

Synergetic Physical Freezing and Chemical Pretreatment of Lignocellulosic Sugarcane Bagasse Followed by Enzymatic Hydrolysis

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Abstract

This study focuses on employing an eco-friendly pretreatment approach for lignocellulosic Sugarcane Bagasse (SCB) as a major problematic solid waste in Egypt, complying with the standard legislation as well. The applied technique depended on SCB physical fractionation via freezing, followed by chemical hydrolysis using alkaline hydrogen peroxide (AHP) and enzymatic hydrolysis. The changes occurred in macrostructure and the entire lignocellulosic compounds during the pretreatment stages were evaluated. Freezing fractionation resulted in relatively low glucose yield and saccharification ratio at -20°C for 2 h of 307.52 mg/gm_{native SCB} and 48.5%, respectively, where no total reducing sugars (TRS) was obtained. Further AHP pretreatment was performed for the frozen-fractionated SCB at -20°C and 2 h with assistance of Box–Behnken Design response surface methodology (RSM). The investigated key parameters were H_2O_2 concentration (3, 5.5 and 8 %v/v), temperature (25, 42.5 and 60°C) and pretreatment duration (1, 3 and 5 h). The results revealed that the statistical modelling was able to predict the response of glucose yield and TRS production with $R^2 = 0.8221$ and 0.8814, respectively. Applying the optimization tool of RSM, the optimum predicted values of glucose yield and TRS production were (886.51 mg/gm_{native SCB} and 1.44 mg/mL), respectively; confirmed by the experimental analysis (898.5 mg/gm_{native SCB} and 1.32 mg/mL), respectively. The coincided saccharification ratio was 97.5%. These results were obtained at H_2O_2 of 3 % (v/v), 56.93°C and 1 h which were 4.32 and 2.01 times higher than that obtained during the freezing pretreatment phase for glucose yield and saccharification ratio, respectively.

1. Introduction

Solid waste management is a major public health and environmental concern especially in developing countries like Egypt. Solid waste is increasingly accumulating in open areas and along water canals' banks causing considerable air, soil and ground water contamination, and thus indirect detrimental health impacts. Its accumulation contributes to the global greenhouse gases (GHG) emissions for up to 0.57% based on emission increase rate of 5.1% annually and population increase rate of 1.7% – 2.3%. The trend of GHG emissions in Egypt for the agricultural solid waste within years 2000–2010 shows dramatic increasing trends from 31.7- 52.18 million tons/year (1). Solid waste is categorized according to its origin namely domestic, industrial, and agricultural, etc. These categories accord to the solid waste contents like organic materials, glass, metal, plastic and paper, or their hazardous potentials such as toxic, flammable, radioactive, infectious substances, etc. In addition, the density of solid waste varies based on the point of measurement (at source, during transportation or at disposal) (2).

The Egyptian agricultural waste amount ranges from 30–35 million tons per year. About 7 million tons of them are utilized as animal feed and other 4 million as organic manure (3). The remainder is burnt directly on the fields or used for heating in small villages (4). They represent however important source of bioenergy and valuable products. Sugarcane bagasse (SCB) is one of the most important parts of agricultural waste in Egypt and all over the world. It is resulted from sugarcane after extraction of juice, amounts up to 4.7 million tons per year across the country. Sugar mills generate bagasse about 500 g/kg water content at a rate of 270 kg/t of harvested cane (5). In south Egypt, for instance, Quena has 3 mills: in (Naga Hammadi, Doshna and Qous) with a total production capacity of 4.3 million tons of SCB per season. Despite being good for business, this huge amount of sugarcane production brings some environmental inconvenience. Most of the SCB is used for heat generation in the sugar mills with very poor efficiency and emits black smoke (1).

SCB is a recalcitrant lignocellulosic waste against biodegradation and bioconversion. Lignocelluloses are composed of cellulose, hemicellulose and lignin in an intricate structure, which is hard to be decomposed (6). It must be

pretreated to increase their biodigestibility and make cellulose more accessible to the cellulolytic enzymes, affording a wealthy bioethanol as a clean energy source. Furthermore, the polymers contained in lignocelluloses are themselves relatively difficult to be hydrolyzed to their sugar monomers. Lignin has very complicated structure which covers both cellulose and hemicellulose and plays the role of cement between them, forming a rigid three-dimensional structure of the cell wall. Lignin can be used though the production of chemicals, as a source of combined heat and power, pharmaceutical industry, etc. (7).

There are several pretreatment methods of SCB which can be classified into four categories: physical, chemical, biological and physicochemical pretreatment (4) and (6). The main objective of the pretreatment is to increase the cellulose digestibility and open its recalcitrant crystal structure. This could be done by disrupting hydrogen bonds in crystalline cellulose. Furthermore, hemicellulose and lignin would be disrupted and solubilized. This facilitates rapid and efficient hydrolysis of carbohydrates (cellulose and hemicellulose) to fermentable sugars via enzymatic hydrolysis.

The choice of the optimum pretreatment process depends mainly on the objective of the solid waste pretreatment, composition, economic assessment and environmental impact. According to the literature, application of freezing for SCB fractionation possesses important advantages. Significant lower environmental impact due to less discharge of hazardous derivatives and null used chemicals are examples of these advantages (8). Therefore, inhibition of the subsequent hydrolysis and fermentation steps due to those derivatives would be avoided, and thus, less costs and clean environment can be gained. The mechanism of freezing pretreatment is based on the expanding of liquid volume as it freezes related to its crystal structure (9). When water freezes, it stacks on the crystalline lattice configuration and in turn stretches the rotational and vibrational components of the bond. Consequently as observed by Pinsky et al. (10), the freeze-thaw process could disrupt bulk hydration layer, making the crystal lattice of ice and protein inactive. Franceschini et al. (11) have shown that the best results of fractionation were obtained after one cycle of freeze-thaw below -10°C . However, Vahur Rooni (13) found that the optimum results were obtained after 4 times of the freeze-thaw. Ken-Lin Chang et al. (9) observed a significant increase in the enzyme digestibility of rice straw from 48–84% after freeze-thaw. Their obtained reducing sugar yield of native and pretreated rice straw after 48 h were 93.84 g/kg and 226.77 g/kg substrate, respectively. However, there is no common conclusion about the required total time and the resulted biomass characteristics after fractionation.

On the other hand, alkaline hydrogen peroxide (AHP) proved promising results in SCB pretreatment especially for lignin dissolution. In such a process, the ester and ether bonds in lignin-carbohydrate complexes are broken, while the internal surface area of biomass increased (14). Besides, it is a typical and relatively safe eco-friendly agent used for delignification during wood pulping processes. Previous researches (15) and (16) found that dilute alkaline solutions of (1–5% (v/v)) H_2O_2 removed about 50% of the lignin present in lignocelluloses; yielding a cellulose-rich insoluble residue that can be enzymatically converted to glucose with up to 90% overall efficiency. Irfan et al. (17) experienced that SCB pretreatment using 3% (v/v) H_2O_2 yielded about 1.55 mg/mL of total and reducing sugars with maximum delignification ratio was 36%.

Therefore, the objective of this research is to check a promising eco-friendly strategy for SCB fractionation and further pretreatment. This was achieved in two successive sets: 1. to fractionate the physical composition of SCB by means of freeze-thaw, followed by 2. dissolution of the lignin by dilute H_2O_2 to release cellulose-rich insoluble residue for further enzymatic hydrolysis. To more come up with a reliable approach, different key operating conditions were investigated taking the interaction between them into consideration. This target was handled in this study by using response surface methodology (RSM) investigating duration, temperature and H_2O_2 concentrations

for the best fractionated sample during freezing. In addition, enzymatic hydrolysis and compositional analysis with (XRD, FTIR and SEM) have been conducted for assuring the efficiency of the studied pretreatment approach.

