

Effect of Combination of Microwave and Hydrogen Peroxide (H₂O₂) Pretreatment on Enzymatic Saccharification of Rice Straw

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Abstract

Rapid industrialization, increasing energy demand and climate change all these conditions compel the researchers to develop a clean, efficient, renewable and sustainable source of energy which have a potential to replace fossil fuels. Biofuels are essentials as they can provide impending substitute for fossil fuels. Rice straw has gained much attention from researchers because of its usability as a potential feedstock for production of bioethanol. Pretreatments are crucial for enzymatic hydrolysis of rice straw. In present study, effectiveness of microwave pretreatment in combination with hydrogen peroxide (H₂O₂) for increasing enzymatic hydrolysis of rice straw has been investigated. The Response surface methodology is used to optimize the pretreatment conditions with respect to H₂O₂ concentration, microwave power and irradiation time. Under optimum conditions, maximum reducing sugar obtained through microwave assisted H₂O₂ is 1453.64 µg/ml. The chemical and morphological analysis ascertained that the surface of the samples treated with microwave assisted H₂O₂ was more ruptured and have a significantly high crystalline index (63.64%) than untreated rice straw sample. Microwave assisted H₂O₂ disrupted the silicon waxy structure and broken down all ether linkages between lignin and carbohydrates and thus, efficiently remove lignin. Hence, the present study proves that microwave assisted H₂O₂ pretreatment can effectively enhanced enzymatic saccharification of rice straw and it is feasible to convert rice straw for bio-ethanol production.

Keywords: Enzymatic saccharification; Rice straw; Biofuel; Microwave; Hydrogen peroxide.

1. Introduction

In recent times, increasing human population and fuel demand have forced the researchers to develop a sustainable and renewable energy resource which can solve the crisis of energy scarcity. Biofuels are good alternative for liquid transportation fuels with powerful economic, environmental and strategic attributes. Biofuels are of several types such as bioethanol, biodiesel, biohydrogen etc. Bioethanol has gained much attention as an alternative fuel or oxygenate additive for fossil fuels. Its production reduces the energy dependency on fossil fuels as well as saves the earth from environment pollution. In 2005 and 2006, worldwide production capacity of ethanol were about 45 and 49 billion litres respectively and the total projected demand in 2015 is over 115 billion litres (Talebinia et al., 2010). Ligno-cellulosic feedstocks are abundant, cheap, and renewable and therefore, can be used for ethanol production through microbial intervention (Zhao et al., 2007). But, ligno-cellulosic materials are comprised of cellulose, hemicelluloses and lignin and the main challenge is to alter the ligno-cellulosic structure to make it accessible to enzymatic hydrolysis. Therefore, effective utilization requires separation of polymeric components i.e. cellulose, hemicellulose and lignin (Sun and Cheng, 2002). Among ligno-cellulosic materials, cereal straws are available in large quantities that can provide potential substrate as a starting constituent for biofuel production (Sun et al., 2010). Rice straw is one of the most abundant ligno-cellulosic crops that can be converted into fermentable sugars, ethanol and other useful products (Jeya et al., 2009; Zhu et al., 2005a; Lo et al., 2010). It is one of the major crops of India and contains 23% straw of its total weight (Gadde et al., 2009). Rice straw consists of cellulose, hemicellulose, lignin, silicon and ash content. Among all the constituents, cellulose is present in larger quantity which can be hydrolyzed into glucose by cellulose enzyme. Different methods for hydrolysis of ligno-cellulosic materials have been developed, enzymatic hydrolysis is the most common method because of its mild reaction conditions, lack of corrosion and positive environmental effects (Gong et al., 2010). Hydrolysis of rice straw is a complicated process due to the presence of lignin and complex structure of polysaccharides. Thus, pretreatments are essential for increasing the digestibility of polysaccharides and removal of lignin (Jorgensen et al., 2007). It decreases the crystallinity of cellulose, increases the surface area and removes or alters the hemicellulose or lignin (Mosier et al., 2005; Karimi et al., 2006). Various pretreatment methods including steam explosion, liquid hot water, dilute acid, flow through acid pretreatment, lime, wet oxidation and ammonia fiber /freeze explosion milling and grinding, microwave energy, steam explosion, wet oxidation and high energy radiation etc. has been developed for this purpose (Liu et al., 2005; Fan et al., 1982)

