MICROWAVE-ASSISTED ALKALI PRETREATMENT OF *HAPLOPHRAGMA ADENOPHYLLUM* LEAVES FOR BIOETHANOL PRODUCTION

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The current study is focused on the use of *Haplophragma adenophyllum* leaves for bioethanol production. The biomass was pretreated with two types of alkalis, *i.e.* KOH and NaOH. The Box-Behnken Design (BBD), with three levels and three variables, of the response surface methodology (RSM) was employed to determine the effects of alkali concentration, biomass loading and soaking time in the microwave oven on the release of total phenolic compounds, total sugars, reducing sugars and exposure of cellulose. NaOH was found more efficient in the removal of lignin from cellulose. Maximum cellulose (55%) was observed after the treatment with 0.5% NaOH concentration, 10% substrate concentration and 5 s residence time. FTIR spectroscopy of the raw and pretreated samples depicted the chemical changes incurred by the pretreatments. Commercial cellulase (40 FPU) was used for saccharification and maximum hydrolysis (50.1%) was obtained after 28 h. The hydrolysate was fermented using *Saccharomyces cerevisiae* and the highest ethanol yield (4.11%) was recorded after 96 h of fermentation.

Keywords: bioethanol, biomass, cellulose, pretreatment, alkali, assisted

INTRODUCTION

Considering the unsustainable and declining supply of fossil fuels, as well as their negative environmental effects, it is necessary to create technologies to increase the use and production of renewable energy. Abundant and ubiquitous biomass is generated by photosynthesis, which uses sunlight and CO_2, therefore, modifying bio-refineries to produce biofuels, especially bioethanol, could be one of the most practical approaches to solve the above-mentioned challenges. Bioethanol burning causes no net carbon addition to the atmosphere. Lignocellulosic biomass is one of most common feedstocks for second generation bioethanol.[1-4] However, despite considerable advances in biochemical conversion of biomass to biofuels, one of the biggest barriers to producing cheap biofuels from renewable feedstock is the need of suitable pretreatment for degrading plant cell walls in order to succeed saccharification and fermentation.

Lignocellulosic biomass is a plentiful organic polymer source found in plants, the bulk of which remains unused. With a global production of $1x10^{10}$ MT of lignocellulosic biomass, it represents a significant resource for energy generation.[4] The need for biomass-based goods will rise globally in the coming centuries. Biofuels will gradually drive the economic future, in addition to assuring food safety for a bigger and wealthier world population. Wastes and residues from a variety of industries, including forestry and agriculture,[5,6] make up lignocellulosic biomass. Biomass is mostly made of cellulose, hemicelluloses and lignin, depending on the source.[7] In nature, hydrogen and van der

Waals bonds bind cellulose fibres to hemicelluloses and lignin.

Cellulose may be converted into ethanol by hydrolysing it into glucose and then fermenting it. Due to its minor environmental effect, ethanol derived from lignocellulose biomass is widely recognised as an alternative or supplement to fossil fuel.[8] However, the poor bioconversion yield of lignocellulosic biomass into simple sugars presents a serious challenge to biotechnology. Due to the presence of lignin and the crystalline structure of the cellulose, untreated lignocellulose is difficult to break. A considerable quantity of phenolic chemicals is found in lignin.[9,10] Lignin prevents cellulolytic enzymes from accessing cellulose. A pretreatment is required to alter the structural and chemical composition of lignocellulosic biomass in order to facilitate rapid and effective conversion of the carbohydrate to sugars.[11]

The objective of the pretreatment is to break down the inherently resistant structure of the lignocellulosic biomass, which inhibits cellulose and hemicellulose bioconversion *via* enzymatic and microbiological processes. Chemical and physical structure factors regulate the enzymatic hydrolysis of cellulose. Chemical characteristics include the structure and composition of cellulose, while physical characteristics include cellulose crystallinity, polymerization, lignin and hemicellulose barrier, surface area, and hemicellulose acetylation.[12,13] Different pretreatment procedures have varying degrees of impact on these characteristics. Dilute acid pretreatment, for example, can primarily eliminate hemicelluloses and alter available surface area, while having little effect on lignin, whereas alkaline pretreatment can significantly decrease the lignin content, while having a moderate or slight effect on cellulose and hemicelluloses.[14]

Haplophragma adenophyllum, an elegant 30-50 feet tree with lovely flowers and foliage that is planted in gardens and avenues, and belongs to *Bignoniaceae* family. It is native to Assam, Myanmar and Bangladesh as well. The lower section of the plant's stem is woody, and the higher portion is often cylindrical and solid. The leaflets are elliptical, entire, acute, glabrous above and pubescent underneath. The leaves of this tree are enticing feedstock for biofuel generation die to their availability and high carbohydrate content as well. The aims of the current study are to employ these leaves as a substrate for bioethanol production. Response surface methodology (RSM) has been used to optimise microbial growth and enzyme synthesis in biological processes, notably in the manufacture of enzymes.[15] In this work, different concentrations of dilute potassium hydroxide were utilised to improve the pretreatment of *Haplophragma adenophylum* leaves, using Box-Behnken design (BBD), utilising three variables with three levels for bioethanol production.

EXPERIMENTAL

Substrate preparation

Leaves of *Haplophragma adenophyllum* were collected from the University of Sargodha, Punjab, Pakistan. The leaves were washed, dried and ground to powder form for further use.[16]

Pretreatment

The *H. adenophyllum* biomass was soaked in 100 mL of KOH and NaOH, separately, in Erlenmeyer flasks for certain time spans in a microwave oven, as stated in the experimental design described in Table 1.[16]

FTIR analysis of substrate

FTIR analysis of untreated and treated substrates was performed as described in our earlier reports.[17]

Analytical methods

The reducing sugars (RS) in the filtrate were determined using the 3,5-dinitrosalicylic acid (DNS) method.[18] The total sugar (TS) content was calculated using the method of Dubois.[19] The method of Carralero *et al*. was used to measure the total phenols in the filtrate to test the degradation of lignin by pretreatment.[20] Also, Gopal and Ranjhan's method was used to calculate the cellulose content of the solid residual portion.[21]

Experimental design

BBD was used to design experiments for optimization of alkali pretreatment following Ghazanfar *et al*.[16]

Saccharification

One percent pretreated substrate loading was hydrolyzed with 40 FPU commercial cellulase (Optiflow RC 2.0 (Danisco Genencor, Belguim)) in citrate buffer (pH 5) in a 250 mL Erlenmeyer flask. Hydrolysis was conducted as reported earlier.[1]

Inoculum preparation of *Saccharomyces cerevisiae*

Inoculum of a *S. cerevisiae* was prepared as described in our earlier report.[1]

Fermentation

The sugars obtained from hydrolysis of raw and treated biomass were fermented in different flasks for production of bioethanol.[23]

Statistical analysis

Minitab software (ANOVA) was used for statistical analysis of data.

