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Synergistic physic-chemical pretreatment of lignocellulosic sugarcane bagasse via freezing and alkaline processes

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Keywords
Sugarcane Bagasse; Lignocellulose; Freezing; Hydrogen peroxide; Response surface methodology.

Abstract
This study focuses on employing a hybrid pre-treatment approach for lignocellulosic Sugarcane Bagasse (SCB) as a major problematic solid waste. The applied technique depended on SCB physical pre-treatment 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 pre-treatment stages were evaluated. Freezing pre-treatment 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. Further AHP pre-treatment was performed for the frozen-pre-treated SCB at -20 °C and 2 h with assistance of Box–Behnken Design response surface methodology (RSM). The investigated key parameters were H2O2 concentration (3, 5.5 and 8 %v/v), temperature (25, 42.5 and 60 °C) and pre-treatment 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 R2 = 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 H2O2 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 pre-treatment phase for glucose yield and saccharification ratio, respectively. The results are higher that obtained in the literature for SCB with 2% (v/v) H2O2 at 30°C for 8 h. Therefore, The AHP following freezing pre-treatment proved the efficient break down of esterified linkage in the lignocellulosic matrix and deacetylation of hemicellulose during the process.

List of Abbreviations and Acronyms

SCB	Sugarcane Bagasse	XRD	X-ray diffraction
AHP	Alkaline Hydrogen Peroxide	FTIR	Fourier transform infrared
RSM	Response Surface Methodology	SEM	Scanning electron microscopy
BBD	Box–Behnken Design	TRS	Total reducing sugars
GHG	Greenhouse gas		

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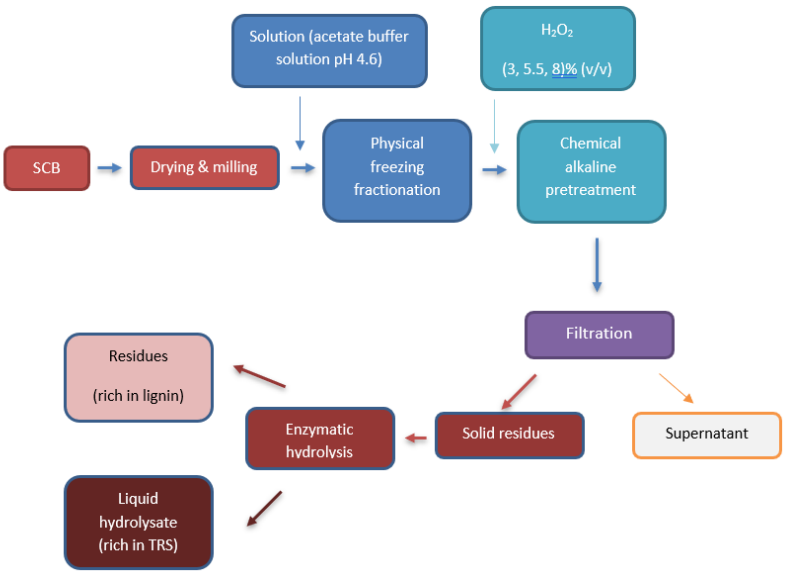
Graphical Abstract



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% (El-dorghamy 2007). 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 (Nakhla et al. 2013). 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) (Elfeki et al. 2017).

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 (Abou Hussein and Sawan 2010). The remainder is burnt directly on the fields or used for heating in small villages (El-Mashad et al. 2003). 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 (Rabelo et al. 2011a). In south Egypt, for instance, Quena has 3 mills: in (Naga Hammadi, Deshna 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 (Nakhla et al. 2013).

SCB is a recalcitrant lignocellulosic waste against biodegradation and bioconversion. Lignocelluloses are composed of cellulose, hemicelluloses and lignin in an intricate structure, which is hard to be decomposed (Haghighi Mood et al. 2013). 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. (Harmsen et al. 2010).

There are several pretreatment methods of SCB which can be classified into four categories: physical, chemical, biological and physicochemical pretreatment (El-Mashad et al. 2003; Haghighi Mood et al. 2013). 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 pretreatment possesses important advantages. Significant lower environmental impact due to less discharge of hazardous derivatives and null used chemicals are examples of these advantages (Chen et al. 2014). 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 (Chang et al. 2011). When water freezes, it stacks on the crystalline lattice configuration and in turn stretches the rotational and vibrational components of the bond. Owczuk et al. (2014) have shown that the best results of pretreatment were obtained after one cycle of freeze-thaw below -10 °C. However, Rooni et al. (2017) found that the optimum results were obtained after 4 times of the freeze-thaw. Chang et al. (2011) observed a significant increase in the enzyme digestibility of rice straw from 48% to 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 freezing pretreatment.

