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EFFECT OF LIGNOCELLULOLYTIC FUNGUS ON ENZIMATIC ACTIVITY, FIBER FRACTION, AND DIGESTIBILITY ON FERMENTATION PROCESS OF COCOA POD

PENGARUH KAPANG LIGNOSELULOLITIK TERHADAP AKTIVITAS ENZIM, FRAKSI SERAT, DAN KECERNAAN PADA PROSES FERMENTASI KULIT BUAH KAKAO

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ABSTRACT

The study was conducted to determine the enzyme activity, fiber fraction and digestibility in the fermentation process of cocoa pod. The substrate used was the cocoa pod while the fungi were *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Schizophyllum commune*. Cocoa pod was chopped, finely ground and then dried. The solid state fermentation was conducted in a 250 ml Erlenmeyer flask as the fermenter. Erlenmeyer flask was filled with 100 g of cocoa pod, which was then inoculated with mold as much as 5% of the weight of the substrate based on the dry matter. The fermentation process used a shaker incubator at a speed of 150 rpm and temperature of 30°C for 7 days. Fungi was grown in liquid medium for preparation. Fermentation conducted with four different fungi as treatment and five replications, those are T1 = fermentation of cocoa pod without fungi addition, T2 = fermentation of cocoa pod with *P. chrysosporium* addition, T3 = fermentation of cocoa pod with *P. ostreatus* addition, and T4 = fermentation of cocoa pod with *S. commune.* Fermentation was performed by adding 100 g of cocoa pod and 5% of fungi (dry matter bassis) on a 250 ml Erlenmeyer. Variables observed were enzyme activity, fiber fraction and digestibility. This study was designed using completely randomized design with a unidirectional pattern analysis of variance, followed with Duncan's multiple range test. The results showed that fermentation using *P. chrysosporium* has highest lignin peroxidase enzyme activity of 0.52±0.04 U/ml and mangan peroxidase 0.06±0.00 U/ml, neutral detergent fiber 75.54±0.41%, acid detergent fiber 68.10±0.30%, lignin 26.86±0.19%, cellulose 27.17±0.25%, hemicellulose 6.77±0.52%, dry matter digestibility 69.70±0.43% and organic matter digestibility 69.59±1.03%. The conclusion from this research is the fermentation by using fungi *P. chrysosporium* addition has the best result to degradate lignin.

(Keywords: Cocoa pod, Digestibility, Enzyme activity, Fermentation, Fiber fraction)

INTISARI

Penelitian ini dilakukan untuk mengetahui aktivitas enzim, fraksi serat dan kecernaan dalam proses fermentasi pod kakao. Substrat yang digunakan yaitu kulit buah kakao (KBK) sedangkan kapang yang digunakan adalah Phanerochaete chrysosporium, Pleurotus ostreatus dan Schizophyllum commune. Kulit buah kakao dipotong dan ditumbuk halus kemudian dikeringkan. Sebanyak 100 g kulit buah kakao dimasukkan dalam 250 ml Erlenmeyer kemdian diinokulasikan kapang sebanyak 5% berdasar bahan kering. Persiapan jamur dengan menumbuhkan jamur dalam medium cair. Metodologi penelitian adalah fermentasi dilakukan dengan jamur yang berbeda digunakan empat perlakuan dan lima ulangan. T1 = fermentasi KBK tanpa penambahan kapang, T2 = fermentasi KBK dengan penambahan P. chrysosporium, T3 = fermentasi KBK dengan penambahan P. ostreatus, dan T4 = fermentasi KBK dengan penambahan S. commune.Proses fermentasi menggunakan shaker incubator pada kecepatan 150 rpm dan temperatur 30° C selama 7 hari.Penelitian ini dirancang menggunakan desain penelitian rancangan acak lengkap dengan analisis pola searah yang dilanjutkan dengan uji jarak Duncan apabila terdapat perbedaan. Hasil penelitian menunjukkan fermentasi digunakan P. chrysosporium memiliki aktivitas enzim lignin peroksidase tertinggi 0,52±0,04 U/mL dan aktivitas enzim mangan peroksidase 0,06±0,00 U/mL, neutral detergent fiber 75,54±0,41%, acid detergent fiber 68,10±0,30%, lignin 26,86±0,19% selulosa 27,17±0,25%, hemiselulosa 6,77±0,52%, kecernaan bahan kering 69,70±0,43%

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dan kecernaan bahan organik 69,59±1,03%. Kesimpulan dari penelitian ini bahwa fermentasi dengan menggunakan kapang P. chrysosporium memiliki hasil terbaik untuk mendegradasi lignin.

