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RESEARCH PAPER

Production of Bioethanol from Sugarcane Bagasse Using NH_4OH-H_2O_2 Pretreatment and Simultaneous Saccharification and Co-fermentation

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Abstract In this study, we investigated the production of bioethanol from sugarcane bagasse (SCB) using an NH_4OH-H_2O_2 pretreatment and simultaneous saccharification and co-fermentation (SScF). Response surface methodology and a 2^3 Box-Behnken design were used to evaluate the effect of different liquid mixture concentrations, liquid-to-solid ratios (LSRs) and pretreatment temperatures on the production of ethanol. The liquid mixture concentration and LSR significantly influenced the fermentation efficiency. Based on ridge max analysis, the following pretreatment conditions resulted in a fermentation efficiency of 95.79 ± 0.01%: liquid mixture concentration 53%, LSR 28, and a temperature of 63°C. A morphological analysis performed using scanning electron microscopy (SEM) and chemical characterization revealed that these pretreatment conditions were effective in disrupting the sugarcane fibers and removing lignin. Ethanol fermentation with the pretreated SCB using SScF in yeast SHY 07-1 resulted in an ethanol concentration of 14.65 ± 0.17 g/L, an ethanol yield of 0.48 ± 0.01 g/g, and an ethanol productivity of 0.12 ± 0.01 g/(L/h), which represents increases of 106.02, 89.98, and 107.02%, respectively, over the values obtained from SScF with untreated SCB.

Keywords: sugarcane bagasse, NH_4OH-H_2O_2 pretreatment, response surface methodology, simultaneous saccharification and co-fermentation, ethanol fermentation

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1. Introduction

The depletion of fossil fuels has led to an increasing interest in the use of renewable resources, for example, agricultural waste for the production of alternative fuels such as bioethanol [1,2]. However, it is not sustainable to produce bioethanol from grains such as corn and wheat in several countries, including China [3,4]. Lignocellulosic materials are attractive feedstocks for the production of bioethanol because they are abundant and cheap. One of the major lignocellulosic materials considered for bioethanol production in tropical countries is sugarcane bagasse (SCB), which comprises the fibrous residue that remains after extracting the juice, mainly sucrose, from sugarcane in the sugar production process. More than 70% of SCB consists of hydrolyzable carbohydrates that can yield fermentable sugars for the production of value-added bio-products [5-7]. It is estimated that each year approximately 100 million dry tons of SCB are produced around the world. Although most SCB is burned to produce steam power, there is still a surplus of this material leftover that can be used for bioethanol production [8].

To overcome the innate recalcitrance of the biomass and to effectively convert lignocellulosic feedstocks to fermentable sugars *via* enzymatic hydrolysis, the raw biomass should be pretreated. Several pretreatment methods exist, depending on the type and composition of biomass, which include different types of chemical, physical and physico-chemical techniques [1,4,6]. Regardless of the exact method used, the goal of pretreatment is to alter or remove lignin and/or hemicellulose, disrupt the crystallinity of cellulose, and increase porosity so that the cellulose becomes more accessible to cellulase enzymes [5,7,9-11].

Of these methods, alkali pretreatment of lignocellulosic materials is one of the most effective as it increases the exposed surface area for enzymatic hydrolysis by removing

the acetyl and uronic acid substituents that may be present on the hemicellulose backbone [12]. Unlike steam and acid pretreatments, alkali pretreatments solubilize the lignin and a small percentage of the hemicellulose [13,14]. Alkali pretreatments are generally more effective on agricultural residues and herbaceous crops than on wood materials, with NaOH and lime being the most common chemicals used for alkali pretreatment. A yield of 74.9% of the original hemicelluloses has been previously achieved by pretreatment of dewaxed SCB with 3% NaOH at a solid to liquid ratio of 1:25 (g/mL) at 50°C for 3 h [15]. Beukes *et al.* studied the effect of lime pretreatment on the synergistic hydrolysis of SCB with hemicellulases and achieved a degree of synergy of 2.14 for the lime-pretreated substrate, a 14.44% increase over the value achieved with untreated SCB [1]. NH_4OH, a weak alkali, has been used by other research groups to extract lignin from lignocellulosic materials and has been known to retain a high amount of both glucan and xylan in the solid fraction [16,17]. High retention of these carbohydrates helps increase the overall ethanol yield and simplifies the bioconversion process [18]. Moreover, NH_4OH pretreatment is also advantageous because the mild reaction conditions afforded by NH_4OH prevent the formation of various toxic compounds, such as furfural and hydroxymethyl furfural (HMF), that are generated from the decomposition of sugars in most other pretreatments involving harsh reaction conditions [19,20]. H_2O_2, a strong oxidant and bleaching agent used in the paper industry, can also be used to enhance the hydrolysis of biomass. Radicals, including singlet oxygen, superoxide, and hydroxyl radicals generated from H_2O_2, have been known to cause damage to DNA, proteins, and lipids. By combining NH_4OH with H_2O_2 as a pretreatment, the recalcitrance of lignocellulosic materials can be even further reduced [21]. In this study, the pretreatment of SCB with NH_4OH-H_2O_2 was investigated by evaluating the amount of bioethanol produced using simultaneous saccharification and co-fermentation (SScF) in yeast.

In addition, we evaluated the effect of different liquid mixture concentrations, liquid-to-solid ratios (LSRs), and temperatures on the pretreatment of SCB with NH_4OH-H_2O_2 by using a response surface methodology and a 2^3 Box-Behnken design. The feasibility of bioethanol production from pretreated SCB using SScF in yeast SHY 07-1 was also assessed.

