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Performance study of combined microwave and acid pretreatment method for enhancing enzymatic digestibility of rice straw for bioethanol production

Renu Singh[*1], SapnaTiwari[1], Monika Srivastava[1], Ashish Shukla[2]

[1]Centre for Environment Science and Climate Resilient Agriculture, Indian Agricultural Research Institute, New Delhi, 110012, India;
[2]Civil Engineering, Architecture and Building (CAB), Faculty of Engineering and Computing, Coventry University, Priory Street, Coventry CV1 5FB, UK

[*]Corresponding author: renu_icar@yahoo.com

Abstract

Utilization of fossil fuel resources at large scale led to global warming alerts and threats. Bio-ethanol as an alternative fuel source can provide combat against these threats. Conversion of rice straw to fuel ethanol involves pretreatments followed by enzyme-catalyzed hydrolysis. In the present study, microwave-assisted H_2SO_4 is employed for pretreatment in order to enhance enzymatic digestibility of rice straw. Prepared samples of rice straw have first gone under microwave treatment followed by chemical treatment using H_2SO_4. Chemical concentration of 0.1-2.0 % for the treatment time of 1 to 5 minutes have been used utilizing microwave power range of 70-700 W. Under optimum conditions e.g. reaction time, power, concentration of chemical, maximum reducing sugar obtained through microwave assisted H_2SO_4 is 1376.99 µg/ml. The XRD analyses showed that the crystallinity index of microwave assisted H_2SO_4 rice straw samples is significantly high (61.36 %) as compared to untreated sample (52.2%). The morphological study performed using scanning electron microscope (SEM) certified that the surface of the samples treated with microwave assisted H_2SO_4 was more ruptured. Also the silicon waxy structure, ether linkages between lignin and carbohydrates were either damaged or broken down. Study on Indian rice straw proved that microwave assisted H_2SO_4 pretreatment can successfully remove lignin and increase the enzymatic accessibility.

Keywords: Enzymatic hydrolysis; Rice straw; Biofuel; Microwave; lignin.
Abbreviations: µg/ml_Microgram per mililitre; SEM_Scanning electron microscope; XRD_X-ray Diffraction; RSM_Response surface methodology; H_2SO_4_ Sulphuric acid.

Introduction

In the present time, countries in the developing world are facing two major problems energy shortage and climate change. The depletion of fossil fuel resources, increasing demand of the energy and the environmental protection lead to an increasing interest in alternative fuels. Bioethanol represents an alternative energy resource which might be able to present clean and green solution to ever growing energy demand. Lignocellulosic biomasses are the most abundant and provide cheap raw materials for the production of ethanol. It includes crop residues, hard wood, soft wood, cellulose wastes and municipal solid wastes. Annual production of lignocellulosic materials is over 150 billion tons (Zhu et al., 2005). Among all lignocellulosic biomass, cereal straws are promising feedstock for bioethanol production because of its abundance and relatively low cost (Sun, 2010). Rice is one of the major crops grown in India and it contains 23% of rice straw by its weight. In conventional ways wither rice straw is left in the field or burnt to clear the field for next crop and results in the air pollution. In India, the contribution of open-field burning of rice straw in GHG emissions is 0.05% (Gadde et al., 2009). Therefore, use of rice straw as a feedstock for bioethanol production not only provides an alternative fuel but also mitigates climate change.

Rice straw mainly consists of cellulose, hemi-cellulose, lignin, silica and ash contents. Conversion of rice straw to fermentable sugar is hindered due to the presence of complex structure of lignin and hemicelluloses with cellulose (Sun and Cheng, 2002; Zhu, 2005). The presence of complex structures causes a challenge for the use of rice straw as feedstock for ethanol production. Hence, pretreatment of rice straw is required to remove or to modify the surrounding matrix of lignin and hemicellulose prior to the enzymatic hydrolysis of the polysaccharides (cellulose and hemicellulose). It reduces cellulose crystallinity and increases the porosity of the materials (Karimi et al., 2006). Various types of pretreatment technologies have been found for conversion of lignocellulosic biomass into ethanol such as steam explosion, liquid hot water, dilute acid, flow through acid pretreatment, lime, wet oxidation and ammonia fiber /freeze explosion milling and grinding, microwave energy, wet oxidation and high energy radiation etc. (Liu et al., 2005; Fan et al., 1982). Enzymatic hydrolysis is the most common method used for the hydrolysis of lignocellulosic biomass for obtaining fermentable sugars. Mild reaction conditions, lack of corrosion and positive environmental effects (Gong et al., 2010) makes enzymatic hydrolysis more promising with comparison to other techniques.

