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Research Article

Statistical modeling of alkali pretreatment for degradation of saccharum

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ABSTRACT

Saccharum spontaneum (Kans grass) is consistent grass of South Asian descent. It is a source of cheap, renewable and common sugars that microorganisms can easily use to substantive and cost-effective compounds. The objective of the present study was to establish better conditions for Kan's grass pretreatment using the response surface methodology. In the present study, chemical (NaOH) and thermochemical (NaOH followed by steam) pretreatment of the Kans grass was performed. Three independent variables with three different levels, such as: the concentration of NaOH (0.6, 0.8, 1 % w/v), substrate loading (5, 10, 15 %), and time of reaction (4, 6, 8 hours) were employed for the liberation of total sugars (mg/ml), and total phenol content (mg/ml). In the pretreatment with alkaline steam, the substrate was immersed in an alkaline solution for 4, 6, and 8 hours, followed by autoclaving at 121 °C for 2 hours and at 15 psi. The maximum total sugar value released was 115.58 mg/ml in chemical pretreatment when the optimal conditions were 1 % NaOH concentration, 10 % loading of the substrate, and 4 hours of reaction time. The maximum total phenol value released was 65.25 mg/ml in thermochemical pretreatment when the optimal conditions were 1 % NaOH concentration, 10 % loading of the substrate, and 4 hours of reaction time. Statistical analysis of regression model equations and coefficient of determination (R^2) tested model significance. Higher F value supports the model's significance. This study provides a novel approach to optimizing the pretreatment of *Saccharum spontaneum* for enhanced sugar and phenol extraction, positioning it as a viable feedstock for biofuel production and other sustainable applications.

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INTRODUCTION

Limited supply and the growing demand for fuel have led to the exploration of alternative bioenergy sources. Among the alternative bioenergy sources, lignocellulose has been recognized as a major source of biofuels [1-3]. There should be short-term potential substitutes for fossil fuels as they are rapidly depleted. Among biofuels, cellulose-ethanol can be manufactured on an industrial scale

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and is also compatible with current automobiles and supply systems [4]. In this scenario, the wild plant *Saccharum spontaneum* could be a better choice for fuel ethanol manufacture. Lignocellulose sources are among the greatest upcoming raw materials for ethanol manufacture.

S. spontaneum is a common weed growing in a vast amount of available non-agricultural land next to roads, canals, and banks. It is a tall perennial grass that spreads in many tropical nations' naturally deserted and pastoral areas. Despite having many possible uses and functions, this species must still be appreciated and utilized. In Hindi, it is referred to as "Kans" and in English as "Wild cane" [5]. Cellulose, hemicellulose, and lignin are the main components of lignocellulose [6]. Lignocellulosic raw materials show elastic resistance to enzymes, so an appropriate pretreatment is required to help contact the plant polysaccharides with the enzymes. Specifically, the pretreatment results typically include a reduction of lignin content, an increase in surface area, and a drop in crystallinity of biomass; all results improved the enzyme rate of hydrolysis and gain [7-9]. Physical treatments are designed to maximize the available surface area of the lignocellulose components by dropping their mechanical consistency and disrupting their basic dimensions. Chemical pretreatment is the most common technique for removing lignin and hemicellulose from lignocellulose compounds and distributing lignocellulose components of lignocellulosic fibers. Chemical pretreatment involves acid and base pretreatment. Steam explosion (steam cracking and explosive decompression) is one of the best physicochemical techniques frequently used for the pretreatment of lignocellulose components. Also, microorganisms or enzymes are functional to pretreat lignocellulosic components. Certain microbes rejected for a selective preparation to prevent hemicellulose and lignin are white, brown, and soft rot fungi [10].

Response surface methodology (RSM) is a precise and arithmetic exploration that is beneficial for demonstrating and investigating issues with the response of concern being affected by numerous variables. RSM is abundantly used to optimize various steps in enzyme hydrolysis and biotechnological processes. RSM's primary objective is to examine response variation by adjusting the factors to get an ideal answer. In contrast to conventional experiment designs, RSM lessens both the number of experiments and the interacting effects of the variables being researched. As a result, there is a corresponding decrease in material use [11-13]. Box-Behnken design (BBD) was selected because of three variables and three levels. Three Box-Behnken designs were used to improve delignification conditions in subsequent enzymatic hydrolysis and obtain a new polynomial of the equation, and three days were used to build feedback plans. Box-Behnken-suggestions are test methods of reaction surfaces, with relationships being examined in a much more descriptive variable and whether there is more than one variable [14].

The main objective of this study was to find the optimum conditions for saccharification of a novel feedstock, Kans grass, to release maximum cellulosic contents. The purpose was to enhance chemical and thermochemical pretreatment by BBD of RSM to increase the total phenol and total sugar content released from Kans grass so that this study could help in utilizing this novel substrate in different industries, especially biofuel industry for renewable energy, in agriculture for organic fertilizers and animal feed, and biochemistry for the development of bioplastics and other bio-based chemicals. These applications promote sustainability and resource efficiency while reducing reliance on fossil fuels.

MATERIALS AND METHODS

Substrate Processing

Kans grass was taken from Sargodha, Pakistan. The substrate was washed, dried, and milled into powder for further processing [15].

Pretreatment

Two pretreatments, chemical and thermochemical pretreatment using NaOH in 13 different experimental runs, were optimized through the Box-Behnken design. Pretreatment experiments were performed in 500 ml Erlenmeyer flasks. BBD was used to explore the synchronized effect of NaOH concentration, biomass used, and reaction time on aggregate phenol and aggregate sugar amount. There were three different ranks for every variable: -1, 0, and 1 (shown in Table 1).

The pretreatment of Kans grass was performed as described in an earlier report [16]. In base pretreatment, 100 mL solutions at various concentrations (0.6, 0.8, 1% w/v) of NaOH were used to pretreat 5, 10, and 15 g chopped substrate and soaked at room temperature for various time

Table 1. Box–Behnken design codes and levels of the variables

Independent variables	Symbols	Coded and actual values		
		-1	0	+1
NaOH concentration (%)	X_1	0.6	0.8	1
Substrate concentration (%)	X_2	5	10	15
Time (h)	X_3	4	6	8

intervals (4, 6, and 8 h). After completion of the pretreatment, all the samples were filtered and cleaned with distilled water till they acquired a neutral condition. Then, they were dried up at a temperature of 70°C for one day and kept in small-sized storage bags at 25°C. The filtrates of all experimental runs were kept in a freezer to analyze phenol and sugar contents.

