Aqueous Ammonia With Sodium Sulfite Pretreatment For Enhancing Enzymatic Saccharification and Bioethanol Production of Sugarcane Bagasse

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Research

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Aqueous ammonia with sodium sulfite pretreatment for enhancing enzymatic saccharification and bioethanol production of sugarcane bagasse

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Abstract

Background: Bioethanol is considered as a promising alternative fuel. Lignocellulosic biomass can be used for the production of bioethanol, but its recalcitrant structure makes it difficult to be utilized. Thus, proper pretreatment is a crucial step to break this structure and enhance enzymatic saccharification. Aqueous ammonia with sodium sulfite pretreatment (AAWSSP) was first applied to enhance the enzymatic saccharification and bioethanol production of sugarcane bagasse (SCB) in this research.

Results: Response surface methodology was applied to optimize the conditions of pretreatment. Under optimal parameters, 16.92 g/L of total sugar concentration (P1 SCB: 202.08℃, 11.06% aqueous ammonia, 13.37% sodium sulfite, 1.22 h) and 0.51 g/g of total sugar yield (P2 SCB: 199.47℃, 10.17% aqueous ammonia, 13.11% sodium sulfite, 1.17 h) were achieved, respectively. The results of ethanol fermentation showed that separate hydrolysis and fermentation performed better than that of simultaneous

saccharification and fermentation, and the maximum ethanol yields of 143.30 g/kg for P1 SCB and 145.33 g/kg for P2 SCB, were obtained, respectively.

Conclusions: This research indicated that aqueous ammonia and sodium sulfite in pretreatment solution might have a synergistic effect on delignification and enzymatic saccharification. AAWSSP might be a prospective method for enhancing enzymatic saccharification and bioethanol production of SCB, which provided new guidance for the bio-refinery of lignocellulose.

Keywords: Sugarcane bagasse, Pretreatment, Aqueous ammonia, Sodium sulfite, Enzymatic saccharification, Ethanol fermentation

Background

Fossil fuels are the most important energy sources, but their unreasonable exploration and utilization have caused energy shortage and environmental pollution [1]. Thus, alternative energy sources need to be clean, low-carbon, pollution-free and renewable [2]. Recently, lignocellulosic biomass has attracted great attention for it can be utilized for producing renewable biofuels while alleviating environmental problem [3, 4]. As an alternative fuel, bioethanol has many advantages, such as high oxygen content, high octane value, excellent burning performance and lower emission of pollutants [5]. The production of bioethanol from biomass mainly includes pretreatment, enzymatic saccharification and microbial fermentation [6]. Cellulose, hemicellulose and lignin are the primary components of lignocellulose [7]. The recalcitrant structure of these three components makes it difficult to be hydrolyzed by enzymes or biodegraded, which brings great challenges to the utilization of lignocellulose [8, 9]. Proper pretreatment is the crucial step to break down this recalcitrant structure, remove lignin or/and

hemicellulose, and enhance enzymatic saccharification and bioethanol fermentation [10].

Compared with acid pretreatment, alkali pretreatment is conducted under relatively mild conditions, leading to less carbohydrates losses while providing higher digestibility of lignocellulose because of the lignin removal or decomposition of lignin-carbohydrates complex [11]. It enhances the accessibility of enzyme to lignocellulose mainly by modifying and removing lignin, solubilizing hemicellulose, and swelling cellulose [11]. The mechanism of delignification involves the destruction of aryl-ether bonds and the solubilization of lignin after ionization and solvation of aromatic hydroxyl groups [12]. Pretreatment aims to change or remove structural and compositional barriers of enzymatic saccharification and to enhance the enzymatic digestibility [13]. Alkali pretreatment might produce intermediate products such as black liquor containing lignocellulosic material, acids greases, polyphenolic compounds, aliphatic acids, and resinous compounds, which cannot be reused, but direct discharge will cause environmental pollution [14].

Ammonia pretreatment has been applied to the delignification of lignocellulose successfully. It can enhance the surface area of cellulose, destroy the crystalline structures, retain most of carbohydrates, and has shown better delignification along with production of minimum toxic compounds, so as to improve the enzymatic efficiency [15]. Ammonolysis of lignocellulose occurs during ammonia pretreatment, which acts specifically on the lignin-carbohydrates complex by cleaving ether and ester bonds for purpose of removing lignin and increasing the accessibility of cellulase to holocellulose [16, 17]. It has higher selectivity with lignin than that of carbohydrates. Delignification of lignocellulose results in the increasing content of cellulose which can be transformed

into fermentable sugar and then bioethanol [17]. Ammonia is an effective expansion reagent for biomass and one of the most widely used chemicals with lower cost [18]. Furthermore, ammonia is a pollution-free and non-corrosive reagent, and its high volatility makes it easier to be recycled [18]. In conclusion, ammonia is a suitable reagent for pretreatment because of a large amount of excellent features. However, the delignification and enzymatic saccharification of biomass pretreated by ammonia alone are still not as good as that of strong alkali.

It was found that pretreatment system containing a higher proportion of sodium sulfite could soften the lignocellulose better via delignification, hemicellulose degradation, and lignin sulfonation [19]. Sulfonation can increase the hydrophilicity of lignin and the swelling of substrate, reduce the tendency of non-productive combination of lignin and cellulase, which will improve the enzymatic saccharification [20]. Sulfite pretreatment in alkaline environment can also increase the sulfonation and dissolubility of lignin [21].

In this study, sugarcane bagasse was subjected to aqueous ammonia with sodium sulfite pretreatment (AAWSSP), in order to obtain higher total sugar concentration (TSC) and total sugar yield (TSY) while selectively removing lignin and remaining most of carbohydrates. Besides, aqueous ammonia can be recycled. Later the pretreatment conditions, including pretreatment temperature, aqueous ammonia concentration, sodium sulfite concentration and pretreatment time, were optimized using response surface methodology (RSM) based on single factor experiments. Ultimately, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were carried out for the production of bioethanol. To our best knowledge, it is the first time to pretreat sugarcane bagasse by

aqueous ammonia with sodium sulfite.

Results and discussion

Effect of pretreatment temperature on components and enzymatic saccharification of pretreated SCB

Temperature acts an critical part in the pretreatment of biomass for it has a direct impact on the energy input, the capacity of pretreatment and pretreatment cycle [22]. Hence, the impact of temperatures of 120℃, 140℃, 160℃, 180℃, 200℃, 220℃ on SCB with AAWSSP was first investigated. The biomass recovery, components, TSC and TSY were compared (Additional file 1: Table S1). Most of the pretreatment groups had a significant influence on the biomass recovery and the components. Besides, AAWSSP improved the enzymatic saccharification of pretreated SCBs compared to native SCB.

