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Research Article

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Isolation of Cellulolytic Bacteria from Rumen Liquit of Buffalo Both as a Probiotics Properties and has Cmc-Ase Activity to Improve Nutrient Quality of Soybean Distillery By-Product as Feed

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ABSTRACT

This experiment was carried out to study implementation of Cellulolytic bacteria culture (isolation from rumen liquit of buffalo samples) as a probiotics agent can be used in order to alleviate the negative effect of soybean distillery by-product as feed. Eighteen of male Bali duckling was assigned to three treatments in a completely randomized design. Each treatment has six replications with one bird per replication (individual cage). The treatments were (i) unfermented soybean distillery by-product as control; (ii) fermented soybean distillery by-product by 0.30% Cellulolytics bacteria culture; and (iii) 0.60% Cellulolytics bacteria culture, respectively. The report on the first experiment showed that five isolates of cellulolytic bacteria (B-3, B-6, B-7, B-10, and S-13) were isolated from isolation from rumen liquit of buffalo samples in the first experiment. The whole isolates of cellulolytic bacteria showed resistant grew on both in different temperature (20^0-55^0C), acid conditions (3.0-6.0), and bile salt (0.20-0.60 NaDC). Isolates cellulolytics bacteria B-6 was potensial as probiotics sources and has CMC-ase activity. The study showed that fermentation of soybean distillery by-product using of isolates Cellulolytics bacteria B-6 isolate culture could improve significant differences ($P<0.05$) on digestibility of its dry matter (DM), organic matter (OM), crude protein (CP), crude fibre (CF), and increased its metabolizable energy of soybean distillery by-product. The content of dry matter, organic matter, and gross energy of soybean distillery by-product were no effected significantly different ($P>0.05$) by fermentation. On the other hand, fermentation caused increasing crude protein (CP) of the soybean ditellery by-product. It was concluded that fermentation of soybean distillery by-product by Cellulolytics bacteria B-6 isolate culture (isolation from rumen liquit of buffalo samples) could increase nutrient composition and digestibility of soybean distillery by-product as feed.

Key words: *Cellulolytic bacteria, soybean distillery by-product, crude fiber, digestibility.*

INTRODUCTION

Soybean distillery by-product is a waste of household industry, still containing proteins with amino acids lysine and methionine. However, the high fiber content, so the limiting factor in the use of poultry rations[16]. Availability of soybean distillery by-product out quite a lot, especially in the manufacture of household industry centers knows. Sometimes tofu of this problem is often less pleasant odor and thus disturbing the environment, because the water content of soybean distillery by-product is very high, so it is easy to decompose. It would be wise if the soybean distillery by-product is used as feed. Before you are given, the first fermented seed inoculants capable of acting as a probiotic microbes in the digestive tract of poultry (pass the test pH, temperature, acid and bile salts). Therefore, to empower soybean distillery by-product needs to be treated and one of them is the probiotic biotechnology.

Interesting to study is the use of microbes in the rumen of buffalo, because most contain cellulolytic microbes and has the highest cellulolytic activity compared with other livestock cellulolytic microbes,

such as cattle[21]. At the buffalo rumen fluid found seven cellulolytic bacterial colonies, whereas in only four colonies in bali cattle. Through isolation and testing capability of microbial isolates selected as probiotics and fiber degrading (CMC-ase) and when implemented through continuous fermentation products through feed, presumably will be able to assist in digesting of poultry in the ration based soybean distillery by-product, from the aspects of dry matter digestibility values, organic matter, and crude fiber.

Improvement of the nutritional value and digestibility soybean distillery by-product is done by adding the buffalo rumen cellulolytic microbes in the fermentation process soybean distillery by-product out or given directly as a supplement in feed. Evaluation conducted on Bali ducks and treatment is based on an approach that potential cellulose-digesting microbes isolated from the rumen of a kind in the other livestock will provide a positive interaction in digesting crude fiber in the digestive tract of the host animal.

