

Article

# Saccharification Yield through Enzymatic Hydrolysis of the Steam-Exploded Pinewood

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**Abstract:** Pressure, temperature, and retention time are the most studied parameters in steam explosion pretreatment. However, this work aimed to fix these parameters and to evaluate the influences of several less investigated steam explosion parameters on the saccharification yield in hydrolysis. In this study, firstly, pinewood samples smaller than 200  $\mu\text{m}$  were treated with steam explosion at 190  $^{\circ}\text{C}$  for 10 min. The variable parameters were biomass loading,  $\text{N}_2$  pressure, and release time. Steam-exploded samples were hydrolyzed with the *Trichoderma reesei* enzyme for saccharification for 72 h. The sugar content of the resultant products was analyzed to estimate the yield of sugars (such as glucose, xylose, galactose, mannose, and arabinose). The best glucose yield in the pulp was achieved with 4 g of sample,  $\text{N}_2$  pressure of 0.44 MPa, and short release time (22 s). These conditions gave a glucose yield of 97.72% in the pulp, and the xylose, mannose, galactose, and arabinose yields in the liquid fraction were found to be 85.59%, 87.76%, 86.43%, and 90.3%, respectively.

**Keywords:** lignocellulosic biomass; hydrothermal pretreatment; enzymatic hydrolysis; sugar yield; high-performance liquid chromatography (HPLC) analysis

## 1. Introduction

Recently, attention on biorefineries has increased dramatically because of the environmental and economic impacts of fossil sources. Biorefineries use biomass as a feedstock to produce biofuels, biopower, biomaterials, and biochemicals instead of fossil sources [1,2]. Biorefineries can use a wide variety of biomasses, from microalgae to wood. Biorefineries provide flexibility in terms of both feedstock and resultant products, i.e., the different feedstocks that can be used and the different products that can be produced in one biorefinery [3,4]. Biorefineries that use wood as feedstock are called lignocellulosic feedstock biorefineries (LCFs). LCFs are one of the primary alternative candidates to fossil-based biorefineries due to their wide availability, easy accessibility, low cost, and the diverse nature of their feedstock [5]. Lignocellulosic biomass contains mainly cellulose (25–50%), hemicellulose (15–30%), and lignin (10–35%) [6–8]. Cellulose is the leading sugar source for lignocellulosic bioethanol production. Whereas cellulose has polymeric glucan chains that contain glucose monomers, hemicellulose has polymeric structures that are formed from hexoses and pentose, and it may involve sugar acids [6,9]. Lignin, a chemical compound that provides rigidity to plants, is formed from phenolic monomers [6]. Its higher lignin ratio makes the lignocellulose structure more rigid, and the recalcitrant structure of lignocellulosic biomass makes bioconversion more complicated compared to that of first-generation bioethanol feedstocks [10]. Despite its potential, its recalcitrant

structure is a significant barrier to the commercialization of lignocellulosic bioethanol. Therefore, pretreatment methods should be applied to eliminate these barriers.

The main objectives of pretreatment methods can be summarized as breaking the structure of lignocellulose, reducing the crystalline structure of cellulose, increasing the porosity of the surface of the lignocellulosic feedstock, increasing the surface area, and providing enzyme accessibility in the cellulose [11,12]. The cost of lignocellulosic biomass pretreatment accounts for nearly 33% of the total bioethanol production cost [13]. Therefore, the application of efficient and less chemical and energy-dependent pretreatment methods will decrease the total cost of bioethanol production.

Steam explosion, a commonly used physicochemical pretreatment technique, is a hydrothermal method in which lignocellulosic biomass is treated with high-pressure saturated steam, and later, the pressure is suddenly dropped [13]. Therefore, it causes explosive decompression of the structure of lignocellulosic biomass. In the steam explosion method, both physical forces and chemical processes are applied to lignocellulosic biomass [14]. The presence of water creates an acidity effect in the medium, and a sudden pressure drop causes the separation of fibers. It quickly provides enzyme accessibility to the cellulose structure. A liquid fraction and pulp are formed during steam explosion pretreatment. Whereas the liquid fraction contains mainly sugars from the hemicellulose structure, the pulp contains both cellulose and lignin. The pretreatment temperature and pressure are usually set between 160 and 260 °C, and 0.69 and 4.83 MPa, respectively [15]. Higher temperatures could cause hemicellulose degradation and lignin transformation. The retention time differs from seconds to minutes, and later, the pressure is suddenly decreased to atmospheric pressure. The main benefits of steam explosion pretreatment can be summarized as significantly lower environmental effects, a lower capital investment cost, lower usage of hazardous chemicals, and the opportunity to operate under moderate conditions to produce a higher saccharification yield [16]. In addition, the steam explosion process causes a reduction in energy consumption compared with conventional physical pretreatment methods to obtain a similar particle size reduction [17,18]. However, the main disadvantage of the steam explosion process is the production of some inhibitory compounds, such as furfural and 5-Hydroxymethylfurfural (HMF), which result from the degradation of sugars during pretreatment. These compounds decrease the efficiency of enzymatic hydrolysis and fermentation [16,19].

