Majalah Ilmiah Biologi Biosfera : *A Scientific Journal* DOI: 10.20884/1.mib.2019.36.2.967
Vol 36, No 2 Mei 2019 : 85 – 89

Ligninolytic Activity of Fungi Isolated from Empty Fruit Bunch of Oil Palm (*Elaesis guineensis* Jacq.)

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Abstract

Lignin is a natural polymer and plays an important role as a compound of plant cell wall constituent. A study about the degradation of lignin in the environment has been receiving considerable attention because the complex structure and difficult to be degraded compared to the degradation of others plant cell wall constituent. A study to determine the activity of the ligninolytic enzyme (lignin peroxidase, manganese peroxidase and laccase) of fungi isolated from oil palm empty fruit bunch. This study has been done with a screening of ligninolytic activity using potato dextrose agar supplemented with tannic acid (0,1%), showed that two out of five fungal isolates have ligninolytic activity. The highest activity of lignin peroxidase was produced by SN2 isolates i.e. 9.677U ml^{-1}, whereas the highest activity of manganese peroxidase and laccase was produced by SN3 isolates i.e. 1.942 U ml^{-1} and 1.846 U m^{-1} respectively.

Key words: laccase, ligninolytic, lignin peroxidase, manganese peroxidase, oil palm empty fruit bunch.

Introduction

One of the biggest commodities in Indonesia is palm oil, due to the high demand for cooking oil and many derivatives which are made from palm oil. This condition leads to the dependence of the Indonesian community on oil palm availability.

Naturally oil palm can only grow and produce well in tropical climates like Indonesia. According to Direktorat Jendral Perkebunan, Indonesia has reached 11 million Ha of oil palm plantation areas and produced 31 million tons of oil palm in 2017. With the rapid increment of the oil palm plantation area and production, waste generation is expected to be increasing.

The palm oil production process eventually will produce untreated oil palm empty fruit bunches (OPEFB) which is considered as the largest waste accounts for 34.37 % (Isroi *et al*., 2012). Generally, OPEFB waste contains highly organic matter content which has an impact on environmental pollution. Fortunately, aside from being a waste, OPFEB waste can be used to produce bioethanol (Sudiyani *et al*., 2010) and have potential as reinforcing fibre for energy absorption application (Faizi *et al*., 2017). In general, the solid waste of OPEFB contains lignocellulose. OPEFB contains 33.25 % of cellulose, 23.24 % hemicellulose and 25.83 % lignin (Sudiyani *et al*., 2010). Naturally, lignin is difficult to decompose and only a few microorganisms are able to decompose it.

White rot fungi are a group of fungi known to produce extracellular ligninolytic enzymes, which are able to degrade lignin to obtain nutrient which is required for the growth system. The main extracellular ligninolytic enzymes are Lignin Peroxide (LiP), manganese peroxide (MnP) and Laccase. Some fungal species that can degrade lignin is *Irpex lacteus* CD2 (Xu *et al*., 2009) (*Phellimus pini* dan *Pleurotus* spp. (Wong, 2009). Fungal species from Ascomycetes class also have the potential to degrade lignin, such as *Penicillium* sp KSt3, *Aspergillus* sp I3, *Penicilium* sp I3I (Subowo & Corazon, 2010). The diversity of fungi in Indonesia is abundantly scattered. In addition, Indonesia's tropical climate provides the best habitat for fungal growth. However, they are not yet known for their ability to produce ligninolytic enzymes. This study was aimed to obtain ligninolytic fungi from OPEFB and examine the ligninolytic activity of selected isolates. The study conveyed a feasible of isolating fungi from OPEFB which have a high ligninolytic activity. The long term goal is to use the isolated fungi for lignocellulose waste treatment especially OPEFB.

Methods

Raw material

OPEFB was collected from an Oil Palm Plantation belongs to the domestic plantation, in Deli Serdang, North Sumatera, Indonesia. The OPEFB was taken and stored in sealed plastic.

