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Comparative Study of Using Sodium Chlorite and Tartaric Acid Pretreatment on Waste Rice Straw for Enhanced Production of Bioethanol

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Abstract: *This is comparative study of developing bioethanol from age old waste rice straw (WRS) collected from thatched roofs pretreated with tartaric acid and sodium chlorite separately. WRS of different ages, collected from nearby village, they were dried, grinded and screened. Proximate analysis was conducted by (Neutral detergent fibre) NDF and (Acid detergent fibre) ADF methods for estimating the hemicellulose, cellulose and lignin content of variable age (not more than 6, 12, 24, 36 and 48 months), with least content 11.35% in 48 months age old sample. Pre-treatment was conducted on least lignin content biomass sample by using different concentrations of tartaric acid and sodium chlorite under variable physical parameters such as soaking temperature, soaking time and agitation speed which were optimized by response surface methodology design tool, followed by moist heat treatment. The sample resulting best lignin degradation estimated by total phenol content was subjected to saccharification by using both xylanase and cellulase. The time yielding maximum hexose and pentose was 36th hour resulting in 494.8 mg/g and 274.9 mg/g glucose and 134.8 mg/g and 124 mg/g xylose for tartaric acid and sodium chlorite pretreated samples respectively. After saccharification the hydrolysate was collected and set for fermentation by Pichia stipitis. Ethanol yield was obtained to be 20.226% and 25.467%.*

Keywords: *Response surface methodology, Sodium chlorite, Tartaric acid, Thatched, Waste rice straw (WRS)*

I. Introduction

Rice straw, commonly known as paddy straw is available widespread in and around India. It is the vegetative part of Rice plant (Oryza sativa) which is cut during harvesting. The huge amounts of these straws are burnt and are left in the fields till the next ploughing as Rice straw has its important role as soil improver. Fresh rice straw is also used as a feed for livestock .It is also used as the interim material for idol structuring and thatch making. So after few years these rice straws would add on to the bulk of waste and have absolutely no role otherwise. They are either being thrown here and there or are being unwisely burnt off increasing the load on greenhouse gases. [1,2]

So, an alternative reuse of rice straw from thatches are to be introduced which can be of utility to mankind. Rice straw is an attractive lignocellulosic biomass for the production of bioethanol since it can be one of the most abundant renewable resources available. It has several characteristics, such as high cellulose and hemicelluloses content that can be readily hydrolyzed into fermentable sugars.

The choice of pretreatment methods plays an important role to increase the efficiency of enzymatic saccharification thereby making the whole process economically viable Nature and its resources has resulted in the scientific attraction in using biological renewable resources as a replacement to the use of non-renewable resources which are rapidly depleting such as fossil fuels. Lignocellulosic biomass can be used as an alternative to the automobile fuels due to its availability, abundance and relatively low cost. Lignocellulosic biomass from old thatched roofs are considered to be effective as lignin content is very less compared to that of fresh rice straw, on addition that fresh rice straw adds to the livestock and plays an important role in the food chain, but waste rice straw on the other hand can be utilized for fermentation process for production of bioethanol as renewable resource that stores energy from sunlight in its chemical bonds. It has been reported that ethanol produced from lignocellulosic biomass resources has the potential to cut greenhouse gas emissions by 86% as it is burnt more completely as compared to petrol. Ethanol has a much higher latent heat of vaporization (855 MJ/kg) than petrol (293 MJ/kg) as well as a higher octane number (99) than petrol (80–100) as a result, pre-ignition does not occur when ethanol is used. Moreover, it has no participation in the global warming crisis because the carbon dioxide produced in the combustion process of ethanol is utilized by the plants for their growth adding up to the carbon cycle balance in the nature.[2,3,4]

Successive yield of cellulose and hemicellulose abundance was observed on physiochemical pre-treatment of rice straw. Both acid hydrolysis and alkaline hydrolysis were conducted on rice straw for progressive lignin degradation. On acid hydrolysis, lignin degradation was a success so as with alkali pretreatment, but not a cost effective method for degrading lignin. Hence, the concept of using tartaric acid fulfills the purpose of cost effectiveness. Tartaric acid was used on pre-treating wheat straw for lignin degradation. Tartaric acid is found in remnants of tamarind, as we know abundance of both rice straw and tamarind is drastic in southern parts of India, rural people can easily harvest tamarind and use it effectively for acid hydrolysis of rice straw, which is definitely cost effective, and resulting in effective lignin degradation.