As demonstrated, adding sodium sulfite (Na_2SO_3) to the aqueous ammonia pretreatment could increase the lignin removal and enzymatic saccharification to some extent at 120℃ when compared with aqueous ammonia pretreatment alone. When the temperature increased from 120℃ to 220℃, the biomass recovery declined from 75.39% to 60.93%, principally because of the lignin removal. A large proportion of carbohydrates were remained, the cellulose recoveries were higher than 89% and hemicellulose recoveries were within the range of 67.31-87.71%, which implied that AAWSSP could selectively remove lignin while remaining most carbohydrates [23]. Hemicellulose was more likely to be degraded at high temperature compared to cellulose, indicating that the structural intensity of hemicellulose is more weaker [24]. In addition, the contents of cellulose and hemicellulose after AAWSSP were significantly higher than native SCB ($p < 0.05$). The delignification rates of pretreated

SCBs were 51.63-91.13%, and the values increased with the elevation of temperature from 120℃ to 220℃, indicating that high temperature resulted in the higher removal of lignin [25]. As is well-known, higher temperature could cleave α-ether and ester bonds between lignin and hemicellulose, contributing to the synergistic degradation of biomass pretreated by alkaline [26]. However, higher temperature will lead to a good deal of hemicellulose degradation on account of the weak structure of amorphous hemicellulose.

The TSCs and TSYs of SCBs after AAWSSP were significantly higher than that of native SCB ($p < 0.05$). The TSCs and TSYs significantly elevated with the increment of pretreatment temperature ($p < 0.05$). The highest TSC of 15.04 g/L and TSY of 0.47 g/g were both gained at 200℃, which probably resulted from the higher delignification efficiency and the destruction of the recalcitrant structure, leading to the higher accessibility of enzyme to cellulose. As the temperature increased to 220℃, the cellulose decreased from 62.38% to 60.70% compared to that of 200℃, resulting in the lower recovery of cellulose. Though the delignification improved at 220℃, the TSC and TSY both decreased. Therefore, 200℃ might be the proper temperature for subsequent investigation of AAWSSP conditions.

Effect of aqueous ammonia concentration on components and enzymatic saccharification of pretreated SCB

Aqueous ammonia pretreatment can partially break the chemical linkages between hemicellulose and lignin, change the physical characteristics of biomass making it more pliable and increase its uptake of water, which plays a crucial part in the digestibility of lignocellulose [27]. Hence, it is likely that the increasing concentration of aqueous

ammonia might contribute to higher lignin removal and enzymatic saccharification efficiency.

Fig. 1

The impact of diverse aqueous ammonia concentrations on componential changes and enzymatic saccharification of SCB was shown in Fig. 1. Clear changes were found in the main components of SCBs pretreated with diverse aqueous ammonia concentrations compared to native SCB. Along with the increment of aqueous ammonia concentration from 5% to 25%, the biomass recoveries decreased from 66.57% to 58.24%, while the cellulose contents increased from 57.48% to 67.09%. Over 95% of cellulose was maintained after AAWSSP. Similar to pretreatment temperature, aqueous ammonia concentration has less impact on the hemicellulose contents of SCBs. When aqueous ammonia concentration enhanced from 5% to 15%, the contents of hemicellulose were not significant ($p > 0.05$) and decreased significantly with 20% and 25% of aqueous ammonia ($p < 0.05$). The AAWSSP process significantly reduced the lignin content ($p < 0.05$), which declined from 22.83% of the native SCB to 8.78-4.20% of pretreated SCBs. With a rise in aqueous ammonia concentration from 5% to 20%, the delignification increased significantly ($p < 0.05$) but no significant difference was shown between 25% and 20% of aqueous ammonia ($p > 0.05$). When the concentration of aqueous ammonia was 20%, the maximum delignification of 89.17% was obtained, but the lower TSC and TSY were obtained. The highest TSC (15.04 g/L) and highest TSY (0.47 g/g) were achieved with 10% of aqueous ammonia. With the concentration of aqueous ammonia increasing from 10% to 25%, the TSC and TSY decreased slightly. Therefore, 10% (w/w) of aqueous ammonia was appropriate for AAWSSP.

Effect of Na_2SO_3 concentration on components and enzymatic saccharification of pretreated SCB

It is acknowledged that sulfite can cleave α-alkyl ether bonds, α-benzyl ether bonds, and β-benzyl ether linkages on phenolic lignin groups as well as sulfonate the lignin, thus improving the enzymatic digestibility of substrates [28]. If sulfite was added to pretreatment, the sulfonation of lignin occurred, resulting in the increasing swelling of fiber and higher solubility of lignin.

Fig. 2

The Na_2SO_3 concentration varied from 4 to 20% (w/w, based on dry SCB) supplemented with 10% (w/w) aqueous ammonia to investigate the impact on AAWSSP at 200℃ for 1 h. As presented in Fig. 2, the maximum biomass recovery of 64.95% was achieved with 4% Na_2SO_3, followed by that of 63.57% when the concentration of Na_2SO_3 was 12%, but the Na_2SO_3 concentration doesn't showed significant influence on the biomass recovery in the range from 8% to 20% ($p > 0.05$). Previous study reported that high biomass recovery made a sense because the economic feasibility of pretreatment relies extremely on high biomass recovery [29]. When Na_2SO_3 concentration increased from 8% to 12%, the hemicellulose contents increased from 26.53% to 28.06% although the cellulose content and delignification declined a little. And the maximum hemicellulose recovery of 77.75% was achieved when the loading of Na_2SO_3 was 12%. Delignification increased with the chemical loading increasing from 12% to 20% in pretreatment step. The maximum delignification corresponding to 88.03% of AAWSSP with 20% sodium sulfite, indicating that the sulfonation of lignin enhanced with the increasing loading of Na_2SO_3. The TSCs gradually improved when the loading of Na_2SO_3 increased from 4% to 12%. The improved TSCs partly resulted

from the modification/degradation and the increasing hydrophilicity of lignin [21]. This could result in the easier accessibility of enzyme to carbohydrates, leading to the release of sugar when compared to the native group. When the chemical charge was 12%, the delignification of 81.39% was achieved. The increasing Na_2SO_3 concentration during AAWSSP resulted in higher delignification since 88.03% of delignification was achieved when the chemical loading was 20%. The highest TSC of 16.48 g/L and TSY of 0.52 g/g were both obtained with 20% Na_2SO_3 pretreatment, followed by 16.19 g/L for TSC and 0.51 g/g for TSY with 12% Na_2SO_3. But no significant difference was shown as the concentration of Na_2SO_3 increased from 12% to 20% ($p > 0.05$). Therefore, 12% (w/w) sodium sulfite was selected for further investigation of the AAWSSP conditions.

