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Research Article
Effect of Manganese (Mn²⁺) Addition on Cocoa Pod Fermentation with *Phanerochaete chrysosporium*

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Abstract

Objective: The study was conducted to determine the effect of manganese (Mn²⁺) addition on cocoa pod fermentation using *Phanerochaete chrysosporium* Pc2804. The enzyme activities of LiP and MnP, as well as the fiber fraction and digestibility of the cocoa pod, were evaluated. **Methodology:** The treatments were as follows: T1 = Cocoa pod fermentation without Mn²⁺, T2 = Cocoa pod fermentation with the addition of 200 µg Mn²⁺ g⁻¹ substrate, T3 = Cocoa pod fermentation with the addition of 400 µg Mn²⁺ g⁻¹ substrate, T4 = Cocoa pod fermentation with the addition of 600 µg Mn²⁺ g⁻¹ substrate and T5 = Cocoa pod fermentation with the addition of 800 µg Mn²⁺ g⁻¹ substrate. The observed variables were LiP and MnP activity, analysis of the fiber fraction including NDF, Acid Detergent Fiber (ADF), lignin, cellulose and hemicellulose and an analysis of *in vitro* digestibility. **Results:** The results showed that the addition of 600 µg Mn²⁺ g⁻¹ substrate to cocoa pod fermentation with *Phanerochaete chrysosporium* produces the highest LiP and MnP activities: 0.199±0.00 and 0.098±0.00 U mL⁻¹, respectively (p<0.05). The addition of 600 µg Mn²⁺ g⁻¹ substrate to cocoa pod fermentation with *Phanerochaete chrysosporium* Pc2804 causes a decrease in NDF (75.83±0.78%), ADF (67.49±0.41%), lignin (25.27±0.41%), hemicellulose (8.34±1.05%) and cellulose (31.53±0.68%) (p<0.05). The addition of 600 µg Mn²⁺ g⁻¹ substrate to cocoa pod fermentation with *Phanerochaete chrysosporium* Pc2804 produced the highest DM digestibility, which was 70.86±0.22% and the highest OM digestibility, which was 70.47±0.47% (p<0.05). **Conclusion:** The conclusion of this study is that the addition of 600 µg Mn²⁺ g⁻¹ substrate to cocoa pod fermentation with *Phanerochaete chrysosporium* Pc2804 gives the best digestibility of the cocoa pod.

INTRODUCTION

Wastes from food crops and plantations have an important role and potential in the supply of forages for ruminants such as cattle, goats, sheep and buffalo, especially in the dry season. In the dry season, forage grasses are stunted, reducing the availability of forages in terms of both quantity and quality. Even in areas of specific fodder, grass will dry up and die, causing a crisis of forage feed in the dry season. In addition, the ruminant rearing system is still largely dependent on forages in the form of grasses and other forages, with little or no additional feed.

One of the potential supplies of ruminant feeds in the dry season is cocoa pod, as the solid waste of cocoa plantations (*Theobroma cacao* L.). Indonesia has the world's third largest cocoa production. Indonesian cocoa production is[1] 740,513 t year^{-1}. Cocoa pod accounts for approximately 74% of the cocoa. Using this number, generation of cocoa pods is[1] 535,000 t year^{-1}. Cocoa pods can be utilized as animal feed either fresh or in the form of flour after processing. Cocoa pod contains 7.75% protein, 40.15% crude fiber and 3900 kcal kg^{-1} energy, exceeding the composition of elephant grass, which has 6.9% of protein and a total energy of 3800 kcal kg^{-1} [2]. By composition, cocoa pod is a potential source of fiber for ruminant feed. However, cocoa pod has low digestibility due to its lignin content, which is approximately 35%[3]. Hence, delignification is necessary to enhance its digestibility.

