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Poplar (*Populus deltoides* L.): The Effect of Washing Pretreated Biomass on Enzymatic Hydrolysis and Fermentation to Ethanol

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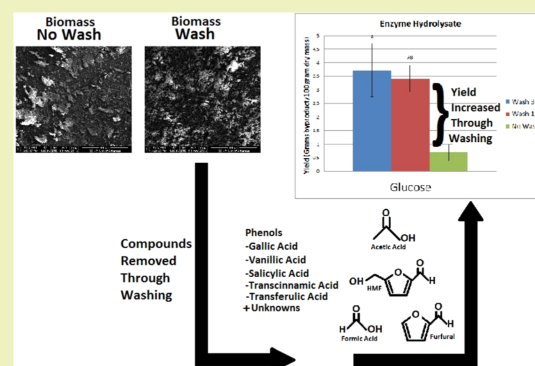
ABSTRACT: In this work, *Populus deltoides* L. biomass was pretreated in 0.98% (v/v) sulfuric acid at 140 °C for 40 min. Prior to enzymatic hydrolysis, pretreated biomass was either not washed or washed with 1.5 or 3 volumes of water, as compared to biomass. Rinsing the pretreated biomass with 1.5 or 3 volumes of water resulted in glucose yields that were seven times greater than the nonwashed treatment. Pretreatment hydrolyzates, wash waters, and enzymatic hydrolysis hydrolyzates were analyzed for carbohydrate, aliphatic acid, aldehyde, and phenolic content. An analysis of the wash waters showed the presence of gallic, vanillic, syringic, *p*-coumaric, ferrulic, *trans*-cinnamic, and salicylic acids at concentrations below 0.07 mg mL^{-1}. Washed and nonwashed enzymatic hydrolyzates showed significant differences in gallic, vanillic, ferrulic, and salicylic acid concentrations, indicating that these compounds could be in part responsible for inhibiting enzymatic hydrolysis. Nonwashed and washed enzymatic hydrolysates were fermented to ethanol with self-flocculating SPSC01 and nonflocculating ATCC4126 yeasts. While the biomass washed with 3 volumes of water produced the highest ethanol yields (up to 0.43 g g^{-1} glucose) and were significantly higher than those from the nonwashed sample (≤0.28 g g^{-1} glucose), the ensuing differences between samples washed with 3 and 1.5 volumes of water were not significant. The SPSC01 strain generally outperformed the ATCC4126 strain in ethanol fermentation efficiency, in particular when the nonwashed hydrolysates were used as feedstock.



KEYWORDS: *Biomass, Cellulosic ethanol, Wash waters, Dilute acid pretreatment*

INTRODUCTION

Although horizontal drilling is currently providing ample oil and gas supplies, sustainable energy still needs to be developed from a long-term perspective, especially for liquid transportation fuels. One pathway for sustainable energy production is the biochemical deconstruction of feedstocks into sugars, which can then be fermented to biobased fuels or chemicals. This approach requires pretreatment of biomass to remove hemicellulose or lignin components prior to enzymatic hydrolysis,[1] but this pretreatment step often produces byproducts that inhibit the downstream biochemical conversion steps. Pretreatment of plant cell wall components leads to the formation of degradation compounds, such as aliphatic acids, furans, and lignin-derived phenolic compounds,[2,3] which inhibit enzymatic hydrolysis by at least 50%.[4]

Dilute acid pretreatment is a promising candidate for eventual implementation in second-generation biomass ethanol plants.[5] However, dilute acid pretreatment causes the formation of inhibitory compounds through the degradation of cellulose, hemicellulose, and lignin.[6] These inhibitory compounds can compete or delay enzyme activity, as well as inhibit the fermentation.[4] Washing pretreated biomass with successive volumes of water circumvents the inhibitory action of degradation compounds that are produced during dilute acid pretreatment. However, this washing step necessitates the usage of large quantities of water that could be difficult to implement at the manufacturing scale. On the other hand, pretreatment cannot be omitted from the biochemical processing train because, without pretreatment, the expensive enzymes cannot access and hydrolytically cleave complex carbohydrates into coveted monomeric sugars.

Washing the biomass is recommended after dilute acid pretreatment with up to 12 volumes of water.[7] Water solubilizes and removes many of the inhibitory compounds, allowing the enzymes to release the plant cell wall sugars. The effect of wash water temperature was also examined; 25 and 90 °C water was

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tested for the removal of inhibitory compounds that were present in steam pretreated mix hardwood hydrolyzates.[8] Water at 90 °C was more effective in removing inhibitory compounds. On a laboratory scale, washing of pretreated biomass can be accomplished with ease, but difficulties can exist in implementation during scale-up. There is a need to minimize water usage in all steps of the biochemical conversion process. To reduce the use of fresh water, city wastewater effluent was investigated for biorefinery water supply.[9] Minimization of water usage for biomass washing that simultaneously does not restrict saccharification will be critical for this technology to move forward.

