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Temperature dependent cellulase adsorption on lignin from sugarcane bagasse

Ariane Zanchetta[a,b], Antonio Carlos Freitas dos Santos[b,c], Eduardo Ximenes[b,c], Christiane da Costa Carreira Nunes[a], Maurício Boscolo[a], Eleni Gomes[a], Michael R. Ladisch[b,c,*]

[a] *Sao Paulo State University-Unesp, IBILCE, São José do Rio Preto, São Paulo, Brazil*
[b] *Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN, USA*
[c] *Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN, USA*

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ABSTRACT

Extents of adsorption of cellulolytic enzymes on lignin, derived from sugarcane bagasse, were an inverse function of incubation temperature and varied with type of lignin extraction. At 45 °C, lignin derived from acid hydrolyzed liquid hot water pretreated bagasse completely adsorbed cellulolytic enzymes from *Trichoderma reesei* within 90 min. Lignin derived from enzyme hydrolyzed liquid hot water pretreated bagasse adsorbed only 60% of *T. reesei* endoglucanase, exoglucanase and β-glucosidase activities. β-Glucosidase from *Aspergillus niger* was not adsorbed. At 30 °C, adsorption of all of the enzymes was minimal and enzyme hydrolysis at 30 °C approached that at 45 °C after 168 h. Hence, temperature provided an approach to decrease loss of enzyme activity by reducing enzyme adsorption on lignin. This helps to explain why simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP), both carried out at 30–32 °C, could offer viable options for mitigating lignin-derived inhibition effects.

1. Introduction

Lignin confers rigidity to the cell wall and protects the cellulose and hemicellulose against hydrolytic attack by plant pathogens (Eriksson and Bermek, 2009). The stable aromatic structure of lignin is a major obstacle to the hydrolysis of lignocellulose and necessitates pretreatment of lignocellulose to make the cellulose accessible and susceptible to enzyme hydrolysis (Bonawitz et al., 2014; Ko et al., 2009; Ladisch et al., 1978). While both lignin composition and its distribution in different plant tissues are factors in defining recalcitrance of pretreated lignocellulosic materials with respect to enzyme hydrolysis (Bonawitz et al., 2014; Chapple et al., 2007; Xu et al., 2014, Zeng et al., 2007, 2012a,b), pretreatment also affects hydrolysis through release of molecules from lignin that inhibit or deactivate cellulolytic enzymes (Kim et al., 2015).

During lignocellulose pretreatment, lignin and hemicellulose are solubilized, cellulose crystallinity, degree of polymerization, and particle size are reduced, while porosity and accessibility of cellulose to enzymes are increased (Sousa et al., 2009). For hardwood pretreated in liquid hot water (Weil et al., 1998), there is a particularly strong correlation of increased enzyme hydrolysis with decreased particle size and cellulose degree of polymerization (Kim et al., 2015; Ximenes et al., 2017). These changes allow greater access of enzymes to cellulose and hemicellulose, but may also alter how cell wall components and particularly lignin, interact with enzymes (Ximenes et al., 2017).

Pretreatment carried out at temperatures above 160 °C cause lignin to melt. Upon cooling lignin is redeposited as micron-sized beads or globular granules on plant cell wall structures and cellulose fibers (Donohoe et al., 2008, Li et al., 2014). This increases both exposed hydrophobic surface area of lignin and the adsorption of protein resulting in significant loss of enzyme activities (Kaparaju and Felby, 2010; Ko et al., 2015a, 2015b; Yarbrough et al., 2015). Prior work showed that liquid hot water (LHW) pretreatment changed hardwood derived lignin resulting in a more condensed and syringyl deficient form with higher guaiacyl content that adsorbed more enzymes, mainly β-glucosidases at 45 °C (Ko et al., 2015a,b).

The current work addresses the effect of temperature on enzyme adsorption on lignin-rich fractions derived from liquid hot water pretreated sugarcane bagasse. Enzyme adsorption was minimal at 30 °C and moderate at 45 °C when β-glucosidase, exoglucanases, and endoglucanases from *Asperigillus niger* and *Trichoderma reesei* were contacted with the enzyme hydrolyzed liquid hot water pretreated sugarcane bagasse lignin. Acid hydrolyzed liquid hot water pretreated sugarcane bagasse lignin completely adsorbed cellulases at 45 °C. The

* Corresponding author at: 500 Central Dr., West Lafayette, IN 47907, USA.
E-mail address: ladisch@purdue.edu (M.R. Ladisch).

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Table 1
Compositional Analysis of untreated and LHW pretreated sugarcane bagasse.

