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Effects of dilute acid and flowthrough pretreatments and BSA supplementation on enzymatic deconstruction of poplar by cellulase and xylanase

Samarthya Bhagia[a,b,c], Rajeev Kumar[b,c], Charles E. Wyman[a,b,c,*]

[a] Department of Chemical and Environmental Engineering, Bourns College of Engineering, University of California Riverside, 900 University Ave, Riverside, CA 92521, USA
[b] Center for Environmental Research and Technology, Bourns College of Engineering, University of California Riverside, 1084 Columbia Ave, Riverside, CA 92507, USA
[c] BioEnergy Science Center (BESC), Oak Ridge National Laboratory (ORNL), Oak Ridge, TN 37831, USA

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ABSTRACT

To help understand factors controlling the recalcitrance of lignocellulosic biomass to deconstruction to sugars, poplar was pretreated with liquid hot water (LHW) and extremely dilute acid (EDA) at 140 °C and 180 °C in batch and flowthrough reactors. The resulting solids were then subjected to enzymatic hydrolysis by eight combinations of cellulase, xylanase, and bovine serum albumin (BSA). Co-addition of xylanase to cellulase resulted in up to 11 percentage points higher overall sugar yield than their sequential addition. In general, supplementation of BSA to enzymes had a larger impact on flowthrough solids with reduced lignin content than batch solids with high lignin content. BSA did not affect xylan yields and while it had low impact on LHW solids, it caused large increases in sugar yields from EDA solids. Flowthrough pretreatment produced less recalcitrant solids than did batch operation, but using very dilute acid reduced recalcitrance even more.

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1. Introduction

Production of sustainable liquid fuels from lignocellulosic biomass is vital for reducing greenhouse gas emissions and attaining energy sustainability and independence (Gupta, 2008). The sugars in cellulose and hemicellulose offer an enormous potential resource for fermentation into ethanol and other products, and the aromatics in lignin could be valuable for conversion into chemicals or fuels (Lynd, Wyman, & Gerngross, 1999). However, deconstruction of biomass into sugars and aromatics from lignocellulosic biomass requires overcoming its recalcitrance to thermal or biological processing that currently results in high overall conversion costs (Wyman, 2007). Hemicellulose and lignin in particular present important obstacles to biological processing as they cover cellulose in unprocessed biomass and impede access of bulky enzymes to cellulose (Converse, 1993; Kumar & Wyman, 2013; Mansfield, Mooney, & Saddler, 1999). As a result, some type of pretreatment is generally needed to disrupt the hemicellulose-lignin shield and achieve economically viable sugar yields (Yang & Wyman, 2008).

Aqueous batch pretreatments such as liquid hot water and dilute acid solubilize a large portion of hemicellulose, only a small amount of lignin, and limited quantities of cellulose (Bobleter, 1994). Through this process, pretreated solids have much increased cellulose surface area and pore accessibility due to swelling, hemicellulose solubilization and shrinking of lignin through coalescence (Kumar & Wyman, 2009a). Although pretreatment lowers recalcitrance and aids in enzymatic hydrolysis of cellulose into glucose that may reach near theoretical yields (Wyman, Decker et al., 2005), pretreatment and enzymatic hydrolysis are the most expensive steps for recovery of fermentable sugars for biological processing and require improvements to reduce pretreatment and/or enzyme costs (Klein-Marcuschamer, Oleskowicz-Popiel, Simmons, & Blanch, 2012) while still achieving commercially viable sugar yields from cellulose and hemicellulose combined.

Several factors can be involved in reducing enzymatic saccharification of biomass polysaccharides. Enzymes can be inhibited

Abbreviations: LHW, liquid hot water; EDA, extremely dilute acid; BSA, bovine serum albumin; BESC, Bioenergy Science Center; BESC STD, BESC standard poplar; CAZy, carbohydrate active enzymes.

* Corresponding author at: 1084 Columbia Avenue Riverside, CA 92507, USA.
E-mail addresses: sbhag001@ucr.edu (S. Bhagia), rkumar@cert.ucr.edu (R. Kumar), cewyman@engr.ucr.edu (C.E. Wyman).

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by glucose (Holtzapple, Cognata, Shu, & Hendrickson, 1990; Xiao, Zhang, Gregg, & Saddler, 2004), cellobiose (Zhao, Wu, Yan, Gao, 2004), structural polysaccharides and sugar oligomers (Kumar & Wayman, 2014; Qing et al., 2010b), and pretreatment derived inhibitors (Kim, Ximenes, Mosier, & Ladisch, 2011; Palmqvist, Hahn-Hägerdal, Galbe, & Zacchi, 1996; Ximenes, Kim, Mosier, Dien, & Ladisch, 2011; Ximenes, Kim, Mosier, Dien, & Ladisch, 2010). Further, enzyme accessibility to cellulose can be limited by due to hemicellulose and lignin (Kumar & Wyman, 2009a; Kumar & Wyman, 2009b). Enzymes can adsorb onto lignin which lowers the amount of active enzyme available for hydrolysis (Kaar & Holtzapple, 1998). One strategy to prevent this loss can be through blocking of the surface of lignin by bovine serum albumin (BSA) or Tween® 20 (non-ionic surfactant) (Eriksson, Börjesson, & Tjerneld, 2002 ; Kumar & Wyman, 2009c; Yang & Wyman, 2006). In one-study, pre-addition of 1% solution of BSA in enzymatic hydrolysis of dilute acid pretreated corn stover (1% H_2SO_4, 140 °C, 40 min) increased yield from 82 to 92% at an enzyme loading of 15 FPU cellulase per gram cellulose after 72 h (Yang & Wyman, 2006). In another study, pre-addition of 300 mg BSA per g glucan in unpretreated biomass to enzymatic hydrolysis of dilute acid pretreated poplar (0.02% H_2SO_4, 190 °C) raised glucose yields from about 55–63% at a dose of 20 mg enzyme per gram glucan in unpretreated biomass after 72 h (Kumar & Wyman, 2009d). In yet another study, blocking lignin through pre-addition of 50 mg BSA per gram of mixed hardwood chips pretreated in liquid hot water at 220 °C for 15 min increased enzymatic hydrolysis glucose yield from around 18%–72% at an enzyme loading of 8 mg protein per gram glucan (Ko, Kim, Ximenes, & Ladisch, 2015; Ko, Ximenes, Kim, & Ladisch, 2015). However, these studies that employed BSA were carried out on solids pretreated at severe conditions. Unproductive loss to lignin for solids prepared at less severe pretreatments and lower temperatures has not been studied. Moreover, solids pretreated in aqueous conditions at high pretreatment severities have low xylan content. It is not known how residual xylan in moderately pretreated solids affects cellulose deconstruction by cellulase. Moreover, such studies have never been carried out on flowthrough pretreated biomass. This is the first report of effect of cellulase loadings, effect of xylanase, and supplementation of BSA on extremely dilute acid pretreated solids. The effect of BSA supplementation to enzymes on sugar yields for flowthrough solids prepared at low severity has rarely been reported.

