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Optimization of two steps pretreatment techniques on lignin elimination in wheat straw to improve the bio-oil quality

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■ **ABSTRACT :** Cross-linking of degraded lignin components present to bio-oil increases the density and causes instability during storage. Bio-oil quality and quantity from wheat straw was improved by alkaline and enzymatic treatment before pyrolysis. Alkaline pretreatment reduced the lignin content from 18 per cent to 10 per cent. Enzymatic pretreatment using β-glucosidase and cellulase in ratio 5:10, 10:10, 15:10 U for 24, 48, 72 h was optimized by using Response Surface Methodology (RSM) and eliminated 3.1 per cent lignin. The maximum reducing sugar and glucose content was found 24.2 g/L and 15.488 g/L, respectively. Bio-oil yield by fast pyrolysis of treated and untreated wheat straw was 30 per cent and 27 per cent, respectively. Bio-oil of the treated wheat straw exhibited increased in pH, higher density and decreased in viscosity. The flash point of bio-oil from treated wheat straw was very close to commercial diesel. Bio-oil was characterized by FTIR and GC-MS analysis.

■ **KEY WORDS :** Bio-oil, Alkali pretreatment, Enzymatic pretreatment, Wheat straw, Lignin reduction, Pyrolysis, FTIR of bio-oil, GC-MS of bio-oil

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Biomass is the third largest global source of energy in the world could be one of suitable renewable resource to solve the problem of depletion of fossil fuel (Zou *et al.,* 2016). Numerous fuels that are being generated from biomass include methane, hydrogen, methanol, ethanol, Fischer-Tropsch diesel and biodiesel (Demirbas, 2007). There are different methods such as thermal, chemical, and biochemical for conversion of biomass to biofuel. Bio-oil is obtained through a single step thermochemical conversion of nearly all kinds of wood biomass with a chemically complex liquid product. Bio-oil is a dark colored liquid obtained by the irreversible breakdown of organic matter at elevated temperature (400-500°C) in the absence of oxygen. It is also a promising source of numerous value-added chemicals (Chiaramonti *et al.*, 2007). Considerable efforts are being continuously put in to develop upgrading strategies to transform it to engine fuel applications. Bio-oils are often termed 'clean' compared to fossil fuels since they offer several ecological benefits (Mohan *et al.,* 2006).

Bio-oil upon storage undergoes series of chemical reactions triggered by organic acids and intermediates in addition to the re-polymerization of reactive olefinic compounds (Oasmaa *et al.*, 2010). Aging also reduces bio-oil quality, increases its viscosity and ultimately leads to the separation of continuous and dispersed phases (Adjaye *et al.*, 1992). Presence of substantial amounts of organic acids often imparts acidity to the crude bio-oil

whose pH ranges between 2 and 4 (Oasmaa *et al.,* 2010). Under these circumstances, storage, handling, and transportation of bio-oils become highly challenging (Zhang *et al.*, 2007). Bio-oil from different lignocellulosic waste have been reported (Lyu *et al.*, 2015). It was hypothesized, that removal of lignin from lignocellulosic material and then the pyrolysis process could improve the bio-oil properties. Hence, the main aim was to remove lignin chemically and enzymatically in wheat straw to improve the bio-oil quality. The usage of the alkaline chemical in the pre-treatment of wheat straw and enzymatic hydrolysis to degrade lignocelluloses biomass and saccharification of cellulose is expected to alter the original biomass structure. Response surface methodology by Box–Behnken design employing the multivariate approach enables substantial improvement in the method development using fewer experiments, without wastage of large volumes of organic solvents, which leads to high analysis cost (Wani *et al.*, 2012). It can be used to get optimum process conditions considering single response or multiple responses.

METHODOLOGY

Plant materials and chemicals:

Wheat straw was collected from Crop Research Centre (C.R.C.) Govind Ballabh Pant University of Agriculture and Technology, Pantnagar and dried at 50°C for 12 h (Curreli *et al.*, 2002). The dried wheat straw was subjected to size reduction process in the hammer mill by using a mesh size of 2 mm. Enzymes β-glucosidase, Cellulase, and other chemicals were procured from Hi-media Pvt. Ltd. India.

Alkali and enzymatic pre-treatment:

The wheat straw was treated with 2 per cent NaOH w/v in 10 per cent substrate concentration at 121°C for 1h (Silverstein *et al.*, 2007) and was washed with water until to neutralized the pH. The wheat straw was oven dried at 60^0 C for 72h to remove the moisture. Proximate analysis such as hemicellulose, cellulose, and lignin content was observed after alkali pre-treatment of wheat straw (Ververis *et al.,* 2007).

Experimental design:

Seventeen experiments were designed by Design-Expert software using Box-Behnken for enzymatic pretreatment process. The preliminary trial experiment was conducted to find the optimum condition for enzymes for improved the saccharification. Since we used the two enzymes β-glucosidase (optimum temperature 35°C) and Cellulase (optimum temperature 40°C) for single step saccharification, the conditions were optimized. The single step enzyme addition included the addition of enzymes *viz.,* cellulase (10 FPU) and β-glucosidase (10 U) at conditions mean to their optimum activity in an individual action for 24 h. On the other hand in two-step enzyme addition, the enzyme in similar amounts was added but at varied conditions as provided by manufacturer's recommendation on the optimum activity of individual enzymes in the intervals of 12 h. The substrate used in the experiment was a cellulose powder (5% w/v concentration) with the two enzymes *viz.,* cellulase and β-glucosidase. The operating conditions of each enzyme and used in one step enzyme addition are shown in Table A.

Table A : Optimization of operating conditions for enzymes by preliminary experiments

Method	Temperature	pH
One step enzyme addition	37.5°C	4.8
Two step enzyme addition:		
Cellulase	40°C	4.5
β-glucosidase	35°C	5.0

Response surface methodology (RSM) was used for the design and analysis of all experiments for three predicted variables at three levels. Box-Behnken model was selected for the optimization of the process variables (Sharma *et al.,* 2014). Box-Behnken is a class of rotatable second order design based on three levels of incomplete factorial design. This design does not contain for which all factors are simultaneously at their highest and lowest levels. So this design is useful in avoiding experiments performed under extreme conditions for which unsatisfactory results might occur (Bezerra *et al.,* 2008).

The number of experiments (N) required for the development of Box-Behnken Design. This design is defined as

$$N=2K(K-1)+C_0 \quad \text{..... (1)}$$

where,

N= Total no. of experiments,

K = No. of variables,

C_0= Centre point

In order to determine a critical point (maximum,

minimum or saddle) it is necessary for the polynomial function to contain the quadratic terms. According to the equation presented below:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_{i^2} + \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j \quad \text{..... (2)}$$

where, β_{ii}, β_0 and β_{ij} are the co-efficients of regression (i=1, 2, 3, ...n) (j=1, 2, 3, ...n).