Microwave pretreatment technology has been widely used by several researchers as a potential method for pretreatment of various ligno-cellulosic materials (Eskicioglu et al., 2007a, b; Taherzadeh and Karimi, 2008; Alvira et al., 2010; Shi et al.,

Table 1. ANOVA of the quadratic model H_2SO_4 and its influential factors.

Source	Sum of squares	Degree of freedom	Mean square	F- value	P-value	
Microwave and H_2SO_4 treatment						
Model	19299.36	9	2144.37	13.87	0.0001	Significant
Time (C)	1151.89	1	1151.89	74.06	0.0001	
Power (B)	741.27	1	741.27	47.66	0.0002	
Quadratic terms H_2SO_4 concentration (A^2)	9281.90	1	9281.90	596.77	0.0001	
Power (B^2)	6680.06	1	6680.06	429.49	0.0001	
Interaction term (AC)	522.40	1	522.40	33.59	0.0007	
Residual	108.88	7	15.55			
Lack of fit	29.40	3	9.80	0.49	0.7061	Not Significant

Table 2. Optimum condition for delignification of rice straw.

Pretreatment Methods	Chemicals concentration (%)	Power (Watt)	Time (Min)
H_2SO_4-microwave pretreatment	2	115	3.32

2011; Jackowiak et al., 2011). Microwave heating is preferred over conduction/convection heating as it heats the target object directly by applying an electromagnetic field to dielectric molecules (Newnham, 1991). It can not only degrade lignin and hemicelluloses but it can also increase enzymatic hydrolysis (Zhu et al., 2005; Azuma et al., 1984; Ooshima et al., 1984; Kitchaiya et al., 2003).

In current study, microwave assisted- sulphuric acid (H_2SO_4) pretreatment is employed for enzymatic hydrolysis of rice straw. Statistical analysis is performed using Response Surface methodology (RSM). By careful design of experiments, the objective is to optimize output response which is influenced by several independent input variables. For optimization, the user is required to supply minimum and maximum values for each factor (Ferreira et al., 2007). For optimization, a Box- Behnken design (BBD) was selected. The design would help in investigating the effects of power (W), treatment time (min) and concentration of chemicals (%) on reducing sugar yield. Further, the morphological characteristics of rice straw are also analyzed through scanning electron microscope (SEM) and biomass crystallinity through X-ray diffraction (XRD). The goal of the present study is to optimize an efficient, microwave pretreatment technology for the hydrolysis of rice straw.

Results and Discussion

Response surface methodology (RSM) results

For optimization of microwave effect and other factors on saccharification of rice straw, experiments based on BBD model are employed. Design expert software is used for data analysis, analysis of variance (ANOVA), regression coefficients and regression equations. ANOVA model has showed that the model is significant for H_2SO_4 at Fisher's F-test value 13.87 (Table 1).

The coefficient of variation (R^2) for H_2SO_4 has been found 0.98. The model appears statistically sound as the lack of fit test used for testing of model shows p value of 0.7061 and it is not significant. The most significant parameter for H_2SO_4 is time (C), power (B) and quadratic terms H_2SO_4 concentration (A^2), power (B^2) and interaction term (AC) [H_2SO_4 concentration (A) and time (C)]. Analysis of residuals showed no abnormality.

