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Effects of Humic Acid Dosage and Fermentation Time with *Bacillus subtilis* on Nutrient Content and Quality of Palm Kernel Cake

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Abstract: This study aims to determine the effects of humic acid dosage and fermentation time with *Bacillus subtilis* on the nutrient content and quality of Palm Kernel Cake (PKC). A Completely Randomized Design (CRD) with a 3 × 4 factorial pattern and 3 replications was applied. The present experiment considered two major parameters, termed A and B. Factor A represents the humic acid dose in three different compositions, including A1 (100), A2 (200) and A3 (300 ppm), while B refers to the fermentation time, comprising B1 (3), B2 (4), B3 (5) and B4 (6 days). Variables employed were enzyme activities of cellulase, manannase and protease, as well as crude protein content, crude fiber, crude fat, nitrogen retention, crude fiber digestibility and metabolic energy. The variance analysis results showed a significant effect ($p<0.05$) between factors A and B on cellulase, manannase and protease performances as well as crude protein, crude fiber, crude fat, nitrogen retention and crude fiber digestibility. Each component also observed a substantial effect ($p<0.05$) on these parameters. In summary, the PKC samples fermented with *B. subtilis* at 300 ppm humic acid and 6 days fermentation period generated the optimal results, as indicated by the activities of cellulase 17.86, manannase 25.37 and protease 10. 63 U/mL, as well as the percentage composition of crude protein 28.78, crude fiber 13.55, crude fat 0.76, nitrogen retention 68.76 and crude fiber digestibility 57.47%.

Keywords: *Bacillus subtilis*, Broiler, Enzyme, Palm Kernel Cake

Introduction

The importance of feed to livestock farming cannot be overemphasized, despite incurring up to 70% of the overall production costs. A potential substitute involves the application of palm oil by-products, such as Palm Kernel Cake (PKC). Indonesia has an abundant supply of Crude Palm Oil (CPO) as its 2015 production estimate of 23,096,541 tons steadily attained 31,070,015 tons by 2017. This increment was also accompanied by a corresponding extension in by-products, including PKC. These samples demonstrate a significant potential as feed ingredients for poultry rations, due to the high nutrient contents.

A previous study outlined the percentage nutrient composition of PKC as crude protein 17.31, fiber 27.62, fat 7.14, Ca 0.27 and P 0.94%, in addition to Cu 48.04 ppm (Mirnawati *et al*., 2019a). Meanwhile, the carbohydrate, protein, fat and ash contents were reportedly 65.8, 16.5, 5.1 and 5.2%, respectively (Bello *et al*., 2018). Despite the relatively high crude protein, the current use of PKC in livestock feed appears minimal and is only 10% in broiler rations (Sinurat, 2003).

This insignificant utilization is attributed to high mannan samples. Biochemical studies on PKC composition indicated that 57.8% of its hemicellulose structure was composed of β-Mannan (Azman *et al*., 2016). Consequently, mannan degradation is performed using fermentation with the support of cellulolytic and mannanolytic enzymes. Mirnawati *et al*., (2017) conducted PKC fermentation with mannanolytic molds, where the nutrient content and quality increased in crude protein 26.96, crude fiber 12.72, crude fat 0.22, Ca 0.75, P 0.85 and nitrogen retention 57.16%, in addition to 2,511 kcal/kg metabolic energy. Also, the broiler rations were tested and the maximum consumption of PKC

fermented with *Sclerotium rolfsii* was estimated at 25% (Mirnawati *et al*., 2018a).

However, the fermentation time in this mold appeared relatively extensive. For this reason, the present study employed faster microorganisms, such as bacteria. Mananolytic bacteria are very necessary and as a result, *Bacillus subtilis* was selected. This microbe produces manannase enzymes capable of hydrolyzing the mannan substrate content into mannose (Dhawan and Kaur, 2007). The finding also matched a previous investigation where *B. subtilis* was known to generate several enzymes to aid food digestion, including proteases and manannase (Hooge, 2003). Furthermore, the bacteria tend to develop in the digestive tract of chickens and fulfills one of the probiotic criteria (Stephen *et al*., 2008).