2. Materials And Methods

2.1. SCB characteristics

Fresh SCB was provided from a local sugarcane juice facility in Assuit, Egypt. It was dried at 105°C for 24 h (Binder oven), left overnight at room temperature, put into plastic bags and kept in a freezer until being used. Grinding with a house mixer to reach the particle size around 1.5 cm was subsequently done.

2.2. Compositional analysis of SCB

The chemical compositions of the native and pretreated SCB were determined as described previously (18). One g sample was weighed into a 300 mL flask, and 100 mL neutral detergent was added. The flask was heated for 1 h, and then the sample was filtrated by 3# filter funnel Buchner. The residue “a” was resulted after being washed using distilled water and acetone. The weight of residue “a” was obtained after dried at 60°C for 72 h. Residue “a” was then placed into a 300 mL flask, and 100 mL 2M HCl was added. The flask was heated for 50 min at 100°C. Residue “b” was obtained after filtration and washing by distilled water to adjust the pH to 6.5–7.0. The residue “b” was weighed after drying at 72°C for 72 h. Residue “b” was washed twice by acetone, thereafter dried at 60°C, and placed into a 300 mL flask. After added 10 mL 72% H₂SO₄, residue “b” was hydrolyzed for 3 h at 20°C, and then 90 mL by tap water was applied to keep in room temperature overnight. Residue “c” was obtained after filtrated at pH of 6.5. The weight of residue “c” was got after dried at 60°C for 72 h. The remainder “d” was harvested and ashed at 550°C. The calculation formula were as follows: -

$$\text{Hemicellulose(\%)} = \frac{W_a - W_b}{\text{sampleweight}} \times 100\%$$

$$\text{Cellulose(\%)} = \frac{W_b - W_c}{\text{sampleweight}} \times 100\%$$

$$\text{Lignin(\%)} = \frac{W_c - W_d}{\text{sampleweight}} \times 100\%$$

2.3. Pretreatment of SCB

The combined physico-chemical pretreatment was conducted in two sets as shown in (Fig. 1). The first set was dedicated to study the physical freezing fractionation at different temperatures and durations. The second set was conducted to study the effect of chemical alkaline pretreatment using dilute H₂O₂ at different concentrations, temperatures and durations. Subsequently, all the residues from both sets were exposed to enzymatic hydrolysis.

2.3.1. Physical freezing fractionation of SCB

The dried SCB was mixed with acetate buffer solution (pH 4.6) for 1 h at solid to liquid ratio of 5 (w/v) % [15]. Three sets of mixtures were then frozen at -10, -20 and -30°C for 1, 2 and 3 h in a freezing apparatus (Heto CBN 8–30). Afterward, the frozen SCB samples were thawed at room temperature overnight. The samples were filtered and washed with distilled water and dried at 105°C for 24 h to conduct further analysis. A control sample at room

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2.3.2. Chemical alkaline pretreatment

At this set, response surface methodology (RSM) using Box–Behnken Design (BBD), Minitab® 17.1.0 was applied to study the performance of dilute H₂O₂ pretreatment on the frozen-fractionated SCB. The investigated parameters were H₂O₂ concentration, temperature and duration mainly on the glucose yield and TRS production as listed in Table 1. The pretreatment solution of alkaline H₂O₂ was prepared using distilled water at pH 11.5 with sodium hydroxide (NaOH) according to Rabelo et al. (5). Four grams of SCB was soaked in 100 mL of the pretreatment solution in 250 mL flasks at H₂O₂ concentration of 3, 5.5 and 8 (v/v)% using “a Clifton water bath”, operated at 150 rpm at temperatures of 25, 42.5 and 60°C for a period of 1, 3 and 5 h. After the pretreatment step, the residues were collected by filtration, washed with distilled water until the pH of the filtrate became neutral and then dried at 105°C for 24 h.

To correlate the relationship between variables and response, a quadratic polynomial equation was used for fitting. The general form of the predictive polynomial quadratic equation was as following (Eq. 1) (19):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$
 Eq. (1)

Y is the predicted response of the (glucose yield mg/gm_{native SCB}) and (TRS Production mg/mL) after the enzymatic hydrolysis. X₁, X₂ and X₃ are the independent variables corresponding to H₂O₂ concentration, temperature and duration, respectively. β₀ is a constant term: β₁, β₂ and β₃ are linear coefficients, β₁₁, β₂₂, β₃₃ are square coefficients, β₁₂, β₁₃ and β₂₃ are cross product coefficients.

Table 1. Experimental range and levels of the independent process variables.

Parameter	Value		
H ₂ O ₂ concentration (v/v) % (X ₁)	3	5.5	8
Temperature (°C)..... (X ₂)	25	42.5	60
Time (h)..... (X ₃)	1	3	5

2.4. Enzymatic hydrolysis of the pretreated residues

The following enzyme was provided from Novozymes (Denmark, Lot # SLBS6227): Cellic®CTec2 enzyme and has activity of 100 FPU/ml (FPU: filter paper units).

The enzymatic hydrolysis was carried out by incubation of native and pretreated SCB from the both aforementioned sets using 20 FPU/g pretreated bagasse (Cellic®CTec2, Lot # SLBS6227) enzyme with 5% solid load (1 gm biomass/20 mL buffer solution (with phosphate buffer, pH 4.8). The flasks were sealed with aluminum foil and the hydrolytic mixture was incubated in “GFL shaking incubator” operated at 135 rpm at 40°C for 48 h. Boiling at 90°C was then conducted to stop the biological activity. Remainders after enzymatic hydrolysis were separated by centrifugation (5000 rpm, 15 min) “SIGMA 3K30 Centrifuge” and filtered for further analysis.

2.5. Analytical methods

The glucose yield and TRS production from the hydrolysates produced at each case was analyzed by glucose oxidase reaction using the 3,5 dinitrosalicylic acid (DNS) method Miller 1959 (20). For glucose quantification, 10 µL of the sample and 1 mL of the mono-reagent glucose oxidase were added in assay tubes and put in a water bath at 37°C for 10 min. At the end of the reaction, the absorbance was estimated in a spectrophotometer “NICOLET evolution 100” at 540 nm. For the TRS quantification, 0.5 mL of the samples and 1.5 mL of DNS reagent were added in assay tubes and put in a water bath at 95°C for 5 min. Afterward, the samples were cooled immediately by the immersion in an ice bath. The absorbance was read in a spectrophotometer at 540 nm. In both methods, the standard glucose (Merck) was used for the preparation of the standard curve (21).

2.5.1. X-ray diffraction analysis (XRD)

XRD measurements were performed on a Philips PW 1710. The diffracted intensity of Cu K α radiation ($\lambda = 0.1542$ nm; 40 kV and 30 mA) was measured in a 2θ range between 5° and 35°.

In previous studies (22) and (23), it was reported that a major diffraction peak of the cellulose crystallographic planes can be identified for 2θ ranging between 22° and 23°, and for amorphous cellulose ranging between 18° and 20° (24). The crystallinity index (CI) was calculated from the ratio of the maximum peak intensity (I_{002} , $2\theta = 22^\circ$) and minimal depression (I_{am} , $2\theta = 18.5^\circ$) between peaks 001 and 002 (Segal et al., 1959) (24) according to Eq. (2).

$$\text{crystallinityindex(\%)} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \text{Eq. (2)}$$

where I_{002} is the maximum intensity of the 002 peak and I_{am} minimal depression of the amorphous structure.