Hydrolysis breaks the polymeric cellulose structure and produces glucose monomers. After the hydrolysis of lignocellulosic feedstock, the hexose sugar yield can range from 0.5 to 0.75 g/g cellulose [20]. The saccharification yield indicates the hydrolysis yield for bioethanol production. Enzymatic hydrolysis is performed with the cellulase enzyme, which is commonly produced by soft-rot fungi such as *Trichoderma* sp., *Penicillium* sp., and *Aspergillus* sp. [20,21].

In steam explosion studies, mostly, reaction temperature and retention time have been investigated. Alvira et al. (2016) examined different reaction temperatures and retention times on the sugar yield for wheat straw. The highest glucose and xylose yield were found to be 97.9% and 91.1% at 190 °C reaction temperature for a ten-minute retention time, respectively. It was indicated that at this pretreatment temperature and retention time, sugar degradation and toxic formation were decreased [17]. Simangunsong et al. (2020) pretreated beech wood with steam explosion at 150, 170, 190, and 210 °C for 2.5, 5, 10, and 15 min. They stated that the highest sugar recovery was achieved when the severity factor was 3.65 (190 °C and 10 min) [22]. Bondesson and Galbe (2016) found the highest total glucose and xylose yield to be 0.32 g/g biomass at 190 °C and 10 min [23]. Therefore, the reaction temperature and the retention time were selected to be 190 °C and 10 min in this study [17,22,23].

In the literature, no detailed study was found regarding other pretreatment parameters such as biomass loading, N<sub>2</sub> pressure, and release time. Thus, the aim of this work was to fix reaction temperature and retention time and to evaluate the influences of biomass loading, N<sub>2</sub> pressure, and release time on the saccharification yield in hydrolysis.

## 2. Materials and Methods

### 2.1. Feedstock Characterization

In the experimental part of this study, Northern US Pinewood from Moscow/Idaho was used. To analyze saccharification, pinewood was shaved with chopper twice and then sieved. Samples smaller than 200  $\mu\text{m}$  were used in the experiment. All experiments were conducted with the same pine wood timber. The moisture, ash, lignin, and extractive content analysis were conducted according to American Society for Testing and Materials (ASTM) E1756-01, ASTM E1755-01, ASTM E1758-01, and ASTM E 1690, respectively [24–27]. For moisture content analysis, 2 g of sample was weighed accurately and placed in an oven at 104 °C. The oven-dried sample was reweighed every 4 h until a  $\pm 0.1\%$  change in the weight percent solids from the previous measurement was recorded. The difference between the final and initial weight represented the moisture content of the sample [24]. For ash content analysis, a 1 mg sample was measured and placed in a tarred crucible, and crucibles were placed in a muffle furnace at 600 °C for 24 h. The difference between final and initial weight represented the ash content of the sample [25]. For lignin content analysis, a 200 mg sample was weighed and placed in a test tube. Then 2 mL 72% sulfuric acid was added and incubated for 60 min at 30 °C with regular stirring. Primary hydrolysate was then transferred into a 200 mL Erlenmeyer flask with 56 mL of distilled water (final sulfuric acid concentration of *v/v* 4%). The flask was placed in a pressure tube in a water bath for incubating at 30 °C for 60 min. The secondary hydrolysate was filtered through the crucibles and washed with 40 mL of distilled water. The solid fraction (lignin) was oven-dried for one night and weighed. The secondary hydrolysate was used for sugar analysis [26]. The content of individual sugars was analyzed using high-performance liquid chromatography (HPLC) according to the “Analysis of Biomass Sugars Using a Novel HPLC Method”, which is detailed in Section 2.4 [28]. The Soxhlet extraction was used to determine extractive content analysis. Approximately 3 g of the sample was mixed with 150 mL of solvent. Dichloromethane (DCM), ethanol, and water were used as the solvent. The extraction time was 12 h for DCM and 24 h for ethanol and water. Then, extracted solids were washed with fresh ethanol and oven-dried for one night at 40 °C [27]. The chemical composition of untreated pinewood is presented in Table 1.

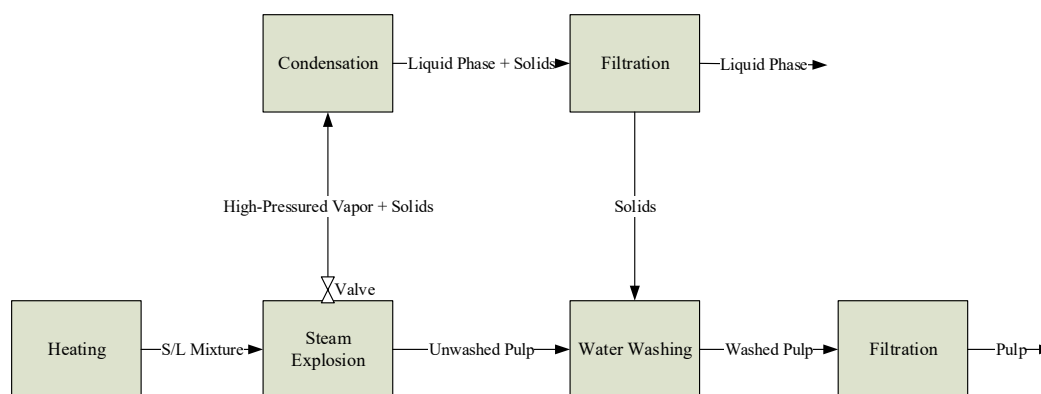
**Table 1.** The chemical composition of untreated pinewood.