Mirnawati *et al*. (2019a) fermented PKC using *B. subtilis* under a 6-day fermentation time. The studies obtained the optimal results with increasing crude protein content 24.65, crude fiber 17.35, nitrogen retention 68.47 and crude fiber digestibility 53.25%. Also, the activities of mannanase, cellulase and protease were specified at 24.27, 17.13 and 10.27 U/mL, respectively (Mirnawati *et al*., 2019b). Furthermore, the biological testing on broilers showed the maximum PKC consumption at 25% (Mirnawati *et al*., 2020). Apart from the high mannan composition, the presence of heavy metals, including Cu, Zn and Mn, poses another obstacle in applying palm kernel cake. Vidal *et al*. (2001) mentioned that Cu was a limiting factor in the fermentation process. Therefore, substances/compounds capable of reducing the Cu, such as humic acids, are applied. This particular substance is effective in binding micronutrients, including Cu, Zn and Mn (Tan, 1998), leading to the formation of chelate compounds (Tate and Theng, 1980). The acid supplies certain elements, termed N, P and S, to the soil and formed the energy for enzyme activity (Manal *et al*., 2016). Furthermore, the application of humic acid in animal feed offers several health and growth benefits, including the ability to metabolize carbohydrates and proteins by catalysis (Kocukersan *et al*., 2005).

Mirnawati *et al*. (2017) stated that the addition of 200 ppm humic acid in PKC fermentation with *S. rolfsii* generated superior quality and nutrient value, where the percentage crude protein, nitrogen retention, crude fiber and fiber digestibility were estimated at 27.43, 59.17, 11.53 and 55.40%, respectively. Subsequently, the resulting product was tested on broiler rations to obtain a maximum utilization of 32% (Mirnawati *et al*., 2018b). Under this circumstance, the need to conduct a study on Palm Kernel Cake (PKC) to determine the effects of humic acid dosage and fermentation time on nutrient content and quality, using *B. subtilis*, appears very significant. Therefore, the combination is expected to increase the quality and nutritional value of the samples.

Materials and Methods

Feed Preparation and Analysis

The primary study material, PKC, was acquired from PT. Incasi Raya, humic acid and *B. subtilis* were obtained from Indonesian Institute of Sciences (LIPI) Bogor. Nutrient Agar (NA) medium, rice bran, aquades, NaOH, H_2SO_4, acetone and other laboratory supplies were provided for proximate analysis. The crude components of protein, fiber and fat were evaluated by proximate analysis, while nitrogen retention, fiber digestibility and metabolic energy were assessed with the Sibbald (1976) method. Analytical balance, autoclave, needle loop, test-tube, plastic container, porcelain dish, metal cup, erlenmeyer, oven, furnace, desiccator, incubator, beaker, filter paper, bomb calorimeter and metabolic cage, Kjehdal flask and Soxhlet tube, were the tools utilized for separate purposes. Measurement of enzyme activity (cellulase, mannanase, protease) followed Mirnawati *et al*. (2019b).

Experimental Animal

This experiment involved 40 broilers of 4 weeks old weighing 1.5 kg were also involved that were initially placed under fasting for 6 hours to avoid the influence of previous ration. Each treated chicken was fed with fermented products up to 20 grams per individual and subsequently transferred into a metabolic cage equipped with a drinking container (*ad-libitum*) and feces storage. Furthermore, the manure was collected hourly for 30 intervals and simultaneously sprayed with 0.3 N of sulfuric acid to avoid nitrogen evaporation. Sample collecting period was carried up for 15 days. The resulting feces were oven dried between 50-60°C, followed by grounding into fine powder, before chemical analyzing.

Study Design and Data Analysis

This study was conducted by experimental method using a Completely Randomized Design (CRD) with a 3 × 4 factorial pattern and 3 replications. The applied treatments include factor A (humic acid), comprising A1 (100), A2 (200) and A3 (300 ppm), while B (fermentation period) consisting of B1 (3), B2 (4), B3 (5) and B4 (6 days). Crude protein, fiber, fat, as well nitrogen retention, crude fiber digestibility and metabolic energy served as the measured parameters. Subsequent data were statistically processed by analysis of variance (Steel and Torrie, 1991), while the variations between treatments were examined using Duncan Multiple Range est (DMRT).

Results and Discussion

Cellulase Activity

Extensive cellulase activity was detected due to increasing humic acid dosage with a longer fermentation

time (Table 1). This condition is also due to the ability of the acid containing N, S and P elements, to instigate microbial growth (Manal *et al*., 2016). The resulting cellulase enzyme activity intensifies as the microbes develop and reproduce. Mirnawati (2010) also stated that the addition of humic acid tends to accelerate cellulase performance in the feedstuff fermentation process.

