**Alkaline hydrogen peroxide (AHP) pretreatment of softwood: Enhanced enzymatic
hydrolysability at low peroxide loadings**

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1 **Abstract**

2 This study investigated the specific effects of hydrogen peroxide (H_2O_2), alkali and
3 temperature on substrate hydrolysability during alkaline hydrogen peroxide (AHP)
4 pretreatment of Douglas fir. We demonstrated that the presence of a small amount of
5 hydrogen peroxide is essential for effective pretreatment of Douglas fir by alkali. However
6 contradictory to previous finding from others, we found that increasing H_2O_2 did not
7 directly correlate to enhanced substrate hydrolysability, whereas the alkali charge played a
8 more dominant role. The paper illustrated a strategy to apply AHP for softwood
9 pretreatment with low peroxide loadings and achieve high cellulose-to-glucose yield (up to
10 95 %). It was also found that glucomannan gelation (physicochemical change) occurred
11 during AHP pretreatment of softwood which presents a newly identified recalcitrance
12 factor to substrate hydrolysability. The resulting glucomannan derived hydrogel-like
13 material has a high affinity toward cellulases and can cause a non-productive binding
14 effect.

15 **Keywords:** Alkaline hydrogen peroxide, Softwood, Enzymatic hydrolysis, Glucomannan,
16 hydrogel, low peroxide loading

1. Introduction

Implementing cost effective softwood deconstruction processes to maximize fermentable sugars production remains a huge challenge [1, 2]. Among a large selection of plant biomass pretreatment technologies developed to date, only a few of them, including acid or SO_2 pre-impregnated steam explosion [3], alkaline or sulfite pulping based pretreatments [4], wet explosion [5] and organosolv pretreatment [6], are able to deconstruct softwood for fermentable sugar production. However the cost associated with these processes is still formidable to justify for a biorefinery operation.

In recent years, alkaline hydrogen peroxide (AHP) treatment, a pulping and bleaching method employed in papermaking industry [7, 8] has drawn attention for biomass pretreatment of hardwoods and grasses [9-11]. AHP pretreatment has shown to remove lignin while selectively retaining the cellulose [12]. In addition, AHP has a potential to simultaneously convert hemicelluloses to valuable organic acids (e.g. lactic, succinic and glycolic) products [13]. Results from most AHP studies suggest that increasing hydrogen peroxide (H_2O_2) loading is beneficial for improving substrate hydrolysability. Therefore, high optimum H_2O_2 loadings (mass fraction of H_2O_2 to biomass $w_{\text{H}_2\text{O}_2}$) have been proposed for treating most lignocellulosic biomasses including cashew apple bagasse ($w_{\text{H}_2\text{O}_2} = 0.86$) [14], rice hulls and straw ($w_{\text{H}_2\text{O}_2} = 0.80$) [15], and corn stover ($w_{\text{H}_2\text{O}_2} = 0.50$) [9]. Due to the high recalcitrance of softwood, the implementation of AHP for this biomass would require a high peroxide loading, which presents a significant cost factor of AHP treatment [16]. In this paper, we evaluated the use of different pretreatment conditions during AHP of

1 softwood to with an objective to obtain high cellulose-to-glucose yields during enzymatic
2 hydrolysis while maintaining a low peroxide loading ($w_{\text{H}_2\text{O}_2} < 0.10$).

3 It is generally recognized that AHP pretreatment is more effective under basic
4 conditions at a pH around 11.6 [17]. However, this pH was identified based on optimum
5 conditions for pulp bleaching. Applying AHP bleaching at mild temperatures (up to 90°C)
6 is another common practice during papermaking. A number of AHP pretreatment
7 processes were conducted at mild temperatures ($< 50\text{ }^\circ\text{C}$) [9, 15]. However, it has not been
8 corroborated that conditions used during AHP for pulp and paper production are optimum
9 for biomass pretreatment. It is conceivable that conditions aimed at obtaining strong (high
10 crystallinity) and long fibers (low disruption of biomass matrix) for papermaking may not
11 be ideal for biomass pretreatment for fermentable sugar production.