| Component   | Content (%) |
|-------------|-------------|
| Glucan      | 26.43       |
| Xylan       | 18.86       |
| Mannan      | 6.45        |
| Galactan    | 3.92        |
| Arabinan    | 1.54        |
| Lignin      | 34.25       |
| Extractives | 6.77        |
| Ash         | 1.78        |

### 2.2. Steam Explosion Pretreatment

A Parr reactor (High-Pressure/High-Temperature reactor, 600 mL, Parr Instrument Company, Moline, IL, USA) was used for steam explosion pretreatment. Figure 1 shows the experiment diagram of the steam explosion pretreatment. First, for solid loading, the sample (4 or 8 g) was measured accurately. Then, it was mixed with 200 mL of deionized water and heated to steam explosion temperature with constant stirring. The hot mixture that included both solid/liquid mixture was then placed in a hydrothermal steel reactor. The reactor was closed tightly, the temperature was set at 190 °C, the mixer speed in the reactor was set to 1000 rpm, and the retention time was 10 min. The pressure of the reactor at 190 °C was measured as being between 1.6 and 1.8 MPa. The pressure in the reactor consisted of the pressure of the water in the sample and the pressure of nitrogen. The valve that releases the gas

inside the hydrothermal reactor was opened suddenly, and the pressure was equalized to atmospheric pressure in 22 or 46 s. The shortest release time of 22 s was selected because sudden pressure drops can damage the moving equipment, such as the mixer in the reactor. The released pressurized gas was collected and condensed in a vessel, and the liquid fraction was obtained. While the gas was releasing, some solid particles passed through the valve due to the high-pressure drop. Thus, filtration was applied to collect the solid particles from the liquid phase. Therefore, solid particles were separated from the liquid phase. For purification of the solid phase, deionized water wash and vacuum filters were applied.



**Figure 1.** The experiment diagram of the steam explosion pretreatment.

In the steam explosion pretreatment method, the variable parameters were the solid loading (g), the presence of N<sub>2</sub> pressure, and the release time, as shown in Table 2. The optimal hemicellulose solubilization and hydrolysis was found at the temperature of 190 °C and the retention time of 10 min [12]. At these conditions, the severity factor ( $R_0$ ) was calculated as 3.65.

**Table 2.** Steam explosion experiment design.

| Experimental Group | Temperature (°C) | Biomass Loading (g) | Nitrogen Pressure (MPa) | Release Time (s) |
|--------------------|------------------|---------------------|-------------------------|------------------|
| S1                 | 190              | 4                   | 0                       | 22               |
| S2                 | 190              | 4                   | 0.44                    | 22               |
| S3                 | 190              | 4                   | 0.44                    | 46               |
| S4                 | 190              | 4                   | 0                       | 46               |
| S5                 | 190              | 8                   | 0.44                    | 46               |
| S6                 | 190              | 8                   | 0                       | 22               |
| S7                 | 190              | 8                   | 0.44                    | 22               |
| S8                 | 190              | 8                   | 0                       | 46               |

A 2<sup>3</sup> experimental design was applied, and eight experiments were conducted. All experiments were conducted in triplicate. Experimental groups were coded based on experimental conditions.

### 2.3. Hydrolysis of Pretreated Samples

In the enzymatic reaction of steam-exploded samples, commercial cellulase (*Trichoderma reesei* ATCC 26921, lyophilized powder, ≥1 unit/mg solid) was used as an enzyme and purchased from Sigma-Aldrich, Merck Group, Darmstadt, Germany. The enzymatic hydrolysis method is described in detail by the analytical procedure for the enzymatic saccharification of lignocellulosic biomass [29]. The enzyme concentration was prepared to be 4 FPU/mL. The enzyme was diluted with 50 mM of citrate buffer solution [30]. Steam-exploded samples of 400 milligrams were placed in individual plastic shaker bath tubes. Then, 40 mL of enzyme solution was added to the samples. The biomass-to-enzyme solution ratio was set at 10:1 (*w/v*). Plastic shaker bath tubes were placed in an orbital shaker bath for 72 h, and the temperature and speed were set at 50 °C and 300 rpm, respectively

## 2.4. Sugar Analysis

For sugar analysis, the sample was cooled to room temperature. Then, 5 mL of filtrate was placed in a test tube, inositol was added, and the solution was stirred. Then, 0.161 g of  $\text{PbCO}_3$  was added and mixed. After centrifuging all samples, the liquid part was transferred to a small ion-exchange cartridge (0.5 mL of both IR 120  $\text{H}^+$  resin and IR 402 OH resin). The supernatant was filtered, and the filtrate was collected in an HPLC vial. To prepare the standard solution, 10 mg of glucose and 5 mg of each sugar were mixed and diluted to 100 mL with DI water. The solution was filtered into samples [28]. HPLC was used to measure the concentrations of sugar monomers such as glucose, xylose, galactose, mannose, and arabinose. For HPLC, the Waters Breeze system and two Rezex RPM columns in series (7.8 mm  $\times$  30 cm, Phenomenex, Torrance, CA, USA) and Waters HPLC (Waters, Milford, MA, USA) columns for carbohydrate were used, on elution with water (mobile phase) with a flow rate of 0.5 mL/min and operated at 90 °C. Thirty-milliliter samples were injected. A refractive index detector (ERC RefractoMax 520, Thermo Scientific, Waltham, MA, USA) was used for the non-chromophoric sugars. Data analysis was conducted using the Breeze software package.