In thermochemical pretreatment, the whole above procedure was followed by autoclaving at 121 121˚C and 15 psi after the completion of their residence time. The autoclaved samples were filtered, and residues were cleaned with distilled H_2O 5 to 6 times to neutralize them. The filtrate of every pretreated material was kept in the fridge, and residues were dried up at 70 70˚C for further study.

Experimental Design

The investigations were performed on a BBD (Box-Behnken design) with a quadratic model employed to find the combined result of three self-determining variables: concentration of NaOH, biomass loading, and reaction time. The 13 experimental runs were optimized to study the effects of three variables in the experimental reactions because of unnecessary features. The following Equation can be used to calculate the coded and actual values of independent variables:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

Here, x_i is the unit less assessment of an autonomous variable, X_i is the actual value of an autonomous variable, X_0 represents the central point value X_i, and ΔX_i indicates the phase variation [17,18]. A second degree polynomial equation was used for the experimental figures using the statistical software Minitab v. 17.0 to calculate the response of the dependent variable and predict the optimal conditions. The second-degree polynomial was stated as:

$$Y=\beta_0 + \beta_1 X_I + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

In equation (2), Y is the predicted response, X_1, X_2, and X_3 are independent variables, β_0 is the offset term, β_1, β_2, and β_3 are linear effects, and β_{11}, β_{22}, and β_{13} are interaction terms.

The substrate was filtered through muslin cloth, cleaned with water, dried at 60 60°C for 6 hours, and kept in slider storage bags after the pretreatment time was completed. The material was analyzed to calculate the total sugar and total phenol.

Total Phenol Estimation

As described by the method of Carralero [19], Total phenolic compounds of pretreated biomass were calculated. Samples were diluted, and each run was executed triplicate. In test tubes, 0.5ml of the sample was taken, then 2.5ml of Folin's reagent solution (1:10 solution of Folin's reagent in water) and 2 ml of 7.5% Na_2CO_3 solution were added. The reaction mixture was stayed at room temperature for 2 hours. The optical density of the solution was taken at 765 nm using a spectrophotometer. Vanillin was taken as standard.

Total Sugar Estimation

As described by Dubois [20], the total sugar content of the pretreated substrate was measured. In a test tube, 0.5 ml of filtrate and 0.5 ml of fresh 5% phenol solution were added and tenderly mixed. After that, 2.5 ml of Conc. Sulphuric acid was added gently, which changed the solution color to dark brown. The test tubes were kept at a temperature of 25 °C for 30 minutes, and then absorbance was measured using a spectrophotometer at a wavelength of 490nm. Glucose was taken as standard. The whole methodology is described in Figure No. 1.

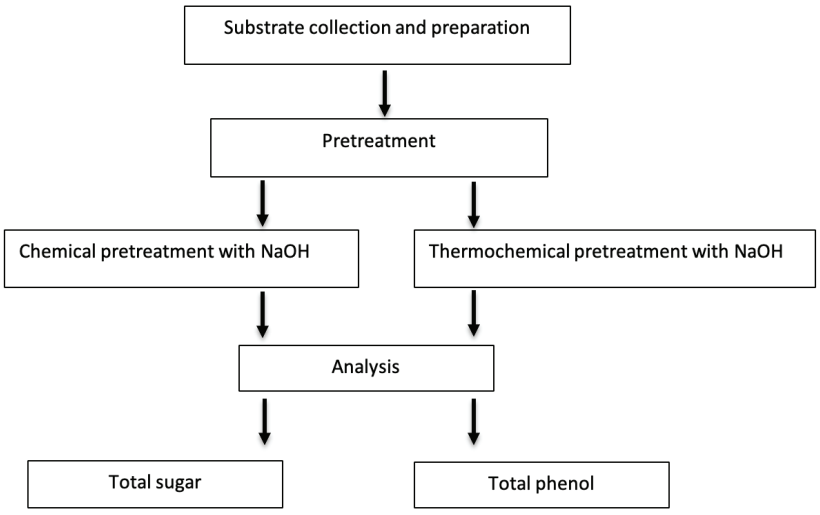

Figure 1. Flow diagram of the methodology used.

RESULTS AND DISCUSSION

Pretreatment experimentation with or without steam for the different concentrations of sodium hydroxide (0.6, 0.8, 1%) time of reaction (4, 6, and 8 h) at a loading of biomass (5, 10, and 15 %) were monitored. With the help of hydrogen bonds, carbohydrate units are strongly attached to lignin while some are covalent bonds. The basic purpose of pretreatment/hydrolysis processes is delignification to break down cellulosic and hemicellulosic biomass into lignin and degradation of the carbohydrates to form simple sugars. Lignin is made up of phenolic compounds, and it is the cause of hardness. The aim of the present investigation was to find out the influence of concentration of base, loading of biomass, and time of exposure on the discharge of phenolic compounds. RSM with median complex as the statistical model was used to increase the formation of phenolic compounds in the hydrolysate optimization process. In run no. 8, the highest total phenolic compounds (62.52 mg/ml) was produced in NaOH pretreatment, when NaOH concentration was 0.6%, time 8h, and substrate loading was 10g (Table 2). While in run no. 4, the highest total phenolic compounds (65.24 mg/ml) was observed in base steam pretreatment when 10g of the substrate and 1% NaOH were used for a reaction time of 4 hours.

The total amount of phenol and total sugar manufactured was calculated using experimental evidence. Total phenolic compounds and total sugar produced using NaOH pretreatments ranged from 10.94 mg/ml to 62.52 mg/ml and 38.39 mg/ml to 115.5 mg/ml, respectively. By using NaOH steam pretreatment, the amount of total phenolic compounds and total sugar produced fluctuated from 16.34 mg/ml to 65.24 mg/ml and 27.89 mg/ml to 93.24mg/ml, respectively. In base and base steam pretreatment, run no. 8 (62.52mg/ml), and Run no. 4 (65.24 mg/ml) produced the maximum phenol content, respectively. By the pretreatment without and with steam, the maximum total sugar contents produced are 115.58mg/ml and 93.24 mg/ml, respectively.

