Screening of Basidiomycetes with laccase activity for lignin degradation on POME

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Abstract. Laccase producing Basidiomycetes were selected to degrade lignin on Palm Oil Mill Effluent (POME). The selection to obtain fungal isolates which produce high activity ligninolytic enzymes has been done continuously. The objective of the research was to isolate high activity enzymes producing fungus and obtain the data on enzyme activity changes after the addition of inducer. Fruiting body of fungus were collected in surrounding area of Cibinong Sciences Centre and Bogor Botanical Garden. Twelve isolates were obtained and all of the isolates could grow on Poly R-478. Isolate Mycena sp J24 produced the highest laccase activity. The fungus was able to decolorize Poly R-478 as much as 89.73% in 21 days incubation. The addition of CuSO$_4$ increased decolorization rate as much as 2.32%. Mycena sp J24 reduced 33.82% of POME color in fifteen days incubation. The addition of CuSO$_4$ increased POME decolorization as much as 72.11%. This fungus could also reduce COD level on POME as much as 33.14% in fifteen days incubation. The addition of veratryl alcohol increased the reduction of COD level as much as 55.15%. Mycena sp J24 produced the highest amount of laccase and could be applied on POME degradation as well.

1. Introduction
Basidiomycetes is a group of fungus which a part of its member has the ability to secrete laccase. White rot is known as effective ligninolytic-producer basidiomycetes. These are some white rot species which has shown the ability to generate enzymes; Phanerochaeta chrysosporium, Coriolus hirsutus, Phlebia radiata, Coriolus versicolor, Agaricus bisporus, Pleurotus ostreatus. White rot secretes one to three types of extracellular enzymes that represent important role in lignin decomposition. Those enzymes are Lignin Peroxidase (EC 1.11.1.14), Mn-dependent Peroxidase (EC 1.11.1.13) and Cu containing-Phenoloxidase, Laccase (EC 1.10.3.2) [1]. Lignin, a complex aromatic biopolymer, strengthens wood rigidity and protect it against microbial infections [2]. Laccase, one of ligninolytic enzyme, oxidizes organic and anorganic compound such as phenol (catechol, hydroquinone, 2,6-dimethoxyphenol and syringaldazine) [3].

Most of white rot fungus produce enzymes with low activity. However, it can be improved by adding inducers such as aromatic or phenolic compound, metal, alcohol and detergent [4]. Higher enzyme activity ensures faster substrate reaction and increase the applicability and effectiveness of enzyme-catalyzed processes [5]. The addition of 200 mL of CuSO$_4$ on a submerged Volvariella volvacea culture produced the highest laccase activity [6]. The addition of sucrose on submerged P. ostreatus increase the activity of laccase [7]. Other than frying oil as its main product, palm oil industries also produce waste in a form of effluent and solid waste. Palm Oil Mill Effluent (POME) is a viscous, brownish, putrid, high colloid-containing liquid waste [8]. POME is the residual wastewater which was used for boiling fresh palm fruits bunches. Fresh POME contains 38.36% cellulose,
23.21% hemicellulose and 26.72% lignin [9]. As the component supporting plants tissues, lignin is recalcitrant. Direct waste disposal into the water body will result in many problems for the environment [10]. Environmental pollution corresponds to the level of organic matters in the water body (COD= 40,000-50,000 mg/L, BOD= 20,000-25,000 mg/L). Indonesia’s production of POME is estimated to be 28.7 juta tonnes/year [11].

Some members of Basidiomycetes especially the white rot have the ability to produce ligninolytic enzymes with high enzyme activity. However, there has not been a successful research to obtain it. Therefore, the objectives of this research was to isolate and select Basidiomycetes fungi with the ability to produce high laccase activity to degrade lignin on POME.

2. Materials and Methods

2.1. Microorganism
Fresh fruiting body of Basidiomycetes was collected from two different places; Cibinong Science Center (CSC) and Bogor Botanical Garden (BBG). The samples were brought to the laboratory to be isolated until pure isolates were obtained.

