Indian Journal of Chemical Technology
Vol. 25, September 2018, pp. 431-440

Sequential acid-autoclave and microwave-alkali pretreatment of rice straw for bioethanol production

Jaspreet Kaur[1], Monica S Taggar*[, 2], Gurvinder S Kocher[3] & Mohammed Javed[4]

[1]Department of Biochemistry, Punjab Agricultural University, Ludhiana 141 004, Punjab, India
Department Renewable Energy Engineering, Punjab Agricultural University, Ludhiana 141 004, Punjab, India
[3]Department of Microbiology, Punjab Agricultural University, Ludhiana 141 004, Punjab, India
[4]Department of Mathematics, Statistics & Physics, Punjab Agricultural University, Ludhiana 141 004, Punjab, India
E-mail: monicasachdeva@pau.edu

Received 25 December 2016; accepted 13 July 2017

Two-step acid-autoclave and microwave-alkali pre-treatment process of rice straw have been carried out for bioethanol production. The reaction conditions of both the steps are optimized using Response Surface Methodology. The studies reveal that the optimum conditions for acid-autoclave pretreatment are found to be 1.90 per cent acid concentration, 51.85 min time and 1:17.51 solid:liquid ratio with maximum release of reducing sugars (16.94 g per 100 g) and minimum release of furfurals (0.93 g per 100 g). For second step pretreatment i.e. microwave-alkali, optimized conditions are observed to be 3.75 percent alkali concentration, 9.16 min time and 475W microwave power with minimum lignin content of 2.96 percent in pre-treated straw. Scanning electron micrographs reveal extensive damage of silicified waxy surface and disruption of the cell wall structure of straw after two step pre-treatment process. It has been concluded that sequential pre-treatment of rice straw using acid-autoclave and microwave-alkali process efficiently remove hemicellulose and lignin from straw, thereby increasing accessibility of cellulose to enzymatic hydrolysis for enhanced production of fermentable sugars and bioethanol.

Keywords: Rice straw, Acid-autoclave, Microwave-alkali, Bioethanol

Burning of fossil fuels has increased the greenhouse gases and has thus resulted in air pollution and climate change[1]. There is an increased interest in alternative fuels globally; especially liquid transportation fuels with increasing demand for energy and fast depleting petroleum resources[2]. Biofuels have a number of environmental advantages, including lower emissions of harmful pollutants, decreased greenhouse gas emissions and increased energy security, especially in rural areas of India[3]. The lignocellulosic biomass conversion into biofuels and biochemicals has gained impetus due to the feasibility of alternative processes available to convert the complex biomass into biofuels and biomaterials. Cereal straws are promising feedstock among all lignocellulosic biomass for bioethanol production because of their relatively low cost and abundance[4]. Rice is one of the major crops grown in India and produces 23 percent of rice straw by its weight[5]. Rice straw has many characteristics that make it a potential feedstock for production of fuel ethanol. It has high cellulose and hemicellulose content that can be readily hydrolyzed into fermentable sugars. The chemical composition of rice straw mainly contains cellulose (32-47%), hemicellulose (19-27%), lignin (5-24%), silica and ash content[6]. The conversion of rice straw into ethanol involves hydrolysis of cellulose into fermentable sugars and subsequently fermentation of these sugars into ethanol. The hydrolysis is carried out by using cellulase enzyme. Due to the presence of complex structure of lignin and hemicellulose with cellulose, the conversion of rice straw to fermentable sugar is hindered[6,7]. The presence of these complex structures poses a great challenge for the use of rice straw as feedstock for ethanol production. Hence, efficient pre-treatment of rice straw is required to remove the surrounding matrix of lignin and hemicellulose prior to enzymatic hydrolysis of the cellulose. Plant cell walls could be hydrolyzed by using acid pre-treatments, especially for removal of hemicellulose component. For acid pre-treatments, sulphuric acid, nitric acid and hydrogen chloride have been widely used in dilute form[8]. Cell walls have also been modified by using alkaline pre-treatments. During such pre-treatments, solvation and saponification reactions take place resulting in swelling of biomass and increased access of saccharification enzymes to its inner space[9]. Such pretreatments selectively remove lignin from biomass. Sodium hydroxide and potassium hydroxide have been evaluated for use in alkali pre-treatments because they are cheaper than other reagents[10].

Removal of hemicellulose and lignin could dramatically improve enzyme digestibility by enhancing the enzyme accessibility to cellulose. The combined pre-treatment process using sequential pre-treatment steps have gained great attention as a promising strategy to reduce process severity factors such as chemical dosage and temperature in each step[11]. Combinations of pre-treatments such as alkaline/ per acetic acid[12], formic acid/aqueous ammonia[13], liquid hot water/ammonia[13] and acid/alkali[14] with different effects on biomass have been studied by many researchers. These pre-treatments lead to increased pretreatment efficiency with improved separation of different lignocellulosic components in a sequential manner. The present study focuses on optimization of an efficient two-step acid-autoclave and microwave-alkali pre-treatment process for the hydrolysis of rice straw for bioethanol production.