Thus, moderately pretreated poplar hardwood solids were produced through liquid hot water and extremely dilute acid pretreatments (0.05% acid) in batch and flowthrough reactors at 140 °C for 192 min and 180 °C for 12 min. Our previous study focused on sugar and lignin mass balances of poplar from these pretreatments, and enzymatic hydrolysis at high enzyme loading of 100 mg cellulase per gram glucan in unpretreated biomass (Bhagia, Li, Gao, Kumar, & Wyman, 2016). A distinct characteristic of flowthrough pretreated solids was that they were lower in lignin content than the batch pretreated solids as flowthrough pretreatment reduces or prevents lignin re-deposition (Yang & Wyman, 2004). First, three cellulase loadings of 5, 15, and 100 mg cellulase (per gram glucan in unpretreated biomass) were employed to learn how cellulase loading affects deconstruction in these solids. Since many solids had significant amount of xylan in pretreated solids, 30 mg xylanase was supplemented through co-addition or sequential addition to 15 mg cellulase for their effect on xylan as well as glucan yields. Next, 30 mg of xylanase co-added to 15 mg cellulase was replaced with 30 mg BSA to distinguish between its catalytic effects and unproductive loss to lignin. Finally, pre-addition of high loading of 100 mg BSA to poplar solids was done to block lignin followed by enzymatic hydrolysis with just 15 mg cellulase or co-addition of 15 mg cellulase and 30 mg xylanase (Supplemental Table 3). BSA was used to investigate the impact of unproductive binding enzymes to lignin on cellulose enzymatic saccharification. However, in the industry, such high non-catalytic protein loadings would only increase in cost. In all, these experiments explore deconstruction of polysaccharides in poplar layer by layer through moderate pretreatment followed by enzymatic hydrolysis in many enzyme formulations and protein supplementation strategies.

2. Materials and methods

2.1. Materials

Bioenergy Science Center standard poplar (BESC STD) that had been provided to University of California at Riverside (UCR) by Oak Ridge National Laboratory (ORNL) (Oak Ridge, TN, USA) was used in this study after knife milling through a 1 mm screen (Model 4 Wiley Mill of Thomas Scientific Company at Swedesboro NJ). DuPont Industrial Biosciences (previously Genencor, Palo Alto, CA) graciously supplied Accellerase® 1500 cellulase (Batch# 4901298419) and Multifect® Xylanase (Lot# 301-04021-015,). Protein contents of these commercial preparations as determined by the standard BCA method (Smith et al., 1985) were 82 mg/ml for Accellerase® 1500 and 42 mg/ml for Multifect® Xylanase. Bovine serum albumin (BSA, 98% purity, Batch# 078K0730) was purchased from Sigma-Aldrich (St. Louis MO).

2.2. Pretreatments

Flowthrough and batch pretreatments were applied with just hot water (LHW) and 0.05 wt% sulfuric acid (EDA) at 140 and 180 °C in the combinations summarized in Supplemental Table 1 (please see Supplemental File 1) with the times adjusted to keep the severity parameter value at 3.4 in LHW and combined severity of 1.4 in EDA to gain a perspective of the effect of temperature on recalcitrance. Details of the pretreatment methods have been previously described (Bhagia, Li et al., 2016)

2.3. Solids composition

Prior to compositional analysis, biomass solids were dried at 37 °C for several days that reduced the moisture content to between 4 and 7%. Moisture content was taken into account for calculation of dry weight. Composition of biomass was then measured according to the NREL standard procedure "Determination of Structural Carbohydrates and Lignin in Biomass (Sluiter et al., 2008). Composition data are shown in Supplemental Table 2. A UV–vis spectrophotometry with a Spectramax® M2e Plate Reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMax® Pro data acquisition software in a Costar® UV 96 well-plate with absorbance of water blank taken into account for correction of absorbance in sample was employed to measure acid soluble lignin. Three replicates for each sample in 96 well-plate were kept for measurements. An absorption coefficient of 25 l/gram cm for a 240 nm wavelength was applied per the NREL standard procedure to calculate acid soluble lignin concentration of poplar using the Beer Lambert Bouguer law (Sluiter et al., 2008).

As reported in our previous sugar and lignin mass balances for pretreatment (Stage 1) coupled with enzymatic hydrolysis (Stage 2) at high enzyme loadings (Bhagia, Li et al., 2016), lignin can reprecipitate onto solids after solubilization during batch pretreatment, while continuous flow of liquid during flowthrough pretreatment removes a large portion of solubilized lignin from the reactor before it can reprecipitate. Thus, flowthrough solids have a much lower lignin content, as shown by the balance among glucan, xylan, and total lignin for solids in Supplemental Table 2.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in duplicates according to the NREL standard procedure 'Enzymatic saccharification of lignocellulosic biomass' (Selig, Weiss, Ji, 2008) at a 1% (w/v) glucan loading, 50 °C, 150 rpm, and a total reaction volume of 10 ml. Solids loading equal to 1% glucan loading was chosen to focus on understanding the intrinsic factors of biomass that affect enzymatic yields, as higher solids loading will increase mass transfer resistance and enzyme inhibition (Bhagia, Muchero, Kumar, Tuskan, & Wyman, 2016). However, commercial processes will require high biomass loading for low cost operation. Enzymatic hydrolysis was then carried out on solids that were thoroughly washed with room temperature deionized water after pretreatment. Composition data in Supplemental Table 2 shows glucan content of all solids. All enzymatic hydrolysis runs were carried out for 120 h after cellulase addition. To follow enzymatic hydrolysis progress, 0.5 ml aliquots were withdrawn 4 h after addition of enzymes and then after every 24 h, and only 120 h yields are shown. All protein loadings mentioned throughout this study were based on mg of protein per gram glucan in the poplar prior to pretreatment. These 8 conditions are mentioned in Supplemental Table 3. Statistical analysis was carried out, however, in several cases, where standard deviation was very low, hypothesis testing with α level of 0.05 based on normal distribution predicted significant difference in sugar yield. However, it is known that a 1–2% difference in sugar yield may not be significant. Consequently, the statistical analysis results are not included and instead the standard deviations for all xylan and glucan yields are reported in Supplemental Tables 7 and 8, respectively.

