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Statistical Optimization of Thermo-Alkaline Pretreatment of Corn Stover for Efficient Enzymatic Hydrolysis

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***Abstract*—Corn stover is the most sustainable agricultural residue that can be efficiently utilized for the production of cellulosic biofuel. In the present study, statistical optimization of the thermochemical pretreatment of corn stover was carried out by evaluating the effects of NaOH concentration, pressure of steam and treatment time by using central composite design of response surface methodology for achieving maximum release of reducing sugars and glucose upon enzymatic hydrolysis. The enzymatic hydrolysis of the pretreated biomass was carried out by in-house produced cellulase preparation from *Penicillium sclerotiorum* SKN-11 in a constant solid-to-liquid ratio of 1:10 for 96 h at 50°C. Optimum conditions of thermochemical pretreatment were found to NaOH concentration of 1.34 % w/v, steam treatment at 15 psi for 140 min and the same resulted the total reducing sugars yield of 750.66 mg/g and glucose yield of 306.50 mg/g upon enzymatic hydrolysis thus revealing the hydrolysis efficiency of 86.82 %.**

***Keywords*—Corn Stover; Alkali Pretreatment; Response Surface Methodology; Penicillium sclerotiorum; Enzymatic Hydrolysis**

I. INTRODUCTION

The increasing concentration of green house gases (GHGs) in the biosphere is a paramount concern of the current time. Various researchers around the world have proposed that the increased usage of primary sources of energy such as fossil fuels and coal, would lead to their rapid depletion in the near future [1]. Continuous depletion of fossil fuel reserves and alarmingly increasing rate of air pollution has shifted the interest of the scientific community to look for some renewable energy resources that can be converted into biofuels to meet the increasing energy demands of the society in a sustainable manner [2].

One of such potentially sustainable and renewable feedstock for biofuel production is agricultural lignocellulosic biomass. In India, the total surplus agricultural lignocellulosic material available annually is 382.7 million metric tons (MMT) in which corn cobs contribute 27 MMT and the estimated ethanol production from corn cobs is observed to be 9.1 billion litres per year [3]. The cobs, stalks, leaves and stem of the maize plant which are left in the plant after harvesting of the crop are collectively known as corn stover. In India, stem and leaves of the maize plant are being used as fodder but some left over including cobs remain in the field which are generally burnt in the field itself and contribute to the GHGs and air pollution. However, corn stover seems to be a lucrative and sustainable lignocellulosic material for the production of cellulosic ethanol. The rate of ethanol production depends upon the amount of fermentable sugars released from the hydrolysis of the lignocellulosic biomass [4]. However, the complex lignin-cellulosic association and the crystalline structure of the cellulose are the physical barriers that restrict the enzymatic access to the cellulose fibers and thus it offers a biggest hurdle for the efficient hydrolysis for the release of fermentable sugars. Therefore, a systematic and effective pretreatment strategy is a mandatory step before hydrolysis, for the efficient removal of the lignin from the biomass which simultaneously disorganizes the crystalline structure of the cellulose fibres. The main purpose of the pretreatment is to remove lignin, cause minimum or no loss of sugars (hexoses and pentoses), avoid formation of inhibitors which may affect fermentation and must be economical in terms of solid recovery and energy input [5]. Till date, several thermal [6], [7], and thermo-chemical pretreatment strategies involving the use of dilute acids including hydrochloric acid, sulphuric acid and nitric acid [8], [9], [10], alkali [11], [12], ammonia, [13] alkaline hydrogen peroxide [14], supercritical CO_2, [15], dilute acid followed by lime [16] and biological agents [17] have been studied for the delignification of corn stover. Amongst all these strategies, alkali pretreatment with NaOH is found to be better as it can remove lignin, cause less degradation of carbohydrate polymers, reduce the crystallinity of the cellulose thus making it more vulnerable to enzyme attack. Therefore, in the present study, statistical optimization of alkali pretreatment in combination with steam was carried out to optimize

the alkali concentration, steam pressure and resident time for the maximum removal of lignin from the corn stover for efficient release of sugars during enzymatic hydrolysis.

