Optimization and Modeling of Alkali Pretreatment using Response surface methodology for Bioethanol Production from Guinea grass (*Panicum maxicum*)

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In the present demand for renewable and sustainable sources of energy to overcome the burden on world energy crisis, perrenial grasses have presented exciting options. *Panicum maximum* (Guinea grass) is a perennial grass and can be utilized for the production of bio-fuel in the form of cellulosic ethanol as it is a cost effective and efficient feedstock for bioethanol production. The conversion of biomass into glucose, an important step for the bioethanol production and it requires optimum pretreatment. Among various pretreatment methods available, alkali treatment reduces the lignin content and decreases the crystallinity of cellulose efficiently. In the present paper Response surface methodology was applied to optimize the alkali pretreatment of *P.maximum* (Guinea grass) for maximum reducing sugar production. Joint effects of five independent variables; Sodium Hydroxide concentration, Temperature, Substrate loading, pH and Reaction time, were investigated. The optimum conditions in which maximum reducing sugar yield (57.42%) obtained were: 1.8% substrate, 1% Sodium Hydroxide loading, 9.5 pH, a reaction time of 127.5 min, and a temperature of 110 ºC. This result has been statistically analyzed with a second order polynomial equation. This study reveals the promising use of *P.maximum* biomass as a feedstock for getting reducing sugar, which is required for bioethanol production.

Keywords: Response surface methodology, Feedstock, Biomass, Pretreatment

1. Introduction

In the last few years , the demand of alternative fuel sources is accelerated due to the excessive consumption of fossil fuels (Zaldivar J. *et al*,2001).Currently, ethanol production depends on crops such as sugar cane and corn but they have social issues as they are potential food or feed resources (Ferreira S. *et al* ,2009).Therefore, the utilization of nonfood biomass, that is, lignocellulosic biomass for bioethanol production , is creating interest worldwide (Moukamnerd C. *et al.*,2010; Zhang *et al*, 2010).The lignocellulosic biomass has so many advantages for e.g. huge availability, being economical, and reduced emissions of greenhouse gases and does not have any social and economic concerns regarding the use of food resources. All these factors make them one of the most promising sources for bioethanol production.

There are three processes involved in conversion of lignocelluloses to ethanol: pretreatment of raw materials, enzymatic hydrolysis of pretreated raw materials into monomers (fermentable sugars) and fermentation of sugars into ethanol. Among all of these steps, pretreatment process utilizes as much as 30% of the total ethanol production cost (Yang B.and Wyman C.E., 2008). Number of methods have been developed like uncatalyzed steam explosion, liquid hot water, dilute acid, flow-through acid pretreatment, lime, wet oxidation and ammonia fibre/freeze explosion, for the pretreatment of lignocellulosic biomass (Liu and Wyman, 2005). Amongst all, most commonly used chemical pretreatment technique is use of alkali. Additionally, most of these pretreatment methods require high-temperature or high-pressure. Alkali treatment is comparatively cost effective. Apart from this alkali treatment increases the internal surface area of lignocellulosic materials, decreases crystalline nature of cellulose and separates the structural linkages between lignin and carbohydrates (Hendriks A.T.W.M. and Zeeman G., 2009).

Panicum maximum belonging to the family Poaceae, is indigenous to Africa and distributed in tropics and subtropics and commonly known as guinea grass (Aganga and Tshwenyane, 2004). Guinea grass is cultivated in Haryana, Punjab, Himachal Pradesh. It has also wide adaptability in eastern and southern India. Guinea grass grows well in warm moist climate. The grass tolerates shade and grows under trees and bushes. The grass is well adapted to a wide range of soils and also has ability to grow on wasteland. Its harvesting period is short as compare to other sources of bioethanol for e.g. corn and sugarcane. The first cut is usually ready in 9-10 weeks after planting and subsequent cuts are taken at 45 to 60 days intervals. About six to seven harvests can

be made in a year which means more feedstock will be available for bioethanol production. Approximately 80-100 t/ha of green fodder is obtained per year. So the excessive growth rate, multicut nature, high yield and wider adaptability of this grass without any fertilizer input shows it as a potential renewable source of lignocellulosic biomass available for ethanol production.

