Valorization of *Bombax ceiba* Waste into Bioethanol Production through Separate Hydrolysis and Fermentation and Simultaneous Saccharification and Fermentation

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Abstract: In this study, Seed pods of *B. ceiba* were used as a novel, cheap, and sustainable feedstock for second-generation bioethanol production. *B. ceiba* waste was pretreated with NaOH under different conditions using a Box–Behnken design (BBD) with three factors and three levels. Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), and scanning electron microscopy (SEM) were used to investigate the chemical, structural, and morphological modifications made by pretreatment. NaOH pretreatment followed by steam was more effective as it offered 60% cellulose and 9% lignin at 10% substrate loading, 5% NaOH conc., and 4 h residence time. Samples with maximum cellulose were employed for ethanol production by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) using indigenously produced cellulase as well as commercial cellulase. HPLC analysis revealed the best saccharification (50.9%) at 24 h and the best ethanol yield (54.51 g/L) at 96 h of fermentation in SSF using commercial cellulose by *Saccharomyces cerevisiae*. SSF offered a better production of bioethanol from seed pods than SHF. The implications of the work support the notion that *B. ceiba* waste could be utilized for large-scale bioethanol production.

Keywords: *B. ceiba*; pretreatment; NaOH; bioethanol; saccharification

1. Introduction

The reserves of non-renewable energy, such as fossil fuels, have been declining day by day because of the growing population and vast industrialization. Serious ecological and environmental issues have appeared due to the immense exploitation of fossil fuels. There is a need to search for an alternative renewable energy source to maintain the growth of society. Ethanol from cellulosic biomass could be a promising substitute for petrol and has good octane, which leads to lessened emissions of air pollutants [1–4]. Previously, sugar-rich biomasses from food crops, e.g., corn and sugarcane, have been employed for bioethanol production. However, the growing demand for bioethanol induced competition for these food crops, resulting in increased prices of food products, and it also disturbed the food chain for animals and humans [5,6]. Lignocellulosic biomass could be an encouraging and non-food feedstock for bioethanol production. Such biomass is fairly low-priced as well as abundantly and locally accessible [7–9].
Cellulose is the major component of lignocellulosic biomass and is considered to be a perpetual source of raw material for green energy. It is the most ubiquitous waste matter and inexhaustible biopolymer from agriculture and is abundantly found in nature [10,11]. It can be obtained from solid waste, remnants of forest and agriculture, and woody and herbaceous crops. Bioethanol production from lignocellulosic material involves three major steps, including pretreatment, saccharification, and fermentation [12–14]. Various physical, chemical, and thermochemical pretreatment techniques have been employed to remove lignin from lignocellulosic biomass and to enhance the accessibility of cellulase enzyme to the cellulosic fibers [2]. Cellulases are hydrolytic enzymes with an important role in the biocconversion of lignocellulosic biomass into fermentable sugars. Numerous microbes, e.g., bacteria and fungi, produce hydrolytic enzymes [15,16]. Bacteria are potential producers of cellulases as they have a higher growth rate compared to fungi. Several bacterial genera, including Bacillus, Cellulomonas, Micrococcus, Cellvibrio, and Pseudomonas sp., have cellulase-degrading abilities [17].

Glucose obtained from saccharified material is converted into ethanol by the fermentation process [18]. The yeast Saccharomyces can convert glucose into ethanol with approximately 90% of the theoretical yield [19]. Separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are the two most common processes used in the fermentation of lignocellulosic hydrolysate. The optimization of the pretreatment processes is required for greater ethanol yield and low production costs. The Box–Behnken design (BBD) of response surface methodology (RSM) was used in this study to optimize pretreatment conditions. RSM is a mathematical and statistical modeling approach that is used to examine the effect of different parameters and their interactions on productivity. Different biotechnological processes are optimized by using this technique. The optimization of conditions by the commonly used one-factor-at-a-time approach is time-consuming and strenuous and may cause inaccurate results in the end. However, RSM delivers the interaction of multiple variables on the response in less time. Therefore, scientists are now using this technique to optimize several process conditions for maximum production [2].

Due to its large size and flaunting flowers, Bombax ceiba is known as King of the Forest (Figure 1). It is a deciduous tree with a straight cylindrical stem and horizontally spreading branches. Its large size, horizontally branching system, and buttress at the base are the first perceived features to distinguish the species in the forest. The tree reaches up to 40 m in height and 2 m in diameter with a vibrant trunk of 24–30 m [20]. It is locally found in tropical regions of western Africa, Southeast Asia, Pakistan, Bangladesh, Sri Lanka, Bhutan, India, Maldives, and Nepal (Aguoru et al., 2015). Its seeds are covered in seed pods that fall on ripening. Seeds are dispersed via silky hairs, whereas seed pods remain as waste. The enormous amount of fallen seed pods is usually found as waste around the B. ceiba tree. We selected this tree as a feedstock because of its abundant, easy, and cheap availability. The use of waste seed pods with good polysaccharide content (25% cellulose, 34% lignin) would be a step toward waste management [21]. The aim of this study was to obtain optimized conditions for the NaOH pretreatment of B. ceiba (seed pods), saccharification with commercial and indigenous cellulase to obtain maximum sugars, and fermentation of sugars for bioethanol production using SSF and SHF.
2. Materials and Methods

2.1. Biomass

*B. ceiba* seed pods were collected, washed, dried, and milled to powder form and kept in plastic bags for further use [2].

2.2. Pretreatment of Biomass

Powdered seed pods were pretreated by the method reported earlier [2]. Substrate (10 g) was soaked in different concentrations of NaOH solution at the ratio of 1:10 (solid:liquid) for different durations at room temperature. Then, the samples were subjected to pressurized heat treatment as per experimental design. After steaming, the samples were filtered, and solid residues were washed up to neutrality. Levels of variables are mentioned in Table 1.

| Independent Variables Symbols | Codes and Their Values |
|-------------------------------|------------------------|
| Concentration of NaOH (%) $X_1$ | $-1$ $3$ $5$ |
| Concentration of *B. ceiba* (%) $X_2$ | $5$ $10$ $15$ |
| Time (h) $X_3$ | $4$ $6$ $8$ |

2.3. Cellulose and Lignin Estimation

Cellulose was analyzed from solid residues (dried in oven) by method described by Gopal and Ranjhan [23]. Then, the samples containing maximum cellulose were estimated for lignin [24].