Microwave irradiation has been extensively used because of its high heating efficiency and easy operation. It heats the target object directly by applying an

electromagnetic field to dielectric molecules as compared to conduction/convection heating which is based on intra-molecular heat transfer (Newnham.,1991). Several studies have explored microwave pretreatment technology as an impending method for pretreatment of ligno-cellulosic materials (Eskicioglu et al., 2007a, b; Taherzadeh and Karimi, 2008; Alvira et al., 2010; Shi et al., 2011; Jackowiak et al., 2011) as well as to damage the recalcitrant lignin (Hu and Wen, 2008). It can degrade lignin and hemicelluloses as well as increases enzymatic susceptibility (Zhu et al., 2005b). Also, it has been reported that microwave pretreatment significantly enhances the enzymatic saccharification of rice straw (Azuma et al., 1984; Ooshima et al., 1984; Kitchaiya et al., 2003). Pretreatment process can be improved by the combination of microwave irradiation and chemical pretreatment technique.

In present research work, microwave assisted-hydrogen peroxide (H₂O₂) pretreatment is employed for enzymatic hydrolysis of rice straw. Response Surface methodology (RSM) is used for statistical analysis. It is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). For optimization, the user required to supply minimum and maximum values for each factor (Ferreira et al., 2007). Since, for rice straw peroxide pretreatment along with microwave treatment is not yet explored. In the current study, a systematic study of microwave assisted H₂O₂ was carried out by optimizing the pretreatment conditions. For optimization, a Box-Behnken design 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 analyzed through scanning electron microscope (SEM) and biomass crystallinity through X-ray diffraction (XRD). The goal of this study is to optimize an efficient, microwave pretreatment technology for the hydrolysis of rice straw for ethanol production.

2. Material and Methods

2.1 Raw materials and microwave- peroxide Pretreatment

In the present research work Indian straw from rice variety "PUSA SUGANDH" has been used. The samples of rice straw were 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 1. Microwave pretreatment is one of efficient way and modified type 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 H₂O₂ 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.

Table 1: Chemical composition of Rice straw variety (PusaSugandh) (Manjunath et al., 2008)

S. No	Characteristics of rice straw	Values (%)
1	Cellulose	39.04 ± 0.50
2	Hemi-cellulose	21.64 ± 0.50
3	Lignin	16.2 ± 0.30
4	Ash	18 ± 1.1

2.2 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 50°C and 150 rpm in a constant temperature shaker water bath. Samples has 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.

2.3 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 45° C for 24 hour and 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.

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

Crystallinity of untreated and pretreated rice straw samples has 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);

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

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 this research work, intensity of crystalline portion was at $2\theta = 22.4$ and intensity for amorphous portion was at $2\theta = 10.1$.

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 (Gumuskeya and Usta, 2002).

$$d_{002} = \frac{0.9\lambda}{\beta \cos \theta} \quad (2)$$

Where, λ is wavelength of X-ray tube ($\lambda = 1.5406 \text{ \AA}$), β is FWHM (full width at half maximum) of (002) peak, θ is diffraction angle of (002) plane.

2.5 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 60 °C till the constant weight has been achieved.

2.6 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_2O_2 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:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Where, Y is the predicted response; β_0 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.

3. Results and Discussion

3.1 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 represents that model is significant for H₂O₂ at Fisher's F-test value 8.54 (Table 2). The coefficient of variation (R²) for H₂O₂ has been found 0.92. The model appears statistically sound as the lack of fit test used for testing of model shows p value of 0.2893 and it is not significant. The most significant parameter for H₂O₂ is quadratic terms H₂O₂ concentration (A²) and power (B²). Analysis of residuals showed no abnormality. The 3D response surfaces for H₂O₂ treatment is shown in Fig 1. To depict the interactive effect of independent variables on responses, one variable should be 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.