The objective of this work was to minimize the volume of water necessary to rinse dilute acid pretreated *Populus deltoides* L. biomass, such that a fermentable sugar stream was produced. The pretreatment conditions consisted of 0.98% dilute sulfuric acid pretreatment (v/v) at 140 °C for 40 min, as previously described.[10] The resulting pretreatment hydrolysates, wash waters, and enzymatic hydrolysis were analyzed by high performance liquid chromatography (HPLC) and ultrahigh performance liquid chromatography (UPLC) in order to determine which compounds could possibly be responsible for enzymatic hydrolysis inhibition. Nonwashed and washed enzymatic hydrolyzates were fermented to ethanol with self-flocculating and ATCC4126 yeast strains.

■ MATERIALS AND METHODS

Biomass. *Populus deltoides* L. clone S13C20[11] was obtained from the University of Arkansas Pine Tree Branch Station (34° 03′ 83″ N and 92° 22′ 22″ W) and stored at 4 °C; similar material was previously used.[12,13] The composition of the biomass was glucose, 49.7; xylose, 12.3; extractives, 1.5; lignin/ash, 16.4; and ash, 0.4 g per 100 g of biomass[12] The specific gravity was 0.48, using methods that were previously reported.[13] All biomass was dried at room temperature before being ground; moisture content was measured using an Ohaus MB45 Moisture Analyzer (Pine Brook, NJ). The biomass was ground to 20 mesh in a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ).[14]

Pretreatment. Pretreatment was carried out in a 1 L Parr (Moline, IL) 4525 reaction vessel. A working volume of 250 mL was used at a solids loading of 10% (wet basis) ratio, where the reactor was loaded with 25 g of biomass. The average moisture content of the biomass was between 7% and 13%, dry basis. The working volume was 250 mL with a sulfuric acid concentration (EMD, Gibbstown, NJ) of 0.98% (v/v). The reactor was heated to 140 °C over the course of 20−30 min before starting the 40 min pretreatment cycle. When the pretreatment was completed, the vessel was quenched under cold running tap water, where the temperatures dropped below 100 °C in approximately 4−5 min. Once the temperature dropped below 60 °C, usually taking between 10 to 12 min, the vessel was opened and the contents were filtered, using a Buchner funnel fitted with a Whatman type 1 filter connected to a vacuum pump (Niles, IL). The filtrate was collected, its volume recorded,and the pH measured on a Mettler-Toledo pH meter (Schwerzenbach, Switzerland) and set aside for high performance liquid chromatography analysis (HPLC) and ultrahigh performance liquid chromatography (UPLC) analysis. The solid fraction was removed from the filter and set aside for the washing step. Hydrolysates, including liquid and solid portions, were stored at 4 °C.

Wash. The washing step was conducted with either 0, 1.5, or 3 volumes of Millipore Direct Q3 (Molsheim, France) filtered water; these volumes translated to 0, 38, and 75 mL of water per 25 g of filtered biomass, respectively. The slurry was stirred for 5 to 10 min by hand when the 1.5 rinsing volume was used or with a stir bar when the 3 rinsing volume was used. Slurries were filtered as described above. The filtrate was collected, its volume and pH measured, and set aside for HPLC analysis. The filtrate was stored at 4 °C.

Enzymatic Hydrolysis. Enzymatic hydrolysis was conducted in a 600 mL Parr reactor using the previously reported methods.[12,13] A working volume of 400 mL loaded to 10% solids on a wet basis was used, corresponding to 40 g of pretreated filtered washed or nonwashed biomass. The final composition of the resulting enzyme slurry was 20 mL Accellerase 1500 (Genencor, Rochester, NY), 180 mL of Millipore filtered water, 40 g of filtered poplar biomass (wet basis), and 200 mL of a 4.9 pH sodium citrate buffer solution (sodium citrate EMD Gibbstown, NJ). Enzymatic hydrolysis was conducted over a 24 h period, with stirring at approximately 180 rpm at 50 °C. The slurry was collected at the end of the 24 h hydrolysis cycle and analyzed by HPLC for carbohydrates, organic acids, and aliphatic acids. When not in use, the slurries were stored at 4 °C. The enzyme preparation had a previously published protein concentration of 82 mg mL^{-1}.[15]

HPLC Analysis. Samples obtained from dilute acid pretreatment hydrolyzates, wash/rinsing waters, and enzyme hydrolyzates were analyzed by HPLC for carbohydrates and organic acid content. Carbohydrates were quantified by a Waters 2695 Separations module (Milford, MA) equipped with a Shodex (Waters, Milford, MA) precolumn (SP-G, 8 μm, 6 × 60 mm) and Shodex column (SP0810, 8 μm, 6 × 300 mm). The water mobile phase, eluting at 0.2 mL min^{-1}, was heated to 85 °C, using a Waters (WAT038040) external heater. The carbohydrates were detected using a Waters 2414 refractive index detector (Milford, MA), as previously described.[12] Organic acids were detected on a Waters 2695 separation module with a Bio-Rad (Hercules, CA) Aminex HPX-87H ion exclusion (7.8 mm × 30 mm) column at 55 °C. The mobile phase consisted of 0.005 M H_2SO_4, flowing at a rate of 0.6 mL min^{-1}. The organic acids, furfural, and hydromethylfurfural (HMF) were detected at a UV wavelength of 280 nm UV, while acetic and formic acids were detected at 210 nm. All organic acid concentrations were quantified using a Waters 2996 photodiode array detector. An Acquity Waters UPLC fitted with a 2.1 mm × 50 mm C18 1.7 μm column (Milford, MA) was used for phenolic detection and quantification. A Waters UPC UV detection module, adjusted at wavelengths between 210 and 280 nm using a methanol and water gradient solvent system ramping from 15% water to 85% over the course of 8 min was used to detect the phenolics. The injections had a volume of 2.5 μL, and analysis was conducted at 50 °C. The procedure was adapted from a previously described method.[16]

