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Effects of supplementation with phosphorus, calcium and manganese during oil palm frond fermentation by *Phanerochaete chrysosporium* on ligninase enzyme activity

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Abstract. *Pazla R, Jamarun N, Agustin F, Zain M, Cahyani NO. 2020. Effects of supplementation with phosphorus, calcium and manganese during oil palm frond fermentation by* Phanerochaete chrysosporium *on ligninase enzyme activity. Biodiversitas 21: 1833-1838.* The objective of this study was to evaluate the effects of supplementation with phosphorus (P) in combination with calcium (Ca) and manganese (Mn) during oil palm frond (OPF) fermentation by *Phanerochaete chrysosporium* on ligninase enzyme activity and lignin degradation. This study was carried out using a randomized complete design with 3 treatments (addition of P, Ca and Mn) and 5 replicates. The following treatments were performed: T1 (P 1000 + Ca 2000 + Mn 150 ppm), T2 (P 1500 + Ca 2000 + Mn 150 ppm), and T3 (P 2000 + Ca 2000 +Mn 150 ppm). The data were subjected to an analysis of variance (ANOVA), and differences between treatment means were tested using Duncan's multiple range test (DMRT). The parameters measured were as follows: lignin peroxidase (LiP) activity (U/mL), manganese peroxidase (MnP) activity (U/mL), crude protein (CP) content (%), crude fiber (CF) content (%) and the decrease in lignin (%). The results revealed a significant increase in LiP activity and CP content and a decrease in the lignin content ($p<0.05$) by the addition of P in the T3 treatment. However, the treatment nonsignificantly increased ($p>0.05$) MnP activity and significantly decreased ($P<0.05$) the CF content. In conclusion, supplementation of the OPF fermentation process with P 2000, Ca 2000, and Mn 150 ppm resulted in the highest ligninase enzyme activity and in decreased lignin content.

Keywords: Calcium, manganese, MnP and LiP, Oil palm frond, *Phanerochaete chrysosporium*, phosphorus

INTRODUCTION

Livestock ruminants are able to contribute greatly to human welfare by providing the most animal protein through meat and milk. The factors that determine the success of the livestock business is the availability of adequate feed ingredients, which should be available continuously but may be very difficult to obtain. Under those conditions, the need exists for alternative feeds, which may come from the utilization of agricultural and plantation wastes, such as palm oil plantation waste (Jamarun et al. 2016).

Palm oil waste has the potential to be used as an alternative feed because of its large production and ongoing availability. The area of oil palm plantations in Indonesia in 2015 reached approximately 11,300,370 Ha, and palm oil production in West Sumatra in 2015 reached 1,002,920 tons, with growth in the production of crude palm oil increasing by 8.44% from 2014 to 2015 (BPS 2015)

One type of palm oil waste that can be used as an alternative feed is oil palm frond (OPF) waste. It has nutrients that have potential when the waste is provided as fiber feed. The result of the laboratory analysis showed that the chemical composition of the OPF is as follows: dry matter content (DM) 83.96%, organic matter (OM) 94.23%, CP 3.64%, CF 49.80%, NDF 89.98%, ADF 73.21%, hemicellulose 16.78%, cellulose 41.35%, and lignin 30.63% (Jamarun et al. 2018). Although the nutritional content allows the use of OPF as a source of fiber feed, the OPF contains high amounts of lignin, which has limited digestibility (Suryani et al. 2016; Jamarun et al. 2017).

One effort to maximize the use of OPF includes lowering the lignin content. Lignocellulose is a major component of OPF, which consists of lignin, cellulose and hemicellulose (Febrina 2016). Ligninase is a lignin-breaking enzyme that can degrade lignin. The use of microorganisms that produce the enzyme ligninase is highly recommended because this approach is more environmentally friendly (Perez et al. 2002). One such effort to reduce lignin levels is fermentation using *Phanerochaete chrysosporium* mold.

Phanerochaete chrysosporium is the most efficient lignin-degrading fungus (Suparjo 2008). Its growth is influenced by the availability of minerals in the substrate. The minerals P, Ca and Mn are added to OPF fermentation to trigger the growth and extension of the cilia-like mycelium (Pasaribu et al. 2003; Suparjo 2010). Fermentation of the OPF that was fermented with *Phanerochaete chrysosporium* with Ca 2000 ppm and Mn 150 ppm minerals added resulted in a 25.77% decrease in the lignin (Febrina 2016).

