Optimization of Dilute Acid Pretreatment for Enhanced Release of Fermentable Sugars from Sugarcane Bagasse and Validation by Biophysical Characterization

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Abstract

Pretreatment of biomass is one of the most challenging steps in process of second generation (2G) ethanol and biochemicals production. Dilute acid pretreatment is a widely adapted and convenient method to recover pentose (C5) as well as hexose (C6) sugars due to its featured solubilization of hemicellulose and cellulose before and after enzymatic saccharification, respectively. In the present study, dilute sulphuric acid (H_2SO_4) pretreatment of sugarcane bagasse (SCB) was statistically optimized using factorial central composite design (FCCD) of response surface methodology (RSM) in terms of acid concentration (0.1-3% v/v), solid loading (5–20% w/v) and retention time (15–30 min) at constant temperature of 121 ^oC followed by enzymatic hydrolysis using commercial cellulase (Novozymes Cellic CTec2) for enhanced combined sugar yield (CSY) together with C5 and C6 in pretreated as well as saccharified hydrolysates. Optimized process parameters found in the study were 2.18% (v/v) acid; 14.35% (w/v) solid loading; 29.49 min retention time. CSY under optimized conditions was found to be 521.42 ± 7.2 g/kg raw SCB with 72.06 ± 1.0% sugars recovered out of maximum theoretical sugars present in raw biomass. Total reducing sugars yields in pretreated and saccharified hydrolysates were found to be 215.28 ± 2.4 and 306.14 ± 5.3 g/kg raw SCB, respectively. Morphological and structural changes in optimized pretreated and saccharified biomass further validated the efficiency of optimized pretreatment applied in the present study. The maximum ethanol concentration, volumetric productivity, and yield from released sugars were calculated as 10.82 ± 2.2 g/l, 0.45 ± 0.9 g/l/h and 0.42 g/g-glucose consumed or 71.45 ± 2.5 g/kg raw SCB, respectively. Ethanol yields obtained from fermentation of dilute H_2SO_4 pretreated SCB was corresponding to 82.4% of the maximum theoretical yield (0.51 g/g).

1. Introduction

Continuous depletion of non-renewable oil reserves and related pollution issues have raised the demands for bioethanol as an ideal renewable transportation fuel to mitigate environmental impacts i.e. reduced emissions of greenhouse gases (GHGs) and sustainable fuel supply due to higher oxygen content than gasoline, which speeds up the combustion rate [1]. Second generation (2G) or cellulosic ethanol, which is generated from lignocellulosic biomass (LCB) or agricultural wastes is more advantageous over conventionally produced first generation ethanol (from molasses, grains, beet juice) in terms of resourceful management of agricultural wastes generated on large scale, lower cost and abundant availability of feedstock, combating the risk of food scarcity, etc. Most acceptable LCBs for 2G ethanol production are sugarcane bagasse (SCB), sugarcane straw, rice straw, corn stover, dedicated energy crops among others [2]. However, 2G ethanol or chemicals production from any LCB including SCB requires several technological steps: pretreatment to break down the recalcitrant and protective lignin, and/or hemicellulose to make cellulose more accessible; enzymatic hydrolysis to release monomeric sugars; fermentation; and product recovery. Various advanced physical, thermo-chemical, and combined pretreatment strategies for LCB focusing enhanced yield of hexose (C6) sugars (glucose) are being investigated [3, 4] Given that, recovery of pentose (C5) sugars is also desirable in addition to C6 sugars for the efficient yields of bioethanol and renewable chemicals, novelty and efficiency of the pretreatment method lies in its cost effectiveness, easy recovery of chemicals, recovery of 2G sugars, eco-friendly and low energy demanding facility. Based on these novel requirements, dilute acid pretreatment specifically sulphuric acid (H_2SO_4) is the most common, and promising method from industr8ial point of view for bioethanol production particularly from SCB due to the process simplicity, shorter residence time, low-cost, significant recovery of C5 sugars via solubilization of hemicellulose in pretreatment filtrate due to its easier digestion than cellulose, and C6 sugars (glucose from cellulose) in saccharified hydrolysate after enzymatic hydrolysis [5–8]. Due to decisive interactions between the process parameters, optimum pretreatment conditions may be formulated by an effective design of the experiments for getting enhanced sugar yield and recovery from biomass in short time and an economical way.

For biochemical process optimization, response surface methodology (RSM) is considered as an appropriate design software due to its flexibility in navigating the design space, which employs statistics for investigating the optimum process conditions by experiment designing, determining main and interaction effects of controlled factors on response, predicting the response, measuring correlation between experimental and coded values of response, and checking adequacy of the model [9, 10]. Central Composite Design (CCD) is the most popular design used in RSM for biochemical processes optimization, which is associated with flexible rotation of factors in space, and predicts the response more precisely along with the experimental errors [11, 12].

In the present study, optimization of dilute acid pretreatment for the holistic conversion of SCB polysaccharides into fermentable sugars (C6 as well as C5) using CCD of RSM was investigated with respect to operating variables; acid concentration; solid loading; and retention time. Furthermore, to validate the results obtained, optimized pretreated and residual biomass after enzymatic hydrolysis were characterized by biochemical and biophysical techniques to analyze the structural changes. Fermentation of released sugars was also carried out by using thermotolerant pentose utilizing yeast strain *Kluyveromyces marxianus* NIRE K-3 MTCC 5742 for getting enhanced ethanol yield.

2. Materials And Methods

2.1 Feedstock, enzymes and microorganisms

SCB was collected from Wahid Sanghar Sugar Mill, Phagwara (Kapurthala), Punjab, India. Collected biomass was used in the present study without any size reduction to avoid energy and time consumption. Cellic CTec2 with cellulase activity of 150 filter paper units/ml (FPU) was generously provided by Novozymes, Denmark. Fermenting yeast used was isolated and developed thermotolerant strain *K. marxianus* NIRE-K3 (MTCC 5742) in the Biochemical Conversion Division, SSS-NIBE, Kapurthala, Punjab, India.

2.2 Maintenance of culture and inoculum preparation

K. marxianus NIRE-K3.2 was grown in YPD medium with a composition (g/l); yeast extract- 3.0, peptone-5.0, and dextrose- 10.0 at pH 5.5 and 45 ^oC in cotton plugged conical flask, and stored in the refrigerator at 4 ± 0.5 ^oC for further use. The inoculum was prepared in salt medium with a composition (g/l); yeast extract- 4.81, K_2HPO_4- 1.10, NaH_2PO_4- 1.05, $MgSO_4$- 0.95, $(NH_4)_2SO_4$- 1.99 and glucose-10.0 at pH 5.5 and 45 ^oC [13]. About 100 ml medium was inoculated

with 100 µl of mother inoculum from YPD medium and incubated for 16 h at 120 rpm in an orbital incubator shaker (New Brunswick Scientific, Innova 43R). After 16 h, the culture was centrifuged (Centrifuge 5430 R, Eppendorf) at 10,000 rpm for 5 min at 4 ^{o}C, the supernatant was discarded, and pallet was collected in a micro-centrifuge tube. The dry cell weight of cells was determined by drying at 80 ^{o}C for 4 h in a vacuum oven (Macro Scientific Works Pvt. Ltd., Model No. # MSW – 218).

2.3 Pretreatment optimization of SCB

SCB was was treated with 100 ml of H_2SO_4 of variable concentrations (0.1-3.0% v/v) in 500-ml capped flasks at different solid loadings (5–20% w/v) for variable retention times (15–60 min) at 121 ^{o}C and pressure of 15 psi in an autoclave (Macro Scientific Works Pvt. Ltd., Model No. MSW 101). Three independent process variables X1, X2, X3; acid concentration (%) (v/v), solid loading (%) (w/v), and retention time (min) were optimized using face centered CCD (FCCD) design of RSM, Design Expert software version 8.0 (STAT-EASE Inc., Minneapolis, USA) for dependent variable (response) as maximum combined sugar yield (CSY) (g/kg raw SCB) (Y) from pretreated and saccharified hydrolysates. Table 1 shows the coded values of the process factors to be investigated. The design consisted of 20 runs (2n factors) with 14 non-central points along with 2n axial (α = 1) and 6 repeated central points for reliable estimate of the experimental error (pure error variance) and adequate statistical authenticity of the model. During analysis of the results, insignificant response values were eliminated for smaller and fit set of factors. After pretreatment, all batches were cooled down to room temperature, filtered using muslin cloth and vacuum filtered (Tarsons Rockwac 610), washed with distilled water up to pH 6.5-7.0. Pretreated hydrolysate was further neutralized up to pH 6.5-7.0 using sodium hydroxide (NaOH), centrifuged and aanalyzed for sugars, furfural, hydroxyfurfural, acetic acid, formic acid, levullinic acid, etc., whereas a part of pretreated biomass was dried in a hot air oven (Macro Scientific Works Pvt. Ltd., Model No. # OHTT50 SPL) at 105 ^{o}C for 4 h, utilized for mass balance compositional and biophysical analysis, whereas another fraction of wet biomass sample was used for enzymatic saccharification.

Table 1
Coded values for variables of FCCD for pretreatment optimization

Variables	Unit	-1	0	+1
Acid concentration	%	0.1	1.55	3.0
Solid loading	%	5	12.5	20
Retention Time	min	15	37.5	60

2.3 Enzymatic hydrolysis

Pretreated biomass samples after designed experiments were placed in 50 ml of sodium citrate buffer with pH 4.8 in 250-ml capped flasks at 10% (w/v) solid loading. Saccharification was performed using Cellic CTec2 with a loading of 20 FPU/g pretreated biomass at 50 ^{o}C with shaking at 130 rpm in an orbital incubator shaker for 72 h. Samples were withdrawn after each 24 h for measuring total reducing sugars concentration in the enzymatic hydrolysate. A part of residual biomass was dried and utilized for mass balance and structural changes.

2.4 Separate hydrolysis and fermentation (SHF)

SCB samples pretreated with dilute H_2SO_4 under optimized conditions were utilized for ethanol production via SHF in triplicate. In brief, the pretreated biomass was dissolved in 10 ml of 50 mM sodium citrate buffer (pH 5.5) in 100-ml capped flasks, and hydrolyzed using 20 FPU/g-PB (pretreated biomass) of Cellic CTec2 at 10% (w/v) solid loading in an orbital incubator shaker at 50 ^{o}C and 130 rpm for 72 h. After hydrolysis, the hydrolysate was filtered through muslin cloth followed by vacuum filtration, and supplemented with fermentation medium with a composition (g/l); yeast extract- 2.93, K_2HPO_4- 1.99, NaH_2PO_4- 0.24, $MgSO_4$- 0.42, $(NH_4)_2SO_4$- 1.34 and pH 5.5 [13], and subjected to the fermentation by inoculating with 2 g/l cells of *K. marxianus* NIRE-K3 under anaerobic conditions in an orbital incubator shaker at 45 ^{o}C and 130 rpm for 24 h. The samples were withdrawn after every 2 h, centrifuged at 6,000 rpm at 4°C for 10 min, filtered through 0.2 µm syringe filters, supernatants were stored at 4°C, and analyzed for sugars and ethanol. A fraction of the residual biomass was dried at 105 ^{o}C in a hot air for 4 h, and utilized for mass balance after the end of the process.