Effect of pretreatment time on components and enzymatic saccharification of pretreated SCB

Fig. 3

To evaluate the appropriate pretreatment time, 10% (w/w) aqueous ammonia with 12% (w/w) Na_2SO_3 was implemented for SCB pretreatment at 200℃ for different time (0.5, 1, 2, 3 h). The components and enzymatic saccharification of the native SCB and pretreated groups were presented in Fig. 3. With the prolongation of pretreatment time, the biomass recovery declined. All the cellulose contents were significantly higher than that of native SCB ($p < 0.05$), and reached the highest value of 57.59% at 3 h. The contents of cellulose significantly increased from 51.29% to 57.56% with the prolongation of pretreatment time from 0.5h to 2h ($p < 0.05$), but there was no significant difference between 2 h and 3 h ($p > 0.05$). The hemicellulose contents of

SCBs after pretreatment were also significantly higher than that of native SCB ($p < 0.05$). The recovery of hemicellulose declined with the increasing of pretreatment time. Increasing the pretreatment time from 0.5 h to 1 h lowered the content of lignin from 10.92% to 6.68%, and there was no significant difference with the prolongation of pretreatment time from 1 h to 3 h ($p > 0.05$). As presented in Fig. 3 B, all the TSCs and TSYs of SCBs after AAWSSP were significantly enhanced compared with native SCB ($p < 0.05$). As the pretreatment time prolonged from 0.5 h to 2 h, the TSC and TSY significantly enhanced from 12.08 g/L to 17.16 g/L for TSC and from 0.41 g/g to 0.53 g/g for TSY ($p < 0.05$). Both the highest TSC of 17.16 g/L and TSY of 0.53 g/g were obtained at 2 h. With the pretreatment time increasing from 2 h to 3 h, the TSC and TSY showed no significant difference ($p > 0.05$), which might be caused by the similar lignin content and delignification. Although there was significant difference of the TSC and TSY between 1 h and 2 h ($p < 0.05$), it was unworthy to double pretreatment time just to obtain more 1 g/L of TSC and 0.02 g/g of TSY. Hence, taking time and energy consumption into account, 1 h might be the more suitable pretreatment time for further investigation of AAWSSP.

Optimization of AAWSSP using RSM

In order to obtain the highest TSC and TSY, the Box-Behnken design (BBD) was then used to optimize the AAWSSP conditions. Four independent contributions, pretreatment temperature (℃, X_1) from 180 to 220℃, aqueous ammonia concentration (%, X_2) from 5 to 15%, sodium sulfite concentration (%, X_3) from 8 to 16% and pretreatment time (h, X_4) from 0.5 to 1.5 h, were chosen as the variables, and the TSC and TSY were used as the response values. A set of 29 groups of experiments were conducted according to the

software Design-Expert (Additional file 1: Table S2). Based on the experimental data, the quadratic Eqs. (1) and (2) for the TSC (Y_1) and TSY (Y_2) models, respectively, were obtained as follow:

$$Y_1 = 16.04 + 0.61X_1 + 1.02X_2 + 0.84X_3 + 1.58X_4 - 0.20X_1X_2 - 0.46X_1X_3 - 0.46X_1X_4 - 0.64X_2X_3 - 0.45X_2X_4 + 0.047X_3X_4 - 1.02X_1^2 - 1.37X_2^2 - 0.98X_3^2 - 1.65X_4^2 \quad (1)$$

$$Y_2 = 0.51 + 0.006X_1 + 0.017X_2 + 0.018X_3 + 0.037X_4 + 0.000X_1X_2 - 0.018X_1X_3 - 0.010X_1X_4 - 0.020X_2X_3 - 0.025X_2X_4 + 0.000X_3X_4 - 0.046X_1^2 - 0.038X_2^2 - 0.031X_3^2 - 0.053X_4^2 \quad (2)$$

The ANOVA results of the TSC and TSY models (Additional file 1: Table S3 and Table S4) implied that the *p*-values were both less than 0.05 and the F-values were 13.1 and 5.25, respectively, indicating that both the two models regressions were significant at 95% confidence level and the models were suitable to use. The *p*-values of the Lack of Fit (0.1359 and 0.1439, respectively) demonstrated that it is not relative to the accidental error, and the equations fitted well with small experimental error. The Pred R-Squares of 0.9291 for TSC and 0.8400 for TSY were achieved, which meant that 92.91% and 84.00%, of the change (caused by variable changes within the variation range of response variables in this research) of TSC and TSY, respectively, could be explained, so the equations of both models fit well. The “Adeq Precision” represents the signal to noise ratio, which is generally greater than four [30]. The Adeq Precisions of 12.172 for TSC model and 8.165 for TSY model, respectively, indicated the two models were able to navigate the design space. The actual values of responses were near to the predicted ones, further demonstrating that the two models were credible (Additional file 1: Table S2). The results of ANOVA implied that pretreatment time affected TSC and

TSY most, followed by aqueous ammonia concentration and pretreatment temperature, and sodium sulfite concentration the last (Additional file 1: Table S3 and Table S4).

Fig. 4

Fig. 5

The 3D plots of RSM were utilized to present the interactions between two variables factors. It was found that the interaction between each of the four factors was not significant ($p > 0.05$), indicating that each factor was independent and would not interact with each other. Based on the regression quadratic Eq. (1) and (2), the response of TSC and TSY as the function of independent variables of AAWSSP, were shown in Fig. 4 and Fig. 5. It could be found that the maximum TSC and TSY were around 16 g/L and 0.50 g/g, respectively. The highest TSC and TSY were obtained as the following conditions: 13.37% (w/w) sodium sulfite in 11.06% (w/w) aqueous ammonia at 202.08℃ for 1.22 h (P1 SCB) and 13.11% (w/w) of sodium sulfite in 10.17% (w/w) aqueous ammonia at 199.47℃ for 1.17 h (P2 SCB), respectively. Under the optimal parameters, the predicted TSC was 16.67 g/L while the predicted TSY was 0.51 g/g. Confirmatory experiments were carried out for further verification of the predicted results. The actual TSC of 16.92g/L and TSY of 0.51 g/g were obtained (Table 1), which were close to the actual ones and further indicated that the two models fitted well. After RSM optimization, the TSC of SCB pretreated under the optimal pretreatment condition increased by only 4.51% while the TSY was nearly close, but the biomass recovery declined, and the content of cellulose increased to 63.91% and 60.99%, respectively. The results above revealed that the AAWSSP might be an efficient lignocellulose pretreatment method.