Cellulose fraction is the largest component of the cell wall constituent soybean distillery by-product, which is about 40-50% which is very difficult/can not be digested by digestive enzymes of duck. In order to be used, the cellulose must first be broken down into low molecular weight compounds, such as mono, di, and tri-saccharides. This degradation involves complex cellulase enzymes produced by microbes[32], the endo-beta-glucanase and beta-glucosidase.

Polysaccharides in the cell wall such as celluloses, pectins, and oligosaccharides are known as non starch polysaccharides (NSP). NSPs cannot be degraded in the digestive systems of the birds due to lacking of enzymes for the NSPs degradation in their digestive systems[9]. Non starch polysaccharides are the carbohydrate components of CF and are the predominant substrates for anaerobic fermentation. Non starch polysaccharides can be broken down by microflora permanently, colonizing in the gastrointestinal tract, and their breakdown mainly occurs in the hindgut of all non ruminants by microbial fermentation[33]. These enzymes are effective in degradating the complex compounds such as beta glucans and arabinoxylans[11]. Most of the recent studies focus on the effect of the bacterial and fungal enzymes used in cereal. Hong *et al.*[13] reported that fermentation of feed using *Aspergilus oryzae* increased digestibility of its DM and CP. The inclusion of soluble dietary fiber (wheat bran) increased daily NSP excreted from feces[5,15,26,34]. Chen *et al.*[7] reported that addition of 0.20% complex probiotics in basal diets increased digestiblity of DM and CP.

Based on this, interesting to note is the use of cellulolytic microbes from buffalo rumen fluid as the fiber degrading inoculant soybean distillery by-product before given to the duck. This is possible because the buffalo rumen fluid microbes turns out to have the highest cellulolytic activity compared with other livestock cellulolytic microbes. According to Sudirman[24], in addition to determining the source of the microbial digestion of fiber activity, is also determined by the exact microbial inoculum dose, type, and microbial populations were used. Provision of microbial cultures from buffalo rumen fluid to the ducks is expected to lead to a synergistic effect between species of buffalo rumen microbes with microbes of duck in digestive tract, which can lead to the ability to digest fiber.

Fermentation by cellulolytic microbes can simplify the feed material particles, thereby increasing the nutritional value, as well as changing the protein complex into simple amino acid that is easily absorbed[16]. Incomplete fermentation process seems to lead to the development of bacterial pathogens that can cause health problems and death of livestock. Therefore, the selection of a microbial inoculant in the fermentation process should be observed.

Based on the above description of research conducted to assess the cellulolytic microbes selected from the rumen of buffalo for improving the nutritional content of soybean distillery by-product, an increase in the digestibility of dry matter, organic matter, and crude fiber.

METERIAL AND METHODS

Animals and experimental design

Eighteen of male Bali ducklings were assigned to three treatments in a completely randomized design. Each treatment has six replications with one bird per replication (individual cage). All of the birds were fed experimental diets for two days.

The treatments were (i) unfermented soybean distillery by-product as control; (ii) fermented soybean distillery by-product by 0.30% Cellulolytics bacteria B-6 culture; and (iii) fermented soybean distillery by-product by 0.60% Cellulolytics bacteria B-6 culture; respectively. The objectives of this study is to determine the nutrient digestibility and the ME value of soybean distillery by-product using male Bali duckling at 12 weeks of age.

Extraction and Sample Preparation Resources Microbes

Buffalo rumen fluid obtained at slaughterhouses in the area Sangeh, Badung regency. Buffalo rumen fluid taken immediately after the animal is die. Samples were put into a flask full of water that previously contained warm (temperatures around 39^0c) that it has been issued. The full contents of the flask samples, then closed the meeting to be free from contamination of air and immediately used for the study[30]. Rumen fluid were centrifuged for 10 minutes at 3.000 rpm, then filtered with a double satin.

Tofu Waste/soybean distillery by-product

Soybean distillery by-product obtained from domestic industry in the manufacturing at Ubung Kaja, Denpasar-Bali.

