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Utilisation of the spent biomass from the old thatched roofs for production of bioethanol

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Abstract
Increasing demand for alternative biofuel production and the need to address the problems of environmental pollution along with energy security has led to a shift in focus towards lignocellulosic biomass. Rice straw being the most abundant lignocellulosic biomass, most of it is either used as cattle feed or used on thatched roof tops, rest either decomposed or burnt. After a certain span of time those used on roof tops are thrown away for decomposition or burnt leading to air pollution. The present study aims to resolve the food/feed versus fuel problems and to device a cost effective technique for bioethanol production. The study analyses the decrease in lignin content with ageing and optimizes the pretreatment process parameters influencing the output with maximum reducing sugars yield of 348.8 mg/g after saccharification followed by fermentation with *Pichia stipitis* resulting in 20.123 g/l of ethanol.

Keywords: Waste rice straw, pretreatment, enzymes, saccharification, *Pichia stipitis*, ethanol

1. Introduction
Population growth and industrial development has led to a steady increase in energy consumption. By 2030, the demands for crude oil are expected to increase to 116 million barrels. With the growing concern over depleting reserves of fossil fuels along with the need to address the issues of green house effect and global warming, there has been a shift in focus towards alternative cleaner and greener sources called biofuels. The insufficiency of other conventional energy sources to meet the ever increasing demands has led to an intensive study and shift towards a high level of interest in non-conventional fuel originating from bio-renewable sources like food crops and lignocellulosic biomass[1,2].

Lignocellulosic biomass in spite of being the most cheap energy sources, are highly abundant and distributed in a proportionate manner in terms of geographical point of view. Thus, they are more domestic and provide security of supply. Apart from being the most renewable and sustainable energy sources, they also help in fixation of carbon dioxide (responsible for green house effect) and global warming. They minimize potential conflict between land use for food (feed) and energy feedstock production. Thus, they are the most attractive sources for production of second generation biofuels.

Rice straw is the vegetative part of rice plant (*Oryza sativa*) cut and separated after grain harvest. Rice originated in 6500 BC and is now known to be grown in most of the tropical and subtropical regions. Rice is the most abundant lignocellulosic material in the world, with an annual production ranging from 731 million tonnes out of which Asia is one of the largest producer, producing about 667.6 million tonnes followed by America, Europe and Africa which together produce around 62 million tonnes. About 60% mass of rice is composed of straw. Approximately, each kilogram of rice produced is accompanied by 1-1.5 kg of rice straw [2]. Thus, nearly 667 million tonnes of straw is produced per year which has a huge potential of producing 205 billion litres of bioethanol per year, accounting to be the largest ever feedstock source for biofuel [2]. But, majority of straw produced is used as cattle feed, while some are used to prepare thatched roofs which after some years are either wasted and thrown away for decomposition or subjected to field burning. Due to low bulk density and high extent of mineral content the process of decomposition is very slow and unfavourable, resulting in spread of unwanted plant pathogens and paddy diseases. Further, the process of field burning contributes to severe air pollution. Thus, diverting an essential fraction of these unused straw for bioethanol production is an attractive option both from environmental and economic point of view.

Chemical structure analysis of rice straw has led to the presence of cellulose (32-47%), hemicelluloses (19-27%), lignin (5-24%) [2].

The major issues associated with bioconversion of various fermentable sugars to bioethanol is the presence of highly recalcitrant lignin which forms a solid seal around cellulose micro-fibrils, and exhibits limited covalent association with hemicelluloses, preventing enzyme accessibility[3]. Pretreatment increases sugar yields by 90% compared to that without pretreatment[4]. Pretreatment thus constitute an essential step occupying 33% of total operating cost of the entire process as long as economic feasibility is concerned. Thus, pretreatment not only should maximize the digestibility of lignocelluloses but also should have a low capital and operating cost with little/no inhibitory products [2,3,4].