2.4. Analytical Methods

Total sugar (TS) content was analyzed as described by Dubois et al. [25]. Reducing sugar (RS) was determined by DNS method [26]. Total phenolic (TP) contents found in filtrate, liberated during pretreatment, were estimated by the method of [27].

2.5. Experimental Design

Optimization of NaOH pretreatment conditions was designed through BBD following Ghazanfar et al. [2].

2.6. Substrate Characterization

Treated and untreated substrates were characterized by X-ray diffraction (XRD) [28], thermogravimetric analysis (TGA) [29], Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) [30].
2.7. Saccharification and Fermentation Using SHF and SSF

Raw and pretreated substrates from each pretreatment with maximum cellulose contents were employed for bioethanol production through SSF and SHF, as reported earlier [21].

2.8. Statistical Analysis

The data collected from experiments were statistically analyzed through Minitab software (State College, PA, USA), and ANOVA was also performed by Minitab software [31].

3. Results and Discussion

3.1. Pretreatment

In this study, B. ceiba seed pods were pretreated both chemically and thermochemically with different concentrations of NaOH. An analysis of raw seed pods revealed 34% cellulose and 25% lignin. The liberation of TP indicates the degradation of lignin, and the release of RS and TS indicates the hydrolysis of hemicellulose and cellulose contents in the biomass of B. ceiba. To optimize the pretreatment conditions for maximum lignin breakdown and enhanced cellulose exposure, we applied BBD with three parameters and three levels. Second-order polynomial regression equations (Equations (1)–(8)) were applied to calculate the response (Tables 2 and 3).

Maximum TP (435.9400 mg/mL) liberated and cellulose (46%) exposed were obtained at 3% \((w/v)\) NaOH concentration and 15% \((w/v)\) substrate loading while the residence time was 8 h, whereas the maximum RS (28.0 mg/mL) and total sugars (1563.4 mg/mL) released at a 5% \((w/v)\) NaOH concentration and 15% \((w/v)\) substrate loading while the soaking time was 6 h. Autoclaving proceeded by NaOH pretreatment was found to be more effective, releasing maximum TP (246.8 mg/mL) after 4 h soaking time at a 15% substrate concentration, 3% NaOH solution, and reducing sugars up to 87.44 mg/mL at a 1% NaOH solution, 15% substrate concentration, and 6 h residence time. The highest cellulose content exposed (60%) was obtained at a 10% substrate loading, 5% NaOH solution, and 4 h residence time, and TS liberated were 259.57 mg/mL at a 6 h soaking time, 15% substrate concentration, and 5% NaOH solution during thermochemical pretreatment.

Table 2. Observed values for RS, TS, cellulose, and TP after NaOH pretreatment optimized by Box–Behnken design (BBD).

| Run No. | X₁ | X₂ | X₃ | Cellulose (%) | TS (mg/mL) | RS (mg/mL) | TP (mg/mL) |
|---------|----|----|----|---------------|------------|------------|------------|
| 1       | 5  | 15 | 6  | 32            | 1563.4     | 28         | 239.1      |
| 2       | 1  | 5  | 6  | 36            | 408.27     | 8.85       | 330.7      |
| 3       | 5  | 5  | 6  | 36            | 749.32     | 0.23       | 229.7      |
| 4       | 3  | 5  | 8  | 36            | 684.08     | 0.8        | 291.4      |
| 5       | 1  | 15 | 6  | 45            | 816.21     | 5.91       | 342.7      |
| 6       | 3  | 15 | 8  | 46            | 902.47     | 9.23       | 435.9      |
| 7       | 1  | 10 | 4  | 45            | 640.7      | 1.83       | 103.1      |
| 8       | 3  | 10 | 6  | 36            | 519.29     | 4.23       | 190.8      |
| 9       | 5  | 10 | 8  | 36            | 1066.4     | 7.26       | 106.2      |
| 10      | 3  | 10 | 6  | 39            | 521.6      | 3.31       | 191.6      |
| 11      | 5  | 10 | 4  | 34            | 1090.9     | 9.79       | 62.35      |
| 12      | 3  | 5  | 4  | 40            | 419.71     | 1.14       | 46.65      |
| 13      | 3  | 10 | 6  | 35            | 524.04     | 2.97       | 189        |
| 14      | 1  | 10 | 8  | 44            | 425.34     | 7.77       | 550.6      |
| 15      | 5  | 15 | 4  | 44            | 1412.1     | 14.3       | 26.54      |

Pretreatment condition = room temperature, X₁ = base conc., X₂ = substrate conc., X₃ = retention time, TS = total sugar, RS = reducing sugar, TP = total phenol.
Table 3. Observed values for RS, TS, cellulose, and TP after NaOH + steam pretreatment optimized by Box–Behnken design (BBD).

| Run No. | $X_1$ | $X_2$ | $X_3$ | Cellulose (%) | TS (mg/mL) | RS (mg/mL) | TP (mg/mL) |
|--------|-------|-------|-------|--------------|------------|------------|------------|
| 1      | 5     | 15    | 6     | 54           | 259.5      | 53.5       | 119.3      |
| 2      | 1     | 5     | 6     | 58           | 52.55      | 13.6       | 66.6       |
| 3      | 5     | 5     | 6     | 56           | 131.9      | 8.74       | 47.7       |
| 4      | 3     | 5     | 8     | 60           | 84.43      | 10.2       | 67.7       |
| 5      | 1     | 15    | 6     | 36           | 169.1      | 87.4       | 204.6      |
| 6      | 3     | 15    | 8     | 32           | 156.7      | 63.1       | 186.1      |
| 7      | 1     | 10    | 4     | 52           | 117.3      | 29.4       | 122.7      |
| 8      | 3     | 10    | 6     | 45           | 162.4      | 20.5       | 116.1      |
| 9      | 5     | 10    | 8     | 54           | 180.1      | 17.4       | 108.5      |
| 10     | 3     | 10    | 6     | 46           | 163.3      | 23.6       | 117.7      |
| 11     | 5     | 10    | 4     | 60           | 233.7      | 17.8       | 128.1      |
| 12     | 3     | 5     | 4     | 54           | 55.8       | 6.58       | 41.59      |
| 13     | 3     | 10    | 6     | 47           | 164.1      | 19.6       | 115.3      |
| 14     | 1     | 10    | 8     | 45           | 128.5      | 43.8       | 121.9      |
| 15     | 3     | 15    | 4     | 54           | 223.6      | 59.9       | 246.8      |