Chemical Pretreatment

In chemical (NaOH) pretreatment, overall total phenol content fluctuated between 10.94 mg/ml for run no. 10 (0.6% Sodium Hydroxide Concentration, 10% w/v substrate used, 4h reaction interval) to 65.24 mg/ml for run no.8 (0.6% NaOH concentration, 10% w/v substrate used, 8 h reaction interval) by BBD experimental set up. Polynomial equations of second order that symbolize association among phenol content and three autonomous variables of base pretreatment are produced by the demonstration of RSM on data obtained through experiments.

$$\text{Total Phenolic compounds (mg/ml)} = 59.4 - 86.9\,X + 5.52X_2 - 14.81\,X_3 + 67.0\,X_1^2 - 0.1564\,X_2^2 + 1.288\,X_3^2 - 1.759X_1X_2 - 0.00\,X_1X_3 - 0.1413\,X_2X_3 \quad (3)$$

$$\text{Total Sugar (mg/ml)} = -32.3 - 58\,X_1 + 5.85X_2 + 13.5\,X_3 + 81.0\,X_1^2 + 0.142\,X_2^2 - 0.039\,X_3^2 - 3.12\,X_1X_2 - 6.67\,X_1X_3 - 0.830\,X_2X_3 \quad (4)$$

The total phenolic content released in response to NaOH pretreatment was evaluated by applying F-Test for the ANOVA, the arithmetical results fit into the polynomial regression equation of second order. The response surface regression of phenolic compounds for chemical (NaOH) pretreatment of *S. spontaneum* (kans grass) is shown in table no. 4. From the F value of the model, which was 9.58

Table 2. Actual and predicted values of total Phenol and total sugar released from base pretreated kans grass using BBD

Run No.	X1	X2	X3	Total Phenol (mg/ml)			Total Sugar (mg/ml)		
				Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.8	10	6	32.89432	32.89432	-0.0000	69.5912	69.5912	0.0000
2	1.0	10	8	56.41936	48.03029	8.3891	79.464	93.8238	-14.3598
3	1.0	15	6	40.22680	38.55917	1.6676	96.5608	93.8024	2.7584
4	1.0	10	4	45.42064	53.14775	-7.7271	115.584	113.1610	2.4231
5	1.0	5	6	20.87720	23.20679	-2.3296	101.8584	92.6801	9.1783
6	0.6	15	6	19.40052	17.07093	2.3296	44.9952	54.1736	-9.1784
7	0.8	5	4	17.10912	7.05242	10.0567	45.8724	57.4738	-11.6014
8	0.6	10	8	62.52976	54.80265	7.7271	38.3904	40.8134	-2.4230
9	0.8	15	8	19.55328	29.60998	-10.0567	47.3172	35.7158	11.6014
10	0.6	10	4	10.94780	19.33687	-8.3891	91.4352	77.0754	14.3599
11	0.6	5	6	15.98888	17.65651	-1.6676	40.4544	43.2129	-2.7585
12	0.8	5	8	37.62988	43.68936	-6.0595	59.598	54.4165	5.1815
13	0.8	15	4	41.95808	35.89860	6.0595	83.076	88.2575	-5.1815

and a probability value of 0.011, indicated that the model was highly significant. The factor of linear term X_3 was highly significant than X_2 and X_1 by having a p-value of 0.04, which is less than 0.05. Since all other square terms are significant. So, X_1X_2 is significant with a p-value of 0.087. For this model, R^2 coefficient of determination was 94.52%, such a high value of R^2, and fitness of the model with the experimental data was displayed by adjusted R^2 (84.65%). This model was established to calculate the number of phenolic compounds produced by the chemical pretreatment using NaOH. After base pretreatment, the regression equation of second order for the discharge of phenolic compounds was used to construct contour plots and surface plots (Fig No. 5-7).

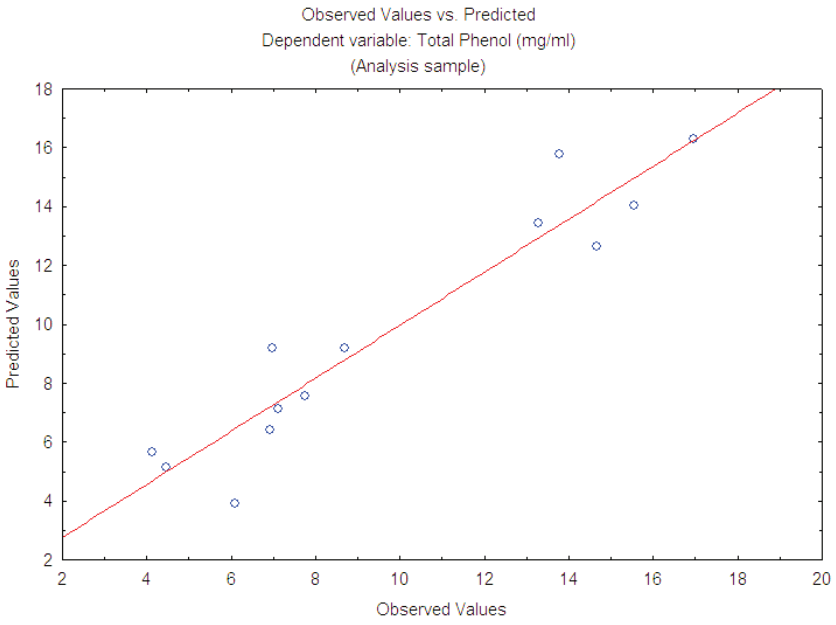

Figure 2. Graph between observed values and predicted values of Total Phenol for chemical (base NaOH) pretreatment of Kans grass.