## 3. Results and Discussion

### 3.1. Sugar Analysis

The optimal solid loading (4 or 8 g), presence of  $\text{N}_2$  (0 or 0.44 MPa), and release time (22 and 46 s) for the pretreatment were tested. The lignin content of steam-exploded samples is presented before the HPLC results because the HPLC analysis was conducted using the second filtrate of lignin determination. All samples were analyzed and are shown in Table 3.

**Table 3.** The chemical composition of steam-exploded pulp.

|    | Lignin<br>(w/w, %) | Ash<br>(w/w, %) | Glucose<br>(w/w, %) | Xylose<br>(w/w, %) | Mannose<br>(w/w, %) | Galactose<br>(w/w, %) | Arabinose<br>(w/w, %) |
|----|--------------------|-----------------|---------------------|--------------------|---------------------|-----------------------|-----------------------|
| S1 | 33.2 $\pm$ 0.18    | 3.38 $\pm$ 0.21 | 21.19 $\pm$ 0.10    | 1.41 $\pm$ 0.12    | 0.62 $\pm$ 0.06     | 0.57 $\pm$ 0.05       | 0.02 $\pm$ 0.00       |
| S2 | 33.85 $\pm$ 0.26   | 3.45 $\pm$ 0.26 | 24.85 $\pm$ 0.18    | 1.74 $\pm$ 0.16    | 0.54 $\pm$ 0.04     | 0.31 $\pm$ 0.06       | 0.03 $\pm$ 0.01       |
| S3 | 34.45 $\pm$ 0.22   | 3.64 $\pm$ 0.28 | 24.55 $\pm$ 0.21    | 1.83 $\pm$ 0.12    | 0.58 $\pm$ 0.09     | 0.43 $\pm$ 0.07       | 0.02 $\pm$ 0.01       |
| S4 | 35.3 $\pm$ 0.19    | 4.09 $\pm$ 0.32 | 21.56 $\pm$ 0.06    | 1.50 $\pm$ 0.16    | 0.61 $\pm$ 0.07     | 0.27 $\pm$ 0.08       | 0.03 $\pm$ 0.01       |
| S5 | 36.2 $\pm$ 0.22    | 4.24 $\pm$ 0.24 | 20.81 $\pm$ 0.20    | 1.50 $\pm$ 0.06    | 0.65 $\pm$ 0.06     | 0.51 $\pm$ 0.04       | 0.03 $\pm$ 0.01       |
| S6 | 35.65 $\pm$ 0.20   | 4.18 $\pm$ 0.20 | 19.99 $\pm$ 0.10    | 1.40 $\pm$ 0.10    | 0.76 $\pm$ 0.04     | 0.49 $\pm$ 0.08       | 0.01 $\pm$ 0.01       |
| S7 | 33.6 $\pm$ 0.21    | 4.04 $\pm$ 0.18 | 21.84 $\pm$ 0.16    | 1.70 $\pm$ 0.18    | 0.73 $\pm$ 0.05     | 0.52 $\pm$ 0.08       | 0.03 $\pm$ 0.00       |
| S8 | 33.2 $\pm$ 0.26    | 3.87 $\pm$ 0.23 | 19.06 $\pm$ 0.12    | 1.32 $\pm$ 0.06    | 1.22 $\pm$ 0.16     | 0.45 $\pm$ 0.04       | 0.03 $\pm$ 0.01       |

Data are presented as mean  $\pm$  SD ( $n = 3$ ).

The lignin contents of S1, S2, S3, and S4 were lower than in S5, S6, S7, and S8. This result indicates that the biomass loading affects the lignin content. Higher lignin content can be explained by hemicellulose solubilization in the pulp, lignin condensation in the surface of the biomass fiber, and the formation of pseudo-lignin because of sugar degradation processes [31]. More biomass loading causes cellulose and hemicellulose degradation and produces more inhibitors such as HMF, furfural, and acetic acid. These inhibitors decrease the yield of bioethanol fermentation [32]. It is expected that mostly insoluble cellulose and lignin particles are to be found in the pulp [31]. When the cellulose ratio decreased due to the degradation of cellulose, the lignin ratio in the pulp increased. Therefore, to reduce the cellulose ratio, degradation should be prevented. A smaller biomass loading produced a higher saccharification yield in the enzymatic hydrolysis step. To compare the effects of the biomass loading on steam explosion pretreatment in the pulp, S1, S2, S3, and S4 included 4 g of biomass loading that was pretreated with steam explosion. In contrast, S5, S6, S7, and S8 included 8 g of biomass loading. The S1–S6, S2–S7, S3–S5, and S4–S8 comparisons, in which every parameter except biomass loading was constant, showed that the saccharification yield was higher when 4 g of the steam-exploded sample was used than when 8 g of the steam-exploded sample was used, because the

