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Research Article
Cocoa Pods with Different Nitrogen Sources Fermented by Using *Pleurotus ostreatus* as Poultry Feed

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Abstract

Background and Objective: Cocoa pods can be used as a nonconventional animal feed but their utilization is limited due to their high crude fiber (lignin and cellulose) and low crude protein content. Fermentation with *Pleurotus ostreatus*, a lignocellulolytic fungus (producing ligninase and cellulase enzymes), has been used to increase crude protein (CP) levels and quality. This study evaluated the nutrient content and quality of cocoa pods fermented in combination with different nitrogen sources, such as tofu waste (TW), soy meal waste (SMW) and rice bran (RB), using *Pleurotus ostreatus*. **Materials and Methods:** The experiment was performed by using a completely randomized design with 4 treatments, namely, A (100% CP), B (80% CP+20% TW), C (80% CP+20% SMW) and D (80% CP+20% RB) and 5 replicates. Subsequently, crude protein, crude fiber (CF), nitrogen retention, crude fiber digestion and cellulose enzyme activity were measured. **Results:** The results of the experiment showed that cocoa pods with the tofu waste nitrogen source fermented with *Pleurotus ostreatus* significantly reduced CF and increased crude protein content, nitrogen retention, crude fiber digestion and cellulase activity (p<0.01). **Conclusion:** An 80% CP with 20% TW combination fermented with *Pleurotus ostreatus* was identified as the best condition for improving the nutrient content and quality of cocoa pods.

Key words: Cocoa pods, fermentation, nutrient quality, *Pleurotus ostreatus*, poultry feed

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

INTRODUCTION

Cocoa pods can be used as a nonconventional animal feed. Cocoa plants are one of the most common plants in Indonesia, especially in West Sumatra. The production of cocoa fruit on smallholder plantations in the West Sumatra region increased in 2016 from that in the previous year. Cocoa production in 2015 was 50,553 t, while in 2016, it was 60,254 t[1]. Cocoa fruit is used as a food, while cocoa pods are thrown away, even though cocoa pods can be used as an alternative to animal feed. Cocoa fruit consists of three parts: cocoa pods (74%), placenta (2%) and seed (24%); therefore, it can be estimated that the quantity of available cocoa pods in 2016 was 44,587 t[2]. The nutritional content of cocoa pods containing 1950 kcal kg^{-1} ME is as follows: crude protein (11.71%), crude fiber (28.75%,including cellulose (22.07%) and lignin (20.13%)), fat (11.80%) and BETN (34.90%)[2]. The utilization of cocoa pods as animal feed is limited, totaling only 5% in broiler diets[2]. The limiting factor for the utilization of cocoa pods is the low crude protein and high crude fiber contents. Another limiting factor is the presence of theobromine (0.16-0.25% content), which causes poisoning in livestock[2].

One way to overcome the limitations of cocoa pods is through fermentation by using *Pleurotus ostreatus*. *Pleurotus ostreatus* is a fungus that produces lignocellulolytic and cellulolytic enzymes and has a hypocholesterolemic effect. *Pleurotus ostreatus* is classified as a white rot fungus that is able to degrade lignin because it produces extracellular ligninolytic enzymes such as laccase, lignin peroxidase and Mn peroxidase[3,4]. In addition, cellulase enzymes and amylase enzymes are produced by *Pleurotus ostreatus*[3,4]. Previous research has shown that *Pleurotus ostreatus* can degrade crude fiber, lignin and cellulose from palm oil sludge[4].

Another advantage of fermentation with *Pleurotus ostreatus* is the presence of lovastatin compounds, which can inhibit the formation of cholesterol[5]. Lovastatin has a hypercholesterolemic effect (reducing blood cholesterol) by inhibiting the action of the enzyme HMG-CoA reductase, which is needed for mevalonate formation in cholesterol synthesis[6,7]. Products fermented with *Pleurotus ostreatus* can be used as a substitute for concentrate or commercial rations and produce meat and eggs low in cholesterol content[2].

The success of solid media fermentation depends on the conditions used, such as the composition of the substrate, the dose of the inoculum given and the length of incubation[2-4]. The most important components that must be present in the fermentation medium are the sources of carbon, nitrogen and other essential elements in the appropriate quantities and proportions[2-4]. Fungal growth requires carbon sources (c) to form structural elements and nitrogen (N) is needed to form amino acids, purines, pyrimidines, carbohydrates and lipids[4,5]. Cocoa pods can be used as a carbon source (c) in the fermentation medium but require an additional nitrogen (N) source to obtain a C:N balance that is suitable for fungal growth. The sources of nitrogen that can be used are tofu waste, soy milk and rice bran.