RESULTS AND DISCUSSION

The pretreatment of powdered leaves of *H. adenophyllum* was done by using different concentrations of KOH and NaOH. It was assumed that maximum release of total sugars and reducing sugars shows maximum hydrolysis of cellulose and hemicelluloses of *H. adenophyllum* biomass, while maximum release of total phenol (TP) shows maximum degradation of lignin. The response obtained was calculated through polynomial regression equations shown below (Eqs. 1-8).

Table 1
BBD codes and the levels of variables

Variables	Symbols	Coded and uncoded levels		
		-1	0	+1
Alkali concentration (%)	X_1	0.1	0.55	1.0
Substrate concentration (%)	X_2	5	10	15
Microwave residence time (s)	X_3	5	10	15

Regression equations for KOH pretreatment:

$$\text{Cellulose (\%)} = 38.33+54.73X_1 - 6.517X_2 + 0.323X_3 - 28.07X_1^2 + 0.1393X_2^2 - 0.1007X_3^2 + 1.160X_1X_2 - 0.720X_1X_3 + 0.2720X_2X_3 \quad (1)$$

$$\text{TS (mg/mL)} = 22.44 + 34.37X_1 - 1.037X_2 - 0.644X_3 - 24.71X_1^2 + 0.3723X_2^2 + 0.0396X_3^2 - 2.165X_1X_2 + 4.448X_1X_3 - 0.2574X_2X_3 \quad (2)$$

$$\text{TP (mg/mL)} = 152.9 - 93.4X_1 - 3.36X_2 - 12.58X_3 + 37.2X_1^2 + 0.129X_2^2 + 0.512X_3^2 + 1.74X_1X_2 + 0.42X_1X_3 + 0.056X_2X_3 \quad (3)$$

$$\text{RS (mg/mL)} = -1.44 - 29.12X_1 + 0.838X_3 + 7.40X_1^2 - 0.1831X_2^2 - 0.0424X_3^2 + 0.645X_1X_2 + 1.210X_1X_3 + 0.0130X_2X_3 \quad (4)$$

Regression equations for NaOH pretreatment:

$$\text{TS (mg/mL)} = 117.96 - 110.33X_1 - 12.554X_2 - 0.275X_3 + 63.497X_1^2 + 0.39137X_2^2 + 0.21437X_3^2 + 3.2560X_1X_2 - 3.1920X_1X_3 + 0.09680\ X_2X_3 \quad (5)$$

$$\text{Cellulose (\%)} = 42.37 - 1.13X_1 + 3.952X_2 - 2.478X_3 - 9.833 - 2.1600X_1X_2 + 4.0000X_1X_3 - 0.16200X_2X_3 \quad (6)$$

$$\text{RS (mg/mL)} = 2.59 - 34.35X_1 + 3.933X_2 + 2.646X_3 + 11.82X_1^2 - 0.2144X_2^2 - 0.1637X_3^2 + 0.943X_1X_2 + 0.008X_1X_3 + 0.0752X_2X_3 \quad (7)$$

$$\text{TP (mg/mL)} = 135.34 - 72.64X_1 - 6.901X_2 + 2.051X_3 + 42.61X_1^2 + 0.4902X_2^2 - 0.3378X_3^2 - 1.197X_1X_2 + 0.229X_2X_3 - 0.0110X_2X_3 \quad (8)$$

Maximum total sugars of 75.88 mg/mL was liberated at 1% KOH concentration and 15% substrate concentration at 15 s residence time, and maximum reducing sugars were released at 1.5% KOH concentration, 10% substrate loading and 15 s of microwave residence time, as shown in Table 2. Maximum total phenol (96.51 mg/mL) was observed at 1% NaOH concentration and 15% substrate concentration at 5 s residence time (Table 5). Maximum cellulose content of 55% was observed at 0.5% NaOH concentration 10% substrate concentration and 5 s of microwave residence time, as shown in Table 5. The coefficient of determination values of 89.05%, 99.45%, 99.52% and 99.92% for total phenol, cellulose, reducing sugar and total sugar, respectively, obtained after KOH pretreatment revealed the accuracy of the model. Furthermore, the credibility of the model was supported by adjusted R^2 values (69.34%, 98.46%, 98.66% and 99.79% for total phenolic compounds, cellulose, reducing sugar and total sugar, respectively). The *F* values for total phenol, cellulose, reducing sugar, and total sugar were 4.52, 100.74, 115.30 and 724.87, respectively, as shown in Tables 6 and 7.

In the case of the NaOH treatment, the *P*-values of 0.000, 0.000, 0.000, and 0.000 for

cellulose, total sugar, total phenols, and reducing sugar, respectively, were found to be very significant and well described by the model. The Fisher's *F*-test values for cellulose, TS, TP, and RS were 914.48, 1822.16, 1936.21, and 224.87, respectively, as shown in Tables 8 and 9.

Table 2
Observed and predicted values of TS (mg/mL) and RS (mg/mL) after microwave-assisted KOH pretreatment of *H. adenophyllum*

Run				Total sugar (mg/mL)			Reducing sugar (mg/mL)		
no.	X_1	X_2	X_3	Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.5	10	15	43.190	43.486	-0.296	19.180	18.739	0.440
2	1.0	15	15	75.880	75.903	-0.023	20.190	20.298	-0.108
3	0.5	10	5	45.280	45.506	-0.226	11.009	11.491	-0.482
4	1.0	10	10	53.100	53.576	-0.476	16.610	16.436	0.173
5	1.0	5	15	72.160	72.066	0.093	13.780	14.594	-0.814
6	1.0	5	5	39.000	38.976	0.023	2.057	1.948	0.108
7	1.0	10	10	54.590	53.576	1.013	16.160	16.436	-0.276
8	0.5	15	10	66.900	66.580	0.320	12.180	12.512	-0.332
9	1.5	10	15	73.740	73.513	0.226	29.490	29.007	0.482
10	1.0	15	5	68.460	68.553	-0.093	7.163	6.348	0.814
11	1.0	10	10	53.040	53.576	-0.536	16.540	16.436	0.103
12	1.5	15	10	63.340	63.542	-0.202	19.580	19.954	-0.374
13	1.5	10	5	31.350	31.053	0.296	9.219	9.659	-0.440
14	0.5	5	10	39.250	39.047	0.202	11.060	10.685	0.374
15	1.5	5	10	57.340	57.660	-0.320	12.010	11.678	0.332

