



Effect of Particle Size and Pretreatment on Cellulose Degradation of Rice Straw from Agricultural Land in Malang

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Abstract : Rice straw is composed of 28-36 % cellulose, 12-16 % lignin, 15-20 % ash, and 9-14 % silica with strong non-covalent and covalent cross linkages between molecules, which are very difficult to degrade. A pretreatment process is needed to degrade these components. The objective of this research is to evaluate the effect of pretreatment and particle sizes on cellulose degradation of rice straw. Rice straw pretreatment consists of no delignification, delignification with thermochemical pretreatment, and delignification with thermochemical pretreatment followed by microbial degradation, while rice straw particle sizes are 38, 53, and 112 μm . The final composition of substrates after pretreatment is observed using SEM and FTIR. The results show that variation of particle size with thermochemical pretreatment does not alter cellulose degradation, but microbial consortium are able to increase cellulose degradation. Rice straw thermochemical pretreatment followed by microbial degradation shows that smaller sizes of particle yield more effective degradation of cellulose. The percentage of cellulose degradation at particle sizes 38 μm , 53 μm and 112 μm are 71.96 %, 50.15 %, and 24.30 % respectively.

Key words : cellulose, rice straw, pretreatment, particle size, SEM and FTIR.

Introduction

Indonesia is an agricultural country producing rice straw abundantly. Until today, rice straw is still regarded as agricultural wastes and it is not fully utilized. Most of rice straw is burned and it contribute to the release of CO_2 in the environment¹. Rice straw is composed of cellulose, hemicellulose and lignin, which are bound very strong with their non-covalent chemical bond and covalently cross linkages among molecules. Generally, rice straw contain 28-36 % cellulose, lignin 12-16 %, 15-20 % ash and 9-14 % silica, which is very difficult to be degraded². Lignocellulose is a renewable biomass that can be harnessed into products with higher values. One of the advantages of lignocellulose waste utilization is because rice straw's abundant availability in nature. Lignocellulose can be used to produce a variety of products such as biofuels, animal fedder, enzymes, and other chemical derivative products^{3,4,5}.

Biodegradation of cellulosic biomass using microbial consortium has been proposed as a highly efficient approach for biotechnology applications. Biodegradation of lignocellulose waste by microbial consortium is a convenient and environment friendly method². In any pretreatment, biomass size reduction allows for a physical structure that is more open and accessible to certain changes. Each pretreatment method

offers advantages and disadvantages. Physical and chemical pretreatment involve energy costs and are relatively more expensive. Microbial pretreatment is the most suitable method to improve the efficiency of the degradation process of rice straw. In nature, there are many microorganisms, such as bacteria and fungi, which live in the neighborhood of lignocellulose⁶. In addition, pretreatment via different methods can be used to enhance the degradation of lignocellulose materials⁷. The objective of this research is to analyze the effect of pretreatment methods, which are physical, thermochemical and microbial consortium, and also the particle size, on the degradation of the complex structure of rice straw. Microbial consortium is expected to provide benefits for agricultural and industrial applications based on rice straw in Indonesia.

Materials and Methods

Preparation of rice straw in variable sizes

Rice straw (*Oryza sativa*) type IR 64 was taken from an agriculture field in Malang, East Java Province – Indonesia. Rice straw is cleaned and cut into small pieces, dried under sunlight for approximately one week, and then dried in an oven at 100°C until the water content was below 10 %. The rice straw was ground and filtered using a vibrating screen to get particle sizes of 38, 53, and 122 µm. The smaller the particle size, the easier it is expected to be to hydrolize/degrade the lignocellulose contained in the rice straw^{8,9}.

Rice straw pretreatment

The experiment was carried out using a Complete Randomized Design with three replications. The variables of the experiment consist of rice straw pretreatment types (without pretreatment, thermochemical pretreatment, and thermochemical pretreatment followed by microbial degradation) and rice straw particle size (38.0, 53.0, and 112.0 µm). The experimental parameters analyzed include ADF, lignin, cellulose, the enzyme activity (Exo-1,4-β-Glukanase, Endo-1,4-β-Glukanase, and β-Glukosidase). The structure of cellulose was analyzed using SEM and FTIR. The data for ADF, lignin and cellulose was variance analyzed with $\alpha = 0.05$.

Rice straw powder (particle sizes of 0.112, 0.053, and 0.038 mm) underwent thermochemical pretreatment using a pressure vessel (autoclave) at 1 bar, 121°C for 30 minutes. A sample of the resulting powder was analyzed for its lignin level prior to the delignification process. Twenty grams of rice straw and 100 g of distilled water were added and it was then heated by the autoclave. The result of the delignification process was filtered by the filter cloth and washed with distilled water up to neutral (pH 7). It was then dried in an oven at a temperature of 105°C for ± 10 hours until its weight was constant, and analyzed for ADF, lignin and cellulose content¹⁰.