Table 1 Comparison before and after RSM optimization

	Components			Biomass recovery (%)	TSC (g/L)	TSY (g/g)
	Cellulose (%)	Hemicellulose (%)	Lignin (%)			
Native SCB	40.21 ± 0.14	22.94 ± 0.35	22.83 ± 0.61	100	0.77 ± 0.02	0.04 ± 0.00
Initial	55.86 ± 0.40	28.06 ± 0.55	6.68 ± 1.06	63.57 ± 0.55	16.19 ± 0.37	0.51 ± 0.02
Optimized (a)	63.91 ± 0.28	27.48 ± 0.00	6.19 ± 0.82	60.85 ± 0.64	16.92 ± 0.15	0.51 ± 0.00
Optimized (b)	60.99 ± 0.88	28.45 ± 0.54	7.42 ± 0.11	61.90 ± 0.18	16.42 ± 0.34	0.51 ± 0.01
Predicted (a)	-	-	-	-	16.67	-
Predicted (b)	-	-	-	-	-	0.51

(a) Optimal condition of AAWSSP for the maximum TSC.

(b) Optimal condition of AAWSSP for the maximum TSY.

Ethanol fermentation

After RSM optimization, ethanol fermentation of native SCB, P1 SCB and P2 SCB were performed. The results of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were shown in Fig. 6.

Fig. 6

The results of SHF (Fig. 6 A-B) showed that the glucose of the native SCB was exhausted after 6 h. The concentration of ethanol reached the maximum value of 0.15 g/L, and the corresponding ethanol yield was 7.5 g/kg native SCB. The glucose of P1 SCB and P2 SCB were both exhausted after 15 h. The highest ethanol concentration of 4.71 g/L for P1 SCB and 4.70 g/L for P2 SCB were gained, and the corresponding ethanol yields were 143.30 g/kg and 145.33 g/kg native SCB, respectively, which were

both significantly higher than that of native SCB ($p < 0.05$).

As presented in Fig. 6 C-D, the highest ethanol concentration (0.13 g/L) of native SCB was obtained after 12 h during SSF, and the yield of ethanol was 6.3 g/kg native SCB. The highest ethanol concentration of 4.57 g/L (P1 SCB) and 4.33 g/L (P2 SCB), respectively, were obtained, and the corresponding ethanol yields were 138.92 g/kg and 133.89 g/kg native SCB, respectively. The ethanol concentrations and yields of P1 SCB and P2 SCB were both significantly higher than that of the native one ($p < 0.05$).

It was also found that xylose cannot be exhausted for ethanol fermentation in both SHF and SSF because *saccharomyces cerevisiae* CICC 1445 is lack of xylose metabolic pathway [31]. Taking the ethanol yield and fermentation time into consideration, SHF might perform more excellently on bioethanol fermentation than that of SSF.

Conclusion

In this research, we put forward AAWSSP for the first time and the pretreatment conditions were optimized by single factor experiments and RSM optimization to enhance the TSC and TSY of SCB. The maximum TSC (16.92 g/L) and TSY (0.51 g/g) were obtained as followed: 11.06% (w/w) aqueous ammonia with 13.37% (w/w) sodium sulfite at 202.08℃ for 1.22 h and 10.17% (w/w) aqueous ammonia with 13.11% (w/w) sodium sulfite at 199.47℃ for 1.17 h, respectively. The results of ethanol fermentation showed that SHF performed more excellently than that of SSF, and the highest ethanol yields of 143.30 g/kg native SCB for P1 SCB and 145.33 g/kg native SCB for P2 SCB were obtained. This study provided a new pretreatment technology (AAWSSP) for enhancing the enzymatic saccharification and ethanol production of SCB, which provided new guidance for the bio-refinery of lignocellulose.

Methods

Biomass and chemicals

Sugarcane bagasse (SCB) was supplied by the Liutang Sugar Factory in Guangxi, China. The particle of dry SCB was between the sizes of 100 and 200 mesh (75-150 μm). The SCB composed of 40.21 ± 0.14% cellulose, 22.94 ± 0.35% hemicellulose and 22.83 ± 0.61% lignin (on a dry weight basis). All the chemicals involved were analytical grade. Enzymes used in this study were Cellic® CTec2, provided by Sigma-Aldrich LLC (Shanghai, China). S*accharomyces cerevisiae* CICC 1445 used for bioethanol fermentation was purchased from China Center of Industrial Culture Collection.

Single factor experiments of sugarcane bagasse by aqueous ammonia with sodium sulfite pretreatment (AAWSSP)

AAWSSP of SCB was carried out in Hydrothermal Synthesis Autoclave Reactor (150 mL, 250℃, 3 MPa). AAWSSP was conducted as follow: (5, 10, 15, 20, 25%, w/w) aqueous ammonia and (4, 8, 12, 16, 20%, w/w, based on dry SCB) sodium sulfite at a solid-to-liquid ratio of 1:10 (w/w) at 120-220℃ for 0.5-3h. The sealed reactor was heated to the target temperature within 5 min in an oil bath and stirred at a low speed to insure the adequate mixture of the substrate. After pretreatment, the solid-solution mixtures were filtered for separation and washed by hot deionized water to remove inhibitors. And then the solid residues were dried in an oven at 80℃ for 10 h. The biomass recovery was then calculated. Samples were kept in sealed plastic bags for later tests. The components of samples were measured, and enzymatic hydrolysis was performed.

Response surface methodology optimization of AAWSSP

Response surface methodology (RSM) was applied to further optimize AAWSSP conditions for purpose of obtaining higher TSC and TSY. There were four numerical factors: pretreatment temperature (X_1), aqueous ammonia concentration (X_2), sodium sulfite concentration (X_3) and pretreatment time (X_4). Three trial levels were studied: -1, 0, 1. The levels (coded and actual) of four factors (X_1, X_2, X_3 and X_4) are listed in Table 2.

