

# The Influence of Inducers on the *Coltricia cinnamomea* Laccase Activity and its Ability to Degrade POME

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**Abstract.** Some species of Basidiomycetes, specifically white rot groups, produce three ligninolytic enzymes, namely, Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase (Lac), which have low activity in degrading Palm Oil Mill Effluent (POME). The research objective was to obtain the data on the ability of the *Coltricia cinnamomea* to produce LiP, MnP, and Lac enzymes to degrade POME. This research also studied the effect of sucrose, alcohol, veratryl alcohol, CuSO<sub>4</sub> and ZnSO<sub>4</sub>, as inducers. Isolates of *Coltricia cinnamomea*, which were stored in a PDA media at -20°C were obtained from the Microbiology section of the Research Center for Biology (LIPI). Furthermore, the growth media used were DM, Bean sprout Extract (TE) and PDB. The result indicated that PDB is the most suitable growth media for the production of ligninolytic enzymes, because in this medium these enzymes showed the highest activity. It was also observed that sucrose increased the laccase activity by 40.80%. Furthermore, *Coltricia cinnamomea* was able to reduce the concentration of Poly R-478 by 60.74%, after the addition of ZnSO<sub>4</sub>. In addition, it degraded and decreased the color and COD of POME, by 72.63% and 91.19% respectively, after the addition of veratryl alcohol, and incubation for 10 days. Therefore, this fungus can be used to degrade POME in order to prevent environmental pollution. *Coltricia cinnamomea* has not been used for POME degradation. By using *Coltricia cinnamomea*, we obtained new data regarding the activity of laccase and its ability to degrade POME.

**Key words:** POME degradation; inducer; laccase; *Coltricia cinnamomea*

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## INTRODUCTION

The size of oil palm plantations in Indonesia keeps increasing every years. In 2019, the area of oil palm plantations in Indonesia increased by 1.88 percent to 14.60 million hectares. Palm oil (CPO) production increased by 12.92% to 48.42 million tons (Indonesian Oil Palm Statistics, 2019). However, besides producing palm oil for daily needs, the palm oil industry also produces liquid and solid waste. Palm Oil Mill Effluent (POME) could potentially cause environmental problem due to its characteristics such as dark brown color, thick, dense, and unpleasant odor. The color of POME is predicted to come from melanoidin, biopolymer pigment resulted from Maillard reaction during the processing. Beside melanoidin, phenolic compounds are detected on POME and these compounds are toxic (Zahra et al., 2020).

POME is the residual waste resulting from condensate water after the boiling of palm oil fresh fruit bunches. Untreated POME contains 38.36% cellulose, 23.21% hemicellulose, and 26.72% lignin (Baharuddin et al., 2010). Lignin is a tissue which strengthens plant structures and is recalcitrant (difficult to decompose). Disposal of this waste in water bodies without proper treatment will have an

impact on the environment. The production of POME in Indonesia is estimated to be at 28.7 million tons, every years (Irvan et al., 2012).

Basidiomycetes is a group of fungi, which some members secrete ligninolytic enzymes, especially those belonging to the white rot group. According to Gonzalez (2013), most ligninolytic fungi produce at least one laccase isoenzyme. Laccases are blue copper oxidases, found on several plants and secreted by some ligninolytic fungi. The enzyme oxidizes some organic compounds, especially phenolic and aromatic amines (Zucca et al., 2015). Laccase degrades phenolics and non-phenolics compounds and also some pollutants in the environment (Upadhyay et al., 2016). Laccase naturally play important roles in catalyzing lignification, delignification, plants' stress management, as well as fungal morphogenesis and virulence (Sitarz et al., 2016).

Some species of white rot fungi secrete poorly active laccase. Thus, various efforts has been done to increase laccase production by inducing laccase gene expression on white rot fungi. Several materials such as aromatic compounds related to lignin and their derivatives, carbon and nitrogen sources, and metal ions regulate the transcription of the laccase gene (Piscitelli et al., 2011). Higher enzyme activities

result in a greater and faster reaction in substrates, and increase the application and efficiency of the enzyme's catalysis process (Rao et al., 2014). In *Pleurotus sajor-caju* strain PS-2001 culture on media with sucrose and added with aromatic syringaldazine, benzoic acid, galic acid, vanillin, and  $\text{CuSO}_4$  increased laccase activity up to 58-80 U/mL (Bettin et al., 2014).