12 The objective of this study is to fill several knowledge gaps within AHP
13 pretreatment of softwood. H_2O_2 is acidic in its native form. Alkali NaOH and Na_2CO_3 are
14 commonly added to adjust and maintain the basic pH of the reaction solution. Therefore, an
15 increase in H_2O_2 loading is always accompanied with an increase in alkaline concentration.
16 The use of high H_2O_2 loadings on previous AHP pretreatment studies [9, 14, 15] was also
17 associated with a high alkaline loading. A clear understanding of the individual effects of
18 hydrogen peroxide (H_2O_2) and alkali (NaOH and Na_2CO_3) on substrate hydrolysability
19 during softwood pretreatment is lacking. Glucomannan has been shown to be susceptible to
20 alkaline degradation. However after pretreatment, an appreciable amount of glucomannan
21 often remained in the residual substrate [13]. The properties of residual glucomannan and
22 its effects on subsequent enzyme hydrolysis have not been investigated. It is conceivable

that applying higher treatment temperature (180 °C) and pH (> 11.6) may improve the AHP pretreatment of softwood for sugar production. However this effect has not been well understood. This study aims at providing new insights into identifying efficient AHP pretreatment method of softwood.

2. Materials and methods

2.1. Description and chemical composition of Douglas fir samples

Two different Douglas fir (*Pseudotsuga menziesii*) samples were used in this study, a saw mill residual pulp chips (FS01) with 1.4% bark collected on October 2011 from a wood chip pile on Southwest Washington (46° 6' N and 122° 57' W, located on Weyerhaeuser Longview Pull Mill) and a forest harvest residue (FS10) with 3.4% bark content collected on January 2013 from a road side pile on Southeast Oregon (43° 6' N and 122° 9' W, owned by Weyerhaeuser). Collected samples were chosen to represent typical forest residues of USA Pacific Northwest region as part of the Northwest Advance Renewable Alliance (NARA) project aimed to the production of fuels and chemicals from biomass. The used nomenclature for softwood residues states the order of their collection from a larger set of forest samples (FS) [18]. Forest samples were milled using a Peterson horizontal drum fixed-hammer grinder to obtain wood chips, and screened over a woven wire screen to remove fines (< 3 mm). The wood chips were first air dried to less than 10 % moisture before shipping for analysis to Washington State University, Bioproducts Science and Engineering Laboratory, Richland, WA.

Prior to analysis, the samples were milled to a specific size (mesh -40 to +60) and their cellulose, glucomannan, arabinoxylan and lignin compositions were determined following standard procedures [19, 20].

2.2. *Experimental conditions for alkaline hydrogen peroxide pretreatment*

Alkaline hydrogen peroxide (AHP) pretreatment of Douglas fir was conducted in pressure tube reactors to allow processing a large number of treatments at identical conditions. Stainless steel pressure tube reactors were purchased from Swagelok Company (Solon, Ohio, U.S.A) with a volumetric capacity of 15 cm³. AHP pretreatments of pulp wood chips (FS01) were carried out at different temperatures (25 °C, 90 °C, 140 °C, 180 °C and 200 °C), reaction times between 0 min to 90 min, peroxide loadings (mass fraction of H₂O₂ to biomass $w_{\text{H}_2\text{O}_2}$) from $w_{\text{H}_2\text{O}_2} = 0$ to $w_{\text{H}_2\text{O}_2} = 0.04$ (gram of peroxide per gram of biomass), and a solid concentration of 10 % g/cm³. Reaction time was recorded after the samples reached the desired temperature as previously reported [13]. The pH of the reaction solution was adjusted first by sodium carbonate to pH 10.6 (pH regulator), and then to pH 11.6 with the addition of sodium hydroxide. Due to the weak acidity of hydrogen peroxide, the amount of sodium carbonate and sodium hydroxide applied increased with the increase in H₂O₂ dosage.

After pretreatment, the solid fraction (water insoluble solids, WIS) and water soluble fraction (WSF) were separated and collected by filtration through a glass filtering crucible. The WIS was washed with water and then subjected to enzymatic hydrolysis to determine the resulting cellulose-to-glucose yields of pretreated Douglas fir samples.