Optimization of pretreatment process is highly challenging as it is necessary to obtain high yield of monomeric sugars which can be converted into bioethanol by fermentation process. Optimization of multifactorial system by conventional techniques is generally done with one-factor at a time. However, this type of method is very time consuming and does not explain the interactive effects between the variables (Jeya M., 2009). RSM is a statistical technique for building model and optimization of multiple variables. It gives maximum information with a minimum number of runs.(Kim J.K. *et al.*,2008)

Therefore ,in the present paper response surface methodology (RSM) was used to optimize the alkali pretreatment process and find out the effects of alkali concentration, substrate loading, temperature, pH and retention time of pretreatment on yield of reducing sugars content in *P.maximum* .For the screening of optimum pretreatment condition a *Plackett* -burman design was used. Then, a Box-behnken design (BBD) was applied in this study to optimize the selected parameter condition with reducing sugars concentration as response variable.

2. Material and methods

All experiments were carried out three times, and the given values are the mean values ± SD.

2.1. Feedstock

The Guinea grass *(Panicum maximum*), was collected from the Micromodel Complex, IIT.Delhi, (77.09°E, 20.45°N). Before any pretreatment biomass was cut to nominally 1–2 cm length and washed thoroughly with tap water until the washings were clean and colorless and then oven-dried, grinded to 1 mm size and stored in a polyethylene black bag at room temperature for further use.

2.2. Analysis

The pretreated samples were neutralized with 1N H_2SO_4, followed by washing with tap water and dried at room temperature (30 ±2°C) for reducing sugar estimation. Reducing sugar content

of the untreated and pretreated guinea grass was identified according to Laboratory Analytical Procedures from dinitrosalicylic acid (Miller G.L., 1972).

2.3. Optimization of parameters for pretreatment

Optimization of parameters for pretreatment of biomass was performed in two stages. Initially five variables were screened using Plackett-burman design to identify parameters that significantly influenced pretreatment. In the second stage, the levels of these parameters optimized using response surface methodology.

2.3.1. Screening of parameters by Plackett-burman design

The Plackett-burman design is an efficient mathematical approach to determine and screen out the parameters. It offers a good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment (Reddy *et al.*2008; Singh *et al.*2010). In this study, temperature, retention time, alkali concentration (NaOH), substrate concentration (grass only) and pH were selected as the independent variables (Zhu *et al.*, 2006; Hu and Wen, 2008; Maa *et al.*, 2009). These variables were investigated and 31 experiments were carried out. Each variable were set at three levels, high level ,middle and low level. The experimental design is given in Table 1a. These variables were investigated by performing 31 runs. Each variable was set at two levels, a low level and high level (Table. 1b).The experimental design is given in Table. 1a .The significance of regression was also done by F-test. (Table.1b)

Table. 1a: *Plackett* t-burman design for screening of variables for *P. maximum*

Run	Solid loading (g/L)	Chemical loading (%)	Temperature (°C)	Retention time (min)	pH	Reducing sugar (%)
1	-0.600	-0.330	1.000	1.000	1.000	20.1515
2	-1.000	-0.309	-0.060	-0.420	0.640	37.8485
3	-1.000	1.000	1.000	-1.000	1.000	18.3333
4	-1.000	-0.309	-0.060	-0.420	0.640	37.1212
5	-0.310	1.000	0.070	-1.000	0.040	28.4848
6	-1.000	-1.000	1.000	-0.600	-1.000	23.1818
7	0.200	-0.110	0.800	-1.000	0.000	13.8182
8	1.000	-0.140	-1.000	-0.100	0.050	31.6667
9	0.900	-1.000	-1.000	0.420	1.000	24.8485
10	0.900	-0.010	-0.230	1.000	1.000	35.4545
11	1.000	1.000	-0.280	-1.000	1.000	20.9091
12	0.800	0.460	0.160	1.000	-0.576	33.1818
13	-0.780	-0.200	-0.186	-1.000	-1.000	30.4545

