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Original Article

The utilization of ink cap mushroom (*Coprinus cinereus*) on palm oil mill effluent degradation

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Abstract

A research on the utilization of ink cap mushroom (*Coprinus cinereus*) on Palm Oil Mill Effluent (POME) degradation was conducted. Palm oil industry is an agricultural industry which keeps on developing in Indonesia. Aside from producing palm oil, this industry also generates POME. POME has high level of BOD and COD value thus it is dangerous for the environment. *Coprinus cinereus* was examined to degrade POME because of its ability to produce ligninases enzymes. The aim of this research was to evaluate ligninases enzymes produced by *C. cinereus* after the addition of inducers and its ability in degrading POME. *Coprinus cinereus* was cultivated on Defined Medium (DM) then the mycelia was harvested Inducers used in this study were $CuSO_4$, KNO_3 and sucrose. The suspension of *Coprinus cinereus* mycelia was used to degrade POME. The result showed that *Coprinus cinereus* could produce Laccase, Mangan Peroxidase and Lignin Peroxidase on DM. The addition of 200 µM $CuSO_4$ and 10 mM KNO_3 on DM could increase laccase activity by 62.92 %. *Coprinus cinereus* has ability to degrade Poly R-478 and POME. The treatment of 200 µM $CuSO_4$ on media could increase the degradation of Poly R-478 by 19.32% after 9 days of incubation. The addition of 15 g/L sucrose on POME could increase decolorization by 75.26 % and COD reduction by 91.26 % after 27 days of incubation. Ink Cap Mushroom (*Coprinus cinereus*) can be used for POME degradation. The addition of sucrose and heating treatment would accelerate COD reduction.

Keywords: *Coprinus cinereus, inducer, POME, Poly R-478, degradation*

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Introduction

As an agricultural country, the sector of agriculture plays an important role in Indonesian economy and palm oil plantation has been a promising industry. The plantation area has been increasing through the years and present in almost all of Indonesian main islands. According to Ministry of Agriculture statistics, total area used for this crop in 2015 reached approximately 11,444,808 hectare with the production capacity was 30,948,931 tons (Anonymous, 2014). The palm oil products will be used to cover the country's need and exported. Besides producing palm oil, this industry also generates solid waste and effluent. Palm oil mill effluent (POME) is waste water characterized by brown in color, suspension-like with high colloid and unpleasant smell (Ahmad and Chan 2009). POME originated from the remnant of palm fresh fruits milling. POME is hazardous to the environment because it has high content of organic compound (COD= 40,000-50,000 mg/L, BOD= 20,000-25,000 mg/L, oil, fat and complex polymer (carbohydrate, protein, lipid, mineral and nitrogen compound)) (Ibegbulam-Njoku and Achi, 2014). The milling of a ton of fresh fruits palm bunch will generate wastewater by 50 % of total waste while empty fruit bunch milling will produce 23 % of total waste. The production of POME in Indonesia reached 28.5 million tons/year (Irvan *et al*., 2012). The release of POME to surrounding waterbody without proper waste treatment will impact largely to the environment (Wu *et al*., 2007).

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There are some species of white-rot fungi that produce ligninases enzymes including *Phanerochaeta chrysosporium, Coriolus hirsutus, Phlebia radiata, Coriolus versicolor, Agaricus bisporus, Pleurotus ostreatus, Trametes versicolor, Trametes villosa, Pycnoporus cinnabarinus*. White-rot fungi secreted one or more extracellular enzymes to degrade lignin: Lignin Peroxidase (LiP) (EC 1.11.1.14), Mn-dependent Peroxidase (MnP) (EC 1.11.1.13) and Cu-containing Phenoloxidase, Laccase (EC 1.10.3.2) (Songulashvili *et al*., 2007). Laccase will oxidize organic and inorganic compounds such as phenol, catechol, hydroquinone, 2,6-dimethoxyphenol, and syringaldazine (Thruston, 1994) and also amina aromatic and ascorbate (Madhavi and Lele, 2009). Shneider et al. (1999) reported that *C. cinereus* produces laccase with the highest titer value from all five detected enzymes. The activity was higher during mycelium growth than during fruiting body formation (Raymond *et al*., 2015). *Coprinus cinereus* also produces peroxidase such as lignin peroxidase and mangan peroxidase (Heinzkill *et al*., 1998).