2.5.2. Fourier transform infrared analysis (FTIR)

FTIR spectroscopy was used for determining chemical functional groups of native and regenerated SCB using “Nicollet 6700” spectrophotometer using potassium bromide. Dried potassium bromide (KBr) and biomass sample were mixed and then pressed uniformly into a disc. The mixed powder was scanned and recorded between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1} . The rubber band correction method was used for baseline correction following the spectrum minima.

2.5.3. Scan electron microscopy (SEM)

Scanning electron microscopy (SEM) was used for investigating the physical structure of the native and regenerated SCB. “A JOEL-JSM 5400 LV (Japan)” Scanning Electron Microscope was used. SEM images were taken at 50x and 200x magnification at acceleration voltages 15 kV.

2.6. Calculations and kinetic studies

The supernatants after experiments were separated by filtration to analyze the glucose yield, TRS production and saccharification ratios was calculated based on Eq. (3) (18).

$$\text{Saccharificationratio(\%)} = \frac{\text{Glucoseyield} \times 0.9}{\text{Celluloseinregenerated}} \times 100 \text{Eq. (3)}$$

2.7. Statistical analysis using Response Surface Methodology

Experimental data was carried out using Box–Behnken Design (BBD) of RSM by Minitab® 17.1.0. A numerical optimization methodology was employed to optimize the glucose yield and TRS production at different phases. RSM is a collection of mathematical and statistical techniques that is useful for developing, improving and optimizing the processes and to evaluate the relative significance of several process parameters even in the presence of complex reaction conditions. Differences were considered statistical significance at $P < 0.05$ (19).

3. Results And Discussion

3.1. SCB composition

Table 3 summarizes the composition of the investigated native SCB compared to previous studies. The data shows relatively similar proportions of the different SCB components to that observed in those studies. Cellulose was the primary component in the SCB accounting 37.19%, showing that SCB is worth to be fractionated and is a promising raw material for bio-energy production. While hemicellulose and lignin were 24.8% and 19.13%, respectively. The depicted differences are related to the growing location, genetics, season and harvesting methods.

Table 3
Composition of native SCB in this study compared to other studies

Component	The current study	Rainey et al. (25)	Bertoti et al. (26)	Da silva et al. (27)	Rabelo et al. (28)	Soccol et al. (29)	Rocha et al. (30)	Canilha et al. (31)	Chandel et al. (32)
Cellulose	37.19%	47%	47.5–51.1%	38.8%	38.4%	32–44%	45.5%	45%	39.53%
Hemicellulose	24.8%	27%	26.7–28.5%	26%	23.2%	27–32%	27%	25.8%	25.63%
Lignin	19.13%	23%	20.2–20.8%	32.4%	25%	19–24%	21.1%	19.1%	30.36%
Extractives	18.88%	-	0.8-3% other	—	—	—	4.6%	9.1%	2.9%
Ashes	< 1%	1%	compounds	2.8%	—	4.5-9%	2.2%	1%	1.44%

3.2. Frozen-fractionated SCB

Table 4
Chemical composition of native and frozen-fractionated SCB

Component	Cellulose%	Hemicellulose%	Lignin%	Others%
Native SCB	37.19%	24.8%	19.13%	18.88%
Frozen-fractionated SCB (-20 °C/ 2h)	57.11%	15.21%	14.81%	15.06%

Chemical compositional analysis was further performed on the frozen-fractionated SCB to investigate the effect of the different employed conditions as shown in (Figs. 2). It was found that cellulose content peaked at 57.11% after 2 h of freezing at -20°C. Whereas, hemicellulose and lignin contents decreased to 15.21% and 14.81%, respectively. This shows that the freezing fractionation significantly altered the SCB composition, resulting in higher cellulose fraction in the regenerated biomass. Ken-Lin Chang et al. (33) showed that during freezing fractionation, ice crystals

Loading [MathJax]/jax/output/CommonHTML/jax.js ng force entire the recalcitrant lignocellulosic biomass. However,

prolonging the freezing time to 3 h at the same temperature was followed by slightly insignificant increment in the lignin and hemicellulose contents to 15.11% and 15.15 %, respectively. Similar trends were observed by Han-Seob Jeong (34), where the major contents of cellulose and hemicellulose and their derivatives in the liquid hydrolysates were affected until a freezing time of 2 h, after which, they remained relatively constant. Likely, the expansion of water volume inside the biomass during freezing fractionation was not critically affected by time.

3.2.2 Enzymatic hydrolysis of the frozen-fractionated SCB

The glucose yield and saccharification ratio after the enzymatic hydrolysis of the frozen-fractionated SCB at 2.0 h and -20 °C were (307.52 mg/ gm_{native SCB} and 48.5%), (Figs. 3) respectively. Chang et al. (9) report that the highest glucose yield was 371.91 g/kg of dry rice straw, when pretreated with acetate buffer at 2.0 h and -20 °C. No TRS production was recorded from all the samples, reflecting the slight effect of freezing fractionation pretreatment on further carbohydrates hydrolysis. These low results during the first set was not satisfied; indicating that the freezing fractionation was not enough alone for efficient treatment performance and needs further step. Thus, AHP was subsequently employed for enhancing the overall SCB pretreatment. The frozen-fractionated SCB at -20 °C and 2 h was chosen for the subsequent experiments.

3.3. AHP pretreatment

3.3.1. Physical compositions

Table 5 presents the increased amount of cellulose after AHP pretreatment by 30.35% (57.11–82%) using 3% (v/v) AHP more than that obtained before. This result was due to the produced amount of foam during AHP reaction, which increased the loss of solid matter with high concentration and high temperature (35). On the other hand, AHP resulted in loss of lignin content from 14.81–5.2% after freezing and AHP pretreatment, respectively, which agreed with the results of Selig et al. (36). They observed a decrease in the lignin content from 15.8% to 5.4 % when corn Stover pretreated with AHP 1% (w/w) at 65°C and 3 h.

Table 5
Chemical composition of the frozen-fractionated SCB after AHP pretreatment

Component	Cellulose%	Hemicellulose%	Lignin%	Others%
After AHP pretreatment (H ₂ O ₂ of 3 % (v/v), 56.93°C and 1 h)	82%	8.9%	5.2%	3%

3.3.2. Statistical analysis

The effects of H₂O₂ concentration, temperature, and duration mainly on the glucose yield and TRS production were examined with aid of RSM. According to ANOVA as shown in Table 6 as well as Eqs. 4 and 5, the established models were found to be significant and stable for predicting glucose yield and TRS production, respectively under the employed operating conditions (p < 0.05). Specifically, it is indicated that the effect of interactions between the investigated variables and their quadratic effects are highly significant. Based on Table 6 data, all terms except the temperature, time and quadratic terms of time have key roles during the AHP pretreatment process for glucose yield. Meanwhile, all terms except the H₂O₂ concentration, the quadratic terms of temperature and time are significant for TRS production.

The model showed a good fit with the experimental data, since the coefficient of determination (R^2) were 0.8221 and 0.8814 for glucose yield and TRS production, respectively. The fitness of the models can be visualized graphically in diagnostics plots of the predicted versus actual values shown in Figs. 4 (a and b) for glucose yield and TRS production, respectively. The small deviation between the values signifies that the models developed are good representations of the real situation.

Table 6

ANOVA for the response surface quadratic model for glucose yield and TRS production by AHP pretreatment.

