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Optimization of Combined Hydrothermal and Mechanical Refining Pretreatment of Forest Residue Biomass for Maximum Sugar Release during Enzymatic Hydrolysis

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Abstract: This study aimed to investigate the effect of chemical-free two-stage hydrothermal and mechanical refining pretreatment on improving the sugar yields during enzymatic hydrolysis of forest residue biomass (FRB) and optimize the pretreatment conditions. Hot-water pretreatment experiments were performed using a central composite design for three variables: temperature (160–200 °C), time (10–20 min), and solid loading (10–20%). Hydrothermally pretreated biomass was subsequently pretreated using three cycles of disk refining. The combined pretreatment was found to be highly effective in enhancing sugar yields during enzymatic hydrolysis, with almost 99% cellulose conversion for biomass pretreated at 213.64 °C, 15 min, and 15% solid loading. However, the xylose concentrations in the hydrolysate were found to be low under these conditions due to sugar degradation. Thus, less severe optimum pretreatment conditions (194.78 °C, 12.90 min, and 13.42% solid loading) were predicted using a second-order polynomial model. The response surface model optimized the hydrothermal pretreatment of FRB and predicted the glucan, xylan, and overall conversions of 94.57%, 79.78%, and 87.84%, respectively, after the enzymatic hydrolysis. The model-predicted biomass conversion values were validated by the experimental results.

Keywords: hydrothermal pretreatment; disk milling; forest residue biomass; enzymatic hydrolysis; response surface methodology (RSM); central composite design (CCD)



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1. Introduction

There is growing concern about the negative environmental impacts of fossil fuel consumption. At the same time, the need for future energy security is forcing the fossil fuel-based energy sector to transition toward alternative renewable resources [1]. Food-based crops, such as sugarcane and corn, are the major feedstock used for biofuel production; however, these feedstocks are not available across the globe [2]. Additionally, the food versus fuel debate and the need for arable land, water, fertilizer, and other resources make these feedstocks unfavorable in terms of environmental sustainability [3]. Alternatively, lignocellulosic biomass presents an attractive and sustainable feedstock to produce biofuels and bioproducts due to its abundant availability and non-food applications. For example, wood harvesting operations produce substantial amounts of waste or underutilized lignocellulosic biomass, including low-grade wood, bark, treetops, slash, and branches [4,5]. Those harvesting residues are known as forest residue biomass (FRB) and are widely available at relatively lower cost [4,6]. More than 150 million Mg of FRB (dry basis) is available annually in the U.S. [7].

Upcycling FRB to biofuel and high-value bioproducts can support the development of a sustainable bioeconomy while simultaneously reducing waste, mitigating wildfire risks,

and minimizing environmental impacts [8,9]. Similarly to other lignocellulosic biomass, FRB is mainly composed of cellulose, hemicellulose, and lignin, as well as a smaller proportion of extractives and ash [10]. Cellulose and hemicellulose can be hydrolyzed into fermentable sugars, which can be later converted into various biofuels, biochemicals, and biopolymers [11]. Lignin, a complex polymer, can be depolymerized into aromatic compounds for the synthesis of specialty chemicals, adhesives, and advanced materials such as carbon fibers [12].

One of the most critical steps in FRB processing is the production of sugars from carbohydrate hydrolysis. Similarly to other lignocellulosic biomass, FRB has a recalcitrance structure due to a complex matrix of structural carbohydrates and lignin. A pretreatment step is necessary to break the recalcitrance structure and improve subsequent hydrolysis efficiency [13]. The pretreatment process must be effective to depolymerize the biomass structure fully to fractionate the hemicellulose and/or lignin and increase the accessibility of enzymes to cellulose chains. At the same point, it should avoid sugar degradation that could result in potential inhibitor generation for the consequent conversion process [1,14,15]. Concurrently, the pretreatment process should utilize inexpensive materials and chemicals to become economically sustainable [16]. Conventional pretreatment uses chemicals (various acids) at high temperatures and pressure to reduce biomass recalcitrance [17,18]. These pretreatment processes are effective in enhancing hydrolysis yields; however, they suffer from the limitations of high energy use, chemical waste generation, and the need for a detoxification step before microbial fermentation. To address these issues, this work studied chemical-free hydrothermal pretreatment (a combination of hot-water pretreatment and disk milling) that has been found effective for several feedstocks, such as corn stover, sugarcane bagasse, and energycane [14].

Hot-water pretreatment uses water as a solvent and follows a similar kinetic mechanism to dilute acid pretreatment [19,20]. During hydrothermal pretreatment, an increased concentration of hydronium ions catalyze the breakdown of hemicellulose into soluble oligosaccharides and monosaccharides [21]. Additionally, lignin is fractionated under elevated temperature and pressure conditions [21]. Such fractionation enhances cellulose accessibility to enzymes during the next hydrolysis step for sugar release. Varied temperature, time, and biomass loading can be used for sugar release from the biomass. Hot-water pretreatment is generally not severe enough to fully convert hemicellulose into xylose. Instead, it primarily produces oligosaccharides, which reduces the likelihood of xylan sugar degradation into furfural inhibitors [20]. However, hot-water pretreatment alone is typically insufficient to achieve high sugar yields unless it is carried out at very high temperatures and extended residence times [14,22,23]. The effectiveness of hydrothermal pretreatment can be increased by a post-treatment step, mechanical refining pretreatment, such as disk milling [21,24].

Mechanical refining pretreatment decreases biomass particle size and increases specific surface area through biomass fiber shortening, curling or straightening, and internal or external fibrillation [24,25]. The higher energy requirements of mechanical refining restrict their implications in biomass pretreatment alone [26]. However, if mechanical refining is coupled after hydrothermal pretreatment, its energy requirement can be decreased up to 95% due to fiber softening at earlier hydrothermal pretreatment [27–29]. The synergistic utilization of hydrothermal and mechanical refining pretreatment has been reported, resulting in several-fold higher monomeric sugar release compared to untreated biomass, resulting in improved economic sustainability of biofuel production [27,30].

The pretreatment process is associated with a wide variability of process parameters, and optimization of these parameters is necessary to develop an effective and economically feasible pretreatment process for maximum biomass sugar release in the hydrolysis process [31]. Response surface methodology (RSM) has been efficiently used for lignocellulosic biomass pretreatment process optimization as it efficiently explores the relationships between multiple process parameters and their effects on desired responses [32]. It allows the modeling of complex interactions between independent parameters, enabling the identifi-

cation of optimal conditions with fewer experiments [33]. Thus, this optimization approach results in reduced experimental cost and time, improved accuracy in identifying optimal conditions, and the ability to visualize response surfaces for a better understanding of the pretreatment process [34,35]. In addition, optimization based on RSM helps in maximizing pretreatment efficiency for biomass conversion, leading to better sugar yield in the hydrolysis process [33,34]. Thus, the objective of this study is to optimize the FRB pretreatment process based on RSM for improved sugar yields in enzymatic hydrolysis. The RSM based on the central composite design (CCD) approach was used to optimize hot-water pretreatment of lignocellulosic FRB. The FRB generated from sugar maple (*Acer saccharum*) harvesting was used as feedstock in this study. Pretreatment temperature, residence time, and solid loading were chosen as the three independent variables. The optimization of pretreatment conditions was carried out to maximize the carbohydrate conversion.

This study presents several novel contributions to the field of woody biomass processing for biofuels and bioproducts. Forest residues from sugar maple offer a high-potential, sustainable carbon source for fermentable sugar production. While sugar maple wood has been studied for biofuel production, research on low-quality residues is scarce, if not entirely absent. In terms of pretreatment, although hydrothermal and mechanical refining pretreatment has been studied in the context of agricultural residues [22,23,29], it has not been thoroughly explored for woody biomass. Furthermore, most previous studies on hot-water disk-milling pretreatment have focused solely on the effects of temperature and time. In contrast, this work explores three key parameters, temperature, time, and solid loading, providing a more comprehensive understanding of the process.