Phanerochaete chrysosporium is a microorganism that has the ability to selectively degrade the fiber fraction[4]. Cellulose and hemicellulose within the cocoa pod can be utilized by the fungus as a carbon source for its growth. Hence, degradation of lignin, the outer barrier of lignocellulosic material, can be expected. The high efficiency of lignin degradation and minimal utilization of cellulose polymer compared to other white rot fungi make *Phanerochaete chrysosporium* a good choice in the treatment of cocoa pod prior to use as ruminant feed.

In general, white-rot fungi synthesize three kinds of enzymes: Lignin-peroxidase (LiP), manganese-peroxidase (MnP) and laccase. These enzymes play an important role in the degradation of lignin[5]. In the process of lignin degradation, manganese plays an important role in the activity of manganese peroxidase and organizes the production of manganese peroxidase, laccase and lignin peroxidase[6]. Previous research proved that the addition of manganese affected lignin degradation with increasing activity. The manganese peroxidase activity level increased up to 24-fold with Mn^{2+} treatment compared with controls[7]. Production of manganese peroxidase by *Pleurotus ostreatus* increased 3-10-fold during solid fermentation on sawdust supplemented with $MnSO_4$[8]. However, research conducted by Camamero *et al.*[8] stated that the addition of manganese increased the degradation of lignin during solid fermentation with *Pleurotus ostreatus*, but the level of manganese peroxidase was not affected. The addition of 825 µg of manganese affected manganese peroxidase activity in three species of *Pleurotus*, namely *Pleurotus eryngii, Pleurotus pulmonarius* and *Pleurotus ostretaus*[9]. Meanwhile, the addition of 660 µg of manganese improved manganese peroxidase activity by 9 times when compared with a sample without added manganese[10].

Fermentation using *Phanerochaete chrysosporium* and the addition of the mineral Mn^{2+} causes a decrease in the fiber fraction comprising hemicellulose, cellulose and ligninase, which rumen microbes have difficulty in digesting. Hence, this study aimed to determine the effects of Mn^{2+} addition on cocoa pod fermentation by *Phanerochaete chrysosporium*. The ultimate goal is to provide an alternative ruminant feed using solid waste available locally in Indonesia.

MATERIALS AND METHODS

Materials: *Phanerochaete chrysosporium* (Pc2804) was maintained at 37°C on Potato Dextrose Agar (PDA) (200 g L^{-1} potato extract, 20 gL^{-1} glucose and 20 gL^{-1} agar) plates. *Phanerochaete chrysosporium* was cultured in an immersed liquid culture system. The culture medium was prepared as described by Tien and Kirk[11] and altered to contain 20 mM acetate buffer (pH 4.4) instead of dimethyl succinate buffer. In addition, 1.5 mM Veratryl Alcohol (VA), 0.2 g L^{-1} yeast extract powder and 1 gL^{-1} Tween 80 were added. A final spore concentration of 1×10^5 spores mL^{-1} was placed into a 250 mL Erlenmeyer flask containing 100 mL medium. Then, the flasks were incubated at 37°C in a rotary shaker and agitated at 150 rpm. The cultures were harvested at the time when the maximum activity of LiP was detected, approximately day 6 and centrifuged at 16200×g for 30 min at 4°C. The supernatant was used directly as crude ligninolytic enzymes in the fermentation experiments.

Cocoa pod: Cocoa pod was obtained from Wonogiri Regency, Central Java Province, Indonesia. Each cocoa pod was chopped into 2 cm pieces and sun-dried until the water content reached approximately 35%.

Solid state fermentation: The solid state fermentation was conducted in a 250 mL Erlenmeyer flask, which was used as the fermenter. The Erlenmeyer flask was filled with 50 g of cocoa pod, which was then inoculated with mold by as much as 5% of the substrate weight, based on the dry weight. The fermentation process occurred in a shaking incubator at a speed of 150 rpm and a temperature of 30°C for 7 days. Before and after fermentation, the cocoa pods were weighted. At the end of fermentation, the cocoa pods were dried at 50°C for 4 days to stop the microorganism activity. The cocoa pods were milled and sieved using a Thomas-Wiley Mill type 4 with a 1 mm diameter sieve.