Steam conditions: temperature = 121 °C, pressure = 15 Psi, time = 15 min, $X_1$ = base conc., $X_2$ = substrate conc., $X_3$ = retention time, TS = total sugar, RS = reducing sugar, TP = total phenol.

Data were analyzed statistically, and regression equations depicted that the results were significant. The thermochemical pretreatment resulted in a greater breakdown of hemicellulose contents and hence liberated more sugars. The Fisher’s F-test values of 31,568.92, 13.05, 28.84, and 38.21 were noticed for TS, cellulose, RS, and TP, respectively, for chemical pretreatment (Tables 4 and 5). The F-test values were found to be 18.09, 101.29, 231.10, and 4398.87 for TP cellulose, RS, and TS, respectively, in the case of thermochemical treatment (Tables 6 and 8). For the chemical treatment, the $R^2$ values were 100.00%, 87.88%, 98.07%, and 96.65% for TS, cellulose, RS, and TP, respectively. Moreover, the integrity of the model was assisted by adjusted $R^2$ values (81.14%, 100.00%, 95.51%, and 93.30% for cellulose, TS, RS, and TP, respectively). Thus, the validity of the model was revealed from these values. Figures 2 and 3 illustrate contour plots for cellulose, RS, TP, and TS liberated during different pretreatments. These plots depict different ranges of responses by keeping one variable constant and varying the other two variables.

Table 4. ANOVA of cellulose (%) and TS (mg/mL) after NaOH treatment.

| Source       | DF | Adj SS | Adj MS | F-Value | p-Value |
|--------------|----|--------|--------|---------|---------|
| Model        | 9  | 282.517| 31.391 | 6.43    | 0.027   |
| Linear       | 3  | 173.250| 57.750 | 11.83   | 0.010   |
| $X_1$        | 1  | 128.000| 128.000| 26.21   | 0.004   |
| $X_2$        | 1  | 45.125 | 45.125 | 9.24    | 0.029   |
| $X_3$        | 1  | 0.125  | 0.125  | 0.03    | 0.879   |
| Square       | 3  | 55.767 | 18.589 | 3.81    | 0.092   |
| $X_1^2$      | 1  | 1.256  | 1.256  | 0.26    | 0.634   |
| $X_2^2$      | 1  | 5.026  | 5.026  | 1.03    | 0.357   |
| $X_3^2$      | 1  | 49.641 | 49.641 | 10.17   | 0.024   |
| 2-Way Interaction | 3  | 53.500 | 17.833 | 3.65    | 0.099   |
| $X_1*X_2$    | 1  | 42.250 | 42.250 | 8.65    | 0.032   |
| $X_1*X_3$    | 1  | 2.250  | 2.250  | 0.46    | 0.527   |
| $X_2*X_3$    | 1  | 9.000  | 9.000  | 1.84    | 0.233   |
| Error        | 5  | 24.417 | 4.883  |         |         |
| Lack-of-Fit  | 3  | 15.750 | 5.250  | 1.21    | 0.482   |
| Pure Error   | 2  | 8.667  | 4.333  |         |         |
| Total        | 14 | 306.933|        |         |         |
Table 4. Cont.

| Source      | DF | Adj SS   | Adj MS   | F-Value | p-Value |
|-------------|----|----------|----------|---------|---------|
| Model       | 9  | 1,831,767| 203,530  | 31,568.92| 0.000   |
| Linear      | 3  | 1,363,114| 454,371  | 70,476.32| 0.000   |
| X₁          | 1  | 593,903  | 593,903  | 92,118.65| 0.000   |
| X₂          | 1  | 739,802  | 739,802  | 114,748.72| 0.000   |
| X₃          | 1  | 29,409   | 29,409   | 4561.58  | 0.000   |
| Square      | 3  | 268,561  | 89,520   | 13,885.27| 0.000   |
| X₁²         | 1  | 91,000   | 91,000   | 14,114.81| 0.000   |
| X₂²         | 1  | 156,204  | 156,204  | 24,228.44| 0.000   |
| X₃²         | 1  | 59,779   | 59,779   | 9272.11  | 0.000   |
| 2-Way Interaction | 3  | 200,091  | 66,697   | 10,345.18| 0.000   |
| X₁*X₂       | 1  | 41,252   | 41,252   | 6398.43  | 0.000   |
| X₁*X₃       | 1  | 9105     | 9105     | 1412.25  | 0.000   |
| X₂*X₃       | 1  | 149,734  | 149,734  | 23,224.86| 0.000   |
| Error       | 5  | 32       |          | 6        | 0.476   |
| Lack-of-Fit | 3  | 21       | 7        | 1.24     | 0.000   |
| Pure Error  | 2  | 11       | 6        |          |         |
| Total       | 14 | 1,831,799|      |          |         |

TS (mg/mL) = base conc., X₂ = substrate conc., X₃ = retention time, TS = total sugars.