Isolation of cellulolytic Microbes from Buffalo Rumen Fluid

Isolation of cellulolytic bacteria was done by culturing the source of isolates/microbial growth medium broth at thioglicollate fluid (medium number 6 in Ogimoto and Imai, 1981) by using the method of Hungate, ie. 1 ml of diluent 10^5-10^9 microbes in immediately inoculated into tubes have contained liquid growth medium at temperatures 39-40^0C and CO_2 contents with a sterile pipette. The next tube was sealed and stirred until homogeneous, then left at room temperature while continuously flowing CO_2 gas to condense conditions. Subsequent cultures were incubated at a temperature of 39^0 C for 7 days. Colonies of bacteria growing observed morphologic and purified by the method of the quadrant streak on solid selective growth medium petri dish[30].

Selection of cellulolytic bacteria isolates as Probiotics Agent

Pure isolates were then selected to obtain isolates of cellulolytic bacteria by testing the ability to grow at various temperatures, acids, and bile salt.

Selection ability to grow at various pH levels

Selection ability of isolates to grow at different pH levels, particularly at low pH is done by inserting 500 mL of pure culture isolates in eppendorf tubes containing 900 mL selective growth medium liquid with a pH of 3; 4; 5; and 6.5. Thus it incubated in a water bath at a temperature of 39^0c for 3 hours, then centrifuged at 7000 rpm for 5 minute and the supernatant discarded. Isolates pellet was washed twice with 1500 mL saline. The next pellet suspended in 1500 mL saline solution and 50 mL of the suspension was inoculated into 6.5 ml of selective growth medium and incubated at pH 7 for 24 hours at a temperature of 39^0c. Growth of the isolates was observed optical density/OD with a spectrophotometer.

Selection ability to grow on bile salt

Selection ability to grow on bile salts is done by inserting 5 ml of liquid growth medium into 4 test tubes and inoculated 50 mL of pure culture isolates. In the first tube (as a control) was not added sodium Dioxicholic/NaDC. While in the second test tube, third, and fourth, respectively added to 10 mL and 30 mL of 100 mM NaDC (0.2 mM, 0.4 mM, and 0.6 mM). Those were incubated at 39^0c tfor 24 hours. Furthermore optical observed density using a spectrophotometer[10].

Cellulolytic bacteria culture

Preparation of buffalo rumen cellulolytic bacterial culture performed by isolation of cellulolytic bacteria from buffalo rumen fluid. Isolates were then made of buffalo rumen cellulolytic bacterial culture using solid media (rice bran), namely: 150 g of molasses, 15 g of urea, 5 g of lime, 5 g of salt, 2 g of vitamin multi-mineral, 400 g of rice bran, and enough water until the mixture reaches a weight of 1 kg, then add the cellulolytic bacterial isolates buffalo rumen fluid as much as 0.30%. Subsequently the mixture was incubated in an incubator space in the anaerobic atmosphere for 1 week at 37-39^0C temperature (the temperature is kept constant). After one week of incubation, then dried in an oven at a temperature of 45^0C and after dried crushed back and cellulolytic bacterial colony number observed in these cultures (ready for use as bacterial cultures of cellulolytic rumen fluid buffalo).

Fermented of soybean distillery by-product

The isolate of Cellulolytics bacteria B-6 which has been approved from bile salt and poultry digestive tract *in vitro* test could assimilate cholesterol for probiotics agency and have CMC-ase activity**.** The study was carried out at the Bioscience Laboratory of Udayana University, Bali, Indonesia. Fermentation of soybean distillery by-product was prepared as follows. The soybean distillery by-product was used. Approximately 0.30% Cellulolytics bacteria B-6 isolate culture was added to 100 g of steamed soybean distillery by-product. Then, water was added to bring the product to 50% content and left up to 2 days for fermentation. After that, fermented soybean distillery by-product was dried at 45^0C for six hours and then it was ground for analysis. Unfermented soybean distillery by-product was also ground for its chemical analysis.