II. Materials and methods

A. *Raw materials*

Corn stover (*Zea mays*) which consist of leaves, stalk and cobs after harvesting of the crop, was obtained from Hamirpur district of Himachal Pradesh, India. It was directly collected from the field, brought to the lab and dried in the oven at 60°C to bring the moisture content to less than 3%. It was then ground to reduce the particle size and stored in plastic bags at room temperature.

B. *Production of cellulases by P. sclerotiorum SKN-11 via solid state fermentation of wheat bran for hydrolysis of pretreated corn stover*

5.0 g of wheat bran moistened with 7.5 ml distilled water having pH 6.0-7.0, dispensed in 250 ml Erlenmeyer flask was used as the medium for the production of cellulases by a natural variant of *P. sclerotiorum* SKN-11 isolated from the biodiversity of Chandigarh city. The medium was autoclaved at 15 psi and inoculated with five discs (7mm) cut from the periphery of actively growing mycelium of the fungal strain on PDA plates followed by incubation at 28^{o}C for 96 h under stationary conditions in the BOD incubator. Enzyme extraction was carried out in 100 ml distilled water in the laboratory blender and the fermented residue was separated by filtration through a metallic sieve followed by centrifugation at 10,000 rpm at 4 °C for 10 min. The clear supernatant thus obtained was used as extracellular cellulase preparation and assayed at 50^{o}C, pH 4.0.

C. *Enzyme assays*

Cellulases in the supernatant were assayed in terms of endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) using carboxymethyl cellulose (CMC), Whatmann filter paper (1×6cm) and salicin respectively, as substrates using 0.1M acetate buffer, pH 4.0 and expressed in terms of International units (IU) of CMCase, Fpase and β-glucosidase activities respectively [19]. One unit of the activity was defined as the amount of enzyme which is required to release 1µmol of glucose per ml from their respective substrates per min using dinitrosalicylic acid reagent (DNSA) [20].

D. *Optimization of thermochemical pretreatment of corn stover using sodium hydroxide and steam by statistical modelling*

NaOH concentration (1-5%), steam pressure (5-15psi) and pretreatment time (60-120 min) were selected for the optimization of thermochemical pretreatment of corn stover with sodium hydroxide and steam by response surface methodology. Solid concentration was kept constant at 10%, in all the experiments on the basis of preliminary studies which stated that high substrate loading leads to insufficient mixing and increased viscosity. Different sets of 500 mL screw capped Erlenmeyer flasks each containing 15 g finely grounded corn stover biomass dispensed in NaOH solutions of variable concentration ranging from 1.0-5.0% w/v. The flasks were placed in autoclave and steamed at different pressures for variable time periods according to the experimental designs. All the flasks were screw capped tightly so as to prevent evaporation during pretreatment. After autoclaving, pretreated samples were filtered through a sieve and thoroughly washed with tap water until neutral pH was achieved. The filtered biomass was dried at 65 °C to determine the dry weight and then stored in the plastic bags at room temperature for further enzymatic hydrolysis experiments.
The central composite design (CCD) for three factors used in the study is presented in Table 1. A total of 20 experimental runs including 8 tests for factorial points, 6 tests for axial points and 6 replication tests at central points were carried out. The effect of three operating variables of NaOH concentration (X_1), steam pressure (X_2) and pretreatment time (X_3) on the two response variables, total reducing sugars and glucose yield was determined. The independent variables were studied at five coded levels (–2 , –1, 0, +1, + 2) in the range of 1-5%, 5-15 psi and 30-150 min for X_1, X_2 and X_3 respectively [21], [22]. The response values were determined by the average of two independent experiments. A second-order quadratic equation (1) was fitted to evaluate the effect of each independent variable on the response [8]:

$$Y= b_0 +\sum b_i x_i +\sum\sum b_{ij} x_i x_j + \sum b_{ii} x^2 i + e \qquad (1)$$

Where Y= measured response; b_0, b_i, b_{ij}, b_{ii} are constant and regression coefficients of model; x_i and x_j are levels (codes values) of independent variables; e is random error. The resulting model was analysed using 'analysis of variance' (ANOVA) with p and F values were also determined. Contour plots were also obtained by using Design Expert software version 9.0 (Stat-Ease, Inc., Minneapolis, MN, USA) to illustrate the relationship and interactive effects between the variables. Accuracy and general ability of polynomial model was evaluated by determination coefficient (R^2).