Table 5. ANOVA of TP (mg/mL) and RS after NaOH treatment.

| Source      | DF | Adj SS   | Adj MS   | F-Value | p-Value |
|-------------|----|----------|----------|---------|---------|
| Model       | 9  | 295,775  | 32,864   | 11.35   | 0.008   |
| Linear      | 3  | 226,136  | 75,379   | 26.03   | 0.002   |
| X₁          | 1  | 59,469   | 59,469   | 20.54   | 0.006   |
| X₂          | 1  | 2656     | 2657     | 0.92    | 0.382   |
| X₃          | 1  | 164,010  | 164,010  | 56.64   | 0.001   |
| Square      | 3  | 22,130   | 7377     | 2.55    | 0.169   |
| X₁²         | 1  | 9328     | 9328     | 3.22    | 0.133   |
| X₂²         | 1  | 7418     | 7418     | 2.56    | 0.170   |
| X₃²         | 1  | 4566     | 4566     | 1.58    | 0.265   |
| 2-Way Interaction | 3  | 47,509   | 15,836   | 5.47    | 0.049   |
| X₁*X₂       | 1  | 2        | 2        | 0.00    | 0.982   |
| X₁*X₃       | 1  | 40,733   | 40,733   | 14.07   | 0.013   |
| X₂*X₃       | 1  | 6774     | 6774     | 2.34    | 0.187   |
| Error       | 5  | 14,479   | 2896     |          |         |
| Lack-of-Fit | 3  | 14,476   | 4825     | 2721.01 | 0.000   |
| Pure Error  | 2  | 4        | 2        |          |         |
| Total       | 14 | 310,255  |          |          |         |

TP (mg/mL) = base conc., X₂ = substrate conc., X₃ = retention time, TP = total phenol, RS = reducing sugar.

| Source      | DF | Adj SS   | Adj MS   | F-Value | p-Value |
|-------------|----|----------|----------|---------|---------|
| Model       | 9  | 678,748  | 75,416   | 22.97   | 0.002   |
| Linear      | 3  | 324,558  | 108,186  | 32.95   | 0.001   |
| X₁          | 1  | 54,706   | 54,706   | 16.66   | 0.010   |
| X₂          | 1  | 269,352  | 269,352  | 82.04   | 0.000   |
| X₃          | 1  | 0.500    | 0.500    | 0.15    | 0.712   |
| Square      | 3  | 94,885   | 31,628   | 9.63    | 0.016   |
| X₁²         | 1  | 52,467   | 52,467   | 15.98   | 0.010   |
| X₂²         | 1  | 44,576   | 44,576   | 13.58   | 0.014   |
| X₃²         | 1  | 1.376    | 1.376    | 0.42    | 0.546   |
| 2-Way Interaction | 3  | 259,304  | 86,435   | 26.33   | 0.002   |
| X₁*X₂       | 1  | 235,776  | 235,776  | 71.81   | 0.000   |
| X₁*X₃       | 1  | 17,935   | 17,935   | 5.46    | 0.067   |
| X₂*X₃       | 1  | 5,993    | 5,993    | 1.70    | 0.249   |
| Error       | 5  | 16,416   | 3,283    |          |         |
| Lack-of-Fit | 3  | 15,566   | 5,189    | 12.21   | 0.077   |
| Pure Error  | 2  | 0.850    | 0.425    |          |         |
| Total       | 14 | 695,164  |          |          |         |

RS (mg/mL) = base conc., X₂ = substrate conc., X₃ = retention time, TP = total phenol, RS = reducing sugar.

X₁ = base conc., X₂ = substrate conc., X₃ = retention time, TS = total sugars.
### Table 6. ANOVA of cellulose (%) and TS (mg/mL) after NaOH + steam pretreatment.

| Source                        | DF | Adj SS  | Adj MS  | F-Value | p-Value |
|-------------------------------|----|---------|---------|---------|---------|
| Model                        | 9  | 957.15  | 106.350 | 101.29  | 0.000   |
| Linear                       | 3  | 579.250 | 193.083 | 183.89  | 0.000   |
| $X_1$                        | 1  | 136.125 | 136.125 | 129.64  | 0.000   |
| $X_2$                        | 1  | 338.000 | 338.000 | 321.90  | 0.000   |
| $X_3$                        | 1  | 105.125 | 105.125 | 100.72  | 0.000   |
| Square                       | 3  | 81.650  | 27.217  | 25.92   | 0.002   |
| $X_1^2$                      | 1  | 55.442  | 55.442  | 52.80   | 0.001   |
| $X_2^2$                      | 1  | 4.673   | 4.673   | 4.45    | 0.089   |
| $X_3^2$                      | 1  | 30.519  | 30.519  | 29.07   | 0.003   |
| 2-Way Interaction            | 3  | 296.250 | 98.750  | 94.05   | 0.000   |
| $X_1^1X_2$                   | 1  | 100.000 | 100.000 | 95.24   | 0.000   |
| $X_1^1X_3$                   | 1  | 0.250   | 0.250   | 0.24    | 0.646   |
| $X_2^1X_3$                   | 1  | 196.000 | 196.000 | 186.67  | 0.000   |
| Error                        | 5  | 5.250   | 1.050   |         |         |
| Lack-of-Fit                  | 3  | 3.250   | 1.08    | 1.08    | 0.513   |
| Pure Error                   | 2  | 2.000   | 1.000   |         |         |
| Total                        | 14 | 962.400 |         |         |         |

### Table 7. ANOVA of TS (mg/mL) and RS after NaOH + steam pretreatment.

| Source                        | DF | Adj SS  | Adj MS  | F-Value | p-Value |
|-------------------------------|----|---------|---------|---------|---------|
| Model                        | 9  | 50,737.2| 5637.5  | 4398.87 | 0.000   |
| Linear                       | 3  | 44,404.2| 14,801.4| 11,549.4| 0.000   |
| $X_1$                        | 1  | 14,257.0| 14,257.0| 11,124.6| 0.000   |
| $X_2$                        | 1  | 29,330.2| 29,330.2| 22,886.10| 0.000  |
| $X_3$                        | 1  | 817.0   | 817.0   | 637.51  | 0.000   |
| Square                       | 3  | 2971.1  | 990.4   | 772.77  | 0.000   |
| $X_1^2$                      | 1  | 568.6   | 568.6   | 443.66  | 0.000   |
| $X_2^2$                      | 1  | 1853.0  | 1853.0  | 1445.89 | 0.000   |
| $X_3^2$                      | 1  | 426.9   | 426.9   | 333.08  | 0.000   |
| 2-Way Interaction            | 3  | 3361.9  | 1120.6  | 874.43  | 0.000   |
| $X_1^1X_2$                   | 1  | 30.4    | 30.4    | 23.75   | 0.000   |
| $X_1^1X_3$                   | 1  | 1048.7  | 1048.7  | 818.26  | 0.000   |
| $X_2^1X_3$                   | 1  | 2282.8  | 2282.8  | 1781.28 | 0.000   |
| Error                        | 5  | 6.4     | 1.3     |         |         |
| Lack-of-Fit                  | 3  | 4.8     | 1.6     | 2.06    | 0.343   |
| Pure Error                   | 2  | 1.6     | 0.8     |         |         |
| Total                        | 14 | 50,743.6|         |         |         |

$X_1$ = base conc., $X_2$ = substrate conc., $X_3$ = retention time, TS = total sugars.