2. Materials and Methods

2.1. Materials

Sugar maple FRB was collected from the SUNY ESF Heiberg Memorial Forest located in the Onondaga and Cortland counties, New York, USA, for this study. To prevent microbial biomass growth, FRB chips were air-dried to reduce the moisture content below 10% and stored in a cold room at 4 °C [36]. The dried chips were ground in a Willey Mill using a 10-mesh screen to maintain a uniform 2.00 mm feedstock size for all experiments. The cellulase and hemicellulase enzymes, Cellic® Ctec2 and Cellic® Htec2, used in this study were donated by Novozymes North America, Inc., Franklinton, NC, USA. All chemicals were purchased from the commercial suppliers VWR (Radnor, PA, USA) and Fisher Scientific (Hampton, NH, USA).

2.2. Composition Analysis

The chemical composition of biomass was determined using standard Laboratory Analytical Procedures (LAPs), developed by the National Renewable Energy Laboratory (NREL) [37–39]. The solid content in the samples was measured using gravimetric analysis. About 1 to 2 g of ground FRB was kept in a convection oven for at least four hours at 105 °C and then weight loss was calculated before and after the heating for moisture content calculation. A similar approach was applied to determine the ash content. A higher temperature (575 °C) and time (24 h) were maintained in ash content measurement for the minerals' and inorganic materials' determination.

Non-structural materials of biomass including inorganics, non-structural sugars, nitrogenous materials, etc., were determined in a two-step (water and ethanol) extraction process outlined by NREL in the LAP NREL/TP-510-42619 [39]. The extractive free biomass was used to determine the structural carbohydrates and lignin, using the two-step acid hydrolysis method [37]. Briefly, 0.3 g of biomass (dry basis) was mixed with 3.0 mL of concentrated sulfuric acid (72% *w/w*) and incubated at 30 °C for one hour in the first step of acid hydrolysis. The acid concentration was diluted to 4% by adding water, and the final slurry was autoclaved at 121 °C for 60 min, the second step of acid hydrolysis. The slurry was cooled down at ambient conditions and then filtered for the residual biomass and liquid fraction separation. The liquid filtrate contained monomeric structural sugars and

acid-soluble lignin which were measured using high-performance liquid chromatography (HPLC) and ultraviolet–visible (UV-Vis) spectroscopy [37]. Residual biomass was dried (at 105 °C for 4 h) and later ashed (at 575 °C for 24 h) for acid-insoluble lignin content determination. Samples were passed through a 0.2 µm nylon filter before the HPLC analysis and all the composition analysis experiments were conducted in triplicate.

2.3. Hydrothermal and Mechanical Refining Pretreatment

Hydrothermal pretreatment of FRB was carried out in a 300 mL Parr Reactor (Parr Instrument Co., Moline, IL, USA) equipped with a 4848-reactor controller for temperature and stirring control. All experiments were performed at a 200 mL working volume. Pretreatment temperature, time, and solid loading were varied during this hydrothermal pretreatment as the effectiveness of the pretreatment was significantly affected by those parameters in previous studies [22,40,41]. High temperatures accelerated the breakdown of hemicellulose, improving cellulose accessibility, but excessively high temperature led to the formation of inhibitory by-products such as furfural and hydroxymethylfurfural [22,40]. Similarly, extended pretreatment time increased the biomass depolymerization, but prolonged exposure resulted in the loss of monomeric sugars [41]. Solid loading affects both the energy efficiency and the viscosity of the pretreatment slurry. High solid loading could potentially improve the process efficiency by reducing water and energy requirements, but too-high solid loading hinders the heat and mass transfer, leading to uneven pretreatment [27,40]. Thus, optimizing these parameters is key to maximizing sugar recovery while minimizing the formation of inhibitors during the pretreatment of biomass.

The RSM based on CCD was used to design the hydrothermal pretreatment process. Table 1 shows the whole matrix of CCD for hydrothermal pretreatment of FRB where a total of 20 pretreatment experiments were carried out. In total, (2^3) 8 factorial points, (2×3) 6 axial points, and 6 central points were considered in the RSM by varying the pretreatment parameters (temperature, time, and solid loading) at three different levels (−1, 0, and +1). Both the coded and real pretreatment values are listed in Table 1, where α is considered equal to 1.68 for axial point generation [42]. Based on the solid loading used in the experiment, pre-calculated amounts of biomass and water were added to the reactor to achieve a total volume of 200 mL. The slurry was heated to a desired temperature (Table 1) and held at that temperature for the set time. At the end of the incubation period, the slurry was brought to the ambient temperature by immersing the reactor in ice-cold water.

The reaction mixture (slurry) was mechanically pretreated “as-is” (without any washing, filtration, or separation) by passing it through a lab-scale disk mill (Quaker City grinding mill model 4E, Straub Co., Philadelphia, PA, USA). A constant 89 rpm was maintained in the disk mill to provide pretreated biomass through two disks at the outlet. One disk remained stationary while the other one was constantly rotating by maintaining the minimum distance between them [22,43]. Based on the recommendations from previous studies [22,43–45], milling was limited to three consecutive cycles in all instances to optimize electricity consumption, reduce costs, and manage heat dissipation.

Table 1. The CCD matrix for the hydrothermal pretreatment conditions of FRB.

Space Type	RSM Experiment Design			Glucan Conversion (%)			Xylan Conversion (%)			Overall Conversion (%)		
	Temperature (°C)	Time (min)	Solid Loading (%)	Exp. ¹	Pred. ²	Resl. ³	Exp. ¹	Pred. ²	Resl. ³	Exp. ¹	Pred. ²	Resl. ³
Factorial points	−1(160)	−1(10)	−1(10)	46.33	48.23	−1.90	41.23	44.81	−3.58	43.66	46.00	−2.34
	+1(200)	−1(10)	−1(10)	92.72	95.62	−2.90	77.46	77.40	0.06	85.90	87.86	−1.96
	+1(200)	−1(10)	+1(20)	93.24	92.81	0.43	75.07	71.54	3.53	85.56	84.23	1.33
	−1(160)	−1(10)	+1(20)	43.02	50.57	−7.55	40.28	52.35	−12.07	41.12	49.80	−8.68
	−1(160)	+1(20)	−1(10)	55.00	58.53	−3.53	54.46	64.80	−10.34	53.44	58.88	−5.44
	+1(200)	+1(20)	−1(10)	98.92	94.47	4.45	62.53	57.27	5.26	85.76	81.19	4.57
	+1(200)	+1(20)	+1(20)	96.56	97.76	−1.20	47.48	50.71	−3.23	79.76	81.52	−1.76
	−1(160)	+1(20)	+1(20)	66.78	66.98	−0.20	64.76	71.63	−6.87	64.49	66.64	−2.15
Axial points	− α (146.36)	0(15)	0(15)	41.57	35.22	6.35	34.14	17.89	16.25	38.34	29.26	9.08
	+ α (213.64)	0(15)	0(15)	99.00	100.96	−1.96	21.08	27.70	−6.62	73.70	76.97	−3.27
	0(180)	− α (6.59)	0(15)	83.27	77.67	5.60	91.76	86.87	4.89	83.34	78.39	4.95
	0(180)	+ α (23.41)	0(15)	89.29	90.50	−1.21	90.92	86.17	4.75	87.79	86.93	0.86
	0(180)	0(15)	− α (6.59)	74.61	73.79	0.82	80.32	78.49	1.83	74.38	73.29	1.09
	0(180)	0(15)	+ α (23.41)	82.10	78.53	3.57	87.12	79.31	7.81	81.48	76.76	4.72
Center points	0(180)	0(15)	0(15)	83.63	84.17	−0.54	87.64	86.22	1.42	82.68	82.63	0.05
	0(180)	0(15)	0(15)	82.40	84.17	−1.77	85.06	86.22	−1.16	81.08	82.63	−1.55
	0(180)	0(15)	0(15)	85.30	84.17	1.13	85.87	86.22	−0.35	83.30	82.63	0.67
	0(180)	0(15)	0(15)	84.49	84.17	0.32	86.28	86.22	0.06	82.87	82.63	0.24
	0(180)	0(15)	0(15)	84.20	84.17	0.03	86.09	86.22	−0.13	82.61	82.63	−0.02
	0(180)	0(15)	0(15)	84.25	84.17	0.08	84.75	86.22	−1.47	82.26	82.63	−0.37

¹ Exp.—experimental value, ² Pred.—predicted value, ³ Resl.—residual value.