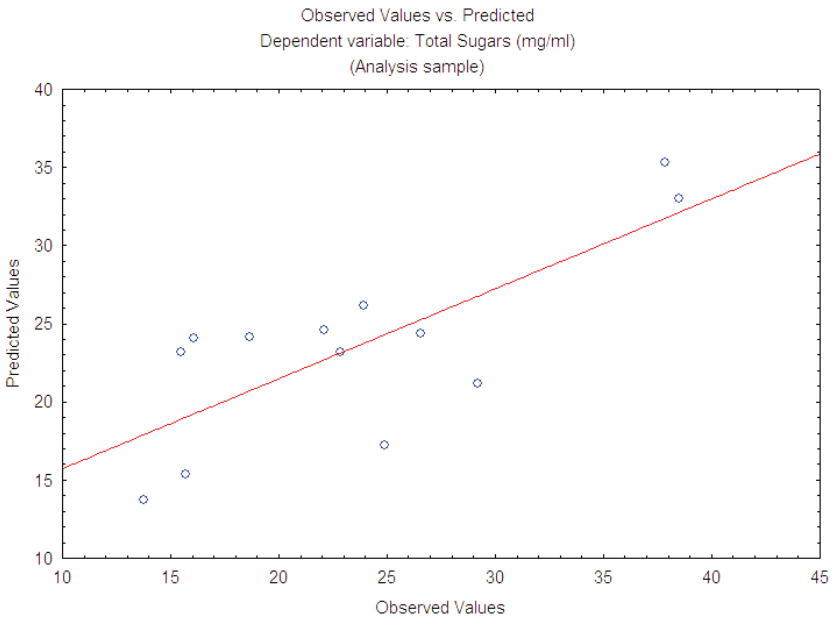

Figure 3. Graph between observed values and predicted values of Total Sugar for chemical (NaOH) pretreatment of Kans grass.

Figure 2 shows a plot between experimental results and predicted values using quadratic model equations for total phenol content after chemical pretreatment of substrate Kans grass. Results showed a strong correlation between actual and predicted values. Figure 3 shows a plot between experimental results and predicted values using quadratic model equations for total sugar content after chemical pretreatment of substrate Kans grass. Results showed a strong correlation between actual and predicted values. Model significance was tested by analysis of variance (ANOVA) of regression model equations and coefficient determination $R^{2,}$ as shown in Table 4. Higher R^2 values 77.64 and 94.52 indicate that the model explained the reaction very well. The higher values of Adj. R^2 37.40 and 84.65 also support the model's significance.

F-value statistically evaluated the significance of terms in polynomial functions at P 0.001, 0.01, and 0.05. The models have F values of 9.58 and 1.93 and probability values of 0.011 and 0.243 for chemical (NaOH) substrate pretreatment for total Phenol and total sugar analysis, respectively.

Table 4. Analysis of variance of total phenol & total sugar after NaOH treatment

Total Phenol (mg/ml)	Sources	DF	Adj SS	Adj MS	F value	P value
	Model	9	237.91	26.35	9.58	0.01
	Linear	3	24.90	8.30	3.02	0.13
	X_1	1	2.24	2.24	0.82	0.40
	X_2	1	3.88	3.88	1.41	0.28
	X_3	1	18.77	18.77	6.82	0.04
	Square	3	191.92	63.97	23.26	0.00
	X_1 2	1	26.50	26.50	9.63	0.02
	X_2 2	1	56.41	56.41	20.51	0.00
	X_3 ^{2}	1	98.02	98.02	36.63	0.00
	2 Way interaction	3	20.36	6.78	2.47	0.17
	X_1*X_2	1	12.37	12.37	4.50	0.08
	X_1*X_3	1	0.00	0.00	0.00	1.00
	X_2*X_3	1	7.98	7.98	2.90	0.14
	Error	5	13.75	2.75		
	Lack of fit	3	13.42	4.47	26.84	0.03
	Pure error	2	0.33	0.16		
	Total	14	250.94			
Total Sugar (mg/ml)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	729.71	81.07	1.93	0.24
	Linear	3	306.39	102.13	2.43	0.18
	X_1	1	0.09	0.09	0.00	0.96
	X_2	1	294.08	294.08	7.00	0.04
	X_3	1	12.22	12.22	0.29	0.61
	Square	3	80.51	26.83	0.64	0.62
	X_1 2	1	38.74	38.74	0.92	0.38
	X_2 2	1	46.27	46.27	1.10	0.34
	X_3 2	1	0.09	0.09	0.00	0.96
	2 way interaction	3	342.79	114.26	2.72	0.15
	X_1*X_2	1	38.87	38.87	0.93	0.38
	X_1*X_3	1	28.43	28.43	0.68	0.44
	X_2*X_3	1	275.49	275.49	6.56	0.05
	Error	5	210.11	42.02		
	Lack of fit	3	114.68	38.22	0.80	0.59
	Pure error	2	95.43	47.71		
	Total	14	939.82			

Table 5. Coded coefficients for total phenol and total sugar after NaOH treatment

	Term	Effect	Coef	SE Coef	T-Value	p
Total Phenol	Constant		7.281	0.958	7.60	0.001
	X_1	1.060	0.530	0.586	0.90	0.407
	X_2	1.394	0.697	0.586	1.19	0.288
	X_3	-3.064	-1.532	0.586	-2.61	0.048
	X_1* X_1	5.358	2.679	0.863	3.10	0.027
	X_2* X_2	-7.818	-3.909	0.863	-4.53	0.006
	X_3* X_3	10.305	5.152	0.863	5.97	0.002
	X_1* X_2	-3.518	-1.759	0.829	-2.12	0.087
	X_1* X_3	-0.000	-0.000	0.829	-0.00	1.000
	X_2* X_3	-2.826	-1.413	0.829	-1.70	0.149
Total Sugar	Constant		18.68	3.74	4.99	0.004
	X_1	0.21	0.11	2.29	0.05	0.964
	X_2	12.13	6.06	2.29	2.65	0.046
	X_3	-2.47	-1.24	2.29	-0.54	0.613
	X_1* X_1	6.48	3.24	3.37	0.96	0.381
	X_2* X_2	7.08	3.54	3.37	1.05	0.342
	X_3* X_3	-0.32	-0.16	3.37	-0.05	0.965
	X_1* X_2	-6.24	-3.12	3.24	-0.96	0.380
	X_1* X_3	-5.33	-2.67	3.24	-0.82	0.448
	X_2* X_3	-16.60	-8.30	3.24	-2.56	0.051

The model's values 9.58, and 1.93 and their respective p values 0.011 and 0.243, F>P>0.001 shows the model's accuracy (Table No.4). A p value of less than 0.05 shows the significance of the model. Model terms X_1, X_2, X_3 and quadratic term interactions X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2, X_3^2 were also found significant. Higher R^2 values of 77.64 and 94.52 and the adjusted R^2 values of 37.40 and 84.65 indicate that actual results are by the model's predicted values.