Rice straw which has been processed by thermochemical pretreatment was then degraded using microbial consortium. Rice straw powder (2.5 g) was added to 225 ml BHM media (0.05 g FeCl₃.6H₂O, 0.2 g MgSO₄.7H₂O, 0.02 g CaCl₂, 1 g K₂HPO₄, 1 g KH₂PO₄, 1 g NH₄NO₃, dissolved in 1L distilled water). Afterwards, it was sterilized at 121°C for 30 minutes in the autoclave. Microbial consortium was then added up to 10% of the volume so that bacterial density was 10⁶ cell/mL after 24 hours. The culture was incubated at 30°C for 10 days. During the incubation period, a sample culture suspension was taken to analyze the enzyme activity¹¹.

ADF, lignin and cellulose analysis

Acid Detergent Fiber (ADF), lignin and cellulose were pretreated physically, thermochemically, degraded using microbial consortium, and then analyzed using the van Soest method. A sample of 1 g (A) was inserted into a beaker and 100 ml ADS solution was added. The solution was refluxed for 1 hour at 100 °C with an electric bath, then heated in the oven with glass filter G4 at 105 °C and stored in a desiccator until the weight was constant (B). The samples were refluxed and then filtered with glass filter and vacuum pumps. Samples were rinsed three times with hot water and acetone. The waste products of filtration were then oven-dried at 105 °C for 8 hours and placed in a desiccator. The sample was dried to a constant weight (C). The ADF value was determined by (equation 1).

$$ADF = \frac{C-B}{A} \times 100\% \dots\dots\dots (1)$$

For lignin and cellulose, ADF residue was weighed (C grams) then placed on a tray filled with water at the height of *ca.* 1 cm. Sulfuric acid (72 %) solution was added to the glass filter for about $\frac{3}{4}$ part of the glass filter, and left for 3 hours with stirring every hour. Samples were separated from the solution by filtration using a vacuum pump. The solution of acetone and hot water. The waste product from filtration was removed and then the remaining sample was dried in an oven at 105 °C for 8 hours. The dried samples were then stored in a desiccator and weighed up to a constant (E). To determine lignin content, the crucible was then reheated in the oven at 105 °C for 8 hours and stored in a desiccator until the crucible weight was constant. Sample results remaining in crucible were analyzed to calculate the % cellulose and the residue was put in the furnace at 800°C for 4 hours to calculate the % ash. The crucible was put in the desiccator until the weight constant was (F)¹⁰.

$$\%Lignin = \frac{E-F}{A} \times 100\% \dots\dots\dots (2)$$

$$\%Selulosa = \% ADF - \% lignin \dots\dots\dots (3)$$

Analysis of Consortium Bacteria Indigenous Enzyme Activity in Rice Straw Degradation

The microbial activity resulting from the degradation of rice straw was analyzed by reducing sugar. The concentration of reducing sugar in the bacteria culture was determined by the DNS method. The suspension of bacteria (1.5 mL) was centrifuged at 10,000 rpm for 5 minutes. The supernatant was then tested for the extract of crude enzyme activity. A total of 1.8 ml of substrate (1% CMC, Avicel, cellobiose) supplemented with 0.2 ml of enzyme crude extract, was shaken by vortex until homogeneous, then incubated for 30 minutes at 30°C. Enzyme activity was then discontinued in boiling water for 15 minutes. Thereafter, the solution was added to a solution of 2 ml DNS, then heated in boiling water for 15 minutes. The absorbance was measured at 540 nm using a spectrophotometer. Blank control treatment was done simultaneously with the same method and stages in the test sample. Blank control is an enzyme that has been inactivated prior to reacting with the substrate, while the form does not use enzymes but using citrate phosphate buffer pH 7 is reacted with the substrate (CMC, Avicel, cellobiose)^{12,13,10}.

Analysis of Rice Straw Physical Structure After Pretreatment

The morphology and physical structure of rice straw after pretreatment was analyzed using electron microscope SEM S-3700 (Hitachi) and the degree of degradation of the rice straw components was determined by *Fourier transform infrared spectroscopy* (FTIR)^{14,7}.

Results and Discussion

The Effect of Pretreatment and Particle Size on Lignocellulose Degradation

The size of the main ingredient, lignocellulose, is an important factor in conversion efficiency. Many research studies suggest that the conversion efficiency is bigger when the particle size is smaller. Lignocellulose is generally ground to a smaller size prior to the next process for producing simple sugars. However, grinding lignocellulose to a smaller particle size requires energy and increases the production cost of the conversion process. In other research studies, it is stated that the conversion result of cellulose from wood powder increases by 50 % in enzymatic hydrolysis when the particle size is one tenth of the actual size for particles between 0,033-0,850 mm⁵. The conversion efficiency of wheat straw cellulose by pretreatment is about 90% and 65% consecutively for the particle size 0,053-0,149 mm and 2-4 cm. The same result for microcrystalline cotton cellulose when converting to cellobiose and glucose².