degradation of components was higher in the latter case. Reducing the solid loading from 70% to 10% decreased the hardness of biomass from 592 to 266 g [33]. According to Lam et al. (2013), reducing biomass hardness results from breaking the crystalline structure of biomass [34]. Balan et al. (2020) tried different biomass loadings (1% and 5%) and evaluated the impact on enzymatic hydrolysis. At the same pretreatment conditions, when higher solid biomass (5%) was applied, glucose and xylose yields were reduced from 88% to 67% and 75% to 59%, respectively [35]. Therefore, lower solid biomass loading caused more breakage of the crystalline structure and higher sugar yield.

S1–S2, S3–S4, S5–S8, and S6–S7 comparisons, in which every parameter except N<sub>2</sub> was constant, were conducted. The addition of N<sub>2</sub> in S2, S3, S5, and S7 was associated with more glucose and xylose recovery than in non-N<sub>2</sub> added samples. N<sub>2</sub> molecules are smaller than water and other gases (CO<sub>2</sub> and SO<sub>2</sub>) that are also used for explosion. Therefore, N<sub>2</sub> penetrates the cells of the biomass, and expands and breaks the cellulosic structure when the pressure drops [36]. When N<sub>2</sub> increases the pressure, the conversion of polymeric to monomeric sugars becomes easier. Stenberg et al. (1998) tested spruce and pine woodchips with steam explosion. SO<sub>2</sub> was added as a catalyst during steam explosion. They applied steam at a temperature of 210 °C and with a residence time of 5.5 min. The total sugar recovery was found to be 42.1%, and only glucose and mannose were detected [37].

For the S1–S4, S2–S3, S5–S7, and S6–S8 comparisons, in which every parameter except the release time was constant, a shorter release time (22 s) (S1, S2, S6, and S7) was associated with a higher saccharification yield than a longer release time (46 s). A sudden pressure drop caused the biomass structure to break easier than a slower pressure drop. Although it has been indicated that the release time varies from seconds to minutes, no studies exist regarding the release time in the literature. The saccharification yields are presented in Figures 2 and 3.

