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Explore the Perennial Kans grass (*Saccharum spontaneum*) Biomass for Releasing Reducing Sugars and its Optimization

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ABSTRACT

Kans grass (Saccharum spontaneum) is a perennial grass native to South Asia. It is a low cost, renewable and prevalent source of reducing sugars which can further be easily utilized by the microorganisms to produce valuable chemicals. The objective of the present study was to investigate the effect of H_2SO_4 concentration, biomass loading and reaction time for releasing reducing sugars from Kans grass. A 2^3 rotatable central composite design was adopted in designing the experiments and response surface methodology was used to optimize the process. The optimum acid concentration, biomass loading and reaction time were found to be 61.10% (w/w), 10.80% (w/v) and 45 min respectively. The batch processing of all the experiments were carried out at normal boiling temperature of water under standard atmospheric pressure. Under these conditions significantly very high total reducing sugar yield was 83.5% (w/w) obtained on the total carbohydrate content basis.

Keywords: Lignocellulose, batch processing, hydrolysis, bioconversion, optimization, response surface methodology.

INTRODUCTION

The largest renewable resource of carbohydrates in nature is lignocellulosic biomaterials. Lignocellulosic biomass is less expensive and more abundant than starch or sucrose containing feedstocks. Lignocellulosic biomaterials are grouped into different categories according to source, such as forest biomass (hard wood, soft wood, saw dust), agricultural residues (corn stover, cob, wheat straw, rice straw, oat hull, sugar bagasse, cotton stalk), herbaceous grass (switch grass, bermuda grass, alfalfa fiber, rye grass) and municipal waste (waste paper, waste food, paper mill sludge) [1]. Besides these lignocellulosic materials some free-floating aquatic

plants like water hyacinth, water lettuce have also been reported for good carbohydrate content [2,3]. Lignocellulosic biomass is composed of majorly carbohydrate polymers (cellulose and hemicellulose), relatively much lesser quantity of lignin and a small fraction of other compounds like extractives, acids, salts and minerals. The cellulose and hemicellulose, which typically comprise two thirds of the dry mass, are polysaccharides that can be hydrolysed to sugars and eventually be converted to valuable products mainly via fermentation [4]. The main products obtained from this route include enzymes, furfural, amino acids, organic acids (e.g. lactic acid, succinic acid), biohydrogen, methane and bioethanol [5]. Bioethanol produced from various lignocellulosic materials is a renewable fuel, is becoming increasingly important today as a consequence of greater concern for the increasing greenhouse effect, depleting oil reserves, and rising oil prices [6,7,8].

Among the various lignocellulosic biomaterials perennial crops (e.g. grasses) are promising feedstock because of high yields, low costs, and good suitability for low-quality land with almost no requirement of water supply for its growth and its availability throughout the year [9]. Kans grass (*Saccharum spontaneum*) having all these properties, was selected as the lignocellulosic raw material for the present work. Kans grass is a perennial grass, native to South Asia and occurs throughout India along the sides of the river. It grows up to three meters in height, with spreading rhizomatous roots. In the Terai-Duar savanna and grasslands, a lowland eco region at the base of the Himalaya range in Nepal, India, and Bhutan, Kans grass quickly colonises exposed silt plains created each year by the retreating monsoon floods, forming almost pure stands on the lowest portions of the floodplain. It is self-seeding, resistant to many diseases and pests, and can produce high yields with low applications of fertilizer and other chemicals. It is also tolerant to poor soils, flooding, and drought; improves soil quality and prevents erosion due to its type of root system. It uses less water per gram of biomass produced than other plants [10]. These characteristics makes Kans grass biomass (KGB) a novel substrate with great potential for the production valuable products.

The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds and also by some covalent bonds. The pretreatment/hydrolysis processes are used for delignification to liberate cellulose and hemicellulose from their complexes with lignin and depolymerization of the carbohydrate polymers to produce free sugars. Various pretreatment options are available now to fractionate, solubilize, hydrolyze and separate cellulose, hemicellulose, and lignin components. These include dilute acid [11], SO_2 [12], hydrogen peroxide [13], steam explosion (autohydrolysis) [14], ammonia fiber explosion (AFEX) [15], wet-oxidation [16], lime [17], liquid hot water [18], CO_2 explosion [19] and organic solvent treatments [20]. The existing pretreatment/hydrolysis technologies suffer from low sugar yields, and/or severe reaction conditions, and/or narrow substrate applicability and high capital investment etc. These are the greatest technical and economic barriers for effectively releasing protected polysaccharides from the complex lignocellulosic biomaterials to fermentable sugars [21].