Table 2 Coded values and levels of variables in BBD

Variables	Symbols	Coded levels of variables		
		-1	0	1
Pretreatment temperature (℃)	X_1	180	200	220
Aqueous ammonia concentration (%, w/w)	X_2	5	10	15
Sodium sulfite concentration (%, w/w)	X_3	8	12	16
Pretreatment time (h)	X_4	0.5	1	1.5

Enzymatic saccharification

Enzymatic saccharification was conducted in the serum bottle (50 mL). Pretreated or native SCBs were mixed with citric acid-citric sodium buffer (0.1 M, pH 4.8), and the solid loading corresponded to 2% (w/w). Tetracycline (0.08 g/L) and nystatin (0.06 g/L) were added to the buffer for the prevention of microbial contamination. The loading of Cellic® CTec2 was 30 FPU/g SCB. The serum bottles were put in an incubator shaker (50℃, 200 rpm). After 72 h enzymatic saccharification, samples were taken and heated to 100℃ for 10 min to terminate the reaction, and then centrifuged (14,000 rpm, 15 min). The supernatants were acidized with 10% sulfuric acid and then filtered through a

membrane (0.22 μm) before analyzing sugars (glucose and xylose) concentrations by the high performance liquid chromatography (HPLC).

Ethanol fermentation

SHF and SSF of pretreated or native SCBs were carried out at 50-mL serum bottles, and *saccharomyces cerevisiae* CICC 1445 was used as the fermentation strain. During SHF, 0.5 g substrate and 25 mL citric acid-citric sodium buffer (0.1 M, pH 4.8) were put into serum bottles, and Cellic® CTec2 (30 FPU/g SCB) was added meanwhile. The serum bottles were put in an incubator shaker (50℃, 200 rpm). After 72 h enzymatic saccharification, samples were collected and peptone was added until the final concentration of 20 g/L was achieved. Then the fermentation strain suspension was inoculated according to the initial OD_{600}=0.5. SHF was conducted in an incubator shaker (30℃, 200 rpm) for 21 h. As for the SSF process, substrate, buffer, Cellic® CTec2, peptone and the fermentation strain suspension were added likewise in serum bottles at the same time. SSF was conducted in an incubator shaker (30℃, 200 rpm) for 108 h.

Calculations and statistical analysis

The components of SCB were measured according to the procedure established by the NREL [32]. Sugars and ethanol concentrations were determined on HPLC system equipped with an Aminex HPX-87H column and refractive index detector at 55℃. The column was operated with 0.6 mL/min H_2SO_4 (5 mM).

The native SCB, TSC and TSY were calculated as followed:

$$\text{Native SCB (g)} = \frac{\text{Solid mass in enzymatic saccharification (g)}}{\text{Biomass recovery (\%)}} \quad (3)$$

$$\text{TSC (g/L)} = \text{Glucose concentration (g/L)} + \text{xylose concentration (g/L)} \quad (4)$$

$$\text{TSY (g/g)} = \frac{\text{TSC (g/L)} \times \text{V (L)}}{\text{Native SCB (g)}} \quad (5)$$

$$\text{Ethanol yield (g/kg)} = \frac{\text{Ethanol concentration (g/L)} \times \text{V (L)}}{\text{Native SCB (kg)}} \quad (6)$$

where the native SCB is equal to the mass of the native SCB in enzymatic saccharification; TSC presents the total concentration of glucose and xylose; TSY presents the total yield of glucose and xylose gained from per gram native SCB; V represents the cubage of enzymatic saccharification.

The data was expressed as the mean value ± standard deviation (SD) and analyzed by one-way ANOVA using Origin (Version 8.6, EA, USA). All the tests were implemented in duplicate.

Abbreviations

AAWSSP: Aqueous ammonia with sodium sulfite pretreatment; SCB: Sugarcane bagasse; TSC: Total sugar concentration; TSY: Total sugar yield; SSF: Simultaneous saccharification and fermentation; SHF: Separate hydrolysis and fermentation; RSM: Response surface methodology; HPLC: High performance liquid chromatography; BBD: Box-Behnken design.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional file.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

MZ designed, conducted experiments and wrote the original manuscript. BT, PL, ZL, XL and JW participated in investigation and analyzed part of data. JL, WX, YX and SH contributed in editing and coordination. ZL conceived the project and helped to revise the manuscript. All authors read and approved the final manuscript.

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Fig. 1 Components (A) and enzymatic saccharification (B) under various ammonia concentrations (8% Na_2SO_3, 200℃, 1 h)

Fig. 2 Components (A) and enzymatic saccharification (B) under different Na_2SO_3 concentrations (10% ammonia, 200℃, 1 h)

Fig. 3 Components (A) and enzymatic saccharification (B) under different pretreatment time (10% ammonia, 12% Na_2SO_3, 200℃）

Fig. 4 3D plots revealing the effect of interaction between various factors on TSC

Fig. 5 3D plots revealing the effect of interaction between various factors on TSY

