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Increased Saccharification Yields from Aspen Biomass Upon Treatment with Enzymatically Generated Peracetic Acid

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Abstract The recalcitrance of lignocellulosic biomass to enzymatic release of sugars (saccharification) currently limits its use as feedstock for biofuels. Enzymatic hydrolysis of untreated aspen wood releases only 21.8% of the available sugars due primarily to the lignin barrier. Nature uses oxidative enzymes to selectively degrade lignin in lignocellulosic biomass, but thus far, natural enzymes have been too slow for industrial use. In this study, oxidative pretreatment with commercial peracetic acid (470 mM) removed 40% of the lignin (from 19.9 to 12.0 wt.% lignin) from aspen and enhanced the sugar yields in subsequent enzymatic hydrolysis to about 90%. Increasing the amount of lignin removed correlated with increasing yields of sugar release. Unfortunately, peracetic acid is expensive, and concentrated forms can be hazardous. To reduce costs and hazards associated with using commercial peracetic acid, we used a hydrolase to catalyze the perhydrolysis of ethyl acetate generating 60–70 mM peracetic acid in situ as a pretreatment to remove lignin from aspen wood. A single pretreatment was insufficient, but multiple cycles (up to eight) removed up to 61.7% of the lignin enabling release of >90% of the sugars during saccharification. This value corresponds to a predicted 581 g of fermentable sugars from 1 kg of aspen wood. Improvements in the enzyme stability are needed before the enzymatically generated peracetic acid is a commercially viable alternative.

Keywords Perhydrolysis · *Pseudomonas fluorescens* esterase · Peracetic acid · Lignocellulosic biomass · Aspen · Pretreatment · Saccharification · Enzyme

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Introduction

Replacing the current biofuels feedstock—corn—with lignocellulosic biomass such as wood, switchgrass or agricultural wastes is a key current goal of bioenergy research. Recalcitrance—inefficient release of sugars from lignocellulosic biomass—is the key problem that must be solved to make this replacement. This inefficiency is largely due to the lignin component of lignocellulosic biomass. The lignin component severely limits the release of sugars in two ways [1]. First, lignin blocks access of the cellulase to its substrate cellulose, and second, lignin nonproductively binds the cellulase [2–6]. Lignin is a hydrophobic aromatic polymer and binds cellulases mainly via their cellulose-binding domain [7].

Nature uses oxidation reactions to selectively remove lignin. For example, white rot fungi degrade lignin in wood and non-wood biomass using a mixture of oxidative enzymes [8, 9]. Unfortunately, these degradations require 2–3 weeks, which is too slow for industrial applications [10].

Researchers currently use various pretreatment methods—typically hot acid—to overcome the recalcitrance of lignocellulosic biomass [11–13]. Current pretreatment technologies involve harsh chemicals and/or high temperatures, which, unfortunately, are not selective for the lignin component. If the pretreatment is too mild, only a small amount of glucose will be released from the cellulose, but if it is too harsh, it will destroy hemicellulose and can remove cellulose fractions thereby wasting some of the sugars. Harsh methods also create substances, for example furfurals, that inhibit subsequent fermentation. Poljak first used peracetic acid to remove lignin from wood pulp [14]. Peracetic acid oxidizes the hydroxyl groups in lignin side chains to carbonyl groups [15] and cleaves the β-aryl bonds, which reduces lignin molecular weight and introduces hydrophilic groups [16–20]. In addition, peracetic acid hydroxylates the phenolic rings in lignin to form hydroquinones [21] and subsequently oxidizes the hydroquinones to quinones which undergo ring opening to yield water-soluble muconic, maleic, and fumaric acid derivatives. These reactions depolymerize the lignin and introduce hydrophilic groups that allow the lignin fragments to dissolve in water. This removal of lignin increases the accessibility of hemicellulose and cellulose to subsequent enzymatic hydrolysis. In addition, the partial oxidation of the remaining lignin reduces its hydrophobicity thereby reducing its ability to bind cellulases [5, 22].

Many researchers have improved saccharification yields from lignocellulose by pretreating with peracetic acid. Gharpuray et al. pretreated wheat straw with peracetic acid [23] and found a tenfold increase in the rate of cellulase-catalyzed glucose release. Other examples include peracetic acid pretreatment of different fibrous wastes such as sugar cane bagasse [24–27]. To reduce the amount of expensive peracetic acid needed, researchers combined peracetic acid pretreatment with other pretreatments, such as steam explosion [28] irradiation by electron beam [29] and alkaline pretreatments [30, 31].

Peracetic acid is too expensive for commercial use. For example, adding 9 wt.% peracetic acid based on hardwood biomass would require 90 kg of peracetic acid per metric ton at a cost of $4,300 or about US $13.4/l of ethanol generated [32]. In addition, concentrated solutions of peracetic acid are explosive, which creates hazards associated with storage and transport of peracetic acid.

A potential solution is enzymatic generation of peracetic acid. An enzymatic synthesis could use less expensive precursors than chemical synthesis to lower costs and could generate peracetic acid in situ to avoid the hazards of concentrated solutions. Perhydrolases are enzymes that catalyze the formation of peracetic acid from hydrogen peroxide and either

acetic acid or acetic acid esters (Fig. 1). Most hydrolases catalyze this reaction inefficiently, since water and hydrogen peroxide have similar structures [33, 34]. Perhydrolases are a subclass of hydrolases that favor hydrogen peroxide in this reaction. Recently, we modified esterase from *Pseudomonas fluorescens* to catalyze perhydrolysis [35] and efficiently generate peracetic acid from acetate and hydrogen peroxide. Two variants, wild type-PFE and a single mutant PFE-L29G, where the leucine residue at position 29 has been replaced by glycine using site-directed mutagenesis, show good perhydrolysis activity.