The present study deals with a modelling based statistical approach named Response surface methodology (RSM) which was successfully implemented to find out the optimum conditions for maximizing the amount of xylose yield and total reducing sugar yield by building up an experimental design. The fitting of the responses extracted from design of experiments (DOEs) to a polynomial function is effectively done by RSM. RSM includes statistical modelling to determine the interaction effects of the factors on the response and serve as an effective tool for evaluating the optimum condition [5].

To solve the issues related to energy and environmental security, the present study analyses decrease in lignin content with ageing as straw is exposed to various environmental factors and uses waste rice straw from thatched roofs for bioethanol production. Further, the study aims to develop an easy and cost effective technique for pretreatment and to optimize various parameters affecting the output of the process.

2. Materials and methods

2.1 Biomass sample preparation

Rice straw samples of various (approximate) ages (6 months, 1 year, 3 years, 4 years) were collected from nearby village using reliable sources. Each sample was dried for a period of 4 h for 2 days in a tray dryer at 60 °C. The dried samples were finely powdered using a grinder and was stored for future use in air tight containers.

2.2 Preliminary analysis of hemicellulose, lignin and cellulose content

Estimation of hemicelluloses and lignin was done by Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) method [6]. Quantification of cellulose was done using Anthrone test [7].

2.3 Pretreatment

Finely powdered waste rice straw samples ageing approximately 4 years from thatched roofs were mixed with 10 ml of 5% sodium hydroxide (10% loading) and incubated for different periods and at different soaking temperatures and at varying agitation rates. After incubation, the samples were subjected to moist heat, autoclaved at 15 psi, 121 °C, for different treatment periods. The samples were then filtered using whatman filter paper 1. The hydrolysate were collected and concentration of reducing sugars and xylose were found using different analytical techniques. Estimation of reducing sugars present in the hydrolysate of biomass was performed using DNS method [8] and the estimation of xylose was done by modified Tollen's method [9].

2.4 Experimental design and optimization strategy

Design of experiments using response surface methodology (RSM) is a highly efficient, structured, pre-designed and reliable method for obtaining relationship between various parameters affecting a process (X_1) and response of the process (Y). Central composite rotatable design (CCRD) is among the principal experimental design technique used to analyse the interaction between the process parameters. Combined effects of various process parameters such as soaking temperature, soaking time, treatment time and agitation speed on reducing sugars and xylose yield after pretreatment with sodium hydroxide was estimated using Response surface Methodology (RSM). The analysis was done by Design Expert 9.0.3 trial software for optimization by Response Surface Methodology. The software was used to estimate the responses of the dependent variable, regression analysis, graphical analysis of the data obtained and to find out optimization efficiency.

The range of the independent parameters for the alkaline pretreatment by sodium hydroxide are mentioned in Table. 1.

Table: 1 Experimental range and levels of independent process variables for sodium hydroxide pretreatment.

Factors	Name	Unit	Low	High
A	Soaking Temperature	°C	10	90
B	Soaking Time	h	0	16
C	Treatment Time	min	-10	30
D	Agitation	rpm	70	190

The input variables are scaled to coded levels based on the following equation Eq. (1)

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i}, \qquad i=1,2,3....k \qquad (1)$$

where 'x_i' is a dimensionless parameter of the independent process variable, 'X_i' indicates the real value of the independent variable, 'X_{cp}' implies the real value of an independent variable at the centre point and 'ΔX_i', represents the step change in the real value of the variable 'i' upon an unit change in the dimensionless value of the variable 'i'.

A second order polynomial equation Eq. (2) is used to estimate the relationship between the independent and the experimental responses:

$$Y=\beta_o+\sum_{i=1}^{k}\beta_i X_i+\sum_{i}^{k}\beta_{ii}X_iX_i+\sum_{i=1}^{k-1}\sum_{j=i+1}^{k}\beta_{ij}X_iX_j \qquad \text{--- (2)}$$

Analysis of variance (ANOVA) was used to find the statistical significance of the ratio of mean square because of regression analysis and mean square due to the presence of residual error or any noise. Fisher F test and P test was used to evaluate the significance of process parameters for a given response. 3-D response surface graphs and contour plots were used to study and further confirm the effect of significantly chosen parameters.

