1 **Comparison of Chemical Treatment Methods**
2 **for Loblolly Pine to Utilize as Enzyme**
3 **Hydrolyzate Feedstock[#]**

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42 **Abstract**

43 The ground pine wood chips (GPWC) were examined through chemical treatment
44 methods employing alkaline hydrogen peroxide treatment (AHP, consisted of 50 g L⁻¹
45 potassium hydroxide, and 50 g L⁻¹ hydrogen peroxide with added water at 78 °C for 24
46 h), and ethyl-hydro-oxides treatment (EHOs, consisted of 60% ethanol (of total solution),
47 50 g L⁻¹ potassium hydroxide, and 50 g L⁻¹ hydrogen peroxide with added water at 78 °C
48 for 24 h). The effects of chemical treatments on GPWC were examined through
49 combination of chemical composition followed by enzymatic hydrolysis (using
50 Accellerase® 1500). The application of EHOs treatment removes the foaming issue that
51 currently occurs due to AHP treatment at the elevated temperature. A removal of the
52 foaming phenomenon increased the enzymatic glucan conversion (>70%) which is
53 important in increasing the efficiency of biorefinery. The interaction of different variables
54 in EHOs solvent formulations including ethanol concentration (0-60%), potassium
55 hydroxide concentration (0-50 g L⁻¹), hydrogen peroxide concentration (0-50 g L⁻¹), and
56 processing temperature (38 °-78 °C) were analyzed through four variables response
57 surface methodology using glucan conversion and total polysaccharide conversion as
58 responses. The glucan conversion and total polysaccharide conversion were analyzed to
59 determine the specific and overall effects of Accellerase® 1500 on the amorphous
60 polysaccharide contents of GPWC resulted through EHOs treatment formulations,
61 respectively. The implications of all these findings are discussed with the available
62 literature.

63 **Keywords**

64 Pine wood chips; alkaline hydrogen peroxide (AHP) treatment; ethyl-hydro-oxides
65 (EHOs) treatment; enzymatic saccharification; Accellerase® 1500; four variable response
66 surface methodology.

67 **Abbreviations**

68 GPWC: Ground pine wood chips
69 AHP: Alkaline hydrogen peroxide
70 EHOs: Ethyl-hydro-oxides

71 **1. Introduction**

72 Softwood such as loblolly pine (*Pinus taeda*) is a fast-growing tree. Loblolly pine can
73 reach a height of 18-30 m with a diameter of 0.3 to 1.5 m between 30-60 years.
74 Softwoods such as pine and spruce consist of 42-45 g of cellulose, 17-22 g of
75 hemicellulose, and 24-32 g of lignin per 100 g of biomass (dry basis) [1-6]. The high
76 polysaccharides content of loblolly pine (approx. 63 g per 100 g of dried biomass) is
77 crucial for increasing both transportation and development of bio-product efficiencies.
78 The conversion of pine wood chips into bio-products is an energy intensive process due
79 to a tight binding of polysaccharides matrix with lignin content. To enhance the release of
80 polysaccharide contents of softwood, both physical and thermochemical treatments are
81 required. A physical treatment involves the size reduction of particles, thereby increasing
82 the surface area per unit volume. Pine wood chips require 1.7-3.8 times more energy
83 compared to corn stover for the same size reduction [7]. The milling energy of pine wood
84 chips increases with the decreasing particle sizes [7,8]. Whereas, thermochemical
85 treatments are required to convert crystalline content to amorphous content with removal
86 of lignin. Several different thermochemical treatments employing steam [2], sulfuric acid

90 based reagents (such as Kraft [4], organosolv [3, 9,10], and alkaline sulfite-
 91 anthraquinone-methanol (ASAM) [4]), alkaline hydroxide [5], and alkaline hydrogen
 92 peroxide (AHP) [6] have been reported for softwood conversion. The effects of
 93 thermochemical treatments on structural-chemical properties of softwoods can be
 94 measured through X-ray diffraction, scattering electron microscopy, kappa number, and
 95 enzyme digestibility [2-6].
 96 Water is the most polar solvent and form hydrogen bonding with cellulosic hydroxyl
 97 groups on interaction. However, the hydrophobic nature of lignin inhibits water molecule
 98 interaction with cellulose. To overcome the physical barrier of lignin in softwood
 99 conversion, steam treatment is utilized at a high temperature of 180 °C for 20 min [2].
 100 Steam treatment at high temperature penetrates lignocellulosic biomass through
 101 formation of a hydronium ion [2]. The steam treatment promotes hemicellulose
 102 degradation and transforms lignin, thereby increasing access to cellulose content [2]. The
 103 application of steam treatment on softwood (particle size: 0.25-0.42 mm) completely
 104 removes xylan content and decreases 22.5% of phenolic compounds without affecting its
 105 cellulose content [2]. During enzyme hydrolysis, steam treated softwood converts up to
 106 4% and 12.5% of total polysaccharide into fermentable sugars at substrate loadings of 50
 107 g L⁻¹ and 200 g L⁻¹, respectively [2]. Sulfuric acid based reactants along with
 108 anthraquinone, organic solvents (such as ethanol and methanol) and alkaline hydroxide
 109 have been used with water to enhance softwood conversion [3-4, 9]. The paper pulp
 110 industry utilizes sulfuric acid based reactants on softwood (size: particle (less than 5 mm)
 111 to chip (20 mm x 2 mm x 2 mm)) at temperature ranges of 160 °-180 °C for 1-3 h [3-4].
 112 Compared to the conventional Kraft treatment, a significant improvement in both pulp
 113 brightness and yield have been reported with the application of ASAM treatment [4].
 114 However, the use of carcinogenic anthraquinone in ASAM treatment formulation set back
 115 the process and its usage at commercial scale production [11]. Organic solvents (such as
 116 ethanol and methanol) are effective in the delignification of lignocellulosic biomass.
 117 Mantanis [12] has reported that the activation energy of softwood swelling (using spruce
 118 and Douglas-fir) ranges from 32.2- 38.9.0 KJ mol⁻¹ and 57.1- 60.4 KJ mol⁻¹ at the
 119 temperature of 23 °-100 °C using water and ethanol as solvents, respectively. The
 120 addition of -CH₃ group causes a subsequent increase in the activation energy of wood
 121 using ethanol compared to water [12]. The maximum swelling of wood in the aqueous
 122 organic solvent is accompanied by solvent basicity, molar volume, and hydrogen bonding
 123 capability [12]. In organosolv treatment, use of ethanol (50-80% of solution) along with
 124 mineral catalysts such as H₂SO₄ (1.0-1.1 g per g of biomass) at elevated temperature (160
 125 °-200 °C) increases both delignification and enzymatic saccharification of softwood [10].
 126 The application of organosolv treatment (65% ethanol of total solution and 1.1 g H₂SO₄
 127 per g of biomass; processing temperature = 170 °C for 1 h) on softwood (particle size:
 128 less than 5 mm) removes 61.2% of phenolic compounds with 79% of glucose recovery,
 129 and increases enzymatic glucan conversion up to 70% [3]. Organosolv treatment involves
 130 the formation of hydro ion [H₃O⁺] similar to the acid treatment [10]. The generated lignin
 131 through organosolv treatment has a potential market value in the development of carbon
 132 fibers [10, 13]. The use of organosolv treatment is limited in the biofuel industry due to a
 133 high price of solvent, high temperature processing, and an expensive infrastructure
 134 development. Moreover, the processing temperature at above 170 °C produces furfural
 135 and hydroxymethyl furfural [3]. The production of these inhibitors affect both a
 136 fermentation step (due to low conversion of sugars into ethanol) and a downstream
 137 processing step (due to low productivity).