The concentrated sulfuric acid treatment for the hydrolysis of lignocellulosic biomass has the advantages over other methods like (1) no enzymes are required; (2) broad range of biomass types can be used; (3) pretreatment at moderate temperature allows the use of plastic construction materials (this is in contrast with dilute acid pre-treatment carried out at temperatures near 180 ºC and high pressure); (4) the low temperature used limits the production

of inhibitory compounds such as furfural [22]. To our knowledge, there is no report on concentrated acid hydrolysis of Kans grass (*Saccharum spontaneum*) biomass. The aim of the present investigation was to determine the effect of acid concentration, biomass loading and reaction time on the release of reducing sugars (glucose and xylose) from KGB. To maximize the formation of reducing sugars in hydrolysate optimization process was followed using response surface methodology (RSM) with central composite as statistical design.

RSM is a collection of statistical and mathematical methods that are useful for the modeling and analyzing engineering problems. In this technique, the main objective is to optimize the response surface that is influenced by various process parameters. RSM also quantifies the relationship between the controllable input parameters and the obtained response surfaces. The application of experimental design and response surface methodology in bioprocesses can result in improved product yields, reduced process variability and development time and overall costs. The central composite design (CCD) is the most popular of the many classes of RSM designs and chosen for the present work due to some of its properties like; a CCD can be run sequentially, it can be naturally partitioned into two subsets of points; the first subset estimates linear and two-factor interaction effects while the second subset estimates curvature effects, it is very efficient, providing much information on experiment variable effects and overall experimental error in a minimum number of required runs and it is very flexible. This technique was successfully used by many workers e.g. Rodrigues et al for xylitol production [23], Baskar et al for L-asparaginase production [24], Scordia et al for bioethanol production [25] etc.

MATERIALS AND METHODS

Raw material

The sample of KGB was collected from the side of the Gang Nahar in Jwalapur (about 20 km from Roorkee), Haridwar (Uttarakhand), India. The sample was chopped in small pieces and air dried. After six days of air drying sample was screened to select the fraction of particles with the size between 2.36 to 5.60 mm and homogenized in a single lot. The homogenized KGB was then oven dried at 70^{o}C for overnight and was analyzed by using standard methods for determination of its main composition.

Acid hydrolysis

Acid hydrolysis experiments of KGB were carried out in Erlenmeyer flasks as per the experimental plan. The experimental range and levels of independent variables i.e. acid concentration (A), biomass loading (B) and reaction time (C) are laid down in **Table 1**. Operating temperature was kept constant for all experiments at 100°C i.e. normal boiling temperature of water at standard atmospheric condition. All the experiments were carried out in triplicate and mean of three readings were taken as result for a particular experiment.

Table 1: Experimental range and levels of independent variables

Independent variables	Symbol	Range and levels				
		-α	-1	0	+1	+ α
Acid Concentration, % (w/w)	A	33.18	40	50	60	66.82
Biomass loading, % (w/v)	B	1.59	5	10	15	18.41
Reaction time (min)	C	9.55	30	60	90	110.45

Analytical methods

The main constituents of KGB were determined by the standard methods. Cellulose content was estimated using semimicro determination method of Updegraff [26]. This is a simple and rapid method for cellulose estimation and is based on colored reaction between cellulose and cold anthrone on heating. Pentose sugar analysis was carried out method proposed by Roe and Rice [27]. The method is based on the formation of furfural from pentoses in acetic acid containing thiourea (an anti-oxidant) at 70°C and the reaction of furfural with p-bromoaniline acetate to form a pink colored product. Modified Klason Lignin method ASTM D1106-96 was used for determination of lignin content. Estimation of ash content was done by ASTM E1755-01. Total reducing sugar (TRS) was estimated by using dinitrosalicylic acid (DNS) reagent [28].