2. Materials and Methods

2.1. Raw materials and NH_4OH pretreatment with H_2O_2

The SCB was a donation from the Guangzhou Sugarcane Industry Research Institute (Guangdong province, China). The SCB was ground and sieved until the SCB particles were able to pass through a 60 mesh (0.3 mm) sieve, and only these particles were used for the pretreatment experiments [4]. The pretreatment process was conducted in a 3,000 mL Erlenmeyer flask immersed in a water bath. NH_4OH and H_2O_2 were mixed at a volume ratio of 2:1, and this concentration was defined as 100%. This stock solution was then diluted to other concentrations with distilled water. 15 g SCB was packed into the flask, and different concentrations of the NH_4OH-H_2O_2 mixture were added. The pretreatment was conducted at different temperatures and durations with various liquid-to-solid ratios (LSRs). After pretreatment, the bagasse solid was separated by centrifugation at 955 *g* for 10 min (CF7D2, HITACHI CENTRIFUGE, Japan) and was then washed with distilled water until the pH was neutral. After washing, the bagasse solids were dried at 60°C for 72 h. The dried solids were collected for compositional analysis and enzymatic saccharification and fermentation.

2.2. Experimental design and statistical analysis

A 2^3 Box-Behnken design with fifteen experiments (each replicated twice) was used to determine the pretreatment conditions. To study the pretreatment performance of the liquid mixture (NH_4OH:H_2O_2 = 2:1, v/v) and to explore the interaction amongst the pretreatment variables, three factors were analyzed. Runs were performed in a random order, with a treatment time of 20 h. The design plan is summarized in Table 1 according to the results of a single factor experimental design. The independent variables were liquid mixture concentration (A, varied from 30 to 50%), the LSR (B, varied from 15 to 35) and pretreatment temperature (C, varied from 40 to 60°C). The ethanol concentration as obtained from SScF over a period of 24 h was considered to be the response variable. Quadratic models of the type described by Eq. (1), in which Y_i represents the response variable, b_0, b_i, b_{ii}, and b_{ij} represent the regression coefficients, and X_i and X_j represent the coded levels of the independent variables, were generated by regression analysis to quantify the influence of the independent variable on the response variable (the ethanol concentration at 24 h using SScF). The regression coefficients used in the models were those found to be statistically significant (5% significance

Table 1. Parameters and levels used in the 2^3 Box-Behnken design

Parameters		Minimum value −1 level	0 level	Maximum value +1 level
Codes	components			
A	Liquid mixture concentration (%)	30	40	50
B	LSR	15	25	35
C	Temperature (°C)	40	50	60

level, $P < 0.05$) [22]. The response surfaces described by the models were plotted with the software Design Expert 8.0.

$$Y_i = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} X_i X_j \quad (1)$$

2.3. Preparation of the microorganism and inoculums

SHY07-1 yeast, an intergeneric protoplast fusant between *Saccharomyces cerevisiae* (donated from the Sanhe ethanol factory, Zanjiang, Guangdong province) and *Pichia stipitis* (donated from the Guangzhou Sugarcane Industry Research Institute, Guangzhou), possesses the ability to convert not only glucose but also xylose into ethanol, and was thus used in this study [23,24]. The SHY07-1 yeast was grown at 30°C for 2 days on a YPX-agar plate containing 20 g/L yeast extract (Y), 10 g/L peptone (P), 20 g/L xylose (X), and 20 g/L agar. The medium was sterilized by steam autoclave at 115°C for 20 min prior to plating. To prepare the inoculums, a loop of active cells was transferred to 10 mL of YPX medium in sterile test tubes and was incubated at 30°C and 200 rpm for 12 ~ 18 h in an incubator shaker (C24KC refrigerated incubator shaker, Edison, NJ, United States). 10 mL of the active cells was then, in turn, aseptically transferred to 100 mL of sterile YPX medium in a 250 mL Erlenmeyer flask. The cells were harvested by centrifugation in 50 mL sterilized centrifuge tubes for 10 min at 2,655 *g* using a TDL-5000B centrifuge (Shanghai Anke Company, Ltd., China). The pellets were re-suspended in sterilized 0.9% NaCl solution to obtain a suspension with a cell mass concentration of 200 g wet cells per liter. Finally, a 6% inoculum of the cell suspension was used for initiating the fermentation experiments.

2.4. Analytical methods

An analysis of the cellulose and hemicellulose content in SCB was conducted according to the methods recommended by the National Renewable Energy Laboratory (NREL) [25]. The other components of SCB were analyzed as follows according to the corresponding Chinese Standards: moisture, GB/T 2677.2-1993; ash, GB/T 2677.3-1993; Klason lignin, GB/T 2677.8-1994; acid-soluble lignin, GB/T 747-2003; and benzene-ethanol extractives, GB/T2677.6-1994.

The total cellulase activity was quantified in filter paper units (FPUs) using a Whatman No. 1 filter paper strip (1.0 × 6.0 cm, approximately 50 mg) as a substrate. Xylanase activity was assayed using 1% (w/w) oat spelts xylan (Sigma, St. Louis, USA) as a substrate. Enzyme activities were expressed in international units (IU) as the amount of enzyme required to release 1 μmol per minute of either glucose (cellulase) or xylose (xylanase) under the assay conditions (pH 4.8, 50°C). The amount of reducing sugars was estimated using the 3, 5-dinitrosalicylic acid (DNS) method [26].

To determine the amount of ethanol and monomeric sugars, 1 -mL fermentation samples were acidified with 10% (w.t) sulfuric acid, centrifuged in 1 mL Eppendorf tubes at 14,580 *g* for 10 min (TGL-16H Centrifuge, HEMA Company, Ltd., Zhuhai, China) and then filtered through a 0.22 μm filter. The supernatant (pH 1-3) was analyzed for the presence of soluble sugars and ethanol using high pressure liquid chromatography (HPLC, Waters 2695, United States) equipped with a refractive index detector (RID, Waters 2414, Millford LA, United States). Glucose, xylose, glycerol, organic acid and ethanol were analyzed using an Aminex HPX-87H column equipped with a Cation H Cartridge Micro-Guard column (Bio-Rad, Hercules CA, United States). The column was operated at 60°C with 2.5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min [27]. Arabinose was analyzed by HPLC using an Aminex HPX-87P column equipped with a Carbo-P Micro-Guard column (Bio-Rad, Hercules, CA, United States). The column temperature was 60°C, and ultrapure water was used for the mobile phase at a flow rate of 0.6 mL/min.

