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Enhancement of total sugar and lignin yields through dissolution of poplar wood by hot water and dilute acid flowthrough pretreatment

Lishi Yan, Libing Zhang and Bin Yang*

Abstract

Background: Pretreatment is a vital but expensive step in biomass biofuel production. Overall, most of this past effort has been directed at maximizing sugar yields from hemicellulose and cellulose through trials with different chemicals, operating conditions, and equipment configurations. Flowthrough pretreatment provides a promising platform to dissolution of lignocellulosic biomass to generate high yields of fermentable sugars and lignin for biofuels productions.

Results: Dissolution of xylan, lignin, and cellulose from poplar wood were significantly enhanced by water-only and dilute acid (0.05% w/w, H₂SO₄) flowthrough pretreatment when the temperature was raised from 200°C to 280°C over a range of flow rates 10-62.5 mL/min, resulting in more than 98% solid removal. Up to 40% of original xylan was converted to xylose in the hydrolyzate and the rest xylan was solubilized into xylooligomers with negligible furfural formation. Up to 100% cellulose was removed into hydrolyzate with the highest glucose yield of 60% and low 5-hydroxymethylfurfural (5-HMF) formation. The maximal recovered insoluble lignin and soluble lignin were 98% and 15% of original lignin, respectively. In addition, enzymatic hydrolysis of pretreated whole slurries was characterized under various enzyme loadings with or without Bovine serum albumin (BSA) treatment. More than 90% glucose yield and 95% xylose yield were obtained from enzymatic hydrolysis of dilute acid pretreated whole slurries with 10 mg protein Ctec 2 with 2 mg Htec2/g glucan + xylan.

Conclusions: Nearly complete dissolution of whole biomass was realized through water-only and dilute acid flowthrough pretreatment under tested conditions. Temperature was considered as the most significant factor for cellulose degradation. The cellulose removal significantly increased as temperature reached 240°C for water-only and 220°C for dilute acid. Dilute acid pretreatment resulted in higher yields of recovered xylan and cellulose as monomeric sugars in the hydrolyzate than that for water-only pretreatment. Enzymes readily hydrolyzed the degraded cellulose and xylooligomers in pretreatment hydrolysate. Results suggested that kinetics controlled the flowthrough pretreatment of biomass dissolution, which was also affected by flow rate to certain extent.

Keywords: Hot water, dilute acid, flowthrough pretreatment, Severity parameter, poplar wood, Enzymatic hydrolysis

Background

Deconstruction of the naturally recalcitrant complex polymers comprising lignocellulosic biomass into simpler molecules that can be converted into useful fuels and chemicals is the major hurdle that needs to be overcome for economic viability [1]. Pretreatment is essential for achieving high yields of desirable products through

overcoming the recalcitrance of lignocellulosic feedstocks, including: (1) hemicellulose, lignin and other compounds coating the surface of the cellulose microfibrils, and (2) the crystalline nature of the cellulose structure [2]. The feasibility of many pretreatment technologies has been proven at bench and pilot scales. However, a promising, less expensive path to improving the technology by the use of very dilute acid or even water-only technologies has been suggested [3]. Apart from their economic viability, these technologies have several powerful attributes including high yields, high cellulose digestibility, low chemical usage, and

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fewer safety and environmental concerns [4]. Unfortunately, these alternative approaches are typically difficult to implement due to the high water consumption [2].

A number of studies over the years have shown that passing liquid hot water with and/or without addition of chemicals (for example, acid, alkali) [5-9] through cellulosic biomass at high temperatures produces highly digestible cellulose, high yields of sugars from hemicelluloses [8,10-12], over 85% lignin removal [13], and liquid hydrolyzate that appears more compatible with fermentative organisms [14]. Increasing the temperature of hot water flowthrough pretreatments to 225 to 270°C within or above saturated steam pressure also solubilizes the cellulose [10,15]. For example, as early as the 1970s and 1980s, Bobleter and his colleagues [16] applied hot water flowthrough process to hydrolyze air-dried pure cellulose at 260 to 270°C. Up to 52% glucose yield and 10% 5-hydroxymethylfurfural (5-HMF) were obtained through hydrolyzing cellulose under 265°C at a flow rate of 12 mL/minute. Furthermore, employing a two-stage (230°C for 15 minutes and 270°C for 15 minutes) semi-flow hot water pretreatment at a flow rate of 10 mL/minute under pressure of 10 Mpa was found to remove 100% xylan, 89.4% lignin and 79.5% cellulose, respectively. However, substantial sugar degradations, including furfural (approximately 6.9%), 5-HMF (approximately 6.9%), glycoaldehyde (approximately 2.7%), were observed [17]. Results from flowthrough pretreatment at elevated temperatures provide invaluable evidence of the deconstruction pattern of biomass and improve understanding of how releases of various biomass fractions are related while providing new fundamental insights into hydrolysis kinetics that are not possible to observe in batch operations.

Enzymatic hydrolysis of pretreated whole slurry, including hydrolyzate and pretreated solid residues, in a simplified single step that could lead to lower capital and operating costs [18], depending on the technologies and conditions applied, was shown to be challenging. The nature of both pretreatment hydrolysate and pretreated residues strongly affect the digestibility of pretreated whole slurries [19,20]. Bovine serum albumin (BSA) treatment with the mechanism attributed to promoting blocking enzymes from non-productive binding [20], stabilizing enzyme [21], and detoxification of hydrolyzate [22] was shown promising in improving the efficacy of enzymatic hydrolysis. Therefore, BSA treatment coupled with advanced pretreatment method has the potential to realize enzymatic hydrolysis of pretreated whole slurry with high yield.

In this study, poplar wood was pretreated in a flowthrough system at elevated temperatures of 200 to 280°C under varied conditions (0 to 30 minutes, H₂SO₄ 0.0 to 0.05% (w/w), and flow rates of 10 to 62.5 mL/minute) to investigate effects on yields of total mass, lignin and

sugars (mono and oligomer), as well as subsequent enzymatic hydrolysis of pretreated whole slurries. In addition, the evaluation of subsequent enzymatic hydrolysis of pretreated whole slurries at varied enzyme loadings, with and without BSA was compared. We seek to understand reasons for differences in the performance, and establish knowledge gained to help apply and improve pretreatment technology.

Results and discussion

Effects of preheating on removal of total mass, xylan, lignin, and cellulose

At the start of reaction, the temperature transients that occur as the reactor is heated from ambient to reaction temperature must be considered [23]. A series of experiments for water-only (220 to 280°C) and 0.05% (w/w) H₂SO₄ operations (200 to 250°C) with a flow rate of 10.0 to 62.5 mL/minute were carried out to determine the poplar wood degradation performance during the preheating process (provided in Additional file 1: Figure S1 and Additional file 2: Table S1). As presented in Figure S1, preheating time from room temperature to 200 to 280°C ranged from 1.2 to 2.8 minutes. Table S1 shows that more than 76% xylan and 52% lignin were removed during preheating to 220°C for water-only pretreatment. Elevating target temperature or adding acid increased the removal of both xylan and lignin. Overall, up to 100% of xylan, 49% cellulose and 87% lignin were removed into the hydrolyzate through the preheating processes under tested conditions. Most of the dissolved xylan and cellulose for these preheating processes was in the form of oligomers with a small amount of xylose and glucose, and negligible degradation compounds.

Effects of pretreatment severity parameter on removal of total mass, xylan, lignin, and cellulose

Removal of xylan, lignin, and cellulose from poplar wood through flowthrough pretreatment under target temperatures ranging from 220 to 280°C for water-only, and 200 to 250°C for dilute acid pretreatment, for 0 to 30 minutes (including preheating time), and at flow rates of 10 mL/minute, 25 mL/minute and 62.5 mL/minute, were investigated. The pH of each liquid sample was promptly measured with a pH meter upon cooling to room temperature. Water-only pretreated hydrolyzates with a pH value of 4.0 to 3.2 and dilute acid pretreated hydrolyzates with a pH value of 2.6 to 2.2 were observed at corresponding severities (Figure 1a).