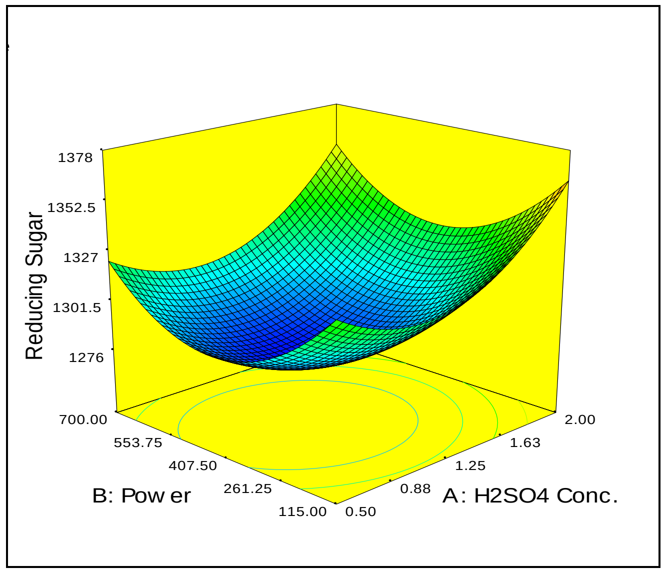


Fig 1. Response surface for the effect on reducing sugar using power (microwave) and H_2SO_4 concentration at constant time.

The 3D response surfaces for H_2SO_4 treatment is shown in Fig 1. To depict the interactive effect of independent variables on responses, one variable has been kept constant while the other two variables were varied at different ranges. The interaction between different factors has been shown through the shape of response surfaces.

Optimum conditions for acid pretreatment to saccharify rice straw

Design expert software is used for deciding optimum conditions (Table 2). The reducing sugar obtained through H_2SO_4-microwave pretreatment under optimum condition is 1376.99 µg/ml. The reducing sugar concentration in the saccharified rice straw under H_2SO_4-microwave pretreatment was increased 1784 % (Table 3) as compared to raw straw.

Table 3. Predicted and experimental reducing sugar obtained under optimum conditions.

Pretreatment in combination with microwave	Control [a] (µg/ml)	Predicted (µg/ml)	Measured[b] (µg/ml)	Increasing rate (%)
H_2SO_4	73	1351	1376 ± 20.3	1784

[a] Rice straw was used in control was untreated. [b] Mean ± standard deviation of five replicates.

Table 4. Chemical composition of Rice straw before and after pretreatment.

S. No	Characteristics of rice straw	Before treatment	H_2SO_4-microwave pretreatment
1	Cellulose	39.04 ± 0.5	42.6 ± 2.4
2	Hemi-cellulose	21.64± 0.50	14.2 ± 1.5
3	Lignin	16.2 ± 0.3	3.8 ± 0.5
4	Ash	18 ± 1.1	13.5 ± 0.2

Scanning electron microscope (SEM) analysis

The morphological changes occurred due to pretreatment has been analyzed with the help of SEM (Namasivayam and Kavitha, 2006). SEM images of the untreated sample showed that there is less number of cracks and the surfaces of the samples are densely packed as compared to H_2SO_4-microwave pretreated sample (Fig. 2). The silicon waxy structure, lignin-hemicellulose complex of rice straw is broken down significantly. Previous studies have also shown that the surface of the samples treated with microwave assisted organic acid become loose and irregular (Gong et al., 2010). It proves that microwave pretreatment has improved the straw digestibility by removing silica content (Rezanka and Sigler, 2008).

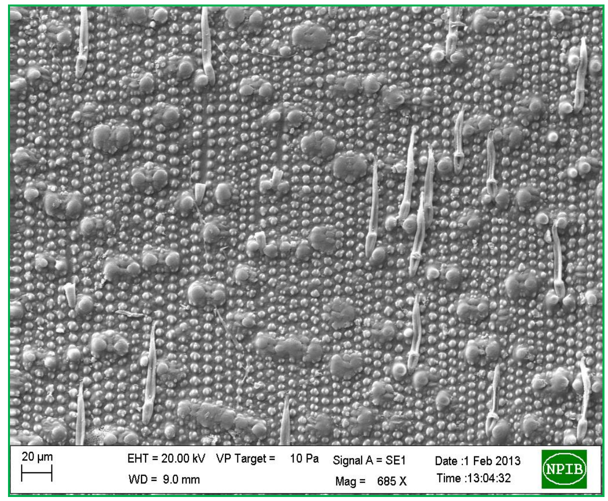
Effect on chemical composition of rice straw

Chemical components of rice straw have been changed after pretreatment with microwave assisted treatment containing H_2SO_4 (Table 4). There is increase in percentage of cellulose contents in treated rice straw samples with comparison to untreated. However other components e.g. lignin, hemi-cellulose and ash has been reduced significantly. This indicates that the pretreatment method is capable of removing lignin and other components. It damaged the cell wall by disrupting the lignin structure. Also increase in the surface area has provided better enzymatic accessibility. All these conditions are greatly beneficial for enzymatic hydrolysis.