Table 3
Observed and predicted values of TP (mg/mL) and cellulose (%) after microwave-assisted KOH pretreatment of *H. adenophyllum*

Run				Total phenol (mg/mL)			Cellulose (%)		
no.	X_1	X_2	X_3	Observed	predicted	Residual	Observed	Predicted	Residual
1	0.5	10	15	40.590	41.587	-0.997	31.400	30.850	0.550
2	1.0	15	15	47.790	46.907	0.882	48.800	48.600	0.200
3	0.5	10	5	61.440	57.287	4.152	24.200	24.150	0.050
4	1.0	10	10	20.060	28.650	-8.590	38.800	38.533	0.266
5	1.0	5	15	33.140	28.872	4.267	32.800	33.500	-0.700
6	1.0	5	5	44.370	45.252	-0.882	43.800	44.000	-0.200
7	1.0	10	10	35.780	28.650	7.130	38.400	38.533	-0.133
8	0.5	15	10	43.260	43.145	0.115	30.600	31.350	-0.750
9	1.5	10	15	42.190	46.342	-4.152	30.200	30.250	-0.050
10	1.0	15	5	53.460	57.727	-4.267	32. 600	31.900	0.700
11	1.0	10	10	30.110	28.650	1.460	38. 400	38.533	-0.133
12	1.5	15	10	57.770	54.500	3.270	40. 000	40.150	-0.150
13	1.5	10	5	58.840	57.842	0.997	30. 200	30.750	-0.550
14	0.5	5	10	33.320	36.590	-3.270	35. 800	35.650	0.150
15	1.5	5	10	30.430	30.545	-0.115	33. 600	32.850	0.750

Table 4
Observed and predicted values of TS (mg/mL) and RS (mg/mL) after microwave-assisted NaOH pretreatment of *H. adenophyllum* substrate

Run				Total sugar(mg/mL)			Reducing sugar(mg/mL)		
no.	X_1	X_2	X_3	Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.5	10	15	43.330	43.230	0.100	25.270	25.168	0.101
2	1	15	15	37.790	37.717	0.072	24.900	24.848	0.051
3	0.5	10	5	9.370	9.390	-0.020	24.160	23.888	0.271
4	1	10	10	14.330	13.726	0.603	24.360	25.063	-0.703

5	1	5	15	37.710	37.902	-0.192	7.810	7.691	0.118
6	1	5	5	24.790	24.862	-0.072	10.080	10.131	-0.051
7	1	10	10	13.100	13.726	-0.626	25.870	25.063	0.806
8	0.5	15	10	19.910	20.082	-0.172	27.450	27.602	-0.152
9	1.5	10	15	44.590	44.570	0.020	23.730	24.001	-0.271
10	1	15	5	15.190	14.997	0.192	19.650	19.768	-0.118
11	1	10	10	13.750	13.726	0.023	24.960	25.063	-0.103
12	1.5	15	10	53.570	53.662	-0.092	31.330	31.110	0.220
13	1.5	10	5	42.550	42.650	-0.100	22.540	22.641	-0.101
14	0.5	5	10	41.480	41.387	0.092	18.700	18.920	-0.220
15	1.5	5	10	42.580	42.407	0.172	13.150	12.997	0.152

Table 5
Observed and predicted values of cellulose (%) and TP (mg/mL) after microwave-assisted NaOH pretreatment of *H. adenophyllum*

Run				Cellulose (%)			Total phenol (mg/mL)		
no.	X_1	X_2	X_3	Observed	Predicted	Residual	Observed	Predicted	Residual
1	0. 5	10	15	36.200	36.375	-0.1750	39.100	38.531	0.568
2	1	15	15	40.800	40.900	-0.100	49.800	49.865	-0.065
3	0.5	10	5	55.000	55.025	-0.0250	85.790	85.528	0.261
4	1	10	10	46.600	46.666	-0.066	61.150	61.273	-0.123
5	1	5	15	53.200	52.950	0.2500	34.210	34.452	-0.242
6	1	5	5	43.600	43.500	0.100	79.820	79.755	0.065
7	1	10	10	46.600	46.666	-0.066	61.130	61.273	-0.143
8	0.5	15	10	48.400	48.125	0.275	93.200	93.703	-0.503
9	1.5	10	15	54.000	53.975	0.025	42.320	42.581	-0.261
10	1	15	5	47.400	47.650	-0.250	96.510	96.267	0.242
11	1	10	10	46.800	46.666	0.133	61.540	61.273	0.266
12	1.5	15	10	35.000	34.925	0.075	90.950	90.623	0.326
13	1.5	10	5	32.800	32.625	0.175	86.720	87.288	-0.568
14	0.5	5	10	41.200	41.275	-0.075	71.430	71.756	-0.326
15	1.5	5	10	49.400	49.675	-0.275	81.150	80.646	0.503

Table 6
ANOVA for TP (mg/mL) and TS (mg/mL) after microwave-assisted KOH pretreatment of *H. adenophyllum*

	Sources	DF	Adj SS	Adj MS	F value	P value
TP (mg/mL)	Model	9	1810.35	201.150	4.52	0.056
	Linear	3	849.45	283.149	6.36	0.037
	X_1	1	14.10	14.098	0.32	0.598
	X_2	1	465.43	465.430	10.45	0.023
	X_3	1	369.92	369.920	8.31	0.034
	Square	3	873.07	291.024	6.54	0.035
	X_1^2	1	320.03	320.035	7.19	0.044
	X_2^2	1	38.64	38.641	0.87	0.394
	X_3^2	1	605.42	605.420	13.60	0.014
	2-Way interaction	3	87.83	29.276	0.66	0.612
	X_1*X_2	1	75.69	75.690	1.70	0.249
	X_1*X_3	1	4.41	4.410	0.10	0.766
	X_2*X_3	1	7.73	7.728	0.17	0.694
	Error	5	222.63	44.525		
	Lack of fit	3	95.87	31.956		
	Pure error	2	126.76	63.378	0.50	0.717
	Total	14	2032.97			
TS (mg/mL)	Model	9	2773.27	308.141	724.87	0.000
	Linear	3	1497.27	499.089	1174.06	0.000