2.3. Enzymatic hydrolysis of pretreated samples

Enzymatic hydrolysis of pretreated Douglas fir FS01 and FS10 were carried out at 2% substrate consistency (2 g odw biomass per 100 cm³ solution) and 50 °C using Cellic[®] CTec2 (Ctec2) cellulase enzymes (Novozymes). Substrate and enzyme were mixed in sodium acetate buffer with a pH of 4.8. Sodium azide (0.02%) was also added to prevent microbial contamination according to NREL enzymatic saccharification procedure [21]. Enzyme dosages ranged from 20 FPU/g to 30 FPU/g (enzyme unit per gram of cellulose in the substrate) were tested from an initial enzyme stock with an activity of 119 FPU/cm³ and 257 g/L protein concentration. Enzymatic hydrolysis experiments were performed at least in duplicates using 2 cm³ Eppendorf tubes incubated in a molecular shaker (Eppendorf Thermomixer R). Hydrolysate samples were collected at different time intervals during 96 h. Glucose in the hydrolysate was measured by the glucose oxidase-peroxidase (GOPOD) assay kit (Megazyme, Ireland). Selected samples were also analyzed by HPLC to measure glucose, xylose, arabinose, galactose and mannose contents following NREL protocol [20].

Supplementing either mannanases NS-51023 (Novozymes) or xylanases (Pulpzyme HC 2500 and Novozymes Cellic[®] HTec2) to Novozymes Ctec2 enzyme for substrate hemicellulose hydrolysis was also tested. Three dosages of hemicellulolytic enzymes were evaluated, 0.3 g (low), 0.6 g (medium) and 1.2 g (high) of enzyme protein per 100 g of substrate. The enzyme protein concentration of mannanase NS-51023 (Mann), Pulpzyme HC 2500 (Pulp) and Novozymes Cellic[®] HTec2 (Htec2) stock solution were (77 ± 7) g/L, (72 ± 3) g/L and (364 ± 17) g/L, respectively. Enzyme protein content were determined

using the Pierce[®] BCA protein assay kit (Thermo Fisher Scientific) following manufacturer's recommendations. In addition, control experiments using enzymes alone without the substrate were conducted to calculate background sugars in commercial enzymes mixtures.

2.4. Cellulase adsorption isotherm on model carbohydrates

Cellulose (Avicel[®] PH-101, Sigma-Aldrich), glucomannan (from Konjac, Megazyme) and xylan (from birch wood, Sigma-Aldrich) were used as model carbohydrate polymers to determine cellulase adsorption on specific biomass fractions. Celluclast (Novozymes) instead of Ctec2 was used for the adsorption isotherm experiments following a previous method [22]. Enzyme solutions with concentrations ranging from 0.05 g/l to 4 g/l were added to the buffer with substrates at 2% consistency. The mixture was incubated at 25 °C for 1 h to reach equilibrium [23], and after centrifugation the protein content in the supernatant was determined by Pierce[®] BCA protein assay kit using bovine serum albumin (BSA) as a standard. Samples were run in duplicates and the adsorbed protein was calculated based on the difference between the initial cellulase content and free cellulase content in the supernatant [22]. The Langmuir adsorption isotherm model was used to study cellulase adsorption on model biomass substrates. The surface concentration of adsorbed cellulases on substrate (Γ) as a function of free cellulase concentration on solution (C) is given by:

$$\Gamma = \frac{\Gamma_{max} KC}{1+KC} \quad (1)$$

Where Γ_{max} is the surface concentration of protein at full coverage and K is the Langmuir constant.

2.5. Statistical analysis

All the experiments were performed at least using two independent replicates. The Data was analyzed by a one-way analysis of variance (ANOVA) using 95 % confidence level ($p < 0.05$) for statistical significance. The Tukey test (with $p < 0.05$) was used to analyze the significances of different pretreatment conditions on ensuing enzymatic hydrolysis or lignin removal. Origin 8.1 software was used to perform the calculations.

3. Results and Discussion

3.1. Effect of pretreatment conditions on enzymatic cellulose-to-glucose yield

The chemical mass fraction composition of Douglas fir pulp chips (FS01) includes (48.0 ± 0.9) % glucan, (11.4 ± 0.8) % mannan and (27.7 ± 0.8) % lignin as main components [13]. Minor hemicellulose components of Douglas fir FS01 included xylan (2.7 ± 0.1) %, galactan (2.2 ± 0.1) %, arabinan (0.5 ± 0.0) % and acetyl groups (2.6 ± 0.2) %. Compared to FS01, FS10 has lower glucan content (43.2 ± 0.6) %, similar mannan (11.3 ± 0.5) % and higher lignin content (30.4 ± 0.1) %. FS10 also contains small amounts of xylan (3.0 ± 0.3) %, galactan (1.6 ± 0.2) %, arabinan (1.2 ± 0.0) % and acetyl groups (3.2 ± 0.1) %.