Microscopy. Samples were dried to 1% moisture content (tested with MB45 Moisture Analyzer) and placed on aluminum specimen mounts, using double-coated carbon conductive PELCO tabs (Ted Pella, Inc., Redding, CA). Samples were sputter-coated with 1−2 nm gold, using a Polaron/Emitech SC7620 sputter coater (Quorum Technologies, Ltd., East Sussex) and viewed at 30 kV under the beam of an SEM using an FEI Nova Nanolab duo-beam SEM/FIB (FEI Company, Hillsboro, OR).

Fermentation. Two different strains of yeast were used in 50 mL fermentation reactions to generate ethanol from the sugars hydrolyzed in the previous step. One strain, SPSC01, was a self-flocculating strain that was provided by the Dalian University of Technology, China.[17] The other was a standard nonflocculating ATCC4126 strain of yeast. A medium containing 30 g L^{-1} of glucose, 5 g L^{-1} of yeast extract, and 5 g L^{-1} of peptone was used to prepare the precultures for fermentation. The yeast strains were harvested using a centrifuge set at 4100*g* for 10 min. The harvested pellets were then washed twice with deionized water and suspended in a 50 mM sodium citrate buffer at a pH of 4.8. The final cell concentration was 2 to 4 × 10^9 mL^{-1}. Ten milliliters of each tested hydrolysate was then inoculated with enough suspended yeast cells to bring the cell concentration to 8 × 10^7 mL^{-1}. For 8 h, the yeast−hydrolysate mixture was allowed to ferment on a 150 rpm rotary shaker at a temperature of 30 °C. The glucose colorimetric assay kit (Cayman Chemical, MI) was used before fermentation to determine the glucose content of the prefermented hydrolysates. After fermentation, ethanol yields were quantified by gas chromatography (GC) on the Shimadzu GC-2010 equipped with a flame ionization detector and a Stabilwax-DA column (cross-band poly(ethylene glycol), 0.25 mm × 0.25 μm × 30 m), as previously described.[18] Fifty microliters of fermentation broth was diluted 10

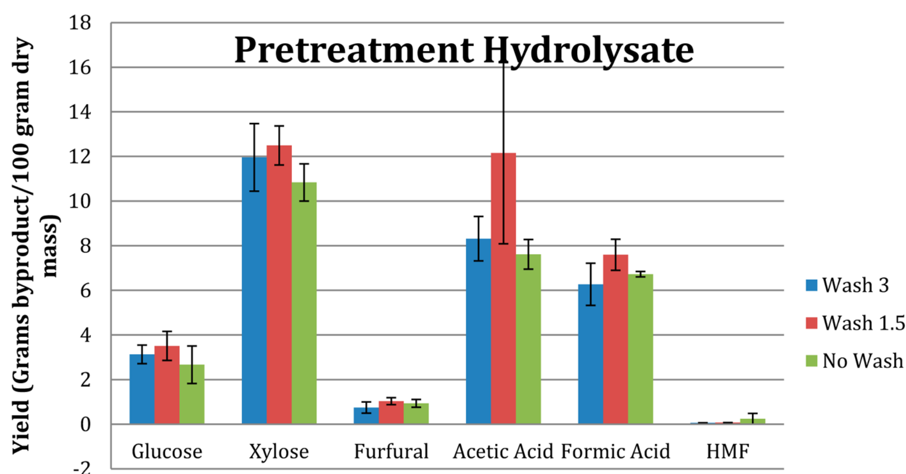

Figure 1. Monosaccharide and inhibitory byproduct yield after 0.98% dilute acid pretreatment for 40 min at 140 °C, at the conditions of pretreatment water volume 0 (no wash control), pretreatment water volume of 1.5, and pretreatment water volume of 3. No significant differences exist between treatments at the $\alpha = 0.05$ level.