2.2. Media
PDA (Difco), PDB and Poly R-478 media [12]. PDA composition: 4.0 g Potato starch; 20.0 g Dextrose; 15.0 g Agar. PDB composition: 4.0 g Potato starch; 20.0 g Dextrose. Poly R-478 media composition: 0.60 g KH₂PO₄; 0.50 g MgSO₄.7H₂O; 0.40 g K₂HPO₄; 0.22 g (NH₄)₂ tartrate; 40.0 g sorbose; 0.20 g Poly R-478 (Sigma); 10.0 mL mineral solution stock added with distilled water until 1 L. Mineral solution stock: 7.4 g CaCl₂.2H₂O; 1.2 g Ferri citrate; 0.7 g ZnSO₄.7H₂O; 0.5 g MnSO₄.4H₂O; 0.1 g CoCl₂.6H₂O; 10.0 mg Thiamin HCl, added with distilled water until 1 L.

2.3. Isolation of fungi
Fungus isolation was done in a laminar airflow by cutting the parts of fresh fruiting body. The part then put on PDA in a petri dish. The cultured petri dish was incubated at the room temperature for two days. The newly grown mycelium was placed on new PDA culture for purification. The pure isolates obtained were stored in agar slants tubes.

2.4. Fungal growth on Poly R-478 liquid media
As much as 5 mL mycelium suspension was inoculated on 45 mL Poly R-478 liquid media in a 100 mL Erlenmeyer flask. It was incubated on a shaker (115 rpm) at the room temperature for five days. The culture then was filtered using Whatman No.1 filter paper, dried in at 80 °C oven for 24 hours [13]. Dried weight was obtained by subtracting initial filter paper weight from the sum of filter paper and mycelium weight.

2.5. Inoculum preparation
Mycelium suspension was produced by making fungal culture on PDA agar slants tubes. As much as 5 mL of sterile aquades was added when the whole agar surface was covered by mycelium. Then, mycelium was taken using a loop inoculation. The mixture of aquades and mycelium was inoculated on PDB (Potato Dextrose Broth) until the total volume reached 50 mL. The culture was incubated on a shaker (115 rpm) at the room temperature for seven days. After incubation, it was centrifugated on 9000 rpm at 4 °C for 15 minutes. Mycelium was washed with aquabidest to form the mycelium suspension.

2.6. Laccase activity measurement
In a test tubes: 0.5 mL buffer citrate pH 6.0 + 0.1 mL ABTS 1 mM and 0.4 mL supernatant enzymes. The tubes were shaken slowly and incubated for 15 minutes at the room temperature. Absorbance was observed on 420 nm [14].
2.7. **Fungal identification**

The fruiting body of the fungal isolate that showed the highest laccase activity was identified using the identification key on Mushrooms and Toadstools afield guide by Geoffrey Kibby [19].

2.8. **Degradation ability of fungi on Poly R-478**

To measure, degradation ability on Poly R-478, as much as 5 mL mycelium suspension was inoculated to 45 mL Poly R-478 media then was incubated on a shaker (115 rpm) at room temperature. Poly R-478 content was determined after seven days incubation. Absorbance was observed on 520 nm.

2.9. **Fungal ability to degrade POME**

2.9.1. **POME decolorization.** Selected fungus was used to decolorize POME. In this research, POME was added with CuSO₄, sucrose, veratryl alcohol to induce laccase activity. Media treatments tested were: 1) 100 mL POME, 2) 100 mL POME + 200 µM CuSO₄, 3) 100 mL POME + 15 g/L sucrose, 4). 100 mL POME+ 40 mM veratryl alcohol. All the media were sterilized and allowed to cool. Then, the as much as 10 mL mycelium suspension was inoculated on media 2-4. Media 1 was the control treatment (without inoculum). All cultures were incubated on a shaker (115 rpm) at room temperature for 15 days. Samples were taken and cetrifugated (9000 rpm) at room temperature to measure the level of decolorization. Supernatant’s absorbance was read at the wavelength of 600 nm using spectrophotometer.

2.9.2. **The decline of COD level on POME.** The decrease of COD level was observed on day 15 with spectrophotometer at 600 nm [15].