X_1	1	121.29	121.290	285.32	0.000
X_2	1	558.28	558.281	1313.30	0.000
X_3	1	817.70	817.697	1923.55	0.000
Square	3	498.57	166.190	390.94	0.000
X_1^2	1	140.89	140.885	331.42	0.000
X_2^2	1	319.89	319.892	752.51	0.000
X_3^2	1	3.62	3.622	8.52	0.033
2-Way interaction	3	777.44	259.145	609.61	0.000
X_1*X_2	1	117.18	117.181	275.66	0.000
X_1*X_3	1	494.62	494.618	1163.54	0.000
X_2*X_3	1	165.64	165.637	389.64	0.000
Error	5	2.13	0.425		
Lack of fit	3	0.58	0.194	0.25	0.856
Pure error	2	1.54	0.771		
Total	14	2775.40			

Table 7
ANOVA for cellulose (%) and RS (mg/mL) after microwave-assisted KOH treatment of *H. adenophyllum*

	Sources	DF	Adj SS	Adj MS	F value	P value
Cellulose (%)	Model	9	534.323	59.369	100.74	0.000
	Linear	3	41.720	13.907	23.60	0.002
	X_1	1	18.000	18.000	30.54	0.003
	X_2	1	4.500	4.500	7.64	0.040
	X_3	1	19.220	19.220	32.61	0.002
	Square	3	261.043	87.014	147.65	0.000
	X_1^2	1	181.786	181.786	308.46	0.000
	X_2^2	1	44.801	44.801	76.02	0.000
	X_3^2	1	23.386	23.386	39.68	0.001
	2-Way interaction	3	231.560	77.187	130.97	0.000
	X_1*X_2	1	33.640	33.640	57.08	0.001
	X_1*X_3	1	12.960	12.960	21.99	0.005
	X_2*X_3	1	184.960	184.960	313.85	0.000
	Error	5	2.947	0.589		
	Lack of fit	3	2.840	0.947	17.75	0.054
	Pure error	2	0.107	0.053		
	Total	14	537.269			
RS (mg/mL)	Model	9	585.894	65. 099	115. 30	0.000
	Linear	3	440.284	146. 761	259. 94	0.000
	X_1	1	35.575	35. 575	63. 01	0.001
	X_2	1	51.035	51. 035	90. 39	0.000
	X_3	1	353.674	353. 674	626. 41	0.000
	Square	3	98.182	32. 727	57. 97	0.000
	X_1^2	1	12.622	12. 622	22. 36	0.005
	X_2^2	1	77.387	77. 387	137. 06	0.000
	X_3^2	1	4.157	4. 157	7. 36	0.042
	2-Way interaction	3	47.428	15. 809	28. 00	0.001
	X_1*X_2	1	10.401	10. 401	18. 42	0.008
	X_1*X_3	1	36.603	36. 603	64. 83	0.000
	X_2*X_3	1	0.425	0. 425	0. 75	0.425
	Error	5	2.823	0. 565		
	Lack of fit	3	2.706	0. 902	15. 38	0.062
	Pure error	2	0.117	0. 059		
	Total	14	588.717			

Table 8
ANOVA for TS (mg/mL) and cellulose (%) after microwave-assisted NaOH pretreatment of *H. adenophyllum*

	Sources	DF	Adj SS	Adj MS	F value	P value
Total sugar (mg/mL)	Model	9	3080.86	342.318	1822.16	0.000
	Linear	3	1288.47	429.490	2286.18	0.000
	X_1	1	598.58	598.580	3186.25	0.000
	X_2	1	50.50	50.501	268.82	0.000
	X_3	1	639.39	639.389	3403.48	0.000
	Square	3	1249.20	416.401	2216.51	0.000
	X_1^2	1	930.42	930.422	4952.65	0.000
	X_2^2	1	353.46	353.464	1881.50	0.000
	X_3^2	1	106.05	106.046	546.48	0.000
	2-Way interaction	3	543.19	181.062	963.80	0.000
	X_1*X_2	1	265.04	265.038	1410.80	0.000
	X_1*X_3	1	254.72	254.722	1355.89	0.000
	X_2*X_3	1	23.43	23.426	124.69	0.000
	Error	5	0.94	0.188		
	Lack of fit	3	0.18	0.061	0.16	0.915
	Pure error	2	0.76	0.379		
	Total	14	3081.80			
Cellulose (%)	Model	9	652.937	72.549	914.48	0.000
	Linear	3	46.370	15.457	194.83	0.000
	X_1	1	11.520	11.520	145.21	0.000
	X_2	1	31.205	31.205	393.34	0.000
	X_3	1	3.645	3.645	45.95	0.001
	Square	3	24.317	8.106	102.17	0.000
	X_1^2	1	22.314	22.314	281.27	0.000
	X_2^2	1	1.853	1.853	23.35	0.005
	X_3^2	1	0.314	0.314	3.96	0.103
	2-Way interaction	3	582.250	194.083	2446.43	0.000
	X_1*X_2	1	116.640	116.640	1470.25	0.000
	X_1*X_3	1	400.000	400.000	5042.02	0.000
	X_2*X_3	1	65.610	65.610	827.02	0.000
	Error	5	0.397	0.079		
	Lack of fit	3	0.370	0.123	9.25	0.099
	Pure error	2	0.027	0.013		
	Total	14	653.333			

According to the contour plot for the KOH treatment, maximum total phenols observed were of 30-55 mg/mL at a fixed substrate concentration of 10%, with varying time and alkali concentration (Fig. 1). Also, in the same situation, more than 25 mg/mL of reducing sugar was observed. When treatment time was fixed to 10 s, while varying substrate and KOH concentrations, more than 40-70 mg/mL total sugars were observed. Under the same conditions, more than 42% cellulose was obtained.

For the NaOH treatment, the contour plot for maximum total sugar shows 20-50 mg/mL at a hold value of time of 10 s and various substrate and alkali concentrations. Also, under these conditions, more than 30 mg/mL reducing sugar was observed. When varying time and alkali concentration, while maintaining the substrate at a fixed value of 10%, more than 55% of cellulose was achieved. Also, at a hold value of time of 10 s and various alkali and substrate concentrations, more than 90 mg/mL total phenol was observed (Fig. 2). To conclude, this pretreatment was extremely efficient in solubilizing lignin and hemicelluloses.

Observing the experimental values and those predicted by the model reveals that most of the results were very close to the predictions, with the maximum number of dots on the line confirming the correctness of the findings (Figs. 3 and 4).

