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## The pattern of lignocellulose degradation from Cacao pod using the brown rot (*Serpula lacrymans*) and white rot (*Schizophyllum commune*) fungi

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# The pattern of lignocellulose degradation from Cacao pod using the brown rot (*Serpula lacrymans*) and white rot (*Schizophyllum commune*) fungi

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**Abstract.** The feasibility of lignocellulosic pretreatment is a key step for the production of biorefinery product, since the breakdown of cell walls can increase the access to sugars present in cellulose and hemicellulose while the aromatic compounds released from lignin. Cacao pod is an example of major agricultural residue, which contains high lignocellulose consisting of cellulose 36%, hemicellulose 38% and lignin 15%. In this study, the efficacy of biological pretreatment degrade lignocellulose from cacao pod using microbes brown rot (*Serpula lacrymans*) and white rot (*Schizophyllum commune*) has been examined and compared. The addition of precursor  $\text{CuSO}_4$  to the media was also tested to identify whether this chemicals had an effect on increasing the ability of fungi to degrade lignocellulose. The results revealed that between *S.lacrymans* dan *S.commune* showed different pattern in degrading lignocellulose of cacao pod as indicated by pH, weight loss, total reducing sugars and phenols released. Compared to white rot fungi *S.commune*, the brown rot fungi *S.lacrymans* produces higher levels of sugars and phenols released from cacao pod. The different concentration of  $\text{CuSO}_4$  added on media also emphasised the distinct change on chemicals released from degradation of lignocellulose of cacao pod.

## 1. Introduction

The production of cacao crop in Indonesia reaches 709.33 million tons in 2016 and produced as much as 75% (w/w) of cacao pod [1]. Cacao pod contains high amount of lignocellulose consisting of 14% lignin, 35% cellulose and 37% hemicellulose [2]. All the derivative compounds of lignocellulose can be used as a substrate for the production of biorefinery products. The breakdown of lignocellulose structure can be carried out through biological pretreatment methods, using both white and brown rot fungi [3], such as *Schizophyllum commune*, and *Serpula lacrymans*. *S. commune* could breakdown lignocellulose enzymatically by synthesizing manganese peroxidase (MnP), laccase, and lignin peroxidase (LiP) whereas the brown rot fungus *S. lacrymans* breakdown lignocellulose non-enzymatically by involving Fenton's reaction to produce hydroxyl radical ( $\text{OH}^*$ ) which acts to breakdown crystalline cellulose and lignin [4]. This biotransformation process has been reported to be able to produce various types of high value added chemicals such as vanillin [3].

The use of fungi as a lignocellulose degrading agent can be optimised by the addition of inducers, such as the addition of metal to the lignocellulose degradation process which enhanced the activity of



lignolytic enzyme [6]. Inducers are specific molecules that are able to induce enzyme formation, which can be obtained from various of organic substrates such as wood, rice straw, and fruits [6] or synthetic chemicals such as ferulic acid, copper and  $\text{MnSO}_4$ . Copper is one of the important micronutrients needed by white rot fungi to produce laccase which will induce laccase activity and MnP. The addition of copper plays several roles on the mechanism of lignocellulose degradation which increased the production  $\text{H}_2\text{O}_2$ , and oxalic acid.  $\text{Fe}^{2+}$  and Cu will react with  $\text{H}_2\text{O}_2$  to produce  $\text{OH}^*$ , while the oxalic acid plays a role in initiating the breakdown of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  and produce  $\text{OH}^*$  in Fenton's reaction [7]. Based on research conducted by [8], the addition of copper about  $40 \text{ mg l}^{-1}$  can increase the decomposition of  $\text{H}_2\text{O}_2$  about 76%. This study was conducted to determine the effect of addition  $\text{CuSO}_4$  towards the activity of *S. lacrymans* and *S. commune* in degrading lignocellulose of cacao pod, indicated by the changes of total soluble phenol ( $\text{mg g}^{-1}$ ), total reducing sugars ( $\text{mg g}^{-1}$ ), pH and weight loss (%).