On the contrary to high temperature chemical treatment processes, moderate temperature treatment processes using alkaline hydroxide [5, 6] and AHP [6] have also been reported for softwood conversion. Using alkaline hydroxide treatment (0.075 g of NaOH per g of biomass; processing temperature = 80 °-100 °C for 2 h) on softwood (particle size: less than 0.8 mm) had no significant effect on the delignification, and the enzymatic glucan conversion stayed below 25% [5]. Furthermore, the change in processing conditions of alkaline hydroxide treatment (80 g L⁻¹ of NaOH, equivalent to 0.8 g NaOH per g of biomass; processing temperature = 70 °C for 20-21 h) on softwood (particle size: 0.125- 0.50 mm), removed up to 25% of the phenolic compounds and the enzymatic glucan conversion stayed up to 37.5% [6]. However, the use of AHP treatment (80 g L⁻¹ of NaOH (equivalent to 0.8 g NaOH per g of biomass) and 204 g L⁻¹ of H₂O₂ (equivalent to 2.04 g H₂O₂ per g of biomass); processing temperature = 70 °C for 20-21 h) on softwood (particle size: 0.125- 0.50 mm), increased the delignification efficiency up to 60% with the enzymatic glucan conversion up to 60% [6]. Using herbaceous feedstock, AHP treatment can increase the enzymatic saccharification efficiency up to 90% [14]. In alkaline condition, hydrogen peroxide produces hydroxyl radicals (OH[•]), superoxide anions (O₂⁻), hydroperoxide anions (HO₂⁻), solvated electrons (aq. e⁻) and atmospheric oxygen (O₂) [15]. The generated hydrogen peroxide reactive species play a

critical role in the delignification of lignocellulosic feedstock, thereby increasing subsequent enzymatic saccharification [14, 19]. The scope of AHP treatment is limited to certain feedstock due to crystallinity, volatility of reaction with the increasing temperature, and safety concerns with a scale-up of the process [6, 19-20]. AHP treatment can be performed using potassium hydroxide (KOH), sodium hydroxide (NaOH), calcium hydroxide (Ca(OH)₂), and ammonium hydroxide (NH₄OH). The efficacy of NH₄OH with H₂O₂ decreases with the increasing crystallinity of lignocellulosic biomass [19]. KOH has both environmental and health benefits compared to NaOH. The recovery of potassium ion (K⁺) in the form of potassium chloride (KCl) and potassium citrate salts have applications in fertilizer, food and beverages, and pharmaceutical products development [21-22].

The objective of the present study was to compare the effect of solvent polarities of EHOs treatment (60% ethanol + 50 g L⁻¹ KOH + 50 g L⁻¹ H₂O₂ with added deionized water), and AHP treatment (50 g L⁻¹ KOH + 50 g L⁻¹ H₂O₂ with added deionized water) using GPWC. The generated pulps were analyzed through chemical composition and enzymatic saccharification efficiency. Furthermore, the four variables response surface methodology was examined to understand the effect of temperature, ethanol, KOH, and H₂O₂ in the enzymatic saccharification of GPWC.

2. Materials and Methods

2.1. Pine Wood Chips Preparation

Pine woods chips were received from Arbogen (Ridgeville, SC). The received pine wood chips were oven dried for 6 days at 40 °C. The moisture content of 10.5 ± 0.6% of total biomass was measured. The dried chips (2 cm x 2.5 cm x 0.75 cm) were reduced to 0.5-1.0 mm size particles using a laboratory Wiley mill (Thomas Model 4 Wiley[®] Mill). The moisture content of the sieved GPWC was 0.1 ± 0.0% before further analyses.

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2.2. Chemical Treatments

Chemical treatments formulation consisted of hydrogen peroxide (350 g L⁻¹ H₂O₂, BDH), potassium hydroxide (85% KOH purity, Alfa Aesar), ethanol, and deionized water. For chemical treatments, screw-capped 1 L media glass bottles (Pyrex) and a Parr reactor (Model 4553) were used similar to the previous research [19]. Initially, 8.07 ± 0.03 g of GPWC was added with AHP and EHOs solvents in the ratio of 1:10 (w/v). AHP treatment formulation consisted of 4g of KOH (equivalent to 50 g L⁻¹ and 0.50 g per g of biomass), 11.4 mL of H₂O₂ (equivalent to 50 g L⁻¹ and 0.50 g per g of biomass) and 64.6 mL of deionized water. Whereas, EHOs treatment formulation consisted of 48 mL of ethanol (equivalent to 60% of total solution), 4 g of KOH (equivalent to 50 g L⁻¹ and 0.50 g per g of biomass), 11.4 mL of H₂O₂ (equivalent to 50 g L⁻¹ and 0.50 g per g of biomass), and 16.6 mL of deionized water. Both, AHP and EHOs treatments were carried out at 78 °C for 24 h. After chemical treatments, the three consecutive hot water washes were performed. Each wash was corresponded to 200 mL of liquid. The chemically treated GPWC were retained using 0.15-0.18 mm sieve. At the end of the washing step, the retained biomass was squeezed (using a lemon squeezer) to remove excessive water. The obtained samples were used for chemical composition and enzymatic saccharification analyses. AHP and EHOs treatments were performed in five replicates such that two replicates for mass balance (including moisture content and chemical composition) and three replicates for enzymatic saccharification analyses.

2.3. Analytical Methods

National renewable energy laboratory (NREL) protocols were followed for the determination of carbohydrates, acid-insoluble and soluble lignin, ash, and extractive contents of GPWC [23-25]. The chemical composition of AHP and EHOs-treated GPWC were analyzed using HPLC (Shimadzu SIL-10AF, Palo Alto, CA) equipped with 355 RID (refractory index detector) and Bio-Rad Aminex 87 P column (operated at 85 °C), using water as a mobile phase. The enzymatic saccharification process, response surface methodology (both chemical composition and enzymatic saccharification), and ethanol concentration (after EHOs treatment), were analyzed using HPLC equipped with 355 RID and Bio-Rad Aminex 87 H column (operated at 65 °C), using 0.01 N H₂SO₄ as a mobile phase. The moisture content of GPWC was determined by oven-drying method at 105 °C to a constant weight. Cellulase enzyme activity (reported in FPU mL⁻¹) was determined [26] using BioTeK® plate reader (BioTeK®, Model Synergy HT) at 540 nm.

2.4. Response Surface Methodology

The effects of variables such as ethanol content (%), KOH content (g L⁻¹), H₂O₂ (g L⁻¹), and operating temperature (°C), on EHOs treatment formulation were studied through the four variables response surface methodology. A response surface methodology can be used to find a combination of levels or settings for four factors that affects GPWC conversion (resulted through a combined chemical composition and enzymatic saccharification analyses). A central composite design of the four factors (ethanol, KOH, H₂O₂, and temperature) was used to create the data set for the response surface analysis. The central composite design with orthogonal coordinates defines the combinations of the four factors that are utilized in the present study. In this study, ethanol, KOH, H₂O₂, and temperature had five levels (Table1). The levels of ethanol, KOH, H₂O₂, and temperature were selected based on the factors EHOs treatments was developed as

described in the results and discussion section. Using all possible combinations of these factor levels would require 5^4 or 625 combinations. However, a central composite design requires only $2k(k-1) + C_0$ combinations, where k is the number of factors (4) and C_0 is the number of “center” combinations (all four factors set at the middle level). In this study, with four factors and four center combinations, only 28 combinations were required (Table 1). GPWC of 8 g was utilized with each sample preparation (Table 1). The ratio of sample to solvent was 1:10 (w/v). Each treatment was performed in five replicates such that three replicates for composition and two replicates for enzyme hydrolysis analyses.

Table 1 - Central composite design for EHOs treatment analysis.