2.4. Enzymatic Hydrolysis of Pretreated Biomass

Enzymatic hydrolysis of pretreated biomass was conducted at 10% solid loading, following the modified protocols of NREL/TP-5100-63351 [22,23,45]. A total of 5 g of pretreated biomass (oven-dried basis) was mixed with pre-calculated water in 125 mL Kimax glass flasks, and the pH of the slurry was adjusted to 5.0 by adding sodium hydroxide. A 1.0 M sodium acetate buffer (pH 5.0) was added to achieve a final concentration of 50 mM and to maintain the pH at 5.0 during enzymatic hydrolysis. Sodium azide (0.2 mL, 0.75 M) was used to prevent microbial growth during hydrolysis. Commercial cellulase (Cellic® Ctec2) and hemicellulase enzymes (Cellic® Htec2) were added at a dosage of 0.21 mL/g biomass (30 FPU/g biomass) and 51 µL/g biomass (one-fourth the volume of cellulase), respectively [23]. The hydrolysis was carried out for 72 h in a shaking incubator maintained at 50 °C and 200 rpm. All hydrolysis experiments were conducted in triplicates. Enzyme blanks (slurry without biomass) were tested to determine background sugar release from the enzyme cocktail in enzymatic hydrolysis.

2.5. Sugar Concentrations, Conversion Calculation

At the end (72 h) of hydrolysis, an approximately 2.0 mL sample was withdrawn for sugar analysis. The sample was heated at 95 °C for 6.0 min to stop enzyme activity and centrifuged at 10,000 × g for 5 min. The supernatant was filtered through a 0.2 µm nylon filter and transferred to 2 mL HPLC vials. An HPLC system equipped with a refractive index (RI) detector was used for the measurement of monosaccharides released (in g/L) from biomass. The analysis was performed using a HyperREZ™ XP Carbohydrate column (Thermo Fisher Scientific, Waltham, MA, USA), operating at 50 °C, and using 5 mM sulfuric acid as a mobile phase at a flow rate of 0.6 mL/min. The concentrations of sugars were quantified by comparing corresponding peak areas to external calibration standards. The glucan, xylan, and total carbohydrate conversion due to combined pretreatment and enzymatic hydrolysis of FRB were calculated using Equations (1)–(3). The glucose and xylose concentration values used here were corrected by deducting the background sugar release from the enzyme cocktail (enzyme blank) from the concentrations measured at the end of hydrolysis.

$$\text{Glucan conversion, } Y_{\text{Gl}} (\%) = \frac{C_{\text{Glu.}} \times 0.90 \times V_L}{M_B \times \left(\frac{\text{Gl}}{100}\right)} \times 100 \quad (1)$$

$$\text{Xylan conversion, } Y_{\text{Xl}} (\%) = \frac{C_{\text{Xyl.}} \times 0.88 \times V_L}{M_B \times \left(\frac{\text{Xy}}{100}\right)} \times 100 \quad (2)$$

$$\text{Overall conversion, } Y_{\text{Ov}} (\%) = \frac{(C_{\text{Glu.}} \times 0.90 \times V_L) + (C_{\text{Xyl.}} \times 0.88 \times V_L)}{M_B \times \left(\frac{\text{Gl}}{100}\right) + M_B \times \left(\frac{\text{Xy}}{100}\right)} \times 100 \quad (3)$$

where $C_{\text{Glu.}}$ and $C_{\text{Xyl.}}$ are the corrected glucose and xylose concentrations (g/L), 0.90 and 0.88 are the anhydrous correction factors for glucose and xylose accordingly, V_L is the volume of the liquid after enzymatic hydrolysis (L), M_B is the weight of the FRB (dry basis) (g), Gl and Xy are the glucan and xylan in FRB (%), and Y_{Gl} , Y_{Xl} , and Y_{Ov} are the glucan, xylan, and overall conversions in percentages (%), accordingly.

2.6. Model Development, Statistical Analysis, and Optimization

A second-order polynomial equation was used to relate the effects of independent process parameters on various responses—glucan conversion, xylan conversion, and overall conversion [33,35,46]. The following second-order equation (Equation (4)) was used to find out the linear, quadratic, and interaction effects of process parameters on responses.

$$Y_i = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j + \varepsilon \quad (4)$$

where Y_i is the predicted responses such as glucan conversion (Y_{Gl}), xylan conversion (Y_{Xl}), and overall conversion (Y_{Ov}) and expressed in percentages (%); X_i and X_j are the independent process parameters, for example, pretreatment temperature ($^{\circ}\text{C}$), time (min), and solid loading (%), affecting responses; β_0 is the interception coefficient; β_i , β_{ii} , and β_{ij} are the linear, quadratic, and interaction regression coefficients correspondingly; and ε is the random error. Design-Expert[®] v13 was used for the regression model's development using RSM based on CCD, shown in Table 1. The in-built analysis of variance (ANOVA) tool of Design-Expert[®] software was used for the statistical analysis of the model. The quality of fit of the model with the experimental conversion values was determined by the coefficient of determination (R^2) value, whereas the significance of the model was expressed in terms of F value. The effect of independent process parameters at various combinations on responses was also predicted by the model, and their relative significance was analyzed based on the F value at $p < 0.05$. Numerical optimization of Design-Expert[®] was used to find the optimum condition of the regression model for the hydrothermal pretreatment.

2.7. Scanning Electron Microscopy (SEM) Analysis

The SEM analysis of biomass was carried out using a JEOL JSM-IT100, Tokyo, Japan, microscope equipped with an energy-dispersive X-ray analyzer. Biomass samples were dried and sputter-coated using gold (22.5 nm thickness) before imaging by a secondary electron detector (SED) under a high vacuum. A constant working distance (WD) of 11 mm and probe current (PC) of 40–50 eV with a variable magnification between 2300 $\times$ and 6000 $\times$ was maintained during the SEM analysis of biomass.

3. Results and Discussion

3.1. Composition of Biomass

Biomass contained 7.94% extractives, 40.77% glucans, and 17.47% xylans. The total lignin content was measured as 31.44%. Biomass contained a low ash content of 1%. Similar glucan (41.8%) and xylan (18.4%) contents were reported previously in the maple wood species [17,47]. Lower lignin and extractives were identified (about 27% and 3% accordingly) whereas a higher ash percentage of 2.3% was measured in the maple wood in those studies [17,47]. In a separate study, Kaakinen et al. [48] found a similar glucan content (41.4%) in the sugar maple while the lignin and extractives were 23.1% and 1.5%, correspondingly, in the biomass. Kaakinen et al. [48] also reported 10.5% hemicellulose in biomass during a composition analysis of sugar maple. The variation in composition analysis of the same biomass species resulted from geographic location, climate, soil conditions, and growth cycles. Variations in harvesting time, storage conditions, and preprocessing methods such as drying or grinding possibly resulted in the different chemical composition of sugar maple biomass.

