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Determination of the Appropriate Ratio of Rice Bran to Cassava Leaf Meal Mixture as an Inoculum of *Rhizopus Oligosporus* in Broiler Chicken Ration

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ABSTRACT

Rice bran (RB) was used as a medium for the fermentation process because it contained complete nutrients necessary for the growth of microbes. The ability to induce a fermentation substrate by microbes dependent on the availability of an inducer in the medium. The enzyme activity would increase when a suitable inducer was in the medium. In order to increase protease and cellulase activities of *Rhizopus oligosporus* in processing Cassava Leaf Meal (CLM), it was necessary to add CLM in medium producing an inoculum *Rhizopus oligosporus*. This study was conducted in a completely randomized design with 4 treatments and 5 replications. The treatments were ratios of RB to CLM as follows: 100:0% (A), 90:10% (B), 80:20% (C), and 70:30% (D). The observed variables were protease activity, cellulase activity, dry matter content, organic matter content and the total colony of *Rhizopus oligosporus* numerically. The different ratios of RB to CLM significantly affected protease and cellulase activities as well as the content of dry matter and organic matter. The addition of 10% CLM to the RB medium increased protease and cellulase activities and reduced dry matter and organic matter contents. However, when more than 10% CLM was added, it reduced the protease and cellulase activities and increased dry matter and organic matter content. The highest total colony of *Rhizopus oligosporus* was numerically in treatment B (90:10% of RB to CLM ratio). The appropriate ratio of RB to CLM mixture as the inoculum of *Rhizopus oligosporus*, based on their enzyme activities, dry matter, and organic matter contents, and the total colony was 90:10% (treatment B).

Key words: Cassava leaf meal, Enzyme activity, Inoculum, *Rhizopus oligosporus,* Rice bran.

INTRODUCTION

Cassava leaf flour (CLM) has the potential to be used as an alternative feed ingredient. Judging from its availability, CLM is quite widely available. Cassava leaf flour is obtained as waste material when harvesting cassava roots from cassava plants. Cassava production in West Sumatra reaches 201,833 tons, in Indonesia 19,341,233 tons (Statistics of Indonesia, 2018).This cassava plant was cultivated on an area of 792,952 Hectare (Ha) or 0.8 million Ha (Statistics of Indonesia, 2018). The production of fresh cassava leaves per Ha was between 7 to 15 tons by Sudaryanto et al., 1982 in Yuniza et al., (2016). From the area of cassava plantation and cassava leaf production per hectare, the availability of fresh cassava leaves as animal feed was plentiful, ranging from 5.6 to 12 million tons or about 1 to 2 million tons under dry conditions per year. If only 1 million tons of dried cassava leaves was used as chicken feed, this amount could be estimated to feed up to 274 million laying hens per year when the cassava leaves were included 8% in the ration.

The potential of CLM as an alternative feed ingredient in terms of nutritional content. The nutritional content of CLM is as follows: dry matter (DM) 25.30%, crude protein (CP) 25.1%, crude fiber (CF) 11.4%, crude fat 12.7%, nitrogen retention 46 , 1% and 9.1% ash (Iheukwumere et al., 2008). On the other hand, CLM has a limiting factor as alternative feed ingredients. The use of CLM in broiler chicken rations can only be used up to 5% because utilization of up to 10 and 15% can reduce the efficiency of feed use (Wyllie and Chamanga, 1979;

Melesse et al. 2018). The use of CLM in broiler chicken rations is still limited due to high CF, tannins, HCN, alkalis, low digestibility (Ravindran et al., 1986) and deficiency of amino acids containing sulfur, especially methionine (Eggum, 1970). Therefore, the use of CLM was still limited. In order to further increase the amount of CLM in poultry rations, the physical, chemical and biological fermentation must be carried out.

The biological fermentation by using microbes is a process of the activity of microorganisms or microbes that can produce the product whose texture, flavor, smell and nutrient quality change better than that of the raw material (Mirnawati et al., 2019a; Mirnawati et al., 2019b; Dewi et al., 2019; Mirnawati et al., 2018; Mirrnawati et al., 2017; Adrizal et al., 2017; Mirnawati et al., 2013; Rizal et al., 2013; Rizal et al., 2012; Mirnawati et al., 2012; Aisjah and Abun, 2012; and Mirnawati et al., 2010). According to Sugiharto (2019), solid-fermentation using fungi could be a simple method to improve the nutrient qualities of cassava pulp and thus increased the production of such a cheap agro-industrial by-product in chicken rations. According to Han et al. (2003), *Rhizopus oligosporus* produced protease, lipase, alpha-amylase, glutaminase, and alpha-galactosidase and (Dewi, 2015) found that *Rhizopus oligosporus* also produces cellulase. The presence of these enzymes were expected to reduce the anti-nutrients and toxins contained in CLM and also increase their nutritional values (Sumiati et al., 2011), thus increasing the use of CLM in broiler chicken rations.

