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Dilute acid pretreatment and enzymatic hydrolysis of sorghum biomass for sugar recovery—A statistical approach

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Sorghum is one of the commercially feasible lignocellulosic biomass and has a great potential of being sustainable feedstock for renewable energy. As with any lignocellulosic biomass, sorghum also requires pretreatment which increases its susceptibility to hydrolysis by enzymes for generating sugars which can be further fermented to alcohol. In the present study, sorghum biomass was evaluated for deriving maximum fermentable sugars by optimizing various pretreatment parameters using statistical optimization methods. Pretreatment studies were done with H_2SO_4, followed by enzymatic saccharification. The efficiency of the process was evaluated on the basis of production of the total reducing sugars released during the process. Compositional analysis was done for native as well as pretreated biomass and compared. The biomass pretreated with the optimized conditions could yield 0.408 g of reducing sugars /g of pretreated biomass upon enzymatic hydrolysis. The cellulose content in the solid portion obtained after pretreatment using optimised conditions was found to be increased by 43.37% with lesser production of inhibitors in acid pretreated liquor.

Keywords: Biofuel, Cellulase, Enzymatic hydrolysis Pretreatment, Sorghum biomass.

In the year 2008, the Government of India announced its National Policy on biofuels, mandating a phase-wise implementation of the programme of ethanol blending with petrol in various states. In India, of the total available ethanol, about 45% is used for the production of potable liquor, about 40% is used in the alcohol-based chemical industry (as a solvent in synthesis of other organic chemicals) and the rest is used for blending with petrol and other purposes[1]. The demand for ethanol has been continually increasing on account of the growth of user industries and use of ethanol as a supplementary fuel in the country. However, the production and availability of ethanol has largely lagged behind. India is the fourth largest producer of ethanol in the world with approximate production of 2000 million litres mainly by fermentation of sugarcane molasses. According to a report of the Committee on Development of Biofuels, Planning Commission of India, the projected demand for ethanol for the year 2016-17 is 3785 million litres[2] and depending on molasses as the only fermentable sugar source will not be sufficient to achieve this demand. Therefore, ethanol from waste lignocellulosic biomass is the only alternative.

Using lignocellulose to produce ethanol avoids competition with the food industry, as opposed to first generation biofuels, which are directly converted from sugar and starch crops found in arable areas[3]. Lignocellulosic biomass are a potential source for bioethanol production and being an agrarian country, India has the potential for generating the required biomass. Sorghum is one of the major crops cultivated in India and among the sorghum growing countries, India ranks first in acreage but second in production. According to a report from Main Sorghum Research Station - Athwa (Navsari Agricultural University), the sorghum accounts for an area of nearly 15.8 million hectares and a production of 11.85 million tonnes in 2012 in India[4]. Sorghum is a C4 crop with high photosynthesis rate. Because of its many advantages like high energy content, drought resistance, adaptation to multiple climates and soil conditions and relatively short harvest window, sorghum proves itself as one of the potential candidates for production

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of biofuels. Pretreatment of the raw material, enzymatic hydrolysis of the pretreated raw materials into fermentable sugars and further fermentation of these sugars into ethanol are the three main steps in conversion of lignocelluloses to ethanol. Out of these, pretreatment process utilizes as much as 30% of the total ethanol production cost[5]. Current challenges in efficient and effective pretreatment process are reducing the cost (capital and operating) of the process, requirement for low particle size so as to increase surface area-to-mass ratio for maximum exposure to contact surfaces, minimizing the accumulation of inhibitory products that could interfere with the subsequent fermentation[6,7]. Different methods, like acid, alkali, organic solvent and heat treatments have been already studied for pretreatment of various biomass. Acid pretreatment is preferred here as it removes hemicellulose portion in liquid fraction after pretreatment[8]. This hemicellulose fraction can be used for production of value added products like amino acids viz lysine, 2, 3-butanediol, xylitol, lactic acid, butanol, etc.