Source	glucose yield					TRS production				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	492597	54733	10.27	0.000	9	0.878741	0.097638	16.52	0.000
Linear	3	121211	40404	7.58	0.001	3	0.540787	0.180262	30.50	0.000
H ₂ O ₂ X ₁	1	88858	88858	16.67	0.001	1	0.012656	0.012656	2.14	0.159
Temperature X ₂	1	22062	22062	4.14	0.055	1	0.275625	0.275625	46.64	0.000
Time X ₃	1	10291	10291	1.93	0.180	1	0.252506	0.252506	42.73	0.000
Square	3	221924	73975	13.87	0.000	3	0.092278	0.030759	5.20	0.008
H ₂ O ₂ x H ₂ O ₂ X ₁ ²	1	56622	56622	10.62	0.004	1	0.078217	0.078217	13.24	0.002
Temp x Temp X ₂ ²	1	179498	179498	33.67	0.000	1	0.016082	0.016082	2.72	0.115
Time x Time X ₃ ²	1	2320	2320	0.44	0.517	1	0.006832	0.006832	1.16	0.295
2-Way Interaction	3	149462	49821	9.34	0.000	3	0.245675	0.081892	13.86	0.000
H ₂ O ₂ x Temp X ₁ * X ₂	1	61229	61229	11.48	0.003	1	0.040613	0.040613	6.87	0.016
H ₂ O ₂ x Time X ₁ * X ₃	1	36182	36182	6.79	0.017	1	0.110450	0.110450	18.69	0.000
Temp x Time X ₂ * X ₃	1	52051	52051	9.76	0.005	1	0.094612	0.094612	16.01	0.001
Error	20	106631	5332			20	0.118196	0.005910		
Lack-of-Fit	3	64033	21344	8.52	0.001	3	0.002413	0.000804	0.12	0.948
Pure Error	17	42598	2506			17	0.115783	0.006811		
Total	29	599228				29	0.996937			
DF: degree of freedom; SS: sum of squares; MS: mean squares										
Glucose yield: mg/g _{native SCB} = -833 + 168.9 X ₁ + 59.06 X ₂ + 63.2 X ₃ - 14.01 X ₁ ² - 0.5091 X ₂ ² - 4.43 X ₃ ² - 2.000 X ₁ * X ₂ + 13.45 X ₁ * X ₃ - 2.305 X ₂ * X ₃ Eq. (4)										

Source	glucose yield					TRS production				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
TRS: mg/ml = $-0.348 + 0.1686 X_1 + 0.03873 X_2 + 0.1112 X_3 - 0.01647 X_1^2 - 0.000152 X_2^2 - 0.00760 X_3^2 - 0.001629 X_1 * X_2 + 0.02350 X_1 * X_3 - 0.003107 X_2 * X_3$ Eq. (5)										

3.3.3. Effect of operation conditions

Figures 5 (a,b,c) and 6 (a,b,c) show the interaction effect of H₂O₂ concentration and time on glucose yield and TRS production. The results revealed that increasing the H₂O₂ concentration has a significant effect on glucose yield (p = 0.001), however a slight or limited effect on TRS production (p = 0.159). However, when H₂O₂ effect incorporated with the time and/or temperature, the pretreatment influence became clearer.

For the contour plot shown in (Fig. 5a, 6a) of glucose yield and TRS production vs. H₂O₂ and time at the optimized temperature value of 56.93°C, it was observed that the optimum H₂O₂ and time which achieve the highest glucose yield (884.52 mg/gm_{native SCB}) was respectively at H₂O₂ = 3 (v/v)% and 1 h, where no significant observed H₂O₂ effect on TRS production (p = 0.159). On the other hand, increasing the H₂O₂ concentration to 8% was followed by deterioration of both glucose yield and TRS production whatever the time and imposed temperature. The glucose yield and TRS production dropped to 430.79 mg/gm_{native SCB} and 0.92 mg/mL with [8 (v/v)% at 60°C for 3 h] and [8 (v/v)% at 42.5°C for 1 h] of AHP pretreatment, respectively. This accorded to the SCB large mass loss of 238% that occurred at H₂O₂ of 8 (v/v)%. A comparable glucose yield of 374 mg / gm_{raw SCB} was obtained after AHP pretreatment of SCB with 7.35% (v/v) at 25°C for 1 h (37). Michael G. et al. (16) explained that H₂O₂ has a powerful ability on the solubilization of hemicellulose, accordingly decreasing the mass weight. Moreover, the combination effect of high H₂O₂ concentration and prolonged time obtained stronger degradation of SCB (38).

For the contour plot shown in (Fig. 5b, 6b) of glucose yield and TRS vs. (H₂O₂ and temperature at time = 1 h), it is worth to note that the average glucose yield and TRS production always augmented with raising the temperature. The average predicted glucose yield increased from 701.98 to 810.66 mg/ gm_{native SCB} and then decreased to 627.72 mg/ gm_{native SCB}, while TRS increased from 1.09 to 1.27 to 1.35 mg/ml with raising the temperature from 25 to 42.5 to 60°C, respectively. Karagöz et al. (37) showed that raising the temperature from 50 to 70°C using AHP for rapeseed straw pretreatment resulted in lower glucose release, which is mainly due to the H₂O₂ decomposition to water at high temperatures (39).

Similar trends were observed with varying the pretreatment time on glucose yield and TRS production vs. time and temperature at H₂O₂ = 3 (v/v)% (Fig. 5c, 6c). Both glucose yield and TRS production accounted slight decrement with prolonging pretreatment time. They decreased from (884.52 mg/ gm_{native SCB} to 800.74 mg/ gm_{native SCB}) and (1.32 to 1.23 mg/ml) when time was extended from 1 to 5 h, respectively. Long reaction time would result in solubilization of cellulose into glucose which then further degraded into smaller compounds such as furfural compounds. The presence of furfural and other inhibitors hinder the hydrolysis process to produce reducing sugar. These observations were in agreement with previous works (40) (36), who experienced increment in sugar yields from maize stems and corn Stover with increasing the pretreatment time.

The SCB samples were finally hydrolyzed at 40°C for 48 h, from which, about 97.5% as a saccharification ratio was obtained for the AHP-pretreated SCB with 3% (v/v) H₂O₂ at 56.93°C for 1 h. The result is slightly higher than that obtained by Azzam et al. (41) for SCB, who reported a saccharification ratio of 95 % with 2% (v/v) H₂O₂ at 30°C for 8 h by cellulase at 45°C for 24 h. This difference of results can be attributed to the improved hydrolysis rates of cellulose thanks to the previous pretreatment phase of freezing as well as the prime importance to take the interaction effect between the operation conditions on the pretreatment process into consideration.

Table 7
Experimental and predicted glucose yield and TRS production for the frozen-fractionated SCB
pretreated with H₂O₂ at different operating conditions

Run	H ₂ O ₂	Temp	Time	glucose yield		TRS	
	(X ₁) (v/v)%	(X ₂) (°C)	(X ₃) (h)	mg/gm _{native SCB}		mg/mL	
Experimental	Predicted	Experimental	Predicted				
1	5.5	42.5	3	885.04	873.40	1.16	1.33
2	3	42.5	5	736.06	800.74	1.1	1.23
3	5.5	42.5	3	864.00	873.40	1.37	1.33
4	5.5	25	5	824.95	842.93	1.31	1.35
5	8	60	3	451.43	430.79	1.29	1.21
6	3	25	3	665.76	654.10	0.97	1.00
7	8	42.5	1	570.97	600.98	0.88	0.92
8	5.5	60	5	733.98	607.33	1.33	1.40
9	5.5	42.5	3	836.26	873.40	1.36	1.33
10	5.5	60	1	714.04	717.94	1.39	1.37
11	5.5	25	1	624.17	630.88	0.88	0.93
12	3	25	3	621.86	654.10	1.03	1.00
13	5.5	25	5	851.14	842.93	1.38	1.35
14	5.5	42.5	3	822.32	873.40	1.41	1.33
15	3	60	3	671.09	754.80	1.41	1.41
16	5.5	60	5	607.60	607.33	1.44	1.40
17	5.5	25	1	510.68	630.88	0.92	0.93
18	8	42.5	5	735.07	786.20	1.42	1.41
29	8	25	3	789.31	680.03	1.15	1.09
20	3	60	3	681.26	754.80	1.41	1.41
21	5.5	60	1	731.60	717.94	1.36	1.37
22	3	42.5	5	895.78	800.74	1.39	1.23
23	8	25	3	728.00	680.03	1.03	1.09
24	5.5	42.5	3	892.01	873.40	1.34	1.33
25	3	42.5	1	933.27	884.52	1.35	1.32
26	3	42.5	1	983.27	884.52	1.36	1.32