### Table 8. ANOVA of TS (mg/mL) and RS after NaOH + steam pretreatment.

| Source                        | DF | Adj SS  | Adj MS  | F-Value | p-Value |
|-------------------------------|----|---------|---------|---------|---------|
| Model                        | 9  | 42,001.4| 4666.8  | 8.30    | 0.016   |
| Linear                       | 3  | 37,490.7| 12,496.9| 22.24   | 0.003   |
| $X_1$                        | 1  | 1573.6  | 1573.6  | 2.80    | 0.155   |
| $X_2$                        | 1  | 35,539.1| 35,539.1| 63.24   | 0.001   |
| $X_3$                        | 1  | 378.0   | 378.0   | 0.67    | 0.449   |
| Square                       | 3  | 1436.1  | 478.7   | 0.85    | 0.523   |
| $X_1^2$                      | 1  | 449.4   | 449.4   | 0.80    | 0.412   |
| $X_2^2$                      | 1  | 65.6    | 65.6    | 0.12    | 0.746   |
| $X_3^2$                      | 1  | 826.9   | 826.9   | 1.47    | 0.279   |
| 2-Way Interaction            | 3  | 3074.6  | 1024.9  | 1.82    | 0.260   |
| $X_1^1X_2$                   | 1  | 1102.2  | 1102.2  | 1.96    | 0.155   |
| $X_1^1X_3$                   | 1  | 88.4    | 88.4    | 0.16    | 0.708   |
| $X_2^1X_3$                   | 1  | 1884.0  | 1884.0  | 3.35    | 0.127   |
| Error                        | 5  | 2809.7  | 561.9   |         |         |
| Lack-of-Fit                  | 3  | 2806.8  | 935.6   | 626.51  | 0.002   |
| Pure Error                   | 2  | 3.0     | 1.5     |         |         |
| Total                        | 14 | 44,811.1|         |         |         |
Table 8. ANOVA of TS (mg/mL) and RS after NaOH + steam pretreatment.

| Source          | DF | Adj SS   | Adj MS   | F-Value | p-Value |
|-----------------|----|----------|----------|---------|---------|
| Model           | 9  | 8159.46  | 906.61   | 131.94  | 0.000   |
| Linear          | 3  | 7106.45  | 2368.82  | 344.73  | 0.000   |
| $X_1$           | 1  | 736.51   | 736.51   | 107.18  | 0.000   |
| $X_2$           | 1  | 6315.76  | 6315.76  | 919.13  | 0.000   |
| $X_3$           | 1  | 54.18    | 54.18    | 7.89    | 0.038   |
| Square          | 3  | 787.37   | 262.46   | 38.20   | 0.001   |
| $X_1^2$         | 1  | 127.04   | 127.04   | 18.49   | 0.008   |
| $X_2^2$         | 1  | 694.11   | 694.11   | 101.01  | 0.000   |
| $X_3^2$         | 1  | 0.00     | 0.00     | 0.00    | 1.000   |
| 2-Way Interaction| 3  | 265.63   | 88.54    | 12.89   | 0.009   |
| $X_1 \times X_2$| 1  | 210.83   | 210.83   | 30.68   | 0.003   |
| $X_2 \times X_3$| 1  | 54.76    | 54.76    | 7.97    | 0.037   |
| Error           | 5  | 34.36    | 6.87     |         |         |
| Lack-of-Fit     | 3  | 25.55    | 8.52     | 1.93    | 0.359   |
| Pure Error      | 2  | 8.81     | 4.40     |         |         |
| Total           | 14 | 8193.82  |          |         |         |

$X_1$ = base conc., $X_2$ = substrate conc., $X_3$ = retention time, TP = total phenol, RS = reducing sugar.
Figure 2. Contour plots for TS, cellulose, TP, and RS liberated after chemical pretreatment of seed pods.

Figure 3. Cont.

X1 = base conc., X2 = substrate conc., X3 = retention time, TP = total phenol, RS = reducing sugar.
Asghar et al. [32] reported maximum cellulose exposure (60.6%) at 2.5% NaOH after 24 h soaking in the cotton stalk, whereas the maximum cellulose (73.19%) was obtained at 121 °C after a 1 h soaking time with 2.5% NaOH, and we found 60% cellulose content in seed pods after 2.5% NaOH pretreatment. Samples with the maximum cellulose content were analyzed for lignin content. It was found that the sample from the chemical treatment had 15% lignin, while the sample from the thermochemical treatment had 9% lignin, whereas the untreated sample had 25% lignin content (Figure 4).