Xylan removal

It is known that hemicellulose and lignin are covalently linked in biomass, and the high solubility of hemicellulose oligomers can facilitate their dissolution, thus these soluble compounds can be removed before any further

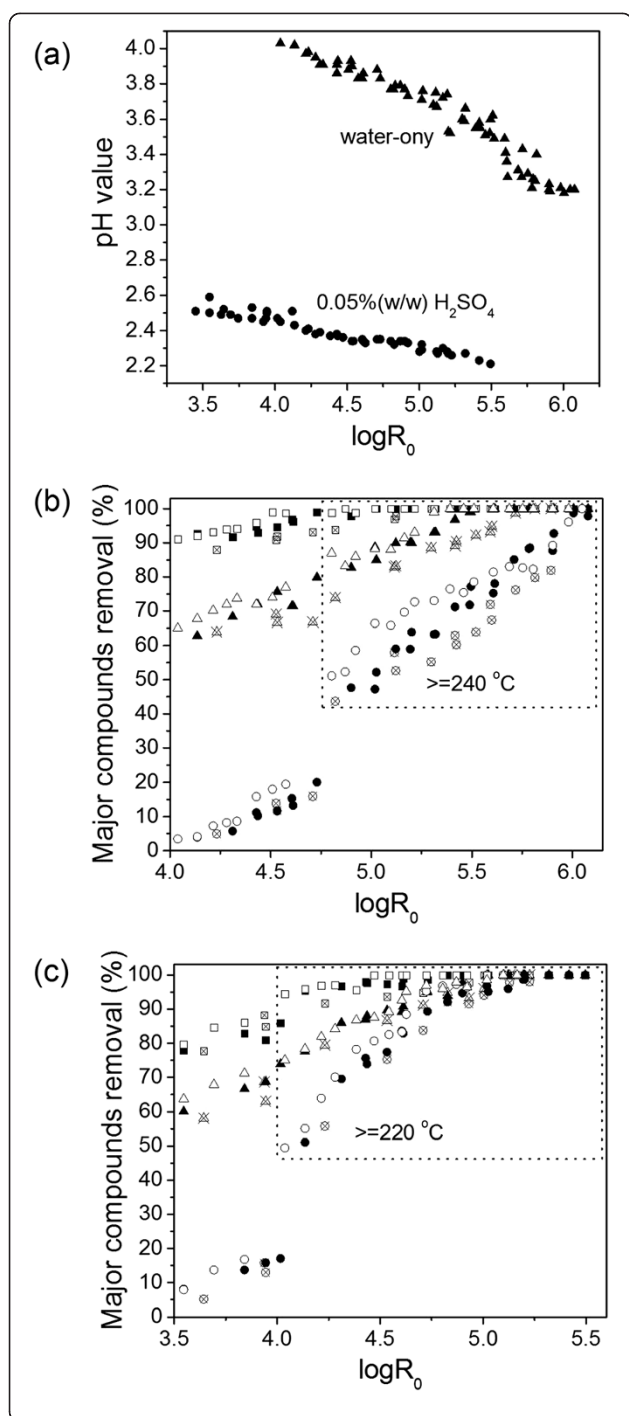


Figure 1 Effect of severity parameter ($\log R_0$) on the removal

of xylan, lignin and cellulose. **(a)** Log R_0 versus pH: solid triangles = water-only pretreatment; solid circles = 0.05% (w/w) H_2SO_4 pretreatment. **(b)** Log R_0 versus removal of xylan, lignin and cellulose with water-only pretreatment. **(c)** Log R_0 versus removal of xylan, lignin and cellulose with 0.05% (w/w) H_2SO_4 . In **(b)** and **(c)**: crossed squares = xylan removal (10 mL/minute), solid squares = xylan removal (25 mL/minute); open squares = xylan removal (62.5 mL/minute); crossed triangle = lignin removal (10 mL/minute), solid triangle = lignin removal (25 mL/minute), open triangle = lignin removal (62.5 mL/minute); crossed circle = cellulose removal (10 mL/minute); solid circle = cellulose removal (25 mL/minute); open circle = cellulose removal (62.5 mL/minute).

reactions occur [24]. Figure 1b and c show that increasing severities for water-only and dilute acid pretreatment enhanced xylan removal. Almost all xylan was removed when reaction severity $\log R_0 > 4.5$ and $\log R_0 > 4.2$ for water-only and dilute acid operations, respectively. As expected, the most readily hydrolyzed constituent, such as xylan, is partially deacetylated as well as depolymerized in the presence of acidic water [7]. The sulfuric acid addition increased the rate of xylan removal for flow-through systems (Figure 1c). However, water-only flow-through pretreatment at lower temperatures (160 to $220^\circ C$) led to almost total xylan removal at analogous severities ($\log R_0 > 4.5$) [13], suggesting that temperature had limited effect on xylan removal. On the other hand, increasing the flow rate from 10 mL/minute to 62.5 mL/minute appeared to have limited effects on xylan solubilization (less than 10% xylan removal increased under experimental conditions).

Lignin removal

The apparent coupling of lignin and hemicellulose release during flowthrough pretreatment suggests that hemicelluloses-lignin oligomers dissolution rates and solubility limitations play key roles in realizing high lignin removal [24]. Results (Figure 1b and c) showed that increasing pretreatment severity improved lignin removal for both water-only and dilute acid pretreatment although less portion of lignin than xylan was removed at a similar severity. At the lowest severity tested, about 65% and 60% lignin was removed by water-only and dilute acid, respectively. For water-only pretreatment, about 85% lignin removal with a flow rate of 10 mL/minute and nearly 100% lignin removal with a flow rate of 62.5 mL/minute at $\log R_0 = 5.3$ were obtained, while for dilute acid pretreatment, nearly 90% lignin removal with a flow rate of 10 mL/minute and 95% lignin removal with a flow rate of 25 mL/minute at $\log R_0 = 4.7$ were observed. At all comparable values of pretreatment severity, higher flow rate resulted in larger portions of lignin being removed by water-only pretreatment. Results suggested that increasing flow rate from 10 mL/minute to

62.5 mL/minute could improve lignin removal by 5 to 15% for water-only and around 5% for dilute acid, respectively.

Cellulose removal

Unlike xylan and lignin, cellulose consists of cellulose I β and I α , which are both held together via a network of hydrogen hydrophobic interactions, causing deconstruction of the crystals challenging [25]. Thus, the removal of cellulose was only 5 to 20% at $\log R_0 = 4.0$ to 4.7 with flow rates ranging from 10 to 62.5 mL/minute for water-only pretreatment, which increased gradually as severity was elevated (Figure 1b). Interestingly, cellulose removal rapidly increased to 40% at 10 mL/minute and 50% with 62.5 mL/minute flow rate when severity $\log R_0$ reached 4.8 at 240°C. As previously reported, cellulose I β underwent a transition into an amorphous structure when temperature increased to around 220 to 230°C [26]. Increasing the temperature of hot water and/or dilute acid flowthrough pretreatments to 220 to 270°C within or above saturated steam pressure solubilizes the cellulose [10,15]. As severity further increased to 6.0 with a temperature ranging from 240 to 270°C, cellulose removal was continuously improved until nearly 100% removal was reached with water-only. The removal of almost all cellulose also corresponded to more than 98% total biomass dissolution. For dilute acid pretreatment, abrupt enhancement of cellulose removal from 16% to about 50% was also observed but at lower severity of 4.0 and lower temperature of 220°C. When $\log R_0$ was higher than 4.0 at a temperature above 220°C, cellulose removal was rapidly improved to nearly 100% at $\log R_0 = 5.0$. Previous water-only or dilute acid flowthrough pretreatment studies also revealed that small amount of cellulose was hydrolyzed at lower temperatures of 180 to 220°C [27], whereas cellulose decomposed significantly at higher

temperatures (that is, >250°C) [5,10]. Results showed that among tested factors (for example, acid concentration, time, etcetera), temperature could play an important role in explaining the effects of acidic aqueous pretreatment on cellulose dissolution. It was reported that a sudden departure of the cellulose degradation rate constants from a normal Arrhenius pattern occurs around 215°C with 0.07% (w/w) H₂SO₄ [28].