2.5 Analytical techniques

Raw, pretreated and saccharified SCB samples were analyzed for total solids (TS) and ash contents using NREL procedures [14, 15]; cellulose, hemicellulose and lignin by acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) tests according to Van Soest method with the help of Fiber-Tech Foss 2022 [16, 17]. Pretreatment and saccharification products (sugars and other metabolites) were analyzed by HPLC (Agilent Technologies 1260 Infinity) using HiPlex H column at 57 ^{o}C with 1 mM H_2SO_4 as the mobile carrier at a flow rate of 0.7 ml/min and Refractive Index Detector (RID) at 50 ^{o}C. Biochemical changes in SCB were analyzed using Fourier transform infrared (FTIR) spectrum analysis (Cary 660 Agilent Tech). The sample was scanned in the MIR (Middle infrared) range of 4000 – 400 cm^{-1} at a spectral resolution of 4 cm^{-1}. Further, morphological changes in biomass were studied using biophysical techniques; confocal laser scanning microscopy (CLSM) using a Zeiss LSM 780 confocal microscope (Zeiss, Jena, Germany), equipped with a 405 nm laser diode and a Coherent Chameleon laser (Ti: sapphire) as the excitation source and apochromatic objective lenses; 13C Solid State Nuclear Magnetic Resonance (^{13}C ssNMR) were performed in a Varian 400 Inova spectrometer operating at frequencies of 100.5 MHz and 400.0 for ^{13}C and ^{1}H respectively. The spectra were acquired using the Total suppression of Spinning Sidebands techniques using MAS rotation frequency of 5 kHz. Typical π/2 pulse lengths were 3.5 and 4.0 µs for ^{13}C and ^{1}H, respectively; ^{1}H Time-Domain NMR experiments were performed in a Bruker Minispec (Bruker, Germany) with a frequency of 20 MHz for ^{1}H, by using a CPMG echo train consisting of 16392 echoes separated by 70 µs. The signal was processed by an Inverse Laplace Transform procedure [18]; Field emission scanning electron microscopy (FESEM) using Scanning Electron Microscope equipped with field emission gun (FESEM-FEI Quanta 650); X-ray diffraction (XRD) spectral studies with the help of Rigaku Rotaflex diffractometer (RU200B) equipped with monochromatic

CuKα radiation (1.54 Å) at 45 kV and 36 mA and equipped with a graphite monochromer according to the procedures followed up in previous studies [19, 20]. All of the biophysical studies were carried out at São Carlos Institute of Physics, University of São Paulo (São Carlos, SP, Brazil).

3. Results And Discussion

RSM is widely used in biorefinery studies to achieve high sugars and ethanol production as well as to make process cost effective, more stable and reliable [21, 22]. In this study, RSM was used to explore the most significant factors involved in H_2SO_4 pretreatment of SCB, possible interactions between the factors to influence the overall process efficiency, and to predict the possible optimal solutions based on the correlation between observed and predicted values. The numerical optimization method was used to investigate the optimal operational parameters by analyzing the standard error (Std. Err.) and from quadratic regressions in the model with desirability function value as 0.999 for accuracy of response.

3.1 Evaluation of optimized parameters

FCCD results obtained with observed and coded values of dependent variable i.e. CSY for independent variables are shown in Table 2. Out of 20 designed experiments, highest CSY was found in range of 509.21-528.34 g/kg raw SCB against predicted value of 517.48 g/kg raw SCB from biomass samples pretreated with 6 central points; 1.55% (v/v) H_2SO_4 and 12.5% (w/v) solid loading for 37.5 min. CSY was determined as the sum of reducing sugars; glucose, xylose, galactose, arabinose, mannose and cellobiose found in the pretreated and saccharified hydrolysate of batches. Second order quadratic model equations of response as a function of independent variables with respect to the coded and actual factors were analyzed with multiple regression analysis for a good fit (Equations 1 and 2):

Table 2
FCCD for pretreatment optimization with dilute H_2SO_4

Run	X1: Acid concentration (%, (v/v)	X2: Solid loading (%, w/v)	X3: Retention time (min)	Y: CSY (g/kg raw SCB)	
				Experimental value	Predicted value
1	0.1	5	15	82.43	90.43
2	3	5	15	232.36	222.99
3	0.1	20	15	12.12	5.88
4	3	20	15	162.33	160.1
5	0.1	5	60	192.77	193.13
6	3	5	60	248.44	252.81
7	0.1	20	60	95.75	103.24
8	3	20	60	194.46	184.58
9	0.1	12.5	37.5	340.25	330.63
10	3	12.5	37.5	420.48	437.58
11	1.55	5	37.5	410.25	406.89
12	1.55	20	37.5	319.65	330.5
13	1.55	12.5	15	392.18	402.01
14	1.55	12.5	60	467.65	465.6
15	1.55	12.5	37.5	528.34	517.48
16	1.55	12.5	37.5	523.57	517.48
17	1.55	12.5	37.5	518.76	517.48
18	1.55	12.5	37.5	509.21	517.48
19	1.55	12.5	37.5	518.67	517.48
20	1.55	12.5	37.5	521.28	517.48
*X1, X2, X3-Process factors; Y-Response; CSY-Combined Sugar Yield					

Second order quadratic model equations of response

Second order quadratic model equations in terms of coded and actual factors:

$$Y = 53.48 \times A - 38.19 \times B + 31.79 \times C + 5.41 \times A \times B - 18.22 \times A \times C - 1.33 \times B \times C - 133.37 \times A^2 - 148.78 \times B^2 - 83.67 \times C^2 (1)$$

Where, Y is CSY, and A, B and C are acid concentration, solid loading and retention time, respectively.

$$CSY = 248.2431 \times H_2SO_4 + 60.5584 \times Solids + 14.77369 \times Time + 0.4979 \times H_2SO_4 \times Solids - 0.5584 \times H_2SO_4 \times Time - 0.0078 \times Solids \times$$

Fisher's "*F*"-test was further applied on data obtained using ANOVA for adequacy of the quadratic model (Table 3). Model was found to be significant due to high *F*-value as 500.01 and lower probability values (*P*-values) as $P < 0.05$ (< 0.0001) with linear and squared effect of all the variables [12]. The regression model was found to be fit and of reasonably high statistical significance with coefficient of determination i.e. $R^2 = 0.9978$, which means that there is 99.78% variation in the response value. R^2 evaluates the variance in values of the response because of variation in the interaction of process parameters. R^2 equal or close to 1 implies the stronger model, and predicts the response value with better precision however, a model could be accepted with $R^2 > 0.75$ [13]. Further, high value of the adjusted coefficient of determination, adj. $R^2 = 0.9958$ again indicates fairly high significance of model by displaying correlation between the experimental and coded values. Ávila-Lara et al. [11] reported a good and average correlation between the experimental and coded values of the model by reporting R^2 as 0.9151 and 0.7270 for alkali and dilute acid pretreatment, respectively using agave bagasse as feedstock. A non-significant lack of fit relative to the pure error with *F*-value as 5.02 further precisely indicated the validity of model. Furthermore, low value of coefficient of variation i.e. *CV* = 3.31 indicated small difference between observed and predicted CSY. Data fitting in the model was further estimated by plotting observed and coded values of response (Fig. 1). Observed values were found to be in close agreement with the coded values in the plot, and concentrated around the slope line.

Table 3
ANOVA for FCCD results

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	551500	9	61282.61	500.01	< 0.0001	Significant
A-Acid concentration	28595.76	1	28595.76	233.31	< 0.0001	
B-Solid loading	14587.82	1	14587.82	119.02	< 0.0001	
C-Retention time	10108.58	1	10108.58	82.48	< 0.0001	
AB	234.58	1	234.58	1.91	0.1966	
AC	2655.75	1	2655.75	21.67	0.0009	
BC	14.2	1	14.2	0.1159	0.7406	
A^2	48915.78	1	48915.78	399.11	< 0.0001	
B^2	60876.68	1	60876.68	496.7	< 0.0001	
C^2	19254.14	1	19254.14	157.1	< 0.0001	
Residual	1225.63	10	122.56			
Lack of Fit	1021.97	5	204.39	5.02	0.0506	not significant
Pure Error	203.66	5	40.73			
Cor Total	5.53E + 05	19				

Std. Dev.-11.07; Mean-334.56; (C.V. %)-3.31; R-Squared-0.9978; Adj R-Squared-0.9958; Pred R-0.9796; [a]df- Degrees of freedom; F-Fisher's variance ratio; P-probability value; P < 0.05- significant at 5% level

On the basis of observed data with maximum value of response, software selected the combination of factors; acid concentration (% v/v)-2.18, solid loading (%)-14.35 and retention time (min)-29.49 with constant temperature kept at 121 ^oC. Model validity experiment was conducted in triplicate under selected conditions. Average observed value of CSY was found to be 521.42 ± 7.2 g/kg raw SCB, whereas predicted value was 517.48 g/kg raw SCB. The reducing sugars (glucose, xylose and total) recovered from biomass pretreated under all conditions are shown in Fig. 2. Although individual xylose and glucose recoveries were found to be 44.89 ± 1.3 and 46.95 ± 0.8%, respectively but total reducing sugar recovery obtained under optimized conditions was found to be 72.06 ± 1.0% of maximum theoretical total sugars present in SCB due to consideration of all monomeric reducing sugars during calculations. Obtained results in this study were almost equal to as reported in previous studies. Soares et al. [8] optimized H_2SO_4 pretreatment of SCB using rotational CCD of RSM, and reported optimized acid concentration, 1.5%; solid loading, 20%; time, 15 min at 170 ^oC with maximum sugar yield of 350 g/kg of pretreated biomass after 72 h of enzymatic hydrolysis with cellulase loading of 0.232 g (Cellucast 1.5 L: 65 FPU/ml + 17 IU/ml of β-glucosidase) and 0.052 g IU/g β-glucosidase (Novozymes 188:376 IU/g) and 5% solid loading at 50 ^oC. In another study, optimized conditions for H_2SO_4 pretreatment for agave bagasse using factorial CCD of RSM were reported as 2.1% (v/v) concentration, 8.5% solid loading and 33.8 min time with sugar yield of ~ 457 g/kg raw SCB after 72 h of saccharification using CTec2 and HTec2 loading at 35 FPU/g biomass and 60 CBU/g biomass, respectively at 55 ^oC with 3% solid loading [11]. Benjamin et al. [23] reported maximum sugar yield of 504 g/kg raw SCB, when the SCB was pretreated with 0.65% (w/w) H_2SO_4 at 180 ^oC for 10 min (optimized using CCD of RSM) at 5% solid loading with 74.5% sugar recovery after 72 h of saccharification with cellulase (Spezyme CP) loading of 15 FPU/g pretreated biomass supplemented with 15 IU/g pretreated biomass of β-glucosidase (Novozym 188) with 2% solid loading at 50°C. In another study, Sritrakul et al. [24] evaluated dilute acid pretreatment of SCB pith with 2% (v/v) H_2SO_4 at 10% solid loading and 121 ^oC for 90 min followed by enzymatic hydrolysis with cellulase loading 15 FPU/g dry biomass (Cellucast 1.5), 5% solid loading at 50 ^oC for 24 h, and reported 537 g/kg of dry biomass with 67% sugar recovery from theoretical sugars.

The present study indicates the optimized dilute acid pretreatment as a promising approach for both C5 and C6 recovery from SCB. This was not only cost effective, but also practically adaptable for industrial production. Effect of reaction parameters on the production of reducing sugars during enzymatic hydrolysis is presented in supplementary material.