(Kata kunci: Aktivitas enzim, Fermentasi, Fraksi serat, Kecernaan, Kulit buah kakao)

Introduction

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

Cocoa pod has an important role and potential in the supply of ruminant feed, especially goats, especially in the dry season. Cocoa pod utilization as animal feed can be given fresh or in the form of flour after processing. Judging from the composition, the cocoa pod contains 7.75% protein and energy of 3900 kcal/kg which exceeded the composition of elephant grass of 6.9% and a total energy of 3800 kcal/kg (Puastuti *et al*., 2009). Cocoa pod was an agro-industrial waste produced cocoa plant (*Theobroma cacao L*.). Cocoa consisting of 74% rind, 2% and 24% seed placenta. Proximate analysis results containing 22% protein and 3-9% fat (Nasrullah and Ella, 1993).

Cocoa pod has a high content of fiber fraction especially lignin content. Therefore if cocoa pod given directly to the ruminant, it will cause low digestibility. *Phanerochaete chrysosporium* was a microorganism that has the ability to selectively degrade lignocellulose that degrades the lignin component first, followed by the cellulose component (Tuomelo *et al*., 2000). Cellulose and hemicellulose utilized by fungi as a carbon source. This fungus also has the ability to grow at a relatively high temperature (36-40^{o}C) so suitable to be used in fermentation processes that produce heat (Tuomelo *et al*., 2000). Lignin degradation of high efficiency and minimal in utilizing cellulose polymers compared to other white rot fungi, make *P. chrysosporium* as the best choice in the treatment of lignin degradation.

Fungi degrade lignin are most active white-rot fungi, such as *P. chrysosporium* and *Coriolus versicolor* able to degrade hemicellulose, cellulose and lignin from plant waste into CO_2 and H_2O (Paul, 1992; Limura *et al*., 1996). In general, white-rot basidiomisetes synthesize three kinds of enzymes, Lignin-peroxidase (LiP), manganese-peroxidase (MnP) and laccase. The all of these enzyme plays an important role in the degradation of lignin (Srinivasan *et al*., 1995).

Materials and Methods

Preparation of the fungus

The fungus was maintained at 37^{o} C on potato dextrose agar (PDA) (200 g L^{-1} potato extract, 20 g L^{-1} glucose and 20 g L^{-1} agar) plates. The fungus was cultured in animmersed liquid culture system. The culture medium was prepared as described by Tien and Kirk (1984) but containing 20 mM acetate buffer (pH 4.4) instead of dimethyl succinate buffer. In addition, 1.5 mM vertryl alcohol (VA), 0.2 g L^{-1} yeast extract powder, and 1 g L^{-1} Tween 80 were added. The final spore concentration of 1 x 10^5 spores mL^{-1} was fed into a 250 mL Erlenmeyer flask containing 100 mL medium. Then the flasks were incubated at 37^{o} C in a rotary shaker with agitation of 150 rpm. The cultures were harvested at the time when the maximum activities of LiP was detected at approximately day-6 and centrifuged at 16.200 g for 30 minute at 4^{o}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. Cocoa pod was chopped into 2 cm in size and sun-dried until the water content reached around 35%.