14	1.000	-0.140	-1.000	-0.100	0.050	31.1515
15	-1.000	0.900	0.340	1.000	-0.580	21.9697
16	-0.060	-1.000	-1.000	-1.000	1.000	11.9697
17	0.646	0.344	0.640	-0.220	1.000	15.303
18	0.230	0.850	0.430	-0.280	-1.000	37.29
19	-1.000	-1.000	-1.000	1.000	1.000	26.8182
20	-0.370	1.000	-0.870	0.320	0.870	28.7879
21	1.000	-1.000	1.000	-0.240	1.000	21.2121
22	0.050	-1.000	0.050	0.650	-0.080	29.2727
23	0.200	-0.110	0.800	-1.000	0.000	13.1818
24	-0.540	-0.370	-0.930	0.390	-1.000	23.9394
25	1.000	-0.500	1.000	1.000	-1.000	37.7273
26	1.000	-1.000	-0.320	-1.000	-1.000	22.7273
27	0.050	-1.000	0.050	0.650	-0.080	29.8485
28	1.000	1.000	-1.000	1.000	-1.000	29.0909
29	-1.000	1.000	-1.000	-1.000	-0.670	1.51515
30	1.000	1.000	1.000	0.660	0.262	16.6667
31	0.230	0.850	0.430	-0.280	-1.000	37.2727

Table. 1b Regression analysis for *Plackett* t Burman design variables *P. maximum*

Term	**Effect**	**Coefficient**	**Standard Error**	***F*- value**	**P-Value**
Intercept		34.42	0.54	3.58	0.0015
Substrate loading (SL)	24.18	1.16	0.54	12.14	0.0018
Chemical Loading (CL)	16.08	0.37	0.54	4.91	0.03
Temperature (TM)	1.24	0.028	0.54	31.03	<0.0001
Retension Time (RT)	0.3	3.15	0.54	37.02	<0.0001
pH	-12.52	- 1.46	0.54	15.27	0.0006

2.3.2. Screening of parameters by Box-Behnken design

A Box-Behnken factorial design with five variables and with their three replicate at the centre point, was used for the optimization of pretreatment conditions. In this experiment ,BBD was used to evaluate the main and interaction effects of the five independent variable SL (A),CL (B),TM(C),RT(D) and pH(E) on reducing sugar content. The range and levels of the variables investigated were given in Table 1c, whereas the experimental designs with the observed

responses for reducing sugar presented in Table 1d. A polynomial quadratic equation was fitted to evaluate the effect of each five independent variable on reducing sugar content.

$$Y=b_0+b_1*A+b_2*B+b_3*C+b_4*D+b_5*E+b_6*A*B+b_7*A*C+b_8*A*D+b_9*A*E+b_{10}*B*C+b_{11}*B*D+ b_{12}*B*E+ b_{13}*C*D+ b_{14}*C*E+ b_{15}*D*E+ b_{16}*A^2+b_{17}*B^2+b_{18}*C^2+b_{19}*D^2 +b_{20}*E^2 \quad \text{…. Eq (1)}$$

where Y is the predicted response; b_0 is a constant; b1,b2,b3,b4 and b5 are the linear coefficients; b6,b7,b8,b9,b10,b11, b12, b23, b13,b14 and b15 are the cross-coefficients; b16,17, b18,b19 and b20 are the quadratic coefficients. The response surfaces of the variables inside the experimental domain were analyzed using Design Expert software. Subsequently, additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

Table. 1c: Actual and coded level of variables tested with Box–Behnken design.