3.2 Effect of reaction parameters on the production of reducing sugars during enzymatic hydrolysis

In a biochemical process, parameters not only affect progress of the process individually in fact, they also exert effects on each other. The main and interactive effects of acid concentration, solid loading, and retention time on CSY were studied by plotting contour curves, three dimensional (3-D) surface and interaction curves between any two variables keeping another variable at its central level (Fig. 3). 3-D response surface plots are crucial way for interpreting interaction and correlation of two different factors, and help to understand interaction effects of independent variables on target responses [13, 25]. The results from this study showed good agreement with FCCD results given in Table 2. All factors studied were found to be critical to the CSY from SCB. Convex 3-D surface suggested valid optimal solutions for the maximum value of response by appropriate fit of variables in space from observed trend of response [9]. SCB was pretreated with acid concentrations of 0.1, 1.55, and 3% (v/v) along with variable solid loading and retention time at constant temperature of 121 ^{o}C. Acid concentration significantly affected the amount of reducing sugars in pretreated as well as saccharified hydrolysate along with solid loading and retention time (Fig. 3a, c, d and f). Biomass solubilization increases with increasing acid concentration and time during pretreatment [26].

The maximum reducing sugars were produced in a range of 319.65 to 528.34 g/kg raw SCB at coded value of 1.55% (v/v) acid in combination of different values of solid loading and retention time however, further decrease or increase in acid concentration led to significant reduction in sugar production. This trend has also been validated in Table 2, where sugar yield was observed to be decreased drastically up to 12.12 g/kg raw SCB against predicted value of 5.88 g/kg raw SCB at lowest concentration of 0.1% (v/v) of acid with 20% (w/v) solid loading for 15 min, whereas sugars production decreased up to 162.33 g/kg raw SCB against predicted value of 160.1 g/kg raw SCB at highest concentration of 3% (v/v) of acid with 20% (w/v) solid loading for 15 min. Further, on the basis of observed trend and model validity experiment, optimal acid concentration was confirmed out to be 2.18% (v/v). It was also reported previously that about 2% (v/v) H_2SO_4 at 121 ^{o}C is the best condition for SCB pretreatment, while process efficiency ceases with increasing acid concentrations above 2% due to continuous rise of inhibitors amount with process inhibition [27, 28]. Similarly in the present study, inhibitors concentration was found to be high in runs with 3% (v/v) acid concentration. Pretreatment retention time was another significant factor determining sugars yield in the pretreated as well as saccharified hydrolysates. The combined effect of retention time-acid concentration and solid loading-retention time on CSY is shown in Fig. 3b, c, e and f. The maximum sugar yields were obtained in runs pretreated for 37.5 min, whereas further decrease or increase in time led to a reduced fermentable sugars production. This trend was again validated in Table 2, where time below 37.5 min is associated with decreased production of sugars up to 12.12 and 95.75 g/kg raw SCB in 15 min and 60 min, respectively. Similar trend was reported in a study of 1% (v/v) H_2SO_4 acid pretreatment of wheat straw at 140 ^{o}C and 10% solid loading in 30 min with maximum sugar recovery of 89% after 48 h of enzymatic hydrolysis [29]. Further, in model validity experiment, optimal retention time was confirmed to be 29.49 min. Generally, higher solid loading is recommended from industrial point of view due to lower capital costs and high feedstock loadings [30, 31]. Moreover, pretreatment with high solid loading enables to recover higher C5 sugars in the pretreated hydrolysate, which was one of the major objectives of this study. High solid loadings generally result into enhanced sugars production up to a certain level, and after that sugars production start declining due to difficulties in mass transfer [26, 32].

The combined effect of solid loading-retention time and solid loading-acid concentration on release of reducing sugars is shown in Fig. 3a, b, d and e. Maximum reducing sugars were produced in runs with 12.5% (w/v) solid loading and sugar yields in the range of 340.25-528.34 g/kg raw SCB, and further decrease or increase in solid loading lead to decreased sugars production. For instance, solid loading below 12.5% (w/v) is associated with decreasing production of sugars up to 82.43 g/kg raw SCB at 5% (w/v) solid loading with 0.1% (v/v) acid for 15 min. On the other hand, above 12.5% (w/v), sugar yield was also found to decrease up to 12.12 g/kg raw SCB at 20% (w/v) solid loading with 0.1% (v/v) acid for 15 min. Based on observations, optimized solid loading was found to be 14.35% (w/v). Optimized solid loading in the present study was higher than reported optimization of solid loading as 4% (w/v) for pretreatment of wheat straw with 0.75% (v/v) H_2SO_4 at 121 ^{o}C for 1 h using FFD (full factorial design) of RSM [33]. In contour plots, shape of the contour provide the information about level of interaction between variables, for instance elliptical contours indicate strong interactions, whereas the circular shape displays the weaker interactions [4, 12]. The surface inside the smallest ellipse with red color demonstrates the maximum response value. From contour plots in the present study, it was observed that there are stronger interactions between solid loading and acid concentration as estimated from elliptical shape of contour (Fig. 3g). However, there are weaker interactions between acid concentration and retention time, and even the weakest interactions between retention time and solid loading due to circular shape of contours in Fig. 3h and i i.e. retention time-acid concentration and retention time-solid loading do not interact significantly to affect the sugar yield in pretreated and saccharified hydrolysates. Thus, it could be concluded that solid loading and acid concentration have significantly more effect on release of reducing sugars from solubilization of hemicellulose and enzymatic hydrolysis as compared to retention time in two-way interaction.

3.3 Sugars and other compounds in pretreated hydrolysates

Dilute H_2SO_4 at high temperature for some residence time acts specifically on hemicellulose in turn solubilizing into sugar monomers in addition to the oligosaccharides. Being a heteropolysaccharide of various sugar units (xylan, galactan, mannan, arabinan, etc.), hemicellulose is depolymerized primarily into C5 sugars (xylose and arabinose) along with some amount of C6 sugars (glucose, galactose, mannose), and some of the inhibitory metabolites [34]. Recently, Silveira et al. [26] studied steam explosion of sugarcane bagasse in the presence of H_2SO_4 catalyst and observed that steam explosion alone of bagasse showed lower xylose (15 g/l) amount and higher xylo- and cello-oligosaccharides (60 g/l) whereas, H_2SO_4 catalysed-steam explosion of bagasse showed higher xylose release of 40 g/l with lower xylo- and cello-oligosaccharides (14 g/l). In the present study, the pretreated hydrolysate was also found to contain a good amount of sugars such as glucose, galactose, mannose, arabinose and cellobiose with xylose as the major monomeric sugar due to the fact that it is the major C5 sugar unit of hemicellulose (Table 4). The maximum xylose concentration was found in hydrolysate of batch with 1.55% acid (v/v), 12.5% (w/v)

solid loading and 37.5 min retention time in a range of 23.12 to 25.96 g/l, which further increased to 25.57 ± 0.6 g/l with yield of 0.10 ± 2.9 g/g raw SCB and 36.42% recovery under optimized conditions. The lowest concentration of xylose (0.15 g/l) and some other sugars were observed in two runs (1 & 3) with 0.1% (v/v) acid for 15 min with 5 and 20% (w/v) solid loading. Xylose concentration was found to increase with increasing acid concentration and time up to certain level where it started decreasing due to degradation of xylose under severe conditions. The xylose yield and recovery in the present study was lower than reported by Sritrakul et al. [24], where they achieved 0.23 g/g raw biomass xylose yield with 56.28% recovery in hydrolysate of SCB pith pretreated with 2% (v/v) acid at 121 ^{o}C for 90 min. Benjamin et al. [23] reported 0.16 g/g raw biomass xylose yield with 76% recovery in hydrolysate of industrial SCB pretreated with 0.99% (w/w) acid at 180 ^{o}C for 10 min. Further, maximum glucose concentration in a range of 4–6 g/l was found in various runs (4, 8, 10, 12, 14–20). However, similar glucose concentration and yield were found under optimized conditions as 4.38 ± 0.3 g/l and 0.02 ± 0.7 g/g raw SCB, respectively, which is very low as compared to xylose thus, leaving cellulose fraction mostly unaltered in biomass. It could be possible that this glucose was released as a result of hemicellulose hydrolysis. However, presence of cellobiose with a concentration of 1.43 ± 0.4 g/l and yield of 0.01 ± 1.6 g/g raw SCB indicates that some of cellulose alteration occurred as well during the pretreatment. Low glucose release in pretreated hydrolysate indicates a beneficial impact of pretreatment keeping high cellulose content in pretreated biomass to be hydrolyzed later during saccharification.

Table 4
Reducing sugars released in pretreated hydrolysate samples of H_2SO_4 pretreated SCB

Run	Chemical concentration (%, v/v)	Solid loading (%, w/v)	Retention time (min)	Glucose (g/l)	Xylose (g/l)	Galactose (g/l)	Arabinose (g/l)	Mannose (g/l)	Cellobiose (g/l)	Total reducing sugars (g/l)	Total reducing sugars (g/kg raw SCB)
1	0.1	5	15	0.44	0.15	0.001	0.15	0.59	1.45	2.78	44.56
2	3	5	15	2.81	10.2	0.1	0.05	0.13	1.55	14.84	207.82
3	0.1	20	15	0.013	0.15	0.02	0.05	1.78	0.86	2.87	0.28
4	3	20	15	6.45	19.55	8.95	5.95	6.98	10.85	58.73	23.49
5	0.1	5	60	1.76	2.97	0.45	0.45	0.19	3.98	9.80	156.87
6	3	5	60	2.072	10.4	0.01	0.01	0.03	0.16	12.67	190.06
7	0.1	20	60	1.53	3.13	0.012	0.01	0.01	6.02	10.69	1.60
8	3	20	60	5.95	20.46	0.46	0.09	0.48	20.82	48.27	12.07
9	0.1	12.5	37.5	2.48	2.70	5.02	3.02	2.51	11.49	27.22	163.34
10	3	12.5	37.5	5.742	20.73	0.06	0.22	1.45	35.85	64.07	230.64
11	1.55	5	37.5	2.935	10.66	0.01	0.42	0.03	7.12	21.16	338.57
12	1.55	20	37.5	6.03	15.97	8.86	8.65	8.65	12.62	60.77	115.47
13	1.55	12.5	15	2.69	8.042	6.79	8.13	8.21	6.03	39.91	191.55
14	1.55	12.5	60	5.54	23.42	2.65	2.45	2.78	19.89	56.73	272.31
15	1.55	12.5	37.5	4.692	25.96	6.48	7.08	10.28	1.462	55.97	223.91
16	1.55	12.5	37.5	4.664	24.95	5.58	7.89	10.28	1.078	54.47	217.88
17	1.55	12.5	37.5	5.55	24.08	6.49	7.34	10.76	1.148	55.39	217.14
18	1.55	12.5	37.5	4.45	23.12	6.12	7.74	10.28	1.068	52.78	206.92
19	1.55	12.5	37.5	6.128	24.99	4.22	7.15	9.23	1.957	53.67	214.71
20	1.55	12.5	37.5	6.403	25.15	5.04	7.08	9.01	1.008	53.69	219.06
Optimized	2.18	14.35	29.49	4.38 ± 0.3	25.57 ± 0.6	6.44 ± 0.02	7.90 ± 0.4	9.64 ± 0.07	1.43 ± 0.4	55.36 ± 0.4	215.28 ± 2.4

Maximum cellobiose concentration of 35.85 g/l with yield of 0.13 g/g raw SCB was found in run 10 (3% v/v acid, 12.5% solid loading, 37.5 min time). From Table 4, it was observed that high acid concentration (1.55, 3% v/v) was responsible for cellulose hydrolysis either into glucose or cellobiose. Arabinose exists in the form of furanose as arabinoxylan in hemicellulose, and it is hydrolyzed faster than pyranose sugars (xylose, glucose) during acid pretreatment [35]. The maximum arabinose concentration 8.65 and 8.13 g/l with yield 0.02 and 0.04 g/g raw SCB in the present study was found at runs 12 and 13, respectively, with acid concentration of 1.55% (v/v). Almost similar arabinose concentration of 7.9 ± 0.4 g/l with yield of 0.03 ± 1.5 g/g raw SCB was observed under optimized conditions. Hydrolysate also contained galactose and mannose with concentrations of 6.44 ± 0.02 and 9.64 ± 0.07 g/l, and yields 0.02 ± 0.5 and 0.04 ± 0.9 g/g raw SCB, respectively under optimized conditions. Total reducing sugar concentration in pretreated hydrolysate under optimized conditions was found to be 55.36 ± 0.4 g/l with a yield of 215.28 ± 2.4 g/kg raw SCB with 29.75 ± 0.3% sugar recovery. Although acid pretreatment is an efficient method of C5 sugar recovery from hemicellulose in pretreated hydrolysate, however, it is also associated with production of toxic inhibitory metabolites due to breakdown of hemicellulose sugar subunits, which not only results into lower sugar yield, but also potentially inhibits the growth of fermenting strain with reduced ethanol

production [36, 37]. Thus, keeping the significance of these toxic metabolites in mind, a profile was prepared for pretreated hydrolysate (Table 5). Acetic acid is well known inhibitory metabolite produced in ample amount during acid pretreatment by hydrolysis of the acetyl group in hemicellulose fraction, whereas furfural and 5-hydroxymethyl furfural (HMF) are generated from degradation of glucose and xylose, respectively. Further breakdown of these metabolites leads to formation of formic and levulinic acids whereas, partial breakdown of lignin produces some phenolic compounds [38].