Some organisms secrete laccase in small amount, but the yield can be increased by the treatment of chemical compounds such as aromatic and phenolic compounds, metal, alcohol and detergent (Leonowicz *et al*., 2001). The effect of copper on laccase synthesis was studied on *Trametes versicolor* (Collins and Dobson, 1997) and *Pleurotus ostreatus* (Palmieri *et al*., 2003). The treatment of 200 µM $CuSO_4$ on submerged culture of *Volvariela volvacea* resulted a high laccase activity (Chen

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et al., 2003). The treatment of 10 mM KNO_3 on submerged culture of *Ganoderma lucidum* increased the production of laccase and MnP (Songulashvili *et al*., 2007). The treatment of sucrose on submerged culture of *Pleurotus ostreatus* increased the activity of laccase (Subowo, 2015). Recently, the utilization of ink cap mushroom (*Coprinus cinereus*) to degrade POME and inducer treatment to increase ligninases activity on the fungi has not been conducted yet. The objective of this research was to evaluate ligninases enzymes produced by *Coprinus cinereus* after the addition of inducers on media and to observe its ability to degrade POME.

Method

Microorganism

The isolate of *Coprinus cinereus* derived from culture collection of Microbiology Department, Research centre of Biology, The Indonesian Intitute of Sciences. The isolate was preserved on slant agar (PDA) and incubated at 4 ºC.

Media

Media used were: Defined Medium (DM) (Songulashvili et al., 2007), Potato Dextrose Broth (PDB), Bean sprout extract (TE), Poly R-478 media (Glenn and Gold 1983). DM composition: 10 g glucose; 1 g NH_4NO_3; 0.8 g KH_2PO_4; 0.2 g Na_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 2 g yeast extract. PDB: 400 g potato, 20 g Dextrose. Bean Sprout Extract (TE): 100 g bean sprout; 60 g sucrose. Media Poly R-478: 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 stock; added with distilled water until 1 L. Mineral solution stock: 7.4 g $CaCl_2.2H_2O$; 1.2 g Ferri 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 Thiamin HCl; added with distilled water until 1 L.

Inoculum Preparation

C. cinereus was cultivated on DM, 20 mL of mycelia suspension was inoculated into 180 mL DM medium, then incubated in shaker at room temperature, 150 rpm for 7 days. Suspension of mycelia was harvested by centrifugation at 9,000 rpm, 4 ºC for 15 min then was washed with double distilled water. The suspension of mycelia (5 mL) was inoculated in DM in a final volume of 50 mL. The culture was incubated in shaker at 150 rpm, room temperature for 4 days. The supernatant was harvested by centrifugation at 9,000 rpm, room temperature for 15 min. Then, the supernatants were used for further analyses.

Ligninases Enzymes Activity of *C. cinereus*

Laccase activity (Lac) was determined via the oxidation of 2,2-azino-bis(3 ethylbenzthiazoline)-6-sulfonate (ABTS) (Papinutti et al., 2003). The reaction mixture consists of 0.5 mL citrate buffer of pH 6, 0.1 mL ABTS 1 mM, 0.4 mL supernatants. ABTS oxidation was monitored by the increase in absorbance at 420 nm.

Manganese Peroxidase activity (MnP) was measured by monitoring the oxidation of guaiacol spectrophotometrically (Yoshida *et al*., 1996). The reaction mixture contained 0.1 mL guaiacol 4 mM, 0.1 mL lactate buffer 50 M of pH 4.5, 0.2 mL $MnSO_4$ 1 mM, 0.3 mL distilled water, 0.1 mL H_2O_2, 0.2 mL supernatants. Guaiacol oxidation was monitored by the increase in absorbance at 465 nm.

Lignin Peroxidase activity (LiP) was estimated by the method of Tien and Kirk (1983). The assay is based on the oxidation of veratryl alcohol to veratryl aldehyde in the presence of H_2O_2. 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 H_2O_2 5 mM, 0.2 mL supernatants. The increasing of absorbance was monitored at 310 nm, with three replications.