In addition, flow rate appeared to affect cellulose removal to some extent. For example, increasing the flow rate from 10 mL/minute to 62.5 mL/minute for water-only pretreatment resulted in 3% to 15% higher cellulose removal at comparable severities (Figure 1b).

Sugars, sugar degradation products and lignin recovery through flowthrough pretreatment

Xylan recovery

Figure 2a presents xylose and xylooligomers yield from poplar wood by water-only flowthrough pretreatment. Results showed that xylooligomers were predominantly recovered xylan in filtered pretreatment hydrolyzate at all tested severities. Higher than 75% xylooligomer yields were observed while xylose yields were less than 25%.

Previous studies reported that recovered xylan pretreated at lower temperatures (200°C) is also primarily composed of xylooligomers with even less xylose (<10% xylose yield) [24]. Xylooligomer yields decreased slightly as $\log R_0$ increased while the corresponding xylose yield increased. It indicated that the increased severity could shift the distribution of generated sugars to monomers. Conversely, increasing the flow rate from 10 mL/minute to 62.5 mL/minute resulted in 10 to 20% improvement of xylooligomer yield while the corresponding xylose yield decreased by about 15%.

Figure 2b revealed that 56.2 to 71.2% xylooligomer yield and 7.2 to 19.2% xylose yield was obtained at around

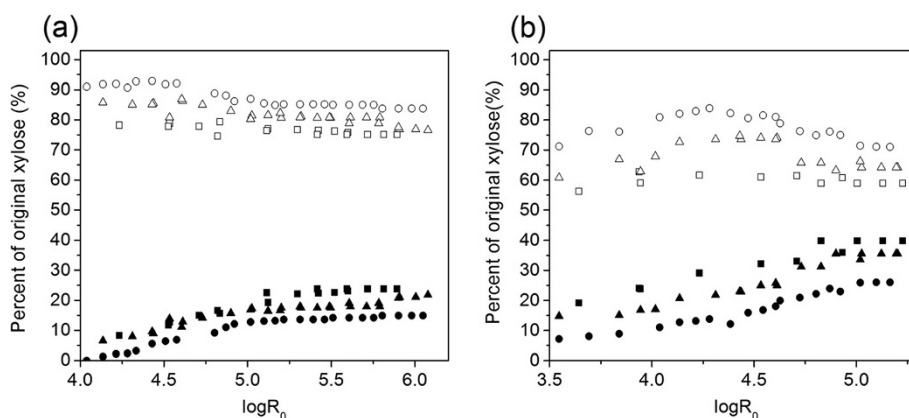


Figure 2 Effect of $\log R_0$ on xylan recovery with (a) water-only and (b) 0.05% (w/w) H₂SO₄. Solid squares = xylose (10 mL/minute); open squares = xylooligomers (10 mL/minute); solid triangles = xylose (25 mL/minute); open triangles = xylooligomers (25 mL/minute); solid circles = xylose (62.5 mL/minute); open circles = xylooligomers (62.5 mL/minute).

$\log R_0 = 3.5$ with flow rates ranging from 10 mL/minute to 62.5 mL/minute for dilute acid pretreatment. As severity increased, xylose yield gradually increased to 39.9, 35.6 and 26.0% at $\log R_0 = 4.9$ with a flow rate of 10 mL/minute, 25 mL/minute and 62.5 mL/minute, respectively, then remained similar value when severity was between 5.0 and 5.5. On the contrary, xylooligomer yield climbed to the peak yields of 74.7 and 83.9% around $\log R_0 = 4.3$ to 4.4 with a flow rate of 25 mL/minute and 62.5 mL/minute, respectively, then it gradually declined as severity increased further. Compared to water-only pretreatment, adding dilute acid increased the xylose yield (Figure 2b). For example, at a flow rate of 25 mL/minute, the xylose yield was observed 14.7 to 35.6% at $\log R_0 = 3.5$ to 5.5, much higher than 6.6 to 21.8% xylose yield obtained with water-only at similar severity parameters. On the contrary, the xylooligomer yield with dilute acid decreased to 60.8 to 74.7% compared with 76.6 to 87.0% for water-only. In addition, results showed that with flow rate of 10 mL/minute, $\log R_0 = 5.9$ was necessary to reach the highest xylose yield of 25% for water-only while dilute acid pretreatment yielded similar xylose at $\log R_0 = 3.9$. It appeared that lower severity value was required to reach similar xylose yield for dilute acid than water-only flowthrough pretreatment.

Results indicated that flow rate had more significant effects on xylose and xylooligomer yield for dilute acid: 10 to 20% increase of xylooligomer yield and 12 to 20% decline in xylose yield when flow rate was increased from 10 mL/minute to 62.5 mL/minute. In addition, results indicated that almost all the removed xylan was recovered as xylose and xylooligomers with negligible amount of degradation compounds. For example, almost 100% xylan removal resulted in 98.2 and 98.8% xylose plus xylooligomers yields for water-only ($\log R_0 = 5.0$, 25 mL/minute) and dilute acid ($\log R_0 = 4.8$, 25 mL/minute), respectively. As in the above discussion of effects of preheating on removal of total mass, xylan, lignin, and cellulose, most of soluble xylooligomers were swept out of the reactor before any further reactions occurred in the preheating procedure, during which the temperature was lower than the target temperature, thus led to low formation of furfural [2].

Cellulose recovery

Both water and dilute acid pretreatments at elevated temperature with increased pretreatment severity (for example, temperature, acid concentration, and reaction time) lead to the decrystallization of cellulose structure and further release of glucose by cleavage of β -1,4-glycosidic bonds hence promote the hydrolysis of cellulosic biomass [10,17,29]. Yields of glucose and soluble glucose oligomers in filtered pretreated hydrolysate can indicate the yields of soluble cellulosic fractions, while the total glucan recovery after enzymatic hydrolysis of unfiltered

hydrolyzate can reveal the total glucan available in pretreated hydrolyzate. In this study, yields of glucose and soluble glucose oligomers in filtered pretreated hydrolysate and the total glucan recovery after enzymatic hydrolysis of unfiltered hydrolyzate were compared. Figure 3a shows that the yields of glucose and soluble glucose oligomers in filtered hydrolyzate and total glucan recovery increased as severity was elevated for water-only pretreatment. Results showed that both glucose and glucose oligomer yields increased gradually as severity increased from 4.0 to 6.0 for all tested flow rates except for glucose oligomer yield, which showed a slightly abrupt increase around $\log R_0 = 4.8$ for a flow rate of 25 mL/minute and 62.5 mL/minute. The highest glucose yield of 16.2% was achieved at a high severity around $\log R_0 = 5.8$ with a flow rate of 10 mL/minute while the highest glucose oligomer yield of 45.0% was found at $\log R_0 = 6.0$ with a flow rate of 62.5 mL/minute. Correspondingly, although the total glucan recovery increased gradually with $\log R_0 < 4.8$, an abrupt increase was observed at about $\log R_0 = 4.8$ and it continuously rose rapidly to around 95% at $\log R_0$ ranging from 4.8 to 6.0 as temperature was higher than 240°C. These results indicated that the total glucan recovery was comparable to that of cellulose removal (Figure 1b). Furthermore, it was noteworthy that the difference between the total glucan recovery and the sum of glucose and glucose oligomer yields, which implied the yield of removed insoluble cellulosic fractions, also showed abrupt enhancement when $\log R_0$ was around 4.8 and temperature was higher than 240°C. At $\log R_0 = 6.0$, nearly 100% cellulose removal merely resulted in 50% glucose plus glucose oligomer yield and 1.6% 5-HMF yield (see Table 1) while the total glucan recovery was about 95% with a flow rate of 62.5 mL/minute. It indicated that when $\log R_0$ was >4.8 , besides glucose and glucose oligomers and the small amount of cellulose in pretreated solid residues, the remainder cellulosic fractions in hydrolyzate was predominately in the form of insoluble cellulosic fractions.