Phosphorus (P) is an essential mineral for metabolic processes and is required by all microbial cells primarily to

maintain the integrity of cell membranes and cell walls, as a component of nucleic acids and as part of high-energy molecules (ATP, ADP and AMP) (Zain et al. 2010). In addition, in the cell, phosphorus is present as a phosphoprotein, phospholipid and nucleic acid compound. The addition of the mineral P is expected to trigger the development of mold.

The addition of phosphorus to fermentation using *Phanerochaete chrysosporium* has not been reported yet. Therefore, based on the description above, a study was conducted to examine the effect of the mineral P on the fermentation of OPF using *Phanerochaete chrysosporium,* with added Ca and Mn minerals, to determine the effects of these additions on the ligninase enzyme activity and lignin degradation.

MATERIALS AND METHODS

Experimental site

For this study, the fermentation of OPFs and the analysis of enzyme activity was conducted at the Laboratory of Technology for Feed Industries, Andalas University, and the nutrient content analysis was conducted at the Laboratory of Dairy Cattle Nutrition, Faculty of Animal Science, Bogor Agricultural University, Indonesia.

Materials

Leaves and palm fronds that have been ground. *P. chrysosporium* mold obtained from the Microbiology Laboratory of the Faculty of Life Sciences, Bandung Institute of Technology (ITB), Indonesia. Minerals P, Ca and Mn derived from the minerals KH_2PO4, $CaSO_4.2H_2O$ and $MnSO_4.2H_2O$. Mineral solution from Brook et al. (Brook et al. 1969). Potato Dextrose Agar (PDA) culture media for the growth of molds (v). Chemicals for proximate analysis of the activity of ligninase according to Van Soest et al. (1991).

Research procedure

OPFs fermentation

The palm fronds used as the raw material in this study were taken from the distal two-thirds of the OPF. The OPF substrates were cut, dried and finely milled. The Ca was obtained from $CaSO_4$, P was obtained from $KHPO_4$, and Mn was obtained from $MnSO_4.H_2O$. *Phanerochaete chrysosporium* was maintained on Potato Dextrose Agar (PDA) slants at 4 °C, transferred to PDA plates at 37 °C for 6 days and subsequently grown on OPFs mixed with rice bran. Equal amounts of OPF leaves and stems were used and then the Brook mineral solution was added (Brook et al. 1969). The fermentation process was initiated by adding water to the OPFs until the water level reached 70%; then, the Ca, P or Mn was added, depending on the treatment. Observations were made every 48 h for 20 days (Febrina 2016). Samples were then taken for proximate analysis and fiber fraction determination. The proximate components were determined as described by AOAC (2010). The predominant fibers (NDF, ADF, cellulose, and lignin) were determined according to the method of Van Soest et al. (1991).

Enzyme extraction of fermented OPFs

Enzyme extraction was initiated with the centrifuge were determined according to the method of Gassara et al. (2010): approximately 1 g of the sample was mixed with 50 mM phosphate buffer (pH 6.5) at a ratio of 10/1 (v/w) and stirred at a speed of 150 rpm for 1 h. The samples were then centrifuged at a speed of 7000 xg for 20 minutes. The resulting supernatant (liquid) was separated and used for analysis of enzyme activity.

Measurement of lignin peroxidase enzyme activity

For determination of lignin peroxidase enzyme activity, 0.1 mL veratryl alcohol 8 mM, 0.2 mL 50 mM acetate buffer pH 3, 0.45 mL distilled water, 0.05 mL 5 mM H_2O_2, and 0.2 mL enzyme filtrate were added to a 2 mL tube, with the reaction being brought to a volume of 1 mL. The cuvette tube was shaken slowly so that all ingredients were mixed. The enzyme activity reaction was carried out at a temperature of 20 ± 1 °C. Absorbance was measured at 0 and 30 minutes at a wavelength (λ) 310 nm.

Calculation formula:

$$\text{Enzyme activity } (\text{U ml}^{-1}) = \frac{\Delta O310 \text{ x Vtot (ml) x } 10^9}{\varepsilon \text{ max x d x Vol enzyme (ml) x t}}$$

Where:

max ε : the verified molarity of the veratryl alcohol (9300 M^{-1} cm^{-1})

d : the thickness of the cuvette (cm)

ΔOD310 : the difference in the absorbance value at λ 310 nm

Measurement of activity of manganese peroxidase enzymes

Two stages of measurement occurred, namely, the measurement following the addition of Mn and the measurement without the addition of Mn.