Perhydrolysis of acetic acid is an equilibrium reaction, which can generate only low concentration of peracetic acid in water. The estimated equilibrium constant for perhydrolysis of acetic acid is 2.8 [36, 37]. To obtain 40 mM peracetic acid, the reaction mixture should contain 2 M sodium acetate (pH 5.5, and about 16% of the total concentration in acetic acid form) and 2 M H_2O_2. These high concentrations of starting material increase the costs. In practice, inactivation of the enzyme by peracetic acid may stop the approach to equilibrium so that less than the predicted amount of peracetic acid forms. In preliminary studies, the reaction conditions above yielded only 27 mM peracetic acid. Due to these low concentrations of peracetic acid, perhydrolysis of acetic acids is not likely to be a practical route to peracetic acid for pretreatment of lignocellulosic biomass.

Perhydrolysis of esters can give higher concentrations of peracid because ester cleavage is thermodynamically favored. For example, hydrolysis of ethyl acetate to unionized products is favored by 1.7 kcal/mol [38], which corresponds to an equilibrium constant >10. We expect that perhydrolysis of ethyl acetate would be similarly favorable. Several enzymes catalyze the efficient perhydrolysis of esters [39–41]. Ethyl acetate was used as substrate in this report, but in the future, endogenous acetyl esters in lignocellulosic biomass might also be used as the ester source. Although the perhydrolysis, in this case, is not a reversible reaction, the product, peracetic acid, could be hydrolyzed to acetic acid in the presence of perhydrolase. There is a competition between the formation and hydrolysis of peracid, which is another limitation to obtain high concentration of peracid.

Aspen (*Populus* spp.) is a hardwood (angiosperm) tree that grows with little maintenance throughout a broad geographic range, with short rotation times between harvests. The aspen species *Populus tremuloides* is attractive as a bioenergy crop, and its wood contains little lignin, ~20 wt.%, low extractives, ~2 wt.%, and high levels of fermentable sugars, 50–70 wt.% combined glucose and xylose [42]. *P. tremuloides*, like other aspen species, also has relatively high degree of acetylation of xylans: ~3.5 wt.% acetyl groups relative to total

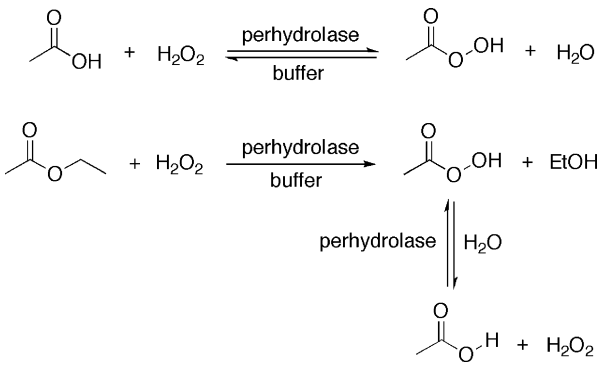

Fig. 1 Perhydrolysis of acetic acid forms equilibrium amounts of peracetic acid, while perhydrolysis of ethyl acetate is an irreversible reaction that makes kinetically controlled amounts of peracetic acid. Subsequent hydrolysis of peracetic acid may limit the amount of peracetic acid generated

biomass [43]. Aspen is also recognized as a recalcitrant biomass where pretreatment is critical.

Materials and Methods

General

Bark-free chips of aspen (*P. tremuloides* Michx.) wood were obtained from a Minnesota paper mill. The chips were air-dried and milled to 40-mesh in a Wiley mill to homogenize the feedstock. Commercial peracetic acid solution (Sigma-Aldrich 32 wt.% peracetic acid containing 5 wt.% hydrogen peroxide) was standardized before use by titration [44]. Hydrogen peroxide was titrated with ceric sulfate, then iodide was added to react with peracetic acid, and the resulting iodine was titrated with thiosulfate. Enzymatically generated peracetic acid was determined by the oxidation of methyl *p*-tolyl sulfide to the sulfoxide [45]. Cellulase (Sigma-Aldrich Celluclast 1.5 L) activity was determined as filter paper units (FPU in units/mL of original undiluted enzyme solution) according to the NREL method LAP-006 [46]. One unit of activity is the release of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 min. β-Glucosidase (Novozyme 188, Sigma) activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside [47]. One unit of activity is the release of 1 μmol of *p*-nitrophenol per minute. Wild-type PFE and PFE L29G were encoded in plasmids (pJOE2792) and expressed in *Escherichia coli* as described previously [35]. The proteins contained a 6×His tag and were purified by nickel affinity chromatography on Ni-NTA agarose.

Composition of Aspen

Dry weights of aspen samples were determined after oven drying for 48 h at 100°C according to NREL protocol for determination of total solids in biomass, LAP-001 [48]. All lignin and carbohydrate content percentages are based on dry weights. The composition of biomass was determined by acid hydrolysis with sulfuric acid (TAPPI T 222 om-23). According to this method, the acid-insoluble lignin was first removed by filtration and weighed after oven drying. The carbohydrates (glucose, xylose, arabinose, galactose, and mannose; cellobiose was added as an internal standard) in the filtrate were determined by high-performance liquid chromatography (HPLC) at 80°C using BIO-RAD De-Ashing Refill Cartridges, 30×4.6 mm (guard column) in line with VARIAN MetaCarb 87P, 300×7.8 mm (analytical column) eluted with deionized and degassed water flowing at 0.3 mL/min and detected with a refractive index detector. The composition of the aspen was determined before and after each pretreatment.

Saccharification of Aspen

Aspen meal was saccharified by treatment with cellulase (60 FPU/g cellulose) and β-glucosidase (64 *p*NPGU/g cellulose) in citrate buffer (50 mM, pH 4.8) for 5–7 days at 50°C according to NREL method for the enzymatic saccharification of lignocellulosic biomass, LAP-009 [49]. The procedure was modified to use 25 mg of cellulose loading instead of 100 mg so that we could use smaller samples. Other components were scaled down fourfold including the final volume (2.5 mL instead of the standard 10 mL). Replicate, comparative saccharifications between both reaction volumes were statistically equal, giving confidence

in our scale-down. The liquids obtained after the saccharification were analyzed with HPLC as above, but without a cellobiose internal standard and using a Varian CarboP guard column instead of the de-ashing column.