Tofu waste has good nutritional value and is classified as a protein source. Tofu waste is an agroindustrial waste product formed during the process of making solid tofu, which is widely available. Tofu waste has the potential to be used as a source of nitrogen for microorganisms and as an animal feed because it contains 28.36% crude protein and 5.52% fat, 7.06% crude fiber and 45.44% BETN[2]. According to Hsieh and Yang[8], soy milk waste is produced when making soy milk, has a 26.5% crude protein content and contains lysine, methionine and vitamin B. Rice bran is a byproduct of rice production in rice mills. Rice bran contains protein (12-14%) and fat (7-9%) and is rich in the vitamin B complex. Rice bran increases the porosity of the substrate, which makes it easier for fungi to grow in the fermentation medium[2]. The main objective of this study was to assess improvements in the nutrient quality of cocoa pods through fermentation by *Pleurotus ostreatus* using several nitrogen sources.

MATERIALS AND METHODS

Experimental design: The experiment was performed by using a completely randomized design with 4 treatments: 100% CP, 80% CP+20% TW, 80% CP+20% SMW and 80% CP+20% RB, with 5 replicates.

Variables: Crude protein, crude fiber (CF), nitrogen retention, crude fiber digestion and cellulose enzyme activity were measured.

Fermented feed preparation: *Lentinus edodes* cultures were reconstituted and sub cultured using potato dextrose agar for 4 days. Inoculated subcultures were maintained at room temperature. CPF was prepared according to the procedure described by Nuraini *et al.*[2]. In this experiment, 100 g of substrate containing an 80% CP and 20% nitrogen source (TW, SMW and RB) mixture per experimental unit was added together with 125 mL of aquadest (moisture content: 70%). The mixture was then sterilized in an autoclave (121°C for 15 min). After cooling to 27-30°C, the sample was inoculated with 8% *Pleurotus ostreatus*. These mixtures were then incubated for 9 days. After incubation, the fermented product was dried in an oven at 80°C for 2 h and 60°C for12 h.

Data analysis: All of the data were statistically analyzed by one-way analysis of variance with completely randomized design (CRD). Significant differences between treatments were determined by using Duncan's multiple range test, with p<0.05 considered significantly different.

RESULTS

Effect of different nitrogen sources on crude fiber and cellulase enzyme activity in cocoa pods fermented with *Pleurotus ostreatus*: The effects of different nitrogen sources on crude fiber and cellulase enzyme activity in cocoa pods fermented with *Pleurotus ostreatus* are shown in Table 1-2, respectively. We found that the different nitrogen sources had a significant effect on the crude fiber content and cellulase enzyme activity of cocoa pods fermented with *Pleurotus ostreatus* (p<0.05).

Effect of the nitrogen source on the crude protein content of cocoa pods fermented with *Pleurotus ostreatus*: The effects of different nitrogen sources on the crude protein content and nitrogen retention in cocoa pods fermented with *Pleurotus ostreatus* are shown in Table 3-4. The nitrogen source in cocoa pods fermented with *Pleurotus ostreatus* had a highly significant effect on crude protein content and nitrogen retention (p<0.01).

Table 1: The effects of *Pleurotus ostreatus* fermentation on crude protein of cocoa pods for use, as poultry feed

Treatments	Crude protein content (%)
A (PC)	15.11[d]
B (80% PC+20% TW)	21.68[a]
C (80% PC+20% SW)	20.36[b]
D (80% PC+20% RB)	16.05[c]
SE	0.20

[a-b]Different superscripts within a column show highly significance (p<0.01), SE: Standard error of the mean

Table 2: The effects of *Pleurotus ostreatus* fermentation on nitrogen retention of cocoa pods for use, as poultry feed

Treatments	Nitrogen retention (%)
A (PC)	55.91[d]
B (80% PC+20% TW)	67.16[a]
C (80% PC+20% SW)	63.23[b]
D (80% PC+20% RB)	59.01[c]
SE	0.28

[a-b]Different superscripts within a column show highly significance (p<0.01), SE: Standard error of the mean

Effect of the nitrogen source on the crude fiber digestibility of cocoa pods fermented with *Pleurotus ostreatus*: The effects of different nitrogen sources on crude fiber digestibility in cocoa pods fermented with *Pleurotus ostreatus* are shown in Table 5. The different nitrogen sources had a significant effect (p<0.05) on crude fiber digestibility of cocoa pods fermented with *Pleorotus ostreatus*.