Measurement upon the addition of Mn

For this measurement, 0.1 buffer lactate (pH 4.5, 50 M), 0.1 mL guaiacol (4 mM), 0.2 mL MnSO4, 0.3 mL Aqua Des™ (1 mM), 0.1 mL H_2O_2 (1 mM), and 0.2 mL filtrate enzyme were added to a 2 mL tube for a total volume of 1 mL. The cuvette tube was shaken slowly so that all ingredients were mixed. The enzyme activity reaction was carried out at a temperature of 20 ± 1 °C. Absorbance was measured at 0 and 30 minutes at wavelength (λ) 465 nm.

Measurement without the addition of Mn

For this measurement, 0.1 mL buffer lactate (pH 4.5 50 M), 0.1 mL guaiacol (4 mM), 0.5 mL distilled water, 0.1 mL H_2O_2 (1 mM), and 0.2 mL filtrate enzyme were added to a 2-mL tube for a total volume of 1 mL. The cuvette tube was shaken slowly so that all the ingredients were mixed. The enzyme activity reaction was carried out at a temperature of 20 ± 1°C. Absorbance was measured at 0 and 30 minutes at wavelength (λ) of 465 nm. The calculation formula is:

$$\text{Enzyme activity (U ml}^{-1}) = \frac{\Delta OD465 \times Vtot\ (ml) \times 10^9}{\varepsilon max \times d \times Vol\ enzyme\ (ml) \times t}$$

Where:

max ε : the molar absorption of the guaiacol (12100 M^{-1} cm^{-1})

d : the thickness of the cuvette (cm)

ΔOD465 : the difference in absorbance value at λ 465 nm

The activity of MnP is the activity observed upon the addition of Mn minus the enzyme activity without the addition of Mn.

Experimental design and statistical analysis

The study was carried out using a randomized complete design consist of three treatments with five replicates (the addition of P, Ca and Mn defined the treatments). The differences between the treatment means were analyzed using Duncan's multiple range test with a confidence interval of 5% ($p<0.05$).

The following treatments were carried out: T1 (1000 ppm P + 2000 ppm Ca + 150 ppm Mn) (i), T2 (1500 ppm P + 2000 ppm Ca + 150 ppm Mn) (ii), T3 (2000 ppm P + 2000 ppm Ca + 150 ppm Mn) (iii).

Parameters measured

The variables measured in this study were LiP activity, MnP activity, the decrease in lignin content, and the contents of CP and CF.

RESULTS AND DISCUSSION

The analysis of the data for the LiP activity, MnP activity, decrease in lignin content, and the contents of CP and CF due to the addition of Ca, P and Mn in the fermentation process of OPFs by *Phanerochaete chrysosporium* is presented in Table 1 and Figure 1.

Phanerochaete chrysosporium is a more complex model than the models provided by other strains for use in the development and understanding of the ligninolytic enzyme production system (Singh and Chen 2008). The resulting ligninolytic enzymes are lignin peroxidase (LiP) and manganese peroxidase (MnP). The ligninolytic peroxidases (LiP and MnP) produced by white-rot fungi oxidize polymer lignin to aromatic radicals. The final stage of this process is the formation of a simple compound of lignin degradation that enters the mold and synchronizes into the intracellular catabolic pathway (Martínez et al. 2005). The rate of ligninase enzyme activity from the fermentation of OPF using *Phanerochaete chrysosporium* supplemented with P mineral in combination with Ca and Mn is presented in Table 1.

Table 1 shows that the highest enzyme activity of ligninase (lignin peroxidase and manganese peroxidase) is found in T3 treatment (P 2000 ppm); the lowest, in the T1 treatment (P 1000 ppm). The enzyme activity of ligninase increases with the increasing dose of P (1000 ppm to 1500 ppm and 2000 ppm). The results of the diversity analysis show that the P dosage significantly affected ($P<0.05$) the enzyme activity of lignin peroxidase but did not significantly affect ($P>0.05$) the activity of the manganese peroxidase enzyme.

