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Research Article
Effects of Calcium, Phosphorus and Manganese Supplementation During Oil Palm Frond Fermentation by *Phanerochaete chrysosporium* on Laccase Activity and *in vitro* Digestibility

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Abstract

Objective: The objective of this study was to evaluate the effects of calcium (Ca), phosphorus (P) and manganese (Mn) supplementation during Oil Palm Frond (OPF) fermentation by *Phanerochaete chrysosporium* on laccase activity and *in vitro* fiber digestibility. **Materials and Methods:** This study was carried out using a randomized block design with 3 treatments (Addition of Ca, P and Mn) and 5 replications. The following treatments were performed: T1 = Ca 2000+P 1000+Mn 150 ppm, T2 = Ca 2000+P 1500+Mn 150 ppm, T3 = Ca 2000+P 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: Laccase activity (U mL $^{-1}$), Neutral Detergent Fiber (NDF) digestibility (%), Acid Detergent Fiber (ADF) digestibility (%), cellulose digestibility (%) and hemicellulose digestibility (%). **Results:** Results revealed that laccase enzyme activity, NDF digestibility, ADF digestibility and cellulose digestibility were significantly increased by the addition of P in T3. However, T2 and T3 non-significantly affected ($p>0.05$) the digestibility of hemicelluloses. **Conclusion:** It is concluded that supplementation of OPFs with Ca 2000, P 2000 and Mn 150 ppm resulted in the highest laccase activity and *in vitro* fiber digestibility.

Key words: Oil palm frond, *Phanerochaete chrysosporium*, calcium, phosphorus, manganese, laccase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oil Palm Fronds (OPFs) are the trimmed waste from oil palm plantations that receive little attention from farmers. The large number of fronds produced annually makes plantations of palm fronds a promising source of fibrous feed for ruminants. The lignin in palm fronds can reach 20% of the dry biomass[1]. The utilization of OPFs as a feedstock is still very limited because OPFs contain a high percentage lignin (30.18%), which results with the low digestibility of OPFs[2,3].

Lignocellulose and lignohemicellulose are the main component of OPFs and are composed of lignin, cellulose and hemicelluloses. The degradation of lignin is the key step in lignocellulose and lignohemicellulose transformation[4]. *Phanerochaete chrysosporium* is a white rot fungus that can degrade lignocellulose and lignohemicellulose[5]. *Phanerochaete chrysosporium* has been reported to liberate lignin from plant tissue and study has shown that lignin is oxidized and degraded by a ligninolytic system[2,6,7] composed of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase[8]. Laccase is an enzyme that can reduce oxygen to water through oxidation and reduction reactions at the same clutch that requires mediators to restore the function of the enzyme to its original shape. Laccase releases antioxidant compounds from the biocatalyst agent guaiacol[9], degrades the lignin and is used in a bleaching biodelignification process in the pulp paper industry[10].

Phanerochaete chrysosporium growth is influenced by the availability of minerals such as calcium (Ca), phosphorus (P) and manganese (Mn). Calcium is an inorganic element that is needed by virtually all organisms and is essential for the stability of protein and cell membrane structures; it enhances the growth of molds in its role of shaping the ends of branch hyphae. Manganese (Mn) is a micro-nutrient that is required as a co-factor in enzyme systems and improves the development of cell filaments. Suparjo[11] reported that the combination of Ca at 1190 and Mn at 100 ppm in the fermentation of cocoa pod husks by *Phanerochaete chrysosporium* was able to increase the growth of mold, with the average colony diameter of 8.39 cm and a mycelium dry weight of 17.49 mg. Ligninolytic enzyme production was also increased by to the addition of these minerals, thereby increasing digestibility. Febrina *et al.*[12] reported a decline in the lignin content in OPFs of 25.77% with the addition of Ca (2000 ppm) and Mn (150 ppm) as the *in vitro* digestibility of OPFs increased significantly. Phosphorous (P) is an essential mineral for metabolic processes that is required by all microbial cells. Phosphorous (P) maintains the integrity of the cell membrane and the cell wall components of nucleic acids and is part of the high-energy molecules ATP, ADP and AMP[13-15]. The addition of P was proven to meet the real needs of microbes and improve the digestibility of the fiber fraction in rice straw ammonization[16] and palm fronds[17].

This study aimed to determine the effects of Ca, P and Mn supplementation in fermented OPFs by *Phanerochaete chrysosporium* and their effects on laccase activity and *in vitro* digestibility.

MATERIALS AND METHODS

Oil palm frond fermentation: Oil Palm Frond (OPF) substrates used in this study were cut, dried and finely milled. Calcium (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. The ratio of the leaves and stems of OPFs was 1:1, the mineral solution from Brook *et al.*[18] was then added. The fermentation process was initiated by adding water to the OPFs until the water level reached 70%, either Ca, P or Mn was added, depending on the treatment. Observations were made every 120 h for 20 days. After 20 days, samples were taken for proximate analysis and fiber fraction determination. The proximate components were determined as described by AOAC[19]. The predominant fiber residues (hemicellulose, cellulose and lignin) were determined according to the method of Van Soest *et al.*[20].