Ligninases Enzymes Activity on Three Media

Three media is often used for growing Basidiomycetes fungi in the laboratory, namely: PDB, TE and DM. These media will be used to evaluate the activity of ligninases enzymes of *C. cinereus*. The suspension of mycelia (20 mL) was inoculated into PBD, TE and DM in a final volume of 200 mL. The cultures were incubated in shaker 150 rpm, room temperature for 16 days. Ligninases enzymes (Lac, MnP, LiP) activity was measured on day: 0, 2, 5, 7, 9, 12, 14, and 16. 2 mL of aliquot was taken for centrifugation at 9,000 rpm at room temperature. The supernatants were used for further analyses like the methods mentioned above, with three replications.

The Ability of *C. cinereus* on Poly R-478 Decolorization

Poly R-478 is a model compound that is often used to determine the ability of microbial decolorization of dye and measuring the ligninases activity. The addition of inducers such as $CuSO_4$, KNO_3 and sucrose in the Poly R-478 media to enhance the ligninases activity of *C. cinereus*. Media were tested include: TB0 with composition: Poly R-478 + suspension of mycelia; TB1: Poly R-478 media + $CuSO_4$ + suspension of mycelia; TB2: Poly R-478 media + KNO_3 + suspension of mycelia; TB3: Poly R-478 media + sucrose + suspension of mycelia; TB4: Poly R-478 media + $CuSO_4$ + KNO_3 + sucrose + suspension of mycelia. The suspension of *C. cinereus* mycelia (20 mL) was inoculated into the media in a final volume of 200 mL. The cultures were incubated in shaker 150 rpm at room temperature for 9 days. 2.0 mL of samples was centrifuged at 9,000 rpm, room temperature for 15 mins. The decolorization of Poly R-478 dye was measured by the percentage reduction of absorbance at 520 nm, with three replications.

The Ability of *C. cinereus* on POME Degradation

Palm Oil Mill Effluent (POME) contains lignin and cellulose. Lignin can be degraded by laccase secreted by *C. cinereus*, that resulted in POME decolorization and the decrease of COD level. POME used in this research was obtained from PT Bangka Putra Mandiri in Bangka Island. Experimental design:

- Induction effect: Two media treatments were used A and B. Media A was composed of 100 mL POME + 15 g/L sucrose, while media B was composed of 100 mL POME + 15 g/L sucrose + 200 µM $CuSO_4$ + 10 mM KNO_3. Both media were sterilized then inoculated with 10 mL of mycelia suspension. Both cultures were incubated in a shaker (150 rpm) at room temperature for 27 days. POME decolorization and the decrease of COD level were observed on day 0 and 27 with spectrophotometer at 600 nm (Li *et al*., 2009)
- Sterilization effect: Media was composed of 100 ml POME + 15 g/L sucrose + 200 µM $CuSO_4$ + 10 mM KNO_3. Sterilization treatments were done. Media D was sterilized in an autoclave (121 ℃, 1 atm, 15 min), while media E was not sterilized. Both media were inoculated with 10 mL of mycelia suspension then incubated in a shaker (150 rpm at room temperature for 27 days). POME decolorization and the decrease of COD level were observed on day 0 and 27 with spectrophotometer at 600 nm (Li *et al*., 2009).
- Shaking effect: Media was composed of 100 ml POME + 15 g/L sucrose + 200 µM $CuSO_4$ + 10 mM KNO_3. Both media were sterilized then inoculated with 10 mL of mycelia suspension. Shaking treatments were done. Culture F was incubated in a shaker (150 rpm at room temperature for 27 days), while culture G was not shaked during incubation. POME decolorization and the decrease of COD level were observed on day 0 and 27 with spectrophotometer at 600 nm (Li *et al*., 2009). The significance between treatments was determined using T-Test analysis at the level of $P<0.05$, with three replications

Results

C. cinereus (ink cap) cultivated in DM medium could produce three ligninases enzymes: Laccase, Manganese Peroxidase and Lignin Peroxidase. Induction using $CuSO_4$ and KNO_3 increased laccase enzyme activity by 62.92% (Table 1).