The high activity of enzyme ligninase in the T3 treatment caused by the P 2000 ppm dose combined with Ca 2000 ppm and Mn 150 ppm was the optimum trigger for the development of the mold hyphae. The increasing amount of mold enabled enzyme production, which also increased, thereby increasing the rate at which the mold can work to degrade the cell walls of the substrate. Febrina (2016) reported that the optimal combination of minerals could produce optimal ligninase enzymes as well as break down the cell wall in the fermentation process. Suparjo and Nelson (2012) stated that the amount of secreted enzymes depends on the ability of the fungus to penetrate into the substrate affected by mycelia and increase mold growth and production of the lignin-degrading enzymes that can be stimulated by mineral addition. Jamarun et al. (2017) reported the supplementation of fermented OPFs by *Phanerochayte chrysosporium* with combination of Ca, P and Mn provided the highest laccase enzyme activity and *in vitro* digestibility.

Phosphorus (P) is an essential mineral for metabolic processes, and the addition of phosphorus from a variety of previously different sources has been attempted in the fermentation of oil palm sludge by *Aspergillus niger* can help the growth of mold mycelia (Pasaribu et al. 2003). The addition of phosphorous minerals to deMan, Rogosa and Sharpe (MRS) medium for the growth of lactic acid bacteria can lead to increased growth and cell density (Subagiyo et al. 2016). Phosphorus is also a mineral that is able to increase the activity of rumen microbes, thereby increasing feed digestibility (Pazla et al. 2018).

Table 1. The decreased lignin and the contents of CP and CF because of the addition of minerals in the fermentation of OPFs by *P. chrysosporium*

Parameters	Treatments		
	T1	T2	T3
Decreased lignin (%)	24.33[a]	29.41[a]	40.08[b]
CP content (%)	7.04[a]	8.22[ab]	8.89[b]
CF content (%)	42.95[a]	40.56[ab]	38.59[b]

Means in the same row with different superscripted letters are significant at $p<0.05$, CP: Crude protein, CF: Crude fiber.

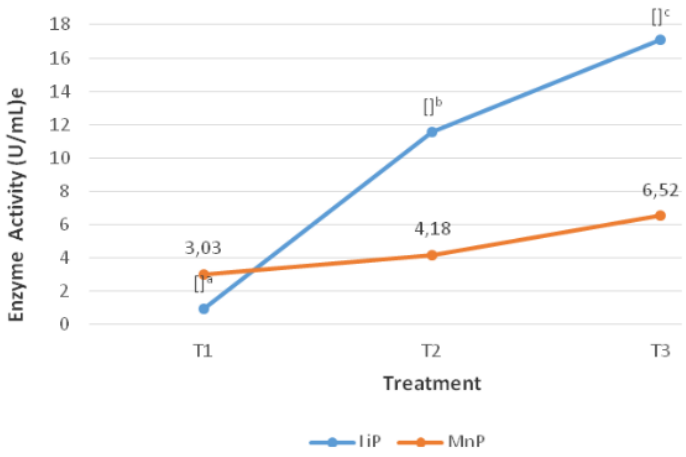

Figure 1. Enzyme activity of lignin peroxidase and manganese peroxidase because of the addition of minerals in the fermentation of OPFs by *P. chrysosporium*

The magnitude of the decrease in the lignin content is visibly different in T3 with the addition of P mineral at 2000 ppm (40.08%). This result is due to the availability of sufficient P so that the population and growth of *Phanerochaete chrysosporium* increase. This increase in mold growth will maximize the production and activity of the ligninolytic enzymes (LiP and MnP), resulting in optimal lignin reshuffle. Fadilah et al. (2009) stated that the addition of nutrients will increase the rate of lignin degradation and increase the growth of the fungi. The better the mold growth is supported by the availability of minerals, as characterized by spores and mycelium multiplication, the more LiP and MnP enzymes produced for the remodeling of the lignin. LiP and MnP are the main catalysts in the lignolysis process by *Phanerochaete chrysosporium* mold because they are able to breakdown the lignin structure. Imsya (2013) added that an increase in the production of fungus spore enzymes will increase the rate at which the mold works in breaking down the cell wall.

The T1 treatment showed the lowest lignin drop. The result of lignin decrease in T1 is not much different from the research of Febrina (2016) who showed a decrease in lignin of 25.77% in OPF fermentation, which is supplemented with Ca and Mn minerals. The slight decrease in lignin in this treatment is due to the nonoptimal growth support of mycelial fungi from the availability of P mineral so that the resulting LiP and MnP enzymes are also low. The low activity of these enzymes means the process by which the lignin is changed is not optimal.

