydrolysis for non-bio-abated samples (controls incubated for 24 h and 48 h, respectively). An increase in cellulose conversion was observed for bio-abated liquors from DA-pretreated corn stover (Fig. 1A). When inhibitors were removed by bio-abatement for 48 h, cellulose conversion increased to 70.2% in DA liquor (Fig. 1A). The improvement in cellulose conversion with decreased inhibitory concentrations for bio-abated liquor may be attributed to reduced formation of enzyme-inhibitor complexes (Jing et al., 2009; Rahikainen et al., 2013) in bio-abated compared to untreated liquors.

Similar to the result observed with DA liquors, improved cellulose conversion was also observed for bio-abatement of LHW liquors. After 48 h incubation at 50 °C in bio-abated LHW liquors at the same enzyme loadings, an increase in cellulose conversion (Fig. 1B) was observed compared to non-bio-abated samples due to the 77% removal of phenolic enzyme inhibitors, previously identified as major enzyme inhibitors and/or deactivators (Kim et al., 2011; Ximenes et al., 2010, 2011). Ximenes et al. (2011) studied the inhibition effects by measurements combining the inhibitors (phenols) with enzyme and substrate immediately at the beginning of the assay. The deactivation effects were determined by pre-incubating phenols with cellulases or β-Glucosidases for specified periods of time, prior to the respective enzyme assays. They found that the strength of the inhibition or deactivation effect depended on the type of enzyme, the microorganism from which the enzyme was derived, and the type of phenolic compounds present. From the enzyme tested, β-Glucosidases were most affected by the phenolic compounds.

Although the LHW pretreatment method is generally considered to generate fewer fermentation inhibitors when compared to DA pretreatment, the inhibitory effect on enzymes was equally or more severe in LHW than in DA liquors. Since the inhibitory compounds found in the DA and LHW liquors (Table 1) after bio-abatement were at a similar level, except for the higher level of xylo-oligosaccharides, their presence is one possible reason for this stronger inhibitory effect. Xylo-oligosaccharides have been reported to be strong inhibitors of cellulase activity (Kumar and Wyman, 2009; Qing et al., 2010). Kim reported that at a concentration of xylo-oligosaccharides of 8 g/L, the cellulose conversion rate decreased by approximately 40% and glucose yield was reduced by 20% (Kim et al., 2011). Because LHW pretreatment produces xylo-oligomers rather than exclusively xylose monomers (Table 1), xylanase, β-xylosidase or a combination of...
both is needed to generate xylose monomers for subsequent fermentation, and may also relieve some of the cellulase inhibition observed in LHW liquors. *C. ligniaria* NRRL30616, a strain originally isolated for its ability to metabolize traditional fermentation inhibitors (López et al., 2004) also produces xylanase activity (López et al., 2007). However, in the experiments described here, the strain was not grown under specific conditions to induce xylanase production, and removal of xylo-oligomers was not observed during the bio-abatement incubations (Table 1). Synergistic addition of xylanase activity, either produced by the bio-abatement microbe or added as an enzyme cocktail to the cellulase and β-Glucosidase used in these experiments, may further improve cellulose hydrolysis in LHW-pretreated liquors.

### 3.3. Effect of bio-abatement on cellulose conversion at reduced enzyme dosage

The bio-abated DA liquor promoted significant saccharification of Solka Floc at 0.1x cellulosic activity (1.5 FPU/g glucan or 2.6 mg protein/g glucan and 3.0 CBU/g glucan) cellulase (Spezyme CP) and 3.0 CBU/g glucan (0.64 mg protein/g glucan) β-Glucosidase (Novozyme 188), with glucose conversion reaching 42.0% within 120 h, whereas conversion of non-bio-abated samples only reached 33.2% over the same incubation time period (Fig. 2A). Improved enzymatic hydrolysis (10.3% higher cellulose conversion to glucose) in bio-abated LHW liquor compared to non-bio-abated samples also occurred (Fig. 2B).

The effectiveness of enzymatic hydrolysis correlates with the dosage of enzyme for hydrolysis in both DA and LHW liquors. In other words, to achieve high overall conversion of cellulose even with lower inhibition levels, elevated enzyme dosage is still needed. The reduction of the contribution of enzymes to biofuel
production costs begins to approach a feasible range when the commercial enzyme loading of 1.5 FPU/g glucan cellulase and 3.0 CBU/g glucan β-Glucosidase is effective (Chen et al., 2012; Nguyen and Saddler, 1991).

3.4. Comparison of bio-abatement versus lime-treatment

The advantages of biological abatement compared with existing detoxification methods include no requirement for added chemicals, suitability for treating liquid–solid mixtures, and minimal generation of waste streams. Bio-abatement, using a microorganism selected for inhibitor tolerance, compares favorably to an alternate method for mitigating fermentation inhibitors (Nichols et al., 2010). To evaluate the relevance of bio-abatement for mitigating enzymatic inhibitors, the results described here for bio-abatement of corn stover liquors were compared with overliming. Fig. 3 shows the effect of reduced inhibitory compounds resulting from bio-abatement and overliming on enzymatic hydrolysis. In general, the two methods of inhibitor abatement were roughly equivalent. There was a concomitant increase in the cellulose conversion in detoxified liquors at both 0.1x and 1x enzyme dosages. As seen in Fig. 3, when using bio-abated versus non-bio-abated DA corn stover liquor, about 8.6% and 16% increases in cellulose conversion were obtained at 0.1x and 1x enzyme dosages, respectively. Similar improvement in cellulose conversion after bio-abatement and overliming at 0.1x and 1x enzyme dosages were also observed in LHW corn stover liquors (Fig. 3B).

The experiments described here were designed to determine the effect of inhibitor abatement on hydrolysis of cellulose, separate from the effects of residual recalcitrance that remain after the corn stover was pretreated using either dilute acid or liquid hot water. By removing the corn stover solids after pretreatment and replacing with a cellulose substrate (Sokla Floc), we clearly see the effect of inhibitors, and also that the inhibitors for the two pretreatments are different, with LHW being more inhibitory than DA. Since the cellulose used in the two sets of runs is the same, the difference is not due to difference in reactivity due to different pretreatments, but rather the inhibitors. This had not been shown previously. Bio-abatement is shown to be another approach for detoxification of lignocellulosic slurries.

4. Conclusions

Bio-abatement removes compounds inhibitory to enzymatic hydrolysis. The relative conversion of cellulose in bio-abated vs. non-abated liquors varied with hydrolysis time, but ranged from 1.21- to 1.47-fold increase in LHW liquor at high and low enzyme loadings, respectively. In DA liquors, relative conversion was 1.32 at high enzyme loading and 1.26 at low enzyme loading. This work further confirms the detrimental effects of phenolics on enzyme inhibition and also describes conditions of bio-abatement that remove them.

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