2.5. Scanning electron microscopy

The morphology of untreated and pretreated SCB was analyzed by scanning electron microscopy (SEM). SEM pictures of untreated and pretreated bagasse were taken at a magnification of 500x and 2, 000x using a ZEISS EVO-18 microscope (51-XMX0003 Special Edition, German) equipped with an Oxford X-Max detector operating at 10 or 50 kV. The samples were first dried in a freeze dryer (FD-1C-50, BOYIKANH, Beijing, China) for 24 h followed by coating with 20 nm of gold in a high vacuum metalizator (Coating System KYKY SBC-12, China) before being kept in a desiccator until analysis.

2.6. Simultaneous saccharification and co-fermentation

A 125 mL serum bottle with a working volume of 50 mL was used as a bioreactor for simultaneous saccharification and co-fermentation (SScF). Untreated or pretreated SCB was added at an initial solid loading of 4% (w/v, expressed in terms of dry mass). $MgCl_2$ (stock concentration 25 g/L) was added as an enzyme activator to a final concentration of 0.5 g/L, after which the fermentation medium was sterilized by steam autoclave at 121°C for 30 min. HCl (1 M) or 10% NaOH was used to adjust the pH to 4.8 ~ 5.1, that is the optimal pH range according to pre-experiment results. Celluclast 1.5 L cellulolytic complex (Novozymes, Denmark) with a cellulase and xylanase activity of 12.54 FPU/mL and 0.97 U/mL, respectively, was also added to an

enzyme loading at 20 FPU/g of dry SCB. The fermentation medium was inoculated with 6% (v/v) inoculum (as described previously in the section on the preparation of the microorganism and inoculum). The fermentation temperature was maintained at 30 ± 0.5°C in an orbital shaker for 120 h, and the broth was kept under agitation at 200 rpm. At appropriate timepoints (24, 48, 72, 96, and 120 h) during the fermentation, 2 mL samples were aseptically withdrawn for analysis. Glucose, xylose, cellobiose, lactic acid, acetic acid and ethanol concentrations were measured using HPLC as described previously. For fermentation experiments, at least one experimental condition was duplicated for each set of experiments to ensure consistency and accuracy in the results. The fermentation efficiency, ethanol yield and productivity were calculated using the following equations:

Fermentation efficiency

$$= \frac{\text{Ethanol formed concentration, g/L}}{\text{Theoretical ethanol concentration, g/L}} \times 100\% \quad (2)$$

Ethanol yield

$$= \frac{\text{Ethanol formed concentration, g/L}}{\text{Theoretical reducing sugar concentration, g/L}} \quad (3)$$

Ethanol productivity

$$= \frac{\text{Ethanol formed concentration, g/L}}{\text{Fermentation time, h}} \quad (4)$$

3. Results and Discussion

3.1. The effect of pretreatment conditions on fermentation efficiency of SScF

There are several factors that affect NH_4OH-H_2O_2 pretreatment, including the liquid mixture concentration, the LSR, and the pretreatment time and temperature. The liquid mixture concentration had the most significant effect on the fermentation efficiency of SScF, as is shown in Fig. 1A. As shown in this figure, the fermentation efficiency of pretreated solid increases dramatically when compared to the control untreated SCB. Increasing the liquid mixture concentration resulted in higher fermentation efficiencies. When the liquid mixture concentration was increased from 10 to 50%, the 120 h fermentation efficiency for SScF increased from 71.48 to 104.70%. Additionally, the concentration of ethanol correspondingly increased from 10.33 to 15.73 g/L, and the fermentation efficiency increased by 41.10 and 106.68% when compared to the values obtained for untreated SCB. As shown in Fig. 2, the degree of delignification increased from 19.80 to 33.93% when the liquid mixture concentration was increased from 10 to 30%. When the LSR was fixed, increasing the liquid mixture concentration caused, to some extent, a higher degree of delignification, which enhanced the enzymatic saccharification [4]. These results indicate that NH_4OH-H_2O_2 solubilizes the SCB lignin network effectively. Other research groups have confirmed the finding that ammonia pretreatment is effective in removing lignin and in improving the cellulase accessibility [11,18]. Adding peroxide to the NH_4OH pretreatment can be expected to enhance enzymatic hydrolysis by oxidative delignification, decreasing the cellulose crystallinity. Wet-oxidation is regarded as an efficient method for opening up the crystalline structure of lignocellulose, solubilizing the hemicellulose fractions, and degrading lignin into CO_2, H_2O, and carboxylic acids [21]. In a previous study, increased lignin solubilization and cellulose availability were observed after pretreating wheat straw, Douglas fir, and oak with peroxide [11]. H_2O_2 can also change the hemicellulose structure and remove the lignin in SCB, resulting in a cellulose-rich insoluble residue that can be converted to glucose [21].

Fig. 1B shows that the fermentation efficiency of the pretreated solid was not affected by varying the LSR between 100 and 40, but a marked increase in efficiency was observed for lower LSRs. The highest fermentation efficiency was obtained with an LSR of 20 and was as high as 94.97% at 120 h, representing an 87.48% increase over the fermentation efficiency with untreated SCB. However, when the LSR was lower than 20, maintaining a homogeneous reaction system became difficult because there was less liquid present.