The increasing cellulase activity was accompanied by a corresponding extension in fermentation time. This interval appears very influential in evaluating the number of generated enzymes, as lengthier fermentation process produces more degradable substrates. In addition, the microbes tend to develop and reproduce to overhaul the extra food substances. Higher cellulase activity in the A3B4 treatment was also caused by the further intensive degradation of cellulose into glucose, subsequently leading to a decline in crude fiber (Mirnawati, 2010).

The present study obtained the best cellulase activity in the A3B4 treatment at approximately 17.86 U/mL. This result was slightly higher, compared to the findings by Mirnawati *et al*. (2019b), where PKC fermentation with *B. subtilis* generated a cellulase activity of 17.13 U/mL without humic acid.

Mannanase Activity

Table 1 indicates that higher humic acid dosage and fermentation time triggered sufficient activity. This circumstance was due to the amount of humic acid instigating higher microbial growth and excess. The percentage acid content including crude protein 7.10, ash 8.33, crude fiber 12.5, carbohydrates 51.20, nitrogen 1.14 and water 8.6%, appear very significant in microbial growth and development (Kompiang and Supriyanti, 2007). In addition, the availability of feed elements N, S and P, are equally important under this perspective (Manal *et al*., 2016). Higher performance was also caused by prolong fermentation at each dose of humic acid, leading to further microbial growth and reproduction to overhaul the surplus food substances. The result matched the outcome of Susanti and Rahmi (2020) under this condition.

Result shows the maximum activity in the A3B4 treatment at 25.37 U/mL. This outcome appears relatively substantial, compared to Mirnawati *et al*. (2019b), where the PKC fermentation with *B. subtilis* obtained a activity of 24.27 U/mL without humic acid.

Protease Activity

Table 1 indicates that extensive fermentation produced higher protease activity, leading to further microbial growth and reproduction. Susanti and Rahmi (2020) confirmed that the fermentation time greatly determines the number of the resulting enzymes. This study obtained an optimal protease activity in the A3B4 treatment at 10.63 U/mL, compared to Mirnawati *et al*. (2019b) that reported 10.27 U/mL without humic acid.

Crude Protein

Table 2 indicates that additional humic acid dosage instigates the fermentation time, leading to an increase in crude protein. This condition was due to higher bacterial growth and development, under the influence of acid contents, including N, S and P (Manal *et al*., 2006). Further introduction of humic acid effectively binds the micronutrients and provides the underlying elements, including the energy for microbial action.

These results are supported by Mirnawati *et al*. (2017), where the addition of 200 ppm humic acid was able to increase the crude protein composition from 17.31-27.43%, using *S. rolfsii*. Extensive fermentation time tends to generate additional enzymes, including protease. This observation corresponds to Mirnawati *et al*. (2017), where the fermentation interval greatly determined the number of the resulting enzymes. In addition, the increase in crude protein content is probably due to protein inclusion derived from the microbial cells. This condition is in accordance with Sudarmadji *et al*. (2010), where several microorganisms tend to develop and reproduce. Apart from producing enzymes, the bacteria also generates extracellular enzyme proteins responsible for the increase in crude protein levels.

Crude Fiber

Based on the data in Table 2, further humic acid addition and lengthy fermentation time obtained lower crude fiber content. This decrease is due to the bacterial ability to properly develop according to Enviromate, (2002), where the humic substance contained crude protein 7.10, ash 8.33, crude fiber 12.5, carbohydrates 51.20, nitrogen 1 .14 and water 8.6%.

The high bacteria growth was related to the addition of humic acid, both at 3, 4, 5 and 6 days fermentation. This is because the substance supplies the necessary nutrients for microbial growth and development (Kompiang and Supriyanti, 2007). The opinion is in accordance with Manal *et al*. (2016), where humic acid contributed N, S, P elements into the soil and energy for the enzyme activities.

The increase in the microbial growth certainly extends the cellulase enzyme in degrading the PKC cellulose into glucose, resulting in lower crude fiber. This observation is also supported by Mirnawati *et al*. (2017), where the addition of 200 ppm humic acid was able to reduce the crude fiber content by 11.53%, using *S. rolfsii*. The minimal fiber content was also caused by higher fermentation time at humic acid doses of 100, 200 and 300 ppm. This also means more opportunities for microbes to grow and generated enzymes, particularly cellulase that disintegrates cellulose into glucose, leading to lesser crude fiber in accordance with Mirnawati *et al*. (2012).