## 2. Materials and Methods

### 2.1. Microorganisms and culture preparation

Pure cultures of *S. lacrymans* and *S. commune* were supplied by Bioindustry Laboratory, Agroindustrial Technology Departement, Faculty of Agricultural Technology, Brawijaya University, and stored at  $4^\circ\text{C}$  (in a freezer). *S. lacrymans* and *S. commune* were grown on malt extract agar (MEA) at  $22 \pm 2^\circ\text{C}$  for 15 days. Agar plugs of mycelia were added to barley grain and grown at  $22 \pm 2^\circ\text{C}$  for 30 days to produce inocula (grain spawn) for the solid state fermentation (SSF).

### 2.2. Solid state fermentation (SSF)

Cacao pod was obtained from farmers in the vicinity of the Universitas Brawijaya, Malang. All cacao pod were chopped into small size (about 1-2 cm length). 10 g of this materials was placed into honey jars (250 ml) and 13 ml of water was added before undergoing a double autoclaved of  $121^\circ\text{C}$  for 1 hour. The prepared materials was then inoculated with 1 g of grain spawn of the appropriate fungal species and incubated for 35 days at the optimal temperatures ( $22 \pm 2^\circ\text{C}$ ).

### 2.3. Aqueous extraction

The first stage of extraction used an aqueous solution to remove any sugars and water-soluble phenolics. 150 ml of purified water was measured into each beaker and boiled to  $80^\circ\text{C}$ , then poured onto each of the samples and mixed at 100 rpm for 15 minutes,  $40^\circ\text{C}$  in an orbital shaker. The jars were subsequently emptied separately into fine muslin netting held in 250 ml beakers. The biomass was squeezed by hand within the muslin netting trapping the liquid produced in a separate beaker. The cake was retained for subsequent solvent extraction. The aqueous was centrifuged at 18,000 rpm for 10 minutes and the centrifuged extract of each sample was filtered separately, through a  $7\mu\text{m}$  glass fibre filter using a Buchner funnel. A second stage of filtration of  $0.65\mu\text{m}$  was then applied to each sample, and filtrate decanted into labelled 50 ml tubes and frozen at  $-20^\circ\text{C}$  until analysis.

### 2.4. Analysis

#### 2.4.1. Total reducing sugar [9]

Total reducing sugar and soluble phenolics analysis were performed on the aqueous extract samples. Reducing sugars were determined colourimetrically by the DNS (dinitrosalicylic acid) method using glucose as the standard and the absorbance was read at 540 nm using a spectrophotometer (UV-Vis merk Thermo Scientific type Genesys 10 UV). The concentration of total reducing sugar was expressed per gram of substrate (dry weight).

#### 2.4.2. Total soluble phenol assay [10]

Phenols were measured colourimetrically using the Folin-Ciocalteu method with gallic acid as the standard and the absorbance was read at 760 nm using a spectrophotometer (UV-Vis merk Thermo Scientific type Genesys 10 UV). The intensity of light absorption at that wavelength is proportional to

the concentration of phenols. The concentration of phenols was expressed per gram of substrate (dry weight).

#### 2.4.3. pH [11]

Fungal culture pH was determined using a pH meter (merk Ohaus type ST20 ). *S. lacrymans* and *S. commune* fungal cultures extract following aqueous extraction (described above). All fungal culture extract were collected sequentially at different time of incubation. 10 ml of fungal extract was added into 250 ml plastic tube and shaking for 15 minute.

#### 2.4.4. Weight loss (dry weight) [12]

Samples were taken at different times (0, 1,2,3,4 and 5 weeks), and three samples were collected at each sampling time. Three samples were removed from the cultivation jars and were oven-dried at 100°C till a constant weight was reached. These were used to determine the weight loss. Weight loss was estimated as the difference between the weight of the whole culture in medium at the beginning and at the end of the pretreatment (Balance Merk Sartorius type GE2102).