Run	H ₂ O ₂	Temp	Time	glucose yield		TRS	
	(X ₁) (v/v)%	(X ₂) (°C)	(X ₃) (h)	mg/gm _{native SCB}		mg/mL	
27	8	60	3	430.73	430.79	1.14	1.21
28	8	42.5	1	600.63	600.98	0.94	0.92
29	8	42.5	5	689.84	786.20	1.42	1.41
30	5.5	42.5	3	940.79	873.40	1.33	1.33

3.3.4. Prediction and verification of optimization point.

Table 8 indicates the verified experimental values of glucose yield and TRS production from the frozen-fractionated SCB pretreated by AHP under the optimized conditions according to RSM. By implementing the experimental condition of 3% H₂O₂ concentration at 56.93°C and 1 h, the experimental value of glucose yield and TRS production were 886.507 mg/ gm_{native SCB} and 1.44 mg/mL respectively. Based on the predicted and experimental results, the experimental values were in good agreement with the predicted values proposed by the model.

Table 8
Results of verification experiments at optimum process condition

Responses	Predicted	Experimental
glucose yield	886.507 mg/ gm _{native SCB}	898.5 mg/ gm _{native SCB}
TRS production	1.44 mg/mL	1.32 mg/mL

3.3.5. FTIR and XRD analyses

The XRD spectra of the native and pretreated SCB showed in (Fig. 7). Crystallinity index (CI) of frozen-fractionated SCB increased to 58.5% instead of 43% of native SCB. While, after AHP pretreatment with (3 v/v % H₂O₂), the CI increased to 63.2%. These results are in accordance to previous research works (42) and (35), which indicates the enhanced modification of the composition of the pretreated SCB. According to Chirayil et al. (43), AHP pretreatment of cellulose with low-concentration removed part of hemicellulose and lignin away from cellulose; causing the increase of CI.

Likewise, FT-IR analysis showed that the pretreatment with AHP have done synergistic activities during the lignin degradation as shown in (Fig. 8). In particular, AHP destroys the ether bonds between lignin and hemicelluloses polysaccharides and decreases the polymerization degree of lignin in the cell wall (39). The vibration at 3405 cm⁻¹ and 3385 cm⁻¹, which belong to a functional group-OH (44), refers structural changes in hydroxyl groups in (alcohol and phenols) structures and in turn changes in the level of inter- and intramolecular structures (hydrogen bonding). In all three FT-IR spectra, the peaks locate between 2924 and 2919 cm⁻¹ with small shoulder bands that are assigned to -CH₂ stretching. The band in the frozen-fractionated SCB sample of 2919 cm⁻¹ shifted to 2899 cm⁻¹ after AHP pretreatment, which implies the improved delignification of SCB (45). Furthermore, the peak at 1732 cm⁻¹ was pronounced in the native and frozen-fractionated SCB samples, but this peak disappeared after AHP pretreatment. This result is attributed to the removal of most hemicelluloses and lignin during the AHP pretreatment as well as the

destruction of bond C = O in hemicellulose (46). In addition, the AHP pretreatment proved the efficient break down of esterified linkage in the lignocellulosic matrix and deacetylation of hemicellulose during the process.

The lignin-associated peaks at 1605 cm^{-1} , 1514 cm^{-1} , 1247 cm^{-1} and 834 cm^{-1} are present in the native and frozen-fractionated SCB samples and were not present in the AHP pretreated SCB. Lv et al. (47) indicates the partial removal of lignin during AHP process and this result is consistent with the composition analysis results. Correia et al. (48) mentioned that it was necessary to remove the bands related to the aromatic ring vibrations in lignin ($1500\text{--}1550\text{ cm}^{-1}$) when the cashew bagasse biomass was analyzed before and after the pretreatment with H_2O_2 , which was comparable to the present results. The band at 1640 cm^{-1} is attributed to the bending mode of the absorbed water (49).

FT-IR spectral area closer to $1424\text{--}1430$, $1371\text{--}1376$, $1162\text{--}1164$, $1050\text{--}1057$ and $897\text{--}898\text{ cm}^{-1}$ were identified as cellulose-related bands (50) and (51). The band at around $1424\text{--}1430\text{ cm}^{-1}$ and 897 cm^{-1} are associated with the amount of crystalline and amorphous structure of the cellulose respectively in addition to those assigned to (C-H deformation in "crystalline" and "amorphous" cellulose. However, the vibration peak at 897 cm^{-1} , which was absent in the native sample, appears in the frozen-fractionated and AHP-SCB samples. This was assigned to the glycosidic bonds (53).

The peak appearing about $1370\text{--}1375\text{ cm}^{-1}$ in native and SCB treated samples was as a result of carbon and hydrogen bond distortion (C-H deformation) of celluloses (50), (51) and (54). The hydroxyl phenolic groups peaks are between 1375 and 1325 cm^{-1} (55), this peaks not observed in this study. Pan et al. (56) mentioned that this groups present an inhibitory effect on cellulases.

This suggests that AHP pretreatment enriched the SCB with cellulose. Cellulose was uncovered as a result of the pretreatment could be evident by the prominence appearing of cellulose related peaks in pretreated SCB. Since cellulases break down β 1–4 glycoside bonds in cellulose, those peaks strength.

3.3.6. SEM analysis

Figure 9.a. shows the undamaged surface of native SCB, which had smooth, contiguous, and clumpy appearance. While Fig. 9.b. showed the frozen-fractionated SCB that depicts the looseness of biomass with a slight disorder and rough surface. This was attributed to the force created by water crystallization during freezing and thawing. On the other hand, SEM picture of SCB after AHP pretreatment in Fig. 9.c shows the increased SCB diffusivity related to the produced foam during reactions.

4. Conclusion

The effect of physical freezing in addition to alkaline hydrogen peroxide (AHP) pretreatments on SCB was studied. The content of cellulose increased from 37.19–57.11% by water volume expansion according to freezing time of 2 h at -20°C and solid to acetate buffer ratio of 5:1. Consequently, the enzymatic hydrolysis was performed which resulted in glucose yield of (307.52 mg/ gm_{native SCB}) with saccharification ratio of 48.5%. Chemical hydrogen peroxide was further investigated using RSM, that proved to be a promising pretreatment method. Three key factors of H_2O_2 concentration, temperature, and time on the glucose yield and TRS production were investigated. The optimum conditions that gave the maximum glucose yield and TRS production were at H_2O_2 concentration of 3 (v/v)

%, temperature of 56.93°C and time of 1 h. Under these conditions, the response predicted values of glucose yield and TRS production were 886.51 mg/gm_{native SCB} and 1.44 mg/mL, respectively. These values were relatively close to the experimentally obtained values of (898.5 mg/gm_{native SCB} and 1.32 mg/ml) with saccharification ratio of 97.5%. The experimental results show moreover that the pretreatments (freezing and alkaline) were efficient synergistically in reducing the hemicellulose from 24.8 to 8.9% and lignin from 19.13 to 5.2%, coincided with an increase in the cellulose content from 37.19–82%.