Yields of glucose and soluble glucose oligomers in pretreatment hydrolyzate increased more rapidly with dilute acid than those with water only at similar severity parameters (Figure 3b). Results suggested that the addition of acid accelerated the hydrolysis rate of cellulose to glucose oligomers, and subsequently to glucose. Glucose yield increased gradually with severity at tested flow rates, then climbed steeply to the maximum yield of 59.6% with a flow rate of 25 mL/minute at $\log R_0 = 4.1$ to 5.5, while soluble glucose oligomer yield continuously increased to the peak yield of 43.3% at $\log R_0 = 4.8$ with a flow rate of 62.5 mL/minute, then declined with all tested flow rates as severity further increased. Within the range of tested severity parameters and flow rates, it was found that the maximum yield of glucose plus

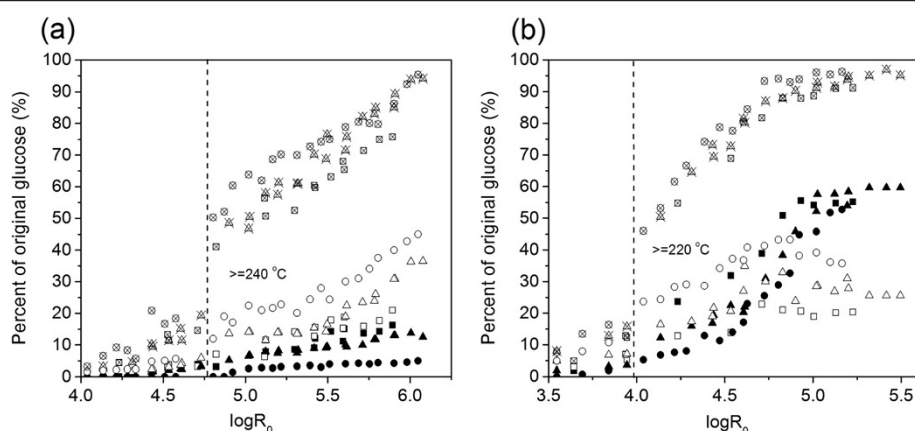


Figure 3 Effect of $\log R_0$ on cellulose recovery by (a) water-only and (b) 0.05% (w/w) H_2SO_4 pretreatment. Solid squares = glucose (10 mL/minute); open squares = glucose oligomers (10 mL/minute); crossed squares = total glucan recovery (10 mL/minute); solid triangles = glucose (25 mL/minute); open triangles = glucose oligomers (25 mL/minute); crossed triangles = total glucan recovery (25 mL/minute); solid circles = glucose (62.5 mL/minute); open circles = glucose oligomers (62.5 mL/minute); crossed circles = total glucan recovery (62.5 mL/minute).

soluble glucose oligomers by dilute acid pretreatment reached 86.3%, much higher than that of 50.2% for water-only operation. In addition, glucose yield by dilute acid was much higher than that by water-only pretreatment. For example, with dilute acid pretreatment, 12.3 to 59.6% glucose yield was obtained at $\log R_0 = 4.1$ to 5.5 with a flow rate of 25 mL/minute. In comparison, under similar conditions (that is, temperature, time, flow rate), glucose yield reached 0.0 to 9.5% for water-only pretreatment. The total glucan recovery pretreated with dilute

acid also increased as severity increased and showed abrupt enhancement at lower severity $\log R_0 = 4.0$ and a lower temperature of 220°C than water-only pretreatment. At $\log R_0 = 5.5$ with a 25 ml/minute flow rate with dilute acid, where 100% cellulose was removed, 84.5% glucose plus soluble glucose oligomer yield with negligible 5-HMF was observed and 98.7% original glucan was recovered in pretreatment hydrolyzate. This indicated around 14.2% insoluble cellulosic fractions were formed.

Table 1 Effects of reaction parameters on the generation of 5-HMF and furfural during flowthrough pretreatment

Flow rate (mL/minute)	Temperature (°C)	Reaction time (minutes) ^a	H_2SO_4 concentration (%)	pH	5-HMF (%)	Furfural (%)
10	220	5	0	3.98	0	0
10	220	10	0	3.90	0	0
10	240	5	0	3.77	1.8	0
10	240	10	0	3.67	3.1	0
10	250	5	0	3.66	2.8	0.7
10	250	10	0	3.56	3.1	0.8
10	220	5	0.05	2.41	1.3	0
10	240	5	0.05	2.36	4.5	1.2
25	240	6	0	3.73	0	0
25	240	10	0	3.67	0	0
25	270	6	0	3.21	0	0
25	270	10	0	3.18	0	0
25	280	6	0	3.19	3.0	0.5
25	240	6	0.05	2.34	0	0
25	250	6	0.05	2.28	2.6	0.3
62.5	270	5.6	0	3.23	0	0
62.5	280	5.6	0	3.12	1.6	0
62.5	250	5.6	0.05	2.33	1.2	0

^aIncluding the preheating time.

Results showed that soluble glucose oligomer yields increased with flow rate for water-only and dilute acid pretreatment (Figure 3a and b). This could be explained by more glucose oligomers dissolving at higher flow rates due to the presence of a greater amount of water. Meanwhile, the faster flow could also rapidly remove dissolved oligomers from the reactor before they further hydrolyze into monomers. On the other hand, a lower flow rate increased the portion of glucose in pretreatment hydrolyzate. For example, at $\log R_0 = 5.9$, when the flow rate decreased from 62.5 mL/minute to 10 mL/minute, the glucose yield increased from 4.2 to 16.3%, while the glucose oligomer yield declined from 40.1 to 21.0% with water-only. The total glucan yield increased 10 to 20% and 5 to 10% for water-only and dilute acid, respectively, when the flow rate was increased from 10 mL/minute to 62.5 mL/minute. Thus, flow rate appeared to influence the generation of glucose and glucose oligomers in a manner similar to its effect on the yields of xylose and xylooligomers.

Sugar degradation patterns

Biomass-derived monomeric sugars can be further dehydrated into furans (furfural and 5-HMF) [30,31], which in turn can degrade into organic acids, such as levulinic acid [32], resulting in reduced fermentable sugar yield. As shown in Table 1, at a flow rate of 10 mL/minute, 3.1% 5-HMF yield was observed at 240°C after 10 minutes with water only, whereas elevating the flow rate to 25 mL/minute resulted in negligible 5-HMF yield. Even when the temperature was raised to 270°C, 5-HMF yield remained negligible with a flow rate of 25 mL/minute: 0.7% furfural was formed under 250°C at 10 minutes when employing a flow rate of 10 mL/minute. However, furfural became imperceptible when the flow rate was raised to 25 mL/minute and 62.5 mL/minute under identical or higher temperatures (for example, 270°C). Results indicated that higher flow rates of 25 mL/minute and 62.5 mL/minute led to both negligible amounts of 5-HMF and furfural at elevated temperatures for both water-only ($\leq 270^\circ\text{C}$) and 0.05% (w/w) H_2SO_4 ($\leq 240^\circ\text{C}$) operations. Compared to other studies conducted at analogous temperatures (265 to 270°C, water only) with lower flow rates (10 to 12 mL/minute), higher amounts of 5-HMF (approximately 10%) and furfural (approximately 6.9%) were observed [16,17]. A flow rate of 25 mL/minute with relatively lower water consumption appeared to be desired for higher sugar concentration. Results suggested that undesirable decomposition reactions of glucose and xylose to 5-HMF and furfural can be limited by controlling severity parameter and flow rate. In line with this reasoning, it is interesting to note that the yields of 5-HMF and furfural observed under water-only and dilute acid operations under analogous

severities were comparable. The yields of furfural were lower than those of 5-HMF under these tested conditions although xylose was much easier to degrade than glucose [33]. The possible explanation was that a much higher fraction of xylan was swept out of a reactor in the preheating period due to a greater solubility when temperature and flow rate increased, and acid was added.