Codification of levels of the variables:

It consists of transforming each studied real value into coordinates inside a scale with dimensionless value, which must be proportional at its localization in the experimental space. Codification is of concern because it enables the investigation of variables of different orders of magnitude without greater influencing the evaluation of the lesser. The following equation can be applied to transform a real value (Zi) into a coded value (Xi) according to a determinate experiment design:

$$X_i = \{(zi - zi^{\circ})/\Delta zi\}\beta d \quad \text{..... (3)}$$

where, Δzi= distance between real value in the centre point and the real value in the superior/inferior level of a variable. βd=Major coded limit value in the matrix for each variable, zi°=real value in the centre point

The final experiments were designed using Box-Behnken design (Table D). Following constant independent and dependent parameters (Table B and C) were taken into consideration.

To find out all possible combinations of parameters, experiments were designed using Response Surface Methodology using Design Expert 8.0.7.1 software. Seventeen experiments were conducted for the samples subjected to induced saccharification for various intervals in h at 37°C and 4.8 pH. Box-Behnken design was used on three independent variables (Time, Enzyme loading ratio, Substrate concentration) at three levels. The detailed values of independent and constant variables of experimental design are given in Table B and C. The detailed experimental design is shown in Table C.

Table B : Levels of independent variables for the enzymatic removal of lignin

Independent variables	Values		
	-1	0	+1
Reaction time (hours)	24	48	72
Enzyme loading ratio (IU/FPU)	5:10 (0.5)	10:10 (1)	15:10 (1.5)
Substrate concentration (g/L)	4	5	6

Table C : The variables kept constant during the experiments

Variables	Values
Temperature	37.5°C
pH	4.8
Buffer concentration	0.1M

Table D : Experimental designmatrix of the model for the maximum lignin elimination by quadratic

Run	Coded values			Actual values		
	X_1	X_2	X_3	Time (h)	Enzyme Loading ratio (IU/FPU)	Substrate Conc. (g/L)
1	-1	-1	0	24	5:10	50
2	-1	0	-1	24	10:10	40
3	0	-1	-1	48	5:10	40
4	1	0	-1	72	10:10	40
5	0	1	-1	48	15:10	40
6	-1	1	0	24	15:10	50
7	1	-1	1	72	5:10	60
8	0	0	0	48	10:10	50
9	-1	0	1	24	10:10	60
10	0	1	1	48	15:10	60
11	0	-1	1	48	5:10	60
12	1	1	0	72	15:10	50
13	0	0	0	48	10:10	50
14	0	0	0	48	10:10	50
15	1	0	1	72	10:10	60
16	0	0	0	48	10:10	50
17	0	0	0	48	10:10	50

X_1: Time (h), X_2 : Substrate conc. (%w/v), X_3 : Enzyme loading ratio

Response analysis:

The following dependent variables were considered to check the quality of the end product.

Reducing sugars:

Reducing sugar was estimated by the DNS method (Miller, 1959).

Ash estimation:

Air dried samples, (0.5 mm, 0.7g each) were boiled with 5 mL of 72% w/w H_2SO_4 solution for 4.5 h in order to hydrolyze the cellulose and hemicellulose. The suspension remaining after the above treatment was filtered through a crucible and the solid residue dried at 105°C for 24 h and weighed (W_1). The residue was then transferred to a pre-weighed dry porcelain crucible and heated at 600°C for 5 h. After cooling down, it was weighed (W_2) and ash content (%) was determined (Ververis *et al.*, 2007).

Lignin estimation:

The sample was hydrolyzed by 72% w/w H_2SO_4 and lignin content was estimated gravimetrically by Klason. Acid insoluble lignin was calculated by the difference in W_1-W_2 (Ververis *et al.*, 2007).

Glucose estimation:

Glucose concentration was analyzed by glucose oxidation (GOD and POD) kit (M/S Autospan Pvt. Ltd. India).

Cellulose estimation:

The filtrate from the H_2SO_4 treatment that contained the sugars released from cellulose and hemicellulose was thoroughly stirred and homogenized. Glucose (C1) and reducing sugar (C2) concentrations in the filtrate were determined according to a GOD and POD kit and the DNS method (Miller, 1959) respectively. The cellulose content in the starting material was calculated using the following equation:

% w/w cellulose content= (0.9/0.96) x C1 x (V/M) x α x 100

where 0.9 is the co-efficient that results from the molecular weight ratio of the polymer and the monomer hexose. The saccharification yield was taken as 0.96, C1 as the glucose concentration (g/L), V the total volume of sugar solution (L), M the dry weight of the wheat straw sample (g) and α the dilution of the sample (Ververis *et al.,* 2007).

Hemicelluloses estimation:

The hemicellulose content was calculated from the following equation:% w/w hemicellulose content = (0.8/ 0.93) x (C2-C1) x α x 100, where 0.88 is the co-efficient that results from the molecular weight ratio of the polymer and the monomer pentose, 0.93 is the saccharification yield of xylan to xylose, C2 is the determined reducing sugars concentration (g/L) from the DNS method, C1 the glucose concentration (g/L) from above, V the total volume of sugar solution (L), M the dry weight of the algal biomass sample (g) and α the dilution of the sample (Ververis *et al.*, 2007).

Bio-oil preparation and analysis:

Bio-oil was prepared at Central Institute of Agricultural Engineering, Bhopal, Madhya Pradesh, India. One kilogram each of untreated and treated wheat straw was taken for the pyrolysis process. Pyrolysis of each sample was carried out as described by our published method (Mandal *et al.*, 2018).

Kinematic viscosity:

The kinematic viscosity of fuels at 38^0 C was determined using Wisdom make Redwood Viscometer No. 1 (IS: 1448 [P: 25] 1976). Kinematic viscosity in centistokes was calculated from time units by using the empirical relation (Nakra and Chaudhary, 1985).

$$Kv = At - \frac{B}{t} \quad \text{.....(4)}$$

where, Kv=Kinematic viscosity, cSt t = Time of efflux, s A and B are constants applicable to the type of the viscometer A= 0.26, B= 172

Dynamic viscosity:

Dynamic viscosity was calculated by using the following relationship between Kinematic viscosity and density

Dynamic viscosity = Kinematic viscosity (cSt) at 38°C × ρ (g/mL) of oil at 15°C ..... (5)

Relative density:

The relative density of the bio-oil at 15^0C was determined as per IS: 1448 [P: 32] 1992.

The relative density was measured by:

$$\text{Relative density} = \frac{\text{Density of fuel at } 15^0\text{ C } (\rho_f)}{\text{Density of distilled water at } 15^0\text{C } (\rho_w)}$$

where, ρ_f= Density of water (0.9992 g/c^3) ρ_w= Density of fuel, (g/c^3)

Flash and fire point:

The flash point and fire point of the test oil samples was determined as per IS: 1448 [P: 21]: 1992 by a Khera make Pensky Martin Flash point (closed) apparatus.