Declarations

- **Funding:** Not applicable. No funding was received for this work.
- **Conflicts of interest/Competing interests:**

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

- **Code availability:** Not applicable.
- **Authors' contributions:**

The paper reflects the authors' own research and analysis in a truthful and complete manner.

- **Ethics approval:**

This manuscript is original and has not been published/under consideration for publication elsewhere or submitted earlier to Arabian Journal for Science and Technology

- **Consent to participate:**

We also confirm that the statement and order of authors listed in the manuscript has been approved by all named authors.

- **Consent for publication:**

We confirm that the manuscript has been read and approved by all named authors for publication.

- **Availability of data and materials:**

Most of the data generated or analysed during this study are included in this published article. The rest of them are available from the corresponding author on reasonable request.

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Figures

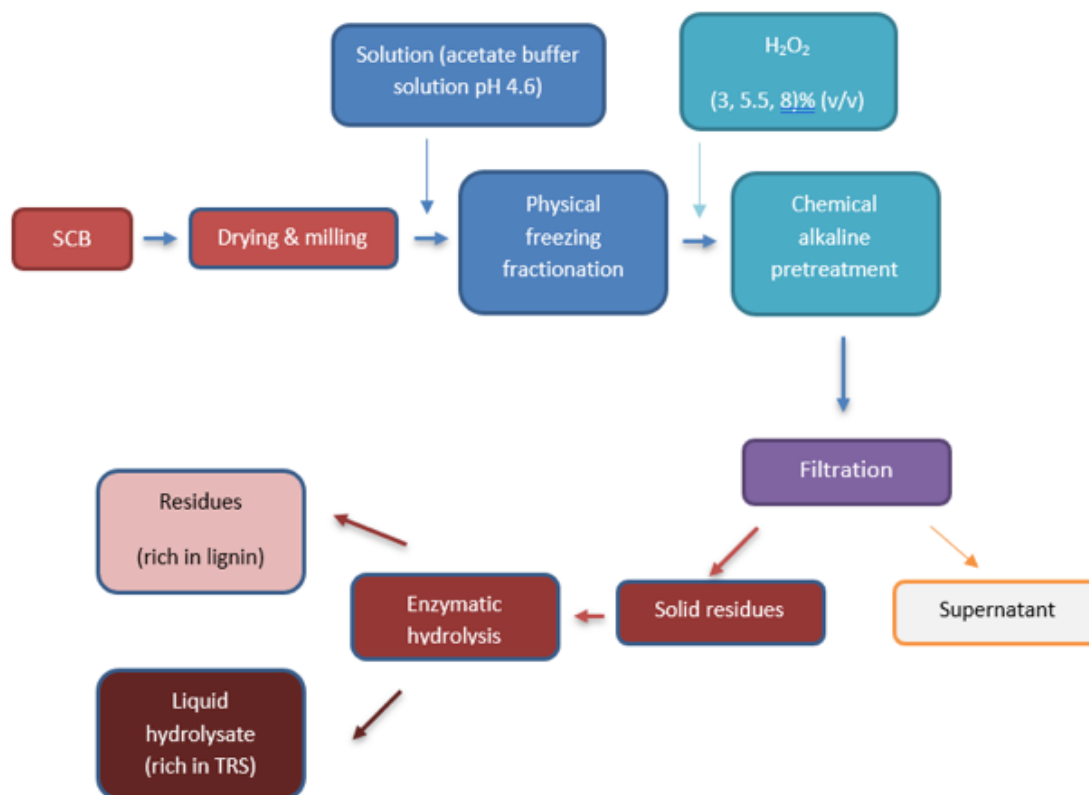


Figure 1

Experimental scheme of the whole process of SCB freezing fractionation and subsequent alkaline pretreatment

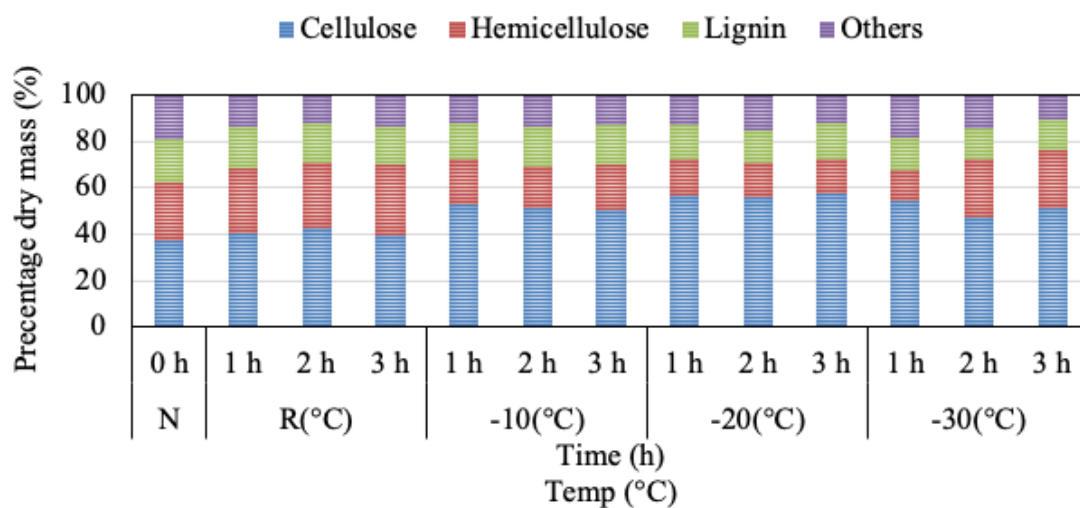


Figure 2

Compositional analysis of native and frozen-fractionated SCB N: native at (R) room temperature $\approx 23\text{ }^{\circ}\text{C}$

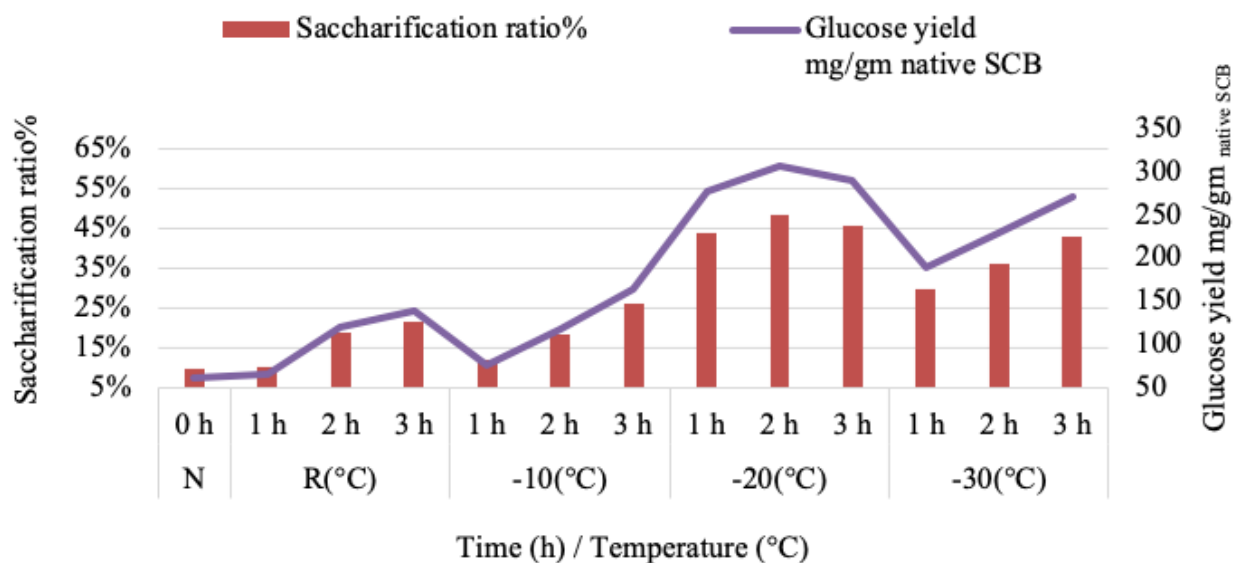


Figure 3

Saccharification ratio and glucose yield of native and freezing-fractionated SCB N: native at (R) room temperature $\approx 23^{\circ}\text{C}$