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 (Li et al. 2016). Besides, it is a typical and relatively safe eco-friendly agent used for delignification during wood pulping processes. Previous researches (Azzam 2008) found that dilute alkaline solutions of (1-5% (v/v)) H2O2 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. (2011) 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 strategy for SCB pretreatment 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 H2O2 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 H2O2 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

Fig. 1 depicts the employed experimental scheme of the whole process of SCB freezing pretreatment and subsequent alkaline pretreatment. In particular, 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. The chemical compositions of the native and pretreated SCB were determined as described previously (Lin et al. 2009; Timung et al. 2015).

2.2. 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 pretreatment 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.

2.2.1. Physical freezing pretreatment of SCB

The dried SCB was mixed with acetate buffer solution (pH 4.6) for 1 h at solid to liquid ratio of 5:1 (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 temperature ≈ 23 °C was also analyzed to check the freezing performance.

2.2.2. 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 pre-treatment on the frozen-pre-treated SCB. The investigated parameters were H2O2 concentration, temperature, and duration mainly on the glucose yield and TRS production as listed in Table 1. The pre-treatment solution of alkaline H2O2 was prepared using distilled water at pH 11.5 with sodium hydroxide (NaOH) according to Rabelo et al. (2011a). Four grams of SCB was soaked in 100 mL of the pre-treatment 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 pre-treatment 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) (Wang et al. 2015):

$$Y= \beta_0+ \beta_1X_1+ \beta_2X_2+ \beta_3X_3+ \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33}X_3^2+ \beta_{12} X_1X_2+ \beta_{13} X_1X_3 + \beta_{23} X_2X_3 \quad \text{Eq. (1)}$$

Y is the predicted response of the (glucose yield mg/gm $_{native\ SCB}$) and (TRS Production mg/mL) after the enzymatic hydrolysis. X_1, X_2 and X_3 are the independent variables corresponding to H_2O_2 concentration, temperature and duration, respectively. β_0 is a constant term: β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are square coefficients, β_{12}, β_{13} and β_{23} are cross product coefficients.

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

Parameter	Value		
H_2O_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

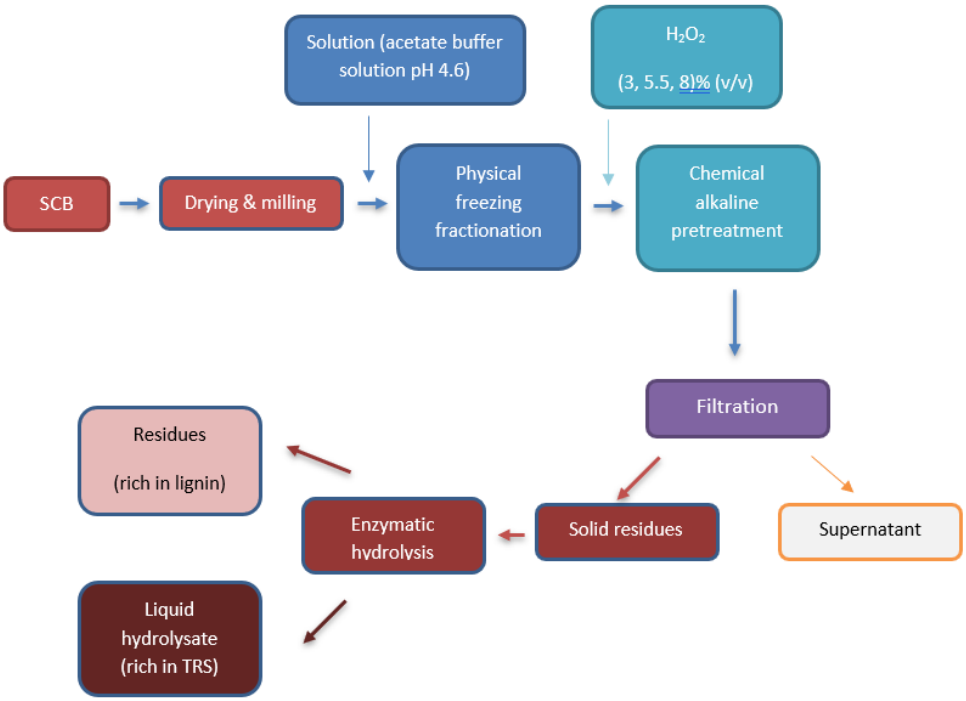


Fig. 1. Experimental scheme of the whole process of SCB freezing pre-treatment and subsequent alkaline pre-treatment

2.3. 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 pre-treated SCB from both aforementioned sets using 20 FPU/g pre-treated 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 aluminium 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.