Experimental Section

Procurement of raw material

The rice straw was collected from Demonstration area, Department of Renewable Energy Engineering, Punjab Agricultural University, Ludhiana. The straw samples were washed with tap water to remove soil, dust, and other unwanted materials prior to sun drying, followed by subsequent drying in an oven at a temperature of 50±2°C. The straw samples were powdered using an electric grinder and were sieved through 30 mesh screen. The samples were stored in polythene bags at room temperature for subsequent studies.

Sequential acid-autoclave and microwave-alkali pre-treatment of rice straw

The straw was subjected to sequential acid-autoclave and microwave-alkali pretreatment. The acid-autoclave pre-treatment was carried out as per the method of Kocher and Kalra[15]. A known amount of rice straw contained in 250 mL reagent bottles was subjected to acid pre-treatment in an autoclave at 15 psi and 121°C. The reaction conditions were 0.5 to 2 percent acid concentrations, 1:10 to 1:20 solid:liquid ratio and 20-60 min reaction time. The treated slurry was filtered through Whatmann No. 1 filter paper and filtrate was analyzed for reducing sugars and furfurals by the method of Nelson(1994)[16] and Horwitz(1980)[17], respectively. The solid residue, thus, obtained after acid-autoclave pre-treatment was subjected to microwave-alkali treatment. The pre-treatment with alkali was carried out at microwave power 160-480 W, reaction time 5-10 min and alkali concentration 2-4 percent. The residue obtained after two step pretreatment i.e. acid-autoclave and microwave- alkali was repeatedly washed with distilled water and dried in an oven at 60°C. The hemicellulose and lignin content of pre-treated straw residue was determined by the method of Goering and Vansoest(1970)[18] while cellulose content was determined by the method of Crampton and Maynard(1938)[19].

Experimental design and statistical analysis

For optimization of two-step acid-autoclave and microwave-alkali pre-treatment of rice straw, a Box-Behnken Design (BBD) was selected and the statistical analysis was performed using Response Surface Methodology (RSM). For acid autoclave pre-treatment, BBD with three factors and three levels including three replicates at centre point was used to evaluate the main and interaction effects of factors like acid concentration (A), time (B) and solid:liquid ratio (C) on the response i.e. reducing sugar and furfural content. The maximum release of reducing sugars and minimum furfural content was taken as response of the designed experiment. The range of independent variables and their coded values have been mentioned in Table 1. The design matrix was obtained with 15 experimental runs in one block with three replicates. Similarly, for microwave-alkali pre-treatment of acid-pretreated straw, BBD was used to evaluate the main and interaction effects of factors like alkali concentration (A), time (B) and microwave power (C) on the response i.e. lignin content of pre-treated straw. The minimum lignin content of pre-treated straw was taken as response of the

Table 1 — Independent variables and coded values for acid autoclave pre-treatment of rice straw

Coded factor	Parameter	Coded levels		
		-1	0	+1
		Actual levels		
A	Acid concentration (%)	0.5	1.25	2
B	Time (min)	20	40	60
C	Solid: liquid ratio	1:10	1:15	1:20

designed experiment. The range of independent variables and their coded values have been mentioned in Table 2. The design matrix with 15 experimental runs in one block with three replicates was obtained.

A quadratic polynomial equation(1) was used to evaluate the effect of each independent variable on the response.

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC \quad \dots (1)$$

where, Y is the predicted response; β_0 is a constant; β_1, β_2, β_3 are the linear coefficients; β_{11}, β_{22}, β_{33} are the quadratic coefficients; β_{12}, β_{13}, β_{23} are the cross coefficients.

The statistical software package Design Expert (version 9.0.1, Stat-ease, Inc. Minneapolis, USA) was used for analysis of variance (ANOVA) and regression analysis of experimental data. The response surface plots were developed by using quadratic polynomial equation obtained from regression analysis. The optimum conditions for two-step pre-treatment were determined by using the same software through numerical optimization. The confirmation experiments for acid autoclave and microwave alkali pre-treatment were conducted under optimized conditions to test the validity of statistical experimental strategies.

Validation of two step pre-treatment of rice straw

The validation experiment was conducted by taking 25 g of rice straw in 500 mL reagent bottle. The rice straw was subjected to sequential pre-treatments, i.e. acid-autoclave, followed by microwave-alkali under optimized conditions. The pre-treated straw was, then, analyzed for cellulose, hemicellulose and lignin content as per methods mentioned above.

Morphological studies of pre-treated rice straw using scanning electron microscope

The morphology of pre-treated rice straw was studied using Scanning Electron Microscope (Model, S-3400 N, SEM-Hitachi, Tokyo, Japan) as per standard protocol of Bozzola and Russell (1999)[20] at Electron Microscopy and Nanoscience (EMN) Laboratory, PAU, Ludhiana.