Pretreatment with Commercial Peracetic Acid

In 50-mL screw-cap bottles, aspen meal was mixed with commercial peracetic acid at charges of 0, 5, 10, and 25% peracetic acid based on oven-dried aspen weight. Next, water or citrate buffer (150 mM, pH 5–6) was added to a solution volume to wood weight ratio of 7:1. The suspensions were mixed by shaking and stored at room temperature for 72 h. The suspensions were then transferred to centrifuge tubes and washed four to five times with distilled water until the supernatant was free of soluble matter (confirmed by no residue detected after evaporation). The pellet was air-dried and saccharified with cellulase and β-glucosidase as described above.

Enzymatic Generation of Peracetic Acid

To a 125 mL flask, materials were added in the following order: water, sodium phosphate buffer (pH 7.0 with final concentration of 200 mM), H_2O_2 (final concentration, 500 mM), and ethyl acetate (final concentration, 600 mM). The enzyme, wild-type PFE (or L29G), was added last with the final concentration of 0.5 mg/mL (total volume of 12 mL). The reaction mix was allowed to react at 37°C for 40 min while shaking at 130 rpm before the peracetic acid concentration was determined using the methyl *p*-tolyl sulfide (MTS) assay [45]. This method consistently generated peracetic acid concentrations of 70±2 mM (or L29G with 60±2 mM).

Pretreatment with Enzymatic Peracetic Acid

The aspen wood 1.2 g (or pretreated by NaOH, see below) was introduced to enzymatic perhydrolysis reaction mixture (12 mL, 40 min reaction time) and incubated at 37°C for 4 h. After filtration and washing with distilled water (15 mL×5), the wood sample was ready for next cycle. Finally, the wood sample was dried at room temperature and saccharified by adding cellulase and β-glucosidase.

Pretreatment with NaOH

Sodium hydroxide solution (1.5 M NaOH (6 wt.%), 12 mL) and aspen wood (1.20 g) in a 125-mL flask were incubated at 37°C for 3 h while shaking at 150 rpm. The suspension was filtered and washed with distilled water (15 mL×6) until the pH of the wash was near neutral. Pretreatments with 0.50 M NaOH (2 wt.%) or 0.125 M NaOH (0.5 wt.%) used the same procedure.

Results

Characterization of Untreated Aspen Wood

Acid hydrolysis revealed the aspen wood had a composition of 23.5% acid-insoluble lignin, 40.4% glucan, 12.7% xylan, 2.7% other polysaccharides, and 20.7% other components.

These values are similar to those reported by other researchers. For example, Zhang et al. reported 19.7% acid-insoluble lignin, 41.6% glucan, 16.6% xylan, 4.0% other polysaccharides and 18.1% other components [50]. One kilogram of our aspen wood sample could theoretically yield 619 g of fermentable sugars (mainly glucose and xylose, but including arabinose, mannose, and galactose). As expected, enzyme-catalyzed release of the sugars from untreated aspen was inefficient. Hydrolysis with commercial cellulases using standard enzyme loadings (60 filter paper units of cellulase and 64 *p*-nitrophenyl-β-D-glucopyranoside units of β-glucosidase per gram cellulose), and reaction times (5–7 days) released an average of only 25.4% of the available glucose and only 13.3% of the available xylose (sample no. 1 in Table 1 below). These values correspond to the release of 114 g of glucose and 19 g of xylose from a kilogram of aspen wood. The total—135 g of sugars—is only 21.8% of the theoretical yield, clearly showing the need for pretreatments to enhance the release of sugars. This untreated aspen saccharification data is the baseline for pretreatments to increase the efficiency of the sugar release.

Pretreatment of Aspen with Commercial Peracetic Acid

Pretreatment of aspen wood with increasing concentrations of commercial peracetic acid decreased the lignin content of the biomass and increased the efficacy of the subsequent saccharification (Fig. 2). Aspen wood meal was suspended in 0, 94, 190, and 470 mM peracetic acid. These values correspond to 0, 5, 10, and 25 wt.% peracetic acid based on wood weight using a solution-to-aspen ratio of 7:1 (*v*/*w*). The suspensions were stirred for 72 h at room temperature, a time after which no residual peracetic acid was detected by titration. The experiments were conducted at room temperature because preliminary experiments showed rapid decomposition of the peracetic acid at 60°C [51, 52]. After the pretreatment, the suspension was centrifuged, and the pellet was washed five times with distilled water. The pellet was dried and saccharified with cellulase and β-glucosidase as above for the untreated aspen wood. A control with only hydrogen peroxide equivalent to the amount in the peracetic acid showed the same residual lignin content and cellulose-to-glucose conversion as a blank without peracetic acid.

Some samples were run without buffer (open symbols in Fig. 2, initial pH of the solutions was 2), while others were run in 150 mM citrate buffer at pH 5–6 (closed symbols in Fig. 2) to be closer to the pH 7 used for the enzymatic generation of peracetic acid below. There was no significant difference in the amount of lignin removed or glucose released from the different pH conditions. At 470 mM (or 25 wt.% based on aspen wood weight) peracetic acid and citrate buffer pH 5–6, the cellulose-to-glucose conversion reached 90%. The blank pretreatment with no peracetic acid, removed no lignin and yielded only 27% cellulose-to-glucose conversion in subsequent saccharification. With increasing concentration of peracetic acid during the pretreatment, the residues had lower amounts of lignin and released higher yields of sugars upon saccharification. At 94 mM peracetic acid and citrate buffer pH 5–6, the lignin content decreased from 19.9 to 19.3; at 188 mM peracetic acid the lignin content further decreased to 18.3; finally, at 470 mM, it decreased to 12.0. At the same time, both the cellulose-to-glucose and the xylan-to-xylose conversion increased from 27.3/17.6 with no pretreatment to 32.4/22.3 with 94 mM, 46.3/36.8 with 188 mM, and finally 79.5/80.3 with 470 mM.