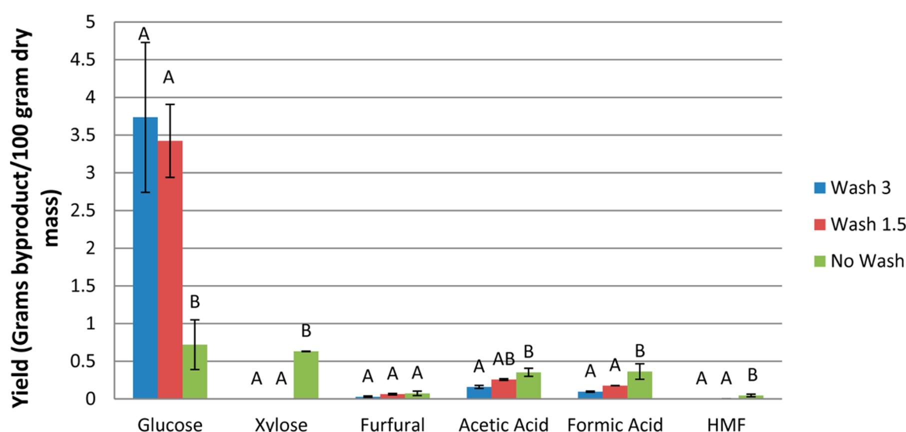

Figure 2. Effect of washing the pretreated biomass (1 pretreatment volume: 0 water volume (no wash); 1 pretreatment volume: 1.5 water volumes; and, 1 pretreatment volume: 3 water volumes) on the concentration of monosaccharaides and inhibitory by products present in the enzyme hydrolysis expressed as grams product/100 gram dry biomass. Letters A and B refer to statistically significant differences between the data, where the letter AB refers to data which is not statistically different from either the A group or the B group. Alpha level is .05.

times with deionized water along with 50 μL of 0.1 mg mL^{-1} *n*-butanol standard prior to GC analysis, following the previously reported procedure, before the samples were run through the gas chromatograph.[10]

Statistical Analysis. All pretreatment and enzymatic hydrolysis experiments were carried out in duplicate, while fermentation experiments were done in triplicate. All concentrations were determined from linear regression analysis using JMP 11.0 and Excel 2007. Analysis of the variances of the pretreatment and enzymatic hydrolysis sample sets was completed using JMP 11.0 software, a business unit of SAS, with a LS-Means difference and an alpha value of 0.05. For fermentation data, two factor analysis of variances (ANOVA) followed by a Tukey *post hoc* range test was used to determine differences between treatments with $p < 0.05$ considered to be significant.

RESULTS AND DISCUSSION

Monosaccharide, Organic Acid, and Aliphatic Acid Release during Pretreatment and Rinsing. During pretreatment, the biomass was subjected to temperatures of 140 °C at an acid loading of 0.98%, which resulted in a calculated severity parameter of 1.16.[19] Using a compositional analysis previously reported for poplar feedstock,[12] the pretreatment step released 6.3% of the available glucose and 96.5% of the available xylose. Recoveries of glucose and xylose are presented in Figure 1. As expected, no significant differences were determined between monosaccharaides, organic acids, and aldehydes as a function of the number of washes because, at this point, there had been no changes in experimental procedures. Statistical analysis showed that the sample sets were within the tolerance range ($\alpha = 0.05$) of each other, establishing the fact that differences observed in enzyme hydrolysis or fermentation would be a result of subsequent changes in experimental parameters.

Figure 2 presents monosaccharide, aliphatic acid, and aldehyde releases after enzymatic hydrolysis when pretreated biomass was not washed or was washed with 1.5 or 3 volumes of water. The results showed that washing was critical in favoring glucose release from pretreated biomass; rinsing with 1.5 or 3 volumes of water resulted in seven times more glucose when compared to the nonwashed treatment. Conversely, acetic and formic acids, as well as HMF, yields were higher in the nonwashed enzyme hydrolyzates. Interestingly, the

