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The Interaction between *Marasmius pulcheripes* J8 and Soil Fungi on Laccase Activity for POME Degradation

Yohanes Bernard Subowo✉, Arwan Sugiharto

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Department of Microbiology, Research Centre of Biology, Lembaga Ilmu Pengetahuan Indonesia

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Abstract

A study on the effect of *Marasmius pulcheripes* J8 and soil fungi interaction on the activity and ability of laccase to degrade palm oil mill effluent (POME) was previously conducted, and some Basidiomycetes fungi were identified capable. Therefore, the aim of this study was to determine the ability of *Marasmius pulcheripes* J8 to degrade POME in the presence of inducers, and interactions with soil fungi. Furthermore, 3 types of inducers were applied to elevate its laccase activity, which include CuSO4, sucrose and Ammonium tartrate. In addition, *M. pulcheripes* J8 was grown together with soil fungi, encompassing *Aspergilus niger* NK and *Penicillium* sp R 75, in order to boost the action. The results showed the highest laccase activity was in *M. pulcheripes* J8 pure culture on a PDB medium of 3566.04 U / mL. Moreover, the POME decolorization was up to 74.25% after 20 days of incubation, and reduced COD level was 81%. Meanwhile, the addition of an inducer has never been performed before, and the outcome of this investigation showed the ability of *M. pulcheripes* J8 to degrade POME, and decrease environmental pollution. POME waste treatment using fungi is more affordable than other methods.

How to Cite

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✉ Correspondence Author:
Jl. Raya Bogor Km 46 Cibinong 16911 Bogor
E-mail: yosubowo@yahoo.com

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INTRODUCTION

Some industries that use plants as raw materials, including for textile and paper, require the use of laccase to degrade their lignin content, in order to obtain a better yield. This enzyme is able to break down wood, plastic, paint and jet fuel into nutrients (Majeau et al., 2010). Similarly, in the palm oil industry, it is also needed in the decomposition of lignin contained in Palm Oil Mill Effluent (POME), which consists of colloidal slurry of water (95 – 96%), oil (0.6 – 0.7%), total solid (4 – 5%), suspended solid (2 – 5%), temperature (80-90 °C), acidic (pH 3.84.5) (Onyia et al., 2001).

Lignin contained in POME is the byproduct of palm oil. Lignin content in palm fruit was found 25 – 28 % in mesocarp (Rizal et al., 2018) and 44 % in endocarp (Zainal et al., 2017). In addition, it spreads on the entire plant tissues in order to strengthen and maintain stability of the cell walls, creating a rigid pipe-like structure, which is necessary for vascular plants to transport water and nutrition. Simultaneously, tissues that consist of cellulose, hemicellulose and lignin form a biocomposite that supports the tree (Janusz et al., 2017). .

Basidiomycetes are a group of fungi that are able to decompose lignin, especially the white rot type. These microorganisms secrete at least one of three extracellular enzymes to enhance the degradation, which include Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase (Lac). Laccase oxidizes organic and inorganic compounds, including phenol (catechol, hydroquinone, 2,6-dimethoxyphenol, and syringaldazine). *Marasmius pulcheripes J8* is a member of the white rot group, small in size and is often found on the surface of litterfall.

Most of this group of fungi secretes laccase with low enzyme activity, which is possibly to be enhanced by the addition of inducers, including aromatic or phenolic compounds, metals, alcohols, and detergents (Leonowicz et al., 2001). Furthermore, elevated functionality results in superior and faster modification in substrate degradation, and also better efficiency in enzyme catalysis (Rao et al., 2014). Moreover, the addition of 200 µM $CuSO_4$ into a submerged *Volvariela volvacea* culture was able to produce the highest laccase activity (Chen et al., 2003), which was also obtained through the application of 15 g / L sucrose to submerged *Pleurotus ostreatus* culture (Subowo, 2015). In addition, laccase activity has also been reported to be influenced by carbon and nitrogen concentrations in the media, D'Agostini et al. (2011) reported that. higher ratio of C/N led to better mycelium growth. However, it lowered laccase production.