The aim of the present study is to optimize the dilute acid pretreatment process and to study the effects of acid concentration, solid loading, temperature and time of pretreatment on the production of maximum fermentable reducing sugars (RS) after the enzymatic hydrolysis of pretreated biomass. Dilute H_2SO_4 has been chosen for study as it has several advantages like low cost and easy availability. The data of maximum sugar production with optimised pretreatment conditions may give idea about maximising ethanol production. The process was statistically optimized using response surface methology (RSM).

Material and Methods

Raw material—The sorghum biomass was received from Directorate Sorghum Research Institute, Hyderabad. The dried sorghum stalks were milled using a knife mill so as to obtain the particle size less than 10 mm. The milled samples were then mixed thoroughly, packed in air tight containers and stored at room temperature till further use.

Optimisation of dilute sulphuric acid pre-treatment—Optimisation of dilute acid pretreatment of sorghum biomass was carried out by statistical method. The experimental design and statistical analysis were performed using Minitab 15.1.1.0 (Minitab Inc USA). A three level Box Behnken factorial design was employed to optimize the combined effect of six independent variables. The parameters for pretreatment as well as enzymatic hydrolysis were optimised using a single model rather than optimising them separately. The six variables in this model were selected as: 4 for acid pretreatment and 2 for enzymatic hydrolysis. Sulphuric acid concentration (0.5-4%) (v/v), solid loading [SL(P)] (10-15%) (w/w), residence time (15-60 min) and temperature of pretreatment (120 – 200 °C) were the factors studied for acid pretreatment while enzyme concentration (6.5-30 FPU/g) and solid loading [SL(E)] (10-20%) (w/w) were studied for enzymatic hydrolysis. The range and levels of variables studied are given in Table 1. Based on the results obtained from the first model, another Central Composite Design was employed for further fine tuning of selected parameters for maximizing the sugar yield (Table 2).

Enzymatic hydrolysis of pretreated sorghum biomass—Each pretreated biomass was subjected to enzymatic hydrolysis using commercial cellulase from Zytex India Pvt. Ltd., Mumbai, India. Experiments were done in 150 mL screw capped flasks and pretreated biomass and enzyme was added as shown in the Table 1 for each run. In addition to this, the mixture contained tween 80 (0.05%, w/v) and antibiotic solution (1%, v/v) in 0.05 M citrate buffer, *p*H 4.8. The flasks were incubated at 50 °C in shaking water bath at 200 rpm. Samples were taken at 48 h of incubation, centrifuged at 10,000 rpm for 5 min and the supernatant was stored in -20 °C till further analysis.

Analytical methods—The total reducing sugar released was measured by 2, 5-dinitrosalicylic acid method[9]. The estimation was carried out in duplicates and values are reported as average values. The liquor obtained subsequent to the optimised pretreatment was analysed for quantification of component sugars as well as inhibitors by HPLC method. Sugars were analysed using Rezex RPM carbohydrate column (Phenomenex) with flow rate of 0.6 mL/min and deionised water as mobile phase, while inhibitors using organic acid column (Phenomenex) with flow rate of 0.6 mL/min and 0.01 N H_2SO_4 as mobile phase.

Characterisation of native and pretreated biomass

Composition analysis—The composition analysis of sorghum, native as well as pretreated one, was carried out as per the NREL protocols with slight modifications[10]. Briefly, the biomass was hydrolysed

Table 1—Reducing sugar yields for individual runs of the RSM design (Box Behnken)