Chemical and Reagent

Chemicals that have been used in this experiment were tartrate buffer (pH 2.5), H2O2, guaiacol, $MnSO_4$, citrate phosphate buffer (pH 5.5), sodium acetate buffer (pH 5.5) veratryl alcohol, Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), chloramphenicol, KH_2PO_4, $MgSO_4$.7H2O, tannic powder, K_2HPO_4, Alkaline Lignin, NH_4NO_3, KCL, $MgSO_4.7H_2O$,$FeSO_4.7H_2O$, $MnCL_2.2H_2O$, $CuSO_4.5H_2O$.

The experiment was carried out through several steps namely: isolation of fungi from OPEFB, Screening of Ligninolytic Enzyme Activity, Preparation of Enz yme Sources and Screening of Ligninolytic Enzyme Activity. Particularly for the ligninolytic activities were measured by UV-vis spectrophotometer.

Isolation of Lignin Degrading Fungi from OPEFB

Empty palm oil bunch was taken aseptically from oil palm plantations. The sample was then cut into a smaller size and weighed as much as 5 g and inoculated into 50 ml PDB medium, incubated at room temperature (26-30 ^{o}C) for 48 hours. PDB medium containing empty palm oil bunch was then taken as much as 10 ml, diluted to 10^5 and spread over PDA medium which has been added with chloramphenicol and incubated at room temperature for 48 hours. The fungi colonies grown was then inoculated on new PDA media to make a single culture.

Screening of Ligninolytic Enzyme Activity

Screening of enzymatic activity was qualitatively performed by using Bavendamm test (Nisidha *et al.,* 1988). The selected isolates were grown on PDA media which has been added with 0.1% tannic acid. When a brown precipitate is formed on the medium, it indicates the presence of phenol oxidase activity, which shows that fungi belong to the group of ligninolytic fungi.

Preparation of Enzyme Sources

The enzyme source for the quantitative test was prepared by culturing the isolates on liquid ligninase medium at room temperature for fourteen days. The fungal suspension was centrifuged at 10,000 rpm at 4°C for 15 minutes to extract the enzyme (Artiningsih, 2006). Crude enzyme extract was used for the quantitative measurement of ligninolytic activity. Measurement of ligninolytic enzyme activity was performed every 2 days for fourteen days.

Measurement of Lignin Peroxidase (LiP), Manganase Peroksidase (MnP) and Laccase Activity

LiP activity was measured according to Bonnen *et al*., 1994, Crude enzyme extract solution of 0.2 ml was added to 2.8 ml of a tartrate buffer solution (pH 2.5). This mixture is then added with 1 ml of veratryl alcohol 2 mM and H_2O_2 0.4 mM. The mixture was further homogenized using vortex and incubated for 30 minutes at room temperature. The amount of veratraldehyde formed was measured using a spectrophotometer at a wavelength of 310 nm. The mixture of 1 ml of veratryl alcohol 2 mM, 1 ml of H_2O_2 0.4 mM and 0.2 ml of distilled water was incubated at 60° C for 5 minutes then used for the blank solution. The amount of veratraldehyde formed is calculated according to the Lambert-Beer formula, written as :

$$\Delta C = \frac{(At - A0}{k\ x\ b}$$

Where ΔC is the amount of veratraldehyde formed during t minutes (mol/liter), A_t is the absorbance value at *t* minutes, A_0 is the absorbance value at the beginning of the reaction, b is the diameter of cuvette (1 cm) and k is the constant (veratraldehyde = 9,300/M/cm). The enzyme activity is expressed in units is equivalent to 1 nmol veratraldehyde produced per minute from the treatment of 1 ml of the enzyme which is reacted.

Measurement of MnP enzyme activity was performed according to Bonnen *et al*., 1994, Crude enzyme extract solution of 0.2 ml was added to 2.5 ml of phosphate citrate buffer solution (pH 5.5). This mixture was then added with 1 ml of 0.1 mM guaiacol, 1 ml of $MnSO_4$ 0.1 mM and 1 ml of H_2O_2 50 mM, then the mixture was homogenized with vortex, and incubated for 15 minutes at room temperature. The loss of guaiacol concentration was measured at a wavelength of 465 nm using a spectrophotometer. The mixture of 1 ml of guaiacol, 1 ml of $MnSO_4$, 1 ml of H_2O_2 and 0.2 ml of distilled water was incubated at 60° C for 15 minutes then used for the blank solution. The amount of guaiacol lost is measured based on the Lambert-Beer formula with guaiacol molar absorptivity of 12,100 / M / cm. The enzyme activity is expressed in the unit. One unit of enzyme activity is equivalent to a reduction of 1 nmol of guaiacol per minute from the treatment of 1 ml of the enzyme to guaiacol.