2.4. Analytical methods

The glucose yield and TRS production from the hydrolysates produced at each case was analyzed by using the 3,5 dinitro salicylic acid (DNS) method (Weerachanchai et al. 2012). For glucose quantification, 10 µL of the sample and 1 mL of the mono-reagent glucose oxidase were added in assays 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. XRD measurements

were performed on a Philips PW 1710. The diffracted intensity of Cu K α radiation (λ = 0.1542 nm; 40 kV and 30 mA) was measured in a 2θ range between 5° and 35°.
In previous studies (Mulinari et al. 2009; Chen et al. 2011), it was reported that a major diffraction peak of the cellulose crystallographic planes can be identified for 2θ ranging between 22º and 23º, and for amorphous cellulose ranging between 18º and 20º. The crystallinity index (CI) was calculated from the ratio of the maximum peak intensity (I002, 2θ = 22º) and minimal depression (Iam 2θ = 18.5º) between peaks 001 and 002(Segal et al. 1959) according to Equation (2).

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

where I002 is the maximum intensity of the 002 peak and Iam minimal depression of the amorphous structure. 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. 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.

2.5. 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) (Farghaly et al. 2017).

$$\text{Saccharification ratio (\%)} = \frac{\text{Glucose yield} * 0.9}{\text{Cellulose in regenerated}} \times 100 \qquad \text{Eq.(3)}$$

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 $P < 0.05$ (Wang et al. 2015).

3. Results and discussion

3.1. SCB composition

Table 2 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 utilized 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.

3.2. Frozen-pre-treated SCB

Chemical compositional analysis was further performed on the frozen-pre-treated SCB to investigate the effect of the different employed conditions as shown in (Fig. 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 pre-treatment significantly altered the SCB composition, resulting in higher cellulose fraction in the regenerated biomass. Tuankriangkrai and Benjakul (2010) showed that during freezing fractionation, ice crystals would be formed and caused, in turn, breaking 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 Jeong et al. (2016), 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.

Table 2. Composition of native SCB in this study compared to other studies

Component	The current study	Rainey et al. (Rainey 2009)	Bertoti et al. (Bertoti et al. 2009)	Da silva et al. (da Silva et al. 2010)	Rabelo et al. (Rabelo et al. 2011b)	Soccol et al. (Soccol et al. 2010)	Rocha et al. (Jackson de Moraes Rocha et al. 2011)	Canilha et al. (Canilha et al. 2012)	Chandel et al. (Chandel et al. 2014)
Cellulose	37.2%	47.0%	47.5-51.1%	38.8%	38.4%	32.0-44.0%	45.5%	45.0%	39.5%
Hemicellulose	24.8%	27.0%	26.7-28.5%	26.0%	23.2%	27.0-32.0%	27.0%	25.8%	25.6%
Lignin	19.1%	23.0%	20.2-20.8%	32.4%	25.0%	19.0-24.0%	21.1%	19.1%	30.4%
Extractives	18.9%	-	0.8-3.0% other	----	----	----	4.6%	9.1%	2.9%
Ashes	<1.0%	1.0%	compounds	2.8%	----	4.5-9%	2.2%	1.0%	1.4%

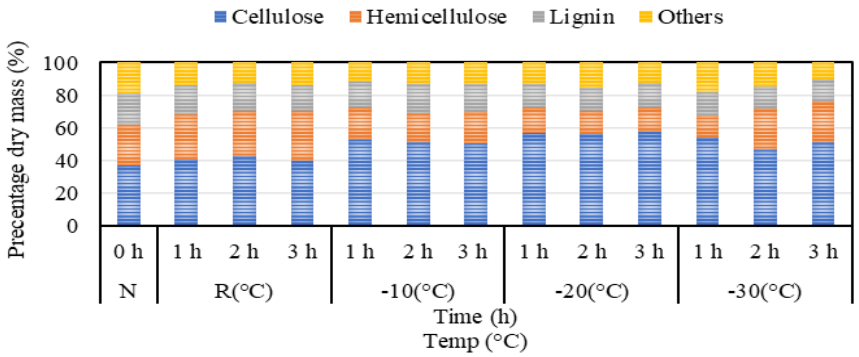


Fig. 2. Compositional analysis of native and frozen-pretreated SCB
N: native at (R) room temperature ≈ 23 ºC