Run order	Acid concentration (% v/v)	SL (P) (% w/w)	Temp (°C)	Residence time (min)	SL (E) (% w/w)	Enzyme conc (FPU/g)	Conc of reducing sugar (mg/g)
1	2.25	15	160	37.5	20	15	268.87
2	4	10	160	15	15	13	343.93
3	2.25	12.5	160	37.5	15	13	11.91
4	4	12.5	160	15	20	10	249.03
5	2.25	10	120	37.5	20	10	253.81
6	2.25	12.5	120	60	15	20	401.29
7	2.25	12.5	160	37.5	15	13	377.06
8	0.5	12.5	200	37.5	15	20	355.04
9	2.25	12.5	200	60	15	20	30.61
10	2.25	15	160	37.5	10	30	366.89
11	2.25	10	120	37.5	10	20	337.80
12	0.5	12.5	200	37.5	15	6.5	304.90
13	2.25	15	120	37.5	10	20	373.82
14	2.25	15	120	37.5	20	10	256.28
15	4	12.5	200	37.5	15	6.5	4.83
16	0.5	12.5	160	15	10	20	347.93
17	4	10	160	60	15	13	260.41
18	2.25	15	200	37.5	10	20	16.60
19	0.5	12.5	120	37.5	15	20	270.83
20	2.25	10	200	37.5	10	20	18.67
21	2.25	15	160	37.5	20	5	64.71
22	2.25	10	200	37.5	20	10	16.31
23	2.25	12.5	120	15	15	6.5	295.24
24	0.5	12.5	160	60	10	20	389.58
25	2.25	12.5	120	15	15	20	537.54
26	4	12.5	200	37.5	15	20	23.71
27	2.25	10	160	37.5	10	30	352.40
28	4	15	160	15	15	13	454.37
29	2.25	12.5	160	37.5	15	13	358.96
30	0.5	10	160	15	15	13	327.36
31	2.25	12.5	200	15	15	20	40.97
32	4	12.5	160	60	20	10	205.03
33	2.25	15	160	37.5	10	10	300.80
34	2.25	12.5	160	37.5	15	13	331.35
35	0.5	12.5	160	60	20	10	375.47
36	0.5	12.5	160	15	20	10	251.62
37	4	12.5	120	37.5	15	6.5	295.24
38	2.25	10	160	37.5	20	5	158.79
39	2.25	10	160	37.5	10	10	289.41
40	2.25	12.5	160	37.5	15	13	345.85
41	0.5	10	160	60	15	13	343.93
42	4	12.5	120	37.5	15	20	32.89
43	2.25	15	200	37.5	20	10	36.07
44	2.25	10	160	37.5	20	15	300.45
45	2.25	12.5	200	15	15	6.5	15.98
46	0.5	15	160	60	15	13	383.81
47	4	12.5	160	15	10	20	327.45
48	2.25	12.5	200	60	15	6.5	6.18
49	2.25	12.5	120	60	15	6.5	335.97
50	2.25	12.5	160	37.5	15	13	519.11
51	4	12.5	160	60	10	20	287.88
52	0.5	15	160	15	15	13	365.18
53	4	15	160	60	15	13	27.63
54	0.5	12.5	120	37.5	15	6.5	462.29

with 64% (v/v) H_2SO_4 until complete dissolution of the sample had occurred. Cellulose and hemicelluloses fractions were analysed from liquid portion by HPLC using Rezex RPM carbohydrate column (Phenomenex) with flow rate of 0.6 mL/min and deionised water as mobile phase. The lignin and ash was estimated from solid portions.

Results and Discussion

Optimisation of pretreatment of sorghum— Pretreatment is the prior step to the enzymatic hydrolysis. In the present study both the pretreatment and enzymatic hydrolysis were optimized in a single experimental model so as to find the possible optimum conditions among the combinations of independent variables. Many different methods have been studied for pretreatment of sorghum biomass such as microwave pretreatment[11], acid pretreatment[12], alkali pretreatment[13] and dilute ammonia pretreatment[14]. Pretreatment with mineral acids lead to hydrolysis of hemicelluloses which results in the reducing sugars being available in the pretreatment liquor[8]. In the present study, dilute acid pretreatment is preferred as hemicellulose fraction obtained in liquid fraction after pretreatment can be used for production of value added products like amino acids.

Initially, various acids such as H_2SO_4, HCl and HNO_3 were tried as pretreatment reagent at 2% (v/v) with biomass loading 5% (w/w) and pretreatment temperature at 120 °C for 30 min. Among these reagents, H_2SO_4 was found to be the best based on the reducing sugar yield (data not shown). Hence further pretreatment process was optimised with dilute H_2SO_4.

A Box Behnken design was used for the optimization of parameters for pretreatment and enzymatic hydrolysis to achieve maximum reducing sugar yield. The 54 experiments were carried out as per the design and the results were analysed. Response surface curves were plotted to find out the interaction of variables and to determine the optimum level of each variable for maximum response. The surface plots showing the interaction between a pair of factors on reducing sugar yield are given in Fig. 1.