Pretreatment of lignocellulosic biomass is required to make saccharifying enzyme accessible to cellulose for hydrolysis to yield high sugar, as lignin is a barrier protecting cellulose against enzyme attack [30]. Hence, pretreatment prior to saccharification is a mandatory step to minimize the lignin content and enhance the surface area for better enzyme action. In this study, we found that NaOH pretreatment followed by steam was more effective in the delignification of seed pods of B. ceiba, revealing 60% cellulose and 9% lignin. Cellulose content increased from 34% to 60% due to a reduction in lignin content (from 25% to 9%). Similarly, total sugar, phenol, and reducing sugars liberated were also greater in thermochemical pretreatment compared to chemical treatment alone. Asghar et al. [32] reported maximum cellulose exposure (60.6%) at 2.5% NaOH after 24 h of soaking in the cotton stalk, whereas the maximum cellulose (73.19%) was obtained at 121 °C after a 1 h soaking time with 2.5% NaOH, and we found 60% cellulose content in seed pods at thermochemical conditions. Ghazanfar and coworkers [2], using another alkali, found the maximum release of RS (50.06 mg/mL) and TP (394.04 mg/mL) at a
15% substrate concentration, 8 h residence time, and 3% KOH concentration at 121 °C and reported maximum TS (206.65 mg/mL) liberated and cellulose exposed (64%) at the same temperature with a 10% substrate loading and 5% KOH concentration for a 8 h soaking time. Their findings also collaborated with our results, as they obtained maximum cellulose (46%), RS (9.0075 mg/mL), TP (300.3901 mg/mL), and TS (146.1480 mg/mL) at a 15% substrate concentration, using 3% KOH solution, and an 8 h soaking time at room temperature.

Another study by Asghar and fellows [33] used BBD to optimize pretreatment conditions and found that Saccharum spontaneum offered maximum cellulose (52.5%) after 24 h of residence time with 2.5% NaOH. In the thermochemical pretreatment, S. spontaneum was soaked in NaOH solution for 2 h followed by autoclaving for different durations (15–60 min) at 121 °C, suggesting 60 min duration as best for maximum cellulose exposure up to 81.2%. Nadeem et al. [34] also found results similar to our findings in that physicochemical treatment resulted in a greater breakdown of hemicellulose and lignin and hence liberated more sugars and phenolic contents. They soaked powdered bagasse for 1 h in a NaOH solution of 2.5% and then autoclaved for 45 min at 126 °C and achieved 9% lignin and 74% cellulose content, whereas untreated substrate had 35% cellulose content and 25% lignin. The results of Sarbishe and coworkers [35] were opposite to our results because they noted a decline in cellulosic contents (from 44% to 27.6%) of tobacco product waste after 10% NaOH pretreatment because of the hydrolysis of carbohydrates by alkali. A recent study by Gunam et al. [36] found maximum cellulose of 65.46% from pretreatment of corn straw with 4% NaOH.

Regression equations for NaOH pretreatment:

\[
\text{Cellulose (\%) = 77.3 + 1.00X_1 - 0.38X_2 - 13.12X_3 - 0.146X_1^2 + 0.0467X_2^2 + 0.917X_3^2 - 0.325X_1 \times X_2 + 0.187X_1 \times X_3 + 0.150X_2 \times X_3}
\]

(1)

\[
\text{TS (mg/mL) = 1366.3 - 272.37X_1 - 18.11X_2 - 254.34X_3 + 39.248X_1^2 + 8.2273X_2^2 + 31.810X_3^2 + 10.155X_1 \times X_2 + 11.927X_1 \times X_3 - 19.348X_2 \times X_3}
\]

(2)

\[
\text{RS (mg/mL) = 12.0 - 8.85X_1 - 3.213X_2 + 4.48X_3 + 0.942X_1^2 + 0.1390X_2^2 - 0.153X_3^2 + 0.767X_1 \times X_2 - 0.529X_1 \times X_3 - 0.1183X_2 \times X_3}
\]

(3)

\[
\text{TP (mg/mL) = -379 + 33.5X_1 - 56.7X_2 + 211.6X_3 + 12.57X_1^2 + 1.79X_2^2 - 8.79X_3^2 - 0.06X_1 \times X_2 - 25.23X_1 \times X_3 + 4.12X_2 \times X_3}
\]

(4)

Regression equations for NaOH steam pretreatment:

\[
\text{Cellulose (\%) = 76.91 - 9.13X_1 + 0.500X_2 - 3.63X_3 + 0.969X_1^2 + 0.0450X_2^2 + 0.719X_3^2 + 0.5000X_1 \times X_2 + 0.062X_1 \times X_3 - 0.7000X_2 \times X_3}
\]

(5)

\[
\text{TS (mg/mL) = -357.19 + 24.02X_1 + 43.538X_2 + 63.24X_3 + 3.102X_1^2 - 0.8961X_2^2 - 2.688X_3^2 + 0.2758X_1 \times X_2 - 4.048X_1 \times X_3 - 2.3889X_2 \times X_3}
\]

(6)

\[
\text{RS (mg/mL) = 0.6 - 0.79X_1 - 3.11X_2 + 4.18X_3 + 1.466X_1^2 + 0.5484X_2^2 + 0.000X_3^2 - 0.726X_1 \times X_2 - 0.925X_1 \times X_3 - 0.011X_2 \times X_3}
\]

(7)

\[
\text{TP (mg/mL) = -50 + 33.2X_1 + 28.0X_2 - 23.1X_3 - 2.76X_1^2 + 0.169X_2^2 + 3.74X_3^2 - 1.66X_1 \times X_2 - 1.18X_1 \times X_3 - 2.17X_2 \times X_3}
\]

(8)

3.2. FTIR

FTIR analysis pointed out chemical changes in pretreated seed pods compared to those untreated (Figure 5). The change in a peak from 3300.6 cm⁻¹ to 3330.4 cm⁻¹ showed −OH band stretching. The intensity of −OH increased, which showed the influence of NaOH on B. cella. The absorption band between 3200 and 3600 cm⁻¹ is usually assigned to the O–H stretching vibrations of alcohols, carboxylic acids, and hydroperoxides [5]. In this
study, the peak changes from 1023.2 cm\(^{-1}\) to 1026.9 cm\(^{-1}\) in samples are related to C–O and C–H deformations that show cellulose breakdown. The peak in untreated \(B. \text{ceiba}\) at 1593.4 denoted lignin ring stretches, but these bands were stretched and decreased in chemical and thermochemical pretreatment, respectively, representing the breakdown of lignin due to pretreatment. The peak at 896.4 cm\(^{-1}\) in both treated samples represented vibrations at \(\beta\)-glucosidic bonds in C–O–C in hemicelluloses and celluloses.