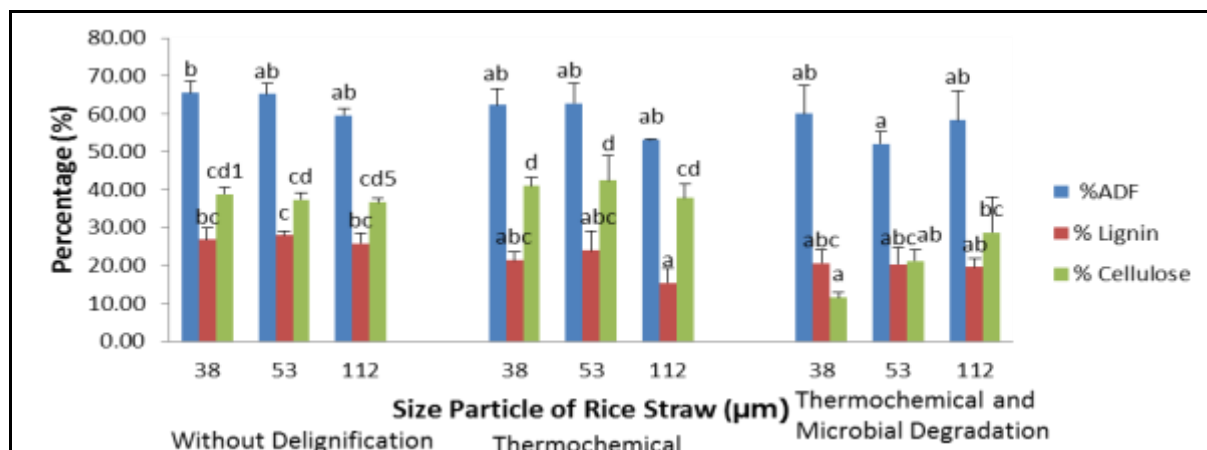


Figure 1. The effect of pretreatment in different particle size of rice straw to ADF value, % lignin and % cellulose

In this research, the values for ADF, lignin and cellulose in rice straw without delignification is independent from particle size, with the values for cellulose, lignin and ADF being 37-39 %, 26-28 %, and 60-66 % consecutively (Figure 1). The values of ADF, lignin and cellulose for rice straw pretreated by thermochemical method (heating by autoclave) is also independent of particle size. A few other research studies have shown that size reduction by using ball mill has the potential for reducing the crystalline characteristic of cellulose¹⁵. However, the result of size reduction and screening process is that the particle size of the material is divided into three levels that are relatively similar, but in a more heterogeneous proportion¹⁶.

The main purpose of the lignocellulose is to eliminate lignin and hemicellulose, change the crystalline structure of cellulose, and increase the porosity so the opportunity for enzymes is also increased, for the purpose of degrading cellulose. Pretreatment is the most beneficial and important phase in the process of biomass lignocellulose conversion to derivative products¹⁷. The results of this research show that the pretreatment process using thermochemical method decreases the lignin value for 14-40% depending on the size of rice straw particle used. Thermochemical pretreatment has a flaw wherein if the lignin value from the biomass is higher than 25%, the delignification process is relatively inefficient¹⁵. However, thermochemical is an effective and efficient pretreatment method, because the steam pressure can disturb the strongly bonded physical structure of rice straw¹⁸.

The result of thermochemical pretreatment that is continued by microbial degradation has a lower concentration of cellulose ($p < 0,05$) compared to that without pretreatment and or solely with thermochemical pretreatment. In the case of thermochemical pretreatment continued by microbial degradation, the smaller the particle size, the higher the reduction in cellulose¹⁹. Rice straw degradation is higher in smaller particle sizes until 100 μm. Previous research has shown that rice straw with smaller particle sizes produces 36% total carbohydrates and 40% glucose after from hydrolysis¹⁵. The cellulose concentration in thermochemical pretreatment continued by microbial degradation shows an increase in degradation related to smaller particle size. Degraded rice straw cellulose percentages by this treatment for particle sizes of 38.0, 53.0, and 112.0 μm are 71,96 %, 50,15 %, and 24,03 % respectively.

The approachment is about degradation of indigenous microbial consortium in the nature can accelerate and enhance the degradation process of rice straw. Microbes in the consortium work synergically in growth, biological process and enzymatic activity to make the process more effective and efficient compare to microba in individual population. The other research showed a simbiotic relation between microbial consortium giving a fundament of stability and efficiency. The biomass degradation ability is based on the functional and structural stability and effectivity. Some microba has a better degradation ability compared to other microba in term of degrading different component of rice straw such as cellulose, hemicellulose and lignin²⁰. The most efficient bacteria in degradation of cellulose, hemicellulose and lignin are combined as a strong and competent consortium. Microbial consortium is more benfical compared that single culture which helps to grow in the low nutrient agricultural residue and protect the plant from polluter. According to te result of the research, the

indigenous microbial consortium can be developed to a more efficient biodegradation process of rice straw². The indigenous microbial consortium can significantly degrade cellulose in a smaller particle size.