This correlation of lignin removal with increased sugar yield from the subsequent hydrolysis is poorest at the lowest amount of peracetic acid, 94 mM. This pretreatment removed little lignin (19.9% to 19.3% or 3% removed), but saccharification increases significantly (glucan from 27.3% to 32.4% or a 19% increase; xylan from 17.6% to 22.3%

Table 1 Composition of and Enzymatic Sugar Release from Aspen Wood Pretreated with Enzymatically Generated Peracetic Acid.[a]

Sample no.	Cycles condition	Lignin content (%)	Glucan content (%)	Xylan content (%)	Glucose released (%)[b]	Xylose released (%)[b]
1	Untreated	23.5	40.4	12.7	25.4	13.3
2[c]	1×PAA	22.4	41.0	10.5	39.1	20.9
3	1×blank	23.6	41.2	10.6	33.0	15.7
4[d]	2×PAA	20.3	43.4	12.3	38.6	40.7
5	2×blank	23.4	43.7	11.6	28.9	32.9
6[c]	3×PAA	19.5	41.8	11.3	48.4	29.9
7	3×blank	23.2	41.7	11.3	32.3	14.8
8[d]	4×PAA	19.3	44.3	12.2	60.9	62.7
9	4×blank	22.8	43.3	12.1	30.2	31.9
10[c]	5×PAA	19.1	42.3	12.9	60.2	38.5
11	5×blank	22.2	43.0	12.2	28.5	13.8
12[d]	6×PAA	14.7	45.9	12.3	81.9	94.3
13	6×blank	22.9	44.0	11.8	29.6	33.9
14[d]	8×PAA	10.8	48.2	13.2	97.8	115[e]
15	8×blank	21.9	43.2	11.5	25.4	29.3

Coefficients of variability: lignin content 4.0%, glucan content 4.2%, xylan content 7.5%, glucose release 9.8%, xylose release 11.1%.

[a] First, perhydrolysis (37°C, phosphate buffer (200 mM, pH7.0), 500 mM H_2O_2, 600 mM ethyl acetate, 0.5 mg/mL enzyme, 40 min) generated 60–70 mM peracetic acid. Next, aspen meal was added and incubated (4 h, 37°C). The blank reactions omitted enzyme and chemical reaction generated 2–4 mM peracid. Saccharification reaction volume was 2.5 mL, with 60 FPU Celluclast 1.5 L and 64 pNPGU Novozyme 188 per g cellulose (68 rpm, 50°C, pH 4.8, 5 days). The two series of aspen samples in this table, marked c and d, used a different perhydrolase

[b] Sugar released after saccharification was calculated as the percentage of cellulose hydrolyzed to glucose from amount of cellulose added to the hydrolysis.

[c] Wild-type-PFE-generated peracetic acid, typically 70 mM.

[d] L29G-PFE-generated peracetic acid, typically 60 mM.

[e] This value corresponds to complete hydrolysis (100%). The value above 100 is likely due to experimental error associated with measuring low concentrations of xylose

or 27% increase). This increase may be due to oxidation of lignin to make it less hydrophobic, thus preventing inhibition of the cellulases by binding to lignin.

Enzymatic Generation of Peracetic Acid

The concentrations of peracetic acid generated enzymatically were 60–70 mM, much lower than the 470 mM commercial peracetic acid that gave the best pretreatment. As catalysts, we used wild-type *P. fluorescens* esterase (PFE) or a variant PFE-L29G, where the leucine residue at position 29 has been replaced by glycine using site-directed mutagenesis. Details on this variant will be reported elsewhere. At pH 5.5, variant PFE-L29G yields slightly more peracetic acid than PFE (data not shown), but at pH 7 (used in this work) the wild-type PFE yields slightly more 70 vs. 60 mM for PFE-L29G. Initial experiments use PFE-L29G, but later experiments used the slightly better enzyme, wild-type PFE. Typical reaction conditions were 0.5 mg/mL perhydrolase, 200 mM

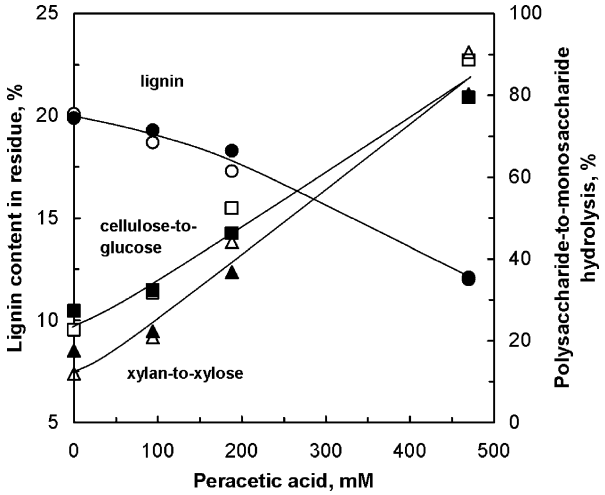

Fig. 2 The lignin content of aspen meal residue decreased with increasing concentrations of peracetic acid in the pretreatment. The amount of glucose released upon subsequent enzymatic saccharification increased with increasing concentrations of peracetic acid in the pretreatment. Peracetic acid pretreatment conditions: room temperature, 72 h, 150 mM citrate buffer pH 5–6 (*filled symbols*); no buffer, pH 2 (*open symbols*). Curves are second-degree polynomial fits to the combined buffered and nonbuffered experiments

pH 7.0 phosphate buffer, 600 mM ethyl acetate (just above its solubility limit) and 500 mM hydrogen peroxide. The enzyme precipitated after 30–40 min of reaction, and the increase in peracetic acid concentration stopped at the same time. We hypothesize that the peracetic acid formed oxidized and inactivated the enzyme at this time. A blank reaction without enzyme generated 2–4 mM peracetic acid under the same conditions. Wild-type PFE typically generated 70 mM PAA in 40 min, while PFE-L29G generated 60 mM.