Varying the pretreatment times between 16 and 28 h had no significant effect on the SScF time profiles and the degree of delignification (Figs. 1C and 2). However, the fermentation efficiency decreased from 61.22 to 58.52% when the treatment time was increased from 16 to 28 h, probably due to the fact that the pretreatment reaction reached equilibrium after 16 h.

Temperature affected the kinetics of delignification and further affected enzymatic hydrolysis and fermentation. The degree of delignification increased rapidly from 18.82 to 49.19% as the temperature rose from 40 to 60°C (Fig. 2). However, as shown in Fig. 1D, the pretreatment temperature had no significant effect on fermentation efficiency below 60°C. As expected, the fermentation efficiency at 60°C for pretreated SCB increased by 46.52% when compared to the value obtained for untreated SCB.

In summary, the complex cellulose-hemicellulose-lignin structure of SCB was disrupted to varying degrees by pretreatment with NH_4OH-H_2O_2, and at different conditions, the NH_4OH and H_2O_2 pretreatment reduced the SCB lignin content to different extents. Rocha *et al.* reported that removing 20 ~ 65% of the lignin was sufficient to increase cellulose accessibility for enzymatic hydrolysis [28]. Thus,

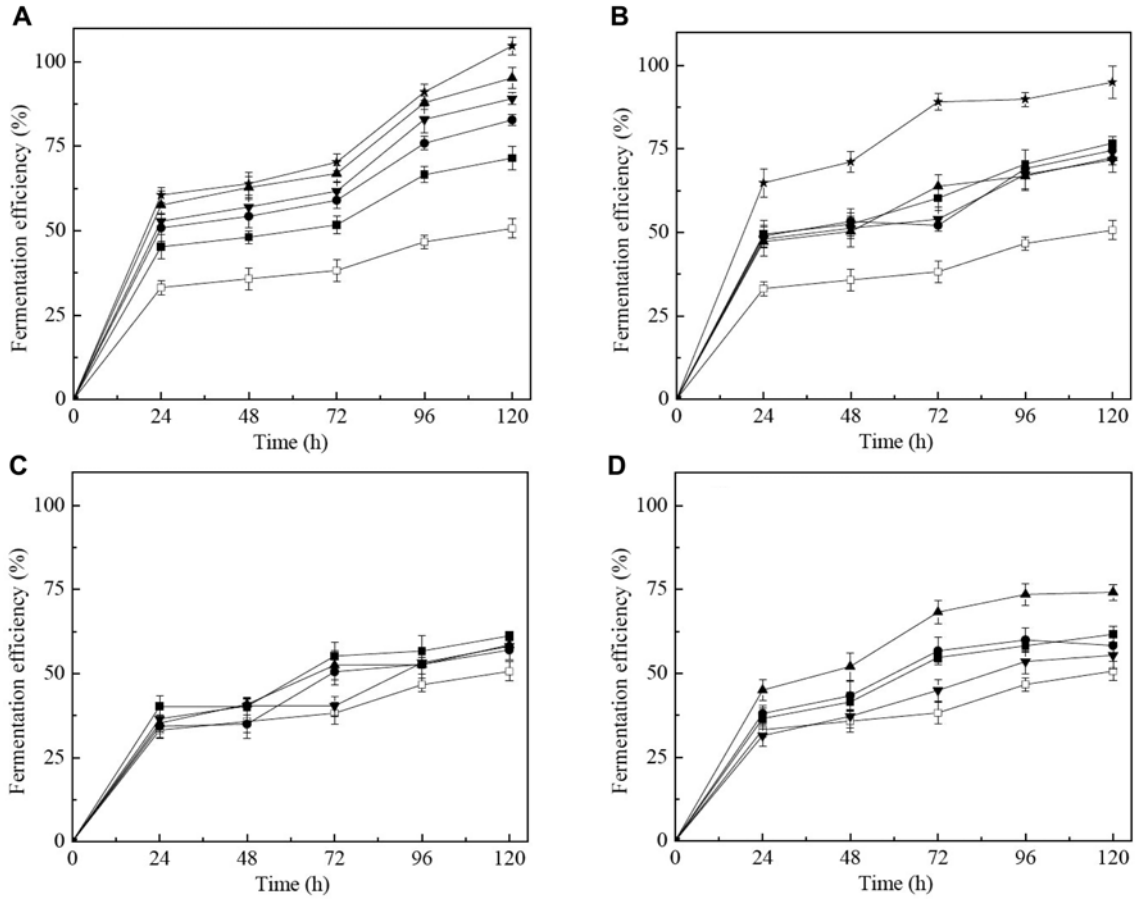

Fig. 1. SScF of untreated SCB and pretreated SCB at different pretreatment conditions. Results are shown as the means of duplicate experiments. Fermentations were conducted at 30°C, an initial pH of 4.8, and 200 rpm agitation under largely anaerobic conditions for 120 h, with an initial substrate loading of 4% (w/v, expressed in terms of dry mass) and the Celluclast 1.5 L enzyme loading was 20 FPU/g of dry SCB. S0: untreated (□). (A): The effect of liquid mixture concentration on fermentation efficiency. Pretreatment conditions: LSR: 50, temperature: 30°C, pretreatment time: 24 h, liquid mixture concentration: S1: 10% (■), S2: 20% (●), S3: 30% (▼), S4: 40% (▲), S5: 50% (★). (B): The effect of LSR on fermentation efficiency. Pretreatment conditions: liquid mixture concentration: 10%, temperature: 30°C, pretreated time: 24 h, LSR: S6: 100 (■), S7: 80 (●), S8: 60 (▼), S9: 40 (▲), S10: 20 (■). (C): The effect of pretreatment time on fermentation efficiency. Pretreatment conditions: liquid mixture concentration: 10%, LSR: 50, temperature: 30°C, pretreatment time: S11: 16 h (■), S12: 20 h (●), S13: 24 h (▼), S14: 28 h (▲). (D): The effect of pretreatment temperature on fermentation efficiency. Pretreatment conditions: liquid mixture concentration: 10%, LSR: 50, pretreatment time: 24 h, temperature: S15: 30°C (■), S16: 40°C (●), S17: 50°C (▼), S18: 60°C (▲).

it is significant that SScF with pretreated SCB resulted in higher ethanol concentration and fermentation efficiency when compared to SScF with untreated SCB.