Table 1. Ligninases activity of *C. cinereus*

Enzymes	Without induction (UmL^{-1})	$CuSO_4$ and KNO_3 induction (UmL^{-1})	Rise of enzymes activity (%)
Laccase	237.84 ±18.10	387.01±187.70	62.92
MnP	692.83±448.92	8201.78±432.08	1083.80
LiP	2867.38±193.59	6667.55±637.62	132.53

The Activity of Ligninases Enzymes Produced

C. cinereus secreted three types of ligninases enzymes on PDB, DM and TE media. Ligninases activity levels on those three media were varies. Laccase secreted on DM and PDB was twice as much of that on TE. MnP secreted on PDB was three times higher than that on TE and four times higher than that on DM. LiP production did not show a significant difference among three media ($P < 0.05$), although the highest production on PDB was observed (Table 2)

Table 2. Ligninases activity of *C. cinereus* on three media

Enzymes	Enzyme concentration (UmL^{-1})		
	DM	PDB	TE
Laccase	377.75 b	431.19 b	168.11 a
MnP	263.88 a	1286.21 b	383.58 a
LiP	3709.92 a	7042.56 a	4737.29 a

DM= Defined Medium, PDB= Potato Dextrose Broth; TE= Beansprout extract. Numbers followed by corresponding letters on the same row at difference column showed no significant difference ($P < 0.05$)

The Ability of *C. cinereus* on Poly R-478 Degradation

Poly R-478 is a chemical material model that often used to understand about ligninases activity. Degradation of Poly R-478 is indicated by color change to a lighter shade. In this study, the laccase ability of *C. cinereus* to degrade Poly R-478 was observed. The decreases of Poly R-478 concentration on media were vary, the highest was observed on media with the addition of $CuSO_4$ (TB1), followed sucrose (TB3). While media with the addition of KNO_3 (TB2) showed the lowest reduction (Table 3)

Table 3. Poly R-478 degradation by *C. cinereus*

No	Treatment	Poly R-478concentration(mg/L)		Poly R-478 Reduction (%)
		Day 0	Day 9	
1	TB0	74.13	66.50	10.50 c
2	TB1	73.32	59.15	19.33 e
3	TB2	71.72	69.10	3.59 a
4	TB3	73.82	62.19	15.83 d
5	TB4	68.54	63.76	6.84 b

TB0 : media + fungi; TB1 : media + $CuSO_4$ + fungi; TB2 : media + KNO_3 + fungi; TB3 : media + sucrose + fungi;TB4 : media + $CuSO_4$ + KNO_3 + sucrose + fungi. Numbers followed by corresponding letters on the same column showed no significant difference ($P < 0.05$).

The Ability of *C. cinereus* on Palm Oil Mill Effluent Degradation.

POME contains lignin and cellulose which resulted in dark brown color and high COD level. The application of ligninases enzymes produced by *C. cinereus* was expected to reduce the brown color and COD level. The addition of sucrose into POME (A) showed higher color reduction (75.26 %) than addition of sucrose, $CuSO_4$, and KNO_3 (B). The COD level reduction on treatment A and B was 85.31% and 72.82%, respectively (Figure 1).