2.5. Products analysis

Waters® e2695 Separations Module with detection on Waters® 2414 RI detector provided the platform for all sugar analyses. This HPLC was equipped with a Biorad® Aminex® HPX-87H column conditioned at 65 °C and used 5 mM sulfuric acid for the mobile phase (Bhagia, Nunez, Wyman, & Kumar, 2016; Sluiter et al., 2006).

2.6. Calculations

Calculations for sugar yields are mentioned in Supplemental File 1. Sugar mass balances used for such calculations have been described previously (Wyman, Dale et al., 2005; Wyman et al., 2009).

2.7. Sugar yields from enzymatic hydrolysis without pretreatment

It is important to note that the poplar used in these experiments was very recalcitrant to enzymatic deconstruction without pretreatment. In particular, sugar yields were 4, 7, and 12% from addition of 5, 15, and 100 mg cellulase protein/g glucan, respectively. In addition, xylanase and BSA supplementation to 15 mg cellulase did not realize sugar yields higher than 8% from unpretreated poplar.

3. Results and discussion

Glucose and xylose yields from LHW and EDA pretreatments and subsequent enzymatic hydrolysis of the pretreated solids with the enzyme loadings outlined in Supplemental Table 3 are pictured as "onion" diagrams in Fig. 1 through 6. Onion diagrams were employed to visually illustrate how different layers of polysaccharides were "peeled off" first by pretreatment and then by enzymes, with the order of release providing a perspective on the transition from less to more recalcitrant components. These diagrams show increasing level of recalcitrance from bottom to top. There is no parallelism of these diagrams with those used in business organization studies with the same name.

3.1. Sugar yields from just pretreatment

Detailed account of comparison of sugar yield and lignin removal through these pretreatments, their mechanism, and theory of hemicellulose assisted lignin solubilization through lignin-carbohydrate complexes have been mentioned elsewhere (Bhagia, Li et al., 2016). Briefly, Fig. 1 shows that Stage 1 (pretreatment) sugar yields were only slightly higher from LHW pretreatment at 180 °C than at 140 °C (same pretreatment severity factor) for both batch and flowthrough reactor configurations. However, flowthrough pretreatment achieved about twice as high Stage 1 sugar yields with LHW than LHW batch operation.

However, Fig. 2 shows that differences in pretreatment sugar yields between batch and flowthrough pretreatment were lower for EDA pretreatments than for LHW conditions in Fig. 1. Comparing temperatures, it can be seen from Fig. 2 that lowering the pretreatment temperature while keeping severity factor constant in EDA batch pretreatments lowered the pretreatment sugar yield by 10 percentage points. However, pretreatment sugar yields were similar (and highest among all pretreatments) for both pretreatment temperatures in EDA flowthrough pretreatments likely due to enhanced mass transfer in flowthrough operation (Bhagia, Li et al., 2016). According to mass transfer models, continuous removal of sugars from the flowthrough reactor creates a concentration gradient that drives hemicellulose solubilization (Brennan & Wyman, 2004).

For both LHW and EDA batch pretreatments, most of the Stage 1 sugar was from hemicellulose xylan as glucan yields were low (2–7%). Nonetheless, flowthrough pretreatment produced higher glucan yields than batch operations (10–13%), as shown in the "pretreatment" columns in Supplemental Tables 4 through 6. Because Stage 1 xylan yields were much higher from flowthrough than batch pretreatment, the xylan contents in flowthrough pretreated solids reported in Supplemental Table 2 were much lower.

3.2. Effect of cellulase loading

Fig. 1 shows overall sugar yields from enzymatic hydrolysis of LHW pretreated solids at cellulase loadings of 5, 15, and 100 mg protein/g glucan in raw poplar. As noted in Figs. 1A and 1B, overall (Stage 1 + Stage 2) sugar yields were slightly higher from batch LHW pretreatment at 180 °C than at 140 °C at 5 and 15 mg cellulase loadings. However, the large difference between overall sugar yields from these LHW batch solids with hydrolysis at high enzyme loading of 100 mg indicates that at moderate pretreatment severity factor, higher pretreatment temperature lowers recalcitrance drastically. On the other hand, solids generated by flowthrough pretreatment showed considerably higher overall sugar yields than realized by batch pretreatment at the same temperatures. Furthermore, sugar yields were much higher for solids from 180 °C flowthrough operation than those produced at 140 °C even at the lower enzyme loadings of 5 and 15 mg cellulase protein unlike LHW batch solids. Solids generated by 180 °C for 12 min flowthrough pretreatment were the only ones that could achieve 100% sugars solubilization among all LHW solids when subjected to the high enzyme dose of 100 mg cellulase protein. Thus, higher temperature LHW pretreatment in both pretreatment configurations produced solids that had lower recalcitrance at the same pretreatment severity factor. It is important to note that glucan has the dominant effect on overall sugar yields due to the higher glucan content in poplar (composition of pretreated solids shown in Supplemental Table 2). Overall xylan yields reported in Supplemental Table 4 reached a