Retention and excretion of nutrients

In order to determine the nutrient digestibility and metabolizable energy (ME) value of the soybean distillery by-product. The amount of soybean distillery by-product used was 50 g. This amount as based on preliminary assays with male Bali duckling using soybean distillery by-product. All the birds were deprived of feed for 24 h to ensure that their alimentary canals were empty from feed residues. They were then force-fed with the specific amount soybean distillery by-product (fermented and unfermented). Stainless steel funnel with 40 cm stem was used in *force feeding technique*[18]. Water was available *ad libitum* during the experimental period

The total excreta were collected in plastic trays. The excreta samples were frozen, allowed to come to equilibrium with the atmospheric moisture, weighed, and groun through a 1 mm sieve. Samples of excreta and soybean ditellery by-product were subjected to appropriate analysis to determine DM, OM, CP, CF, and energy, respectively.

Laboratory analyses

Dry matter (DM), organic matter (OM), CP and ash determinations were done according to the Assocciation of Official Analytical Chemists (l994). The CP content of the diets was determined using the Kjeldahl procedure (AOAC, 1994). Crude fibre in the feeds were determined using the procedure of Van Soest *et al.*[31] on oven-dried samples. Gross energy (GE) was measured with an adiabatic oxygen bomb calorimeter (Parr, USA),

Calculations

The data were used to calculate AME value according to the following formulate[18]: AME (apparent metabolizable energy) = IE – FE. Where IE = ingested energy; FE = fecal energy voided by the fed birds.

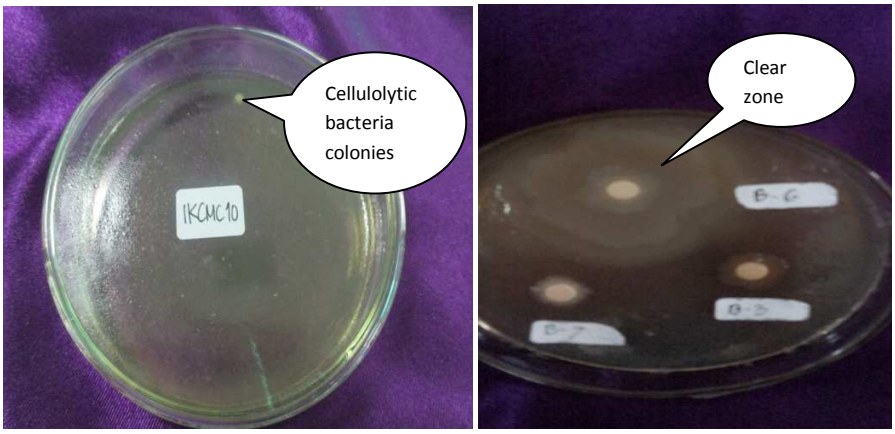
Statistical Analysis

All data were subjected to a one-way analysis of variance test[28]. Statistical significances among treatment means were determined by method of New Multiple Range Test of Duncan when the F value was significant at 5% level.

RESULTS

Clear zone around the colony as typical of cellulolytic microbes do not seem real though distinguishable from other types colonies. On the seventh day of incubation, clear zones began to appear more clearly. Colonies are round with a diameter of between one to two millimeter, beige or brown-white, and not transparent. Other colonies that grow on the medium roll tubes bright white and reddish brown with transverse position opposite to the surface of the growing order. But most of it is white in color with a diameter of less than 1 mm with a position parallel to the surface of the agar medium. The presence of a clear zone around the colonies indicate that the microbes have a strong extracellular cellulase enzyme activity. The size of the clear zone and the apparent absence of a clear zone, an indicator of the ability of the microbes to break down cellulose, as well as fast and slow arise the clear zone[29].

Fig.1: Buffalo rumen cellulolytic bacteria B-6 colonies are round with a diameter of between one to two millimeter, beige or brown, and not transparent (left) and clear zone of CMC-ase activity (right)



The results of the examination of Gram staining showed that the chemical reaction produces a purple color. This suggests that colonies of cellulolytic bacteria including gram-positive group. While the results of microscopic examination showed that the shape of cellulolytic bacteria is spherical (coccus).