FTIR:

The FTIR analysis of bio-oil was carried out using Systonic equipment at IIT Roorkee. Spectrum was measured from 4000 cm^{-1} to 500 cm^{-1} using an oil liquid membrane on KBr pellets with 5 μm spacer.

GC-MS analysis:

The separation and analysis of the organic compounds were achieved with an Agilent 3495 Deer Creek Rd equipment and GC column of same above at IIT Roorkee, India. Micro-filtered of bio-oil was injected into the injection port. A pure nitrogen (>99.999%) was used as carrier gas with a flow rate of 1 mL/ min. The oven's temperature was initially set to 40°C and heated for 2 min, followed by an increase of 5°C min^{-1}, until 180°C, then a 10°C min^{-1} until 280°C and finally held constant for 20 min. The source temperature was 230°C electron ionization was set at 70 eV and spectra were scanned from 33 to 550 m/z. The compounds were identified by NIST database.

■ RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Effect of alkaline pretreatment on wheat straw:

Alkali pretreatment (2% NaOH, 121°C, for 60 min) of wheat straw decreases 60 per cent of the total weight of the sample. The lignin content in the sample was 18 per cent which was decreased to 10 per cent after pretreatment (Table 1). The hemicelluloses content in untreated wheat straw was 26 per cent which was reduced to 15 per cent after alkali pretreatment. However, cellulose content was increased from 36 to 46 per cent. It has reported that in alkali and alkali peroxide treatment decrease in hemicelluloses to 6.3 per cent and 5.3 per cent, and decrease in lignin to 14.6 per cent and 5.6 per cent, respectively (Chen *et al.*, 2008).

The ash content was partially varying before and after pre-treatment. It showed that after alkali treatment ash content of wheat residue was decreased from 11 to 10 % wt. Hence, alkali treatment resulted into a moderate ash removal. The ash content decreased from 11 to 10 % wt. Similar results were shown in a study in which ash content of oil palm mesocarp fibre was reduced after alkali pretreatment. This was due to the removal of lignin after alkali pretreatment (Iberahim *et al.*, 2013). Treatment with 2% NaOH solution reduced lignin to 10 wt. % in wheat straw. The amount of cellulose was higher compared to hemicellulose (15 wt %) and lignin content (10 wt %). These findings were found close to the composition determined in oil palm mesocarp fibre (Iberahim *et al.,* 2013) in which much amount of hemicelluloses degraded and convert to cellulose. Previous works are done by Hendriks and Zeeman (Hendriks and Zeeman, 1998) also agree that alkaline pretreatment could cause cellulose fibres to swell rather than directly degrading it. Gaspar *et al.* (2007) resolved that pretreatment is the best method to avoid further fragmentation of hemicelluloses polymers as well as break up the ester bonds between lignin, hemicelluloses, and cellulose.

Effect of enzymatic pretreatment:

Preliminary trials were conducted using the two enzymes separately and both enzymes together and release of reducing sugars was compared. It was found that cellulase and β-glucosidase enzymes work synergistically at temperature 37.5°C and 4.8 pH, and producing more reducing sugar yield (20 g/L). However, in the two-step enzyme addition experiment, the amount

Table 1 : Proximate of untreated and treated wheat straw

Sugarcane residue biomass	wt %					
	Cellulose	Hemicellulose	Lignin	Ash	Moisture	Others
Untreated % W/ W	36.1	26	18	11	8	8.9
Alkali Treated % W/ W	46.2	15	10	10	7	18.8

of reducing sugar was 18 g/L. This could be due to the accumulation of cellubiose which inhibit the action of cellulase. It had also been reported that the enzymes cellulase and β-glucosidase shows strong synergism when both of them were used together in the same reaction mixture (Sternberg, 1975). The enzymes catalyzing the degradation of lignocellulosic material into fermentable sugars are a mixture of endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases. They act in synergism by targeting lignocellulosic material differently; cellobiohydrolases attack the ends of cellulose and cleave cellobiose units off, endoglucanases randomly attack cellulose chains and releases cello-oligosaccharides thereby 'feeding' the cellobiohydrolases with cellulose ends and finally the β-glucosidases catalyse the hydrolysis of cellobiose and short chain oligosaccharides into glucose (Bhat and Bhat, 1997 and Juhász *et al.,* 2005).

Effect of independent variables on various responses:

Effects of independent variables *i.e.* time, enzyme loading ratio and substrate concentration on responses *viz.,* reducing sugar, glucose concentration, lignin, ash, cellulose, and hemicellulose were determined and statistically analyzed. Experimental data has been presented in Table 2.

Reducing sugar:

In enzymatic pre-treatment, the reducing sugar ranged from 1.433 to 24.2 g/L (Table 2). Maximum reducing sugar was found 24.2 g/L for experiment number 9, with the combination of time 72 h, enzyme loading ratio 5:10 and substrate concentration 5% w/v. Minimum reducing sugar was found 1.433 g/L for experiment number 11, at time 24 h, enzyme loading ratio 5:10 and substrate concentration 5% w/v. Graphical analysis of the reducing sugar represent that there is a slight increase in reducing sugar upto 48 h but after 48 h there is a rapid increase in reducing sugar content (Fig. 1a). The time for incubation was greatly affected by reducing sugar content. The maximum reducing sugar is recorded at 24.2 g/L at 72 h. Saha and Cotta (2007) also reported the release of maximum reducing sugar 428 mg/g at 72 h during saccharification of wheat straw. The statistical analysis of reducing sugar is given in Table (3 a and b).

The model of reducing sugar was found highly significant ($P < 0.01$) because it had higher F-value (187.57). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. According to Table 3a, a high F-value was obtained, indicating the high efficiency of factors. Especially, the P-value which

Table 2 : Experimental data of independent parameters for enzymatic pre-treatment