Activity of Bacterial Enzyme Consortium during the Rice Straw Degradation

The activity of the lignocellulose degrader compound is measured by the amount of glucose produced by the enzyme extract, which breaks the glycosidic bond in cellulose to become glucose according to the DNS method (cellulose is a polymer with cellobiose as a combination of two glucose units). The DNS method is easy to perform and gives satisfying results in measuring reducing sugar produced by microba in the smallest concentrations with higher accuracy.

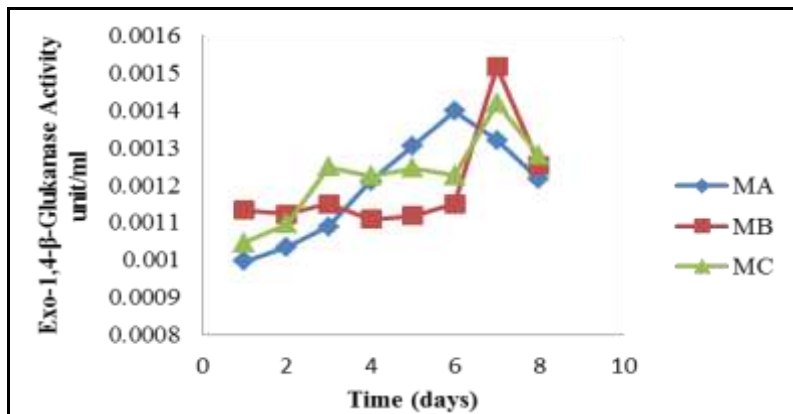


Figure 2. The activity of Exo-1,4-β-Glucanase cellulolytic bacterial consortium in several particle sizes; rice straw particle size MA= 112.0; MB= 53.0 and MC= 38.0 μm

(Figure 2) shows that in rice straw with particle size 112.0 μm, the activity of Exo-1,4-β-Glucanase increased every day until the sixth day. This condition was the highest activity of Exo-1,4-β-Glucanase from a cellulolytic bacterial consortium. On the seventh day, the activity was decreased significantly. This may be caused by the crystalline part in this size being degraded by Exo-1,4-β-Glucanase produced by the consortium. Enzymatic activity for 53 μm sized rice straw from bacterial consortium was constant until the sixth day, increased drastically on the seventh day and decreased after the seventh day. Enzymatic activity for 38 μm sized rice straw from bacterial consortium increased until the third day and stayed constant for the next day until the sixth day. The activity of Exo-1,4-β-Glucanase increased on the seventh day, followed by a decrease in activity on the next day. For the three particle sizes used, enzymatic activity is the highest on the seventh day for particle sizes 53 and 38.0 μm and on the sixth day for 112.0 μm. Enzymatic activity of Exo-1,4-β-Glucanase was not different ($p > 0,05$) for the particle sizes used in this research. From the research, it is concluded that the enzymatic activity of Exo-1,4-β-Glucanase is the highest in rice straw particle size 53.0 μm for 15.15×10^{-4} unit/mL.

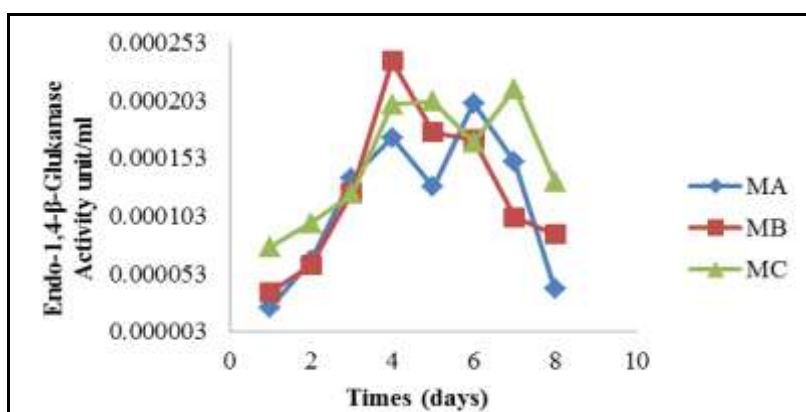


Figure 3. The activity of Endo-1,4-β-Glucanase cellulolytic bacterial consortium in several particle sizes; rice straw particle size MA= 112.0 μm; MB= 53.0 μm and MC= 38.0 μm

According to (Figure 3) the activity of Endo-1,4-- β -Glucanase from cellulolytic bacterial consortium for particle size 112.0 μm increased until the fourth day and decreased on the fifth day. The enzymatic activity increased again on the sixth day then decreased on the seventh day. For the particle size 53.0 μm enzymatic activity increased until the sixth day then decreased on the seventh day. For the particle size 38.0 μm the enzymatic activity fluctuated. The best enzymatic activity for the particle sizes 112.0, 53.0 and 38.0 are on the sixth, fourth and seventh day respectively. Enzymatic activity of Endo-1,4- β -Glucanase was not different ($p>0,05$) for the different particle sizes used in this research. From the research it is concluded that the enzymatic activity of Endo-1,4- β -Glucanase was the highest in rice straw of particle size 53.0 μm for 23.70×10^{-5} unit/mL.