Table 2 — Independent variables and coded values for microwave alkali pre-treatment of rice straw

Coded factor	Parameter	Coded levels -1	0	+1
		Actual levels		
A	Alkali concentration (%)	2.0	3.0	4.0
B	Time (min)	5.0	7.5	10.0
C	Microwave power (W)	160	320	480

Results and Discussion

Optimization of acid autoclave pre-treatment of rice straw

For optimization of acid autoclave pre-treatment of rice straw, the effects of three independent variables, *viz.* acid concentration (A), time (B) and solid:liquid ratio (C) on release of reducing sugars and furfurals were studied. The main objective of pre-treatment was to remove hemicellulose and maximum release of reducing sugars was considered as one of the criteria for optimization of pre-treatment. The high content of furfurals in the fermentation medium strongly inhibited the fermentation, so the minimum release of furfurals was taken as another criterion for optimization. The results obtained from 15 experimental runs, carried according to BBD, RSM have been summarized in Table 3. Under different pre-treatment conditions, the observed reducing sugar content in the hydrolysate of acid autoclave pre-treated straw ranged from of 2.01 to 15.98 g per 100 g straw. The furfural content in hydrolysate during different pre-treatment conditions ranged from 0.08 to 0.95 g per 100 g straw (Table 3).

A second order polynomial was fitted to the data presented in Table 3 using multiple linear regressions to determine the optimum conditions for acid-autoclave pre-treatment of rice straw. The following second order polynomial equation (1) was found to represent the relationship between the reducing sugars and acid concentration, time and solid to liquid ratio.

$$\text{Reducing sugars} = 12.15+3.48A+3.64B+2.68C+1.65AB+1.39BC-1.46A^2-3.34B^2-1.77C^2 \quad \dots (2)$$

The values of reducing sugars as predicted by Equation (2) have been presented in Table 3.

The analysis of variance (ANOVA) was performed to test the significance of statistical model and results of the same have been presented in Table 4. The high model F-value of 54.64 and a very low probability value of 0.0002 revealed that the response model was highly significant. The most significant terms were observed to be A (acid concentration), B (time), C (solid:liquid ratio), AB (acid concentration and time), BC (time and solid: liquid ratio), A^2, B^2 and C^2. The "Lack of Fit F-value" of 0.60 showed that there was insignificant lack of fit. The coefficient of variation

Table 3 — Box-Behnken design for acid autoclave pre-treatment of rice straw

Experimental runs	Factor 1	Factor 2	Factor 3	Response 1		Response 2	
	Actual values			Reducing sugars (g/100g)		Furfurals (g/100g)	
	A: Acid conc. (%)	B: Time (min)	C:Solid: Liquid ratio	Observed values	Predicted values	Observed values	Predicted values
1	0.50	60	1:15	5.23	5.86	0.32	0.40
2	1.25	40	1:15	13.10	12.15	0.67	0.61
3	1.25	20	1:10	1.80	2.11	0.17	0.22
4	1.25	40	1:15	12.13	12.15	0.60	0.61
5	2.00	60	1:15	15.98	16.12	0.95	0.96
6	2.00	40	1:20	14.30	15.08	0.79	0.95
7	2.00	40	1:10	10.02	9.72	0.50	0.65
8	0.50	20	1:15	2.01	1.88	0.08	0.18
9	1.25	20	1:20	4.24	4.69	0.29	0.33
10	0.50	40	1:20	9.03	8.12	0.48	0.57
11	1.25	40	1:15	11.21	12.15	0.56	0.61
12	2.00	20	1:15	6.15	5.54	0.35	0.96
13	1.25	60	1:10	7.04	6.61	0.35	0.43
14	1.25	60	1:20	15.05	14.75	0.85	0.92
15	0.50	40	1:10	2.31	2.76	0.16	0.27

(CV) was observed to be 9.55 percent. This value indicated the degree of precision with which the experimental runs was carried out. The low value of CV suggested a high reliability and reproducibility of design. An adequate precision value of 21.137 for the model indicated an adequate signal to navigate the design space. The coefficient of determination ($R^{2)}$ was observed to be 0.9899, thus, indicating that 98.99 percent of variation in reducing sugar content was explained by the model. High values of R^2 and adjusted R^2 (0.9718) of this model showed a close agreement between the experimental results and the theoretical values predicted by the model.

The following second order polynomial equation (3) was found to represent the relationship between the furfural and acid concentration, time and solid to liquid ratio during acid autoclave pre-treatment.

$$\text{Furfurals} = 0.61+0.19A+0.20B+0.15C +0.090AB+0.090BC-0.13B^2 \quad \ldots (3)$$

The values of furfural as predicted by Equation (3) have been presented in Table 3.

In case of furfurals, the results of ANOVA (Table 4) showed that F-value and probability value was 34.53 and 0.0006, respectively. The low probability value indicated that the response model was highly significant with coefficient of determination (R^2) of 0.9842, coefficient of variation of 11.64 per cent and "Lack of Fit F-value" of 0.98.