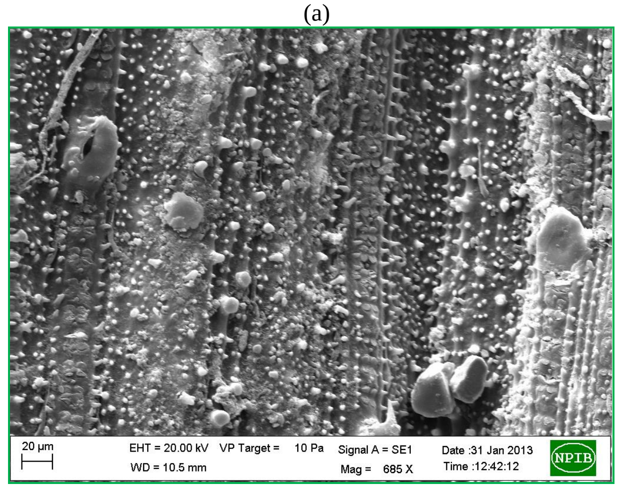
X-ray diffraction (XRD) analysis

Crystallinity index is the percentage of crystalline material in the biomass (Segal et al., 1959). It is one of the major factor that affects enzymatic hydrolysis (Kim and Lee, 2005; O' Dwyer et al., 2007). XRD analysis (Fig 3) shows that the crystallinity index of rice straw treated with microwave-assisted H_2SO_4 is high with comparison to the untreated and blank sample. For untreated and blank (without addition of any chemicals) samples, it is 52.2 % and 49.07% respectively as listed in Table 5.

By disrupting inter and intra chain hydrogen bonding of cellulose fibrils pre-treatments can change the cellulose structure (Mosier et al., 2005). In biomass, Hemi-cellulose and lignin are amorphous in nature however; cellulose is crystalline (Jeoh et al., 2007). The results demonstrated that removal of amorphous parts of the rice straw i.e. lignin, hemi-cellulose was



(a)



(b)

Fig 2. Scanning electron microscope images of (a) untreated sample (b) sample pretreated with microwave assisted sulfuric acid (H_2SO_4) for rice straw

microcrystalline cellulose has been hydrolyzed and larger perfect cellulose has been left in the sample. Several studies have also confirmed the increase in crystalline index value after biomass pretreatment (Chang and Holtzapple, 2000; Kim and Holtzapple, 2006; Bak et al., 2009; Liu et al., 2009).

Table 5. Crystallinity index of rice straw samples.

S. No.	Treatments	Crystalline Index (CrI) %
1	Untreated rice straw	52.2
2	Blank (without addition of any chemicals)	49.07
3	H_2SO_4- microwave pretreatment	61.36

more in samples treated with microwave- assisted H_2SO_4 than untreated and blank. This increase in value showed that pretreatment has potentially removed the amorphous components of rice straw i.e. lignin, hemi-cellulose and increased the crystalline component. It is being found that the cellulose content has been increased because small, imperfect