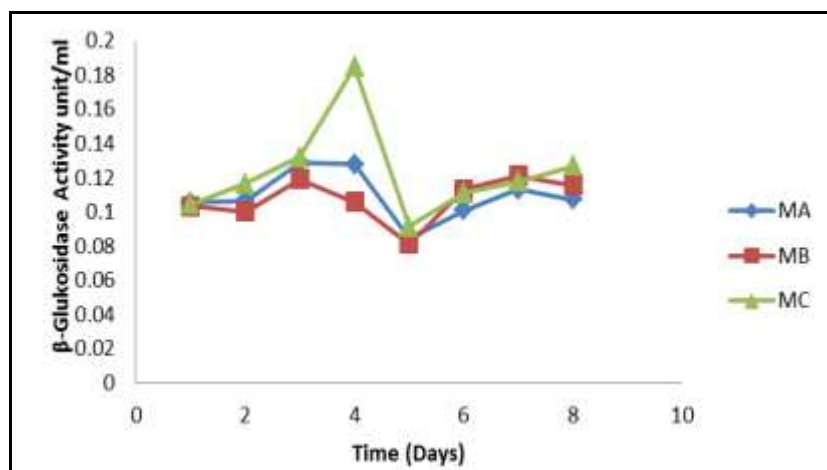


Figure 4. The activity of β -Glucosidase cellulolytic bacterial consortium in several particle sizes; rice straw particle size MA= 112 μm ; MB= 53 μm and MC= 38 μm

(Figure 4) shows that activity of β -Glucosidase for the cellulolytic bacterial consortium for the particle sizes 112.0 μm and 53.0 μm had the same pattern; it increased until the third day and decreased on the fourth and fifth days, followed by an increase of activity on the eighth day. The particle size 38.0 μm has the same pattern as the particle sizes 112.0 and 53.0 μm , but on the fourth day the enzymatic activity increased significantly. This condition also shows the best enzymatic activity is when the particle size used is 38.0 μm . Complex enzyme β -1,4-glucosidase or cellobiase is the third enzyme complex from cellulase enzyme¹³, which has the function of hydrolyzing cellobiose to glucose. From the research it is concluded that the enzymatic activity of enzyme β -1,4-Glucanase is the highest in rice straw of particle size 38.0 μm for 18.48×10^{-2} unit/mL.

Cellulase enzymes consist of Exo- β -1,4-glucanase (avicellase, cellobiohydrolase, C1 cellulase), complex Endo- β -1,4-glucanase (CMCase, Cx cellulase endocellulase, or carboxymethylcellulase)²¹, and β -Glucosidase. Exoglucanase hydrolyzes cellulose by breaking the cellulose chain in the end side to produce cellobiose or glucose as the main product. The success of cellulose hydrolysis using enzymes or microbes depends on the cellulose crystalline degree, cellulase enzyme composition, width of contact surface area, ratio of inoculum and substrate, and substrate purity. Lignocellulose with high crystalline degree is more difficult to degrade compared to an amorphous structure. Cellulose particle size reduction can increase the rate of degradation because it decreases the crystalline degree and increases the surface area in contact with the cellulase enzyme²².

Enzymatic activity of CMCase (Endoglucanase) *Botryosphaeria* sp. is higher for the particle sizes 0.84-1.0 mm compared to FPase (Exoglucanase) and β -Glucosidase, which show the maximum activity with the substrate particle size 0.42 - 0.6 mm. *Pleurotus ostreatus* CP-50 produces maximum FPase and CMCase in different particle sizes of sugar cane waste. The best FPase activity is for a particle size of 0.92 mm and the best CMCase activity is for substrate particle size of 1.68 mm. Two factors that influence the growth rate of *Aspergillus awamori* are particle size and chemical composition of wheat and wheat seed. The growth of fungi in different particle sizes has a significant effect in the production of cellulose¹⁵.

Rice Straw Physical Structure after Pretreatment

Scanning electron microscope (SEM) shows the detail of the structural changes for each of the tissues in biomass lignocellulose as the result of the different pretreatment methods. Figure 5 (a-c) shows no specific change on the surface morphology of rice straw without pretreatment; it can be seen that the straw structure is still stiff and organized. This result is the same as previous studies by other researchers that state the structure of corn stover is very well organized when no pretreatment is performed. After thermochemical pretreatment of rice straw it can be seen that the surface morphology of rice straw is starting to be brittle and disorganized (Figure 5d- 5f).