The glucose yield and saccharification ratio after the enzymatic hydrolysis of the frozen-pre-treated SCB at 2.0 h and -20 °C were (307.52 mg/ gm native SCB and 48.5%), (Fig. 3) respectively. Chang et al. (2011) report that the highest glucose yield was 371.91 g/kg of dry rice straw, when pre-treated with acetate buffer at 2.0 h and -20 °C. These low results during the first set were not satisfying; indicating that the freezing pre-treatment was not enough for efficient treatment performance and needs further step. Thus, AHP was subsequently employed for enhancing the

overall SCB pre-treatment. The frozen-pre-treated SCB at -20 °C and 2 h was chosen for the subsequent experiments.

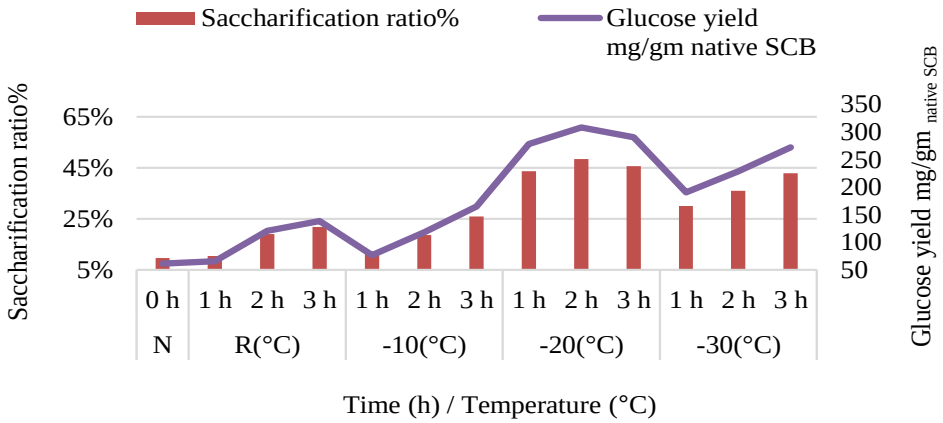


Fig. 3. Saccharification ratio and glucose yield of native and freezing-pretreated SCB
N: native at (R) room temperature ≈ 23 ºC

3.3. AHP pre-treatment

3.3.1. Physical compositions

Table 3 presents the increased amount of cellulose under the synergistic effect of freezing and AHP pre-treatment which peaked from 57.11% to 82% using 3% (v/v) AHP. On the other hand, loss of lignin content from 14.81% to 5.2% was observed, which agreed with the results of Selig et al. (2009).

Table 3. Chemical composition under the synergistic effect of freezing and AHP pre-treatment

Component	Cellulose%	Hemicellulose%	Lignin%	Others%
After AHP pre-treatment (H_2O_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 the aid of RSM. According to ANOVA as shown in Table 4 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 investigated 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 4 data, all terms except the temperature, time and quadratic terms of time have key roles during the AHP pre-treatment 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 (R2) 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 Fig. 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 4 ANOVA for
response surface quadratic model for glucose yield and TRS production by AHP pre-treatment.

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_2O_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_2O_2 x H_2O_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_2O_2 x Temp X_1* X_2	1	61229	61229	11.48	0.003	1	0.040613	0.040613	6.87	0.016
H_2O_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 $_{\text{native SCB}}$ = -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)										
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

All of the experimental and predicted glucose yield and TRS production for the frozen-pretreated SCB pretreated with H2O2 at different operating conditions are summarized in Table 5. Figs. 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 plots shown in (Figs. 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 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 (v/v) % 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 at [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 at 7.35 (v/v)%, 25 °C and 1 h (Karagöz et al. 2012). 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 (Rasmey et al. 2017).

For the contour plot shown in (Figs. 5b, 6b) of glucose yield and TRS vs. (H2O2 and temperature at time = 1 h), 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. (2012) 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.

Similar trends were observed with varying the pretreatment time on glucose yield and TRS production vs. time and temperature at H2O2 = 3 (v/v) % (Figs. 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 (Saha and Cotta 2014; Selig et al. 2009), who experienced increment in sugar yields from maize stems and corn Stover with increasing the pretreatment time.

The SCB samples were finally enzymatically hydrolyzed at 40 °C for 48 h, from which, about 97.5% 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 that obtained by a previous work (Azzam et al. 2008) 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.