Response surface plots and optimization for acid autoclave pre-treatment of rice straw

Response surface curves were plotted to study the effect of interaction between independent variables and to determine the optimum level of variables. The effect of the interaction between acid concentration and time on reducing sugar content was studied at solid: liquid ratio of 1:15 (Fig. 1a). An increase in both acid concentration and time from 0.5 to 2 per cent (w/v) and 20 to 60 min, respectively resulted in enhanced release of reducing sugars at constant solid:liquid ratio. The effect of the interaction between time and solid:liquid ratio on the release of reducing sugars was studied at constant sulphuric acid concentration (Fig. 1b). The release of reducing sugars increased with increase in time (20 to 60 min) and solid:liquid ratio (1:10 to 1:20). The increased release of sugars may be attributed to the high catalytic activity of sulphuric acid. The hydronium ions from H_2SO_4 molecules can breakdown the glycosidic bonds of hemicellulose, thereby causing increased conversion of this fraction of straw into fermentable sugars (Amenaghawon *et al.*)[21].

The effect of the interaction between acid concentration and time on release of furfurals at 1:15 solid to liquid ratio has been shown in Fig. 2a. The increase in both acid concentration and time from 0.5 to 2 percent and 20 to 60 min resulted in enhanced release of furfurals. The increased production of furfurals with increase in time may be due to the

Table 4 — ANOVA for response surface quadratic model for acid autoclave pre-treatment of rice straw

Source	Reducing sugars						Furfurals					
	Sum of squares	df	Mean square	F Value	p-value (Prob > F)	Inference	Sum of squares	df	Mean square	F Value	p-value (Prob > F)	Inference
Model	334.89	9	37.21	54.64	0.0002	Significant	0.95	9	0.11	34.53	0.0006	Significant
A-Acid concentration	97.09	1	97.09	142.56	< 0.0001	Significant	0.30	1	0.30	98.30	0.0002	Significant
B-Time	105.85	1	105.85	155.42	< 0.0001	Significant	0.31	1	0.31	102.14	0.0002	Significant
C-Solid:liquid ratio	57.51	1	57.51	84.45	0.0003	Significant	0.19	1	0.19	61.90	0.0005	Significant
AB	10.92	1	10.92	16.04	0.0103	Significant	0.032	1	0.032	10.61	0.0225	Significant
AC	1.49	1	1.49	2.19	0.1994	Not significant	2.250E-004	1	2.250E-004	0.074	0.7969	Not significant
BC	7.76	1	7.76	11.39	0.0198	Significant	0.036	1	0.036	11.82	0.0185	Significant
A^2	7.88	1	7.88	11.57	0.0192	Significant	0.013	1	0.013	4.17	0.0966	Not significant
B^2	41.27	1	41.27	60.60	0.0006	Significant	0.059	1	0.059	19.26	0.0071	Significant
C^2	11.58	1	11.58	17.00	0.0091	Significant	0.017	1	0.017	5.71	0.0624	Not significant
Residual	3.41	5	0.68				0.015	5	3.055E-003			
Lack of Fit	1.62	3	0.54	0.60	0.6722	Not significant	9.075E-003	3	3.025E-003	0.98	0.5421	Not significant
Pure Error	1.79	2	0.89				6.200E-003	2	3.100E-003			
Cor Total	338.29	14					0.96	14				
C.V. %	9.55							11.64				
Adeq Precision	21.137							17.339				
R-Squared	0.9899							0.9842				
Adj R-Squared	0.9718							0.9557				

fact that reducing sugars in the presence of acid at high temperature get converted into furfurals and hydroxyl-methyl furfurals (Ahi *et al.*)[22]. The effect of the interaction between time and solid:liquid ratio on release of furfurals at 1.25 percent acid concentration has been shown in Fig. 2b. An increase in both pre-treatment time (20 to 60 min) and solid:liquid ratio (1:10 to 1:20) resulted in enhanced furfural production at constant acid concentration.

Optimum conditions for acid-autoclave pretreatment of straw

The optimization studies revealed that the maximum release of reducing sugars (16.94 g per 100 g straw) and minimum release of furfurals (0.93 g per 100 g straw) was observed at an acid concentration of 1.90 per cent, 51.85 min and a solid:liquid ratio of 1:17.51. The validation studies revealed that the experimental values of reducing sugars and furfural i.e. 16.35 and 0.91 g/100 g straw were quite close to the predicted values.