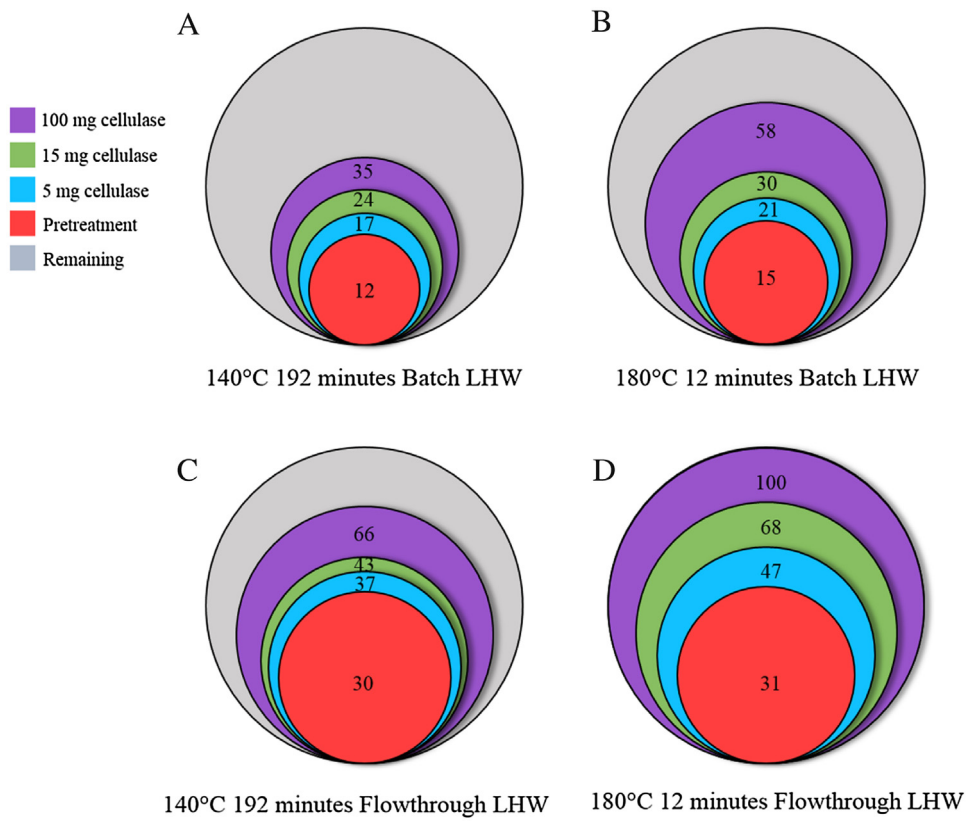

Fig. 1. Effect of cellulase loadings of 5, 15, and 100 mg protein/g glucan in raw poplar on yields of total glucan plus xylan sugars from solids resulting from liquid hot water (LHW) batch and flowthrough pretreatments. Note: Data labels represent overall sugar yields. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. The label "Remaining" means undigested glucan + xylan.

maximum of 48% and 79% (based on xylan in raw poplar) with 100 mg cellulase from solids produced by 140 °C and 180 °C LHW batch pretreatment, respectively. Adding 100 mg of cellulase protein boosted the overall xylan yield to 89% from 140 °C LHW batch pretreatment and 98% from 180 °C LHW operation. The lower sugar yields from enzymatic hydrolysis of the solids produced by batch LHW pretreatment compared to flowthrough LHW operation are likely due to higher xylan and lignin contents in the former (Bhagia, Li et al., 2016).

Fig. 2 also reports the effect of cellulase loadings on overall sugar yields from solids produced by EDA pretreatments. As for LHW pretreatment, yields from pretreatment alone as well as overall yields were significantly higher for solids subjected to 180 °C than 140 °C. In fact, solids from 140 °C batch EDA pretreatment were quite recalcitrant, with the maximum overall yield being only 58% despite an enzyme loading of 100 mg cellulase protein. Unlike LHW for which only the 180 °C flowthrough pretreatment achieved a 100% overall sugar yield, EDA pretreatments at 180 °C achieved 100% overall sugar yields for both batch and flowthrough configurations. Supplemental Table 2 shows that 140 and 180 °C EDA batch solids had xylan contents of 8 and 3%, respectively, and similar lignin content of 30% and 29%, respectively. This coincided with the findings reported earlier that in batch pretreatments removing xylan has a greater effect on enzymatic saccharification than removing lignin (Kumar & Wyman, 2009d; Yang & Wyman, 2004).

Furthermore, even for a low loading of 5 mg cellulase protein, the difference in overall sugar yields between 140 °C (45%) and 180 °C (48%) flowthrough pretreatments was minor. However, the differences increased substantially when the enzyme loading was increased to 15 mg protein. The overall sugar yield from 180 °C flowthrough pretreatment increased to 80% while that from 140 °C flowthrough operation only rose to 59%.

These results provide some insights into features contributing to recalcitrance in hardwoods like poplar pretreated at aqueous conditions. Enzymatic digestibility has a strong positive correlation with xylan removal (Yang & Wyman, 2004). Higher sugar yields for EDA pretreatment can be due to lower xylan content (high xylan removal with pretreatment) and/or altered lignin structure than the hot water pretreated solids. Because the three solids that realized complete deconstruction to sugars were all pretreated at 180 °C, temperature plays a crucial role in reducing recalcitrance. Furthermore, despite high lignin removal (63–67%) by 140 °C flowthrough pretreatments, high yields could not be achieved after 5 days of enzymatic hydrolysis even for the high enzyme loading of 100 mg cellulase protein. On the other hand, near theoretical overall sugar yields were possible from extremely dilute acid pretreated solids with lignin contents as high as 29%, provided the highest enzyme loading was applied. Sugar yields from flowthrough pretreatment were always superior to those from batch operation for the same pretreatment temperature, time, and aqueous condition, likely due to greater xylan and lignin removal. It was unexpected that adding so little acid to water would reduce recalcitrance of pretreated solids to subsequent enzymatic hydrolysis to the extent observed over the wide range of enzyme loadings applied.