Lignin recovery

Lignin is believed to depolymerize and micellarize under acidic conditions via both homolytic and acidolytic cleavage into low molecular-weight lignin globules [34-36]. As acidic water passes through the material, especially at high flow rates, highly reactive nucleophilic carbonium ion intermediates are formed within the lignin structure, and can react further leading to the cleavage of predominant β -O-4 bonds. This realizes efficient depolymerization of lignin, which can be quickly and continuously swept out of the reactor to limit the simultaneous repolymerization reaction and re-precipitation of the depolymerized lignin at ambient temperature [35,36]. As shown in Figure 4, a large fraction of the recovered lignin in the hydrolyzate during flow-through reactions was in the insolubilized form for both water only and dilute acid. For example, insoluble lignin recovery ranged from 59.3 to 87.8% under water-only conditions (25 mL/minute) when the severity was increased from $\log R_0 = 4.1$ to $\log R_0 = 5.5$. In contrast, adding acid significantly enhanced the insoluble lignin recovery from about 75.6 to 98.0% when $\log R_0$ increased from 4.1 to 5.5. Apart from insoluble lignin, a small fraction of removed lignin was solubilized in the hydrolyzates for both water-only and dilute acid flowthrough pretreatment. For water only, the yield of soluble lignin was 3.6% at $\log R_0 = 4.1$, then increased slowly as the severity parameter increased. The highest yield was 11.7% at $\log R_0 = 5.7$, which then decreased when $\log R_0$ was continuously increased. By comparison, soluble lignin recovery for dilute acid pretreatment was much less than that with water only at all severity ranges. Adding acid resulted in the maximal soluble lignin yield of 5.6% at $\log R_0 = 4.7$. Flow rate effected the distribution of the removed lignin to some extent and it was more apparent for water-only pretreatment. Although a higher flow rate (for example, 62.5 mL/minute) resulted in more lignin removal than a lower flow rate (for example, 10 mL/minute), it was found that 3 to 9% increase in soluble lignin yield was realized when the flow rate declined from 62.5 mL/minute to 10 mL/minute for water only. It was plausible that a lower flow rate increased the exposure of removed lignin under high temperature for decomposing into low molecular weight compounds.

The gas chromatography/mass spectrometry (GC/MS) analysis was essentially carried out to determine the chemical components of lignin recovered through water-

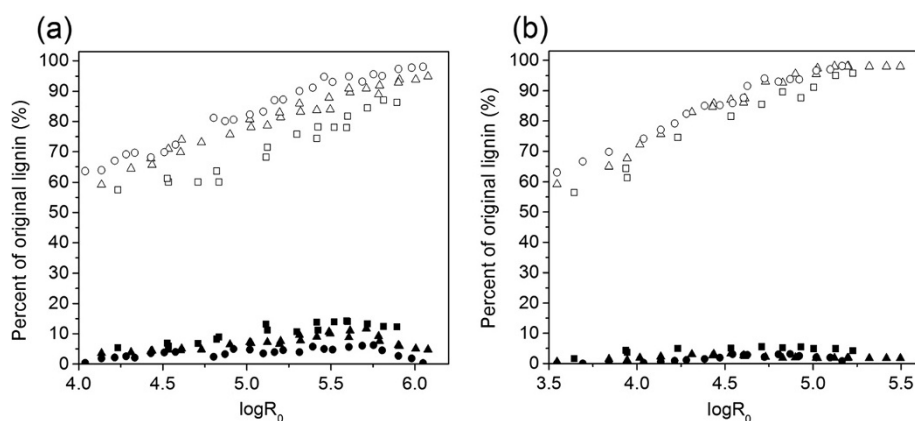


Figure 4 Effect of $\log R_0$ on lignin recovery under (a) water-only and (b) 0.05% (w/w) H_2SO_4 conditions. Solid squares = soluble lignin (10 mL/minute); open squares = insoluble lignin (10 mL/minute); solid triangles = soluble lignin (25 mL/minute); open triangles = insoluble lignin (25 mL/minute); solid circles = soluble lignin (62.5 mL/minute); open circles = insoluble lignin (62.5 mL/minute).

only and dilute acid flowthrough pretreatment. Among the soluble lignin products, vanillin and syringaldehyde were found as the predominant lignin derived aromatic structures pretreated under both water-only and acid conditions (provided in Additional file 3: Figure S2 and Additional file 4: Table S2). These two compounds generally were considered derived from lignin units of coniferyl alcohol (G) and sinapyl alcohol (S). This can be speculated as derived initially from the acidic cleavage of the predominant β -O-4 bonds of lignin to phenylpropanoid structural moieties (for example, sinapaldehyde) and further oxidized to vanillin and syringaldehyde [37,38]. It was noteworthy that dilute acid conditions generated fewer phenylpropanoids than those with water only (provided in Additional file 4: Table S2). For example, no coniferyl alcohol was observed in dilute acid conditions, suggesting that 0.05% (w/w) H_2SO_4 with relatively lower pH was more prone to the oxidation reactions. Most of these soluble lignin compounds presented in hydrolyzates were considered as inhibitory compounds to biocatalysts in the subsequent bioconversion processes. Such hydrolyzates usually require some form of post-pretreatment detoxification to proceed effectively [39,40].

Effects of enzyme loading and BSA addition on enzymatic hydrolysis of pretreated whole slurries

In this study, whole slurries pretreated under water-only or dilute acid conditions were hydrolyzed by enzymes at different enzyme loadings and enzymatic yields of xylose and glucose were investigated. In addition, the enzymatic hydrolysis of pretreated whole slurries with and without BSA was compared to investigate the effects of BSA treatment on digestion of both cellulosic and xylan fractions. Whole slurries pretreated under water-only conditions (that is, 270°C, 10 minutes, 25 mL/minute) and

dilute acid conditions (that is, 240°C, 0.05% (w/w) H_2SO_4 , 8 minutes, 25 mL/minute), which resulted in nearly complete biomass removal, highest total monomeric and oligomeric xylose and glucose yield, negligible sugar degradation products, as well as relatively lower liquid consumption, were applied as substrates for enzymatic hydrolysis evaluation.

As shown in Table 2, results revealed that dilute acid flowthrough pretreatment exhibited overall much better performance in enzymatic hydrolysis than water only. For example, for water-only pretreated whole slurries, about 70% enzymatic glucose yield was reached within 4 hrs at the high enzyme loading and enzymatic glucose yield gradually increased to 95% in 72 hrs. At the medium enzyme loading, only 51.5% enzymatic glucose yield was observed at 4 hrs and it improved to about 65% at 120 hrs. With the low enzyme loading, glucose yields were 41.5 and 49.1% at 4 and 120 hrs, respectively. On the contrary, dilute acid resulted in higher glucose yield of 52.7% at 4 hrs and 73.3% at 120 hrs with the lowest enzyme loading. With the medium enzyme loading, about 93% glucose yield was reached within 120 hrs. At the high enzyme loading, glucose yield was found to be >90% without BSA within 4 hrs. Results indicated that dilute acid pretreatment led to more readily digestible cellulosic derivatives. It could be explained by the fact that the recovered glucan in acid pretreatment hydrolyzate was predominately composed of glucose and soluble glucose oligomers, which totally accounted for 86.3% based on the original glucose in poplar wood. In contrast, only 52.0% yield of glucose plus soluble glucose oligomers was obtained for water-only flowthrough pretreatment, while the rest of removed cellulose (about 50%) was considered as insoluble cellulose derivatives. On the other hand, it is noteworthy that glucose yield within the initial period of 4 hrs of enzymatic hydrolysis