Solid state fermentation

The solid state fermentation was conducted in a 250 mL Erlenmeyer flask as the fermenter. Erlenmeyer flask was filled with 100 g of cocoa pod, which was then inoculated with mold as much as 5% of the weight of the substrate based on the dry

matter. The fermentation process used a shaker incubator at a speed of 150 rpm and temperature of 30°C for 7 days. Before and after fermentation, cocoa pod were weighted. At the end of fermentation, cocoa pod was dried at 50°C for 4 days to stop the activity of microorganisms. Cocoa pod was milled and shieved using a Thomas-Wiley Mill type 4 with a diameter of 1 mm sieve. The research design uses a completely randomized design with four treatments and five replications. The treatments were T1= cocoa pod fermentation without fungi addition, T2= cocoa pod fermentation with *P. chrysosporium* addition, T3= cocoa pod fermentation with *P. ostreotus* addition, and T4= cocoa pod fermentation with *S. commune* addition

Statistical analysis

The activity of LiP and MnP was measured as described by Tien and Kirk (1984), fiber fraction analysis : NDF, ADF, hemicellulose, cellulose, lignin was measured as described by Van Soest (1982) and in vitro digestibility was measured as described by Tilley and Terry (1963) which has been modified by Utomo (2010). Analysis of data obtained from the treatment were then tested by analysis of variance (ANOVA) test pattern in line with Duncan's multiple range test.

Result and Discussion

Enzime activity

Results of Lignin peroxidase (LiP) enzyme activity during the study are presented in Table 1. The mean treatment in a row were T1 = 0.02±0.00 U/mL; T2 = 0.22±0.01 U/mL; T3 = 0.13±0.00 U/mL; and T4 = 0.16±0.00 U/mL. Statistical analysis using the Duncan Multiple showed results significantly different (P<0.05) The results mean Manganese Peroxidase (MnP) enzyme activity are listed in Table 1. The mean treatment in a row were T1 = 0.02±0.00 U/mL; T2 = 0.09±0.00 U/mL; T3 = 0.05±0.00 U/mL; and T4 = 0.05±0.00 U/mL. Statistical analysis using the Duncan Multiple showed results significantly different (P<0.05).

The enzyme activity in T2 where the cocoa pod fermentation was using fungi *P. chrysosporium* produce the highest LiP enzyme activity and MnP among other treatments. Fungus *P. chrysosporium* was one of the white rot fungus that of the timber. These fungi produce extracellular enzymes LiP, MnP and laccase. Cocoa pod fermentation process was performed on all treatments by using temperature and fermentation time same proving that the fungus *P. chrysosporium* shows the results of enzyme activity LiP and MnP higher compared with treatment using other fungi.Lignin peroxidase in treatment T2 was equal to 0.22 U/mL showed higher enzyme activity compared with the previous research (Ilmi, 2013), which reported LiP enzyme activity *P. chrysosporium* fermentation using corncob waste is equal to 0.06 U/mL.

Lignin peroxidase and MnP has the same degradation mechanism on lignin. Lignin peroxidase is a peroxidase enzyme and MnP extracellular using H_2O_2 to degrade lignin. while laccase is an enzyme containing copper using molecular oxygen to degrade lignin (Hattaka, 1994).

LiP and MnP role in the weathering of wood degrading waste and lignin. LiP issued by fungus because cocoa pod was a lignocellulosic organic waste that is on the lignin acts as LiP enzyme inducers. Moreover cocoa pod is also rich in sugars that are naturally easy to be metabolized by the white rot fungus.

Fiber fraction

The results mean the fraction of the fiber fraction during the study are listed in Table 2.

Neutral detergent fiber (NDF). Neutral detergent fiber mean results are listed in Table 2. The mean of NDF were T1 = 80.91±0.80%, T2 = 75.54±0.41%, T3 =

Table 1. Average LiP and MnP enzyme activity and MnP cocoa pod fermentation (U/mL)

Variable	Treatment			
	T1	T2	T3	T4
LiP	0.02±0.00^{a}	0.22^{d}±0.01^{d}	0.13±0.00^{b}	0.16^{c}±0.00^{c}
MnP	0.02±0.00^{a}	0.09^{d}±0.00^{d}	0.05±0.00^{c}	0.06^{c}±0.00^{c}

T1 = fermentation of cocoa pod without fungi addition, T2 = fermentation of cocoa pod + *P. Chrysosporium,* T3 = fermentation of cocoa pod + *P. Ostreatus,* T4 = fermentation of cocoa pod + *S. Commune.*
$^{a.b.c.d}$ Superscript different at the same row indicate significant difference (P<0.05).