% Compositions	Untreated	LHW pretreated	Lignin from enzyme hydrolysis of LHW bagasse	Lignin from acid hydrolysis of LHW bagasse
Cellulose	35.2 ± 2.2	44.1 ± 0.2	42.7 ± 0.2	0.4 ± 0.0
Hemicellulose				
Xylose	18.6 ± 1.6	14.3 ± 0.4	12.7 ± 0.1	1.8 ± 0.1
Arabinose	1.7 ± 0.2	1.5 ± 0.7	0.8 ± 0.1	1.1 ± 0.0
Acetyl groups	3.4 ± 1.0	3.1 ± 1.4	1.4 ± 0.0	0.0 ± 0.0
Total Hemicellulose	23.7	18.9	15.0	2.9
Lignin	24.7 ± 0.4	28.0 ± 0.7	40.3	93.9
Acid Insoluble (AIL)	20.7	23.8	36.8	93.35
Acid Soluble (ASL)	4.0	4.1	3.5	0.5
AIL/ASL	5.2	5.8	10.5	186.7
Ash	3.4 ± 0.1	3.6 ± 0.1	2.5 ± 0.2	3.5 ± 0.1
Total	87.0[1]	94.6	100.5	100.7

[1] Extractives consisting of waxes, nucleic acids, and plant cell wall cytoplasm contents make-up the difference. All analysis ran in triplicate.

work reported in this paper clarifies the effects of temperature and type of lignin extraction method on adsorption of cellulolytic enzymes on lignin in biomass.

2. Materials and methods

2.1. Sugarcane bagasse

Sugarcane bagasse from Usina Alta Mogiana S.A. (Brazil) was washed, oven dried at 45 °C for 48 h, ground in an agricultural crusher and sieved to a particle size fraction of 0.25 mm to 2 mm and pretreated using liquid hot water. Compositions of the sugarcane bagasse before and after pretreatments are given in Table 1.

2.2. Liquid hot water (LHW) pretreatment

Sugarcane bagasse was pretreated at 190 °C for 20 min in a Tecam® SBL-1 fluidized sand bath using 1-inch stainless steel reaction tubes filled with 6.1 g of sugarcane bagasse (7.8% moisture) and 31.4 mL of deionized water to achieve 15% (w/w) dry solids with approximately 20% free space above the liquid level in the tube in order to accommodate thermal expansion of the liquid hot water (Kim et al., 2009, 2014). After pretreatment, the material was washed with 100 mL hot deionized water through a Buchner Funnel with #1 Whatman filter paper, dried at 40 °C overnight, and stored at room temperature until use. The severity factor of 9.67 (Overend and Chornet, 1987) was for ω = 4.6 where ω, from a fit for liquid hot water pretreated hardwood by Kim et al. (2014), also gave a linear correlation of enzyme hydrolysis vs. severity for the sugarcane bagasse used in our work.

2.3. Preparation of lignin fractions

Milling of liquid hot water pretreated sugarcane bagasse in a Wiley mill® at room temperature with a 20 mesh (0.84 mm) sieving screen was followed by enzymatic hydrolysis in 50 mM citrate buffer at pH 4.8 and 50 °C rpm for 7 days in an incubator shaker at 200 rpm or acid hydrolysis. Enzyme hydrolysis of liquid hot water pretreated sugarcane bagasse was carried out with 0.52 mL enzyme (corresponding to 81.6 mg protein/g pretreated sugarcane bagasse, dry weight basis), of an enzyme mixture prepared from 2.27 mL (331 mg protein) Spezyme CP and 0.5 mL (102 mg protein) Novozyme 188. A large excess of cellulase enzyme was used to ensure maximal hydrolysis of the cellulose to obtain a fraction enriched in lignin for further study.

Following enzyme hydrolysis, the protease Protex TM 7 L (54 mg protein/mL) from *Bacillus amyloliquefaciens* (Genencor Division of Danisco, Palo Alto, CA) was used to hydrolyze and remove adsorbed protein from the pretreated sugarcane bagasse. Protease was added at an enzyme concentration of 0.29 mg/mL (equivalent to 5.4 mg protein per g bagasse) (Ko et al., 2015b). The lignin fraction was then dried at 40 °C for 24 h and stored at 8 °C. Protein adsorbed on the solids washed with water and buffer was measured using the micro Kjeldahl method and corresponded to a nitrogen content (N) of 1.2% (elemental analysis based on mass). After protease treatment, the nitrogen content decreased to 0.4%, which was comparable to the nitrogen content associated with the pretreated bagasse before it was treated with cellulase.