DISCUSSION

The crude protein levels of the cocoa pods were increased after fermentation with *Pleurotus ostreatus*. The highest crude protein in treatment B was caused by the addition of tofu waste with a high crude protein content (28.36%), which improved the growth of the fungus due to the balance of C:N being 12.16:1. The proliferation of fungal growth was largely influenced by the availability of carbon and nitrogen in the substrate that was suitable for the growth of *Pleurotus ostreatus*. Nuraini *et al*.[2] reported that 80% of cocoa pod plus 20% of tofu waste with a C:N ratio of 11:1 ratio increased the crude protein when fermented with *Phanerochaete chrysosporium*. According to Musnandar[9], the

Table 3: The effects of *Pleurotus ostreatus* fermentation on crude fiber content of cocoa pods for use, as poultry feed

Treatments (%)	Crude fiber content
A (PC)	23.04[a]
B (80%PC+20% TW)	15.01[d]
C (80%PC+20% SW)	16.16[c]
D (80%PC+20% RB)	17.82[b]
SE	1.50

[a-b]Different superscripts within a column show significance (p<0.05). SE: Standard error of the mean

Table 4: The effects of *Pleurotus ostreatus* fermentation on cellulase enzyme activity of cocoa pods for use, as poultry feed

Treatments	Cellulase enzyme activity (U mL^{-1})
A (PC)	3.56[b]
B (80%PC+20% TW)	6.32[a]
C (80%PC+20% SW)	5.59[ab]
D (80%PC+20% RB)	5.06[bb]
SE	0.17

[a-b]Different superscripts within a column show significance (p<0.05). SE: Standard error of the mean

Table 5: The effects of *Pleurotus ostreatus* fermentation on crude fiber digestion of cocoa pods for use,as poultry feed

Treatments	Crude fiber digestion (%)
A (PC)	40.83[d]
B (80%PC+20% TW)	55.47[a]
C (80%PC+20% SW)	50.14[b]
D (80%PC+20% RB)	47.73[c]
SE	1.09

[a-b]Different superscripts within a column show significance (p<0.05). SE: Standard error of the mean

growth of fungi requires carbon to form the structural elements and nitrogen is needed to form amino acids, purines, pyrimidines, carbohydrates and lipids. According to Crueger and Crueger[10], fungi have a relatively high protein content at 40-60%. The crude protein content of the final fermented product was highly dependent on the crude protein content of the initial substrate. Karmas and Haris[11] argued that changes in the food substance content due to fermentation depend on the availability of food ingredients, the metabolic ability of fermentative microorganisms and the interaction of these elements. According to Carlile and Watkinson[12], an increase in protein content after fermentation is a process of "protein enrichment," which means the process of enriching certain microorganisms because the process is identical to the production of individual cellular proteins and this process is not separated between microbial cells that grow on the substrate.

The low level of crude protein in treatment A occurred because the substrate was only cocoa pods with a C:N ratio of 18:1 and that in treatment D occurred because the C:N ratio was 16.16:1; both substrates had a high carbon content but lacked nitrogen sources, so the growth of *Pleurotus ostreatus* was inhibited. The C:N ratio was related to the added nitrogen source, which contained low amounts of crude protein, so that the growth of microorganisms was uneven and the contribution of proteins from fungi was not as high as in treatment B. Carlile and Watkinson[12]stated that the most important things in the fermentation medium are carbon (c), nitrogen (N) and other essential elements in balanced quantities. According to Pasaribu[13], the increase in protein on solid substrates is due to the molds' own nucleic acid content, which can contribute N. In treatment C, with a C:N balance of 12.42:1, *Pleurotus ostreatus* appears to thrive compared to in treatment A and treatment D but was less fertile than in treatment B. The results of this study revealed a high crude protein content (20.50%) (an increase in crude protein of 33.29%) in treatment B (composition of 80% cocoa pods+20% tofu waste). Nuraini *et al.*[2] reported that 80% of cocoa pods and 20% of tofu waste fermented with *Phanerochaete chrysosporium* increased the crude protein of fermented products by 33.79%.

The high nitrogen retention in treatment B was caused by the high crude protein content consumed by livestock (2.80 g head^{-1}), which is also related to the high crude protein content (20.50%) in treatment B. According to Corzo *et al.*[14], the factors that influence nitrogen retention are feed consumption, especially consumption of protein. If the protein quality (amino acids) is low, nitrogen retention will be low. The nitrogen retention was influenced by an increase in protein levels in feed. Nitrogen retention depends on the level of protein in the feed and nitrogen content, which is retained in line with the protein content of the feed. Nitrogen retention is one way to assess protein quality. The high nitrogen retention in treatment B was 66.53%, which was higher than that detected by Nuraini[15], who fermented cocoa pods with *Lentinus edodes* and obtained a nitrogen retention of 61.74%.