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Figures

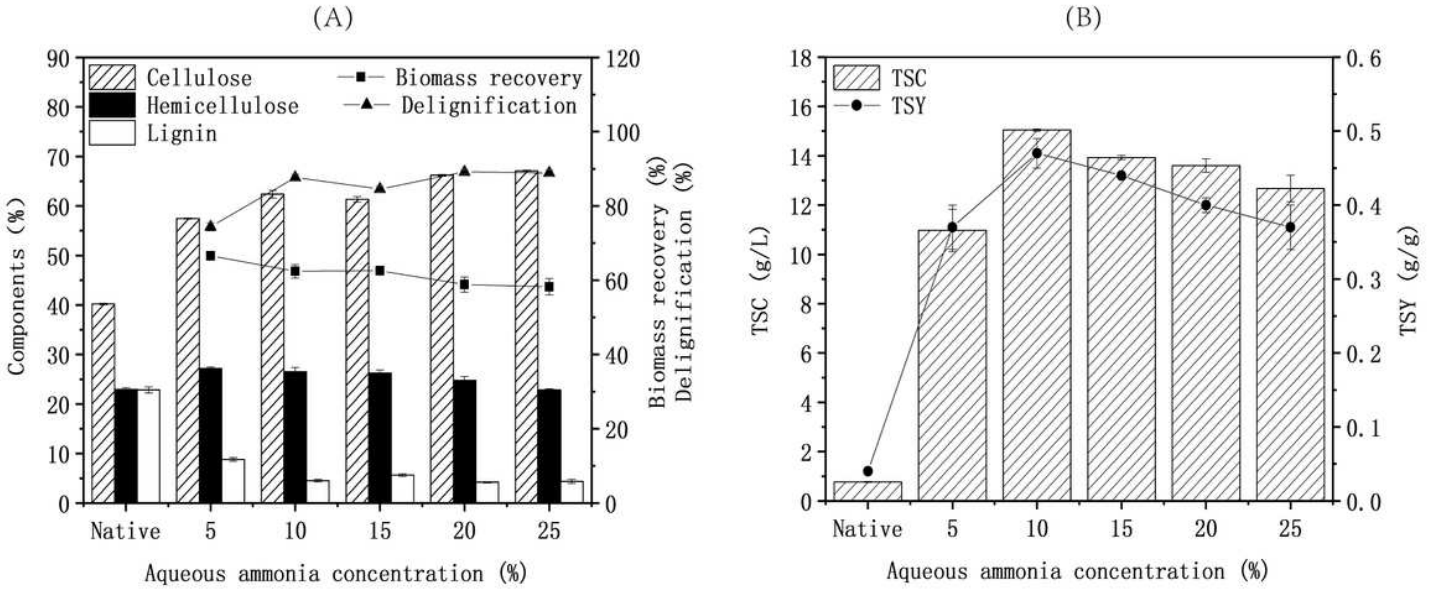

Figure 1

Components (A) and enzymatic saccharification (B) under various ammonia concentrations (8% Na2SO3, 200℃, 1 h)

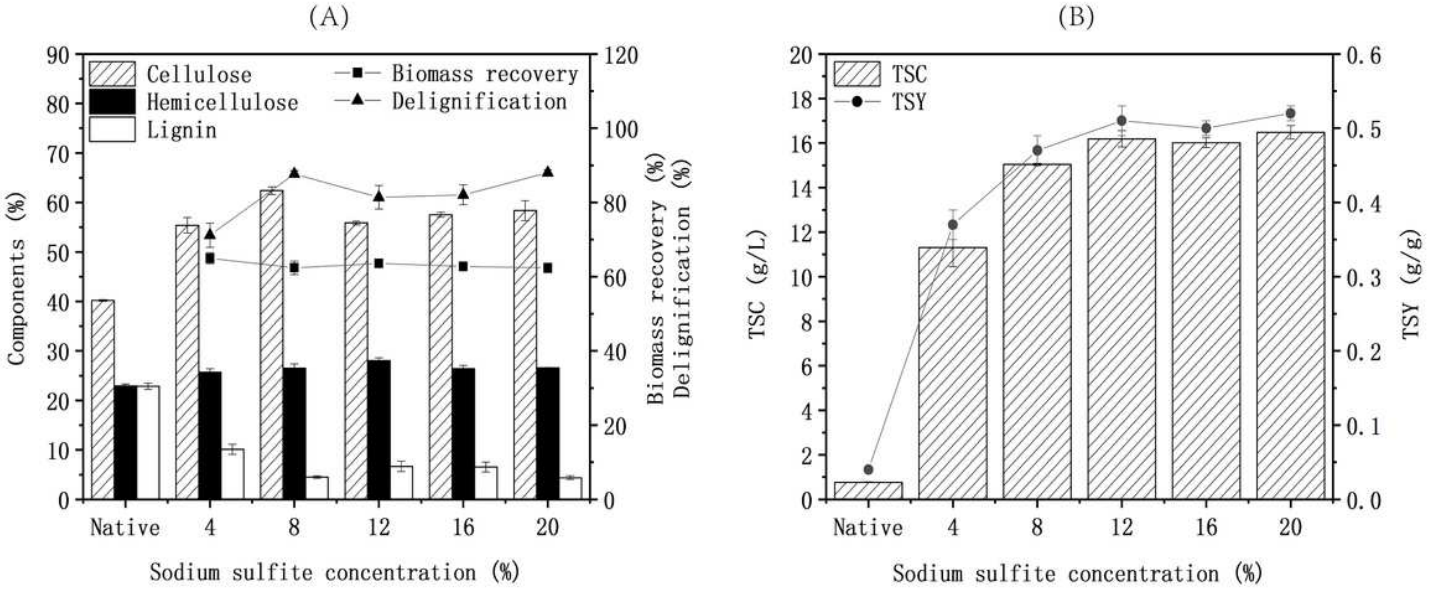

Figure 2

Components (A) and enzymatic saccharification (B) under different Na2SO3 concentrations (10% ammonia, 200℃, 1 h)