The range of total sugar values was from 38.39 mg/ml to 115.58 mg/ml. Minimum and maximum values are respectively yielded by run no. 8 (0.6% NaOH, 10% loading of substrate, 8 hours time of reaction) and run no. 4, 1% Sodium Hydroxide, 10% loading of substrate, 4 hours time of reaction) as shown in Table No.3. Given polynomial equations of second order are produced by the demonstration of RSM based on experimental data that symbolize association among concentration of sugar and 3 autonomous variables of chemical pretreatment. *S. spontaneum* is a novel substrate with an unlimited potential for ethanol production [21]. Kans grass biomass Cell wall has 68%, 43.78%, and 24.22% dry matter of carbohydrate, cellulose, and hemicellulose fractions, respectively, showing the production cost of fuel ethanol [22]. For the maximum release of the phenol content, the optimal conditions for the base pretreatment recorded were 0.6% NaOH, 10% load of the biomass substrate, and 8h reaction or pretreatment time. A maximum discharge of sugar was observed when 1% NaOH, 10% load, and substrate 4 hours reaction time were used. Total phenol and total sugars liberated at this stage were 62.52 mg/ml and 115.58 mg/ml, respectively. The adjoining analysis demonstrated that raw Kans grass comprised 38.5% celluloses, 18.5% hemicelluloses, and 15% lignin compounds. Raw biomass pretreatment is essential for releasing the highest sugar content in successive scarification and decreasing lignin content. The highest value of cellulosic components and delignification of 60.6% and 51.5%, correspondingly, was attained at a concentration of 2.5% NaOH with a reaction time of 24 hours. Increasing the reaction time up to 48 hours led to a decline in cellulosic components. The earlier study revealed that the highest cellulosic components and delignification were attained after 24 h of reaction time, and a decline was detected at 48 hours of reaction time [23].

The results of ANOVA for overall sugar released after chemical (NaOH) pretreatment are displayed in Table No.8. The model's f and p-value, which were 1.93 and 0.24, respectively, illustrate that the model is insignificant. All this shows that the X_1 factor is more significant than X_2 and X_3 by having 0.04 p-values. Except for X_2X_3 with a p-value of 0.05, which is significant, all other square and interaction terms are insignificant. For this model, R^2 and adjusted R^2 values were 77.64% and 37.40% respectively. The model was well accomplished in chemical pretreatment for clarifying a 77.64% deviation in response to the overall sugar content of the studied variables. After the pretreatment by

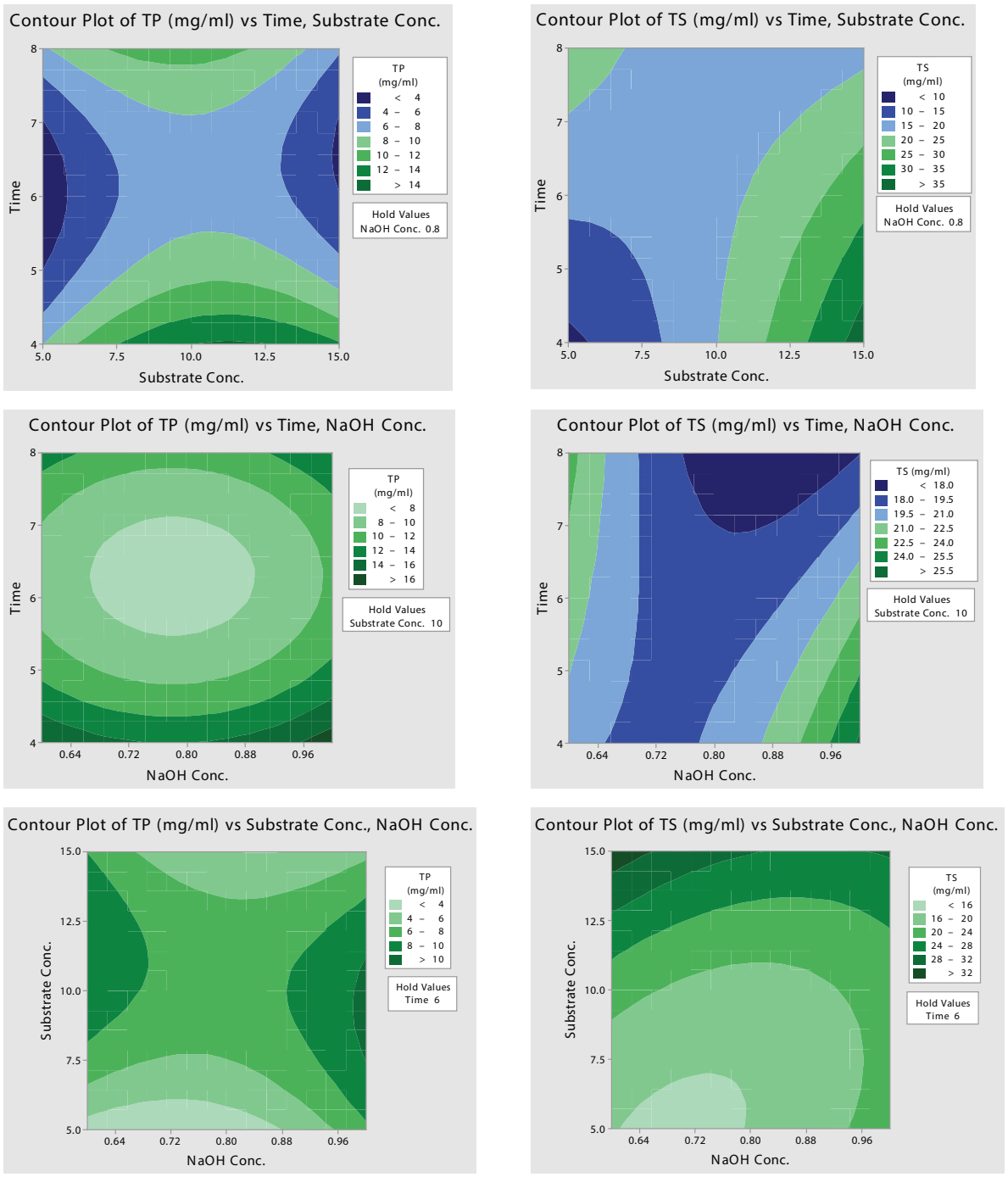

Figure 4. Contour surface plot between substrate concentration, time interval, and NaOH concentration (0.8 %) for chemical (NaOH) pretreatment.