Optimization of microwave alkali pre-treatment (second step) of rice straw

For optimization of microwave alkali pre-treatment of rice straw, the effect of three independent variables, *viz.* alkali concentration (A), time (B) and microwave power (C) on lignin content of treated straw was studied. The main objective of pre-treatment was to remove lignin and thus, lowest lignin content in pre-treated straw was considered as one of the criteria for optimization of pre-treatment. The results obtained from 15 experimental runs carried according to BBD, RSM have been summarized in Table 5. Under different microwave alkali pre-treatment conditions, lignin content in the treated straw ranged from 3.01 to 8.50 percent. A second

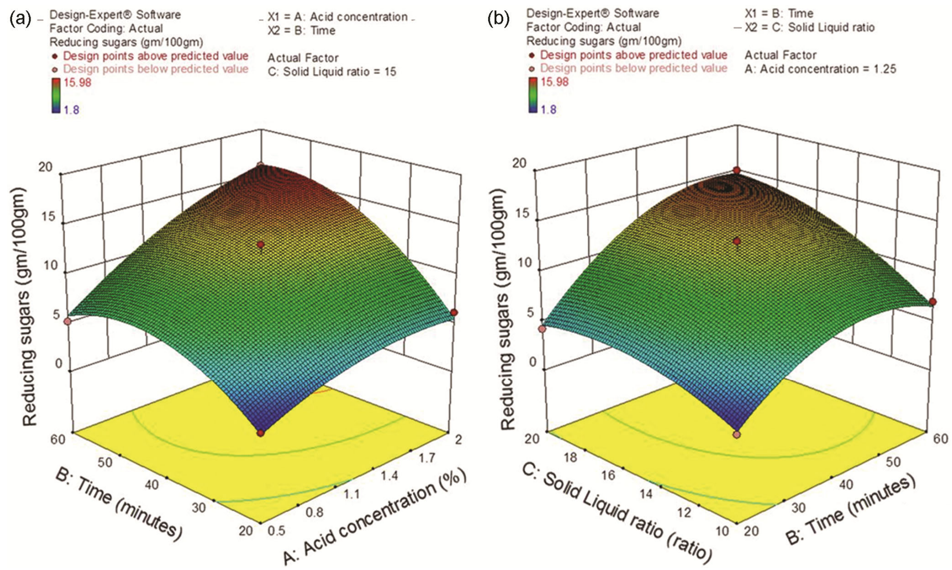

Fig. 1 — Response surface plots showing the effect of: (1 a) acid concentration and time (1 b) solid: liquid ratio and time on reducing sugar content during acid autoclave pre-treatment

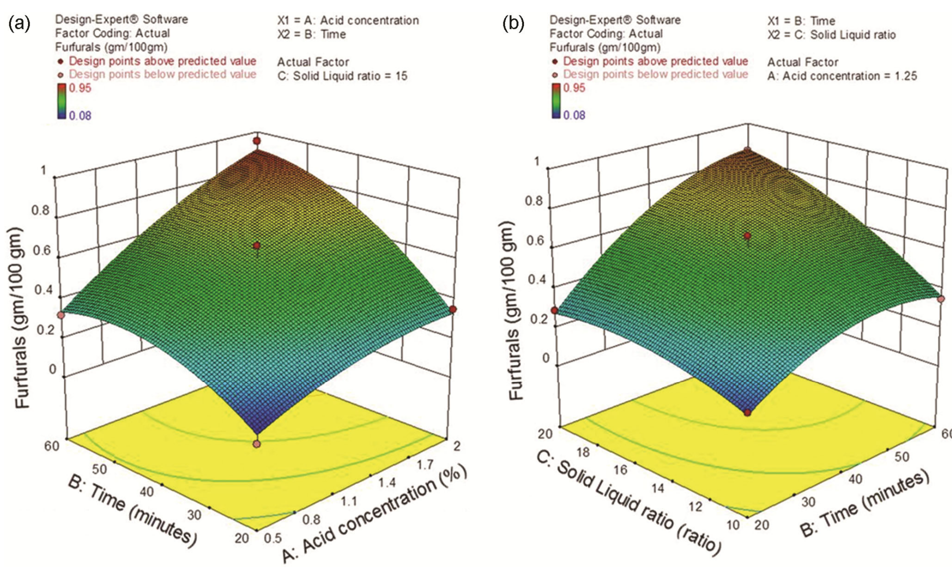

Fig. 2 — Response surface plots showing the effect of: (2 a) acid concentration and time (2 b) solid: liquid ratio and time on furfural content during acid autoclave pre-treatment

order polynomial was fitted to the data presented in Table 5 using multiple linear regression to determine the optimum conditions for microwave- alkali pre-treatment of acid treated rice straw. The following second order polynomial equation (4) was found to represent the relationship between the lignin and alkali concentration, time and microwave power during microwave alkali pre-treatment.

Lignin = 5.69-0.48A-1.14B-1.49C+0.40AB-0.89AC … (4)

The values of lignin as predicted by Eqn (4) have been presented in Table 5.

The fit of the statistical model for the response (lignin) was assessed by carrying out analysis of variance and results have been presented in Table 6. The high model F-value of 43.21 and a very low probability value of 0.0003 revealed that the response model was highly significant. The most significant terms were observed to A (alkali concentration), B (time), C (microwave power), AB (alkali concentration and time), AC (alkali concentration and microwave power). The "Lack of Fit F-value" of 1.09 showed that there was insignificant lack of fit. The coefficient of variation was observed to be 5.09 percent. The low value of CV indicated that the treatments were carried out with high precision and reliability. The high determination coefficient (R^2) of 0.9873 indicated that 98.73 percent of variability was explained by the model. The high values of R^2 and adjusted R^2 of this model (Adj R^2 =0.9645) showed a good correlation between the observed and predicted values.