Expt. No.	Coded levels			Reducing sugar (g/L)	Glucose conc. (g/L)	Lignin (%, w/w)	Ash (%, w/w)	Cellulose (%, w/w)	Hemicellulose (%, w/w)
	Time (X_1, h)	Substrate conc. X_2, %w/v	Enzyme loading ratio (X_3)						
1	0.00	0.00	0.00	1.55	1.007	7.6	7.5	0.629	0.342
2	-1.00	1.00	00.0	1.611	1.007	9.5	9.3	0.053	0.268
3	1.00	1.00	0.00	19.93	13.12	9	96.5	6.562	3.433
4	1.00	0.00	1.00	14.326	8.949	7.9	6.9	8.949	4.45
5	-1.00	0.00	1.00	1.866	1.194	7.6	7.4	0.746	0.44
6	0.00	-1.00	-1.00	2.375	1.472	7.1	6.7	1.148	0.711
7	-1.00	-1.00	0.00	1.645	1.069	7.1	6.5	0.937	0.454
8	0.00	0.00	0.00	1.566	1.018	7.7	7.5	0.635	0.346
9	1.00	0.00	-1.00	24.2	15.488	7.8	7.7	9.675	5.453
10	0.00	0.00	0.00	2.083	1.354	7.7	7.5	0.846	0.459
11	-1.00	0.00	-1.00	1.433	0.946	7.9	7.8	0.591	0.306
12	0.00	1.00	-1.00	2.111	1.33	9.4	9.1	0.664	0.394
13	0.00	0.00	0.00	1.6	1.00	7.7	7.5	0.649	0.353
14	0.00	0.00	0.00	2.566	1.668	7.7	7.5	1.042	0.567
15	0.00	1.00	1.00	2.819	1.804	9.2	9.1	0.901	0.512
16	1.00	-1.00	0.00	21.111	13.3	6.9	6	6.649	3.942
17	0.00	-1.00	1.00	2.708	1.733	7	6.3	0.866	0.768

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

Table 3a : ANOVA for the selected model for reducing sugar

Source	DF	Sum of squares	Mean of squares	F-value	Pr> F
Model	9	1190.13	132.24	187.57	<0.0001
Error	4	0.80	0.20		
Corrected total	16	1195.06			

Co-efficient of variation (CV) = 12.68%

Table 3b : Statistical analysis of factors for reducing sugar

Source	DF	Mean square	F-value	Pr> F
X_1	1	801.38	1136.74	<0.001
X_2	1	0.23	0.33	0.5826
X_3	1	0.23	0.32	0.5894
X_{11}	1	376.75	534.40	<0.0001
X_{22}	1	0.28	0.40	0.5484
X_{33}	1	3.32	4.71	0.0665
X_{12}	1	0.33	0.47	0.5165
X_{13}	1	2.64	3.75	0.0945
X_{23}	1	0.050	0.050	0.8297

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

is important for understanding the pattern of mutual interactions between the variables was below 0.0001. Further, it was found that the factors were considerably variable and also statistically significant. Table 3b shows a statistical analysis of the factors. The time period was found to be the most important factor due to high F-value as 1136.74.

Glucose concentration:

The glucose content ranged from 0.946 to 15.488g/L (Table 2). Maximum glucose content was found 15.488 g/L for experiment number 9, at time 72 h, enzyme loading ratio 5:10 and substrate concentration 5% w/v. Minimum glucose content was observed at 0.946 g/L for experiment number 11, having time 24 h, enzyme loading ratio 5:10 and substrate concentration 5S% w/v. The reaction time and substrate concentration have a clear effect on the amount of glucose concentration. Similar results were also obtained by Teerapatr *et al*. (2013). Maximum glucose released in the experiments having the centre values of enzyme loading ratio and substrate concentration and 48 h of reaction time. Graphical analysis of glucose concentration represent that there is slowly increase in glucose concentration upto 48 h but after that, there is a rapid increase in glucose content (Fig. 1b). The maximum glucose content recorded was 15.488 g/L at 72 h. The reaction time and substrate concentration have a clear effect on the amount of glucose concentration. Similar results were also obtained by (Teerapatr *et al.,* 2013). Maximum glucose released in the experiments having the centre values of enzyme loading ratio and substrate concentration and 48 h of reaction time. The statistical analysis of glucose is given in Table 4 a and b.

The model of glucose was found highly significant ($P < 0.01$) because it had higher F-value (182.34). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. According to Table 4a. a high F-value was obtained, indicating the high efficiency of factors. Especially, the P-value which is important for understanding the pattern of mutual interactions between the variables was below 0.0001. Further, it was found that the factors were considerably variable and also statistically significant. Table 4b. shows a statistical

Table 4a : ANOVA for the selected model for glucose

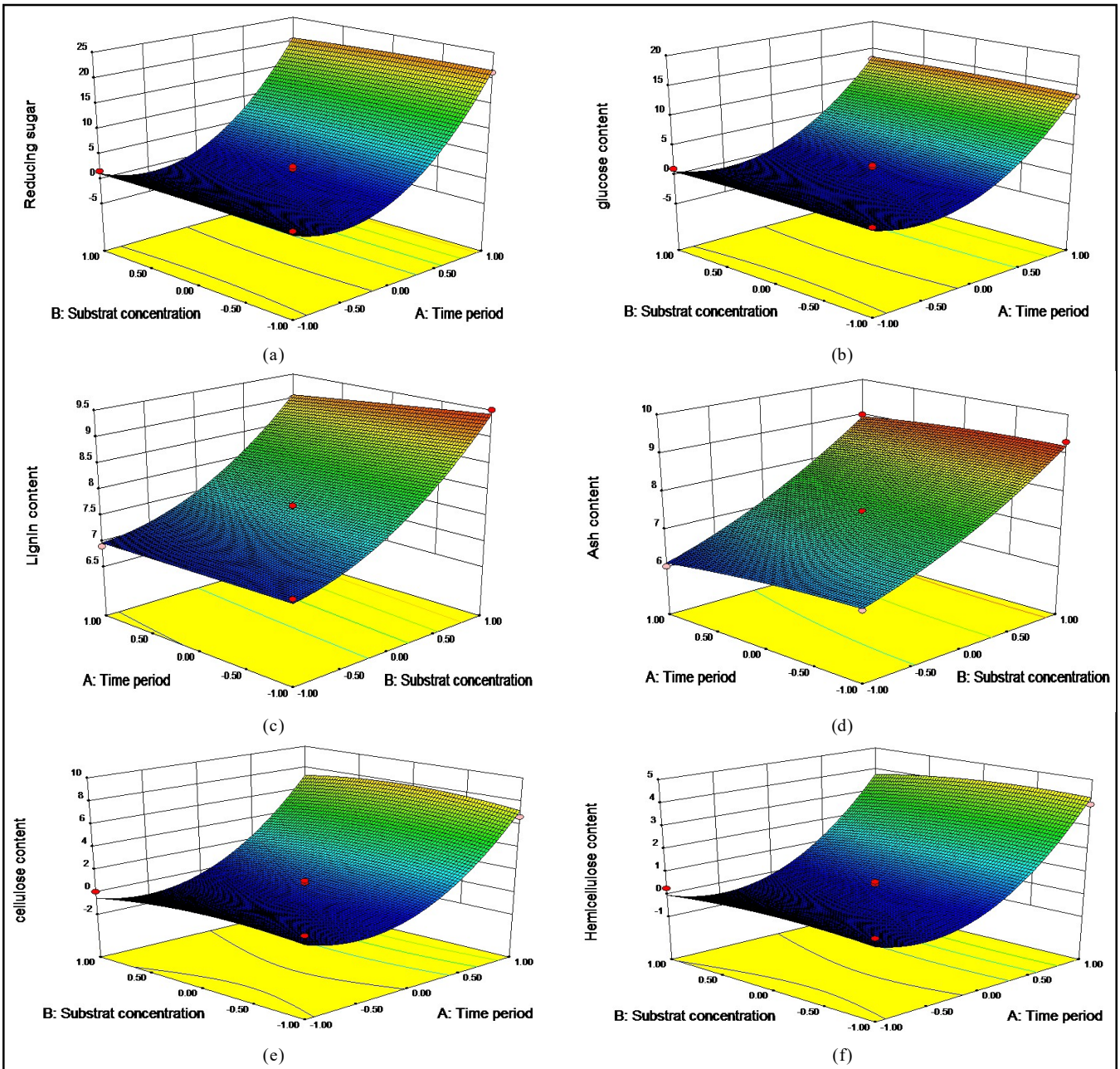
Source	DF	Sum of squares	Mean of squares	F-value	Pr> F
Model	9	501.80	55.76	182.34	<0.0001
Error	4	0.35	0.088		
Corrected total	16	503.94			