E. *Carbohydrate estimation*

Total carbohydrate content in the untreated and alkali pretreated corn stover biomass was carried out by the method of Updegroff [18].

F. *Enzymatic hydrolysis of thermochemically pretreated corn stover biomass*

Different sets of 250 ml screw capped Erlenmeyer flasks containing 2 g alkali pretreated corn stover from various sets of the experimental design of RSM soaked in 15 ml acetate buffer (0.1 M, pH 4.0) were autoclaved at 121°C for 30 min. The contents of the flasks were cooled to 50°C and mixed with 4 ml of crude cellulase preparation produced in-house from the solid state culture of *P. sclerotiorum SKN-11* containing CMCase (40 IU/ml), FPase (5 IU/ml) and β-glucosidase (57 IU/ml) and the total volume was made to 20 ml by adding acetate buffer and supplementing 0.1% sodium azide to prevent bacterial contamination. Enzymatic hydrolysis was carried out at 50°C and 150 rpm in a water bath shaker (Grant, UK) for 96 h. The hydrolysed samples were taken out at regular intervals of time and centrifuged at 10,000 rpm for 10 min. (Thermo Scientific, USA) and the supernatant was analysed for total reducing sugars by DNSA method [20] and glucose content by GOD PAP kit. Hydrolysis efficiency (%) was calculated by using the following formula:

$$\text{Hydrolysis efficiency}(\%) = \frac{\text{Total sugars in the hydrolysate}}{\text{Total obtainable sugars from the substrate}} \times 100 \quad (2)$$

Table 1: Central composite design matrix with experimental values of total reducing sugars and glucose yield

Run	X_1: NaOH conc. (% w/v)	X_2: Steam pressure (psi)	X_3: Treatment time (min)	Response 1: Total reducing sugars yield (mg/g)	Response 2: Glucose yield (mg/g)
1	3	10	90	542.50	290.38
2	2	12.5	60	604.34	297.30
3	2	12.5	120	666.50	310.23
4	4	12.5	120	464.00	240.76
5	2	7.5	120	570.00	256.15
6	4	7.5	120	408.73	251.92
7	3	10	90	540.00	293.07
8	3	10	90	544.50	292.69
9	4	12.5	60	410.50	215.38
10	4	7.5	60	390.28	246.15
11	3	10	90	541.25	293.07
12	2	7.5	60	522.00	258.84
13	3	10	90	537.00	293.84
14	3	5	90	464.07	228.07
15	3	10	150	489.68	269.61
16	3	15	90	605.55	270.00
17	3	10	90	544.50	282.30
18	5	10	90	312.69	195.30
19	1	10	90	644.08	265.26
20	3	10	30	382.50	227.30

G. Scanning electron microscope (SEM) analysis

The surfaces properties of untreated and alkali pretreated corn stover biomass were observed using a Hitachi SU-8010 scanning electron microscope (SEM) (Hitachi Science Systems, Ibaraki, Japan). Specimens were dried at 100°C until the weights were constant, then sputter coated with platinum and observed using a voltage of 10 kV.

III. RESULTS AND DISCUSSION

A. Pretreatment and enzymatic hydrolysis of corn stover biomass

Corn stover was pretreated with NaOH prior to enzymatic hydrolysis. Pretreatment of the corn stover was performed according to the experimental design presented in Table 1. Analysis of samples from liquid fraction showed that total phenolics concentration in the filtrate was almost identical in all samples. However, the concentration of total reducing sugars was slightly increased with increase in NaOH concentration. Dry weight of the pretreated corn stover was found to decrease with increase in concentration of NaOH. The in-house produced cellulase system from *P. sclerotiorum SKN-11* utilized for the hydrolysis of the pretreated corn stover which worked very well as evident from the amount of sugars released.

