ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Optimization of Pretreatment of *Saccharum spontaneum* (Kans Grass) Biomass for Production of Alcoholic Biofuels.

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ABSTRACT

Saccharum spontaneum (Kans grass) is a waste land weed, infesting vast area of Asian continent. The plant stem of Kans grass has rich concentration of cellulose and hemicellulose, which can be converted into alcoholic biofuels. The success of bioconversion of biomass into biofuel is determined by adapting the suitable pretreatment process, which hydrolyze the carbohydrate into fermentable sugar by utilizing less chemical and reaction time. The pretreatment was carried out by chemical (acid/alkali) and physiochemical (pressure cooking) steps. In the chemical step, the pretreatment process was carried out with Sulfuric Acid (H_2SO_4), Hydrochloric Acid (HCl), Sodium Hydroxide (NaOH) and Sodium Carbonate (Na_2CO_3). Among these, dilute Hydrochloric acid (HCl) enhanced a significant increase in reducing sugarfor *S.spontaneum*. Further, the effect of HCl concentration, biomass loading and pressure cooking time on release of reducing sugar was studied and optimized using Central Composite Design (CCD). An estimate of 1.32(%v/v) of HCl, 8.36(%w/v) of biomass and 46.81min of pressure cooking was found be optimum for the release of reducing sugar for *S. spontaneum*. At these optimum conditions, the yield of reducing sugar was observed to be 24.7g/L. The quadratic polynomial models was generated and validated for the release of reducing sugar.

Keywords: Alcoholic Biofuel, Central Composite Design (CCD), Pretreatment, *Saccharum spontaneum*.

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INTRODUCTION

The continuous rising population has increased the demand of energy for urbanization, transportation and industrialization. The current worldwide energy demand was met by the fossil fuel, which is non-renewable, thus causing environmental pollution by the emission of greenhouse gases. This energy problem has enforced the present generation to go for an alternative fuel, which is renewable and will cause less environmental pollution than fossil fuels. One of the alternate fuel is the alcoholic biofuels, having high energy density and reducing carbon emission. Alcoholic biofuels are generated from lignocellulosic biomass, which is abundant on earth and generated from agricultural and non-agricultural residues.

Saccharum spontaneum (Kans Grass) is a well-known waste land weed, generally found near the banks of rivers. The stem of Kans grass contains a high amount of carbohydrate [5-6] and has a potential of being converted into alcoholic biofuels. Although there are few studies on Kans Grass, a previous study has revealed that 67.8% of carbohydrate present in the cell wall of the Kans Grass Biomass (KGB) yielded 12.08 ± 0.72 g/L ethanol concentration [2]. Appropriate pretreatment techniques can be adapted in improving the yield of alcoholic biofuel resulting in the increase of more fermentable sugar for microbial conversion.

Lignocellulosic biomass contains mainly cellulose, hemicelluloses, lignin and pectin. In the biomass, cellulose and hemicellulose are bonded together by lignin, which acts as a protective barrier for microbial attack [8]. Therefore, for the conversion of biomass into alcoholic biofuels, pretreatment of raw biomass is necessitate, wherein it reduces the crystallinity of cellulose and removes the lignin stratum around cellulose and hemicellulose. All these effects maximize the extent of scarification and yield of fermentable sugars[20-21].

The conversion of any biomass (lignocellulse) to alcoholic biofuel requires two steps, such as pretreatment and fermentation. The pretreatment step enhances the bio-digestibility and increase the accessibility of microorganism to the biomass. This process results in enrichment of the biodegradable materials, thus improving the yield of alcoholic biofuel from lignocellulose. Several pretreatment techniques have been successfully developed and adopted for the bio synthesis of ethanol and butanol [1]. Among these methods, chemical treatment such as acid hydrolysis [8, 19, 22], alkaline hydrolysis [9],enzymatic hydrolysis [10], physicochemical techniques such as steam explosion techniques [15], Ammonia fiber explosion[17], CO_2 explosion[15],irradiation techniques by gamma rays or microwave[8]are widely used. In-spite of several researches on these techniques of pretreatment, there exist several obstacles such as improper separation of cellulose and lignin, time of reaction, solvent recycling, corrosion, waste water treatment and energy balance[20].