Factor	Name	Units	Low (-1)	Centre point (0)	High (+1)
A	Solid loading	g/L	0.50	1.31	2.00
B	Chemical loading	%	0.50	1.75	3.0
C	Temperature	°C	40	94.71	150
D	Retention time	min	15	93.32	180
E	pH	-	9	11.10	13

Table. 1d: Box–Behnken design matrix for optimization of parameters identified by *Plackett* t–Burman design.

Run	Solid loading (g/L)	Chemical loading (%)	Temperature (°C)	Time (min)	pH	Response (%)
1	1.000	0.000	0.000	-1.000	0.000	14.4
2	0.000	0.000	0.000	0.000	0.000	56
3	-1.000	0.000	1.000	0.000	0.000	14.54
4	0.000	0.000	0.000	0.000	0.000	56.18
5	0.000	-1.000	0.000	-1.000	0.000	14.1
6	-1.000	0.000	0.000	0.000	-1.000	33.18
7	0.000	1.000	0.000	0.000	-1.000	33.93
8	0.000	-1.000	1.000	0.000	0.000	15.75
9	0.000	-1.000	-1.000	0.000	0.000	55
10	0.000	0.000	-1.000	-1.000	0.000	14.1
11	0.000	1.000	0.000	1.000	0.000	16.5

12	0.000	0.000	0.000	1.000	-1.000	16.34
13	0.000	1.000	0.000	0.000	1.000	43.48
14	0.000	0.000	1.000	-1.000	0.000	15.47
15	0.000	-1.000	0.000	0.000	-1.000	39.39
16	-1.000	0.000	0.000	-1.000	0.000	14.6
17	0.000	0.000	0.000	-1.000	-1.000	15.7
18	0.000	0.000	0.000	0.000	0.000	56.2
19	0.000	0.000	-1.000	0.000	1.000	16.7
20	0.000	1.000	1.000	0.000	0.000	17.5
21	1.000	1.000	0.000	0.000	0.000	54.3
22	0.000	0.000	0.000	1.000	1.000	15.7
23	1.000	0.000	1.000	0.000	0.000	14.84
24	0.000	0.000	-1.000	1.000	0.000	33.33
25	-1.000	-1.000	0.000	0.000	0.000	38.78
26	1.000	0.000	0.000	0.000	1.000	14.6
27	0.000	0.000	0.000	-1.000	1.000	38.18
28	0.000	0.000	1.000	0.000	-1.000	15.3
29	0.000	0.000	1.000	0.000	1.000	15.3
30	1.000	0.000	0.000	0.000	-1.000	36.36
31	0.000	-1.000	0.000	1.000	0.000	14.87
32	-1.000	0.000	-1.000	0.000	0.000	36.96
33	1.000	-1.000	0.000	0.000	0.000	54.5
34	0.000	1.000	-1.000	0.000	0.000	15.1
35	-1.000	0.000	0.000	1.000	0.000	18.48
36	0.000	0.000	0.000	0.000	0.000	56.18
37	1.000	0.000	0.000	1.000	0.000	33.18
38	0.000	1.000	0.000	-1.000	0.000	14.6
39	0.000	-1.000	0.000	0.000	1.000	37.42
40	-1.000	1.000	0.000	0.000	0.000	15.45
41	1.000	0.000	-1.000	0.000	0.000	14.7
42	0.000	0.000	0.000	0.000	0.000	56.21
43	0.000	0.000	-1.000	0.000	-1.000	18.18
44	0.000	0.000	0.000	0.000	0.000	57.1
45	0.000	0.000	1.000	1.000	0.000	16.36
46	-1.000	0.000	0.000	0.000	1.000	14.5

3. Results and discussion

3.1. Optimization of parameters for pretreatment

3.1.1. Preliminary results

Preliminary experiments were performed to determine the effect of main factors and the appropriate ranges by *Plackett* –Burman design. The effects of different factors (chemical loading, substrate loading, retention time, temperature and pH) evaluated on the basis of reducing sugar released after the pretreatment of *P.maximum* (Table 1a). The ranges of 1.2-2.4 g/L, 1-3%, 110-160^{0}C, 70-185 min and 9-10 were chosen for BBD examination as appropriate ranges for substrate loading, chemical loading, temperature, retention time and pH respectively.