2.5. Enzyme Hydrolysis

2.5.1. AHP and EHOs-Treated GPWC

Both AHP-treated and EHOs-treated GPWC were analyzed for the enzymatic saccharification using Accellerase® 1500. In 250 mL stopper conical flasks, AHP-treated GPWC (6.2 ± 0.3 g (dry basis) with moisture content of $70.8 \pm 0.6\%$ of total wet biomass) and EHOs-treated GPWC (5.9 ± 0.2 g (dry basis) with moisture content of $74.5 \pm 0.5\%$ of total wet biomass) were added with 80 mL of 0.1 M citric acid buffer (pH 4.8), 80.2 FPU (filter paper unit of Accellerase® 1500) per gram of biomass [27], along with 2.0 mL of sodium azide buffer solution (stock solution concentration = 20 g L^{-1}). The flasks were incubated at 50°C in a floor shaker incubator (C25KC, New Brunswick Scientific) at 250 revolutions per minute. Sample volumes of 1 mL were taken from the flasks at 0, 24, 48, and 72 h of analyses. All samples were analyzed in three replicates.

2.5.2. Response Surface Methodology

For the response surface study, chemically treated samples were added with 40.1 FPU per gram of biomass, 1 mL of sodium azide buffer solution, followed by the addition of 75 mL of 0.1 M citric acid buffer (pH 4.8). The sample flasks were incubated in the floor shaker incubator at 50°C for 72 h with continuous stirring at 250 revolutions per minute. Samples volume of 1 mL were taken from the flasks at 0 and 72 h of analyses. All samples were analyzed in two replicates.

2.6. Sugars Conversion

The polysaccharide contents of untreated, EHOs-treated, and AHP-treated GPWC were analyzed using glucose, xylose, mannose, galactose and arabinose as standard sugars. Aminex-87 P column was utilized for the analysis. These sugars can be correlated to the polysaccharide contents of GPWC using the following relations.

Glucan = Glucose produced (g) $\times$ 0.9 (hydration factor)

Xylan = Xylose produced (g) $\times$ 0.88 (hydration factor)

Arabinan = Arabinose produced (g) $\times$ 0.88 (hydration factor)

Galactan = Galactose produced (g) $\times$ 0.9 (hydration factor)

Mannan = Mannose produced (g) $\times$ 0.9 (hydration factor)

The enzymatic saccharification and response surface analyses (both chemical composition followed by enzymatic saccharification) were analyzed using Aminex-87 H column. It was observed that the enzyme concentration affects the analysis using Aminex-87 P column (water as a mobile phase) due to increase in the operating pressure

280 of column. To eliminate the pressure effect, Aminex-87 H column was utilized with 0.01
 281 N H₂SO₄ as a mobile phase. The similar retention times of xylose, mannose, and
 282 galactose were observed using 87-H column. Xylose-mannose-galactose concentration
 283 was analyzed using xylose as a standard. For chemical composition determination (for
 284 response surface methodology), xylose-mannose-galactose conversion factor of 0.88 was
 285 used for xylan-mannan-galactan content of GPWC. The enzymatic saccharification
 286 efficiencies such as glucan conversion, xylan-mannan-galactan conversion, and total
 287 polysaccharide conversion, were calculated using the following equations
 288

$$289 \quad \text{Glucan conversion (\%)} = \frac{(\text{Glucose concentration (g L}^{-1}\text{)}) \times \text{Liquid volume (L)} \times 0.9}{\text{Glucan content (g) of treated GPWC}} \times 100\%$$

$$290 \quad \text{Xylan-Mannan-Galactan conversion (\%)} = \frac{\left[\frac{(\text{Xylose} - \text{Mannose} - \text{Galactose concentration (g L}^{-1}\text{)})}{\times \text{Liquid volume (L)} \times 0.88} \right]}{\text{Xylan} - \text{Mannan} - \text{Galactan content (g) of treated GPWC}} \times 100\%$$

$$292 \quad \text{Total polysaccharide conversion (\%)} = \frac{\left[\begin{array}{l} (\text{Glucan conversion} \times \text{Glucan content}) + \\ (\text{Xylan} - \text{Mannan} - \text{Galactan conversion} \\ \times \text{Xylan} - \text{Mannan} - \text{Galactan content}) \end{array} \right]}{(\text{Glucan} + \text{Xylan} - \text{Mannan} - \text{Galactan}) \text{ contents (g) of treated GPWC}} \times 100\%$$

294
 295 Where, glucan, and xylan-mannan-galactan represented the available polysaccharide
 296 content during the enzyme hydrolysis of chemically treated GPWC.

297 2.7. Ethanol Recovery

298 Ethanol content in EHOs treatment (conditions: reactor volume = 3.85 L; solid loading
 299 = 216 g; solvent loading = 2.16 L consisted of 60% ethanol + 50 g L⁻¹ KOH + 50 g L⁻¹
 300 H₂O₂, with volume adjusted using water; processing temperature = 78 °C; processing time
 301 = 24 h) was measured before and after the process using aqueous ethanol standards. After
 302 the treatment, the reactor vessel was cooled down to 60 °C using a water-ice bucket and
 303 then released the pressure of the vessel. Before taking the final sample of the generated
 304 solvent mixture, the reactor vessel was cooled down to the room temperature (25 °C).
 305 The pH of the solvent mixture was adjusted to six (using anhydrous citric acid) before
 306 ethanol content analysis. Internal standard of 60% aqueous ethanol was used as a
 307 reference. Ethanol recovery was defined by the following relationship.

$$308 \quad \text{Ethanol recovery (\%)} = \frac{\text{Final concentration of ethanol after EHOs treatment}}{\text{Initial concentration of ethanol before EHOs treatment}} \times 100\%$$

310 The final concentration of ethanol was analyzed with the external standard of diluted
 311 ethanol concentration with the moisture content of GPWC.

313 2.8. Statistical Analysis

314 Statistical analyses were performed using JMP® Pro software (Windows 10; version
 315 12). Paired *t*-tests were applied for the comparative analysis of enzyme hydrolysis of
 316 EHOs-treated and AHP-treated GPWC at 0.05 significant level. The interaction of
 317 variables (temperature, ethanol, KOH and H₂O₂) were examined using the four variables
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response surface methodology with a second degree factorial analysis. The response surface methodology analyzes a design of statistical experiment, checks the adequacy of the model, estimates the regression coefficients, and predicts the optimal responses. The statistical significance of the model coefficients was determined by the analysis of the variance (ANOVA) combined with the application of Fisher's *F* test. Statistical analyses of responses were included with glucan conversion, and total polysaccharide conversion. Average value was reported with a standard error.

3. Results and Discussion

3.1. Effect of Chemical Treatments on Enzymatic Digestibility of GPWC

The effects of chemical treatments on GPWC were examined through a combination of chemical composition and enzymatic saccharification processes (Table 2 and Fig. 1). At 78 °C, the delignification efficiency/biomass recovery of 43/73% and 49/77% were measured for AHP-treated and EHOs-treated GPWC, respectively (Table 2). A significant difference was found between the enzymatic saccharification efficiencies of AHP-treated and EHOs-treated GPWC (Fig. 1). The enzymatic saccharification of AHP-treated GPWC was resulted in $61.84 \pm 1.08\%$, $31.13 \pm 2.14\%$, and $54.73 \pm 0.57\%$ through conversions of glucan, xylan-mannan-galactan, and total polysaccharide, respectively. Whereas, the enzymatic saccharification of EHOs-treated GPWC was resulted in $75.18 \pm 0.85\%$, $33.28 \pm 2.34\%$, and $66.07 \pm 0.46\%$ through conversions of glucan, xylan-mannan-galactan, and total polysaccharide, respectively. The combined xylose-mannose-galactose yields of $3.86 \pm 0.27 \text{ g L}^{-1}$ and $3.64 \pm 0.26 \text{ g L}^{-1}$ were measured for AHP-treated and EHOs-treated GPWC, respectively.

Table 2 - Effect of chemical treatments on GPWC.

Fig. 1 - Enzyme hydrolysis of AHP-treated and EHOs-treated GPWC using Accellerase® 1500 with the resulted sugar yields and their conversions.