In the present investigation, chemical and physicochemical pretreatment by pressure cooking were explored. The high temperature and pressure during pressure cooking degrades most of the hemicellulose and lignin, thus enhancing the hydrolysis process in short span of time [18]. The factors that affect this process are residence time of biomass inside the reactor, temperature, pressure and moisture content [8]. In this study the factors affecting the pretreatment process were screened and release of reducing sugar was optimized by Central Composite Design (CCD) using Response Surface Methodology (RSM).

MATERIALS AND METHODS

Biomass Collection and Processing

S.spontaneum were collected from the banks of Gurupur river, Mangalore situated in Dakshina Kannada district of Karnataka. The stems were processed by chopping into desired size and sun dried for 2 days. The dried stems werepowdered in laboratory disintegrator and sieved using Taylor series mesh #10. The powdered stems were washed with distilled water untill dust free and dried in the hot air oven at 80^0C for 12h to remove any traces of moisture.The powdered samples were further stored at -20^0C in deep freezer to prevent any microbial degradation.

Analysis of Composition of *S.spontaneum*

The overall efficiency of the process to convert lignocellulose to alcoholic biofuel was determined by the analysis of composition of lignocellulosic feed stock. Lignocellulosic substrate mainly contain cellulose, hemicellulose,insoluble lignin, soluble lignin and ash [7].

The quantitative estimation of cellulose in *S. spontaneum*was determined by the method described inUpdegraff [23]. The ash content was determined by using AOAC (1990) procedures: ash (method 942.05)[4]. Fibre fractions including Neutral Detergent Fibre (NDF)and Acid Detergent fibre (ADF) were assayed according to proceduresas described by Mertens (2002) [13], AOAC (1990)-method 973.18 [4]respectively. The hemicellulose was calculated by substracting NDF by ADF. The total reducing sugar present in the hydrolzate was measured by Miller'smethod[14].

Pretratmet Methods

Pretreatment of Kans Grass Biomass (KGB) was carried out in two steps as described below:

Chemical hydrolysis of KGB

The chemical hydrolysis of 5% *S. spontaneum*(KGB)was carried out with different strengths (1%,3%, 5%,7% and 9%v/v) of HCl, H_2SO_4,NaOH and Na_2CO_3 in different 250ml screw-capped Erlenmeyer flasks. The flasks were pressure cooked for 20min at 15psi pressure in 12L pressure cooker. The KGB suspension was filtered using whatman paper filter grade 4 and reducing sugar concnetration was estimated by Miller's method [14].

Pressure cooking of KGB

5%w/v of KGB was taken in 250ml screw-capped Erlenmeyer flask and subjected to pressure cooking in 12L pressure cooker. Cooking was carried out for different time interval by keeping constant pressure at 15psi. Further the pressure cooked materials were filtered through whatman filter paper grade 4.

Experiment Design and RSM

In the present experimental plan, the factor levels for optimization were selected by carrying out pretreatment by varying One Factor at a Time (OFAT). The factors such as chemical concentration (X_1), biomass loading (X_2) and pressure cooking time (X_3) were varied. The optimization of pretreatment was done by Central Composite Design (CCD) using Response Surface Methodology (RSM). The levels for the CCD were listed in Table 1.