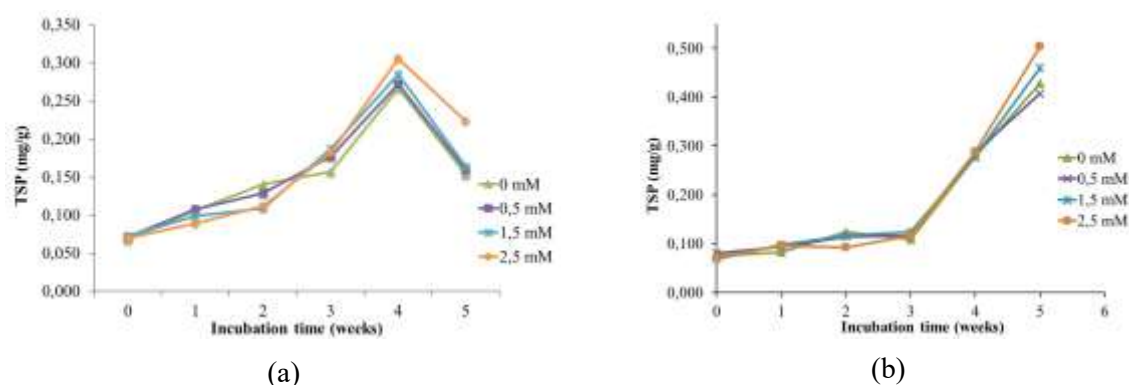
#### 2.4.5. Data Analysis

The research data were analyzed using ANOVA significance test (Minitab 18.0 software) and the significance treatments were tested using least significant difference (LSD) test. In order to minimize results variation, the measurement of each samples was in triplicate.

### 3. Results and Discussion

#### 3.1. Total soluble phenols

The amount of total soluble phenols released from cacao pod indicates the presence of lignin breakdown by lignocellulosic degrading fungi both *S. lacrymans* and *S. commune*. The amount of total soluble phenols released by white rot fungus *S. commune* ranged from 0.067 mg/g to 0.305 mg/g. The degradation of lignocellulose by *S. commune* with addition 2.5mM CuSO<sub>4</sub> at fifth week showed significant amount of phenolic compound released compared to control (without addition CuSO<sub>4</sub>) (Figure 1a), while the highest total soluble phenols produced by *S. lacrymans* achieved at the fourth week. It has been predicted that the longer time of incubation, can produce more lignin derivative product or aromatic compounds. *S. commune* is a white rot fungus that is capable of breaking down lignocellulose structure through enzymatic process. This fungus contains extracellular enzymes such as peroxidase enzymes (LiP and MnP) and laccases [6]. These enzymes play a role in breaking down lignin polymer which produced low molecular weight products.

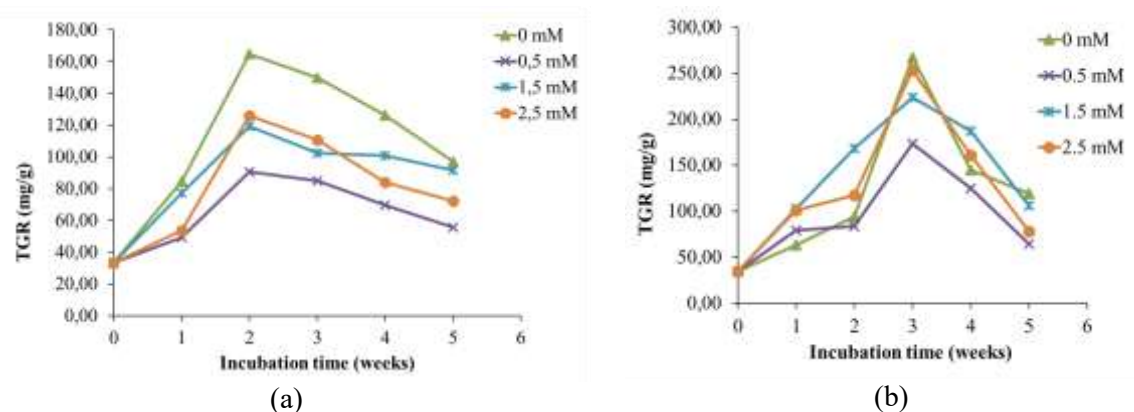


**Figure 1.** The effect of addition CuSO<sub>4</sub> to total soluble phenols (mg/g) from cacao pod extract degraded by (a) *Schizophyllum commune* and (b) *Serpula lacrymans* during 5 weeks incubation.