To increase the productivity of *Rhizopus oligosporus* in producing hydrolytic enzymes (protease and cellulase) and to increase the enzyme activities, it was necessary to produce *Rhizopus oligosporus* starter or inoculum from Rice Bran (RB) and CLM mixture as an inducer. Therefore, the ability of hydrolytic enzymes for the degradation of CP and CF of CLM supposed to increase. The addition of CLM to the inoculum as an inducer should accelerate the adaptation phase and increase the productivity of *Rhizopus oligosporus* hydrolytic enzymes. *Rhizopus oligosporus* would be able to synthesize the necessary enzymes required for metabolism according to the availability of inducer in the starter (Kurnia, 2010; Purkan et al., 2016). The addition of CLM as a substrate in the production of *Rhizopus oligosporus* inoculums could accelerate the adaptation phase (Soeprijanto et al., 2008 and Zakaria et al., 2013).

Therefore, the purpose of this study was to investigate the appropriate ratio of RB to CLM mixture that will be utilized for producing inoculum of *Rhizopus oligosporus*.

MATERIALS AND METHODS

Experimental design

This experiment was conducted in a complete randomized design with 4 treatments and 5 replications. The treatments were the combination ratio of RB to CLM including 100:0% (A), 90:10% (B), 80:20% (C), and 70:30% (D). These combinations of RB and CLM mixtures were fermented by using *Rhizopus oligosporus* at the dose of one test tube for 100 gram (g) dry matter and 2-day fermentation length.

Measured variables

These measured variables wereprotease and cellulase activities as well as dry matter and organic matter contents, and the numericalcalculation ofthe total colony of *Rhizopus oligosporus* in each treatment.

Data analysis

All the data were analyzed by analysis of variance of complete randomized design. The differences among treatment means were detected by Duncan's Multiple Range Test or (DMRT) according to Steel and Torrie (1980).

Preparation of Cassava Leaf Meal sample

After harvesting the tuber roots, cassava leaves were taken from two-third of the entire leaves from the an teriorside. Cassava leaves were dried under the sun and then milled in a two mm screen.

Propagation of fungi

Eight g of Potato Dextrose Agar or (PDA) was weighed and placed into the glass cup and added with 200 milliliter (ml) of distilled water and heated on the hotplate with constant stirring until it was limpid. This PDA solution was poured into 30 test tubes (each test tube contained 5 ml solution) and covered with cotton and aluminum foil, then sterilized by using an autoclave at 2 atmosphere (atm.) pressure and 121^0C for 15 minutes. Other inoculation pieces of equipment (one needle, one mask, one glove) were also sterilized. They were chilled in laminar flow while UV light was turned on a tilted position until solid. *Rhizopus oligosporus* was inoculated by using sterile needle against tilted PDA medium in a sterile environment. Then, the inoculated PDA medium was sealed in test tubes with cotton and aluminum foil and incubated for 36 hours in an incubation container in flowing air under facultative anaerobic condition.

The starters were weighted according to the treatments [ratios of RB to CLM were 100:0% (A), 90:10% (B), 80:20% (C) and 70:30% (D)]. To each starter was added 65 ml of water (until the moisture content was 45%) and homogenized, and then these starters and minerals were prepared according to Brook et al. (1969). The composition of nutrients included Urea 7.5 g, MgSO4 7 H2O 0.375 g, FeSO4 7 H2O 0.15 mg, ZnSO4 7 H2O 0.15 mg, MnSO4 4 H2O 0.15 mg, KH2PO 1.5 g, and thiamine hydrochloride 18.75 mg, which had dissolved in 100 ml equates. The mixture was used, 5 ml in 100 g subtract were sterilized using autoclave at 2 atm. pressure, temperature 121^0c for 15 minutes, and chilled in laminar flow. *Rhizopus oligosporus* in the PDA medium was inoculated in laminar flow into each starter and then was incubated in facultative anaerobe over a period of 36 hours.