3.1.2. RSM results

The results of BBD experiments for studying the effect of five independent variables (Optimized by *Plackett* –Burman Design) were presented along with the mean predicted and observed responses in Table 1b. The regression equations obtained after the ANOVA gave the level of reducing sugar as a function of the initial values of alkali concentration, retention time, substrate concentration, temperature and pH. The final response equation that represented a suitable model for reducing sugar pretreatment is given below:

$$Y= +56.31 + 3.15*A - 3.68*B - 4.94*C + 1.48*D - 0.78*E + 5.78*AB + 5.64*AC + 3.72*AD - 0.77*AE + 10.41*BC + 0.28*BD + 2.88*BE -4.59*CD + 0.37*CE - 5.78*DE -13.12*A^2 - 8.34*B^2 - 20.97*C^2 - 22.91*D^2 - 14.71*E^2 \quad \text{…….Eq.(2)}$$

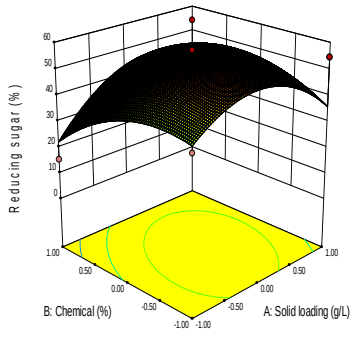
Where Y reducing sugar content (%) and A, B, C, D and E are Substrate loading, chemical loading, temperature, residence time and pH respectively. Table 1e depicts the ANOVA for the fitted model. No abnormality was observed from the diagnoses of residuals. Thus, it can be concluded that the model was statistically sound. The *F* and P-value are 3.58 and 0.0015 for *P. maximum* respectively thus signifies the model with 99% level of confidence ($\alpha = 0.01$) denoting the significance of the coefficients was also important in understanding the pattern of the mutual interactions between the variables. Quality of fit model was estimated by R^2_{adj} values were found to be 0.95 which are fairly high and accurate measures of precision (Ohtani 2000).

Table 1e. ANOVA for selected Box–Behnken Design for P.maximum pretreatment

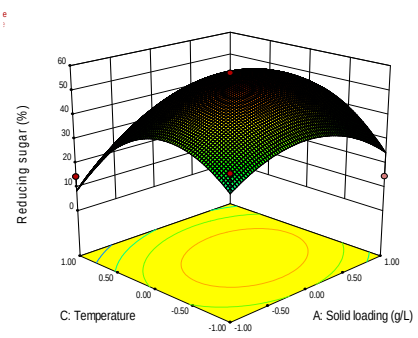
Sources of Variation	**Degrees of freedom**	**Sum of squares (partial)**	**Mean squares (partial)**	**F ratio**	**P value**
Model	20	8856.34	82.3	3.58	0.0015
Linear Effects	35	11137.64	318.22	2050.15	<0.0004
Quadratic Effects	20	3091.95	154.60	996.01	< 0.0001

Residual	25	3092.73	123.71		
Lack of fit	20	3091.95	154.6	1.01	0.157
Pure Error	5	0.78	0.16		
Total	45	11949.07			