2.5. Enzymatic saccharification

The pretreated samples with the best reducing sugars and xylose yield were subjected to saccharification using a combination of enzyme extracts cellulase and xylanase from *Trichoderma reesei* and *Trametes versicolor* respectively. Both these enzymes were obtained from Sigma Aldrich@. 103 U/g of cellulase and 650 U/g of xylanase was used with substrate loading of 4% in Mendel's media. It was then incubated at 50 °C, at (125-130) rpm for a period of 48 h. Sampling was done after 6 h interval and tested for the presence of reducing sugars and pentose sugars by using different analytical techniques to optimize the entire process with time. Estimation of reducing sugars present in the hydrolysate of biomass was performed using DNS method[8] and the estimation of pentose sugars (xylose) was done by modified Tollen's method[9]. Saccharification process period with the maximum reducing sugars and xylose yield was selected for fermentation.

2.6. Fermentation and ethanol production

2.6.1 Inoculum preparation

The fermentation process was carried out with *Pichia stipitis* (NCIM 3500) which was obtained from courtesy of National Collection of Industrial Microorganisms (NCIM). It was used with the aim of fermenting both reducing sugars and pentose sugars simultaneously. Inoculum preparation was done in a media having D-xylose 50 g/l, Glucose 5 g/l, Yeast extract 3 g/l, Malt extract 3 g/l, Peptone 5 g/l, with pH 5.0. The medium was autoclaved at 121 °C and 15 psi for 15 min. The media was inoculated with fresh culture from active plates and kept in a shaker incubator at 30 °C and at 125 rpm for a period of 20 h.

2.6.2 Fermentation

The broth after 20 h was centrifuged at 10000 rpm for 10 min. The pellet was collected and suspended in sterile distil water. The hydrolysate obtained after saccharification by filtering the biomass with muslin cloth and then whatmann filter paper 1. The filtered hydrolysate was supplemented with nutrients as per the standard protocol[10]. The medium was inoculated with 10% inoculum of *Pichia stipitis* at pH 5.0 and incubated at 30 °C and 150 rpm. Sampling was done at regular intervals of time then centrifuged for 15 min and tested for the presence of ethanol, reducing sugars and xylose. Ethanol estimation was done by potassium dichromate method using spectrophotometer[11]. The estimation of reducing sugar present in the hydrolysate of biomass performed using DNS method[8] and the estimation of xylose was done by modified Tollen's method[9].

3. Results and discussion

3.1 Comparative analysis of lignocelluloses content of different age of rice straw samples.

The lignin content was found to be maximum in fresh 6 months rice straw (17.2%) and was subsequently decreased over years and found to be minimum in 4 years old sample (11.35%). The decrease in lignin may be attributed to various environmental factors like temperature fluctuations, moisture content, climatic variations possibly acid rain etc, that ultimately paved the way for the growth of lignin degrading microorganisms like white rot fungus and soft rot fungus. Cellulose content was found to be varying from (32-33)%. The maximum cellulose was found to be present in 48 months old sample (33.23%). The hemicelluloses content was found to be around (24-25)%. It was maximum in 48 months sample (25.3%). The lignocellulose content of various age old rice straw samples has been shown Fig. 1. The values found are in accordance with previous estimations of chemical composition of rice straw, which shows that the straw predominantly contains cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%)[1].

