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Trametes versicolor as Agent for Delignification of Rice Husks

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ABSTRACT

Rice husks contains 33.71% w/w lignocelluloses, the most abundantly available raw material on the earth for the production of biofuels and other valuable products. It is comprised of the carbohydrate polymers, cellulose, hemicellulose, and an aromatic polymer, lignin. One of the methods for removing the lignin component of rice husks is by delignification using white-rot-fungi. The aim of the study was to carry out delignification of rice husks using white-rot-fungi. The white-rot-fungi used here were Trametes versicolor and Phanerochaete chrysosporium. The study consisted of a biomass and microbial preparation, chemical assay of the rice husk, ligninase enzyme tests, and delignification of rice husks. Results showed that T. versicolor and P. chrysosporium have ligninase enzyme. The precentage of lignin from the total biomass rice husks was 23.61% w/w, and following the delignification process by T. versicolor for 20 days, the remaining lignin was 16.20% w/w, making the percentage of rice husks lignin degraded as 7.41% w/w. The biodelignification process also decreased the percentage of holocellullose, cellulose, and other extracted substances, and accordingly this increased the percentage of hemicellulose. Based on the ability of T. versicolor to degrade lignin of the rice husk at room temperature (28ºC) as mentioned above, it can be concluded that T. versicolor has potential to be used for delignification process.

***Keywords**: lignin, rice husks, white-rot-fungi, P. chrysosporium, T. versicolor*

1. INTRODUCTION

Waste is often defined as a material which is not environmentally useful. Agriculture is one sector that produces a large amount of waste, and one of those is rice husks. Rice husks usually comprise approximately 20-30 % of the grain weight. According to Statictics Indonesia (SI) (2013), West Java alone produced about 12,009,422 tons of rice. Increasing the amount of waste can cause problems for the environment. It is therefore considered to be importat to find alternative uses of the rice husk to make it more beneficial.

According to the Indonesian Center for Agricultural Postharvest Research and Development (2001), the rice husk contain 33.71% w/w of the complex carbohydrate lignocellulose. Lignocellulose can be used as a potentially economical source of sugars for the production of ethanol and other valuable products. However, before these polysaccharides can be enzymatically hydrolyzed, they must first be released from their association with lignin (Reid 1985). Utilization of the rice husk, therefore, involves a delignification process to remove the lignin component in order liberate cellulose. The most efficient and famous lignin degraders are white-rot fungi from Basidiomycota phylum. Representatives of many genera of Actinomycetes and other bacteria can also degrade extracted lignin (Tuomela *et al.* 2000; Yu *et al*. 2005; Zeng *et al*. 2006).

Microbes that potentially can be used for delignification of the rice husk are white-rot fungi. These fungi have three types of non-selective extracellular enzymes which are effective in attacking lignin. These enzymes are lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (MnP) (EC 1.11.1.13), and laccase (Lac) (EC 1.10.3.2) (Howard *et al*. 2003), all being known as lignin-modifying enzymes (LMEs). According to Anita *et al.* (2011), *Trametes versicolor* secretes ligninase enzyme faster than *Phanerochaete chrysosporium*.

Delignification by microbes, such as white-rot fungi, is called biodelignification. It is a process to free cellulose by degrading lignin using enzymes from microbes such as fungi and bacteria. This method is advantageous compared to chemical delignification, since the later has drawbacks such as high chemical requirements, high energy cost, and the chemical delignification effluent is classified as hazardous waste because it is toxic and polluting to the environment (Martina *et al.* 2002).

In present study we analyze the ability of *T. versicolor* to degrade lignin of rice husks. Beside it, this method is to develop environmentally-friendly methods for the delignification process of agricultural waste.

2. MATERIALS AND METHODS

Preparation of Rice Husks and Microbes

Rice husks was ground and sieved to obtain biomass particles of 80 mesh. White-rot- fungi *T.versicolor* and *P.chrysosporium* that had been tested in a preliminary tests (ligninase enzyme tests) were grown on potatose dextrose agar (PDA) and incubated at room temperature (28ºC) for 5-7 d. The cultures were stored at 4°C.

The chemical assays of rice husks

Tests performed were the determination of extracted substances, lignin, holoselulosa, hemicellulose, and cellulose (2 repetition).