Table 5
Inhibitors profile of H_2SO_4 pretreated hydrolysates of SCB batches

Run	Chemical concentration (% v/v)	Solid loading (% w/v)	Retention time (min)	Acetic acid (g/l)	Formic acid (g/l)	Furfural (ppm)	HMF (ppm)
1	0.1	5	15	0.15	0.011	38	9
2	3	5	15	2.15	0.83	189	25
3	0.1	20	15	0.05	0.035	25	8
4	3	20	15	2.95	1.15	150	50
5	0.1	5	60	2.25	1.01	98	12
6	3	5	60	5.71	2.15	960	75
7	0.1	20	60	0.98	0.45	120	30
8	3	20	60	8.91	1.12	720	82
9	0.1	12.5	37.5	2.85	0.12	280	6
10	3	12.5	37.5	7.63	1.23	1240	70
11	1.55	5	37.5	3.71	0.95	678	5
12	1.55	20	37.5	2.64	0.75	298	2
13	1.55	12.5	15	4.98	0.09	147	1.5
14	1.55	12.5	60	4.99	0.1	733	50
15	1.55	12.5	37.5	1.58	0.08	515	46
16	1.55	12.5	37.5	1.5	0.08	520	41
17	1.55	12.5	37.5	1.57	0.08	514	4.3
18	1.55	12.5	37.5	1.99	0.08	519	3.9
19	1.55	12.5	37.5	1.56	0.08	520	4.9
20	1.55	12.5	37.5	1.57	0.08	515	4.4
Optimized	2.18	14.35	29.49	4.35 ±0.03	0.07 ± 0.02	510.33 ± 1.5	4.33 ± 0.2

*HMF: 5-hydroxymethyl furfural

Higher concentration of acetic acid was found as 8.91 g/l with yield of 0.002 g/g raw SCB, respectively with 3% (v/v) acid, 20% solid loading and 60 min, while maximum yield of 0.08 g/g raw SCB with concentration of 5.71 g/l was found in hydrolysate of SCB pretreated with 3% (v/v) acid, 5% solid loading for 60 min. On the other hand, maximum concentration and yield of formic acid was found in the same run 6 (3% v/v acid, 5% w/v solid loading, 60 min) as 2.15 g/l and 0.03 g/g raw SCB, respectively. Furfural was observed with maximum concentration of 1240 ppm with yield 0.004 g/g raw SCB, respectively at 3% (v/v) acid, 12.5% solid loading for 37.5 min whereas maximum yield of 0.014 g/g raw SCB with concentration of 960 ppm was found in hydrolysate of SCB pretreated with 3% acid, 5% solid loading for 60 min. HMF with maximum concentration of 82 ppm with yield of 0.00002 g/g raw SCB was found at run with 3% (v/v) acid and 20% solids for 60 min, while along with the trend of other inhibitors, highest yield of HMF was found to be 0.001 g/g raw SCB in same run 6 (3% v/v acid, 60 min). These results indicate that increasing the higher acid concentration and retention time (Runs 6, 8, 10) leads to greater amounts of inhibitors being formed, which could be due to high degree of solubilization of hemicellulose. However, highest yield of all the inhibitors were found in same run (6) due to higher volume of filtrate generated with lesser solid loading of 5% (w/v) (data not shown). Further, average concentration and yields of acetic acid, formic acid, furfural and HMF were found as 4.35 ± 0.03 g/l, 0.07 ± 0.02 g/l, 510.33 ± 1.5 ppm, 4.33 ± 0.2 ppm, 0.02 ± 0.4, 0.0003 ± 0.09, 0.002 ± 0.04 and 0.02 ± 0.9 g/g raw SCB, respectively, in hydrolysate of SCB pretreated under optimized conditions. Low HMF production in hydrolysate (2–82 ppm) again indicates limited degradation of glucose in a liquid fraction. Rajan and Carrier [29] reported production of acetic acid, formic acid, furfural, and HMF as 0.01, 0.03, 0.01 and 0.001 g/g raw biomass, respectively in pretreated filtrate of wheat straw pretreated with 1% (v/v) H_2SO_4 at 140 ^oC and 10% (w/v) solid loading. Due to presence of inhibitors, pretreated hydrolysate needs to be detoxified prior to fermentation [39].

3.4 Sugars in saccharified hydrolysate

The hemicellulose devoid biomass (cellulignin) was washed with tap water to neutralize, and the pretreated biomass was subjected to enzymatic hydrolysis. It was observed that glucose was produced as a major sugar in saccharified hydrolysate along with xylose and cellobiose in smaller concentrations (Table 6). The higher glucose yield than that of xylose in saccharified hydrolysate after acid pretreatment could be due to the removal of hemicellulose in pretreated

hydrolysate, increased porosity and surface area of the cellulose, which provides greater accessibility to the cellulases. Maximum glucose concentrations (25.98–27.75 g/l) with a similar yield of 0.19 g/g raw SCB were found in hydrolysate of SCB pretreated with 1.55% acid and 12.5% (w/v) solid loading for 37.5 min, which increased to 28.25 ± 0.5 g/l concentration and 0.19 ± 4.3 g/g raw SCB yield under optimized conditions. Further, as the retention time and acid concentration was increased, glucose concentration in the enzymatic hydrolysate decreased. This could be attributed to chemical or biophysical changes in the cellulose or lignin with increased chemical concentration or time, which either decreased the enzymatic affinity or surface area for enzymatic hydrolysis [40]. Further, maximum xylose concentrations of about 3 g/l were found in runs 12 and 15–20 with similar acid concentration of 1.55% (v/v) and retention time of 37.5 min, which remained to be almost similar as 3.39 ± 0.2 g/l with yield of 0.02 ± 1.1 g/g raw SCB under optimized conditions.

Table 6: Reducing sugars released in saccharified hydrolysate of H_2SO_4 pretreated SCB using Cellic CTec2 with 15 FPU/g pretreated biomass and solid loading of 10% at 50 ^oC

Run	Pretreatment conditions			Cellobiose (g/l)	Glucose (g/l)	Xylose (g/l)	Total reducing sugars (g/l)	Total reducing sugars (g/kg-raw SCB)
	Chemical concentration (%, v/v)	Solid loading (%, w/v)	Retention time (min)					
1	0.1	5	15	1.26	4.02	2.06	7.34	37.87
2	3	5	15	0.26	10.45	1.09	11.8	24.54
3	0.1	20	15	0.02	1.164	0.02	1.204	11.83
4	3	20	15	4.15	9.55	1.35	15.05	138.84
5	0.1	5	60	1.15	9.45	1.78	12.38	35.90
6	3	5	60	0.05	12.37	1.22	13.64	58.37
7	0.1	20	60	1.75	7.42	1.59	10.76	94.15
8	3	20	60	0.9	15.8	2.25	18.95	182.39
9	0.1	12.5	37.5	5.98	15.95	2.75	24.68	176.91
10	3	12.5	37.5	2.25	20.85	2.28	25.38	189.84
11	1.55	5	37.5	2.85	20.65	2.47	25.97	71.67
12	1.55	20	37.5	8.25	8.94	3.73	20.92	204.18
13	1.55	12.5	15	6.25	15.55	2.2	24.00	200.64
14	1.55	12.5	60	3.45	19.72	1.2	24.37	195.35
15	1.55	12.5	37.5	11.85	27.75	3.4	43.00	304.44
16	1.55	12.5	37.5	12.85	27.05	3.77	43.67	305.69
17	1.55	12.5	37.5	10.58	27.15	3.03	40.76	301.62
18	1.55	12.5	37.5	11.52	26.78	2.07	40.37	302.29
19	1.55	12.5	37.5	11.75	26.52	3.12	41.39	303.97
20	1.55	12.5	37.5	11.65	25.98	3.21	40.84	302.22
optimized	2.18	14.35	29.49	12.96±0.7	28.25±0.5	3.39±0.2	44.6±1.2	306.14±5.3

Lower xylose production in saccharified hydrolysate was due to the fact that large amount of hemicellulose and hence xylose was already removed during the pretreatment process. As the retention time (60 min) and acid concentration (3% v/v) were increased, the xylose concentration in the enzymatic hydrolysis decreased. Cellobiose produced with maximum concentration and yield of 12.96 ± 0.7 g/l and 0.08 ± 3.4 g/g, respectively under optimized conditions. Being a reducing sugar, it could be fermented by some thermophilic strains to bioethanol, therefore, it was included along with other monomeric reducing sugars during calculations [41]. The results obtained in the present study were very similar to that reported by Sritrakul et al. [24] where they evaluated dilute acid pretreatment of SCB pith with 2% (v/v) H_2SO_4 with 10% solid loading at 121 ^oC for 90 min, and reported glucose, xylose and cellobiose yields as 0.17, 0.04 and 0.05 g/g raw SCB, respectively. Benjamin et al. [23] reported maximum glucose yield from enzymatic hydrolysis as 0.29 g/g raw material when the SCB was pretreated at 0.65% (w/w) H_2SO_4, 180 ^oC for 10 min. The total sugar concentration, yield and recovery under optimized conditions in the present study were found to be 44.6 ± 1.2 g/l, 306.14 ± 5.3 g/kg raw SCB and 42.31 ± 0.7%, respectively. Mass balance of the study under optimized conditions is presented in supplementary material.

3.5. Mass balance

Overall mass balance of solid and liquid fractions was determined for pretreatment batch under optimized conditions using 1 kg of raw SCB (Fig. 4). Solid recovery obtained after pretreatment was 68.64% i.e. 686.41 g biomass was recovered out of 1 kg of raw biomass. Overall mass losses found in the study

were 10.3%, which could be due to degradation of hemicellulose into some other undetectable compounds, and underestimation of the acid soluble lignin in the liquid fractions after acid pretreatment [23]. Further, extractives were not determined after pretreatment and saccharification, which also contributed to the mass loss. Despite the overall mass balance losses, the overall balance of cellulose, hemicellulose and acid insoluble lignin were above 92%. However, in spite of significant sugar yield and recovery, a considerable amount of biomass (46.68%) was recovered after enzymatic hydrolysis. Underutilization of considerable biomass part could be due to inefficient hydrolysis of crystalline cellulose as suggested by 93.38 g cellulose in residual biomass, incomplete utilization of hemicellulose (residual-15.83 g) due to presence of cellulase as major enzyme with minor amount of xylanase in Cellic CTec2, and large amount of lignin (184.24 g) in biomass. Optimized pretreatment conditions brought out in this study (2.18% v/v H_2SO_4, 14.35% w/v solid loading, and 29.49 min retention time at 121°C) are sufficient for a good overall sugar recovery with almost similar yields and relatively lower inhibitors production to that obtained in previous studies with the SCB and H_2SO_4 acid but with lower solid loading, higher temperature and retention time, which is a more sustainable and lower energy demanding process.