3.2. Response Surface Model Based on CCD Evaluation

Table 2 shows the estimated regression coefficients of the polynomial model equation (Equation (4)) for each of the responses. The regression coefficient in Table 2 measures the change in response per unit change in process parameter while keeping the other process parameters constant. Coefficients A, B, and C represent the linear regression coefficients (β_i); coefficients AB, AC, and BC represent the interaction regression coefficients (β_{ij}); and A^2 , B^2 , and C^2 represent the quadratic regression coefficients (β_{ii}) of the model equation (Equation (4)). Model intercept (β_0) represents the overall average change in response for all the experimental runs shown in Table 1. The estimated regression coefficients represent the adjustments around the average value based on the variation in process parameters between the maximum and minimum values. The variance inflation factor (VIF) value measures the severity of the correlation of independent pretreatment process parameters. Hasan et al. [49] and Adetoyese et al. [50] reported that a VIF value less than 10 is desirable in the developed quadratic model. Table 2 shows the VIF values less than 10 for all the regression coefficients, meaning that a significant correlation exists between considered

process parameters, hydrothermal pretreatment temperature, time, and solid loading. In addition, the coefficient of determination (R^2), coefficient of variance (CV), and adequate precision values are also included in the same table to show the degree of data fitting and goodness of fit of the model for all three responses. The R^2 values measure the ability of the model to predict variability in response values [51]. A higher R^2 value signifies that the model can accurately predict the correlations between selected independent process parameters [46,52]. The R^2 values achieved in this study are greater than 0.90 for all three responses, meaning that the developed model can explain more than 90% of the variations in glucan, xylan, and overall conversion. The CV measures the estimated errors in responses relative to the mean error, and a desired CV value of less than or around 10% has been reported in previous studies [53,54]. Table 2 shows a CV value of less than 10% for both glucan and overall conversion but it is 12.48% in the case of xylan conversion. It can be said that the xylan conversion model is not that adequate compared to glucan and overall conversion. Adequate precision measures the signal-to-noise ratio, and a higher ratio (greater than 4) is desirable to predict the responses in a better way within the design space [42]. Precision values ranging from 10.98 to 20.88 in this study indicate that the models exhibit an acceptable level of noise, suggesting that the developed models can be reliably used to predict response values within the design space. Table 3 shows the ANOVA data for the models predicting glucan, xylan, and overall conversion. The higher F values of the models indicate the significance of the model. A higher F value for all the models is obtained at lower p values (less than 0.0001). This implies that models are significant with a less than 0.01% probability of resulting in such higher F values due to noise. The linear (X_1 , X_2 , and X_3), interaction (X_1X_2 , X_1X_3 , and X_2X_3), and quadratic (X_1^2 , X_2^2 , and X_3^2) regression parameters are also listed in Table 3 at a p -value less than 0.05. This indicates that all the parameters are significant, but their relative significance to responses varies according to the corresponding F value.

Table 2. Regression coefficient values of the fitted quadratic models for glucan, xylan, and overall conversion.

Factor	Glucan Conversion		Xylan Conversion		Overall Conversion	
	Coefficient	VIF	Coefficient	VIF	Coefficient	VIF
Intercept	84.17	-	86.22	-	82.63	-
A—Temperature	19.54	1.00	2.92	1.00	14.19	1.00
B—Time	3.81	1.00	−0.2094	1.00	2.54	1.00
C—Solid loading	1.41	1.00	0.2450	1.00	1.03	1.00
AB	−2.86	1.00	−10.03	1.00	−4.89	1.00
AC	−1.29	1.00	−3.35	1.00	−1.86	1.00
BC	1.53	1.00	−0.1763	1.00	0.9913	1.00
A ²	−5.69	1.02	−22.43	1.02	−10.44	1.02
B ²	−0.0301	1.02	0.1057	1.02	0.0094	1.02
C ²	−2.83	1.02	−2.59	1.02	−2.69	1.02
R ²	0.97	-	0.91	-	0.94	-
CV (%)	5.68	-	12.48	-	7.30	-
Adequate precision	20.88	-	10.98	-	15.40	-

3.3. Effects of Pretreatment Process Parameters on Glucan Conversion

Table 1 shows the experimental and predicted glucan conversions at different hydrothermal pretreatment conditions while the residual value shows the difference between those two conversions. A lower residual value indicates the ability of the model to predict glucan conversion close to the experimental value. The lowest experimental glucan conversion was calculated as 41.57%, while the lowest predicted glucan conversion was 35.22%. In contrast, the highest experimental and predicted glucan conversions were close to each other (residual value: −1.96). The highest experimental glucan conversion reached in this study was up to 99% under the following hydrothermal pretreatment conditions: 213.64 °C, 15 min, and 15% solid loading. Correspondingly, the glucan concentration in the hydrolysate was 46.29 g/L (Supplementary Table S1). The cellulose conversions

obtained after pretreatment are higher than the cellulose conversions for raw biomass (15.19%, 7.49 g/L glucose). Zhang et al. [17] reported about 90% cellulose conversion from maple wood using dilute oxalic acid pretreatment (0.5 wt%) followed by enzymatic hydrolysis at a higher enzyme loading of 60 FPU/g. Similarly, a comparatively lower cellulose recovery (about 50%) was reported by Brodeur-Campbell et al. [18] during the 0.5 wt% sulfuric acid pretreatment followed by enzymatic hydrolysis of hardwood from aspen. In contrast, a higher cellulose conversion (99%) obtained in this study indicated that combined hot-water and disk-refining pretreatment was effective in reducing biomass recalcitrance and enhancing enzyme accessibility to cellulose for efficient hydrolysis, without the need for any chemicals.

Table 3. ANOVA results of quadratic model for glucan, xylan, and overall conversion.

Source	Glucan Conversion				Xylan Conversion				Overall Conversion			
	Sum of Squares	df	Mean Square	F Value	Sum of Squares	df	Mean Square	F Value	Sum of Squares	df	Mean Square	F Value
Model	6087.74	9	676.42	34.12	8344.19	9	927.13	11.75	4707.95	9	523.11	18.06
X ₁ —Temperature	5215.93	1	5215.93	263.14	116.26	1	116.26	1.47	2748.40	1	2748.40	94.89
X ₂ —Time	198.56	1	198.56	10.02	0.5986	1	0.5986	0.0076	88.14	1	88.14	3.04
X ₃ —Solid loading	27.07	1	27.07	1.37	0.8199	1	0.8199	0.0104	14.58	1	14.58	0.5034
X ₁ X ₂	65.61	1	65.61	3.31	804.61	1	804.61	10.19	191.00	1	191.00	6.59
X ₁ X ₃	13.29	1	13.29	0.6703	89.71	1	89.71	1.14	27.57	1	27.57	0.95
X ₂ X ₃	18.64	1	18.64	0.9402	0.2485	1	0.2485	0.0031	7.86	1	7.86	0.2714
X ₁ ²	465.80	1	465.80	23.50	7247.95	1	7247.95	91.84	1569.64	1	1569.64	54.19
X ₂ ²	0.0131	1	0.0131	0.0007	0.1611	1	0.1611	0.0020	0.0013	1	0.0013	0.0000
X ₃ ²	115.58	1	115.58	5.83	96.55	1	96.55	1.22	104.28	1	104.28	3.60
Residual	198.22	10	19.82		789.22	10	78.92		289.65	10	28.96	
Lack of Fit	193.50	5	38.70	41.02	784.00	5	156.80	150.10	286.76	5	57.35	99.27
Pure Error	4.72	5	0.9435		5.22	5	1.04		2.89	5	0.5777	
Total	6285.96	19			9133.41	19			4997.60	19		