3.2 Optimum conditions

Same Design expert software is used for deciding optimum conditions (Table 3). The reducing sugar obtained through H₂O₂-microwave pretreatment under optimum condition is 1453.64 µg/ml. The reducing sugar concentration in the saccharified rice straw under H₂O₂-microwave pretreatment was increased 2005% (Table 3) as compared to raw straw.

Table 2: ANOVA of the quadratic model H₂O₂ and its influential factors.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value	
Microwave and H₂O₂ treatment						
Model	38409.55	9	4267.73	8.54	0.0049	Significant
Quadratic terms H ₂ O ₂ concentration (A ²)	28075.65	1	28075.65	56.20	0.0001	
Power (B ²)	5203.49	1	5203.49	10.42	0.0145	
Residual	3496.80	7	499.54			
Lack of fit	2001.10	3	667.03	1.78	0.2893	Not Significant

Table 3: Optimum conditions for delignification of rice straw.

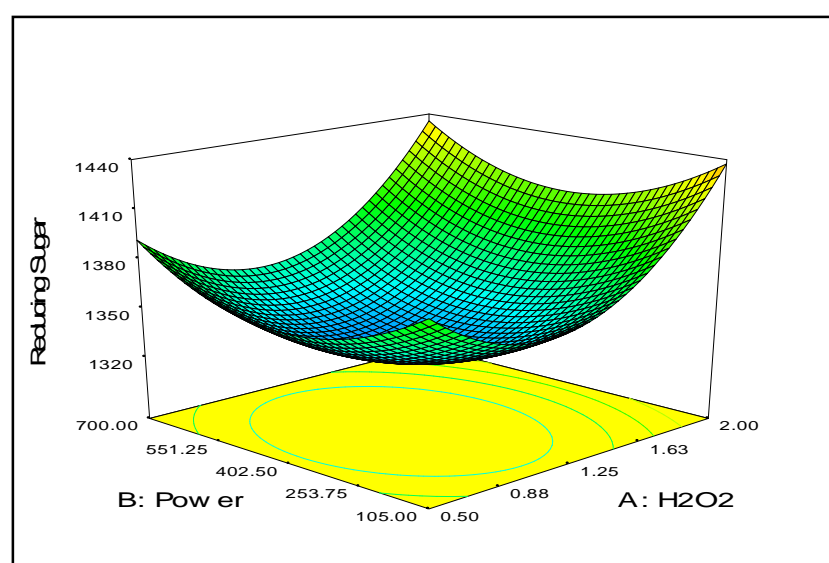
Pretreatment Methods	Chemicals concentration (%)	Power (Watt)	Time (Min)
H_2O_2 -microwave pretreatment	2	100	3

Table 4: Predicted and experimental reducing sugar obtained under optimum conditions.

Pretreatment in combination with microwave	Control ^a ($\mu\text{g/ml}$)	Predicted ($\mu\text{g/ml}$)	Measured ^b ($\mu\text{g/ml}$)	Increasing rate (%)
H_2O_2	69	1436	1453 ± 14.3	2005

^a Rice straw was used in control was untreated.

^b Mean \pm standard deviation of five replicates.

**Figure 1:** Response surface for the effect on reducing sugar using power (microwave) and H_2O_2 concentration at constant time.

3.3 Scanning electron microscope (SEM) analysis

The morphological changes occurred due to pretreatment could be analyzed with the help of scanning electron microscope (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_2O_2 -microwave

pretreated. 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). Also, microwave assisted FeCl_3 damage the cell wall structure and altered the fibrillar structure of rice straw (Lu and Zhou, 2011). It proves that microwave pretreatment has effectively improved the straw digestibility by removing silica content (Rezanka and Sigler, 2008).