The use of *Coltricia cinnamomea* (Tiger's Eye Fungus) to degrade POME has never been done. Similarly the addition of inducers such as veratryl alcohol,  $\text{ZnSO}_4$ , and alcohol to increase laccase activity, for faster degradation process has never been heard of, therefore, this research was conducted. The objective of this study was to obtain valuable data on the ability of the *Coltricia cinnamomea* to degrade POME, and the effect of adding a number of inducers on its laccase activity. We obtained new data regarding the activity of laccase and its ability to degrade POME by used *Coltricia cinnamomea*. It added new insight into the utilization of fungi for POME degradation.

## METHODS

**Microorganism.** Isolates of *Coltricia cinnamomea*, which were stored in a PDA media, at  $-20^\circ\text{C}$ , were obtained from the Microbiology section of the Research Center for Biology (LIPI). Furthermore, the inducers used include: veratryl alcohol, alcohol,  $\text{ZnSO}_4$ , sucrose,  $\text{CuSO}_4$ , while the media used were DM (Defined Medium), TE (Taoge Extract/Bean sprout Extract), PDB (Potato Dextrose Broth) and Poly R-478 media.

**Media.** DM was composed of: 10 g glucose, 1 g  $\text{NH}_4\text{NO}_3$ , 0.8 g  $\text{KH}_2\text{PO}_4$ , 0.2 g  $\text{Na}_2\text{HPO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g of yeast extract (Songulashvili et al., 2007), while Bean Sprout Extract (TE) was composed of 100 g bean sprout, and 60 g sucrose. Furthermore, the compositions of PDB were 4.0 g Potato starch and 20.0 g Dextrose, while that of Poly R-478 were: 0.60 g  $\text{KH}_2\text{PO}_4$ , 0.50 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.40 g  $\text{KH}_2\text{PO}_4$ , 0.22 g  $(\text{NH}_4)_2$  tartrate, 40.0 g sorbose, 0.20 g Poly R-478 (Sigma), and 10.0 mL mineral solution stock plus distilled water, up to 1L (Glend and Gold, 1983). In addition, mineral solution stock composed of: 7.4 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  1.2 g Ferri citrate, 0.7 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.1 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , and 10.0 mg Thiamin HCl, added with distilled water up to 1 L.

### Ligninolytic activity of fungus

Laccase (Lac) activity was calculated using the method of Papinutti et al. (2003). The test is based on the oxidation of ABTS by the enzyme laccase. The reaction mixture contained 0.5 mL of citrate buffer at

pH 6.0, 0.1 mL of 1 mM ABTS and 0.4 mL of the enzyme supernatant. The tube was then shaken slowly to mix all ingredients and allowed to stand for 15 minutes at room temperature. The absorbance was measured at a wavelength of 420 nm.

Manganese Peroxidase (MnP) activity was determined using the method of Yoshida et al. (1996). MnP was measured by monitoring the oxidation of guaiacol spectrophotometrically. The reaction mixture contained 0.1 mL guaiacol 4 mM, 0.1 mL lactate buffer 50 M at pH 4.5, 0.2 mL  $\text{MnSO}_4$  1mM, 0.3 mL distilled water, 0.1 mL  $\text{H}_2\text{O}_2$ , and 0.2 mL of enzyme solution. Guaiacol oxidation was then monitored by watching for any rise in absorbance at 465 nm.

Lignin Peroxidase (LiP) activity was estimated using the Tien and Kirk method (1983). Furthermore, the assay was based on the oxidation of veratryl alcohol to veratryl aldehyde, in the presence of  $\text{H}_2\text{O}_2$ . The reaction mixture contained 0.1 mL of 8 mM veratryl alcohol, 0.2 mL acetate buffer 50 mM of pH 3, 0.45 mL distilled water, 0.05  $\text{H}_2\text{O}_2$  5 mM, and 0.2 mL enzyme solution. The increasing of absorbance was monitored at 310 nm.

### Fungus ability to degrade Poly R-478

To test the ability of fungus to degrade Poly R-478, 5 mL of mycelium suspension was inoculated into 45 mL of Poly R-478 media. The mixture was then incubated on a shaker at a speed of 115 rpm, at room temperature. The R-478 poly content was measured after incubation for 7 days, using a spectrophotometer. The absorbance was read at a wavelength of 520 nm (Moreira et al., 2004).