Extracted substances

This measurement is based on SNI 1032 numbers. Firstly, 2.0 g of dry raw rice husks of 80 mesh were pondered, then put in a thimble of soxlet. The thimbel was covered with fine gauze to avoid the loss of the specimen. Then the rice husk powder was extracted with ethanol-benzene 1:2 v/v for 6.0 h. After that, the chaff was transferred to a Buchner funnel, and the solvent was removed by vacuum. The thimble was then washed with ethanol to remove benzene. Rice husks were then transferred into a thimble, and were extracted with ethanol 95% for 4 hours. Rice husks were transferred to the Buchner funnel and the solvent was removed by vacuum. The thimble was then washed with distilled water to remove ethanol. After that the rice husk were transferred into a 1.0 L beaker followed with addition of 500 ml hot water. Rice husks sample were dried in air and stored in a sealed container (BSN 1989).

$$\text{Extracted substances} = \frac{\text{initial weight - dry weight (g)}}{\text{dry weight of sample (g)}} \times 100\%$$

Klason Lignin Assay

This measurement is based on SNI 0492 numbers. A total of 1.0 g of rice husks dry raw were put in a the beaker. Then 15.0 ml of 72% v/v sulfuric acid was added. The addition of acid was done slowly and gradually while stirring, and the temperature was maintained at 20ºC. Once the content was completely mixed, the beaker was kept at 20ºC for 2 hours with occasional stirring. As much as 300-400 ml of distilled water was added to a 1.0 L Erlenmeyer flask, and the sample was then transferred into an Erlenmeyer flask. The sample was diluted with water to achieve a concentration of 3% w/v with a total volume of 575 ml. The sample was boiled for 4.0 h, and the solution volume was maintained by adding of hot water. Lignin was filtered with a glass filter and washed with hot water until it was free of acid. Samples were dried in an oven at 105ºC to give a constant weight, cooled, and weighed (BSN 2008).

$$\text{Lignin} = \frac{\text{weight of lignin (g)}}{\text{dry weight of sample (g)}} \times 100\%$$

Holocellulose Assay

A total of 2.0 g of rice husks dry raw were put in a 250 ml Erlenmenyer flask, followed by addition of 80 ml distilled water, 1.0 g of sodium chlorite, and 0.5 ml of glacial acetic acid. The solution was heated in a water bath to a temperature of 70ºC. The water surface in the waterbath was maintained so that it was not higher than the solution in the Erlenmeyer flask. Into the solution was added 1.0 g of sodium

chlorite and 0.5 ml of acetic acid at every 1.0 h interval. The addition was done 4x. The mixture was filtered using a glass filter and washed with hot water. To the residue was added 25 ml of 10% v/v acetic acid, and the residue was then washed with hot water until it was free of acid. The residue was then dried in an oven at 105ºC it reached a constant weight (Browning 1967).

$$\text{Holocellulose} = \frac{\text{weight of holocellulose (g)}}{\text{dry weight of sample (g)}} \times 100\%$$

Hemicellulose Assay

This measurement is based on SNI 0444 numbers. Principle of this method is holoselulosa free lignin treated by sodium hydroxide and acetic acid. Residues expressed as alpha cellulose and soluble fractions expressed as levels of hemicellulose. Determination of alpha cellulose are a total of 2.0 g of dry holocellulose sample was put in a 250 ml beaker. A total of 10 ml of 17.5% w/v NaOH solution was added at a temperature of 20ºC and stirred slowly. After that, at an intervals of 5 minutes, as much as 5 ml of 17.5 % w/v NaOH solution was added. The addition was performed 3x making the total volume of 17.5 % w/v NaOH in the mixture to 25 ml. Following the last addition, the mixture was then incubated for 30 min. Following addition of 33 ml of distilled water , the mixture was stirred and incubated for 1.0 h at 20ºC. The mixture was filtered with a filter cup and rinsed with 100 ml of 8.3 % w/v NaOH . Flushing with distilled water was continued until all the mixture was transferred to the filter cup. After that, the mixture was flushed with 250 ml of distilled water. The residue was dried at 105ºC for 24 h, then cooled in a desiccator and weighed to a constant weight (Cross and Bevan 1907).

$$\text{Alpha cellulose} = \frac{\text{weight of alpha cellulose (g)}}{\text{dry weight of sample (g)}} \times 100\ \%$$