The response surface model was used to predict the result by contour plots. Contour plot is the projection of the response surface as a two dimensional plane (Box and Hunter, 1957). The 2D contour plots of the responses using Eq. (2) for the reducing sugar content are shown in Figs.1.A.The shapes of contour plots indicate the nature and extent of the interaction between different factors (Prakash *et al.*, 2008). Less prominent or negligible interactions were shown by the circular nature of the contour plots, while comparatively prominent interactions were otherwise shown by the elliptical nature of the contour plots. The contour plots developed using the fitted quadratic polynomial equation obtained from regression analysis are in Figure 1 (a-e). Each figure presents the effect of two variables on the production of active substances, while other one variable was held at zero level (Zhao et al. 2013). Fig. 1a shows the effect of substrate loading and chemical loading on the reducing sugar production at the fixed temperature, pH and time level. Similarly, other Figures (1b-e) represent the effect of two variables on the reducing sugar production, while third, fourth and fifth variable was held at zero level. In all cases, a clear optimal convergence was observed to find the optimal levels of all the five independent variables on reducing sugar production. Significant interaction was noted between the alkali concentration, retention time and substrate concentration,temperature and pH. Although the actual situation may be more complicated than that, we have reported here, an attempt for the process optimization has been made by RSM.



(a)



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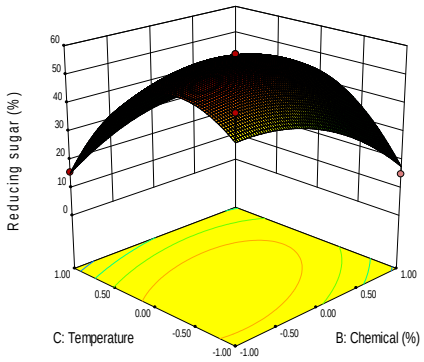

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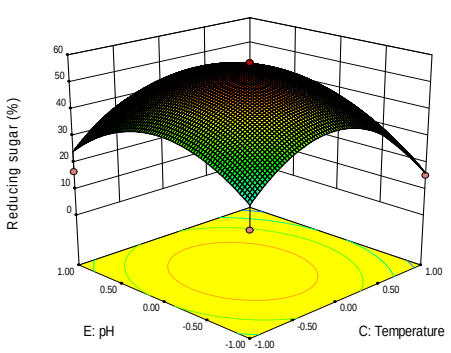

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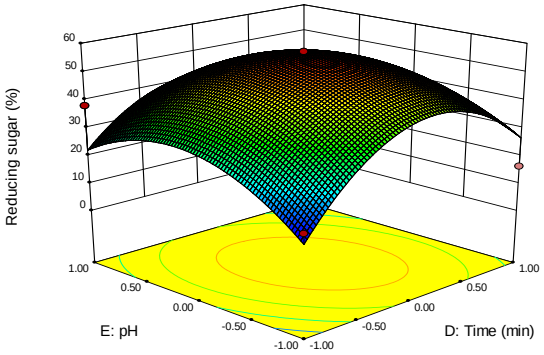

(e)

Fig. 1. Three dimensional response surface and contour plots showing interactive effects between (a) substrate (SL) and chemical loading (CL); (b) temperature (TM) and sample loading (SL); (c) temperature (TM) and chemical loading (CL); (d) pH (PH) and temperature (TM); (e) Ph (PH) and retension time(RT) on reducing sugar production in *P.maximum* .

4. Conclusions

Lignocellulosic biomass utilization for production of valuable product needs cost effective technology development. In the present investigation, alkali pretreatment in improving reducing sugar content was optimized by using Box-Behnken Design. In this study, we examined the influence of NaOH loading, reaction temperature, and pretreatment time on glucose yield. From the statistical analyses, it was revealed that all the independent variables significantly contributed to glucose yield. Good correlations between the experimental and predicted reducing sugar yields were found. The study demonstrated that reducing sugar content was significantly

improved due to lignin removal by alkali pretreatment. The maximum glucose yield of 56.2% was obtained from *P.maximum* at the following optimal conditions: NaOH concentration of 2.0%, temperature of 135 ^{0}C, retention time of 127.5 min, substrate loading 18.0g/L and 9.5 pH. More elaborated study will be performed in our laboratory with the compositional analysis of different sugars in hydrolysate after the enzymatic saccharification, and the use of these residues for the production of bioethanol production to eradicate energy crisis.

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