The interaction between Basidiomycetes and soil fungi has been reported to ultimately elevate the laccase functionality. Baldrian (2004) stated an over 40 times increase in activity, by virtue of the addition of *Trichoderma harzianum* to *Trametes versicolor*. Furthermore, the addition of soil fungi or bacteria causes an upsurge in laccase activity as well, by up to 2–25 times, and the addition of soil or soil extract causes up to 10-15 times intensifications. Meanwhile, the usage of *Marasmius pulcheripes* J8 to decompose POME has never been performed, which was the reason for conducting this investigation. In addition, the objective of the study was to obtain data on the ability of *Marasmius pulcheripes* J8 to degrade palm oil mill effluent with the presence of inducers, as well as its interactions with soil fungi. Waste treatment using fungi will lower the cost needed compared to physical and chemical methods. Fungi are easier and cheaper to grow

METHODS

Marasmius pulcheripes J8 isolates were obtained from the Microbiology department collection, which were stored on Potato Dextrose Agar (PDA) media at -20°C, together with *Aspergillus niger* NK and *Penicillium* sp R 7.5.

The media used were (1) PDA, composed of: 4.0 g Potato starch; 20.0 g Dextrose; 15.0 g Agar. (2) PDB (Potato Dextrose Broth), with the following composition: 400.0 g potatoes; 20.0 g Dextrose. (3) Poly R-478 media composition: 0.60 g KH_2PO_4; 0.50 g $MgSO_4.7H_2O$; 0.40 g K_2HPO_4; 0.22 g $(NH_4)_2$ tartrate; 40.0 g Sorbose; 0.20 g Poly R-478 (Sigma); 15.0 g Agar (Oxoid No. 3); 10.0 ml Mineral solution; added with distilled water to 1L. Mineral solution: 7.4 g $CaCl_2.2H_2O$; 1.2 g Ferric citrate; 0.7 g $ZnSO_4.7H_2O$; 0.5 g $MnSO_4.4H_2O$; 0.1 g $CoCl_2.6H_2O$; 10.0 mg Thiamine HCl; with distilled water to 1L

Up to 5 ml of *M. pulcheripes* mycelium was poured into 45 ml of PDB media, which was termed treatment A. Meanwhile, treatment AB encompassed 5 ml mixture of *M. pulcheripes* and *Penicillium* sp R75 mycelium, which was placed into 45 ml of PDB. In addition, treatment AC included a 5 ml mixture of *M. pulcheripes* and *A. niger* NK mycelium, added into 45 ml of PDB, and treatment ABC comprises of *M. pulcheripes*, *Penicillium* sp R 7.5 and *A. niger* NK myceliums, which were mixed together, and 5 ml of the mixture was poured into 45 ml of PDB. Further-

more, all cultures were incubated on shakers at 115 rpm, at room temperature., Subsequently, samples were taken on day 4, which were then centrifuged at 9000 rpm, and the supernatant was used to measure laccase, MnP and LiP activities.

Laccase activity was determined by the amount of 2,2–azino-bis(3ethylbenzothiazoline)-6-sulfonate (ABTS) oxidized (Papinutti et al., 2003). The reaction mixture consisted of 0.5 mL citrate buffer of pH 6, 0.1 mL ABTS 1 mM, and 0.4 mL supernatants. In addition, ABTS oxidation was monitored through an increase in absorbance at 420 nm.

Mangan Peroxidase activity was measured based on the amount of guaiacol oxidized, using a spectrophotometer (Yoshida et al., 1996). The reaction mixture consisted of 0.1 mL guaiacol 4 mM, 0.1 mL lactate buffer 50 M at a pH of 4.5, 0.2 mL $MnSO_4$ 1 mM, 0.3 mL distilled water, 0.1 mL H_2O_2, and 0.2 mL supernatants. Furthermore, guaiacol oxidation was measured based on the increase in absorbance at 465 nm.