Co-efficient of variation (CV) = 12.89%

analysis of the factors. The time period was found to be the most important factor due to high F-value as 1103.7406.

Lignin content :

Lignin content was from ranged from 6.9 to 9.5% w/w in enzymatic treatment (Table 2). Maximum lignin was found 9.5%w/w for experiment number 2, having time 24 h, enzyme loading ratio 10:10 and substrate concentration 6% w/w. Minimum lignin was found 6.9% w/w for experiment number 16, at time 72 h, enzyme loading ratio 10:10 and substrate concentration 4% w/v. Graphical analysis showed that the maximum lignin was found 9.5% at substrate concentration 6% (Fig. 1c). The



(a) Reducing sugar, (b) Glucose content, (c) Lignin content, (d) Ash content, (e) Cellulose content, (f) Hemicellulose content

Fig. 1 : Effects of independent variables on time and substrate concentration in 3D space

Table 4b : Statistical analysis of factors for glucose

Source	DF	Mean square	F-value	Pr> F
X_1	1	337.30	1103.06	<0.0001
X_2	1	7.260	0.024	0.8819
X_3	1	4.005	0.013	0.9121
X_{11}	1	160.20	523.88	<0.0001
X_{22}	1	0.23	0.76	0.4110
X_{33}	1	1.57	5.14	0.0577
X_{12}	1	9.025	0.030	0.8685
X_{13}	1	0.50	1.63	0.2430
X_{23}	1	0.011	0.037	0.8527

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

substrate concentration had a clear effect on the amount of lignin. Chandel *et al.* (2011) also found the maximum lignin content 12.4 % w/w with the highest value of substrate concentration which showed that higher the substrate concentration higher will the lignin content. The statistical analysis of lignin content was given in Table 5 (a and b).

The model of lignin content was found highly significant ($P < 0.01$) because it had higher F-value (149.92). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. According to Table 5a, a high F-value was obtained, indicating the high efficiency of factors. Especially, the P-value which is important for understanding the pattern of mutual interactions between the variables was below 0.0001. Further, it was found that the factors were considerably variable and also statistically significant. Table 5b shows a statistical analysis of the factors. Substrate concentration was found to be the most important factor due to high F-value as 1221.98.

Ash content:

The ash content ranged from 6 to 9.3 % w/w after enzymatic treatment (Table 2). Maximum ash was found 9.3 %w/w for experiment number 2, at time 24 h, enzyme loading ratio 10:10 and substrate concentration 6 % w/w. Minimum ash was observed 6 w/w for experiment number 16, at time 72 h, enzyme loading ratio 10:10 and substrate concentration 4% w/w.

Graphical representation of ash content showed the maximum ash content is 9.3% was found with substrate concentration which is 6% (Fig 1 d). The ash content

Table 5a : ANOVA for the selected model for lignin

Source	DF	Sum of squares	Mean of squares	F-value	Pr> F
Model	9	11.18	1.24	149.92	<0.0001
Error	4	8.0	2.0		
Corrected total	16	11.24			

Co-efficient of variation (CV) = 1.15%

Table 5b : Statistical analysis of factors for lignin

Source	DF	Mean square	F-value	Pr> F
X_1	1	0.080	9.66	0.0171
X_2	1	10.13	1221.98	<0.0001
X_3	1	0.080	9.66	0.0171
X_{11}	1	2.632	3.176	0.9566
X_{22}	1	0.84	101.76	<0.0001
X_{33}	1	9.500	1.15	0.3198
X_{12}	1	0.023	2.72	0.1434
X_{13}	1	2.500	0.30	0.5999
X_{23}	1	2.500	0.30	0.5999

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

Table 6a : ANOVA for the selected model for ash

Source	DF	Sum of squares	Mean of squares	F-value	Pr> F
Model	9	16.12	1.79	96.44	<0.0001
Error	4	0.000	0.000		
Corrected total	16				

Co-efficient of variation (CV) = 1.79%

Table 6b : Statistical analysis of factors for ash

Source	DF	Mean square	F-value	Pr> F
X_1	1	1.79	96.44	<0.0001
X_2	1	15.13	814.42	<0.0001
X_3	1	0.32	17.23	0.0043
X_{11}	1	0.024	1.28	0.2960
X_{22}	1	0.32	17.15	0.0043
X_{33}	1	2.632	0.14	0.7177
X_{12}	1	0.010	0.54	0.4069
X_{13}	1	0.040	2.15	0.1857
X_{23}	1	0.040	2.15	0.1857

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

was increased linearly with substrate concentration. The substrate concentration had a clear effect on the amount of ash. However the value of ash to increase with the increase in the substrate concentration (Pitarelo, 2007). The statistical analysis of ash content was given in Table 6.

The model of ash content was found highly significant ($P < 0.01$) because it had higher F-value (96.44). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. According to Table 6a, a high F-value was obtained, indicating the high efficiency of factors. Especially, the P-value which is important for understanding the pattern of mutual interactions between the variables was below 0.0001. Further, it was found that the factors were considerably variable and also statistically significant. Table 6b shows a statistical analysis of the factors. Substrate concentration was found to be the most important factor due to high F-value as 814.42.

Cellulose content:

The cellulose content ranged from 0.053 to 9.67 %w/w in the case of enzymatic pre-treatment (Table 2). Maximum cellulose was found 9.67% w/w for experiment number 9, at time 72 h, enzyme loading ratio 5:10 and substrate concentration 5% w/w. Minimum cellulose was found 0.053% w/w for experiment number 2, at time 24 h, enzyme loading ratio 5:10 and substrate concentration 5 % w/w. Graphical representation of cellulose content showed the maximum amount of cellulose degraded was 9.67% 72 h of the incubation period (Fig. 1e). The hydrolysis of cellulose greatly depends on incubation time which was increased rapidly after 48h. The hydrolytic efficiency of cellulase decreased significantly with the increase in substrate concentration. The low cellulose conversion may be ascribed to the high viscosity, uneven slurry distribution and end product inhibition (Chen *et al*., 2008). The statistical analysis of cellulose content was given in Table 7.