In the present study, the concentration of reactants in AHP and EHOs treatments for GPWC were selected based on the previous study on the comparative crystallinity of softwood with sugarcane bagasse [2]. The difference in the crystallinity of untreated softwood (83.8%) compared to untreated sugarcane bagasse (71.8%) indicated the usage of a higher concentration of alkaline hydroxide in the chemical treatment process [2, 6]. For that reason, approximately two fold increased in concentration of KOH in both AHP and EHOs treatments was selected based on the available literature on alkali treatment using sugarcane bagasse [28-29]. The concentration of H_2O_2 in AHP and EHOs treatments was based on the dry weight of GPWC as described by Gould [14] and was selected equivalent to KOH concentration. The ethanol concentration (60% of the total solution) in EHOs treatment was selected based on lignin solubility [30], minimum water usage, and for cellulose-hemicellulose retention [31]. The temperature of 78 °C was selected for both chemical treatments (AHP and EHOs) to achieve moderate activation energy for solvents [12], applicability of solvent mixtures, and to compare analysis of the processes. The processing time of 24 h was selected for both AHP and EHOs treatments based on the previous conducted studies using alkaline hydroxide and AHP treatments [5-6, 14, 19].

The efficiency of chemical treatment method depends on solvent polarity, processing temperature, and time. The solvent polarity assists with solubilization of phenolic

compounds (by extraction), thereby reducing the crystallinity of cellulose and hemicellulose contents of GPWC. A similar phenomenon was examined using AHP and EHOs treatments. The structural-chemical composition of GPWC using a sulfuric acid hydrolysis [25] indicated the quantitative effect of the applied chemical treatments. The sulfuric acid hydrolysis analyzed the chemical composition without distinguishing crystalline and amorphous contents of GPWC resulting through the applied chemical treatments. The chemical composition analysis along with the enzymatic saccharification analysis represented both qualitative and quantitative effects of accessible polysaccharide contents of GPWC. Moreover, the enzymatic saccharification using Accellerase® 1500 mainly hydrolyzes an amorphous glucan content of GPWC [6]. The enzymatic conversions of glucan and total polysaccharide of GPWC represented the specific and overall effect of Accellerase® 1500, respectively. The enzymatic conversion of glucan up to 70% is considered a bench-mark for any process to be commercialized with the economics of the process (such as safety of the process, operating temperature, operating time, biomass conversion efficiency, production of multiple valuable by-products, integration of different unit operations, cost of chemicals, and initial capital investment). The enzymatic glucan conversion efficiency up to 70% had been reported using soaking in aqueous ammonia treatment (ammonium hydroxide concentration = 150-200 g L⁻¹; operating temperature = 60 °-80 °C for 12-24 h) using herbaceous crops [32-35] and currently in commercialization phase for cellulosic ethanol production (leading by Danisco-DuPont). Jain and co-researchers [36] have described the economics of EHOs treatment (25 g L⁻¹ KOH (0.27 g of KOH per g of dried biomass), 25 g L⁻¹ H₂O₂ (0.27 g of H₂O₂ per g of dried biomass), 250 g L⁻¹ water and 525 g L⁻¹ ethanol (70% of the total volume of solvent) at 75 °C for 1 h) compared to soaking in aqueous ammonia treatment (150 g L⁻¹ NH₄OH solution (1.6 g NH₄OH per g of dried biomass) at 75 °C for 15 h) using sugarcane bagasse in terms of reactor loadings, enzyme saccharification at high substrate loadings, electricity production (through generated pressure), development of desalination process (through development of a self-sustainable integrated biorefinery), and anticancer drug development (using lignin fraction).

3.2. Effect of EHOs treatment on GPWC

Based on the achieved results using EHOs treatment of GPWC, the optimization of variables (temperature, ethanol concentration, KOH concentration, and H₂O₂ concentration) were considered within the range that described the effect of the initial selected parameters with the maximum glucan conversion (> 70%) as shown in table 1. Simple statistics of the response surface method using conversions of glucan and total polysaccharide of GPWC as responses are shown in Table 3. The similar R-square values (of 0.96) were examined between the experimental and model based values for conversions of glucan and total polysaccharide of GPWC (Fig. 2). The model based values were estimated from the interaction of temperature, ethanol, KOH and H₂O₂ during the enzymatic saccharification of chemically treated GPWC. The model based values were calculated using the following relationships.

Glucan conversion (%)

$$\begin{aligned}
 &= 54.29 + 1.62 \times (\text{Temp}) + 17.50 \times (\text{KOH}) + 3.37 \times (\text{H}_2\text{O}_2) \\
 &\quad + 1.72 \times (\text{Temp}) \times (\text{H}_2\text{O}_2) + 3.21 \times (\text{KOH}) \times (\text{H}_2\text{O}_2) \\
 &\quad - 4.50 \times (\text{Temp}) \times (\text{Temp}) - 4.43 \times (\text{Ethanol}) \times (\text{Ethanol}) \\
 &\quad - 6.06 \times (\text{KOH}) \times (\text{KOH}) - 6.55 \times (\text{H}_2\text{O}_2) \times (\text{H}_2\text{O}_2)
 \end{aligned}$$

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Total polysaccharide conversion (%)

$$= 41.59 + 2.30 \times (\text{Temp}) + 13.96 \times (\text{KOH}) + 2.74 \times (\text{H}_2\text{O}_2) \\ + 2.51 \times (\text{Temp}) \times (\text{KOH}) + 1.61 \times (\text{Temp}) \times (\text{H}_2\text{O}_2) + 2.69 \times \\ (\text{KOH}) \times (\text{H}_2\text{O}_2) - 3.28 \times (\text{Temp}) \times (\text{Temp}) - 3.35 \times (\text{Ethanol}) \times \\ (\text{Ethanol}) - 4.20 \times (\text{KOH}) \times (\text{KOH}) - 4.48 \times (\text{H}_2\text{O}_2) \times (\text{H}_2\text{O}_2)$$

Table 3 - Analysis of variance (ANOVA) for the quadratic model resulted from glucan conversion (%) and total polysaccharide conversion (%) of GPWC.

Fig. 2 - Analysis of enzymatic saccharification efficiencies of actual values vs. predicted values for conversions of glucan and total polysaccharide of GPWC using Accellerase® 1500.

To understand the interaction of different variables in EHOs treatment formulation, the center points of temperature (48 °, 68 °C), ethanol (15, 45%), KOH (12.5, 37.5 g L⁻¹) and H₂O₂ (12.5, 37.5 g L⁻¹) were analyzed through conversions of glucan and total polysaccharide of GPWC (Figs. 3 and 4a-b). In EHOs treatment formulation, the concentration of KOH (> 37.5 g L⁻¹) had the most significant effect in GPWC conversion (Figs. 3, 4a-b, and 5). In softwood, cellulose mainly occurs in the polymorph form of cellulose I (crystalline) and inaccessible in the native form during enzymatic saccharification [37]. Cellulose I can be converted to cellulose II (amorphous) through regeneration and mercerization processes using KOH solution that enhances cellulose accessibility [38]. The higher pKa values of KOH (pKa value = 16) [39] compared to cellulosic hydroxyl groups (pKa value = 14) [40] would increase the solubility of GPWC. A pKa value represents the strength of acid solution and is directly proportional to Gibbs energy change [41]. The maximum concentration of KOH in optimization of EHOs treatment was measured at 58.2 g L⁻¹ (Figs. 6 and 7a-b). However, the concentration of KOH in EHOs treatment to be effective for GPWC conversion, a significant concentration of H₂O₂ (> 12.5 g L⁻¹) would be required (Figs. 3 and 5). The mixing of KOH with H₂O₂ increased both pH of the solvent [42] and the molecular dissociation of H₂O₂ (pKa value 11.6) [39]. The dissociation of H₂O₂ is both pH and temperature dependent. Moreover, the dissociation of H₂O₂ (in alkaline pH) increased with the increasing temperature (Fig 3). H₂O₂ have a half shelf life of 1 h (at 80 °C) to 5 h (at 60 °C) [43-44]. The temperature and pH dependency of H₂O₂ would be a potential reason corresponding to the optimal concentration of H₂O₂ and the optimal temperature at 42.7 g L⁻¹ and 75.4 °C, respectively (Figs. 6 and 7a-b).