Experimental design and RSM

In the experimental plan, response surface methodology (RSM) was utilized to optimize the hydrolysis process and a 2^3 rotatable central composite design (CCD) was adopted in order to fit a second order model and the design consisted of 20 set of experiments. It included eight experiments for factorial portion (2^k=8, where k is the number of independent variables, 3 in this case), six experiments for axial points (2k=6) and six replications of the center point used to check the reliability of the data for lack of fit test [29]. The second order model was selected for predicting the optima point and expressed as

$$Y=\beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} A B + \beta_{13} A C + \beta_{23} BC \quad (1)$$

where, Y represents response variable i.e. total reducing sugar in % (g/g total carbohydrate content, TCC). β_0 is offset value, β_1, β_2 and β_3 are coefficients of linear terms, β_{11}, β_{22} and β_{33} are coefficients of quadratic terms and β_{12}, β_{13} and β_{23} are coefficients of interactive terms. The effect of variables, acid concentration (A), biomass loading (B) and reaction time (C) were studied on total reducing sugar generation. Regression analysis and graphical analysis were performed using Design Expert v.8.0 (Stat-Ease Inc. Minneapolis) software.

RESULTS AND DISCUSSION

Table 2: Main components of Kans grass (*Saccharum spontaneum)* (on oven-dry basis)

S. no.	Chemical component	% (dry weight basis)
1	Cellulose	43.78±0.4
2	Hemicellulose	24.22±0.5
3	Acid insoluble lignin	23.45±0.3
4	Acid soluble lignin	2.85±0.4
5	Ash	4.62±0.2

Composition of Kans Grass *(Saccharum spontaneum)*

The compositional analysis of KGB was carried out for determination of principal components (% cellulose, % hemicelluloses, % insoluble lignin, % soluble lignin and % ash) using standard methods on the dry weight basis. The chemical composition of KGB is given in **Table 2**. The total carbohydrate content (TCC) of the KGB (i.e. cellulosic and hemicellulosic fraction of the lignocellulosic material) was estimated as 68% on dry weight basis. Other extensively explored potential sugar containing lignocellulosic materials like sugarcane bagasse, 67.15%; corn stover, 58.29%; wheat straw, 54% and sorghum straw, 61% for ethanol production [30] are also

comparable reported data on total carbohydrate content basis of high sugar containing lignocellulosic biomaterials.

Statistical modeling

According to the experimental plan, range and levels of independent variables, acid concentration (A), biomass loading (B) and reaction time (C) studied for the hydrolysis of KGB are shown in Table 1. The value of α was calculated as 1.682 where $\alpha = 2^{k/4}$, (k=3, the number of variables). The coded values of all independent variables and the experimental value of the only response variable Y (% g/g) along with predicted values are presented in **Table 3** where Y was defined by [(g of total reducing sugar obtained/ g of total carbohydrate present initially) x 100]. The coefficients were calculated by using Design Expert v.8.0.

The quadratic model in terms of coded variables was found as

$$Y=+73.98+15.38A+1.22B-6.19C-7.29A^2-7.64B^2-7.55C^2+1.25AB-1.25AC+0.25BC \quad (2)$$

Table 3: Central composite design consisting of 20 experiments with the experimental and predicted response

Run no.	Coded values of the variables			Response (Y) total reducing sugar % (w/w of TCC)	
	A	B	C	Experimental value	Predicted value
1	-1	-1	-1	42.00	41.32
2	1	-1	-1	72.00	72.09
3	-1	1	-1	40.00	40.77
4	1	1	-1	78.00	76.54
5	-1	-1	1	30.00	30.95
6	1	-1	1	58.00	56.72
7	-1	1	1	32.00	31.40
8	1	1	1	62.00	62.17
9	-1.682	0	0	28.00	27.49
10	1.682	0	0	78.00	79.23
11	0	-1.682	0	50.00	50.30
12	0	1.682	0	54.00	54.42
13	0	0	-1.682	62.50	63.02
14	0	0	1.682	42.00	42.21
15	0	0	0	76.00	73.98
16	0	0	0	72.00	73.98
17	0	0	0	76.00	73.98
18	0	0	0	74.00	73.98
19	0	0	0	72.00	73.98
20	0	0	0	74.00	73.98

To fit the response function and experimental data, regression analysis was performed and second order model for the response (Y) was evaluated by ANOVA which is presented in **Table 4**. The regression for the response was statistically significant at 95% of confidence level. The Model F-value of 264.24 and low %CV value of 2.67 implies the model is highly significant. A poor F-value, 0.53 of the lack of fit test (Table 4) confirming the reliability of the experimental data. The "Pred R-Squared" of 0.9838 is in reasonable agreement with the "Adj R-Squared" of 0.9920. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The very high signal to noise ratio of 46.745 indicates that the chance of the values could be due to noise is very less. The goodness of fit of the model was checked by the determination

coefficient (R^2). In this case, the value of the determination coefficient (R^2=0.9958) indicates that only 0.42% of the total variations are not explained by the model.