Table 9
ANOVA for TP (mg/mL) and RS (mg/mL) after microwave-assisted NaOH pretreatment of *H. adenophyllum*

	Sources	DF	Adj SS	Adj MS	F value	P value
Total phenol (mg/mL)	Model	9	6053.03	672.56	1936.21	0.000
	Linear	3	4731.38	1577.13	4540.35	0.000
	X_1	1	16.88	16.88	48.59	0.001
	X_2	1	509.60	509.60	1467.08	0.000
	X_3	1	4204.90	4204.90	12105.38	0.000
	Square	3	1284.22	428.07	1232.36	0.000
	X_1^2	1	419.05	419.05	1206.40	0.000
	X_2^2	1	554.60	554.60	1596.64	0.000
	X_3^2	1	263.28	263.28	757.94	0.000
	2-Way interaction	3	37.43	12.48	35.92	0.001
	X_1*X_2	1	35.82	35.82	103.12	0.000
	X_1*X_3	1	1.31	1.31	3.77	0.110
	X_2*X_3	1	0.30	0.30	0.87	0.394
	Error	5	1.74	0.35		
	Lack of fit	3	1.63	0.54	10.17	0.091
	Pure error	2	0.11	0.05		
	Total	14	6054.77			
Reducing sugar (mg/mL)	Model	9	607. 357	67. 484	224. 87	0.000
	Linear	3	365. 387	121. 796	405. 85	0.000
	X_1	1	2. 916	2. 916	9. 72	0.026
	X_2	1	358. 986	358. 986	1196. 23	0.000
	X_3	1	3. 485	3. 485	11. 61	0.019
	Square	3	205. 600	68. 533	228. 37	0.000
	X_1^2	1	32. 232	32. 232	107. 41	0.000
	X_2^2	1	106. 095	106. 095	353. 53	0.000
	X_3^2	1	61. 853	61. 853	206. 11	0.000
	2-Way interaction	3	36. 370	12. 123	40. 40	0.001
	X_1*X_2	1	22. 231	22. 231	74. 08	0.000
	X_1*X_3	1	0. 002	0. 002	0. 01	0.945
	X_2*X_3	1	14. 138	14. 138	47. 11	0.001
	Error	5	1. 500	0. 300		
	Lack of fit	3	0. 344	0. 115	0. 20	0.890
	Pure error	2	1. 156	0. 578		
	Total	14	608. 858			

In previously published literature, Singh and coworkers reported maximum reducing sugar (1334.79 µg/mL) from saccharification of NaOH+microwave pretreated rice straw.[8] Also, microwave aided pretreatment of *Agropyron elongatum* was performed to improve lignin removal and enhance enzymatic saccharification. The results showed that the optimum pretreatment conditions were: 3% NaOH for 12 minutes at 180 W, leading to the optimum component loss of holocellulose and lignin – of about 24.5% and 74%, respectively.[24] Another study reported improved removal of lignin and hemicelluloses from empty fruit bunch, using microwave-assisted NaOH pretreatment. The authors concluded that such a pretreatment increased the accessibility of hydrolytic enzymes to the cellulose.[25]

The pretreated substrate that yielded the maximum cellulose content was subjected to FTIR for examining the chemical modifications incurred by the pretreatment. Figure 5 presents the FTIR spectra for the untreated and KOH treated substrates. It may be noted a clear difference in the peaks of the untreated and treated substrates, indicating the efficiency of the pretreatment. For instance, the peak at 2922.2 cm^{-1} in the raw biomass sample shifted to 2920.4 cm^{-1} after the KOH pretreatment. This indicated the stretching of -CH_2 bonds in cellulose.[26]

Another significant difference was observed regarding the peak located at 1604.2 cm^{-1} in the untreated substrate, and shifted to 1610.4 cm^{-1} in the KOH pretreated sample. The same peak it remained intact after the NaOH pretreatment. This shifting peak was due to C-O bond stretching, indicating solubilization of hemicelluloses.[17]

Also, the band at 1028.7 cm^{-1} in the untreated sample was shifted to 1047 cm^{-1} and 1026.9 cm^{-1} after the KOH and the NaOH pretreatments, respectively. This can be assigned to the stretching of C-O-C bonds of β-1,4-glycosidic linkage in polysaccharides.[28,29] Ghazanfar and coworkers examined the changes in the FTIR spectra of pretreated *Bombax ceiba* seed pods. The highest peak in raw *B. ceiba* was located at 1023.2 cm^{-1}, which rose up to 1028.7 cm^{-1} and 1026.9 cm^{-1} in KOH pretreated and KOH steam pretreated biomass, respectively. This peak shift represents changes in C-O stretching in cellulose.[1]