Fig. 3 - Interaction profiles of temperature, ethanol, KOH, and H₂O₂ during the enzymatic saccharification of GPWC using Accellerase® 1500.

Fig. 4a – Surface profilers for glucan conversion (%) as functions of temperature (at 58 °C), ethanol (at 30%), KOH (at 25 g L⁻¹) and H₂O₂ (at 25 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

Fig. 4b – Surface profilers for total polysaccharide conversion (%) as functions of temperature (at 58 °C), ethanol (at 30%), KOH (at 25 g L⁻¹) and H₂O₂ (at 25 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

463
464 **Fig. 5 - Sorted estimate parameters for glucan conversion (%) and total**
465 **polysaccharide conversion (%) resulted through the interactions of temperature**
466 **(Temp), ethanol (EtOH), KOH, and H₂O₂ during the enzymatic saccharification of**
467 **GPWC.**

468
469 **Fig. 6 - Optimized EHOs treatment conditions resulted through the enzymatic**
470 **saccharification of GPWC using Accellerase® 1500.**

471
472 **Fig. 7a – Optimized surface profilers for glucan conversion (%) as functions of**
473 **temperature (at 64.2 °C), ethanol (at 32.1%), KOH (at 46.6 g L⁻¹) and H₂O₂ (at 34.5**
474 **g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.**

475
476 **Fig. 7b – Optimized surface profilers for total polysaccharide conversion (%) as**
477 **functions of temperature (at 75.4 °C), ethanol (at 35.7%), KOH (at 58.2 g L⁻¹) and**
478 **H₂O₂ (at 42.7 g L⁻¹) during the enzymatic saccharification of GPWC using**
479 **Accellerase® 1500.**

480
481 Performing a Parr reactor study, the exothermic reaction of KOH and H₂O₂ built up
482 the pressure to 11 bar to 15.2 bar at 60 ° to 80 °C, respectively, in the enclosed vessel
483 (conditions: reactor volume = 3.8 L; solid loading = 216 g; solvent loading = 2.16 L
484 consisted of 60% ethanol + 50 g L⁻¹ KOH + 50 g L⁻¹ H₂O₂ with added deionized water).
485 The pressure in the reactor vessel was increased slowly and gradually after 5 minutes of
486 H₂O₂ addition. Under the similar composition of EHOs-solvent using GPWC at 78 °C,
487 the maximum pressure of 1.6-2.1 bar was calculated (using the ideal gas law) in 1L media
488 bottles (sample 8 g; solvent: sample ratio of 10:1(vol/wt)). Under the alkaline and
489 pressurized conditions, concentration of ethanol (pKa value = 15.5) [16] provided the
490 stability to water molecules (pKa value = 14) [16] and maximized the interaction with
491 GPWC [37-38]. The basicity of the solvent mixture increases with the mixing of ethanol
492 and water [45]. In addition, the basicity of H₂O₂ solution also remained dominant at the
493 lower concentration (up to 0.4 mole fraction of H₂O₂) in aqueous ethanol [45]. Different
494 physiochemical properties of ethanol (such as polarity, thermal conductivity, viscosity
495 and surface tension) compared to water have importance in GPWC conversion. The lower
496 polarity of ethanol (dipole moment = 5.64 x 10⁻³⁰ C m, dielectric constant = 25.3 at 20
497 °C, as in EHOs treatment) compared to water (dipole moment = 6.19 x 10⁻³⁰ C m,
498 dielectric constant = 80.1 at 20 °C, as in AHP treatment) could lead to a higher
499 delignification of GPWC [16]. In EHOs treatment, the interaction of GPWC with the
500 solvent occurs through the formation of ethoxide ion and hydroxide ion in the alkaline
501 condition. Whereas, in AHP treatment, the interaction between the solvent and GPWC
502 mainly occurs due to the formation of hydroxyl ions. The movement of ionic species
503 (hydroxyl and ethoxide ions) govern through the conductivity of a solvent. The thermal
504 conductivities of pure water and pure ethanol differ between 0.61 W m⁻¹ K⁻¹ (at 25 °C, 1
505 bar) to 0.66 W m⁻¹ K⁻¹ (at 75 °C, 1 bar), and 0.17 W m⁻¹ K⁻¹ (at 25 °C, 1 bar) to 0.15 W m⁻¹
506 K⁻¹ (at 75 °C, 1 bar), respectively [16]. The lower thermal conductivity of ethanol
507 solvent (as in EHOs treatment) also indicated that the temperature dependency of ionic
508 species mobility would be smaller compared to water solvent (as in AHP treatment). The
509 thermal conductivity of ethanol (in EHOs treatment) would be a potential reason for the
510 slower and gradual rise in pressure after 5 minutes of H₂O₂ addition in the enclosed

vessel. The concentration of ethanol in EHOs treatment not only slowed down the exothermic reaction (due to KOH and H₂O₂) but also provided safety in the process which is critical with the scale-up studies. Furthermore, both viscosity and surface tension of the solvent have a role in the transportation and interaction of ionic species with an inner matrix of GPWC. The viscosities of pure water and pure ethanol differ between 0.89 mPa s (at 25 °C) to 0.38 mPa s (at 75 °C), and 1.07 mPa s (at 25 °C) to 0.48 mPa s (at 75 °C), respectively [16]. Whereas, the surface tensions of pure water and pure ethanol differ between 72.75 (at 20 °C) to 62.67 mN m⁻¹ (at 80 °C), and 22.39 mN m⁻¹ (at 20 °C) to 18.23 mN m⁻¹ (at 70 °C), respectively [46]. In the present study, the use of 30% aqueous ethanol was found effective in the controlled foaming phenomenon during the chemical treatment processes. The controlled foaming is the prerequisite for the scale-up studies from lab to pilot to commercial scale. For 30% aqueous ethanol, the surface tension of the solvent varies in between 25 mN m⁻¹ (at 20 °C) to 27 mN m⁻¹ (at 50 °C) [47]. The optimal concentration of ethanol in EHOs treatment was measured at 35.7% (Fig. 6). The reduction in the surface tension would be correlated to the decrease of hydrogen bonding capability in aqueous ethanol. The reduced surface tension and the increased pressure would enhance both the conversions of glucan and total polysaccharide of GPWC [38, 48-51]. The use 30%-40% of aqueous ethanol had a significant effect on the swelling of cellulose nanostructure that influences the enzymatic saccharification of lignocellulosic biomass [49-50]. Moreover, the increased content of ethanol ($\geq 70\%$) in EHOs-solvent formulation recover potassium ion as a precipitated salt [52].

After EHOs treatment (conditions: reactor volume = 3.8 L; solid loading = 216 g; solvent loading = 2.16 L consisted of 60% ethanol + 50 g L⁻¹ KOH + 50 g L⁻¹ H₂O₂ with added water; treatment time = 24 h; processing temperature 75 °C), the ethanol recovery of 99.2% was measured. Li and co-researchers [53] have reported that up to 100 g L⁻¹ of NaOH would be utilized along with ethanol during the chemical treatment (temperature = 100 °C, processing time = 30 min) with 99.5% of ethanol recovery. Ethanol can act as either a proton acceptor (in acidic condition) or a proton donor (in alkaline condition as in the present study). The stability of ethanol molecule decreases in the acidic pH compared to the alkaline pH conditions [54-55]. The recovery of ethanol in the alkaline condition (as in EHOs treatment) also indicated that H₂O₂ had no reactivity with ethanol (Fig. 5). Furthermore, the lower polarity of H₂O₂ molecule (dipole moment = 5.25 x 10⁻³⁰ C m, dielectric constant = 74.6 at 17 °C) [16] compared to water, stabilizes in aqueous ethanol [45]. In EHOs treatment, the physiochemical properties of aqueous ethanol, and its interdependency on KOH and H₂O₂ would be a potential reason for the obtained negative curvatures (generated through four variables response surface analysis) (Fig. 5). A further research is required to determine the physiochemical changes in GPWC after EHOs and AHP treatments using X-ray diffraction (for crystallinity), and scattering electron microscopy (for structural changes).