Lignin Peroxidase activity (LiP) was measured using Tien & Kirk (1983) method, based on the oxidation of veratryl alcohol into veratryl aldehyde, in the presence of H_2O_2. Moreover, the reaction mixture consisted of 0.1 mL veratryl alcohol 8 mM, 0.2 mL acetate buffer 50 mM of pH 3, 0.45 mL distilled water, 0.05 H_2O_2 5 mM, and 0.2 mL supernatants. Therefore, the increase in absorbance was observed at 310 nm, using three replications.

A 5 ml measurement of *M. pulcheripes* J8 mycelium suspension was inoculated into the Poly R-478 liquid media in an Erlenmeyer flask, up to the point where the final volume reached 50 ml (treatment A). In addition, *Penicillium* sp R 7.5 was integrated, and 5 ml of this admixture was poured into the Poly R-478 media (treatment of AB). Furthermore, Mycelium of *M. pulcheripes* J8 and *A. niger* NK were mixed and placed in 45 ml of Poly R-478 media (treatment AC). Subsequently, the myceliums of all three isolates (*M. pulcheripes* J8, *Penicillium* sp. R 7.5 and *A. niger* NK) were mixed, and 5 ml of mixture was obtained and placed in the Poly R-478 media (treatment ABC), and all cultures were incubated for 10 days on a shaker, at 115 rpm and room temperature. In addition, the samples were filtered using Whatman paper No. 1, and dried in an oven at 80°C for 24 hours. Therefore, the weight of dry mycelium was obtained from the weight of filter paper and mycelium minus the weight of dry filter paper.

The addition of inducers was conducted on the Poly R-478 media before fungal inoculation, including 200 µM $CuSO_4$, 15 g / L sucrose, and 1.6 g / L Ammonium tartrate. Therefore, all cultures were subsequently incubated on a shaker at 115 rpm, and room temperature, then samples were taken on day 4 and centrifuged, therefore, the supernatants were used to measure the laccase activity.

A total of 5 mL of *Marasmius pulcheripes* J8 mycelium was poured into 45 mL of Poly R-478 media + 200 µM $CuSO_4$ (A). Meanwhile, *M. pulcheripes* and *Penicillium* sp R 7.5 was also mixed, and 5 mL poured into the Poly R-478 media + 200 µM $CuSO_4$ (treatment of AB). Therefore, the test fungi and *A. niger* NK were successively mixed and placed in 45 ml of the media + 200 µM $CuSO_4$ (treatment AC). Furthermore, the myceliums of all three isolates were mixed, and 5 ml was obtained and added to the Poly R-478 media + 200 µM $CuSO_4$ (treatment ABC). In addition, all cultures were incubated for 20 days on a shaker, at 115 rpm and room temperature, the color reduction in the media was evaluated using a spectrophotometer after incubation, and absorbance was assessed at a wavelength of 520 nm.

A total of 5 mL of *Marasmius pulcheripes* J8 mycelium was poured into 45 mL of POME + 200 µM $CuSO_4$ (A). *Penicillium* sp R 7.5 was mixed with the fungi, and 5 mL of the combination was poured into the POME + 200 µM $CuSO_4$ (treatment of AB). Furthermore, *A. niger* NK was mixed with the fungi, and then integrated with 45 mL of POME + 200 µM $CuSO_4$ (treatment AC). Therefore, the myceliums of all three isolates were mixed, and 5 mL was taken to the POME + 200 µM $CuSO_4$ (treatment ABC). Subsequently, all cultures were incubated for 20 days on a shaker, at 115 rpm and room temperature, and therefore, samples were then taken and centrifuged at 9000 rpm, and room temperature. In addition, POME decolorization was evaluated by observing the absorbance of supernatant at 600 nm, using a spectrophotometer, and similarly, COD level was measured with the same procedure.

RESULTS AND DISCUSSIONS

Marasmius pulcheripes J8 produces three ligninolytic enzymes, including Laccase, Lignin Peroxidase and Manganese Peroxidase, in Potato Dextrose Broth (PDB) media during the experiment. A method to increase the laccase activity of the Basidiomycetes was performed by creating an interaction with soil fungi (Baldrian, 2004). However, the interaction between *M. pulcheripes* J8 and soil fungi (*Penicillium* sp R75 and *Aspergil-*

lus niger NK) resulted in no increase in the laccase activity of *M. pulcheripes* J8, and conversely the highest laccase activity (3566.04 U / mL) was produced by *M. pulcheripes* J8 in pure culture (A). Therefore, the mix cultures (AB, AC, and ABC) showed a lower laccase activity (Table 1).