Statistical analysis: The research design uses a completely randomized design with five treatments and six replicates. The treatments were as follows: T1 = Cocoa pod fermentation without Mn^{2+}, T2 = Cocoa pod fermentation with the addition of 200 µg Mn^{2+} g^{-1} substrate, T3 = Cocoa pod fermentation with the addition of 400 µg Mn^{2+} g^{-1} substrate, T4 = Cocoa pod fermentation with the addition of 600 µg Mn^{2+} g^{-1} substrate and T5 = Cocoa pod fermentation with the addition of 800 µg Mn^{2+} g^{-1} substrate.

The activities of LiP and MnP were measured as described by Tien and Kirk[11]. Fiber fraction analysis of NDF, Acid Detergent Fiber (ADF), hemicellulose, cellulose and lignin was performed as described by Van Soest[12]. *In vitro* digestibility was measured as described by Tilley and Terry[13], which was modified by Utomo[14]. Data were analyzed using one way analysis of variance (ANOVA). Duncan's multiple range test was used to test the significant difference among treatments. Statistical significance was set at p<0.05.

RESULTS AND DISCUSSION

Effect of manganese on lignin peroxidase and manganese peroxidase: The enzyme activities of LiP and MnP were measured and the results of the addition of manganese on cocoa pod fermentation using *Phanerochaete chrysosporium* are presented in Table 1.

The enzyme activity of *Phanerochaete chrysosporium* in cocoa pod fermentation with the addition of 600 µg Mn^{2+} g^{-1} substrate produced the highest enzyme activities of LiP and MnP, compared with the other treatments. The activity of LiP in this study was higher than the results reported by Harmini *et al.*[15], who added 400 µg Mn^{2+} g^{-1} oil palm empty fruit bunches using *Pleurotus* sp. that resulted in a LiP activity of 0.0127 U mL^{-1}. Another study showed that LiP activity was 0.06 U mL^{-1} when *Phanerochaete chrysosporium* was used for the fermentation of corn cob[16]. Table 1 also shows that the highest activity of MnP (0.098±0.00 U mL^{-1}), was obtained when 600 µg Mn^{2+} g^{-1} substrate was added.

Phanerochaete chrysosporium is a fungus that specifically degrades lignin by excreting extracellular enzymes. This fungus produces ligninolytic extracellular enzymes such as LiP and MnP. Lignin peroxidase (LiP) and manganese peroxidase (MnP) have the same mechanism of lignin degradation using H_2O_2[17]. The activities of ligninolytic enzymes were slightly affected by the addition of manganese. Manganese peroxidase is able to utilize Mn^{2+} and transform it into Mn^{3+} and H_2O_2. The Mn^{3+} then diffuses into the substrate to activate the oxidation of lignin[8]. The addition of Mn^{2+} could enhance the growth and the extension of the mycelium of *Basidiomycetes*, *Lentinus squarrosulus* and *Psathyrella atroumbonata*[18].

Effect of manganese on fiber fractions: In the process of lignin degradation, manganese plays an important role in the activity of manganese peroxidase and organizes the production of manganese peroxidase, laccase and lignin peroxidase. The results regarding the fiber fraction resulting from cocoa pod fermentation with Mn^{2+} addition are presented in Table 2.

Table 2 shows that the addition of Mn^{2+} to the fermentation decreased the value of Neutral Detergent Fiber (NDF) of the cocoa pod up to 1.8-6.7%. In contrast, the NDF content of cocoa pod before fermentation was 82.13%. The addition of 600 µg Mn^{2+} g^{-1} substrate resulted in an NDF value of 75.83±0.78% (or 6.3% reduction), which was the lowest of

Table 1: LiP and MnP activities of cocoa pods fermented with *Phanerochaete chrysosporium*