3.3. *Effect of adding xylanase*

Two strategies were employed for supplementing cellulase with xylanase. First, 15 mg of cellulase protein together with 30 mg of xylanase protein per gram glucan in unpretreated biomass were added at the same time. In the second strategy, 30 mg of xylanase protein was added 4 days before addition of 15 mg of cellulase protein. Fig. 3 shows that either of these strategies increased overall

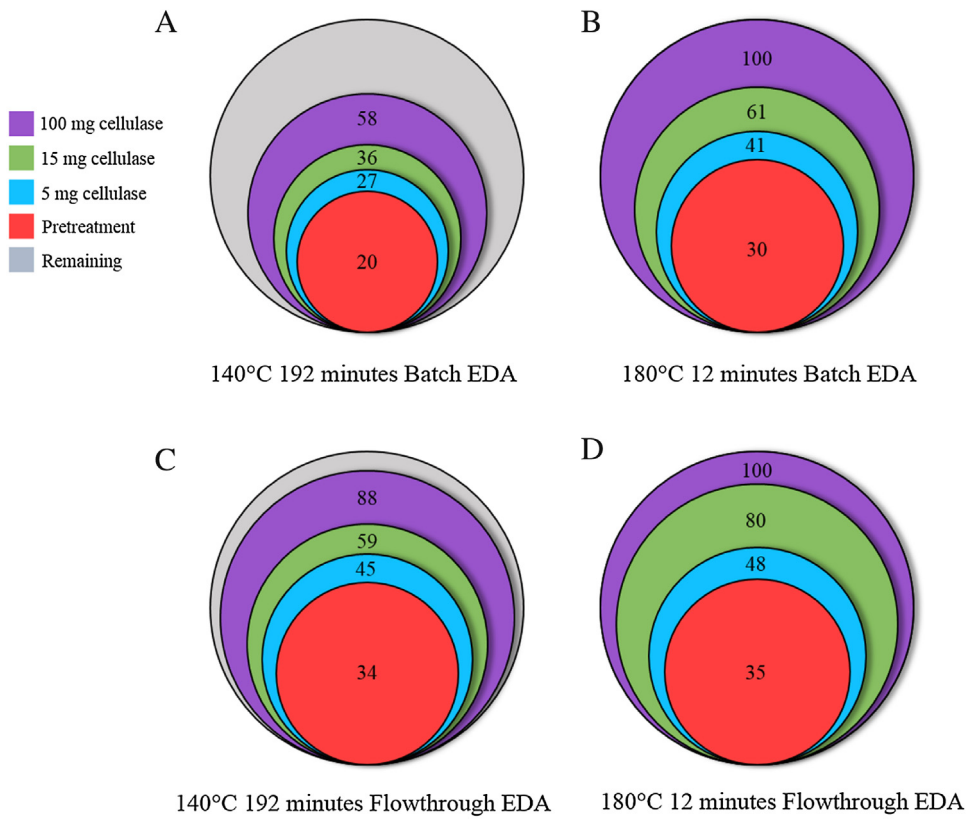

Fig. 2. Effect of cellulase loadings of 5, 15, and 100 mg protein/g glucan in raw poplar on yields of total glucan plus xylan sugars from solids resulting from extremely dilute acid (EDA) batch and flowthrough pretreatments. Note: Data labels represent overall sugar yields. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. The label "Remaining" means undigested glucan + xylan.

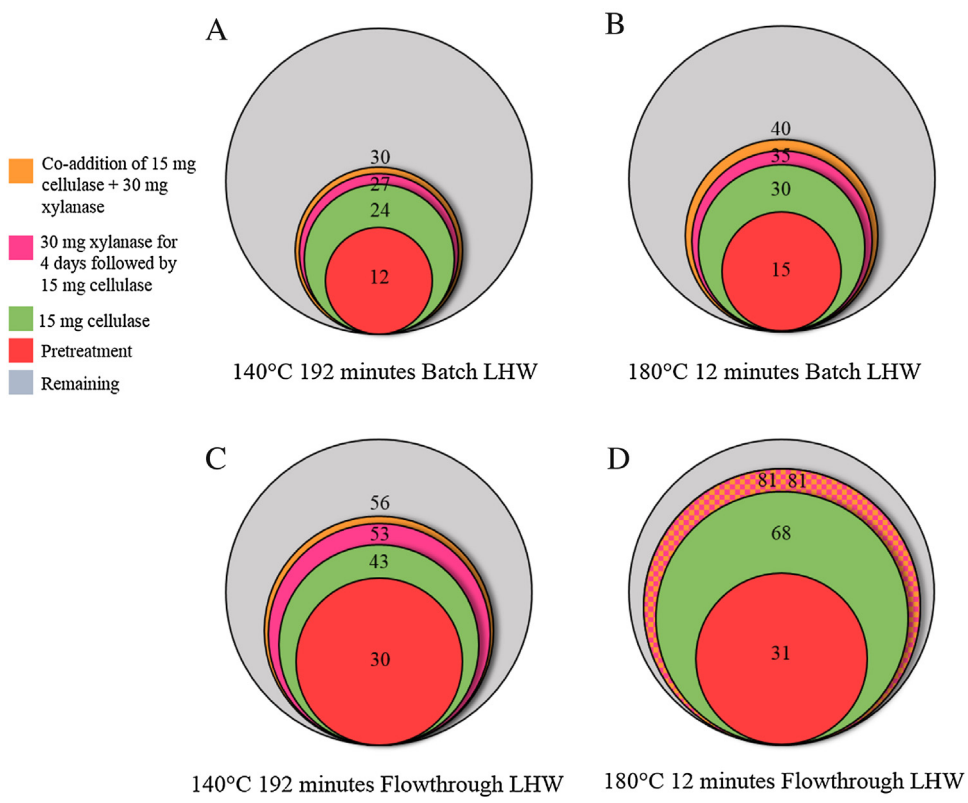

Fig. 3. Effect of adding xylanase to cellulase on yields of total glucan plus xylan sugars from solids resulting from liquid hot water (LHW) batch and flowthrough pretreatments. Note: Data labels represent overall sugar yields. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. The label "Remaining" means undigested glucan + xylan.