Previous AHP pretreatments of hardwoods and grasses were mostly conducted using relatively low temperatures (< 50 °C) and high peroxide loadings (up to $w_{H_2O_2} = 0.86$)

[15, 24]. However, due to the high recalcitrance of softwood, the AHP pretreatment of Douglas fir under mild temperatures (25 °C, 90 °C and 140 °C) and low peroxide loadings ($w_{\text{H}_2\text{O}_2} = 0.04$) were ineffective in producing substrates with good hydrolysability and the ensuing cellulose-to-glucose yields were below 25 % (data not shown). A higher treatment temperature (around 180 °C) is therefore required to achieve high delignification rates during alkaline treatment [25]. Two sets of pretreatment temperatures 180 °C and 200 °C were further used in this study. The enzymatic hydrolysability of softwood pretreated at different H_2O_2 loadings ($w_{\text{H}_2\text{O}_2} = 0$ to $w_{\text{H}_2\text{O}_2} = 0.04$) and treatment times (30 min to 90 min) were evaluated by measuring the cellulose-to-glucose conversion yields after cellulase (Ctec2) hydrolysis during 96 h with an enzyme loading of 20 FPU/g. Fig. 1A shows the enzymatic hydrolysability of pretreated FS01 substrate at 180 °C. Up to 34 % cellulose-to-glucose conversion yield was obtained at $w_{\text{H}_2\text{O}_2} = 0.04$, and 180 °C, independent of treatment time. It appears that 30 min pretreatment of Douglas fir at 180 °C is sufficient to decrease biomass recalcitrance toward enzymatic digestion under tested conditions. Increasing the reaction time from 30 min to 60 min, or 90 min does not lead to statistically significant differences on glucose yields according to ANOVA ($p < 0.05$). It has been demonstrated that hemicellulose and lignin degradation from softwood during AHP reached a “saturation” point during the first 30 min of reaction at 180 °C [13].

Fig. 1 here

The hydrolysability of pretreated Douglas fir at a higher temperature 200 °C was also conducted at the same H_2O_2 loadings ($w_{\text{H}_2\text{O}_2} = 0$ to $w_{\text{H}_2\text{O}_2} = 0.04$) and treatment times (30 min to 90 min) (Fig. 1B). Only a marginal improvement in hydrolysability was

1 obtained, up to 35 % total (at $w_{\text{H}_2\text{O}_2} = 0.04$) by temperature increase which was not
2 statistically significant according to ANOVA ($p < 0.05$). In previous papers, AHP has
3 shown promising results for grass (e.g., corn stover) and hardwood (e.g., hybrid poplar)
4 feedstocks with cellulose-to-glucose conversion yields up to 95 % [9-11]. The low
5 enzymatic hydrolysability of AHP pretreated Douglas fir pulp chips prompted further
6 investigation to identify the underlying recalcitrance factors of softwood and determine
7 strategies to improve the ensuing hydrolysability at low peroxide loading. The hypothesis
8 of using AHP is due to its potential effect on delignification (up to 22 %) without
9 significant cellulose degradation ($< 8\%$) [13]. It was also shown that glucomannan, the
10 most predominant hemicellulose in Douglas fir, was susceptible to alkaline degradation
11 [13]. In order to understand softwood recalcitrance and improve its hydrolysability, we then
12 conducted several subsequent experiments: evaluate the hemicellulases supplementation
13 during enzymatic hydrolysis of AHP pretreated D. fir; determine Langmuir adsorption
14 isotherms of biomass model compounds; and test AHP pretreatment using different H_2O_2
15 and alkaline (pH) combinations.