A relative effect of different hydrothermal pretreatment parameters on glucan conversion can be observed in Table 3. Although pretreatment temperature, time, and solid loading have a significant effect on glucan conversion, higher F values of the temperature (263.14) and time (10.02) represent that only those parameters have a significantly higher effect. The effect of all the pretreatment parameters in linear, interaction, and quadratic combinations can be explained by the regression equation (Equation (5)). The regression equation shows that hydrothermal pretreatment temperature, time, and solid loading have positive impacts on glucan conversion. Of those parameters, pretreatment temperature has the highest impact. All the quadratic parameters of the regression equation negatively influence the glucan conversion. Except for pretreatment time and solid loading interaction, other interaction parameters also have negative effects on glucan conversion. Effects of interaction parameters on glucan conversion can be visualized further in the response surface plots shown in Figure 1.

$$Y_{GL} = 84.17 + 19.54 X_1 + 3.81 X_2 + 1.41 X_3 - 2.86 X_1 X_2 - 1.29 X_1 X_3 + 1.53 X_2 X_3 - 5.69 X_1^2 - 0.0301 X_2^2 - 2.83 X_3^2 \quad (5)$$

The response surface graphs shown in Figure 1 illustrate the effects and interactions of hydrothermal pretreatment temperature, time, and solid loading on the glucan conversion. Each response surface graph shows how two pretreatment parameters influence glucan conversion, keeping the other parameter constant. It can be observed from Figure 1a that higher hydrothermal pretreatment temperatures and longer pretreatment times increased the glucan conversion. Pretreatment at a high temperature for a longer time results in a synergistic effect by breaking down the lignin–carbohydrate complex and enhancing the cellulose’s accessibility to enzymes, which leads to higher cellulose conversion [55]. Figure 1b illustrates that increasing pretreatment temperature and solid loading also increased the glucan conversion. A combination of higher temperature and solid loading intensifies biomass disruption, increasing the concentration of solubles and degradation

products, which further accelerates FRB breakdown [55,56]. The interaction between pretreatment time and solid loading in Figure 1c shows that there is an optimal region where the glucose conversion is maximized. Longer pretreatment times and higher solid loading maximize biomass exposure and interaction during hydrothermal pretreatment conditions, promoting more extensive lignin and hemicellulose fractionation [56]. These combined effects significantly boost glucan conversion by over 98% during enzymatic hydrolysis by improving enzyme–substrate interactions and cellulose accessibility.

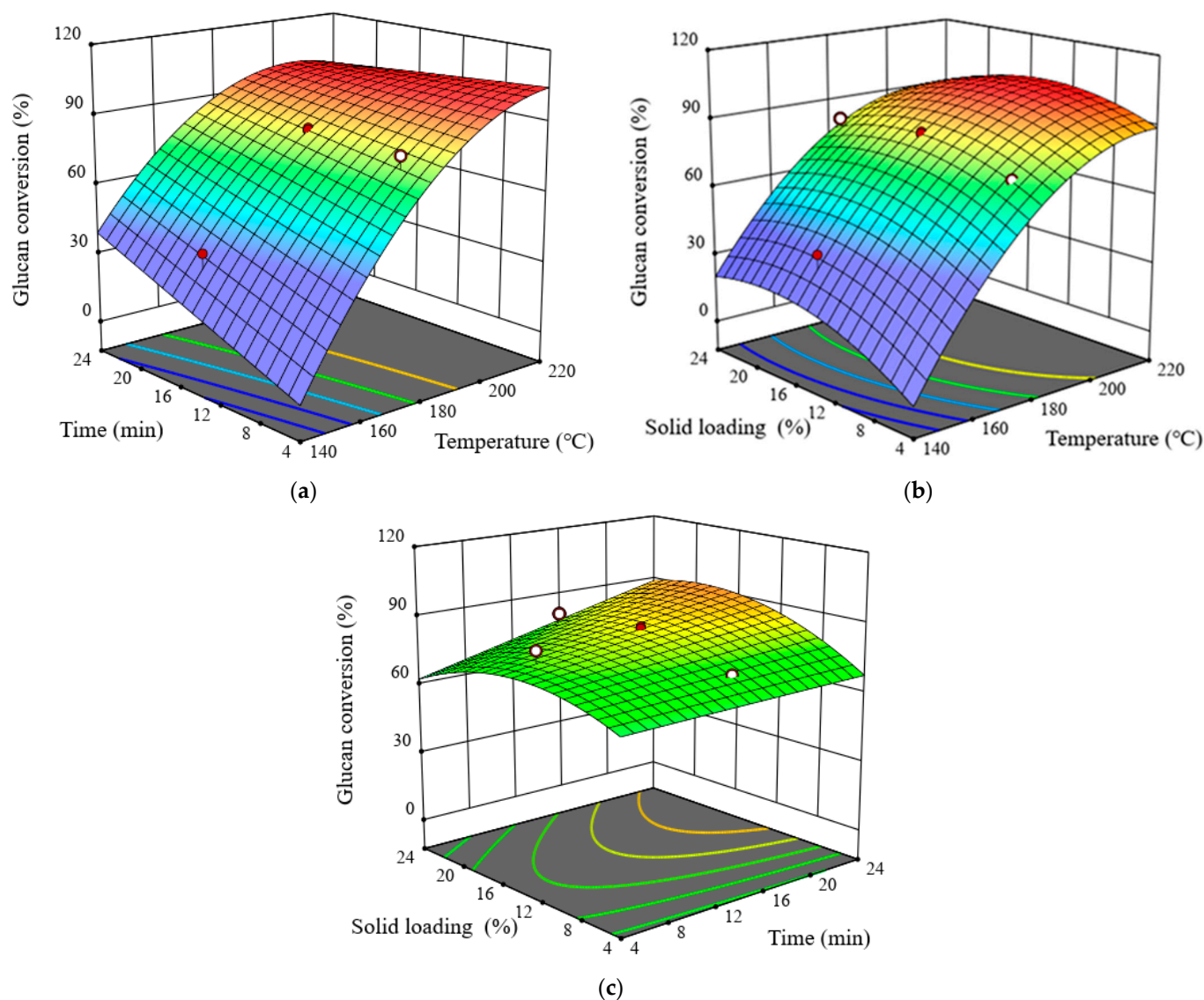


Figure 1. Response surfaces showing the interactions of pretreatment process parameters on glucan conversion: (a) temperature and time, (b) temperature and solid loading, and (c) time and solid loading.

3.4. Effects of Pretreatment Process Parameters on Xylan Conversion

Table 1 shows that experimental xylan conversion varies between 21.08% and 91.76%. In contrast, the developed quadratic model predicts xylan conversion in the range of 17.89–86.87%. It is seen from Table 1 that lower xylan conversions have comparatively higher residual values. This implies that the quadratic model cannot predict the lower xylan conversion values sufficiently, which has already been explained in the model evaluation in Section 3.2. In addition to that, Table 3 shows that the xylan conversion model has a significantly higher “Lack of Fit” value, which might have resulted from the inaccurate

xylan conversion prediction at lower levels. However, there is close agreement between the experimental and predicted xylan conversion values at higher levels. The highest experimental xylan conversion (91.76%) was observed for the FRB pretreated at 180 °C, 6.59 min, and 15% solid loading. Table 3 also shows that hydrothermal pretreatment temperature has the highest contribution, followed by the solid loading and pretreatment time, to xylan conversion. The F value analysis shows that, of the interaction parameters, pretreatment temperature and time interaction parameters have the highest influence while the pretreatment temperature and solid loading interaction also have a significant contribution to the xylan conversion. It can also be said that the quadratic interaction of pretreatment temperature has the biggest contribution to xylan conversion, as indicated by the F value of 91.48, presented in Table 3. Different combinations of independent pretreatment parameters are presented by the regression equation (Equation (6)) for xylan conversion prediction. The regression equation shows that the linear pretreatment parameters such as temperature and solid loading have a positive impact, while the pretreatment time has a negative effect on xylan conversion. Among the quadratic parameters of the hydrothermal pretreatment, temperature has the highest impact, but it contributes negatively to xylan conversion, the same as pretreatment solid loading. All the interaction parameters of the regression equation have a negative impact on xylan conversion. A better representation of the interaction parameters is presented in response surface plots in Figure 2.