Pretreatment of Aspen with Enzymatically Generated Peracetic Acid

A single 4-h treatment of aspen meal with enzymatically generated peracetic acid increased the amount of sugar released only slightly, Table 1. After the enzymatic generation of peracetic acid, 10*w/v*.% of aspen meal was added, and the suspension stirred for 4 h at 37°C. We used 37°C for the peracetic acid pretreatments to minimize the decomposition of peracetic acid [51, 52]. During this time, the concentration of peracetic acid dropped from 70 to ~30 mM. Extending the reaction time to 10 h dropped the concentration to 20 mM. It did not seem worthwhile to extend the treatment time by 6 h for only 10 mM additional peracetic acid consumption. For this reason, the pretreatment time was set to 4 h. The percent of glucose released increased from 25.4% for the untreated sample to 39.1% for the sample treated with enzymatically generated peracetic acid (sample no. 2). The blank sample—same reaction conditions, but no enzyme added—also increased to 33% glucose release (sample no. 3). The small increase in lignin and glucose content of the blank (from 23.5% to 23.6% lignin and from 40.4% to 41.2%) is not likely significant. It may be due to rinsing of minor components or due to variation in measurements. The coefficients of variability were 4.0% for the lignin content and 4.2% for the glucan content. The effect of this one-step pretreatment (25.4 increasing to 39.1% glucose released) is slightly better than using 94 mM commercial peracetic acid (27.3% up to 31.1%).

The efficacy of the pretreatment with enzymatically generated peracetic acid increased dramatically upon repeating it multiple times. For each cycle, the aspen meal was filtered and washed with distilled water, and a freshly prepared peracetic acid solution was added. With increasing cycles of pretreatment, the lignin content decreased, and the fraction of sugar released increased, Table 1. After eight 4-h cycles, the lignin content decreased from 23.5% for the untreated sample to 10.8% indicating removal of 61.7% of the lignin. From these samples, the glucose released increased from 25.4% to 97.8%. The amount of xylose released increased from 13.3% to complete conversion. The measured value of 115% reflects the increase in errors as the xylose levels decrease close to the detection limit. Although one cycle of the blank reaction did increase sugar release slightly, multiple cycles of the blank reaction had no further improvement. After eight cycles, the blank reaction yielded 21.9% lignin, 25.4% of the glucose released and 29.3% of the xylose released. The ineffectiveness of the blank reaction demonstrated that the enzymatically generated peracetic acid caused the removal of lignin and enhanced sugar release.

Enzymatically generated peracetic acid applied in multiple cycles removed lignin slightly more effectively, and enzymatic sugar release was slightly higher compared to commercial peracetic acid. The eight cycles of enzymatically generated peracetic acid consumed approximately 3.20 mol of peracetic acid per kilogram of wood (eight cycles of 70 mM starting concentration, 30 mM final concentration corresponds to a consumption of 320 mM peracetic acid or 24.3 wt.% based on dry wood) and removed 61.7% of the lignin in the aspen wood. In comparison, the commercial peracetic acid experiment consumed 3.28 moles per kg of wood (25 wt.% on dry wood) and removed only 40% of the lignin. The cellulose to glucose conversion also was higher for the enzymatic generated peracetic acid pretreatment (97.8%) than for the commercial peracetic acid (79.5%). The washing steps during the multiple cycles may have contributed to the increased effectiveness.

Cellulose-to-glucose conversion was inversely related to lignin content in aspen wood (Fig. 3), similar to the correlation observed using commercial PAA (Fig. 2 above). Increasing cycles of pretreatment decreased the lignin content and increased the fraction of sugars released. After eight 4-h cycles, the lignin content decreased to a level where the glucose released was close to 100% of available cellulose.

Pretreatment of Aspen with Sodium Hydroxide Followed by Enzymatically Generated Peracetic Acid

Previous researchers reported a combined pretreatment—alkaline solution followed by commercial peracetic acid. Adding the alkaline treatment step reduced the amount of peracetic acid needed [30, 31]. The alkaline solution removes acetyl groups from the xylan, but does not reduce the amount of lignin. We similarly found that the alkaline treatment step significantly enhanced saccharification and reduced the number of peracetic acid cycles needed, Table 2. Pretreatment of aspen with 1.5 M NaOH (60% *w*/*w* based on biomass with a 10:1 solution to biomass) gave good saccharification by itself: release of 72% of the glucose present (Table 2, Sample 16). Unfortunately, this pretreatment used large amounts of caustic reagent and also dissolved 28% of our biomass, which lowered the fermentable sugar yield. Lower concentration of NaOH—0.50 M (20% *w*/*w* based on biomass) and 0.125 M (5% *w*/*w* based on biomass) gave similar results. Pretreatment with 0.50 M NaOH yielded 57% glucose released with 16% biomass loss and 0.125 M NaOH yielded 60% hydrolyzed glucose with 10% biomass loss. Xylan was selectively removed, with 1.5 M NaOH removing 71% of the xylan fraction, 0.50 M NaOH removing 28%, and 0.125 M removing 12% (Fig. 4a).