3.2. Determination of the pretreatment conditions

The results from the 2^3 Box-Behnken experimental design with three variables are presented in Table 2. A set of fifteen trials was generated (12 independent and 3 repetitions of the central point). Statistical significance of the respective model equations was verified using analysis of variance (ANOVA). The ethanol concentration for SScF at 24 h ranged from 7.15 to 11.06 g/L. The highest ethanol concentrations (experiments 1, 5, 7, and 12) occurred when the liquid mixture concentration was over 40% and when the pretreatment temperature was over 50°C, and the maximum ethanol concentration (11.06 g/L) was achieved using the following conditions: liquid mixture concentration 40%, LSR 15, and a temperature of 60°C.

According to the ANOVA results, the quadratic model for ethanol concentration was the best, and a good correlation coefficient ($R^2 = 0.9411$) and small pure error value (0.064) showed that there was close agreement between the experimental results and the predicted theoretical values. A regression analysis was performed to attain a mathematical model that could better describe the relationship between the independent variables and the studied response. Statistical testing of the model was conducted in the form of an ANOVA (Table 3). ANOVA of the regression model

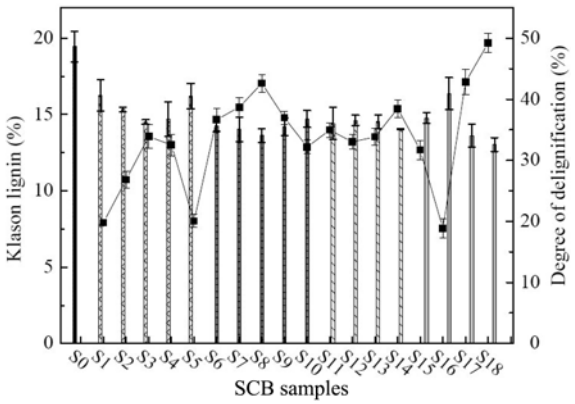

Fig. 2. The Klason lignin content and degree of delignification for the SCB samples pretreated for different pretreatment conditions. Results are means for duplicate experiments. S0: untreated. Pretreatment conditions: A: LSR: 50, temperature: 30°C, pretreatment time: 24 h, liquid mixture concentration: S1: 10%, S2: 20%, S3: 30%, S4: 40%, S5: 50%. B: liquid mixture concentration: 10%, temperature: 30°C, pretreatment time: 24 h, LSR: S6: 100, S7: 80, S8: 60, S9: 40, S10: 20. C: liquid mixture concentration: 10%, LSR: 50, temperature: 30°C, pretreatment time: S11: 16 h, S12: 20 h, S13: 24 h, S14: 28 h. D: liquid mixture concentration: 10%, LSR: 50, pretreatment time: 24 h, temperature: S15: 30°C, S16: 40°C, S17: 50°C, S18: 60°C.

: S0, : a, : b, : c, : d Klason lignin content. : Degree of delignification.

Table 2. Matrix of the 2^3 Box-Behnken design and the corresponding results for ethanol concentration of a 24 h SScF

Runs*	Parameters			Measured response
	A	B	C	
	Liquid mixture concentration (%)	LSR	Temperature (°C)	Ethanol concentration (g/L)
1	50	35	50	10.62
2 (CP)	40	25	50	9.48
3	30	35	50	9.30
4 (CP)	40	25	50	9.88
5	40	35	60	10.35
6 (CP)	40	25	50	9.95
7	50	25	60	10.94
8	50	15	50	9.85
9	50	25	40	8.70
10	30	15	50	8.58
11	30	25	40	7.15
12	40	15	60	11.06
13	40	15	40	8.28
14	40	35	40	7.76
15	30	25	60	9.75

*Runs: twelve independent and three repetitions of the center point (CP).

indicates that the model is highly significant as evidenced by the calculated *F*-value (8.88). The high *F*-value indicates that most of the variation in the response could be explained by the regression model equation. At the same time, Table 3 reveals that temperature had a significant effect on ethanol concentration in SScF. By contrast, the LSR showed no significant effect, and moreover, no obvious interaction between liquid mixture concentration and LSR, liquid mixture concentration and temperature, and LSR and temperature was observed, with all the values of $P > F$ being more than 0.1.

Fig. 3 shows the three-dimensional (3D) response contour and surface plots that represent the regression equations. An analysis of Figs. 3A and 3B shows that a minor increase in either the liquid mixture concentration or the pretreatment temperature could result in an increase in the

Table 3. Analysis of variance (ANOVA) of the model proposed for predicting ethanol concentration after a 24 h SScF

Source*	Sum of squares	*df*	Mean square	*F*-value	*P* > F	Significance
Model	17.4400	9	1.94000	8.88000	0.01350	significant
A	3.55000	1	3.55000	16.2700	0.01000	
B	0.00845	1	0.00845	0.03900	0.85180	
C	13.0300	1	13.0300	59.7000	0.00060	
AB	0.00625	1	0.00625	0.00286	0.95940	
AC	0.03200	1	0.03200	0.15000	0.71590	
BC	0.00903	1	0.00903	0.04100	0.86490	
A^2	0.16000	1	0.16000	0.71000	0.43760	
B^2	0.00187	1	0.00187	0.00856	0.92990	
C^2	0.68000	1	0.68000	3.13000	0.13720	
Residual	1.09000	5	0.22000			
Lack of fit	0.96000	3	0.32000	4.99000	0.17140	not significant
Pure error	0.13000	2	0.06400			
Correlation total	18.5300	14				
[Ethanol concentration]: $[R^2] = 0.9411$						

*The code for variables A, B, C were A: liquid mixture concentration (%), B: LSR, C: temperature (°C).

