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Investigation of Aqueous Ammonia Assisted Sodium Carbonate Pretreatment for Enhancing Enzymatic Saccharification of Sugarcane Bagasse

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Abstract

In this work, the enzymatic saccharification of sugarcane bagasse (SCB) was enhanced after Na_2CO_3 with aqueous ammonia (NWAA) pretreatment. To achieve the highest total fermentable sugar yield (TFSY) and total fermentable sugar concentration (TFSC), NWAA parameters were optimized by response surface methodology. Under the optimum NWAA parameters, 18.16 g/L of TFSC (8.16% Na_2CO_3, 20.44% ammonia, 221.66 ℃, 1.39 h) and 0.4777 g/g of TFSY (7.21% Na_2CO_3, 20.42% ammonia, 201.92 ℃, 1.38 h) were obtained, with delignification of 94.19% and 92.80%, respectively. NWAA pretreatment significantly improved the digestibility of SCB as compared to pretreatment using Na_2CO_3 or aqueous ammonia alone. XRD, FTIR, and SEM were used for analyzing the chemical and physical characteristics of SCB samples, whose results indicated that NWAA could effectively enhance the accessibility of cellulases to celluloses. These results confirm that NWAA is a promising method for pretreatment of SCB.

Keywords

Sugarcane Bagasse; Na_2CO_3; Aqueous Ammonia; Enzymatic Saccharification; Surface Response Methodology.

1. Introduction

As a result of the rapid growth of the human population and economic development as well as the increasingly serious environmental problems, the development of renewable energy has been paid more and more attention [1]. Demand for alternative biofuels will increase sharply in the coming years as the rising demand for fuel drives up the price of crude oil [2]. Bioethanol, as an excellent alternative energy of fossil fuel, could be produced sustainably by using abundant and cheap lignocellulosic biomass [3,4]. In the lignocellulosic biomass, the cellulose is embedded in the interwoven net structure of hemicellulose and lignin, indicating that it is recalcitrant to be degraded [5]. Owing to the recalcitrant and complex net structure of lignocellulose, pretreatment is needed to be carried out with the purpose of improving the accessibility for cellulases to cellulose before the bioconversion of lignocellulose to bioethanol [6,7]. Over the past decades, extensive pretreatment methods including chemical, physical, biological, physicochemical, and combinations of these methods, have been reported [8]. Some disadvantages, such as the uneconomic cost and the unfriendliness to the environment, have existed in these methods which need to be further studied [9].

Regarding alkali-based pretreatment, recovering sodium hydroxide needs expensive cost because of the combustion of black liquor and subsequent causticization stage [10,11]. Recently, mild and inexpensive sodium carbonate was used as a chemical reagent to pretreat lignocellulosic materials for effective delignification since it could retain more carbohydrates without requirement of alkalization for recovery [12,13]. Therefore, pretreatment cost in the

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process could be reduced by using sodium carbonate as a reaction reagent. Most studies of Na_2CO_3 pretreatment have been concentrated on herbaceous biomass, especially rice straw, and few studies have focused on sugarcane bagasse (SCB) [14,15]. However, due to the weak alkalinity of sodium carbonate, the removal rate of lignin was not significantly increased even at a high concentration of Na_2CO_3. In consequence, the addition of H_2O_2, Na_2S or Na_2SO_3 as chemical reagent has effectively enhanced the effect of Na_2CO_3 pretreatment and improved the removal of lignin [16,17,18].

Aqueous ammonia, a weakly alkaline and relatively non-corrosive reagent, is easy for recovery with its high volatility. Shi et al. (2019) recycled 32.52% of ammonia used in aqueous ammonia with glycerol pretreatment process. Moreover, the remaining ammonia during the process of the pretreatment is non-toxic, and it could be used as a feasible nitrogen source during fermentation [20]. Pretreatment using aqueous ammonia could cause delignification through cracking the ester and ether bonds in lignin or between carbohydrates and lignin, resulting in the enhancement of the accessibility for cellulases to cellulose [21]. A variety of methods using ammonia were carried out to pretreat lignocellulosic biomass, including ammonia fiber explosion, ammonia freeze, aqueous ammonia soaking, and ammonia recycles percolation [22]. Both ammonia and Na_2CO_3 could be recovered and recycled, meeting the requirements of the green process. As far as we know, this is the first research to use Na_2CO_3 with aqueous ammonia to pretreat SCB with the purpose of improving its enzymatic hydrolysis efficiency.

At first, the effects of Na_2CO_3 with aqueous ammonia (NWAA) pretreatment on SCB were assessed in this work. Subsequently, response surface methodology (RSM) was utilized for optimizing NWAA conditions, including Na_2CO_3 concentration, ammonia concentration, pretreatment temperature and time. In the end, the analyses of untreated and pretreated SCB samples in terms of chemical and physical characteristics were conducted using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD).

2. Materials and methods

2.1. Materials

SCB materials were friendly furnished by Liutang Sugar Refinery in Liuzhou, Guangxi province, China. At first, SCB materials were air-dried in the laboratory. After that, SCB materials were ground and filtered by passing through a 100-mesh sieve and retaining by a 200-mesh sieve. The contents of the main composition in the raw SCB were as follows: 39.96 ± 0.38% glucan, 23.59 ± 0.14% xylan, 24.69 ± 0.13% lignin. Cellic Ctec2 (75 FPU/mL) used in this work was purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade aqueous ammonia solution and analytical grade sodium carbonate were both purchased from Guangzhou Chemical Reagent Co., Ltd (Guangzhou, China).

2.2. Experimental design for optimization of NWAA pretreatment

NWAA pretreatment was carried out in the Hydrothermal Synthesis Autoclave Reactor (150 mL, 250 ℃, 3.0 MPa). In each reactor, 5 g oven-dried SCB was soaked in 50 g solution different concentrations (based on 50 g solution) of Na_2CO_3 and/or ammonia. The hermetic reactor was placed into the middle of an oven (SFG-02B.400; Huangshi Hengfeng Medical Equipment Co., Ltd., Hubei, China). The incubation time started as the desired temperature was reached in the oven. The pretreated SCB residue was washed using hot deionized water until neutral pH after pretreatment. Then, wet SCB residue was air-dried and stored in a plastic bag for further use.

2.2.1. NWAA pretreatment conditions optimized by single factor tests

The effect of Na_2CO_3 concentration in 15% aqueous ammonia solution on SCB was firstly evaluated. Based on the above-mentioned process, the oven-dried SCBs were pretreated by

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different concentrations of Na_2CO_3 (0, 1, 3, 5, 7, and 9%, w/w) in 15% aqueous ammonia solution at 200 ℃ for 1 h.

With the purpose of assessing the effect of the concentration of ammonia on NWAA pretreatment, the oven-dried SCBs were pretreated by different concentrations of ammonia (0, 10, 15, 20, and 25%, w/w) in 7% Na_2CO_3 at 200 ℃ for 1 h.

Then, the effect of pretreatment temperature was evaluated. The SCBs were pretreated by 7% Na_2CO_3 in 20% aqueous ammonia for 1 h at temperatures of 160 ℃, 180 ℃, 200 ℃, 220 ℃, and 240 ℃, respectively.