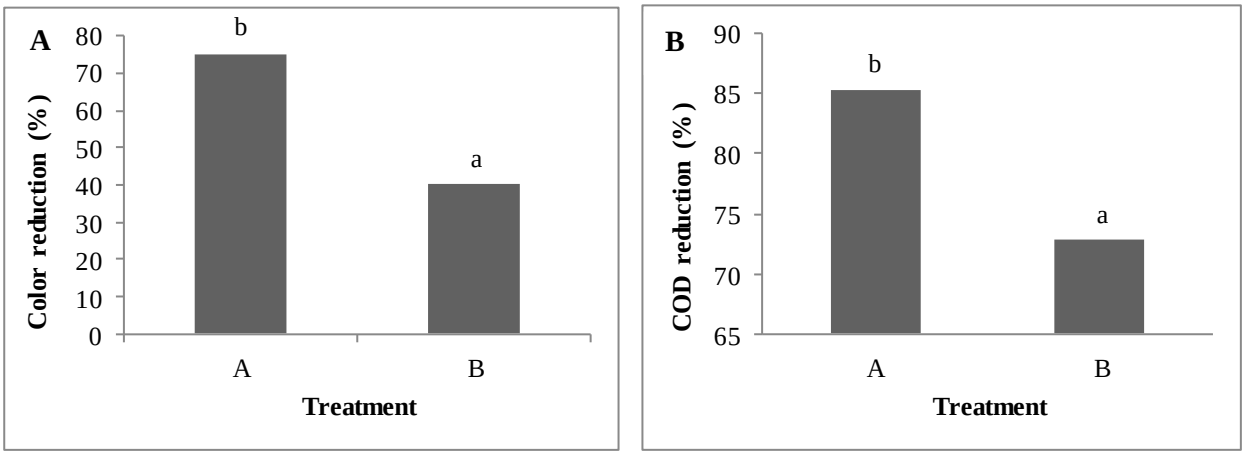

Figure 1. Color reduction percentage (A) and COD reduction percentage (B) on POME inoculated with *C. cinereus*. A: POME + sucrose, B: POME + sucrose + $CuSO_4$ + KNO_3 Bars marked with same letter showed no significant difference ($P<0.05$)

Sterilization treatments on POME did not improve the process of decolorization. Color decolorization observed in sterilized POME (D) inclined to be higher than unsterilized POME (E), but the difference was not significant ($P < 0.05$). The decrease of COD level was significantly different on sterilized POME (D) and unsterilized POME (E) ($P < 0.05$), with the decrease on D was higher than that on E (Figure 2).

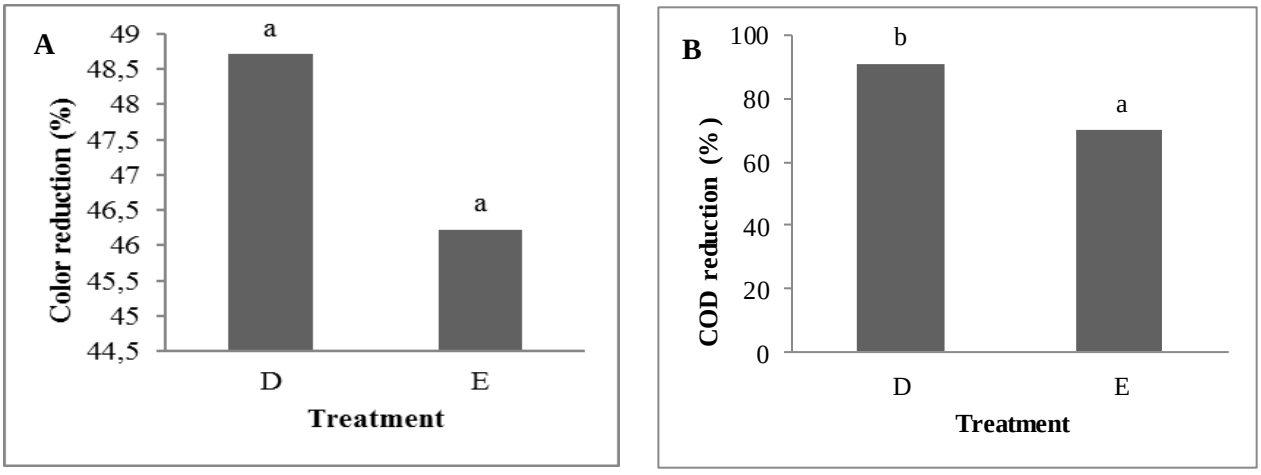

Figure 2. Color reduction percentage (A) and COD reduction percentage (B) on sterilized POME (D) and unsterilized POME (E) inoculated with *C. cinereus*. Bars marked with same letter showed no significant difference ($P<0.05$)

Shaking treatment also influenced POME degradation by *C. cinereus*. The color reduction of POME with shaking treatment (F) showed higher (46.13 %) than without shaking (G). The COD level reduction showed a contradictory result. POME without shaking (G) (69.31 %) showed a higher COD level reduction than POME with shaking (F) (Figure 3).