Table 1: Effects of treatment on cellulase, mannanase and protease activities

Parameters	Factor A (humic acid)	Factor B (fermentation time) B1 (3 days)	B2 (4 days)	B3 (3 days)	B4 (6 days)	Mean	SEM	p
Cellulase activity (U/ml)	A1 (100 ppm)	0.58^{dC}	5.16^{cC}	9.04^{bC}	9.89^{aC}	6.39	0.15	<0.05
	A2 (200 ppm)	1.45^{dB}	5.68^{cB}	9.32^{bB}	10.24^{aB}	6.81	0.40	<0.05
	A3 (300 ppm)	4.69^{dA}	7.34^{cA}	9.82^{bA}	10.63^{aA}	8.25	0.06	<0.05
Mannanase activity (U/ml)	A1 (100 ppm)	10.96^{dC}	12.80^{cB}	14.07^{bC}	21.37^{aC}	14.80	0.27	<0.05
	A2 (200 ppm)	12.30^{dB}	13.08^{cB}	19.15^{bB}	24.88^{aB}	17.35	0.30	<0.05
	A3 (300 ppm)	12.79^{dA}	13.75^{cA}	20.68^{bA}	25.37^{aA}	18.15	0.14	<0.05
Proteaseactivity (U/ml)	A1 (100 ppm)	0.58^{dC}	5.16^{cC}	9.04^{bC}	9.89^{aC}	6.17	0.70	<0.05
	A2 (200 ppm)	1.45^{dB}	5.68^{cB}	9.32^{bB}	10.24^{aB}	6.67	0.22	<0.05
	A3 (300 ppm)	4.69^{dA}	7.34^{cA}	9.82^{bA}	10.63^{aA}	8.12	0.11	<0.05

Lowercase superscripts in the same column and uppercase letters in the same row showed significant differences

Table 2: Effects of treatment on nutrient content, nitrogen retention and crude fiber digestibility

Parameters	Factor A (humic acid dose)	Factor B (time fermentation) B1 (3 days)	B2 (4 days)	B3 (5 days)	B4 (6 days)	Mean	SEM	p
Crude protein (%)	A1 (100 ppm)	18.36^{dC}	22.54^{cB}	24.08^{bB}	27.11^{aB}	23.02	0.34	p<0.05
	A2 (200 ppm)	19.73^{dB}	23.58^{cA}	24.54^{bB}	28.33^{aA}	24.04	0.16	p<0.05
	A3 (300 ppm)	20.83^{dA}	23.58^{cA}	25.40^{bA}	28.78^{aA}	24.64	0.22	p<0.05
Crude fiber (%)	A1 (100 ppm)	24.75^{aA}	21.42^{bA}	18.36^{cA}	15.32^{dA}	19.96	0.77	p<0.05
	A2 (200 ppm)	24.30^{aB}	20.56^{bB}	17.32^{cB}	14.29^{dB}	19.12	0.43	p<0.05
	A3 (300 ppm)	22.51^{aC}	19.47^{bC}	16.67^{cC}	13.55^{dC}	18.05	0.44	p<0.05
Crude fat (%)	A1 (100 ppm)	6.66^{aA}	4.35^{bA}	2.85^{cA}	1.99^{dA}	3.96	0.22	p<0.05
	A2 (200 ppm)	5.37^{aB}	3.17^{bB}	2.58^{cB}	1.41^{dB}	3.13	0.57	p<0.05
	A3 (300 ppm)	4.95^{aC}	2.99^{bC}	2.16^{cC}	0.76^{dC}	2.72	0.03	p<0.05
Nitrogen retention (%)	A1 (100 ppm)	50.92^{dC}	54.05^{cC}	61.74^{bC}	65.39^{Ab}	57.82	0.86	p<0.05
	A2 (200 ppm)	52.12^{dB}	56.23^{cB}	62.58^{bB}	68.20^{aA}	59.60	0.89	p<0.05
	A3 (300 ppm)	53.23^{dA}	57.86^{cA}	63.58^{bA}	68.76^{aA}	60.72	0.93	p<0.05
Crude fiber digestibility (%)	A1 (100 ppm)	46.51^{dC}	49.45^{cC}	52.15^{bC}	55.41^{aC}	50.88	0.20	p<0.05
	A2 (200 ppm)	47.90^{dB}	50.55^{cB}	52.79^{bB}	56.13^{aB}	51.84	0.43	p<0.05
	A3 (300 ppm)	48.56^{dA}	51.79^{cA}	54.83^{bA}	57.47^{aA}	53.16	0.02	p<0.05

Lowercase superscripts in the same column and uppercase letters in the same row showed significant differences.