Cellulose Assay

A total of 2.0 g of dry raw was put into a 300 ml Erlenmeyer flask to which was added 125 ml of 3.5% v/v nitric acid solution and incubated in a water bath for 12.0 h at a temperature of 80ºC. Then the mixture was rinsed with distilled water until colorless, then dried in air. The mixture was transferred to an Erlenmeyer flask, followed by addition of 125 ml of solution containing NaOH and Na_2SO_3 and incubated for 2.0 h at 50ºC. The mixture was filtered with a filter cup and rinsed with distilled water until the filtrate was colorless. Then 50 ml of 10% w/v sodium chlorite was added and the filtrate was washed with water to obtain a white precipitate. Following the addition of 100 ml of 10% v/v acetic acid the resultant precipitate was washed until free of acid. The precipitate was then dried in an oven at a temperature of 105ºC until reaching a constant weight (Cross and Bevan 191).

$$\text{Cellulose} = \frac{\text{weight of cellulose (g)}}{\text{dry weight of sample (g)}} \times 100\%$$

Ligninase Tests

To test for the presence of lignin degrading enzymes (ligninase) which includes lignin peroxidase (EC 1.11.1.14) and laccase (EC 1.10.3.2), a test spot was conducted by applying a drop of 1.0% w/v pyrogallol solution mixed with 0.4% v/v H_2O_2 (1:1) at the edge of the white rot fungal cultures tested (which is still actively growing). Culture was observed following incubation of 3.0 h, 24 h, and 72 h. A brownish yellow color at the point of the pyrogallol spilled indicated the presence of lignin peroxidase enzyme activity (Agustini *et al*. 2011). Laccase activity was detected using 100 mM 1-naphthol dissolved in 96% w/v ethanol, by conducting a spot test at the edge of the white rot fungal cultures tested (which is still actively growing). The presence of a purplish-red color at the point of the spilled 100 mM 1-naphthol reagent indicated the presence of laccase enzyme synthesized by white -rot -fungi in the culture (Stalpers 1978).

Delignification of Rice Husks

Delignification process was conducted using a type of white-rot fungi, the *T.versicolor*. This fungus was selected based on its excellent growth and ligninase activity. A total of 240 g of rice husks were soaked in distilled water for 24 h, and then sterilized using an autoclave at 121^0C for 15 min. After that, the mixture was inoculated with 100 ml of *T.versicolor* suspension (the *T.versicolor* was previously grown on potato dextrose broth (PDB) for 7 d). The mixture was incubated at room temperature (28ºC) for 20 d followed by analysis of the extractable substances, lignin, holocellulose, hemicellulose, and cellulose (modified Akhtar *et al.* 1997).

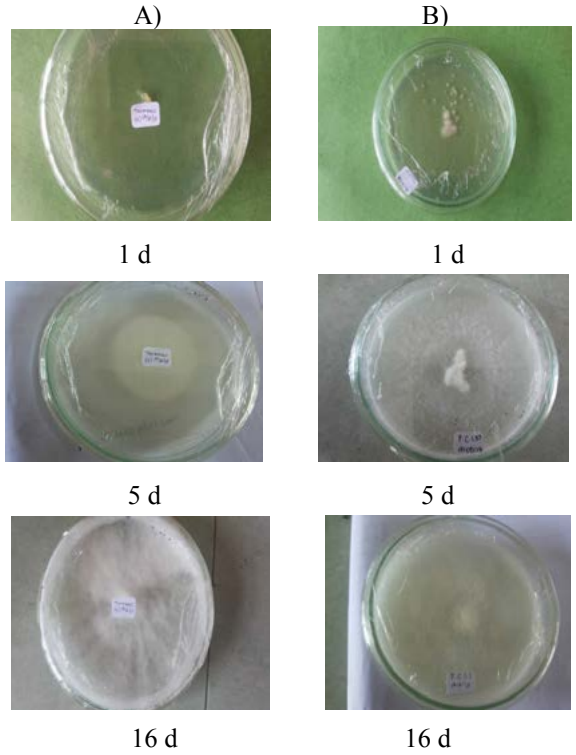

Figure 1. (A) Culture of *T.versicolor* after 1, 5, and 16 d of incubation. (B) Culture of *P. chrysosporium* after 1, 5, and 16 d of incubation.

3. RESULTS

Growth of *T. versicolor* and *P. chrysosporium*

The fungi *T.versicolor* and *P. chrysosporium* were grown on PDA at room temperature (28ºC) prior to their storage in the refrigerator. Isolates with strong growth were used for the following tests. The fungal cultures can be seen in Fig 1. Results showed that both T. *versicolor* and *P. chrysosporium* grew well on PDA at room temperature (28ºC). These fungi were then employed for ligninase tests.