B. Statistical modeling by RSM and analysis of the experimental results

Central composite design of response surface methodology was used to evaluate the optimum response regions of total reducing sugars, glucose yields and to optimize the corresponding variables. The variables of CCD were fitted with second order quadratic equation for both the responses. The equations 3 and 4 depict the relationship of the response variables on the total reducing sugars and glucose yields respectively and can be expressed as follows:

$$\text{Total reducing sugars yield} = 540.928 - 84.4969 \times X_1 + 33.5894 \times X_2 + 24.7794 \times X_3 - 12.9188 \times X_1X_2 - 4.77623 \times X_1X_3 + 6.15123 \times X_2X_3 - 14.6444 \times X_1^2 - 0.536872 \times X_2^2 - 25.2081 \times X_3^2 \quad (3)$$

$$\text{Glucose yield} = 290.07 - 19.2644 \times X_1 + 8.40438 \times X_2 + 7.87562 \times X_3 - 16.8088 \times X_1X_2 + 2.61375 \times X_1X_3 + 4.40375 \times X_2X_3 - 14.2144 \times X_1^2 + -9.52562 \times X_2^2 - 9.67062 \times X_3^2 \quad (4)$$

Analysis of variance (ANOVA) of the quadratic equations for total reducing sugars and glucose yields was performed by Fisher's statistical test (Table 2), which shows that the regressions are statistically significant.

C. Interaction among the factors

Student's t-test was carried out to determine the knowledge of the error's mean square which is essential in testing the significance of the estimated coefficient of the regression equation. A larger t- value and smaller p value, represent the more significant corresponding coefficient [23]. The coefficient estimate and t-values of both the responses are depicted in the Table 2, which indicate that the factors X_2 (steam pressure) and X_3 (treatment time) have positive effect on total reducing sugars and glucose

yields, with factor steam pressure (X_2) exhibiting the highest effect on both the responses.

TABLE 2: ANOVA RESULTS SHOWING TOTAL REDUCING SUGARS AND GLUCOSE YIELDS UNDER RESPONSE SURFACE QUADRATIC MODEL AND MODEL COEFFICIENTS ESTIMATED BY MULTIPLE LINEAR REGRESSIONS

Source of variation	Sum of squares	Coefficient estimate	t-test	F value	P value	Confidence level
ANOVA for total reducing sugars yield						
Model	162431.15	540.26	285.85	663.72	<0.0001	100.00
X_1 : NaOH	114235.52	-84.50	-61.11	3734.27	<0.0001	100.00
X_2 : Steam pressure	18051.93	33.59	24.29	590.10	<0.0001	100.00
X_3 : Treatment Time	9824.29	24.78	17.92	321.15	<0.0001	100.00
X_1X_2	1335.16	-12.92	-6.61	43.65	<0.0001	99.99
X_1X_3	182.50	-4.78	-2.44	5.97	0.03	96.53
X_2X_3	302.70	6.15	3.15	9.90	0.01	98.96
X_1^2	5389.94	-14.51	-13.27	176.19	<0.0001	100.00
X_2^2	16094.74	-25.07	-22.94	526.12	<0.0001	100.00
X_3^2	162431.15	540.26	285.85	663.72	<0.0001	100.00
ANOVA for glucose yield						
Model	16884.20	290.07	112.13	57.49	<0.0001	100.00
X_1 : NaOH	5937.86	-19.26	-12.72	161.73	<0.0001	100.00
X_2 : Steam pressure	1130.14	8.40	5.55	30.78	0.000	99.98
X_3 : Treatment Time	992.41	7.88	5.20	27.03	0.0004	99.96
X_1X_2	2260.27	-16.81	-7.85	61.56	<0.0001	100.00
X_2X_3	155.14	4.40	2.06	4.23	0.0669	93.31
X_1^2	4849.16	-14.21	-11.49	132.08	<0.0001	100.00
X_2^2	2177.70	-9.53	-7.70	59.32	<0.0001	100.00
X_3^2	2244.50	-9.67	-7.82	61.14	<0.0001	100.00