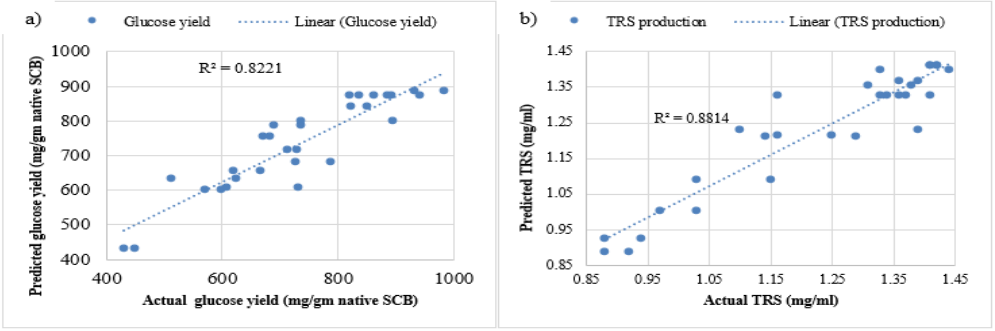


Fig. 4. Predicted versus actual values plots for: (a) glucose yield and (b) TRS production.

Table 5. Experimental and predicted glucose yield and TRS production for the frozen-pre-treated SCB pre-treated with H_2O_2 at different operating conditions

Run	H_2O_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
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 the optimized values

Table 6 indicates the verified experimental values of glucose yield and TRS production under the synergistic effect of freezing and AHP pretreatment at the optimized conditions according to RSM. By implementing the experimental condition of 3 (v/v) % H2O2 concentration at 56.93°C and 1 h, the experimental values 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 6: 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

Triplicate samples have been experimentally analysed, where only the average value was presented