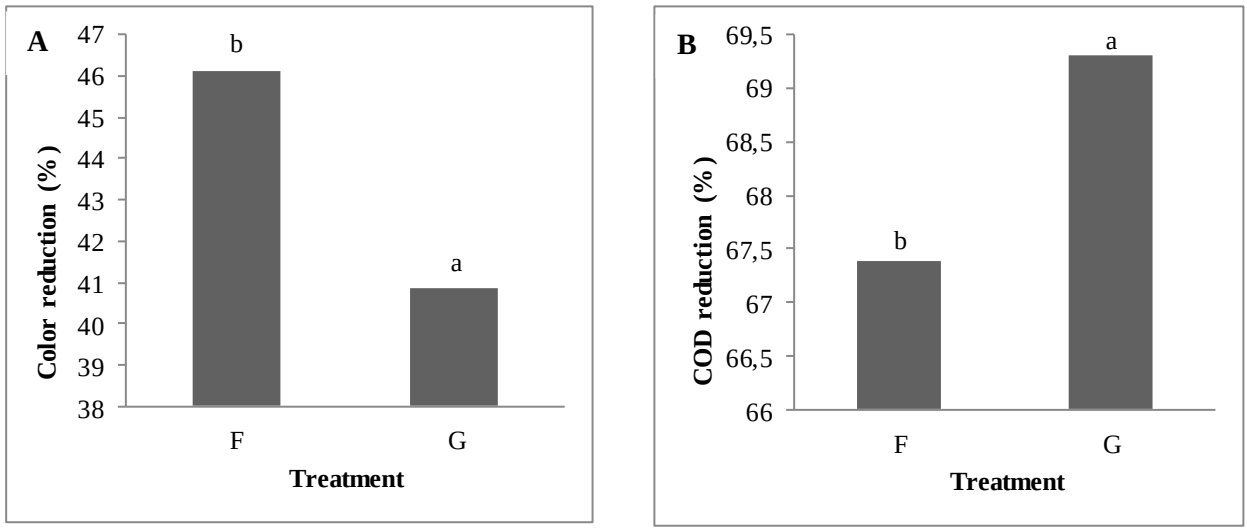

Figure 3. Color reduction percentage (A) and COD reduction percentage (B) on POME with shaking (F) and POME without shaking (G) inoculated with *C. cinereus*. Bars marked with same letter showed no significant difference (*P<0.05*)

Discussions

Defined Medium was used by Songulashvili *et al*. (2007) in the inoculum preparation of some Basidiomycetes fungi such as *Cerrena maxima, Fomes fomentarius, Funalia trogii, Ganoderma adspersum, Ganoderma lucidum, Hypsizygus marmoreus, et ceter.* It is also used in the laccase production by *Ganoderma lucidum.* In this research, DM media was used to examine the ability of *C. cinereus* to produce ligninases. The result showed that *C. cinereus* secreted three types of ligninases, which were laccase, manganese peroxidise, and lignin peroxidise. The addition of $CuSO_4$ and KNO_3 as inducers into DM media was done to increase laccase activity. With inducer addition, activity of laccase increased by 62,92 % (387.01 U/mL). The presence of Cu affected laccase production while KNO_3 influenced the production of biomass. According to Baldrian and Gabriel (2002), Cu not only induced laccase gene but also affected the activity and stability of enzyme. The addition of KNO_3 as source of N increased biomass production by 41-69 % and increased the production of laccase and MnP (Songulashvili et al., 2007). According to Chen et al. (2003) *Volvariella volvacea* in high N concentration showed a high laccase activity and vice versa. In this research, laccase activity produced by *C. cinereus* was 237.84 ± 18.10 U/mL. It was higher than that on Raymond *et al*. (2015) which cultivated *C.cinereus* in textile waste. Laccase activity during mycelium growth was higher than during fruiting body formation. The highest laccase activity was 39,45 ±12,05 U/g. This difference is caused by the type of substrates used and the type of strains.

The cultivation of *C. cinereus* on DM, PDB and TE was conducted to determine the best media to produce ligninase. *C. cinereus* was able to grow and produced three ligninases (laccase, LiP, MnP) on all three media. The highest production of laccase was observed on PDB and DM, while the laccase activity in both media showed no significant difference. The highest production of MnP was observed on PDB while the production of LiP on all three media showed no significant difference. Generally, PDB was the most effective media for ligninases production by *C. cinereus.*

Poly R-478 containing media was used to determine the ability of ligninases produced by *C. cinereus* to degrade Poly R-478. It was observed that there was a difference in Poly R-478 degrading ability after the addition of inducers. The presence of inducers increased ligninases activity especially laccase. These are media with the highest decline of Poly R-478 concentration to the lowest: media + $CuSO_4$ (TB1), media + sucrose (TB3), media without inducer (TB0). In the media added with KNO_3, either individually or mixed, degradation occurred in much slower rate (TB2 and TB4). The addition of $CuSO_4$ increased laccase activity that resulted in a higher Poly R-478 degradation rate. The presence of $CuSO_4$ also increased the activity of MnP (Table 1) which also accelerated the degradation of Poly R-478. The study of Moreira *et al*. (2001) used pure enzymes showed that MnP was the major factor responsible for the reduction of color in Poly R-478. The addition of sucrose can also increase the degradation rate of Poly R-478. Sucrose is one of the source of C on growth and production of antifungal agent of *Gymnopilus spectabilis* (Vahidi *et al*., 2006).