The effect of solid loading in pretreatment and acid concentration on sugar yield is shown in Fig. 1a. At

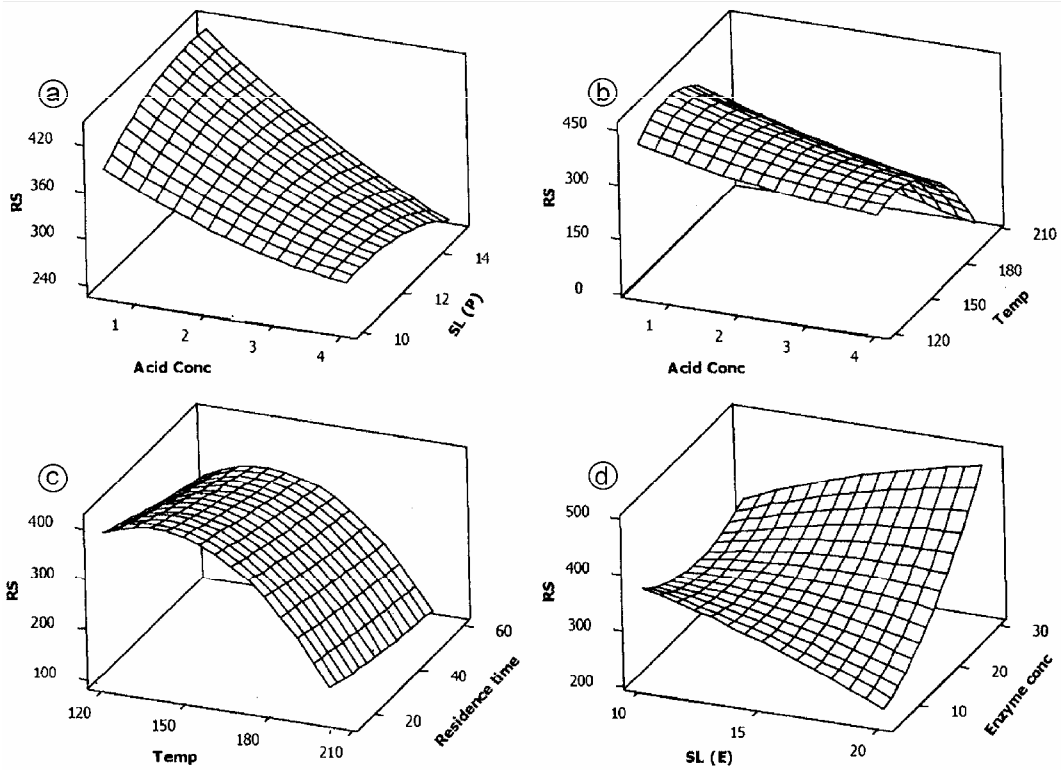

Fig. 1—Interaction plots of Box Behnken design for effect of various parameters on reducing sugar (RS) yield, (a) Surface plot on effect of acid concentration and pretreatment biomass loading SL(P), (b) Surface plot on effect of acid concentration and temperature, (c) Surface plot on effect of residence time and temperature, (d) Surface plot on effect of enzyme concentration and solid loading SL(E) during enzymatic hydrolysis.

biomass loading of 11-14% and acid concentration of 0.5-1%, maximum reducing sugar yield was observed (>0.4g/g). Further increase in biomass loading did not show any further increase in the sugar level. For pretreatment, an optimal substrate concentration is required and any further increase in substrate concentration results in lesser availability of moisture which at high temperature results in charring of the substrate.

Figure 1b shows reducing sugar yield as a function of acid concentration and pretreatment temperature. It was observed that at high acid concentration the reducing sugar yield was less. Maximum reducing sugar yield was observed at 1% (v/v) H_2SO_4 concentration. The lesser yield of sugars at higher acid concentration might be due to the formation of inhibitors.

Pretreatment temperature and residence time have a significant impact on sugar yield. The study showed that the increase of pretreatment temperature beyond 150 °C has a negative impact on sugar yield. It was also observed that the residence time should be less (15 min) for maximizing the sugar yield (Fig. 1c).