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sugar yields from LHW pretreatment. However, only solids produced by 180 °C LHW flowthrough pretreatment realized the same overall yields (81%) for either xylanase supplementation strategy. It is interesting to note that these solids had the lowest xylan content (5%) of all LHW solids. For the other three LHW pretreatment conditions, overall yields were higher 5 days after co-addition of cellulase and xylanase than when xylanase was allowed to act alone for 4 days prior to adding cellulase followed by another 5 days of reaction. These trends for supplementing cellulase with xylanase were similar to those seen in Fig. 1 for varying cellulase doses, that is to say, xylanase supplementation resulted in larger improvements in overall yields from solids that pretreatment had rendered less recalcitrant.

Crude cellulase preparations have some xylanase activity, and crude xylanase preparations have some cellulase activity (Chundawat et al., 2011). Moreover, endoglucanase (EG 1) is highly active on xylan and not just on amorphous cellulose. For example, Gao, Chundawat, Krishnan, Balan, and Dale (2010) reported that purified EG1 had a specific activity of 5.08 U on xylan compared to 8.24 U for endoxylanase. As noted in Supplemental Table 4, 4 days of hydrolysis with xylanase alone resulted in some improvement in xylan saccharification but negligible improvement in glucan saccharification. Interestingly, overall xylan yields with 30 mg of xylanase alone were the same as with 15 mg of cellulase alone. Since crude xylanase preparation (Multifect® Xylanase) without cellulase (Accellerase® 1500) did not show significant glucan hydrolyzing ability (Supplemental Table 5), xylan yield with just 30 mg xylanase can mean that this portion of xylan was accessible to xylanase and was not obstructed by cellulose.

Fig. 4 shows results from supplementing cellulase with xylanase for enzymatic hydrolysis of solids pretreated at EDA conditions. It can be seen that xylanase supplementation improved overall sugar yields from solids produced by flowthrough pretreatment more than from solids resulting from batch operations. Similar to results with solids produced by LHW pretreatments, higher overall yields were achieved through co-addition of cellulase and xylanase than xylanase followed by cellulase. Significant improvement seen in overall yield by supplementation of xylanase to cellulase in these solids was largely due to improvements in glucan yields as contribution of xylan yield to overall yield was small due to low xylan contents of EDA solids (Supplemental Table 2).

In fact, 180 °C batch and 140 and 180 °C flowthrough pretreatments removed close to 90% of the original xylan with the result that of the 17.5 mg of xylan in 100 mg of unpretreated poplar, only about 1.75 mg xylan was left in these three solids. On the other hand, because 140 °C EDA batch pretreatment solubilized only 68% of xylan, about 5.6 mg of xylan would be left in 100 mg of pretreated solids. However, combining 15 mg of cellulase protein with 30 mg of xylanase protein did not improve yields from any of the EDA pretreated solids compared results with 15 mg of cellulase protein alone.

The similar or in most cases better sugar yields from co-addition of cellulase and xylanase compared to addition of xylanase prior to adding cellulase 4 days later could result from loss of activity of xylanase over the extended hydrolysis for sequential addition. The differences could also be due to differences in adsorption for co-addition versus sequential addition of xylanase to cellulase. The third possibility is that cellulase and xylanase work in synergy. It has been previously found that mechanism of cellulase and xylanase maybe synergistic on steam pretreated corn stover as sugar yields were higher in the simultaneous strategy than sequential strategy (Hu, Arantes, & Saddler, 2011). Synergism has been previously shown for bacterial xylanase and cellulase which worked better in the co-addition strategy than sequential addition strategy (Murashima, Kosugi, & Doi, 2003). However, this outcome contradicts finding in one study that reported no difference in co-addition or sequential addition of xylanase followed by cellulase (Kumar & Wyman, 2009d). Also, because it has been reported that better or similar yields can be achieved from enzymatic hydrolysis through replacement of cellulase by xylanase (Hu et al., 2011) an extra experiment with 45 mg cellulase alone was applied to see if these enzymes were interchangeable for the solids used in this study and gave similar yields as co-addition of 15 mg of cellulase protein with 30 mg of xylanase.

3.4. Effect of BSA

It is known that a portion of carbohydrate active enzymes (CAZys) may be lost through adsorption to hydrophobic lignin sites, while binding of Tween 20 (polysorbate type non-ionic surfactant) and bovine serum albumin (BSA) to lignin can block CAZys adsorption and improve cellulose saccharification (Eriksson et al., 2002; Kumar & Wyman, 2009d; Qing et al., 2010a; Yang & Wyman, 2006). Detailed discussion of this topic can be found elsewhere in a review article (Liu, Sun, Leu, & Chen, 2016). Similarly, the improvement in sugar yields by adding xylanase could result from some of the cellulase or xylanase or both blocking lignin and making the remaining enzymes more effective. Thus, to differentiate catalytic effects from lignin blocking effects of xylanases from enzyme loss to lignin, 30 mg BSA was used in place of the 30 mg of xylanase. Although xylanases and BSA likely have somewhat different affinities for lignin, similar performance by both would suggest that reducing enzyme losses to unproductive binding might explain why adding xylanase enhanced yields. Supplemental Tables 4 through 6 show that there was no significant difference in glucan, xylan, and glucan + xylan overall yields when 30 mg of BSA was added at the same time as 15 mg cellulase to LHW solids compared to yields from adding 15 mg cellulase alone. Thus, the improvement in sugar yields by xylanase addition can be attributed to the catalytic effect of xylanase on LHW solids. Similar to solids from LHW batch pretreatments, 30 mg BSA did not enhance sugar yields from solids prepared by EDA batch pretreatment at either temperature. However, adding 30 mg of BSA increased overall sugar yields from 59 to 68% with 15 mg cellulase from solids produced by 140 °C EDA flowthrough pretreatment and from 80 to 91% with 15 mg cellulase from solids resulting from 180 °C EDA flowthrough operation. Because addition of BSA did not improve the xylan yields reported in Supplemental Table 4, the increase in overall sugar yields was just due to improved glucan release. In all, the catalytic effect of xylanases was dominant over unproductive losses to lignin in all solids other than EDA flowthrough solids.