Table 1: Coded levels and actual values of the independent variables for CCD

Independent Variables		Coded Levels				
	Symbol	$-\alpha$	-1	0	$+1$	$+\alpha$
Concentration of HCl (%v/v)	X1	1.32	2	3	4	4.68
KGB (%w/v)	X2	1.63	3	5	7	8.36
Pressure cooking Time min	X3	13.18	20	30	40	46.81

The Central Composite Design for three factors encompassing 16runs+4center points was applied for optimization of pretreatment of *S. spontaneum*. The 3 factors namely concentration of HCl (X_1 %v/v), KGB (X_2 %w/v) and Pressure cooking time (X_3 min). The concentration of reducing sugar (Y g/L) was taken as response (dependent variable). These factors were taken in 3 levels (-α, -1, 0, +1, +α). The quadratic polynomial model was fitted to the response data obtained from the CCD by regression analysis. Eq.1 below gives the quadratic polynomial model for the response variable.

$$Y = \alpha_0 + \sum \alpha_i X_i + \sum \alpha_{ii} X_i^2 + \sum \alpha_{ij} X_i X_j \; (i,j=1,2,3.....k) \qquad (1)$$

Where, Y is the response of dependent variable (Reducing sugar, g/L), $\alpha_0$is the regression constant, $\alpha_i$and α_{ij} is the linear and quadratic effect of three variables. X_i is independent variables.

RESULTS AND DISCUSSION

S.spontaneum Composition

The chemical compositions of most widely used biomass were compared with Kans Grass Biomass (KGB) and is presented in Table 2 as a % dry weight basis. The results obained resembles the composition reported by Lalith et al.(2011) [12]. However the least difference in the values could be attributed to agronomic factors including characteristics of soil, geographic location, season of harvest etc.

Table 2: Chemical Comparison of different biomass

Chemical Composition	% of Dry Weight			
	KGB	**Wheat straw**	**Sugar cane Bagasse**	**Corn stover**
Cellulose	45.25±0.5	37.1±1	38.3±2	34.3±1.6
Hemicellulose	24.96±3.5	34.0±0.5	30.9±1	32.8±0.8
Neutral Detergent Fibre	81.50±6.5	77.25±5.6	80.29±2.5	75.97±2.5
Acid Detergent fibre	56.55±7.5	42.89±3.7	50.39±3.4	40.17±5.3
Lignin	7.40 ±2.5	18.0±0.5	20.9±1	15.1±0.5
Ash	4.62±1.2	3.7±1.5	2±1	3.5±1

The rich concnetration of cellulose present in KGB, makes a potential source of reducing sugar for the synthesis of alchololic biofuels. The cellulose concetration was compared with other potential lignocellulosic resources such as sugar cane bagasse[3], wheat straw[11], corn stover[16] and sorghum straw [11].