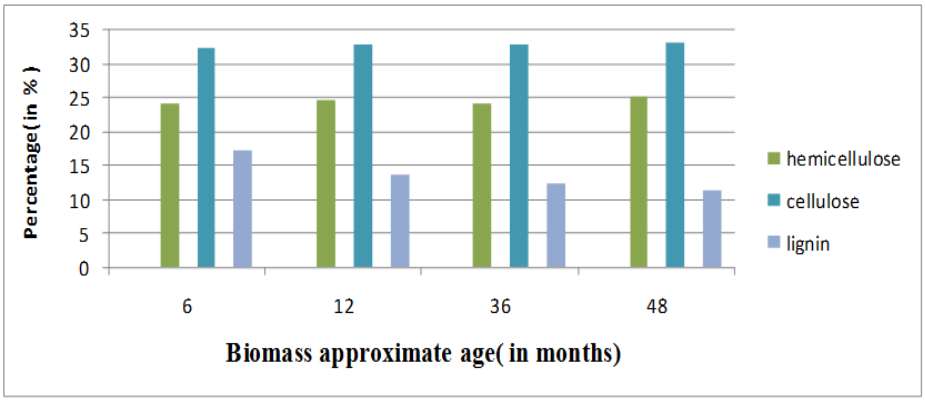

Fig: 1 Lignocellulose content of different rice straw samples

3.2 Pretreatment

Alkaline pretreatment was done using relatively mild reaction parameters like soaking temperature ranging from 30 °C- 90 °C, soaking time varying from 4 h to 16 h, and treatment time of 0 min, 20 min, and 30 min, keeping the sodium hydroxide concentration fixed at 5% (10% loading rate). An additional deciding parameter used was agitation varying from 70 rpm to 190 rpm. For the selected parameters, maximum reducing sugars yield (318.306 mg/g) was obtained with soaking time of 8 h, soaking temperature of 50 °C, treatment time of 10 min and agitation of 190 rpm. The xylose yield (pentose sugars) was 113.63 mg/g for the same set of conditions. Minimum reducing sugars yield (203.656 mg/g) was obtained with soaking time of 8 h, soaking temperature of 70°C, treatment time of 10 min and agitation of 130 rpm. Pretreatment with alkali changes the crystallinity, decreasing the degree of polymerisation and increases the porosity of straw by breaking the ester linkages between xylan hemicelluloses and other components like lignin. [2,3].

3.3 Experimental design and fitting of quadratic model

concentration of sodium hydroxide constant, other operating parameters like soaking temperature, treatment time, soaking time and agitation were the important parameters affecting the reducing sugars and xylose yield. The complete design matrix were produced using CCRD and the experimental setup for pretreatment with sodium hydroxide is shown in Table. 2.

From the experimental analysis it was found that keeping the

Table: 2 Experimental setup for NaOH pretreatment and reducing sugars and pentose sugars (xylose) yield after pretreatment.

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2
Run	A: Soaking time	B:Soaking temp	C:Agitation	D:Treatment Time	Xylose yield	Total Reducing Sugars
	h	oC	rpm	min.	mg/g	mg/g
1	4	30	100	0	106.095	244.036
2	12	30	100	0	80.1905	253.405
3	4	70	100	0	121.905	226.511
4	12	70	100	0	110.723	232.109
5	4	30	160	0	118.582	257.122
6	12	30	160	0	94.25	291.021
7	4	70	160	0	102.63	233.214
8	12	70	160	0	91.33	262.517
9	4	30	100	20	112.646	307.025
10	12	30	100	20	104.974	288.418
11	4	70	100	20	96.822	298.616
12	12	70	100	20	103.799	274.813
13	4	30	160	20	142.65	294.234
14	12	30	160	20	137.09	301.036
15	4	70	160	20	92.86	278.816
16	12	70	160	20	101.111	279.646
17	0	50	130	10	115.34	291.65
18	16	50	130	10	98.587	301.589
19	8	10	130	10	114.43	241.36
20	8	90	130	10	97.123	203.656
21	8	50	70	10	104.513	298.418
22	8	50	190	10	113.63	318.306
23	8	50	130	10	98.23	212.254
24	8	50	130	30	115.51	292.793
25	8	50	130	10	108.466	280.306
26	8	50	130	10	108.466	280.316
27	8	50	130	10	108.466	279.906
28	8	50	130	10	108.466	280.326
29	8	50	130	10	108.466	280.126
30	8	50	130	10	108.466	279.367