16 As shown in Fig. 1, the higher enzymatic glucose yields were obtained at $w_{\text{H}_2\text{O}_2} =$
17 0.04 loading without significant variations from different temperature (180 °C to 200 °C) or
18 reaction time (30 min to 90 min). Therefore, AHP pretreatment of Douglas fir at $w_{\text{H}_2\text{O}_2} =$
19 0.04, 180 °C and 30 min was selected as pretreatment condition for hemicellulase
20 supplementation experiments. The specific effects of residual xylan on cellulase hydrolysis
21 are well understood [22], whereas the effect of residual mannan on cellulase hydrolysis is

not clear yet. We then conducted enzymatic hydrolysis (CTec2) with hemicellulase supplementation.

3.2. Enzymatic hydrolysis with the addition of hemicellulases

Three hemicellulase enzyme preparations, including NS-51023 (mannanase from Novozymes), Pulpzyme HC 2500 (xylanases from Novo Nordisk A/S) and Htec2 (xylanases from Novozymes), were supplemented to cellulose Ctec2 for the hydrolysis of pretreated Douglas fir FS01 ($w_{H_2O_2} = 0.04$, 180 °C and 30min). Pretreated FS01 consists of 52.0 % glucan, 30.7 % lignin, 5.5 % mannan, xylan 1.3 % and 0.6 % arabinan. Three dosages of hemicellulases were tested (0.3 %, 0.6 % and 1.2 %).

It was found that none of the hemicellulase preparations led to a statistically significant improvement in cellulose-to-glucose yields (ANOVA $p < 0.05$) even at the highest (1.2 %) hemicellulases loading (Fig. 2A). In comparison, a significant improvement of glucose yield was obtained by increasing cellulase Ctec2 loading from 20 FPU/g to 30 FPU/g. Further increasing hemicellulolytic enzymes loading was not tested as it will unlikely be a practical strategy for commercial application [26]. Monosaccharides released after 96 h enzymatic hydrolysis were determined (Fig. 2B). A significant amount of xylose and arabinose, representing more than 88 % of residual xylan and arabinan were detected in all hydrolysate samples even with cellulose Ctec2 alone. Ctec2 enzymatic cocktail is known to contain a high xylanase activity, which can explain the fact that additional xylanase did not bring any significant effect on xylan and arabinan removal. There was some mannose, representing 16 % of the mannan in pretreated substrate, released during

Ctec2 treatment alone. The addition of mannanase and pulpyzyme to Ctec2 slightly increased mannose release (up to 38 %). However, more than 60 % of mannan still remained in the hydrolysis residue after mannanase treatment. This suggested that the structure of residual mannan in pretreated substrates was altered and became more recalcitrant to enzymatic hydrolysis.

Fig. 2 here

In addition to alkaline degradation reactions, It has been reported that glucomannan gelation can occur under alkaline treatment conditions [27]. Glucomannan gelation is a physiochemical change in its 3D structure caused by the release of acetyl groups in the alkaline environment which subsequently creates electrostatic repulsions between individual chains leading to its self-aggregation (hydrogel) [27]. This little information on the effect of glucomannan gelation on subsequent enzymatic hydrolysis reported in the literature. To understand this effect, a model glucomannan sample, Konjac glucomannan (Megazyme, Ireland), was treated by AHP using the same conditions as for Douglas fir. After the treatment, approximately 30 % of glucomannan was dissolved and converted to organic acids, whereas 70 % remained as a hydrogel. This hydrogel was subjected to enzymatic hydrolysis (cellulose Ctec2 and mannanases) and negligible amount of releasing sugars was obtained. However, it was found that this glucomannan hydrogel had a strong affinity to cellulase enzymes. Adsorption of cellulases to glucomannan and its hydrogel was then tested to determine the maximum surface coverage of enzyme (Γ_{max}) using Langmuir adsorption isotherm [22]. The Γ_{max} of Ctec2 on untreated glucomannan was 14.1

mg/g whereas this value increased to 72.5 mg/g when tested on glucomannan hydrogel obtained after AHP treatment (Supplementary Fig. 1 and Table 1). For comparison, the Γ_{max} obtained from AHP treated Cellulose (Avicel) was 62.9 mg/g (Supplementary Table 1). This clearly shows a significant increase in the competitive adsorption of cellulase enzymes on glucomannan after AHP treatment. This result agrees with the earlier observation that higher Ctec2 loading (30 FPU/g) could improve glucose release (by providing more enzymes to cleave available cellulose) but hemicellulase additions could not.