The interaction between *M. pulcheripes* J8 and both soil fungi showed no increase in laccase activity, which occurred in all mixed cultures, while the highest activity was observed in the pure culture. That condition is because there is no competition occurred between both microbes in an attempt to obtain resources and space in a pure culture, thus, physiological processes including the production of laccase enzymes ensued optimally. Meanwhile, in mixed cultures, competition arose between microbes, which inhibited each other, leading to reduced enzymes activity. In contrast, in Baldrian's (2004) study which included the interaction between *Trametes versicolor* and *Trichoderma harzianum*, showed that *T. versicolor* laccase activity increased by up to 40 times which the soil fungi used was , known to be a potential mycoparasite. Members of the Trichoderma genus have been confirmed to inhibit several types of white rot fungi (Freitag and Morrell, 1992), whereas *Penicillium* sp R 7.5 and *Aspergillus niger* NK are not included in mycoparasites. The stage of mixing two fungal isolates was also observed to be different, as the Baldrian study reported the addition of *Trichoderma harzianum* on the 11th day after *T. versicolor* grew, while the mixing of *M. pulcheripes* J8 and soil fungi ensued from the first day.

Poly R-478 media, a selective medium, was used to determine ligninase activity in fungi, where the microbe growth indicates the usage of lignin as a source of C and energy. This is also interpreted as such that there is a production of ligninolytic enzymes, which decompose lignin into simple compounds, thus, satisfying their needs. *M. pulcheripes* J8 was observed to have grown on Poly R-478 media, both as pure and mixed cultures and the interaction treatment with soil fungi lead to the creation of different mycelium yields individually. Furthermore, the highest yield of mycelium was obtained from the mixed culture were in the order of AB (1.78 g / L), followed by A, treatment of AC, and finally the mixture of all three (ABC) as seen in Figure 1.

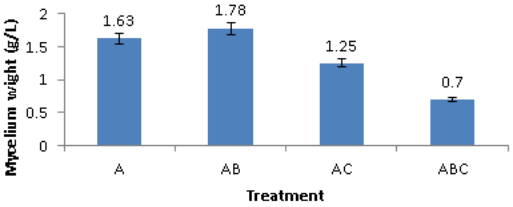

Figure 1. Mycelium yield of *M. pulcheripes* J8 on Poly R-478. A :*Marasmius pulcheripes* J8, B: *Penicillium* sp R 75, C: *Aspergillus niger* NK.

The yield of mycelium is directly correlated to fungal growth, and this study showed the highest to be produced was by AB mixed culture (mixture of *M. pulcheripes* and *Penicillium* sp R7.5), followed by the pure culture of *M. pulcheripes* J8. However, the results of both treatments were not significantly different, thus, it is assumed that fungal growth in pure cultures is generally better than that mixed cultures. In addition, competition for growth and nutrients occured in mixed cultures resulted in inhibited growth of examined

Table 1. Ligninolytic enzymes activity of *Marasmius pulcheripes* J8 and its mixed cultures

	A (U/mL)	AB (U/mL)	AC (U/mL)	ABC (U/mL)
Laccase	3566.04±524.71	1222.37±201.30	1453.08±129.32	1090.12±205.49
LiP	24764.63±2939.19	17745.51±2201.46	16064.51±762.46	13062.12±212.05
MnP	14336.08±1401.35	10785.11±1516.94	8677.68±453.67	6831.95±750.54

Notes: A= *M. pulcheripes* J8; B= *Penicillium sp* R 75; C= *A. niger* NK

Table 2. *M. pulcheripes* J8 laccase activity with the addition of inducer and interaction with soil fungi