Figure 5. FTIR analysis of (a) untreated, (b) NaOH-treated, (c) NaOH + steam-treated seed pods.
FTIR analysis suggested that NaOH pretreatment efficiently alters the linkages in biomass. The peak 3334 cm\(^{-1}\) depicts the absorption of –OH of alcoholic hydroxyl [37]. Another study by Irfan et al. [38] reported a peak shift from 3336 cm\(^{-1}\) (raw) to 3315.26 cm\(^{-1}\) (pretreated). This change indicated –OH band extension in the pretreated substrate. The peak at 1315 cm\(^{-1}\) depicted hemicellulose in the untreated sample. CH\(_2\) stretching in cellulose is indicated by the peaks from 1370 to 1430 cm\(^{-1}\). The peaks at 1500 cm\(^{-1}\) are related to the extension in the C=C bond from the lignin’s aromatic ring. The peak at 1030 cm\(^{-1}\) and 1034 cm\(^{-1}\) found in the untreated and pretreated substrate is associated with C–C–O, C=O, and C–O of cellulose. The peak at 890 cm\(^{-1}\) showed C–O–C vibrations at \(\beta\)-glucosidic bonds in cellulose. Zhang et al. [39] labeled the band at 1032 cm\(^{-1}\) with polysaccharides. The reduction in the crystallinity of cellulose might be characterized by the breakdown of structural hydrogen bonding of cellulose chains and the partial conversion of crystalline parts to amorphous ones. The decreased band intensity around 3350 cm\(^{-1}\) could be linked with the slackening of the intra- and inter-molecular O–H bond of cellulose, proposing that the extremely ordered cellulosic system was converted to a more amorphous form.

### 3.3. XRD

Figure 6 revealed the XRD spectra of raw and pretreated (both chemical and thermochemical) samples. The crystallinity index (CI) demonstrated the crystalline structure of the cellulose. The CI of the untreated substrate was 34.5%, which improved in NaOH-treated (51.2%), and NaOH + steam-treated seed pods (51.1%). The increase in CI showed the removal of amorphous components, such as hemicellulose and lignin, from the biomass. Earlier, it was described that the concentrations of the peak were linked with the crystallinity, which increases with pretreatment and is associated with the decline in the surface area. Increasing CI depicted cellulose exposure.

![Figure 6. XRD analysis of seed pods of B. ceiba treated with NaOH and NaOH + steam.](image)

XRD revealed that the CI of pretreated samples was improved compared to the raw samples, which indicated the removal of hemicellulose and lignin from the crystalline part of the biomass, cellulose [40]. Our results were in accordance with earlier studies describing increased CI after different types of pretreatments using various agricultural wastes [40–43]. A recent XRD study by Gunam et al. [36] revealed changes in the crystallinity degree of NaOH-pretreated corn straw. Research by Singh and coworkers [44] reported that the CI (36.96%) of untreated jute biomass declined to 23.61% and 18.42% after 2% NaOH and 2% H\(_2\)SO\(_4\) treatment, respectively. This decrease in CI may be due to the degradation of intra- and inter-hydrogen bonding in the crystalline cellulose resulting in an altered crystal structure. Awoyale and Lokhat [5] observed peaks of reduced intensities in the pretreated biomass samples, a representation of incomplete breakdown of the cellulose with pretreatment.

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**Figure 6.** XRD analysis of seed pods of *B. ceiba* treated with NaOH and NaOH + steam.
3.4. TGA

To study the thermal degradation behavior of raw and treated (with maximum cellulose from each pretreatment) B. ceiba, TGA was performed. Figure 7a revealed decomposition in line with temperature and time; 9.194% decomposition was observed at 100–200 °C (first stage), 50.02% at 300–400 °C (second stage), and 33.39% at 500–600 °C (third stage). During the first stage, the NaOH-treated substrate presented 7.94% conversion, 44.50% during the second stage, and 28.81% during the third stage (Figure 7b), whereas NaOH + steam-pretreated seed pods showed degradation of 7.291% during the first stage, 65.62% during the second stage, and 26.38% at 500–600 °C (Figure 7c).

(a)

(b)

Figure 7. Cont.
TGA depicted maximum weight loss at the temperature range of the second stage in all substrates, whereas maximum degradation of 65.62% was observed in the NaOH + steam-treated biomass. Another study found the highest (74.48%) degradation of \textit{Pinus ponderosa} (sawdust), followed by \textit{Shorea robusta} (sawdust) (70.03%) and \textit{Areca catechu} (nut husk) (69.09%) at a temperature range of 200–500 °C (second stage). Hemicellulose is degraded at temperatures of 180–340 °C, cellulose at 230–450 °C, and lignin decomposed at temperatures greater than 500 °C [45]. A recent study by Tsegaye and others [29] reported that the rate of loss of weight was very high (nearly 80%) at a temperature range of 200–500 °C for all the employed treatments.

3.5. SEM

The structural modifications that appeared after pretreatment were observed by SEM images. Micrographs verified the surface characteristics and structural changes in seed pods of \textit{B. ceiba} caused by pretreatment. The NaOH reaction damaged the surface of \textit{B. ceiba}, generating some irregular cracks and pores. SEM images revealed a significant difference between the surface structures of pretreated and untreated \textit{B. ceiba} (Figure 8). The raw sample exhibited a complex order and dense structure, while both the treated specimens revealed a greater degree of porosity. The number and size of pores confirmed the effectiveness of NaOH + steam pretreatment in delignification.