Table 5 — Box-Behnken design for microwave alkali pre-treatment of rice straw

Run	Factor 1	Factor 2	Factor 3	Response	
	Actual values			Lignin	
	A: Alkali conc. (%)	B: Time (min)	C: Microwave power (W)	Observed values (%)	Predicted values (%)
1	3.0	7.5	320	6.02	5.69
2	3.0	10.0	480	3.01	3.06
3	4.0	5.0	320	6.01	5.95
4	3.0	10.0	160	6.02	6.04
5	3.0	5.0	480	5.01	5.34
6	2.0	7.5	160	6.93	6.77
7	4.0	10.0	320	4.49	4.47
4	3.0	7.5	320	5.50	5.69
9	4.0	7.5	160	7.99	7.59
10	4.0	7.5	480	3.51	2.83
11	2.0	10.0	320	4.91	4.63
12	3.0	7.5	320	5.54	5.69
13	2.0	7.5	480	6.00	5.57
14	2.0	5.0	320	8.01	7.71
15	3.0	5.0	160	8.50	8.32

Response surface plots and optimization for microwave alkali pre-treatment of rice straw

Response surface curves were plotted to study the effect of interaction between independent variables and to determine the optimum level of variables. The effect of the interaction between alkali concentration and time on lignin content was studied at microwave power of 320W and shown in Fig. 3a. An increase in both alkali concentration and time from 2 to 4 per cent (w/v) and 5 to 10 min, respectively resulted in decreased lignin content at constant microwave power.

The effect of the interaction between alkali concentration and microwave power (Fig. 3b) on lignin content was studied at constant time (7.5 min). An increase in both alkali concentration and microwave power from 2 to 4 percent (w/v) and 160 to 480W, respectively resulted in decreased lignin content at constant time. The highest delignification observed with increased alkali concentration was probably due to increased digestibility of the substrates. This might be due to fact that the hydroxyl ions (OH^-) of sodium hydroxide may break the lignin-lignin bonds and dissolve lignin, thereby, causing oxidation loss of lignin polymers to their aromatic derivatives (Shankarappa and Geeta)[23]. Similarly, Zhu

Table 6 — ANOVA for response surface quadratic model for microwave-alkali pre-treatment of rice straw

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	Inference
			Lignin			
Model	34.29	9	3.81	43.21	0.0003	Significant
A-alkali conc.	1.85	1	1.85	21.01	0.0059	Significant
B-time	10.35	1	10.35	117.38	0.0001	Significant
C-microwave power	17.73	1	17.73	201.06	< 0.0001	Significant
AB	0.62	1	0.62	7.08	0.0449	Significant
AC	3.15	1	3.15	35.73	0.0019	Significant
BC	0.058	1	0.058	0.65	0.4557	Not significant
A^2	0.38	1	0.38	4.30	0.0929	Not significant
B^2	0.085	1	0.085	0.97	0.3703	Not significant
C^2	0.037	1	0.037	0.42	0.5445	Not significant
Residual	0.44	5	0.088			
Lack of Fit	0.27	3	0.091	1.09	0.5116	Not significant
Pure Error	0.17	2	0.084			
Cor Total	34.73	14				
C.V. %	5.09					
Adeq precision	21.662					
R^2	0.9873					
Adj R^2	0.9645					

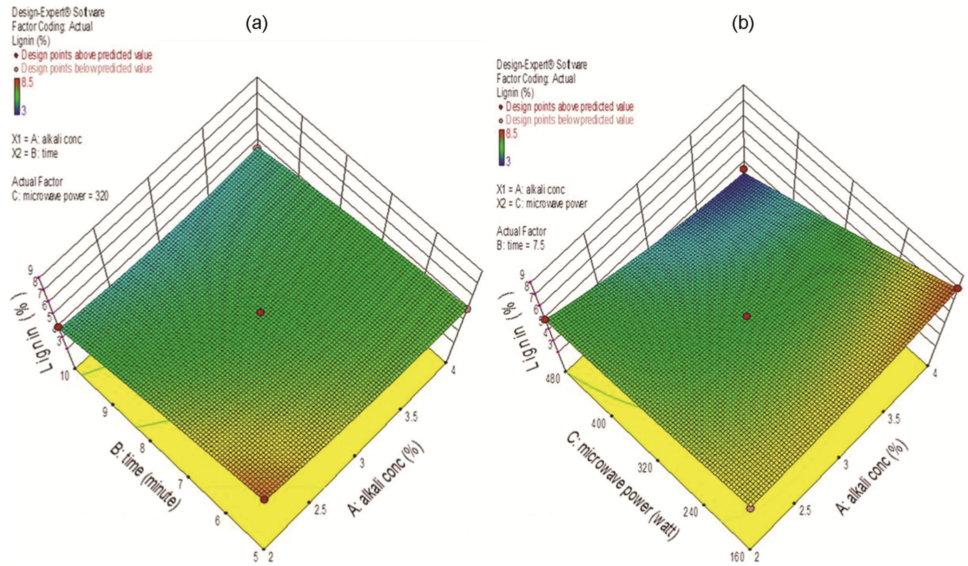

Fig. 3 — Response surface plots showing the effect of: (3 a) alkali concentration and time (3 b) microwave power and alkali concentration on lignin content during microwave alkali pre-treatment

et al.[7] reported that lignin content decreased to 2.9 percent with an increase in microwave power from 300 to 700W for 15 min and by increasing the microwave irradiation time from 15 to 70 min at 300W.