The optimal GPWC conversions of glucan and total polysaccharide were predicted at 71.2% (conditions: 32.1% ethanol + 46.6 g L⁻¹ KOH + 34.5 g L⁻¹ H₂O₂ at 64.2 °C for 24 h) and at 64.2% (conditions: 35.7% ethanol + 58.2 g L⁻¹ KOH + 42.7 g L⁻¹ H₂O₂ at 75.4 °C for 24 h), respectively. Moreover, the optimal conversions of glucan and total polysaccharide were reported in 95% and 87% confidence intervals, respectively (Fig. 6). The lower confidence interval for the total polysaccharide conversion would be resulted due to both detection of different sugars (such as xylose, mannose and galactose) at the single peak (using Biorad Aminex 87-H column), and the lower hemicellulose conversion

559 efficiency of Accellerase® 1500. The interdependency of chemical composition on the
560 enzymatic saccharification would affect analysis of the total polysaccharide conversion of
561 GPWC. However, the optimized GPWC conversions of glucan and total polysaccharide
562 were found comparable to the initial EHOs treatment condition (60% ethanol + 50 g L⁻¹
563 KOH+ 50 g L⁻¹ H₂O₂ at 78 °C for 24 h). The difference between obtained results would
564 be explained on the basis of different enzyme loadings used in the preliminary study
565 (using EHOs and AHP treatments at 80.2 FPU/g of biomass) and the response surface
566 study (at 40.1 FPU/g of biomass). The use of optimal enzyme mixture (blend of xylanase
567 with cellulase) would be useful in the further optimization of EHOs-treatment conditions
568 compared to Accellerase® 1500 alone.

569 The generated pressure in EHOs treatment (conditions: reactor volume = 3.8 L; solid
570 loading = 216 g; solvent loading = 2.16 L consisted of 60% ethanol + 50 g L⁻¹ KOH + 50
571 g L⁻¹ H₂O₂ with added water; treatment time = 24 h; processing temperature 75 °C) was
572 equivalent to the processing pressure of the Kraft process (at 160 ° to 180 °C). In the
573 paper pulp industry, a chemical treatment vessel is pressurized using oxygen or air to
574 improve the delignification process. The application of EHOs treatment eliminated the
575 external pressure requirement in the temperature ranges of 64 °-78 °C. The lower
576 temperature is less energy intensive and would be useful in the biorefinery operations.
577 However, a further research is required with different particle size distribution of GPWC
578 (< 0.15 mm, 0.15 mm-0.25 mm, 0.25 mm-0.5 mm), chemical treatment processing time
579 (1 h -5 h, based on H₂O₂ shelf life), and reactor loading (at 20%, 40%, 60%, and 80%, to
580 determine effective ethanol concentration under the pressurized conditions and for safety
581 concerns of the process) in the optimal GPWC conversion before implication of EHOs
582 treatment at the commercial level. The lignin produced during EHOs treatment process
583 would have commercial application in the formation of carbon fibers and is subjected to
584 further research [13, 56]. Integration of EHOs treatment technology with biofuel
585 development would have better environmental outcomes. The integrated paper pulp and
586 biofuel biorefinery could save up to 20% in the energy lost [57]. The fact that ethanol,
587 KOH, and H₂O₂ are eco-friendly compounds, and the proper recycling of the solvent
588 mixtures (after treatment) makes EHOs solvent system interesting and beneficial for large
589 scale applications.

590 591 **4. Conclusions**

592 The very first time EHOs treatment was performed with GPWC. The glucan
593 conversion efficiency of GPWC increased with the application of EHOs treatment
594 compared to AHP treatment. The enzymatic saccharification of EHOs-treated GPWC had
595 exceeded 70% of the total glucan conversion which is critical in lignocellulosic biomass
596 based biorefinery development. Moreover, the utilization of multiple lignocellulosic
597 feedstocks (ranging from woods to agriculture residues) using EHOs treatment method
598 (with varying composition of solvent based on the crystallinity of lignocellulosic
599 biomass) provided both the flexibility and continuity of biorefinery operation compared
600 to soaking in aqueous ammonia treatment. Furthermore, the examined four variables
601 response surface methodology provided the statistical optimization of EHOs treatment.
602 The use of ethanol content up to 36% in EHOs solvent formulation had the most
603 significant effect in the controlled foaming and would be useful in further scale-up
604 studies.

605
606

607 **Author and Co-Authors Contributions**

608 Author Arpan Jain conceptualized the research, worked on the research, and wrote the
609 manuscript. William C Bridges assisted with the statistical analysis. Karl Kelly was the
610 primary investigator on the project and managed the research project.

611

612 **Competing Interests**

613 The author and co-authors declare that they have no competing interests.

614

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626

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Tables

Table 1 - Central composite design for EHOs treatment analysis.

A. Variables with their coded and uncoded values.

		Coded					Uncoded
		(-2) ^[a]	(-1)	(0)	(+1)	(+2)	
Variables	Temperature (°C)	38 ^[b]	48	58	68	78	
	Ethanol (%)	0	15	30	45	60	
	KOH (g L ⁻¹)	0	12.5	25	37.5	50	
	H ₂ O ₂ (g L ⁻¹)	0	12.5	25	37.5	50	

B. Experimental design and sample preparations based on the response surface methodology.

P #	Variables				
	Temperature	Ethanol	KOH	H ₂ O ₂	Water
1	(-2) 38	(0) ^[a] 30 ^[b] [24.0] ^[c]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[48.29]
2	(-1) 48	(+1) 45 [36.0]	(+1) 37.5 [3.0]	(+1) 37.5 [8.57]	[32.43]
3	(-1) 48	(+1) 45 [36.0]	(+1) 37.5 [3.0]	(-1) 12.5 [2.86]	[38.14]
4	(-1) 48	(+1) 45 [36.0]	(-1) 12.5 [1.0]	(+1) 37.5 [8.57]	[34.43]
5	(-1) 48	(+1) 45 [36.0]	(-1) 12.5 [1.0]	(-1) 12.5 [2.86]	[40.14]
6	(-1) 48	(-1) 15 [12.0]	(+1) 37.5 [3.0]	(+1) 37.5 [8.57]	[56.43]
7	(-1) 48	(-1) 15 [12.0]	(+1) 37.5 [3.0]	(-1) 12.5 [2.86]	[62.14]
8	(-1) 48	(-1) 15 [12.0]	(-1) 12.5 [1.0]	(+1) 37.5 [8.57]	[58.43]
9	(-1) 48	(-1) 15 [12.0]	(-1) 12.5 [1.0]	(-1) 12.5 [2.86]	[64.14]
10	(0) 58	(0) 30 [24.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[48.29]
11	(0) 58	(0) 30 [24.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[48.29]
12	(0) 58	(0) 30 [24.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[48.29]
13	(0) 58	(0) 30 [24.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[48.29]
14	(0) 58	(-2) 0 [0.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[72.29]
15	(0) 58	(+2) 60 [48.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[24.29]
16	(0) 58	(0) 30 [24.0]	(-2) 0.0 [0.0]	(0) 25.0 [5.71]	[50.29]
17	(0) 58	(0) 30 [24.0]	(+2) 50.0 [4.0]	(0) 25.0 [5.71]	[46.29]
18	(0) 58	(0) 30 [24.0]	(0) 25.0 [2.0]	(-2) 0.0 [0.00]	[54.00]
19	(0) 58	(0) 30 [24.0]	(0) 25.0 [2.0]	(+2) 50.0 [11.43]	[42.57]
20	(+1) 68	(+1) 45 [36.0]	(+1) 37.5 [3.0]	(+1) 37.5 [8.57]	[32.43]
21	(+1) 68	(+1) 45 [36.0]	(+1) 37.5 [3.0]	(-1) 12.5 [2.86]	[38.14]
22	(+1) 68	(+1) 45 [36.0]	(-1) 12.5 [1.0]	(+1) 37.5 [8.57]	[34.43]
23	(+1) 68	(+1) 45 [36.0]	(-1) 12.5 [1.0]	(-1) 12.5 [2.86]	[40.14]
24	(+1) 68	(-1) 15 [12.0]	(+1) 37.5 [3.0]	(+1) 37.5 [8.57]	[56.43]
25	(+1) 68	(-1) 15 [12.0]	(+1) 37.5 [3.0]	(-1) 12.5 [2.86]	[62.14]
26	(+1) 68	(-1) 15 [12.0]	(-1) 12.5 [1.0]	(+1) 37.5 [8.57]	[58.43]
27	(+1) 68	(-1) 15 [12.0]	(-1) 12.5 [1.0]	(-1) 12.5 [2.86]	[64.14]
28	(+2) 78	(0) 30 [24.0]	(0) 25.0 [2.0]	(0) 25.0 [5.71]	[48.29]