Finally, to assess the effect of pretreatment time on SCB, the dried SCBs were pretreated by 7% Na_2CO_3 in 20% aqueous ammonia for a series of time (0.5, 1, 1.5, 2, and 3 h) at 220 ℃.

For each single-factor experiment, the main composition and the solid recovery of the solid residue were analyzed; meanwhile, the enzymatic saccharification experiment was performed after pretreatment.

2.2.2. Response surface methodology

In order to gain the highest TFSY or TFSC, NWAA pretreatment parameters were further optimized using RSM in the light of the result of the single factor test. According to the Box-Behnken design (BBD), four independent variables, namely, Na_2CO_3 concentration (X_1), ammonia concentration (X_2), pretreatment temperature (X_3) and pretreatment time (X_4), were chosen to optimize NWAA pretreatment [23]. The chosen variables were investigated at three levels (Table 1). 29 experiments (Table 1) of the four variables containing five center points were drafted using Design-Expert (Stat-Ease, Inc., USA).

2.3. Enzymatic hydrolysis

0.5 g NWAA pretreated SCB was put into the 50-mL serum bottle with citric acid/citric sodium buffer (volume of 25mL, molarity of 0.1 M, pH 4.8). In order to prevent microbial contamination, 60 μg/mL nystatin and 80 μg/mL tetracycline were added to the serum bottle. Cellulases Celic CTec2 (75 FPU/mL) was utilized to degrade the SCB with a loading of 30 FPU/g substrate. The enzymatic reaction was conducted at 200 rpm in a rotary shaker for 72 h at 50 ℃. The sample was taken at 72 h and then boiled until the cellulases were inactive with a time of 10 min. After that, the sample was centrifuged for 10 min with a rotate speed of 12000 rpm. The supernatant was used to detect xylose concentration and glucose concentration by using HPLC (LC-15C, Shimadzu, Japan).

2.4. Analytical methods

2.4.1. Main component analysis

The two-step acid hydrolysis method was applied to detect the contents of the main compositions of SCB [24]. Xylose and glucose in the hydrolysate were detected by HPLC equipped with a refractive index detector (RID-10A, Shimadzu) and a Cation H^+ Cartridge Micro-Guard column (Bio-Rad, Richmond, CA, USA) connected to an Aminex HPX-87X column (Bio-Rad). Xylose and glucose were detected by the column at 55 ℃ with 5 mM H_2SO_4 (0.6 mL/min) as eluates.

2.4.2. FTIR, SEM and XRD analyses of raw and NWAA pretreated SCB samples

Analyses of the chemical structural modification and surface morphology of pretreated SCBs and raw SCB were performed by FTIR, XRD and SEM analyses. FTIR spectrophotometer (Nicolet Is50, Thermo Fisher Scientific, USA) was employed to record FTIR spectra of SCBs. The spectrophotometer was operated with a resolution of 4 cm^{-1} in the wave-number range of 4000-500 cm^{-1} in 64 scans. 1% sample was contained in KBr translucent disk in the FTIR analysis. SEM (Ultra 55, Zeiss, Germany) was employed for recording the surface structural

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characteristics of the raw and NWAA pretreated SCBs with magnification at 507 X and 1010 X, and the acceleration voltage was set at 5 kV. The crystallinity indices (CrI) of SCB samples were determined by XRD equipped with the XD-2 diffractometer (Beijing Purkinje General Instrument Co., Ltd, China) which was set at 40 mA and 40 kV. XRD patterns of the SCBs were recorded with a step size of 0.02° at a scan speed of 2° min^{-1} over the range of 2θ = 5-35°. The CrI of SCB samples were calculated based on the following equation [25]:

$$\text{CrI (\%)} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (1)$$

in which I_{002} represents the scattered intensity of the crystalline portion around 22.5°, while I_{am} represents the scattered intensity of the peak corresponding to the amorphous portion around 18°.

2.4.3. Calculations and statistical analysis

According to the previous study, the mass of raw SCB, TFSC and TFSY were calculated based on equations as follows:

$$\text{Raw SCB } (g) = \frac{\text{Residue mass } (g)}{\text{Solid recovery}} \quad (2)$$

$$\text{TFSC (g/L)} = \text{Xylose concentration (g/L)} + \text{Glucose concentration (g/L)} \quad (3)$$

$$\text{TFSY (g/g)} = \frac{\text{TFSC (g/L)} \times \text{V } (L)}{\text{Raw SCB } (g)} \quad (4)$$

Raw SCB is equal to the mass of untreated SCB used in enzymatic saccharification. Residue mass represents the mass of NWAA pretreated SCB utilized in enzymatic saccharification. The definition of solid recovery (%) can be found in Table 2. The TFSC represents the sum of xylose concentration and glucose concentration contained in the hydrolysate. The TFSY represents the gross of xylose and glucose attained from per gram raw SCB. The V represents the total volume of reaction solution used in enzymatic saccharification. As described above, xylose concentration and glucose concentration were detected using HPLC.

Table 1. The experimental design and results of the BBD.

Run	Experiment variables				Actual response		Predicted response	
	Sodium carbonate concentration (X_1, w/w)	Ammonia concentration (X_2, w/w)	Pretreatment temperature (X_3, °C)	Pretreatment time (X_4, h)	Total fermentable sugar concentration (g/L)	Total fermentable sugar yield (g/g)	Total fermentable sugar concentration (g/L)	Total fermentable sugar yield (g/g)
1	5 (-1)	25 (+1)	220 (0)	1 (0)	14.19	0.41	14.86	0.43
2	9 (+1)	15 (-1)	220 (0)	1 (0)	15.20	0.43	15.39	0.43
3	7 (0)	15 (-1)	220 (0)	1.5 (-1)	15.31	0.41	15.79	0.41
4	7 (0)	15 (-1)	220 (0)	0.5 (-1)	12.33	0.39	12.51	0.40
5	7 (0)	20 (0)	240 (+1)	1.5 (+1)	16.11	0.36	16.99	0.39
6	5 (-1)	20 (0)	240 (+1)	1 (0)	16.66	0.43	16.53	0.43
7	7 (0)	20 (0)	220 (0)	1 (0)	16.84	0.46	16.89	0.46
8	7 (0)	15 (-1)	240 (+1)	1 (0)	16.62	0.42	15.75	0.41
9	5 (-1)	20 (0)	220 (0)	0.5 (-1)	13.67	0.41	13.16	0.40
10	9 (+1)	20 (0)	220 (0)	1.5 (+1)	18.06	0.45	17.64	0.44
11	7 (0)	20 (0)	200 (-1)	0.5 (-1)	11.64	0.37	11.62	0.36
12	7 (0)	20 (0)	240 (+1)	0.5 (-1)	13.91	0.41	14.68	0.42
13	7 (0)	20 (0)	220 (0)	1 (0)	17.15	0.47	16.89	0.46
14	7 (0)	20 (0)	200 (-1)	1.5 (+1)	17.37	0.46	17.46	0.47
15	5 (-1)	20 (0)	200 (-1)	1 (0)	15.20	0.43	15.52	0.44
16	5 (-1)	20 (0)	220 (0)	1.5 (+1)	17.70	0.46	16.78	0.44
17	9 (+1)	20 (0)	220 (0)	0.5 (-1)	13.11	0.40	13.11	0.40
18	9 (+1)	20 (0)	240 (+1)	1 (0)	17.48	0.44	17.22	0.43
19	7 (0)	25 (+1)	220 (0)	0.5 (-1)	12.00	0.37	11.58	0.37
20	9 (+1)	20 (0)	200 (-1)	1 (0)	15.45	0.44	15.64	0.44
21	7 (0)	25 (+1)	240 (+1)	1 (0)	16.20	0.43	15.82	0.42
22	5 (-1)	15 (-1)	220 (0)	1 (0)	14.42	0.42	14.99	0.43
23	7 (0)	25 (+1)	200 (-1)	1 (0)	14.38	0.43	14.33	0.42
24	9 (+1)	25 (+1)	220 (0)	1 (0)	14.97	0.42	15.26	0.43
25	7 (0)	20 (0)	220 (0)	1 (0)	16.60	0.46	16.89	0.46
26	7 (0)	15 (-1)	200 (-1)	1 (0)	15.20	0.44	14.66	0.43
27	7 (0)	20 (0)	220 (0)	1 (0)	16.59	0.45	16.89	0.46
28	7 (0)	25 (+1)	220 (0)	1.5 (+1)	16.57	0.44	16.46	0.43
29	7 (0)	20 (0)	220 (0)	1 (0)	17.26	0.47	16.89	0.46