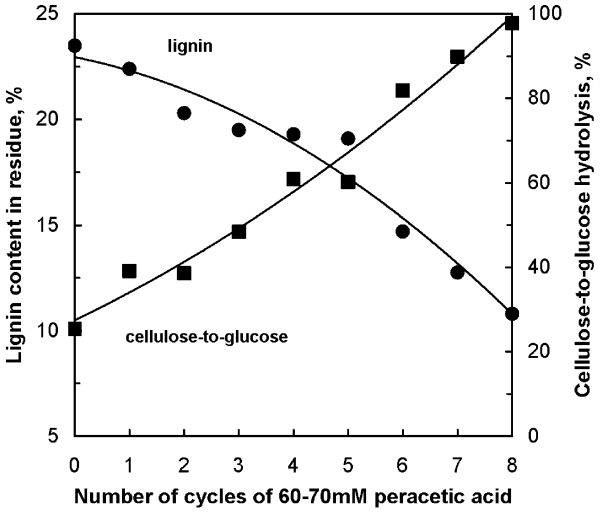

Fig. 3 The lignin content (*circles*) of aspen meal residue decreased with increasing cycles of pretreatment with enzymatically generated peracetic acid. The amount of glucose released (*squares*) upon subsequent enzymatic saccharification increased with increasing cycles of pretreatment with enzymatically generated peracetic acid. Table 1 lists the data and reaction conditions. Curves are second-degree polynomial fits

Adding the enzymatically generated peracetic acid pretreatment to the sodium hydroxide pretreatment enhanced saccharification: 0.125 M NaOH enhanced saccharification by 15%, 0.5 M NaOH by 25% and 1.5 M NaOH by 12% (Table 2 and Fig. 4b). When the NaOH treatment was followed by two cycles of enzymatically generated peracetic acid (60–70 mM

Table 2 Composition of and Enzymatic Sugar Release from Aspen Wood Pretreated with Sodium Hydroxide with and Without a Subsequent Treatment with Enzymatically Generated Peracetic Acid.[a]

Sample no.	Pretreatment method	Glucose released (%)[b]	Xylose released (%)[b]	Biomass loss (%)
16	1.5 M NaOH, 3 h	71.9	74.6	27.7
17	1.5 M NaOH, 3 h+2×60 mM PAA[c]	80.6	106	29.4
18	1.5 M NaOH, 3 h+2×Blank[d]	68.9	81.4	27.2
19	0.50 M NaOH, 3 h	56.9	103	15.8
20	0.50 M NaOH, 3 h+2×70 mM PAA[c]	70.2	135	18.8
21	0.50 M NaOH, 3 h+2×Blank[d]	56.7	107	16.2
22	0.125 M NaOH, 4 h	60.6	61.3	10.2
23	0.125 M NaOH, 4 h+2×70 mM PAA[c]	85.1	85.3	12.1
24	0.125 M NaOH, 4 h+2×Blank[d]	67.4	75.4	10.1
25	Untreated	25.4	13.3	–

[a] All pretreatments were performed at 37°C and peracetic acid (PAA) pretreatment for 4 h

[b] Sugar released after saccharificaiton and the value more than 100% indicate systematic errors in the measurement

[c] The samples were pretreated with NaOH followed by two-cycle PAA pretreatment

[d] The samples were pretreated with NaOH followed by two-cycle blank reaction pretreatment

PAA, 3–4 h, 37°C), cellulose-to-glucose increased to 85%, 70%, and 81% for 0.125, 0.50 and 1.5 M NaOH, respectively. Following NaOH treatment with blank reagents did not improve hydrolysis efficiency.

The lowest sodium hydroxide concentration—0.125 M—mainly removed acetyl groups. The acetyl content decreased from 4.0% to 0.6% after the sodium hydroxide treatment. The additional 6.8% weight loss includes hemicellulose, lignin, and extractives. The sharp increase in yield of sugar release after this treatment (from 25% to 60% glucose release) indicates that the acetyl groups hinder sugar release. Both Grohmann et al. [53] and Kong et al. [43] found similar results. Grohmann et al. removed 90% of the acetyl groups from aspen wood using 4 *w*/*v* % hydroxylamine solution [53]. This treatment removed only a small amount (10%) of the lignin and did not affect either cellulose or hemicellulose content. Enzymatic digestion released 26% of the glucose from cellulose fraction compared to 12% without pretreatment. Similarly, Kong et al. treated aspen with 0.15 M NaOH (room temperature, 24 h, solution volume to wood weight ratio of 5:1) to decrease the acetyl content from 2.6% and 0.54%. This treatment increased the enzymatic hydrolysis from 11% to 46% [54, 55].

Material Balances

Material balance flow diagrams track changes in feedstock composition throughout the pretreatment and saccharification steps, integrating the composition of material removed at each step. The values in Fig. 5 are for 1 kg dry weight of aspen meal, based on our 1.2-g scale experiments and the eight-cycle pretreatment with enzymatically generated peracetic acid and its subsequent hydrolysis using the standard NREL LAP-009 procedure for saccharification [49]. During the pretreatment, 156 g of biomass was lost with the majority (145 g) being lignin (61.7% of total lignin available). We observed no loss of cellulose and only a small loss of the xylose fraction of the hemicellulose. After saccharification, 439 g of glucose was released, yielding 97.8% cellulose-to-glucose conversion, 127 g of xylose,

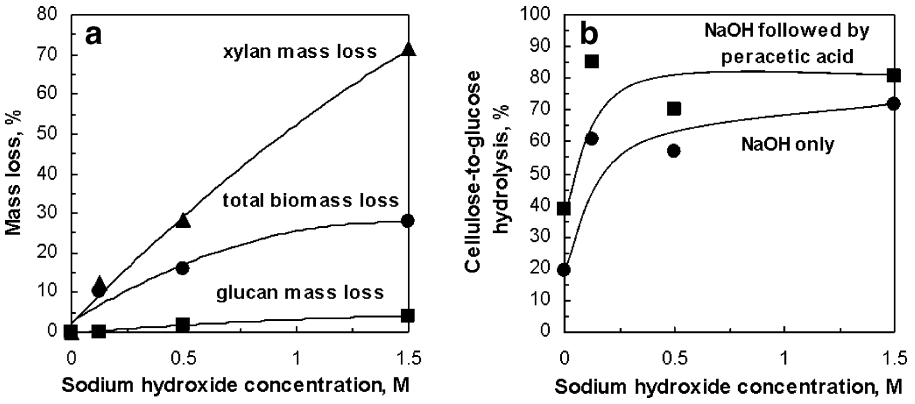

Fig. 4 Sodium hydroxide pretreatment of aspen meal. **a** Treatment with NaOH causes significant loss of hemicellulose (xylose, triangle), but little loss of cellulose (glucose, squares). The *circles* show the total biomass loss, which takes into account the lower proportion of xylose compared to cellulose. **b** Sodium hydroxide pretreatment enhanced the saccharification of cellulose-to-glucose (*circles*). Adding two subsequent cycles of enzymatically generated peracetic acid further enhanced saccharification slightly (*squares*). Data are listed in Table 2. Conditions: NaOH at a ratio 1/10 (*w*/*w*, wood powder/solution) for 3–4 h at 37°C with shaking at 250 rpm