Note:

^[a] Values represent coded variables for the response surface methodology analysis.

^[b] Values represent the uncoded variables used in the response surface analysis.

^[c] Values represent the actual composition of ethanol (mL), KOH (g), H₂O₂ (mL), and added water (mL) at the specific temperature ranges in the sample preparations for the response surface analysis.

Table 2 - Effect of chemical treatments on GPWC.**A. Sample weight before and after treatments**

Chemical Treatments	Water	AHP ^[a]	EHOs ^[b]
Before treatment (g)	8.07 ± 0.02	8.06 ± 0.02	8.07 ± 0.03
After treatment (g)	7.93 ± 0.01	5.92 ± 0.13	6.21 ± 0.20

B. Compositional analysis of GPWC under different treatment conditions

Components	Composition (g (100 g dried GPWC) ⁻¹)		
	Untreated	AHP	EHOs
Cellulose	46.38 ± 0.73	59.16 ± 1.75	61.31 ± 1.04
Glucan	46.38 ± 0.73	59.16 ± 1.75	61.31 ± 1.04
Hemicellulose	22.46 ± 0.52	18.30 ± 0.35	17.42 ± 0.18
Xylan	7.51 ± 0.44	4.70 ± 0.33	4.45 ± 0.41
Arabinan	0.77 ± 0.05	0.48 ± 0.05	0.39 ± 0.06
Galactan	1.76 ± 0.44	0.42 ± 0.03	0.41 ± 0.05
Mannan	12.40 ± 0.30	12.70 ± 0.10	12.19 ± 0.23
Lignin	29.90 ± 1.12	22.01 ± 1.42	20.69 ± 0.85
AIL ^[c]	29.37 ± 1.14	21.48 ± 1.45	20.08 ± 0.87
ASL ^[d]	0.53 ± 0.01	0.54 ± 0.03	0.61 ± 0.02
Ash	0.47 ± 0.04	0.58 ± 0.07	0.59 ± 0.04
Extractive	0.81 ± 0.10		
Solid residues	100.02 ± 2.51	100.04 ± 3.59	100.01 ± 2.11

Note:^[a] AHP = Alkaline hydrogen peroxide.^[b] EHOs = Ethyl-hydro-oxides.

Chemical treatment conditions: solvent: sample ratio 1:10 (wt/vol) at 78 °C for 24 h.

Cellulose: Glucan

Hemicellulose: Xylan + Arabinan + Galactan + Mannan

Lignin: ^[c] AIL (Acid insoluble lignin) + ^[d] ASL (Acid-soluble lignin).

Values are expressed as mean and standard error with two observations made at each combination.

Table 3 - Analysis of variance (ANOVA) for the quadratic model resulted from glucan conversion (%) and total polysaccharide conversion (%) of GPWC.

A. Based on glucan conversion (%)

Residual	DF	Sum of Squares	Mean Square	F Ratio	Pr > F
Model	14	19304.83	1378.92	75.66	<.0001*
Lack of Fit	10	663.38	66.34	24.52	<.0001*
Pure Error	31	83.88	2.71		
Total Error	41	747.26		R ² =0.9958	

B. Based on total polysaccharide conversion (%)

Residual	DF	Sum of Squares	Mean Square	F Ratio	Pr > F
Model	14	12191.42	870.82	76.93	<.0001*
Lack of Fit	10	403.66	40.37	20.71	<.0001*
Pure Error	31	60.41	1.95		
Total Error	41	464.08		R ² =0.9952	

Figures

Fig. 1 - Enzyme hydrolysis of AHP-treated and EHOs-treated GPWC using Accellerase® 1500 with the resulted sugar yields and their conversions. **Note:** Values are average of three replicates. Error bars represent standard errors.

A. Based on glucan conversion (%)

B. Based on total polysaccharide conversion (%)

Fig. 2 - Analysis of enzymatic saccharification efficiencies of actual values vs. predicted values for glucan conversion (%) and total polysaccharide conversion (%) of GPWC using Accellerase® 1500.

A. Based on glucan conversion (%)

B. Based on total polysaccharide conversion (%)

Fig. 3 - Interaction profiles for glucan conversion (%) and total polysaccharide conversion (%) as functions of temperature (48, 68 °C), ethanol (15, 45%), KOH (12.5, 37.5 g L⁻¹) and H₂O₂ (12.5, 37.5 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

Fig. 4a – Surface profilers for glucan conversion (%) as functions of temperature (at 58 °C), ethanol (at 30%), KOH (at 25 g L⁻¹) and H₂O₂ (at 25 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

Fig. 4b – Surface profilers for total polysaccharide conversion (%) as functions of temperature (at 58 °C), ethanol (at 30%), KOH (at 25 g L⁻¹) and H₂O₂ (at 25 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

A. Based on glucan conversion (%)

Term	Estimate	Std Error	t Ratio	Profile	Prob> t
KOH (12.5, 37.5 g L ⁻¹)	17.50	0.62	28.41		<.0001*
H ₂ O ₂ x H ₂ O ₂	-6.55	0.62	-10.63		<.0001*
KOH x KOH	-6.06	0.62	-9.83		<.0001*
Temp x Temp	-4.50	0.62	-7.30		<.0001*
EtOH x EtOH	-4.43	0.62	-7.19		<.0001*
H ₂ O ₂ (12.5, 37.5 g L ⁻¹)	3.37	0.62	5.47		<.0001*
KOH x H ₂ O ₂	3.21	0.75	4.26		0.0001*
Temp (48 °, 68 °C)	1.62	0.62	2.63		0.0121*
Temp x H ₂ O ₂	1.72	0.75	2.28		0.0278*
Temp x KOH	1.49	0.75	1.98		0.0546
Temp x EtOH	0.60	0.75	0.80		0.4290
EtOH x KOH	0.42	0.75	0.56		0.5778
EtOH (15, 45%)	0.30	0.62	0.49		0.6282
EtOH x H ₂ O ₂	-0.22	0.75	-0.29		0.7750

B. Based on total polysaccharide conversion (%)

Term	Estimate	Std Error	t Ratio	Profile	Prob> t
KOH (12.5, 37.5 g L ⁻¹)	13.96	0.49	28.75		<.0001*
H ₂ O ₂ x H ₂ O ₂	-4.48	0.49	-9.23		<.0001*
KOH x KOH	-4.20	0.49	-8.66		<.0001*
EtOH x EtOH	-3.36	0.49	-6.91		<.0001*
Temp x Temp	-3.28	0.49	-6.76		<.0001*
H ₂ O ₂ (12.5, 37.5 g L ⁻¹)	2.74	0.49	5.65		<.0001*
Temp (48 °, 68 °C)	2.30	0.49	4.75		<.0001*
KOH x H ₂ O ₂	2.69	0.59	4.52		<.0001*
Temp x KOH	2.51	0.59	4.22		0.0001*
Temp x H ₂ O ₂	1.61	0.59	2.71		0.0098*
EtOH x KOH	0.53	0.59	0.89		0.3794
Temp x EtOH	0.52	0.59	0.87		0.3913
EtOH (15, 45%)	0.34	0.49	0.70		0.4902
EtOH x H ₂ O ₂	-0.05	0.59	-0.08		0.9338

Fig. 5 - Sorted estimate parameters for glucan conversion (%) and total polysaccharide conversion (%) resulted through the interactions of temperature (Temp), ethanol (EtOH), KOH, and H₂O₂ during the enzymatic saccharification of GPWC. **Note:** A bar chart shows t-ratio with the lines at 0.05 significance level.