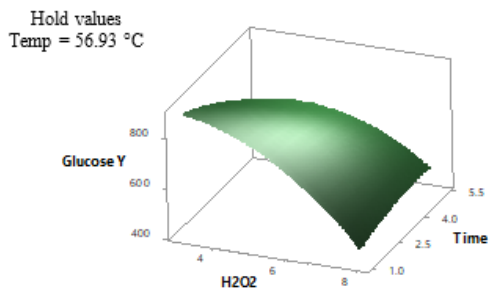


Fig.5a. Glucose Y vs. H_2O_2, Time

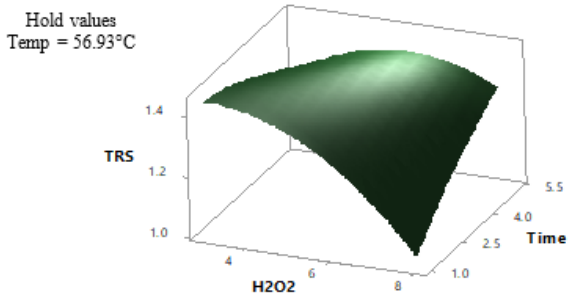


Fig.6a. TRS vs. H_2O_2, Time

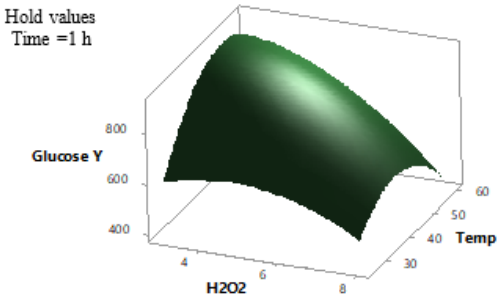


Fig. 5b. Glucose Y vs. H_2O_2, Temperature

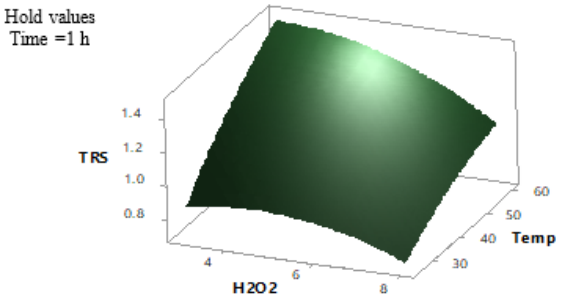


Fig.6b. TRS vs. H_2O_2, Temperature

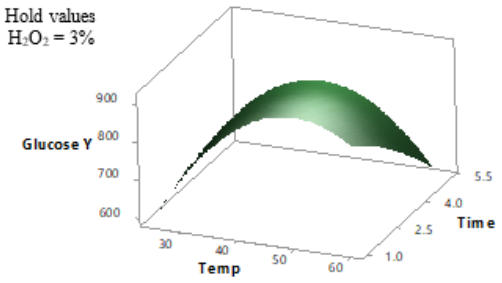


Fig. 5c. Glucose Y vs. Time, Temperature

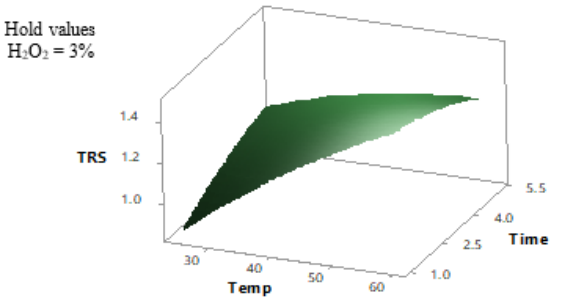


Fig. 6c. TRS vs. Time, Temperature

Fig. 5. Three-dimensional response surface plots of Glucose yield at different operation conditions

Fig. 6. Three-dimensional response surface plots of TRS at different operation conditions

3.3.5. FTIR and XRD analyses

The XRD spectra of the native and pretreated SCB showed in (Fig. 7a). Crystallinity index (CI) of frozen-pretreated SCB increased to 58.5% instead of 43% of native SCB. While, after AHP pretreatment at 3 (v/v) % H2O2, the CI increased to 63.2%. These results are in accordance to previous research works (Reis et al. 2016; De Guilherme et al. 2017), which indicates the enhanced modification of the composition of the pretreated SCB. According to Chirayil et al. (2014), 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. 7b). In particular, AHP destroys the ether bonds between lignin and hemicelluloses polysaccharides and decreases the polymerization degree of lignin in the cell wall (Silverstein et al. 2007). The vibration at 3405 cm-1 and 3385 cm-1, which belong to a functional group-OH (Rojith et al. 2012) refers to 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 located between 2924 and 2919 cm-1 with small shoulder bands that are assigned to -CH2 stretching. The band in the frozen-pretreated SCB sample of 2919 cm-1 shifted to 2899 cm-1 after AHP pretreatment, which implies the improved delignification of SCB (Maryana et al. 2014). Furthermore, the peak at 1732 cm-1 was pronounced in the native and frozen-pretreated SCB samples, but this peak disappeared after AHP pretreatment. This result is attributed to the removal of great percentages of hemicelluloses and lignin during the AHP pretreatment as well as the destroy of bond C=O in hemicellulose (Zhang et al. 2011). 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-pretreated SCB samples and were not present in the AHP pretreated SCB. Lv et al. (2018) indicates the partial removal of lignin during AHP process that is consistent with the composition analysis results. Costa Correia et al. (2013) observed the removal of the bands related to the aromatic ring vibrations in lignin (1500–1550 cm-1) when the cashew bagasse biomass was analyzed before and after the pretreatment with H2O2, which was comparable to the present results. The band at 1640 cm-1 is attributed to the bending mode of the absorbed water (Wójciak et al. 2010). FT-IR spectral area closer to 1424 - 1430, 1371-1376, 1162-1164, 1050-1057 and 897-898 cm-1were identified as cellulose-related bands (Alriols et al. 2010). The band at around 1424 -1430 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-pretreated and AHP-SCB samples. This was assigned to the glycosidic bonds (Trache et al. 2016).

The peak appearing about 1370-1375 cm-1 in native and SCB treated samples was as a result of carbon and hydrogen bond distortion (C-H deformation) of celluloses (Alriols et al. 2010; Corridor et al. 2009). The hydroxyl phenolic groups peaks are between 1375 and 1325 cm-1 (Guo et al. 2009), this peaks not observed in this study. Pan et al. (2008) mentioned that this groups present an inhibitory effect on cellulases. This suggests that AHP pretreatment enriched the SCB with cellulose.