The addition of KNO_3 obstructed the degradation of Poly-R. It was shown in TB0 (without inducer) whose degradation level was higher than that in media + KNO_3. High concentration of N in the media inhibits Poly R decolorization process by *C. cinereus* corresponding to Moreira *et al*. (2004) that used *Trametes versicolor* to decolorize Poly-R478. The best decolorization efficiency was obtained in culture with limited nitrogen in an aerobic environment. Another factor is the derivate (NO_2^-) of

KNO_3 which not only toxic but also can affect ligninases activity (Moreira *et al*., 2004)

The addition of sucrose into POME (A) increased the activity of laccase and MnP, resulted in accelerated POME degradation. This can be seen from reduction of COD level and color of POME. Sucrose is a source of C and energy for *C. cinereus* which resulted in a higher activity. This result is in accondance to Subowo (2015) which stated that the addition of $CuSO_4$ and sucrose increased laccase activity which lead to accelerated decolorization and degradation of POME by *Pleurotus ostreatus*. While the addition of $CuSO_4$, KNO_3 and sucrose simultaneously did not effect on POME degradation. POME degradation is a complex process involving many enzymes such as ligninases, cellulases, lipases, etc. NO_2^- which derivated from KNO_3 was toxic and antimicrobial effect on soil microbial activity (Bancroft *et al*., 1979).

Heating in autoclave reduced initial microbes which resulted in a condition that only enzymes from *C. cinereus* present to degrade POME, but decolorization level from treatment D and E showed no significant difference. While a significant difference of COD level can be observed on media with and without sterilization. Thus, it can be concluded that POME sterilization do not have effect on decolorization but affect COD level. The increase of laccase activity due to microbe activity was observed. The result is in accordance with Baldrian (2004) which reported that laccase activity of *Trametes versicolor* and *Pleurotus ostreatus* increased after interacting with soil fungi, yeasts, bacteria or unsterilized soil. The reduction of COD level in treatment D was the highest (91.26 %) after 27 days. The COD reduction in this study took longer time compare to POME degradation using *Candida rugosa* and *Geotrichum candidum* with the result of *Candida rugosa*: COD (48.6 %), BOD (74.5 %) O&G (41.8 %) and *G.candidum*: COD (59.1 %), BOD (75.7 %), O&G (59.1 %) after 144 h (Ibegbulam-Njoku and Achi, 2014).

Shaker treatment during incubation affected decolorization of POME. POME with shaker treatment (F) showed a faster decolorization. Shaking influences the availability of oxygen in the media, the distribution of nutrition and the homogenity of substrates. On the contrary, POME without shaker treatment (G) showed a higher decline of COD level compare to shaking condition (F). Degradation of lignicellulose occured faster in a static condition which might be caused by the unification of enzymes in static state. The result is contradictory with the study of Rohilla *et al* (2012) using various microorganisms (*Aspergillus flavus, A. niger, A. oryzae, A. tereus*) which reported that dye decolorization was more effective on shaker.

Based on this research, it can be concluded that *C. cineres* produced three ligninases enzymes (Laccase, LiP and MnP) on DM. The addition of 200 µM $CuSO_4$, 10 mM KNO_3 and 15 g/L sucrose increased laccase. PDB and DM were suitable media for *C. cinereus* to produce ligninases enzymes compared to TE. The addition of $CuSO_4$, KNO_3 and sucrose simultaneously into Poly-R media did not effect on Poly R-478 degradation. POME supplemented with sucrose increased decolorization and reduction of COD level. Sterilization and shaking treatments increased color reduction. For future research, POME degradation using other Basidyomycetes that have higher laccase activity and other inducers might show better result.

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