$$Y_{XL} = 86.22 + 2.92 X_1 - 0.2094 X_2 + 0.245 X_3 - 10.03 X_1 X_2 - 3.35 X_1 X_3 - 0.1763 X_2 X_3 - 22.43 X_1^2 + 0.1057 X_2^2 - 2.59 X_3^2 \quad (6)$$

Figure 2a shows that xylan conversion increases with the increase in pretreatment temperature up to 180 °C. Further increases in temperature lower the xylan conversion. Similarly, pretreatment time alongside the increased pretreatment temperature reduced the xylan conversion. This can be explained by the accelerated hemicellulose depolymerization around 180 °C and 15 min pretreatment time followed by the sugar degradation under comparatively severe pretreatment conditions. Similar results have been reported in the literature on hot-water pretreatment of other lignocellulosic biomass [23,57]. Hydrothermal pretreatment around and below 180 °C significantly accelerated the hemicellulose depolymerization by releasing acetal groups from β -1-4 xylan backbone linkages [58,59]. Acetyl groups further formed acetic acid which catalyzed the hydrolysis of β -1-4 glycosidic bonds in the hemicellulose backbone. In addition, hydronium ion generation from the ionization of water used in hydrothermal pretreatment enhanced the depolymerization of hemicellulose. In addition, previous studies reported that uronic acid and formic acid contribute to hydronium ion formation, which resulted in accelerated depolymerization of hemicellulose of FRB into soluble sugar [58,60]. However, higher temperatures and time beyond 180 °C produced sugar degradation products such as furans, levulinic acid, and others in pretreatment liquor. Kang X et al. [61] and Jamilah S et al. [62] also reported that xylan degradation product formation starts at a hydrothermal pretreatment temperature of 190 °C. Thus, high pretreatment temperatures and time accelerate xylan degradation product formation, which results in lower xylan conversion values. Figure 2b shows that a gradual increase in both solid loading and pretreatment temperature gradually increases the xylan conversion to about 85% around 180 °C and 15% solid loading. It is also seen that increasing pretreatment severity beyond 180 °C and 15% solid loading can lower the xylan conversion to about 20%. It can be said that (from Figure 2c) the interaction of pretreatment time and solid loading has comparatively less contribution to xylan conversion, which is also seen in the corresponding regression coefficient of Equation (6).

3.5. Effects of Pretreatment Process Parameters on Overall Conversion

The experimental and predicted overall conversion values at different hydrothermal pretreatment conditions are presented in Table 1. The low residual values indicate that the regression model can predict overall conversion closely to the experimental values. A higher R^2 value (0.94) in Table 2 also confirms the degree of a good fit for the overall conversion regression model. The regression equation, relating all the pretreatment parameters under

various linear, interaction, and quadratic combinations, is shown in Equation (7). It is seen that hydrothermal pretreatment temperature, time, and solid loading have a positive impact on overall conversion prediction. Among those parameters, pretreatment temperature has the highest influence as the F value is 94.89 (Table 3). Although the parameters of pretreatment temperature and time interaction (F value 6.59) and temperature and solid loading interaction (F value 0.95) have significant contributions to overall conversion, both interaction parameters impact conversion negatively. Similar negative but significant impacts are observed in the case of the temperature (F value 54.19) and solid loading (F value 3.60) quadratic parameters of the regression equation (Equation (7)) on overall conversion.

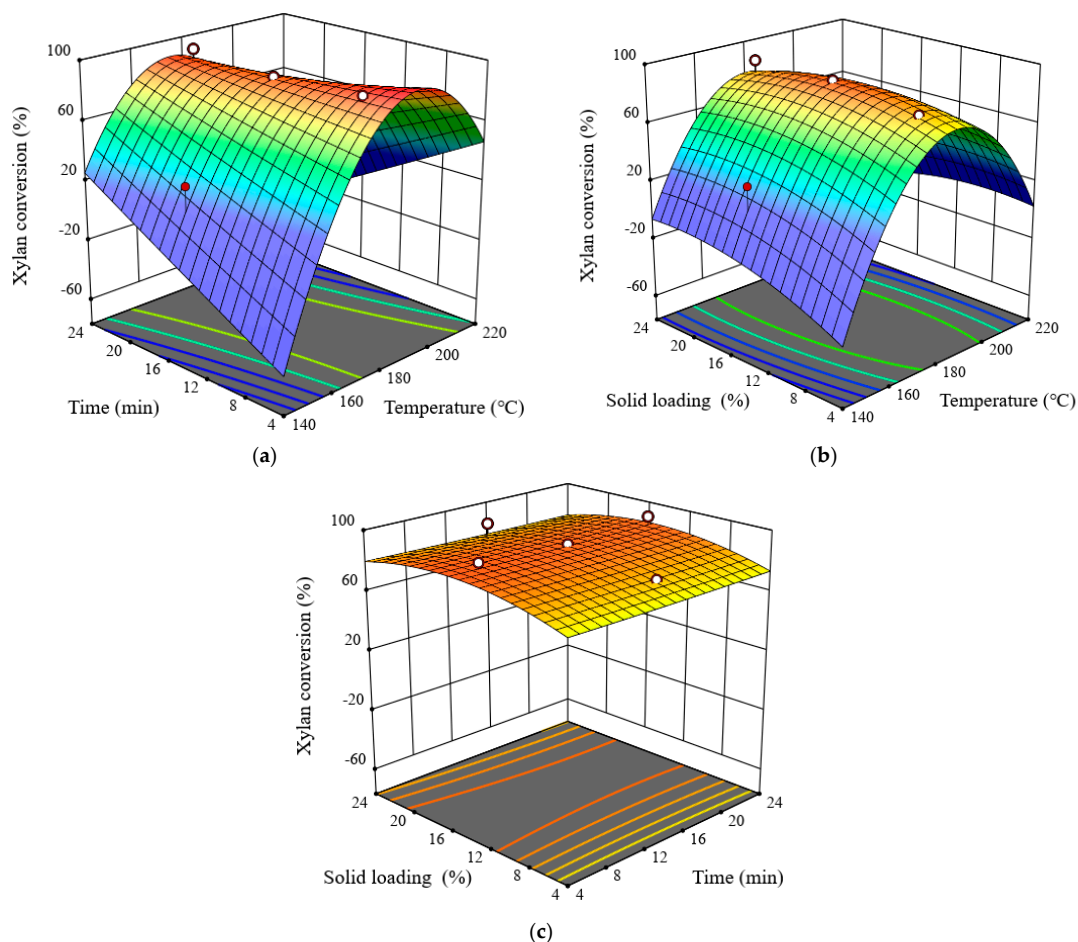


Figure 2. Response surfaces showing the interactions of pretreatment process parameters on xyylan conversion: (a) temperature and time, (b) temperature and solid loading, and (c) time and solid loading.

$$Y_{Ov.} = 82.63 + 14.19 X_1 + 2.54 X_2 + 1.03 X_3 - 4.89 X_1 X_2 - 1.86 X_1 X_3 + 0.9913 X_2 X_3 - 10.44 X_1^2 + 0.0094 X_2^2 - 2.69 X_3^2 \quad (7)$$

Response surface plots illustrating the influence of interaction parameters on overall conversion prediction are presented in Figure 3. Figure 3a shows that overall sugar conversion increases with the increase in pretreatment temperature until 180 °C, where it reaches a steady overall conversion of about 80%. This could be the reason for the partial dissolution of lignin in the hydrothermal pretreatment of FRB. The acidic conditions of hydrothermal pretreatment cleaved the α -o-4 and β -o-4 bonds, which accelerated the partial dissolution of lignin in the pretreatment liquor [63]. At the same time, p-coumaric acid and ferulic acid present in FRB might be hydrolyzed into the pretreatment slurry [64]. Those lignin dissolution features along with the hemicellulose depolymerization through hydrothermal pretreatment enhanced the overall sugar conversion of FRB in enzymatic hydrolysis, which

was reported by H Zabed et al. [65] and N Das et al. [66]. At temperatures above 180 °C, the overall conversion decreases with an increase in pretreatment temperature and time (Figure 3a). Since the overall conversion is calculated from the combination of glucan and xylan conversion, a lower xylan conversion above 180 °C resulted in lower values of overall conversion. A similar trend can be observed in Figure 3b that overall conversion increases to about 80% with the gradual increase in pretreatment temperature and solid loading; however, it reduces to below 70% with the higher pretreatment temperature and solid loading interactions. As discussed earlier in this section, pretreatment time and solid loading interaction parameters have a relatively lower influence on overall conversion, which can be seen in Figure 3c as well.