Materials and Methods

Raw materials and microwave-acid pretreatment

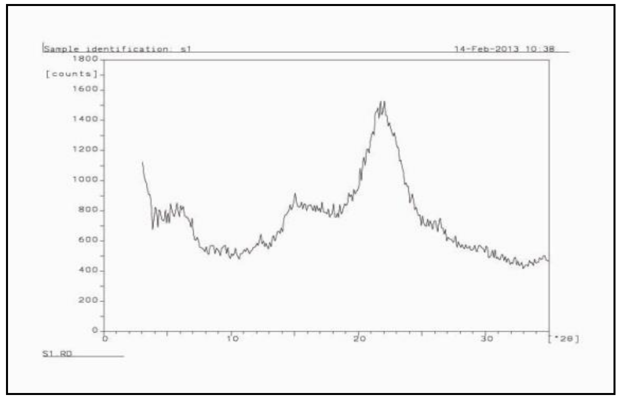
In the present research work Indian straw from rice variety "PUSA SUGANDH" has been used. The samples of rice straw have been locally harvested at Indian Agriculture Research Institute. Firstly, rice straw has been cut into pieces of size 1-2cm. Now the prepared samples of rice straw are cleaned thoroughly using tap water until the washings became clean and colorless. Before any pretreatment, samples have been air dried. The chemical composition of rice straw is given in Table 5. Microwave pretreatment is one of efficient way and modified domestic microwave oven is used in the present study. The microwave power is varied between 70 to 700W respectively. About 5g of rice straw was suspended in 30 ml of H_2SO_4 concentration ranged from 0.1 to 2% and left for overnight as per RSM fitted design. It was then radiated at in the range of 70-700W for 1-5 min in microwave. All the pretreatment conditions i.e. power 70-700 W, concentration of chemicals 0.1 to 2% and treatment time 1 to 5 min is designed by Response Surface Model (RSM), Design Expert software Version 7.

Enzymatic saccharification of pretreated rice straw

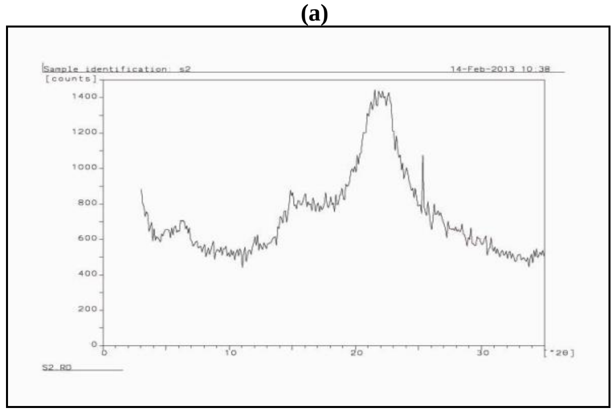
Saccharification or hydrolysis of the wet pretreated paddy straw samples is carried out using E-CELAN, endo-1, 4-β-glucanase from *Aspergillusniger* supplemented with EBGLUC (endo-β-glucosidase), β-glucosidase from *Aspergillusniger* (Megazyme International and Genecor) (Saritha et al., 2012). All other chemicals employed in this study are of reagent grade. Enzyme saccharification is carried out in 50 ml screw capped bottles, which consisted of 1.0 g microwave treated rice straw, 10 units of E-CELAN, and 5 units of EBGLUC. The final volume of reaction mixture has been made using 10 ml of citrate buffer (pH 4.8). Bottles are kept at 50ºC and 150 rpm in a constant temperature shaker water bath. Samples have been collected from reaction mixture at different time intervals and analyzed for sugar by DNSA method (Miller, 1959). All the experiments have been performed in triplicate and the average values are reported.

Morphological characterization through scanning electron microscope (SEM)

In this study, the morphology of rice straw is examined through scanning electron microscope (ZEISS, Evoma-10). Firstly, samples are dried in a vacuum dryer oven at 45ºC for 24 h and



(a)



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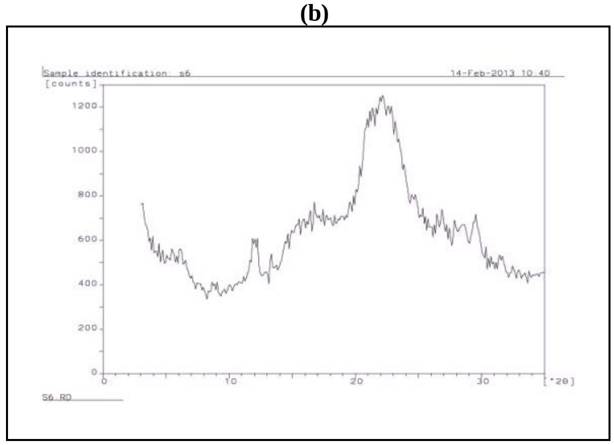

(c)

Fig 3. X- ray diffraction pattern of (a) untreated sample (b) blank (c) H_2SO_4-microwave treated sample.

then gradually dehydrated using acetone-water mixtures. Same process is being repeated with 50% - 100% acetone. The samples have been mounted on aluminium stubs and coated with gold and platinum mixtures prior to imaging under SEM.