For total reducing sugars yield: Std.Dev. =5.76, R^2 =0 .9982, Mean =509.23, Adj R^2 = 0.9963, C.V. % = 1.13, Pred. R^2 =0.9841, Adeq Precision = 84.21
For glucoe yield: Std.Dev. = 5.89, R^2 = 0.9819, Mean =263.88, Adj R^2 = 0.9638, C.V. % = 2.23, Pred. R^2 = 0.8347, Adeq Precision = 27.80

The interaction between factors X_1X_2 and X_1X_3 showed the negative effects on the total reducing sugars yield and X_2X_3 showed positive effect on the total reducing sugars yield. However, X_1X_2 showed negative effect whereas X_1X_3 and X_2X_3 showed positive effects on glucose yield.

Coefficient of determination (R^2) value determines the quality of the model. The value of R^2 is always between 0 and 1. It is known that the R^2 value greater than 0.75 indicates the accuracy of the model. The R^2 value for the total reducing sugars yield (Response 1) was 0.9982 indicating that 99.82 % of variables fit the response. The "Pred R^2" of 0.9841 is in reasonable agreement with the "Adj R^2 Squared" of 0.9963. For response 2 (glucose yield), R^2 value was observed to be 0.9819 indicating that 98.19 % of variables fit the response. The model's F-value for total reducing sugars was 543.28 and for glucose yield was 54.21 which indicate that both the models are significant and there is only 0.01 % chance that the F-value this large could occur due to noise. Coefficient of variation (CV) indicates the degree of precision of the experiments performed. High CV value indicates low reproducibility of experiments. Both the responses obtained in the study have lower value of coefficient of variation, (CV= 1.13% for total reducing sugars and CV=2.23% for glucose yield) indicated the greater reproducibility and accuracy of the performed experiments.

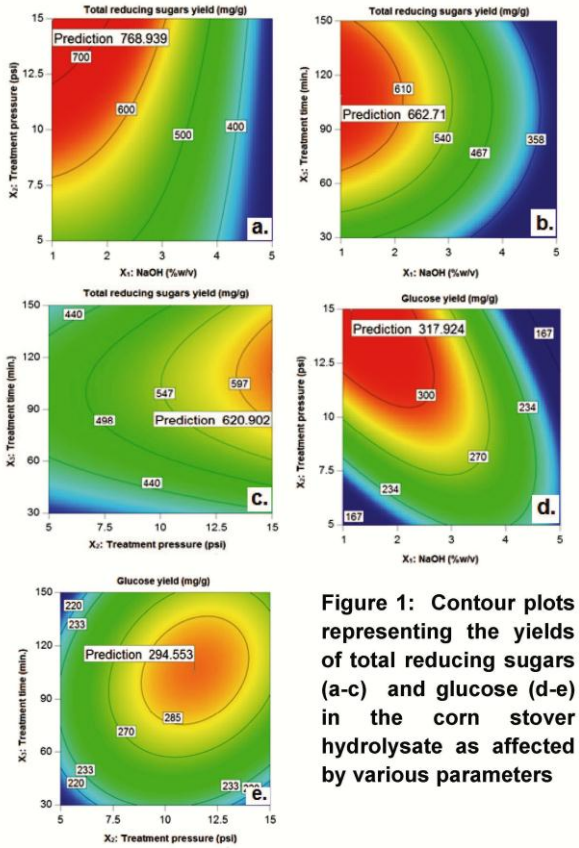

Figure 1: Contour plots representing the yields of total reducing sugars (a-c) and glucose (d-e) in the corn stover hydrolysate as affected by various parameters

A high adequate precision value for both the models (total reducing sugars yield =84.21, glucose yield = 27.80) suggested an adequate signal to noise ratio, hence, both the models can be used to navigate the design space.