Table 2 Enzymatic hydrolysis of flowthrough pretreated whole slurries

Pretreatment conditions ^a	Enzyme loading ^b	1% (w/w) Bovine serum albumin	Enzymatic glucose yield (%)						Enzymatic xylose yield (%)					
			t ₁	t ₂	t ₃	t ₄	t ₅	t ₆	t ₁	t ₂	t ₃	t ₄	t ₅	t ₆
A	Low	Yes	45.9	48.7	51.5	51.5	53.6	54.9	43.7	79.3	88.0	89.8	90.2	91.3
A	Medium	Yes	56.0	58.5	63.6	66.3	70.8	73.0	62.8	88.1	92.8	95.0	96.0	97.8
A	High	Yes	81.6	93.1	100	100	100	100	94.9	96.8	97.0	97.8	98.0	98.3
A	Low	No	41.5	42.8	43.0	44.9	47.2	49.1	38.6	74.0	84.9	89.2	90.0	90.8
A	Medium	No	51.5	53.8	56.5	59.9	62.5	66.4	58.1	85.0	88.1	92.2	95.0	96.1
A	High	No	73.4	86.5	92.7	95.6	98.1	99.0	89.7	94.1	95.7	96.0	97.1	97.8
B	Low	Yes	57.1	72.6	75.4	77.4	77.6	79.6	48.9	82.5	90.3	91.8	92.1	92.8
B	Medium	Yes	67.3	83.1	88.3	92.8	94.7	97.7	68.3	90.6	91.0	93.2	95.5	97.1
B	High	Yes	99.5	99.8	100	100	100	100	93.7	96.7	97.8	98.1	98.2	98.8
B	Low	No	52.7	65.6	66.2	69.8	71.3	73.3	43.9	77.8	86.3	91.0	91.7	92.2
B	Medium	No	62.9	77.9	82.9	86.5	90.3	93.3	63.8	87.6	89.8	93.0	94.9	97.0
B	High	No	91.2	94.4	97.8	100	100	100	89.0	96.2	96.9	97.2	98.0	98.6

^aA: 270°C, 10 minutes, 25 mL/minute, water only; B: 240°C, 8 minutes, 25 mL/minute, 0.05% (w/w) H₂SO₄. ^bLow enzyme loading: 3 mg protein Ctec2 (2.8 filter paper units (FPU)) with 0.6 mg protein Htec2/g glucan + xylan; medium enzyme loading: 10 mg protein Ctec 2 (9.3 FPU) with 2 mg Htec2/g glucan + xylan; high enzyme loading: 100 mg protein Ctec 2 (93 FPU) with 20 mg Htec2/g glucan + xylan. Enzymatic hydrolysis time-t₁: 4 hrs; t₂: 24 hrs; t₃: 48 hrs; t₄: 72 hrs; t₅: 96 hrs; t₆: 120 hrs.

of pretreated whole slurries with various enzyme loading was 41.5% to 91.2% for both water only and dilute acid, then glucose yield gradually increased to 49.1% to 100% at 120 hrs with prolonged hydrolysis. It indicated that when nearly complete biomass dissolution was achieved, the large portion of soluble glucose oligomers and insoluble cellulose derivatives in pretreatment hydrolyzate were quickly hydrolyzed by enzymes thus enzymatic hydrolysis of such whole slurries was more effective than hydrolysis of cellulose remained in the pretreated solid residues.

Enzymatic xylose yield of pretreated whole slurries reached 94.1 and 96.8% for water only and dilute acid, respectively, within 24 hrs at the high enzyme loading (Table 2). The medium enzyme loading resulted in 92.2 and 89.2% of enzymatic xylose yield for water-only pretreated whole slurries in 72 hrs, respectively. Similar enzymatic xylose yields were found at these lower enzyme loadings for dilute acid pretreated whole slurries. Results suggested that xylooligomers in pretreated whole slurries were effectively hydrolyzed by enzymes even with low enzyme loading and that both water-only and dilute acid flowthrough pretreatment led to high yield of xylose by enzymatic hydrolysis.

It was reported that BSA treatment resulted in substantial improvement of enzymatic glucose yield from enzymatic hydrolysis of solid residues pretreated by various pretreatments [20,21]. Effects of BSA treatment on enzymatic hydrolysis of pretreated whole slurries by water only and dilute acid were investigated. Results showed that enzymatic glucose yield of water-only pretreated whole slurries with low to high enzyme loading

was enhanced around 5 to 10% more than that without BSA treatment. Comparatively, the effectiveness of BSA treatment was less apparent on enzymatic hydrolysis of dilute acid pretreated whole slurries (0 to 6% enhancement) than that of water-only pretreated slurries. Slight improvement of around 0 to 5% in enzymatic xylose yield was observed with BSA addition for both water-only and dilute acid pretreated slurries. It was proposed that BSA blocked non-specific binding of cellulases, reduced inhibitory effects of pretreatment generated compounds and stabilized enzymes [41]. With most of glucan and xylan recovered in pretreatment hydrolyzate in forms of monomers, soluble oligomers and insoluble derivatives in this study, benefits of BSA treatment on improving enzymatic sugar yield were less apparent than that with pretreated solid residues in previous studies [20].

Combined total monomer sugar yields through flowthrough pretreatment followed by enzymatic hydrolysis

The pretreated whole slurries after water-only and dilute acid flowthrough pretreatment (stage 1) subsequently underwent enzymatic hydrolysis (stage 2) to maximize mono sugar yield. Table 3 compares and summarizes the sugar yields obtained from stage 1 and stage 2 under water-only (that is, 270°C, 10 minutes, 25 mL/minute) and dilute acid (that is, 240°C, 0.05% (w/w) H₂SO₄, 8 minutes, 25 mL/minute) conditions that resulted in the highest total sugar yields (monomers and soluble oligomers), negligible sugar degradation products,

Table 3 Material balance^a of flowthrough pretreatment (stage 1) and enzymatic hydrolysis (stage 2)

Pretreatment conditions	Enzyme loading ^b	Stage 1							Stage 1 + stage 2			
		Soluble fractions					Insoluble fractions		Soluble fractions			Insoluble fractions
		Glu (g)	GOS(g)	Xyl (g)	XOS (g)	SL (g)	L-GOS (g)	ISL (g)	Glu (g)	Xyl (g)	SL (g)	ISL (g)
A	High	7.5	19.7	4.0	14.7	1.2	23.8	21.8	52.7	18.6	1.2	21.8
B	Medium	31.2	15.5	6.8	12.2	0.5	4.9	23.0	50.8	18.7	0.5	23.0

^aThe calculation is based on 100 g dry weight poplar wood, which contains 54.2 g glucose, 19.1 g xylose and 23.7 g lignin; A: 270°C, water only, 25 mL/minute, 10 minutes; B: 240°C, 0.05% (w/w) H₂SO₄, 25 mL/minute, 8 minutes; the water consumption for water only (that is, 270°C, 10 minutes, 25 mL/minute) and dilute acid (that is, 240°C, 0.05% (w/w) H₂SO₄, 8 minutes, 25 mL/minute) operations was approximately 250 mL and 200 mL, respectively, which led to around 0.2 to 0.25% (w/w) overall solid to liquid ratio with a solid loading of 0.5 g. ^bHigh enzyme loading: 100 mg protein Ctec 2 (93 filter paper units (FPU)) with 20 mg Htec2/g glucan + xylan; medium enzyme loading: 10 mg protein Ctec 2 (9.3 FPU) with 2 mg Htec2/g glucan + xylan. Glu, glucose; Xyl, xylose; XOS, xylooligomers; GOS, soluble glucose oligomers; L-GOS, insoluble glucan derivatives; SL, soluble lignin; ISL, insoluble lignin.

nearly complete biomass removal and relatively lower water consumption at stage 1.