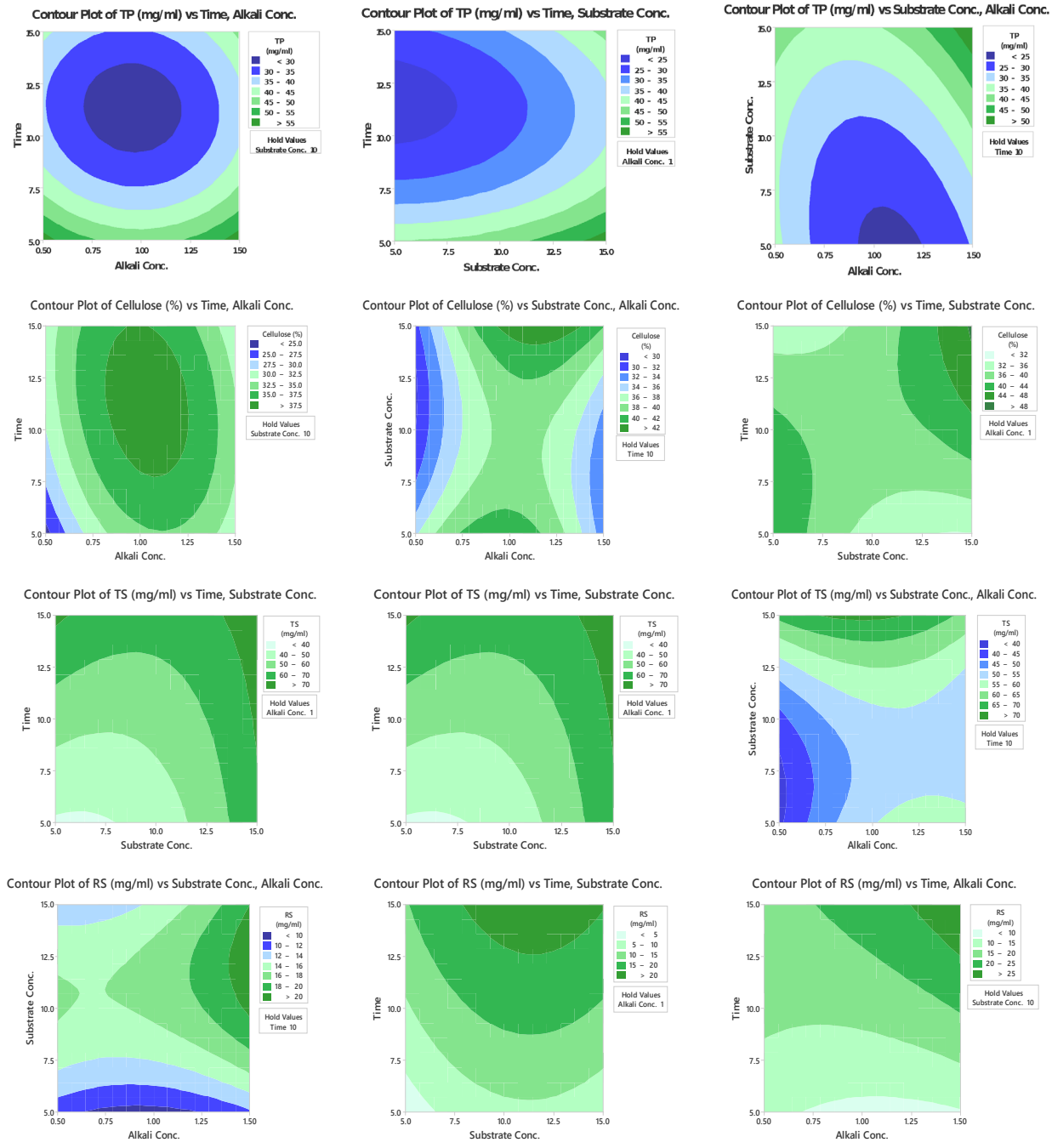

Figure 1: Contour plots for TP (mg/mL), cellulose (%), TS (mg/mL) and RS (mg/mL) after microwave-assisted KOH pretreatment of *H. adenophyllum*

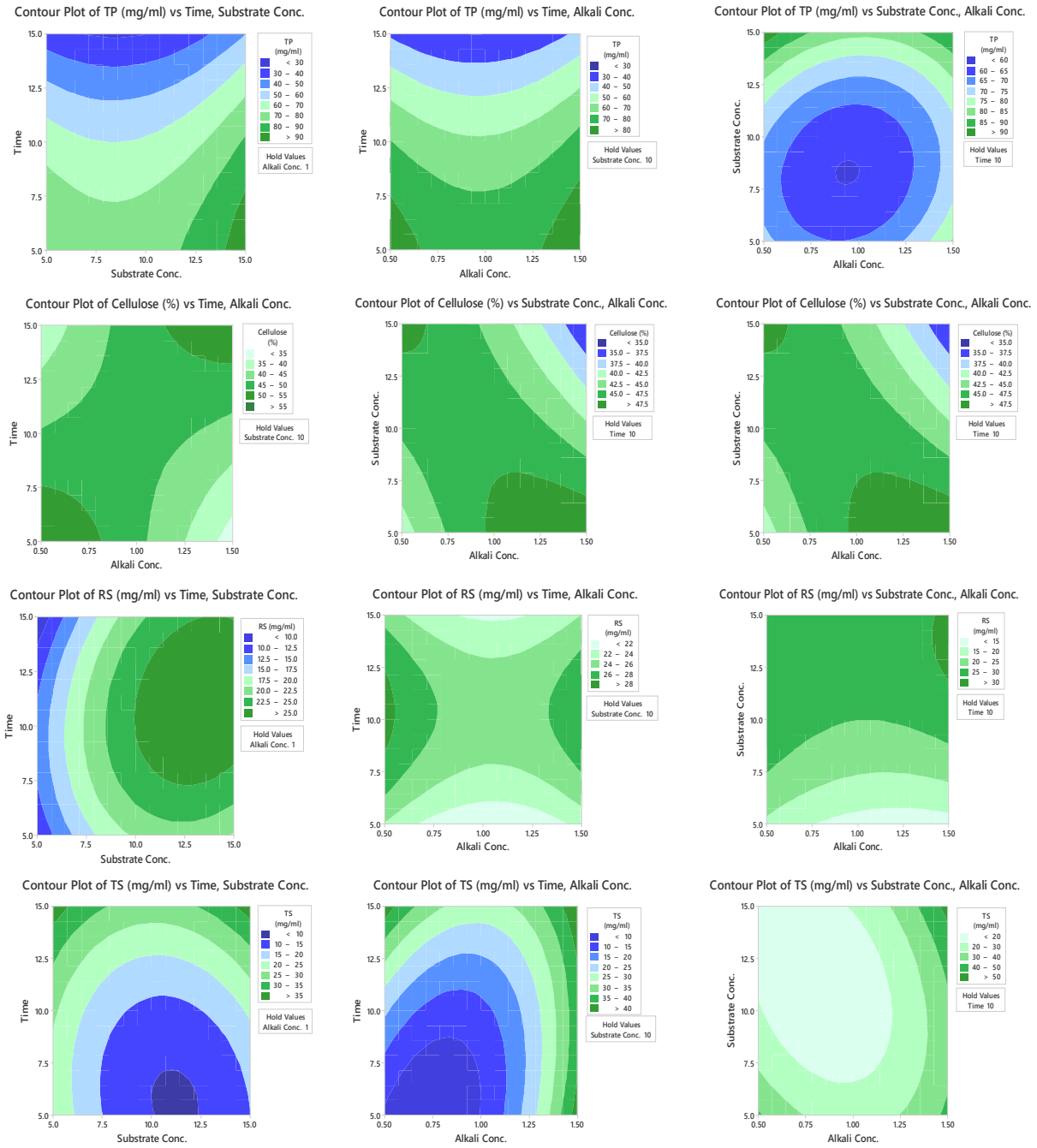

Figure 2: Contour plots for TP (mg/mL), cellulose (%), TS (mg/mL) and RS (mg/mL) after microwave-assisted NaOH pretreatment of *H. adenophyllum*