3.6 Structural and morphological analysis of biomass

3.6.1 Compositional analysis

Chemical composition of raw SCB sample and residual biomass after pretreatment and saccharification under optimized conditions is summarized in Table 7. The sum of all components measured was found as 100 ± 1.6, 91.88 ± 2.4 and 93.43 ± 1.6% for untreated, pretreated and saccharified biomass, respectively. This could be attributed to the fact that extractives were not quantified after pretreatment and saccharification, moreover, some degradation of the sugars due to acid hydrolysis during analysis could also contribute to lower value of components. High contents of cellulose (40.45%) and hemicellulose (24.12%) found in raw SCB used in the study are considered very significant from biorefinery concept for 2G ethanol production [42]. The similar cellulose, hemicellulose, lignin and ash content but extractives (10.72%) were observed higher to that reported in the previous studies literature [20, 42, 43]. Lower ash content of SCB in comparison to the other crop residues provides greater opportunities in biorefinery approaches [34]. After pretreatment, cellulose was increased by 36.34%, whereas hemicellulose was reduced by 74.96%. However, lignin content was increased by 42.31%. Proportional increase in cellulose and lignin contents was due to the significant removal of hemicellulose. Ash content was reduced to 3.85 ± 0.3%. Residual biomass after saccharification was found to contain 29.14 ± 0.3% cellulose, 4.94 ± 0.2% hemicellulose and 1.85 ± 0.5% ash content. However, lignin was found to increase to 57.5 ± 0.6% due to utilization of most of the carbohydrate content of biomass.

Table 7
Compositional data of native, dilute H_2SO_4 pretreated and enzymatically hydrolyzed SCB

Components	Untreated bagasse (%)	Pretreated bagasse (%)	Saccharified bagasse (%)
Cellulose	40.45 ± 0.2	55.15 ± 0.4	29.14 ± 0.3
Hemicellulose	24.12 ± 0.2	06.04 ± 0.9	04.94 ± 0.2
Lignin	18.86 ± 0.1	26.84 ± 0.8	57.5 ± 0.6
Ash	5.85 ± 0.7	3.85 ± 0.3	1.85 ± 0.5
Extractives	10.72 ± 0.4	---	---
Total	100 ± 1.6	91.88 ± 2.4	93.43 ± 1.6

3.6.2 Fourier transform infrared (FTIR)

FTIR spectrum bands provide evidence of structural modifications of the biomass after pretreatment. FTIR analysis of untreated, optimized pretreated and saccharified bagasse was carried out in the present study to understand the structural changes in biomass precisely. The spectrum obtained for raw SCB (Fig. 5 and Table 8) was almost similar to reported studies [44, 45]. The FTIR spectra of the pretreated bagasse (2.18% v/v acid, 14.35% solid loading, 121 ^oC, 29.49 min) showed a peak at 664 cm^{-1}, which is generally assigned as contamination of atmospheric CO_2 due to vibration and deformation (Fig. 6). New peak found at 1101 cm^{-1} as compared to raw sample, which could be attributed to the hemicelluloses removal by C-O-C stretching vibration and O-H association of saccharides, alteration of lignin structure via CO deformation of lignin (also belong to glucose) as a result of acid pretreatment. Similar results were reported by for SCB pretreated with dilute H_2SO_4 acid. Intensity of peaks at 1317 and 1366 cm^{-1} were less affected in relation to the raw sample due to less alteration in lignin structure and content by acid pretreatment. Further, peak at 1240 cm^{-1} (C-O of a guaiacyl ring in lignin) was decreased from raw sample to 1238 cm^{-1}, which is generally considered as indicator of the lignin removal during delignification processes [46]. However, decrease in the intensity was very small, which is a typical feature of acid pretreatment as this small lignin removal was attributed to the loss of acid soluble lignin. Increase in intensities of peaks at 1452 cm^{-1} (1449 cm^{-1} in raw SCB) could be due to proportional increase in lignin due to removal of hemicellulose after pretreatment, which was validated from compositional changes as shown in Table 7. Complete disappearance of peak at 1632 cm^{-1}, and a new peak at 1688 cm^{-1} were due to alterations in lignin aromatic groups (methoxyl and phenolic).

Table 8
Organic groups assigned to different peaks in FTIR spectra of SCB

Wavenumber (cm^{-1})	Organic groups
832.59	p-hydroxyphenyl unit of lignin
898.07	C-O-C of glycosidic linkage
1032.53	C-O of cellulose and hemicellulose
1159.84	C-O-C (β-1,4) of cellulose and hemicellulose
1240.28	C-O of a guaiacyl ring in lignin
1318.64	C-H of cellulose and C-O of syringyl unit of lignin, C-H deformations in cellulose and hemicellulose
1370.79	O-H phenoliccompounds
1424.10	Aromatic C-H of lignin
1449.74	C-H of lignin and saccharides
1513.18	C = C in lignin
1604.07	Aromaticgroups in lignin
1632.88	Aromaticgroups in lignin
1729.71	Acetyl and uronic ester groups of hemicellulose
3338.69	O-H of cellulose and hemicellulose

Further, peak at 1730 cm^{-1} (C = O stretching vibration) was disappeared as compared to untreated SCB. The removal of arabinoxylan particularly acetyl and uronic ester groups contributed to this drastic disappearance of peak at this region, which further elaborated the severity of pretreatment [45]. Presence of high concentration of arabinose in pretreated hydrolysate further validated the fact. A new peak at 2360 cm^{-1} in pretreated sample may represented the increased accessibility of cellulose as a result of pretreatment as this region is characteristic for C-H stretching in cellulose and hemicellulose. Figure 6 also displays the FTIR spectra of residual biomass after saccharification. A small change in intensity of peaks at 1322 and 1363 cm^{-1} in relation to raw sample was observed probably due to formation of aggregates of lignin remained undigested during enzymatic hydrolysis. Intensity peak at 1238 cm^{-1} was further decreased from pretreated sample to 1234 cm^{-1}, which might be due to some altered form of lignin, because acid pretreatment may also cause breaking of bonds in the lignin molecule by radical condensation, which leads to precipitation of partially altered lignin on the surface of the cellulose fibre, and this modification further allows better catalytic adsorption of enzyme to the later [47]. More increased intensity of peak at 1455 cm^{-1} (1452 cm^{-1} in pretreated sample) was again due to proportional increase in lignin content due to digestion of cellulose and hemicellulose, which was also shown in compositional analysis of saccharified biomass (Table 7). Smaller decrease in peak intensity at 1686 cm^{-1} was again shown the lesser effects in lignin content. A new peak at 2922 cm^{-1} in saccharified sample represents absorptions due to stretching vibrations of C-H of alkyl and aromatic groups of lignin, which indicated further proportional enrichment of lignin in biomass.

3.6.3 X-Ray diffraction (XRD)

The crystallinity of a biomass is related to its composition wherein; hemicellulose and lignin are amorphous fractions, whereas cellulose mostly forms crystalline regions [48]. After pretreatment, cellulose structure can undergo changes in crystallinity, and severity of pretreatment could be related to changes in crystallinity index (CI) of pretreated sample as compared to untreated sample. CI by XRD is determined by the diffraction intensities of crystalline cellulose in comparison to the total amorphous fraction of the biomass including lignin as well as amorphous hemicelluloses. The CI and average crystallite size of untreated, optimized pretreated and saccharified SCB were calculated using XRD. CIs of the pretreated and saccharified samples were found to be increased as compared to the untreated SCB (Table 9). Furthermore, a significant increase in crystallite sizes was observed for pretreated and saccharified samples (Table 9). This could be related to the fact that pretreatment and indeed saccharification processes led to a removal of hemicellulose, and partially cellulose fractions of the biomass. Remaining cellulose seems to aggregate and collapse leading to a large crystallite observed in the residual biomass samples.

Table 9
Crystallinity Index of SCB samples under optimized conditions.

Sr. No.	Sample	CI (%)	Crystallite (nm)
1	Untreated SCB	50 ± 2	2.5 ± 0.1
2	Pretreated SCB	58 ± 3	3.3 ± 0.2
3	Saccharified SCB	58 ± 3	3.9 ± 0.3

3.6.4 Confocal laser scanning microscopy (CLSM)

Fluorescence emission spectrum and its decay time for lignin as a chromophore within the cell wall could be significantly altered by pretreatments. Therefore, cell wall structures of optimized pretreated and saccharified SCB were analyzed to detect fluorescence spectra of lignin and time of its decay in reference to the raw biomass using CLSM to explore its redistribution caused by pretreatment and enzymatic hydrolysis (Fig. 7). Biomass pretreated with 2.18% (v/v)

H_2SO_4 at 121 °C and 14.35% (w/v) solid loading for 29.49 min showed significant spectral modifications, and emitted green color as compared to blue color of raw sample (Fig. 7a & b). This is consistent with previous studies of untreated SCB showing that it emits fluorescence in a range of 400–500 nm as reported by Santo et al. [20] (Fig. 7a). The blue color of untreated biomass could be due to unaltered native lignin in cell wall as cellulose is not responsible for emission at the wavelength in this range [49]. Green color of pretreated sample might be due to redistribution of lignin carried out by acid pretreatment and proportional increase in total lignin content after pretreatment as indicated in Table 7. Similar observation was reported in a study of pretreatment of SCB with 1% H_2SO_4, where lignin was mostly not removed but rather underwent molecular rearrangements forming aggregates [49]. Redistributed or aggregated lignin molecules emit between green and red color of longer wavelengths (500–700 nm), thus lower energy emissions. On similar trends, saccharified sample emitted red color totally different from untreated as well as pretreated samples (Fig. 7c). This could be attributed to the lignin molecular aggregates after enzymatic degradation of almost all significant sources of sugars; cellulose and hemicellulose, which is validated from compositional analysis (Table 7). Enzymatic hydrolysis and removal of polysaccharide fractions of the biomass lead to more aggregated and dense lignin deprived interactions with hemicellulose and cellulose which manifest itself in red shift of its intrinsic fluorescence.

3.6.5 Field emission scanning electron microscopy (FESEM)

Available surface area of the cellulose fiber structure is a critical factor in enzymatic hydrolysis of lignocellulosic materials. Figure 8 displays morphology of raw, pretreated and saccharified SCB samples under optimized conditions via FESEM images. Significant differences existed between untreated, pretreated and saccharified biomass samples due to removal of hemicellulose and lignin. For the raw SCB, a complete, smooth, compact and uniform lignocellulosic structure is clearly observed at both 5,000 and 10,000 X magnifications that are common for SCB (Fig. 8a & b). After pretreatment with 2.18% (v/v) H_2SO_4 acid and 14.35% (w/v) solid loading for 29.49 min at 121 °C, the cell wall structure turned into irregular and disorganized indicated by rough, loosened fibrous network and certain cracks on the surface which can be observed at 5,000 X magnification (Fig. 8c). At high magnification (10,000 X), cell wall tubes or pores could be observed as a result of the pretreatment, which showed significant external as well as internal breakdown of the lignocellulosic structure (Fig. 8d). These porous spaces in biomass are generated by structural changes in lignin carried out after acid pretreatment, which allow more enzyme accessibility and adsorption through the surface resulting into enhanced enzymatic hydrolysis, since the pore volume is considered as a key factor for improved enzymatic saccharification. The coarse, disordered cellulose fibers and wide pores with exposed surface area of biomass are more accessible for cellulase during saccharification [50]. These changes could expose more sites on the surface to increase the effect of enzymatic hydrolysis. Similar structural changes were found in a reported study using rice straw [51]. Thus, this optimized acid pretreatment was observed to be efficient in terms of providing an extended degree of degradation in structure of SCB. Furthermore, in images of residual biomass samples left after saccharification, more disruption of cell wall could be seen, that was indicated by more loose, irregular fibril network observed at 5,000 X magnification (Fig. 8e), which revealed degradation of cellulose during hydrolysis. Moreover, number of holes on the surface at higher magnification of 10,000 X (Fig. 8f) indicated that after almost complete degradation of cellulose as a result of hydrolysis, residual biomass consisted of lignin as a major component along with non-hydrolyzed cellulose fibers called enzymatic hydrolysis residue lignin (EHRL) [52]. Thus, FESEM analysis of SCB samples revealed significant effects of optimized pretreatment on SCB structure and morphology.