Table 1 shows the nutrient digestibility and metabolizable energy of unfermented soybean distillery by-product (0.0% *Cellulolytic bacteria* B-6 *isolates* culture) and fermented soybean distillery by-product. The content of crude protein soybean distillery by-product were slightly increased significantly different (P<0.05) by *Cellulolytic bacteria* B-6 *isolates* culture fermentation.

The digestibility of DM, OM, CP, and CF were slightly increased significantly different (P<0.05) by the fermentation. The metabolizable energy of fermented soybean distillery by-product were higher ie. 12.93% and 13.56%, resvectively than unfermented soybean distillery by-product.

The metabolizable energy of soybean distillery by-product fermented was increased significantly different (P<005) than metabolizable energy of unfermented soybean distillery by-product (UFS). Fermented of soybean distillery by-product ingredient was caused increased significantly different (P<0.05) of crude protein soybean distillery by-product 9.53% and 10.32% than unfermented soybean distillery by-product ingredients, resvectively.

Chemical composition and nutrient digestibility of soybean distillery by-product (fermented compared to the unfermented) were shown in Table 1 as below:

Table 1. Chemical composition and nutrient digestibility of unfermented and fermented soybean distillery by-product by *Cellulolytic bacteria* B-6 *isolates* culture (in % Dry Matter)

Parameters	*Levels of Cellulolytic bacteria* B-6 *isolates* culture on soybean distillery by-product Fermentation			SEM
	0.0%	0.30%	0.60%	
Chemical composition:				
Dry Matter (%)	88.37a	87.51a	87.28a	1.092
Organic Matter (%)	89.72a	89.51a	89.27a	1.105
Crude Protein (%)	20.26b	22.19a	22.35a	0.318
Crude Fibre (%)	13.92a	13.71a	13.31a	0.503
Gross Energy (Kcal/kg)	3518.36a	3602.81a	3663.42a	67.820
Nutrient digestibility (%):				
Dry matter digestibility (%)	46.72b	49.92a	50.17a	0.503
Organic Matter digestibility (%)	47.81b	50.03a	50.38a	0.629
Crude Protein digestibility (%)	45.75b	54.84a	54.95a	1.507
Crude fibre digestibility (%)	16.07b	22.18	22.31a	1.075
Metabolizable energy (Kcal/kg)	2708.36b	3058.60a	3075.62a	50.925

Note:

- *SEM = Standart Error of The Treatment means*
- The different superscript at the same row is significantly different (P<0.05)

DISCUSSION

Some advantages of Cellulolytic bacteria fermentation process, these microorganisms are rapidly proliferating, resistant to high alcohol content, resistant to high temperatures, has held steady and rapid nature of adaptation. According to Ahmad[1], temperature optimum environment for microbe growth is 25-30^{0}C and a maximum temperature of 35-47^{0}C. Some advantages Cellulolytic bacteria microorganisms in the fermentation process that is rapidly proliferating, resistant to high alcohol content, resistant to high temperatures, has held steady and rapid nature of the adaptation.

Soybean distillery by-product fermented will be able to increase the microbial biomass, so that the crude protein content of soybean distillery by-product increased. Fermentation process and the product is affected by the type and number of microbes, substrat types, pH, and temperature during the fermentation process. Biomass is a form of mass from the biological processes of microorganisms. Microorganisms capable of converting material into proteins[25]. The fermentation process has the objective to produce a product (material feed) that have a nutrient content, texture, better biological availability, and reduce substance antinutrisi[4]. Suparjo *et al.*[27] stated that the fermentation of rice bran with 0.2% *Aspergillus niger* cultured for 72 hours can markedly increase protein and phosphorus content of rice bran, on the contrary lower crude fiber content and acid phytat rice bran.