3. **Results and Discussion**

The fruiting body of Basidiomycetes was taken from two different places; Cibinong Science Centre (CSC) and Bogor Botanical Garden (BBG). The fungus grow on wood log, branches, trunks, bamboos and plants litter on the forest floor. Fresh samples were collected then brought to the laboratorium for isolation. Isolation is the process to culture the sample on media agar until pure isolates were obtained, twelve isolates were acquired from both places (Table 1).

| No | Isolates code | Medium       | Location |
|----|---------------|--------------|----------|
| 1  | J2            | Decayed wood | CSC      |
| 2  | J6            | Decayed wood | CSC      |
| 3  | J8            | Forest litter| CSC      |
| 4  | J10           | Decayed wood | CSC      |
| 5  | J14           | Decayed wood | BBG      |
| 6  | J15           | Decayed wood | BBG      |
| 7  | J17           | Decayed wood | BBG      |
| 8  | J19           | Decayed wood | BBG      |
| 9  | J20           | Bamboo       | BBG      |
| 10 | J21           | Decayed wood | BBG      |
| 11 | J22           | Decayed wood | BBG      |
| 12 | J24           | Bamboo       | BBG      |

Fungal collection process was done during dry season consequently only few fruiting bodies were found. Fungus availability heavily depends on substrate accessibility. If there were abundant amount of decayed wood, in certain range of time fungus will appear rapidly. Wood type also affect the growth of fungi. Aside from those factors, weather and season play important role in fungal growth. The humidity is high on wet season therefore fungus can be found plentifully. Low humidity on dry season does not support the growth of mycelium. Few species of Basidiomycetes can still develop in
low humidity. In concise, factors affecting fungal growth are nutrition, gas, light intensity, microclimate, obstacles and interaction between mycelium [16].

Pure isolates were inoculated on Poly R-478 liquid media. The isolate capability to utilize Poly R-478 as carbon source was observed. The result showed that all isolates were able to grow on Poly R-478 liquid media, proven by the rise of mycelium dry weight. The highest dry weight represented by J20 (20.83 g/L) while J15 had the lowest number (3.99 g/L) (Figure 1).

Figure 1. Mycelia dry weight on Poly R-478 liquid media

Poly R-478 liquid media was used on the selection of fungal isolate capability to grow on that media. It is related to each isolates ability to utilize Poly R-478 liquid media as carbon and energy source. Isolates that produced high dry weight were able to degrade and consume Poly R-478 liquid media. These isolates possibly produce higher amount of ligninolytic enzyme than other isolates. *Streptomyces violaceoruber* and *Streptomyces spiroverticillatus* can also use Poly R-478 as the main source of carbon for their growth. *S. violaceoruber* is able to decolorize Poly R-478 faster than *S. spiroverticillatus* [17].

Laccase is essential in POME lignin degradation. Therefore isolates selection based on laccase activity was done. The result was observed using spectrophotometer. The result showed that the range of laccase activity was between 368.98Unit/mL (J24) and 146.75 Unit/mL (J17) (Figure 2).

Figure 2. Laccase activity of some fungal isolates

Isolate number J24 was confirmed to exhibit the highest laccase activity. Therefore, it was selected for the next step. Laccase is an important enzyme in the simplification of lignin. Fungal laccase can oxidize monomer to dimer similar to that produced by chemical lignin degradation. Basically, any substrate with similar characteristics to p-diphenol will be oxidized by laccase. At the least, fungal laccase is able to oxidized monophenol such as cresol and some of them can oxidize ascorbic acid [18].

Isolate J24 was selected to be applied on Poly R-478 degradation complimented by inducer treatments such as CuSO₄, veratryl alcohol and sucrose. Mushrooms and Toadstools a field guide [19]-based identification confirmed that J24 species name was *Mycena* sp. Different percentage of
The highest level of decolorization was observed on CuSO₄ addition treatment (89.73%), followed by fungi + veratryl alcohol, fungi without inducers treatment and fungi + sucrose treatment (Table 2).