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 analyzing the amount of lignin 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].

For the purpose of producing ligninase, ligninolytic fungi will have a higher activity when incubation condition using submerged fermentation technique or Submerged fermentation (SMF) compared to solid fermentation or Solid State Fermentation (SSF).

II. Materials and methodology

2.1 Biomass estimation

Rice straw samples from thatched roofs were collected from settlings over a long period of time (6 months, 1 year, 3 years, 4 years approximately) from nearby village from reliable sources. These samples were sun dried for a week and further moisture content were removed in a tray dryer for period 4 hours for consecutive days at 60° C. These dried samples were finely grinded to powder and stored in air tight containers. These powdered samples were used for experimental drives. The Hemicellulose and lignin content of the dried and powdered biomass was estimated by NDF (Neutral Detergent Fibre) and ADF (Acid Detergent Fiber) method. After estimating the lignin and hemicellulose content, theoretical value of cellulose and minerals were obtained. To verify the actual amount of cellulose content present in waste rice straw of a particular age, anthrone test was conducted as confirmatory quantitative test.[7]

2.2 Pretreatment

Finely powdered dried rice straw biomass were mixed with 10 mL of variable concentrations of tartaric acid and sodium chlorite incubated for different incubation time, different agitation speed and at variable soaking temperatures, these variable parameters were designed using a tool Design Expert which is based on RSM (Response Surface Methodology) algorithm. After incubation, these samples were subjected to moist heat (autoclaved at 15 psi, 121°C, for 5 min. The series of treated samples were then filtered using Whatmann's filter paper No. 1. Hydrolysate or filtrate was collected and concentration of lignin was determined by estimating total phenol content by Folin-Cialcateau method and concentrations of reducing sugars both hexose and pentose sugars were analyzed by DNSA and Phloroglucinol assay. [9]

2.4 Experimental design

structured, pre-designed and reliable method for obtaining relationship between various parameters affecting a process (X_i) 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)

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

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:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i}^{k} \beta_{ii} X_i X_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \tag{2}$$

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.

2.3 Saccharification

The pretreated samples with maximum phenol yield were subjected to enzymatic 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. The presence of reducing sugars and pentoses as well as phenol content using different analytical techniques to optimize the time taken to reach saturation point. Estimation of reducing sugar present in the hydrolysate of biomass performed using DNS method, and the estimation of xylose was conducted by modified Tollen's method. The saccharification process with the maximum reducing sugars and xylose yield was selected for further bioprocess. [8,9]