The highest cellulase enzyme activity in the treatment B was due to the addition of a nitrogen source in the form of tofu waste, which provided an additional source of nutrients to the fermentation medium; as a result, the growth of *Pleurotus ostreatus* increased and the cellulase enzyme activity increased accordingly. In treatment A, there was no additional source of N, so the nutrients in the fermentation medium were limited; as a result, the growth of *Pleurotus ostreatus* was not optimal and the activity of the cellulase enzymes decreased. Cellulase enzymes work in 3 ways, namely, (1) Endo-1,4-β-D-glucanase (endocellulase, carboxymethyl cellulase or CMCase), which breaks down the cellulose polymers by randomly breaking the internal bonds of α-1,4-glycosides to produce oligodextrins with varying chain lengths. (2) Exo-1,4-β-D-glucanase (cellobiohydrolase), which breaks down cellulose from the reducing and nonreducing ends to produce cellobiose for glucose (3). β-Glucosidase enzyme (cellobiase), which breaks down cellobiose to produce glucose[16]. Cellulase enzyme activity is influenced by several factors including temperature, pH, substrate concentration, presence of inhibitors and incubation time[17].

The crude fiber levels of the cocoa pods were decreased after fermentation with *Pleurotus ostreatus*, which was due to degradation by ligninase and cellulase produced by the fungi. The lowest CP crude fiber content was observed in treatment B substrate composition after 9 days of incubation with *Pleurotus ostreatus* (reduction of 30.64%). The lowest crude fiber in treatment B was caused by the increased fungal growth in response to the balance of C:N, which was suitable for the growth of *Pleurotus ostreatus*. This C:N balance was achieved by the addition of tofu waste to the substrate so that the fungus grew and there was more degradation of crude fiber from the cocoa pods during fermentation by *Pleurotus ostreatus*. The increased mold growth caused an increase in the enzymes produced, resulting in a concomitant decrease in crude fiber. This result is consistent with the opinion of Pasaribu[13] who stated that, it is necessary to add nitrogen and minerals for the optimal growth of fungi. *Pleurotus ostreatus* also produces lignin peroxidase (LiP), manganese peroxidase (MnP) and lactase (Lac) enzymes[18], which can hydrolyze lignin, cellulose and hemicellulose into simpler components, resulting in a decrease in crude fiber content.

The small reduction in crude fiber content in treatment A occurred because the substrate used was only cocoa pods and there was no addition of N sources, preventing the fungus from growing well. In treatment D, bran was added, which contained crude protein and a low level of nutrients; consequently, the fungus did not grow optimally, which resulted in low resurfacing of crude fiber in the fermented cocoa pods. From the results of this study, we obtained an optimal crude fiber reduction of 13.01% in treatment B (composition of 80% pod cocoa+20% tofu waste). Nuraini[2] achieved 80% fermentation of cocoa pods plus 20% tofu pulp with *Phanerochaete chrysosporium* and reduced the crude fiber fermentation products by 33.02% (from 33.40-22.37%).

The high digestibility of crude fiber in treatment B was caused by the crude fiber content, which was also lower in treatment B, at 13.01%. The low crude fiber content is due to the lignin and cellulose contained in fermented cocoa pods being broken down by ligninase and cellulase enzymes secreted by *Pleurotus ostreatus* mold, which increases the digestibility. According to Wahju[19], crude fiber consists of cellulose, hemicellulose and lignin, which are mostly indigestible by poultry and act as boosters or bulking agents. The lower the crude fiber content of feed ingredients is, the higher the digestibility of crude fiber feed ingredients is. Foods that undergo fermentation usually have better nutritional value than the original ingredients due to microorganisms that are catabolic or break down complex components into simpler substances.

The low digestibility of crude fiber in treatment A was related to the crude fiber content, which was high compared to that in other treatments, namely, treatment A (23.04%). The high crude fiber content in feed ingredients is of little value, not utilized properly and widely released through excreta. Crude fiber can only be digested by monogastric animals and a high crude fiber content in the feed will reduce the efficiency of the use of other food substances; in addition to the effect of crude fiber that it cannot be digested and excreted through feces, poultry will reproduce and grow imperfectly[19].

CONCLUSION

The 80% CP+20% TW combination of substrate and fermentation with *Pleurotus ostreatus* was identified as the best condition for improving the nutrient content (reducing crude fiber, lignin and cellulose levels as well as increasing crude protein and cellulose enzyme activity) and nutrient quality of cocoa pods.

SIGNIFICANCE STATEMENT

This study assessed the utilization of cocoa pods through fermentation using lignocellulolytic fungi. The use of CP waste as an alternative poultry feed is still limited due to the high crude fiber content and low protein content. This study found that fermenting cocoa pods using *Pleurotus ostreatus* (lignocellulolytic fungi) reduced the crude fiber content.

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