Furthermore, a high BSA loading was employed similar to other studies (Brethauer, Studer, Yang, & Wyman, 2011; Eriksson et al., 2002; Ko, Ximenes et al., 2015; Yang & Wyman, 2006) to block the surface of lignin from unproductive loss of enzyme. 100 mg of BSA was added to LHW pretreated solids 24 h prior to addition of 15 mg of cellulase or to co-addition of 15 mg cellulase and 30 mg xylanase. The results plotted in Fig. 5 reveal that adding BSA before enzymes had a negligible effect on overall sugar yields from solids pretreated by LHW batch reactors at both temperatures. However, pre-addition of BSA did enhance sugar yields from solids produced by LHW flowthrough pretreatment.

Data are also included in Fig. 6 on sugar yields from EDA pretreatment followed by addition of 100 mg of BSA per gram glucan 24 h prior to addition of 15 mg cellulase and co-addition of 15 mg cellulase and 30 mg xylanase to the EDA solids. For batch EDA operation, BSA outperformed xylanase for poplar solids pretreated at 180 °C, however its impact was low on 140 °C EDA batch solids. Like 180 °C EDA batch solids, both flowthrough solids had a large impact of BSA supplementation to cellulase. Comparing Figs. 4 and 6, sugar yields were higher through supplementation of BSA to cellulase and xylanse to EDA solids, unlike LHW solids where supplementation

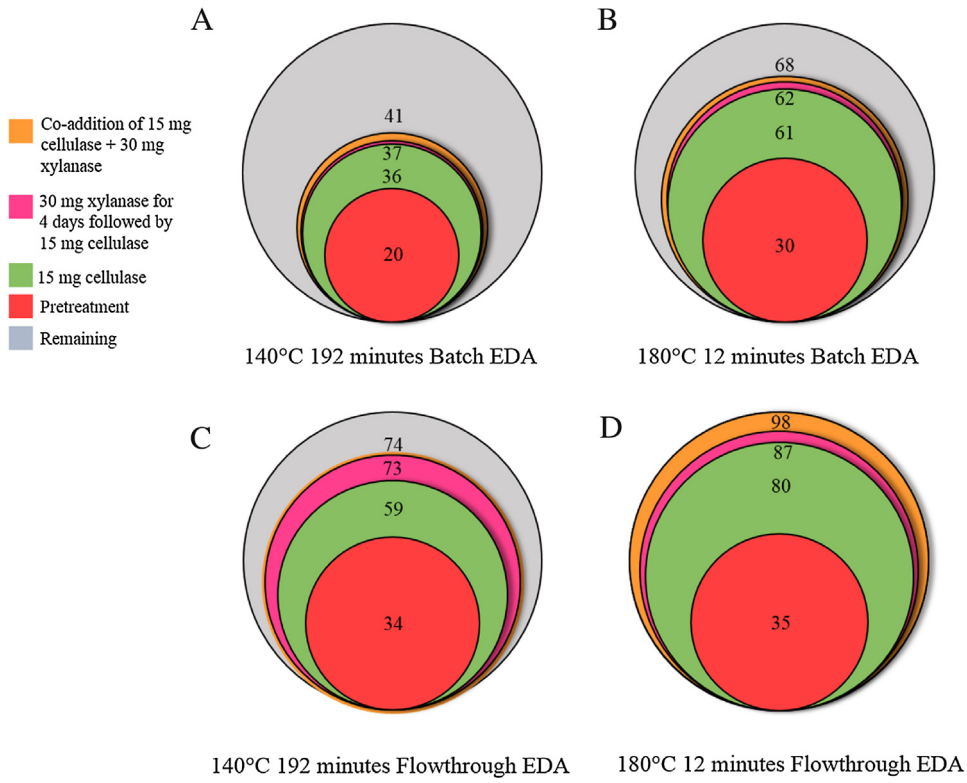

Fig. 4. Effect of adding xylanase to cellulase on yields of total glucan plus xylan sugars from solids resulting from extremely dilute acid (EDA) batch and flowthrough pretreatments. Note: Data labels represent overall sugar yields. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. The label "Remaining" means undigested glucan + xylan.

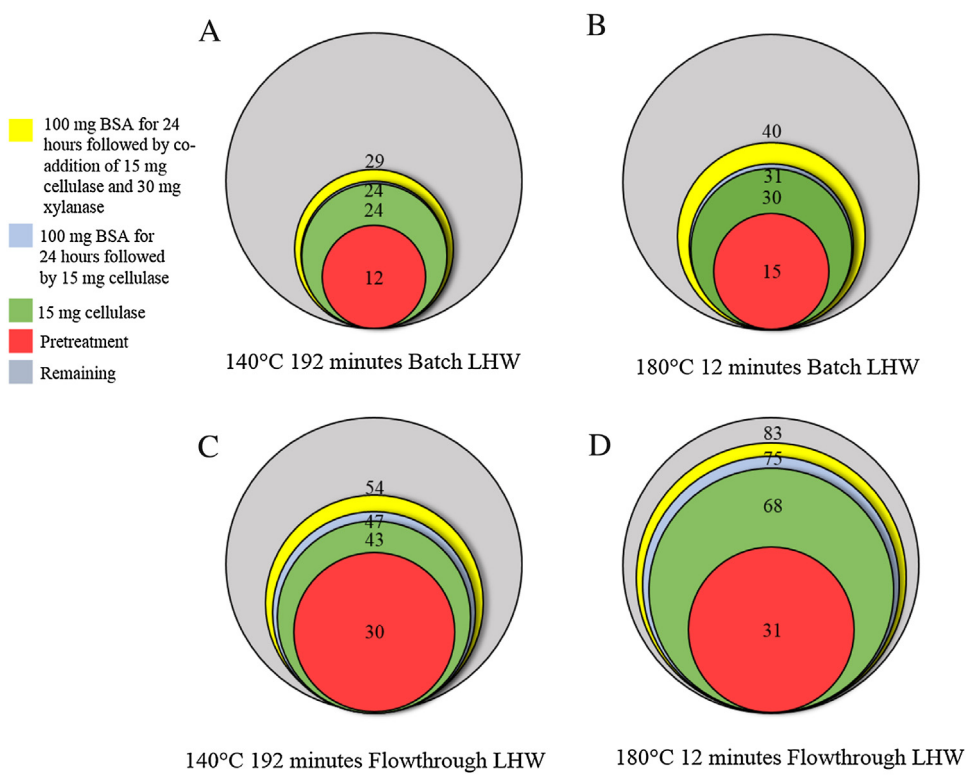

Fig. 5. Effect of adding BSA to cellulase on yields of total glucan plus xylan sugars from solids resulting from liquid hot water (LHW) batch and flowthrough pretreatments. Note: Data labels represent overall sugar yields. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. The label "Remaining" means undigested glucan + xylan.