2.4 Fermentation

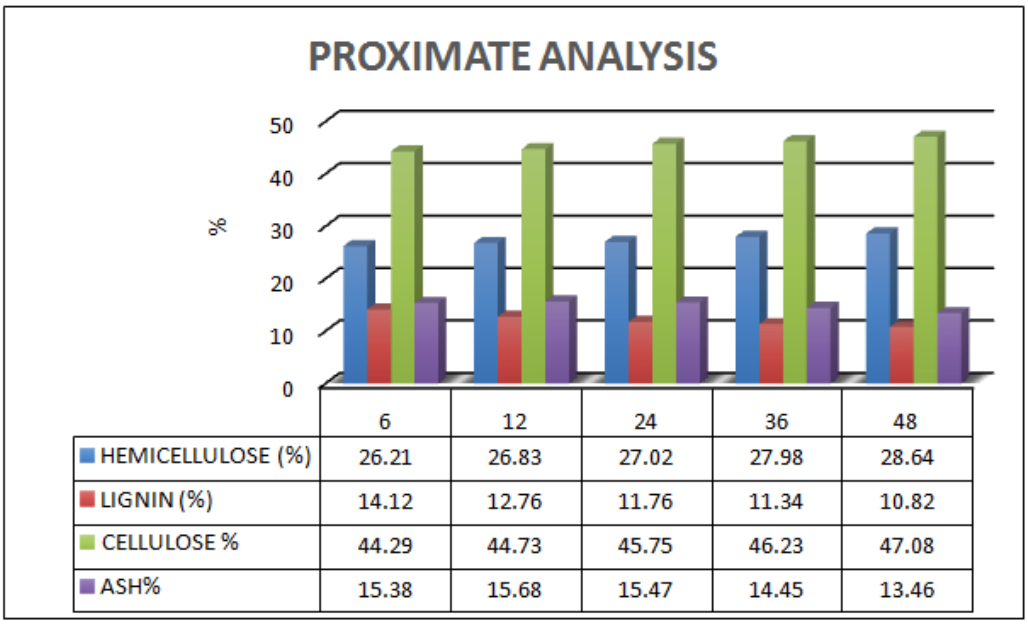
The microbial species Pichia stipitis (NCIM 3500) was used for fermentation purpose as it has the ability to utilize both xylose and glucose. These microbial samples were obtained by the courtesy of National Collection of Industrial Microorganisms. The inoculated flask was incubated at 30^{o}C on a rotating shaker at 125 rpm inside a BOD incubator. After 20 h of incubation, broth was collected in centrifuge tubes and each centrifuge tubes were centrifuged at 10000 rpm for 10 mins. After centrifugation, the cell pellet formed at the bottom of the tube were aseptically washed and suspended in sterile distilled water under pre-sterilized LAF hood or a biosafety cabinet. For fermentation media **(**Ammonium chloride; 0.5 g/l, Potassium dihydrogen phosphate; 2.0 g/l , Magnesium sulphateheptahydrate;0.5 g/l, Yeast extract; 1.5 g/l, Calcium chloride dihydrate;0.1 g/l, Ferric chloride dihydrate;0.1 g/l, Zinc sulphateheptahydrate 0.001 g/l) was prepared and autoclaved at 121.1 ^{o}C, 1.5 psi for 20 min. The hydrolysate obtained after saccharification by filtering the biomass with muslin cloth and then Whatmann's filter paper 1 and it was supplemented into the media before sterilization. The medium was inoculated with 10% inoculum of Pischia stipitis at pH 5.0 after sterilization under LAF or aseptic condition and incubated at 30 °C and 150 rpm. Sampling was done at regular intervals of time then centrifuged with 15 minutes and tested for the presence of ethanol, reducing sugars and xylose. Ethanol estimation was done by potassium dichromate method with the assistance of spectrophotometer. The Estimation of reducing sugar present in the hydrolysate of biomass performed using DNS method, and the estimation of xylose was done by modified tollen's method. [9,11]

III. Results and discussion

3.1 Proximate analysis

On conducting the NDF and ADF method the hemicellulose content and lignin content was estimated for varying sample ages collected from old thatched roofs. Fig. 1 shows the hemicellulose content, Lignin

content and cellulose content. It was observed clearly that with increase in age of the samples, its lignin content decreases thereby exposing hemicellulose and cellulose, increasing their availability. The possibility of naturally degrading lignin is maybe due to physiochemical or biochemical process or by both. The sample with least lignin content was chosen for further bioprocessing. The lignin content was found to be 10.82% on 48 months age old rice straw sample which was least among all. [1]



	6	12	24	36	48
HEMICELLULOSE (%)	26.21	26.83	27.02	27.98	28.64
LIGNIN (%)	14.12	12.76	11.76	11.34	10.82
CELLULOSE %	44.29	44.73	45.75	46.23	47.08
ASH%	15.38	15.68	15.47	14.45	13.46

Figure 1: Represents the variation in rice straw components with age of biomass.