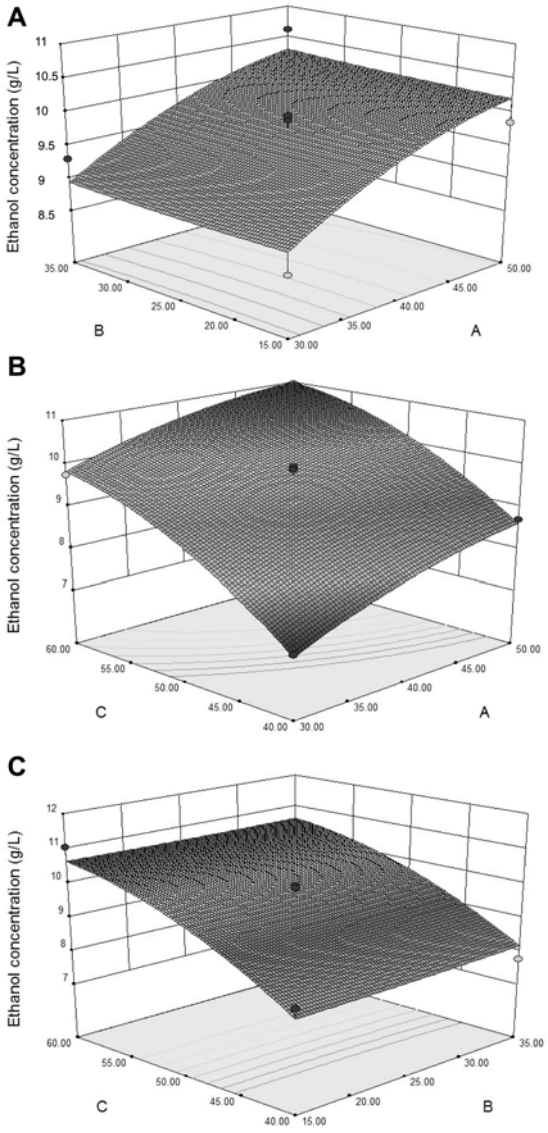

Fig. 3. Response surface plots showing the effect of parameters and their combined effects on ethanol concentration. Interaction between the parameters AB (A), AC (B), and BC (C). The code for the variables A, B, and C were A: liquid mixture concentration (%), B: LSR, C: temperature (°C).

final ethanol concentration. Fig. 3C shows that an increase in the LSR has no significant effect on ethanol production. In addition, Fig. 3 shows that the interaction between liquid mixture concentration and temperature was higher than liquid mixture concentration and LSR or LSR and temperature, confirming the statistical analysis shown in Table 3. For all experiments, increasing the liquid mixture concentration and pretreatment temperature resulted in an increase in ethanol concentration.

The ethanol concentration was correlated to the liquid mixture concentration (A), LSR (B) and temperature (C), resulting in Eq. (5) after multiple regression analyses were conducted on the experimental data. Accordingly, the final equation in terms of actual factors was Eq. (3), where x_1, x_2 and x_3 were the coded values of the test variables, liquid mixture concentration (%), LSR, and temperature (°C), respectively. To determine the pretreatment conditions, the partial derivatives of these three variables in Eq. (6) were calculated using MATLAB 7.0, and the results were as follows: $x_1 = 53.43$, $x_2 = 28.05$, $x_3 = 63.26$. In addition, the predicted value was $Y = 11.07$. This means that the pretreatment conditions were liquid mixture concentration 53%, LSR 28, and temperature 63°C, and the ethanol concentration after 24 h of SScF was 11.07 g/L under these conditions.

$$\begin{aligned}&\text{Ethanol concentration}\\&= 9.77 + 0.67 \cdot \mathrm{A} + 0.032 \cdot \mathrm{B} + 1.28 \cdot \mathrm{C} + 0.013 \cdot \mathrm{A} \cdot \mathrm{B} - 0.09 \cdot \mathrm{A} \cdot \mathrm{C} - 0.048 \cdot \mathrm{B} \cdot \mathrm{C} - 0.2 \cdot \mathrm{A}^2 + 0.023 \cdot \mathrm{B}^2 - 0.43 \cdot \mathrm{C}^2\\ Y &= -15.5162 + 0.2725 \cdot x_1 + 0.01075 \cdot x_2 + 0.6055 \cdot x_3 + 0.000125 \cdot x_1 \cdot x_2 - 0.0009 \cdot x_1 \cdot x_3 - 0.000475 \cdot x_2 \cdot x_3 - 0.00205 \cdot x_1^2 + 0.000225 \cdot x_2^2 - 0.0043 \cdot x_3^2 \qquad (6)\end{aligned}$$

3.3. Ethanol production using SScF with pretreated SCB

The time profiles of the fermentation experiments are presented in Fig. 4. It is apparent that the ethanol concentration at 120 h for SScF with the pretreated SCB was 106.92% higher than that of the untreated SCB, and the fermentation efficiency correspondingly improved by 89.08%. The acetic acid concentration after 120 h of SScF was reduced by 21.97% when the pretreated SCB was used, due to the removal of acetyl groups in the lignocellulose by the NH_4OH-H_2O_2 pretreatment. Accumulation of glucose and xylose of within 24 h of SScF with the pretreated SCB was more than that with the untreated SCB, revealing that the NH_4OH-H_2O_2 pretreatment was effective in improving the enzymatic conversion of cellulose and hemicellulose. Similar results were reported by Beukes *et al.* [1]. By the end of a 120 h SScF with pretreated SCB, the ethanol concentration, ethanol yield, and ethanol productivity were 14.65 ± 0.17 g/L, 0.48 ± 0.01 g/g, and 0.12 ± 0.01 g/(L/h), respectively, and the fermentation efficiency was 95.79 ± 0.01%. Moreover, the ethanol concentration after 24 h of SScF with the pretreated SCB was 11.14 ± 0.11 g/L (with a relative standard error of 0.67%), compared with the predicted value of 11.07 g/L, thus validating our proposed model.