### The ability of fungi in POME degradation

This study used *Coltricia cinnamomea* in order to remediate the immense concentration of colorant in palm oil mill effluent (POME). Furthermore, the inducers,  $\text{CuSO}_4$ , sucrose, veratryl alcohol, alcohol and  $\text{ZnSO}_4$  were also added to boost the laccase activity. In total, six different media treatments were prepared including the untreated POME for 100 mL (1), which were POME with the addition of  $\text{CuSO}_4$  200  $\mu\text{M}$  (2), 15 g/L sucrose (3), 40 mM veratryl alcohol (4), 40 g/L alcohol (5), and 100  $\mu\text{M}$   $\text{ZnSO}_4$  (6). All media were then inoculated with 10 mL of *Coltricia cinnamomea* mycelium suspension and incubated and shook (115 rpm) at room temperature for 10 days. After incubation, mycelium and supernatant were separated by centrifugation (9000 rpm) and the supernatant layer was collected. POME decolorization was then measured by Spectrophotometer at a wavelength of 600 nm.

### COD degradation

COD levels in POME were measured using the method of Xia et al. (2009). Observations were made using a spectrophotometer by reading the absorbance reduction on 0 to day 10, at a wavelength of 600 nm

### Data analysis

At a level of  $p < 0.05$ . The values were expressed as the mean  $\pm$  SD.

## RESULT AND DISCUSSION

*Coltricia cinnamomea* belongs to the family of Polyporaceae. Furthermore, it secreted all three ligninolytic enzymes, namely, Laccase (Lac), Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP), in the three media. However, the activity of each enzyme was different from one media to another. The highest amount of Lac and MnP were obtained in PDB media, with concentrations of 245.36 and 1735.53 units/mL respectively, while LiP was found in DM media with a concentration of 1415.76 units/mL (Table 1).

**Table 1.** Ligninolytic activity of *Coltricia cinnamomea* on several growth media

Media	Lac Activity (unit/mL)	MnP Activity (unit/mL)	LiP Activity (unit/mL)
DM	108.79 $\pm$ 5.30	1584.02 $\pm$ 101.91	1415.76 $\pm$ 108.63
TE	189.81 $\pm$ 7.22	1480.71 $\pm$ 83.50	913.97 $\pm$ 80.64
PDB	245.36 $\pm$ 34.25	1735.53 $\pm$ 20.66	1218.63 $\pm$ 121.21



**Figure 1.** *Coltricia cinnamomea* (Photo: Arwan/Subowo)

PDB (Potato Dextrose Broth) was considered the most suitable for the production of Laccase and Manganese peroxidase enzymes because both enzymes showed the highest activity compared to the

other media, while the highest LiP activity was on DM media. This is consistent with a research conducted by Subowo. In that study, *Coprinus cinereus* was inoculated in DM, PDB and TE, and the result obtained showed that the highest enzyme activity of Lac, LiP, and MnP occurred in the PDB media (Subowo, 2017). Potato extract in PDB contains carbohydrates which affect fungal growth and enzymes production. Correspondingly, research by Taurisia that inoculated *Alternaria alternata* (Fries) Keissler on seven types of media: Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Sabouraud Dextrose Agar (SDA), Czapek Dox Agar (CDA), Corn Meal Agar (CMA), Nutrient Agar (NA), and Malt Extract Agar (MEA), showed that *Alternaria alternata* grew well on PDA (20g), PCA (20g), and SDA (40g) media. It was due to the high content of dextrose and carbohydrates in the 3 media which play an important role in the metabolic process of the fungi (Taurisia et al., 2015).

In this study, inducers were added, to increase the activity of laccase produced by *Coltricia cinnamomea* in the PDB media. Inducers added: including CuSO<sub>4</sub>, sucrose, alcohol, ZnSO<sub>4</sub>, and veratryl alcohol. The result showed that inducers increased the fungal laccase activity. However, the highest increase occurred in media inoculated with sucrose (40.8%), because it is a source of carbon (C), followed by CuSO<sub>4</sub>, and finally alcohol (13.48%) (Table 2).

**Table 2.** The activity of laccase produced by *Coltricia cinnamomea* on PDB after the addition of various inducers

Inducer	Laccase Activity (U/mL)	Increase (%)
Control	138.18 $\pm$ 11.94	-
Alcohol	159.72 $\pm$ 38.48	13.48
Veratryl alcohol	163.76 $\pm$ 26.18	15.62
ZnSO <sub>4</sub>	165.62 $\pm$ 3.47	16.56
CuSO <sub>4</sub>	185.99 $\pm$ 30.75	25.70
Sucrose	233.44 $\pm$ 74.77	40.80

According to Dana glucose, maltose, sucrose, fructose, glycerol, and lactose are common carbon sources. Sucrose in the media is hydrolyzed by the fungus into fructose and glucose. Fructose impacts laccase activity (Dana et al., 2017). Hu reported that fructose produced by *Gongronella* sp. w5 was more readily available to be used by *Coprinopsis cinerea* as carbon sources contributing to the growth and laccase secretion up to 110.6  $\pm$  3.3 U/L (Hu et al., 2019). Dhakar and Pandey (2013) showed similar results that fructose and ammonium sulfate that are suitable carbon and nitrogen sources to increase the laccase production by *Trametes hirsuta* (MTCC 11397).