NaOH (base), the second-order regression equation was used to construct 2D contour plots and surface plots to release overall sugar, as shown in Figure 4.

Thermochemical Pretreatment

The predicted and experimental results for base steam pretreatment of kans grass are shown in Table No.6. The observed phenol values ranged from 16.34mg/ml to 65.24 mg/ml. Minimum and maximum phenol content was observed in run no. 6 (0.6% NaOH, 15% biomass, 6h reaction time) and run no. 4 (1% NaOH, 10% loading of substrate, 4h time of reaction). The given below polynomial equation of 2nd order symbolizes the association among overall Phenol and three autonomous variables of thermochemical pretreatment, which are constructed by demonstration of RSM on data obtained through experiments.

$$\text{Total Phenolic contents} = -106 + 270\,X_1 + 6.54\,X_2 + 2.1\,X_3 - 53\,X_1^2 - 0.086\,X_2^2 + 2.90\,X_3^2 + 1.31\,X_1X_2 - 37.9X_1X_3 - 0.782\,X_2X_3 \quad (5)$$

$$\text{Total Sugar} = 337 - 525\,X_1 + 8.6\,X_2 - 40.4\,X_3 + 335\,X_1^2 - 0.228\,X_2^2 + 4.84\,X_3^2 + 5.25\,X_1X_2 - 14.2\,X_1X_3 - 0.986\,X_2X_3 \quad (6)$$

Conclusions drawn using analysis of variance (ANOVA) for thermochemical pretreatment are displayed in Table 8. The f-value of 1.49 and a P-value of 0.34 for total Phenol demonstrate that the model was insignificant. As all the linear terms and quadrates are insignificant, so the X_1X_3 term was more significant than other interactions by having a p-value of 0.07. To display a model using the investigational information, the determination coefficient (R^2) was 72.78%, and the value of R^2 and R^2 adjusted was 23.79%.

Figure No. 5 represents a plot between experimental results and predicted values using quadratic model equations for total phenol content after thermochemical pretreatment of substrate Kans grass. Results showed a strong correlation between actual and predicted values. Figure No. 6 showed a plot between experimental results and predicted values using quadratic model equations for total sugar content after thermochemical pretreatment of substrate Kans grass. Results showed a strong correlation between actual

Table 6. Actual and predicted values of total Phenol and total sugar released by thermochemical (NaOH steam) pretreatment of Kans grass using BBD

Run No.	X_1	X_2	X_3	Total Phenol (mg/ml)			Total Sugar (mg/ml)		
				Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.8	10	6	33.324	33.324	0.000	44.634	44.634	0.000
2	1.0	10	8	42.316	42.976	-0.659	65.291	68.325	-3.034
3	1.0	15	6	65.088	63.571	1.516	93.241	83.240	10.000
4	1.0	10	4	65.247	66.024	-0.777	89.182	88.937	0.244
5	1.0	5	6	35.381	35.460	-0.078	85.57	92.780	-7.210
6	0.6	15	6	16.349	16.270	0.078	71.552	64.341	7.210
7	0.8	5	4	30.784	29.928	0.856	46.827	39.861	6.966
8	0.6	10	8	30.638	29.860	0.777	50.224	50.468	-0.244
9	0.8	15	8	21.412	22.268	-0.856	39.766	46.732	-6.966
10	0.6	10	4	32.105	31.445	0.659	36.051	33.016	3.034
11	0.6	5	6	33.549	35.066	-1.516	27.898	37.899	-10.000
12	0.8	5	8	22.172	21.433	0.7386	49.983	39.738	10.244
13	0.8	15	4	37.670	38.408	-0.738	39.525	49.770	-10.244

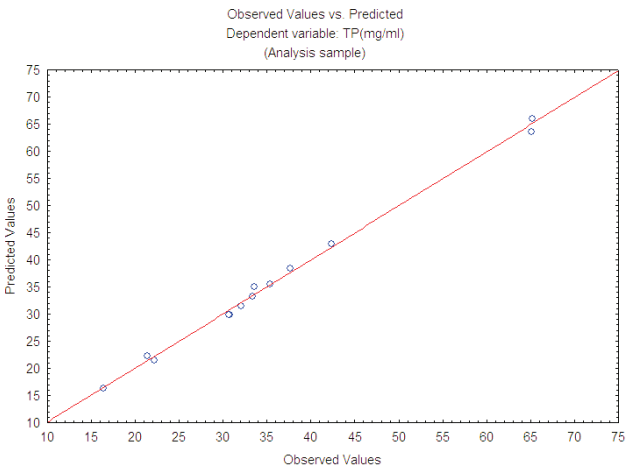

Figure 5. Graph between observed values and predicted values of Total Phenol for thermochemical (NaOH followed by steam) pretreatment of Kans grass.