During breaking down of lignocellulose, the peroxidase enzymes will react with  $\text{H}_2\text{O}_2$  to oxidize the substrate ( $\text{Fe}^{3+}$ ), while laccase will oxidize phenolic to phenoxyl radical by reducing  $\text{O}_2$  to  $\text{H}_2\text{O}$  [3]. The ability of *S. lacrymans* degrades lignin structure has increased at fourth and fifth week. During the breakdown of lignin, *S. lacrymans* utilizes  $\text{OH}^*$  via Fenton reaction. Meanwhile Xu and Goodell [7] stated that the brown rot fungi are able to produce  $\text{H}_2\text{O}_2$  which then react with iron (Fe) naturally contained in lignocellulose fraction and generates  $\text{OH}^*$  and this  $\text{OH}^*$  will damage the structure of lignin.

### 3.2. Total reducing sugars

The average value of the total reducing sugar produced, from cacao pod degrade by *S. lacrymans* (Figure 2b) higher than *S. commune* (Figure 2a). The total reducing sugar released by *S. lacrymans* and *S. commune* ranged from 33.413 mg/g to 266.921 mg/g. By the addition of 1.5 mM  $\text{CuSO}_4$  on media, *S. lacrymans* showed the most significant results on sugar released compared to *S. commune*. The addition of  $\text{CuSO}_4$  on media may possibly increase the enzyme activity in breaking down the structure of lignocellulose by *S. lacrymans* [3]. Cellulase or endo-4-1,4-glucanase (EC 3.2.1.4) is one of the enzymes produced by white rot fungi especially in *S. commune*. This enzyme plays a role in breaking  $\beta$ -1,4-glycosidic bonds in cellulose into simple sugars such as glucose [13].

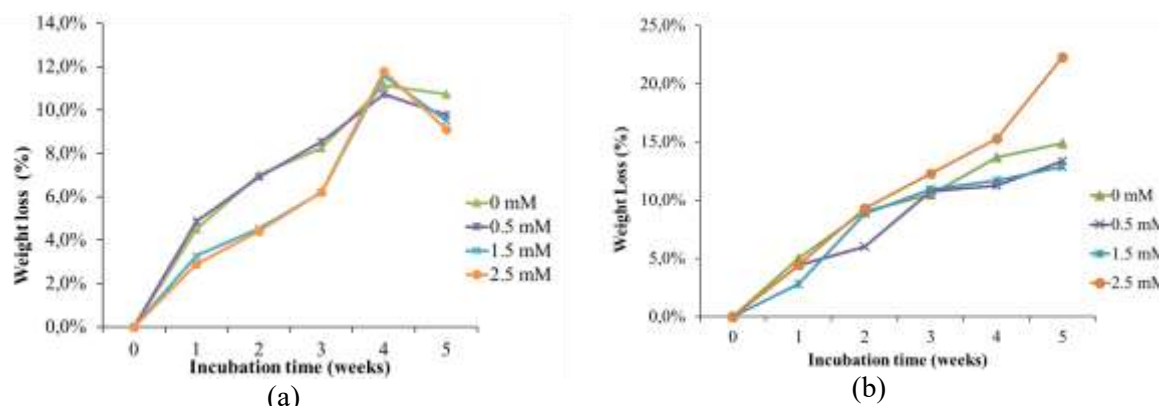


**Figure 2.** The effect of addition  $\text{CuSO}_4$  to total reducing sugars (mg/g) from cacao pod extract degraded by (a) *Schizophyllum commune* and (b) *Serpula lacrymans* during 5 weeks incubation.

Meanwhile *S. lacrymans* also played a role in producing  $\text{OH}^*$  compounds and in breakdown of lignocellulose of cacao pod. The results depicted that the total yield of reducing sugars increase until third week and decreased on the following week (Figure 2b). It is suspected that after three weeks, the fungi also consumed the yield of total reducing sugar released as a fungal nutrient.

### 3.3. Weight loss

The weight loss of the material indicates the loss of lignocellulose structure on the substrate. The average weight loss value during fungal incubated in cacao pod for five weeks ranged 0% to 22.3%. The percentage of weight loss in lignocellulosic materials such as wheat, rice straw, and corn cobs on six days incubation resulted 35%, 24% and 50% respectively [13]. Wheat straw solid state fermentation by *Phanerochaete chrysosporium* was observed for 28 days resulted on weight loss 52% after 28 days incubation [14]. The results show that the longer of the incubation time, the higher percentage of weight loss material obtained (Figure 3a and 3b).