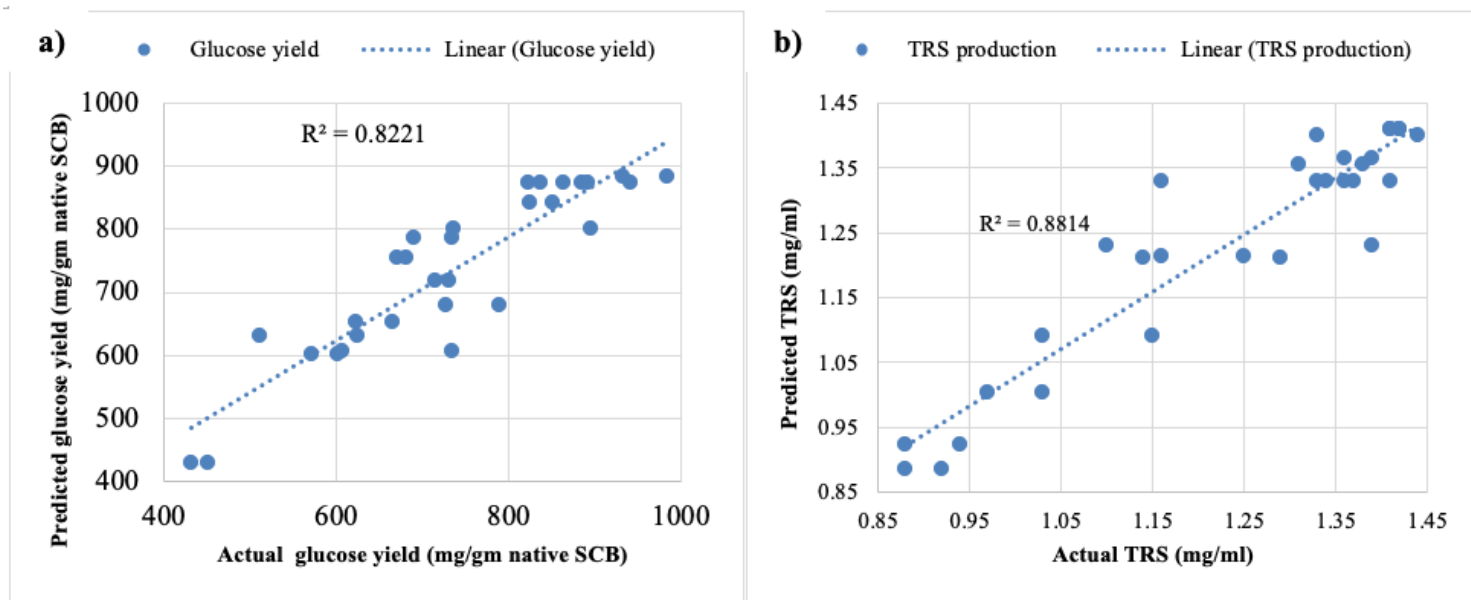


Figure 4

Predicted versus actual values plots for: (a) glucose yield and (b) TRS production.

Hold values
Temp = 56.93 °C

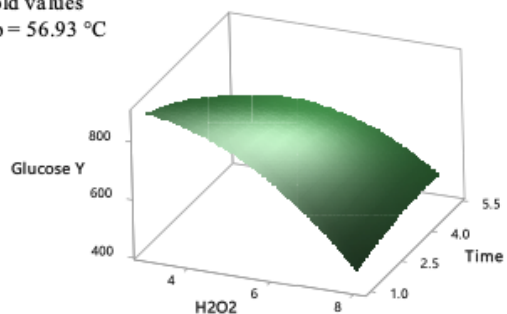


Fig.5a. Glucose Y vs. H₂O₂, Time

Hold values
Time = 1 h

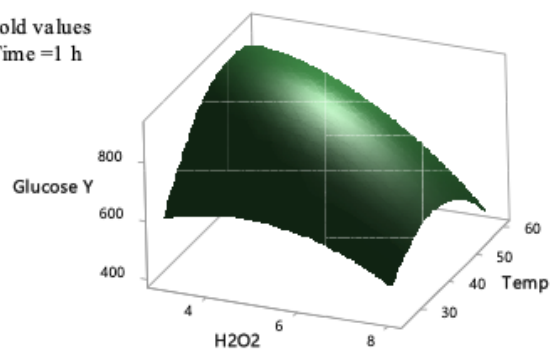


Fig. 5b. Glucose Y vs. H₂O₂, Temperature

Hold values
H₂O₂ = 3%

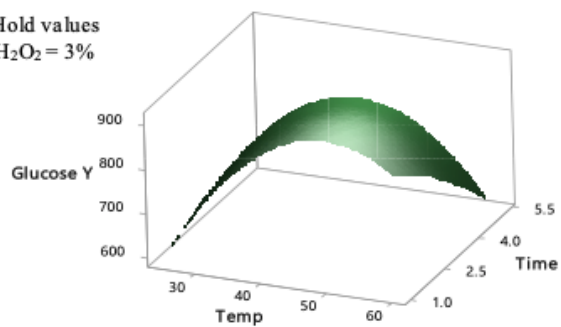


Fig. 5c. Glucose Y vs. Time, Temperature

Figure 5

Three-dimensional response surface plots of Glucose yield at different operation conditions

Hold values
Temp = 56.93°C

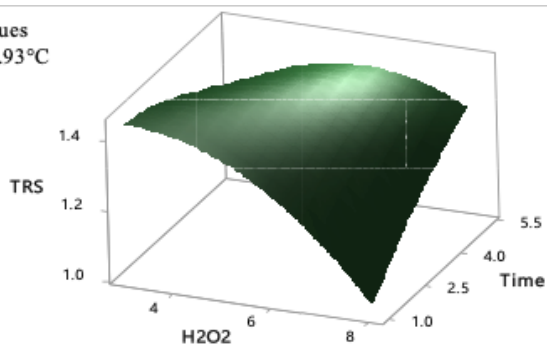


Fig.6a. TRS vs. H₂O₂, Time

Hold values
Time = 1 h

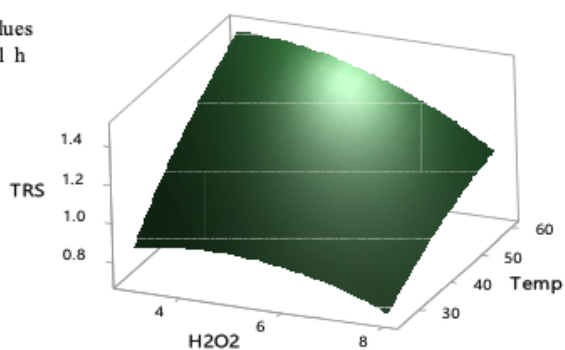


Fig.6b. TRS vs. H₂O₂, Temperature

Hold values
H₂O₂ = 3%

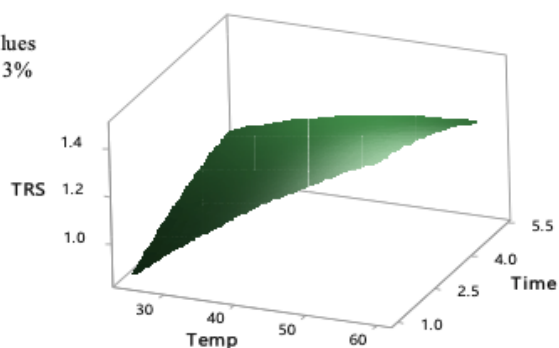


Fig. 6c. TRS vs. Time, Temperature

Figure 6

Three-dimensional response surface plots of TRS at different operation conditions