Measurement of lacase enzyme activity was performed according to Kalra *et al*., (2013) A crude enzyme extract solution of 1 ml was added to a 3 ml sodium acetate buffer solution (pH 5). The mixture was then added with 1 ml of 2 mM guaiacol, homogenized by using a vortex, and incubated for 15 minutes at room temperature. The reduction of guaiacol concentration was measured using a spectrophotometer at a wavelength of 450 nm. For the blank solution 1 ml of aquadest, 1 ml of 2 mM guaiacol and 3 ml of sodium acetate buffer were mixed then incubated at 30 ° C for 15 minutes. The amount of guaiacol lost is calculated by using the Lambert-Beer equation, with guaiacol molar absortivity of 12,100 / M / cm. The enzyme activity is expressed in the unit. A unit of enzyme activity is equivalent to a reduction of 1 nmol of guaiacol per minute from the treatment of 1 ml of the enzyme to guaiacol under normal conditions (U/ml).

Result and Discussion

Screening of Ligninolytic Enzyme Activity with Bavendamm Test

The screening test result for ligninolytic enzyme activity using the Bavendamm test on five fungal isolates, found that two isolates were positive showing ligninolytic activity, SN2 and SN3 (Figure 1). Positive results were indicated by the presence of brown deposits in the media.

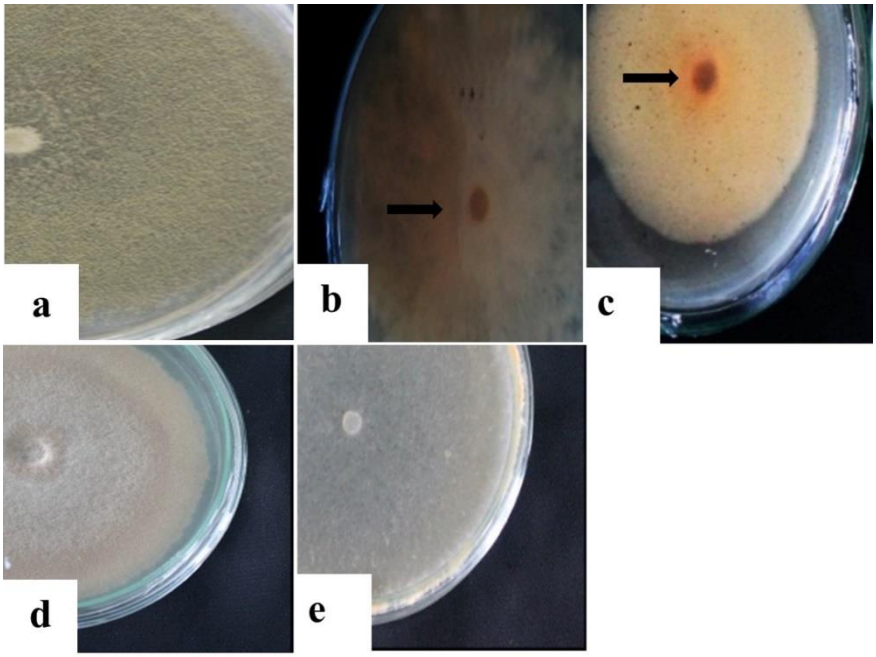

Figure 1. The results of Bavendamm test of fungal isolates from OPEFB; (b) SN2 isolates and (c) SN3 isolates showed positive results in the Bavendamm test where brown deposits were formed (→). (a) SN1 Isolates, d SN4 isolates and (e) SN5 isolates showed a negative result.

White root fungi (WRF) have been successfully studied in terms of lignin decomposition, due to the ability to produce the ligninolytic enzyme. The activity of a ligninolytic enzyme was screened by using bavendamm test. According to (Nishida *et al.,* 1988), banvendamm test was carried out to determine the ability of fungi on producing extracellular phenol oxidase. When brown deposits are formed on the growth solid media the fungus belongs to the group of white rot fungi (WRF). In this study we successfully obtained two WRF isolates. SN2 and SN3 isolates were able to produce extracellular phenol oxidase which is essential for lignin degradation. Badalyan *et al.,* (2011) used Bavendamm test to detect the production of extracellular polyphenol oxidase activities and growth on the lignocellulosic substrate.