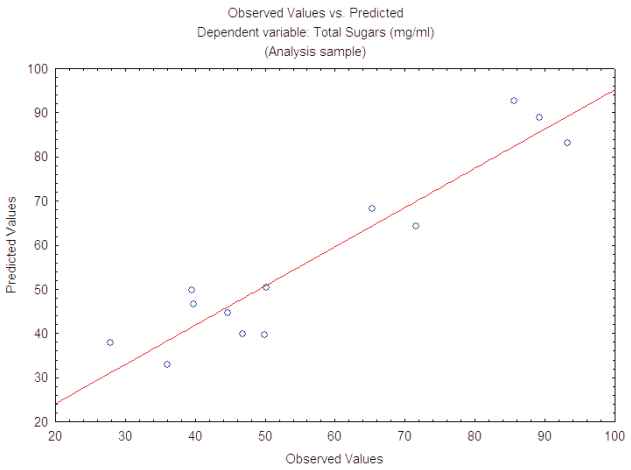

Figure 6. Graph between observed values and predicted values of Total Sugar for thermochemical (NaOH followed by steam) pretreatment of Kans grass.

and predicted values. Model significance was tested by analysis of variance (ANOVA) of regression model equations and coefficient determination R^2. Higher R^2 values, 73.98 and 99.65, indicate that the model explained the reaction very well. The higher values of Adj. R^2 27.16 and 99.01 also support the model's significance.

F-value statistically evaluated the significance of terms in polynomial functions at P 0.001, 0.01, and 0.05. The models have F values of 157.36 and 1.75 and probability values of 0.000 and 0.273 for thermochemical (NaOH followed by steam) substrate pretreatment for total Phenol and total sugar analysis, respectively. The model's values, 157.36, 1.75, and their respective p values 0.000 and 0.273, F>P>0.000, shows the model's accuracy (Table no. 6). Small P value shows the significance of the model. Model terms X_1, X_2, X_3 and quadratic term interactions X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2, X_3^2 were also found significant. Higher R^2 values of 73.98 and 99.65

Table 7. Analysis of Variance (ANOVA) of Total Phenol and Total Sugar released after thermochemical (NaOH steam) pretreatment

Total Phenol (mg/ml)	Sources	DF	Adj SS	Adj MS	F value	P value
	Model	9	2620.50	291.17	157.36	0.000
	Linear	3	1484.22	494.74	267.38	0.000
	X_1	1	1137.41	1137.41	614.70	0.000
	X_2	1	43.39	43.39	23.45	0.005
	X_3	1	303.42	303.42	163.98	0.000
	Square	3	456.43	152.14	82.22	0.000
	X_1 2	1	327.44	327.44	176.96	0.000
	X_2 2	1	97.92	97.92	52.92	0.001
	X_3 2	1	0.10	0.10	0.05	0.825
	2 Way interaction	3	679.85	226.62	122.47	0.000
	X_1*X_2	1	550.07	550.07	297.28	0.000
	X_1*X_3	1	115.17	115.17	62.24	0.001
	X_2*X_3	1	14.61	14.61	7.90	0.038
	Error	5	9.25	1.85		0.000
	Lack of fit	3	9.25	3.08		0.000
	Pure error	2	0.00	0.00		0.000
	Total	14	2629.75			0.000
Total Sugar (mg/ml)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	4399.4	488.8	1.58	0.320
	Linear	3	1621.8	540.6	1.75	0.273
	X_1	1	151.9	151.9	0.49	0.515
	X_2	1	1063.0	1063.0	3.44	0.123
	X_3	1	406.9	406.9	1.32	0.303
	Square	3	2149.1	716.4	2.32	0.193
	X_1 2	1	662.9	662.9	2.14	0.203
	X_2 2	1	119.5	119.5	0.39	0.562
	X_3 2	1	1381.6	1381.6	4.47	0.088
	2 way interaction	3	628.5	209.5	0.68	0.602
	X_1*X_2	1	110.4	110.4	0.36	0.576
	X_1*X_3	1	129.3	129.3	0.42	0.547
	X_2*X_3	1	388.9	388.9	1.26	0.313
	Error	5	1547.0	309.4		
	Lack of fit	3	1281.5	427.2	3.22	0.246
	Pure error	2	265.5	132.7		
	Total	14	5946.3			

Table 8. Coded coefficients for total Phenol and total sugar released after NaOH steam treatment

Total Phenol	Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
	Constant		33.325	0.785	42.43	0.000	
	X_1	23.848	11.924	0.481	24.79	0.000	1.00
	X_2	4.658	2.329	0. 481	4.84	0.005	1.00
	X_3	-12.317	-6.159	0. 481	-12.81	0.000	1.00
	X_1.* X_1	18.834	9.417	0.708	13.30	0.000	1.01
	X_2* X_2	-10.300	-5.150	0.708	-7.27	0.001	1.01
	X_3* X_3	-0.330	-0.165	0.708	-0.23	0.825	1.01
	X_1* X_2	23.454	11.727	0.680	17.24	0.000	1.00
	X_1* X_3	-10.732	-5.366	0.680	-7.89	0.001	1.00
	X_2.* X_3	-3.823	-1.911	0.680	-2.81	0.000	1.00
Total Sugar	**Constant**		**40.80**	**10.22**	**4.02**	**0.010**	
	X_1	-8.71	-4.36	6.22	-0.70	0.515	1.00
	X_2	23.05	11.53	6.22	1.85	0.123	1.00
	X_3	-14.26	-7.13	6.22	-1.15	0.303	1.00
	X_1.* X_1	26.80	13.40	9.15	1.46	0.203	1.01
	X_2* X_2	-11.38	-5-69	9.15	-0.62	0.562	1.01
	X_3* X_3	38.69	19.34	9.15	2.11	0.088	1.01
	X_1* X_2	10.50	5.25	8.79	0.60	0.576	1.00
	X_1* X_3	-11.37	-5.68	8.79	-0.65	0.547	1.00
	X_2.* X_3	-19-72	-9.86	8.79	-1.12	0.313	1.00

and the adjusted R^2 values of 27.16 and 99.01 indicate that actual results are by the predicted values of the model.