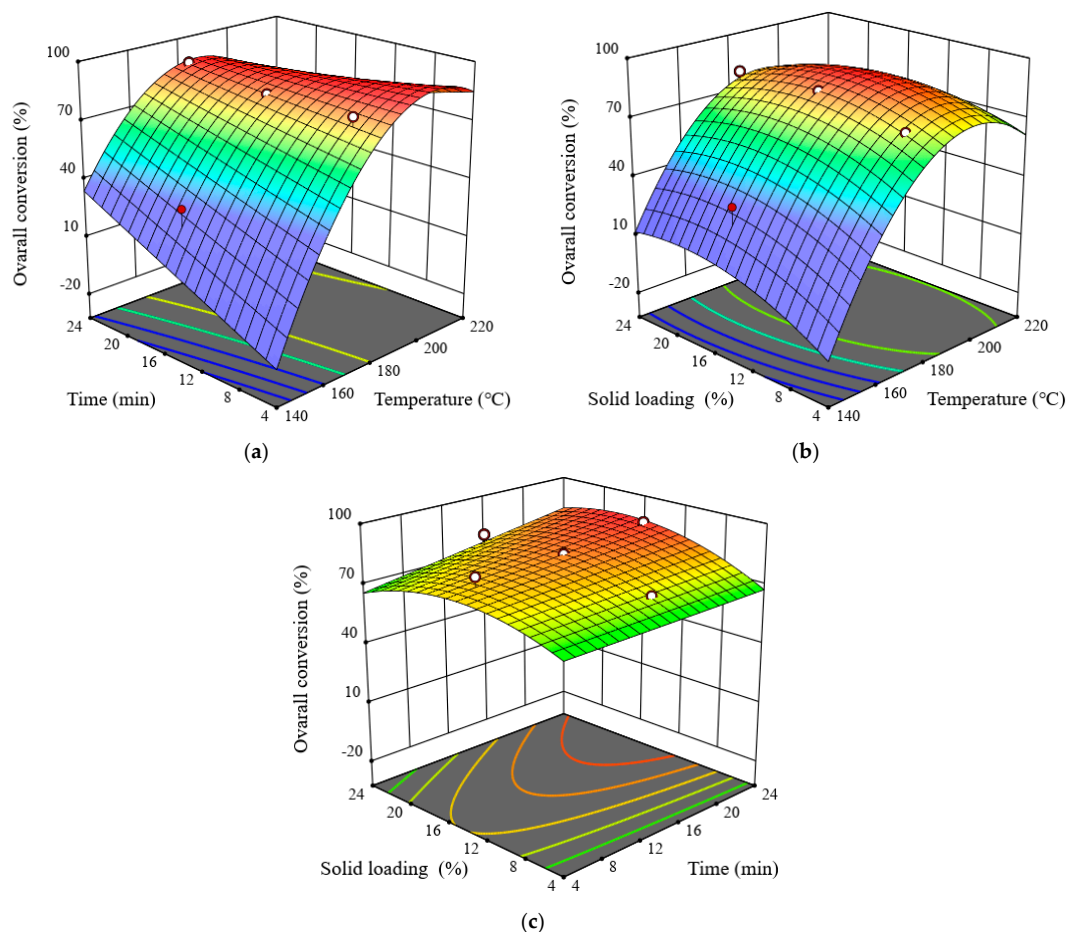


Figure 3. Response surfaces showing the interactions of pretreatment process parameters on overall conversion: (a) temperature and time, (b) temperature and solid loading, and (c) time and solid loading.

3.6. Response Surface Model Optimization

The in-built numerical optimization technique of Design-Expert® v13 was used to optimize the responses. Table 4 shows the specified goals (objectives) of the pretreatment parameters and responses. The highest weight was assigned to glucan conversion followed by overall and xylan conversion during the optimization. The range of the parameters and responses was set from the CCD matrix and RSM study of this work accordingly.

The model-predicted optimum hydrothermal pretreatment conditions with a desirability factor of 0.923 are the following: 194.78 °C, 12.90 min, and 13.42% solid loading. The corresponding glucan, xylan, and overall conversions at those optimum conditions were 94.57%, 79.78%, and 87.84%, respectively. Table 5 shows the experimental glucan, xylan, and overall conversion were 92.43%, 79.07%, and 86.17%, respectively, at those optimum

conditions. A close agreement between model-predicted and experimental conversion values was observed, which validated the developed model for hydrothermal pretreatment of FRB. This also enables the model to accurately predict the biomass pretreatment process parameter interactions for sugar yield analysis from enzymatic hydrolysis and consecutive conversions.

Table 4. Numerical optimization criteria for process parameters and responses.

Parameters and Responses	Goal	Lower Limit	Upper Limit
Temperature (°C)	In range	146	214
Time (min)	In range	6	24
Solid loading (%)	In range	6	24
Glucan conversion (%)	Maximize	41.57	99.0
Xylan conversion (%)	Maximize	21.08	91.92
Overall conversion (%)	Maximize	38.34	87.79

Table 5. Experimental and model-predicted biomass conversion values at optimum pretreatment conditions.

	Glucan Conversion (%)	Xylan Conversion (%)	Overall Conversion (%)
Model predicted values	94.57	79.78	87.84
Experimental values	92.43	79.07	86.17

3.7. Inhibitor Formation

As discussed earlier in the manuscript, biomass pretreatment results in the formation of degradation products, such as acetic acid and furfural, which are inhibitory to the microbes during subsequent fermentation. Although hot-water pretreatment generates relatively lower amounts of these compounds compared to chemical pretreatment, the values can increase under severe conditions. This study investigated 20 pretreatment conditions. Instead of showing inhibitor generation in all those conditions, three conditions were selected to represent low (L)-, medium (M)-, and high (H)-severity pretreatment, and the results are presented in Figure 4. It can be observed that with an increase in pretreatment severity, the generation of inhibitors also increases. A similar trend has been reported in previous studies that conducted hot-water pretreatment of lignocellulosic biomass [67,68]. Previous studies also concluded that the cellulosic fraction of lignocellulosic biomass remains mostly intact during hydrothermal pretreatment, so significant 5-hydroxymethylfurfural (HMF) and levulinic acid generation was not observed [41].

It can be observed from Figure 4 that furfural generation started at a pretreatment temperature of 180 °C, which was increased considerably to 2.59 g/L at 200 °C. In a study on combined hot-water pretreatment and disk refining of sugarcane bagasse, Wang et al. [22] reported that no furfural was detected at pretreatment temperatures of 140 °C and 160 °C, but a significant amount (1.42 g/L) was observed at a pretreatment temperature of 200 °C. The inhibition levels of furfural depend on the type of fermentation and microbes used in the process. Studies have reported that during bioethanol production, furfural inhibition on yeast species starts at about 6 g/L, and the values observed in the current study are well below the inhibition limit [41,69]. Similarly, the inhibition effect of acetic acid on yeast performance starts at about 5 g/L [70,71]. Figure 4 shows that the higher-severity pretreatment condition has acetic acid content above the inhibition limit, while the medium- and lower-severity pretreatment conditions have that far below the inhibition concentration.