Tabel 2. Average fiber fraction cocoa pod fermentation (%)

Variable	Treatment			
	T1	T2	T3	T4
NDF	80.91±0.80^{c}	75.54±0.41^{a}	77.78±0.56^{b}	78.63^{b}±1.08^{b}
ADF	73.30±0.34^{d}	68.10±0.30^{a}	70.53±0.30^{c}	69.70^{b}±0.55^{b}
Lignin	36.00±0.66^{d}	26.86±0.19^{a}	28.45±0.22^{b}	30.07^{c}±0.59^{b}
Cellulosa	30.04±0.24^{c}	27.17±0.25^{a}	28.49±0.61^{b}	28.58^{b}±1.03^{b}
Hemicellulosa	7.61±0.45^{ab}	6.77±0.52^{a}	7.25±0.30^{ab}	8.92^{c}±0.19^{b}

T1 = fermentation of cocoa pod without fungi addition, T2 = fermentation of cocoa pod + *P. chrysosporium*, T3 = fermentation of cocoa pod + *P. Ostreatus,* T4 = fermentation of cocoa pod + *S. commune.*
$^{a.b.c.d}$ Superscript different at the same row indicate significant difference (P<0.05).

77.78±0.56%, T4 = 78.63±1.08%. Statistical analysis using the Duncan multiple showed highly significant results (P<0.01).

Table 2 shows that the average content of NDF on cocoa pod T2 treatment was the lowest amounted to 75.54% and the highest was T1 amounted to 80.91%. The decline of the NDF content might be caused by lignocellulosic enzymes produced by the fungus *P. chrysosporium* were able to loosen the lignin and hemicellulose. The decline in NDF content means the content of the substrate is reduced which then affect the composition of the fiber component. The decline occurred because the NDF content during fermentation of lignocellulosic bonds termination occurs and thriving microbial activity (Akmal, 2003).

Neural detergent fiber is the main part of the cell wall such as hemicellulose, cellulose and lignin (Van Soest *et al.,* 1982). Changes NDF content of cocoa pod in this study followed by reduction of the content of other nutrients that are used by fungi to ferment activity. According to Suparjo *et al.* (2009) changes of the cocoa pod NDF content was due to the utilization of the contents of cell components that contain lipids, sugars, organic acids, non-protein nitrogen, pectin, soluble proteins, and other materials dissolved in the water by the fungus *P. chrysosporium*. In the process of fermentation. molds remodel or breaking the cell wall of the cocoa pod through the action of the cellulase enzyme. Cracking of these cell wall will increase the digestibility of rumen fermentation when cocoa pod livestock consumption.

Acid detergent fiber (ADF). Acid detergent fiber mean results are listed in Table 2. The mean of ADF were T1 = 73.30±0.34%, T2 = 68.10±0.30%, T3 = 70.53±0.30%, and T4 = 69.70±0.55%. Statistical analysis using the Duncan Multiple showed highly significant results (P<0.01).

Table 2 shows that the average of ADF content of cocoa pod of T2 treatment was the lowest amounted to 68.10% and the highest was T1 amounted to 73.30%. The decrease in ADF content of the cocoa pod was due to the enzymes produced by the fungus *P. chrysosporium* capable of loosen the bond of lignocelluloses enzymes so that a strong bond that had become tenuous. The decline in ADF content was highest in treatment T2, because the fermentation process and the production of cellulase enzymes operate optimally. The decrease in ADF content on cocoa pod fermented caused it's lignin degradation caused increase cellulose and hemicellulose. This depolimeryzation product dissolved in acid detergent at fiber analysis with Van Soest method.

Acid detergent fiber value is the estimated value of the fraction of the fibers that are difficult to be degraded by microbes in the rumen (Kustantinah *et al*., 2008). The fraction of fiber was difficult to degrade cellulose and lignin, but the white rot fungus has the ability to degrade lignin. This is supported by Mudgal and Pradhan (1988) which states that white rot fungi have the ability to break down tough fibers degraded fractions such as lignin. In addition, White rot fungus has lignolitic activity and cellulase activity that can also degrade cellulose.