Laccase activity: Enzyme extraction was initiated with the centrifuge method[21]; about 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 g for 20 min. The resulting supernatant (liquid) was separated and used for analysis of enzyme activity.

Laccase enzyme activity was determined by the oxidation of 2.2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS). The reaction mixture consisted of 0.1 mL ABTS, acetate buffer (0.5 mL, pH 5) at a concentration of 0.5 M and 0.4 mL of filtrate enzymes, the total volume was 1 mL. The cuvettes were shaken to ensure 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 min at a wavelength of 420 nm using a spectrophotometer. Enzyme activity can be calculated by following formula:

$$\text{Enzyme activity}(\text{Um L}^{-1}) = \frac{\Delta OD \times 420\,Vtot\,(mL) \times 10^9}{\varepsilon max \times d \times Vol.\ enzyme\,(mL) \times t}$$

where, ϵ max is molar absorptivity ABTS (36000 $M^{-1}cm^{-1}$), Vol. Is volume and d is wave length.

***In vitro* digestibility assay:** *In vitro* digestibility was analyzed by Tilley and Terry[22]. Fistula cow rumen fluid was diluted using McDougal Buffer (1:4) and dispensed into a 1 g substrate-prepared incubation tube, which was purged with CO_2 to maintain an aerobic condition. The tubes were incubated in a water bath at 39°C for 24 h. After fermentation, the erlenmeyer tube containing the sample was inserted into ice water to stop the fermentation. All samples were then centrifuged at 1,200 rpm for 15 min. The pH, NH_3-N and total VFA of the supernatants were then recorded. The NH_3-N concentration was measured using the micro-diffusion conway method and the total VFA concentration was measured using the steam distillation method. The previously incubated samples were vacuum filtered (Whatman No. 41) and dried at 60°C in an oven. The dried samples were used to analyze the NDF, ADF and cellulose in the *in vitro* digestibility assay.

Experimental design and statistical analysis: The study was carried out using a randomized block design (3×5) with five replications (Addition of Ca, P and Mn as treatments). Differences between treatment means were analyzed using Duncan's multiple range test.

The following treatments were carried out:

T1 = Ca 2000 ppm+P 1000 ppm+Mn 150 ppm
T2 = Ca 2000 ppm+P 1500 ppm+Mn 150 ppm
T3 = Ca 2000 ppm+P 2000 ppm+Mn 150 ppm

RESULTS AND DISCUSSION

The analysis of the data for the laccase activity and digestibility of NDF, ADF, cellulose and hemicellulose due to the addition of Ca, P and Mn in the fermentation process of OPFs by *Phanerochaete chrysosporium* is presented in Table 1.

Laccase enzyme is one of the ligninase enzymes produced by *Phanerochayte chrisosporium*. Treatment T3 shows the significant effect ($p<0.05$) against laccase enzyme activity, which indicates that the treatment is able to improve the activity of the laccase enzyme. Laccase enzyme activity was lowest for T1 (1.18 U mL^{-1}). It is suspected that the T1 treatment combination was not optimal for the mineral to extend to the fungi mycelia and was not optimal for the growth of molds; therefore, the resulting laccase enzyme activity was low. With increasing doses of P, the enzyme activity also increased due to laccase, in which P plays an important role in the metabolism of molds[23]. Laccase activity was highest in the T3 treatment (2.88 U mL^{-1}). The high activity of laccase in T3 indicates that fungus grows better and the fungi mycelia are longer; therefore, more enzymes are secreted. The high activity of laccase indicates that the degradation of lignocellulose and lignohemicellulose also increased, making it easier to utilize the rumen microbial cellulose and hemicellulose, which in turn increases digestibility. The laccase activity obtained in this study is higher than in previous study, in which Apriyani[24] was obtaining laccase activity of 2.002 U mL^{-1} in palm fronds fermented by *Pleurotus ostreatus*. However, the present results were lower than previous study which reported by Perdana[25], in which the laccase activity was 3.1 U mL^{-1} in fermented bagasse by *Ganoderma lucidum*.

Table 1: Laccase activity and digestibility of NDF, ADF, cellulose and hemicellulose due to the addition of minerals in the fermentation of OPFs by *Phanerochaete chrysosporium*

	Treatments		
Parameters	T1	T2	T3
Laccase activity (U mL^{-1})	1.18[a]	1.84[a]	2.88[b]
NDF (%)	65.32[a]	66.47[a]	69.10[b]
ADF (%)	48.50[a]	51.50[a]	59.97[b]
Cellulose (%)	57.05[a]	59.24[a]	64.77[b]
Hemicellulose (%)	72.57[a]	78.84[b]	79.78[b]

Means in the same row with different [a,b]letters are significant at $p<0.05$, NDF: Neutral detergent fiber, ADF: Acid detergent fiber