The model of cellulose content was found highly significant ($P < 0.01$) because it had higher F-value (37.46). There is only a 0.01 per cent chance that a "Model F-Value" this large could occur due to noise.

Table 7a : ANOVA for the selected model for cellulose

Source	DF	Sum of squares	Mean of squares	F-value	Pr> F
Model	9	164.17	18.24	37.46	<0.0001
Error	4	0.13	0.033		
Corrected total	16	167.57			

Co-efficient of variation (CV) = 28.56%

Table 7b : Statistical analysis of factors for cellulose

Source	DF	Mean square	F-value	Pr> F
X_1	1	108.84	223.51	<0.0001
X_2	1	0.25	0.52	0.4953
X_3	1	0.047	0.097	0.7641
X_{11}	1	49.91	102.49	<0.0001
X_{22}	1	1.79	5.38	0.0964
X_{33}	1	2.61	3.68	0.0538
X_{12}	1	0.16	0.33	0.5858
X_{13}	1	0.19	0.40	0.5479
X_{23}	1	0.067	0.14	0.7210

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

According to Table 7a, a high F-value was obtained, indicating the high efficiency of factors. Especially, the P-value which is important to understanding the pattern of mutual interactions between the variables was below 0.0001. Further, it was found that the factors were considerably variable and also statistically significant. Table 7b shows a statistical analysis of the factors. The time period was found to be the most important factor due to high F-value as 223.51.

Hemicelluloses content:

The hemicellulose content ranged from 0.26 to 5.45 % w/w in case of enzymatic pre-treatment (Table 2). Maximum hemicellulose was found 5.45% w/w for experiment number 9, at time 72 h, enzyme loading ratio 5:10 and substrate concentration 5% w/w. Minimum hemicellulose was observed at 0.26% w/w for experiment number 2, at time 24 h, enzyme loading ratio 5:10 and substrate concentration 5% w/w. The hydrolysis of hemicellulose is gradually increased after 48 h. Graphical representation of hemicelluloses content showed the maximum amount of hemicellulose hydrolyzed was 5.45% at 72 h of the incubation period (Fig.1 f). The hydrolysis of hemicellulose was greatly depended on incubation time which was increased rapidly after 48 h. Hydrolysis of hemicellulose could be the slow action of a mixture of enzymes. The statistical analysis of hemicellulose content was given in Table 8.

The model of hemicellulose was found highly significant ($P < 0.01$) because it had higher F-value (38.22). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. According to

Table 8a : ANOVA for the selected model for hemicellulose

Source	DF	Sum of squares	Mean of squares	F-value	Pr> F
Model	9	47.22	5.25	38.22	<0.0001
Error	4	0.039	9.740		
Corrected total	16	48.18			

Co-efficient of variation (CV) = 27.15%

Table 8b : Statistical analysis of factors for hemicellulose

Source	DF	Mean square	F-value	Pr> F
X_1	1	31.24	227.63	<0.0001
X_2	1	0.20	1.46	0.2655
X_3	1	0.060	0.44	0.5290
X_{11}	1	14.23	103.68	<0.0001
X_{22}	1	0.22	1.59	0.2479
X_{33}	1	0.71	5.17	0.0572
X_{12}	1	0.026	0.19	0.6760
X_{13}	1	0.32	2.35	0.1688
X_{23}	1	9.303	6.777	0.9367

X_1: Time (h), X_2:Substrate conc. (%w/v), X_3 : Enzyme loading ratio

Table 8a, a high F-value was obtained, indicating the high efficiency of factors. Especially, the P-value which is important to understanding the pattern of mutual interactions between the variables was below 0.0001. Further, it was found that the factors were considerably variable and also statistically significant. Table 8b shows a statistical analysis of the factors. The time period was found to be the most important factor due to high F-value as 227.63.

Optimized parameters:

The experimental values of enzymatic pre-treatment of all samples are reported in Table 2. The numerical optimization was carried out using Design-Expert 8.1.7.1 statistical software. The individual goals for different predicted factors and dependent variables are as given in Table 9.

Based on the above-mentioned criteria, optimization was done for individual responses. The numerical optimization was done to optimize the combination of one or more goals. The goals may apply to either factor or response. The possible goals were to maximize, minimize, within range, target or none for response only and set to an exact value for factors only. Total 6 possible solutions were obtained in all cases, out of which the one possible solution that suited the criteria and was most desirable among other possible was selected.

Compromise optimization of response:

The results were compromised with among response, to get optimum predicted variables using Design-expert 8.0.7.1 software. The goal was fixed in range for time, unit of enzyme loading ratio and substrate concentration, while among the response reducing sugar, glucose concentration, ash, cellulose, hemicelluloses, and lignin. The goal seeking begins at a random starting point and proceeds up and down the steepest slope on the response surface for the maximum and minimum value of the response, respectively. All response and predicted variables were given similar importance. During optimization 10 solutions were obtained, out of which the one that suited the criteria most was selected (Table 10).

For the optimized set (60, 5:10, 6) of three levels, the experiment was conducted. Corresponding responses were 11, 7.5, 8, 8.2, 3.9 and 2.4 for reducing sugar, glucose concentration, lignin, ash, cellulose, and hemicelluloses respectively.

Recovery and properties of bio-oil:

The main objective of the study was to reduce the lignin content by chemical and enzymatic pretreatment of wheat straw. However, it was found that after 2% NaOH pretreatment the lignin content was reduced from 18% w/w to 10% w/w. in enzymatic treatment the lignin content was reduced from 10% to 6.9% w/w. Hence, it was observed in the enzymatic treatment of wheat straw the lignin content was not significantly reduced. Further, the enzyme application could increase the cost of bio-oil production. Untreated and alkali treated wheat straw was

Table 9 : Individual goals for different predicted factors and dependent variables

Factors	Goal	Lower limit	Upper limit
Time (X_1, h)	minimize	-1	1
Substrate concentration (X_3, % w/v)	In range	-1	1
Enzyme loading ratio (X_2)	Minimize	-1	1
Reducing sugar (g/L)	Maximize	1.55	24.2
Glucose concentration (g/L)	Maximize	0.946	15.488
Lignin (%, w/w)	Maximize	6.9	8.5
Ash (%, w/w)	Maximize	6	9.3
Cellulose (%, w/w)	Minimize	0.053	9.675
Hemicellulose (%, w/w)	Minimize	0.268	5.453