The highest CP content was obtained at T3. The increase in the CP content in T3 is due to an increase in the number of mold cells in the mass. The secretion of extracellular enzymes by *Phanerochaete chrysosporium* can also play a role in increasing the protein content of the fermented substrate biomass (Nelson and Suparjo 2011). Nelson and Suparjo (2011) stated that the increase in protein content is due to the bioconversion of sugar into protein mycelium or single cell protein; that is, the more that mycelium becomes available due to the growth of the mass, the more nitrogen available to the fungi and the more proteins being contributed to the fermented substrate (Musnandar 2003). In addition, Jonathan et al. (2008) noted that the increase in crude protein content in the fermentation process is likely due to the result of the addition of the mold biomass to the fermentation substrate. High protein contents provide more Nitrogen (N) for microbial growth. Nearly 80% of rumen microbes require N for protein synthesis (Jamarun and Zain 2013). Good microbial growth also improves feed digestibility (Pazla et al. 2018). The growth and development of rumen microorganisms are highly dependent on the availability of nutrients and precursors, such as amino acids, N and minerals (Elihasridas 2012).

The T1 treatment showed the lowest crude protein content. This may be attributed to the nonoptimal growth of mold with supplementation P 1000 ppm so that the amount of enzyme produced is low, and the activity is low. Low enzyme activity of LiP and MnP contribute to a lignin degradation process that is not optimal. High lignin will prevent the reshuffle of nutrients by molds. Suparjo (2008) stated that LiP and MnP are the major enzymes in the lignin degradation process because they are able to oxidize nonphenolic lignin units because of nonphenolic lignin units constitute approximately 90% of the lignin structure.

Table 1 shows CF content ranging from 38.59% to 42.95%. The addition of P mineral to the OPF fermentation by *Phanerochaete chrysosporium* shoots significantly affected ($P < 0.05$) the reduction in CF content of the fermented OPF. The lowest CF content (38.59%) was

found for the T3 treatment (P> 0.05) compared with the T1 treatment, but the content from the T3 treatment did not differ significantly (P <0.05) from that of the T2 treatment. The low content of CF in the T3 treatment with 2000 ppm P is assumed to be capable of triggering growth and extension of the fungus mycelia, thereby resulting in a reshuffle in the fermentation palm cell wall section. Cell wall reshuffling occurs during the fermentation process due to the role of *Phanerochaete chrysosporium,* which utilizes cell contents as nutrients for growth. According to Tuomela et al. (2000), the utilization of soluble material occurs in the early phase of fermentation, and the reshuffle of cell wall components occurs as required by the mold (Suparjo and Nelson 2012). The greater the dose of P used, the greater the decrease observed in the CF content. In the fermentation process, microorganisms need macro- and micronutrients for growth. One of the required macronutrients is phosphorus (Pazla et al. 2018). The process of glycolysis produces chemical energy in the form of ATP compounds that contain high-energy phosphate groups because glycolysis is instrumental in the process of phosphorylation through the transfer of radical phosphate groups during metabolism (Rodwell et al. 2019). In addition, in the cell, phosphorus is present as a phosphoprotein, phospholipid and nucleic acid compound. The combination of Ca, P and Mn minerals in the T3 treatment succeeded in producing optimum mold growth, enabling enzyme production to be even greater so that the mold work process in degrading the cell wall will increase. Febrina (2016) stated that the optimal combination of Ca and Mn minerals can produce optimal ligninase enzymes as well as break down the cell wall in the fermentation process.

The P (2000 ppm), Ca (2000 ppm) and Mn (150 ppm) minerals in T3 give the best results in increasing the supply of the ligninase enzyme to achieve a decrease in lignin content during OPF fermentation. This result has positive implications for the digestibility of cellulose and hemicellulose by rumen microbes on the palm frond. The main challenge to the implementation of this research is the fermentation process, which is difficult to carry out in rural areas.

To conclude, supplementation of fermented OPFs by *Phanerochaete chrysosporium* with treatments of Ca (2000 ppm), P (2000 ppm) and Mn (150 ppm) in T3 provided the lowest crude fiber content, the highest ligninase enzyme activity, a decreased lignin content, and the highest crude protein content.

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