The enzyme loading employed during stage 2 for selected water-only and dilute acid pretreated slurries were high and medium enzyme loading, respectively, both of which led to >90% enzymatic glucose yield and >95% enzymatic xylose yield from corresponding samples. Results showed that on the basis of 100 g poplar wood, more than half of cellulose and nearly all xylan was converted to soluble sugars at stage 1 for the selected water-only operation: 4.0 g xylose plus 14.7 g xylooligomers, and 7.5 g glucose plus 19.7 g glucose oligomers were obtained. For dilute acid pretreatment, nearly complete polysaccharides solubilization (approximately 100% xylan and approximately 90% cellulose) led to slightly higher xylose content (6.8 g) accompanied with 12.2 g xylooligomers and much higher glucose content of 31.2 g plus 15.5 g glucose oligomers. Predominate soluble sugar fractions and insoluble sugar fractions were converted into sugar monomers at stage 2 for both selected water-only and dilute acid operations, resulting in 52.7 g glucose plus 18.6 g xylose, and 50.8 g glucose plus 18.7 g xylose, respectively. Although the material balance implied slight loss of some mass during pretreatment, the selected flowthrough conditions resulted in more than 93% glucose and 97% xylose yields after stage 1 and stage 2. Particularly, merely less than 10 filter paper units (FPU)/g glucan + xylan enzyme was required to reach over 90% total sugar (C6 and C5) yield during stage 2 for dilute acid pretreated whole slurries because around 90% cellulose was solubilized as glucose and glucose oligomers by pretreatment (stage 1).

Conclusion

Poplar wood was pretreated through water-only and dilute acid flowthrough approaches at a temperature of 200 to 280°C and it resulted in more than 98% solid removal. Temperature was considered as the most significant factor for cellulose degradation. The cellulose removal significantly increased as temperature reached 240°C for water only and 220°C for dilute acid. Up to 100% xylan and 90% cellulose were hydrolyzed with

negligible furfural and 5-HMF formation during pretreatment. Dilute acid pretreatment also resulted in higher yields of recovered xylan and cellulose as monomeric sugars in the hydrolyzate than that for water-only pretreatment. The insoluble lignin accounted for the majority of the original lignin (approximately 90%) while a small amount (approximately 15%) became soluble in the pretreated whole slurries. A larger fraction of recovered lignin was soluble with water-only pretreatment. Increasing severity enhanced total mass removal, xylan removal, lignin removal, and cellulose removal, and adding dilute sulfuric acid significantly accelerated all of the above. Dissolution of almost all biomass in hydrolyzate was obtained at logR₀ around 6.0 without acid added while a faster rate was achieved with dilute acid (logR₀ around 5.0). Comparatively, flow rate appeared to have a less significant effect on removal of xylan, lignin, cellulose, and total mass as well as recovery yields although flow rate was associated with reaction time to affect pretreatment kinetics. Enzymatic hydrolysis of the pretreated whole slurries obtained under desired conditions for water only (270°C, 25 mL/minute, 10 minutes) and dilute acid (240°C, 0.05% (w/w) H₂SO₄, 25 mL/minute, 8 minutes) revealed that 93 to 97% glucose yield and 97 to 98% xylose yield were obtained. The pretreated whole slurries under selected dilute acid conditions (240°C, 0.05% (w/w) H₂SO₄, 25 mL/minute, 8 minutes) that resulted in much higher soluble glucose plus glucose oligomers yield (approximately 90%) at stage 1 than the water-only operation (270°C, 25 mL/minute, 10 minutes) merely required less than 10 FPU/g glucan + xylan enzyme to achieve >90% glucose yield and >95% xylose yield. The limited inhibitory compounds in the pretreated slurries showed insignificant impact on the performance of enzymes on pretreated whole slurries through BSA testing, especially for dilute acid pretreatment. In addition, the insoluble lignin was recovered from hydrolyzate with low molecular weight (<1800 Dalton). We also developed catalytic techniques to convert such technical lignin into C7- to C9-range hydrocarbons through a novel hydrodeoxygenation process in our research group [35,42,43]. Overall, both of water-only and

dilute acid flowthrough pretreatments of poplar wood followed by enzymatic hydrolysis significantly enhanced monomeric sugars and lignin yields. This study proved not only high xylan and cellulose recovery due to the decrystallization of cellulose combined with solubilization of total biomass through pretreatment but also high lignin yield [10,13,15,27,44]. More importantly, the comprehensive characterization of all three major components of biomass during pretreatment under the tested conditions was reported for the first time. These findings also imply that the fundamental interactions of biomass and water and acid can be applied to understand other aqueous chemical pretreatments - their successes, pitfalls, and best optimization strategies can lend considerable insight into their sensitivity. The new insight gained will lead to obtaining of even higher yields of fermentable sugars and reactive lignin for biofuels production.

Methods

Feedstocks

Poplar wood provided by Forest concepts (Auburn, WA, USA) contains 48.8% cellulose, 16.8% xylan and 23.7% Klason lignin as determined by standard National Renewable Energy Laboratory Analytical Procedures (NREL LAPs) [45]. Poplar wood material was grounded with Hammermill (Hammer1067-A-1, Buffalo, NY, USA) at 4500 rpm with a 1.59-mm screen. Then the particles were collected to pass between sieve 20 mesh and sieve 40 mesh to obtain particles over a size range of 0.425 to 0.850 mm for experiments and analysis. The materials were sealed in heavy-duty zipped bags and stored at -20°C in a laboratory freezer.

Flowthrough pretreatment

The flowthrough reactor is 1.3 cm i.d. × 15.2 cm length with an internal volume of 20.2 mL. It is constructed of 316 stainless steel parts using Vacuum Coupling Radius Seal (VCR) fittings, including one VCR male union (1.3 cm), two gasket filters (average pore size 5 μm), two VCR glands (1.3 cm × 1.3 cm), two VCR nuts, and two VCR reducing fittings (1.3 cm × 0.3 cm). All reactor parts are obtained from Swagelok Co., Richland, WA, USA. A preheating coil (0.6 cm o.d. × 0.1 cm wall, stainless steel) is connected with the reactor system and the cooling coil (0.3 cm o.d. × 0.1 cm wall). A high-pressure pump (Acuflow Series III Pumps, Fisher, Pittsburgh, PA, USA) with a flow rate range of 0 to 100 mL/minute, a pressure gauge (pressure range 0 to 1500 psi; Cole-Parmer Instrument Co., IL, USA), and a back-pressure regulator (Valve and Fitting Co., WA, USA) are used to control the flowthrough system. Biomass substrate, 0.5 g, is loaded into the reactor. Distilled water or 0.05% (w/w) sulfuric acid is pumped through the reactor to

purge air and then used to pressurize the reactor to a set pressure of 225 to 1,245 psi. The reactors are heated to the target temperature (200 to 280°C) in a 4-kW fluidized sand bath (model SBL-2D, Omega engineering, Inc., CT). A thermal monitor combined with a 0.3-cm stainless steel thermocouple (Omega Engineering Co., Stamford, CT) was connected to the outlet of the flow reactor to precisely control the reaction temperature.

Analytical methods

The pH of each liquid sample was promptly measured with a pH meter (model pH510 Series, Oakton Instruments, IL) upon cooling to room temperature. All the experiments were performed in duplicate, with the average value reported.