Table 10 : Optimum levels of predicted variables for enzymatic pre-treatment to improve quality of bio-oil

Predicted variables	Coded level	Actual level
Time (X_1)	0.52	60 hour
Substrate concentration (X_2, %, w/v)	1	6 (g)
Enzyme loading ratio (X_3)	-1	5:10 (units)

pyrolyzed and collected. The bio-oil yield from untreated and treated wheat straw was 27 per cent and 30 per cent, respectively. The bio-oil of the untreated sample was found acidic (pH 3.0) then treated sample (pH 4.0). Meanwhile, the density of the bio-oil derived from the treated sample (1.4 kg/L) was slightly higher than the original untreated sample (1.38 kg/L) and also higher than petroleum diesel (0.812 kg/L). The pretreated wheat straw produced oil with a lower viscosity (8.89 cSt) compared to 9.22 cSt for the untreated sample. The flash point of pretreated wheat straw (59°C) was very close to diesel (61°C) comparatively with untreated bio-oil (95°C). The fire point of treated bio-oil (64°C) is also very close to diesel (64°C) comparatively with untreated bio-oil (98°C). It showed that the quality of treated bio-oil was appropriate (Table 11).

The functional groups present in treated and untreated bio-oil samples were analyzed using FTIR spectrophotometer (Fig. 2). The broadband in both spectra at 3000-4000 cm $^{-1}$ was due to absorption of the hydroxyl group as characterized by the phenolic group. The N-H peak is sharp in the untreated sample (*i.e.* 617.83) which is reduced in the treated sample (*i.e.* 611.96). It means the nitrogenous compound is reduced after pretreatment of bio-oil. The presence of phenol was verified by the existence of O-H stretching vibrations at 3435 and 3445 cm^{-1} for both treated and untreated bio-oil samples. Phenolic compounds are mostly produced by the degradation of lignin. It simply means that the lignin is degraded and removed at the

Table 11 : Characteristic properties of diesel, bio-oil from untreated and treated wheat straw

Sr. No.	Property	Diesel	Bio-oil from untreated wheat straw	Bio-oil from treated wheat straw
1.	Kinematic Viscosity (cSt)	4.12	9.22	8.89
2.	Dynamic viscosity (cSt)	3.35	12.778	12.446
3.	Density (g/ml)	0.812	1.38	1.4
4.	Relative density (g/ml)	0.826	0.99	1.00
5.	Flash point (°C)	61	95	59
6.	Fire point (°C)	65	98	64
7.	Moisture content (wt %)	0.05	42	38
8.	pH	5.6	3	4

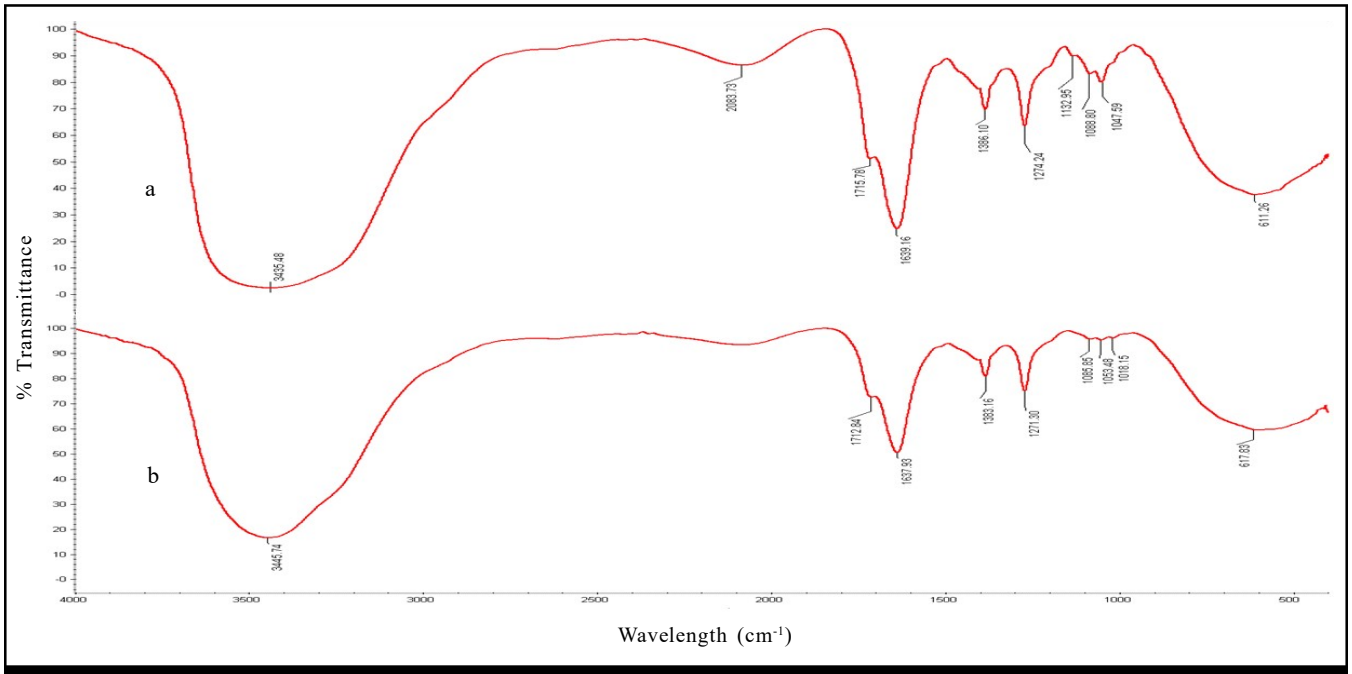


Fig. 2 : FTIR spectra of bio-oil from wheat straw. a. alkali treated wheat straw, b. untreated wheat straw

Table 12 : Selected major compounds after GC-MS analysis of untreated wheat straw bio-oil