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All tests and measurements were implemented in duplicate. Experimental data were expressed as the mean ± standard deviation. LSD multiple range test, *t*-test and one-way ANOVA using SPSS Statistics were used for analyzing the data. The *p*-value < 0.05 represented the result was statistically significant.

3. Results and discussion

3.1. Effect of Na_2CO_3 concentration on NWAA pretreated SCBs

SCBs were pretreated by various Na_2CO_3 concentrations (0%, 3%, 5%, 7% and 9%) with 15% (w/w) aqueous ammonia at 200 ℃ for 1 h to evaluate the effect of Na_2CO_3 concentration on the main compositions and the results of enzymatic hydrolysis of SCBs (Table 2). As a result, the main compositions, TFSYs, and TFSCs of pretreated SCBs were effectively affected by the pretreatment processes.

As showed in Table 2, the solid recoveries of SCBs pretreated by NWAA pretreatment significantly decreased to 56.19% from 64.47% ($p < 0.05$) as Na_2CO_3 concentration increased to 9% from 0%, primarily due to the partial degradation of xylan and the dissolution/removal of lignin. As compared to the pretreatment using ammonia alone, it could be seen that most of the glucan (86.43%-92.27%) and xylan (60.48%-67.51%) were retained in the process of the NWAA pretreatment, which indicated that pretreatment of Na_2CO_3 with ammonia could selectively remove lignin and retain most of the carbohydrate [26]. Simultaneously, all the values of xylan contents and glucan contents in SCB samples pretreated by NWAA were significantly greater than those in the raw substrate ($p < 0.05$). The semblable result has been reported in ammonia-glycerol pretreatment using 9% ammonia (w/w) as a catalyst and 50% glycerol (w/w) as a solvent, resulting in massive degradation of lignin without significant dissolution of carbohydrate [19]. Compared to raw SCB, the lignin content of SCB obtained from pretreatment with ammonia alone (0% Na_2CO_3) reduced significantly ($p < 0.05$), suggesting that pretreatment with ammonia alone could enhance the delignification to a degree [25]. As Na_2CO_3 concentration increased to 7%, the lignin content of pretreated substrate decreased to 4.59% (the lowest lignin content), which indicated that the addition of Na_2CO_3 in aqueous ammonia could significantly enhance the removal of lignin [3]. Meanwhile, the delignification rate increased to 89.30% significantly ($p < 0.05$) as Na_2CO_3 concentration increased to 7%, and then the change was not significant as Na_2CO_3 concentration increased to 9%.

The enzymatic saccharification of the raw and NWAA pretreated SCBs was performed with the purpose of further evaluating the effect of Na_2CO_3 concentration on NWAA pretreatment. As noted in Table 2, the TFSY and TFSC of the raw SCB and the SCB pretreated by Na_2CO_3 alone were significantly lower than those of NWAA pretreated SCB residues ($p < 0.05$). Along with the increase of Na_2CO_3 concentration from 1% to 7%, the TFSY and TFSC of pretreated SCBs increased step by step, and 0.4412 g/g of the highest TFSY and 15.29 g/L of the highest TFSC were respectively attained as Na_2CO_3 concentration was 7%, followed by 0.4126 g/g of TFSY and 14.69 g/L of TFSC from SCB residue pretreated with 9% Na_2CO_3, respectively. The increases of TFSY and TFSC were primarily due to the removal of lignin which could significantly enhance the accessibility of enzymes to substrate, resulting in the enhancement of enzymatic digestibility of the SCB residues [27]. In a word, the maximum delignification, TFSY, and TFSC were achieved as the concentration of Na_2CO_3 was 7%. Therefore, 7% (w/w) Na_2CO_3 was chosen to further investigate the NWAA pretreatment conditions.

ISSN: 2710-0588 DOI: 10.29556/FSE.202106_1(3).0020

Table 2. Main components and enzymatic saccharification analyses of SCBs pretreated with different concentrations of Na_2CO_3.

Entry	Na_2CO_3 (w/w, %)	Solid recovery[a] (%)	Component analysis						Enzymatic saccharification analysis	
			Glucan (%)	Recovery[b]	Xylan (%)	Recovery[c]	Lignin (%)	Delignification[d] (%)	TFSY (g/g)	TFSC (g/L)
1	Raw SCB	100	39.96 ± 0.38D	-	23.59 ± 0.14C	-	24.69 ± 0.13A	-	0.0526 ± 0.0004F	1.05 ± 0.01G
2	0[e]	64.47 ± 0.90A	54.51 ± 1.17C	86.33 ± 1.10C	24.15 ± 0.45C	64.73 ± 1.39B	9.69 ± 0.29B	74.69 ± 0.88E	0.3069 ± 0.0022E	9.52 ± 0.07F
3	1[f]	62.12 ± 0.36B	59.44 ± 0.19B	92.27 ± 0.70A	25.28 ± 0.48B	67.51 ± 0.39A	7.34 ± 0.45C	81.50 ± 1.13D	0.3640 ± 0.0036D	11.72 ± 0.12E
4	3[f]	58.34 ± 0.34C	60.38 ± 0.33B	89.73 ± 2.39B	25.69 ± 0.38B	65.01 ± 1.74B	6.22 ± 0.22D	85.28 + 0.55C	0.3899 ± 0.0070C	13.37 ± 0.24D
5	5[f]	57.66 ± 0.53C	61.99 ± 0.52A	89.48 ± 0.84B	26.45 ± 0.32A	64.76 ± 0.74B	5.49 ± 0.26E	87.20 ± 0.64B	0.4121 ± 0.0075B	14.30 ± 0.26C
6	7[f]	57.72 ± 0.73C	62.44 ± 0.85A	90.35 ± 1.89AB	25.52 ± 0.29B	61.78 ± 0.85C	4.59 ± 0.11F	89.30 ± 0.14A	0.4412 ± 0.0027A	15.29 ± 0.09A
7	9[f]	56.19 ± 0.35D	62.64 ± 0.36A	86.43 ± 0.72C	25.52 ± 0.28B	60.48 ± 0.71C	4.86 ± 0.27F	88.92 ± 0.68A	0.4126 ± 0.0031B	14.69 ± 0.11B

Values with the same capital letter are considered no significant differences ($p > 0.05$), analyzed by LSD multiple range test and one-way ANOVA (n = 2).