According to BBD in table no.6, for NaOH steam pretreatment, total sugar ranged from 27.89 mg/ml at run no. 11 (0.6% Sodium Hydroxide concentration, 5% loading of substrate, 6h time of reaction) to 93.24 mg/ml at run no. 3 (1% Sodium Hydroxide concentration, 15% substrate used, 6h time of reaction). Given polynomial equations of second order are produced by the demonstration of RSM based on experimental data that symbolize association among sugar concentration and 3 autonomous variables of thermochemical pretreatment. The results of ANOVA for overall sugar released after thermochemical pretreatment are displayed in Table 7. The model's f and p-values, which were 1.93 and 0.24, respectively, illustrate that the model is insignificant. All this shows that the X_1 factor is more significant than X_2 and X_3 by having 0.04 p-values. Except for X_2X_3 with a p-value of 0.05, which is significant, all other square and interaction terms are insignificant. For this model, R^2 and adjusted R^2 values were 77.64% and 37.40% respectively. The model was well accomplished in chemical pretreatment for clarifying a 77.64% deviation in response to the overall sugar content of the studied variables. After the pretreatment by NaOH steam, the second-order regression equation was used to construct contour plots for the release of overall sugar, as shown in Figure No. 7. Silverstein [24] described that the highest delignification of 65.63% was attained in 2% NaOH pretreatment for 90 minutes at a temperature of 121°C and pressure of 15 psi. Nadeem also described the results of attaining the highest delignification at a steaming time of 60 minutes [25]. Another study showed that 43% elimination of lignin was attained with 3.5% NaOH at 90°C for 90 minutes of reaction time [26].

In NaOH steam pretreatment, when minimum and maximum phenol content was obtained, the range of overall phenol produced was from 16.34 mg/ml to 65.24 mg/ml. Run no. 6 (0.6% NaOH), 15% substrate, 6 h time of reaction) and run no. 4 (1% NaOH), 10% substrate, 4h time of reaction) respectively. For base steam pretreatment, the range of total sugar was from 27.89 mg/ml in run no.11 (0.6 % NaOH), 5% loading of the substrate, 6h time of reaction) to 93.24 mg/ml at run no. 3 (1% NaOH, 15% biomass loading, 6h reaction time). To test NaOH concentration, reaction time, and temperature, response surface methodology was used by Chittibabu [27], who stated that the production of reducing sugar was improved by increasing the temperature and concentration of base. Maximum reducing sugar content was obtained when the base used was 1 %, the temperature was 110°C, and the time of reaction was 6 h. There was a decline in reducing sugar content over time because of sugar breakdown. These outcomes related to current work in which the highest delignification was perceived at an extreme concentration of base that is 1%, and steam also

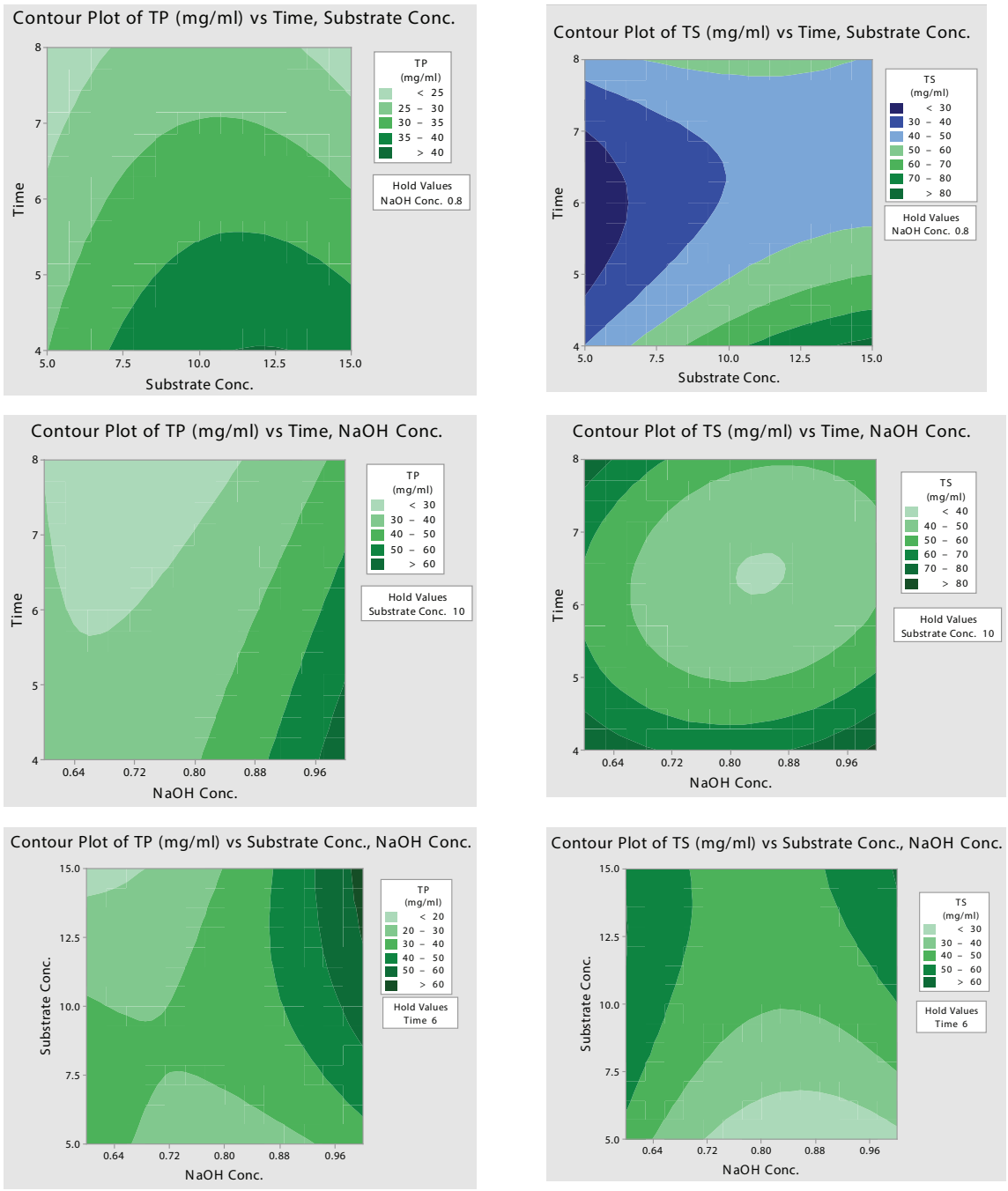

Figure 7. Contour plot between substrate concentration, time interval, and NaOH concentration (0.8 %) for the thermo-chemically pretreated substrate.

enhanced the delignification process as a consequence of elevated temperature.

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AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw

data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

STATEMENT ON THE USE OF ARTIFICIAL INTELLIGENCE

Artificial intelligence was not used in the preparation of the article.

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