Sugar and sugar degradation products analysis

Glucose, xylose, furfural, and 5-HMF in hydrolyzates of pretreatment and enzymatic hydrolysis were analyzed using a Waters HPLC system (model 2695) equipped with a 410 refractive detector and a Waters 2695 autosampler using Waters Empower Build 1154 software (Waters Co., Milford, MA, USA). Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) was operated under 65°C. Yields of glucose, xylose, furfural, and 5-HMF were calculated as follows [46]:

$$\text{Glucose\%} = \frac{W_G \times MW_{Gn}}{W_{Gn} \times MW_G} \times 100\% \quad (1)$$

$$\text{Xylose\%} = \frac{W_X \times MW_{Xn}}{W_{Xn} \times MW_X} \times 100\% \quad (2)$$

$$\text{5-HMF\%} = \frac{W_{5\text{-HMF}} \times MW_{GN}}{W_{Gn} \times MW_{5\text{-HMF}}} \times 100\% \quad (3)$$

$$\text{Furfural\%} = \frac{W_{Fur} \times MW_{Xn}}{W_{Xn} \times MW_{Fur}} \times 100\% \quad (4)$$

In these equations, W_{Gn} and W_{Xn} represent the initial weight of glucan and xylan, respectively. W_G , W_X , $W_{5\text{-HMF}}$ and W_{Fur} represent the weight of glucose, xylose, 5-HMF and furfural, respectively. The unit of W consistently refers to g/100 g dw raw biomass. Molecular weight: $MW_{Gn} = 162$, $MW_{Xn} = 132$, $MW_G = 180$, $MW_X = 150$, $MW_{5\text{-HMF}} = 126$, $MW_{Fur} = 96$.

Pretreatment hydrolyzate flowing out of the flowthrough system was collected then filtered through a 0.45-μm polypropylene membrane filter (VWR, Radnor, PA, USA). The filtrate was autoclaved in 4% (w/w) sulfuric acid for 1 hr at 121°C to breakdown glucose oligomers and xylooligomers into their monomeric sugars based on standard NREL LAPs [47]. Yields of soluble glucose oligomers and xylooligomers were then calculated as follows [48]:

$$\text{Glucose oligomers\%} = \frac{W_{TG} - W_G}{W_{OG}} \times 100\% \quad (5)$$

$$\text{Xylooligomers\%} = \frac{W_{TX} - W_X}{W_{OX}} \times 100\% \quad (6)$$

In these equations, W_{TG} and W_{TX} represent the total glucose and total xylose after autoclaving of filtrate; W_G and W_X represent glucose and xylose in the pretreatment filtrate before autoclaving; W_{OG} and W_{OX} represent the original glucan (as glucose) and original xylan (as xylose); The unit of W consistently refers to g/100 g dw raw biomass.

Pretreatment hydrolyzate (without filtration, not including solid residue in the reactor) was presoaked with 1% (w/w) BSA at pH 4.8 and then followed by enzymatic hydrolysis at 50°C for 168 hours with a high enzyme loading (100 mg protein Ctec 2 (93 FPU) with 20 mg Htec2/g glucan + xylan) that could guarantee maximum glucan conversion. The final glucose concentration after enzymatic hydrolysis was used to determine the total glucan recovery in pretreatment hydrolyzate. The total glucan recovery by pretreatment was calculated as follows:

$$\text{Total glucan recovery\%} = \frac{W_{EG}}{W_{OG}} \times 100\% \quad (7)$$

In this equation, W_{EG} is the total glucose after enzymatic hydrolysis; W_{OG} is the original glucan as glucose. The unit of W consistently refers to g/100 g dw raw biomass.

Lignin analysis

Insoluble lignin content was measured by K-lignin method [49]. Soluble lignin was estimated by UV analysis measuring absorbance at 320 nm using similar calculation of acid soluble lignin method [50]. The structure characterization of soluble lignin was determined by GC/MS analysis. The pretreated samples were filtered and extracted with dichloromethane [51], then analyzed with an Agilent gas chromatography mass spectrometer (GC, Agilent 7890A; MS, Agilent 5975C) equipped with a DB-5MS column (30 m × 320 μm × 0.25 μm). The oven temperature was programmed from 45 to 250°C at a ramping rate of 5°C/minute. Both the initial and final temperature was held for 5 minutes. The flow rate of carrier gas (helium) was 1.3 mL/minute.

Enzymes

Commercial preparations of Novozymes Cellic® CTec2 (220 mg protein/mL, preserve 200 mg glucose/mL, 205 FPU/mL) and Novozymes Cellic® HTec2 (230 mg protein/mL, preserve 180 mg xylose/mL) were generously

provided by Dr Melvin Tucker from NERL for all hydrolysis experiments. The filter paper activity of CTec2 was determined according to the standard filter paper assay [52].

Enzymatic hydrolysis

All enzymatic hydrolysis experiments were run in duplicate under standard conditions (50°C, pH 4.8). The pretreated whole slurries (including solid residue) from flowthrough pretreatment were adjusted to the set pH with 0.1 N NaOH. A mixture of Ctec2 and Htec2 enzymes at a ratio of 5:1 based on protein weight was added at three different enzyme loadings: (1) low enzyme loading: the loadings of 3 mg protein Ctec2 (2.8 FPU) with 0.6 mg protein Htec2/g glucan + xylan; (2) medium enzyme loading: 10 mg protein Ctec 2 (9.3 FPU) with 2 mg Htec2/g glucan + xylan; and (3) high enzyme loading: 100 mg protein Ctec 2 (93 FPU) with 20 mg Htec2/g glucan + xylan, respectively. Liquid samples were taken at 4, 24, 48, 72, 96 and 120 hrs and measured directly by HPLC for monomeric sugars. In addition, BSA treatment was conducted for parts of experiments. Prior to enzyme addition to start hydrolysis, the whole pretreated slurries were presoaked with 1% (w/w) BSA 10 mg/L sodium azide for 24 hrs [20].

$$\text{Enzymatic glucose yield\%} = \frac{W_{G2}}{W_{TG} - W_{G1}} \times 100\% \quad (8)$$

$$\text{Enzymatic xylose yield\%} = \frac{W_{X2}}{W_{TX} - W_{X1}} \times 100\% \quad (9)$$

In these equations, W_{G1} and W_{X1} are the glucose and xylose released in the pretreatment; W_{G2} and W_{X2} are the glucose and xylose released in enzymatic hydrolysis; W_{TG} and W_{TX} are the total potential glucose and xylose released after enzymatic hydrolysis of whole pretreated slurries (including solid residue) with the high enzyme loading (100 mg protein Ctec 2 (93 FPU) with 20 mg Htec2/g glucan + xylan) in 168 hrs. The unit of W consistently refers to g/100 g dw raw biomass.

Severity parameters

A severity parameter $\log R_0$, which is widely applied in hot water and dilute acid pretreatment [13,53,54], was used to unify our data obtained at different combinations of temperature and reaction time, which includes the preheating time.

The severity $\log R_0$ is defined as follows [55]:

$$\log R_0 = \log \left[t \times \exp \left(\frac{T-100}{14.75} \right) \right] \quad (10)$$

In which t is reaction time in minutes (including the preheating time); T is the hydrolysis temperature in °C, and 100°C is the reference temperature.

Because $\log R_0$ is the function of temperature and time as described in Equation 10, its value was calculated based on Equation 10 using the measured value of target reaction temperature from the thermal monitor and the reaction time.

Additional files

Additional file 1: Figure S1. Preheating time for the target temperatures 200°C to 280°C for both water-only and 0.05% (w/w) H₂SO₄ flowthrough pretreatment.

Additional file 2: Table S1. Preheating analysis for both water-only and 0.05% (w/w) H₂SO₄ flowthrough pretreatment.

Additional file 3: Figure S2. Major structure of soluble lignin with water-only or 0.05% (w/w) H₂SO₄ flowthrough pretreatment at flow rate of 25 mL/minute within 6 minutes under (a) 220°C, water only; (b) 240°C, water only; (c) 260°C, water only; (d) 280°C, water only; (e) 200°C, 0.05% (w/w) H₂SO₄; (f) 240°C, 0.05% (w/w) H₂SO₄.

Additional file 4: Table S2. Major soluble aromatic compounds detected in hydrolysate by flowthrough pretreatment of poplar wood with water only and 0.05% (w/w) H₂SO₄.

Abbreviations

5-HMF: 5-hydroxymethylfurfural; BSA: bovine serum albumin; DP: degree of polymerization; FPU: filter paper units; GC/MS: gas chromatography/mass spectrometry; HPLC: high-performance liquid chromatography.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LY and TZ carried out this study under the supervision of BY. All the authors read and accepted this final manuscript.

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