Determination of Enzyme Activity

Crude enzyme extract

10 g of inoculum sample was weighed and then immersed in 90 ml of 0.05 M phosphate buffer at pH 7 in 250 ml of Erlenmeyer and inserted into the incubator shaker for 2 hours at 27^0C at 100 round per minute (rpm). Afterwards, the solution was filtered and the filtrate was taken out, and then was centrifuged at a speed of 5000 rpm for 15 minutes, thus the supernatant was taken out as a crude enzyme extract for the purposes of analyzing enzyme activity by the following methods.

Protease activity

One percent casein solution was pipette up to 2.5 ml and phosphate buffer (0.1 M pH 7) was pipetted up to 1.5 ml and then placed into a test tube and then mixed by using a vortex, then was incubated in a water bath at 37^0C for 10 minutes. One ml of crude enzyme extract was added and then incubated for 10 minutes in a water bath at 50 ^{0}C. To obtain the blank, enzyme activity was stopped by adding 5 ml of 20% TCA solution, then mixed by using a vortex and then refrigerated for 30 minutes to agglomerate the protein. On the other hand, the sample was centrifuged at 5000 rpm for 15 minutes at 4^0C, then filtered and the supernatant was taken. Furthermore, two ml of supernatant was pipetted into the test tube and 0.5 ml of NaOH up to 5 ml and folinciocalteu reagent was added to 0.5 ml and then allowed to stand for 10 minutes. Furthermore, the absorbance was measured using a spectrophotometer at a wavelength of 650 nm. According to Henriette (1993**),** the calculation is done with the following equation;

Activity of protease enzyme (U/ml) = (Y x a)/b x 1/t

Y = Absorbance of sample

a= the a value of the regression curve Y: a + bx

b= value b of the regression curve Y: a + bx

t= incubation time

Casein regression curve = y = 0.031x + 1.136R^2 = 0.986

Cellulase activity

One ml crude enzyme extract was pipetted and added one ml (0.5 ml CMC + 10 ml phosphate buffer) and then poured into the microtube, and incubated for 30 minutes at 40^0C in a water bath shaker. Then one ml of this mixture solution was taken out and poured into the test tube, and then one ml of Nelson AB (25 ml Nelson A and one ml Nelson B)was added. Nelson A was consisted of 2.5 g of Na2CO3, 2 g of NaHCO3, 2 g of NaSO4, 2.5 g of KNaCaH4O6. 4H2O and 100 ml of distilled water. Nelson B was consisted of 75 g of COSO4 and one drop of H2SO4. Thus, the mixture was heated in boiling water for 20 minutes, and after the solution got cold, one ml of phosphor-molybdate and seven ml of distilled water were added. The absorbance was measured by using a spectrophotometer Simadzu 1800 from Kyoto, Japan at a wavelength of 575 nm (Nelson, 1994). The calculation is done with the following equation;

Cellulase activity (U / ml) = (X × P × 1000) / (T× M)

X = the result of standard curve conversion

P = Dilution

T = Time

BM = Molecular weight of glucose

Glucose standard curve =y = 0.045x - 0.010 R^2 = 0.994

Calculation of Total Colonies

The total colonies were calculated by the Pour Plate Method (Putri and Kurnia, 2018). The counting of the colonies had tube sterile from the beginning to the end. All equipments were moistened with 70% alcohol. In addition, the process was carried out near a flame to reduce or prevent microbial contaminants to the equipment and media. The calculation of whole colony was startedwith the productionof physiological solutions. In the first step, oneg of sample was mixed with nine ml of distilled water, then it was homogenized. One ml of this solution was taken with a micropipette and added to nine ml of distilled water to obtain a 1:10 dilution. The solution of 1:10 homogenized dilutions was taken as much as one ml and then poured into a test tube with nine ml of distilled water to made a 2:10 dilution, and the same procedure was used

to prepare the solution until 10:10 dilutions. In the next process, media plate count agar was made using Potato Dextrose Agar (PDA) solution. The PDA solution was sterilized together with Petri dishes and other equipment by using an autoclave at a pressure of 2 atm., 121 ^{0}C for 15 minutes. Then, 0.1 ml of the physiological solution from the dilution tube (10-6) was pipetted into a sterile Petri dish, after which the PDA solution was poured under warm conditions. It was also placed on a flat table and gently shaken like the number eight. This procedure was also repeated for physiological solutions resulting from dilutions of 10-8 and 10-10. All planted Petri dishes were labeled and sealed with stretch film. All Petri dishes were incubated for 24 hours in an incubator jar. After the incubation, the fungi colonies were observed and counted using a colony counter. Total colonies were calculated according to Standard Plate Count (SPC). Several colonies that had been assembled from a large collection of colonies counted as one colony, and a row or chain of colonies considered as a combined row were also counted as a colony. The amount per gram colony was determined using the equation according to Sukmawati and Hardianti (2018).