Although in this study only two out of five isolates showed a positive result of Bavendamm test, we still examined the isolates which had a negative result, in this case SN1.This isolate was tested to observe whether a bavendamm test-negative isolate produces ligninolytic enzym.

Lignin Peroxidase (LiP) Enzyme Activity.

The LiP enzyme production activity was determined by measuring the conversion of veratryl alcohol to veratraldehyde at a wavelength of 310 nm. Isolates were cultured on liquid ligninase medium for fourteen days showed varying LiP enzyme activities (Figure 2). The highest LiP enzyme activity was found in SN2 isolates with the maximum activity achieved on the sixth day which was 9,677 U ml^{-1} and then decreased steeply on the eighth day and continuously decreased until the end of culture. The LiP enzyme activity decreased may be due to several factors such as a decrease in growth and accumulation of cell death. Cell death could also increase the accumulation of toxic in a culture medium which leads to the degradation of the enzyme. In addition, accumulation of metabolic products such as NO_3, NO_2 and NH_3 decreased the ligninolytic activity (Kanwal & Reddy, 2011). Similarly, SN3 isolates reached maximum activity on the eighth day (5.556 U ml^{-1}) while LiP activity in SN3 isolates only reached maximum activity on the sixth day (1.703 U ml^{-1}), then the enzyme activity continuously decreased until the end of culture.

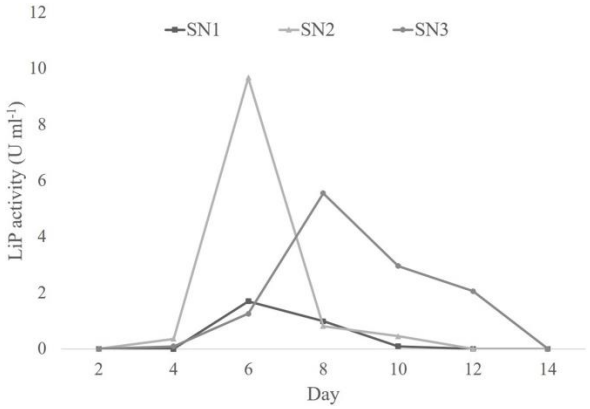

Figure 2. Lignin peroxidase (LiP) enzyme activities of fungal isolates isolated from OPEFB.

The highest activity of LiP was found in SN2 isolates on the sixth day. The LiP activity started to decline steeply on the eighth day. Similarly, Masalu (2016) reported that activity of LiP from *Tricoderma* sp increased on the sixth day of incubation and the highest of LiP activity was 1.44 U ml^{-1} on the eighth day of incubation in the submerged culture fermentation grown in Kirk's medium. Although the LiP activity of this study was bigger (9,677 U ml^{-1}) but it showed a similar pattern of enzymatic activity.

Manganase Peroxidase (MnP) Enzyme Activity

The activity of the MnP enzyme is precisely determined by measuring the concentration loss of guaiacol at a wavelength of 465 nm. From the measurement of MnP activity, isolates cultured on liquid ligninase medium for fourteen days showed varying activity in each fungal isolates (Figure 3), the highest MnP enzyme activity was found in SN3 isolates. The SN3 isolate resulted in a maximum MnP activity on the sixth day (1.942 U ml^{-1}), followed by SN2 isolates which reached the maximum enzymatic activity on the twelfth day with an activity of 0.777 U ml^{-1}. MnP activity in SN1 isolates only reached maximum activity on the sixth day of 0.518 U ml^{-1}, subsequently decreased until the end of culture.