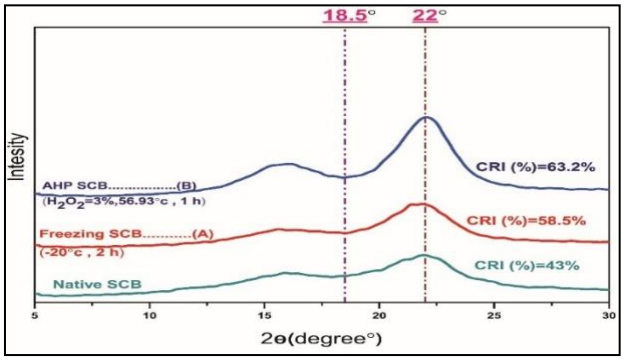


Fig. 7a. XRD spectra of sugarcane bagasse SCB

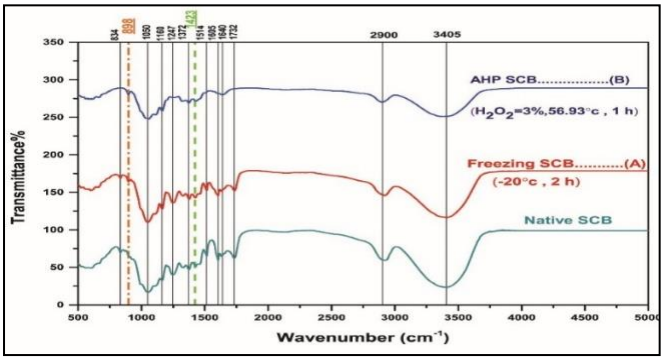


Fig. 7b. FTIR spectra of sugarcane bagasse SCB

3.3.6. SEM analysis

Fig. 8a. shows the undamaged surface of native SCB, which had smooth, contiguous, and clumpy appearance. While Fig. 8b. showed the frozen-pre-treated 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 under the

synergistic effect of freezing followed by AHP pre-treatment in Fig. 8c shows the increased SCB diffusivity.

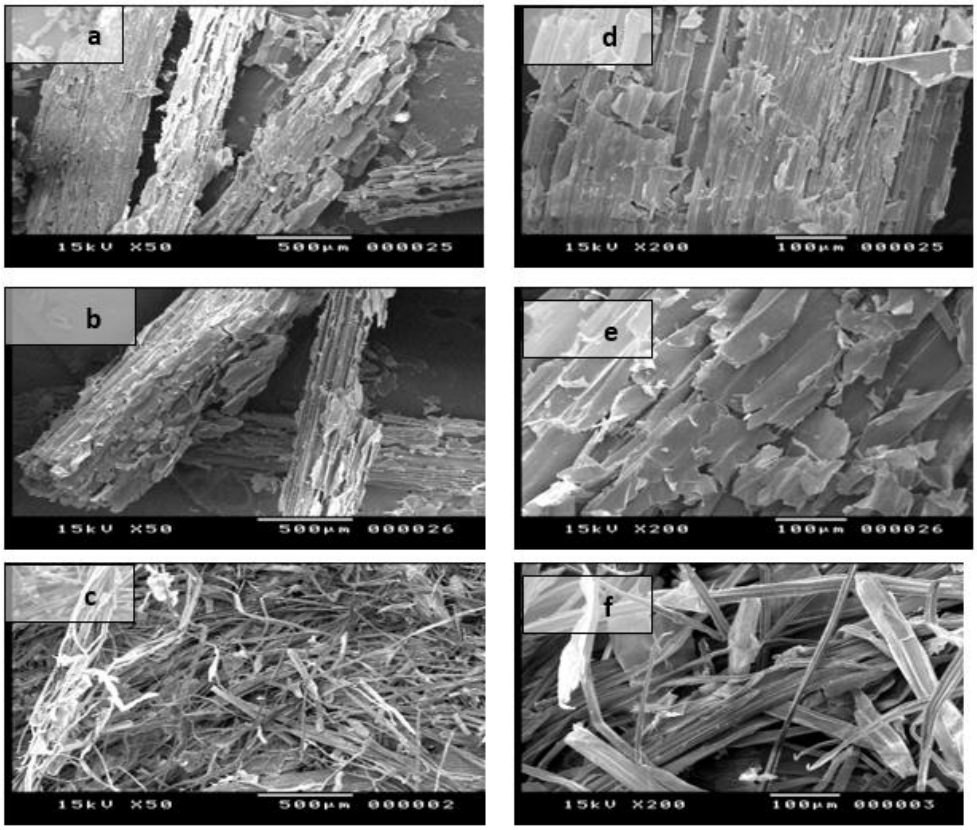


Fig 8. SEM images of SCB before and after freezing and AHP pre-treatments (a-c: X50, d-f: X200 magnification)

4. Conclusion

The effect of physical freezing in addition to alkaline hydrogen peroxide (AHP) pre-treatments on SCB was studied. The content of cellulose increased from 37.19% to 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 (w/v). Consequently, the enzymatic hydrolysis was performed which resulted in glucose yield of (307.52 mg/ gm $_{\text{native SCB}}$) with saccharification ratio of 48.5%. Further chemical pre-treatment using hydrogen peroxide (AHP) was further employed with the help of statistical response surface methodology (RSM), that proved to be a promising pre-treatment 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 obtained 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 $_{\text{native SCB}}$ and 1.44 mg/mL, respectively. These values were relatively close to the experimentally obtained values of (898.5 mg/gm $_{\text{native SCB}}$ and 1.32 mg/ml) with saccharification ratio of 97.5%. The experimental results show that the employed pre-treatment strategy was efficient 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% to 82%.