[a] Solid recovery = regenerated substrate/untreated substrate.

[b] Glucan recovery = (solid recovery × glucan content of regenerated substrate)/glucan content of raw substrate.

[c] Xylan recovery = (solid recovery × xylan content of regenerated substrate)/xylan content of raw substrate.

[d] Delignification = (lignin content of raw substrate – solid recovery × lignin content of regenerated substrate)/lignin content of raw substrate.

[e] SCB sample was pretreated with 15% of aqueous ammonia alone (0% Na_2CO_3) at 200 ℃ for 1h.

[f] SCBs were pretreated with different concentrations of Na_2CO_3 in 15% of aqueous ammonia at 200 ℃ for 1h.

3.2. Effect of ammonia concentration on NWAA pretreated SCBs

To assess the influence of ammonia concentration on SCB, SCBs were pretreated with 7% Na_2CO_3 mixed with different concentrations of ammonia (0, 10, 15, 20 and 25%, w/w) for 1 h at 200 ℃. As noted in Fig. 1A, all the contents of glucan in SCB samples pretreated with different concentrations of ammonia were significantly greater than the glucan content in the raw SCB ($p < 0.05$). The lignin content significantly decreased to 4.24% from 11.94% ($p < 0.05$) as ammonia concentration increased to 20% from 0%. However, the content of lignin significantly increased to 5.73% ($p < 0.05$) as 25% of aqueous ammonia was loaded. Therefore, the maximum delignification rate of 90.25% was achieved as 20% ammonia concentration was loaded. Moreover, the xylan content of the group with 20% ammonia was significantly higher than that of the group with 25% ammonia, but the difference between the glucan contents was not significant. The enzymatic saccharification experiments were conducted with the goal of further evaluating the effect of ammonia concentration on NWAA pretreatment. As shown in Fig. 1B, the ammonia concentration had a significant influence on both TFSC and TFSY which had the same tendency. As the concentration of ammonia increased from 0% to 20%, the TFSY and the TFSC of pretreated SCB gradually increased ($p < 0.05$). When 20% ammonia was loaded, 0.4512 g/g and 15.85 g/L of the highest TFSY and TFSC were achieved, respectively. When the concentration of ammonia increased to 25%, the TFSC and the TFSY significantly declined to 14.22 g/L and 0.4219 g/g ($p < 0.05$), respectively. In conclusion, 20% (w/w) ammonia was chosen as an NWAA pretreatment condition.

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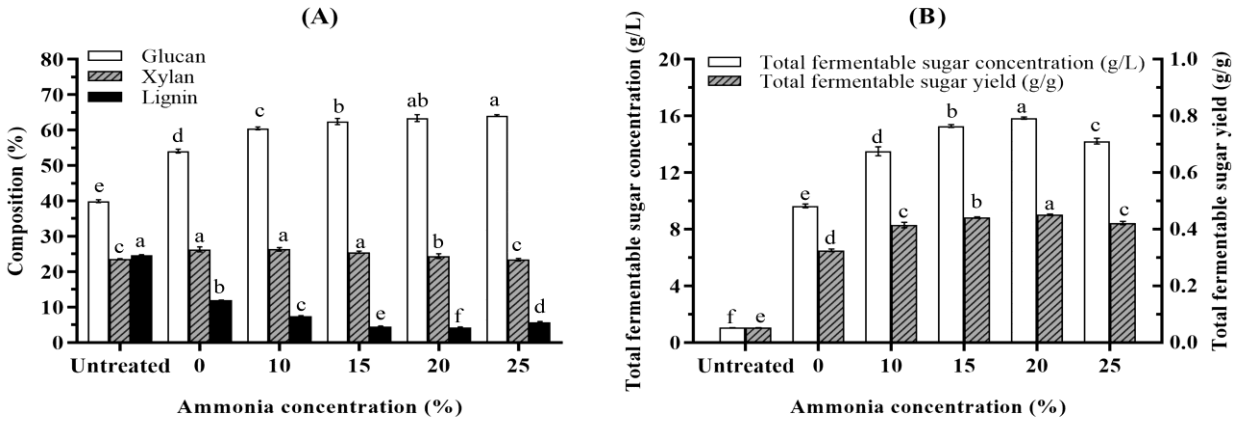

Figure 1. The main compositions (A) and enzymatic saccharification (B) analyses of SCB samples pretreated with different concentrations of aqueous ammonia containing 7% Na_2CO_3 at 200 ℃ for 1 h. The same letter suggests no significant difference ($p > 0.05$) at various concentrations of aqueous ammonia among the same composition, TFSY, or TFSC of SCB samples (n = 2), respectively.

As compared to raw SCB, the contents of xylan and glucan in the group with 0% ammonia (Na_2CO_3 alone) significantly ($p < 0.05$) increased, while the TFSY and TFSC also significantly increased, implying that pretreatment with Na_2CO_3 alone could enhance the digestibility of SCB. Based on the above-mentioned results, the delignification and the enzymatic saccharification performance of NWAA pretreated SCBs were better than those of the solid residue obtained from pretreatment with Na_2CO_3 or ammonia alone, indicating that pretreatment with Na_2CO_3 and ammonia could improve enzymatic digestibility of SCB through removing more lignin. Therefore, NWAA pretreatment is a positive method for improving the enzymatic hydrolysis efficiency of lignocellulose.

3.3. Effect of pretreatment temperature on NWAA pretreated SCBs

In order to investigate an appropriate temperature, SCBs were pretreated with 7% Na_2CO_3 in 20% aqueous ammonia at various temperatures ranging from 160 ℃ to 240 ℃ for 1 h. As noted in Fig. 2A, the temperature had an effective influence on the decrease of lignin content and the increase of glucan content. As the temperature rose from 160 ℃ to 240 ℃, the glucan content of the NWAA pretreated SCB significantly increased from 59.63% to 68.55% ($p < 0.05$), while the lignin content significantly decreased from 6.90 to 2.90% ($p < 0.05$). In addition, the content of xylan was slightly influenced by the temperature, and it decreased from 25.01% to 22.55% as the temperature was elevated from 160 ℃ to 240 ℃. As noted in Fig. 2B, 18.43 g/L of the highest TFSC and 0.4698 g/g of the highest TFSY were respectively achieved at 240 ℃ and 220 ℃, and the difference between the two TFSYs obtained at 220 ℃ and 240 ℃ was not significant ($p > 0.05$). TFSY has more economical significance than TFSC as it is the gross of fermentable sugar attained from 1 g raw substrate, and higher economic viability for lignocellulose pretreatment could be achieved as the TFSY is higher [28]. Therefore, 220 ℃ was suitable for the following investigation of NWAA conditions.