Table 4: Analysis of variance (ANOVA) for total reducing sugar yield

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value Prob > F
Model	5827.93	9	647.55	264.24	< 0.0001
A-Acid conc.	*3231.91*	*1*	*3231.91*	*1318.83*	*< 0.0001*
B-Biomass loading	*20.49*	*1*	*20.49*	*8.36*	*0.0161*
C-Time	*522.55*	*1*	*522.55*	*213.23*	*< 0.0001*
AB	*12.50*	*1*	*12.50*	*5.10*	*0.0475*
AC	*12.50*	*1*	*12.50*	*5.10*	*0.0475*
BC	*0.50*	*1*	*0.50*	*0.20*	*0.6611*
A^2	*765.67*	*1*	*765.67*	*312.44*	*< 0.0001*
B^2	*841.75*	*1*	*841.75*	*343.49*	*< 0.0001*
C^2	*822.39*	*1*	*822.39*	*335.59*	*< 0.0001*
Residual	24.51	10	2.45		
Lack of Fit	*8.51*	*5*	*1.70*	*0.53*	*0.7476*
Pure Error	*16.00*	*5*	*3.20*		
Cor Total	5852.44	19			

The interactive behavior of the variables involved on the total reducing sugar release from KGB is given in Figure 1, 2 and 3. The highly parabolic nature of the contours in Figure 1 (interaction between acid concentration and biomass loading) and Figure 2 (interaction between acid concentration and reaction time) states that total reducing sugar production is highly dependent on the interactive behavior on the respective parameters.

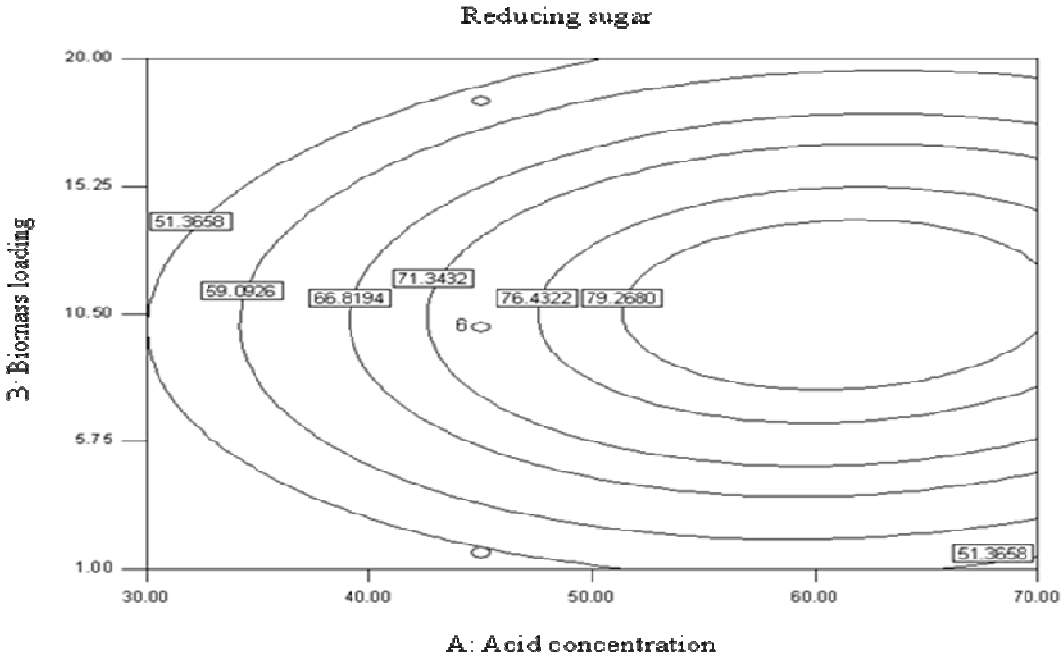

Figure 1. Effect of acid concentration and biomass loading on the release of total reducing sugar from Kans grass (*Saccharum spontaneum*) biomass, reaction time 60 min.