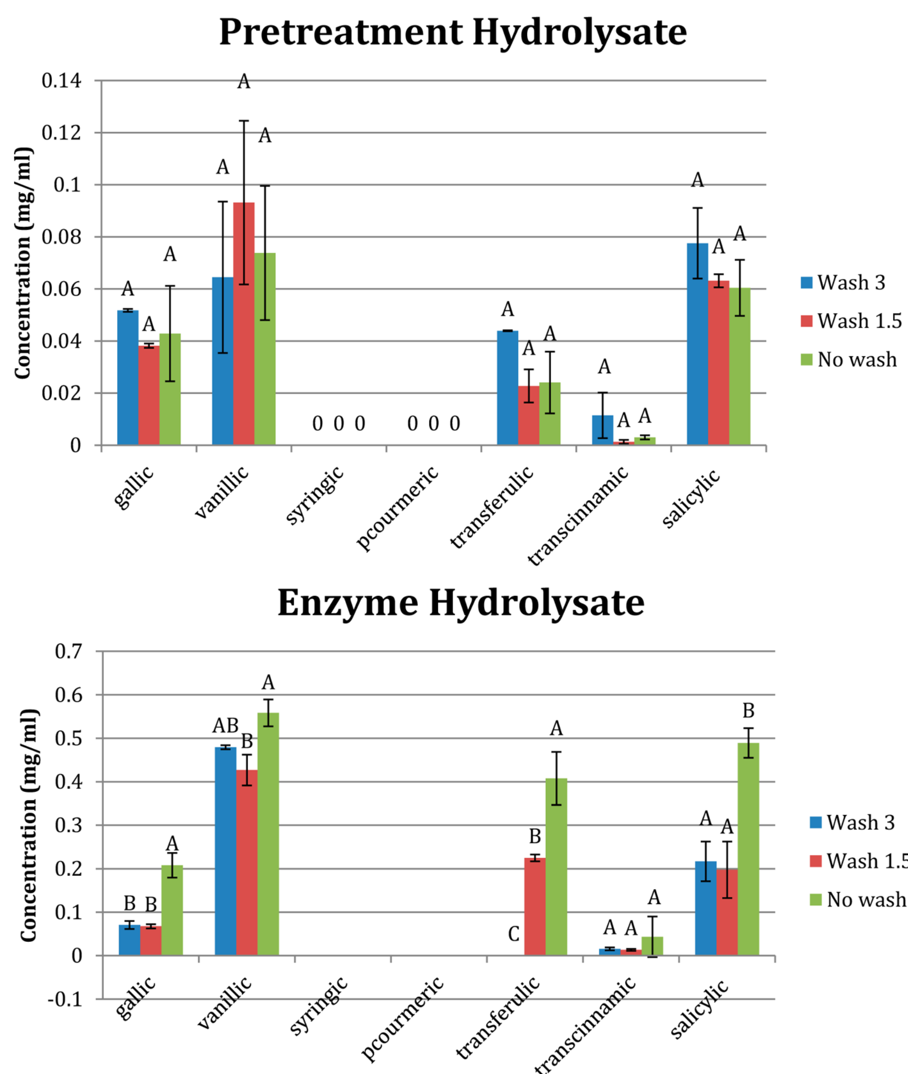

Figure 3. Concentrations of selected phenolic degradation compounds in pretreatment (Top; A), and enzyme hydrolysates (Bottom; B). Letters A, B, and C refer to statistically significant differences between the data, where the letter AB refers to data which is not statistically different from either the A group or the B group. Alpha level is .05.

measured concentrations of organic acids, less than 1 mg mL^{-1} as shown in Figure 2, were lower than the inhibition threshold previously reported.[20,21] Results presented in Figure 2 show that a minimum wash volume of 1.5 is critical for successful enzymatic hydrolysis. On the other hand, statistical analysis showed that there were no significant differences in carbohydrate recovery when the pellet was rinsed with 1.5 or 3 volumes of water, indicating that minimal washing with a 1.5 volume of water could be beneficial.

Characterization of Hydrolyzates and Wash Waters. Total yields from the enzymatic hydrolysis step (of possible sugars available after pretreatment) were 1.6%, 7.5%, and 8.1% for 0, 1.5, and 3 wash volumes, respectively. Figure 3 presents aromatic and aliphatic acids concentrations in pretreatment and enzymatic hydrolyzates. Similar to the results of Figure 1, no significant differences were determined in Figure 3A between aromatic and aliphatic acids concentrations as a function of the number of washes because at this point there had been no changes in experimental procedures. A separate analysis of wash waters showed the presence of gallic, vanillic, syringic, *p*-coumaric, ferrulic, *trans*-cinnamic, and salicylic acids at concentrations below 0.07 mg mL^{-1}. Occurrence of these compounds was previously reported in dilute acid pretreated poplar hydrolyzates where, among others, ferrulic, gallic, and syringic acids were shown to be present in steam-pretreated hardwood wash waters.[3] Figure 3B presents the concentrations of aromatic and aliphatic acids present in enzymatic hydrolyzates. Washed and nonwashed enzymatic hydrolyzates presented significant differences in gallic, vanillic, ferrulic, and salicylic acid concentrations, indicating that these compounds could be in part responsible for inhibiting enzymatic hydrolysis, as observed in Figure 2. It is also possible that gallic, vanillic, ferrulic, and salicylic acid, at the concentrations determined in the hydrolyzates, impede enzymatic activity.

However, the determination of which compounds inhibit enzymatic hydrolysis may be more complicated than initially anticipated. In addition to ferrulic, gallic, and syringic acids, more than 19 additional compounds were solubilized in wash waters through extractions with successive portions of methanol and acetone.[8] These 19 additional compounds were determined to be mostly of phenolic origin, as determined by the Folin−Ciocalteau assay.[8] By treating wash waters with AmberliteXAD-7 resins, Kim et al. removed up to 78% of the phenolics from their washates.[8] Testing of treated washates on

the enzymatic hydrolysis step, using Avicel as substrate and Cellic Ctec and Cellic Ctec 2 as enzymes, was implemented.[8] Phenolics from washates were concentrated by vacuum evaporation. Their results showed that the addition of 2 mg of total phenolics per mg of cellulose protein reduced glucose yields by 35% when compared to their control.[8] The addition of 2 mg of total phenolics per mg of cellulose protein stemming from the resin treated washate, which was devoid of 22 mostly phenolic compounds, essentially yielded similar outcomes.[8] These results indicate that although compounds were removed the resin-treated preparation still remained inhibitory to the enzymatic hydrolysis system