$$N = n \times \frac{1}{fP}$$

N = total colony per gram of sample (CFU/g);

n = Number of colonies counted in the colony counter

*f*P =Dilution factor of the sample (initial dilution × amount of colonies grown)

RESULT

The effect of RB and CLM ratios in the production of *Rhizopus oligosporus* inoculum on their protease and cellulase activities, total colony, and dry matter and organic matter contents were shown in table 1. The effect of ratios and RB to CLM for producing inoculum of *Rhizopus oligosporus* on protease activity was statistically significant ($p < 0.05$). The highest protease activity was found in treatment B (9.84 U/ml), in which it was not different from treatment C (9.90 U/ml), and treatment D (9.79 U/ml) but it was different from treatment A (9.13 U/ml)

The effect of the ratio of RB and CLM to produce the inoculum *Rhizopus oligosporus* on cellulase activity was statistically significant ($p < 0.05$) (table 1). The highest activity of cellulase was at treatment B (1.50 U/ml). It was not different compared to treatment C (1.51 U/ml), D (1.52 U/ml), but it was different from treatment A (1.38 U/ml). The ratio of RB to CLM for assembling the inoculum of *Rhizopus oligosporus* significantly affected the dry matter content ($p < 0.05$). The lowest dry matter content was found in treatment B (91.63%). It was different from treatments A (92.67%), C (92.59%) and D (92.94%). Meanwhile, treatments A, C, and D were not different. The effect of the ratio of RB and CLM to produce an inoculum of *Rhizopus oligosporus* on the content organic matter was statistically significant ($p < 0.05$). The lowest organic matter content was found in treatment B (76.48%), but it was not different compared to treatment C (77.66%). Whereas, this treatment was different from treatments A (78.55) and D (79.00%).

Table 1. The effects of the ratios of Rice bran and Cassava leaf meal for producing *Rhizopus oligosporus* starter, on their protease and cellulase activities, and the content of dry matter, organic matter, and the numerical form of total colony, in the Non-Ruminant Nutrition Laboratory, at the Faculty of Animal Science, Andalas University, Padang, Indonesia.

Ratios of	**Measured Variables**				
RB:CLM	**Protease activity (U/ml)**	**Cellulase activity (U/ml)**	**Dry matter (%)**	**Organic matter (%)**	**Total colony (CFU/g)**
A (100:0)	9.13^b	1.38^b	92.67^a	78.55^a	0.77x10^{10}
B (90:10)	9.84^a	1.50^a	91.63^b	76.48^b	3.00x10^{10}
C (80:20)	9.80^a	1.51^a	92.59^a	77.66^b	2.08 x10^{10}
D (70:30)	9.79^a	1.52^a	92.94^a	79.00^a	1.03x10^{10}
Standar error	0.02	0.01	0.17	0.30	

$^{a, b}$ = Different superscripts at the same columns indicates significantly different effects ($p < 0.05$). U/ml = Unit/ milliliters (%): percentage. (CFU/g) = colony forming unit/ gram. RB:CLM = Rice bran: Cassava leaf meal.

DISCUSSION

The activity of protease

The highest protease activity was founded by the addition of cassava leaves as a source of protein up to 10%. Although increasing the addition of cassava leaves up to 20% and 30% did not significantly alter the activity of the protease, it decreased numerically since the addition of 10% inducer reached optimal. Thus, if the addition of the inducer was increased, it did not increase the protease activity, but reduced it. The protease activity increased to an optimal level according to the inducer contained in the substrate in the starter. According to Kurnia (2010), the protease activity increased to optimal level depending on the inducer contained in the substrate in the starter.

The mechanism of increasing protease activity in accordance with the available inducer to the optimal point was that cassava leaves would bind to the repressor protein as an available inducer so that the repressor protein underwent allosteric changes that could change its shape and cause the repressor to cease the ability to bind to the operator. As a result, the RNA polymerase could copy the genes required for cassava leaf degradation so that the *Rhizopus oligosporus* could synthesize the enzymes necessary to degrade the available inducers. In this case, cassava leaves were used as a source of protein because the abundant crude protein content was 21.59%.