3.3. Effect of pretreatment chemicals on substrate hydrolysability

The effects of different chemical reagents and their loadings (i.e., H_2O_2 , NaOH, Na_2CO_3) on the AHP pretreatment of Douglas fir were next investigated. Maintaining the pH of solution at 11.6 during the reaction is critical to promote the formation of perhydroxyl anion (HOO^-) which is a key reactive species in conventional AHP pretreatment (Equation 2).

NaOH / Na_2CO_3 buffer solution is typically added during the industrial bleaching process to control reaction pH [7, 9]. Sodium hydroxide is a also common delignification agent widely used in pulping. The addition of sodium hydroxide [7] helps active lignin toward nucleophilic reactions and dissolve lignin fragments. As a weak alkali, sodium carbonate has been applied alone or with sodium hydroxide for biomass pretreatment [28].

Therefore, all these three chemicals can contribute to biomass deconstruction. However, the specific role of each reagent on biomass delignification and subsequent enzymatic hydrolysability of pretreated substrates has not been clarified.

Previous studies generally concluded that increasing H_2O_2 loading can promote delignification and improve the enzymatic hydrolysability of pretreated substrates. As a result, high optimal H_2O_2 dosages, from $w_{\text{H}_2\text{O}_2} = 0.50$ to $w_{\text{H}_2\text{O}_2} = 0.86$, have been proposed for biomass pretreatment [9, 15, 24]. It was also observed in this study that the hydrolysability of both pretreated softwood substrates, FS01 and FS10, was improved significantly by raising the H_2O_2 dosage from $w_{\text{H}_2\text{O}_2} = 0.04$ to $w_{\text{H}_2\text{O}_2} = 0.10$ (Fig. 3). During wood pulp bleaching, H_2O_2 loading is often below 100 kg per ton of wood pulp (equivalent to 0.10g H_2O_2 /g wood) which already represents a significant cost to papermaking. It is conceivable that applying high H_2O_2 dosages for biomass pretreatment will face significant economic challenges for industrial applications.

Fig. 3 here

It is apparent that increasing H_2O_2 loadings will require the increasing of the amount of alkali (NaOH / Na_2CO_3) applied to the pretreatment due to the acidic nature of H_2O_2 . In other words, the total active alkaline (NaOH) and total titratable alkaline (NaOH / Na_2CO_3) will also increase with the increase in H_2O_2 charge. For instance, 4.3 % (mass density) of total titratable alkaline is needed to obtain a pH of 11.6 in water, whereas 18.9 % and 46.9 % of total titratable alkaline are required to reach the same pH in $w_{\text{H}_2\text{O}_2} = 0.04$ and $w_{\text{H}_2\text{O}_2} = 0.10$ solutions, respectively.

A better understanding of the specific effects of peroxide, sodium hydroxide, sodium carbonate and soaking times during AHP pretreatment can help identify a viable AHP pretreatment strategy. The effect of alkaline H₂O₂ impregnation of softwood for delignification was first examined (Table 1). Impregnation (or soaking) of biomass in alkaline H₂O₂ solution at room temperature has been evaluated as a pretreatment procedure previously. Overnight and longer impregnation times (12 h to 48 h) at room temperature have been shown to improve hydrolysis yield of AHP pretreated corn stover [9, 10] and wheat straw [29]. It is generally hypothesized that this H₂O₂ impregnation process promoted delignification as indicated by a significant brightening effect on impregnated samples.

Table 1 here

Impregnation of Douglas fir in AHP solutions for 6 h, 12 h, 18 h and 38 h were investigated with variable amounts of H₂O₂, NaOH and Na₂CO₃ (Table 1). Their effects on lignin removal, bleaching and enzymatic hydrolysability of AHP pretreated softwood were subsequently determined (Table 1, Supplementary Fig. 2 and 3, respectively). It was interesting to find that neither the amount of H₂O₂, NaOH and Na₂CO₃ nor the length of impregnation had resulted in any statistically significant lignin removal (ANOVA $p < 0.05$) between treatments (Table 1). However, the addition of H₂O₂ (treatments A and C) clearly led to a bleaching effect on substrates (Supplement Fig. 2). However, this bleaching effect was not due to the lignin removal from Douglas fir, but a consequence of the reduction of lignin chromophore structures instead. Using H₂O₂ as a brightening agent to destroy or modify chromophore structures in pulp and paper products is a conventional practice [7].