Effect of chemical pretreatment on KGB

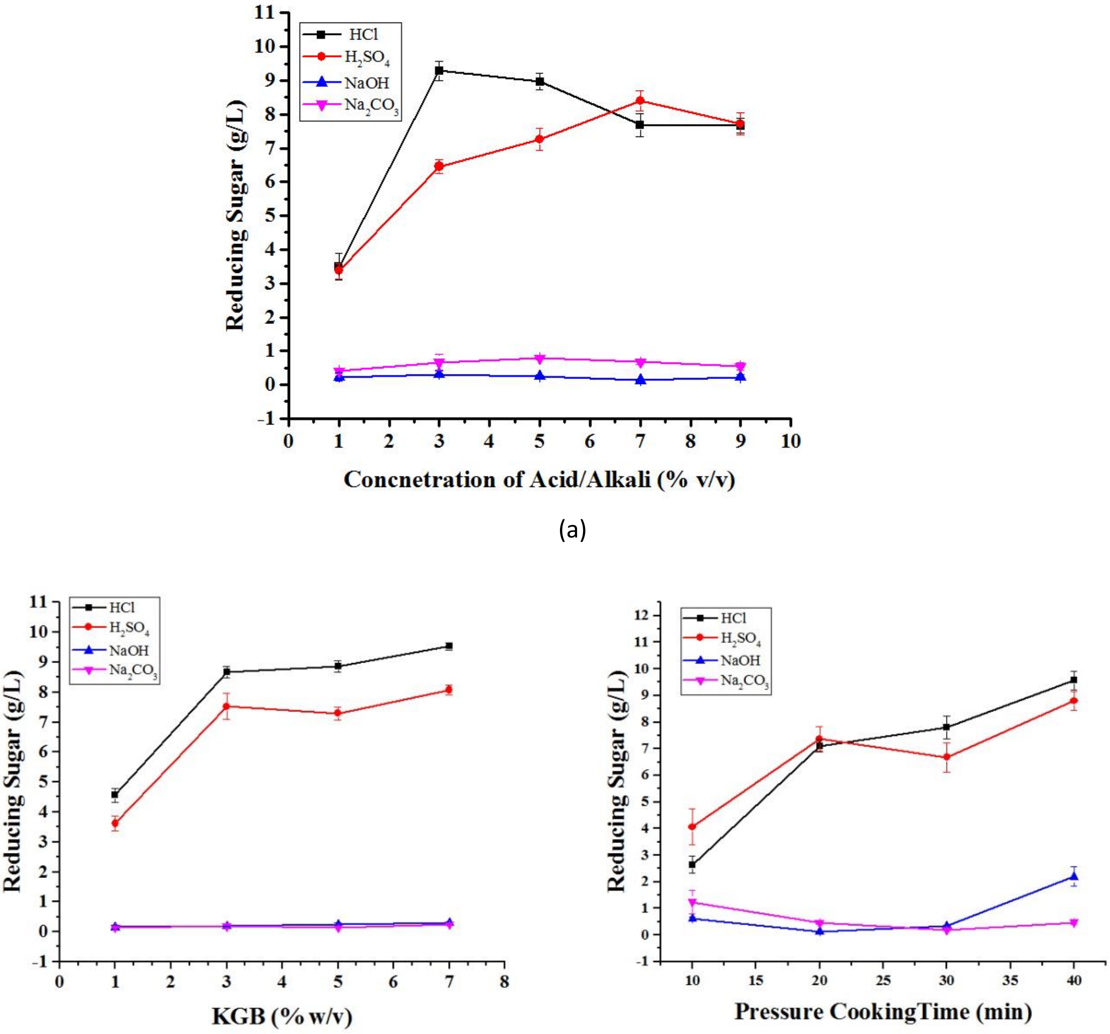
The success of chemical hydrolysis depends upon utilizing the low concentration of acid/alkali to achieve greater release of reducing sugar. The hydrolysis carried out with alkali such as NaOH and Na_2CO_3resulted in poor release of reducing sugar (Figure 1(a)). However acids (HCl and H_2SO_4) yielded greater release of reducing sugar of 9g/L and the trend was similar up to acid concentration of 7%v/v. From the Figure 1(a), the rate of sugar release was found to be high for HCl when compared to H_2SO_4. The high concentration of HCl&H_2SO_4has a negative effect on sugar release, due to the fact that at high concentration reducing sugar degrades and converts it to furfural. Further studies were carried out by taking HCl for chemical pretreatment.

Effect of Biomass Loading on release of Reducing Sugar

The variation in sugar release by changing the biomass load for pretreatment is presented in Figure 1(b). During the hydrolysis acid/alkali comes in contact with the biomass and causes cellulose swelling, structural alteration of lignin, partial solvation of hemicellulose and partial decrystallization of cellulose. The rate of sugar release was high when the biomass load increases from 1% w/v to3%w/v. As the biomass concentration was increased further, the rate of sugar release decreases. When the density of biomass in the flask increases, volume of acid/ alkali present in the flask poorly wets the biomass surface and reduces the contact area between acid/alkali and biomass. This condition is called overloading of biomass and not feasible for fermentation.