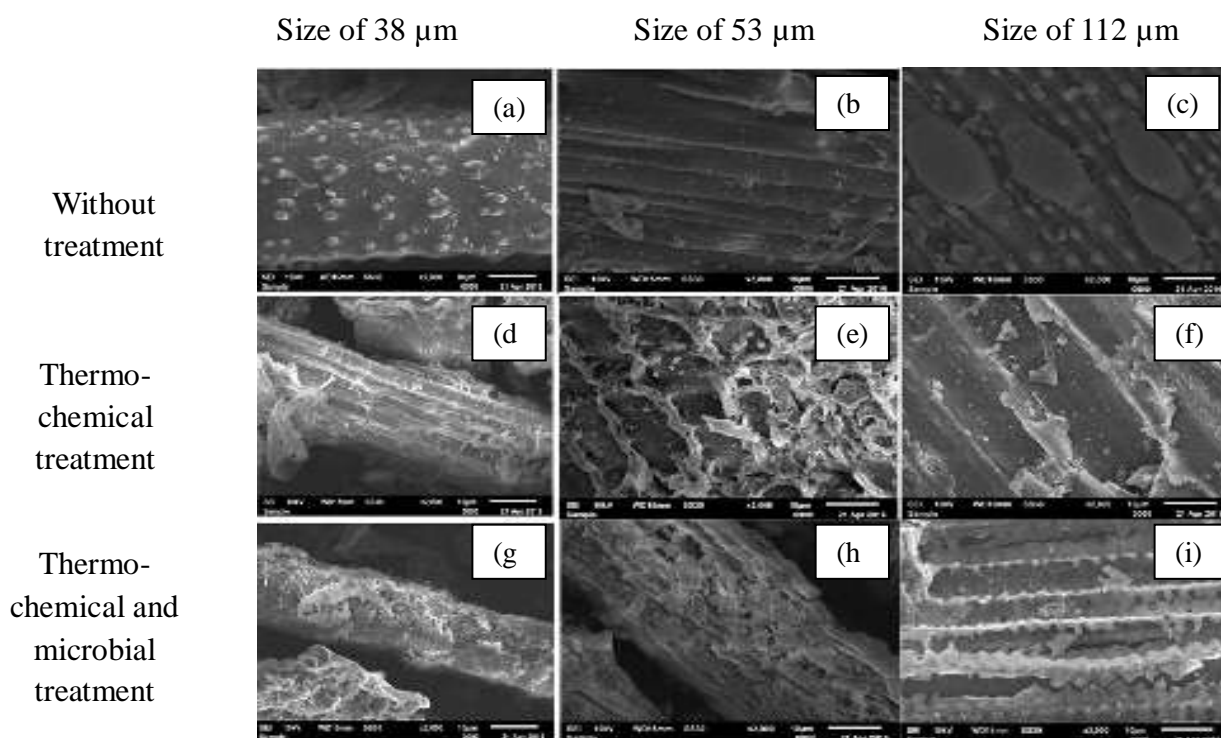


Figure 5. Rice straw morphology can be analyzed by SEM: (a) Without pretreatment for particle size 38.0 μm , (b) 53.0 μm , (c) 112.0 μm , (d) Thermochemical pretreatment for particle size 38.0 μm , (e) 53.0 μm , (f) 112.0 μm , (g) Thermochemical pretreatment followed by microbial degradation for particle size 38.0 μm , (h) 53.0 μm , and (i) 112.0 μm

Spectrometer FTIR analysis using KBr pellets, within the range 4000-400 cm^{-1} with 4 cm^{-1} resolution, shows the lignin and aromatic group are on *wavenumbers* between 1514-1499 under every treatment condition. For the straw size 38.0 μm : (a) without pretreatment, the lignin and aromatic group are on *wavenumber* 1514,02 cm^{-1} , (b) with thermochemical pretreatment they are on 1546,8 cm^{-1} , and (c) with thermochemical pretreatment followed by microbial degradation they are on 1525,29 cm^{-1} . Under the same conditions applied to rice straw with a particle size of 53.0 μm , the lignin and aromatic group are on *wavenumbers*: (a) 1460,01; (b) 1512,09 cm^{-1} , and (c) 1515,94 cm^{-1} . For particle size 112.0 μm lignin and aromatic group are on *wavenumbers*: (a) and (b) 1512,09 cm^{-1} , and (c) 1514,02 cm^{-1} . Lignin and aromatic group, which exist in every particle size of rice straw without pretreatment, start to disappear after thermochemical pretreatment followed by microbial consortium degradation is performed, indicating the reduction of the aromatic ring in lignin²³.

The C-H bond on *wavenumber* 901-873 cm^{-1} exists in particle size of 38.0 μm under every type of treatment. After thermochemical treatment followed by microbial consortium degradation it is found on *wavenumber* 464,81 cm^{-1} and 798,47 cm^{-1} . For the same particle size without pretreatment it is found on *wavenumber* 466,74 cm^{-1} and 794,62 cm^{-1} . For thermochemical pretreatment it is found on *wavenumber* 468,67 and 709,76 cm^{-1} . For the rice straw particle sizes of 53.0 μm and 112.0 μm this bond is also found under every treatment type. From the FTIR result it can be seen that the cellulose and saccharide C-H bonds on *wavenumber* 901-873 cm^{-1} are already deformed and weakened when the rice straw is processed by thermochemical

pretreatment followed by microbial consortium degradation for every particle size of rice straw. This indicates that the cellulose content has decreased after the delignification process⁵.