Optimum conditions for microwave-alkali pre-treatment of straw

The optimal microwave alkali pre-treatment conditions were determined by Design Expert software. The optimization results showed that minimum lignin content of 2.96 percent was recorded in the pre-treated straw under optimized conditions i.e. 3.75 per cent alkali concentration; 9.16 min time and 475W microwave power which was quite close to the experimental value i.e. 3.12 per cent lignin in pre-treated straw.

Effect of sequential pre-treatment (acid autoclave followed by microwave alkali) on chemical composition of rice straw

The chemical composition of rice straw after sequential pre-treatment has been presented in Table 7. The relative proportion of cellulose (68.33%) increased, while that of hemicellulose and lignin was drastically reduced (8.21 and 2.98%, respectively) in pre-treated rice straw as compared to the relative proportion of cellulose, hemicellulose and lignin of 30.33, 23.40 and 9.26 percent, respectively in untreated rice straw. The results thus, indicated that a two-step pre-treatment process efficiently removed hemicelluloses and lignin from rice straw. Similarly, Akhtar *et al.*[24] compared sequential acid/microwave-assisted alkali pre-treatment of oil palm empty fruit bunch. The conventional acid autoclave heating removed 90 per cent of hemicellulose. More than twice the rate of delignification, i.e. 71.9 percent was attained with acid/microwave alkali pre-treatment as compared to conventional alkali autoclave pre-treatment (34.6%). Similarly, Weerasai *et al.*[11] developed a sequential acid and alkali process for pre-treatment of rice straw for ethanol production. The reducing sugar yield of 353.1 mg g^{-1} was obtained from enzymatic hydrolysis after combined intermediate acid followed by mild alkaline pre-treatment as compared to reducing sugar yield of 227.1 mg g^{-1} and 285.0 mg g^{-1} native straw obtained after single step acid and alkali pre-treatments, respectively.

Table 7 — Chemical composition of rice straw after two step pre-treatment

S. No.	Chemical constituents	Pre-treated straw (two step pre-treatment) (%)	Untreated straw (%)
1.	Cellulose	68.33	30.33
2.	Hemicellulose	8.21	23.40
3.	Lignin	2.98	9.26
4.	Reducing sugars	14.37	-

Scanning electron microscope (SEM) studies of pre-treated rice straw

The morphological changes that occurred during two-step pre-treatment of rice straw were assessed with the help of Scanning Electron Microscope (SEM). The micrographs of the untreated rice straw (Fig. 4a) showed that initially, the surface of the

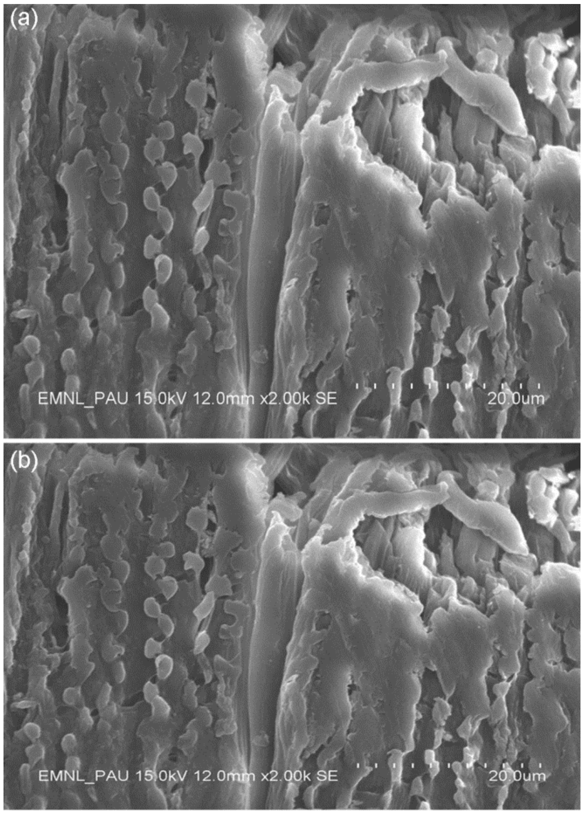

Fig. 4 — Scanning Electron Micrographs of rice straw (2000 X): (4 a) untreated straw (4 b) pre-treated straw

straw was compact and densely packed as compared to pre-treated rice straw. The combined two-step pre-treatment lead to an extensive damage of the silicified waxy surface and the disruption of the cell wall structure (Fig. 4b). These physical disruptions of straw were more pronounced with a two-step pre-treatment of straw as compared to single step pre-treatment. This conferred greater susceptibility of the cellulose fibers to enzymatic action in pre-treated rice straw as compared to untreated rice straw (Weerasai *et al.*)[11].