3.6.6 ^{13}C solid-state nuclear magnetic resonance (^{13}C ssNMR) studies

Chemical changes occurred in SCB after optimized pretreatment and saccharification were further studied by high resolution ^{13}C solid-state NMR in reference to the untreated sample. This technique gives information about modifications taking place among specific chemical groups. Figure 9a shows the ^{13}C CPMAS-TOSS spectra of the raw and pretreated SCB under optimized conditions (2.18% (v/v) H_2SO_4, 14.35% (w/v) solid loading, 121 °C and 29.49 min). The chemical shifts corresponding to the major components of the SCB were assigned based on previous literature reports (peaks or lines 1–17). The spectra of untreated and pretreated samples were normalized by the signal of the C1 carbon of cellulose, at 105 ppm. The signals in the spectral region around 50–120 ppm has major contribution from carbons from cellulose followed by hemicellulose and lignin. Peaks 3 at 62.5 and 7 at 83.5 ppm are attributed to C6 and C4 carbons from amorphous carbohydrates (cellulose and hemicellulose), respectively, whereas the lines 4 at 64.8 and 8 at 87.9 ppm are assigned to C4 and C6 of crystalline fraction of cellulose, respectively. There is also minor contribution of lignin signals at the C6 region (~ 62.5 ppm). Furthermore, hemicellulose peaks are arranged throughout the spectral regions assigned with the numbers as 1, 4, 6, 7, 9 and 17. Lignin peaks are distributed along the broader region from 100 to 200 ppm and a peak at 56.2 ppm throughout the spectral region denoted by lines 2, 11, 12, 13, 14, 15 and 16 [20, 53]. The absence of the hemicellulose peaks 1, 9 and 17 on the pretreated SCB sample as well as the reduction in the intensities of lines 4, 5, 6, 7 and 9 indicates an efficient removal of hemicellulose as a result of the applied acid treatment.

Figure 9b shows the ^{13}C CPMAS-TOSS spectrum of the pretreated SCB sample and the residual biomass after enzymatic saccharification. Because the peaks of lignin on the pretreated SCB and residual biomass spectra are virtually identical, now the normalization was done by using the signal of the lignin peak at 56 ppm. The C4 carbon peaks are of particular interest in this case, since they are in a region with almost no superposition with lignin peaks and the hemicelluloses contribution was removed by the pretreatment [54]. Thus, The C4 carbon peaks have valuable information about the amount of surface (amorphous) and bulk (crystalline) cellulose, encoded on the peaks intensities (areas) at 83.5 and 87.9 ppm, respectively. For the pretreated sample, these areas are 8.4 ± 0.3 for the amorphous peak and 4.0 ± 0.2 for the crystalline one. On residual biomass, they are 5.5 ± 0.2 and 3.6 ± 0.2, respectively. The conversion of cellulose into fermentable sugars can be quantified by the ratio of the areas, for the saccharified and pretreated samples, of the peaks assigned to the cellulose carbons. This ratio is 0.90 ± 0.07 for the bulk and 0.65 ± 0.04 for the surface cellulose regions. It indicates that the conversion takes place mainly on the surface of the cellulose, while most of the bulk cellulose is kept unaffected. It must be remarked that while the CPMAS-TOSS sequence is not quantitative among different chemical groups, the comparison between signals for the same type of carbons used in the C4 carbon analysis is quite reliable.

3.6.7 ^{1}H Time-domain nuclear resonance magnetic studies

In proton NMR relaxation experiments of confined fluids, the interaction between the molecules of the fluid inside a pore results in an enhancement of the spin-spin relaxation. On the fast diffusion limit, the relaxation constant T_2 directly associated to the pore size by the linear relation $r = 2\rho T_2$, with the surface to volume ratio given by r and ρ is the surface relaxivity, a constant which is usually unknown and depends heavily on properties of the sample [55]. On realist scenarios, the CPMG decay is described by a multi-exponential, and the resulting T_2 distribution is obtained through an Inverse Laplace Transform (ILT) procedure [56]. In our case, (NxN)-dimethilacetamide (DMAc, HPLC grade, NEON Labs, Brazil) was used as the molecular probe, due to its weaker interaction with cellulose, in comparison to water [57].

For lignocellulosic biomass, three different length scales are observed, related to pores at microfibril surfaces (shortest T_2 values, about 1 ms), within the lignin-hemicellulose matrix on the surface of the fibers (about dozens of milliseconds) and small luminal pores or between the fibers (hundreds of milliseconds) [58]. Each of these components are deconvoluted on the T_2 distributions by log-Gaussian functions, and the relative area indicates the amount of solvent on the respective interstitial scale. Thus, by measuring the T_2 distributions for samples which underwent different biochemical processes, it is possible to assess how the microstructure of the biomass is modified on different length scales. For the three samples analyzed here, the raw and optimized acid pretreated SCB exhibit an almost identical CPMG decay, as exhibited on Fig. 10a. Thus, no significant differences on the T_2 distribution of these two samples are observed on any of the three length scales, irrespective to the removal of hemicelluloses by the acid pretreatment, as shown in Fig. 10b. However, the saccharified sample showed an increase of the accessibility on the intermediate length scale, mainly related with pores on the surface of the lignocellulose fibers, as shown in Table 10. Such feature is in accordance with the ssNMR results, which indicates that most of the cellulose removed by the enzymatic hydrolysis is the amorphous one, located on the surface of the fibers. For all samples, we did not observe a noticeable change on the average T_2 values for all components. However, this result cannot be taken as a clear indicative that the pore sizes are not affected because the compositional changes over the process modify the surface relaxivity constant. It is worth mention that the modifications observed in the shorter T_2 components cannot be taken as significant because of their small relative area which makes the ILT analysis unreliable.

Table 10
Relative areas and central T_2 for each interstitial scale

Sample	Microfibril surfaces		Cell wall		Lumen	
	Area (%)	T_2 (ms)	Area (%)	T_2 (ms)	Area (%)	T_2 (ms)
Raw SCB	5.0 ± 1.0	4.9 ± 0.2	20 ± 3.0	57 ± 5.0	75 ± 2.0	280 ± 10.0
Pretreated SCB	2.2 ± 0.6	8.6 ± 0.4	19 ± 3.0	56 ± 6.0	79 ± 3.0	250 ± 10.0.
Saccharified SCB	4.0 ± 1.0	7.7 ± 0.6	30 ± 4.0	61 ± 6.0	67 ± 4.0	230 ± 10.0

3.7 Separate hydrolysis and fermentation (SHF)

Enzymatic hydrolysis of optimally dilute H_2SO_4 pretreated biomass resulted into the total reducing sugar concentration of 32.48 g/l in 72 h. The filtered saccharified hydrolysate was fermented using *K. marxianus* NIRE-K3. Figure 11 presents the trend of sugars and products with time during the fermentation process. In Fig. 4.1, it could be seen that the glucose (initial concentration: 25.76 g/l) was consumed almost completely within 8th h of fermentation of SHF of dilute H_2SO_4 pretreated SCB. On the other hand, the rate of xylose (initial concentration: 6.72 g/l) utilization was fast till 6th h but it was lesser than the glucose utilization rate till 6th h, and decreased thereafter. The remaining xylose concentration was calculated to be 2.05 g/l in 8th h, which was not further utilized even at 24 h. Xylose was utilized until glucose was present in the broth, and there was no further utilization of xylose was observed once glucose diminished, although, the rate of xylose utilization was slower than glucose. This observation is obvious because cells take up xylose through hexose transporters, which are active in the presence of hexose sugars, once they are consumed, associated transporters also get inactivated, and do not lead to the transport of pentoses i.e. xylose [59]. After 24 h of fermentation, the maximum ethanol concentration, volumetric productivity, and yield were found to be 10.82 ± 2.2 g/l, 0.45 ± 0.9 g/l/h and 0.42 g/g-glucose consumed, respectively. Ethanol yields obtained from fermentation of dilute H_2SO_4 pretreated SCB was found to be 82.4%, respectively of the maximum theoretical yield (0.51 g/g).

Apart from the ethanol, acetic acid and glycerol were also produced with the maximum concentrations of 0.85 and 0.34 g/l, respectively. Acetic acid and glycerol are the major by-products representing 4–5% of the carbon utilization in addition to the CO_2 formation, and sometimes pyruvic acid or succinic acid [60]. Glycerol is formed during anaerobic fermentation to balance NAD^+/NADH ratio by re-oxidizing NADH to NAD^+, thus prevent the imbalance supposed to be occurred due to non-functioning of the respiratory chain [61]. Biochemical conversion of dilute H_2SO_4 pretreated SCB resulted in the ethanol yield of 71.45 ± 2.5 g/kg raw SCB. Total time taken in the process accomplishment was 96 h including 72 h of enzymatic hydrolysis and 24 h of fermentation however as strain gave maximum ethanol concentration in 16 h, thus total time duration of SHF in the present study may be summed up to 88 h. The results obtained in the present study were more significant than the reported studies for example, in a SHF study, SCB was pretreated with 2.5% (w/v) NaOH (initial soaking of 1 h at room temperature) at 10% (w/v) solid loading at 121°C for 30 min followed by hydrolysis using 1 ml of cellulase loading (CMCase activity of 2900 IU/ml and filter paper activity of 1500 FPU/ml) at 2% (w/v) solid loading at 50°C and 140 rpm for 8 h resulted into the hydrolysate containing the maximum reducing sugars of 140 g/l, which were subsequently fermented using 1 ml cells of *S. cerevisiae* and *Pichia stipitis* separately at 30 ^oC, and resulted into the maximum ethanol yields of 0.49 and 0.29 g/g- glucose consumed, respectively after 96 h. However, both the strains gave extremely lower ethanol yields of 0.37 and 0.22 g/g-glucose consumed, respectively after 24 h as compared to the present study [62]. In another study, the KOH pretreated corncob was used for the enzymatic hydrolysis using 22.04 FPU/g-PB of endoxylanase (from *Streptomyces thermovulgaris* TISTR1948) and cellulase (iKnowZyMe AC) at 50 ^oC for 72 h with subsequent fermentation using of *Candida glabrata* KY618709 at 40 ^oC for 96 h, which resulted into 21.92 g/l of ethanol concentration, 0.23 g/l/h of

volumetric productivity and ethanol yield of 0.37 g/g-glucose consumed [63]. Thus, it could be concluded that optimized dilute H_2SO_4 pretreatment of SCB carried out in the present study proved to be promising for enhaned ethanol production using pentose utilizing thermotolerant yeast strain.