3.4. Effect of pretreatment time on NWAA pretreated SCBs

To obtain the appropriate NWAA pretreatment time, SCBs were pretreated with 7% Na_2CO_3 in 20% aqueous ammonia at 220 ℃ for different time (0.5, 1, 1.5, 2, and 3 h). As shown in Fig. 3A, all the contents of glucan in the NWAA pretreated SCB residues were significantly greater than that in raw SCB ($p < 0.05$). As pretreatment time was prolonged to 3 h from 0.5 h, the glucan

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content significantly increased to 77.77% from 64.60% ($p < 0.05$), while the xylan content significantly decreased to 17.25% from 25.50% ($p < 0.05$). The decrease of xylan content might be that the reaction became stronger when the reaction time was extended, resulting in more hemicellulose were degraded [29]. As the time was extended to 1.5 h, the lignin content declined to 2.57%. However, as the time was extended to 3 h from 1.5 h, the lignin content increased to 5.88% from 2.57%, suggesting that the formation of pseudo lignin or the modification of the structure of the raw material would occur and increase the lignin content as the reaction time was extended [30].

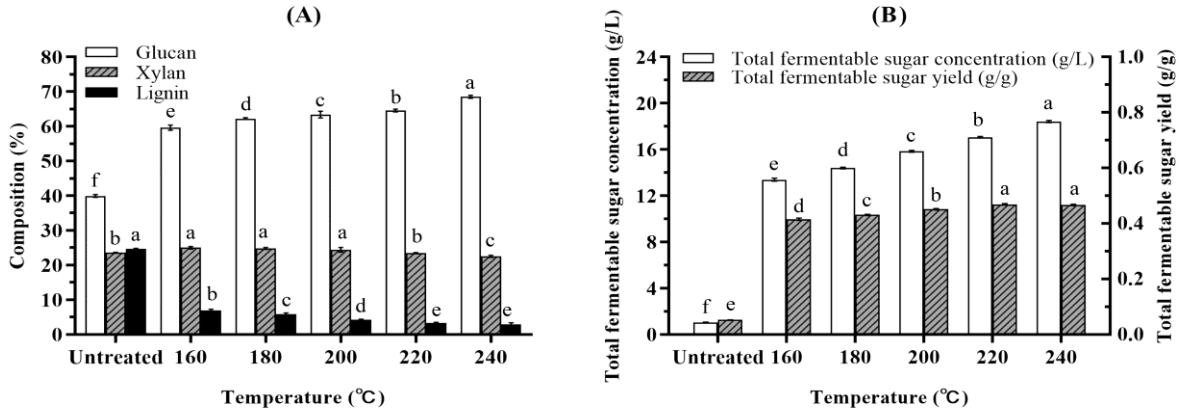

Figure 2. The main compositions (A) and enzymatic saccharification (B) analyses of SCB samples pretreated with 7% Na_2CO_3 in 20% aqueous ammonia at different temperatures for 1 h. The same letter suggests no significant difference ($p > 0.05$) at various temperatures among the same composition, TFSY, or TFSC of SCB samples (n = 2), respectively.

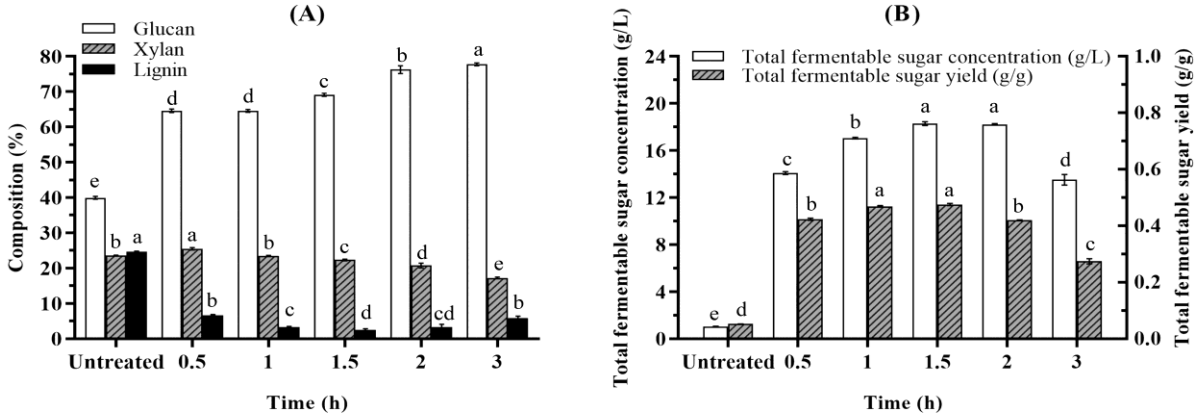

Figure 3. The main compositions (A) and enzymatic saccharification (B) analyses of SCB samples pretreated with 7% Na_2CO_3 in 20% aqueous ammonia for different time at 220 ℃. The same letter suggests no significant difference ($p > 0.05$) at various time among the same composition, TFSY, or TFSC of SCB samples (n = 2), respectively.

As noted in Fig. 3B, the TFSY and the TFSC had the same tendency. As the time was extended to 1.5 h from 0.5 h, the TFSY and TFSC gradually increased, and 0.4757 g/g of the highest TFSY and 18.29 g/L of the highest TFSC were respectively obtained as the pretreatment time was 1.5 h. But the difference between the two TFSYs (0.4698 g/g versus 0.4757 g/g) obtained at 1 h and 1.5 h was not significant ($p > 0.05$). In view of time cost and economic benefit, a pretreatment time of 1 h was suitable for the NWAA method.

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3.5. Optimization of Na_2CO_3 with aqueous ammonia pretreatment using RSM

In order to obtain the maximum TFSY and TFSC which were set as response values, NWAA pretreatment conditions were optimized using a specific BBD model in RSM on the basis of the result of the single factor test. In the supplementary materials, the coded and actual values of the four factors (Na_2CO_3 concentration, ammonia concentration, pretreatment temperature and time) at three levels are enumerated. A BBD of 29 runs containing five center points, resulting in a series of experiments, was carried out at random. According to the results corresponding to RSM, multivariate regression analyses were implemented (Table 3 and Table 4). After that, the predictive TFSY and TFSC could be calculated by the two equations as follow:

$$Y_1 = 16.89 + 0.20X_1 - 0.06X_2 + 0.65X_3 + 2.04X_4 + 0.000X_1X_2 + 0.14X_1X_3 + 0.23X_1X_4 + 0.100X_2X_3 + 0.40X_2X_4 - 0.88X_3X_4 - 0.34X_1^2 - 1.43X_2^2 - 0.32X_3^2 - 1.38X_4^2 \quad (5)$$

$$Y_2 = 0.46 + 0.001667X_1 - 0.0008333X_2 - 0.006667X_3 + 0.019X_4 + 0.000X_1X_2 + 0.000X_1X_3 + 0.000X_1X_4 + 0.005000X_2X_3 + 0.013X_2X_4 - 0.035X_3X_4 - 0.008083X_1^2 - 0.024X_2^2 - 0.018X_3^2 - 0.034X_4^2 \quad (6)$$

in which Y_1 and Y_2 stand for predictive TFSC and TFSY, respectively; and where X_1, X_2, X_3 and X_4 stand for Na_2CO_3 concentration, ammonia concentration, pretreatment temperature and time, respectively.