Effect of Pressure cooking on release of Reducing Sugar

The pressure cooking of chemically treated biomass weekens the liginin bond, which makes acid to penetrate better into the cellulose microfibrils and hydrolyze in short period of time. The release of sugar shows an accelerating effect with pressure cooking time (Figure 1(c)). This could be due to the fact that at high temperature and pressure, the structure of the fibrils disrupts and exposes cellulose and hemicellulose to acid/alkali. This physicochemical process reduces the pretreatment time and also need for concentrated chemicals. During pressure cooking the temperature will go up to 130^{0}C and pressure up to 15psi. These parameters were kept constant and only exposure time was varied.



(a)

(b)

(c)

Figure 1: Effect of pretreatment on release of Reducing Sugar, (a) Chemical Pretreatment of KGB (b) Effect of KGB Loading (c) Effect of pressure cooking Time

Central Composite Design (CCD) for optimizing the release of Reducing Sugar

The experimental conditions for pretreatment in terms of uncoded variables, observed and predicted response of CCD are presented in Table 3. The response data obtained from the experiments were fitted to quadratic polynomial equation by least square techniques.

Table 3: Experiment Design and observed and predicted response of CCD design for optimization of release of reducing sugar

Runs	Factors			Response	
	HCl (%v/v) X_1	**KGB (%w/v) X_2**	**Pressure Cooking Time(min) X_3**	**Reducing sugar (g/L) Y**	
				Observed	**Predicted**
1	2.000000	3.000000	20.00000	3.94	3.61080
2	2.000000	3.000000	40.00000	6.6	5.63713

3	2.000000	7.000000	20.00000	11.54	10.97721
4	2.000000	7.000000	40.00000	18.04	18.41354
5	4.000000	3.000000	20.00000	6.37	5.95774
6	4.000000	3.000000	40.00000	7.19	7.71407
7	4.000000	7.000000	20.00000	8.94	9.86415
8	4.000000	7.000000	40.00000	16.74	17.03048
9	1.318207	5.000000	30.00000	8.71	9.57212
10	4.681793	5.000000	30.00000	11.19	10.38263
11	3.000000	1.636414	30.00000	3.65	4.33312
12	3.000000	8.363586	30.00000	18.99	18.36164
13	3.000000	5.000000	13.18207	4.5	4.70734
14	3.000000	5.000000	46.81793	12.59	12.43742
15	3.000000	5.000000	30.00000	9.5	11.03843
16	3.000000	5.000000	30.00000	11.44	11.03843
17	3.000000	5.000000	30.00000	10.86	11.03843
18	3.000000	5.000000	30.00000	12.33	11.03843
19	3.000000	5.000000	30.00000	11.76	11.03843
20	3.000000	5.000000	30.00000	10.35	11.03843

Among the independent variables the linear effect of KGB (X_2) and pressure cooking time (X_3) showed high significance ($p<0.05$) on sugar release (Y). However, the linear and quadratic effect on HCl and quadratic effect of biomass showed insignificant effect. In addition, the quadratic coefficient X_3X_3 and interaction coefficient X_1X_2 and X_2X_3 were also significant on release of sugar.

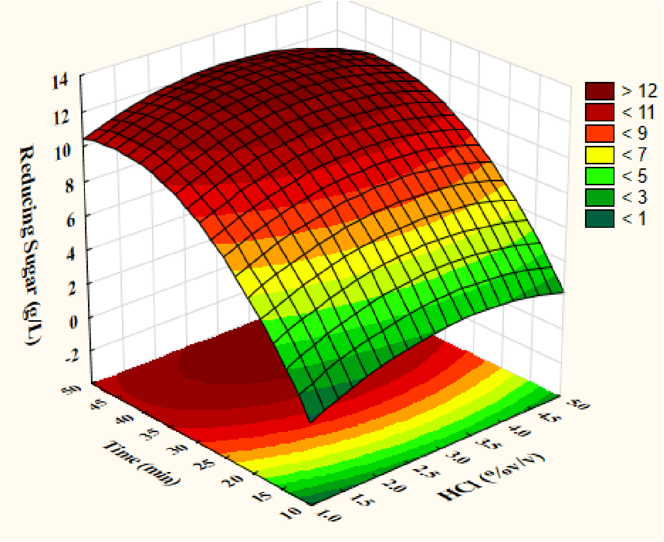
Table 4: ANOVA results for release of reducing sugar, g/L; R-Sqr=0.9706; Adj: 0.94414, 16Runs+4 Center points=20 Runs; MS Residual=1.042024