3.2 Saccharification

The samples pretreated with tartaric acid and sodium chlorite were subjected to saccharification process, where the cellulose and hemicellulose content were enzymatically de-polymerized into their monosaccharides, glucose, pentose and other reducing sugars with the involvement of cellulase (EC 3.2.1.4) and xylanase (EC 3.2.1.8), majorly in order to break down cellulose and hemicellulose polysaccharides into is monomer glucose and xylose. Xylose and glucose yield was optimized with time in hours (Table no. 5) and compared with each pre-treated samples.[12]

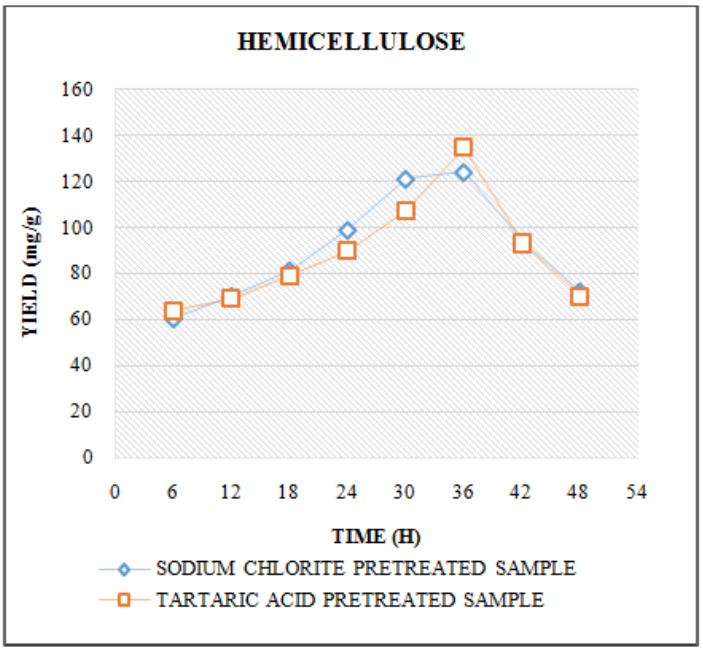


Figure 2: Comparative saccharification of hemicellulose by xylanase for sodium chlorite and tartaric acid pre-treated sample.

Table 2: Comparative yields of pentose sugars after saccharification.

Time (H)	Sodium Chlorite Pretreated Sample	Tartaric Acid Pretreated Sample
6	60.55	63.8
12	70.313	69.01
18	81.4	78.8
24	98.96	89.84
30	121.1	107.5
36	124	134.8
42	93.8	93.1
48	72.3	70.3

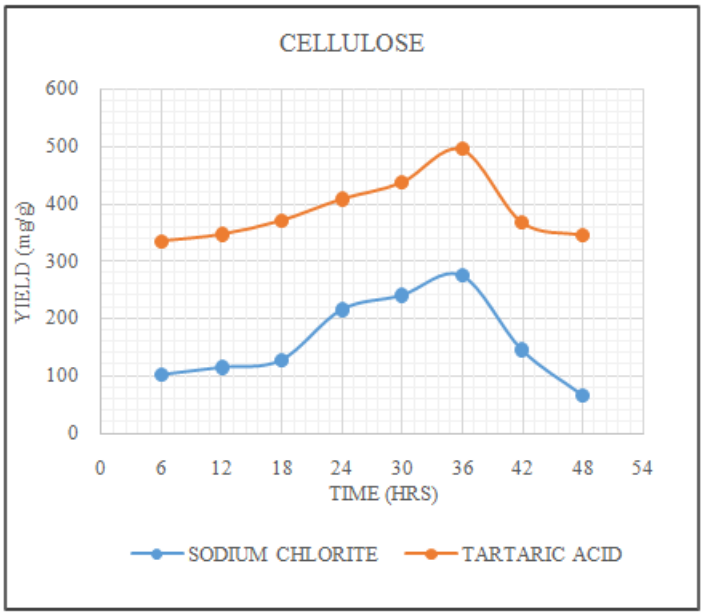


Figure 3: Comparative saccharification of cellulose content of sodium chlorite and tartaric acid pretreated sample by cellulose