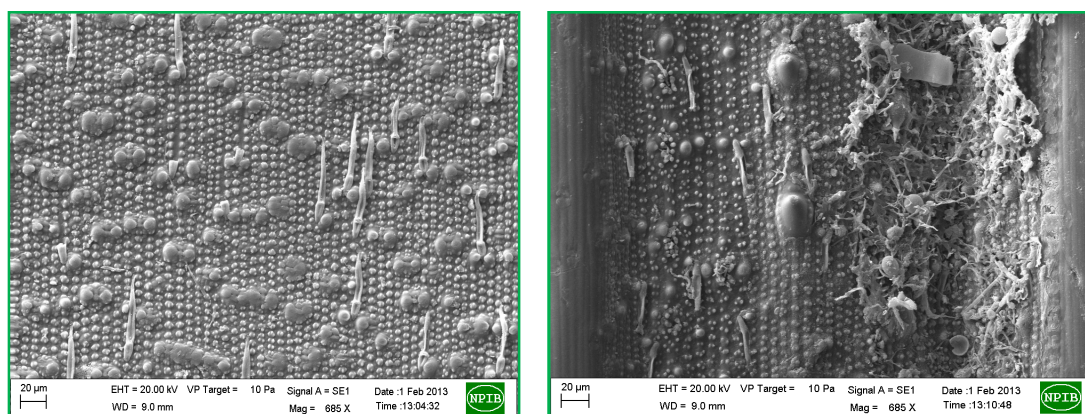


Figure 2: SEM images of (a) untreated sample (b) sample pretreated with microwave assisted Hydrogen peroxide (H_2O_2).

3.4 Effect on Chemical composition of rice straw

Chemical components of rice straw changed after pretreatment with microwave assisted treatment containing H_2O_2 (Table 5). 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. It led to increase in the surface area and thereby better enzymatic accessibility. All these conditions are greatly beneficial for enzymatic hydrolysis.

Table 5: Chemical composition of Rice straw after pretreatment.

S. No	Characteristics of rice straw	H_2O_2 -microwave pretreatment
1	Cellulose	45.3 ± 1.5
2	Hemi-cellulose	51.2 ± 1.9
3	Lignin	3.2 ± 0.4
4	Ash	13.2 ± 0.1

3.5 X-ray Diffraction (XRD) analysis

Crystallinity index is the percentage of crystalline material in the biomass (Segal et al., 1959). It is a major factor that affected 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_2O_2 is high as compared to the untreated and blank sample. For untreated and blank (without addition of any chemicals) sample, it is 52.2 % and 49.07% respectively as listed in table 6. 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 while 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 more in sample treated with microwave- assisted H_2O_2 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 i.e. cellulose in the rice straw. It is being found that cellulose content has been increased but only in small amount, whereas imperfect microcrystalline cellulose has been hydrolyzed and large perfect cellulose was left. Previous research has also suggested that the crystallinity index of rice straw could enhance by hot acid treatment (Yu et al., 2009). Several studies showed increase in crystalline index value after biomass pretreatment (Chang and Holtzapple, 2000; Kim and Holtzapple, 2006; Bak et al., 2009; Liu et al., 2009).

Scanning electron microscope (SEM) analysis, changes on chemical composition of rice straw, X-ray Diffraction (XRD) analysis, used in the present study proved that microwave assisted hydrogen peroxide pretreatment method had the potential of exposing cellulose and increasing cellulose contents. The study also proves that huge availability of rice straw in Indian livestock have tremendous potential for ethanol conversion using microwave-chemical pretreatment methods and technology is working well for them in Indian conditions and varieties of rice straw.

Table 6: 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_2O_2 - microwave pretreatment	63.64