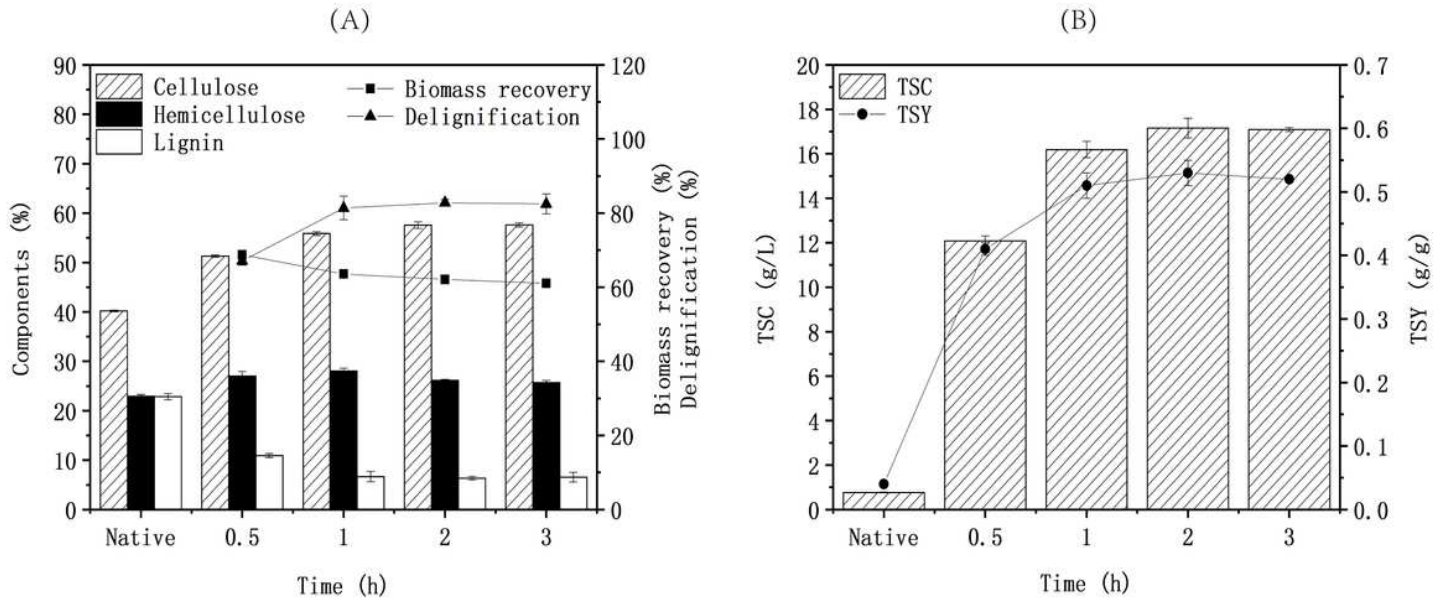

Figure 3

Components (A) and enzymatic saccharification (B) under different pretreatment time (10% ammonia, 12% Na2SO3, 200□)