A. Based on glucan conversion (%)

B. Based on total polysaccharide conversion (%)

Fig. 6 - Optimized EHOs treatment conditions resulted through the enzymatic saccharification of GPWC using Accellerase® 1500.

Fig. 7a – Optimized surface profilers for glucan conversion (%) as functions of temperature (at 64.2 °C), ethanol (at 32.1%), KOH (at 46.6 g L⁻¹) and H₂O₂ (at 34.5 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

Fig. 7b – Optimized surface profilers for total polysaccharide conversion (%) as functions of temperature (at 75.4 °C), ethanol (at 35.7%), KOH (at 58.2 g L⁻¹) and H₂O₂ (at 42.7 g L⁻¹) during the enzymatic saccharification of GPWC using Accellerase® 1500.

Appendix-A

Table A1 - Central composite design: Chemical composition and enzymatic saccharification of GPWC.

P #	Biomass recovery after treatment ^[1] (%)	Moisture content ^[1] (g)	Chemical Composition ^[1] g (100 g dried GPWC) ⁻¹		Enzyme Hydrolysis ^[2] (%)		
			G	XMG	GC	XMGC	TPC
1	86.2 (0.5)	19.7 (0.6)	48.0 (0.4)	21.4 (0.3)	35.0 (1.0)	10.4 (0.3)	27.5 (0.8)
2	82.5 (0.3)	16.8 (0.7)	49.2 (0.7)	24.5 (0.5)	51.5 (0.8)	11.0 (0.1)	38.0 (0.6)
3	82.7 (0.2)	17.7 (0.2)	48.2 (0.6)	23.6 (0.8)	45.7 (0.7)	9.1 (0.2)	33.7 (0.4)
4	86.2 (0.2)	17.9 (0.3)	46.0 (0.2)	24.9 (0.6)	11.9 (0.4)	4.9 (0.6)	9.4 (0.0)
5	86.7 (0.1)	17.9 (0.5)	46.4 (0.5)	24.2 (0.6)	11.5 (0.0)	5.0 (0.3)	9.2 (0.1)
6	81.2 (0.2)	16.5 (0.5)	47.9 (0.5)	24.5 (0.6)	52.7 (0.2)	10.7 (0.1)	38.5 (0.1)
7	80.9 (0.1)	16.2 (0.9)	48.3 (0.8)	24.3 (1.0)	45.9 (0.4)	9.0 (0.1)	33.6 (0.2)
8	86.6 (0.1)	20.0 (1.0)	45.6 (0.7)	24.6 (0.9)	13.1 (0.3)	5.5 (0.4)	10.4 (0.1)
9	87.3 (0.4)	19.3 (0.9)	44.8 (0.3)	25.2 (0.7)	12.5 (0.5)	5.8 (0.2)	10.0 (0.2)
10	80.4 (0.1)	15.9 (0.3)	57.9 (0.2)	23.5 (0.7)	52.4 (1.3)	8.5 (0.2)	39.7 (1.0)
11	80.8 (0.2)	18.4 (0.6)	57.2 (0.2)	23.1 (0.4)	51.0 (0.7)	8.8 (0.1)	38.9 (0.5)
12	80.3 (0.4)	18.3 (0.7)	56.7 (1.0)	21.7 (1.5)	55.8 (1.5)	9.5 (0.2)	43.0 (1.0)
13	80.3 (0.3)	18.3 (0.6)	57.8 (0.5)	22.9 (0.2)	57.9 (0.4)	11.7 (0.8)	44.8 (0.5)
14	81.0 (0.7)	15.4 (0.4)	55.2 (0.3)	22.4 (0.3)	35.5 (1.0)	9.2 (0.8)	27.9 (0.5)
15	80.6 (0.3)	15.2 (0.6)	57.1 (0.2)	23.4 (1.2)	37.9 (0.3)	10.8 (0.4)	30.0 (0.1)
16	91.2 (0.3)	18.8 (0.2)	50.2 (0.5)	19.3 (0.5)	4.6 (0.5)	2.9 (0.3)	4.1 (0.3)
17	77.5 (0.4)	14.1 (0.3)	58.1 (0.6)	17.8 (0.3)	55.8 (1.0)	18.5 (0.5)	47.0 (0.9)
18	84.0 (0.1)	17.2 (0.7)	54.8 (0.6)	18.2 (0.4)	21.7 (0.2)	12.4 (0.7)	19.4 (0.3)
19	81.5 (0.2)	16.4 (1.0)	54.5 (1.4)	18.3 (0.4)	34.8 (0.2)	14.0 (0.2)	29.6 (0.2)
20	75.1 (1.2)	16.9 (0.8)	58.9 (1.5)	16.8 (0.1)	67.1 (0.2)	23.4 (0.2)	57.4 (0.2)
21	73.6 (0.2)	14.6 (0.2)	62.3 (0.3)	20.3 (0.9)	48.2 (0.5)	14.1 (0.8)	39.8 (0.6)
22	85.7 (0.4)	18.5 (0.2)	53.4 (0.6)	23.5 (0.8)	13.7 (0.4)	4.2 (0.2)	10.8 (0.3)
23	85.1 (0.4)	19.2 (0.5)	52.8 (0.4)	20.9 (0.3)	13.1 (0.1)	4.0 (0.3)	10.6 (0.0)
24	74.9 (0.7)	16.1 (0.4)	60.0 (0.9)	18.5 (1.4)	65.4 (0.1)	17.2 (1.0)	54.1 (0.3)
25	73.3 (0.7)	16.1 (0.6)	62.4 (0.6)	18.9 (1.2)	43.8 (0.9)	13.0 (0.1)	36.6 (0.7)
26	86.1 (0.1)	18.7 (0.1)	51.5 (0.2)	22.4 (1.7)	13.5 (0.5)	5.7 (0.5)	11.1 (0.2)
27	86.0 (0.2)	21.0 (0.7)	51.2 (0.5)	25.0 (0.4)	13.4 (0.0)	5.3 (0.3)	10.7 (0.1)
28	75.8 (0.3)	15.8 (0.4)	56.0 (0.6)	22.3 (0.4)	37.8 (0.5)	14.1 (0.9)	31.1 (0.6)

Abbreviations:

G = Extractable glucan content; XMG = Extractable xylan-mannan-galactan content.

GC = Glucan conversion; XMGC = Xylan-mannan-galactan conversion; TPC = Total polysaccharide conversion.

Note:

P# represented the sequential orders of the treatment (see Table 1).

^[1] Three and ^[2] two observations were made at each combination.

Values represented the average with the bracketed standard error.

Appendix-B

Fig. B1 - Experimental set up for EHOs treatment with GPWC using Parr reactor (Total volume = 3.8 L, working volume = approximately 2.2 L).

Note:

1. Weighing of GPWC before treatment.
2. A mixture of ethanol-water-KOH solution with GPWC.
3. An enclosed Parr reactor vessel before addition of H_2O_2 to the vessel.
4. The final pressure after 5 minutes of H_2O_2 addition.
5. EHOs-treated GPWC in the solvent.
6. EHOs-treated GPWC after washing.