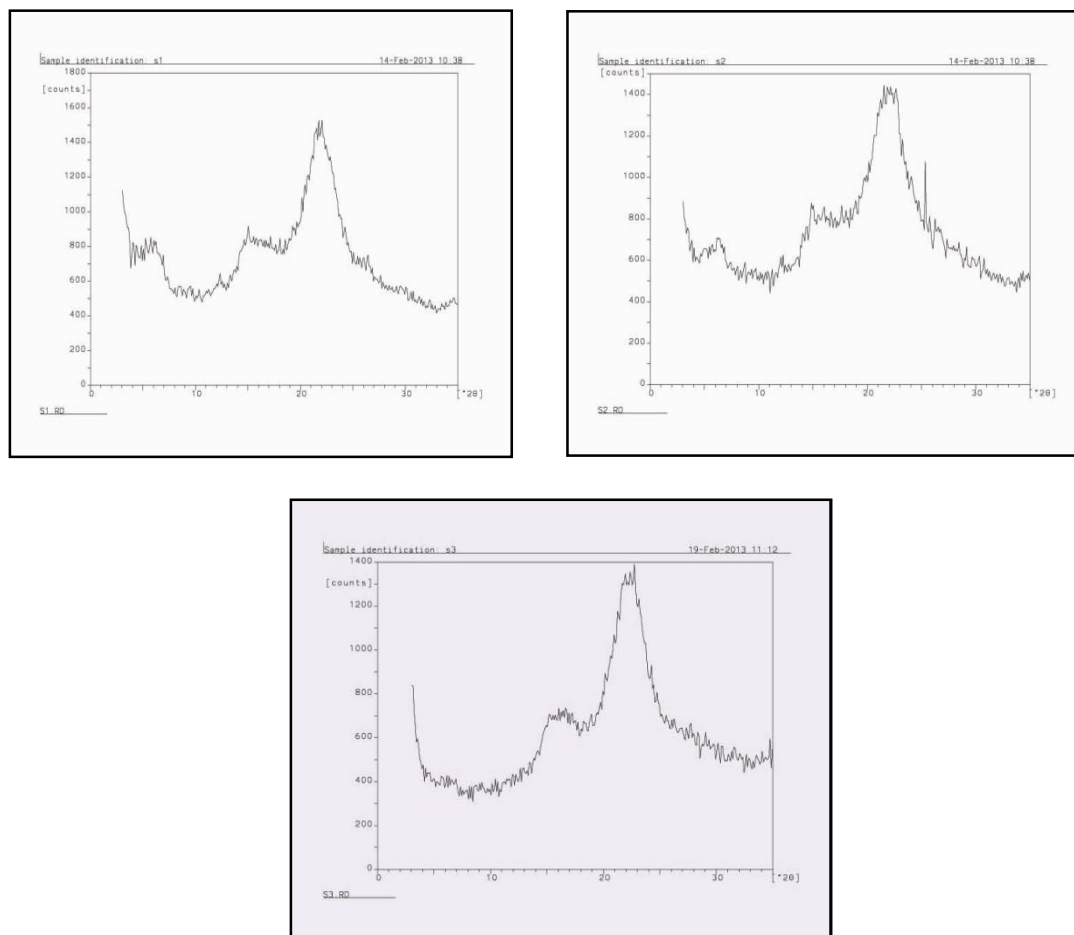


Figure 3: X- ray diffraction pattern of (a) untreated sample (b) blank (c) H₂O₂-microwave treated sample.

4. Conclusions

The current research work substantiate that microwave is an efficient heating method for the pretreatment of rice straw. Microwave assisted H₂O₂ enhances the saccharification of rice straw by removing lignin and hemicelluloses in large quantity which in turn increases enzyme accessibility. The optimal conditions have been deduced by using Box- Behnken design (BBD). Maximum reducing sugar was obtained through microwave assisted H₂O₂ pretreatment (1453.64 µg/ml) using 2% H₂O₂ at 100 W for 3 minute. Analysis of chemical composition of rice straw, the images obtained through scanning electron microscope (SEM) and X-ray diffraction (XRD) analysis showed the eradication of lignin and hemi-cellulose, although lignin has not been recovered significantly. SEM images certified that the surface is more ruptured and damaged in microwave assisted H₂O₂ pretreatment than blank sample. Moreover, crystallinity index for rice straw samples treated with microwave assisted

H₂O₂ is significantly high 63.64 % in comparison to untreated sample 52.2 %. The removal of lignin and hemi-cellulose increased the enzyme accessibility with microwave treatment and thus, the enzymatic saccharification of rice straw can be assisted with microwave efficiently.

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