The table 1 indicated that the increase in the addition of cassava leaves (inducer) was directly proportional to the high production of the enzyme protease. This stage lasted until the optimal inducer concentration, which was the addition of 10% of cassava leaves with an enzyme activity of 9.84 (U/ml). The addition of 20% and 30% of cassava leaves did not significantly increase the protease activity, but instead decreased it numerically since the addition of cassava leaves as an inducer had exceeded the optimal concentration. This excessive inducer concentration caused saturation of the protease enzyme productivity, thus inhibited the formation of enzyme complexes on the substrate (Purkan et al., 2016).

The cellulase activity

Cellulase activity increased to the optimum at the time of addition of 10% cassava leaves as an inducer, then there was no significant increase on addition of 20% and 30% of cassava leaves. This happened because at the point of addition of 10% of cassava leaves had reached the optimal point of adding inducers, increasing the addition of inducers (cassava leaves) did not increase enzymatic productivity. According to Kurnia (2010), the activity of cellulase enzymes increased to the optimum point according to the inducers available in the substrate in the starter. The mechanism of increasing cellulase activity by adding an inducer of cassava leaves to the optimal point was that cassava leaves as an inducer will bind the repressor protein so that the repressor protein undergoes allosteric changes which can change its shape and cause the repressor can no longer bind to the operator. As a result, the RNA polymerase could copy the genes needed for cassava leaf degradation so that the *Rhizopus oligosporus* could synthesize the enzymes needed for metabolism. In this case, cassava leaves are used as fiber inducers because of the high CF content of 14.59%.

In the table, it can be seen that the increase in the concentration of the inducer is directly proportional to the high activity of cellulase. This lasted until the optimum point of adding 10% inducer (cassava leaves) with enzyme activity (1.50 U / mL), then at an increase of 20% and 30%, there was no significant increase in cellulase activity. This happens because, the addition of an inducer of more than 10% causes saturation in the productivity of cellulase enzymes, because the concentration of the inducer is too large to inhibit the formation of enzyme complexes on the substrate, so that enzyme production does not run optimally (Purkan et al., 2016).

Dry matter content

The low dry matter content at treatment B was due to the high protease activity in this treatment, in which it hydrolyzed the protein of the substrate. Thus, the dry matter content of the substrate was reduced. The decrease in dry matter after fermentation was an indicator of the success of fermentation, as the retaining of dry matter in the fermentation process was affected by the use of nutrients from the substrate (dry matter) by microbes as a source of carbon, nitrogen and minerals, and the release of CO2 and energy in the form of heat that evaporated with water particles. According to Astuti et al. (2017), the fermentation process could result in a reduction in the amount of dry matter. The water molecule was formed through a catabolic process that remodeled complex compounds into simpler materials

Organic matter content

The high and low content of organic matter in the treatment was caused by microbial activity in the fermentation process, which caused the breakdown of the substrate content, whereby microorganisms could easily

digest organic matter. The fermentation of organic substances in the form of glucose, alcohol, and amino acids led to changes that affecting the nutritional value. According to Astuti et al. (2017), the fermentation process carried out by microorganisms, so that the carbohydrates were converted into alcohol, organic acids, water, and CO_2. The use of tofu waste in addition to nitrogen sources was also a source of carbohydrates for microbes used in the fermentation, which causes an increase in water content and leaded to the loss of organic matter.

Total colony

The total colonies of *Rhizopus oligosporus* in treatment A ($0.77x10^{10}$ CFU / g), B ($3.00x10^{10}$ CFU / g), C ($2.08x10^{10}$ CFU / g), and D ($1.03x10^{10}$ CFU / g) differ numerically. The highest total of *Rhizopus oligosporu*s colonies in treatment B was due to the availability of inducers needed to synthesize enzymes by *Rhizopus oligosporus*, so that the entire colony became stable and continued to grow (Kurnia, 2010). Conversely, too much inducer concentration inhibited the formation of substrate enzyme complexes, so that enzyme production was not optimal. It also disrupted the stability and growth of the colony (Purkan et al., 2016).

CONCLUSION

The best ratio of rice bran and cassava leaf meal for producing the inoculum of *Rhizopus oligosporus,* based on the enzyme activities, the dry matter and the content of organic matter, and the whole colony was 90% rice bran and 10% cassava leaves (B treatment).

DECLARATIONS

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Author's contribution

Annisa, Yose Rizal, Mirnawati, Irfan Suliansyah and Amri Bakhtiar participated in design, experimental procedure, writing, revised, and reviewing the final edition of manuscript.

Competing interests

The authors have declared that no competing interest exists.

Consent to publish

The authors guarantee that this work has not been published elsewhere and any person named as a coauthor of this study is aware of the facts and has agreed to be named.

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