Table 3: Comparative yield of hexose sugars after saccharification

TIME (h)	Sodium Chlorite Pretreated Sample	Tartaric Acid Pretreated Sample
6	102.7	335.3
12	115.37	347
18	128	371
24	215.4	408.3
30	240.6	437.1
36	274.9	494.8
42	146	368
48	67.6	345

By estimating the yield of total reducing sugars and pentose sugars on various sampling taken on variable time period while incubation lead to an optimized condition (Table no. 4) resulting maximum yield (Figure no. 2) which was at 36th hour resulting in 494.8 mg/g glucose and 134.8 mg/g xylose for tartaric acid pretreated sample. [12]

3.3 Fermentation

Fermentation was conducted for 12 hours, and based on graphical observations a relation can be drawn that on gradual decrease of sugar contents there is an increase in ethanol production. By analyzing the graphical representation of yield% versus time in hours, it is evident that Pichia stipitis is utilizing both pentose and hexose for fermentation, as there is a peak increase in ethanol yield with an abyssal drop of sugar content. Fermentation was set for 12 hours, and the maximum yield of ethanol 20.226%, was obtained on 9th hour of fermentation sampling with an abyss drop of sugar content 14.5% and 12.9% hexose and pentose respectively (Table no. 2). It is evident that Pischia stipitis utilizes both pentose and hexose sugar for fermentation, as there is

gradual decrease of pentose sugar and hexose sugar and simultaneous increment in ethanol yield (Figure no. 3).. The results obtained are in accordance with the ethanol yield of 21.1 g/l as obtained from rice straw in other studies.[13]

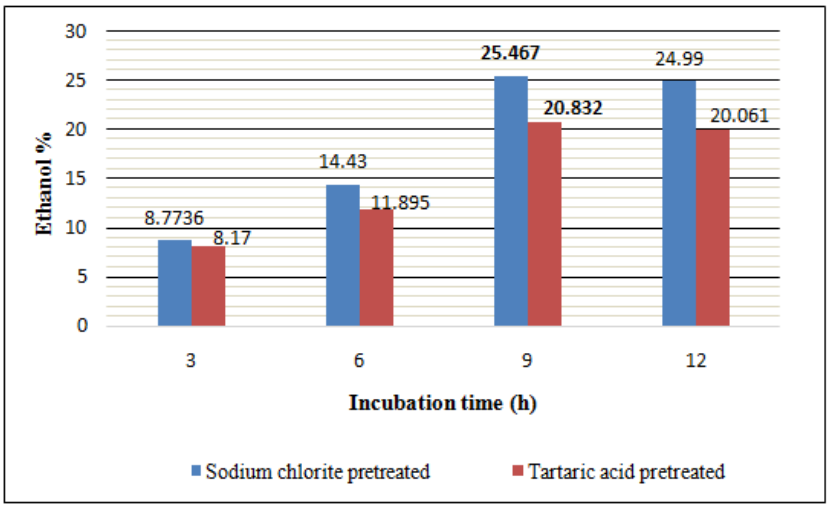


Figure 4: Comparative ethanol % obtained for sodium chlorite and tartaric acid pre-treated rice straw biomass.

3.4 ANOVA Experimental design

The Model F-value of 561.76 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case B, D, AB, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "Lack of Fit F-value" of 51911502.43 implies the Lack of Fit is significant. There is only a 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise. Significant lack of fit is considered to be bad condition, which should be analyzed and made to fit. Standard deviation was obtained to be 6.63 with R-Squared value 0.9981 and a mean of 356.89 adjusted R-Squared value was 0.9963, a C.V. of 1.86%, Predicted R-Squared 0.9890, Adequate Precision was obtained to be 79.549. The "Pred R-Squared" of 0.9890 is in reasonable agreement with the "Adj R-Squared" of 0.9963; i.e. the difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 79.549 indicates an adequate signal. This model can be used to navigate the design space.

Table 4: Experimental range and levels of independent process variables for sodium hydroxide pretreatment.