Variables	Treatments				
	T1	T2	T3	T4	T5
LiP (U mL^{-1})	0.140±0.02^a	0.170±0.00^b	0.165±0.01^b	0.199±0.00^c	0.169±0.00^b
MnP (U mL^{-1})	0.072±0.00^a	0.082±0.00^b	0.082±0.00^b	0.098±0.00^c	0.083±0.00^b

LiP: Lignin peroxidase, MnP: Manganese peroxidase, Different superscripts in the same row indicate significant differences (p<0.05), T1: Cocoa pod fermentation without Mn^{2+} addition, T2: Cocoa pod fermentation+200 µg Mn^{2+} g^{-1} substrate, T3: Cocoa pod fermentation+400 µg Mn^{2+} g^{-1} substrate, T4: Cocoa pod fermentation +600 µg Mn^{2+} g^{-1} substrate, T5: Cocoa pod fermentation+800 µg Mn^{2+} g^{-1} substrate

Table 2: Average fiber fraction of cocoa pods fermented with *Phanerochaete chrysosporium*

Variables	Treatments					Raw material
	T1	T2	T3	T4	T5	
NDF (%)	80.31±0.87[c]	77.70±0.30[b]	78.11±0.27[b]	75.83±0.78[a]	77.86±0.78[b]	82.13
ADF (%)	72.13±0.77[d]	68.94±0.75[b]	70.19±0.64[c]	67.49±0.41[a]	68.71±0.47[b]	72.58
Lignin (%)	27.59±0.45[c]	26.63±0.13[b]	26.79±0.49[b]	25.27±0.41[a]	26.71±0.48[b]	36.46
Cellulose (%)	27.70±0.62[a]	26.71±0.59[a]	29.14±1.16[b]	31.53±0.68[c]	28.93±0.83[b]	32.27
Hemicellulose[ns] (%)	8.18±0.77	8.45±0.47	7.93±0.87	8.34±1.05	9.15±0.99	11.63

NDF: Neutral detergent fiber, ADF: Acid detergent fiber, Different superscripts in the same row indicate significant differences (p<0.05), T1: Cocoa pod fermentation without Mn^{2+} addition, T2: Cocoa pod fermentation+200 μg Mn^{2+} g^{-1} substrate, T3: Cocoa pod fermentation+400 μg Mn^{2+} g^{-1} substrate, T4: Cocoa pod fermentation +600 μg Mn^{2+} g^{-1} substrate, T5: Cocoa pod fermentation+ 800 μg Mn^{2+} g^{-1} substrate, ns: Non significant

the treatments. The decline in NDF content means that the digestibility of the cocoa pod increased. During fermentation, lignocellulosic bonds are broken down by ligninolytic enzymes[19]. According to Suparjo *et al.*[20], changes in the NDF content of the cocoa pod were due to the utilization of cell components that contain lipids, sugars, organic acids, non-protein nitrogen, pectin, soluble proteins and other materials that are dissolved in water by *Phanerochaete chrysosporium*.

The acid detergent fiber in cocoa pod fermentation using *Phanerochaete chrysosporium* with the addition of 600 μg Mn^{2+} g^{-1} substrate produced an ADF value of 67.49±0.41%, which was significantly lower than other treatments (p<0.05).

The content of ADF in the cocoa pod before fermentation was equal to 72.58%. This means that the cocoa pod fermentation with the fungus *Phanerochaete chrysosporium* and the addition of Mn^{2+} cation caused a decrease in the ADF content ranging from 0.45-5.09%. The ADF declined by 5.09% in T4 and the smallest reduction in ADF content was 0.45% in treatment T1. The value of ADF is a fraction of the estimated value of fibers that are difficult for microbes in the rumen to degrade[21]. Hemicellulose is not expected to be degraded because hemicellulose is a polysaccharide that has a degree of degradation that is better than cellulose and lignin[20], so it can be utilized by microbes in the rumen as an energy source. For the measurement of feed quality, the higher the number, the worse the ADF feed quality.