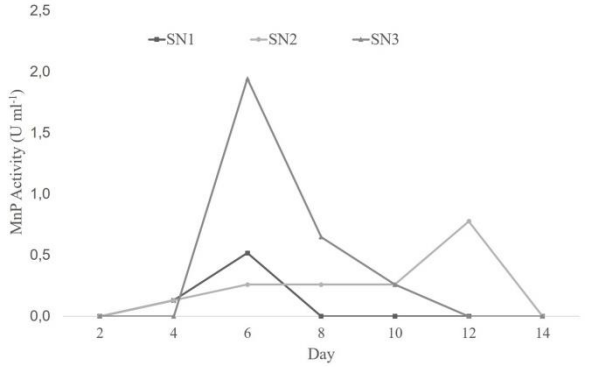

Figure 3. Manganese peroxidase (MnP) enzyme activities of fungal isolates isolated from OPEFB.

Previously, Masalu (2016) reported that the highest activity of MnP by fungus isolated from soil contaminated with cow dung reached on day 7 of incubati on with an activity of 0.30 U/ml.

Laccase Activity

Laccase activity was determined by measuring the loss of concentration of guaiacol by a wavelength measurement of 450 nm. The results of ligninolytic activity produced by isolates cultured on liquid ligninase medium for fourteen days, showed varied activity (Figure 4). The highest laccase activity was found in SN3 isolates, similar with the activity of MnP, where SN3 isolate performed a maximum enzymatic activity on the tenth day (1,846 U ml^{-1}), followed by SN2 isolates which reached the maximum on the sixth day (0.248 U ml^{-1}). Laccase activity of SN1 isolates reached the highest activity the fourteenth day with an activity of 0.055 U ml^{-1}. Laccase activity was declined after eight days of incubation for SN3.

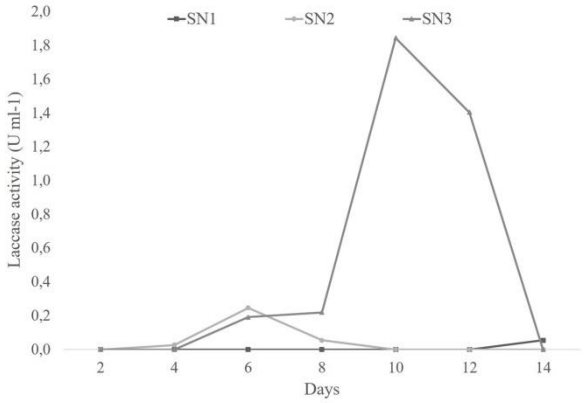

Figure 4. Laccase enzyme activities of fungal isolates isolated from OPEFB.

The results of this study as shown in Figures 2, 3, and 4, there were different patterns of ligninolytic activity in the incubation time. The LiP and MnP produced the highest activity in the initial incubation time which was the sixth day but Laccase reached the highest activity at the end of incubation time which was the tenth day. These results showed a similar pattern in nature where LiP, MnP and Laccase work gradually in the degradation of lignin.

In line with the ligninolytic screening test, SN2 and SN3 fungi showed a positive result in the Bavendamm test. Positive tests are characterized by the formation of brown deposits on the media, which indicate the ligninolytic activity. SN1 isolates which previously showed the negative result on Bavendamm test, but still produced a low LiP activity (Figure 1). The ligninolytic enzyme activity screening test performed on SN1 isolates showed lower enzyme activity and almost none for the lacase enzyme. The low ligninolytic activity

produced by SN1 isolates is assumed to be classified as brown-rot fungi. Dey *et al.* (1994) stated that *Polyporus ostreiformis* molds belonging to the brown-rot fungi were able to produce MnP and LiP enzymes, but their abilities were lower than those of *P. chrysosporium*.

The results obtained in this study showed that SN 1, SN 2 and SN3 isolates were able to produce all three types of ligninolytic enzyme which are LiP, MnP and Laccase.

Conclusion

The results showed that Bavendamm test was useful in determining lignin degrading fungi. In this study, two isolates were white root fungi. The highest yield of lignin peroxidase (LiP) enzyme activity was produced by SN2 isolates (9,677 U ml^{-1}), while the highest manganase peroxidase (MnP) and lacase activity were produced by SN3 isolates with an activity of 1,942 U ml^{-1} and 1,846 U ml^{-1}, respectively. This study provides baseline information on Ligninolytic activity of fungi isolated from OPEFB. It showed that using OPEFB as isolation sources has a big potential of obtaining lignin degrading fungi which can be optimized well for lignocellulose waste treatment. To our knowledge this is the first study to isolate fungi from OPEFB that have ligninolytic activity.

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