D. Effect of the parameters on total reducing sugars and glucose yields

Effect of different independent variables and their interaction on the yields of total reducing sugars and glucose was depicted by contour plots. In these plots, two factors were varied at a time while the other factor was kept at center level. From the plots, the interaction between the two variables and the optimum level can be easily located. Each curve in the plot depicts an infinite number of combinations of two variables whereas the other variable maintained at constant level. The contour graph obtained as a function of NaOH concentration versus steam pressure indicated that total reducing sugars yield increased with increase in treatment pressure and decreased with increase in NaOH concentration. The maximum reducing sugars yield corresponding to 768.939 mg/g of hydrolysed substrate was obtained with 1% w/v NaOH concentration and steam pressure of 15 psi while treatment time held at 0 coded level (1a).The contour graph obtained as a function of NaOH concentration versus treatment time showed that the total reducing sugars yield increased with the increase in treatment time upto 115 min and then decreased with further increase in treatment time. The maximum total reducing sugars yield of 662.71 mg/g

was obtained with 1.0% (w/v) NaOH and treatment time of 109 min while treatment pressure held at 0 coded level (2b). The maximum total reducing sugars yield of 620.902 mg/g was obtained at treatment pressure of 15 psi and treatment time of 112 min while NaOH conc. held at 0 coded level (2c). Maximum glucose yield of 317.92 mg/g was obtained with NaOH conc. of 1.15% and treatment pressure of 15 psi while treatment time held at 0 coded level (2d). The maximum glucose yield of 294.55 mg/g of substrate was obtained with treatment pressure of 12 psi and treatment time of 105 min while NaOH conc. held at 0 coded level (2e).

E. Model validation

To evaluate the accuracy of statistical experimental model of response surface methodology, attempts were made to optimize the process parameters for maximizing the total reducing sugars and glucose yields. Point optimization for total reducing sugars and glucose yields was attempted with Design Expert version 9.0, which predicted the highest yields of total reducing sugar and glucose equivalent to 753.77 mg/g and 310.23 mg/g after enzymatic hydrolysis of the corn stover biomass pretreated with 1.34 % (w/v) of NaOH in presence of steam at a pressure of 15 psi for 140 min. To validate the optimum concentrations, an experiment with the above specified conditions was performed and resulted the total reducing sugars yield of 750.66 mg/g and glucose yield of 306.50 mg/g which are close to the predicted values.

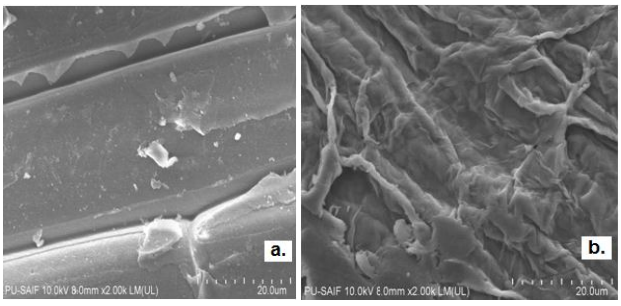

Figure 2: FE-SEM images of untreated (a.) and alkali pretreated corn stover biomass (b.)

FE-SEM micrographs of the untreated and alkali pretreated corn stover clearly indicate the smooth surface of untreated corn stover which was changed to more porous structure with increased surface area and ruptured cell walls following NaOH pretreatment (Fig 2).

IV. CONCLUSION

The thermo-alkali pretreatment of corn stover with dilute NaOH and steam under pressure has been found to be very effective for the enzymatic hydrolysis with in-house produced cellulase system by a natural variant of *P. sclerotiorum* exhibiting the sugar yields comparable to those reported in the literature with expensive commercial cellulases. The statistical optimization of pre-hydrolysis conditions by response surface methodology led to 87.0 % hydrolysis efficiency yielding reducing sugars equivalent to 751 mg/g of pretreated corn stover biomass of which glucose amounted to 307 mg/g thus suggesting an indigenous low cost alternative to enzymatic hydrolysis using in-house produced enzyme system for the production of second generation bioethanol.

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