Peak#	R. Time	Concentration wt.%	Name
1.	4.143	5.32	Hydroquinone
2.	4.262	2.77	1,2-Benzenediol, 3-methoxy-
3.	4.454	1.50	Guaiacol, 4-ethyl-
4.	4.510	1.72	1,2-Benzenediol, 4-methyl-
5.	4.660	0.29	1h-inden-1-one, 2,3-dihydro-
6.	5.406	1.83	1,4-Benzenediol, 2-methyl-
7.	5.673	6.97	Phenol, 2,6-dimethoxy-
8.	6.033	0.08	Furan, 2,2'-methylenebis-
9.	6.129	0.21	1-hexyn-3-ol, 3-methyl-
10.	6.260	0.40	2-Buten-1-ol, propanoate
11.	6.466	0.20	Dodecane
12.	6.583	0.38	2-(1,5-dimethyl-hexyl)-cyclobutanone
13.	6.793	0.41	Benzaldehyde, 4-hydroxy-3-methoxy-
14.	7.192	0.29	1-hydroxy-2-methoxy-4-methylbenzene
15.	7.750	0.87	3,5-Dimethoxy-4-hydroxytoluene
16.	8.846	0.76	Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-
17.	9.282	0.17	Phenol, 2,4-bis(1,1-dimethylethyl)-
18.	9.531	0.19	7-Hexadecenal, (Z)-
19.	9.737	0.87	Benzene, 1,2,3-trimethoxy-5-methyl-
20.	9.936	1.35	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-
21.	10.492	1.79	Dodecanoic acid
22.	11.495	0.49	Pentadecane
23.	14.188	0.41	Heptadecane, 2,6,10,15-tetramethyl-
24.	15.315	0.62	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-
25.	15.854	0.99	Tetradecanoic acid
26.	16.271	0.28	Heptafluorobutyric acid, pentadecyl ester
27.	16.836	0.71	Heptadecane
28.	17.517	0.34	Isopropyl myristate
29.	18.690	0.30	1,2-benzenedicarboxylicacid, bis (2-methylp
30.	20.008	0.63	2-Methylhexacosane
31.	20.746	0.20	1-Decanol, 2-hexyl-
32.	21.048	13.81	n-Hexadecanoic acid
33.	21.843	0.52	Iron, tricarbonyl[n-(phenyl-2-pyridinylmet
34.	22.339	0.23	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione
35.	22.457	0.18	Isopropyl palmitate
36.	25.050	0.04	Heptadecane, 3-methyl-
37.	25.728	12.53	Octadecanoic acid
38.	25.980	1.09	Eicosane
39.	28.575	16.55	Pentacosane
40.	30.479	0.24	Tetracosane
41.	30.634	5.9	Heneicosane
42.	33.615	0.30	1,2-benzenedicarboxylic acid
43.	33.816	0.18	Hentriacontane
44.	35.221	5.68	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)
45.	35.677	0.32	2-methyloctacosane
46.	38.678	0.48	Squalene
47.	46.195	1.03	Tetrapentacontane
48.	49.245	3.13	Tetracontane
		100.00	

Table 10 contd…

Contd… Table 12

time of pretreatment. The presence of C=O stretching vibration at 1712 and 1715 cm^{-1} in untreated and treated bio oil sample is compatible with the presence of ketone, aldehyde, carboxylic and an ester group. Meanwhile, the peak at 1132 in treated and 1085 cm^{-1} in untreated sample is due to the presence of primary, secondary, tertiary alcohols indicated the C-O stretching. The peaks at 1088 in treated and 1053 cm^{-1} in the untreated sample indicate the presence of aromatic groups (Putun *et al.,* 2008). Other weak absorbance observed at nearly 650-900 (cm^{-1}) shows the existence of some substituted aromatic groups.

GC-MS analysis:

GC-MS analysis of untreated bio-oil from wheat straw had 48 compounds with major components pentacosane (16.55%) and there are n-hexadecanoic acid (13.81%), Octadecanoic acid (12.83%), phenols and its derivatives were 17.97% (Table 12).

However, in treated wheat straw bio-oil, the amount of pentacosane became increased to 58.18% followed by heneicosane 15.67% (Table 13). The quantity of n-hexadecanoic acid became decreased to 1.07%. Pentacosane and heneicosane are long chain hydrocarbons. They can be fragmented to form short

Table 13 : Selected major compounds after GC-MS analysis of treated wheat straw bio-oil

Peak#	R. Time	Concentration wt.%	Name
1.	4.540	0.70	Tridecane
2.	5.686	1.77	Phenol, 2,6-dimethoxy-
3.	6.470	0.37	Heptadecane
4.	7.759	0.57	3,5-Dimethoxy-4-hydroxytoluene
5.	8.859	0.41	Nonane, 3,7-dimethyl-
6.	9.953	0.41	Sulfurous acid, hexyl octyl ester
7.	10.487	0.69	Dodecanoic acid
8.	11.497	0.70	Sulfurous acid, 2-ethylhexyl hexyl ester
9.	15.635	0.34	Octadecane
10.	18.696	0.21	1,2-benzenedicarboxylic acid, bis (2-methylp
11.	19.390	0.26	Nonane, 3,7-dimethyl-
12.	20.011	0.32	Tetracosane, 1-iodo-
13.	20.962	1.07	n-Hexadecanoic acid
14.	21.031	0.46	2-Bromotetradecane
15.	21.842	0.73	Iron,tricarbonyl[n-(phenyl-2-pyridinylmet
16.	24.188	15.67	Heneicosane
17.	25.658	1.06	9-octadecenoic acid (z)-
18.	25.980	0.65	Eicosane
19.	28.575	58.18	Pentacosane
20.	33.618	0.37	1,2-benzenedicarboxylic acid, diisooctyles
21.	35.683	1.97	2-methyloctacosane
22.	38.677	0.41	Squalene
23.	39.200	0.41	Octacosane
24.	39.396	0.21	Heptadecane, 3-methyl-
25.	41.609	12.09	Tetracontane
		100.00	

chain hydrocarbon to increase the volatility. The Octadecanoic acid and n-hexadecanoic acid was decreased from untreated to treated wheat straw bio-oil respectively. Phenols and its derivatives were decreased in treated wheat straw bio-oil. Phenol is a product of degradation of lignin (Amin *et al.,* 2010). It showed that in the untreated bio-oil sample the lignin was present in high amount compared with the treated sample. The oil contains the defragmented parts of the oxygenated components of the original biomass structure (mainly cellulose, hemicellulose, and lignin). The compounds identified such as phenol, syringol and dimethylcyclopentanone were present in untreated bio-oil but not present in treated bio-oil sample. However, Furans, ketones, acids, aldehydes, and phenols are also reported in rice husk pyrolysis bio-oil (Zhang *et al.*, 2014).

Conclusion:

Pretreatment of wheat straw with 2% NaOH, the lignin content decreases half of the untreated sample, while in enzymatic treatment 3 dimethylcyclopentanone reduction in lignin content was observed with the increase of the reducing sugars. The bio-oil obtained after removal of lignin had an appreciable property compare to diesel as the flashpoint of the treated bio-oil sample was nearly the same as diesel. On the basis of GC-MS study in the untreated sample the main compound identified pentacosane and heneicosane, they are long-chain hydrocarbons and could be fragmented to form short chain hydrocarbon to increase the volatility. Beside this untreated wheat, straw bio-oil contains (13.81%) n-hexadecanoic and (12.83%) octadecanoic acids. In GC-MS, several compounds were formed from fragmentation and degradation of lignocelluloses such as phenols, furans, aldehydes, ketones, acids, etc. Further, these compounds can also extract to value-added products from bio-oil in commercial scale.

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