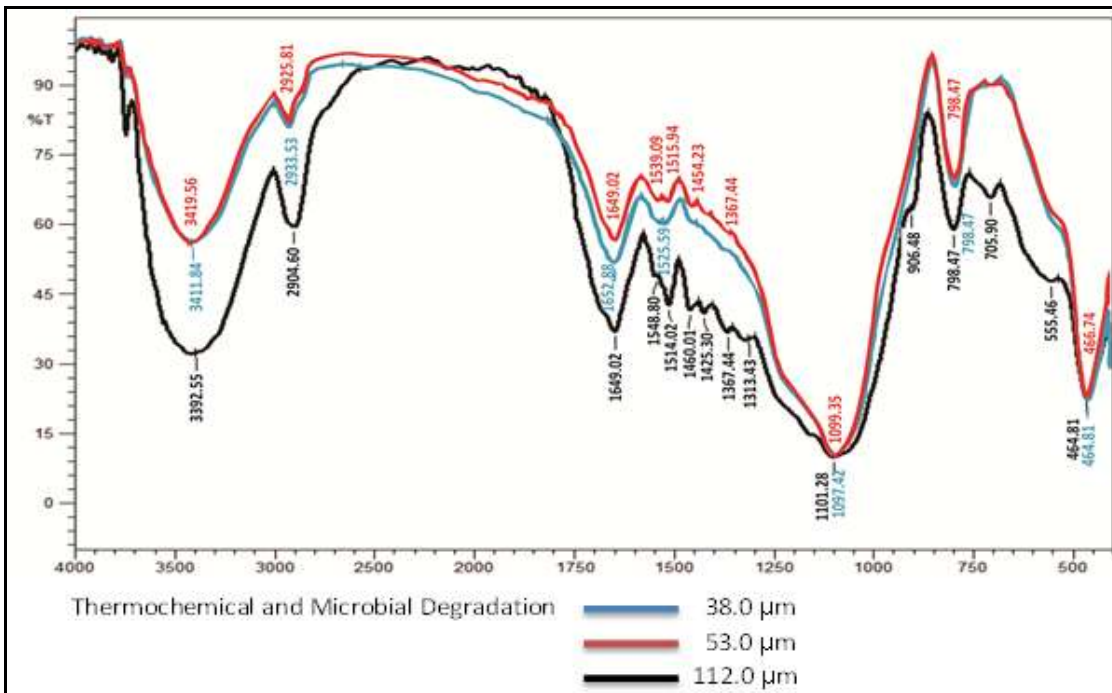


Figure 6. The FTIR result of the several particle sized rice straw with delignification thermochemical and microbial degradation

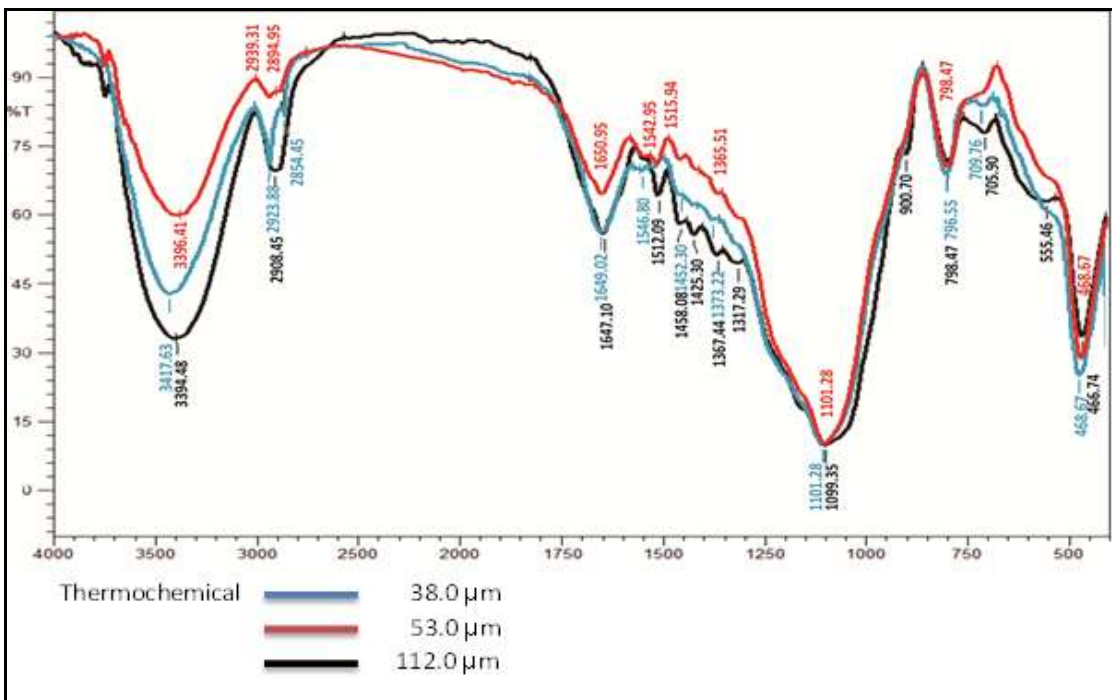


Figure 7. The FTIR result of the several particle sized rice straw with Thermochemical treatment