3.4. Characterization of SCB

The increase in the accessibility of cellulose and hemicellulose by ammonia is believed to be due to an alteration in the lignin structure that makes lignin soluble and easily removed [10], which could be confirmed when the chemical composition of the untreated and pretreated bagasse were

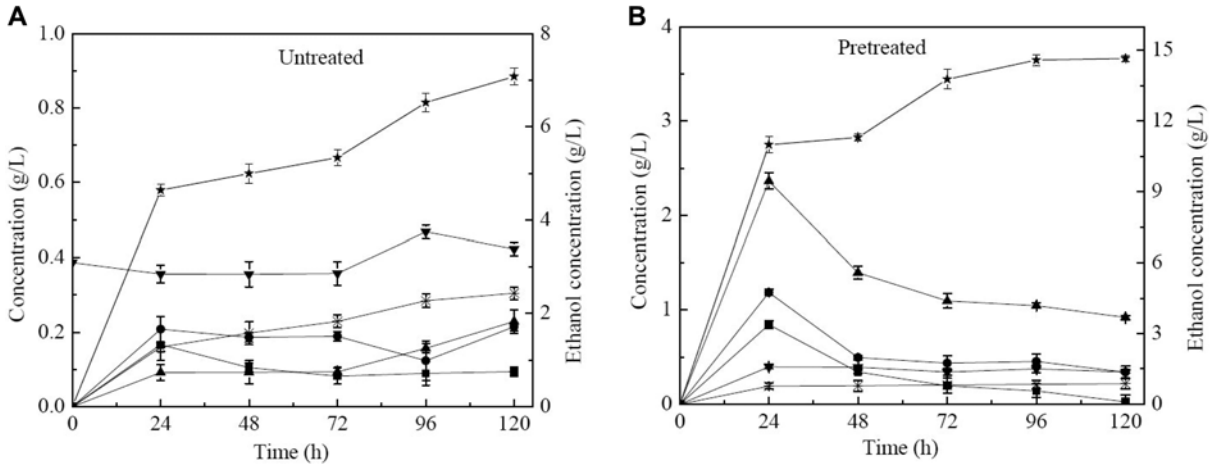

Fig. 4. Time courses for substrate and product concentrations during SScF of untreated SCB and pretreated SCB. (A): Untreated, (B): Pretreated, pretreatment conditions: liquid mixture concentration: 53%, LSR: 28, temperature: 63°C, pretreatment time: 20 h. Results are means for duplicate experiments. Fermentations were conducted at 30°C, an initial pH of 4.8, and 200 rpm agitation under largely anaerobic conditions for 120 h, with an initial substrate loading of 4% (w/v, expressed in terms of dry mass) and the Celluclast 1.5 L enzyme loading was 20 FPU/g of dry SCB. cellobiose (■), glucose (●), xylose (▲), lactic acid (▼), acetic acid (*), ethanol (★).

compared (Table 4). The NH_4OH-H_2O_2 pretreated SCB had approximately 49.19% less Klason lignin and 27.62% higher glucan (relative to the percentage of dry mass) than that of the untreated SCB. The total carbohydrate in the pretreated SCB was 70.38%, an increase of 14.83% over that of the untreated SCB. Thus, Table 4 potentially illustrates that the NH_4OH-H_2O_2 pretreatment had partially solubilized and removed some of the lignin present on the hemicellulose backbone, exposing the lignocellulose structure for enzymatic hydrolysis. An increase in the solubilization of SCB lignin was expected, as similar results were obtained by others with alkali pretreatment [12,14].

To elucidate the physical changes occurring after pretreatment, the morphology of the untreated and pretreated SCB (under different conditions) and the solid residue remaining after a 120 h SScF with pretreated SCB were analyzed by SEM. As shown in Figs. 5A, 5B, 5C, and 5D the surface of the untreated SCB was smoother than that of the pretreated SCB, and there is a noticeable difference in porosity between the two samples. The effect of the pretreatment is clearly evident in the SEM image of the vascular bundle shown in Fig. 5C. It is clear that pretreated SCB is considerably more exposed to the action of cellulases, as deduced by the fragments of pith flocks distributed over the entire surface of the vascular bundle. Empty spaces between the fibers can be easily observed in Fig. 5E and are a consequence of the removal of hemicelluloses and cellulose flocks during the SScF. Pith flocks are easily hydrolyzed because they are composed of parenchyma cells, which have a high content of amorphous and/or low-crystallinity cellulose [29]. This is also evident in another region of the same bundle of fibers, in which a similar morphology can be observed (Fig. 5F).

4. Conclusion

The results of this study demonstrate that NH_4OH-H_2O_2 pretreatment can selectively remove lignin and improve the enzymatic saccharification of SCB, resulting in enhanced ethanol concentration as well as fermentation efficiency for SScF. A response surface methodology and a 2^3 Box-Behnken experimental design were successfully implemented to improve the enzymatic saccharification and fermentation efficiency of a SCB sample pretreated with NH_4OH-H_2O_2 under fixed conditions on a small scale. The two independent variables, namely the liquid mixture concentration and the LSR, significantly influenced the

Table 4. Chemical characterization of the SCB samples as a percentage of dry mass

	Glucan	Xylan	Arabian	Klason lignin	Acid-soluble lignin	Moisture	Ash	Benzene-ethanol extractives
Untreated	39.50 ± 0.66	19.77 ± 0.03	2.02 ± 0.95	21.04 ± 0.01	4.89 ± 0.21	6.85 ± 0.01	5.69 ± 0.01	2.55 ± 0.01
Pretreated*	50.41 ± 3.38	18.57 ± 0.13	1.40 ± 0.04	10.69 ± 0.02	3.14 ± 0.07	2.92 ± 0.01	5.93 ± 0.01	1.14 ± 0.01

*Pretreatment conditions: liquid mixture concentration: 53%, LSR: 28, temperature: 63 °C, pretreatment time: 20 h.