Table 3. Analysis of variance of the response surface quadratic model for the TFSC of SCB samples pretreated by Na_2CO_3 with aqueous ammonia.

Source	Sum of squares	Degrees of freedom	Mean squares	*F*-value	*P*-value (Prob > F)
Model	81.09	14	5.79	14.00	< 0.0001 Significant
X_1-Na_2CO_3 concentration	0.49	1	0.49	1.19	0.2939
X_2-Ammonia concentration	0.049	1	0.049	0.12	0.7348
X_3-Pretreatment temperature	4.99	1	4.99	12.06	0.0037
X_4-Pretreatment time	49.86	1	49.86	120.49	< 0.0001
X_1X_2	1.421E-014	1	1.421E-014	3.434E-014	1.0000
X_1X_3	0.081	1	0.081	0.20	0.6645
X_1X_4	0.21	1	0.21	0.51	0.4863
X_2X_3	0.040	1	0.040	0.097	0.7605
X_2X_4	0.63	1	0.63	1.53	0.2368
X_3X_4	3.12	1	3.12	7.53	0.0158
X_1^2	0.73	1	0.73	1.77	0.2041
X_2^2	13.20	1	13.20	31.90	< 0.0001
X_3^2	0.68	1	0.68	1.63	0.2221
X_4^2	12.31	1	12.31	29.75	< 0.0001
Residual	5.79	14	0.41		
Lack of fit	5.41	10	0.54	5.68	0.0543 Not significant
Pure error	0.38	4	0.095		
Cor Total	86.89	28			

After that, the ANOVA was carried out, whose results (Table 3 and Table 4) showed the *F*-values of TFSY and TFSC models were 6.44 and 14.00, respectively, with *p*-values which were less than 0.05. This suggested that the TFSC and TFSY models were both significant. Moreover, *p*-values > 0.05 of lack-of-fit for TFSC and TFSY models indicated that the two equations obtained from the results of RSM were suitable for small test error, and the response values would not be significantly influenced by the accidental factors. The determinant coefficients (R^2) of TFSY and TFSC models were 0.8656 and 0.9333, respectively, suggesting that 86.56% and 93.33% of the variability of the experimental variables within the scope of research could be explained. The observed signal to noise ratios of TFSY and TFSC models were 9.879 and 13.101, respectively, suggesting that these two models could be used for navigating the design space. Moreover, the similar results between observed values and predicted values (Table 1) further confirmed the

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quality of the models of TFSC and TFSY. According to the results of ANOVA, pretreatment time was the most important factor influencing the response values, following by the pretreatment temperature, Na_2CO_3 concentration, and ammonia concentration.

Table 4. Analysis of variance of the response surface quadratic model for the TFSY of SCB samples pretreated by Na_2CO_3 with aqueous ammonia.

Source	Sum of squares	Degrees of freedom	Mean squares	*F*-value	*P*-value (Prob > F)
Model	0.021	14	1.498E-003	6.44	0.0006 Significant
X_1-Na_2CO_3 concentration	3.333E-005	1	3.333E-005	0.14	0.7106
X_2-Ammonia concentration	8.333E-006	1	8.333E-006	0.036	0.8526
X_3-Pretreatment temperature	5.333E-004	1	5.333E-004	2.29	0.1521
X_4-Pretreatment time	4.408E-003	1	4.408E-003	18.96	0.0007
X_1X_2	0.000	1	0.000	0.000	1.0000
X_1X_3	0.000	1	0.000	0.000	1.0000
X_1X_4	0.000	1	0.000	0.000	1.0000
X_2X_3	1.000E-004	1	1.000E-004	0.43	0.5226
X_2X_4	6.250E-004	1	6.250E-004	2.69	0.1234
X_3X_4	4.900E-003	1	4.900E-003	21.08	0.0004
X_1^2	4.238E-004	1	4.238E-004	1.82	0.1984
X_2^2	3.841E-003	1	3.841E-003	16.52	0.0012
X_3^2	2.121E-003	1	2.121E-003	9.12	0.0092
X_4^2	7.646E-003	1	7.646E-003	32.89	< 0.0001
Residual	3.255E-003	14	2.325E-004		
Lack of fit	2.975E-003	10	2.975E-004	4.25	0.0879 Not significant
Pure error	2.800E-004	4	7.000E-005		
Cor Total	0.024	28			

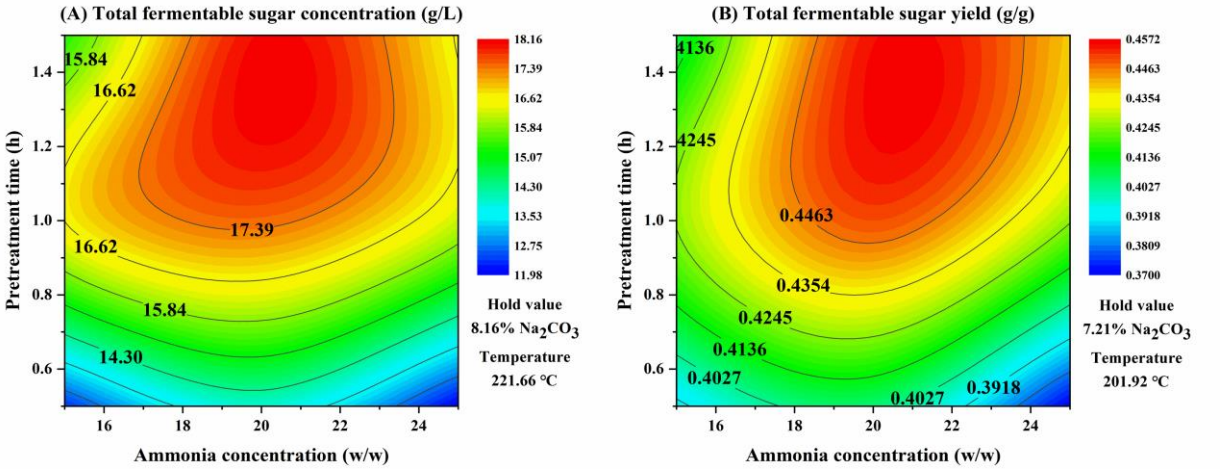

Figure 4. Contour charts revealing the effects of ammonia concentrations and pretreatment time on (A) TFSC with the hold values of 8.16% Na_2CO_3 and 221.66 ℃ (optimal conditions were pretreatment time of 1.39 h and ammonia concentration of 20.44%) and (B) TFSY with the hold values of 7.21% Na_2CO_3 and 201.92 ℃ (optimal conditions were pretreatment time of 1.38 h and ammonia concentration of 20.42%).