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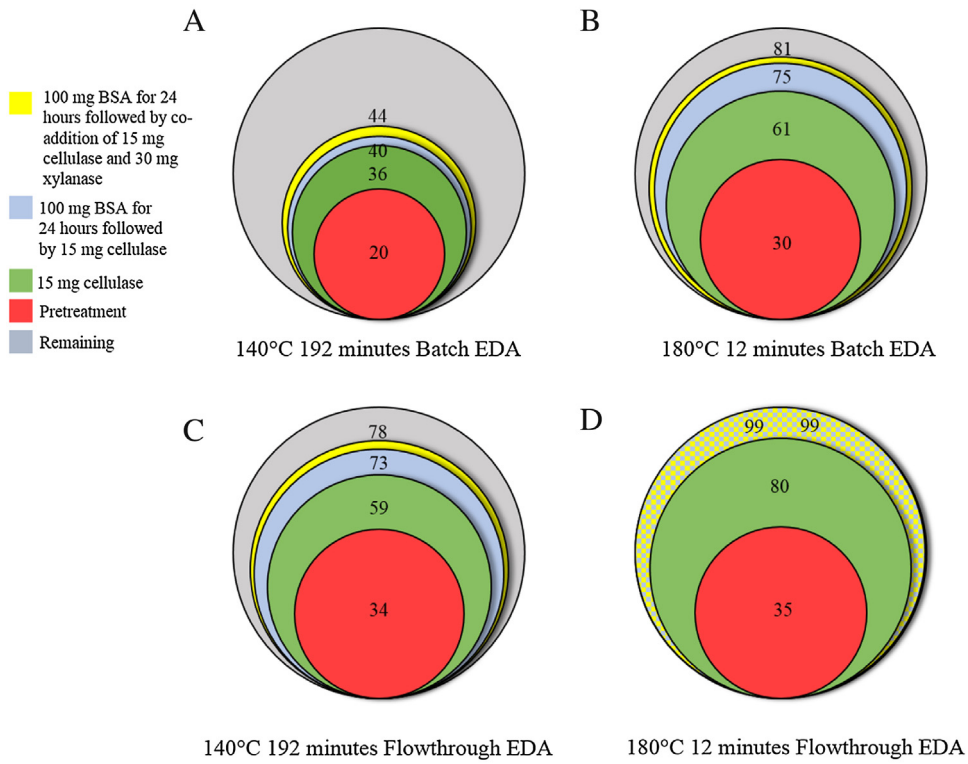

Fig. 6. Effect of adding BSA to cellulase on yields of total glucan plus xylan sugars from solids resulting from extremely dilute acid (EDA) batch and flowthrough pretreatments. Note: Data labels represent overall sugar yields. Enzymes were loaded as mg of protein per gram glucan in unpretreated biomass. The label "Remaining" means undigested glucan + xylan.

of BSA to cellulase and xylanase had low impact on sugar yields. This result might be due to higher hydrophobicity of lignin (Sun, Huang, Sun, & Tu, 2016; Yang & Pan, 2016) in EDA solids than LHW solids and needs further investigation.

Overall for LHW solids, adding xylanase to cellulase improved overall sugar yield more than adding BSA to cellulase, while for EDA solids, adding BSA to cellulase had a similar or better effect as adding xylanase to cellulase. It was surprising to see the significant effect of BSA on flowthrough solids that have much lower lignin content than batch solids. Thus, BSA seems to have a larger role than just reducing unproductive binding of enzyme to lignin. Moreover, co-addition of 30 mg BSA with 15 mg cellulase only improved sugar yields significantly for solids produced by EDA flowthrough pretreatment. Furthermore, adding 30 or 100 mg of BSA to 15 mg cellulase only enhanced sugar yields from glucan but not xylan (Supplemental Tables 4 and 5).

4. Conclusions

Co-addition of cellulase and xylanase produced higher yields than sequential addition of xylanase followed by cellulase suggesting loss of xylanase activity or differences in adsorption or synergism between the two enzyme preparations. The catalytic effect of xylanase had a greater role in improving sugar yields than in blocking lignin in all solids except in extremely dilute acid flowthrough solids. Furthermore, bovine serum albumin (BSA) supplementation largely outperformed co-addition of cellulase and xylanase only when applied to solids produced by 180 °C extremely dilute acid batch pretreatment. In general, BSA improved sugar yields more from solids produced by flowthrough pretreatment than by batch pretreatment despite the lower lignin content in flowthrough solids. Thus, it appears to have a larger role in enhancing sugar yields than just reducing unproductive binding. BSA had significant impact on extremely dilute acid solids but low impact on liquid hot water solids probably due to differences in hydrophobicity of lignin. It is not known why BSA only affected glucan yields while having no effect on xylan yields. Flowthrough pretreatment realized higher sugar yields from pretreatment as well as enzymatic hydrolysis compared to batch pretreatment at otherwise identical conditions likely due to high lignin removal. Extremely dilute acid pretreatment reduced recalcitrance to enzymes significantly compared to liquid hot water operation suggesting the profound effect of lowering of pH on biomass recalcitrance.

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Competing interests

CEW is a cofounder of Mascoma Corporation and former chair of their Scientific Advisory Board. CEW is also founding Editor in Chief of this Journal BfB. He is also a co-founder, president, and CEO of Vertimass LLC, a startup focused on catalytic conversion of ethanol to fungible hydrocarbon fuels. The other authors declare that they have no competing interests.

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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.carbpol.2016.11.085.

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