	SS	df	MS	F	p
			Independent Variables		
(1)HCL (L)	0.7930	1	0.7930	0.7610	0.403474
HCL (Q)	2.0281	1	2.0281	1.9463	0.193194
(2)BIOMASS (L)	**237.5579**	**1**	**237.5579**	**227.9773**	**0.000000**
BIOMASS (Q)	0.1719	1	0.1719	0.1650	0.693147
(3)TIME (L)	**72.1295**	**1**	**72.1295**	**69.2206**	**0.000008**
TIME (Q)	**10.9552**	**1**	**10.9552**	**10.5133**	**0.008833**
			Interactions		
1L by 2L	**5.9858**	**1**	**5.9858**	**5.7444**	**0.037524**
1L by 3L	0.0365	1	0.0365	0.0350	0.855378
2L by 3L	**14.6340**	**1**	**14.6340**	**14.0439**	**0.003799**
Error	10.4202	10	1.0420		
Total SS	354.4465	19			

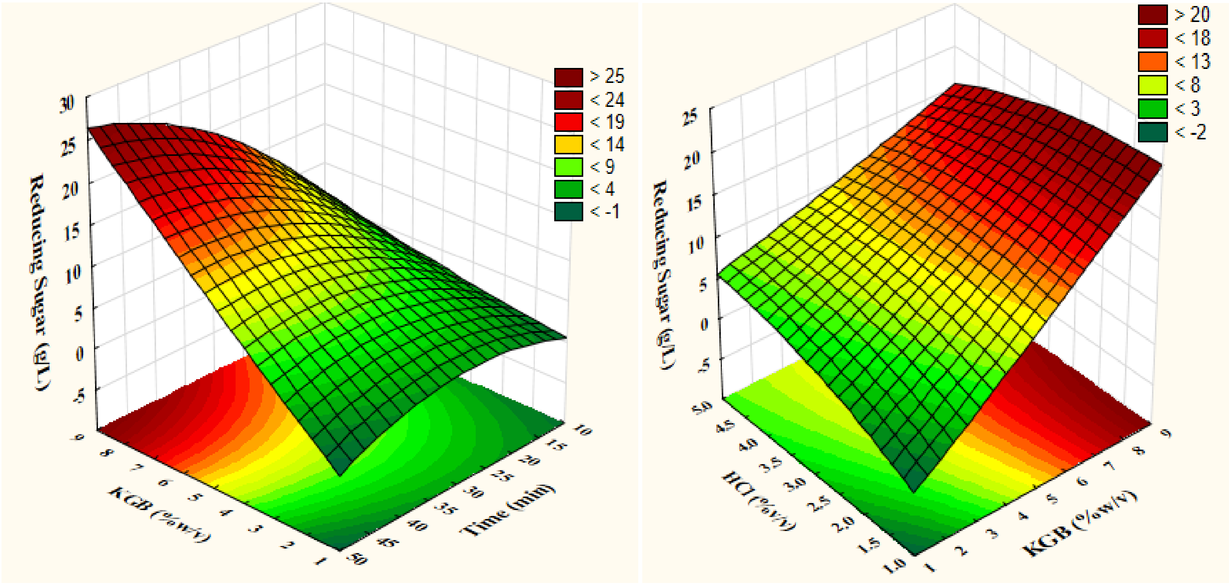
*Values less than 0.05 indicates significance at 95%confident interval

The statistical significance of the quadratic polynomial equation was done by F-test (ANNOVA). Table 4 shows that the analysis of variance (ANNOVA) for the central composite design. The result showed that the regression effect was significant ($p<0.5$). The R^2value of 0.97 obtained for present study shows that the predicted model is a good fit to the experiment data.