X-ray diffraction (XRD) of the pretreated raw materials

Crystallinity of untreated and pretreated rice straw samples have been determined using X-ray diffraction (PW 1710, copper Kα radiation). Rice straw treated with water-microwave served as a control. Crystallinity index is calculated by using following formulae (Segal et al., 1959);

$$C_r I = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \qquad (1)$$

Where; I_{002} is intensity for the crystalline part of the biomass (i.e. cellulose) and I_{am} is intensity for the amorphous part of the biomass (i.e. cellulose, hemi-cellulose and lignin). In the present research, intensity of crystalline portion was at 2θ= 22.4 and intensity for amorphous portion was at 2θ =10.1.

For the estimation of comprising crystalline area in plant (*d*002) Eq. 2 is used to calculate crystalline size of (002) plane based on Scherrer equation (Gumuskaya and Usta, 2002).

$$d002 = \frac{0.9\lambda}{\beta \cos\theta} \qquad (2)$$

Where, λ is wavelength of X-ray tube (λ =1.5406 Å), β is FWHM (full width at half maximum) of (002) peak, θ is diffraction angle of (002) plane.

Removal and recovery of lignin

The extent of lignin removal is mainly determined on the basis of lignin fragments and monomers present in the alkali extract according to the NREL LAP-004. The absorbance is measured at 205 nm through spectrophotometer (Ehrman, 1996). Through acidification, value added acid-precipitable polymeric lignins are recuperated from the extracts (Pometto and Crawford, 1986). In the next step extract is acidified to pH 1-2 with concentrated sulphuric acid. Centrifugation process took 30 minute at 13000 rpm. The precipitates are washed with distilled water and dried at 60ºC till the constant weight has been achieved.

Experimental designs and data analysis

Design Expert software Ver. 7 naming Box- Behnken factorial design (BBD) is used with three factors and three levels, including three replicated at centre point to evaluate the effect of concentration of chemicals (A), power (B) and treatment time (C) on hydrolysis of rice straw (Y) obtained from the pretreatment experiments. The range of variables for H_2SO_4 is power 70-700 W, concentration of chemicals 0.1 to 2% and treatment time 1 to 5 min. The design matrix with 17 experimental runs in one block with five replicates. A polynomial quadratic equation was fitted to evaluate the effect of each independent variable to the response:

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Where, Y is the predicted response; β_o is a constant; $\beta_1, \beta_2, \beta_3$ are the linear coefficients; $\beta_{12}, \beta_{23}, \beta_{13}$ are the cross-coefficients; $\beta_{11}, \beta_{22}, \beta_{33}$ are the quadratic coefficients. The response surfaces of the variables inside the experimental domain were analyzed using Design Expert. Subsequently, five additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

Conclusions

The current research work substantiate that microwave is an efficient heating method for the pretreatment of rice straw. Microwave assisted H_2SO_4 enhances the saccharification of rice straw by removing lignin and hemicelluloses in large quantity which in turn increases enzyme accessibility. Maximum reducing sugar was obtained through microwave assisted H_2SO_4 pretreatment (1376.99 µg/ml) using 2% H_2SO_4 at 115 W for 3.32 minute. Images obtained through SEM and XRD shows that the eradication of lignin and hemi-cellulose, although lignin has not been recovered significantly. SEM images have proved that the surfaces are more ruptured and damaged in microwave assisted H_2SO_4 pretreatment than blank sample. Moreover, crystallinity index for rice straw samples treated with microwave assisted H_2SO_4 is significantly high 61.36 % in comparison to untreated sample 52.2 %. Thus, microwave assisted H_2SO_4 pretreatment technology efficiently removes lignin and increases the enzymatic saccharification of rice straw.

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