Similarly, glucose influences laccase activity. Schneider investigated suitable C and N sources and concentration to support laccase, peroxidase, and manganese peroxidase activity by *Marasmiellus palmivorus* VE111. Combination of glucose and casein resulted in the highest laccase activity (5134 U/mL), total peroxidase (187 U/mL) and manganese peroxidase (57 U/mL) (Schneider et al., 2018).

The addition of inducers to poly R, which is a polymeric dye, improved the decolorization effect of ligninolytic enzyme. The highest increase (up to 60.74%) occurred with the addition of ZnSO<sub>4</sub> (27.79 ppm), followed by veratryl alcohol (22.44 ppm), while the lowest (17.30%) occurred with the addition of alcohol (7.92 ppm) (Table 3).

**Table 3.** The decrease of Poly R-478 concentration by *Coltricia cinnamomea* enzymes

Inducer	Absorbance	Decrease (%)	Poly R-478 concentration decrease (ppm)
Poly R + Fungi	0.0991	5.79	2.66
Poly R + Fungi + Alcohol	0.0870	17.30	7.92
Poly R + Fungi + Sucrose	0.0768	26.99	12.35
Poly R + Fungi + CuSO <sub>4</sub>	0.0648	38.40	17.57
Poly R + Fungi + Ver. alcohol	0.0536	49.04	22.44
Poly R + Fungi + ZnSO <sub>4</sub>	0.0413	60.74	27.79

The addition of ZnSO<sub>4</sub> to the Poly R-478 media increased *Coltricia cinnamomea* laccase activity. Therefore, the concentration of poly R showed the greatest decrease compared to that of the control, which was 60.74%, after incubation for 10 days. Furthermore, the addition of low concentration metal ions to the media, increased the activity of laccase and consequently caused the faster degradation of Poly R-478. Yang reported that MnSO<sub>4</sub>, CoCl<sub>2</sub>, FeSO<sub>4</sub>, NaMoO<sub>4</sub>, and H<sub>3</sub>BO<sub>3</sub> showed no effects on laccase production by *Cerrena* sp. HYB07, while Cu<sup>2+</sup> and Zn<sup>2+</sup> induced laccase production. When Cu<sup>++</sup> or Zn<sup>++</sup> ions were not added to the fermentation media, laccase activity showed a decrease as much as 99.95 and 31.78% (Yang et al., 2016). The activity of LiP and MnP increased on *Phanerochaete*

*chrysosporium* culture when Zn<sup>++</sup> and Cu<sup>++</sup> at low concentrations were added to metal-free culture media (Asgher, 2011). The presence of FeSO<sub>4</sub> and ZnSO<sub>4</sub> as trace elements in the media increased the maximum production of laccase on *Bacillus* sp. PK4 isolate (Rajeswari & Bhuvaneshwari, 2016).

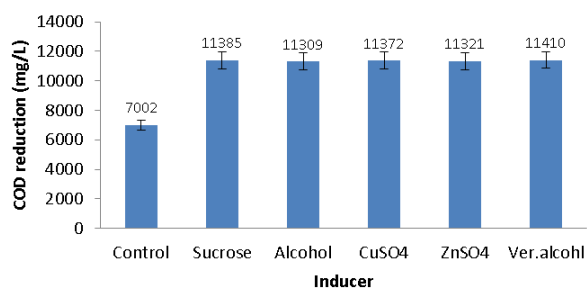
The enzymes produced by *Coltricia cinnamomea* decolorized POME by 13.96% after incubation for 10 days. Moreover, the addition of inducers increased POME decolorization became 37.79-72.63%. The most significant increase (72.63%) occurred with the addition of veratryl alcohol (Table 4).