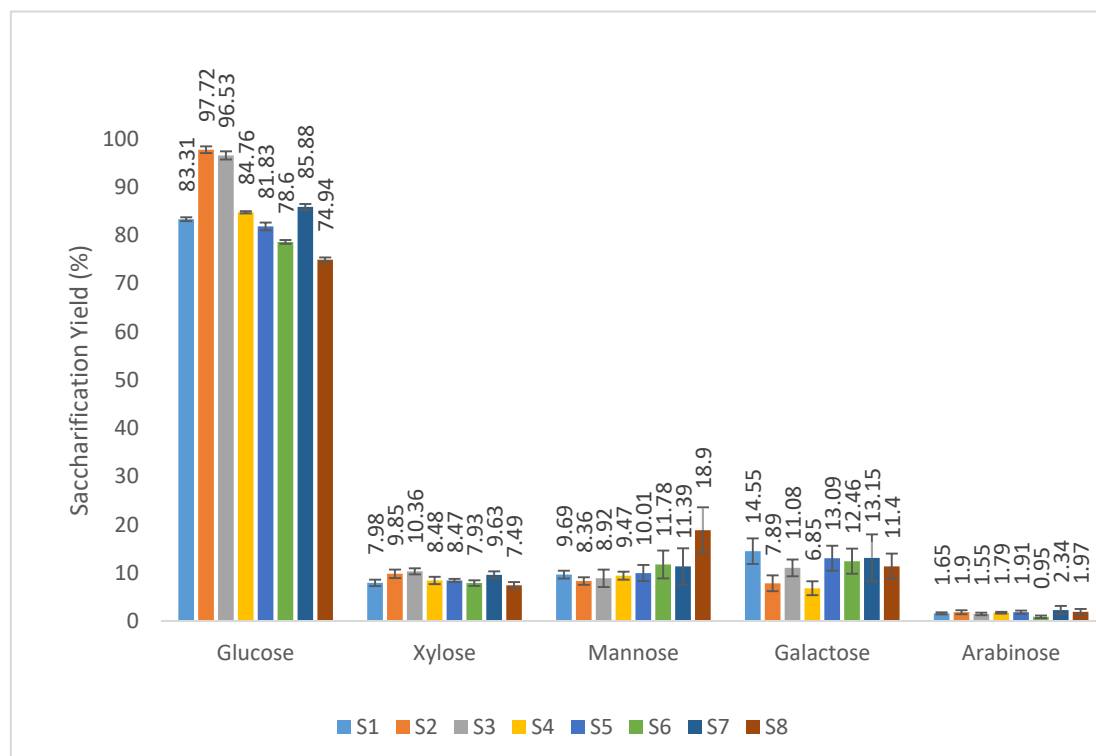
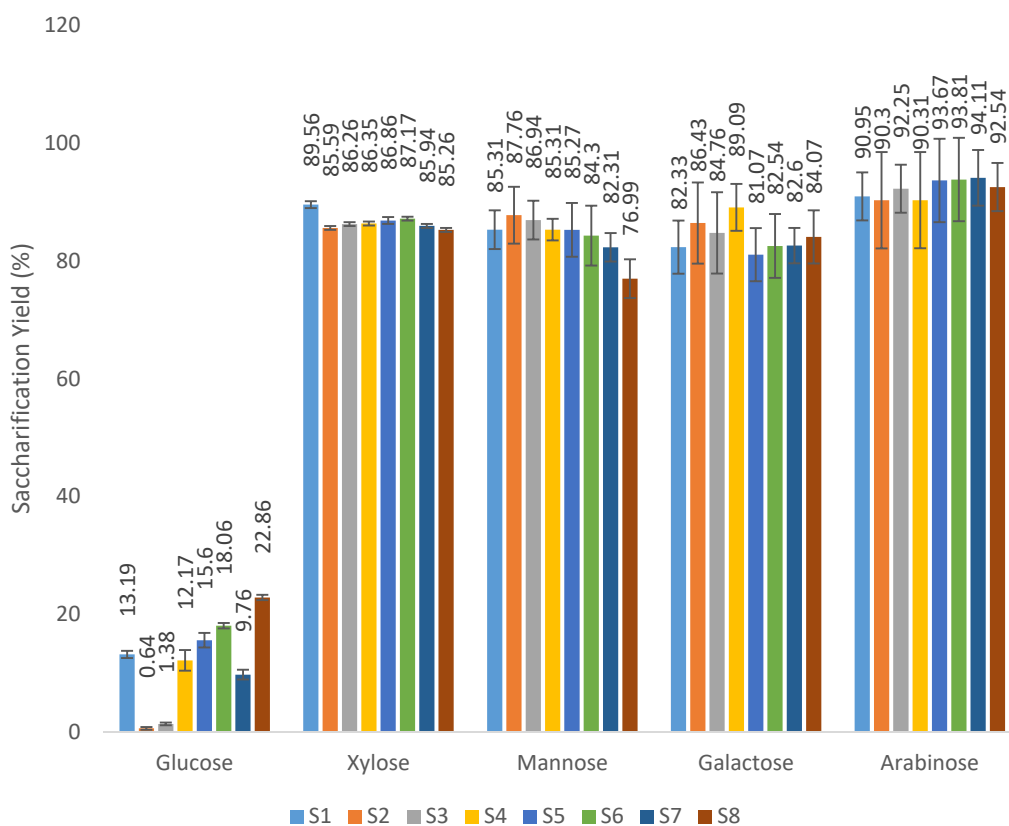


Figure 2. Saccharification yield in pulp.



**Figure 3.** Saccharification yield in liquid fraction.

Figures 2 and 3 show that, under all conditions, the majority of the glucose was found in the pulp, while hemicellulosic sugars were found in the liquid fraction. Thus, these results are applicable to steam explosion. S2 had the maximum glucose yield (97.72%), while S1 had the maximum xylose yield (89.56%). According to the conditions applied in this study, the highest glucose recovery occurred when the temperature was 190 °C, the biomass loading was 4 g, the N<sub>2</sub> pressure was 0.44 MPa, the retention time was 10 min, and the release time was 22 s. In addition, the lowest glucose occurred in the liquid phase at the S2 condition. The maximum xylose recovery was found when the biomass loading was 4 g, the N<sub>2</sub> pressure was 0 MPa, the retention time was 10 min, and the release time was 22 s. Alfani et al. (2000) compared the glucose yield under different steam explosion pretreatment conditions, such as temperature and retention time. They used wheat straw as feedstock, and the best glucose yield was found to be 89% after 48 h of hydrolysis when the steam explosion parameters were a temperature of 217 °C and a retention time of 10 min [38]. Spruce wood was subjected to steam explosion and enzymatic hydrolysis by Pielhop et al. (2016). The maximum glucose yield was found to be 81% at a temperature of 235 °C with a retention time of 10 min [39]. Enzymatic hydrolysis of steam-exploded cotton stalk was tested by Keshav et al. (2016). The steam explosion parameters were set as 200 °C, 5 min, and 4.13 MPa. Then, alkali extraction with 3% NaOH was applied at room temperature for six hours on the steam-exploded cotton stalk. This study only gave the overall saccharification yield (82.13%), instead of the yields of individual sugars such as glucose and xylose [40]. Tabata et al. (2017) used rice husks for steam explosion. Different parameters were tested, and they found that the steam explosion treatment conditions of 3.0 MPa, 235–236 °C, and 3–5 min were optimal for enzymatic saccharification of rice husk. Under these conditions, a maximum glucose yield of 87.7% was achieved [41]. Alvira et al. (2016) examined influences of reaction temperature (170, 180, 190, 200, and 210 °C) and retention time (5, 10, 20, 25, and 30 min) on sugar yield for wheat straw. The maximum glucose and xylose yield were found to be 97.9% and 91.1% at 190 °C reaction temperature for ten-minute retention time, respectively [17]. Another temperature comparison was made by Raud et al.