Thus, pretreatment could efficiently lessen the crystallinity of the \textit{B. ceiba}, as reported earlier for corn leaf and cattail’s narrow leaf by Donghai et al. [46] and Ruangmee and Sangwichien [47]. This shows that alkali pretreatment can remove a significant percentage of lignin and hemicellulose. Jabasingh and Nachiyar [48] also detected such variations in bagasse. Awoyale and Lokhat [5] reported that the micrographs of the alkali-treated lignocellulosic substrate demonstrate more fragmentation and degradation, indicating the efficacy of alkali pretreatment over the other pretreatment processes performed in the research. Kusmiyati et al. [49] observed uneven surfaces with pores in treated palm tree stem waste through SEM. Sindhu et al. [50] also observed differences in the surface structure of native and pretreated bamboo substrates.
3.6. Hydrolysis and Fermentation

Samples with maximum cellulose contents were used for saccharification and production of ethanol via SHF and SSF. In SHF, substrates with maximum cellulose content from both treatments were saccharified by using indigenously produced cellulase as well as commercial cellulase. Sugars obtained from these saccharifications were then fermented by using Saccharomyces cerevisiae. During SSF, the pretreated biomass was added with indigenously produced enzyme to form the maximum sugars needed for the production of ethanol and then incorporated with yeast culture. Similar SSF was repeated with commercial cellulase. The results of SHF with commercial cellulase showed maximum saccharification after 24 h in NaOH + steam-pretreated substrate (52.6%), followed by NaOH-treated (40.8%) and untreated substrates (16.4%). Maximum saccharification of 37% with indigenously produced cellulase was recorded in NaOH + steam-pretreated seed pods. The fermentation of these hydrolysates resulted in the production of ethanol. Hydrolysates obtained with indigenous cellulase offered maximum bioethanol titer (g/L) of 28.7 in NaOH + steam-treated, 16.01 in NaOH, and 8.73 in untreated B. ceiba after 96 h of fermentation. Sugars obtained with commercial cellulase presented a maximum ethanol yield of 48.8 g/L in NaOH + steam-treated, 39.63 g/L in NaOH-treated, and 15.6 g/L in untreated biomass (Figure 9).
of fermentation. Sugars obtained with commercial cellulase presented a maximum ethanol yield of 48.8 g/L in NaOH + steam-treated, 39.63 g/L in NaOH-treated, and 15.6 g/L in untreated biomass (Figure 9).

The results of SSF showed a decrease in sugar every 24 h; this could be due to the sugar consumption by yeast and subsequent ethanol production increase every 24 h due to fermentation. After adding indigenously produced cellulase and 1% *S. cerevisiae,* maximum saccharification in untreated lignocellulosic biomass (17.3%) was observed after 48 h of inoculation, while chemically and thermochemically pretreated substrates showed maximum saccharification (31.12% and 40.8%, respectively) after 24 h. Maximum ethanol production was observed at 11.2 g/L, 18.7 g/L, and 38.8 g/L in raw, chemically treated, and thermochemically pretreated substrates, respectively, after 96 h of fermentation (Figure 10). Sugars obtained with commercial cellulase offered the highest ethanol production in SSF. Maximum saccharification (50.9%) in SSF was seen with commercial cellulase in NaOH + steam-treated seed pods, which subsequently resulted in the highest ethanol (54.51 g/L) production after 96 h of fermentation. Maximum bioethanol yield in NaOH-treated (29.76 g/L) and raw seed pods (19.87 g/L) was also obtained after 96 h fermentation (Figure 11).

![Graph a](image1.png)

**Figure 9.** Separate hydrolysis and fermentation. (a) Saccharification after 24 h. (b) Ethanol produced after 96 h.
We used two different approaches, e.g., SHF and SSF, for saccharification and fermentation of the pretreated substrate. In this study, the highest fermentable sugars in terms of saccharification, 50.9% (after 24 h), and the highest bioethanol yield, 54.51 g/L (after 96 h), were obtained from SSF with the commercial enzyme and S. cerevisiae. SSF with indigenous cellulase gave a relatively low yield. In the case of SHF, results with commercial cellulase were better than that of indigenous cellulase, but the overall yield was less than SSF. The results of Sukhang et al. [51] and Vintila et al. [52] corroborated our findings that the SSF contributed to higher ethanol production from lignocellulosic biomass than that of SHF. A study by Triwahyuni [53] noticed a notable ethanol titer from SHF of oil palm empty fruit bunch. Hydrolysis for 96 h produced 75.48% glucose, which subsequently yielded 78.95% ethanol. Ballesteros et al. [54] found the highest ethanol yield in SSF after 72 h of fermentation. The reason for the good yield of ethanol in SSF could be the direct conversion of produced sugars into ethanol, thus evading any feedback inhibition. Barron et al. [55] employed other strains of yeast for ethanol production and found that Kluyveromyces marxianus produced 10 g/L ethanol after a 60 h fermentation period, and Pachysolen tannophilus produced 11.8 g/L ethanol from the hydrolysate of wheat straw.
Tan and Lee [56] noticed a better yield of bioethanol in SSF (90.9%) compared to SHF (55.9%). SSF of seaweed biomass by means of *S. cerevisiae* had several advantages over SHF, as the earlier approach is a simple single-step process that can save time, costs, and energy while achieving a high bioethanol titer. A study found a maximum ethanol yield of 85.71% at 30 °C with 2% wheat straw and 30 FPU of enzyme loading in SSF Ruiz et al. [57]. Findings of research on bioethanol production from rice husk also supported our results that SSF was better than SHF in yielding a good ethanol titer [58]. Ahmad et al. [59] showed that sweet sorghum and sago biomasses yielded maximum ethanol at the end of 72 h of fermentation. Peace and others [60] used wood shavings for bioethanol production. They noticed that *S. cerevisiae* was able to convert 60.97% brix in wood extract into bioethanol at optimized conditions of 15.66 g wood concentration, 4.47% NaOH concentration, 0.85 inoculum size, 72 h incubation time, and a 40 °C incubation temperature. The ethanol titer obtained was 1.68–2.25%. As various studies have used different plant biomasses in order to produce bioethanol, this study suggested seed pods of *B. ceiba* as a potential renewable source of green energy to be used on a pilot scale.

**Figure 11.** SSF with commercial cellulase and *S. cerevisiae*. (a) Saccharification. (%) (b) Ethanol produced (g/L).
4. Conclusion

The results of this study revealed that *B. ceiba* could be utilized as a potential lignocellulosic biomass source for bioethanol production. NaOH pretreatment significantly exposed cellulose (60%), which was further hydrolyzed to fermentable sugars (50.9% saccharification), which yielded better ethanol (54.51 g/L) production by *Saccharomyces cerevisae* in SSF. The findings of this research recommended that this biomass could be an encouraging feedstock for the large-scale production of second-generation bioethanol.

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