An empirical relationship for response of actual factors for total reducing sugars after sodium hydroxide pretreatment are shown in Eq. (3)

Total reducing sugars = +302.19038 – 7.63355 * soaking time + 3.52587 soaking temperature – 1.94266 * agitation + 7.08087 * treatment time - 0.015262 *soaking time * soaking temperature + 0.051186 * soaking time*treatment time – 2.95680E – 003 * soaking temperature + 0.010128 * soaking temperature * treatment time - 0.021448 * agitation * treatment time + 0.26039 * soaking time^2 - 0.0359041 * soaking temperature^2 + 7.89104E – 003 * agitation^2 - 0.068577 * treatment time^2 --------- (3)

An empirical relationship for response of actual factors for xylose yield after sodium hydroxide pretreatment are shown in Eq. (4)

Xylose yield = + 47.70214 – 4.43165 * soaking time + 1.80152 * soaking temperature + 0.56984 * agitation – 0.23588 * treatment time + 0.43918* soaking time * soaking temperature +2.52105E-003*soaking time * agitation + 0.11674 * treatment time – 0.013957 * soaking temperature * agitation – 0.040699 * treatment time + 0.014082 * agitation * treatment time – 0.021569 * soaking time^2 – 1.60463E-003* soaking temperature^2 + 2.02111E-004 * agitation^2 – 3.68475E – 003 * treatment time^2 -------(4)

3.4 ANOVA study

3.4.1 ANOVA study for total reducing sugars and pentose sugars (Xylose) yield after sodium hydroxide pretreatment

Statistical analysis obtained from second order model and the analysis of variance (ANOVA) from response surface model for total reducing sugars yield is shown in Table. 3. The significance of each coefficient was determined by F-values and P-values. The coefficients for the effects of soaking temperature, soaking time, treatment time and agitation and square effects of these variables were highly significant ($P < 0.0001$) in all the cases. The model F-value of 8660.64 for total reducing sugars indicates that the regression model is highly significant. The goodness of fit of all the models were checked by the multiple regression coefficients (R^2). For total reducing sugars yield from sodium hydroxide pretreatment, value of predicted regression coefficient ($R^2 = 0.9994$) was in reasonable agreement with actual R^2 which was 0.9998 with the difference of less than 0.2 indicating that the signal was adequate. The coefficient of variation (%CV) is a measure of deviation from mean. Lower the

value of CV, higher is the reliability of experiment and better is the precision. The %CV was 0.17 for total reducing sugars. The non significant value of lack of fit, showed that the quadratic model was valid. The predicted residual sum of squares (PRESS) was 14.17 and standard deviation was 0.45 in case of total reducing sugars yield. Similarly, for pentose sugars (xylose) yield the predicted regression coefficient (R^2 = 0.9951) was in reasonable agreement with actual R^2 which was 0.9984. The coefficient of variation (%CV) for xylose yield was 0.17. For xylose yield PRESS was 22.41 and standard deviation was 0.51. The Fig. 2 also clearly shows that model predicted results are very close in agreement with the experimental ones showing the fitness of the model to be excellently accurate. The Fig. 3 gives clear evidence that model predicted results are very close in agreement with the experimental ones.

Table: 3 Analysis of variance (ANOVA) table for total reducing sugars yield after sodium hydroxide pretreatment.