Others reported inhibition of saccharification enzymes by released phenolics.[22−24] Lignin-based compounds have been known to have inhibitory effects in fermentation.[25] Using a clever system composed of filter paper and a Spezyme cellulose cocktail supplemented with *β*-glucosidase, the inhibitory effect of tannic acid, gallic acid, cinnamic acid, ferulic acid, *p*-coumaric acid, sinapic acid, vanillin, syringaldehyde, and 4-hydroxybenzoic acid was investigated.[22] The system was designed to test 34.5 mg of inhibitor (mg protein)$^{-1}$, and only tannic acid displayed inhibitory activity in this system.[22] It is important to note that tannic acid is a generalized term and can be composed of several ellagitannins, leading to difficulties as to the identification of which form of tannic acid displayed enhanced inhibitory effect.[26] Furthermore, Table 1 presents the calculated ratio of inhibitor to mg protein of enzymatic hydrolyzates. The maximum ratio was 0.16, which was 216-fold less than what was previously reported.[22] The use of the filter paper system, as compared to authentic pretreated biomass, may be an oversimplification, allowing for an increased inhibitor tolerance.[22] The inhibitory effect of 5 mg mL^{-1} of formic acid added to cellulose powder or to dilute acid-pretreated washed biomass was tested.[27] After a 48 h incubation period, 10% and 50% glucose recoveries, respectively, were determined for the poplar and cellulose systems, indicating that identical concentrations of supplemented formic acid had different effects.[27] Although a useful system, the use of filter paper for testing inhibition may prove an oversimplification for the identification of inhibitory compounds in enzymatic hydrolysis.[22,27]

Table 1. Ratio of Milligram of Phenolic:Milligram of Protein in Nonwashed, 1.5, and 3 Volumes Washed Enzymatic Hydrolyzates

compounds	nonwashed	1.5 volume wash	3 volume wash
gallic acid	0.06	0.02	0.02
vanillic acid	0.16	1.12	0.14
syringic acid	0.00	0.00	0.00
p-coumaric acid	0.00	0.00	0.00
ferullic acid	0.12	0.07	0.00
cinnamic acid	0.01	0.00	0.00
salicylic acid	0.14	0.06	0.06

Figure 4 presents scanning electron microscopy illustrations of poplar wood: intact before pretreatment, dilute acid pretreated, rinsed with 3 volumes of water, and enzymatically hydrolyzed. All images are presented at approximately identical magnifications. Interestingly, no physical difference was observed between washed (Figure 4C) and nonwashed (Figure 4B) images. The images show that the grinding has broken up the poplar biomass by fracturing and separating the biomass along the large and relatively fragile vessel elements that are distributed uniformly in poplar, a diffuse porous hardwood. Fragments of those vessel walls with pits are shown in Figure 4A, B, and C. Figure 4D and E are biomass after enzymatic hydrolysis, with D being the no wash and E being the washed. Figure 4E, the washed samples, has an enhanced granular aspect as compared to Figure 4D, the nonwashed sample; the granular aspect could possibly be attributed increased enzymatic hydrolysis, as shown in Figure 2.

On the other hand, there are noticeable differences between nonwashed (Figure 4D) and washed (Figure 4E) enzymatically hydrolyzed biomass; the former was reduced to granular shapes devoid of their fibril characteristics, while the later retained more of a mat aspect. The fact that there were no apparent physical differences between washed and nonwashed images points to the fact that the need for rinsing, as shown in Figure 2, is necessary for removing chemicals rather than detaching physical components from the pretreated biomass. Nonwashed biomass (Figure 3B) can become accessible to enzymatic hydrolysis by rinsing, indicating that whichever compounds inhibit enzymatic hydrolysis were removed by rinsing.

Because wash waters in this work were not extracted with organic solvents, only water-soluble compounds were detected. The solubilites of compounds found in wash water, such as phenolics, may be a critical factor for their detection. Removed compounds from pretreated hydrolyzates with 90 °C water could precipitate when the water temperature decreases and not be extracted with organic solvents as earlier reported.[8] In this work, compounds with low solubilities, which were not identified in this wash water analysis, could be removed from pretreated biomass but not detected by HPLC or UPLC. Identification, minimization, or removal of these inhibitors is key to a successful enzymatic hydrolysis step. However, this may prove challenging as these compounds become insoluble with decreased wash water temperatures, thwarting their detection.

Fermentation. Ethanol yields showed a similar relationship, as presented in Table 2. While the biomass washed with 3 volumes of water produced the highest ethanol yields, the ensuing differences between the 3 and 1.5 wash were not significant. The ethanol yields obtained at higher sugar concentrations did not exceed 0.430 g g^{-1}, consistent with those found in other biomass carbohydrates fermented in a similar manner.[18,28] The significantly higher ethanol yields in the washed samples, 0.385−0.421 g g^{-1}, than in the nonwashed hydrolysates, lower than 0.281 g g^{-1}, can be attributed to higher sugar concentrations and lower levels of inhibitors, such as acetic and formic acid, present in the hydrolysates. These results are consistent with the understood mechanism of acetic and formic acids inhibition, which slows cell mass productivity.[20] Except for organic acids, some nonorganic acid-based fermentation inhibitors were also generated from the biomass pretreatment. Lignin is another major byproduct of the acid hydrolysis of plant matter and has been known as the larger contributor to low yields in second generation biomass for enzyme inhibition.[24] Lignin derivatives, such as vanillin and coniferyl aldehydes, are known to inhibit fermentation, as well as causing as much as a 70% decrease in the total ethanol produced.[25] In addition, the concentrations of furfural and HMF were below inhibition thresholds.