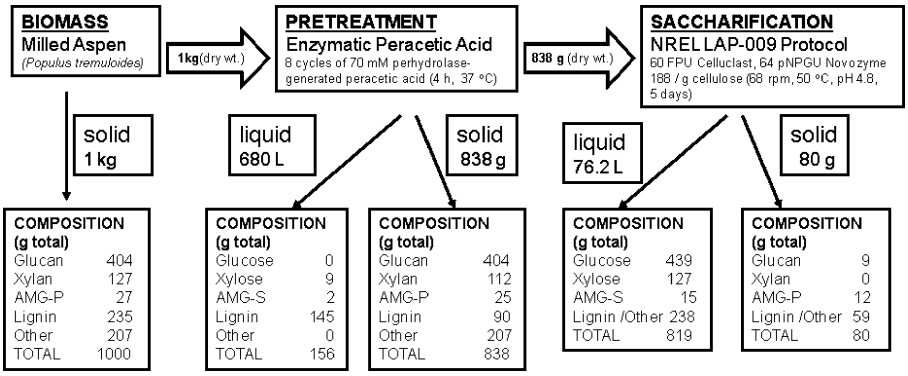

Fig. 5 Material balances for 1 kg of milled aspen wood extrapolated from the results of 1.2-g scale experiments. Eight cycles of perhydrolase-generated peracetic acid (each cycle 70 mM peracetic acid, 4 h, 37°C) and corresponds to sample no. 14 in Table 1. Saccharification released 439 g (97.8%) of glucose from the aspen wood powder into the liquid for further processing, with the loss of 156 g biomass (145 g lignin). Polysaccharides in the solid are give as glucan or xylan, while the sugars in liquids are given as glucose or xylose. A given mass of glucan will yield upon hydrolysis a slightly larger mass of glucose due to the gain of the mass of the water. AMG-S: total of arabinose, mannose, and galactose contents. AMG-P: total of arabinan, mannan, and galactan contents

yielding 100% xylan to xylose. The fermentable sugar release was 581 g. The polysaccharides in solid biomass gain the weight of a water molecule upon hydrolysis to form sugars. Conversion of glucan to glucose and xylan to xylose increased the mass by a factor of 1.11 and 1.14, respectively. For example, complete hydrolysis of 100 g of cellulose yields 111 g of glucose.

The mass balance in Fig. 6 is for one cycle of 0.125 M sodium hydroxide followed by two cycles of perhydrolase-generated peracetic acid and its subsequent hydrolysis using the standard NREL LAP-009 procedure for saccharification [49]. During the pretreatment, 128 g of biomass was lost with the loss being a mixture of lignin and xylose, (53 g lignin and 27 g xylose). After saccharification, 381 g of glucose was released, yielding 85.1% cellulose-to-glucose conversion, 101 g of xylose, yielding 85.3% xylan to xylose. The total fermentable sugar release was 498 g.

Both pretreatments removed some biomass, but differ in which components were removed. The peracid-only pretreatment removed 156 g, mainly lignin (61.7% of total lignin present). Little (1.5%) of the glucose and xylose sugars were lost. The sodium hydroxide pretreatment followed by peracetic acid removed 128 g, but most of this was xylose (22.5% of total lignin present and 21.2% of total xylose present). Although dry weight after pretreatment was higher for the pretreatment that included sodium hydroxide (877 g vs. 838 g), it yielded less fermentable sugar after saccharification (498 g vs. 581 g of total fermentable sugar) both due to some loss of xylose, but also to a less complete digestion of the cellulose.

Discussion

Untreated aspen wood showed typical composition and enzymatic saccharification released only 25.4% of the available glucose and 13.3% of the xylose. Pretreatment of aspen wood

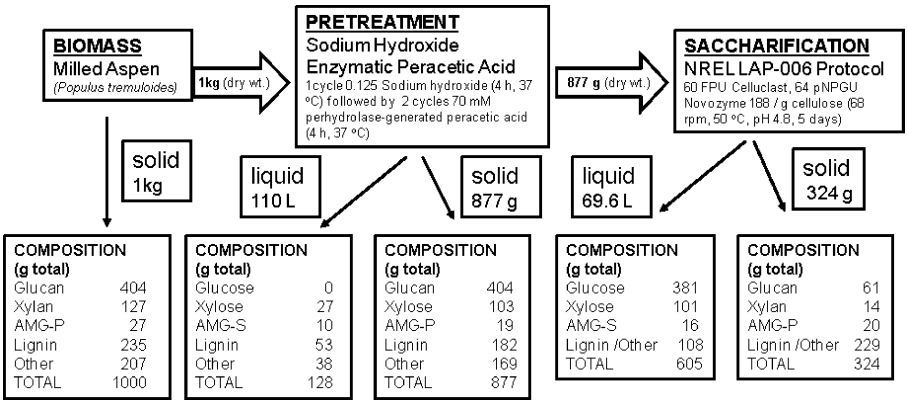

Fig. 6 Material balances for 1 kg of milled aspen wood extrapolated from the results of 1.2-g scale experiments. One cycle of 0.125 M sodium hydroxide (4 h, 37°C) followed by two cycles of perhydrolase-generated peracetic acid (70 mM, 4 h, 37°C) and corresponds to sample no. 23 in Table 2. Saccharification released 381 g (85.1%) of glucose from the aspen wood powder into the liquid for further processing, with the loss of 128 g biomass (53 g lignin and 27 g xylose). Polysaccharides in the solid are give as glucan or xylan, while the sugars in liquids are given as glucose or xylose. A given mass of glucan will yield upon hydrolysis a slightly larger mass of glucose due to the gain of the mass of the water. AMG-S: total of arabinose, mannose, and galactose contents. AMG-P: total of arabinan, mannan, and galactan contents