The Cellulolytic bacteria is a bacteria capable of producing the enzymes amylase and selulolase, so as to increase the digestibility of protein and crude fiber such as cellulose and hemicellulose, as has been overhauled in the form of a simple monosaccharide[32]. Cellulolytic bacteria capable of producing the enzyme 1,4 beta-endo-glukonase, 1,4 beta-exo glukonase, and beta-glucosidase that can degrade components of crude fiber into soluble carbohydrates[14]. The increase of ME content on palm kernel cake (palm kernel cake/meal) as a result of fermentation by the fungus *T. reesei* of 1824.13 kcal/kg to 1930.44 kcal/kg suspected because of the degradation of mannan polysaccharides exist in palm kernel by fungus *T. reesei* into simpler forms (monosaccharides) that produces enough energy value better than in the form of polysaccharides mannan[15]. Sabini *et al.*[23] reported that the fungus *T. reesei* is able to degrade a polysaccharide mannan mannotriosa, mannobiosa, and monnosa. According to Jaelani *et al.*[15], fermented palm kernel cake can markedly increase the crude protein content compared to palm kernel cake without fermentation.

The digestibility of dry matter, organic matter, crude protein, crude fibre, and metabolizable energy were slightly increased by 0.30-0.60% *Cellulolytic bacteria B-6 isolates* culture fermentation. *Cellulolytic bacteria B-6 isolates* culture can effect on crude fiber digestibility of soybean distillery by-product. Becouse, among the cell wall polysaccharides of soybean distillery by-product known as nonstarch polysaccharides (NSP) are celluloses, pectins, and oligosaccharides. NSPs can not be degraded enzymitically in the digestive systems of the birds due to the lacking of enzymes degrading the NSPs in their digestive systems[9]. These results indicated that carbohydrates other than fibres were used for microbial growth and the reduction of nitrogen free extract resulted in increased concentration of the other components.

Fermented of soybean distillery by-product ingredient by 0.30-0.60% Cellulolytic bacteria B-6 isolates culture had better digestibilities, because Cellulolytic bacteria in the gastrointestinal tract can part of an probiotics sourches. Also, Piao *et al.*[20] suggested that probiotics in the gastro intestinal tract can improve protein and energy retention on the body of birds. These bacteria are effective in degrading of the complex compounds such as beta-glucans and arabinoxylans[3]. Cho *et al.*[8] reported that supplementation of microbe in diet could improve the bioavailability of dietary. Wang *et al.*[34] reported that the inclusion of fiber sources such as wheat bran or potato starch reduced the maintenance of energy requirement. Chen *et al.*[7] reported that dietary supplementation of complex probiotic slightly improved digestibility of nutrients.

The high level of non-starch polysaccharides (NSP) in soybean distillery by-product is limiting its unrestricted use in poultry feeding. The NSP is known increasing the gut viscosity, reduce nutrient absorption in the intestine and affect indirectly the growth and performance of bird[8,22]. Many studies have clearly demonstrated that the addition of probiotics culture or enzymes to diets rich in NSP resulted in a

significant reduction of the intestinal viscosity, enhances energy and protein utilization. Wang *et al.*[34] reported that degree of microbial fermentation in the large intestine improves the bioavailability of dietary. Hong *et al.*[13] reported that fermentation of feed using *Aspergilus oryzae* increased digestibility of its DM and CP. The inclusion of soluble dietary fiber (wheat bran) increased daily NSP excreted from feces[26,34] increased both of metabolizable energy and crude protein contents of palm kornel meal[15]. Chen *et al.*[7] reported that dietary supplementation of complex probiotic slightly improved digestibility of nutrients.

CONCLUSION

It was concluded that there are five isolate Cellulolytic bacteria (B-3; B-6; B-7; B-10; and B-13) isolates culture were isolated from rumen liquit of buffalo samples, both were the potensial as a probiotics sources and has CMC-ase activity and its utilization in the soybean distillery by-product fermentation could increase digestibility and metabolizable energy of soybean distillery by-product as feed.

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