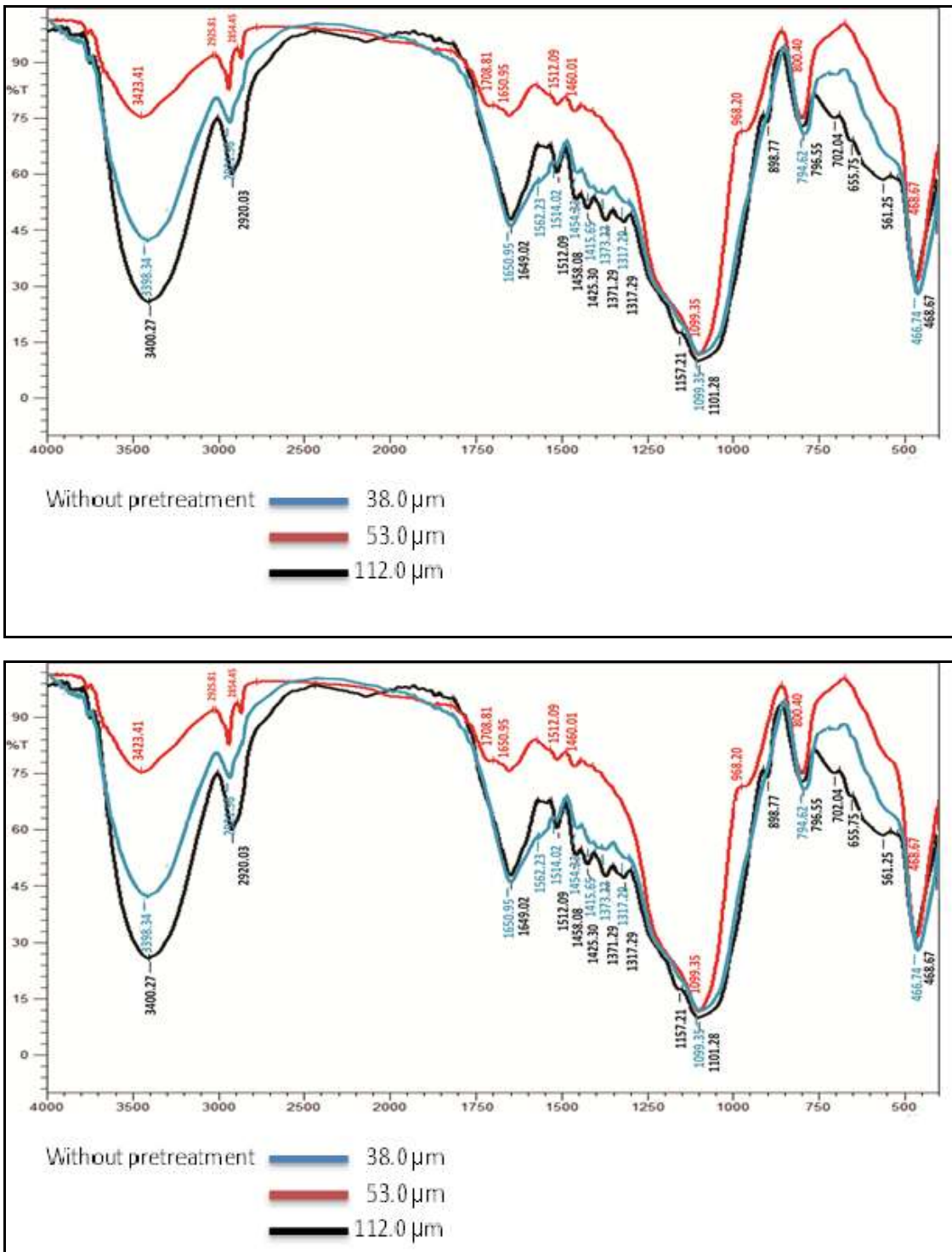


Figure 8. The FTIR result of the several particle sized rice straw without delignification treatment

Conclusion

Thermochemical pretreatment followed by microbial consortium degradation has the ability to degrade rice straw cellulose significantly. The smaller the particle size, the bigger the potential for rice straw cellulose degradation. For particle size 38.0, 53.0, and 112.0 μm the cellulose degradation is 71.96 %, 50.15 %, and 24.03 % respectively.

Acknowledgement

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