Figure 1d shows the interaction between enzyme concentration and solid loading during enzymatic hydrolysis. It was found that lower solid loading is favourable for enzymatic hydrolysis. At high solid loading, the amount of available free water becomes less, which in turn decreases the hydrolysis efficiency. High biomass loading is associated with difficulties in mixing as well as end-product inhibition.

From the optimisation plot (Fig. 2), it was observed that there is a linear decrease in residual sugar as acid concentration was increased from 0.5 to 4%. In case of solid loading during hydrolysis as well as pretreatment process, a linear rise in reducing sugar was observed as the solid loading was increased. A highest value of reducing sugar was obtained at the temperature of 150 °C. At lower temperatures, pretreatment was not that effective and at

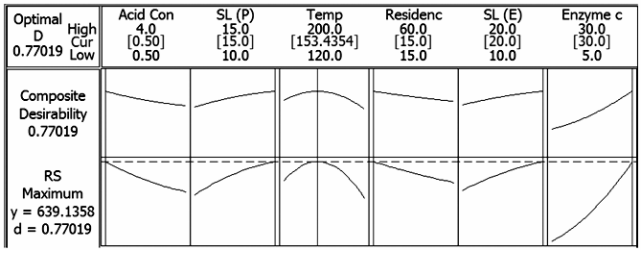

Fig. 2—Optimization plot obtained from analysis of individual run from Box Behnken model.

temperatures higher than 150 °C the sugars may have been degraded further to furfurals and other toxic compounds. A linear decrease in reducing sugar was observed for increase in residence time of pretreatment. High concentration of acid and high temperature of pretreatment within short time produced better hydrolysis yield[15,16]. In contradiction, maximum production of sugars was obtained at moderate conditions like medium temperature, low acid concentration and less time.

There was a linear increase in reducing sugar yield as the enzyme concentration was increased from 5 to 30 FPU/g of substrate. The results from this study were consistent with Jeya *et al*[17]. From the optimisation plot, the optimum saccharification condition was 20% (w/w) of the acid pretreated sorghum and 30 FPU/g-substrate of cellulase at 50 °C to get the maximum yield of sugar. It is a well known that as enzyme concentration increases, the hydrolysis yields will also increase[18, 19]. But, the cost of enzyme being one of the bottlenecks in the process, it should be as minimum as possible so as to get economically viable process. A high saccharification rate should be achieved at minimum enzyme concentration over short times. So, for the rest of process optimisation, the hydrolysis conditions were maintained as 20 FPU/g substrate of enzyme with 10% solid loading.

From the model, the optimised pretreatment conditions obtained were 15% (w/w) biomass loading with 0.5% (v/v) H_2SO_4 for 15 min at 150 °C to get maximum yield of RS. The optimum H_2SO_4 concentration obtained, i.e. 0.5%, was the lowest value of acid concentration used in this model. Therefore, the range of acid concentration below 0.5% needs to be screened. Also, the coefficient of determination (R^2) value obtained for this model was comparatively low i.e. 65.52% with composite desirability (D value) 0.779. Therefore, another model was designed where only two variables i.e. solid loading and acid concentration for pretreatment were optimised (Table 2). The surface plot of sugar yield based on acid concentration and solid loading shows that as the acid concentration decreases, solid loading can be further increased (Fig. 3). As shown in Fig. 4, the optimised conditions predicted with this model were 0.37% (v/v) acid concentration and 16% solid loading to get 0.325 (g/g) predicted yield, while the actual obtained yield was 0.408 (g/g) which shows 66.66% efficiency (considering theoretical maximum yield .i.e. 0.612 g/g). The significance of each of the

Table 2—Reducing sugar yields for individual runs of the CCD model

Run order	Acid concentration (% v/v)	SL (P) (% w/w)	Conc of reducing sugar (mg/g)
1	2.5	12.5	250.23
2	2.5	8.96	258.44
3	2.5	12.5	240.42
4	2.5	12.5	242.02
5	0.37	12.5	267.51
6	2.5	16.03	284.42
7	1	15	302.31
8	1	10	260.40
9	4.62	12.5	207.58
10	4	10	221.18
11	2.5	12.5	245.94
12	4	15	245.82
13	2.5	12.5	246.80