ANOVA for Response Surface Quadratic model						
Analysis of variance table for reducing sugars after NaOH pretreatment						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	24723.13	14	1765.94	8660.64	< 0.0001	significant
A-Soaking time	166.79	1	166.79	818.00	< 0.0001	
B-Soaking temp	2118.08	1	2118.08	10387.63	< 0.0001	
C-Agitation	526.87	1	526.87	2583.91	< 0.0001	
D-Treatment Time	9750.49	1	9750.49	47819.08	< 0.0001	
AB	23.85	1	23.85	116.98	< 0.0001	
AC	603.65	1	603.65	2960.47	< 0.0001	
AD	797.30	1	797.30	3910.20	< 0.0001	
BC	50.36	1	50.36	246.97	< 0.0001	
BD	65.65	1	65.65	321.97	< 0.0001	
CD	662.45	1	662.45	3248.85	< 0.0001	
A^2	476.11	1	476.11	2334.97	< 0.0001	
B^2	5657.26	1	5657.26	27744.77	< 0.0001	
C^2	1383.43	1	1383.43	6784.72	< 0.0001	
D^2	1289.91	1	1289.91	6326.06	< 0.0001	
Residual	3.06	15	0.20			
Lack of Fit	2.35	10	0.24	1.67	0.2979	not significant
Pure Error	0.71	5	0.14			
Total	24726.19	29				

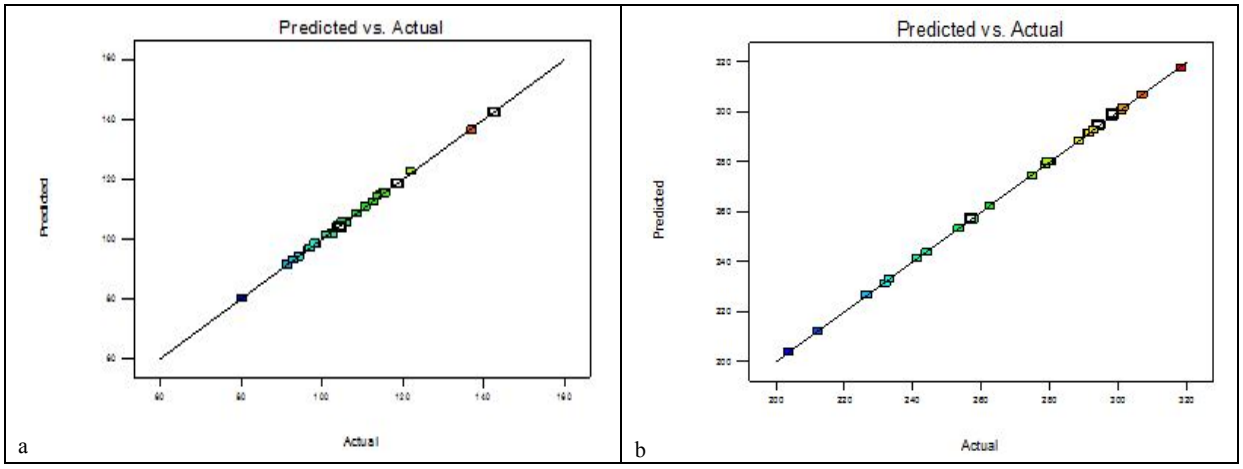

Fig: 2 Predicted vs. Actual graph for reducing sugars yield (a) and xylose yield (b) after sodium hydroxide pretreatment.

3.3.2 Contour plots

Contour plots as shown in Fig. 3 for total reducing sugars yields after sodium hydroxide pretreatment are used to determine the interaction of optimum level of each component with other components for maximum response. These plots shows probable interaction between pair wise combination of independent factors keeping other factors at centre point. Elliptical contour plots showed that mutual interactions were quite prominent between various operating variables. The maximum reducing sugars yield for sodium hydroxide pretreatment was 318.306mg/g. Regression equation was used to calculate maximal predictable response.