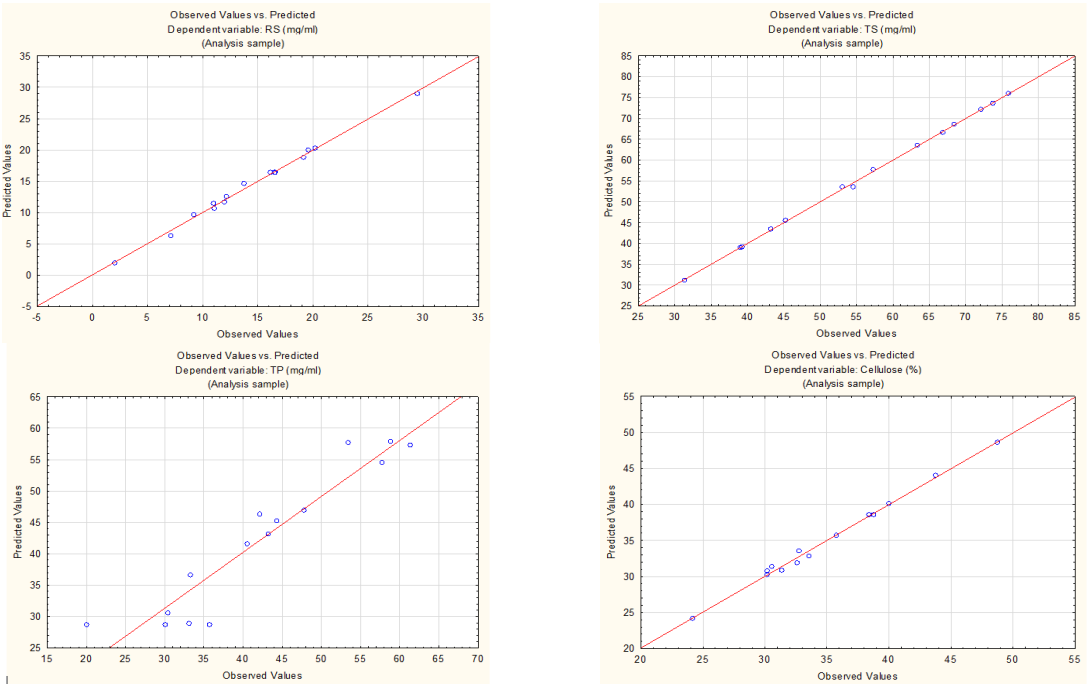

Figure 3: Graph for observed and predicted values of TS, RS, TP and cellulose from microwave-assisted KOH treated *H. adenophyllum*

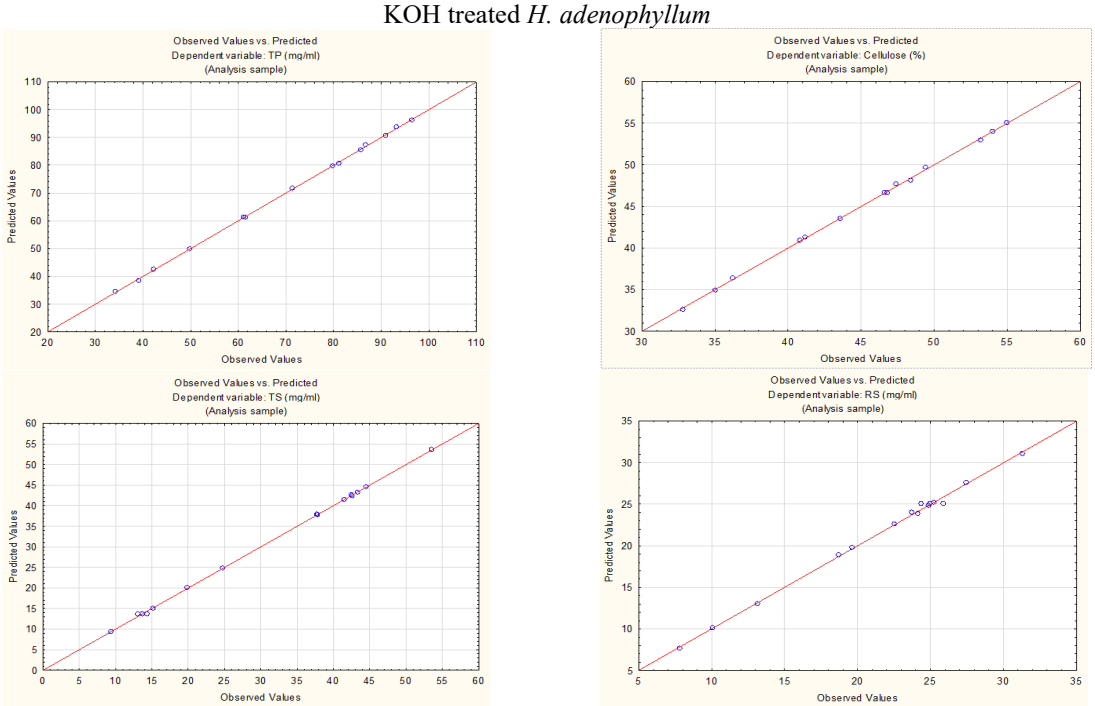

Figure 4: Graph for observed and predicted values of TS, RS, TP and cellulose from microwave-assisted NaOH *H. adenophyllum*

Further, the pretreated substrates that yielded high cellulose content, from each pretreatment, were saccharified with commercial cellulase, at a loading of 40 FPU. The results (Fig. 6) revealed that saccharification in the pretreated biomass was greater than that in the raw substrate. Maximum saccharification was recorded after 28 h in NaOH treated substrate (50.1%), followed by KOH treated (49.5%) and then, by the raw substrate (22.2%). A recent study also found better results of hydrolysis with commercial cellulase in separate hydrolysis and fermentation.[30] Another previous study reported that microwave-assisted alkali pretreated *Miscanthus* sp. yielded high sugars, as compared to conventional pretreatment.[31] Singh *et al*. obtained enhanced enzymatic digestibility of wheat straw with 2% NaOH for 3.16 min of microwave pretreatment

time.[32] The glucose yield was also enhanced in oat hull and canola straw by microwave-assisted alkali pretreatment.[33] According to Irfan *et al*. sugarcane bagasse treated with potassium hydroxide at a concentration of 1.5%, and with an autoclaving period of 30 minutes, generated the total sugar content of 192.32 mg/mL.[14] According to Mikulski *et al*. microwave radiation could be a substitute to traditional biomass heating that results in maximum exposure of cellulose.[34] Pooja *et al*. reported that microwave-assited alkali pretreatment was the most effective technique for ethanol production from cassava residues.[35]