Conclusion

Statistical optimization of process variables for dilute acid pretreatment using RSM was proved to be a promising approach for enhanced CSY from SCB. The optimum values for variables were investigated as 2.18% (v/v) H_2SO_4, 14.35% (w/v) solid loading and 29.49 min at 121 ^oC. CSY in model validity of optimized pretreatment batch was observed to be 521.42 g/kg of raw SCB with total reducing sugar yield in pretreated and saccharified hydrolysates of 215.28 ± 2.4 and 306.14 ± 5.3 g/kg of raw SCB, respectively. Furthermore, CSY was observed to be affected more by solid loading and acid concentration than retention time in a two-way interaction as investigated by 3-D and contour plots. Biochemical and biophysical analysis of biomass further validated the optimization results obtained in the present study. Thus, process optimization using statistical designs is proved to be a significant strategy for efficient recovery of both C6 and C5 sugars from SCB as well as enhanced ethanol yield from biomass.

Declarations

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Author'scontributions:

MH conceived, planned, executed experiments and wrote original draft; VOAP, JGF, ERA and FECG analyzed biophysical parameters; AKC edited; IP reviewed and supervised; BSC reviewed; and SK: conceived, planned, reviewed, edited and supervised.

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Figures

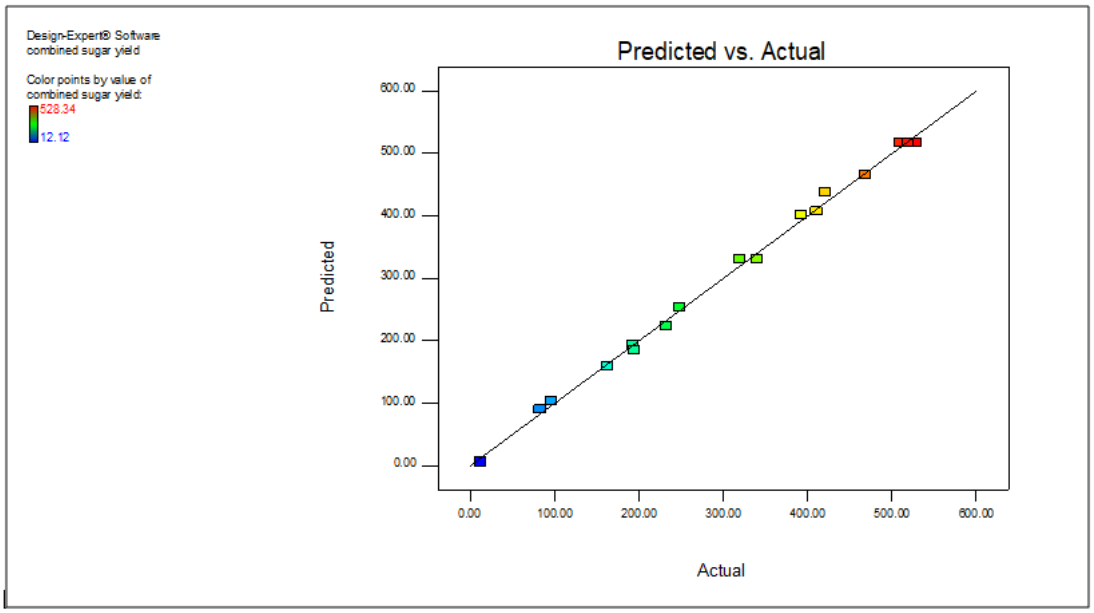

Figure 1

Diagnostic plot of the actual and predicted values of CSY for pretreatment optimization with dilute H_2SO_4.

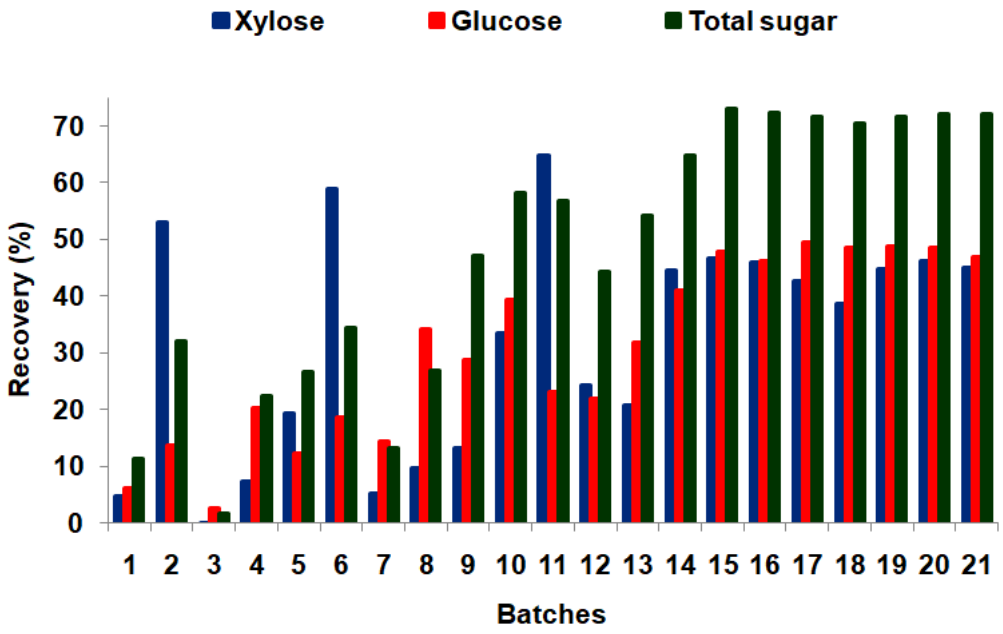

Figure 2

Graphical representation of % recovery of xylose, glucose and total sugars in pretreatment followed by enzymatic saccharification for different runs (described in Table 2) of pretreatment with dilute H_2SO_4.

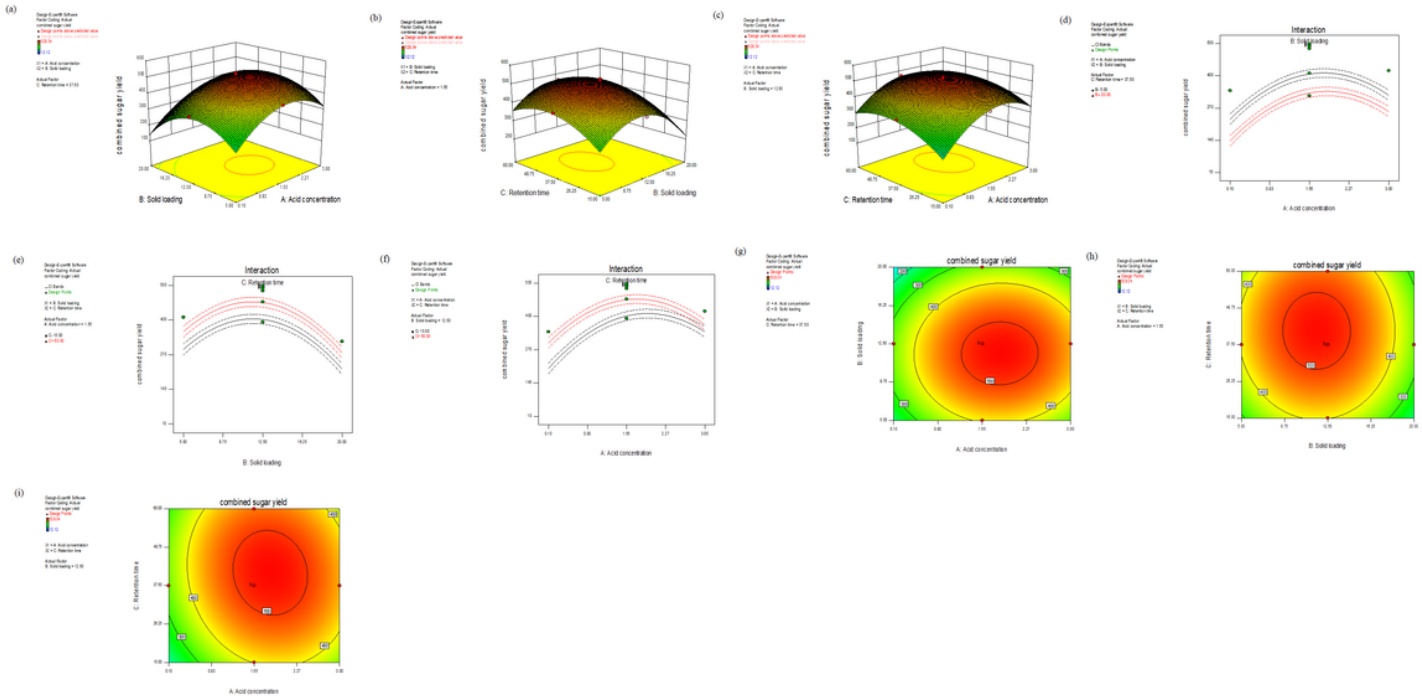

Figure 3

3-D, interaction and contour plots for interaction between process factors during pretreatment with dilute H_2SO_4.

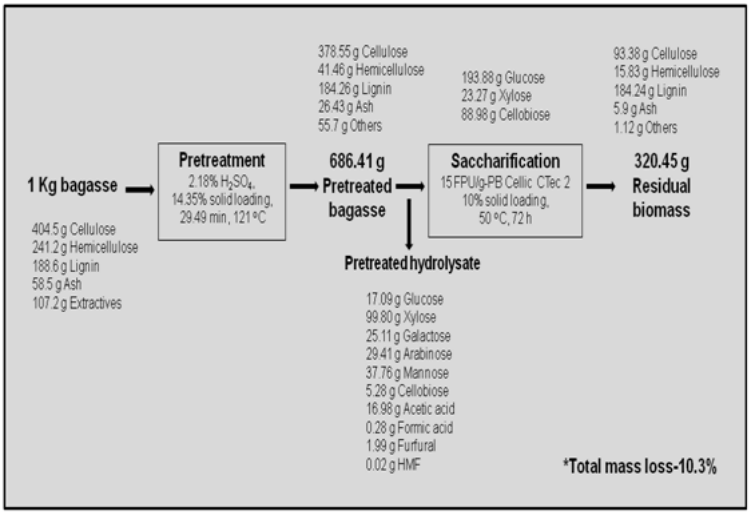

Figure 4

Overall mass balance of pretreatment process of SCB with dilute H_2SO_4 under optimized conditions.

Fourier transform infrared (FTIR)

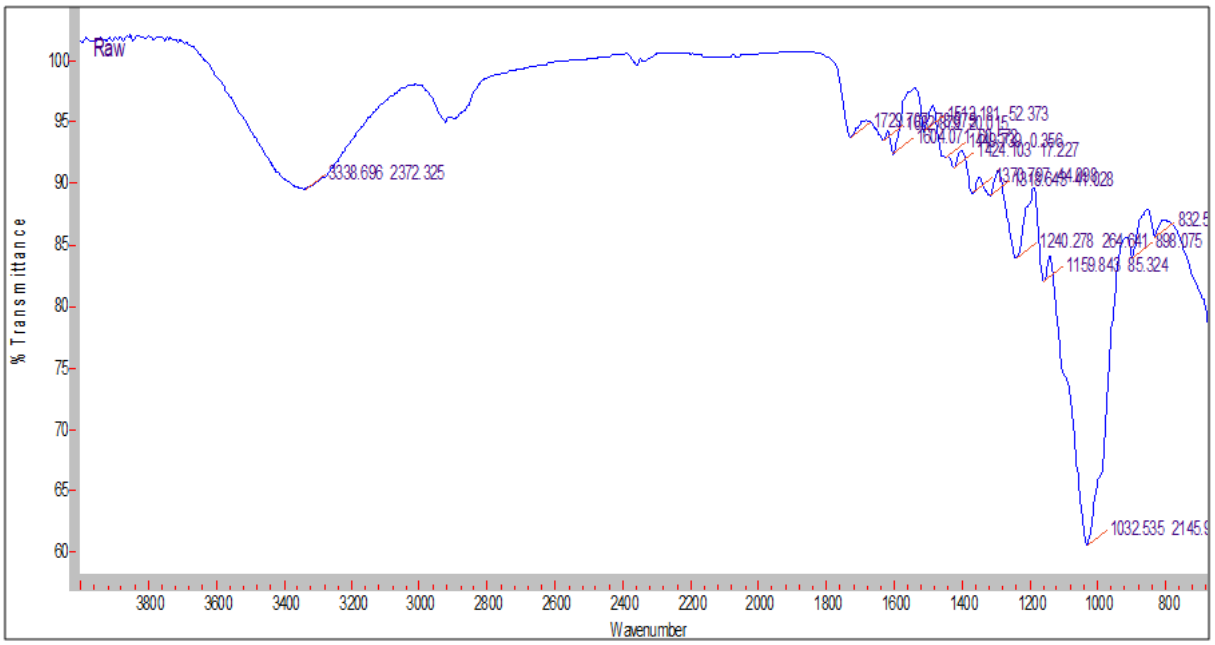

Figure 5

FTIR spectra of raw SCB.