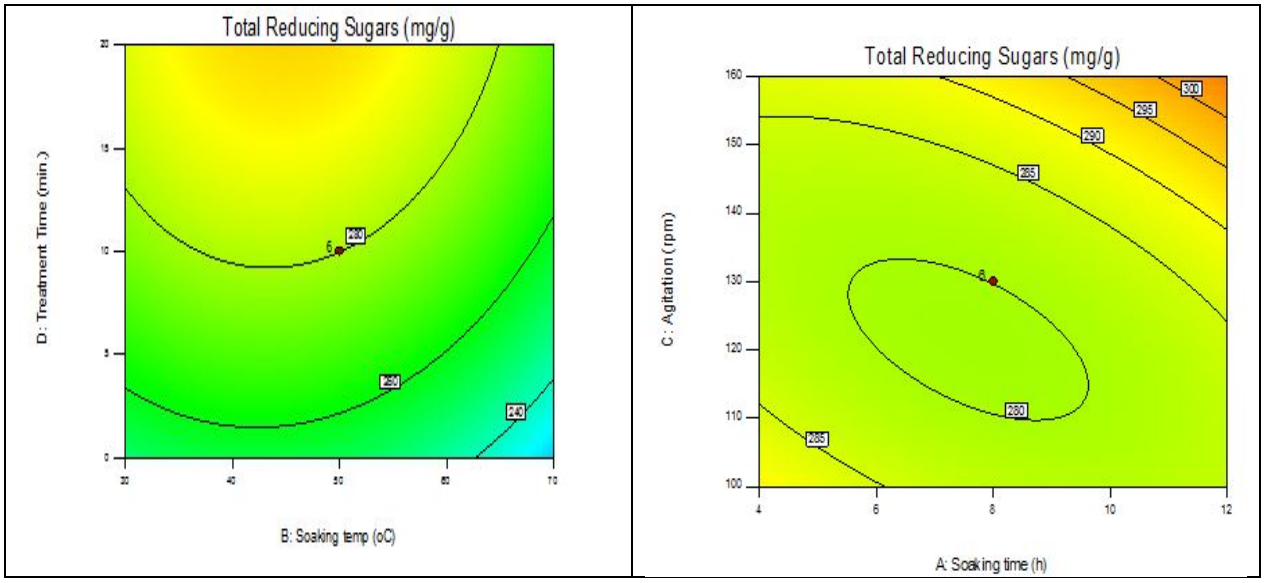

Fig. 3 Elliptical contour plots for sodium hydroxide pretreatment.

3.4. Enzymatic saccharification

The best pretreated waste rice straw samples from sodium hydroxide were used as substrate for saccharification by commercial enzymes. The synergistic action of two different commercial enzymes cellulase and xylanase were used to enhance the yield of reducing sugars and pentose sugars (mainly xylose). The saccharification was performed over a period of 48 h, in order to study the effect of enzymes with time and also to optimize the time period showing the best xylose and reducing sugar yield. The reducing sugars and xylose yield increased with increase in treatment time until 36 h and then showed a decrease in trend due to the formation of inhibitory by products (Fig. 4). Maximum reducing sugars yield was 348.8 mg/g and xylose yield was 155.84 mg/g, after 36 h of pre-treatment. The saccharification percentage after pretreatment was found to be 77.59%, which in accordance with other studies [12]. As a result of alkaline treatment where least amount of saccharide portions are solubilised and synergistic enzyme treatment results in better yields.