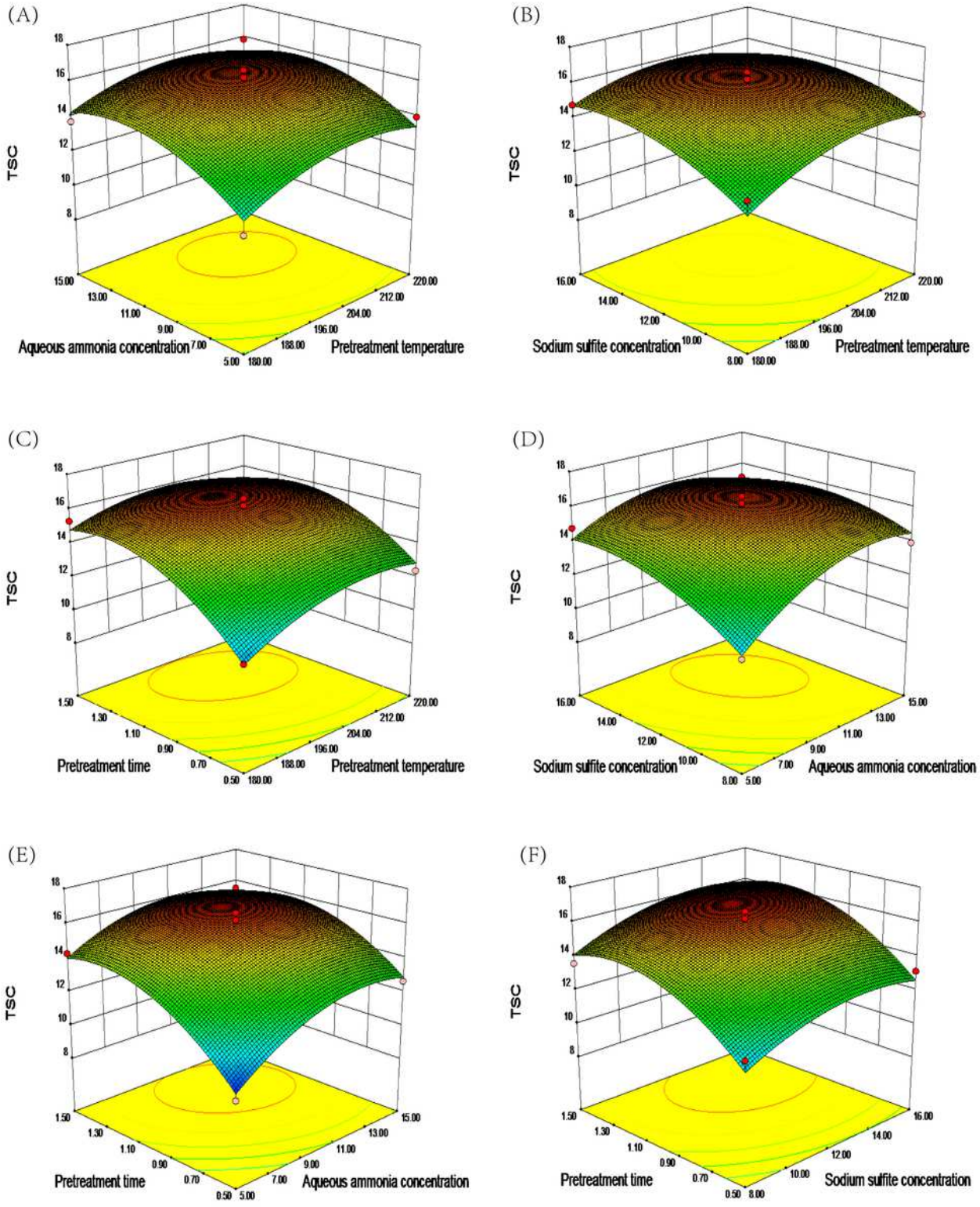

Figure 4

3D plots revealing the effect of interaction between various factors on TSC

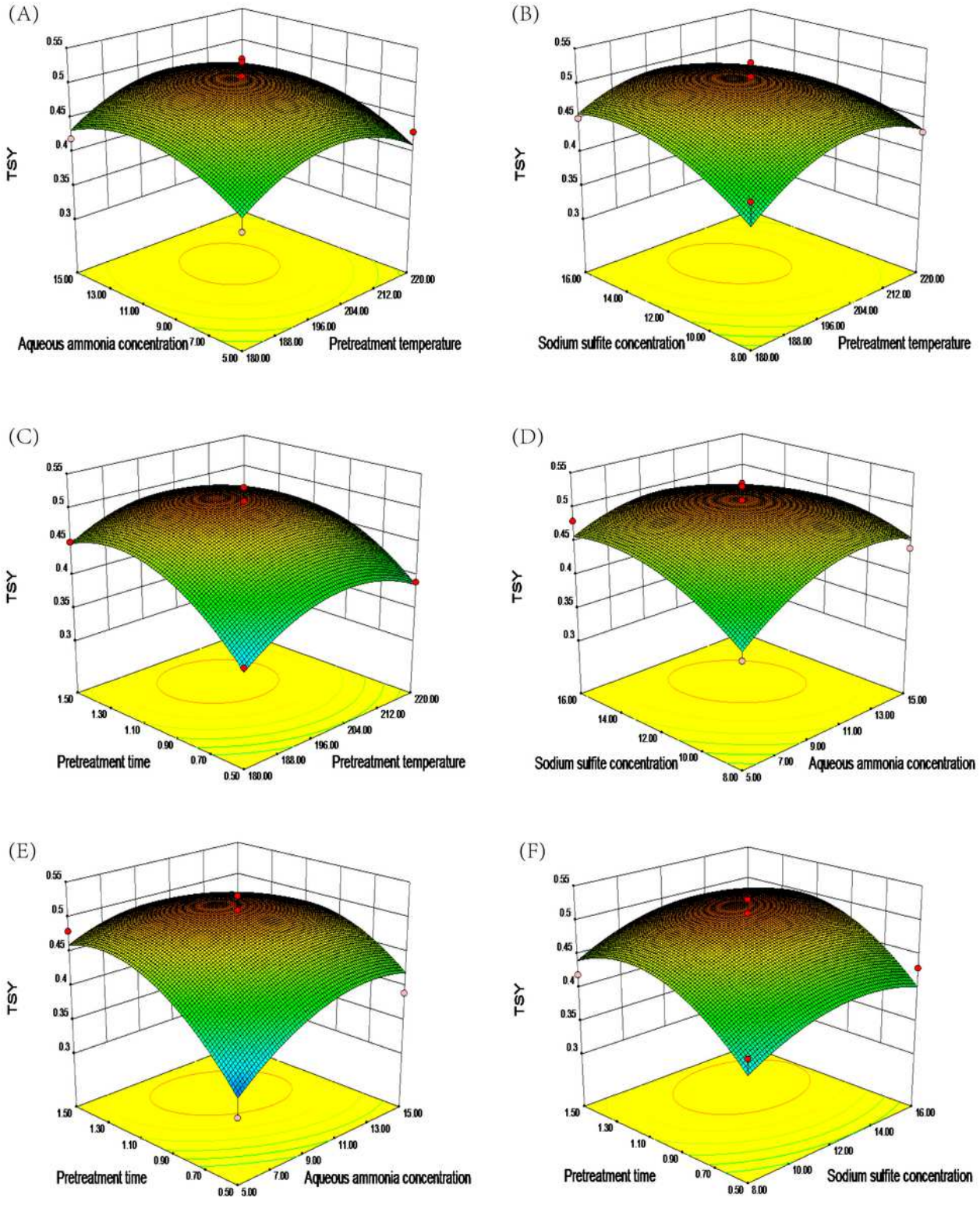

Figure 5

3D plots revealing the effect of interaction between various factors on TSY

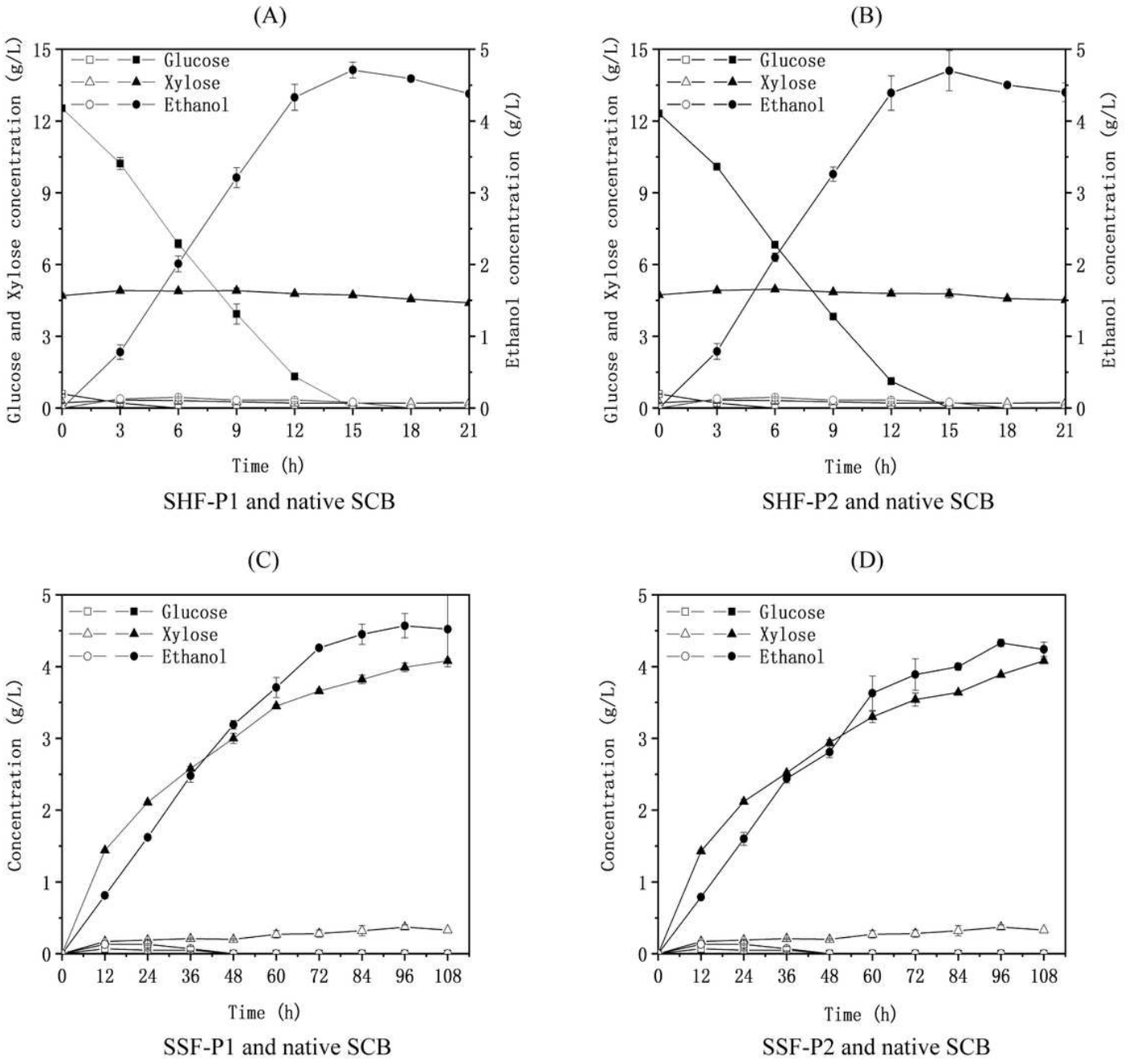

Figure 6

SHF (A-B) and SSF (C-D) for ethanol production. The hollow and solid represent native SCB and P1/P2 SCB, respectively.