Lignin in cocoa pod fermentation using *Phanerochaete chrysosporium* with a 600 μg Mn^{2+} g^{-1} substrate addition produced the lowest ADF value (25.27±0.41%), which was significantly different from other treatments (p<0.05). Cocoa pod fermentation with *Phanerochaete chrysosporium* and the addition of Mn^{2+} caused the highest reduction in lignin. The cocoa pod lignin content before fermentation was equal to 36.46%. This means that the cocoa pod fermentation with the fungus *Phanerochaete chrysosporium* and the addition of Mn^{2+} causes a decrease in the lignin content ranging from

8.87-11.19%. The decrease in the lignin content in T4 was equal to 11.19% and the smallest reduction in the lignin content occurred in the T1 treatment and was equal to 8.87%. The decrease the lignin content during fermentation is expected to be large because lignin is heavily degraded. The greater the degradation of lignin in the feed, the greater the improvement in digestibility of the feed.

Cocoa pod fermentation with *Phanerochaete chrysosporium* and the addition of Mn^{2+} has high MnP and LiP enzyme activities. This occurs because the MnP enzyme is able to utilize Mn^{2+} and convert it into Mn^{3+} and H_2O_2. The Mn^{3+} then diffuses into the substrate and activates the oxidation process[8]. The LiP enzyme has the ability to catalyze some oxidation reactions, including breakage of the Cα-Cβ side chain propyl non-phenolic aromatic components of lignin, oxidation of benzyl alcohol, oxidation of phenols, hydroxylation of benzylic methylene groups and a breakdown of the aromatic ring component of non-phenolic compound lignin[2]. Changes in the lignin content of the substrate occur due to the overhaul of the structure of lignin into simpler components, namely CO_2 and H_2O[22].

The effect of cellulose on cocoa pod fermentation using *Phanerochaete chrysosporium* with the addition of 600 μg Mn^{2+} g^{-1} substrate produces the highest cellulose value (31.53±0.68%), which is significantly different from other treatments (p<0.05).

The cocoa pod cellulose content before fermentation was equal to 32.27%. This means that cocoa pod fermentation with the fungus *Phanerochaete chrysosporium* and the addition of Mn^{2+} cations causes a decrease in the cellulose content ranging from 0.74-4.57%. The decrease in the cellulose content occurring in T1 is equal to 4.57% and the smallest reduction in the cellulose content occurred in treatment T4 was equal to 0.74%. The decline in the cellulose content before and after fermentation is expected to be small because the degraded cellulose content is also small. The smaller the degradation of cellulose in the diet, the better microbes can utilize cellulose in the rumen as an energy source.

Table 3: Average *in vitro* digestibility of cocoa pods fermented with *Phanerochaete chrysosporium*

Variables	Treatments					Raw material
	T1	T2	T3	T4	T5	
DM digestibility (%)	68.29±0.18[a]	69.50±0.38[b]	69.55±0.78[b]	70.86±0.22[c]	69.59±0.46[b]	57.42
OM digestibility (%)	67.99±0.66[a]	69.16±0.10[b]	68.99±0.27[b]	70.47±0.47[c]	69.24±0.70[b]	55.75

DM: Dry mater, OM: Organic matter, Different superscripts in the same row indicate significant differences (p<0.05), T1: Cocoa pod fermentation without Mn^{2+} addition, T2: Cocoa pod fermentation+ 200 µg Mn^{2+} g^{-1} substrate, T3: Cocoa pod fermentation+400 µg Mn^{2+} g^{-1} substrate, T4: Cocoa pod fermentation+600 µg Mn^{2+} g^{-1} substrate, T5: Cocoa pod fermentation+800 µg Mn^{2+} g^{-1} substrate

Hemicellulose during cocoa pod fermentation using *Phanerochaete chrysosporium* and the addition of Mn^{2+} showed no significant differences across all treatments. The hemicellulose content of the cocoa pod before fermentation was equal to 11.63%. This means that cocoa pod fermentation with *Phanerochaete chrysosporium* and the addition of Mn^{2+} cations causes a decrease in the hemicellulose content ranging from 2.48-3.70% and it shows no significant differences between the treatments. The decrease in the hemicellulose content before and after fermentation is expected to be small because the degraded hemicellulose content is also small. The smaller the degradation of hemicellulose in the diet, better the utilization of rumen microbes as an energy source.