Furthermore, it was found that bleached samples did not show any significant improvement on enzymatic hydrolysability (Supplementary Fig. 3). The cellulose-to-glucose conversion yields of treated Douglas fir resulted in non-statistically significant differences for the three tested impregnation treatments (A,B,C), (ANOVA $p < 0.05$).

Next, we investigated the effect of changing H_2O_2 loadings (from $w_{H_2O_2} = 0$ to $w_{H_2O_2} = 0.20$) under a constant alkaline loading during pretreatment at 180 °C for 30 min. The alkaline loading was selected based on the amounts of NaOH ($w_{NaOH} = 0.25$) and Na_2CO_3 ($w_{Na_2CO_3} = 0.47$) that are required to reach a pH of 11.6 for an aqueous solution with peroxide loading of $w_{H_2O_2} = 0.10$ (based on biomass). As shown in Fig. 4A, the addition of H_2O_2 in pretreatment has a clear benefit toward improving substrate hydrolysability.

However further increasing H_2O_2 from $w_{H_2O_2} = 0.04$ to $w_{H_2O_2} = 0.10$, and $w_{H_2O_2} = 0.20$ only shows a marginal improvement on glucose yield. This appears to contradict the results shown in Fig. 3 where $w_{H_2O_2} = 0.10$ pretreated Douglas fir samples had a considerably higher hydrolysability than those obtained from $w_{H_2O_2} = 0.04$. The difference is from the alkaline charge, the hydrolysis curve of $w_{H_2O_2} = 0.04$ in Fig. 4A was obtained using a significantly higher alkaline charge ($w_{NaOH} = 0.25$; $w_{Na_2CO_3} = 0.47$) than that used for $w_{H_2O_2} = 0.04$ treatment in Fig. 3 ($w_{NaOH} = 0.11$; $w_{Na_2CO_3} = 0.19$). This suggests that substrate hydrolysability can be improved by raising the alkaline charge and the reaction pH. In a similar manner, the hydrolysis curve just with the twice the amount of peroxide loading (from $w_{H_2O_2} = 0.10$ to $w_{H_2O_2} = 0.20$) and the same alkaline charge does not result on any significant improvement in enzymatic hydrolysability. The starting pH of the $w_{H_2O_2} = 0.20$ curve on Fig. 4A was below pH 11.0 and resulted even detrimental for the process. This

demonstrates a synergistic effect between H_2O_2 and alkaline (reaction pH) necessary during AHP pretreatment of biomass.

Fig. 4 here

Another set of experiments was conducted using a fixed H_2O_2 loading of $w_{\text{H}_2\text{O}_2} = 0.10$, with varying amounts of NaOH ranging from $w_{\text{NaOH}} = 0.11$ to 0.25 and 0.5 (mass fraction) (Fig. 4B). At the low NaOH loading ($w_{\text{NaOH}} = 0.11$), the cellulose-to-glucose yield of pretreated Douglas fir remains below 30 % (reaction pH was below 11.6). Increasing NaOH loading to $w_{\text{NaOH}} = 0.25$ led to an enzymatic cellulose-to-glucose yield of 73 % (reaction pH around 11.6). As shown in Fig. 4B, increasing alkaline dosage to $w_{\text{NaOH}} = 0.5$ led to 95 % cellulose-to-glucose conversion yield after 96 h hydrolysis with a Ctec2 cellulose loading of 20 FPU/g. The $w_{\text{NaOH}} = 0.50$ loading supplied excessive alkali and kept the solution pH slightly higher than 11.9. The additional alkaline species can cause H_2O_2 decomposition to produce hydroxyl radicals (HO^*) which are recognized to promote lignin and hemicellulose deconstruction [7]. In fact, the lowest lignin (21.8 %) and glucomannan (1.7 %) contents among all tested conditions were obtained after AHP pretreatment at $w_{\text{NaOH}} = 0.50$ and $w_{\text{H}_2\text{O}_2} = 0.10$ loading (180 °C, 30 min). Hemicellulose and lignin degradation during AHP contributed to the higher cellulose-to-glucose yield. To further understand the effect of NaOH on substrate hydrolysability, pretreatment was then conducted at 180 °C for 30 min with $w_{\text{NaOH}} = 0.50$ loading without H_2O_2 . The cellulose-to-glucose yield of the ensuing substrate reached 83 % after 96 h hydrolysis (Supplementary Fig. 4). Based on these data, it is apparent that both H_2O_2 and NaOH contributed to substrate hydrolysability. Balancing the dosages of these two chemicals can achieve an

efficient enzymatic hydrolysability of AHP pretreated softwood at low peroxide loadings which can lead to an economically viable AHP pretreatment process for fermentable sugar production.