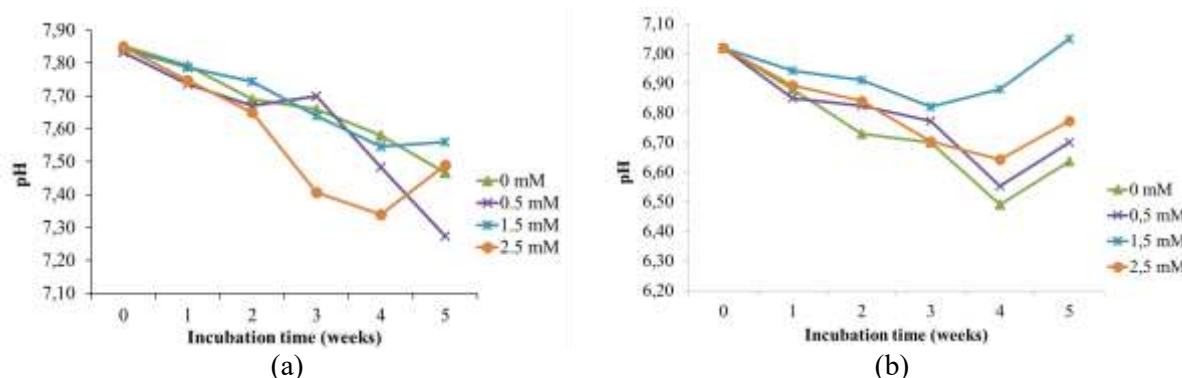


**Figure 3.** The effect of addition CuSO<sub>4</sub> toward weight loss (%) from cacao pod extract degraded by (a) *Schyzophyllum commune* and (b) *Serpula lacrymans* during 5 weeks incubation.

In this study it was found that the weight loss percentage at fifth week by *S. lacrymans* reached 12% while *S. commune* 22.3%. The decreasing of materials weight is due to the degradation of the lignocellulosic of cacao pod by *S. lacrymans* and *S. commune*. The difference pattern on weight loss percentage of the material is thought to be due to differences in the ability of the fungus to breakdown lignocellulose structure especially lignin.

### 3.4. pH

The change of pH is very important factor influences the microbial growth and enzymes activity during solid state fermentation. Figure 4 shows the effect of addition CuSO<sub>4</sub> on the pH extract resulted from degradation substrates by *S. lacrymans* and *S. commune* for 5 weeks. *S. commune* is one type of white rot fungus that is capable of producing oxalic acid. This oxalic acid directly acts to breakdown the structure by generating the hydroxyl radical (OH\*) [3]. *S. lacrymans* is also known to produce oxalic acid which acts to initiate the breakdown of Fe<sup>3+</sup> into Fe<sup>2+</sup> and generate OH\*. According to Nurika [14], the decrease in pH during the SSF on wheat straw by *S. lacrymans* as a result of the production of oxalic acid. In addition, it is suspected that the addition of CuSO<sub>4</sub> can affect the production of organic acids which generated the release of OH\* [15]. During the metabolism, most fungus produces oxalic acid, which caused the decrease on pH [3].



**Figure 4.** The effect of addition CuSO<sub>4</sub> toward pH from cacao pod extract degraded by (a) *Schyzophyllum commune* and (b) *Serpula lacrymans* during 5 weeks incubation.

After substrate was incubated for four weeks, the pH in media incubated by *S. lacrymans* is lower than the pH in *S. commune*. It has been discussed above that both white and brown rot fungi have different mechanism to degrade lignocellulose as well as the pathway to produce oxalic acid, which

affected on the change of pH during incubation. The longer the incubation, the more likely substrate will produce more organic acids.

#### 4. Conclusion

The addition of CuSO<sub>4</sub> affects to the ability of both *S. commune* and *S. lacrymans* breakdown the structure of lignocellulose in cacao pod. With the addition of 2.5 mM CuSO<sub>4</sub> *S. lacrymans* showed a better ability on releasing phenols compared to *S. commune*. However, the pattern of both fungi *S. lacrymans* and *S. commune* on releasing sugars, the change of pH and weight loss were varies during incubation.

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