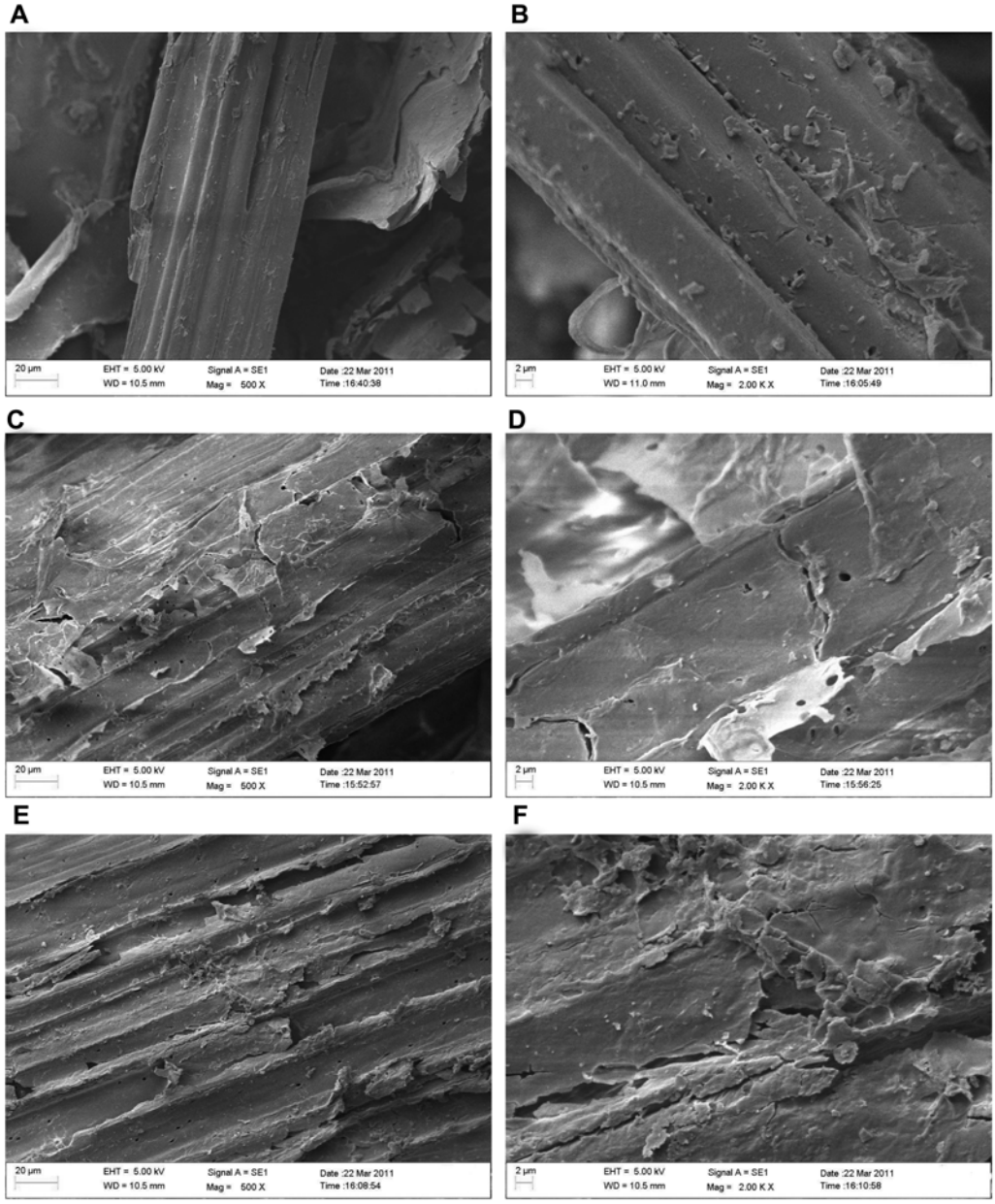

Fig. 5. SEM images of a bundle of fibers in the SCB samples. (A) and (B): untreated SCB, magnification: (A): 500x, (B): 2000x; (C) and (D): pretreated SCB using the following pretreatment conditions: liquid mixture concentration: 53%, LSR: 28, temperature: 63°C, pretreatment time: 20 h, magnification: (C): 500x, (D): 2000x; (E) and (F): solid residues remaining after a 120 h SScF with pretreated SCB, magnification: (E): 500x, (F): 2000x.

fermentation efficiency of SScF. The proposed model can precisely predict the ethanol concentrations resulting from SScF of pretreated SCB. Morphological analysis of the pretreated SCB by SEM revealed that the pretreatment was effective in disrupting the fibers and confirmed the results from a chemical characterization. The results of this study can thus serve as a foundation for further optimization of SCB pretreatment and the production of bioethanol by SScF.

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Nomenclature

ANOVA : Variance analysis
DNS : 3, 5-dinitrosalicylic acid
FPU : Filter paper unit
HMF : Hydroxymethyl furfural
HPLC : High pressure liquid chromatography
LSR : Liquid-to-solid ratio
NREL : National renewable energy laboratory
RID : Refractive index detector
SCB : Sugarcane bagasse
SEM : Scanning electron microscopy
SScF : Simultaneous saccharification and co-fermentation

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