The response surface graph of Y as a function of concentration of HCl (X_1), KGB (X_2) and pressure cooking time (X_3 min) is shown in Figure 2(a,b,c).It was observed that, the reducing sugar concentration increases with increase in concentration of HCl and the pressure cooking time. From Figure 2(a) it is evident that, the pressure cooking time plays a key role in release of reducing sugar. Further increase in the two factors results in decrease of the reducing sugar concentration as the sugar is converted to furfural. The influence of biomass load on reducing sugar concentration was found to increase with increase in pressure cooking time (Figure 2(b)). Similar trend was observed by varying Kans grass biomass and HCl concentration (Figure 2(c)).



(a)



(b) (c)

Figure 2: 3D plot showing the effect of (a) HCl, Time (b) KGB, Time (c) HCl, KGB on reducing sugar

The regression equation for the reducing sugar (Y) as a function of concentration of HCl (X_1, %v/v) KGB (X_2, % w/v) and pressure cooking time (X_3, min) is represented by the following equation (Eq.2).

$$Y = 11.03 + 4.17X_2 + 2.29X_3 - 0.87X_3^2 - 0.86X_1X_2 + 1.35X_2X_3 \qquad (2)$$

The optimized levels of variables (X_1, X_2 and X_3) are determined by using desirability profiles for Y. The optimized factors for obtaining high yield of reducing sugar were1.32%v/v of HCl, 8.36%w/v of KGB and 46.81min of pressure cooking time.

The response of reducing sugar (Y) is assigned a desirability of 1.0 for the highest observed value of Y=18.99g/L and desirability of 0 for Y=3.65g/L to get overall desirability. The levels of variable giving the highest desirability were selected as the optimum levels for reducing sugar.

Validation of Model

The model was validated by carrying out experiment at different values of HCl concentration, biomass loading and pressure cooking time. The results were compared with the model predicted values for reducing sugar. Table 5 shows the comparison of experimental and predicted results. From the Table 5 it is thus observed that the experimental and predicted results were almost matching. For reducing sugar the strength of linear relationship between predicted and experimental values was found to be R^2=0.98. The high R-square values for HCl shows that the model is a good fit.

Table 5: Comparison of observed and predicted values of reducing sugar

HCl, (%v/v)	Biomass, (%w/v)	Time, min	Observed	Predicted
4.4	4	15	4.76	2.94
5.3	4.5	35	12.32	10.13
4.4	5.5	25	13.33	9.84
6.2	3	25	7.67	5.77
6.2	6	25	14.76	11.44
		R-Square	***=0.98***	
	At Optimum Condition			
1.32	8.36	46.	19.426	20.7399