(2019). They tried 150, 170, 190, and 200 °C. The glucose yield was found to be 3.5, 8, 21, and 24.3 g glucose/100 g biomass, respectively [36]. Guerrero et al. (2017) pretreated agricultural waste of banana trees with steam explosion. They tried different pretreatment temperatures. The results of this study show that glucose and xylose yields were 95.7% and 74.9% at 177 °C, and 90.8% and 75.1% at 198 °C, respectively [42]. Russ et al. (2016) evaluated the steam explosion temperature effects on wheat straw. They tested 175, 195, 215, and 230 °C for 10 min. They found that the highest glucose conversion (97%) at 215 °C. In addition, they indicated that the glucose conversion decreased when the temperature was above 215 °C [43]. Yang et al. (2011) tested the conversion of unwashed steam-exploded corn stover to bioethanol. The natural corn stalk was chipped to a size of 1–2 cm and steam exploded at 1.5 MPa and 210 °C for 10 min. Steam-exploded corn stover was used directly for enzymatic hydrolysis. Therefore, inhibitors did not separate from corn stover. The glucose and xylose yields were found to be 71.48% and 86.66%, respectively. The reason for the low result is the inhibitors found in the raw material during the enzymatic hydrolysis [44]. Dekker and Wallis (1983) indicated that steam explosion for 4 min at 200 °C removed 90% of the xylan present in sugarcane bagasse [45]. In this study, xylan removal from the pulp was calculated to be at least 89.64% (S3) and was a maximum of 92.51% (S8).

Because the main component for bioethanol production is glucose, S2 is the best choice for steam explosion. As shown by the results for S2, when 100 g of pinewood was steam exploded, hydrolyzed with the enzyme, and then fermented, 12.698 g of bioethanol (499 mg bioethanol/g cellulose) was theoretically produced. For this calculation, the theoretical conversion of glucose to bioethanol was accepted as 0.511 g bioethanol/g glucose [46,47]. Raud et al. (2018) investigated nitrogen and flue gas during explosion pretreatment. Their best results were found to be 9.0–9.4 g of ethanol/100 g of biomass [48]. Kumagai et al. (2014) pretreated softwood and hardwood samples with steam explosion and collected 359.3 mg bioethanol/g cellulose (87% of the glucose yield) and 75.4 mg bioethanol/g cellulose (92% of the glucose yield) for Hinoki cypress (softwood) and eucalyptus (hardwood), respectively [49]. Safari et al. (2017) used the pine tree for dilute alkali pretreatment. According to their results, the best result (211.452 mg bioethanol/g biomass) was found with 2% (*w/v*) NaOH at 180 °C [50].

### 3.2. Experimental Design and Statistical Analysis

Central Composite Design (CCD) was used for the optimization of glucose yield in the solid fraction and xylose yield in the liquid fraction. The release time (X1), N<sub>2</sub> pressure (X2), and biomass loading (actual factor) were selected for the independent variables, as shown in Table 4. Glucose yield (R1) and xylose yield were used as the dependent output variables. A statistical package (Design-Expert 12, Stat-Ease, Minneapolis, MN, USA) was used to evaluate CCD.

**Table 4.** The chemical composition of steam-exploded pulp.

| Independent Variables   | Unit | Symbol | Low Level (−1) | High Level (+1) |
|-------------------------|------|--------|----------------|-----------------|
| Retention time          | s    | X1     | 22             | 46              |
| N <sub>2</sub> pressure | MPa  | X2     | 0              | 0.44            |
| Biomass loading         | g    | AF     | 4              | 8               |

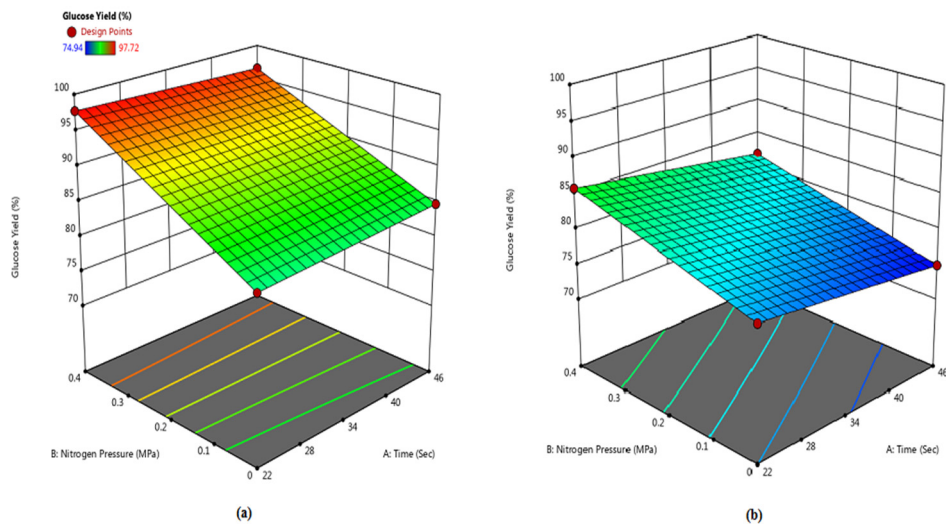
Figures 4 and 5 present the response surface of glucose yield in the solid fraction and xylose yield in the liquid fraction. In both figures, it is clearly seen that lower biomass loading and shorter release time cause higher yield. However, nitrogen pressure increases the glucose in the solid fraction yield while decreases the xylose yield in the liquid fraction. According to response surface results, glucose yield (Equation (1)) and xylose yield (Equation (2)) equations were calculated as below where *A*: release time (s), *B*: nitrogen pressure (MPa), and *C*: biomass loading (g).