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob> F	
Model	3.453E+005	14	24663.68	561.76	<0.0001	Significant
A-SOAKING TEMP	6.483E-003	1	6.483E-003	1.477E-004	0.9905	
B-SOAKING TIME	17602.83	1	17602.83	400.94	<0.0001	
C-AGITATION SPEED	113.22	1	113.22	2.58	0.1291	
D-CONC. OF SODIUM CHLORITE	7886.20	1	7886.20	179.62	<0.0001	
AB	38704.31	1	38704.31	881.57	<0.0001	
AC	44.67	1	44.67	1.02	0.3291	
AD	6717.83	1	6717.83	153.01	<0.0001	
BC	9589.27	1	9589.27	218.41	<0.0001	
BD	4568.02	1	4568.02	104.05	<0.0001	
CD	2807.15	1	2807.15	63.94	<0.0001	
A^2	3372.85	1	3372.85	76.82	<0.0001	
B^2	19205.23	1	19205.23	437.44	<0.0001	
C^2	92463.02	1	92463.02	2106.02	<0.0001	
D^2	1.941E+005	1	1.941E+005	4420.17	<0.0001	
Residual	658.56	15	43.90			
Lack of Fit	658.56	10	0.76			not significant
Pure Error	6.343E-006	5	1.269E-006			
Core Total	3.460E+005	29				

The Model F-value of 88299.72 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Pred R-Squared" of 0.9999 is in reasonable agreement with the "Adj R-Squared" of 1.0000; i.e. the difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 888.435 indicates an adequate signal. This model can be used to navigate the design space. The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space. The surface plots were analyzed to determine the interaction between process parameters on the total phenolic content for the tartaric acid hydrolysis of pretreated waste rice straw biomass. The Response Surface plots shown in Figure nos. 4 (a), (b) and (c) which indicates the mutual interaction between the process parameters are prominent.

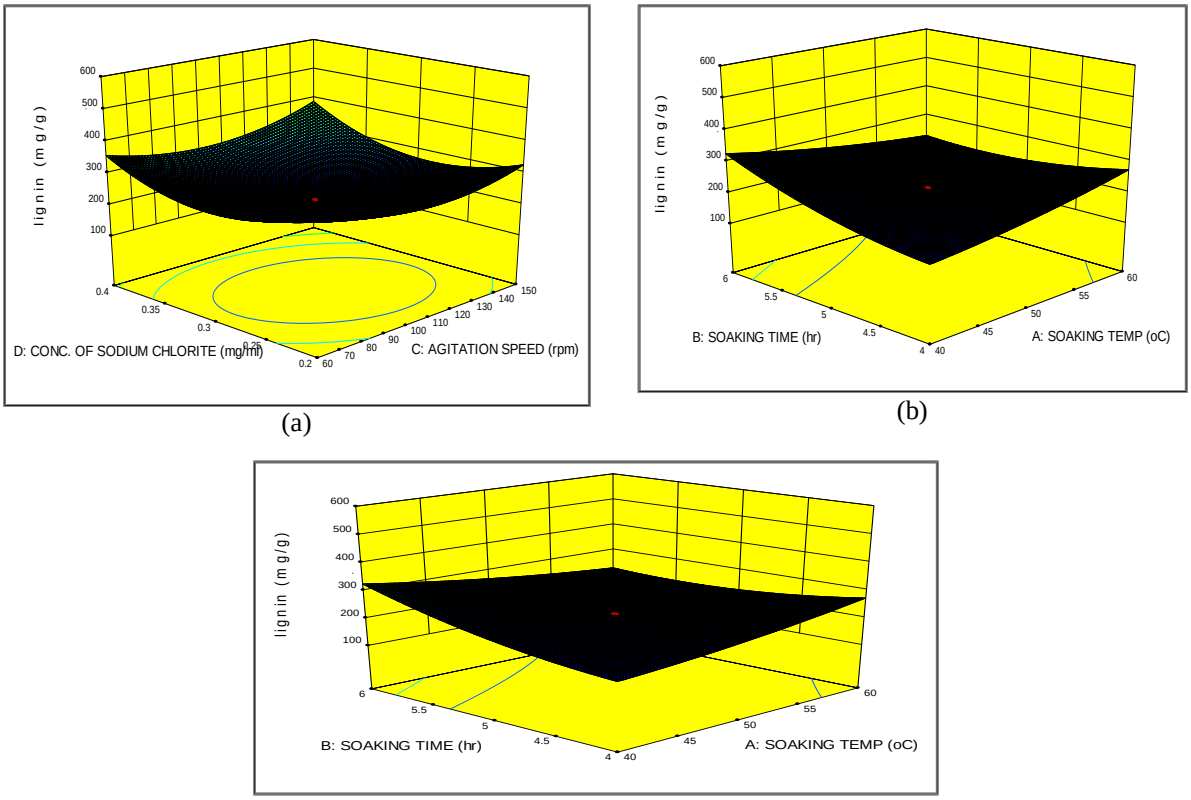
Table 5: The data set analyzed by ANOVA (Analysis of variance) for response 1 lignin of tartaric acid pretreated sample.