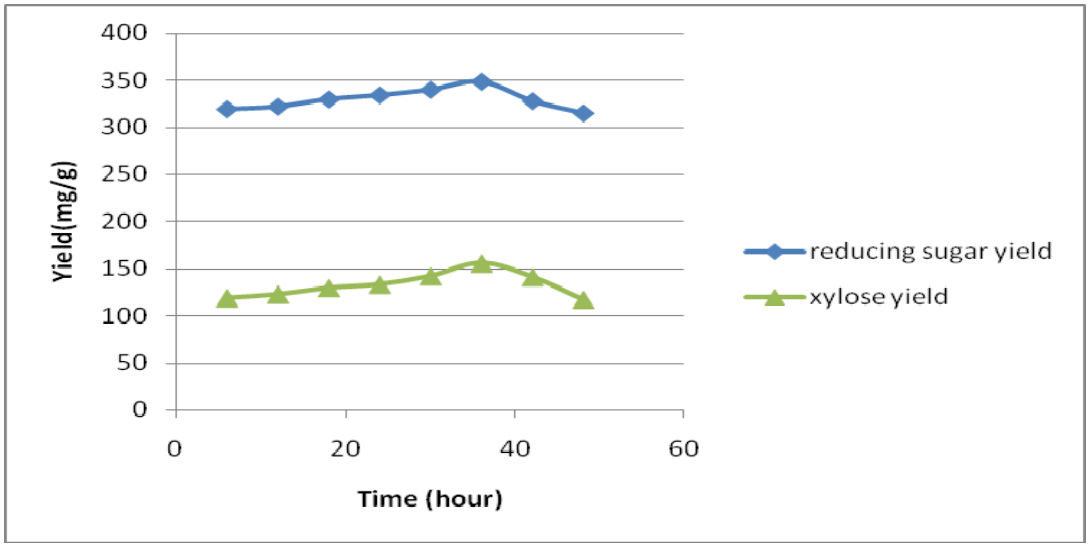

Fig: 4 Comparative analysis of reducing sugar and pentose sugars (xylose) yields of sodium hydroxide pretreated waste rice straw samples with time after saccharification.

3.5. Fermentation

The saccharified samples with the best glucose and xylose yield, after 36 h were subjected to fermentation with *Pichia stipitis* for a period of 12 h and ethanol yield was monitored along with the yield of reducing sugars and xylose. For sodium hydroxide, the ethanol yield showed a relatively increasing trend and maximum ethanol yield was 20.123 g/l after 9 h which then decreased to 19.660 g/l after 12 h of fermentation as shown in Fig. 5. The results obtained are in accordance with the ethanol yield of 21.1 g/l as obtained from rice straw in other studies[13].

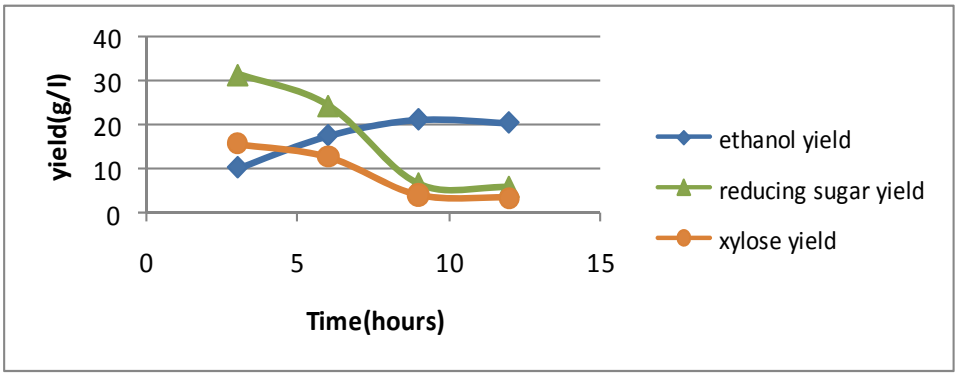

Fig: 5 Comparative analysis of yields of ethanol, reducing sugars and pentose sugars (xylose) with time for sodium hydroxide pretreated samples after fermentation.

4. Conclusion

The present study shows a subsequent decrease in lignin content with ageing of rice straw and with increase in time period of exposure to fluctuations in environmental parameters (like heat, moisture) that ultimately results in growth of various lignin degrading fungus. With the growing need to address the food versus feed waste rice straw was used as the substrate for bioethanol production. Further, in order to make the process more feasible and cost efficient pretreatment was done with sodium hydroxide and various process parameters affecting the reducing sugars and xylose yield was optimized. Since, the pretreated samples along with reducing sugars showed appreciable amount of pentose sugars, saccharification was taken forward with a combined effect of cellulase and xylanase and subsequently fermentation was carried out with *Pichia stipitis* and subsequently high ethanol yield was reported as the strain could utilize both hexose sugars and pentose sugars together.

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