Acid hydrolysis of liquid hot water pretreated bagasse was carried out using a two-step sulfuric acid treatment following NREL standard laboratory procedures with temperatures and hold times of 30 °C (for 60 min) and 120 °C (for 60 min). After the second hydrolysis step, samples were cooled and solids were washed with water and recovered by vacuum filtration through a porcelain-filtering crucible. The solids were then dried at 40 °C for 24 h.

Liquid hot water pretreatment of the sugarcane bagasse removed some hemicellulose and extractives, resulting in increases in the glucan (cellulose) and lignin fractions in the remaining solids (Table 1). The ratio of acid insoluble lignin (AIL) to acid soluble lignin remained constant during pretreatment but increased by 2 or 35-fold when the pretreated material was hydrolyzed using enzyme or acid, respectively. The lignin content of acid treated bagasse was about 2.3× higher than that of enzyme treated lignocellulose (Table 1) due to removal of cellulose and hemicellulose during acid treatment (compare columns 3 and 4 in Table 1).

2.4. Brunauer, Emmett, and Teller (BET) surface area analysis

BET surface areas of isolated lignins and LHW pretreated sugarcane bagasse were determined by nitrogen adsorption, using a Micromeritics TriStar II 3020 at Micromeritics Analytical Services (Norcross, GA) according to the multi-point BET procedure. Samples were dried under vacuum at 40 °C overnight before analysis. Data was based on single measurements.

2.5. Enzymes for adsorption studies

Cellic Ctec2 (90 FPU/mL, 247 mg protein/mL) was provided by Novozyme, North America Inc. (Franklinton, NC). Novozyme 188 (381 ρNPGase/mL, 203 mg protein/mL) was purchased from Sigma-Aldrich, St. Louis, MO. The activity of purified β-glucosidase from Megazyme (Wicklow, Ireland) was 80 U/mg at 40 °C and pH 4.0 with *p*-nitrophenyl β-glucoside as substrates. SDS-gel electrophoresis gave a single band of MW = 121,000, with pI = 4.0 as determined by isoelectric focusing.

2.6. Enzyme activity assays

Endoglucanase activity was measured using 1% (w/v) carboxymethyl cellulose (CMC, Sigma-Aldrich, St. Louis, MO) as substrate (Dien et al., 2008). Exoglucanase and β-glucosidase activities were measured using 2.5 and 10 mM of ρ-nitrophenyl-β-D-cellobioside (pNPC, Sigma-Aldrich, St. Louis, MO) and ρ-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma-Aldrich, St. Louis, MO), respectively, as substrates. One unit is defined as the amount of enzyme that releases 1 µmol of ρ-nitrophenol per min under specified conditions (Dien et al., 2008). Proteins in the supernatant were measured using a bicinchoninic

acid (BCA) protein assay reagent kit (Thermo-Scientific, Rockford, IL). The amount of protein adsorbed was measured by difference between the protein initially in solution and that remained after contact with added lignin. Changes in enzyme activity in solution were fitted by the model of Sadana and Henley (1987), and differences determined with software Origin Pro 2015 (OriginLab, Northampton, MA).

2.7. Protein adsorption experiments

Adsorption measurements of cellulolytic enzymes on the isolated lignin fractions prepared from enzyme hydrolyzed sugarcane bagasse were carried out at a protein concentration of 0.5 mg/mL (equivalent to initial concentrations of 50 mg protein/g dry lignin) using the procedures of Ko et al. (2015a). For assays comparing adsorption of non-purified (Novozyme 188) and purified β-glucosidase (both from *A. niger*), protein concentrations were selected based on maintaining an initial constant ratio of β-glucosidase activity to lignin solids equivalent to 3040 ρNPGase/g lignin. This activity corresponds to concentrations of 203 mg/mL and 0.3 mg/mL for non- purified and purified β-glucosidases, respectively, which is equivalent to 1622 and 10 mg protein/g lignin solids, respectively. All adsorption reactions were run in triplicate, with values averaged for reporting purposes.

Adsorption assays were carried out using 1.5 mL Eppendorf tubes containing 10 mg/mL of lignin, enzymes and 0.05 M citrate buffer (pH 4.8) for a total volume of 500 μL. Lignin was dispersed by sonication at room temperature for 1 min using a Branson 2510 sonicator. The slurry was gently mixed in a hybridization incubator (FinePCR, GyeongGi, Korea) rotating at 20 inversions per min at 30 °C and 45 °C for 1.5 h, 24 h and 48 h, respectively. After incubation, the supernatant was separated from the solid fraction by centrifugation at 4700 × *g* for 10 min and ambient temperature. Controls consisted of enzyme in buffer and confirmed that thermal inactivation and binding of enzymes on tube walls did not occur during the 48 h time period over which adsorption measurements were carried out.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor changes in enzyme compositions remaining in solution after contact with lignin. The samples were prepared from supernatant, loaded to 12% TGX mini-gel (Bio-Rad) and run using Mini-PROTEIN II cell electrophoresis system. The gel was visualized by staining with silver (Bio-Rad). The supernatant fractions of enzyme of lignin controls were run in parallel.