The interaction between H_2SO_4 concentration and biomass loading on reducing sugar release from KGB while reaction time was selected as 60 min is shown in **Figure 1**. From the figure and experimental data it can be interpreted that maximum and minimum reducing sugar yield of 78% and 28% can be obtained by conducting hydrolysis experiment for 10% biomass loading and 70.23% and 19.77% acid concentration respectively.

The effect of acid concentration and reaction time on reducing sugar release is shown in **Fig 2**, when biomass loading was selected at 10% as the centre point. From the figure and experimental data it is evident that as the acid concentration increases, the response also increases significantly but when the time increases, response decreases because some amount of sugar degraded into furfural and hydroxymethyl furfural.

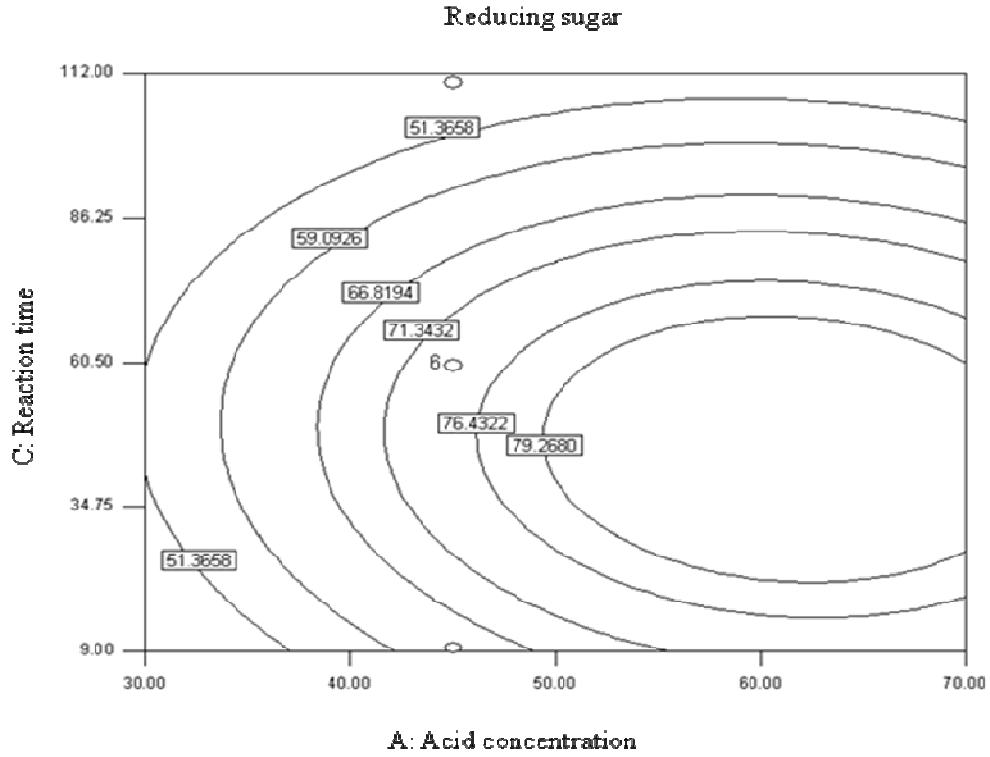

Figure 2. Effect of acid concentration and reaction time on the release of total reducing sugar from Kans grass (*Saccharum spontaneum*) biomass, biomass loading 10%.

Interaction between biomass loading and reaction time is shown in **Fig 3**, while acid concentration was kept constant at 45%. More circular nature of contours shown in the figure signifies that the production of reducing sugars is least dependent on interactive behavior of these two parameters.