The SEM images (Figure 5) also support the inhibitor generation in low-, medium-, and higher-severity hydrothermal pretreatment conditions. Carbohydrate fibers, mostly hemicellulose, were destroyed to some extent during the low-severity pretreatment conditions, resulting in inhibitor generation in low concentrations. Figure 5b shows that a significant number of carbohydrate fibers (presumably hemicellulose) are destructed in the medium-severity pretreatment. In contrast, destruction of hemicellulose fibers is

observed in high-severity pretreatment conditions, which might be the reason for higher inhibitor generation.

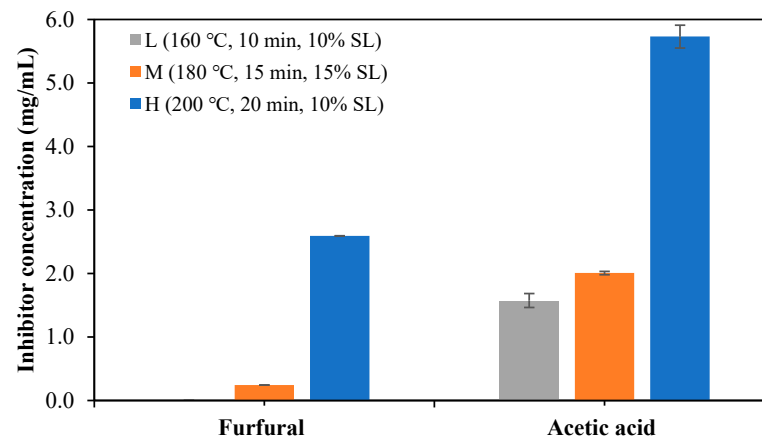


Figure 4. Inhibitor generation at various hydrothermal pretreatment conditions. (L—low-severity pretreatment, M—medium-severity pretreatment, and H—high-severity pretreatment).

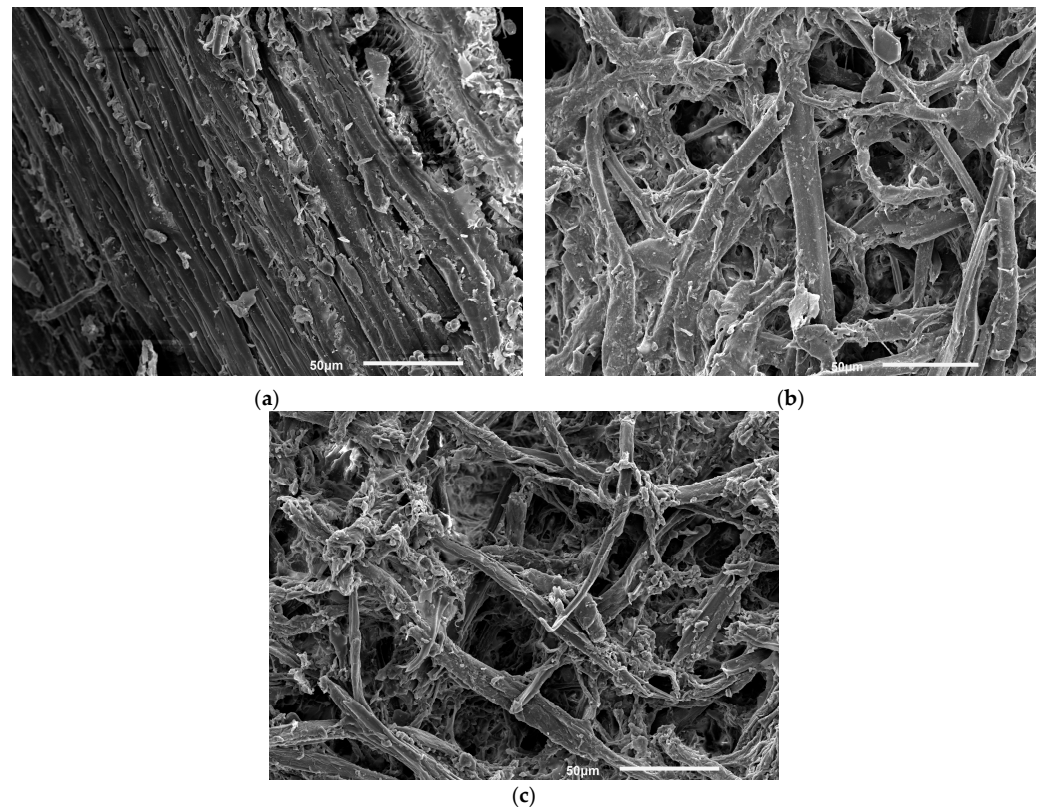


Figure 5. Structural deformation analysis of FRB under (a) low-, (b) medium-, and (c) high-severity pretreatment conditions using SEM analysis at 2300–6000 $\times$ magnification.

4. Conclusions

A two-step hydrothermal and mechanical refining pretreatment was investigated to enhance the enzymatic hydrolysis of forest residue biomass. Three hydrothermal pretreatment conditions, temperature, time, and solid loading, were varied and optimized by RSM based on the CCD matrix. Statistical analysis showed that developed regression models have a good degree of fit ($R^2 > 0.90$) for glucan and overall conversion prediction with lower CV (<10) and higher adequate precision values (>4), but the xylan conversion model showed less capability to predict lower conversion values. It is also seen that hydrothermal

pretreatment process parameters such as temperature, time, and solid loading have a significantly positive impact on conversions, except that pretreatment temperature and time negatively affect xylan conversion. The pretreatment was found to be highly effective and resulted in several-fold higher sugar production compared to raw biomass. The maximum glucan conversion of 99% was achieved for the biomass pretreated at 213.64 °C, 15 min, and 15% solid loading, followed by three cycles of disk milling. On the contrary, comparatively less-severe hydrothermal pretreatment conditions at 180 °C, 6.59 min, and 15% solid loading resulted in the highest xylan conversion of 91.76%. The highest overall conversion of 87.79% was achieved for the biomass pretreated at 180 °C, 23.41 min, and 15% solid loading. Based on the process optimization, the optimum hydrothermal pretreatment conditions were found to be 194.78 °C, 12.90 min, and 13.42% solid loading, where glucan, xylan, and overall conversion were predicted to be 94.57%, 79.78%, and 87.84%, respectively, with a desirability factor greater than 0.92. The experimental glucan, xylan, and overall conversions were found to be 92.43%, 79.07%, and 86.17%, accordingly, at the optimum pretreatment conditions, validating the model-predicted conversion values. This demonstrates the effectiveness of the developed second-order polynomial model in predicting sugar yields and conversion efficiency during the pretreatment process. Although hydrothermal–mechanical refining pretreatment has been found to be highly effective in enhancing sugar yields, commercial feasibility and environmental impacts have not yet been assessed, which will be analyzed in future studies through techno-economic analysis (TEA) and life-cycle assessment (LCA).

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/en17194929/s1>: Table S1: Glucan, xylan, and overall conversion calculation at different hydrothermal pretreatment conditions.

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Nomenclature

The abbreviations used in this text are detailed below:

FRB	Forest residue biomass
RSM	Response surface methodology
CCD	Central composite design
LAPs	Laboratory Analytical Procedures
NREL	National Renewable Energy Laboratory
HPLC	High-performance liquid chromatography
UV-Vis spectroscopy	Ultraviolet–visible (UV-Vis) spectroscopy
SEM	Scanning electron microscopy
SED	Secondary electron detector
WD	Working distance
PC	Probe current
ANOVA	Analysis of variance
VIF	Variance inflation factor
CV	Coefficient of variance
L	Low
M	Medium

H	High
HMF	5-hydroxymethylfurfural
TEA	Techno-economic analysis
LCA	Life-cycle assessment

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