3. Results and discussion

Optimal temperatures for enzyme hydrolysis of cellulose are generally between 45 and 50 °C except for some types of bacterial thermophiles where optimal temperatures are 60–70 °C (Lynd et al., 2005; Ladisch et al., 1981). Hence, adsorption studies were carried out at 45 °C and compared to 30 °C, since simultaneous saccharification and fermentation (SSF), or consolidated bioprocessing (CBP), is typically performed at between 30 and 32 °C. The adsorption studies were also performed with isolated (enzyme or acid derived) lignin from liquid hot water pretreated bagasse to make sure the results observed were related to the adsorption on lignin and not on other lignocellulose derived compounds.

3.1. Adsorption of enzymes on lignin derived from liquid hot water pretreated and enzyme hydrolyzed sugarcane bagasse at 45 °C (enzyme derived lignin)

Endoglucanase activity in the supernatant decreased to 34% of its original activity after 1.5 h incubation with liquid hot water pretreated and enzyme hydrolyzed bagasse at pH 4.8 in 50 mM citrate buffer. After 1.5 h, enzyme activity leveled out (Fig. 1(A)) indicating inhibition (Mosier and Ladisch, 2009; Sadana and Henley, 1987). Exoglucanase activity was 73% after 24 h and decreased to 60% at 48 h with the continuing decrease in activity indicating deactivation (Fig. 1(B)). While *T. reesei* β-glucosidase rapidly decreased to < 60% after 1.5 h, (dark squares in Fig. 1 (C)), *A. niger* β-glucosidase activity decreased by only 2% (dark squares, Fig. 1(D)) confirming its previously reported stability (Ko et al., 2015b). Differentiation between inhibition and deactivation was based on fitting the data with the first-order unimolecular irreversible reaction (Eq. (2)) and a pseudo first-order reaction (Eq. (1)) (Sadana and Henley, 1987). Fits were evaluated using an F-test with a 0.05 significance level. The pseudo first-order model (Eq. (1)) gave the best fit as shown by the lines in Fig. 1(A)–(D):

$$A = (1-\alpha_1)*\exp(-k_1 t) + \alpha_1 \tag{1}$$

$$A = A_0*\exp(-t/\tau) \tag{2}$$

where t = time; τ = characteristic deactivation time; k_1 = kinetic stability constant; α_1 = ratio two different forms of the same enzyme; A_0 = initial activity of enzyme.

3.2. Adsorption of enzymes on lignin derived from liquid hot water pretreated and acid hydrolyzed sugarcane bagasse at 45 °C (acid derived lignin)

Endoglucanase activity from *T. reesei* decreased to 45, 26, and 14% of its initial activity, respectively, after 1.5, 24 and 48 h of incubation indicating deactivation (Fig. 1(A)). Complete disappearance of *T. reesei* exoglucanase and β-glucosidase activities from the supernatant occurred almost immediately indicating deactivation after contacting the acid treated lignin (open circles at the bottom of Fig. 1(B) and (C)). Lignin Derived from LHW pretreated and acid hydrolyzed sugarcane bagasse caused significant inhibition (open circles, Fig. 1(D)) for *A. niger* β-glucosidase which decreased to 57% within 1.5 h and leveled off at 43% after 24 h, again indicating an inhibition effect (open circles in Fig. 1(D)).

3.3. Comparison of enzyme activities adsorbed by enzymatic and acid hydrolyzed lignin at 45 °C

The gel in Fig. 2 is divided into 10 lanes as indicated in the horizontal direction from left to right, and 12 bands identified between lanes 1 and 2, and lanes 4 and 5. Cellulase proteins shown by the bands in Lane 2 are endoglucanase (band 9), exoglucanase (band 7) and β-glucosidase (bands 5 for *T. reesei* in lane 2; 12 and 13 for *A. niger* in lane 5) (identification based on UniProt Consortium, 2017; Himmel et al., 1993). Molecular weights of the proteins ranged from 6.5 to 200 kD based on comparison to protein standards in lanes 1 and 10. Pretreated acid hydrolyzed bagasse (i.e., acid derived lignin, Table 1) adsorbs *T. reesei* proteins to a greater extent than lignin derived from enzyme hydrolysis (compare lane 2 against lane 3 and 4, respectively). This is consistent with Rahikainen et al. (2011) who found that lignin from acid hydrolyzed softwood adsorbed > 95% of *T. reesei* proteins while the residual lignin from enzyme hydrolysis adsorbed 70% protein.