Lignin. Lignin mean results are listed in Table 2. The average of lignin content were T1 = 36.00±0.19%, T2 = 26.86±0.12%, T3 = 28.45±0.22%, and T4 = 30.07±0.59%. Statistical analysis using the Duncan Multiple showed highly significant results (P<0.01).

Table 2 shows that the average content of lignin cocoa pod fermented of T2 was the lowest among treatments, amounted to 26.86% and T1 was the highest amounted to 36.00%. The decrease in lignin content of the cocoa pod of the fermentation treatment group of *P. chrysosporium* fungus was due to fiber degradation process run optimally,

resulting lignin content also decreased. Fungus *P. chrysosporium* can degrade lignin effectively by producing extracellular enzymes peroxidase that LiP and MnP.

Fermented cocoa pod by using fungi *P. chrysosporium* provide the opportunity for mold to grow well so that the production of the enzyme was also high that affected lignin degradation in the pod fermentation process. Lignin was a component of plant cell walls that had been developed after maturation process. Cocoa pod as old crop waste has lignified advanced stages. Changes in lignin content of the substrate occurs due to overhaul the structure of lignin into simpler components. namely CO_2 and H_2O (Kaal *et al*., 1995).

The lignin content in the fermentation process relates to the production of the enzyme ligninase. The lignin degradation will pave the way for an overhaul of cellulose and hemicellulose. White rot fungus *P. chrysosporium* can degrade lignin that promotes lower lignin content. The decrease of lignin levels by the fungus showed that white rot fungi able to degrade lignin. According Kaal *et al*. (1995), that white rot fungus has the ability to depolimerization lignin and lignin metabolizes into CO_2 and H_2O.

Cellulose. The results mean the cellulose is presented in Table 2. The average of cellulose content were T1 = 30.04±0.24%, T2 = 27.17±0.25%, T3 = 28.49±0.61%, T4 = 28.56±1.03%. Statistical analysis using the Duncan Multiple showed highly significant results (P<0.01).

Table 2 shows that the average cellulose content of T2 was the lowest among treatment amounted to 21.17% and T1 was the highest amounted to 31.04%. Cellulose binds tightly with hemicellulose and lignin. Cellulose consists of monomer units of D-glucose bonded through bonding β-1-4-glycoside. Fungus *P. chrysosporium* in addition producing the enzyme ligninase also produce enzymes cellulase and hemicellulase group, in which each plays a role in the hydrolysis of cellulose and hemicellulose. Enzymatic cellulose compounds degradation produced oligosaccharide, a disaccharide and glucose monomers that are soluble. Enzymatic breakdown process occurs in the presence of cellulase enzymes.

Kregel and Dijkstra (2000) said cellulase enzyme was composed of three kinds of enzymes that work synergistically to degrade cellulose. The enzyme endo 1-4 β-glucanase, exo 1-4 β-glucanase and β-glucosidase. The mechanism of cellulose degradation begins with the action of the enzyme exo 1-4-β-D glucanasefollowed by work endo 1-4-β-D glucanase enzymes and enzyme β-D-glucosidase to form glucose products.

Hemicellulose. The average of hemicellulose content are presented in Table 2. The average of hemicellulose content were T1 = 7.61±0.45%, T2 = 6.77±0.52%, T3 = 7.25±0.75%, T4 = 8.92±0.53%. Statistical analysis using the Duncan Multiple showed highly significant results (P<0.01). Table 2 shows that the average of hemicellulose content of T2 was the lowest among treatments amounted to 6.77% and T4 was the highest amounted to 8.92%.

Hemicellulose was a heterogeneous group of polysaccharides with low molecular weight. Number of hemicellulose is usually between 15 and 30 percent of the dry weight of lignocellulosic material (Taherzadeh, 1999). Hemicellulose is relatively easy to be hydrolyzed with acid monomers containing glucose, mannose, galactose, xylose, and arabinose. Hemicellulose binds cellulose fiber sheets to form microfibrils which increases the stability of the cell wall. Hemicellulose. lignin was also crosslinked to form a complex network and provide a strong structure.