In comparing the two yeast strains for fermentation performance, the flocculating SPSC01 strain generally outperformed the ATCC4126 strain, in particular in the fermentation of the nonwashed (and implicitly high-inhibitor)

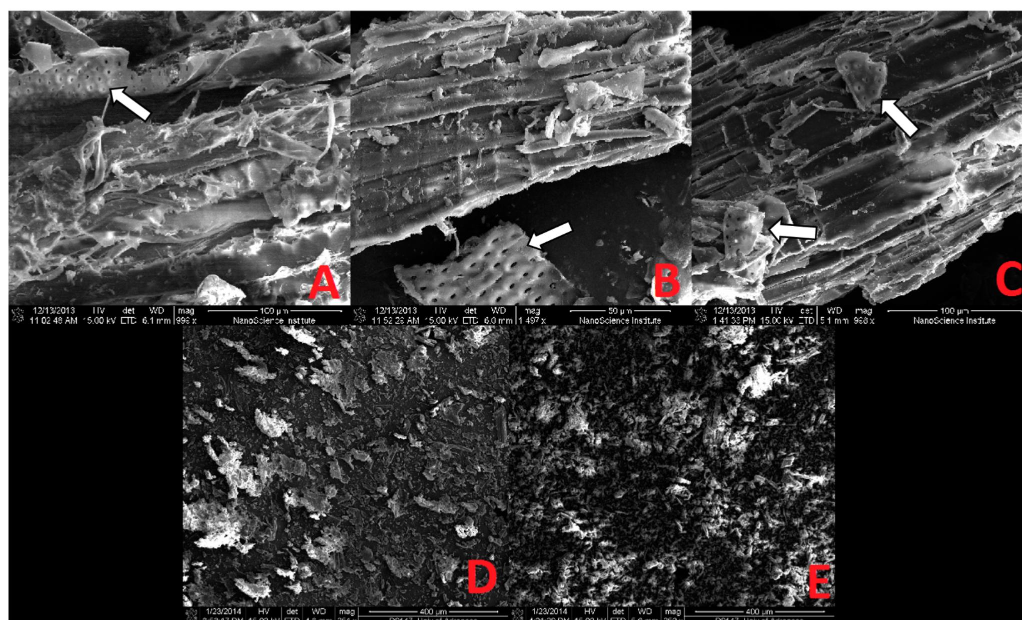

Figure 4. (A, B, C, D, and E) SEM images of biomass. (A) Unpretreated poplar, ground to 20 mesh. (B) Acid pretreated poplar prior to washing. (C) Acid pretreated poplar after washing. (D) Enzymatic hydrolysis of acid pretreated poplar with no wash. (E) Same conditions as D, except with a 3 volume wash. Micrographs A, B, and C are shown in longitudinal sections of the poplar wood, with remnants of bordered pits (arrows). However, after enzymatic hydrolysis, the bordered pits are no longer obvious due to degradation of the cellulosic material.

Table 2. Glucose Content of Different Enzymatic Hydrolysates and Ethanol Yields of Fermentation with Two Yeast Strains (ATCC4126 and SPSC01)[a]

samples	no wash	1.5× wash	3× wash
initial glucose (g/L)			
	0.67 ± 0.04	3.12 ± 0.15	3.43 ± 0.22
Ethanol (g/L)			
ATCC4126	0.14 ± 0.01^{E}	1.19 ± 0.02^{C}	1.36 ± 0.04^{AB}
SPSC01	0.19 ± 0.02^{D}	1.25 ± 0.03^{BC}	1.43 ± 0.03^{A}
$Y_{E/G}$[b] (g/g)			
ATCC4126	0.21 ± 0.03^{d}	0.39 ± 0.01^{b}	0.40 ± 0.01^{ab}
SPSC01	0.28 ± 0.02^{c}	0.40 ± 0.01^{ab}	0.42 ± 0.01^{b}

[a]Different upper case letters (A, B, C, D) indicate significant difference in ethanol concentration (p<0.05); different lower case letters (a, b, c, d) indicate significant difference in ethanol yields, $Y_{E/G}$ (p<0.05). [b]Ethanol yields calculated as ethanol produced/sugar consumed, g g^{-1}.

hydrolysates, wherein the ethanol concentration and ethanol yield generated by the SPSC01 strain, 0.190 g L^{-1} and 0.281 g g^{-1}, respectively, were significantly higher than those by the ATCC4126 strain, 0.136 g L^{-1} and 0.209 g g^{-1}, respectively. As the samples for both yeast strains started with identical concentrations of glucose and more than likely identical concentrations of byproducts, it would appear that the SPSC01 strain outperformed the ATCC4126 in environments with higher inhibitor concentrations.