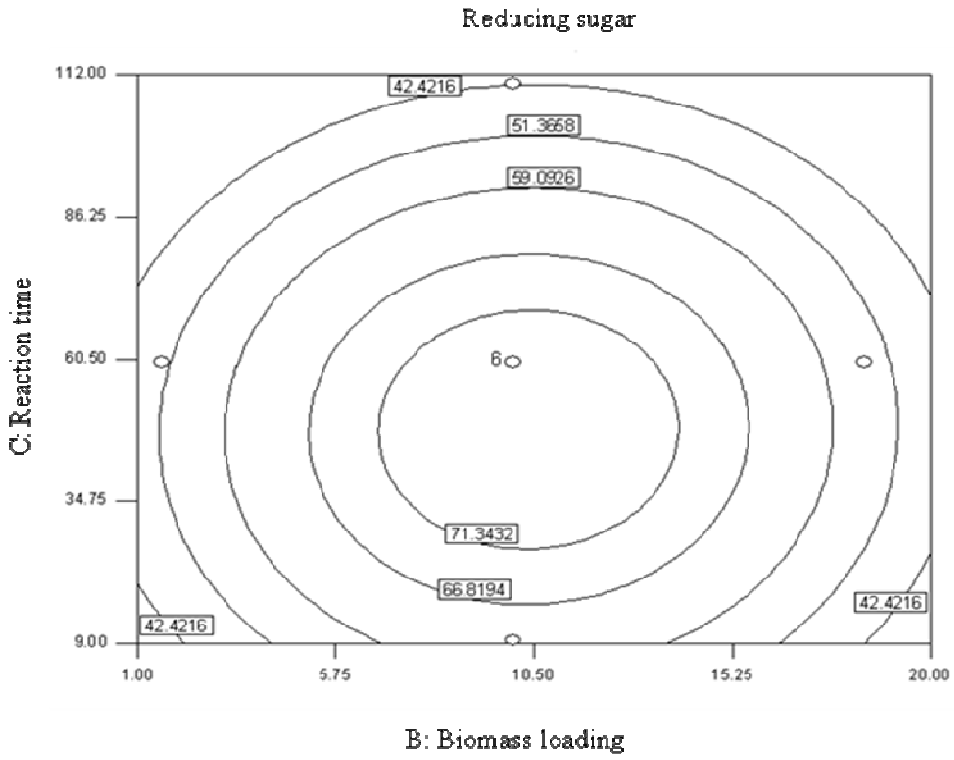

Figure 3. Effect of biomass loading and reaction time on the release of total reducing sugar from Kans grass (*Saccharum spontaneum*) biomass, acid concentration 45%.

On the basis of model, the optimum values of the parameters were calculated by setting the first order derivatives of the equation (2) (dY/dA , dY/dB and dY/dC) as zero. The optimum values of the variables A, B and C in coded and actual form thus obtained are given in **Table 5**.

Table 5: Solution for optimum condition

Parameters	Coded value	Actual Value
Acid concentration % (g/g)	+1.11	61.10
Biomass loading % (g/ml)	+0.16	10.80
Reaction time (min)	-0.50	45.00

Validation of model and significance of study

Three sets of experiments were conducted at the optimum conditions and the mean value of the reducing sugar produced was found to be 83.5% on total carbohydrate content basis. The same was theoretically evaluated from equation 2 for optimum value of A, B, C and was found to be 84.14%. The closeness of the theoretical value to that of experimental value validates the model.

In the present investigation we have used a single step hydrolysis process to convert the cellulosic and hemicellulosic fractions of very low cost KGB to reducing sugars. In many studies it was observed that two or more steps have been used to fractionate the various lignocellulosic materials to obtain the monomeric form of the polysaccharides. Saha *et al* [31] used dilute acid treatment (0.75% v/v, 121°C, 1 h) and cocktail of four commercial enzyme preparations for saccharifying wheat straw and obtained 74% saccharification yield. Coastal Bermuda grass was pretreated using autohydrolysis process (150°C, 60 min) followed by enzymatic hydrolysis and

70% of the theoretical sugar yield was obtained by Lee *et al* [32]. Xu *et al* [33] used sodium hydroxide pretreatment (1% NaOH, 50°C, 12h) followed by enzymatic saccharification of switch grass and obtained 70.8% sugars on total available carbohydrate basis. In our study 83.5% yield of total reducing sugar was obtained which was significantly higher than the reported investigations. Also the proposed generalized model was successful in explaining the experimental facts and can be utilized for further large scale studies.

CONCLUSION

Kans grass, being a potential candidate containing 68% (w/w) total carbohydrate on oven dry basis was used for the present study. The rotatable central composite design and response surface technique that was employed for optimization work in many studies was successfully used in the present investigation. The validation of the model equation developed was done and it was successful in explaining the experimental and theoretical values. The optimum conditions for acid hydrolysis of Kans grass biomass were found as acid concentration 61.10%, biomass loading 10.80% and reaction time 45 min. The maximum amount of released reducing sugars 83.5% on total carbohydrate content basis was obtained at the optimized conditions which is significantly very high. Thus it is concluded that under controlled hydrolysis conditions, Kans grass biomass can be effectively utilized as a potential source of reducing sugars, which can be a staring material for production of various chemicals by microbial conversion process.

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