Based on observations during 16 d, the growth of *T. versicolor* is slower than the growth of *P. chrysosporium*. On the first day, the fungus *T. versicolor* showed growth yet, but *P. chrysosporium* already have sign of growth. On the fifth day, the hyphae of *P. chrysosporium* was already nearly covered most of the surface of a petri dish, whereas *T. versicolor* growing circle formed in the middle of the petri dish. On the sixteen day, the growth of both fungi already covered the entire surface of a petri dish, hyphae of *T. versicolor* look thicker than the hyphae of *P. chrysosporium*.

Ligninase Activity

This test was performed to detect the present of ligninase enzymes of *T. versicolor* and *P. chrysosporium*. The results are presented in Fig 2. Results showed that both *T. versicolor* and *P. chrysosporium* have ligninase enzymes present indicated by the appearance of brownish-yellow color as an indication of lignin peroxidase and purplish-red color as an indication of laccase. These results indicated that the white-rot fungi have the ability to degrade lignin.

Delignification of Rice Husks by *T.versicolor*

The delignification process was carried out using *T.versicolor* in a heat resistant plastic bag as shown in Fig 3 for 20 d. The effectiveness of the delignification process was determined by comparing the chemical content of the rice husk before and after delignification process (20 d). The chemical content of rice husks before and after delignification process is shown in Table 1 and Fig 4.

Fungus *T.versicolor* grows well during the process of delignification. The fungal hyphae covered the surface of the rice husk powder pile. This fungus grow on the top to the middle of the rice husk powder pile. This fungus grows little on the bottom of the rice husk powder pile. This was probably due to the bottom pile of rice husks powder is not enough air for fungal respiration process. It also occurs in the white-rot-fungus *Omphalina sp.* (Loebis 2008).

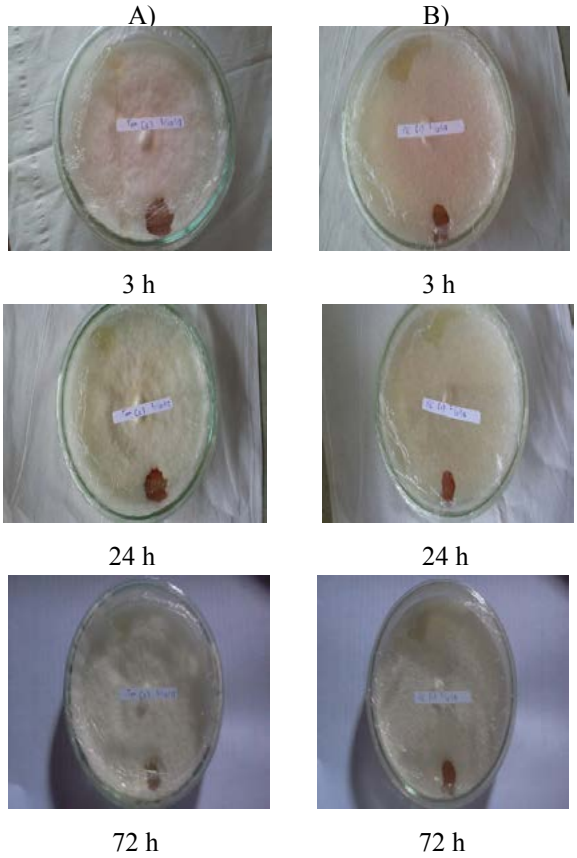

Figure 2. (A) Detection of ligninase of *T.versicolor* after 3, 24, and 72 h of incubation. (B) Detection of ligninase of *P. chrysosporium* after 3, 24, and 72 h of incubation.

Results showed that *T. versicolor* has the ability to degrade lignin. The chemical assay of the rice husk before and after delignification showed that there was reduction of lignin content. Prior to delignification process the percentage of lignin out of the total biomass of rice husks was 23.61 % w/w. Following delignification the percentage of lignin was reduce to 16.20 % w/w.

The delignification process also reduced the cellulose and holocellulose content. The cellulose content of rice husks was reduced from 33.07% w/w to 13.02 % w/w. The percentage of holocellulose was also reduced from 67.08 % w/w to 50.65 % w/w. Hemicellulose of rice husks was calculated by difference from the percentage of holocellulose and cellulose. The percentage of hemicellulose from the total biomassa of rice husks was increased by 3.62% w/w. Percentage of extractable substances on rice husks biomass was reduced from 5.87 % w/w to 3.69% w/w.