with commercial peracetic acid (470 mM, 25 wt.% based on wood) removed 39.7% of the lignin (from 19.9% initially to 12.0% after pretreatment) and increased the enzymatic cellulose-to-glucose conversion to 90%. Previous researchers found similar improvements using peracetic acid to pretreat hybrid polar [30, 56] or sugar cane bagasse [30]. Despite the effectiveness of these peracetic acid pretreatments, the high cost of commercial peracetic acid precludes their use.

Perhydrolases catalyze the conversion of acetic acid or acetic acid esters and hydrogen peroxide to peracetic acid [57]. We used *P. fluorescens* esterase (PFE) or a genetically modified variant PFE-L29G as the perhydrolase. These enzymes generated 60–70 mM peracetic acid. After 30 min, the enzyme precipitated, and no more peracetic acid was generated. We hypothesize that the peracetic acid produced oxidizes and inactivates the enzyme, preventing further catalysis.

Since the concentration of peracetic acid generated enzymatically was lower than the concentrations used in the successful pretreatments using commercial peracetic acid, we repeated the treatment up to eight times. Enzymatically generated peracetic acid, like commercial peracetic acid, progressively and selectively removed lignin, leaving hemicellulose and cellulose intact. Eight cycles of enzymatically generated peracetic acid resulted in lignin content of 10.8% and a cellulose-to-glucose conversion rate of 97.8%.

Enzymatically generated peracetic acid applied in multiple cycles was slightly more effective in removing lignin and increasing enzymatic sugar release than commercial peracetic acid. This difference most likely is due to the increased number of washes in the enzymatically generated peracetic acid pretreatments compared to the pretreatment with commercial peracetic acid. The eight cycles of enzymatically generated peracetic acid consumed approximately 3.20 mol of peracetic acid per kilogram of wood (24.3 wt.% based on dry wood). This estimate assumes that each cycle consumed 40 mM peracetic acid, that is, a drop from 70 to 30 mM peracetic acid during each cycle. In comparison, the commercial peracetic acid pretreatment consumed marginally more peracetic acid: 3.28 mol

kg^{-1} of wood (25 wt.% on dry wood). This estimate assumes that pretreatment with commercial peracetic acid consumed all the acid, since the preliminary experiments showed none remaining. The enzymatically generated peracetic acid removed more lignin (10.8% vs. 12% remaining lignin) and released more glucose from cellulose (97.8% vs. 79.5%).

Although decreasing lignin content correlated with increasing sugar release [58], this correlation is imperfect at the early stages. At the early stages where little lignin has been removed, more sugar is released than expected based on lignin removal (see Figs. 2 and 3). Other oxidative pretreatments have also noted an increase in sugar release even when little lignin was removed: hydrogen peroxide treatment [59], wet oxidation [60], and laccase application [61]. These oxidative pretreatments, like peracetic acid pretreatment, likely add polar substituents to lignin to reduce its hydrophobicity. This reduced hydrophobicity prevents binding of the cellulase to lignin, thereby increasing the release of sugars even when the lignin remains. Another contributor to increased sugar release independent of lignin content is decreased cellulose crystallinity. Peracetic acid pretreatment may reduce cellulose crystallinity [23], which could increase enzymatic hydrolysis of cellulose.

Pretreatment of aspen wood with NaOH improved sugar release, but at the cost of losing significant amounts of xylan. The reason for the improved sugar release may be the removal of acetyl groups. Acetyl group removal increased enzymatic sugar release in poplar wood [43, 56] and in Douglas fir wood [62]. Conversely, adding acetyl groups to cellulose decreased enzymatic sugar release [62], likely by disrupting hydrogen bonds between the enzyme and cellulose or by increasing the diameter of the cellulose chains thus hindering their fit in the active site.

The current enzymatically generated peracetic acid pretreatment needs significant improvement before it can be commercially competitive. The first limitation is the large amount of enzyme needed: 40 g of enzyme to treat 1 kg of aspen wood with eight cycles. Improving the stability of enzyme to peracetic acid or increasing rate of perhydrolysis could reduce the amount needed. Protein engineering improved the stability of a peroxidase more than a 100-fold [63]. A similar improvement to this perhydrolase would reduce the amount of enzyme to only 400 mg/kg. In addition, a more stable enzyme would allow generation of higher concentrations of peracetic acid and eliminate the need for multiple peracetic acid cycles. Other potential improvements are to use all the enzymatically generated peracetic acid, not just 40 of the 70 mM generated (57%) or to use a flow-through reactor that would minimizes contact of the peracetic acid with enzyme and thus increase its effective lifetime. Another potential improvement is the use of the acetyl xylan esters as the acetyl source for the peracetic acid. This would reduce the amount of acetate ester needed and also remove the acetyl groups, which enhances the sugar release.

Enzymatic generation of peracetic acid as a pretreatment for biomass offers at least three significant advantages. First, it generates peracetic acid as needed, thereby eliminating issues related to storage (explosion and stability). Second, it potentially could use acetyl groups in biomass feedstock to reduce chemical costs. Third, it will sterilize the biomass and prevent microbial contamination problems in biomass storage and fermentation. At this time, the amount of enzyme needed is too high to be economical, but improvements in enzyme stability and activity could overcome this barrier.

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