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob> F**	
Model	4.113E+005	14	29378.86	88299.72	<0.0001	significant
A-SOAKING TEMP	26582.43	1	26582.43	79894.89	<0.0001	
B-SOAKING TIME	4408.43	1	4408.43	13249.76	<0.0001	
C-AGITATION SPEED	7350.44	1	7350.44	22092.14	<0.0001	
D-Conc OF Tartaric acid	18.17	1	18.17	54.62	<0.0001	
AB	10139.11	1	10139.11	30473.63	<0.0001	
AC	1.305E+005	1	1.305E+005	3.922E+005	<0.0001	
AD	999.44	1	999.44	3003.86	<0.0001	
BC	7291.26	1	7291.26	21914.25	<0.0001	
BD	666.58	1	666.58	2003.43	<0.0001	
CD	550.75	1	550.75	1655.32	<0.0001	
A^2	30150.96	1	30150.96	90620.29	<0.0001	
B^2	52979.88	1	52979.88	1.592E+005	<0.0001	
C^2	1.837E+005	1	1.837E+005	5.522E+005	<0.0001	
D^2	16389.15	1	16389.15	49258.45	<0.0001	
Residual	4.99	15	0.33			
Lack of Fit	4.99	10	0.50			not significant
Pure Error	0.000	5	0.000			
Cor Total	4.113E+005	29				

The equation in terms of actual factors for Sodium chlorite and tartaric acid pre-treated samples (Equation 3 and 4) can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space. [5]