$$\begin{aligned} \text{Glucose Yield (\%)} = & 84.398 + 0.203A + 53.103B - 0.405C - 0.158A \times B \\ & + 0.042A \times C - 3.753B \times C \end{aligned} \quad (1)$$

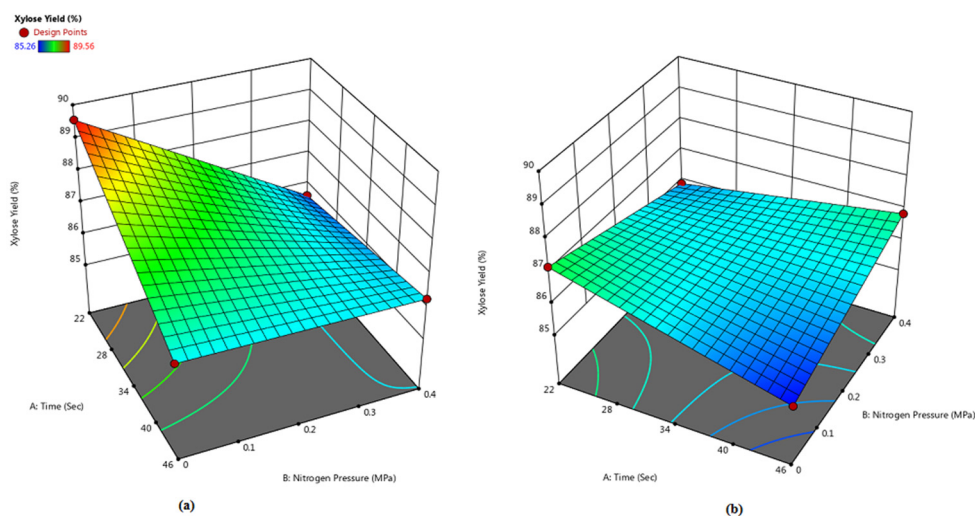


$$\begin{aligned} \text{Xylose Yield (\%)} = & 95.032 - 0.157A - 22.526B - 0.720C + 0.353A \times B \\ & + 0.008A \times C + 1.366B \times C \end{aligned} \quad (2)$$

*t*-Tests were applied between experimental groups for each sugar to determine whether factors such as biomass loading, nitrogen pressure, and release time are important. To understand the importance of biomass loading, S1–S6, S2–S7, S3–S5, and S4–S8 comparisons were conducted. S1–S2, S3–S4, S5–S8, and S6–S7 comparisons were conducted to determine the importance of nitrogen pressure, and S1–S4, S2–S3, S5–S7, and S6–S8 comparisons were performed to determine the significance of the release time. The results indicate significance differences among all experiment groups for glucose ( $p < 0.05$ ). Changes in the biomass loading, nitrogen pressure, and release time affected the glucose yield. While comparing the results, it was found that a lower biomass loading (4 g of the sample instead of 8 g of sample), nitrogen gas addition in the reactor, and a short release time (22 s) increased the glucose yield. However, the results did not indicate any significance differences among experimental groups for hemicellulose (xylose, mannose, galactose, and arabinose) ( $p > 0.05$ ).



**Figure 4.** Response surface of glucose yield in solid fraction (a) 4 g biomass loading (b) 8 g biomass loading.



**Figure 5.** Response surface of xylose yield in solid fraction (a) 4 g biomass loading (b) 8 g biomass loading.

#### 4. Conclusions

In this work the influences of N<sub>2</sub> pressure, biomass loading, and release time during steam explosion pretreatment on the saccharification yield in hydrolysis was evaluated. For the steam explosion pretreatment, particles with a maximum size of 200 µm were used. The solid and liquid phases occurred after the steam explosion. The chemical characterization results show that the solid phase included mainly glucose and the liquid fraction contained mainly hemicellulose. According to the results, the highest glucose yield was found to be 97.72% in the pulp with an N<sub>2</sub> pressure of 0.44 MPa, biomass loading of 4 g, and a release time of 22 s, while the highest xylose yield was found to be 89.56% in the liquid fraction with an N<sub>2</sub> pressure of 0 MPa, biomass loading of 4 g, and a release time of 22 s. These results indicate that lower biomass loading (4 g) and shorter release time have higher glucose and xylose yield than higher biomass loading (8 g) and longer release time (46 s). Although nitrogen pressure increases the yield of glucose in the solid phase, it decreases the yield of xylose in the liquid phase.

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