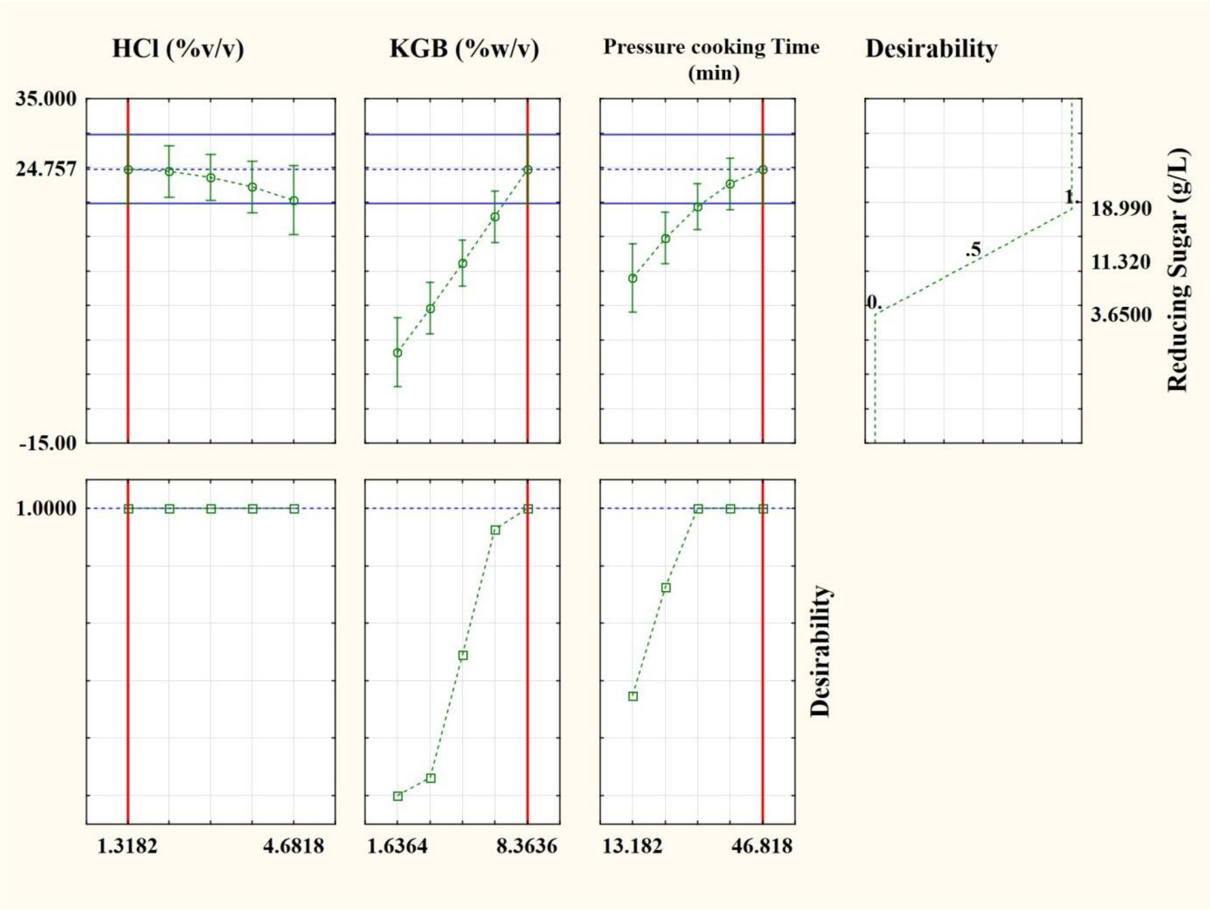


Figure 3: Profiles for desirability levels of different factors (concentration of HCl (X_1, %v/v), KGB (X_2, %w/v) and pressure cooking time (X_3 min) for the optimum reducing sugar.

CONCLUSION

S. spontaneum comprises of high concentration of cellulose that can be effectively used as a source of reducing sugar for the production of alcoholic biofuels. The optimum pretreatment condition for KGB was explored. The results obtained revealed that the pretreatment done by using HCl, releases higher concentration of reducing sugar than H_2SO_4,NaOH and Na_2CO_3 and also physicochemical method (pressure cooking of biomass)aids faster release of reducing sugar than conventional cold treatment.

The rotatable central composite design was employed to get the optimum condition for pretreatment of KGB. The optimum condition was found to be 1.32(%v/v) of HCl, 8.36(%w/v) of KGBand46.81min of pressure cooking. At these optimum conditions, the yield of reducing sugar was found to be 24.7g/L. The model equation was generated and validated with R^2=0.98. The high value of R^2 depicts the strength of linear relationship between observed and predicted reducing sugar concentration.

ACKNOWLEDGEMENT

The authors are grateful to Vision Group of Science and Technology (VGST) and Karnataka State Council for Science and Technology (KSCST) Biofuel board for providing the financial support.

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