4. Conclusion

This study has shown that soaking Douglas fir in alkaline hydrogen peroxide solution at room temperature caused biomass bleaching, but it did not promote delignification nor improve hydrolysability. While the presence of H_2O_2 is essential for AHP treatment of softwood, maintaining a high alkaline loading and a pH stays above 11.6 are more effective than increasing hydrogen peroxide loading to achieve a good substrate hydrolysability.

Substrate with 95 % cellulose-to-glucose yield was obtained from AHP treatment of Douglas fir at 180 °C and 30 min using a low peroxide dosage (at $w_{H_2O_2} = 0.10$). Balancing alkaline and peroxide loadings is a key to optimizing AHP pretreatment of softwood to decrease cellulose recalcitrance while minimizing chemicals cost. We also demonstrated that during AHP pretreatment, softwood glucomannan was transformed to a hydrogel-like material which reduced substrate hydrolysability by non-productive binding to cellulases representing a major recalcitrance factor.

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Figure captions

Fig. 1 - Cellulose-to-glucose yields as a function of AHP pretreatment time at different H_2O_2 loadings and reaction temperatures: (A) 180 °C, (B) 200 °C.

Fig. 2 - Enzymatic hydrolysis of AHP pretreated Douglas fir samples at variable enzyme loadings: (A) Samples FS01 at $w_{\text{H}_2\text{O}_2} = 0.04$ with variable Ctec2 and 1.2 % hemicellulolytic enzymes loading, (B) Effect of hemicellulolytic enzyme addition on hemicellulose solubilization.

Fig. 3 - Effect of H_2O_2 loading ($w_{\text{H}_2\text{O}_2} = 0.04$ and $w_{\text{H}_2\text{O}_2} = 0.10$) on the enzymatic cellulose-to-glucose yield of pretreated Douglas fir.

Fig. 4 - Effect of chemical loadings on the enzymatic cellulose-to-glucose yield of pretreated Douglas fir at constant soaking (18 h), temperature (180 °C), and reaction time (30 min): A) Variable H_2O_2 loading, B) Variable alkaline loading (without Na_2CO_3).

Table 1 - Effect of impregnation times and chemical loadings on Douglas fir delignification

Label	Chemical loading (mass fraction)	Soaking Time (h)	Klason lignin (% mass fraction)
A	$w_{\text{H}_2\text{O}_2} = 0.10,$	6	31.9 ± 0.9
	$w_{\text{NaOH}} = 0.25,$	12	30.4 ± 0.8
	$w_{\text{Na}_2\text{CO}_3} = 0.47$	18	29.4 ± 0.5
		38	28.6 ± 0.7
B	$w_{\text{H}_2\text{O}_2} = 0,$	6	29.7 ± 0.8
	$w_{\text{NaOH}} = 0.25,$	12	29.6 ± 0.8
	$w_{\text{Na}_2\text{CO}_3} = 0.47$	18	29.2 ± 0.9
		38	29.1 ± 0.8
C	$w_{\text{H}_2\text{O}_2} = 0.10,$	6	29.0 ± 0.9
	$w_{\text{NaOH}} = 0.25,$	12	28.7 ± 0.9
	$w_{\text{Na}_2\text{CO}_3} = 0$	18	28.0 ± 1.0
		38	28.2 ± 1.0

1 $w_{\text{H}_2\text{O}_2} : 0$ ■, 0.01 ■, 0.02 ■, 0.04 ■

2 **Fig. 1**

3

1 **Fig. 2**

2

FS01($w_{H_2O_2} = 0.04$) -♦-, FS10($w_{H_2O_2} = 0.04$) ▲, FS01($w_{H_2O_2} = 0.10$) ■, FS10($w_{H_2O_2} = 0.10$) ×

Fig. 3

A)

B)

1 **Fig. 4**

2