The interactive effects of pretreatment time and the concentration of aqueous ammonia on the response values of TFSC and TFSY are presented in Fig. 4. As predicted, 17.76 g/L of the highest TFSC was attained with the condition of 8.16% Na_2CO_3 with 20.44% ammonia at 221.66 ℃ for 1.39 h (pretreatment T1), and 0.4723 g/g of the highest TFSY was attained as SCB was pretreated by 7.21% Na_2CO_3 with 20.42% ammonia at 201.92 ℃ for 1.38 h (pretreatment T2). Confirmatory tests were conducted with the purpose of validating the predicted models, and 18.16 g/L of actual TFSC and 0.4777 g/g of actual TFSY were achieved, respectively (Table 5).

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The difference between the actual values and predicted values was not significant ($p > 0.05$), further suggesting that the TFSC model and TFSY model were both adequate. In comparison to the initial results obtained from single-factor tests, the TFSC and the TFSY obtained from the optimal pretreatment conditions significantly increased from 17.05 g/L to 18.16 g/L and 0.4698 g/g to 0.4777 g/g, respectively ($p < 0.05$). Interestingly, the temperature used in the T2 pretreatment conditions was significantly decreased from 220 ℃ to 201.92 ℃, which was beneficial to reduce the cost of the pretreatment process for obtaining high economic effectiveness. In addition, the delignification efficiencies of pretreatment T1 and T2 were 93.94% and 92.47%, respectively, which were significantly higher than those of our previous study (79.34% and 77.81%) ($p < 0.05$) [19,31]. According to the above results, the NWAA pretreatment presented in this investigation could be an efficient method to remove the lignin for enhancing the enzymatic hydrolysis efficiency of SCB.

Table 5. Main compositions and enzymatic saccharification of SCB samples before and after RSM optimization.

	Component			Solid recovery (%)	TFSC (g/L)	TFSY (g/g)
	Glucan (%)	Xylan (%)	Lignin (%)			
Raw SCB	39.96 ± 0.38	23.59 ± 0.14	24.69 ± 0.13	100	1.05 ± 0.01	0.0526 ± 0.0004
Initial	64.57 ± 0.37	23.51 ± 0.16	3.36 ± 0.14	55.10 ± 0.13	17.05 ± 0.07	0.4698 ± 0.0020
Optimized[a]	66.94 ± 0.18	20.97 ± 0.49	2.91 ± 0.43	51.63 ± 0.54	18.16 ± 0.08	0.4688 ± 0.0023
Optimized[b]	67.45 ± 0.18	21.42 ± 1.17	3.41 ± 0.06	54.44 ± 1.10	17.56 ± 0.01	0.4777 ± 0.0014
Predicted[a]	-	-	-	-	17.76	-
Predicted[b]	-	-	-	-	-	0.4723

[a] The optimal conditions of NWAA pretreatment for the maximum TFSC.

[b] The optimal conditions of NWAA pretreatment for the maximum TFSY.

3.6. Characterization of the raw and NWAA pretreated SCBs

According to the above results, the NWAA method could be effective in enhancing the enzymatic saccharification of SCB, which indicated that the NWAA pretreatment might modify or deconstruct the structure of SCB. With the purpose of verifying this hypothesis and understanding the mechanism of NWAA pretreatment in improving the enzymatic saccharification, the methods of SEM, FTIR, and XRD were used for analyzing the chemical and physical structural characteristics of raw and NWAA pretreated SCB samples. The SCBs obtained from pretreatment T1 and T2 were defined as T1 and T2, respectively. The results of characterization are presented as follows.

3.6.1. SEM observation

Regarding the enhancement of enzymatic hydrolysis efficiency, the available surface area of lignocellulose may be an extremely crucial factor [32]. With the purpose of observing the alteration of ultrastructure in NWAA pretreated SCB, the surface characteristics and morphological features of raw and pretreated SCBs (T1 and T2) were observed using SEM. In keeping with previous research, the SEM images (Fig. 5) showed that the fiber structure of raw SCB was rigid, smooth and highly ordered, which restricted the accessibility for enzymes to cellulose [31]. However, the fiber structures of T1 and T2 were deconstructed to form an irregular structure with coarse, cracky and porous surface, which could increase the susceptibility of cellulose to cellulases. The probable reason of forming the irregular and porous surface is the effect of perforation on the fiber by Na_2CO_3 assisted with aqueous ammonia and the degradation of the lignin and partial xylan in the NWAA pretreatment process. On the surface of the substrate, the morphological changes played a decisive role in exposing more reactive sites, leading to the enhancement of enzymatic saccharification [32].

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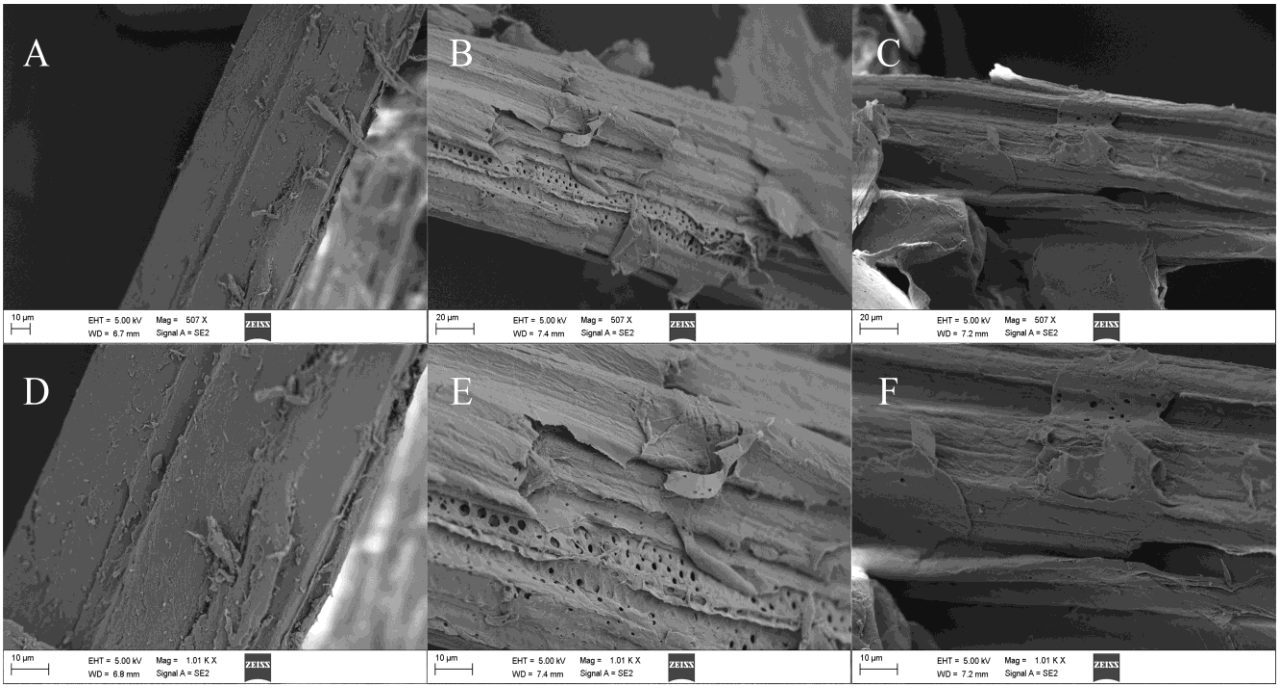

Figure 5. SEM images of raw SCB (A, D), the SCB pretreated by pretreatment T1 to obtain the highest TFSC (B, E), and the SCB pretreated by pretreatment T2 to achieve the highest TFSY (C, F) in two magnifications of 507 and 1010, respectively.