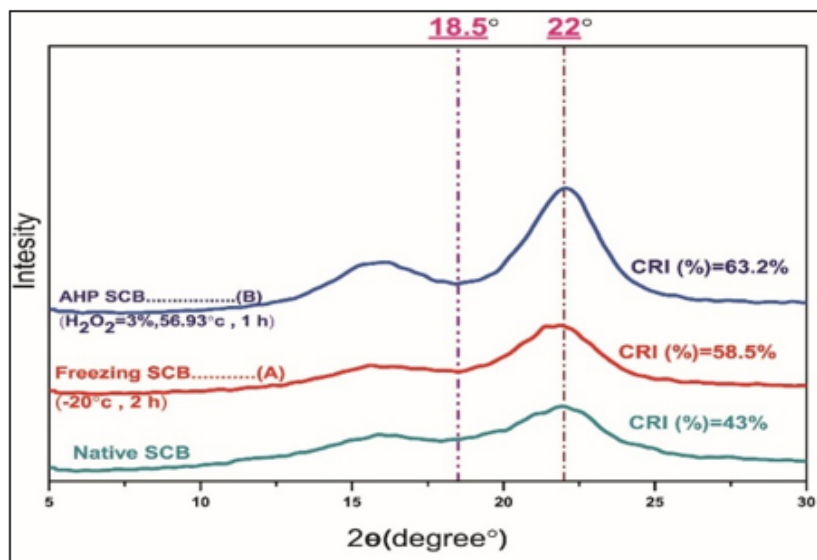


Figure 7

XRD spectra of sugarcane bagasse SCB

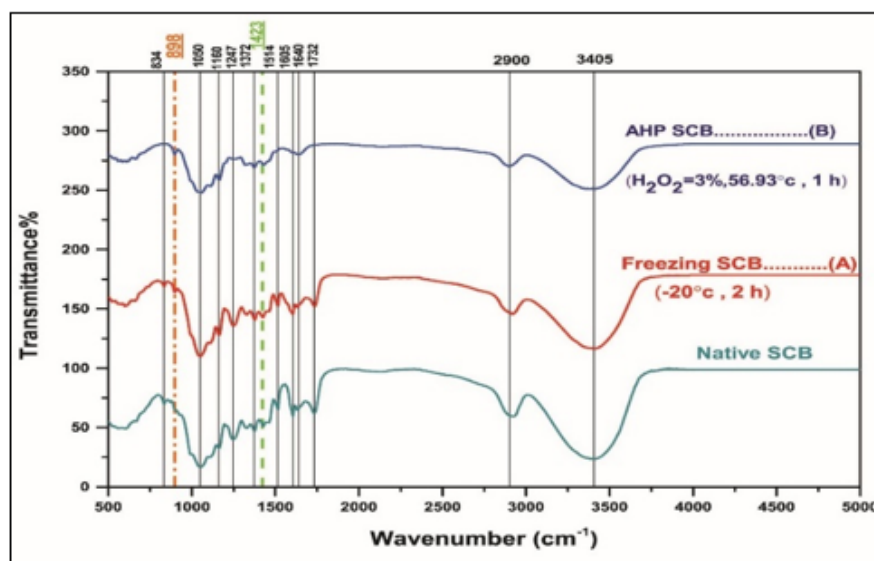


Figure 8

FTIR spectra of sugarcane bagasse SCB

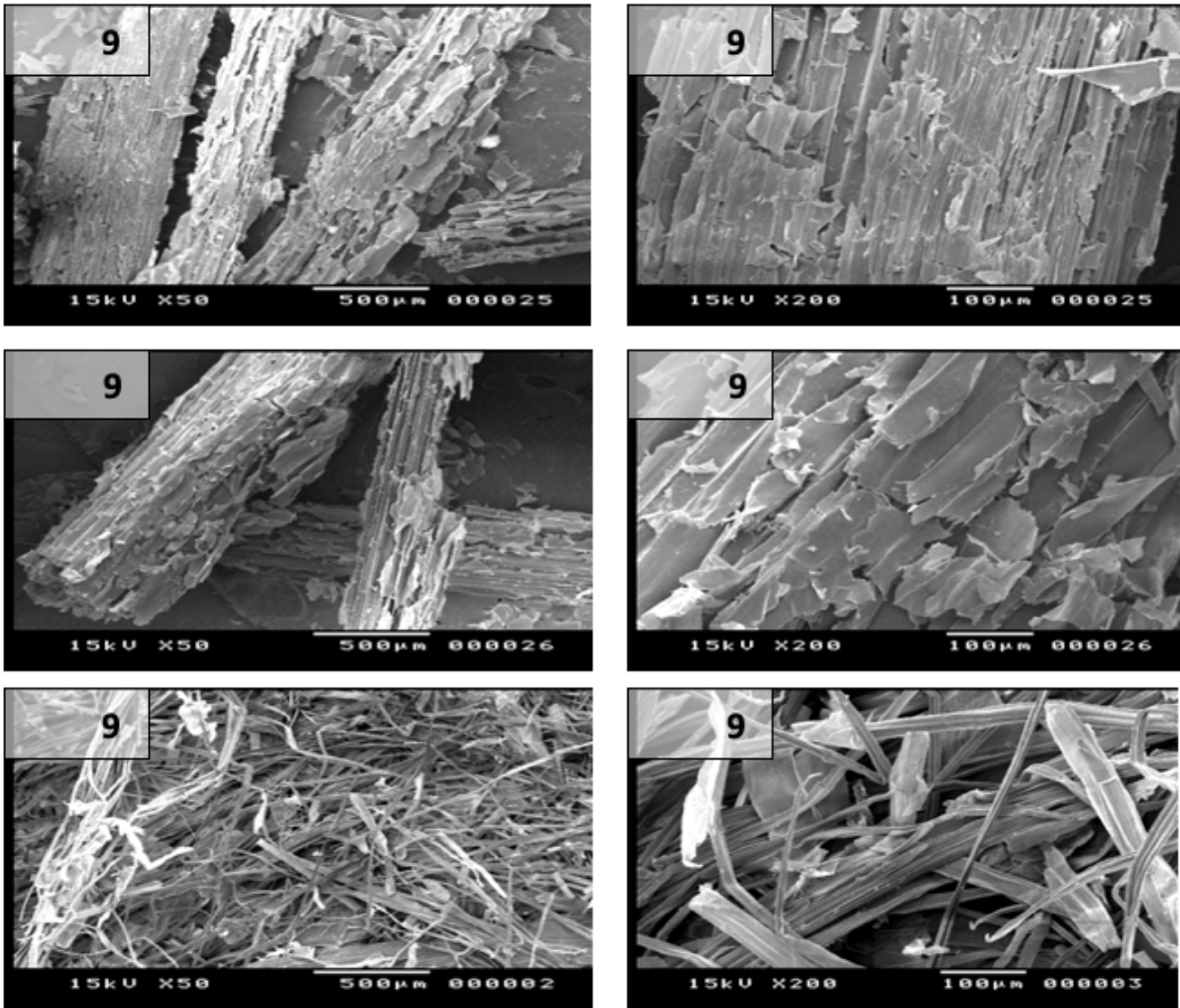


Figure 9

SEM images of SCB before and after freezing and AHP pretreatments (X50, 200 magnification)