Treatment	A (U/mL)	AB (U/mL)	AC (U/mL)	ABC (U/mL)
$CuSO_4$	590.73±52.01	589.35±73.03	589.19±98.80	607.71±69.24
Sucrose	460.79±43.83	452.00±54.06	535.18±46.61	489.96±33.89
Ammonium tartrate	222.68±19.25	233.94±11.94	234.71±43.97	211.26±41.30
Control	313.26±16.57	312.65±18.55	294.13±4.66	253.54±29.43

Notes: A= *M. pulcheripes* J8; B= *Penicillium* sp R 75; C= *A. niger* NK

fungi. This result was in accordance with the study by Chatterjee et al. (2016), where *Aspergillus niger* and *Fusarium verticilloides* were grown on a minimal media with asparagine as the N source. Moreover, the biomass of both fungi increased in pure culture during the growing period and decreased in mixed cultures from the 10th to the 30th day of incubation.

The addition of inducer to the *Marasmius pulcheripes* J8 increased the activity of laccase, and the most significant occurred on the addition of 200 µM $CuSO_4$, followed by 15 g / L sucrose, while the addition of 1.6 g / L Ammonium tartrate lead to a lower laccase activity than the control. Furthermore, the interaction with soil fungi was not capable of elevating this effect, and the value of the four treatments (A, AB, AC, ABC) were not significantly different (Table 2).

The additions of inducer increased the laccase activity of *M. pulcherioes* J8, e.g., 200µM $CuSO_4$ on Poly R-478 media contributed an 88.57% increase; 15 g / L of sucrose increased it by 47.09%, while the addition of Ammonium tartrate resulted in a decline in activity, which was observed to be lower than the control. Meanwhile, the interaction treatment with soil fungi had no impact on laccase activity. $CuSO_4$ is often used as inducer to grow, some white rot fungi types. According to Baldrian and Gabriel (2002), some compounds are responsible for causing a positive response in the production of laccase, and they are known to consist of metal, copper and cadmium ions. Cu^{+2} has been reported to possess inductive capabilities by forming an inseparable prosthetic group (Soden & Dobson, 2001). Meanwhile, the addition of sucrose increases activity by many factors, one of which is the presence of carbon, nitrogen and inducing compounds (Majeau et al., 2010). Furthermore, carbon concentration in nutrient media and lignocellulose substrates have also been investigated to play an important role in enzyme activity (Elisashvili et al, 2002), which does not always occur with the addition of ammonium tartrate as a source of nitrogen.

The degradative ability of *M. pulcheripes* J8 is observed on Poly R-478 media, by measuring the color change that occured. After incubation for 20 days, the highest color degradation occurred in the treatment of *M. pulcheripes* J8 pure culture at 68.89%, then mixed AC cultures (*M. pulcheripes* and *A. niger* NK), ABC treatment (*M. pulcheripes*, *Penicillium* sp R 75 and *A. niger* NK) and the last treatment AB (*M. pulcheripes* J8 and *Penicillium* sp R 75) at 61.63% (Figure 2).

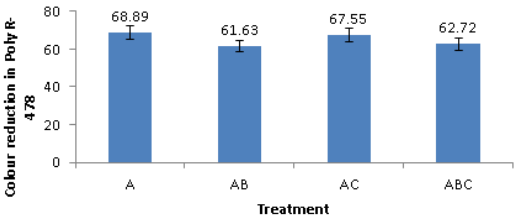

Figure 2. *M. pulcheripes* J8 ability to decolorize Poly R-478 after 20 days incubation. A= *M. pulcheripes* J8; B= *Penicillium* sp R 75; C= *A. niger* NK

Poly R-478 is an anthraquinone-based polymeric dye with high molecular weight that is used to observe ligninolytic ability of an enzyme produced by microbes. In addition, the reduction of color indicates the fungus ability of decomposition, and the result showed a positive effect, which was different from one treatment to another. Furthermore, the most significant influence was observed in the treatment of pure *M. pulcheripes* J8 culture, which was reduced in mixed cultures. This happened because the single sample tends to produce more MnP as seen in Table 1, which is known according to Moreira et al. (2001), as the main factor initiating color modification in Poly R-478. Furthermore, the addition of H_2O_2 with a semicontinuous technique is able to boost the decolorization process.