The addition of mineral Ca, P and Mn significantly ($p<0.05$) affects the NDF digestibility of OPF fermentation. The NDF digestibility was lowest at 65.32% in the T1 treatment. Low NDF digestibility and the resulting mycelium growth of mold and production of enzymes for degrading the ligninolytic substrate was not optimal, which was evident from the low laccase enzyme activity (1:18 U mL^{-1}). The lower enzyme activity was a result of the limit of the lignin degradation process. The lignification process limits the microbes that break down cellulose and hemicellulose as a source of energy for growth it[26]. The combination in the T3 mineral treatment produces the highest NDF digestibility. The increased digestibility of NDFs in the treatment with T3 was due to the combination of these minerals triggering an extension of mycelial fungi and ligninolytic enzymes with the resulting mold *Phanerochayte chrysosporium.* This optimized condition can loosen the bonds of lignocellulose and lignohemicellulose, which causes the cellulose and

hemicellulose to be utilized by microbes. This is supported by the study of Febrina *et al.*[12], in which *Phanerochayte chrysosporium* could decompose lignin in the substrate, penetrating the cellulose and hemicellulose attached to the lignin matrix. Release of the lignocellulosic bonds and lignohemicellulose result in cellulose and hemicellulose that can be used by fungi to grow and develop so that the process of fermentation in the rumen can better function. This is evident by the high digestibility of NDFs in this treatment. The biodegradation of lignin is a key process for lignocellulosic waste composting, in which the increased use of carbon increases the activity of micro-organisms that cause the enzyme activity of ligninolytics, thus increasing the degradation of lignin[27]. Increased doses of P also greatly aid the growth of microbes because P is instrumental in the process of glycolysis. Glycolysis produces chemical energy in the form of ATP compounds. Compounds of ATP (AP~P~P) contain a high-energy phosphate group that plays an important role in the metabolic process of phosphorylation via the radical transfer of phosphate groups. The cell also contains phosphoproteins, phospholipids and nucleic acids. The NDF digestibility obtained in this study is higher than the 66.429% reported by Febrina *et al.*[12] that only added Ca and Mn without P, in the fermentation of OPFs by *Phanerochayte chrysosporium.*

The treatments had a significant effect ($p<0.05$) on the ADF digestibility of OPFs. Treatment T1 provided the lowest ADF digestibility at 48.50%. The low activity of the laccase enzyme in this treatment shows that it is not optimal for fungi to degrade lignocellulose and lignohemicellulose, causing difficulty for the rumen microbes to degrade the feed to the low digestibility of ADF. The ADF digestibility was highest at 59.97% in the T3 treatment. The high digestibility in this treatment is associated with the higher activity of the enzyme, with optimal conditions for laccase in the lignin degradation process. The optimal level of lignin degradation causes the release of lignin from cellulose and hemicellulose. Sanchhez[28] noted that *Phanerochayte chrysosporium* is a white rot fungi that can degrade the lignocellulose components simultaneously. The ADF digestibility value in this treatment is higher than the results obtained by Pulungan[29], in which fermented oil palm trunks using *Phanerochayte chrysosporium* with 0.3 g urea achieved 36.50%.

Treatment T3 significantly affects ($p<0.05$) the digestibility of cellulose and hemicellulose. The treatment T3 causes the release of the lignocellulosic bonds and lignohemicellulose that cellulose and hemicellulose can use as an energy source with the rumen microbes that ultimately improve rumen microbial populations. The increasing population and cellulolytic microbe activity increased NDF, ADF and cellulose digestibility[30]. The addition of minerals into the substrate is still within normal levels, as seen from the digestibility of cellulose. Dias *et al.*[31] reported Ca, P, Mg and Mo at normal levels in the rumen microbes increase activity in digesting cellulose fibers. The digestibility of cellulose and hemicellulose was lowest at 57.05 and 72.57% in treatment T1, low digestibility showed low activity and the number of rumen microbes in the remodeled lignocellulose and lignohemicellulose resulted in still bound lignin with cellulose and hemicellulose. This is in accordance with Zain *et al.*[32] and Genther and Hansen[33], who noted that the digestibility of feed in ruminants was closely related to the amount and activity of rumen microbes. The digestibility of cellulose and hemicellulose increases with increasing laccase activity. High enzyme activity would increase the level of degradation of lignin so that the cellulose and hemicellulose were more widely used by the rumen microbes. The combination of minerals in T3 optimized the digestibility of cellulose and hemicellulose. Mineral supplementation was sufficient and appropriate microbial activity was indispensable in fulfilling normal functions to stimulate the proliferation and activity of fermentation[32]. The digestibility rates of cellulose and hemicellulose in this study were higher than in Febrina *et al.*[17], who added P, S and Mg in the fermentation of OPFs by *Phanerochayte chrysosporium*, at 55.97 and 78.63%. Hemicellulose digestibility is higher than the digestibility of cellulose because hemicellulose has a smaller molecular weight than cellulose due to the short chain branch that consists of different sugars, thus making it easier to be hydrolyzed[34].

CONCLUSION

Supplementation of fermented OPFs by *Phanerochayte chrysosporium* with treatments of Ca (2000 ppm), P (2000 ppm) and Mn (150 ppm) (T3) provided the highest laccase activity and *in vitro* digestibility.

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