Table 2. The ability of Mycena sp. J24 to degrade Poly R-478

| No | Treatment                      | Percentage of decolorization (%) |
|----|--------------------------------|----------------------------------|
| 1  | Fungi                          | 87.69 ± 0.0665                   |
| 2  | Fungi + CuSO₄                  | 89.73 ± 0.9823                   |
| 3  | Fungi + Veratryl alcohol       | 88.79 ± 1.3926                   |
| 4  | Fungi + Sucrose                | 89.64 ± 3.3606                   |

Laccase that produced by Mycena sp. J24 has the ability to degrade Poly R-478. It was shown by Poly R-478 decolorization as much as 87.69% after 21 days incubation. Some researches reported that only lignin decomposing microbes could decolorize polymer colorant. Decolorization efficiency is related to the ability to degrade some lignin models [20]. Polymer decolorization is a simple and quick method to investigate ligninolytic system on microorganisms [21].

Therefore, it is not surprising to find structure similarity between widely used commercial colorant and lignin that can be transformed by ligninolytic enzymes [22]. The addition of CuSO₄ increase ligninolytic enzyme activity thus the process of Poly R-478 decolorization occurred faster. The same effect as also observed on sucrose and veratryl alcohol treatments. The addition of CuSO₄ until 1 mM did not affect the growth of Trametes trogii but prompt the production of ligninolytic enzyme consequently increase the speed of polymer decolorization [23].

It was presented that Mycena sp. J24 has the ability to degrade Poly R-478. This species was chosen to degrade POME with the addition of some inducers addition. The highest decolorization percentage was observed on CuSO₄ addition followed by veratryl alcohol, sucrose and without inducer treatment. Mycena sp. J24 and CuSO₄ as inducer could decolorize POME as much as 58.21%. The lowest number was shown by only fungi (without inducer) treatment (33.82%).

Table 3. POME degradation by Mycena sp J24

| No | Treatment                  | POME decolorization (%) |
|----|----------------------------|-------------------------|
| 1  | Fungi                      | 33.82 b                 |
| 2  | Fungi + CuSO₄              | 58.21 a                 |
| 3  | Fungi + Veratryl alcohol   | 37.21 b                 |
| 4  | Fungi + Sucrose            | 37.17 b                 |

Note: Numbers followed by corresponding letters on the same column showed no significant difference (P<0.05)

The pigment of POME is compound composing plants tissues like lignin and phenolic [24]. It can be degraded by laccase produced by Mycena sp. J24. The treatment of CuSO₄ inducer on Mycena sp. J24 increase the rate of POME decolorization as much as 24.39%. It was the highest rise compared to other inducer’s result. The addition of CuSO₄, sucrose and KNO₃ could increase the speed of POME decolorization by Coprinuscinereus as well [25]. As much as 25.0 mM of CuSO₄ on P. pulmonarius was used to ferment corn cob. A rise on laccase activity was observed, from 270 U/L to 1,420 U/L [26].

The decrease of COD level on POME degradation by Mycena sp J24 with the addition of inducers showed different trend compared to decolorization. The highest COD level reduction was presented by veratryl alcohol inducer treatment (51.42%), followed by fungi without inducer treatment, CuSO₄ and sucrose (5.71%) (Figure 3).
Figure 3. The decrease of COD level on POME by *Mycena* sp. J24

The reduction trend of COD level on POME was different from decolorization. Not only ligninolytic but also cellulolytic and lipase played important role in COD level reduction. The addition of inducers did not increase COD reduction rate. It merely increased laccase activity. Therefore only lignin degradation showed a rise. However, *Mycena* sp J24 was able to reduce COD level as much as 32.95% since this fungi not only produce ligninolytic enzyme but also cellulolytic. *Mycena galopus* produces mannase and xylase if planted on conifer litters [27]. The usage of microbial consortium taken from compost can enhance COD reduction rate from 10.350 mg/L to 1000 mg/L in 7 days incubation [28]. *Candida rugosa* can reduce COD level on POME as much as 50.7% in 6 days incubation [29].

4. Conclusions

Twelve Basidiomycetes isolates were obtain from two different spots. All the isolates grew on Poly R-478 liquid media. *Mycena* sp J24 produce the highest laccase activity and decolorized Poly R-478 as much as 89.73% in 21 days incubation. *Mycena* sp J24 could also degrade POME and decolorized it as much as 33.82% after 15 days incubation. The addition of inducers increased POME decolorization rate. *Mycena* sp J24 decolorized POME as much as 33.14% after 15 days incubation.

5. References
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