Bands 12 and 13 in lanes 5, 6 and 7 representing protein from *A. niger* β-glucosidase (Novo 188) are about the same indicating minimal adsorption (Fig. 2). Differences in activity levels of β-glucosidases (Fig. 1), however, show losses of activities and confirm that the interaction of enzymes with lignin depends on the microbial source from which the enzyme is derived, consistent with previous reports (Berlin et al., 2006; Ximenes et al., 2010), as well as the relative amounts of lignin in enzyme and acid treated bagasse (Table 1). β-glucosidase from *A. niger* is also stable with respect to soluble, lignin derived inhibitors unlike *T. reesei* β-glucosidase which is inhibited (Haven and Jørgensen, 2013; Ko et al., 2015b; Ximenes et al., 2010; 2011).

Both ionic and hydrophobic interactions of β-glucosidase with lignin determine adsorption. β-glucosidase from *T. reesei* has pI values of 5.7–6.4, compared to 4.6 for *A. niger* (Ko et al., 2015a). At pH 4.8, *T. reesei* β-glucosidase is positively charged, which promotes binding with

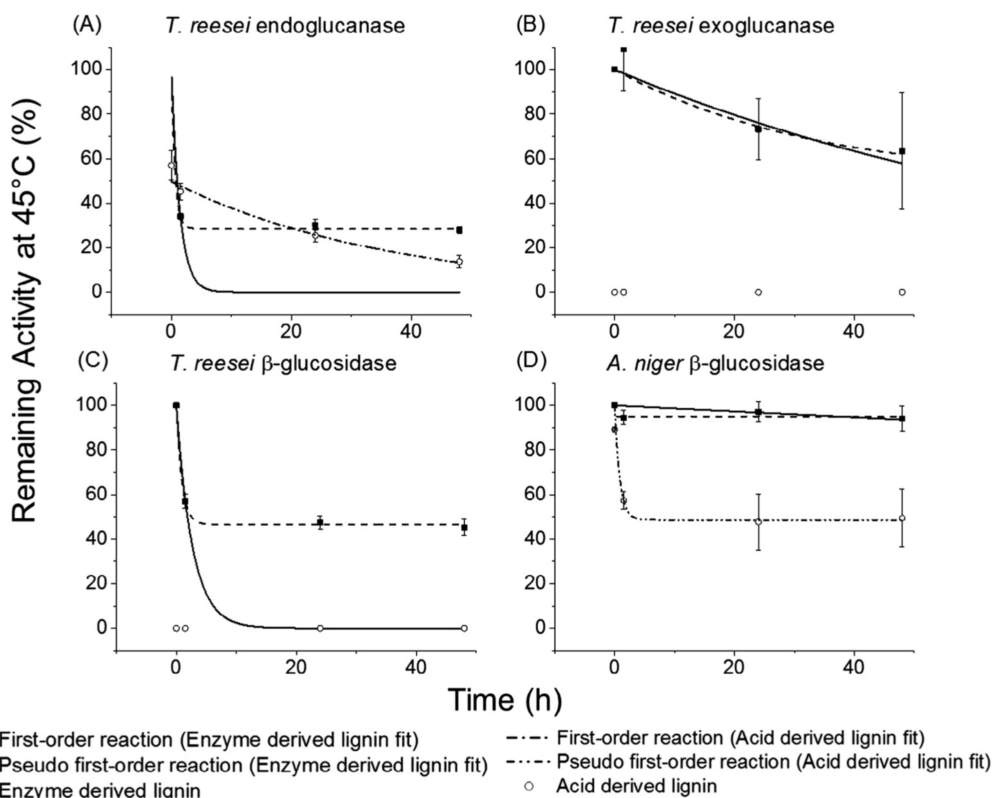

Fig. 1. Comparison of different inhibition models and experimental data for activity after exposure to enzyme hydrolyzed residual lignin (dark symbols) and acid hydrolysis (open symbols) residual lignin at 45 °C in 50 mM citrate buffer at pH 4.8, over time. A–C, CTEC2; D, Novozyme 188. First-order reaction model and Pseudo first-order reaction of Sadana and Henley (1987) fitted to the experimental data are represented by lines as indicated in the figure. Values of fitted constants for indicated best fit lines are: (A) *T. reesei* endoglucanase on enzyme-derived lignin (data given by dark square) Eq. (1), Prob (F) < 0.05: $\alpha_1 = 0.28424$; $k_1 = 1.70156$; and on acid derived lignin (data given by open circles) Eq. (2), Prob (F) < 0.05: $\tau = 36.5575$; $A_0 = 49.71113\%$ (B) *T. reesei* exoglucanase on enzyme-derived