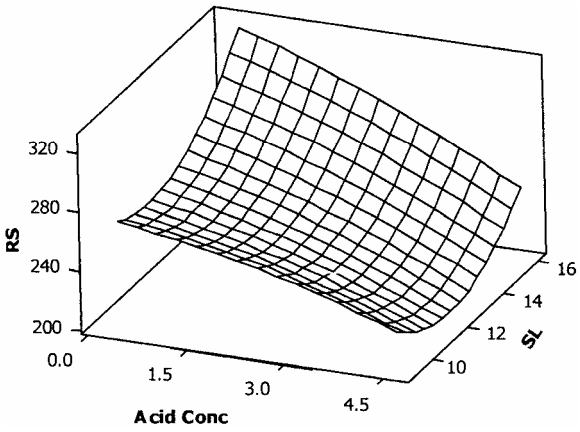

Fig. 3—Interaction between acid concentration and solid loading (SL) in pretreatment for reducing sugar yield (RS) from CCD model.

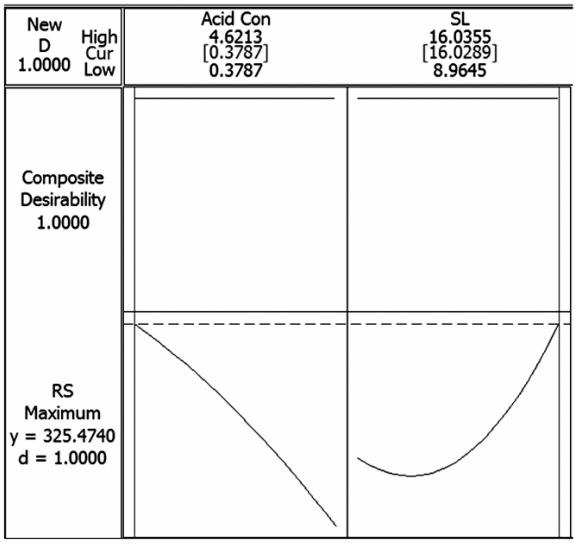

Fig. 4—Optimization plot obtained from analysis of individual run from central composite design

coefficients was checked by P-value. The regression coefficient for reducing sugar yield was found to be best with both acid concentration and solid loading with P-value 0.000 for both of them. The coefficient of determination (R^2) was found to be 97.16% and 1.00 was the composite desirability (D value) observed with this model, which proves the significance of the model (Table 3). From the above total study, the optimum pretreatment conditions obtained were 16% of sorghum biomass, 0.37% H_2SO_4 at 150 °C for 15 min.

The yield of total reducing sugars obtained here (0.408 (g/g) of sorghum) is comparatively higher than the other pretreatment methods tried earlier with the sorghum biomass. A report by Phuengjayaem *et al*[20] , where the pretreatment conditions for sorghum biomass employed were 3% dilute H_2SO_4 with 10% of solid loading pretreated at 120 °C for 10 min and saccharification conditions were 30 FPU/g substrate, 2.5% loading, at 40 °C and *p*H 4 in 96 h gave the total sugar of 0.366 (g/g) of sorghum biomass. Choudhary *et al.*[11] reached sugar concentration 0.398 (g/ g) bagasse from microwave pretreated sweet sorghum bagasse followed by enzymatic saccharification. Ban *et al.*[21] reported the largest yield of reducing sugar of 0.3024 (g/g) of sorghum with phosphoric acid pretreatment at concentration 80 g/L, temperature 120 °C, 10 % solid loading and 80 min time.

Increase in the temperature and acid concentration during pretreatment leads to increased formation of inhibitors, such as acetic acid, formic acid, furfural and 5-hydroxymethyl furfural (HMF) in pretreatment liquor[22]. However, the concentrations of the inhibiting compounds detected in liquor, in the present study, were lower than those reported to inhibit the bioconversion of lignocellulosics to ethanol[23]. The concentrations of inhibitors were measured in pretreatment liquor obtained from the optimum pretreatment conditions (0.37% v/v acid; 150 °C; 15 min at 16% w/w sorghum loading) and found to be 4.32, 2.46, and 0.66 g/L for acetic acid, formic acid and HMF, respectively. These pretreatment conditions are thus giving rise to formation of inhibitors in low concentrations and are, therefore, advantageous as the liquor obtained can be further used for production of value added products.