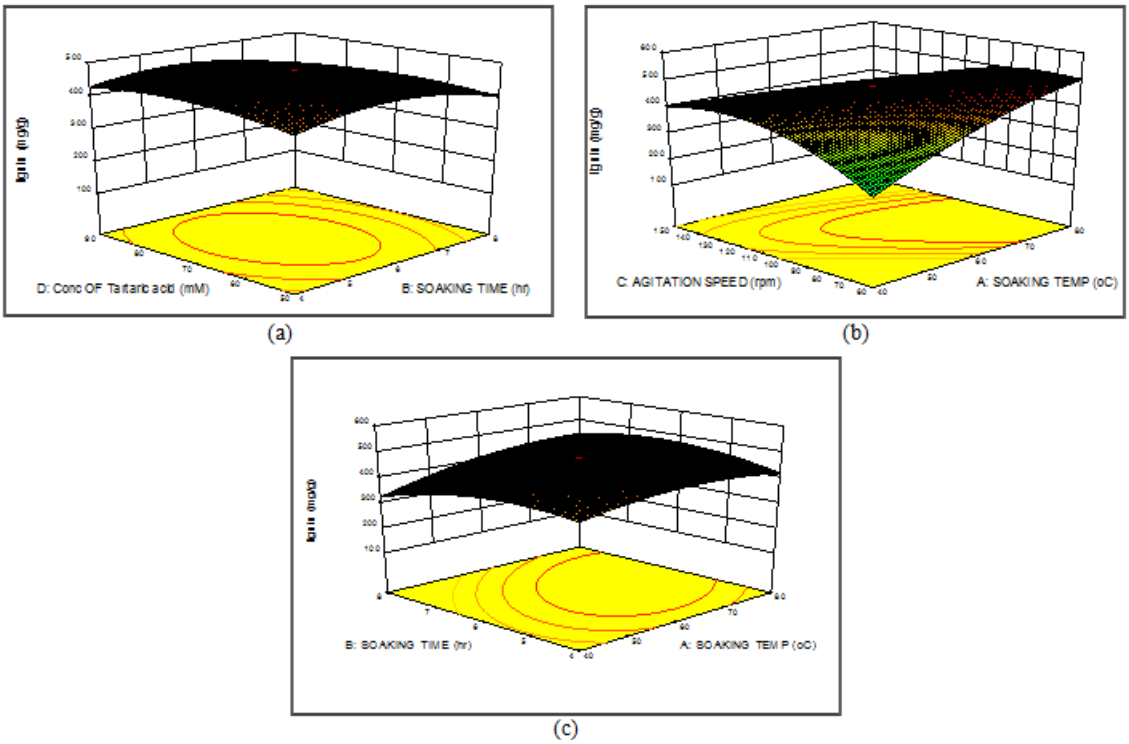
Lignin=+1105.56701+20.03806* SOAKING TEMP-99.42441* SOAKING TIME-9.39037* AGITATION SPEED-4994.96573* CONC. OF SODIUM CHLORITE-4.91835* SOAKING TEMP * SOAKING TIME-3.71312E-003* SOAKING TEMP * AGITATION SPEED-20.49060* SOAKING TEMP * CONC. OF SODIUM CHLORITE+0.54403* SOAKING TIME * AGITATION SPEED+168.96791* SOAKING TIME * CONC. OF SODIUM CHLORITE+2.94348* AGITATION SPEED * CONC. OF SODIUM CHLORITE+0.11089* SOAKING TEMP^2+26.46112* SOAKING TIME^2+0.028672* AGITATION SPEED^2+8411.43667* CONC. OF SODIUM CHLORITE^2 …Eq 3

Lignin = -1573.10912 +16.98686 * SOAKING TEMP +123.51256 * SOAKING TIME +15.99809 * AGITATION SPEED +9.06614 * Conc OF Tartaric acid +0.62933 * SOAKING TEMP * SOAKING TIME -0.10034 * SOAKING TEMP * AGITATION SPEED +0.019759 * SOAKING TEMP * Conc OF Tartaric acid -0.23719* SOAKING TIME * AGITATION SPEED -0.16136 * SOAKING TIME * Conc OF Tartaric acid -6.51893E-003 * AGITATION SPEED * Conc OF Tartaric acid -0.082887 * SOAKING TEMP^2 -10.98737 * SOAKING TIME^2 -0.040416 * AGITATION SPEED^2 -0.061111 * Conc OF Tartaric acid^2 …Eq 4



(a) (b) (c)

Figure 5: RSM plots sodium chlorite: projecting the co-relation between variable parameters and lignin. (a) Conc. of sodium chlorite with agitation speed; (b) Soaking time with soaking temperature; and (c) Soaking time with soaking temperature.



(a) (b) (c)

Figure 6: RSM plots tartaric acid: projecting the co-relation between variable parameters and lignin. (a) Conc. of tartaric acid with soaking time; (b) Agitation speed with soaking temperature; and (c) Soaking time with soaking temperature.

IV. Conclusion

It can be concluded that on an age old waste rice straw there was less lignin barrier compared to fresh rice straw. The tartaric acid pretreatment resulted a better yield of ethanol compared to that of sodium chlorite pretreatment. But on saccharification tartaric acid pre-treated sample resulted a better yield of xylose and glucose compared to that of sodium chlorite pre-treated sample. Sodium chlorite can be considered as a better solution than for delignification than tartaric acid. Lignin was removed effectively, as observed by estimating the total phenol content. Optimization of parameters and yields for lignin were designed by Box-Behnken design, this method also analyzed the relations between the parameters and yields. The experiments thus conducted projects a clear image that for effective delignification sodium chlorite pre-treatment can be implied resulting in a better ethanol yield using Pichiastipitis.

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