lignin (dark square) Eq. (2), Prob (F) = 0.671: $\tau = 87.74496$, $A_0 = 100\%$; and on acid derived lignin (open circles, all points equal zero). (C) *T. reesei* β-glucosidase on enzyme derived lignin (dark square) Eq. (1), Prob (F) < 0.05: $\alpha_1 = 0.46477$; $k_1 = 1.08436$; and on acid derived lignin (open circles, all points equal zero). (D) *A. niger* β-glucosidase on enzyme derived lignin (dark square), Eq. (2), Prob (F) = 0.054: $\tau = 743.90164$; $A_0 = 100\%$; and on acid derived lignin (open circle), Eq. (1), Prob (F) < 0.05: $\alpha_1 = 0.48565$; $k_1 = 1.17438$.

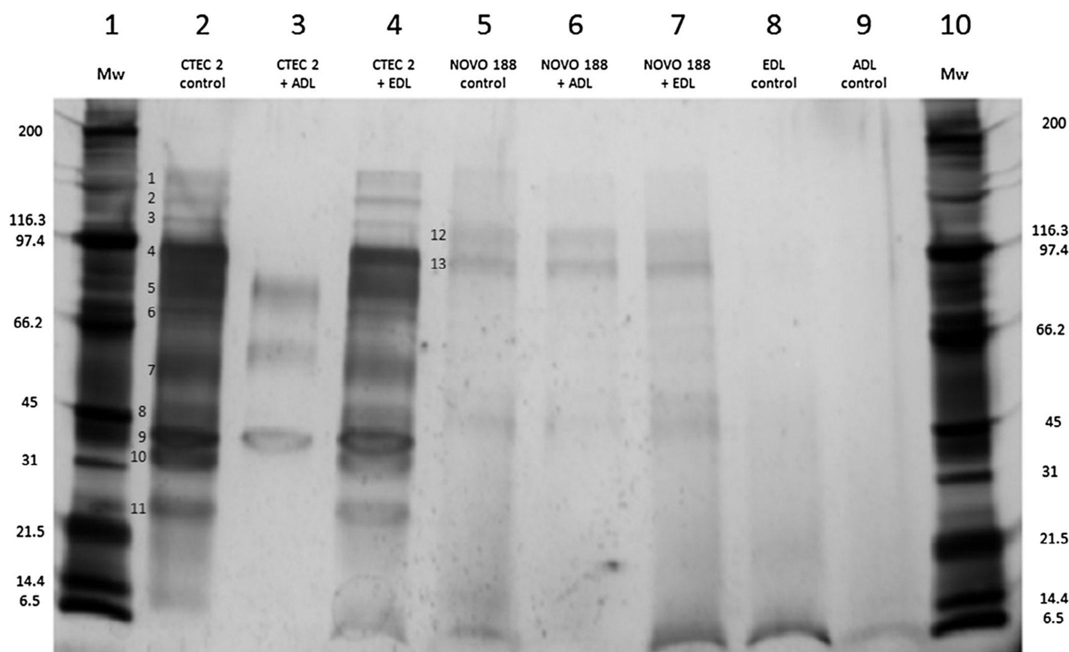

Fig. 2. SDS-PAGE analysis of free proteins (Cellic Ctec2 and Novozyme 188) in the supernatant after 1.5 h-adsorption on acid or enzyme hydrolyzed residual lignin at 45 °C. The band numbers indicate the proteins of the specific molecular weights of between 6.5 and 200 kD. ADL denotes acid digested lignin and EDL enzyme digested lignin. Lanes are 1 and 10 – standard protein mixture; 2 – Cellic CTEC 2; 3, 4 – Cellic CTEC 2 after incubation with acid derived lignin and EDL, respectively, 5 – Novozyme 188; 6, 7 – Novozyme 188 after incubation with ADL and EDL, respectively; 8, 9 – washed EDL and ADL, respectively controls showing absence of proteins.

negatively charged lignin (Rahikainen et al., 2013), unlike *A. niger* β-glucosidase which is negatively charged at the same pH, leading to the observed losses of *T. reesei* enzyme activity (compare Fig. 1(C) and (D)). Ko et al. (2015b) showed that when the pH was increased from 4.0 to 6.0, the extent of adsorption of β-glucosidase from *T. reesei* decreased, and when NaCl was added, some β-glucosidase activity was desorbed from the lignin, thus confirming electrostatic interactions. Since 60% of β-glucosidases remained bound to lignin at 200 mM NaCl, hydrophobic interactions between protein and lignin were also indicated to play a role.