Analysis of sugars in the liquor obtained after pretreatment with optimized condition revealed the release of the total sugar 53.634±0.1 mg/g of sorghum biomass during pretreatment. Out of this total sugar, xylose was contributing the major fraction i.e.

Table 3—Estimated regression coefficients and analysis of variance for reducing sugar yield for CCD model

Term	Coef	SE Coef	T	P
Constant	245.082	2.418	101.351	0.000
Acid conc.	-22.558	1.912	-11.800	0.000
SL	12.911	1.912	6.754	0.000
Acid conc.*Acid conc.	-3.034	2.050	-1.480	0.182
SL*SL	13.909	2.050	6.785	0.000
Acid conc.*SL	-4.318	2.704	-1.597	0.154

R-Sq = 97.16% R-Sq(pred) = 84.50% R-Sq(adj) = 95.12%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	6991.22	6991.22	1398.24	47.82	0.000
Linear	2	5404.53	5404.53	2702.27	92.43	0.000
Square	2	1512.12	1512.12	756.06	25.86	0.001
Interaction	1	74.56	74.56	74.56	2.55	0.154
Residual error	7	204.66	204.66	29.24		
Lack-of-Fit	3	143.36	143.36	47.79	3.12	0.150
Pure Error	4	61.30	61.30	15.32		
Total	12	7195.88				

Table 4—Comparative compositional analysis of native and acid pretreated sorghum

Type of biomass	Composition analysis (%)			
	Cellulose	Hemicellulose	Lignin	Others
Native Sorghum	39.57	13.63	20.63	26.30
Acid pretreated Sorghum (Optimised conditions)	56.73	8.96	30.21	6.92

42.736±0.067 mg/g while rest were 10.436±0.004 mg/g glucose, 0.459±0.002 mg/g cellobiose and 0.003±0.000 mg/g arabinose. It is apparent from these results that the dilute H_2SO_4 pretreatment was more directed to hemicellulose hydrolysis into pentose sugars, while the cellulose fraction was least affected. This condition is also idyllic for pretreatment as cellulose is intact in pretreated solid fraction which will be available as hexose sugars after saccharification.

Compositional analysis of native and pretreated sorghum biomass—Chemical compositional analysis of lignocellulosic biomass is important in order to know the percent constituent polymers (cellulose, hemicelluloses and lignin) present in them. It may provide an insight into the yield of bioethanol depending on the percentage of these polymers. The comparison of percentages of the different polymers in both native as well as acid pretreated sorghum is shown in Table 4. From this data, it can be concluded that the changes occurring in composition of sorghum during pretreatment were desirable as the cellulose percentage was found to be increased in pretreated one. The dilute sulfuric acid pretreatment in sorghum biomass exhibited the augmentation of cellulose composition by 43.37%, while the hemicellulose composition decreased by 34.26% as compared to native sorghum biomass.

Conclusion

The optimised pretreatment conditions for dilute acid pretreatment of sorghum biomass were 0.37% (v/v) H_2SO_4 with 16% (w/w) loading of sorghum at 150 °C for 15 min. Total reducing sugars reached the maximum of 0.408 (g/g) after 48 h of enzymatic hydrolysis at 20 FPU/g of solid at 10% loading. The dilute acid pretreatment partially removed hemicellulose which led to increase the total hydrolysis efficiency up to 66.74%. Dilute H_2SO_4 pretreatment in sorghum biomass exhibited the increase in cellulose composition by 43.37%, while the hemicellulose composition decreased by 34.26% in pretreated sorghum biomass. The optimised pretreatment process is effective and efficient because of mild conditions, directed hemicellulose hydrolysis and low inhibitor production during pretreatment and thus, eventually leading to higher efficiency of process. The process will also be cost effective as the residence time of pretreatment process is comparatively less.

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