Conclusion

The sequential pre-treatment of rice straw was carried out using acid-autoclave and microwave-alkali process. The optimization studies using Box-Behnken Design (BBD) reveal that the optimum conditions for acid-autoclave pre-treatment were observed to be 1.90 percent acid concentration, 51.85 min time and 1:17.51 solid:liquid ratio with maximum release of reducing sugars (16.94 g per 100 g) and minimum release of furfurals (0.93 g per 100 g). For second step pre-treatment i.e. microwave-alkali, optimized conditions were observed to be 3.75 percent alkali concentration, 9.16 min time and 475W microwave power with minimum lignin content of 2.96 percent in pre-treated straw. Thus, sequential acid-autoclave and microwave- alkali pretreatment could enhance the saccharification of rice straw by efficiently removing lignin and hemicelluloses from the straw. This, in turn, would likely increase the bioethanol production from straw.

Acknowledgement

Special thanks are expressed to Head, DREE, PAU, Ludhiana for providing necessary facilities for carrying out the present studies. The authors also acknowledge the generous technical help and valuable assistance rendered by the Director (EMN Laboratory) and Dr Anu Kalia, PAU, Ludhiana.

References

1 IPCC, Summary for Policymakers, in *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, edited by S Solomon, D Qin, M Manning, Z Chen, M Marquis, K Averyt, M Tignor & H L Miller (Cambridge University Press, Cambridge, UK and New York, USA), 2007, 2.

2 Sukumaran R K, Surender V J, Sindhu R, Binod P, Janu K U, Sajna K V, Rajasree K P & Pandey A, *Bioresour Technol*, 101 (2010) 7.

3 Gonsalves J B, An assessment of the biofuels industry in India, *United Nations Conference on Trade and* Development, Geneva, 2006. *www.unctad.org/en/docs/ditcted 20066_en.pdf.*

4 Sun R C, *Cereal straw as a resource for sustainable biomaterials and biofuels,* 1st Edn, (Elsevier Publications, Amsterdam, The Netherlands), 2010.

5 Gadde B, Menke C & Wassmann R, *Biomass Bioenergy*, 33 (2009) 1532.

6 Sun Y & Cheng J, *Bioresour Technol*, 83 (2002) 10.

7 Zhu S, Wu Y, Yu Z, Liao J & Zhang Y, *Proc Biochem*, 40 (2005) 4.

8 Idrees M, Adnan A, Sheikh S & Qureshic F A, *EXCLI J*, 12 (2013) 10.

9 Kim T H, Taylor F & Hicks K B, *Bioresour Technol*, 99 (2008) 8.

10 Hu Z, Wang Y & Wen Z, *Appl Biochem Biotechnol*, 148 (2008) 10.

11 Weerasai K, Suriyachai N, Poonsrisaswat A, Arnthong J, Unrean P, Laosiripojana N & Champreda V, *Bioresour*, 9 (2014) 13.

12 Zhao X, Song Y & Liu D, *Enzym Microb Technol*, 49 (2011) 6.

13 Zhang M, Wang F, Su R, Qi W & He Z, *Bioresour Technol*, 101 (2010) 5.

14 Kim T H & Lee Y Y, *Bioresour Technol*, 97 (2006) 8.

15 Kocher G S & Kalra K L, *Microbiology*, 3 (2013) 2.

16 Nelson N, *J Biol Chem*, 153 (1944) 5.

17 Horwitz W, *Official Methods of Analysis of the Association of Official Analytical Chemists,* (Published by Association of Official Analytical Chemists, Washington, DC), 1980.

18 Goering H K, Van Soest P J & Peter J *Forage Fiber Analyses (apparatus, reagents, procedures, and some applications)* (US Agricultural Research Service, Washington, DC), 1970.
19 Crampton E W & Maynard I A, *J Nutr,* 15 (1938) 383.
20 Bozzola J J & Russel L D, *Electron Microscopy: Principles and Technique for Biologists,2nd edn,* (Jones and Bartlett Publishers, Inc Sudbury, MA, USA), 1999.
21 Amenaghawon N A, Ogbeide S E & Okieimen C O, *Acta Polytech Hung*, 11 (2014) 239.
22 Ahi M, Azin M, Shojaosadati S A, Farahani E V & Nosrati M, *Chem Eng Technol*, 36 (2013) 8.
23 Shankarappa T H & Geeta G S, *J Agric Sci*, 26 (2013) 8.
24 Akhtar J, Teo C L, Lai L W, Hassan N, Idris A, Aziz R A & Iqbal T, *Bioresources*, 10 (2015) 8.