Crude Fat

Table 2 reveals that the higher humic acid dosage and fermentation time generate minimal crude fat content, but stimulate bacterial growth. This microbial increase produces several enzymes, such as lipase. Morikawa (2006) reported that *B. subtilis* tends to develop various types of extracellular enzymes, including proteases, cellulases, lipases, amylase, nucleases, phosphatases and xylanase.

Crude fat content in the A3B4 treatment decreased from 7.14 to 0.76% after fermentation. This occurrence was due to the presence of *B. subtilis*, that is responsible for lipase formation. Based on Yuneta and Putra (2009), the decrease in fat content was attributed to the lipolytic nature of *B. subtilis* capable of hydrolyzing fat. Furthermore, the lipase has the ability to decompose triglycerides into free fatty acids and glycerol.

Extensive fermentation generates sufficient microbes to disintegrate food substances, particularly fats into fatty acids and glycerol, leading to a decrease in crude fat content. As a consequence, the free fatty acids become easily damaged (Oktavia *et al*., 2012).

Nitrogen Retention

Based on the data in Table 2, further addition of humic acid and fermentation time obtained an increased crude protein component and nitrogen retention. McDonald *et al*. (2010) showed that nitrogen retention was greatly dependent on the feed protein content. This high crude protein was due to the microbial action, where the protein structure was modified to amino acids. Consequently, the protein quality was determined by the completeness of its amino acid content (Setiawan, 2006). Meanwhile, nitrogen retention tends to increase with sufficient protein quality and therefore, instigates high palatability.

Essentially, the increment in nitrogen retention was caused by suitable nutrient quality and sufficient amino acids. Setiawan (2006) reported that a positive response to nitrogen retention was obtained with additional amino acids. Also, nitrogen retention tends to increase, due to higher consumption of the feed ingredients. This conclusion is in accordance with Dady *et al*. (2015), where further utilization provided an opportunity for the animal's body to retain nutrient compounds.

Crude Fiber Digestibility

Table 2 also shows that higher humic acid dosage and fermentation time resulted in an improved crude fiber digestibility. This increase is related to the minimal fiber content that aids proper utilization. The finding was supported by Mirnawati *et al*., (2017), where crude fiber digestibility was greatly dependent on its content in the feed ingredients. This phenomenon poses as a limitation for livestock, due to lower digestibility as a result of the high fiber composition.

Digestibility is influenced by several factors, including the amount of crude fiber in the feed, its material composition and microbial activity (Maynard and Loosly, 2005). The increase in digestibility at higher humic acid dosage (A3B4) was also triggered by the presence of excess intestinal microflora. Melati and Sunarno (2016) showed that further disintegration of cellulose into glucose by additional cellulase enzymes tends to increase crude fiber digestibility. Positive relationship was observed between bacterial growth and cellulase production, where the enzyme supported the decomposition of cellulose into glucose, leading to an increased crude fiber digestibility (Mirnawati *et al*., 2013). Koni *et al*. (2021) reported using fermented *Amorphophallus companulatus* with *B. subtilis* showed an increase in broiler production performance at level of 15% ration. For comparison, another agriculture by-product such as palm oil sludge treated with inocula mixture between *Phanerochaete chrysosporium* and *Neurospora crassa* was capable to be used up to 25% in rations of broiler (Mirnawati *et al*., 2021).

Conclusion

This study confirmed the effects of humic acid and duration of Palm Kernel Cake (PKC) fermentation with *Bacillus subtilis* on enzyme activity and nutrient content. The acid dosage of 300 ppm and 6 days fermentation period obtained the optimal result, with increased activities of cellulase (17.86), mannanase (25.37) and protease (10.63 U/mL). Similar percentage increment was reported in crude protein content (28.76), nitrogen retention (68.76) and crude fiber digestibility (57.47%). However, the crude fiber and fat compositions decreased by 13.55 and 0.76%, respectively. Future research is directed at *in vivo* evaluation to determine the effect of fermented PKC diet on growth and production performance in broilers and layers.

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Author's Contributions

Mirnawati: Supervised study and writing original paper.

Gita Ciptaan: Conducted the study and collecting data.

Yoga Seftiadi: Analyzed the data.

Malik Makmur: Finalize the paper.

Ethics

Animal experiment were carried out based on the Republic of Indonesia Law No. 18 of 2009 that related to holding, rearing and proper treatment and care for animals.

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