4. DISCUSSION

The present study has shown the ability of white-rot-fungus *T. versicolor* to degrade the lignin component of the rice husk which potentially facilitates the ultilization of the cellulose component for production of biofuels and other valuable products. Two white-rot fungi, *T. versicolor* and *P. chrysosporium,* were tested for detecting ligninase enzyme and then the *T. versicolor* was selected

Table 1 The chemical content of rice husks before and after delignification over 20 d

Material	Before (0 d)	After (20 d)	Difference
Lignin (% w/w)	23.61	16.20	-7.41
Holocellulose (% w/w)	67.08	50.65	-16.43
Cellulose (% w/w)	33.07	13.02	-20.05
Hemicellulose (% w/w)	34.01	37.63	+ 3.62
Extractive substances (% w/w)	5.87	3.69	-2.18

Figure 3. Delignification process of rice husks using *T.versicolor*

and employed for lignification process. Both *T. versicolor* and *P. chrysosporium* grew on PDA at room temperature and both showed ligninase enzyme. The ligninase tests showed positive results following incubation for 3, 24, and 72 h marked by the appearance a brownish-yellow color indicating the presence of lignin peroxidase and the purplish-red color indicating the presence of laccase enzyme. According to Howard *et al*. (2003), there are three types of non-selective extracellular enzymes produced by white-rot- fungi all of which are effective in attacking lignin. These enzymes are are lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (MnP) (EC 1.11.1.13), and laccase (Lac) (EC 1.10.3.2).

During and after the ligninase tests, the growth of *T. versicolor* and *P. chrysosporium* was observed. *T. versicolor* showed better growth marked by numerous growing hyphae and was adaptive to room temperature. The fungus *P. chrysosporium* grew at room temperature, but it looked less optimal possibly due to its optimum growth temperature is 35-40 ºC (Irawati 2006). Therefore, the fungus *T. versicolor* was selected and used for the delignification process of rice husks.

Rice husks comprised of organic compound such as lignin, chitin, cellulose, hemicellulose, nitrogen substances, lipid, vitamin B, and organic acid. Rice husks also has anorganic compound such as silica (Ismunadji *et al.*1988). On degradation process, using rice husks as a substrat have to through several steps that are delignification for releasing cellulose and henicellulose from lignin complex and depolimerization to get free sugar (Anindyawati 2010).

Prior to its use in the delignification process, the *T. versicolor* was activated by re-culturing on PDA for 7 d. Delignification by *T. versicolor* was performed at room temperature over 20 d. The results of the chemical assays of the rice husk before and after delignification showed measurable reduction of lignin content. The initial lignin percentage from the total biomass was 23.61 % w/w, after delignification the lignin percentage was 16.20 % w/w. Therefore the lignin percentage of the rice husk was reduced by 7.41 % w/w from the total biomass. If the reduction was calculated based on the total lignin, the percent lignin reduction was 31.38 % w/w. Irawati (2006), found that the percentage lignin of sengon wood was reduced 2.51 to 12.59 % w/w following delignification process using *P. chrysosporium*. The present data showed that *T. versicolor* can effectively degrade the lignin component of rice husks, although rice husks is not its natural carbon source. Other white-rot fungi commonly used for delignification processes are *P. chrysosporium*, *Omphalina sp.*, *Marasmus sp.* (Lobos *et al*. 2001; Sun and Cheng 2002; Siswanto *et al.* 2007).

The delignification process not only reduces the percentage of lignin in rice husks, but also the holocellulose and cellulose content. This is because

Figure 4. Chemical content of rice husks before and after delignification

fungi use cellulose as a carbon source for growth. In the present study the percentage of cellulose from total biomass of rice husks was reduced 20.05 % w/w. These results are supported by Irawati (2006) who found that sengon wood cellulose decreases from 21.06 to 42.41 % w/w following delignification process. The percentage of holocellulose was reduced by 16.43% w/w.

Hemicellulose of the rice husk were calculated by difference from the percentage of holocellulose and cellulose. Following delignification process the percentage of hemicellulose increased 3.62 % w/w. This was due to the consumption of cellulose in fungal growth. These data are supported by Irawati (2006), who found that the hemicellulose increases up to 38.91 % w/w following delignification process over 20 days. In addition, the percentage of extractable substances reduced 2.18 % w/w after delignification.

The present study shows that delignification rice husks biomass by *T. versicolor* was successful. This fungus, therefore, has the potential to be employed in the large scale delignification process to degrade the lignin component of lignocellulose of rice husks in order to generate sugar sources for biofuels, industrial fermentations, ruminant animal energy feed, and other valuable products. Delignification using this fungus can also be developed as an alternative environmentally friendly method for lignin degradation.

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