Declaration

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- **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 your journal.
- **Consent to participate**:
  We also confirm that the statement and order of authors listed in the manuscript has been approved by all named authors.

Authors' contributions:

Dr. Ahmed Farghaly has made substantial contributions to the conception and design of the research work and paper editing. Ms. Kareman Ahmed has conducted the experimental work and the related analysis of results, i.e, figures and tables. Prof. Dr. Ali Abdelrahman was involved in revising the manuscript for important scientific content. All authors reviewed and approved the final version of the manuscript.

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- **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 analyzed 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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Figure legends

- Fig. 1. Experimental scheme of the whole process of SCB freezing pretreatment and subsequent alkaline pretreatment
- Fig. 2. Compositional analysis of native and frozen-pretreated SCB
  N: native at (R) room temperature ≈ 23 ºC
- Fig. 3. Saccharification ratio and glucose yield of native and freezing-fractionated SCB
  N: native at (R) room temperature ≈ 23 ºC
- Fig. 4. Predicted versus actual values plots for: (a) glucose yield and (b) TRS production
- Fig. 5. Three-dimensional response surface plots of Glucose yield at different operation conditions
- Fig. 6. Three-dimensional response surface plots of TRS at different operation conditions
- Fig. 7. XRD and FTIR spectra of sugarcane bagasse SCB
- Fig. 8-a-c. SEM images of SCB before and after freezing and AHP pretreatments
  (X50, 200 magnification)

المعالجة الفيزيائية والكيميائية التآزرية لتفل قصب السكر الليجنوسليلوز عبر عمليات التجميد والقلوية

الملخص بالعربي:

تتناول هذا البحث موضوع تقدير التردد اللحظي (IF) لنظام الطاقة الذي سيتم استخدامه على سبيل المثال في وحدات قياس الطور (PMUs) المستخدمة للمراقبة والتحكم والحماية .الهدف الرئيسي هو عمل تقدير دقيق لتردد نظام الطاقة في الوقت الفعلي وبأقل تأخير.

نقدم خوارزمية جديدة تعتمد على تحويل جابور Gabor Transform لتقدير التردد اللحظي. كما قمنا بمراجعة عدد من خوارزميات تقدير التردد التي تم تناولها في الأبحاث والمقترح تطبيقها في أنظمة الطاقة وهي خوارزمية Zero Crossing وخوارزمية تحويل فوربيه المنفصلة ثلاثية المستويات Three Level DFT وخوارزمية التطور التفاضلي .Differential Evaluation تم عمل المحاكاة لمقارنة الأداء النسبي للخوارزميات الأربعة. تشمل هذه الاختبارات اختبار التردد الثابت؛ تتبع التردد المتغير، والاختبار عندما يتغير كل من التردد والسعة، وأيضًا عندما تحتوي الإشارة على التوافقيات الثالثة والخامسة والسابعة أو الضوضاء البيضاء أو إشارة التيار المستمر.

كشفت اختبارات المحاكاة أنه في ظل العديد من الظروف، قدمت خوارزمية Gabor أدنى مستوى لـ RMSE تقريبًا في جميع الحالات أو على الأقل في المرتبة الثانية. تحتاج خوارزمية Gabor إطارًا من ٤ دورات أو أقل بمعدل أخذ عينات يبلغ ٢٠٠ عينة/ثانية لتقدير التردد اللحظي بدقة. ويمكن من خلال تداخل الإطارات، يمكن حتى استنتاج تقدير دقيق بعد كل دورة. وجدنا أن خطأ مربع متوسط الجذر (RMSE) يكون صفراً بواسطة خوارزمية Gabor في حالة التردد الثابت، في ظل الظروف المذكورة أعلاه لمعدل أخذ العينات وعدد الدورات. ومع ذلك، يمكن أيضًا الوصول إلى هذا الصفر للـ RMSE من خلال ظروف الإشارة الأخرى إذا تم رفع تردد أخذ العينات و / أو عدد الدورات قليلاً.