Biodegradable hemicellulose into monomeric sugars and acetic acid by the enzyme hemicellulase. Hemicellulase like most other enzymes that can hydrolyze plant cell wall is a multi-domain protein. Xylan was the main carbohydrate constituent of hemicellulose and xylanase was a hemicellulase primary β-1.4 bond hydrolyze xylan chains. Fungus *P. chrysosporium* produces endoxylanase that play a role in the breakdown of xylan into oligosaccharides (Perez *et al*., 2002).

In vitro digestibility

The results of dry matter and organic matter digestibility during the study are presented in Table 3. Dry matter digestibility were T1 = 64.63±0.55%, T2 = 69.70±0.43%, T3 = 67.04±0.60%, and T4 = 66.84±0.61%. Statistical analysis using the Duncan Multiple showed highly significant results (P<0.01). Organic matter digestibility were T1 = 64.11±0.12%, T2 = 69.59±1.03%, T3 =

Tabel 3. Average in vitro digestibility on Cocoa pod fermentation (%)

Variable	Treatment			
	T1	T2	T3	T4
DM	64.63±0.55[a]	69.70±0.43[c]	67.04±0.60[b]	66.84±0.61[b]
OM	64.11±0.12[a]	69.59±1.03[c]	67.11±0.58[b]	67.09±0.60[b]

T1 = fermentation of cocoa pod without fungi addition, T2 = fermentation of cocoa pod + *P. chrysosporium*, T3 = fermentation of cocoa pod + *P. Ostreatus,* T4 = fermentation of cocoa pod + *S. commune.*
[a.b.c] Superscript different at the same row indicate significant difference (P<0.05).

67.11±0.58%, and T4 = 67.09±0.60%. Statistical analysis using the Duncan Multiple showed highly significant results (P<0.01).

Table 3 shows that the dry matter digestibility of T1 was the lowest among treatment amounted to 64.63% and the highest was T2 amounted to 69.70%. Dry matter digestibility of Pangola in this study amounted to 64.18% showed different results with research Pramono *et al*. (2013) which states dry matter digestibility of pangola grass was 63.65%.

Table 4 shows that the average organic matter digestibility of T1 was the lowest was T1 among treatments amounted to 64.11% and the highest was T2 amounted to 69.59%. Pangola organic matter digestibility results in this study amounted to 64.78% showed different results with research Pramono *et al*. (2013) which states that the organic matter digestibility of pangola grass was 65.52%.

Dry matter digestibility and organic matter digestibility of cocoa pod fermented with *P. crysosporium* has a higher digestibility values. This is because the fraction of the fiber was capable of optimally degraded by white rot fungus so that it resulted in an increase dry matter digestibility and organic matter digestibility. Tabel 2 shows that fiber fraction on cocoa pod fermentation with *P. crysosporium* has lower value so that the digestibility become higher in Table 3.

Value dry matter digestibility and organic matter digestibility on cocoa pod fermentation process was closely related to white rot fungus enzyme activity. With the activity of this enzyme then the plant cell wall fraction fibers such as lignin. cellulose and hemicellulose can be degraded by either causing the rising value of the digestibility of the cocoa pod. The higher the value of the cocoa pod. the potential of the cocoa pod as animal feed ruminasia the better. High digestibility value which means the proportion of cocoa pod on the feed becomes larger.Dry matter digestibility pattern consistent with organic matter digestibility because most of the dry matter consists of organic matter and the only difference being the ash content (Tillman *et al*., 1998).

Conclusion

Fermentation of cocoa pod with fungus *P. chrysosporium* produce LiP enzyme activity and the highest MnP consecutively 0.22±0.01 U/ml and 0.09±0.00 U/ml. Fermentation of cocoa pod with the addition of *P. chrysosporium* causes a decrease in content of NDF 75.54±0.41%, ADF 68.10±0.30%, lignin 26.86±0.19%, cellulose 27.17±0.25 %, and hemicellulose 6.77±0.52%. Fermentation of cocoa pod with *P. chrysosporium* produces the highest dry matter digestibility 69.70±0.43% and the highest organic matter digestibility 69.59±1.03%.

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