M. pulcheripes J8 was applied in the degradation of Palm Oil Mill Effluent (POME) pigment by adding 200 µM $CuSO_4$ to the liquid waste. Therefore, after a 20 days of incubation period, the maximum color reduction was observed in treatment A by 74.25%, which was followed by AC, then ABC, and the lowest was in treatment AB, by 24.97% (Table 3).

The degradation of POME is indicated by a reduction in color, where the maximum decline occurred in the pure culture by up to 74.25%, while the mixed cultures resulted in lower extent. This result was consistent with the most significant ligninolytic enzyme activity produced by the pure culture of *M. pulcheripes* J8. The color of POME is usually generated by compounds in plant tissues, including lignin, which is degraded by laccase, into simpler compound, and phenolic compound (Chanida & Poonsuk, 2011). Moreover, a more significant effect is observed when compared to *Coprinus cinereus,* which initiated a 75.26% reduction after 27 days of incubation (Subowo, 2017).

The treatment of *M. pulcheripes* J8 was observed to also reduce the level of COD (Chemical Oxygen Demand) in POME, where the most sig-

Table 3. The ability of *Marasmius pulcheripes* J8 to degrade POME after 20 days of incubation

A (%)	AB (%)	AC (%)	ABC (%)
73.99	17.55	67.51	55.37
74.44	32.77	62.35	56.83
74.34	24.61	66.06	56.65
74.25±0.23	24.97±7.61	65.30±2.66	56.28±0.79

Notes: A= *M. pulcheripes* J8; B= *M. pulcheripes* J8; C= *A. niger* NK

nificant decline occurred in treatment A by 81% after 20 days of incubation period, followed by treatment AC, ABC and the lowest was AB by 77.79% (Figure 3).

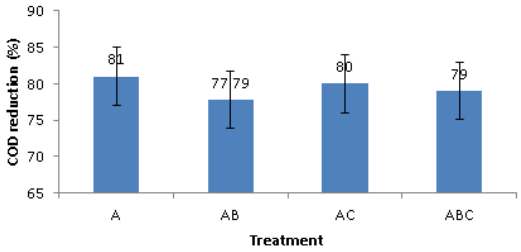

Figure 3. The decline in COD level of POME with fungal treatment after a 20 day incubation period. A: *M. pulcheripes* J8, B: *Penicillium* sp R 75, C: *A. niger* NK.

The decline of COD (Chemical Oxygen Demand) level in the POME also showed that the degradation process occurred. The optimum decrease occurred in the treatment of pure culture of *M. pulcheripes* J8 which was 81% after incubation for 20 days, whereas in mixed cultures the results were lower. The COD decline occurred because of the degradation process by enzymes produced by *M. pulcheripes* J8 on the compound dissolved in POME. Environmental pollution was associated with high concentration of organic matter (COD = 40000-50000 mg / L, BOD = 20000-25000 mg / L) (Najafpour et al., 2005). Moreover, the ability *M. pulcheripes* J8 to reduce COD level by 81% lead to the diminution by up to 32,400 mg / L after 20 days of incubation period. Meanwhile, in comparison with *Coprinus cinereus*, a reduction of about 91.26% was reported after 27 days of incubation period (Subowo, 2017).

Researches on the use of *M. pulcheripes* J8 to degrade POME has not been done. The addition of $CuSO_4$ as inducer to increase laccase activity has not been as well. The result of this research provided new knowledge on the capability of *M. pulcheripes* J8 on the degradation of POME. This method is less expensive and easier compared to chemical and physical methods.

CONCLUSION

Pure culture of *Marasmius pulcheripes* J8 on PDB media showed the highest laccase activity (3566.04 U / mL) whereas it was lower in mixed cultures. The addition of $CuSO_4$ on Poly R-478 media increased laccase activity by 88.57%. This fungus was able to reduce color in POME by 74.25% and reduce POME COD level by 81% after incubation for 20 days.

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