Because the glucose concentrations of the original enzymatic hydrolysates were lower than 3.5 g L^{-1} and varied among different hydrolysates, the real effects of inhibitors on the fermentation efficiency might not be suitably evaluated. To overcome this problem, fermentations were also conducted in the enzymatic hydrolyzates supplemented with 30 g L^{-1} glucose, so that the initial glucose concentration in the different hydrolyzates became high and comparable. As shown in Figure 5, higher ethanol yields (up to 0.46 g g^{-1}) were obtained from the glucose-added hydrolyzates than those from the original hydrolyzates. However, the nonwashed hydrolysates took longer to reach the maximum ethanol concentration, indicating higher levels of cell growth inhibition being present in these hydrolyzates than in the washed hydrolyzates. Both yeast strains performed similarly, and no significant differences were calculated. Figure 5 and Table 2 both show yields relative to sugar present, with the theoretical maximum values for $Y_{p/s}$ being 0.51 g g $^{-1}$. Conditions within the shake flasks were maintained as anaerobic, but ethanol yields dropped after 6 to 9 h possibly due to volatilization during each sampling period. Oxygen contamination likely occurred during sampling, creating slightly aerobic conditions. Despite these sources of error, these results indicate that the low yields of ethanol retrieved from original enzymatic hydrolyzates with no glucose supplementation, as shown in Table 2, were possibly the result of low sugar concentrations being unable to support the fermenting organism, as well as the presence of fermentation inhibitors.

■ CONCLUSION

Although the objective of this work was not to maximize carbohydrate recovery, there were significant differences in the glucose concentrations and ethanol yields in the enzyme hydrolyzate between washed and nonwashed pretreated biomass, indicating that in the pretreatment conditions used in this work, washing was necessary for efficient saccharification and subsequent fermentation. When the pretreated biomass was not washed, the glucose yields were reduced 7-fold. However, there were no significant differences between the use of 1.5 and 3 wash volumes in terms of carbohydrate recoveries and ethanol yields. Analysis of enzymatic hydrolyzates that resulted in saccharification showed that there were significant differences in terms of the gallic, vanillic, *trans*-ferrulic, and salicylic acid concentrations, indicating that these compounds could possibly be responsible for enzymatic hydrolysis inhibition and are removed during rinsing steps. Interestingly,

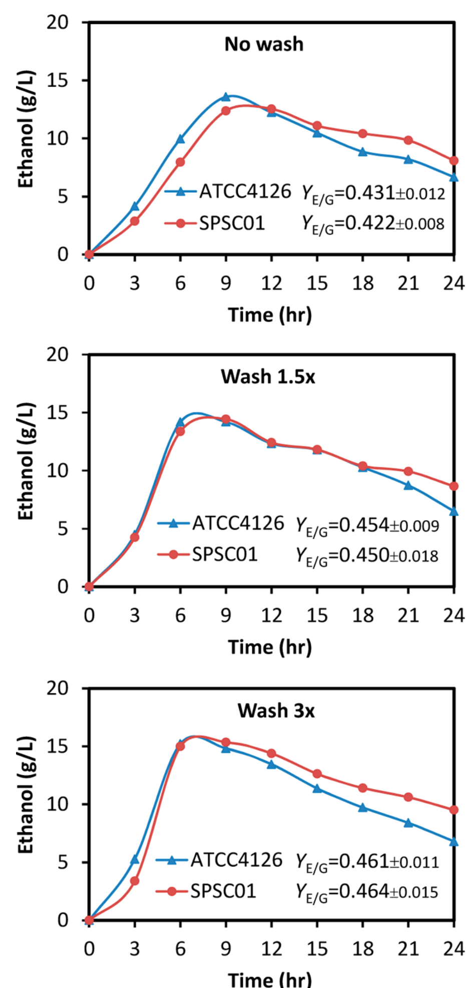

Figure 5. Fermentation of three different hydrolysates supplemented with 30 g L^{-1} glucose with the ATCC4126 and SPSC01 yeast strains. Ethanol yields ($Y_{E/G}$) were calculated as ethanol produced/sugar consumed, g g^{-1}. The theoretical maximum for $Y_{E/G}$ in all cases is 0.51 g g^{-1}.

the ratio of these compounds were up to 216-fold less than what was previously reported, indicating that byproducts may be more potent in authentic hydrolyzates[2] However, it is more than likely that these compounds are not the sole compounds responsible for enzymatic inhibition because some compounds removed by washing may have precipitated in the wash waters, thwarting their detection. The long-term goal of this research is to elucidate which generated byproduct inhibits enzymatic hydrolysis. Determining which compound(s) and their ensuing combinations is critical for designing the enzymatic hydrolysis unit operation that minimizes enzyme loading and water rinsing volumes.

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Notes

The authors declare no competing financial interest.

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