Ko et al. (2015a,b) and Kim et al. (2015) clearly explained the role of BSA in adsorbing on lignin and blocking non-productive binding of cellulolytic enzymes for liquid hot water pretreated hardwood. Subsequently, Siqueira et al. (2017) confirmed similar phenomena for sugarcane bagasse pretreated by other methods. Studies with cellulose binding domain (CBM), bovine serum albumin (BSA), soy proteins, and surfactants (Tween or polyethyleneglycol) show these act to block protein adsorption (Florencio et al., 2016). These macromolecules when added to the enzyme compete with the cellulases for lignin adsorption, and significantly decreased non-productive binding between lignin and cellulolytic enzymes (Zhang et al., 2017). Hydrophobic interactions of these agents hindered adsorption of enzymes on lignin and increased the amount of active cellulases and β-glucosidases left in the supernatant (Florencio et al., 2016; Haven and Jørgensen, 2013; Kumar et al., 2013).

3.4. Specific surface area and surface characteristics of lignocellulose particles

Liquid hot water pretreated bagasse had a specific surface area of 1.7 m^2/g compared to enzyme hydrolysis derived lignin at 2.5 m^2/g. The surface area attributed to small pores is more pronounced for sulfuric acid hydrolysis derived lignin with a BET specific surface area of 86 m^2/g. Similar results were reported for spruce with 80 m^2/g vs. 2.5 m^2/g for acid and enzyme hydrolyzed softwood, respectively (Rahikainen et al., 2011). This is consistent with the greater extents of cellulose and hemicellulose removal for acid treated bagasse compared to enzyme treated bagasse. Gama et al. (1994) showed that cellulolytic enzymes do not enter into the micropores for five studied celluloses, and concluded that hydrolysis occurs initially at the external surface of the fibers. Lin et al. (1985) measured wet pore sizes and pore size distributions in corn stover, and showed that external or macropore surface properties determine the extent of interaction between enzymes and substrate, where the enzymes in this case were from cellulolytic bacteria. Since the small pores cannot be penetrated by enzymes from *T. reesei* with molecular weights between 20 and 70 kD (Gong et al., 1979), we would expect the intraparticle access of enzyme preparations in this work (20–150 kD, Fig. 2) to be limited and the external area to be a key determinant for contact of enzyme with solid substrate.

Previous reports showed that increasing temperature of dilute acid pretreatment resulted in 8× lower adsorption of *T. reesei* cellulase by lignin (Ooshima et al., 1990) and 6× higher adsorption on cellulose at 50 °C (Lynd et al., 2002). Conversely Zheng et al. (2013) found adsorption of enzymes by lignin to be 10× higher at 50 °C than at 4 °C. We found acid treatment causes a significant increase in the fractional amount of lignin remaining in the bagasse, an increase in accessible specific surface area, and a decrease in structural carbohydrates that partially shield the lignin from the contact with enzymes. Hence, enzyme adsorption is rapid and extensive.

3.5. Adsorption of T. reesei and A. niger enzymes at 30 °C

Adsorption of enzymes at 30 °C onto lignin derived from liquid hot water pretreated and enzyme hydrolyzed bagasse was examined at 30 °C, since this temperature is within the range where simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) with yeast is carried out. The lower temperature must be used in these fermentations since many microorganisms have limited thermal stability. We found that endoglucanase and β-glucosidase from *T. reesei* and *A. niger* were not significantly adsorbed on enzyme derived lignin as indicated by minimal changes in enzyme activity and total protein concentration at 30 °C. The only activity that changed was exoglucanase (cellobiohydrolase), which initially decreased by 20% within 1.5 h of incubation, but then remained stable. Hence, temperature provided an approach to reducing loss in enzyme activity by reducing enzyme adsorption on lignin. More importantly, for SSF or CBP the temperature of about 30 °C has unintentionally offered a viable option for mitigating lignin-derived inhibition effects when liquid hot water pretreatment is used to prepare the lignocellulose feedstock for hydrolysis.

3.6. Hydrolysis of cellulose and pretreated sugarcane bagasse at 30 °C and 45 °C

We tested the hypothesis that lower temperature and longer reaction times mitigate inhibitory effects of lignin for large proteins. Lignin residues and pretreated sugarcane bagasse were hydrolyzed using 5 FPU or 13 mg protein/g glucan (Cellic CTEC2) for 72 and 168 h at 30 and 45 °C (Fig. 3) in 50 mM citrate buffer, pH 4.8. Enzyme loadings are based on total glucan in the reaction mixture. The amount of added solka floc was adjusted against the residual cellulose in the enzyme or acid digested lignin to give equivalent total glucan of 1% (w/w) and 30% (w/w) lignin.