Phanerochaete chrysosporium showed little degradation of hemicellulose. This suggests that *Phanerochaete chrysosporium* selectively degrades the fiber, preferring lignin over hemicellulose.

Effect of manganese on the feed digestibility of cocoa pod: Table 3 presents the values of dry matter digestibility and organic matter digestibility of the cocoa pod after fermentation using *Phanerochaete chrysosporium* and with the addition of Mn^{2+}.

Table 3 shows that T4 had the highest value of DM digestibility (70.86±0.22%), which was significantly different (p<0.05) from T1, T2, T3 and T5. Likewise, treatment T4 has the highest value of OM digestibility (70.47±0.47%) which was significantly different (p<0.05) from T1, T2, T3 and T5.

Table 3 shows that the average cocoa pod fermentation DM digestibility with the fungus *Phanerochaete chrysosporium* ranged from 68.29% (T1 treatment) to 70.86% (T4 treatment). Table 3 shows that the average OM digestibility of the cocoa pod fermented with *Phanerochaete chrysosporium* ranged from 67.99% (T1 treatment) to 70.47% (T4 treatment).

Dry matter digestibility and OM digestibility of the cocoa pod fermented with the fungus *Phanerochaete chrysosporium* with the addition of 600 µg Mn^{2+} g^{-1} substrate have the highest digestibility values. This is because the enzyme activities of LiP and MnP are higher than the other treatments,

causing the best degradation of the fiber fraction and resulting in an increased level of digestibility. The fiber fraction is capable of being optimally degraded by the fungus *Phanerochaete chrysosporium* such that it results in an increase in DM and OM digestibility.

Dry matter digestibility of the cocoa pod before fermentation is equal to 57.42%. This means that the cocoa pod fermentation with the fungus *Phanerochaete chrysosporium* and the addition of Mn^{2+} causes an increase in DM digestibility ranging from 10.87-15.44%. The increase in DM digestibility was highest in T4 (15.44%) and smallest in T1 (10.87%). The increase in DM digestibility during fermentation is expected to be large so that more feed can be digested.

Organic matter digestibility of the cocoa pod before fermentation is equal to 55.75%. This means that the cocoa pod fermentation with the fungus *Phanerochaete chrysosporium* and the addition of Mn^{2+} causes an increase in OM digestibility ranging from 12.24-14.72%. The increase in OM digestibility was greatest in T4 (14.72%) and smallest in T1 (12.24%). The increase in OM digestibility before and after fermentation is expected to be large so that more feed can be digested.

Values of DM digestibility and OM digestibility during the cocoa pod fermentation process are closely related to the activities of the enzymes of *Phanerochaete chrysosporium*. With the activities of these enzymes, fibers of the plant cell wall fraction of lignin degrade particularly well, increasing the digestibility value of the cocoa pod. With higher values of the DM and OM digestibilities, the potential of the cocoa pod as animal feed for ruminansia is high. High digestibility values mean that the proportion of cocoa pod for the feed becomes larger.

CONCLUSION

The addition of the mineral Mn^{2+} to cocoa pod fermentation with the *Phanerochaete chrysosporium* fungus produces the enzymatic activities of LiP and MnP and improves the digestibility of the fiber fraction better than the cocoa pod fermentation with *Phanerochaete chrysosporium* fungus without the addition of Mn^{2+}. The addition of

600 µg Mn^{2+} g^{-1} substrate to cocoa pod fermentation with *Phanerochaete chrysosporium* fungus produces the highest enzyme activities of LiP and MnP and the highest dry matter and organic matter digestibility. The addition of 600 µg Mn^{2+} g^{-1} substrate to cocoa pod fermentation with the *Phanerochaete chrysosporium* fungus produced the lowest NDF.

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