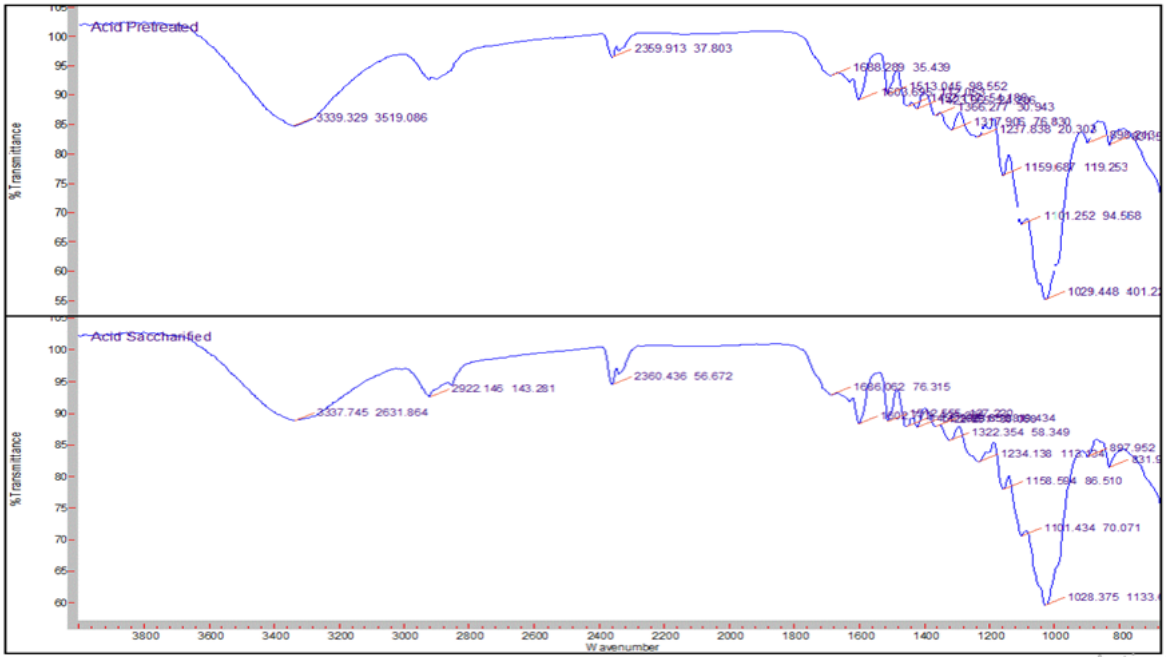

Figure 6

FTIR spectra of SCB pretreated with dilute H_2SO_4 under optimized conditions and residual biomass after enzymatic hydrolysis.

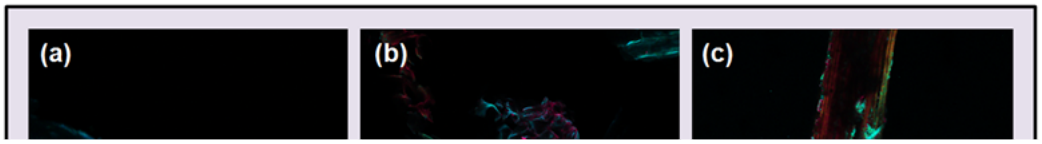

Figure 7

CLSM images of (a) untreated SCB; (b) optimized pretreated SCB with dilute H_2SO_4; (c) residual biomass after enzymatic hydrolysis.

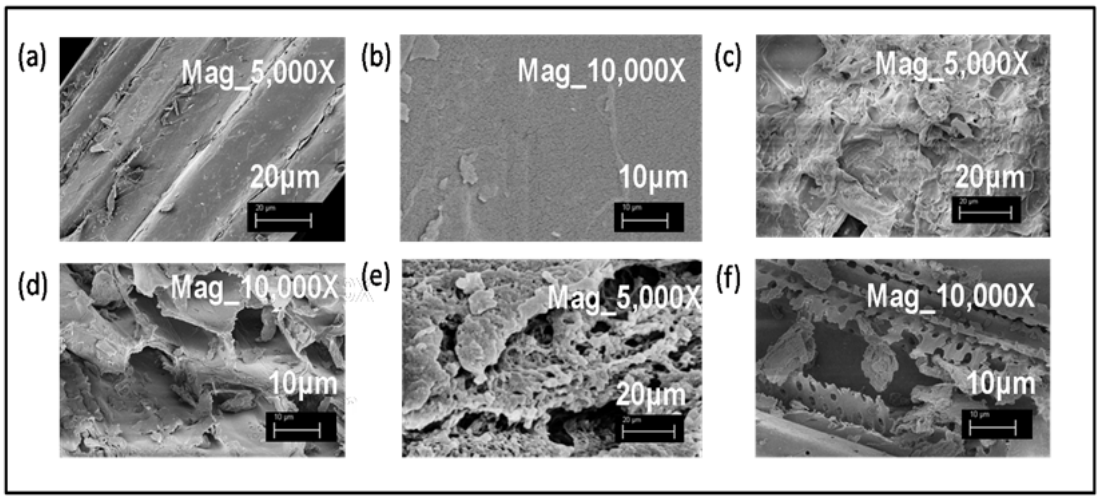

Figure 8

FESEM images of (a), (b) untreated SCB; (c), (d) optimized pretreated SCB with dilute H_2SO_4; (e), (f) Residual biomass after enzymatic hydrolysis.

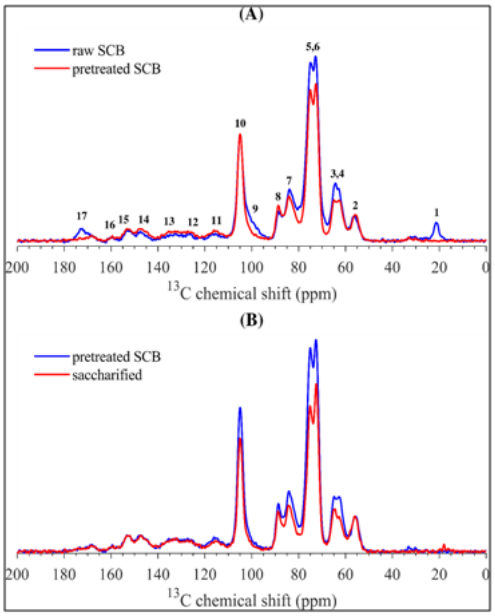

Figure 9

^{13}C CPMAST-TOSS NMR spectra obtained for (a) raw and optimized pretreated SCB samples with dilute H_2SO_4, normalized the signal C1 carbon of cellulose at 105 ppm (line 10); (b) optimized pretreated SCB and residual biomass after enzymatic hydrolysis, normalized by lignin signal at 56.2 ppm (line 2).

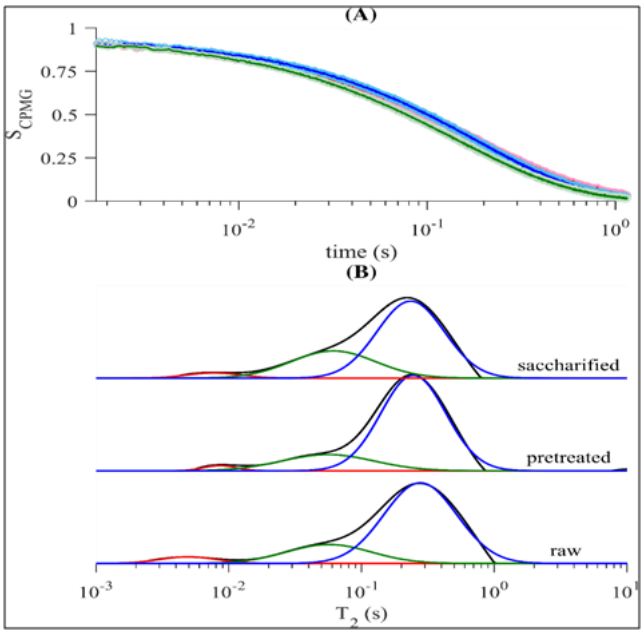

Figure 10

(a) Measured CPMG decays for the raw (red, almost completely covered), pretreated (blue) and saccharified (green) SCB samples. The solid lines are the best fit of the data using an ILT procedure; (b) T_2 distributions curves for raw, pretreated and saccharified SCB with their respective deconvulations. The black line is the distribution obtained from the ILT procedure. The red, green and blue lines denote the deconvoluted components, describing fluid on the inter-microfiber, cell wall and luminal interstitial spaces, respectively. All distributions are normalized by the total area.

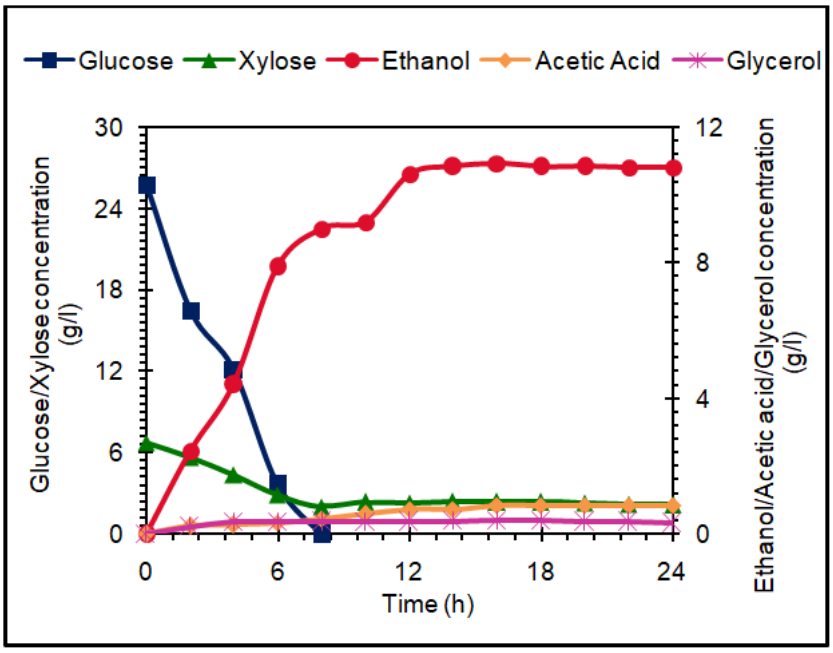

Figure 11

SHF of dilute H_2SO_4 pretreated SCB