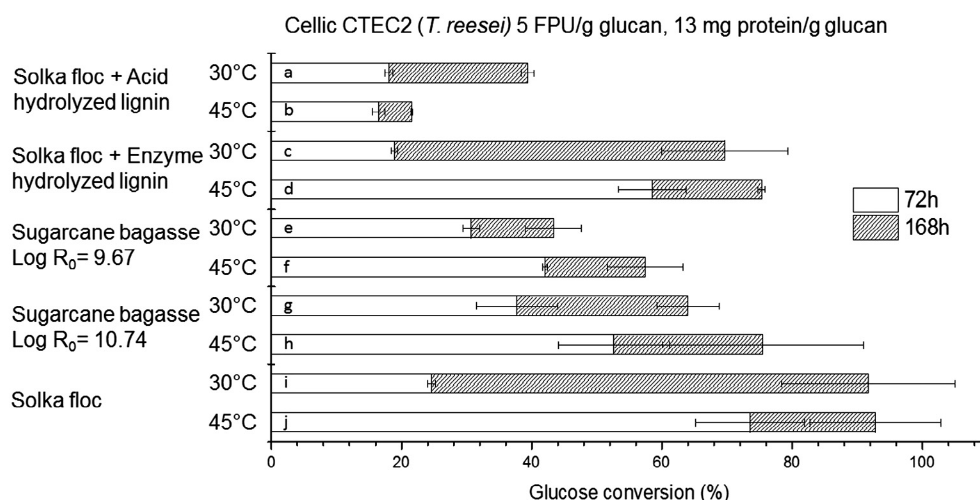

Fig. 3. Comparison of cellulose and sugarcane bagasse hydrolysis at 30 and 45 °C glucose conversion. Enzyme loading was 5 FPU/g glucan or 13 mg protein/g glucan. (a, b): Solka floc to which acid hydrolyzed lignin was added with enzyme hydrolysis at 30 and 45 °C – open bar denotes 72 h hydrolysis and shaded bar 168 h. (c, d) solka floc with enzyme hydrolyzed lignin added. (e, f): enzyme hydrolysis of liquid hot water pretreated sugarcane bagasse – pretreatment at severity R_o = 9.67; (g, h): enzyme hydrolysis of sugarcane bagasse pretreated at R_o = 10.74; (i, j): enzyme hydrolysis of solka floc.

Addition of acid derived lignin to solka floc dramatically decreased maximum conversion, with lower yields at 45 °C evident after 168 h (Fig. 3(a) and (b)). Addition of enzyme hydrolyzed lignin to solka floc gave higher conversion and showed only a small difference in extent of hydrolysis after 168 h (Fig. 3(c) and (d)), although the slower rate at 30 °C was evident at 72 h. While the presence of inhibitors derived from biomass or generated during pretreatment lowers the overall extents of cellulose hydrolysis (Ximenes et al., 2010; 2011), these soluble molecules may be removed by washing (Kim et al., 2013, 2015).

Hydrolysis of liquid hot water pretreated sugarcane bagasse (at log R_0 = 9.67 or log R_0 = 10.74) (Fig. 3(e) and (f) or (g) and (h)), give smaller differences in hydrolysis since restricted access of the cellulase to the lignin as well as recalcitrance due to structural features (Fig. 3) is a factor, unlike the runs with solka floc as the cellulose substrate. These proteins have small diffusion coefficients of 10^{-6} cm^2 and kinetically hindered adsorption (Ladisch, 2001). Based on a simple Arrhenius approximation that each 10 °C reduction in temperature reduces reaction rate 2×, cellulases would be approximately 3× less active at 30 °C than at 45 °C, leading to lower rates of hydrolysis. In the case of solka floc, hydrolysis in the absence of inhibitors gave cellulose conversion to glucose of 24% at 30 °C vs. 72% at 45 °C after 72 h (Fig. 3(i) and (j)). After a total of 168 h, hydrolysis at 30 °C caught up to hydrolysis at 45 °C.

4. Conclusions

Adsorption of cellulolytic enzymes on lignin was significant at 45 °C, with *A. niger* β-glucosidase less affected than *T. reesei* enzymes. At 30 °C, adsorption of β-glucosidase, endocellulases and exocellulases is lower. Hence, hydrolysis of accessible cellulose in solka floc or liquid hot water pretreated sugarcane bagasse at 30 °C, while slower, catches up to hydrolysis at 45 °C, except where inhibition due to adsorption of enzyme onto added acid treated lignin dominates. These results help to explain why inhibition effects have not been noted during SSF or CBP carried out at 30 °C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.12.061.

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