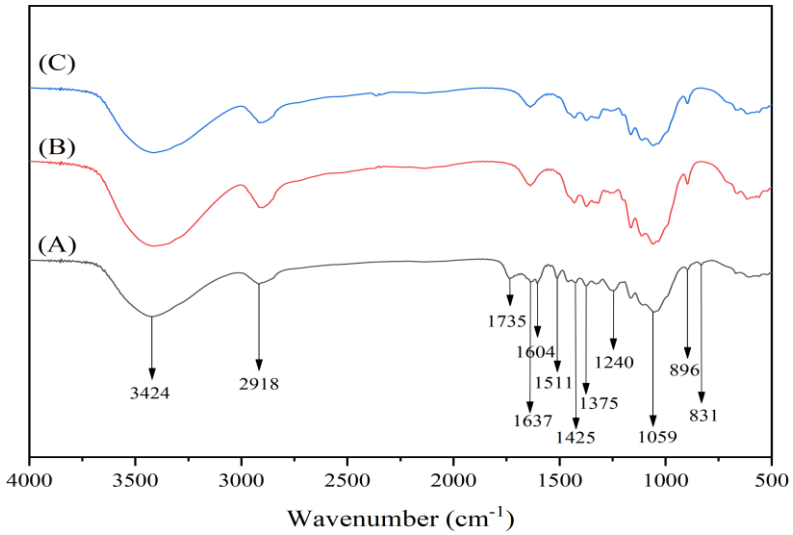

Figure 6. FTIR spectra of raw SCB (A), the SCB pretreated by pretreatment T1 to obtain the highest TFSC (B), and the SCB pretreated by pretreatment T2 to achieve the highest TFSY (C).

3.6.2. FTIR measurement

FTIR spectroscopy was used for analyzing the alterations of structural composition and chemical functional groups in the untreated and the NWAA pretreated SCBs. The chemical changes of carbohydrates and lignin were analyzed using the characteristic peaks (Fig. 6). The variations of T1 and T2 could be clearly observed as compared to the raw SCB; however, the difference between T1 and T2 was not significant.

Compared to the raw SCB, the lignin-associated peaks of T1 and T2 at 831 cm^{-1}, 1240 cm^{-1}, 1511 cm^{-1}, 1604 cm^{-1} and 1735 cm^{-1} vanished, suggesting that the most part of lignin was removed during NWAA pretreatment. In regard to these peaks associated with lignin, the peak at 831 cm^{-1} represents syringyl lignin vibration, and the peak near 1240 cm^{-1} is related to β-ether

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bonds in lignin and between carbohydrates and lignin [32]. The peak at 1735 cm^{-1} is associated with the phenolic hydroxyl groups in lignin and the vibrations of carbonyl/acetyl groups in hemicellulose [33]. Additionally, the bands at 1511 cm^{-1} and 1604 cm^{-1} represent the skeletal vibration of aromatic lignin ring.

The bands at 896 cm^{-1}, 1059 cm^{-1},1425 cm^{-1}, 2918 cm^{-1} and 3424 cm^{-1} are associated with the β-(1-4)-glycosidic bond; C-O, C-C, C-O-C stretching; the C-H_2 vibration; the C-H vibration; the O-H stretching, respectively [34]. These bands are relevant to hemicellulose and/or cellulose. The intensities of these peaks in T1 and T2 were relatively higher than those of the raw substrate, which indicated the content of total carbohydrates in the SCBs after NWAA pretreatment could be greater than the carbohydrate content of untreated solid. The band at 1637 cm^{-1} is in connection with the bending vibrations of absorbed water, and the peaks of T1 and T2 became stronger [35]. In addition, the intensity of the peak at 1375 cm^{-1} (acetylation of hydroxyl groups) was declined in NWAA pretreated samples, suggesting deacetylation in the process of NWAA pretreatment [36].

3.6.3. XRD measurement

Another important indicator of the performance of enzymatic saccharification is the crystallinity of cellulose [34]. An X-ray diffractometer was employed for determining the CrI of the raw and NWAA pretreated (T1 and T2) SCB samples (Fig. 7). After calculation based on Eq. (1), the CrI of raw SCB was 34.24%, while those of T1 and T2 were 38.62% and 41.29%, respectively. The increase of CrI was consistent with many published reports, and the result might be ascribed to the dissolution/removal of hemicellulose and lignin during the process of pretreatment [32,36]. Moreover, NWAA possibly dissociated hydrogen bonds among crystalline cellulose and hemicellulose/lignin, bringing about the increase of the CrI [33].

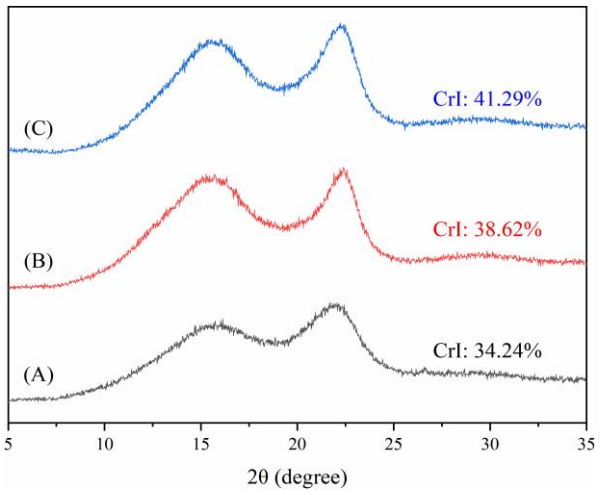

Figure 7. XRD patterns and corresponding CrI of raw SCB (A), the SCB pretreated by pretreatment T1 to obtain the highest TFSC (B), and the SCB pretreated by pretreatment T2 to achieve the highest TFSY (C).

4. Conclusions

Under the optimal NWAA conditions optimized by RSM, 18.16 g/L of TFSC and 0.4777 g/g of TFSY were obtained with the delignification of 93.94% and 92.47%, respectively. Structural characteristics analyses of the pretreated SCBs indicated that the NWAA pretreatment could deconstruct the recalcitrant structure of SCB and remove the lignin, which is crucial to improve the enzymatic digestibility of SCBs significantly. Thus, NWAA pretreatment could be a prospective candidate for the pretreatment process of enzyme-based lignocellulosic biorefineries.

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