**Table 4.** POME degradation by *Coltricia cinnamomea* after inducers addition

Treatments	POME decolorization (%)
POME + Fungi	13.96 ± 0.91 <sup>a</sup>
POME + Fungi + sucrose	37.79 ± 0.82 <sup>b</sup>
POME + Fungi + alcohol	46.88 ± 4.97 <sup>c</sup>
POME + Fungi + CuSO <sub>4</sub>	47.88 ± 7.65 <sup>c</sup>
POME + Fungi + ZnSO <sub>4</sub>	54.68 ± 5.23 <sup>c</sup>
POME + Fungi + veratryl alcohol	72.63 ± 3.96 <sup>d</sup>

Several aromatic compounds have been used to increase laccase production including veratryl alcohol, guaiacol, ferulic acid and 1-hydroxybenzotriazole (Kocyigit et al., 2012). Saraiva reported that laccase production by *Trametes versicolor* increased by two, four, and eight-fold due to the presence of ligninosulphonates, veratryl alcohol, and xyloidine inducer, respectively (Saraiva et al., 2012). Usha reported that 0.02% veratryl alcohol added to *Stereum ostrea* culture increased the production of 3 enzymes. Laccase activity increased 1.9 times compared to control and MnP and LiP production increased by 50% (Usha et al., 2014). The POME color reduction of 72.63% after incubation for 10 days was the highest result of degradation by laccase produced by *C. cinnamomea*. Neoh reported that *Curvularia clavata* reduced the color of POME by 80% with 54% as a result of biosorption and 46% as a result of degradation after 5 days of treatment (Neoh et al., 2013).

COD decreased from 12512 mg/L (fresh POME) to 5510 mg/L with *Coltricia cinnamomea* treatment without an inducer, in the POME degradation process. The addition of inducer accelerated the reduction of COD, alcohol (1203 mg/L), ZnSO<sub>4</sub> (1191 mg/L), CuSO<sub>4</sub> (1140 mg/L), sucrose 1127 mg/L, and veratryl alcohol (1102 mg/L). The greatest decrease (91.19%) occurred after the addition of veratryl alcohol, while the lowest occurred in the control, where no inducer was added (Figure 2).



**Figure 2.** The decrease of COD level of POME by *Coltricia cinnamomea* after the addition of inducers

Ligninolytic enzyme produced by the *Coltricia cinnamomea* fungus is proven to reduce COD levels in POME. Furthermore, untreated POME had a relatively high COD level. The addition of inducers to POME caused an increase in enzyme activities, which led to faster degradation process. However, all the inducers tested did not cause a significant alteration on COD level. According to Soleimaninanadegani and Manshad, POME contains high organic matter, COD concentrations ranged from 45,000–65,000 mg/L and BOD of 18,000–48,000 mg/L (Soleimaninanadegani & Manshad, 2014). Furthermore, *Coltricia cinnamomea* reduced COD levels by 91.19% after the addition of veratryl alcohol. This result is still higher than that of *Marasmius pulcheripes* J8, which was able to reduce COD level by 81% after incubation for 20 days (Subowo & Sugiharto, 2019). Lanka and Pydipalli reported that *Emericella nidulans* NFCCI 3643 revealed to be an excellent biological agent in degrading organic matters in POME. This fungus reduced COD by 80.28%, BOD by 88.23%, and fat content by 87.34% (Lanka & Pydipalli, 2018). Bala reported that POME can be degraded by a mixture of indigenous POME microbes consisting of *Micrococcus luteus* 101PB, *Stenotrophomonas maltophilia* 102PB, *Bacillus cereus* 103PB, *Providencia vermicola* 104PB, *Klebsiella pneumonia* 105PB, *Bacillus subtilis* 106PB, *Aspergillus fumigatus* 107PF, *Aspergillus nomius* 108PF, *Aspergillus niger* 109PF, and *Meyerozyma guilliermondii* 110PF. These microbes decreased BOD level as much as 90.23%, COD level up to 91.06% and TSS as much as 92.23% (Bala et al., 2018).

*Coltricia cinnamomea* is a member of the Basidiomycetes group. There has not been research that explains the ability of *Coltricia cinnamomea* to produce the ligninolytic enzymes. Therefore, this research will obtain new information about this fungus and the possibility of POME degradation. This new information can be used to treat palm oil industry waste, especially POME and add new knowledge about this fungus.

## CONCLUSION

*Coltricia cinnamomea* produced three ligninolytic enzymes (Laccase, Manganese Peroxidase, Lignin Peroxidase) on PDB medium. This fungus can reduce the color of POME by 13.96% after incubation for 10 days. The addition of veratryl alcohol increased the POME color reduction by 72.63%. This fungus also reduced COD at POME by 91.19% after adding veratryl alcohol.

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