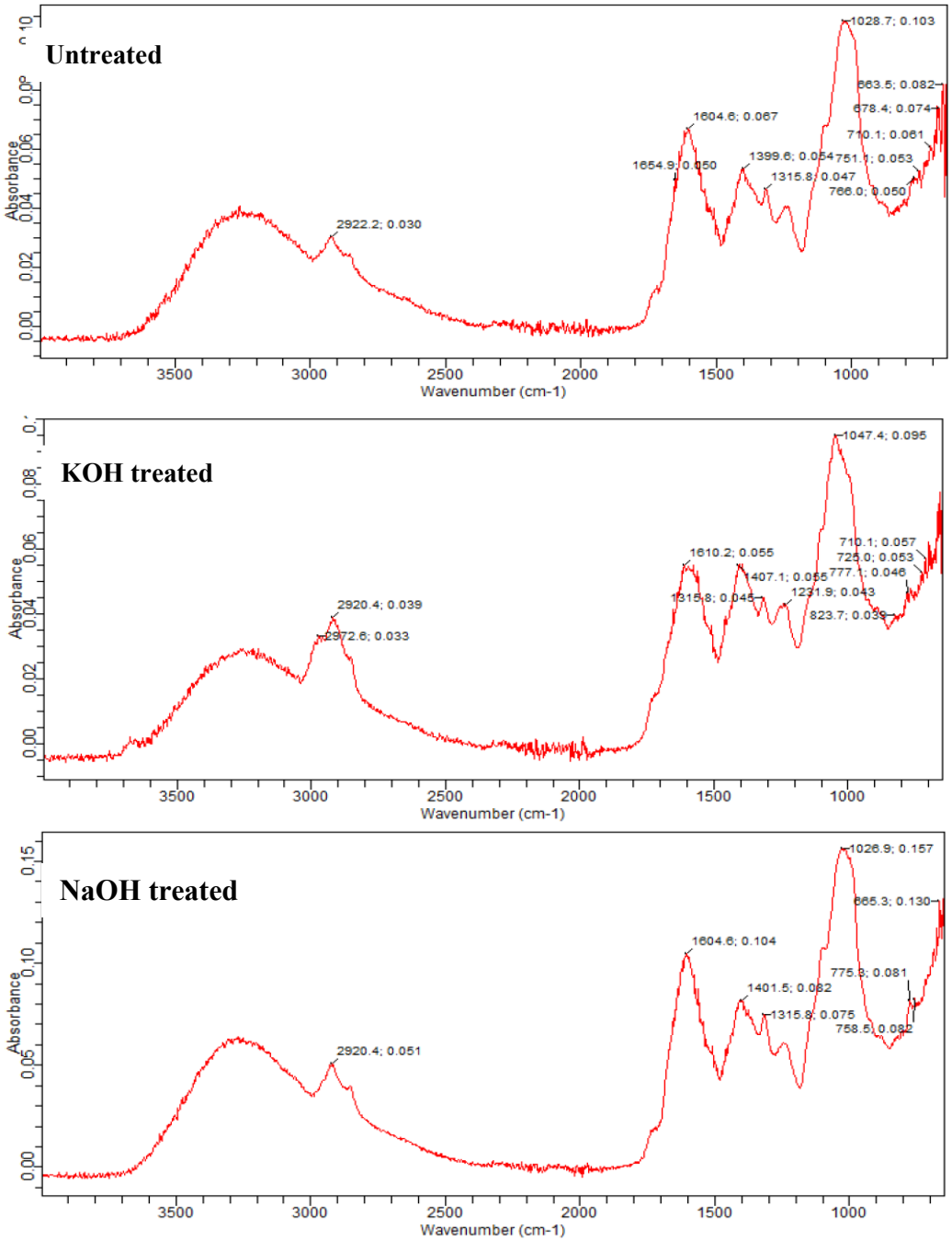

Figure 5: FTIR spectra of untreated and pretreated *H. adenophyllum*

After saccharification, the obtained sugars were supplemented with nutrients and further used for ethanol fermentation by *Saccharomyces cerevisiae*. Maximum ethanol titers were observed in untreated, KOH pretreated and NaOH pretreated substrate (4.06%) after 96 h of

fermentation (Fig. 7). After 96 h of fermentation, ethanol production tended to decline. This could be explained by the consumption of resources. In earlier studies, the hydrolysate obtained by the action of commercial cellulase offered a maximum ethanol titer in NaOH and steam treated biomass (48.8 g/L), followed by NaOH-treated (39.63 g/L) and untreated biomass (15.6 g/L).[30] Ethanol production in the hydrolysate of KOH pretreated and raw biomass reached 18.04 g/L and 8.73 g/L, respectively.[1] A study by Triwahyuni recorded a significant ethanol production from separate hydrolysis and fermentation of oil palm empty fruit bunch. Saccharification for four days formed 75.48% sugars, which later produced 78.95% ethanol.[36]

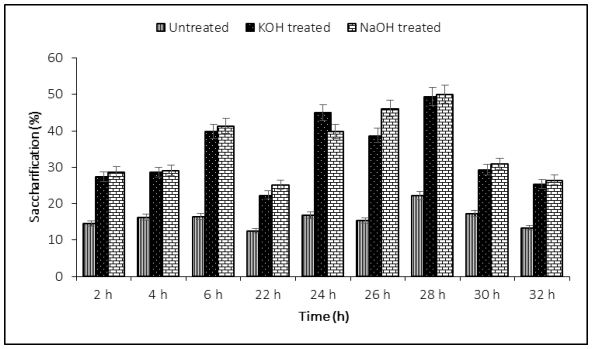

Figure 6: Saccharification at different time period using commercial cellulase

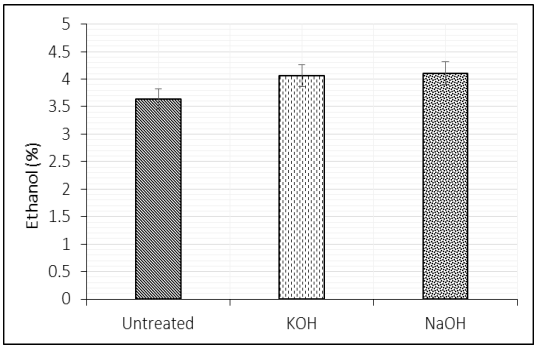

Figure 7: Ethanol production after 96 h in different substrates

CONCLUSION

The present research was aimed to optimize microwave-assisted KOH and NaOH pretreatments of *Haplophragma adenophyllum* leaves for bioethanol production. The findings of this study suggested that microwave-assisted sodium hydroxide pretreatment is more efficient in removing lignin and hemicelluloses, resulting in maximum exposure of cellulose, which then leads to the production of 4.11% ethanol.

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